American Institute of Integrative Oncology
RESEARCH & EDUCATION

Foundations of Integrative Oncology
COURSE

**GRAND ROUNDS CALL**
**With Dr. Nalini Chilkov**
**October 16th, 2019**
Second Wednesday of Every Month
5:30 PM Pacific / 6:30 PM Mountain / 7:30 PM Central / 8:30 PM Eastern

**Agenda**

- Clinical Pearl:
  - Osteoporosis Risk: Effect of Selected Chinese Herbs on Bone Metabolism

- Case Report:
  - Unexpectedly Long Survival of Patient With Chronic Lymphocytic Leukemia: Why Integrative Methods Matter.

- Research Highlights:
  - Oral Curcumin Reduces Radiation-Induced Dermatitis (Ph 1)
  - Oral curcumin for radiation dermatitis: A URCC NCORP study of 686 breast cancer patients (Ph II)
  - Curcumin Appears Safe and Tolerable in Combination with FOLFOX Chemotherapy
  - Pharmacological Uses and Health Benefits of Ginger Zingiber officinale in Traditional Asian and Ancient Chinese Medicine and Modern Practice
  - Topical silymarin administration for prevention of acute radiodermatitis in breast cancer patients: A randomized, double-blind, placebo-controlled clinical trial.
  - Effect of Oral Silymarin Administration on Prevention of Radiotherapy Induced Mucositis: A Randomized, Double-Blinded, Placebo-Controlled Clinical Trial.
  - Topical Silymarin Administration for Prevention of Capecitabine-Induced Hand-Foot Syndrome: A Randomized, Double-Blinded, Placebo-Controlled Clinical Trial.

| **Clinical Pearl: Osteoporosis Risk: Effect of Selected Chinese Herbs on Bone Metabolism** |
| --- |
| **See Resource Library for Slides and Recording** |

| **Case Study:** |
| --- |
| Submitted by: No Case Study Submitted<br><br>This is a Case Report published in Integrative Medicine •Vol. 17, No.1 •February 2018<br>https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6380985/pdf/imcj-17-51.pdf |

**Case Report of Unexpectedly Long Survival of Patient With Chronic Lymphocytic Leukemia: Why Integrative Methods Matter**

Gregory Haskin, MS; Mikhail Kogan, MD

**We have presented a case of a woman whose CLL has been well managed for more than 15 years without the use of chemotherapy or other forms of conventional treatment.**

**Introduction:**

**Chronic lymphocytic leukemia (CLL)** is one of the most common types of leukemia. In the present case, an evidence-guided treatment plan of supplements and lifestyle changes were used to support the patient.

**Case Presentation:**

A 56-y-old female presented to her primary care physician for a routine physical in 2001. Complete blood cell results suggest pathology among white blood cells. Flow cytometry was used to confirm the presence of CLL. Other than an episode of splenomegaly in 2005 and mild lymphadenopathy, **the patient has remained asymptomatic since diagnosis in 2001.** In late 2001, the patient began a physician-assisted regimen of alternative dietary supplements and lifestyle changes.

**Conclusion:**

**Nutritional supplementation along with lifestyle changes appears to have supported the maintenance of stable and indolent CLL in this patient. It is important for physicians to be prepared to engage with their patients on the use of supplements and lifestyle changes in managing their disease.**

The patient's oncologist never discouraged her to take supplements. However, when the patient approached the oncologist for guidance on diet and supplements, the oncologist did not provide any recommendations, and moreover expressed that diet and supplements are not going to alter her illness course. Although the patient learned how to navigate her care between 2 different providers, she often felt uneasy that her oncologist did not want to engage in any discussion about integrative approaches. Although this case points toward a possible way of slowing down the CLL progression, it also underscores the dire need for field of oncology to embrace lifestyle strategies of managing indolent cancers by either adding these methods to the treatment toolbox or at least aggressively engaging into collaboration with integrative medicine providers, who often care for such patients. Fortunately, many academic centers and large medical systems have begun integrating lifestyle and alternative modalities into care of cancer patients. **The authors do hope that in the future, integrative oncology strategies will be available to every cancer patient**

**Table 2.** Dietary Supplements Utilized in Case

| Product Description | | Daily Dosage | Indications for Use |
|---|---|---|---|
| **Vitamin Supplements** | | | |
| Vitamin $K_2$ | | | Vitamin $K_3$ induces apoptosis leukemia cells.[15] |
| Vitamin $D_3$ | A fat soluble vitamin | | Induced apoptosis in primary CLL cells in vitro and is also known to be important in calcium and bone homeostasis.[16,17] |
| Vitamin E | Mixed tocopherols and carotenoids | | Tocopherols have been found to slow the growth of various cancer types.[18] |
| **Plant Extract Supplements** | | | |
| Methylation support | Methyl $B_{12}$, methylfolate, riboflavin, vitamin $B_6$, and trimethylglycin (methyl-guard plus) | | Vitamin $B_{12}$ and folate deficiency have been associated with anemia in CLL symptomatology.[19] |
| DHEA | Dehydroepiandrosterone made from yam or soy extract | 25 mg | Research suggests that DHEA supplements may help increase bone density in older adults.[20] |
| Green tea extract | High-dose epigallocatechin-3-gallate | 1800 mg | Increases apoptosis among CLL cells.[21,22] |
| Safflower and flax seed oil | 4:1 ratio of omega 6:omega 3 (BodyBio Oil) | | Inhibition of nuclear factor kappa B activation.[23] |
| Curcumin | Curcumin phytosome (Meriva-500) | 1000 mg | Curcumin is immune supportive and also has anti-inflammatory effects on the body.[24] |
| **Antioxidant Supplements** | | | |
| *N*-acetyl-cysteine | Antioxidant amino acid | | *N*-acetyl-cysteine has potent antioxidant effects and is believed to assist in the detoxification process as well as prevent CLL cell-mediated T-cell dysfunction.[25] |
| Milk thistle | The flavonolignan silybin is the major constituent of silymarin, a complex extracted from milk thistle fruit[26] | | Milk thistle is believed to conserve tissue glutathione, which is thought to be liver-protective and have anticancer potential.[26] |

Abbreviations: CLL, chronic lymphocytic leukemia; DHEA, dehydroepiandrosterone.

**Comment from Dr. Nalini:**
I would replace Tocopherols with Tocotrienols and add Vitamin A 25, 000iu per week and Andrographis 2 grams daily)

**CAUTION:**
With leukemias and leukocytosis do not use botanicals and beta glucan rich polysaccharides from fungi that promote White Blood Cells, especially leukocytes and neutrophils including but not limited to Astragalus, Echinacea, Guggul (Commiphora mukul) and Medicinal and edible mushrooms such as Coriolus, Cordyceps, Poria cocos, Chaga, Shitake (Lentinula edodes), Maitake (Grifola frondosa), Agaricus spp.

## Questions & Answers

No Questions Submitted

**CURCUMIN Curcuma Longa Rhizome**

## Research: Oral Curcumin Reduces Radiation-Induced Dermatitis (Ph 1)

Ryan JL, et al. *Curcumin for Radiation Dermatitis: A Randomized, Double-Blind, Placebo-Controlled Clinical Trial of 30 Breast Cancer Patients. Journal of Clinical Oncology.* 2012. 20 May 2012; 30 (suppl; abstr 9027)

**BACKGROUND:**
**Radiation dermatitis occurs in approximately 95% of patients receiving radiation therapy for cancer and often leads to pain and treatment delays.** There is no standard treatment with demonstrated effectiveness for the prevention of radiation dermatitis. We conducted a randomized, double-blind, placebo-control clinical trial to assess the efficacy of curcumin, a potent antioxidant and antiinflammatory component of turmeric, to reduce radiation dermatitis in **30 breast cancer patients.**

**METHODS:**
Eligible patients included adult females with non-inflammatory breast cancer or carcinoma in situ prescribed radiation therapy without concurrent chemotherapy. After randomization, patients took four 500 mg capsules of curcumin or placebo three times daily throughout their course of radiation therapy (total daily dose = 6.0 g). Weekly assessments included Radiation Dermatitis Severity (RDS) Score, presence/absence of moist desquamation, erythema measure, and McGill Pain and Symptom Inventory (SI) questionnaires.

**RESULTS:**
The **30 evaluable patients** were white (90%; mean age = 58.1 years) with ER+PR+ breast cancer (76.7%) who did not have total mastectomy (90%) or chemotherapy prior to start of radiation therapy (56.7%). No significant differences were observed between arms for demographics, compliance, erythema, pain, symptoms, or radiation skin dose.

**Standard pooled variances t-test showed that curcumin reduced RDS at end of treatment compared to placebo (mean RDS = 2.6 vs 3.4; p=0.008)**. Fisher's exact test showed that **curcumin significantly reduced the presence of moist desquamation at the end of radiation therapy** (28.6% vs. 87.5 %; p=0.002). Repeated measures analysis confirmed divergence of RDS between curcumin and placebo arms at Week 5.

**CONCLUSIONS:**
**Oral curcumin, 6.0 g daily during radiation therapy, reduced radiation dermatitis severity and moist desquamation in breast cancer patients.** A multi-site CCOP trial (N=700) is underway to confirm the effectiveness of curcumin to reduce radiation dermatitis severity during various radiation therapy regimens for breast cancer.

## Research: Oral curcumin for radiation dermatitis: A URCC NCORP study of 686 breast cancer patients (Ph II)

Julie Ryan Wolf, Support Care Cancer. 2018 May ; 26(5): 1543–1552. doi:10.1007/s00520-017-3957-4

**Abstract**

**Purpose**

Despite advances in medical technology, **radiation dermatitis occurs in 95% of patients receiving radiation therapy (RT) for cancer.** Currently, there is no standard and effective treatment for the prevention or control of radiation dermatitis. **The goal of the study was to determine the efficacy of oral curcumin, one of the biologically active components in turmeric, at reducing radiation dermatitis severity (RDS) at the end of RT, using the RDS scale, compared to placebo**.

**Methods**

**This was a multisite, randomized, double-blinded, placebo-controlled trial of 686 breast cancer patients**. **Patients took four 500 mg capsules of placebo or curcumin three times daily throughout their prescribed course of RT until one week post-RT.**

**Results**

**A total of 686 patients were included in the final analyses (87.5% white females, mean age = 58).** Linear mixed model analyses demonstrated that **curcumin did not reduce radiation dermatitis severity at the end of RT compared to placebo** (B (95% CI) =0.044 (−0.101, 0.188), p=0.552). **Fewer curcumin patients with RDS > 3.0 suggested a trend toward reduced severity** (7.4% vs. 12.9%, p=0.082). **Patient-reported changes in pain, symptoms, and quality of life were not statistically significant between arms. Conclusions—Oral curcumin did not significantly reduce radiation dermatitis severity compared to placebo.** The skin rating variation and broad eligibility criteria could not account for the undetectable therapeutic effect. An objective measure for radiation dermatitis severity and further exploration for an effective treatment for radiation dermatitis is warranted.

**Comment from Dr. Nalini:** *The source and composition of curcumin and whether or not it is in a lipid soluble form with phospholipids and piperine and taken with a meal with fats and oils may determine absorption and efficacy. One of the problems with botanical studies in the research setting is the lack of deep knowledge on the part of some researchers about product quality, absorbable and low absorption forms and how best to take oral supplements and botanicals to enhance absorption. This was a multi-site study. Patients dosed themselves at home.*

## Research: Curcumin Appears Safe and Tolerable in Combination with FOLFOX Chemotherapy

James MI, et al. *Curcumin inhibits cancer stem cell phenotypes in ex vivo models of colorectal liver metastases, and is clinically safe and tolerable in combination with FOLFOX chemotherapy.* Cancer Lett. 2015 Aug 10;364(2):135-41. doi: 10.1016/j.canlet.2015.05.005. Epub 2015 May 12.

**ABSTRACT:**

In vitro and pre-clinical studies have suggested that a**ddition of the diet-derived agent curcumin may provide a suitable adjunct to enhance efficacy of chemotherapy in models of colorectal cancer.** However, the majority of evidence for this currently derives from established cell lines. Here, we utilised patient-derived colorectal liver metastases (CRLM) to assess whether curcumin may provide added benefit over 5-fluorouracil (5-FU) and oxaliplatin (FOLFOX) in cancer stem cell (CSC) models. Combination of curcumin with FOLFOX chemotherapy was then assessed clinically in a phase I dose escalation study.

Curcumin alone and in combination significantly reduced spheroid number in CRLM CSC models, and decreased the number of cells with high aldehyde dehydrogenase activity (ALDH(high)/CD133(-)). **Addition of curcumin to oxaliplatin/5-FU enhanced anti-proliferative and pro-apoptotic effects in a proportion**

**of patient-derived explants, whilst reducing expression of stem cell-associated markers ALDH and CD133.** The phase I dose escalation study revealed **curcumin to be a safe and tolerable adjunct to FOLFOX chemotherapy in patients with CRLM (n = 12) at doses up to 2 grams daily.** Curcumin may provide added benefit in subsets of patients when administered with FOLFOX, and is a well-tolerated chemotherapy adjunct.

**GINGER: Zingiber off rhizome**

**Research: Pharmacological Uses and Health Benefits of Ginger Zingiber officinale in Traditional Asian and Ancient Chinese Medicine and Modern Practice**

Mohamad Hesam SHAHRAJABIAN, Wenli SUN, Qi CHENG Not Sci Biol, 2019, 11(3):309-319. DOI: 10.15835/nsb11310419

**Abstract Ginger** (Zingiber officinale)
Has been used as a spice and a medicine for over 200 years in traditional Chinese medicine. Ginger is an important plant with several medicinal and nutritional values used in Asian and Chinese traditional medicine.Ginger and its general compounds such as **Fe, Mg, Ca, Vitamin C, flavonoids, phenolic compounds (gingerdiol,gingerol,gingerdione and shogaols), sesquiterpenes, paradols** has long been used as an herbal medicine to treat various symptoms including **vomiting, pain, cold symptoms and it has been shown to have anti-inflammatory, anti-apoptotic, anti-tumor activities, antipyretic, anti-platelet, anti- tumorigenic, anti-hyperglycaemic, antioxidant anti-diabetic, anti-clotting and analgesic properties, cardiotonic, cytotoxic**. It has been widely used for **arthritis, cramps, sprains, sore throats, rheumatism, muscular aches, pains, vomiting, constipation, indigestion, hypertension, dementia, fever and infectious diseases**. Ginger leaves
have also been used for **food flavouring** and Asian traditional medicine, especially in China. Ginger oil also used as a food flavouring agent in soft drink, as spices in bakery products, confectionary items, pickles, sauces and as a preservative. **Ginger is available in three forms, namely fresh root ginger, preserved ginger and dried ginger. The pharmacological activities of ginger
were mainly attributed to its active phytocompounds 6-gingerol, 6-shogaol, zingerone beside other phenolics and flavonoids.**
**Gingerol and shogaol in particular, is known to have anti-oxidant and anti-inflammatory properties.** In **both traditional Chinese medicine, and modern China, Ginger is used in about half of all herbal prescriptions.** Traditional medicinal plants are often cheaper, locally available and easily consumable raw and as simple medicinal preparations. The obtained findings suggest potential of ginger extract as an additive in the food and pharmaceutical industries.

**MILK THISTLE: SILYMARIN Silybum marianum seeds**

**Caution**
**Cytochrome P450 2C9 (CYP2C9) substrates. Taking milk thistle might affect this enzyme and drugs it processes, such as diazepam (Valium), warfarin (Coumadin, Jantoven) and others.**

**Research: Topical silymarin administration for prevention of acute radiodermatitis in breast cancer patients: A randomized, double-blind, placebo-controlled clinical trial.**

Phytother Res. 2019 Feb;33(2):379-386. doi: 10.1002/ptr.6231. Epub 2018 Nov 27. Karbasforooshan H1,

Hosseini S2, Elyasi S1, Fani Pakdel A2, Karimi G3,4.

**Abstract**
Radiation-induced dermatitis is one of the most common side effects of radiotherapy. **Silymarin, a flavonoid extracted from Silybum marianum, exhibits antioxidant and anti-inflammatory activities**. **The purpose of this study was to investigate the efficacy of silymarin gel in prevention of radiodermatitis in patients with breast cancer.** During this **randomized, double-blinded, placebo-controlled clinical trial, the preventive effect of silymarin 1% gel was assessed in comparison with placebo, on radiodermatitis occurrence.** Forty patients randomly received silymarin gel or placebo formulation on chest wall skin following modified radical mastectomy, once daily starting at the first day of radiotherapy for 5 weeks. Radiodermatitis severity was assessed weekly based on Radiation Therapy Oncology Group (RTOG) and National Cancer Institute Common Terminology for Adverse Events (NCI-CTCAE) criteria radio dermatitis grading scale for 5 weeks. The median NCI-CTCAE and RTOG **scores were significantly lower in silymarin group at the end of the third to fifth weeks (p value < 0.05)**. The scores increased significantly in both placebo and silymarin groups during radiotherapy, but t**here was a delay in radiodermatitis development and progression in silymarin group.**

**Prophylactic administration of silymarin gel could significantly reduce the severity of radiodermatitis and delay its occurrence after 5 weeks of a**pplication.

***Recommend Nano Emulsified Milk Thistle by Quicksilver Scientific (milk thistle seed extract plus phospholipids) or have a compounding pharmacy make a hydrosol gel***

## Research: Effect of Oral Silymarin Administration on Prevention of Radiotherapy Induced Mucositis: A Randomized, Double-Blinded, Placebo-Controlled Clinical Trial.

Phytother Res. 2016 Nov;30(11):1879-1885. doi: 10.1002/ptr.5704. Epub 2016 Aug 23.
Elyasi S1, Hosseini S2, Niazi Moghadam MR1, Aledavood SA3, Karimi G4.

**Abstract**
Mucositis is a frequent severe complication of radiation therapy in patients with head and neck cancer. Silymarin is a polyphenolic flavonoid extracted from the milk thistle that exhibits strong antioxidant and antiinflammatory activities. In this study, we **evaluate silymarin efficacy in the prevention of radiotherapy induced mucositis in patients with head and neck cancer,** as the first human study. During this pilot, **randomized, double-blinded, placebo-controlled clinical trial, the effect of oral silymarin 420 mg daily in three divided doses starting at the first day of radiotherapy for 6 weeks, on oral mucositis occurrence was assessed.** Twenty-seven patients fulfilled the inclusion criteria assigned to the silymarin or placebo group. World Health Organization and National Cancer Institute-Common Terminology Criteria oral mucositis grading scale scores were recorded at baseline and weekly during these 6 weeks. The median World Health Organization and National Cancer Institute Common Terminology Criteria **scores were significantly lower in silymarin group at the end of the first to sixth week (p < 0.05).** The scores increased significantly in both placebo and silymarin groups during radiotherapy, but there was **a delay for mucositis development and progression in silymarin group**. **Prophylactic administration of conventional form of silymarin tablets could significantly reduce the severity of radiotherapy induced mucositis and delay its occurrence in patients with head and neck cancer.**

**Research: Topical Silymarin Administration for Prevention of Capecitabine-Induced Hand-Foot Syndrome: A Randomized, Double-Blinded, Placebo-Controlled Clinical Trial.**

Phytother Res. 2017 Sep;31(9):1323-1329. doi: 10.1002/ptr.5857. Epub 2017 Jun 21. Elyasi S1, Shojaee FSR1, Allahyari A2, Karimi G

**ABSTRACT**

**Hand-foot syndrome (HFS) is a frequent dose-limiting adverse reaction of capecitabine in patient with gastrointestinal cancers. Silymarin is a polyphenolic flavonoid extracted from the Silybum marianum that exhibits strong antioxidant and antiinflammatory activities.** In this study, we evaluated silymarin efficacy in prevention of capecitabine-induced HFS in patients with gastrointestinal cancers, as the first human study. During this pilot, randomized, double-blinded, placebo-controlled clinical trial, the effect of s**ilymarin gel 1%, which is applied on the palms and soles twice daily starting at the first day of chemotherapy for 9 weeks, on HFS occurrence was assessed**. Forty patients fulfilled the inclusion criteria assigned to the silymarin or placebo group. World Health Organization HFS grading scale scores were recorded at baseline and every 3 weeks during these 9 weeks. **The median WHO HFS scores were significantly lower in silymarin group at the end of the 9th week ($p < 0.05$)**. The scores increased significantly in both placebo and silymarin groups during chemotherapy, but there was **a delay for HFS development and progression in silymarin group. Prophylactic administration of silymarin topical formulation could significantly reduce the severity of capecitabine-induced HFS and delays its occurrence in patients with gastrointestinal cancer after 9 weeks of application.**

***Recommend Nano Emulsified Milk Thistle by Quicksilver Scientific (milk thistle seed extract plus phospholipids) or have a compounding pharmacy make a hydrosol gel***

**References:**

1. ChenyueLiua, JianzhaoNiub, Tcm, & Tcm. (2017, January 19). Rehmanniae Radix in osteoporosis: **A review of traditional Chinese medicinal uses, phytochemistry, pharmacokinetics and pharmacology.** Retrieved fro https://www.sciencedirect.com/science/article/pii/S0378874116306250.

2. Chin, K.-Y., & Ima-Nirwana, S. (2015, April 8). **The biological effects of tocotrienol on bone: a review on evidence from rodent models.** Retrieved from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4396581/.

3. **Effect of serum from postmenopausal women with** ... (n.d.). Retrieved from https://www.spandidos-publications.com/10.3892/etm.2015.2616/download.

4. Elyasi, S., Hosseini, S., Moghadam, M. R. N., Aledavood, S. A., & Karimi, G. (2016, August 23). **Effect of Oral Silymarin Administration on Prevention of Radiotherapy Induced Mucositis: A Randomized, Double-Blinded, Placebo-Controlled Clinical Trial.** Retrieved from https://onlinelibrary.wiley.com/doi/10.1002/ptr.5704.

5. Elyasi, S., Shojaee, F. S. R., Allahyari, A., & Karimi, G. (2017, June 21). **Topical Silymarin Administration for Prevention of Capecitabine-Induced Hand–Foot Syndrome: A Randomized, Double-Blinded, Placebo-Controlled Clinical Trial.** Retrieved from https://onlinelibrary.wiley.com/doi/full/10.1002/ptr.5857.

6. Guo, T., Chen, X., Wu, X., Shan, E., Jin, Y., Tai, X., … Chen, Z. (2016, September 21). **Acupuncture for osteoporosis: a systematic review protocol. Retrieved from** https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5031268/.

7. Hadji, P., Asmar, L., Nes, J. G. H. van, Menschik, T., Hasenburg, A., Kuck, J., … Ziller, M. (2010, December 18). **The effect of exemestane and tamoxifen on bone health within the Tamoxifen Exemestane Adjuvant Multinational (TEAM) trial: a meta-analysis of the US, German, Netherlands, and Belgium sub-studies.** Retrieved from https://link.springer.com/article/10.1007/s00432-010-0964-y.

8. Hadji, P. (2015, May 20). **Cancer Treatment-Induced Bone Loss in women with breast cancer.** Retrieved from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4440228/.

9. **Intestinal microbiota: a potential target for the** ... (n.d.). Retrieved from https://www.nature.com/articles/boneres201746.

10. James, M. I., Iwuji, C., Irving, G., Karmokar, A., Higgins, J. A., Griffin-Teal, N., … Brown, K. (2015, August 10). **Curcumin inhibits cancer stem cell phenotypes in ex vivo models of colorectal liver metastases, and is clinically safe and tolerable in combination with FOLFOX chemotherapy.** Retrieved from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4510144/.

11. Jia, M., Nie, Y., Cao, D.-P., Xue, Y.-Y., Wang, J.-S., Zhao, L., … Qin, L.-P. (2012). **Potential antiosteoporotic agents from plants: a comprehensive review.** Retrieved from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3551255/.

12. Jin, R., Li, M., Liu, Z., Xie, Q., Xie, Q., Wang, P., … Department of Rehabilitation Medicine. (n.d.). **The Effectiveness of Acupuncture for Osteoporosis: A Systematic Review and Meta-Analysis.** Retrieved from https://www.worldscientific.com/doi/10.1142/S0192415X18500258.

13. Karbasforooshan, H., Hosseini, S., Elyasi, S., Pakdel, A. F., & Karimi, G. (2018, November 27). **Topical silymarin administration for prevention of acute radiodermatitis in breast cancer patients: A randomized, double-blind, placebo-controlled clinical trial.** Retrieved from https://onlinelibrary.wiley.com/doi/full/10.1002/ptr.6231.

14. Kwi-OkOha. (2003, June 25). **Effect of Rehmannia glutinosa Libosch extracts on bone metabolism.** Retrieved from https://www.sciencedirect.com/science/article/pii/S0009898103002389.

15. Luo, D., Liu, Y., Wu, Y., Ma, R., Wang, L., Gu, R., & Fu, W. (n.d.). **Warm Needle Acupuncture in Primary Osteoporosis Management: A Systematic Review and Meta-Analysis** - Ding Luo, Yue Liu, Yanan Wu, Rui Ma, Lin Wang, Ronghe Gu, Wenbin Fu, 2018. Retrieved from https://journals.sagepub.com/doi/abs/10.1136/acupmed-2016-011227.

16. Ryan, J. L., Heckler, C. E., Ling, M., Katz, A., Williams, J. P., Pentland, A. P., & Morrow, G. R. (2013, July). **Curcumin for radiation dermatitis: a randomized, double-blind, placebo-controlled clinical trial of thirty breast cancer patients.** Retrieved from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3998827/.

17. Sacco, S. M., Horcajada, M. N., & Offord, E. (2013, February 5). BPS Publications. Retrieved from https://bpspubs.onlinelibrary.wiley.com/doi/pdf/10.1111/bcp.12033.

18. Shahrajabian, M. H., Sun, W., & Cheng, Q. (2019). **Pharmacological Uses and Health Benefits of Ginger (Zingiber officinale) in Traditional Asian and Ancient Chinese Medicine, and Modern**

**Practice.** *Notulae Scientia Biologicae*, *11*(3), 309–319. doi: 10.15835/nsb11310419

19. Wolf, J. R., Heckler, C. E., Guido, J. J., Peoples, A. R., Gewandter, J. S., Ling, M., … Morrow, G. R. (2017, December 1). **Oral curcumin for radiation dermatitis: a URCC NCORP study of 686 breast cancer patients.** Retrieved from https://link.springer.com/article/10.1007/s00520-017-3957-4.

20. Yu-Chi Wang1, J.-H. C. (2019, February 4). **Decreased fracture incidence with traditional Chinese medicine therapy in patients with osteoporosis: a nationwide population-based cohort study.** Retrieved from https://bmccomplementalternmed.biomedcentral.com/articles/10.1186/s12906-019-2446-3.

# Osteoporosis
## Selected Chinese Herbs that Support Bone Health

Dr. Nalini Chilkov, L.Ac., OMD, Founder



# OSTEOPOROSIS

**Potential Antiosteoporotic Agents from Plants: A Comprehensive Review**
*Evidence-Based Complementary and Alternative Medicine Volume 2012, Article ID 364604, 28 pages*

**Osteoporosis is a systemic skeletal disease characterized by low bone mineral density (BMD) and microarchitectural deterioration of bone tissue, leading to a consequent increase in bone fragility and fracture risk.**

The amount of bone formed during each remodeling cycle decreases with age in both sexes.

Based on the principles of physiological bone regeneration and the role of osteoblasts and osteoclasts in the process, the rate of supply of new osteoblasts and osteoclasts, and the timing of the death of these cells by apoptosis are critical determinants of bone regeneration.

**The activities of these cells are mainly associated with sex steroid deficiency, senescence, and glucocorticoid excess; furthermore, at menopause, the rate of bone remodeling increases precipitously.**

**The loss of sex steroids upregulates the formation of osteoclasts and osteoblasts in the marrow by upregulating the production and action of cytokines, including IL-6, TNF, IL-1, and macrophage colony stimulating factors (M-CSF) which mediate osteoclastogenesis and osteoblastogenesis**

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Bonekey Rep. 2015; 4: 692. Published online 2015 May 20. doi: 10.1038/bonekey.2015.60

**Cancer Treatment-Induced Bone Loss in women with breast cancer.** Peyman Hadji[a.]

Breast cancer is the most frequent cancer in women

**Osteoporosis is one of the most frequent diseases in postmenopausal women, leading to an increased fracture risk due to the physiologic loss of the bone protective effects of estrogen.**

**Risk factors for fracture**

Prevalent fracture Low bone mineral density Age Low body mass index
Family history Tendency to falls Smoking Use of SSRI Glucocorticoids

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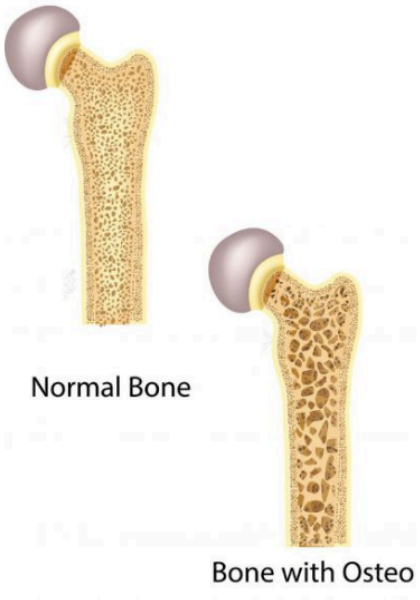
## Review Protective Effects of Selected Botanical Agents on Bone

Int J Environ Res Public Health. 2018 May 11;15(5). pii: E963.
doi: 10.3390/ijerph15050963.

Anti-Osteoporotic effects of phytochemicals includes actions on

- Osteoblasts (bone forming cells)
- Osteoclasts (bone resorbing cells)
- Bone Remodeling

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# Protective Effects of Selected Botanical Agents on Bone

The role of botanical bioactive compounds in regulating bone metabolism. They may act directly on the bone cells, or through reducing inflammation and oxidative stress, or indirectly via increasing the level of sex hormones and interacting with sex hormone receptors on bone cells.

*Int. J. Environ. Res. Public Health* **2018**, *15*(5),963; https://doi.org/10.3390/ijerph15050963

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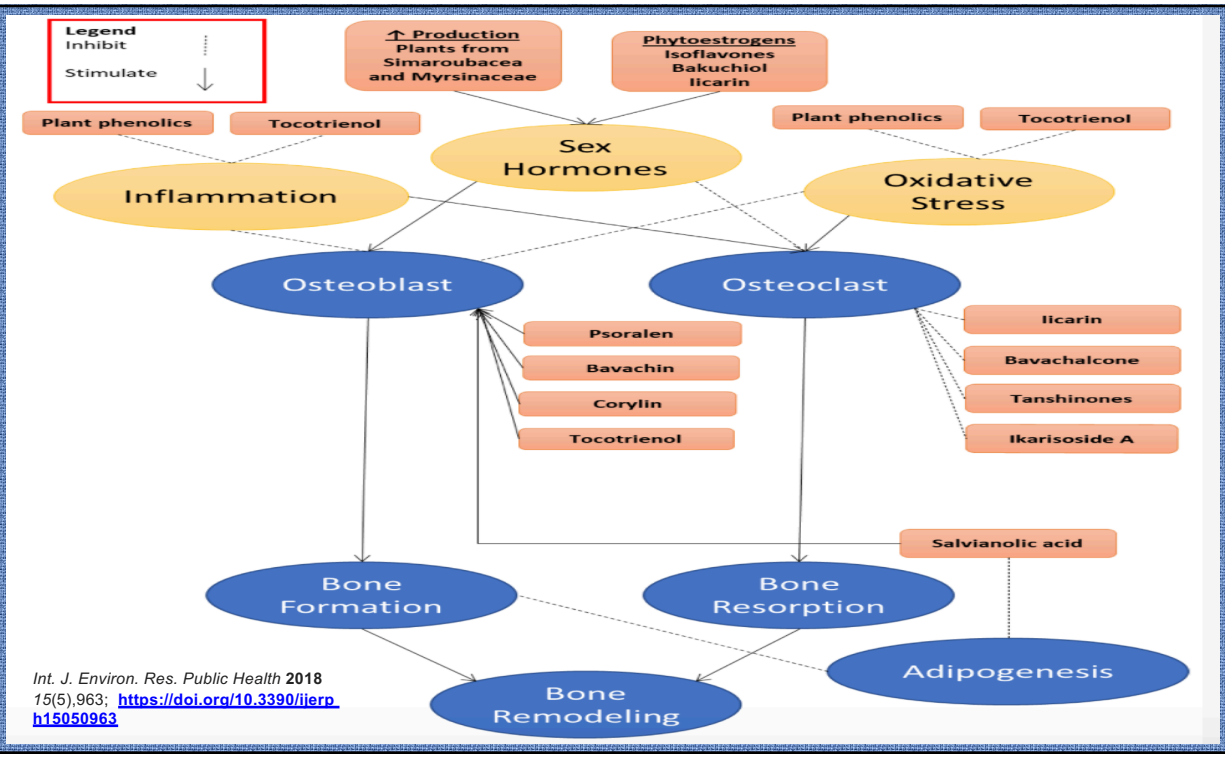
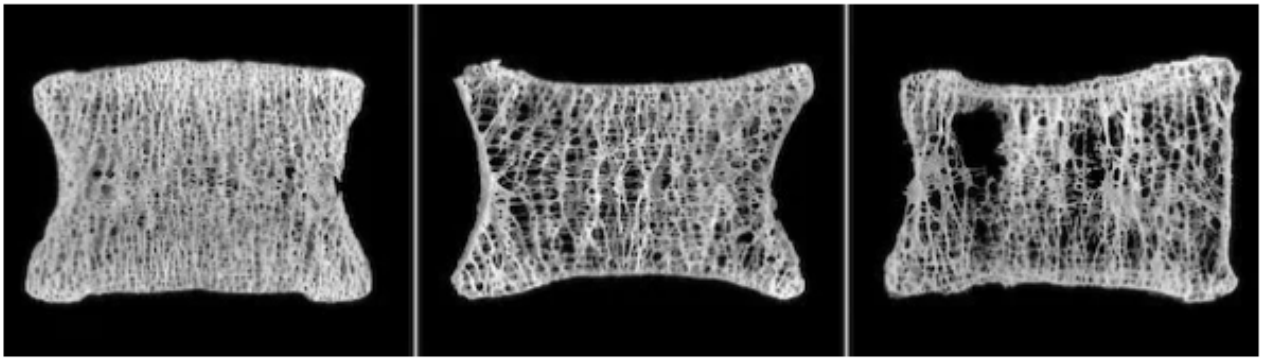



*Int. J. Environ. Res. Public Health* **2018** *15*(5),963; https://doi.org/10.3390/ijerph15050963



*These scans show the progression of a person's vertebra over an 8 year period, from normal bone density (far left) to moderate osteoporosis (middle) to severe osteoporosis (far right)*



## Cancer treatment-induced bone loss (CTIBL)

## Some but not all of these cancer-specific treatments interfere with bone turnover, leading to an accelerated bone loss

Further reduction in endogenous estrogens with chemotherapy (CHT), GnRH analoga or aromatase inhibitors (AIs) continuously increases fracture risk.

| | |
|---|---|
| **Radiation** | **Antibody treatment** |
| **Chemotherapy** | **Endocrine treatment** |

- CHT leads to an unspecific increase in bone resorption
- AI reduces residual serum endogenous estrogen level and is associated with a decrease in BMD and increased fracture risk.
- **Bone loss is 2–3-fold increased compared with healthy, age-matched postmenopausal controls**.

# Bone Density: Tamoxifen vs Aromatase Inhibitors

**Exemestane (AI) resulted in decreases in BMD and increases in bone turnover markers. BMD increased and bone turnover markers decreased with tamoxifen.**

- Patients receiving tamoxifen showed a mean increase from baseline in lumbar spine BMD of 1.2% at month 12 and 0.2% at month 24.
- Patients receiving exemestane showed a mean decrease from baseline of 2.6% after 12 months and 3.5% after 24 months.
- There were significant differences in the changes in lumbar spine BMD between treatment groups ($P < 0.0001$ at both time points).
- Changes in BMD from baseline at the total hip were also significantly different between exemestane and tamoxifen ($P < 0.05$ at both time points).
- **Bone turnover markers decreased from baseline with tamoxifen and increased with exemestane.**

J Cancer Res Clin Oncol. 2011 Jun;137(6):1015-25. doi: 10.1007/s00432-010-0964-y. Epub 2010 Dec 18.

**The effect of exemestane and tamoxifen on bone health within the Tamoxifen Exemestane Adjuvant Multinational (TEAM) trial: a meta-analysis of the US, German, Netherlands, and Belgium sub-studies**.

Hadji P[1], Asmar L, van Nes JG, Menschik T, Hasenburg A, Kuck J, Nortier JW, van de Velde CJ, Jones SE, Ziller M.

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## Selected Botanicals from the Chinese Medicine Materia Medica That Promote Bone Health

Evid Based Complement Alternat Med. 2012; 2012: 364604.
Published online 2012 Dec 31. doi: 10.1155/2012/364604

# Potential Anti-Osteoporotic Agents from Plants: A Comprehensive Review

Min Jia,[1] Yan Nie,[1,2] Da-Peng Cao,[1] Yun-Yun Xue, et al

Numerous medicinal plants
can modulate bone metabolism
to reduce bone loss

Proper nutrition and lifestyle can promote bone health
and pharmacotherapy can slow bone loss or even build new bone



# Radix REHMANNIA GLUTINOSA preparata (cooked) Shu Di Huang. (warms Kidney Yang

J Ethnopharmacol. 2017 Feb 23;198:351-362. doe: 10.1016/j.jep.2017.01.021. Epub 2017 Jan 19.

**Rehmanniae Radix in osteoporosis:**
**A review of traditional Chinese medicinal uses, phytochemistry, pharmacokinetics and pharmacology**. Liu C, etal

Rehmannia Radix displays **bone protecting** features in the osteoporosis models via the **delicate balance between osteoclastogenesis and osteoblastogenesis** through single herb extracts and its isolated compounds.

Clin Chim Acta. 2003 Aug;334(1-2):185-95.

## Effect of Rehmannia glutinosa Libosch extracts on bone metabolism.

Oh KO[1], Kim SW, Kim JY, Ko SY, Kim HM, Baek JH, Ryoo HM, Kim JK.

Rehmannia glutinosa extract **stimulates the proliferation and activities of osteoblasts, while inhibiting the generation and resorptive activities of osteoclasts.**

It also shows **preventive effects on osteoporotic bone loss induced by an ovariectomy.**



## EPIMEDIUM Yin Yang Huo

### Prevents osteoporosis without causing uterine hyperplasia in ovariectomized rats.

➢**Inhibits bone resorption, triggers bone formation, and blocks urinary calcium excretion.**

➢Increases the messenger ribonucleic acid expressions of bone morphogenetic protein and cyclin D.

➢**Stimulates osteoblast proliferation** via **estrogen receptor-dependent** mechanism.

➢**Possesses estrogenic activity and is able to regulate bone metabolism and improve the maturation of osteoblasts** by inducing alkaline phosphatase, bone morphogenetic protein-2, macrophage colony stimulating factor, osteoprotegerin, receptor activator of nuclear factor-κB ligand, core binding factor α1, and interliukin-6 and signaling effectors against decapentaplegic protein 4.

## EPIMEDIUM: Icariin

➢**Inhibits bone loss in** the distal femur and tibia of the rat model and **postmenopausal women.**

➢**Decreases** tartrate-resistant acid phosphatase **activity of osteoclasts**, decreases the size of lipopolysaccharide-induced osteoclasts formation, **prevents** lipopolysaccharide-induced **bone resorption and interleukin-6 and tumor necrosis factor-α expression.**

➢**Inhibits cyclooxygenase** type-2 synthesis, expression of lipopolysaccharide-induced **hypoxia inducible factor-1α**, and lipopolysaccharide-mediated activation of the p38 and Jun N-terminal kinase involved in osteoclasts differentiation.

➢Reduces extracellular regulated-kinases 1/2 and lipopolysaccharide-induced activation.

➢**Reduces specific genes of osteoclasts**: tartrate-resistant acid phosphatase**, matrix metalloproteinase-9,** cathepsin K and **receptor activator NF-kappa-B ligand**.



## EPIMEDIUM: Ikarisoside A

➢Shows **antioxidant and anti-inflammatory prop**erties in lipopolysaccharide-stimulated bone marrow-derived macrophage precursor cells and in RAW264.7 cells.

➢**Inhibits** activation of nuclear factor kappa-light-chain-enhancer of activated B cells, Jun N-terminal kinase, protein kinase B-receptor activator of **NFκB** ligand pathway **in osteoclasts and their resorbing activity.**

## EPIMEDIUM SELECTED REFERENCES

Sheng, M.; Chen, Q.; Wang, L.; Tian, X. Hybridization among **epimedium** (berberidaceae) species native to china. *Sci. Hortic.* **2011**, *128*, 342–351. []

Indran, I.R.; Liang, R.L.Z.; Min, T.E.; Yong, E.-L. Preclinical studies and clinical evaluation of compounds from the genus **epimedium f**or osteoporosis and bone health. *Pharmacol. Ther.* **2016**, *162*, 188–205. ]

Ma, H.; He, X.; Yang, Y.; Li, M.; Hao, D.; Jia, Z. The genus **epimedium**: An ethnopharmacological and phytochemical review. *J. Ethnopharmacol.* **2011**, *134*, 519–541.

Hsieh, T.P.; Sheu, S.Y.; Sun, J.S.; Chen, M.H.; Liu, M.H. Icariin isolated from **epimedium** pubescens regulates osteoblasts anabolism through bmp-2, smad4, and cbfa1 expression. *Phytomedicine* **2010**, *17*, 414–423

Tantry, M.A.; Dar, J.A.; Idris, A.; Akbar, S.; Shawl, A.S. Acylated flavonol glycosides from **epimedium** elatum, a plant endemic to the western himalayas. *Fitoterapia* **2012**, *83*, 665–670. []



# PSORALEA CORYFOLIA Bu Gu Zhi

## BAVACHALCONE

➢**Inhibits osteoclastogenesis.**

➢Inhibits the extracellular regulated-kinases and protein kinase B signaling

➢Inhibits chromosome-Fos and nuclear factor of activated T cells c1 induction during differentiation

# PSORALEA CORYFOLIA

| | |
|---|---|
| **Psoralidin, Isobavachin** | Strong antioxidant. |
| **Bavachin Corylin** | **Stimulates osteoblastic proliferation**. |
| **Bakuchiol**<br>➢**Has high binding affinity for ERα.**<br>➢**Shows no significant uterotrophic activity.**<br>➢Stimulates estrogenic activity in vitro.<br>➢**Reduces postmenopausal bone loss** by **increasing** alkaline phosphatase, calcium concentrations, serum estrogen concentration, and **bone mineral density.** | |
| **Psoralen**<br>➢**Stimulates new bone formation.**<br>➢Stimulates differentiation of osteoblasts in a dose-dependent manner<br>➢**Upregulates osteoblast-specific genes** expression of osteocalcin, type I collagen and sialoprotein.<br>➢**Stimulates bone morphogenetic protein-**2 and bone morphogenetic protein-4 gene expression. | |

# Psoralea Selected References


Chopra, B.; Dhingra, A.K.; Dhar, K.L. Psoralea corylifolia l. (buguchi)—Folklore to modern evidence: Review. *Fitoterapia* **2013**, *90*, 44–56.

Weng, Z.-B.; Gao, Q.-Q.; Wang, F.; Zhao, G.-H.; Yin, F.-Z.; Cai, B.-C.; Chen, Z.-P.; Li, W.-D. Positive skeletal effect of two ingredients of *Psoralea corylifolia* L. On estrogen deficiency-induced osteoporosis and the possible mechanisms of action. *Mol. Cell. Endocrinol.* **2015**, *417*, 103–113

Tang, D.-Z.; Yang, F.; Yang, Z.; Huang, J.; Shi, Q.; Chen, D.; Wang, Y.-J. Psoralen stimulates osteoblast differentiation through activation of bmp signaling. *Biochem. Biophys. Res. Commun.* **2011**, *405*, 256–261.

Li, F.; Li, Q.; Huang, X.; Wang, Y.; Ge, C.; Qi, Y.; Guo, W.; Sun, H. Psoralen stimulates osteoblast proliferation through the activation of nuclear factor-κb-mitogen-activated protein kinase signaling. *Exp. Ther. Med.* **2017**, *14*, 2385–2391.

## Soy Isoflavone Genistein (Glycine Max)

➢ Dietary soybean protein supplementation is **effective in reducing loss of bone mineral density** in ovariectomized rats.

➢**Improves bone turnover markers, bone mineral density, and bone strength among postmenopausal women.**

➢**Modulates bone metabolism-related gene expressio**n of collagen type I, osteocalcin, calciotropic receptor, alkaline phosphatase, cytokines, and growth factors.
➢**Induces bone calcification** in rats.

➢Increases the concentration of inorganic phosphorus in serum.

➢**Regulates the trabecular microstructure and prevent bone loss in postmenopausal women** and animal models.



## Soy Isoflavone Genistein (Glycine Max)

➢Shows estrogenic effects in the bone but not in the uterus.

➢Modulates B-lymphopoiesis.

➢Inhibits bone degradation.

## Soy Isoflavone References


1.Hidaka, S.; Okamoto, Y.; Miyazaki, K.; Uesugi, T. Evaluation of a soybean product fujiflavone p40 as an antiosteoporotic agent in rats. *Phytother. Res.* **2003**, *17*, 112–119.

2.Ye, S.F.; Saga, I.; Ichimura, K.; Nagai, T.; Shinoda, M.; Matsuzaki, S. Coumestrol as well as isoflavones in soybean extract prevent bone resorption in ovariectomized rats. *Endocr. Regul.* **2003**, *37*, 145–152.

3.Chin, K.Y.; Ima-Nirwana, S. Can soy prevent male osteoporosis? A review of the current evidence. *Curr. Drug Targets* **2013**, *14*, 1632–1641

4.Lambert, M.N.T.; Thybo, C.B.; Lykkeboe, S.; Rasmussen, L.M.; Frette, X.; Christensen, L.P.; Jeppesen, P.B. Combined bioavailable isoflavones and probiotics improve bone status and estrogen metabolism in postmenopausal osteopenic women: A randomized controlled trial. *Am. J. Clin. Nutr.* **2017**, *106*, 909–920.

5.Ricci, E.; Cipriani, S.; Chiaffarino, F.; Malvezzi, M.; Parazzini, F. Soy isoflavones and bone mineral density in perimenopausal and postmenopausal western women: A systematic review and meta-analysis of randomized controlled trials. *J. Womens Health (Larchmt)* **2010**, *19*, 1609–1617.]

6.Taku, K.; Melby, M.K.; Kurzer, M.S.; Mizuno, S.; Watanabe, S.; Ishimi, Y. Effects of soy isoflavone supplements on bone turnover markers in menopausal women: Systematic review and meta-analysis of randomized controlled trials. *Bone* **2010**, *47*, 413–423. [

7.Arcoraci, V.; Atteritano, M.; Squadrito, F.; D'Anna, R.; Marini, H.; Santoro, D.; Minutoli, L.; Messina, S.; Altavilla, D.; Bitto, A. Antiosteoporotic activity of genistein aglycone in postmenopausal women: Evidence from a post-hoc analysis of a multicenter randomized controlled trial. *Nutrients* **2017**, *9*, 179.




## TOCOTRIENOLS Annatto Palm *Elaeis guineensis*


➢ **Antioxidant, anti-oxidative stress, anti-inflammatory** and **anti-osteoporotic** agent.

➢**Suppresses the proinflammatory cytokines** expression.

➢Effective in **retaining trabecular bone structure** in the nicotine-induced bone loss model.

➢**Increases bone mineral density** at the **femur and vertebrae** of the rats **in testosterone deficiency and the glucocorticoid bone loss**

➢**Restores bone calcium level at the femur and vertebra** of orchidectomized and ovariectomized rats.

➢**Improves biomechanical strength of the femur** in normal male rats.

# Tocotrienol Selected References


Peh, H.Y.; Tan, W.D.; Liao, W.; Wong, W.F. Vitamin e therapy beyond cancer: Tocopherol versus tocotrienol. *Pharmacol. Ther.* 2016, *162*, 152–169.

Ahsan, H.; Ahad, A.; Iqbal, J.; Siddiqui, W.A. Pharmacological potential of tocotrienols: A review. *Nutr. Metab.* 2014, *11*, 52.

Zhao, L.; Fang, X.; Marshall, M.R.; Chung, S. Regulation of obesity and metabolic complications by gamma and delta tocotrienols. *Molecules* 2016, *21*, 344.

Shen, C.L.; Klein, A.; Chin, K.Y.; Mo, H.; Tsai, P.; Yang, R.S.; Chyu, M.C.; Ima-Nirwana, S. Tocotrienols for bone health: A translational approach. *Ann. N. Y. Acad. Sci.* 2017, *1401*, 150–165.

Ng, M.H.; Choo, Y.M.; Ma, A.N.; Chuah, C.H.; Hashim, M.A. Separation of vitamin e (tocopherol, tocotrienol, and tocomonoenol) in palm oil. *Lipids* 2004, *39*, 1031–1035.

Frega, N.; Mozzon, M.; Bocci, F. Identification and estimation of tocotrienols in the annatto lipid fraction by gas chromatography-mass spectrometry. *J. Am. Oil Chem. Soc.* 1998, *75*, 1723–1727. [

Chin, K.-Y.; Ima-Nirwana, S. The biological effects of tocotrienol on bone: A review on evidence from rodent models. *Drug Des. Dev. Ther.* 2015, *9*, 2049.
Wong, S.K.; Chin, K.-Y.; Suhaimi, F.H.; Ahmad, F.; Ima-Nirwana, S. The effects of palm tocotrienol on metabolic syndrome and bone loss in male rats induced by high-carbohydrate high-fat diet. *J. Funct. Foods* 2018, *44*, 246–254.




# SALVIA MILTIORRHIZA Dan Shen

In ovariectomized rats

➢**Prevents the decrease in trabecular bone mass and bone mineral density.**

➢**Reduces the tartrate-resistant acid phosphatase activity.**

➢**Decreases oxidative stress**.

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## SALVIA MILTIORRHIZA Dan Shen

| | |
|---|---|
| **Tanshinones** | ➢**Reduces** the tartrate-resistant acid phosphatase-positive multinucleated **osteoclast formation** |
| **Tanshinones IIA** | ➢Partially **inhibits ovariectomy-induced bone loss by reducing bone turnover.** |



## SALVIA MILTIORRHIZA Dan Shen

| | |
|---|---|
| **Salvianolic Acid A** | ➢**Inhibits bone loss** in rats given long-term prednisone.<br>➢**Stimulates osteogenesis**.<br>➢**Suppresses adipogenesis in bone marrow** stromal cells. |
| **Salvianolic Acid B** | ➢**Inhibits glucocorticoid-induced** cancellous **bone loss.**<br>➢**Suppresses adipogenesis.**<br>➢**Stimulates bone marrow** stromal cell differentiation to osteoblasts.<br>➢**Upregulates osteoblastic activities**.<br>➢**Modulates the expression PPARgamma and β-catenin in mesenchymal stem cell.** |

## Selected References: Tanshinones & Salvionolic Acids

1.Kim, H.-K.; Woo, E.-R.; Lee, H.-W.; Park, H.-R.; Kim, H.-N.; Jung, Y.-K.; Choi, J.-Y.; Chae, S.-W.; Kim, H.-R.; Chae, H.-J. The correlation of salvia miltiorrhiza extract–induced regulation of osteoclastogenesis with the amount of components tanshinone i, tanshinone iia, cryptotanshinone, and dihydrotanshinone. *Immunopharmacol. Immunotoxicol.* **2008**, *30*, 347–364

2.Lee, S.-Y.; Choi, D.-Y.; Woo, E.-R. Inhibition of osteoclast differentiation by tanshinones from the root ofsalvia miltiorrhiza bunge. *Arch. Pharm. Res.* **2005**, *28*, 909–913.

3.Kwak, H.B.; Yang, D.; Ha, H.; Lee, J.-H.; Kim, H.-N.; Woo, E.-R.; Lee, S.; Kim, H.-H.; Lee, Z.H. Tanshinone iia inhibits osteoclast differentiation through down-regulation of c-fos and nfatc1. *Exp. Mol. Med.* **2006**, *38*, 256.

4.Cui, L.; Liu, Y.-Y.; Wu, T.; Ai, C.-M.; Chen, H.-Q. Osteogenic effects of d (+) β-3, 4-dihydroxyphenyl lactic acid (salvianic acid a, saa) on osteoblasts and bone marrow stromal cells of intact and prednisone-treated rats. *Acta Pharmacol. Sin.* **2009**, *30*, 321.

5.Cui, L.; Li, T.; Liu, Y.; Zhou, L.; Li, P.; Xu, B.; Huang, L.; Chen, Y.; Liu, Y.; Tian, X. Salvianolic acid b prevents bone loss in prednisone-treated rats through stimulation of osteogenesis and bone marrow angiogenesis. *PLoS ONE* **2012**, *7*, e34647.



## Selected References: Acupuncture and Osteoporosis

**Acupuncture** for **osteoporosis**: a systematic review protocol.
Guo T, Chen X, Wu X, Shan E, Jin Y, Tai X, Liu Z, Zhu B, Yuan K, Chen Z.
Syst Rev. 2016 Sep 21;5(1):161.

The Effectiveness of **Acupuncture** for **Osteoporosis**: A Systematic Review and Meta-Analysis.
Pan H, Jin R, Li M, Liu Z, Xie Q, Wang P.
Am J Chin Med. 2018;46(3):489-513. doi: 10.1142/S0192415X18500258. Epub 2018 Apr 4.

Warm needle **acupuncture** in primary **osteoporosis** management: a systematic review and meta-analysis.
Luo D Jr, Liu Y Jr, Wu Y Jr, Ma R Jr, Wang L Jr, Gu R Jr, Fu W Sr.
Acupunct Med. 2018 Aug;36(4):215-221. doi: 10.1136/acupmed-2016-011227. Epub 2018 Jul 9.

# Dr. Nalini's Custom Bone Support Tonic

480 ml

| | | |
|---|---|---|
| 180 | You Gui Tang | Restore Right Decoction |
| 80 | Bu Gu Zhi | Fr. Psoralea coryfolia |
| 80 | Yin Yang Huo | H. Epimedium |
| 50 | Dan Shen | Rdx Salvia miltiorrhiza |
| 30 | Shan Zhu Yu | Fr. Corni |
| 30 | Bai Shao | Rdx Paeonia alba |
| 30 | Xu Duan | Rdx Dispaci |

2 teaspoons daily

## ACUPOINTS

K3, K7, CV 4
Sp3, St 36
UB 20, UB 23,
GV3, GV 4

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CASE REPORT

# Case Report of Unexpectedly Long Survival of Patient With Chronic Lymphocytic Leukemia: Why Integrative Methods Matter

*Gregory Haskin, MS; Mikhail Kogan, MD*

**Abstract**

**Introduction:** Chronic lymphocytic leukemia (CLL) is one of the most common types of leukemia. In the present case, an evidence-guided treatment plan of supplements and lifestyle changes were used to support the patient.

**Case Presentation:** A 56-y-old female presented to her primary care physician for a routine physical in 2001. Complete blood cell results suggest pathology among white blood cells. Flow cytometry was used to confirm the presence of CLL. Other than an episode of splenomegaly in 2005 and mild lymphadenopathy, the patient has remained asymptomatic since diagnosis in 2001. In late 2001, the patient began a physician-assisted regimen of alternative dietary supplements and lifestyle changes.

**Conclusion:** Nutritional supplementation along with lifestyle changes appears to have supported the maintenance of stable and indolent CLL in this patient. It is important for physicians to be prepared to engage with their patients on use of supplements and lifestyle changes in managing their disease.

**Gregory Haskin is a current student at Drexel Medical center and a graduate of the Complementary and Alternative Medicine Program in the Department of Physiology and Biophysics, Georgetown University, in Washington, DC. Mikhail Kogan, MD, is the medical director of the George Washington Center for Integrative Medicine in Washington, DC.**

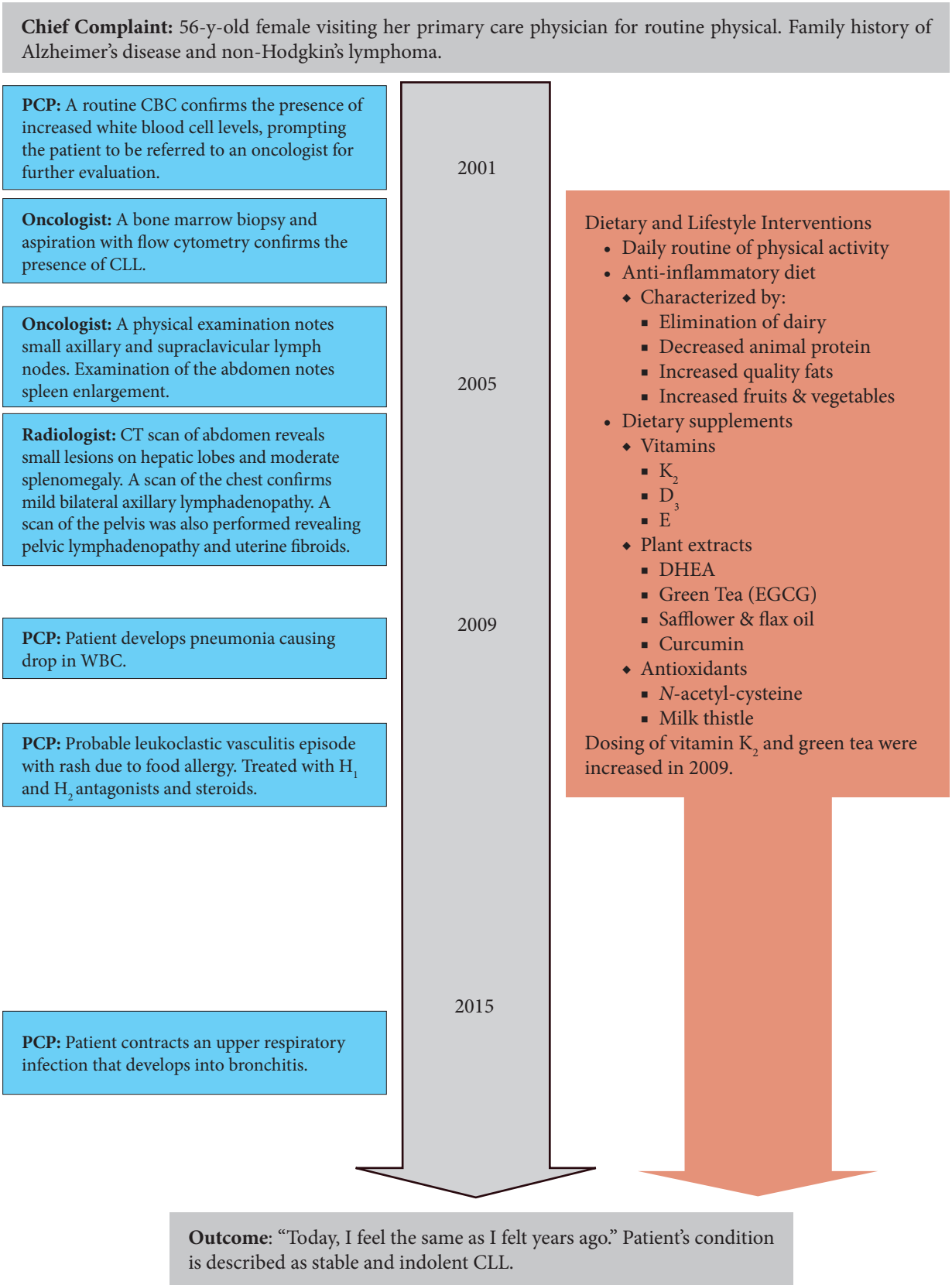
*Corresponding author: Mikhail Kogan, MD*
*E-mail address: mkogan@gwcim.com*

Chronic lymphocytic leukemia (CLL) is one of the most common types of leukemia among adults in the United States and is still considered incurable.[1,2] It affects B and T lymphocytes as well as natural killer cells, but the majority of CLL cases diagnosed are of the B-cell phenotype.[3] CLL results from the uncontrolled clonal growth of small B lymphocytes in a manner that often leads to the crowding out of healthy cells. The disease affects bone marrow and peripheral blood, which can lead to pathology in the lymph nodes, liver, and spleen.[4] The initial symptoms of CLL vary but may include loss of energy, weight loss, enlarged lymph nodes, and splenomegaly.[4] Despite this, many patients remain asymptomatic for a number of years. Physicians typically monitor patients with CLL for signs of infection, autoimmunity, and bone marrow failure, which are common long-term complications.[5]

CLL is often found after a routine complete blood count (CBC) that exhibits an abnormally high white blood cell (WBC) count. This elevation in WBC counts often occurs long before the patient experiences any illness from the disease. A number of prognostic markers are used in tracking the progression of CLL, including lymphocyte doubling time, level of immunoglobulin variable region of the heavy chain variation, CD-38 expression, Zap-70 expression, β-2-microglobulin levels, and serum CD-23 levels.[6,7] The staging of CLL progression is typically determined using the Rai and Binet classification systems.[8,9,10] Both staging systems depend on the following factors: spleen and liver size, platelet counts, hemoglobin levels, and the number of affected lymph nodes.[9,10]

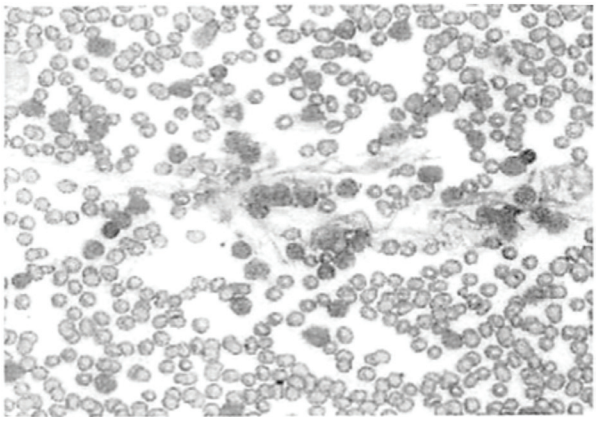
Our goal is to inform clinicians on the value of integrating life style and alternative modalities into care of cancer patients. This case report was prepared in accordance with the CAse REport (CARE) guidelines.[11] A timeline of the patient's medical history and course of care is presented in Figure 1.

**Figure 1.** Timeline of Patient's Medical History and Course of Care




Abbreviations: PCP, primary care provider; CBC, complete blood count; CLL, chronic lymphocytic leukemia; CT, computed tomography; WBC, white blood count; DHEA; dehydroepiandrosterone; EGCG, epigallocatechin-3-gallate.

**Figure 2.** Peripheral Blood Smear Image With Smudge Cells and a Chronic Lymphocytic Leukemia Cell Population



## Patient Information

The patient was a 56-year-old female visiting her primary care physician for a routine physical in 2001. The initial CBC gave the following results: hemoglobin, 13.7; hematocrit, 42; WBC count, 53.7; and platelets, 204. The patient was then referred to an oncologist in the area for a definitive diagnosis of CLL. The patient was self-referred to the George Washington Center for Integrative Medicine (Washington, DC, USA) following her diagnosis in September 2001. Prior to her diagnosis in 2001, she had been relatively healthy with no major illnesses or surgeries to report. Other than her brother being diagnosed with non-Hodgkin's lymphoma, she had no family history related to the disease.

## Clinical Findings

The physical exam performed by her oncologist was unremarkable at the time of diagnosis.

**Table 1.** Rai and Binet Staging Systems[7,8,9,10,12]

| | Stage | Description | Median Survival (mo) | Median Survival (y) |
|---|---|---|---|---|
| **Rai[9]** | | | | |
| Low risk | 0 | Lymphocytosis only | 140 | >10 |
| Intermediate risk | I | Lymphocytosis and lymphadenopathy | 100 | 6 |
| | II | Lymphocytosis in blood and marrow with splenomegaly and/or hepatomegaly, with or without lymphadenopathy | 70 | |
| High risk | III | Lymphocytosis with anemia (hemoglobin <11 g/dL or hematocrit <33%) | 20 | 2 |
| | IV | Lymphocytosis with thrombocytopenia (platelet count <100 000/mm$^3$) | 20 | |
| **Binet[10]** | | | | |
| | A | Enlargement of <3 lymphoid areas; no anemia or thrombocytopenia | 140 | >7 |
| | B | Enlargement of ≥3 lymphoid areas; no anemia or thrombocytopenia | 60 | <5 |
| | C | Anemia (hemoglobin <10g/dL) and/or thrombocytopenia (platelet count <100 000/mm$^3$) | 24 | <2 |

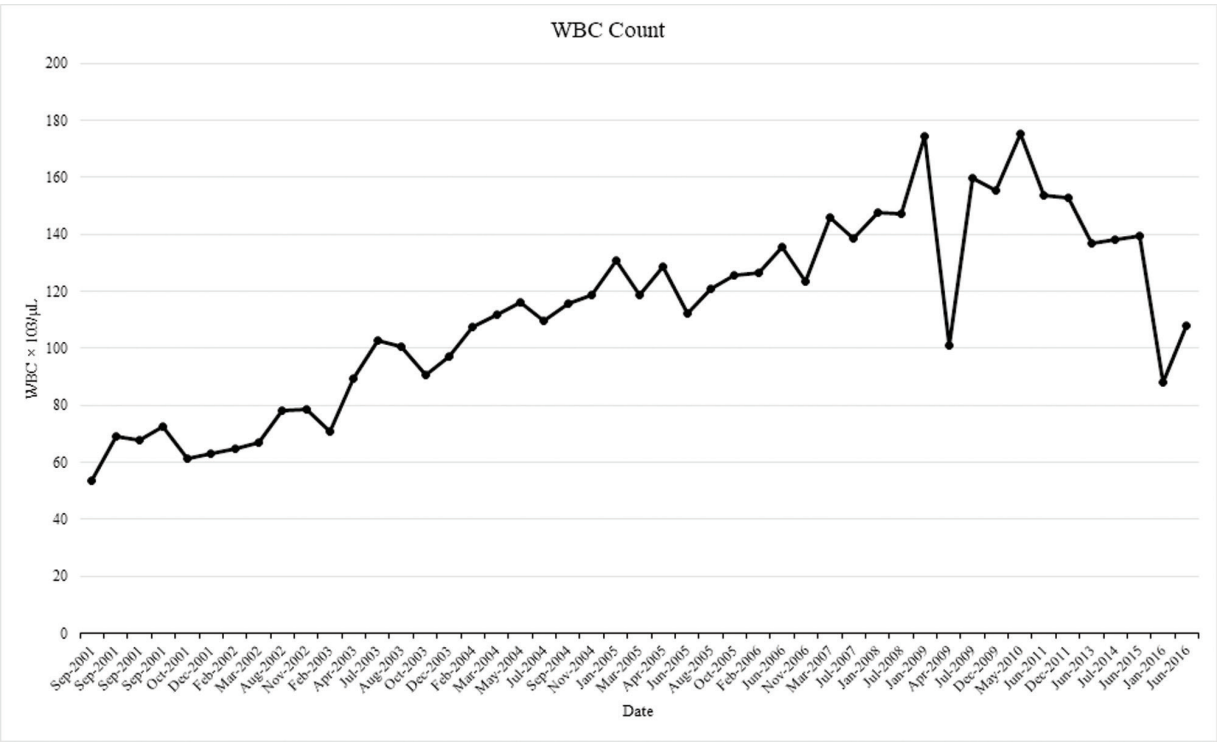
## Diagnostic Assessment

A flow cytometry report showed the presence of a monoclonal B-cell population, which variably expressed CD19, CD20, CD11C, CD23, and aberrant CD5. The report also found a positive but dim population of kappa molecules. FISH was also performed, which showed normal CCND1-IgH, ataxia-telangiectasia mutated, chromosome 12, 13q, and TP53. The blood smear sample shows smudge cells as well as CLL cells.

Based on the workup, her CLL was characterized as stable Rai stage II and Binet stage A. Binet clinical stage A is characterized by no anemia (Hb ≥ 10.0 g/dL) or thrombocytopenia (platelets ≥ 100 × 109/L) and less than 3 areas of lymphoid involvement.[10] Binet stage A patients have a median survival of more than 10 years.[10] Rai stage II CLL is characterized by lymphocytosis with either hepatomegaly or splenomegaly with or without lymphadenopathy.[9]

Since diagnosis in 2001, the patient has remained asymptomatic for more than 15 years. Lab results demonstrate a gradual increase in her WBC during this period, doubling in comparison with her count at diagnosis 3 years later in 2004. She did experience an episode of splenomegaly in 2005. A computed tomography scan of the abdomen was performed confirming the presence of moderate splenomegaly. The scan also revealed small lesions on the anterior

**Figure 3.** Fluctuations in WBC Count by Month Between 2001 and 2016[a]




[a]The patient's WBC count reached 175.3 × 103/μL; however, it stabilized and later decreased slightly, plateauing at approximately 130 to 140 × 103/μL after high-dose epigallocatechin-3-gallate was added to her diet and supplements regimen in the later part of 2009. Of note, the sharp drop in mid-2008 and mid-2015 both correlate with acute infections. Pneumonia in 2008 and upper respiratory infection turned into bronchitis in the second part of 2015.

Abbreviation: WBC, white blood cell.

left and posterior right hepatic lobes. Overall, this trend of increasing WBC has remained with in normal limits. Conversely, her platelet counts show a decreasing trend that appears to have stabilized.

The patient's maximum WBC reached 175 000; however, it stabilized and later began to slowly decrease, possibly plateauing in the 120 000 to 130 000 range after high-dose epigallocatechin-3-gallate (EGCG) was added to her diet and supplements regimen.

## Therapeutic Interventions and Follow-up

Given the lack of effective treatment for early stage CLL in asymptomatic patients, a "watch and wait" approach to treatment was taken.[4,5] Meaning, the physician observed the patient's condition with physical exams and lab tests withholding the use of drugs or other therapies. The decision to wait and observe was made weighing the risks and side effects of chemotherapy with the patient's need for intervention based on disease-staging measures.

During this period, the patient also began a physician-assisted regimen of alternative dietary supplements. The complete list of supplements included the following: vitamin $K_2$; mixed omega-3/omega-6 oil; vitamin $D_3$; meriva-500 (curcumin); combination of milk thistle and broccoli extract; *N*-acetyl-cysteine, methylation support product combining methyl-$B_{12}$, methylfolate, riboflavin, vitamin $B_6$, and trimethylglycin; high-potency multivitamin with activated B vitamins, mixed tocopherols, and carotenoids; low-dose dehydroepiandrosterone; and high-dose EGCG green tea extract (equivalent of approximately 1800 mg of EGCG per day).

As part of her health regimen, the patient also adopted an anti-inflammatory diet. Anti-inflammatory diets are characterized by eliminating dairy; increasing the consumption of quality fats, fruits, vegetables; and decreased animal protein. She also began walking daily to maintain a level of physical activity. The patient's last visit was in June 2016 for her regular check-up, and no new findings were reported. She agreed with the approach and agreed to continue to adhere to the regimen recommended by her physician.

## Discussion

We have presented a case of a woman whose CLL has been well managed for more than 15 years without the use of chemotherapy or other forms of conventional treatment.

**Table 2.** Dietary Supplements Utilized in Case

| Product Description | | Daily Dosage | Indications for Use |
|---|---|---|---|
| **Vitamin Supplements** | | | |
| Vitamin $K_2$ | | | Vitamin $K_3$ induces apoptosis leukemia cells.[15] |
| Vitamin $D_3$ | A fat soluble vitamin | | Induced apoptosis in primary CLL cells in vitro and is also known to be important in calcium and bone homeostasis.[16,17] |
| Vitamin E | Mixed tocopherols and carotenoids | | Tocopherols have been found to slow the growth of various cancer types.[18] |
| **Plant Extract Supplements** | | | |
| Methylation support | Methyl $B_{12}$, methylfolate, riboflavin, vitamin $B_6$, and trimethylglycin (methyl-guard plus) | | Vitamin $B_{12}$ and folate deficiency have been associated with anemia in CLL symptomatology.[19] |
| DHEA | Dehydroepiandrosterone made from yam or soy extract | 25 mg | Research suggests that DHEA supplements may help increase bone density in older adults.[20] |
| Green tea extract | High-dose epigallocatechin-3-gallate | 1800 mg | Increases apoptosis among CLL cells.[21,22] |
| Safflower and flax seed oil | 4:1 ratio of omega 6:omega 3 (BodyBio Oil) | | Inhibition of nuclear factor kappa B activation.[23] |
| Curcumin | Curcumin phytosome (Meriva-500) | 1000 mg | Curcumin is immune supportive and also has anti-inflammatory effects on the body.[24] |
| **Antioxidant Supplements** | | | |
| *N*-acetyl-cysteine | Antioxidant amino acid | | *N*-acetyl-cysteine has potent antioxidant effects and is believed to assist in the detoxification process as well as prevent CLL cell-mediated T-cell dysfunction.[25] |
| Milk thistle | The flavonolignan silybin is the major constituent of silymarin, a complex extracted from milk thistle fruit[26] | | Milk thistle is believed to conserve tissue glutathione, which is thought to be liver-protective and have anticancer potential.[26] |

Abbreviations: CLL, chronic lymphocytic leukemia; DHEA, dehydroepiandrosterone.

There are a number of individuals whose CLL does not progress to the point of requiring chemotherapy. Given that there are currently no conventional treatments for early stage CLL, patients should feel comfortable exploring the body of literature on natural medicines available. It is not uncommon for patients with leukemia to seek out other forms of therapy not prescribed by their oncologists.[27] For this reason, it is important for oncologists to be knowledgeable of popular therapies. We hope to highlight the value of a concerted effort between patients and physicians in devising a health regimen with thoughtful and evidenced nutritional supplementation. The management of this patient's CLL can be explained by the explained in the context of the supplements she was prescribed, including omega-3, EGCG, meriva-500, and vitamin $D_3$.

The blend of organic safflower and flax seed oil with a 4:1 ratio of omega-6 to omega-3. Omega-3 polyunsaturated fatty acids are essential fatty acids that are believed to downregulate nuclear factor kappa B (NF-κB), a key mediator of inflammatory processes in the body.[28] Chronic inflammation as a result of the upregulation proinflammatory molecules such as NF-κB are believed to provide a cellular environment favorable for malignant cell growth.[23] The activation of NF-κB has been associated with more aggressive tumor growth and resistance to both chemotherapy and radiotherapy.[23] So, the use of the BodyBio Balance oil, which contains omega-3 may have aided in dimming the population of κ-positive lymphocytes observed on the flow cytometry report in 2015.

An in vitro study demonstrated that vitamin D analogs caused preferential apoptosis in primary CLL cells

through a p53-independent mechanism.[17] It is also suggested that vitamin D insufficiency is a risk factor for the disease, and high vitamin D levels are predictive of a longer time to first treatment in CLL.[17,29]

A clinical trial found EGCG to be effective against CLL.[31] Preclinical research on EGCG, the active ingredient in green tea, suggests that it may interfere with vascular endothelial growth factor (VEGF) receptors in these cells.[21,22] CLL cells are characterized by their resistance to apoptosis, which is believed to be maintained by the secretion and binding of VEGF. There is also preclinical evidence indicating that curcumin may potentiate the effects of EGCG on CLL.[26,32,33] The patient consumed 1000 mg of curcumin phytosome daily. Furthermore, results from a clinical trial on Rai stage 0/1 CLL patients suggests these patients may benefit from curcumin therapy.[33]

## Conclusion

The patient's oncologist never discouraged her to take supplements. However, when the patient approached the oncologist for guidance on diet and supplements, the oncologist did not provide any recommendations, and, moreover expressed that diet and supplements are not going to alter her illness course. Although the patient learned how to navigate her care between 2 different providers, she often felt uneasy that her oncologist did not want to engage into any discussion about integrative approaches. Although this case points toward a possible way of slowing down the CLL progression, it also underscores the dire need for field of oncology to embrace lifestyle strategies of managing indolent cancers by either adding these methods to the treatment toolbox or at least aggressively engaging into collaboration with integrative medicine providers, who often care for such patients. Fortunately, many academic centers and large medical systems have begun integrating lifestyle and alternative modalities into care of cancer patients. The authors do hope that in the future, integrative oncology strategies will be available to every cancer patient.

### Author Disclosure Statement

This case report was prepared according to the CARE guidelines. Written consent was obtained from the patient for submission of this case report.

### References


1. American Cancer Society. *Cancer Facts and Figures 2016*. Atlanta, GA: American Cancer Society; 2016.
2. Dighiero G, Hamblin TJ. Chronic lymphocytic leukaemia. *Lancet*. 2008;371(9617):1017-1029.
3. Boelens J, Lust S, Vanhoecke B, Offner F. Chronic lymphocytic leukaemia. *Anticancer Res*. 2009;29:605-615.
4. Zelenetz AD, Gordon LI, Wierda WG, et al. Chronic lymphocytic leukemia/small lymphocytic lymphoma, version 1.2015: Clinical practice guidelines in oncology. *J Nat Comprehensive Cancer Net.* 2015;13(3):326-362.
5. Eichhorst B, Dreyling M, Robak T, Montserrat E, Hallek M. ESMO Guidelines Working Group: Chronic lymphocytic leukemia: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. *Ann Oncol*. 2011;22(suppl 6):vi50-vi54.
6. Kalil N, Cheson BD. Chronic lymphocytic leukaemia. *Oncologist*. 1999;4;352-369.
7. Ferrarini M, Cutrona G, Neri A, Morabito F. Prognostic factors in CLL. *Leukemia Supp*. 2012;1(suppl 2):S29-S30.
8. Schroers R, Griesinger F, Trumper L, et al. Combined analysis of ZAP-70 and CD38 expression as a predictor of disease progression in B-cell chronic lymphocytic leukemia. *Leukemia*. 2005;19:750-758.
9. Rai KR, Sawitsky A, Cronkite EP, et al. Clinical staging of chronic lymphocytic leukemia. *Blood*. 1975;46(2):219-234.
10. Binet JL, Auquier A, Dighiero G, et al. A new prognostic classification of chronic lymphocytic leukemia derived from a multivariate survival analysis. *Cancer*. 1981;48(1):198-206.
11. Gagnier JJ, Kienle G, Altman DG, Moher D, Sox H, Riley D; CARE Group. The CARE Guidelines: Consensus-based clinical case reporting guideline development. *Glob Adv Health Med*. 2013;2(5):38-43.
12. Hallek M, Cheson BD, Catovsky D. Guidelines for the diagnosis and treatment of chronic lymphocytic leukemia: A report from the International Workshop on Chronic Lymphocytic Leukemia updating the National Cancer Institute-Working Group 1996 guidelines. *Blood*. 2008;111:5446-5456.
13. Ghia P, Hallek M. Management of chronic lymphocytic leukemia. *Haematologica*. 2014;99(6):965-972.
14. Malek S. Molecular biomarkers in chronic lymphocytic leukemia. *Adv Exp Med Biol*. 2013;792:193-214.
15. Lin C, Kang J, Zheng R. Vitamin K3 triggers human leukemia cell death through hydrogen peroxide generation and histone hyperacetylation. *Pharmazie*. 2005;60(10):765-771.
16. Pepper C, Fegan C. CLL: A supplementary question?. *Blood*. 2011;117(5):1439-1440.
17. Molica S, Digiesi G, Antenucci A, et al. Vitamin D insufficiency predicts time to first treatment (TFT) in early chronic lymphocytic leukemia (chronic lymphocytic leukemia). *Leuk Res*. 2012;36:443-447.
18. Hardman WE. Diet components can suppress inflammation and reduce cancer risk. *Nutr Res Pract*. 2014;8(3):233-240.
19. Mauro FR, Gentile M, Foa R. Erythropoietin and chronic lymphocytic leukemia. *Rev Clin Exp Hematol*. 2002;suppl 1:21-31.
20. Villareal DT, Holloszy JO, Kohrt WM. Effects of DHEA replacement on bone mineral density and body composition in elderly women and men. *Clin Endocrinol*. 2000;53:561-568.
21. Beliveau R, Gingras D. Green tea: Prevention and treatment of cancer by nutraceuticals. *Lancet*. 2004;364:1021-1022.
22. Forester SC, Lambert JD. Antioxidant effects of green tea. *Mol Nutr Food Res*. 2011;55(6):844-854.
23. Li F, Sethi. Targeting transcription factor NF-kappaB to overcome chemoresistance and radioresistance in cancer therapy. *Biochem Biophys Acta*. 2010;1805(2):167-180.
24. Rayburn ER, Ezell SJ, Zhang R. Anti-Inflammatory agents for cancer therapy. *Mol Cell Pharmacol*. 2009;1(1):29-43.
25. Jia L, Gribben JG. Dangerous power: Mitochondria in CLL cells. *Blood Apr.* 2014;123(17):2596-2597.
26. Kidd PM. Bioavailability and activity of phytosome complexes from botanical polyphenols: The silymarin, curcumin, green tea, and grape seed extracts. *Altern Med Rev.* 2009;14:226-246.
27. Wesa K, Cassileth BR. Is there a role for complementary therapy in the management of leukemia? *Expert Rev Anticancer Ther*. 2009;9(9):1241-1249.
28. Fahrmann JF, Ballester OF, Ballester G, et al. Inhibition of nuclear factor kappa B activation in early-stage chronic lymphocytic leukemia by omega-3 fatty acids. *Cancer Invest.* 2013;31:24-38.
29. Shanafelt TD, Drake MT, Maurer MJ, et al. Vitamin D insufficiency and prognosis in chronic lymphocytic leukemia. *Blood*. 2011;117(5):1492-1498.
30. Lemanne D, Block KI, Kressel BR, Sukhatme VP, White JD. A case of complete and durable molecular remission of chronic lymphocytic leukemia following treatment with epigallocatechin-3-gallate, an extract of green tea. *Cureus*. 2015;7(12):e441.
31. Shanafelt TD, Call TG, Zent CS, et al. Phase 2 trial of daily, oral Polyphenon E in patients with asymptomatic, Rai stage 0 to II chronic lymphocytic leukemia. *Cancer*. 2013;119:363-370.
32. Ghosh AK, Kay NE, Secreto CR, Shanafelt TD. Curcumin inhibits prosurvival pathways in chronic lymphocytic leukemia B cells and may overcome their stromal protection in combination with epigallocatechin-3-gallate. *Clin Cancer Res*. 2009;15:1250-1258.
33. Golombick T, Diamond T, Manoharan A, Ramakrishna R. The effect of curcumin (as Meriva) on absolute lymphocyte count (ALC), NK cells and T cell populations in patients with stage 0/1 chronic lymphocytic leukemia. *J Cancer Ther*. 2015;6:566-571.

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RESEARCH ARTICLE Open Access

# Decreased fracture incidence with traditional Chinese medicine therapy in patients with osteoporosis: a nationwide population-based cohort study

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## Abstract

**Background:** There are no published studies regarding the efficacy of traditional Chinese medicine (TCM) for the prevention of osteoporotic fracture. Therefore, we conducted this nationwide, population-based cohort study to investigate the probable effect of TCM to decrease the fracture rate.

**Methods:** We identified cases with osteoporosis and selected a comparison group that was frequency-matched according to sex, age (per 5 years), diagnosis year of osteoporosis, and index year. The difference between the two groups in the development of fracture was estimated using the Kaplan–Meier method and the log-rank test.

**Results:** After inserting age, gender, urbanization level, and comorbidities into the Cox's proportional hazard model, patients who used TCM had a lower hazard ratio (HR) of fracture (adjusted HR: 0.47, 95% CI: 0.37–0.59) compared to the non-TCM user group. The Kaplan-Meier curves showed that osteoporosis patients who used TCM had a lower incidence of fracture events than those who did not ($p < 0.00001$). Our study also demonstrated that the longer the TCM use, the lesser the fracture rate.

**Conclusion:** Our study showed that TCM might have a positive impact on the prevention of osteoporotic fracture.

**Keywords:** National Health Insurance Research Database, Osteoporotic fracture, Traditional Chinese medicine

## Background

Osteoporosis is defined as a skeletal disorder that occurs with the decrease in bone density and quality, leading to an increased risk of fracture [1]. The most frequent fracture areas are the hip, wrist, and spine. In the United States, 1.5 million osteoporotic patients over 50 years of age suffer from hip fractures each year [2] and over 3.5 million fragility fractures occur each year in Europe [3]. Osteoporotic fracture is an economic burden which cost US$17 billion annually in the United States in 2005 and €37 billion in Europe in 2010 [3, 4]. In Taiwan, the incidence of hip fracture increases 9.3% annually, with 13,892 women and 8616 men over 50 years of age suffering from hip fracture in 2010. According to the data from Health Promotion Administration, approximately 40% of women over the age of 70 need long-term bed rest and care due to hip fracture, and 10% die from hip fracture in Taiwan. In Taiwan, every case requires more than NT$100,000 during the acute phase, and more resources are needed in the long run [5]. The incidence of fractures is expected to increase over the next 30 years because of the increase in the aged population [6]. Half of all hip fractures will occur in Asia by 2050 where the amount of older people will be most markedly increased [7].

There are numerous drugs available for treating osteoporosis; however, only bisphosphonates and denosumab have been demonstrated to have antifracture efficacy. Besides, only teriparatide and intact Parathyroid hormone (PTH) are approved to stimulate bone formation; the others are antiresorptive agents. In view of this,

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people might wonder whether other therapies have been ignored. There are no published studies regarding the efficacy of traditional Chinese medicine (TCM) for the prevention of osteoporotic fracture. Some studies revealed the effects of TCM, such as *Cistanche deserticola* extract, Baicalin, Semen Astragali Complanati decoction, and Rhizoma Cibotii decoction, in regard to promoting bone formation, mineralization, and decreasing bone loss [8–10]. However, these studies used cell-lines and animal models. Therefore, large-scale, population-based analyses examining the preventative effect of TCM herbal products for osteoporotic fracture are needed.

To investigate the probable effect of TCM to decrease the fracture rate in patients with osteoporosis, we analyzed the National Health Insurance Research Database (NHIRD) of Taiwan from 2000 to 2010. TCM has been reimbursed by the National Health Insurance (NHI) program since 1996 in Taiwan, including Chinese herbal products, acupuncture/moxibustion, and manipulative therapy [11]. At the end of 2011, more than 99% of the population were enrolled in the NHI program [12]. This study provides important information for clinicians and shows that Chinese herbal prescriptions could also be useful for further pharmacological investigation or clinical trials.

## Methods

### Data source

Taiwan launched the mandatory National Health Insurance (NHI) program in 1995 and has been reimbursing Western and TCM since 1996. TCM treatment includes Chinese herbal medicine, acupuncture, and moxibustion therapy in ambulatory clinics. Large computerized data (NHIRD) was used to perform our nationwide population-based cohort study. We used the LHID2000 (Longitudinal Health Insurance Database 2000) provided by the National Health Insurance Administration, which is managed by the National Health Research Institutes. The LHID2000 includes data from 1 million randomly selected patients who were NHI beneficiaries in 2000. Similar distributions of beneficiaries based on age and gender of beneficiary age and gender in the LHID2000 and the general NHI database were observed. The registration and claim dataset from the LHID2000 spans the years 2000 to 2011. Ambulatory care claims contain an individual's gender, date of birth, visit date, and the International Classification of Disease, Ninth Revision, Clinical Modification (ICD-9-CM) codes for three primary diagnoses. Inpatient claims contain ICD-9-CM codes for the principal diagnosis and up to four secondary diagnoses. A disease diagnosis without valid supporting clinical findings may be considered as medical fraud by the NHI with a penalty of 100-fold the payment claimed by the treating physician or hospital. This study was approved by the Institutional Review Board of China Medical University (CMUH104--REC2–115).

### Study population

Our population cohort study used newly diagnosed osteoporosis patients (aged ≥18 years) identified between 2000 and 2010 and followed up until the December 31, 2011 or the first manifestation of fracture. Subjects with osteoporosis were required to have at least two outpatient claims or at least one inpatient claim with the diagnosis of ICD—CM code 733.0 during the study period. The exclusion criteria included less than 18 years old, incomplete information of age and sex, and withdrawal from the NHIRD during the follow-up period. Patients who received TCM treatment for their osteoporosis from the initial diagnosis to December 31, 2010, were identified as the TCM user cohort. The date of the first TCM treatment after a new diagnosis of osteoporosis was used as the index date for the cohort group. No diagnosis and TCM treatment code in the database was categorized as non-TCM users. Figure 1 shows the subject recruitment flowchart of osteoporosis patients from the NHIRD in Taiwan.

### Covariate assessment

Sociodemographic factors included age and sex. Patients were divided into two groups based on their age; < 65 years and ≥ 65 years old. The townships in which subjects registered for insurance were grouped into four levels of urbanization based on a score calculated by incorporating variables indicating population density (people/km$^2$), population ratio of different educational levels, population ratio of elderly, population ratio of agriculture workers, and the number of physicians per 100,000 people [13]. Baseline comorbidities comprised alcohol-related disease (ICD-9-CM: 291, 303, 305.00, 305.01, 305.02, 305.03, 571.0–571.3, 790.3, V11.3), cancer (140–208), cardiovascular disease (410–414, 428, 430–438, 440–448), chronic kidney disease (585–586, 588.8–588.9), chronic obstructive pulmonary disease (491, 492, 493, 496), diabetes mellitus (250), dementia (290.0, 290.1, 290.2, 290.3, 290.4, 294.1, 331.0), depression (296.2, 296.3, 300.4, 311), hyperlipidemia (272.0, 272.1, 272.2, 272.3, 272.4), hypertension (401–405), and Parkinson's disease (332.x).

### Data analysis

Categorical variables were reported as numbers and percentages. The difference in proportions was assessed using the chi-square test. Cox's proportional hazard model estimated hazard ratios (HR) of TCM usage on fractures. The difference in fracture development between the two groups was estimated using the Kaplan–Meier method and the log-rank test. Statistical analysis was performed and figures were created using SAS 9.4

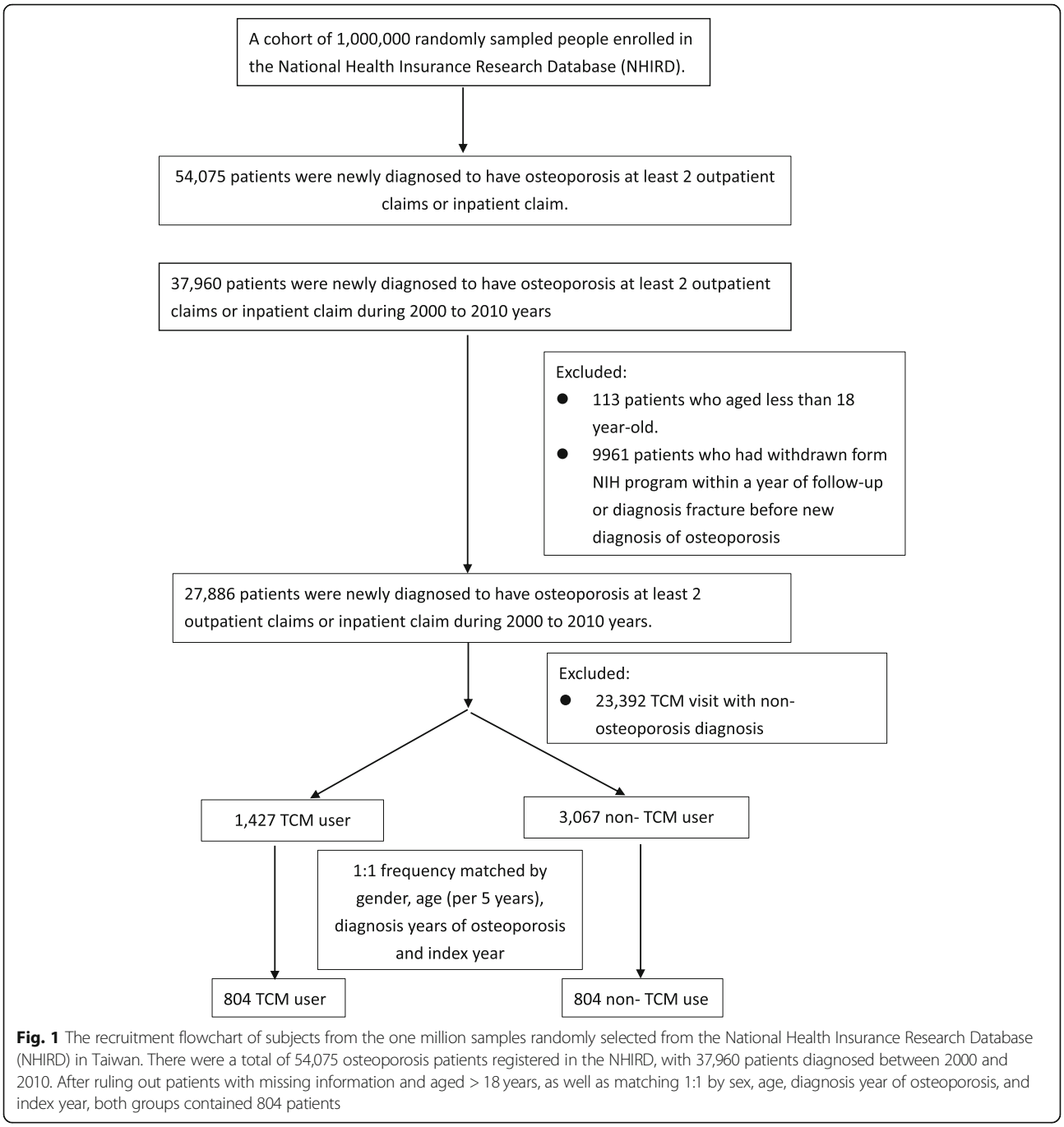


**Fig. 1** The recruitment flowchart of subjects from the one million samples randomly selected from the National Health Insurance Research Database (NHIRD) in Taiwan. There were a total of 54,075 osteoporosis patients registered in the NHIRD, with 37,960 patients diagnosed between 2000 and 2010. After ruling out patients with missing information and aged > 18 years, as well as matching 1:1 by sex, age, diagnosis year of osteoporosis, and index year, both groups contained 804 patients

(SAS Institute, Cary, NC, U.S.A.) and R software, with $p < 0.05$ in two-tailed tests indicating statistical significance.

## Results

Overall, there were 1427 TCM users and 3067 non-TCM users among the osteoporosis patients. After frequency matching, there were 804 patients in the TCM user and non-TCM user groups. Table 1 shows the baseline characteristics according to TCM usage. Osteoporosis patients in both groups had a similar distribution of gender and age. The mean age of the TCM user and non-TCM user groups was 64.48 ± 11.08 and 64.57 ± 11.08, respectively, and the female and male percentages were 76.49 and 23.51%, respectively. Compared with non-TCM user group, TCM users had a higher proportion of chronic obstructive pulmonary disease and hyperlipidemia, but had a lower proportion of cancer.

A total of 323 patients were newly diagnosed with a fracture during the follow-up period (59% non-TCM

**Table 1** Characteristics of osteoporosis patients according to use of traditional Chinese medicine

| Variable | TCM | | | | |
|---|---|---|---|---|---|
| | No ($N = 804$) | | Yes ($N = 804$) | | *p*-value |
| | n | % | n | % | |
| Gender | | | | | 0.99* |
| Female | 615 | 76.49 | 615 | 76.49 | |
| Male | 189 | 23.51 | 189 | 23.51 | |
| Age group, year | | | | | 0.99* |
| < 65 | 386 | 48.01 | 386 | 48.01 | |
| ≥ 65 | 418 | 51.99 | 418 | 51.99 | |
| Mean (SD) | 64.57 (11.08) | | 64.48 (11.08) | | 0.8813[a] |
| Urbanization level[†] | | | | | 0.0104* |
| 1 (highest) | 207 | 25.75 | 240 | 29.85 | |
| 2 | 238 | 29.6 | 226 | 28.11 | |
| 3 | 106 | 13.18 | 134 | 16.67 | |
| 4 (lowest) | 253 | 31.47 | 204 | 25.37 | |
| Baseline comorbidity | | | | | |
| Alcohol-related disease | 4 | 0.5 | 1 | 0.12 | 0.3742[b] |
| Cancer | 45 | 5.6 | 22 | 2.74 | 0.0041* |
| Cardiovascular disease | 307 | 38.18 | 305 | 37.94 | 0.9182* |
| Chronic kidney disease | 25 | 3.11 | 25 | 3.11 | 0.99* |
| Chronic obstructive pulmonary disease | 188 | 23.38 | 241 | 29.98 | 0.0028* |
| Diabetes mellitus | 212 | 26.37 | 185 | 23.01 | 0.1184* |
| Dementia | 19 | 2.36 | 12 | 1.49 | 0.2043* |
| Depression | 44 | 5.47 | 54 | 6.72 | 0.2972* |
| Hyperlipidemia | 168 | 20.9 | 218 | 27.11 | 0.0035* |
| Hypertension | 447 | 55.6 | 416 | 51.74 | 0.1211* |
| Parkinson's disease | 8 | 1 | 11 | 1.37 | 0.4887* |
| Interval between diagnosis and initial TCM use, mean (days) | | | 611 | | |
| Follow-up time, mean (median; years) | 3.75 (2.86) | | 5.38 (5.18) | | |

*Chi-Square Test, [a] t-test, [b]Fisher's exact test

[†]: The urbanization level was categorized into four levels based on the population density of the residential area, with level 1 as the most urbanized and level 4 as the least urbanized

Traditional Chinese medicine (TCM) included Chinese herbal remedies, acupuncture, and manipulative

users and 40% TCM users). A reduced risk of fracture recurrence was associated with TCM use (Crude HR: 0.50, 95% CI: 0.4–0.63). After inserting age, gender, urbanization level, alcohol-related disease, cancer, cardiovascular disease, chronic kidney disease, chronic obstructive pulmonary disease, diabetes mellitus, dementia, depression, hyperlipidemia, hypertension, and Parkinson's disease into the Cox's proportional hazard model, TCM use had a lower HR of fracture (adjusted HR: 0.47, 95% CI: 0.37–0.59) compared to the non-TCM user group (Table 2).

The Kaplan-Meier curves showed that osteoporosis patients using TCM had a lower incidence rate of fracture events than those not using it ($p < 0.0001$; Fig. 2).

Table 3 shows the distribution of TCM users according to their accumulated days of herbal use. Patients with < 30 days of Chinese herb medicine use per year (including non-TCM users) were selected as the reference. Patients who accumulated between 30 and 180 days of herbal use showed an aHR of 0.60 (95% CI: 0.43–0.84), and those with more than 180 days of herbal use showed an aHR of 0.37 (0.20–0.68). When analyzing patients with more than 30 accumulated days of herbal use, those who cumulatively used herbal prescriptions for more than 180 days had a lower risk of fracture (aHR: 0.63, 95% CI: 0.32–1.24) than in the compared cohort; however, this was not statistically significant.

**Table 2** Cox model with hazard ratios and 95% confidence intervals of fracture associated with TCM and covariates among osteoporosis patients

| Variable | Fracture no. ($n = 323$) | Crude* | | | Adjusted† | | |
|---|---|---|---|---|---|---|---|
| | | HR | (95%CI) | p-value | HR | (95%CI) | *p*-value |
| TCM use | | | | | | | |
| No | 193 | 1.00 | reference | | 1.00 | reference | |
| Yes | 130 | 0.50 | (0.4–0.63) | <.0001 | 0.47 | (0.37–0.59) | <.0001 |
| Gender | | | | | | | |
| Female | 266 | 1.00 | reference | | 1.00 | reference | |
| Male | 57 | 0.78 | (0.59–1.04) | 0.0939 | 0.58 | (0.43–0.79) | 0.0004 |
| Age group, year | | | | | | | |
| < 65 | 107 | 1.00 | reference | | 1.00 | reference | |
| ≥ 65 | 216 | 2.28 | (1.81–2.87) | <.0001 | 2.62 | (2.03–3.39) | <.0001 |
| Urbanization level | | | | | | | |
| 1 (highest) | 73 | 1.00 | reference | | 1.00 | reference | |
| 2 | 94 | 1.28 | (0.94–1.73) | 0.1173 | 1.24 | (0.91–1.69) | 0.1665 |
| 3 | 52 | 1.46 | (1.02–2.08) | 0.0372 | 1.43 | (0.99–2.05) | 0.0534 |
| 4 (lowest) | 104 | 1.54 | (1.14–2.07) | 0.0049 | 1.31 | (0.97–1.77) | 0.0824 |
| Baseline comorbidity | | | | | | | |
| Alcohol-related disease (Yes vs. No) | 4 | 5.20 | (1.94–13.96) | 0.0011 | 4.38 | (1.6–12.02) | 0.0041 |
| Cancer (Yes vs. No) | 9 | 0.83 | (0.43–1.61) | 0.5758 | 0.70 | (0.36–1.37) | 0.2979 |
| Cardiovascular disease (Yes vs. No) | 139 | 1.38 | (1.1–1.71) | 0.0046 | 1.07 | (0.83–1.38) | 0.5856 |
| Chronic kidney disease (Yes vs. No) | 9 | 1.22 | (0.63–2.36) | 0.5608 | 1.05 | (0.53–2.07) | 0.8909 |
| Chronic obstructive pulmonary (Yes vs. No)disease | 92 | 1.25 | (0.98–1.59) | 0.0759 | 1.20 | (0.93–1.54) | 0.1688 |
| Diabetes mellitus (Yes vs. No) | 87 | 1.20 | (0.94–1.54) | 0.1442 | 1.11 | (0.85–1.44) | 0.4518 |
| Dementia (Yes vs. No) | 6 | 1.62 | (0.72–3.64) | 0.2432 | 1.00 | (0.43–2.29) | 0.9909 |
| Depression (Yes vs. No) | 25 | 1.41 | (0.94–2.12) | 0.0972 | 1.62 | (1.05–2.5) | 0.0285 |
| Hyperlipidemia (Yes vs. No) | 62 | 0.79 | (0.6–1.04) | 0.0922 | 0.73 | (0.54–0.98) | 0.0352 |
| Hypertension (Yes vs. No) | 186 | 1.28 | (1.03–1.59) | 0.0292 | 0.92 | (0.72–1.19) | 0.5362 |
| Parkinson's disease (Yes vs. No) | 5 | 1.25 | (0.52–3.02) | 0.6248 | 0.92 | (0.37–2.27) | 0.8498 |

Crude HR* represented relative hazard ratio; Adjusted HR† represented adjusted hazard ratio: mutually adjusted for TCM use, age, gender, urbanization level and baseline comorbidity in Cox proportional hazard regression

The HR of the 10-single herb and multiherb products most commonly prescribed for the treatment of osteoporosis are listed in Table 4. Frequency meant how many times the single herb or multiple herb formula was used during the research. Number of person-days meant how many days the single herb or multiple herb formula was used during the research.

## Discussion

With the increase in osteoporosis prevalence and incidence, prevention of osteoporotic fracture is of great importance [14]. People have become more interested in natural products in recent years and TCM is becoming a common choice in complementary and alternative medicine. There are some difficulties when surveying the preventive effect of TCM for osteoporotic fracture in operational studies. First, a long follow-up time is required for bone mass and fracture events. The longest reported follow-up time in Western medicine for osteoporosis was ten years [15]. Therefore, most studies focus on the benefit to bone health [16] rather than the fracture rate. Secondly, a common problem of studying TCM is that it is difficult to quantify Chinese herbs. Current Chinese herb studies focus on extracts or simple herbs [9, 17, 18], which greatly differ from those used in the clinical setting. For the above reasons, there are no studies on the fracture incidence following TCM therapy in patients with osteoporosis. Therefore, we could conduct this survey using NHIRD analysis.

Our results showed a decreased risk of fracture following the use of TCM therapy among osteoporosis patients (HR: 0.47, 95% CI: 0.37–0.59; $p < 0.0001$). The follow-up

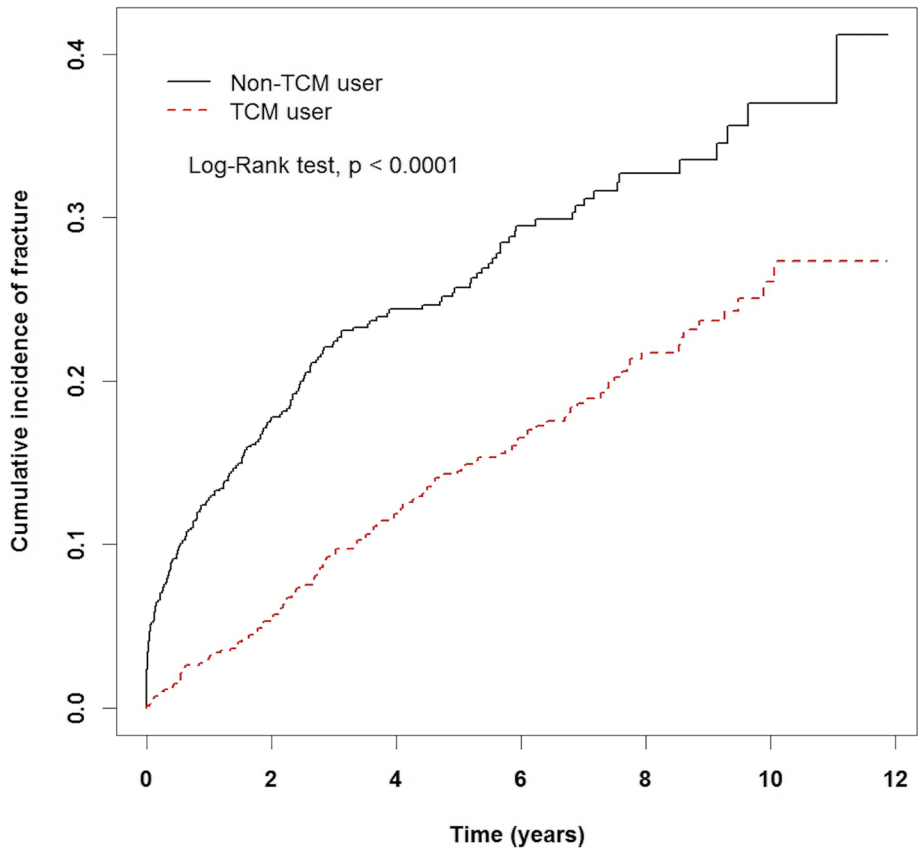


**Fig. 2** Kaplan–Meier curve of the difference between the TCM user and non-TCM user groups in the development of fracture

period was also longer in the TCM user group than in the non-TCM user group (5.38 and 3.75 years, respectively). It means TCM use might delayed the occurrence of fracture after osteoporosis was diagnosed. The Kaplan–Meier curve also demonstrated that patients who took TCM had a lower incidence of fracture. In addition, our study demonstrated that the longer the use of TCM, the lesser the fracture rate. Patients who took TCM between 30 to 180 days were at less risk than those who took TCM for less than 30 days. Similarly, patients who took TCM for more than 180 days were at less risk than those who took TCM between 30 to 180 days. This result strengthens the relationship between TCM and osteoporotic fracture.

We should consider the possibility of a decreased fracture rate after using TCM therapy. First, TCM may improve bone strength [16], while falls are also a prominent factor for which one tenth lead to fracture [19]. Some studies demonstrate that the most important cause of a fracture is a fall rather than bone strength [20–22]. TCM may improve the quality of life by reducing limb pain or strengthening muscle endurance [23–

**Table 3** Hazard Ratios and 95% confidence intervals of fracture risk associated with cumulative use day of traditional Chinese herb medicine among osteoporosis patients

| TCM used (days per year) | N | No. of Event | Hazard Ratio (95% CI) | | Hazard Ratio (95% CI) | |
|---|---|---|---|---|---|---|
| | | | Crude | Adjusted† | Crude | Adjusted† |
| Non-TCM users or Chinese herb users < 30 days per year | 1245 | 269 | 1(reference) | 1(reference) | – | – |
| Chinese herb users (≥ 30 days per year) ‡ | | | | | | |
| 30–180 days per year | 270 | 43 | 0.63 (0.46–0.87)** | 0.60 (0.43–0.84)** | 1(reference) | 1(reference) |
| 180 days per year | 93 | 11 | 0.42 (0.23–0.78)** | 0.37 (0.20–0.68)** | 0.65 (0.33–1.25) | 0.63 (0.32–1.24) |

Crude HR* represented relative hazard ratio; Adjusted HR† represented adjusted hazard ratio: mutually adjusted for age, gender, baseline comorbidity, and urbanization level in Cox proportional hazard regression
*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

**Table 4** Ten most common herbal formulas prescribed

| Herbal formula | Frequency | Number of person-days | Average daily dose (g) | Average duration for prescription (days) |
|---|---|---|---|---|
| Single Herb | | | | |
| Eucommiae Cortex (Du-Zhong) | 803 | 10,832 | 1.2 | 13.5 |
| Salviaemiltiorrhizae Radix (Dan-shen) | 592 | 7850 | 1.3 | 13.3 |
| Chaenomelis Fructus (Mu-gua) | 520 | 6651 | 1.0 | 12.8 |
| Achuranthes (Huai-niu-xi) | 432 | 5181 | 1.1 | 12.0 |
| Dipsaci Radix (Xu-Duan) | 393 | 4701 | 1.3 | 12.0 |
| Sepiae Endoconcha (Haipiaoxiao) | 337 | 4476 | 1.4 | 13.3 |
| Corydalis Rhizoma (Yan-hu-suo) | 346 | 4284 | 1.5 | 12.4 |
| Spatholobi Caulis | 272 | 3570 | 1.4 | 13.1 |
| Testudinis Plastrum (Gui-ban) | 279 | 3477 | 0.9 | 12.5 |
| Drynariae Rhizoma (Gu sui-bu) | 259 | 3282 | 1.3 | 12.7 |
| Multiple Herb Formula | | | | |
| Du Huo Ji Sheng Tang | 1109 | 13,795 | 5.7 | 12.4 |
| Gui Lu Er Xian Jiao | 433 | 8083 | 7.0 | 18.7 |
| Shu Jing Huo Xue Tang | 564 | 7427 | 4.6 | 13.2 |
| zuo Gui Wan | 516 | 5625 | 5.2 | 10.9 |
| ji Sheng Shen Qi Wan | 308 | 4478 | 5.1 | 14.5 |
| Zhi Bai Di Huang Yin | 322 | 4365 | 4.7 | 13.6 |
| Hu Qian Wan Without Hugu | 312 | 4211 | 7.2 | 13.5 |
| You Gui Wan | 302 | 3982 | 4.3 | 13.2 |
| Ma Zi Ren Wan | 219 | 3572 | 1.8 | 16.3 |
| Xiang Sha Liu Jun Zi Tang | 220 | 3423 | 4.2 | 15.6 |

25]. Some Chinese herbal clinical trials conducted in China showed that people who took Chinese herbs had a better quality of life or reduced symptoms including pain, muscle fatigue, and limited mobility. While these trials were included in a review study [26], they did not match the standard of peer-reviewed journals.

The frequency of major osteoporotic fractures varies in different races, especially in hip fractures, with rates varying by > 200-fold. White women have a higher fracture risk than black women. Furthermore, variation was also observed in different regions with northern Europe and Mediterranean areas experience the highest rates and the lowest rates, respectively [27]. Based on these differences, it is difficult to conclude the benefit of TCM when comparing ethnic groups.

The most commonly prescribed single herbs and formulas were presented in Table 4. While this is not the discussion point of our research, it may be important for clinicians and researchers. The results provide future research candidates for basic and clinical trials. Some herbs have been proven to be beneficial to bone health, such as Eucommiae Cortex (Du-Zhong) [28], Achuranthes (Huai-niu-xi) [29, 30], Salviae miltiorrhizae Radix [18], Dipsaci Radix (Xu-Duan) [31], Testudinis Plastrum (Gui-ban) [32–34], Drynariae Rhizoma (Gu sui-bu) [35–37], Du Huo Ji Sheng Tang [38], and zuo Gui Wan [39]. We found patients lived in highly urbanized areas were more likely to receive TCM treatment. In addition, some comorbidities showed significant difference between two groups in baseline. It might mean the patients with chronic obstructive pulmonary disease and hyperlipidemia preferred to receive TCM, and the patients with cancer were not disposed to use TCM. Besides, less cancer rate might also mean patients in TCM group take care themselves better than another group usually. Because of this, they go further to seek TCM treatment when osteoporosis is diagnosed.

There are some limitations to our research. Firstly, we were unable to include medicines taken at the patient's own expense. According to the specification of the NHI program, Western medicine for osteoporosis can only be applied after a fracture occurred. It is possible that the patients source such medicines at their own expense when they were diagnosed with osteoporosis before a fracture happens. Secondly, some data related to fractures, such as a patient's exercise, lifestyle, BMI, alcohol, and cigarette use is not available from the NHI program. Thirdly, the Kaplan–Meier curve might be influenced by economic

levels and patient severity. However, we can conclude that TCM might have a positive impact on the prevention of osteoporotic fracture from Tables 2 and 3. Moreover, this study is derived from a very large, well-indicated data set, which provided a practical method to investigate the effect of TCM in osteoporotic patients. Our study not only reveals the preventative value of TCM use for patients with osteoporosis in the clinical setting, but also provides valuable information regarding the most common prescriptions provided to osteoporotic patients.

## Conclusions

In conclusion, our study had a relatively large population and long follow-up time, which demonstrated that TCM might have a positive impact on the prevention of osteoporotic fracture. Further research is needed to verify the causal relationship between TCM and the outcomes. More clinical trials are also required to confirm whether this relationship is true in non-Asian patients.

### Abbreviations

HR: Hazard ratios; LHID2000: Longitudinal Health Insurance Database 2000; NHI: National Health Insurance; NHIRD: National Health Insurance Research Database; PTH: Parathyroid hormone; TCM: Traditional Chinese medicine

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### Availability of data and materials

Large computerized data (NHIRD; [40]) was used to perform our nationwide population-based cohort study. We used the LHID2000 (Longitudinal Health Insurance Database 2000) provided by the National Health Insurance Administration, which is managed by the National Health Research Institutes.

### Authors' contributions

All authors made substantive intellectual contributions to this study to qualify as authors. YCW and CHT designed the study. YCW and CHT collected subject data. JHC performed statistical analysis. An initial draft of the manuscript was written by YCW. JHC, HCH, and CHT re-drafted parts of the manuscript and provided helpful advice on the final revision. All authors were involved in writing the manuscript. All authors have read and approved the final manuscript.

### Ethics approval and consent to participate

Not applicable.

### Consent for publication

Not applicable.

### Competing interests

The authors declare that they have no competing interests.

## Publisher's Note



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### References

1. NCD P. Osteoporosis prevention, diagnosis, and therapy. JAMA. 2001;6:285–785.
2. (US) OotSG. Bone Health and Osteoporosis: A Report of the Surgeon General. In: Reports of the Surgeon General. edn. Edited by Rockville; 2004.
3. Hernlund E, Svedbom A, Ivergård M, Compston J, Cooper C, Stenmark J, McCloskey EV, Jönsson B, Kanis JA. K: Osteoporosis in the European Union: medical management, epidemiology and economic burden. A report prepared in collaboration with the International Osteoporosis Foundation (IOF) and the European Federation of Pharmaceutical Industry Associations (EFPIA). Arch Osteoporos. 2013;8(136).
4. Burge R, Dawson-Hughes B, Solomon DH, Wong JB, King A, Tosteson A. Incidence and economic burden of osteoporosis-related fractures in the United States, 2005–2025. J Bone Miner Res. 2007;22:465–75.
5. 2015 Taiwan consensus guidelines and prevention of osteoporosis in adults. https://www.puzih.mohw.gov.tw/public/hygiene/ebf03aeee1551307ea349553c0726840.pdf.
6. Kinsella K, He W: U.S. Census Bureau, International Population Reports, P95/09–1. An Aging World: 2008, US Government Printing Office, Washington, DC 2009.
7. Cooper C, Campion G, Melton LJ. Hip fractures in the elderly: a world-wide projection. Osteoporos Int. 1992;2:285–9.
8. Li TM, Huang HC, Su CM, Ho TY, Wu CM, Chen WC, Fong YC, Tang CH. Cistanche deserticola extract increases bone formation in osteoblasts. J Pharm Pharmacol. 2012;64(6):897–907.
9. Guo AJ, Choi RC, Cheung AW, Chen VP, Xu SL, Dong TT, Chen JJ, Tsim KW. Baicalin, a flavone, induces the differentiation of cultured osteoblasts: an action via the Wnt/beta-catenin signaling pathway. J Biol Chem. 2011;286(32):27882–93.
10. GGX ML, Rong P, Dong J, Zhang Z, Zhao H, Teng J, Zhao H, Pan J, Li Y, Zha Q, Zhang Y, Ju D. Semen astragali complanati- and rhizoma cibotii-enhanced bone formation in osteoporosis rats. BMC Complement Altern Med. 2013;13(141).
11. TP H, PH L, AS L, SL Y, HH C, Yen HR. Y: A nationwide population-based study of traditional Chinese medicine usage in children in Taiwan. Complement Ther Med. 2014;22(3):500–10.
12. 2011 National Health Insurance Annual Statistical Report [http://www.mohw.gov.tw/en/Ministry/Statistic.aspx?f_list_no=474&fod_list_no=5236]. Accessed 25 Jan 2019
13. Liu CYHY, Chuang YL, Chen YJ, Weng WS, Liu JS, Liang KY. Incorporating development stratification of Taiwan townships into sampling design of large scale health interview survey. J Health Manag. 2006;14:1–22.
14. International Osteoporosis Foundation. https://www.iofbonehealth.org/impact-osteoporosis. Accessed 25 Jan 2019
15. Black DM, AVS KEE, Cauley JA, Levis S, Quandt SA, Satterfield S, Wallace RB, Bauer DC, Palermo L, Wehren LE, Lombardi A, Santora AC, Cummings SR. Effects of continuing or stopping alendronate after 5 years of treatment. JAMA. 2006;296(24).
16. Che CT, Wong MS, Lam CW. Natural products from Chinese medicines with potential benefits to bone health. Molecules. 2016;21(3):239.
17. Feng XLY, Wu Z, Fang Y, Xu H, Zhao P, Xu Y, Feng H. Fructus ligustri lucidi ethanol extract improves bone mineral density and properties through modulating calcium absorption-related gene expression in kidney and duodenum of growing rats. Calcif Tissue Int. 2014;94(4):433–41.

18. Guo YLY, Xue L, Severino RP, Gao S, Niu J, Qin LP, Zhang D, Bromme D. Salvia miltiorrhiza: an ancient Chinese herbal medicine as a source for anti-osteoporotic drugs. J Ethnopharmacol. 2014;155(3):1401–16.
19. Berry SD, Miller RR. Falls: epidemiology, pathophysiology, and relationship to fracture. Curr Osteoporos Rep. 2008;6(4):149–54.
20. Kannus PU-RK, Palvanen M, Parkkari J. Non-pharmacological means to prevent fractures among older adults. Ann Med. 2005;37:302–10.
21. Svenhjalmar van Helden ACMvG, Piet P. Geusens, Alfons Kessels, Arie C. Nieuwenhuijzen Kruseman, Peter R.G. Brink: Bone and fall-related fracture risks in women and men with a recent clinical fracture. J Bone Joint Surg Am 2008, 90(241–248).
22. Jä r TLSH, Khan KM, Heinonen A, Kannus P. Shifting the focus in fracture prevention from osteoporosis to falls. BMJ. 2008;336:124–6.
23. Chen BZH, Marszalek J, Chung M, Lin X, Zhang M, Pang J, Wang C. Traditional Chinese medications for knee osteoarthritis pain: a meta-analysis of randomized controlled trials. Am J Chin Med. 2016;44(4):677–703.
24. Chen BZH, Chung M, Lin X, Zhang M, Pang J, Wang C. Chinese herbal Bath therapy for the treatment of knee osteoarthritis: meta-analysis of randomized controlled trials. Evid Based Complement Alternat Med. 2015;2015.
25. Yeh TSHC, Chuang HL, Hsu MC. Angelica sinensis improves exercise performance and protects against physical fatigue in trained mice. Molecules. 2014;19(4):3926–39.
26. Liu Y, Liu JP, Xia Y. Chinese herbal medicines for treating osteoporosis. Cochrane Database Syst Rev. 2014;3:CD005467.
27. Johnell OGB, Allander E, Kanis JA. The apparent incidence of hip fracture in Europe: a study of national register sources. Osteoporos Int. 1992;2:298–302.
28. Qi S, Zheng H, Chen C, Jiang H. Du-Zhong (Eucommia ulmoides Oliv.) cortex extract alleviates Lead acetate-induced bone loss in rats. Biol Trace Elem Res. 2018.
29. Zhang SZQ, Zhang D, Wang C, Yan C. Anti-osteoporosis activity of a novel Achyranthes bidentata polysaccharide via stimulating bone formation. Carbohydr Polym. 2018;184:288–98.
30. D S, Z C, S H, J T, N L, H Q, X C, C W, K C, Y S, et al. Achyranthes bidentata polysaccharide suppresses osteoclastogenesis and bone resorption via inhibiting RANKL signaling. J Cell Biochem. 2018;119(6):4826–35.
31. K K, Q L, X Y, Z X, Y W, J S, L C, W X, L H, H S: Asperosaponin VI promotes bone marrow stromal cell osteogenic differentiation through the PI3K/AKT signaling pathway in an osteoporosis model**.** Sci Rep 2016, 19(6):35233.
32. GY S, H R, JJ H, ZD Z, WH Z, X Y, Q S, T Q, YZ Z, JJ T, et al. Plastrum Testudinis extracts promote BMSC proliferation and osteogenic differentiation by regulating let-7f-5p and the TNFR2/PI3K/AKT signaling pathway. Cell Physiol Biochem. 2018;47(6):2307–18.
33. H R, G S, J T, T Q, Z Z, W Z, X Y, J H, Liang ZY, et al. Promotion effect of extracts from plastrum testudinis on alendronate against glucocorticoid-induced osteoporosis in rat spine. Sci Rep. 2017;7(1):10617.
34. D L, H R, T Q, G S, B X, Q W, Z Y, J T, Z Z, X J: Extracts from plastrum testudinis reverse glucocorticoid-induced spinal. Biomed Pharmacother 2016, 82:151–160.
35. S S, Z G, X L, Y N, Y Z, C L, Y L, Z W. Total flavonoids of Drynariae Rhizoma prevent bone loss induced by Hindlimb unloading in rats. Molecules. 2017; 22(7).
36. SH S, YK Z, CQ L, Q Y, Y L, Y Z, WP H, ZZ W. Effects of total flavonoids from Drynariae Rhizoma prevent bone loss in vivo and in vitro. Bone Rep. 2016; 10(5):262–73.
37. ZC Q, XL D, Y D GKX, XL W, KC W, MS W, XS Y. Discovery of a new class of Cathepsin K inhibitors in Rhizoma Drynariae as potential candidates for the treatment of osteoporosis. Int J Mol Sci. 2016;17(12).
38. JY W, WM C, CS W, SC H, PW C, JH C. Du-Huo-Ji-sheng-Tang and its active component Ligusticum chuanxiong promote osteogenic differentiation and decrease the aging process of human mesenchymal stem cells. J Ethnopharmacol. 2017;198:64–72.
39. N L, Z Z, B W, X M, Y G, C Y, Z W, L W, R M, X L, et al. Regulatory effect of traditional Chinese medicinal formula Zuo-Gui-wan on the. J Ethnopharmacol. 2015;166:228–39.
40. NHIRD [ http://nhird.nhri.org.tw/en/]. Accessed 25 Jan 2019

# Effect of serum from postmenopausal women with osteoporosis exhibiting the Kidney-Yang deficiency pattern on bone formation in an hFOB 1.19 human osteoblastic cell line

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**Abstract.** The aim of the present study was to investigate the underlying mechanism of the Kidney-Yang deficiency (KYD) pattern of osteoporosis in postmenopausal women of a certain age range by comparing the effect of serum from postmenopausal women with osteoporosis exhibiting the KYD pattern with that of serum from postmenopausal women without osteoporosis on bone formation in an hFOB 1.19 human osteoblastic cell line. A random selection of 30 female, postmenopausal volunteers aged 60-70 years, including 15 cases without osteoporosis and 15 cases with the KYD pattern of osteoporosis, were enrolled at the Physical Examination Center of the Second Affiliated Hospital of Fujian University of Traditional Chinese Medicine. Venous blood was extracted and the serum was separated. The hFOB 1.19 cells were treated with 10% KYD pattern-serum or control serum from postmenopausal women of the same age range without osteoporosis. It was found that the KYD pattern-serum significantly decreased the cell viability, activity of alkaline phosphatase and number of calcified nodules, as well as downregulated the expression of osteocalcin and osteoprotegerin (OPG) and upregulated that of receptor activator of nuclear factor κB ligand (RANKL) in the hFOB 1.19 cells. In addition, the present results showed that the concentrations of estradiol ($E_2$), OPG and insulin-like factor-1 (IGF-1) in the KYD pattern-serum were lower than those in the control serum. In combination, these findings suggest that the downregulation of $E_2$, OPG and IGF-1 in the KYD pattern-serum inhibits the OPG/RANKL system, leading to a decrease in bone formation in the hFOB 1.19 cells. This indicates that the alterations in $E_2$, OPG and IGF-1 may account for the susceptibility of certain postmenopausal women to the KYD pattern of osteoporosis.

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*Key words:* bone formation, osteoblast, postmenopausal osteoporosis, Kidney-Yang deficiency pattern

## Introduction

Postmenopausal osteoporosis (PMO), a common disease characterized by bone reduction and microarchitectural deterioration of the bone, has a serious effect on the quality of life of the patients, particularly the elderly (1,2). PMO is considered to be the result of an imbalance between bone resorption and formation, which are regulated by osteoclasts and osteoblasts, respectively. This imbalance leads to increased bone fragility and susceptibility to fractures (3-5). The mechanism underlying the pathogenesis of PMO is multifactorial and complicated. Gonadal steroids play an important role in bone remodeling and skeletal structure maintenance (6-8).

According to Traditional Chinese Medicine (TCM) theory, PMO can be classified into different TCM patterns (Zheng), including the Kidney-Yang deficiency (KYD), Kidney-Yin deficiency and Kidney-Yin and Yang deficiency patterns (9-11). Zheng, the body's overall response to different factors in the evolution of a disease, is intrinsically linked to a group of signs and symptoms at a certain stage of the disease (12,13). Zheng is based on factors including pulse feeling and tongue appearance and can be used as a guideline in TCM disease classification; however, it is not simply a collection of disease symptoms but rather can be defined as the TCM theoretical abstraction of the symptom profiles of patients (14-16). The KYD pattern (Shen-Yang-Xu Zheng) is an important syndrome of PMO (17); while some postmenopausal women are prone to forming the KYD pattern of osteoporosis, others of the same age group exhibit no development of osteoporosis. The underlying mechanism of this phenomenon remains to be elucidated. We hypothesized that the serum taken from patients with the KYD pattern of osteoporosis contains bioactive molecules in the metabolic products of the disease. The collection of this

serum is easy, and the serum can be used to objectively imitate the interaction between the serum and cells, thus generating an effective approach for the mechanistic study of the disease. In the present study, the susceptibility of certain postmenopausal women of the same age group to the KYD pattern of osteoporosis, as well as the associated underlying mechanism, was investigated.

## Materials and methods

*Ethics statement*. Ethical approval for the present study was obtained from the Clinical Trial Ethics Committee of the Second Affiliated Hospital of Fujian University of TCM (Fuzhou, China), and written informed consent was obtained from all participants prior to the experiment.

*Participants*. A random selection of 30 postmenopausal female volunteers aged 60-70 years, including 15 women with and 15 without osteoporosis, was enrolled in the study from the Physical Examination Center of the Second Affiliated Hospital of Fujian University of TCM. The diagnosis of PMO was defined by a bone mineral density (BMD) T-score of ≥2.5 standard deviations below the young normal gender-matched BMD of the reference database, in accordance with the World Health Organization criteria (18). Participants receiving any medications known to affect the calcium or bone metabolism, such as current use or history of a ≥3-month use of exogenous estrogens, thiazine or corticosteroids, were excluded from the study. Participants with any other disorder known to affect bone metabolism were also excluded.

The TCM diagnosis of the participants was based on the information obtained from four diagnostic processes, including looking, smelling, asking and touching. The diagnostic criteria of the KYD pattern included a sensation of cold and aching in the loins and knees, cold limbs and body, sexual hypoesthesia, infertility due to cold in the uterus, dispiritedness and lassitude, early morning diarrhea or frequent micturition, clear and profuse urine, profuse nocturnal urine, loose stools, bright whitish or blackish complexion and a light-colored tongue with white fur, as well as a deep and weak pulse (19).

*Serum preparation*. Venous blood was collected in the morning between 8:00 and 9:00 a.m. and centrifuged for 10 min at 1,200 x g within 30 min, and the serum was separated and stored at -80˚C.

*Cell culture*. An hFOB 1.19 human osteoblastic cell line from the Institute of Biochemistry and Cell Biology (Chinese Academy of Sciences, Shanghai, China) was cultured in Dulbecco's modified Eagle's medium (Gibco-BRL, Grand Island, NY, USA), supplemented with 10% (v/v) fetal bovine serum (FBS) (Gibco-BRL), penicillin (100 U/ml) and streptomycin (100 $\mu$g/ml) at 37˚C in humidified incubator with 5% $CO_2$. When the cells reached 80% confluence, they were harvested with 0.25% trypsin-EDTA solution and then seeded in 96- and 12-well plates at a density of $6x10^3$ and $1x10^5$ cells/well, respectively, in a medium of 10% FBS. Twenty-four hours after stabilization, the cells were washed in phosphate-buffered saline solution twice and treated with the KYD pattern-serum or control serum from postmenopausal women without osteoporosis.

*Analysis of cell viability using MTT assay*. The cells were treated with 10% KYD pattern-serum for different periods of time. The medium was discarded and replaced with 10 $\mu$l MTT (Sigma-Aldrich, St. Louis, MO, USA) at 37˚C for 4 h and then 100 $\mu$l dimethylsulfoxide was added. The absorbance at 490 nm was measured on an ELISA reader (Model EXL800; BioTek Instruments, Inc., Winooski, VT, USA).

*Alkaline phosphatase (ALP) activity assay*. Following treatment with the KYD pattern-serum for 72 h, the cells were lysed with 0.05% Triton X-100 (Amresco, Inc., Solon, USA). The activity of ALP was determined by the conversion of *p*-nitrophenyl phosphate to *p*-nitrophenol using a commercial kit (Nanjing Jiancheng Biological Technology Co., Ltd., Nanjing, China). The total protein concentration was evaluated with a bicinchoninic acid protein assay kit (Bio-Rad, Hercules, CA, USA). An equal quantity of protein was mixed with 100 $\mu$l substrate at 37˚C for 15 min and 80 $\mu$l reaction-stop solution was added. The results were determined at 405 nm. The absorbance was normalized based on the protein content.

*Alizarin red S staining for mineralization*. Calcified nodules of the hFOB 1.19 cells treated with 10% KYD pattern-serum were demonstrated by Alizarin red S staining. The cells were seeded into 48-well plates at a density of $2x10^5$ cells per well. The cells were subsequently treated with 10% KYD pattern-serum for 14 days and then fixed with 0.5 ml/well formalin:methanol:$H_2O$ (1:1:1.5) for 30 min at room temperature. The cells were stained with 0.1% Alizarin red S (Sigma-Aldrich) at 37˚C for 30 min and images of the stained calcified nodules were captured using microscopy.

*RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis*. Total RNA from the cells was isolated using TRIzol® reagent (Invitrogen Life Technologies, Carlsbad, CA, USA). RT was performed using random primers and the SuperScript™ III First-Strand Synthesis system (Invitrogen Life Technologies). qPCR was conducted in an ABI Prism 7700 Sequence Detection System using the SYBR® Green PCR Master Mix (Invitrogen Life Technologies). The sequences of the PCR primers for the amplification of the ALP, osteocalcin, osteoprotegerin (OPG), receptor activator of nuclear factor κB ligand (RANKL) and GAPDH transcripts were as follows: ALP forward, 5'-AGC CCTTCACTGCCATCCTGT-3' and reverse, 5'-ATTCTCTCG TTCACCGCCCAC-3', 68 bp; osteocalcin forward, 5'-CAA AGGTGCAGCCTTTGTGTC-3' and reverse, 5'-TCACAG TCCGGATTGAGCTCA-3', 150 bp; OPG forward, 5'-AGT ACGTCAAGCAGGAGTGCAAT-3' and reverse, 5'-CCAGCT TGCACCACTCCAA-3', 129 bp; RANKL forward, 5'-AGA GCGCAGATGGATCCTAA-3' and reverse, 5'-TTCCTTTTG CACAGCTCCTT-3', 180 bp; GAPDH forward, 5'-CAACTA CATGGTTTACATGTTC-3' and reverse, 5'-GCCAGTGGA CTCCACGAC-3', 163 bp. The amplification protocol was as follows: Denaturation at 95˚C for 10 min and 40 cycles of 95˚C for 20 sec, 57˚C for 10 sec, and 72˚C for 30 sec. The amplification and melting curve data were collected. Fold-changes of the genes expression were estimated according to the comparative $2^{-\Delta\Delta Ct}$ method.

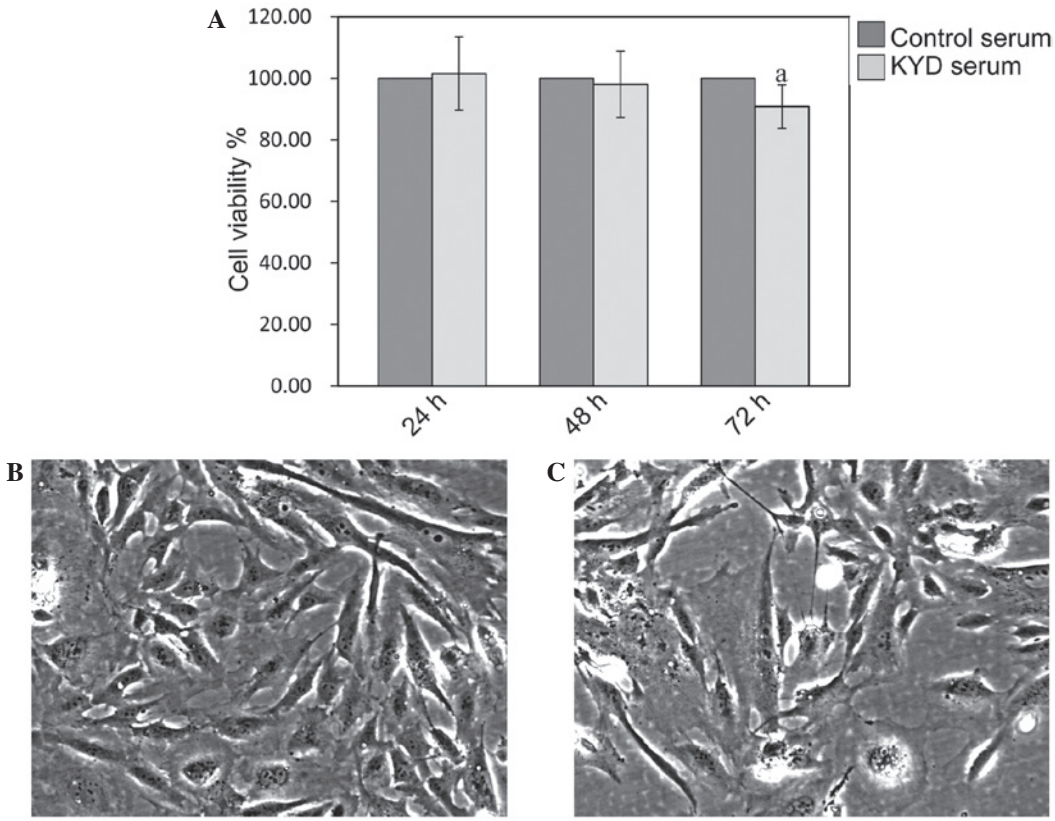


Figure 1. KYD pattern-serum inhibits cell viability. (A) The viability of hFOB 1.19 cells was determined via MTT assay, and a statistically significant difference was observed between the cells treated with the KYD pattern-serum and those treated with control serum for 72 h (magnification, x200). Data are presented as the mean ± standard deviation (error bars) from at least three independent experiments. [a]P<0.05, significant vs. control serum. (B and C) Morphology of hFOB 1.19 cells treated with the (B) KYD pattern-serum and (C) control serum for 72 h. KYD, Kidney-Yang deficiency.

*Western blot analysis*. Total cellular protein was extracted from the cells using radioimmunoprecipitation assay buffer (Beyotime Biotechnology Co., Ltd., Shanghai, China), and the total protein concentration was determined using a Bio-Rad protein assay. Equal quantities of protein were separated using SDS-PAGE and transferred onto polyvinylidene fluoride membranes (Invitrogen Life Technologies). The blots were blocked with 5% skimmed milk powder (Sigma-Aldrich) for 2 h at room temperature and were incubated with rabbit polyclonal antibodies against osteocalcin (1:800; sc-30044), OPG (1:1,000; sc-11383), RANKL (1:800; sc-9073) and β-actin (1:1,000; sc-130657) antibodies (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) overnight at 4˚C followed by a goat anti-rabbit horseradish peroxidase (HRP)-conjugated secondary antibody IgG (1:10,000; ZB-2301; Zhongshan Golden Bridge Biotechnology Co., Ltd., Beijing, China) at room temperature for 1 h. The immunoreactive proteins were visualized using Western Blot Chemiluminescence Luminol Reagent (Santa Cruz Biotechnology, Inc.). Immunoblot bands were quantified using the Tocan 190 protein assay system (Bio-Rad). β-actin was used as the loading control.

*ELISA*. The serum concentration of estradiol ($E_2$), OPG, and insulin-like growth factor 1 (IGF-1) was assessed using ELISA (Shanghai Jinma Biological Technology Co., Ltd, Shanghai, China). All commercial assays were performed according to the manufacturer's instructions. Briefly, ELISA plates were percolated with mouse anti-human immunoglobulin G, and standards, and samples were loaded into the wells and incubated for 1 h at room temperature. HRP-conjugated anti-human $E_2$, OPG and IGF-1 detection antibodies were added and incubated at room temperature for 1 h. The reaction was visualized through color development and the absorbance (optical density) was measured at a 450-nm wavelength on an ELISA reader (Model EXL800; BioTek Instruments, Inc.). The conversion of optical density units for the study samples to concentration was achieved through a computer software-mediated comparison with a standard curve using the KC Junior (BioTek Instruments, Inc.).

*Statistical analysis*. Data were analyzed using the SPSS 19.0 software for Windows (IBM SPSS, Armonk, NY, USA). The quantitative data are expressed as the mean ± standard deviation. The statistical analysis of the data was performed using nonparametric tests for two independent samples. P<0.05 was considered to indicate a statistically significant difference.

## Results

*KYD pattern-serum inhibits cell viability of the hFOB 1.19 cells*. As shown in Fig. 1A, the viability of the hFOB 1.19 cells was not affected by treatment with the KYD pattern-serum at 24 and 48 h (P>0.05), but was significantly decreased at 72 h (P=0.025), compared with the viability of cells treated with control serum. Cells treated with the KYD pattern-serum decreased in number following treatment and underwent

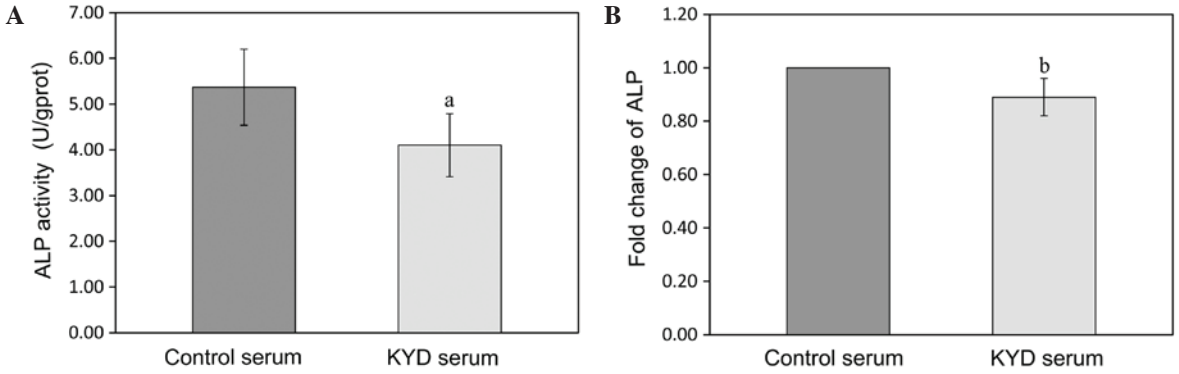


Figure 2. KYD pattern-serum decreases ALP activity and mRNA expression in the hFOB 1.19 cells. The hFOB 1.19 cells were treated with the KYD pattern-serum for 72 h, and the (A) ALP activity and (B) mRNA expression were measured. Data are presented as the mean ± standard deviation (error bars) from at least three independent experiments. [a]P<0.05 and [b]P<0.01 vs. control serum. ALP, alkaline phosphatase; KYD, Kidney-Yang deficiency.

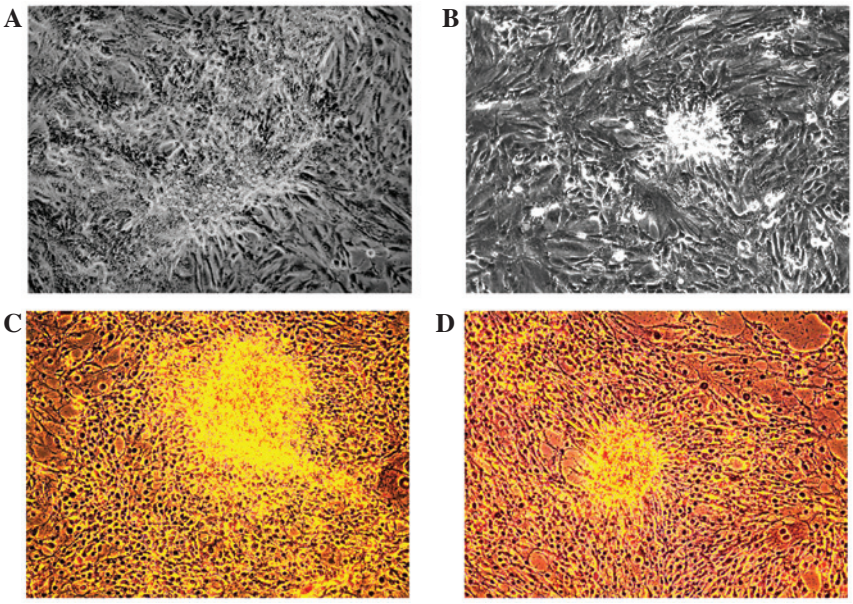


Figure 3. Effect of the KYD pattern-serum on calcified nodules in hFOB 1.19 cells (magnification, x100). (A and B) Cultivation of cells on day 14; the hFOB 1.19 cells were treated with the (A) KYD pattern-serum or (B) control serum for 14 days. (C and D) Alizarin red S staining for calcified nodules: (C) KYD pattern-serum-treated cells and (D) control serum-treated cells. KYD, Kidney-Yang deficiency.

morphological changes (Fig. 1B and C), including cell size and shape, indicating that the KYD pattern-serum inhibited the osteoblast viability significantly, contributing to the progression of bone loss in PMO.

*KYD pattern-serum decreases ALP activity and mRNA expression in the hFOB 1.19 cells*. The activity of ALP was downregulated in the hFOB 1.19 cells treated with the KYD pattern-serum, compared with that in the cells treated with control serum (P=0.037) (Fig. 2A). qPCR also showed that the mRNA expression of ALP was clearly decreased following treatment with the KYD pattern-serum compared with that following treatment with control serum (P=0.008) (Fig. 2B). The calcified nodules appeared bright red in color following Alizarin red S staining (Fig. 3A-D). The KYD pattern-serum could significantly inhibit the formation of calcified nodules compared with the control serum, which suggests that the KYD pattern-serum reduced bone formation.

*KYD pattern-serum downregulates the expression of osteocalcin and OPG and upregulates the expression of RANKL in the hFOB 1.19 cells*. In order to further explore the mechanism of the KYD pattern in bone formation, the mRNA and protein expression of osteocalcin, OPG and RANKL was analyzed following KYD pattern-serum treatment using RT-qPCR and western blotting, respectively. The protein levels of osteocalcin and OPG in the hFOB 1.19 cells treated with the KYD pattern-serum were downregulated (P=0.047 and P=0.009), and the protein level of RANKL was upregulated (P=0.006), compared with the protein levels following treatment with control serum (Fig. 4A-D). The changes in the mRNA expression of osteocalcin, OPG and RANKL following treatment with the KYD pattern-serum were similar to the changes in the protein levels (Fig. 4E-G) (P=0.002, P<0.001 and P=0.004 versus control, respectively), which suggested that the KYD pattern-serum regulated the bone metabolism via the OPG/RANKL system.

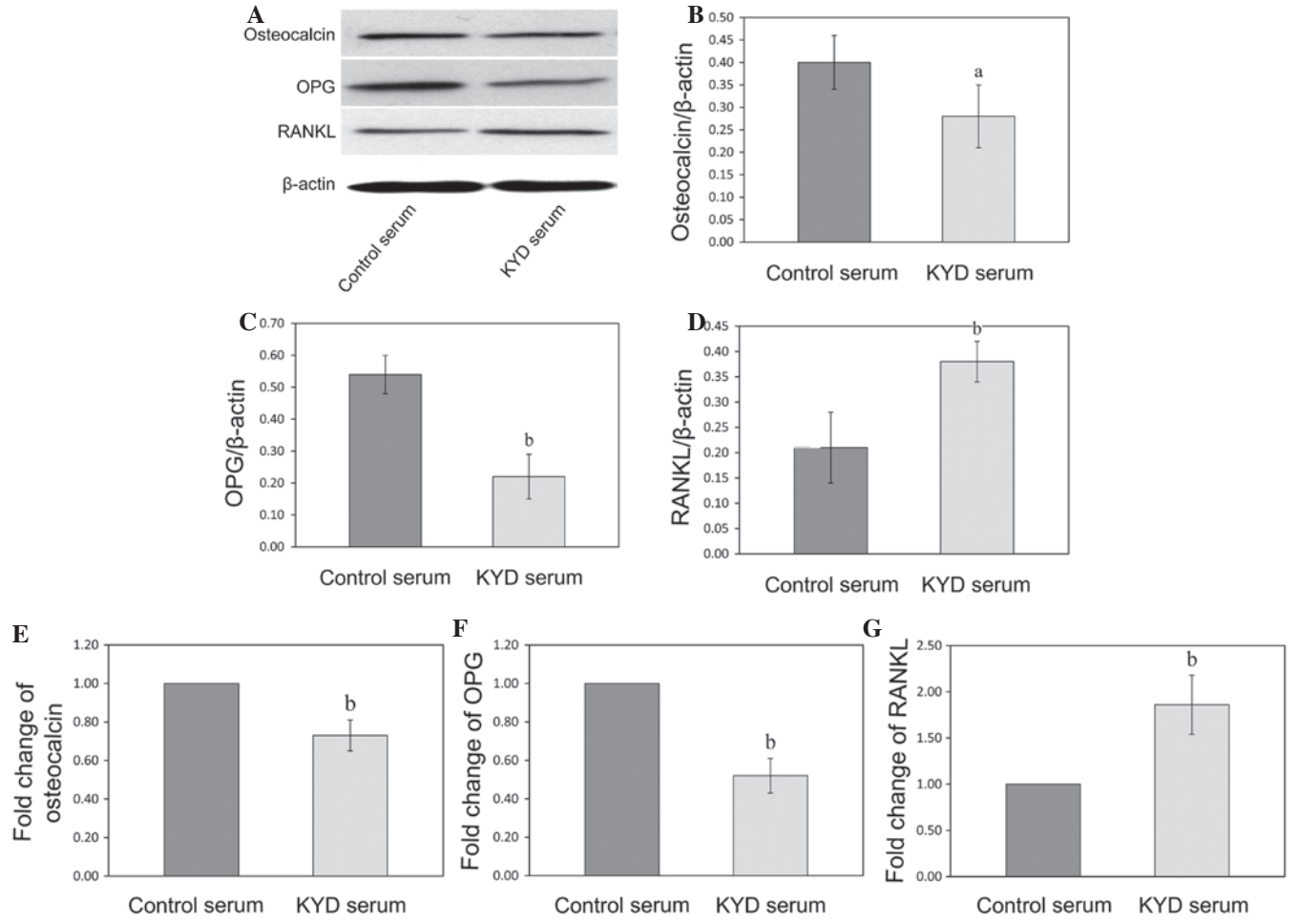


Figure 4. Effect of KYD pattern-serum on the expression of osteocalcin, OPG and RANKL. Total protein was isolated from the hFOB 1.19 cells treated with the KYD pattern-serum for 72 h, and (A) western blotting was performed in order to determine the protein levels of (B) osteocalcin, (C) OPG and (D) RANKL, which were normalized to the levels of β-actin. Total RNA was isolated and the quantitative polymerase chain reaction was performed to determine the mRNA expression of (E) osteocalcin, (F) OPG and (G) RANKL, which was normalized to that of GAPDH. Data are presented as the mean ± standard deviation (error bars) from at least three independent experiments. [a]P<0.05 and [b]P<0.01 vs. control serum. KYD, Kidney-Yang deficiency; OPG, osteoprotegerin; RANKL, receptor activator of nuclear factor κB ligand.

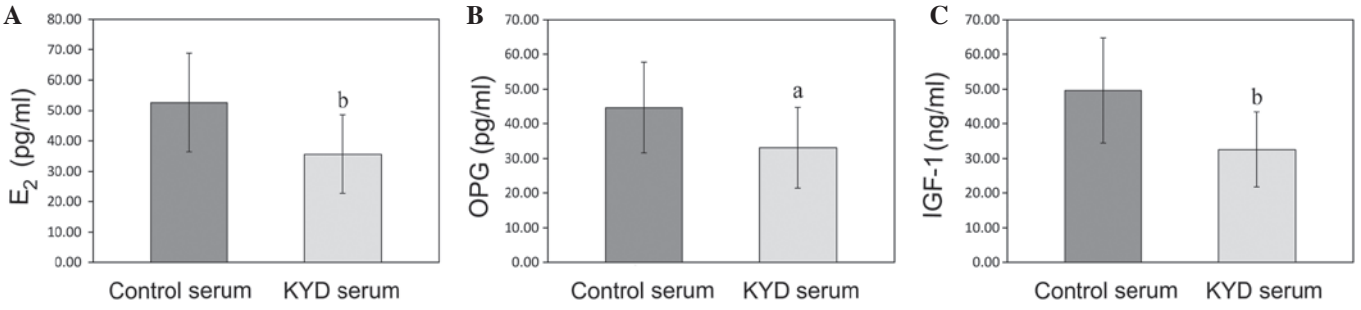


Figure 5. Concentrations of $E_2$, OPG and IGF-1 in the KYD pattern-serum and control serum. (A-C) The concentrations of (A) $E_2$, (B) OPG and (C) IGF-1 in the KYD pattern- and control serums were assessed by ELISA. Data are presented as the mean ± standard deviation (error bars). [a]P<0.05 and [b]P<0.01 vs. control serum. $E_2$, estradiol; OPG, osteoprotegerin; IGF-1, insulin-like growth factor-1; KYD, Kidney-Yang deficiency.

*Downregulation of $E_2$, OPG and IGF-1 in the KYD pattern-serum leads to an inhibition of bone formation in the hFOB 1.19 cells*. In order to obtain some insight into the underlying mechanism of the inhibition of bone formation by the KYD pattern-serum, the concentrations of $E_2$, OPG and IGF-1 in the KYD pattern- and control serums were analyzed. As shown in Fig. 5A-C, the concentrations of $E_2$, OPG and IGF-1 in the KYD pattern-serum were lower than those in the control serum (P=0.003, P=0.012 and P=0.001, respectively), indicating that the alteration in the serum levels of $E_2$, OPG and IGF-1 may be responsible for the formation of the KYD pattern in postmenopausal women.

## Discussion

According to TCM theory, the kidney regulates bone formation and development. Kidney deficiency leading to bone loss is associated with the pathological process of PMO (17,20,21). Among all kidney deficiency patterns, the KYD pattern is a common clinical type of PMO; however, the precise mechanism behind its formation remains unclear. The present results revealed that alterations in $E_2$, OPG and IGF-1 may account for the susceptibility of certain postmenopausal women to the KYD pattern of osteoporosis.

Using the MTT assay, it was shown that the KYD pattern-serum significantly inhibited the viability of the hFOB 1.19 cells, suggesting that it also inhibited the proliferation of these cells. The possibility of the KYD pattern-serum controlling the mineralization of osteoblasts was explored by measuring the ALP activity, osteocalcin expression and formation of calcified nodules in the hFOB 1.19 cells. ALP, a classic biomarker of osteoblast cell differentiation, plays a crucial role in the early stage of extracellular matrix mineralization (22,23). When cultured in appropriate osteogenic media, osteoblastic cells form a calcified extracellular compartment and express osteocalcin; thus calcified nodules are indicative of osteoblast differentiation and mineralization (24,25). In the present study, it was found that the KYD pattern-serum significantly decreased the ALP activity and formation of calcified nodules and downregulated the expression of osteocalcin. It has been reported that, in PMO patients, the altered bone microarchitecture and low BMD result in an increased risk of bone fractures due to decreased proliferation and mineralization of osteoblasts (26,27), and the results of the present study were in accordance with this conclusion.

Previous studies showing that OPG mediates bone formation and RANKL mediates bone resorption have enhanced the understanding of bone remodeling regulation (28-30). A number of studies have suggested that the binding of RANKL to RANK results in the activation of signaling pathways, which control the function of osteoclasts; however, OPG protects the bones from excessive resorption by inhibiting the binding of RANKL to RANK (31-33). In order to investigate the effects of the KYD pattern-serum on the OPG/RANKL system in the hFOB 1.19 cells, the expression of OPG and RANKL was examined. The present results showed that the KYD pattern-serum could reduce bone formation through the downregulation of OPG and upregulation of RANKL.

The risk of PMO develops increasingly with estrogen deficiency, which causes a series of changes in the blood and interrupts the balance between bone formation and resorption (34). The suppression of $E_2$, OPG and IGF-1 production is closely associated with an increase in bone turnover and an accelerated bone loss, as shown by a decrease in the BMD (35,36). IGF-1, a growth-promoting polypeptide that is essential for normal growth and development directly regulates bone growth and density; therefore, the possibility that the changes in the serum levels of $E_2$, OPG and IGF-1 could account for the formation of the KYD pattern was explored in the present study by measuring the concentrations of $E_2$, OPG and IGF-1 in the KYD pattern-serum and control serum. The findings showed that the concentrations of $E_2$, OPG and IGF-1 were downregulated in the KYD pattern-serum, compared with those in the control serum. Although it is clear that the alterations in the $E_2$, OPG and IGF-1 serum levels affect bone formation, the other proteins in the serum may also play a crucial role in bone remodeling and therefore warrant future investigation.

In conclusion, the present study has provided data showing that the alterations in the concentrations of $E_2$, OPG and IGF-1 may account for the susceptibility of certain postmenopausal women to the KYD pattern of osteoporosis by inhibiting the OPG/RANKL system, which leads to a reduction in bone formation. The major limitation of this study was the small sample size, and thus a randomized, controlled trial with a larger sample size needs to be conducted. Furthermore, the fact that the KYD pattern-serum was the only pattern of kidney deficiency investigated, with regard to its effects on the function of osteoblasts, could be considered one-sided; therefore experiments on the other patterns will be carried out in the future.

## Acknowledgements

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## References

1. Reid IR: Should we prescribe calcium supplements for osteoporosis prevention? J Bone Metab 21: 21-28, 2014.
2. Lagari VS and Levis S: Phytoestrogens in the prevention of postmenopausal bone loss. J Clin Densitom 16: 445-449, 2013.
3. Liang W, Lin M, Li X, Li C, Gao B, Gan H, Yang Z, Lin X, Liao L and Yang M: Icariin promotes bone formation via the BMP-2/Smad4 signal transduction pathway in the hFOB 1.19 human osteoblastic cell line. Int J Mol Med 30: 889-895, 2012.
4. Hayden RS, Quinn KP, Alonzo CA, Georgakoudi I and Kaplan DL: Quantitative characterization of mineralized silk film remodeling during long-term osteoblast-osteoclast co-culture. Biomaterials 35: 3794-3802, 2014.
5. Huang S, Eleniste PP, Wayakanon K, Mandela P, Eipper BA, Mains RE, Allen MR and Bruzzaniti A: The Rho-GEF Kalirin regulates bone mass and the function of osteoblasts and osteoclasts. Bone 60: 235-245, 2014.
6. Zhao R: Immune regulation of bone loss by Th17 cells in oestrogen-deficient osteoporosis. Eur J Clin Invest 43: 1195-1202, 2013.
7. Frenkel B, Hong A, Baniwal SK, Coetzee GA, Ohlsson C, Khalid O and Gabet Y: Regulation of adult bone turnover by sex steroids. J Cell Physiol 224: 305-310, 2010.
8. Baek KH, Oh KW, Lee WY, *et al*: Changes in the serum sex steroids, IL-7 and RANKL-OPG system after bone marrow transplantation: Influences on bone and mineral metabolism. Bone 39: 1352-1360, 2006.
9. Cheng M, Wang Q, Fan Y, Liu X, Wang L, Xie R, Ho CC and Sun W: A traditional Chinese herbal preparation, Er-Zhi-Wan, prevent ovariectomy-induced osteoporosis in rats. J Ethnopharmacol 138: 279-285, 2011.
10. Xu H and Lawson D: Theories and practice in prevention and treatment principles in relation to Chinese herbal medicine and bone loss. J Tradit Chin Med 24: 88-92, 2004.
11. Damiani G: The Yin and Yang of anti-Darwinian epigenetics and Darwinian genetics. Riv Biol 100: 361-402, 2007.
12. Wang P and Chen Z: Traditional Chinese medicine ZHENG and Omics convergence: A systems approach to post-genomics medicine in a global world. OMICS 17: 451-459, 2013.
13. Jiang M, Lu C, Zhang C, Yang J, Tan Y, Lu A and Chan K: Syndrome differentiation in modern research of traditional Chinese medicine. J Ethnopharmacol 140: 634-642, 2012.
14. Lu A, Jiang M, Zhang C and Chan K: An integrative approach of linking traditional Chinese medicine pattern classification and biomedicine diagnosis. J Ethnopharmacol 141: 549-556, 2012.

15. Li S, Zhang ZQ, Wu LJ, Zhang XG, Li YD and Wang YY: Understanding ZHENG in traditional Chinese medicine in the context of neuro-endocrine-immune network. IET Syst Biol 1: 51-60, 2007.
16. Jiang M, Zha Q, Lu C, He Y and Lu A: Association between tongue appearance in Traditional Chinese Medicine and effective response in treatment of rheumatoid arthritis. Complement Ther Med 19: 115-121, 2011.
17. Liang W, Li X, Li Y, Li C, Gao B, Gan H, Li S, Shen J, Kang J, Ding S, *et al*: Tongue coating microbiome regulates the changes in tongue texture and coating in patients with post-menopausal osteoporosis of Gan-shen deficiency syndrome type. Int J Mol Med 32: 1069-1076, 2013.
18. Méndez JP, Rojano-Mejía D, Pedraza J, Coral-Vázquez RM, Soriano R, García-García E, Aguirre-García Mdel C, Coronel A and Canto P: Bone mineral density in postmenopausal Mexican-Mestizo women with normal body mass index, overweight, or obesity. Menopause 20: 568-572, 2013.
19. An S, Li E and Tong X: Study on relationship between estrogen receptor gene polymorphism and syndrome differentiation typing of female postmenopausal osteoporosis in Traditional Chinese medicine. Zhongguo Zhong Xi Yi Jie He Za Zhi 20: 907-910, 2000 (In Chinese).
20. Leung PC and Siu WS: Herbal treatment for osteoporosis: A current review. J Tradit Complement Med 3: 82-87, 2013.
21. Gao Z, Lu Y, Halmurat Upur, Jing J and Xu D: Study of osteoporosis treatment principles used historically by ancient physicians in Chinese Medicine. Chin J Integr Med 19: 862-868, 2013.
22. Matsumoto Y, Otsuka F, Takano-Narazaki M, Katsuyama T, Nakamura E, Tsukamoto N, Inagaki K, Sada KE and Makino H: Estrogen facilitates osteoblast differentiation by upregulating bone morphogenetic protein-4 signaling. Steroids 78: 513-520, 2013.
23. Coelho MJ and Fernandes MH: Human bone cell cultures in biocompatibility testing. Part II: Effect of ascorbic acid, beta-glycerophosphate and dexamethasone on osteoblastic differentiation. Biomaterials 21: 1095-1102, 2000.
24. Melville KM, Kelly NH, Khan SA, Schimenti JC, Ross FP, Main RP and van der Meulen MC: Female mice lacking estrogen receptor-alpha in osteoblasts have compromised bone mass and strength. J Bone Miner Res 29: 370-379, 2014.
25. Parker BD, Bauer DC, Ensrud KE and Ix JH: Association of osteocalcin and abdominal aortic calcification in older women: The study of osteoporotic fractures. Calcif Tissue Int 86: 185-191, 2010.
26. Sapir-Koren R and Livshits G: Is interaction between age-dependent decline in mechanical stimulation and osteocyte-estrogen receptor levels the culprit for postmenopausal-impaired bone formation? Osteoporos Int 24: 1771-1789, 2013.
27. Oh SM, Kim HR and Chung KH: Effects of ginkgo biloba on in vitro osteoblast cells and ovariectomized rat osteoclast cells. Arch Pharm Res 31: 216-224, 2008.
28. Botella S, Restituto P, Monreal I, Colina I, Calleja A and Varo N: Traditional and novel bone remodeling markers in premenopausal and postmenopausal women. J Clin Endocrinol Metab 98: E1740-E1748, 2013.
29. Stuss M, Rieske P, Cegłowska A, Stêpień-Kłos W, Liberski PP, Brzeziańska E and Sewerynek E: Assessment of OPG/RANK/RANKL gene expression levels in peripheral blood mononuclear cells (PBMC) after treatment with strontium ranelate and ibandronate in patients with postmenopausal osteoporosis. J Clin Endocrinol Metab 98: E1007-E1011, 2013.
30. Shoji S, Tabuchi M, Miyazawa K, Yabumoto T, Tanaka M, Kadota M, Maeda H and Goto S: Bisphosphonate inhibits bone turnover in OPG (-/-) mice via a depressive effect on both osteoclasts and osteoblasts. Calcif Tissue Int 87: 181-192, 2010.
31. Mazière C, Salle V, Gomila C and Mazière JC: Oxidized low density lipoprotein enhanced RANKL expression in human osteoblast-like cells. Involvement of ERK, NFkappaB and NFAT. Biochim Biophys Acta 1832: 1756-1764, 2013.
32. Yasuda H: RANKL, a necessary chance for clinical application to osteoporosis and cancer-related bone diseases. World J Orthop 4: 207-217, 2013.
33. Aubin JE and Bonnelye E: Osteoprotegerin and its ligand: A new paradigm for regulation of osteoclastogenesis and bone resorption. Osteoporos Int 11: 905-913, 2000.
34. Mozaffari-Khosravi H, Hesabgar HA, Owlia MB, Hadinedoushan H, Barzegar K and Fllahzadeh MH: The effect of garlic tablet on pro-inflammatory cytokines in postmenopausal osteoporotic women: A randomized controlled clinical trial. J Diet Suppl 9: 262-271, 2012.
35. Yakar S, Rosen CJ, Beamer WG, Ackert-Bicknell CL, Wu Y, Liu JL, Ooi GT, Setser J, Frystyk J, Boisclair YR, *et al*: Circulating levels of IGF-1 directly regulate bone growth and density. J Clin Invest 110: 771-781, 2002.
36. Papierska L, Rabijewski M, Kasperlik-Załuska A and Zgliczyński W: Effect of DHEA supplementation on serum IGF-1, osteocalcin and bone mineral density in postmenopausal, glucocorticoid-treated women. Adv Med Sci 57: 51-57, 2012.

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REVIEW ARTICLE

# Intestinal microbiota: a potential target for the treatment of postmenopausal osteoporosis

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**Postmenopausal osteoporosis (PMO) is a prevalent metabolic bone disease characterized by bone loss and structural destruction, which increases the risk of fracture in postmenopausal women. Owing to the high morbidity and serious complications of PMO, many efforts have been devoted to its prophylaxis and treatment. The intestinal microbiota is the complex community of microorganisms colonizing the gastrointestinal tract. Probiotics, which are dietary or medical supplements consisting of beneficial intestinal bacteria, work in concert with endogenous intestinal microorganisms to maintain host health. Recent studies have revealed that bone loss in PMO is closely related to host immunity, which is influenced by the intestinal microbiota. The curative effects of probiotics on metabolic bone diseases have also been demonstrated. The effects of the intestinal microbiota on bone metabolism suggest a promising target for PMO management. This review seeks to summarize the critical effects of the intestinal microbiota and probiotics on PMO, with a focus on the molecular mechanisms underlying the pathogenic relationship between bacteria and host, and to define the possible treatment options.**

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## INTRODUCTION

Postmenopausal osteoporosis (PMO) is an estrogen deficiency-induced metabolic bone disorder characterized by reduced bone strength, which increases the risk of fracture in postmenopausal women.[1] The onset of PMO is occult, without any obvious symptoms until a fracture occurs. The most prevalent complication is a fragility fracture, which often occurs in the hip, femur, or spine under non-traumatic or mildly traumatic conditions, resulting in pain, malformation, dysfunction, and even death. Studies showed that the mortality rate associated with a hip fracture was 17% in the first year[2] and ~12%–20% within the two following years.[3] PMO is also a potential risk factor for oral bone loss and aggressive periodontitis in postmenopausal females. PMO animal models showed an equivalent bone loss in alveolar bone and femurs.[4] Compared with healthy postmenopausal women, patients afflicted with PMO also exhibited an inclination to more bone loss and lower bone mineral density (BMD) in the jaw, especially in postmenopausal females with preexisting periodontitis who suffered from accelerated alveolar bone loss under routine treatment.[5–7] In addition to bone loss and microstructural deterioration, PMO affects the osseous formation processes. Delayed osseous maturation and reduced bone regeneration during bone healing in ovariectomized (OVX) rats were reported.[8–9] The high morbidity and serious complications of PMO have attracted major research efforts on its prophylaxis and treatment for decades. Current medications for the treatment of PMO include bisphosphonates, raloxifene, teriparatide and calcitonin, denosumab, estrogen and menopausal hormone therapy, and so on. These medications can prevent bone loss and increase bone mineral density, with a decreased risk of fractures in the vertebra, hip, or long bones.[1,10] All of these pharmacological agents can reduce bone resorption by inhibiting osteoclasts,

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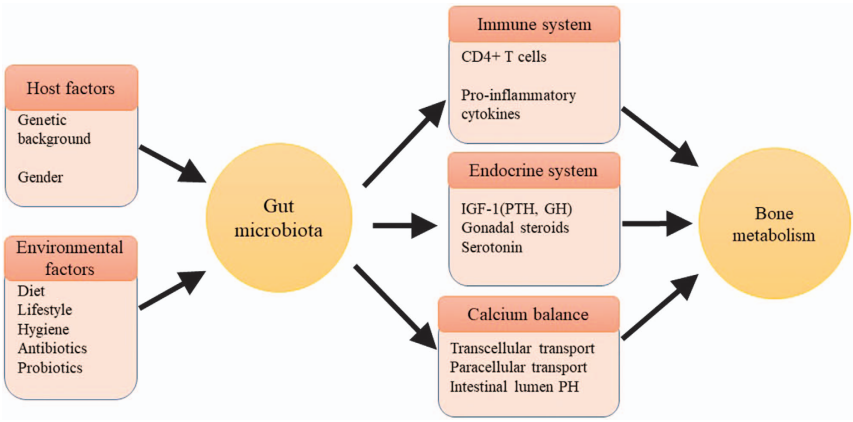


**Figure 1.** Regulators of the gut microbiota and mechanisms by which the gut microbiota regulates bone metabolism. Shaped by both host and environmental factors, the gut microbiota regulates bone metabolism through various pathways, including the immune system, endocrine system, and influences on calcium balance.

except teriparatide, which acts as an anabolic agent by activating or increasing osteoblast activity and prompting bone formation.[1,11] Recent studies have demonstrated a close relationship between the intestinal microbiota and bone metabolism,[12–15] providing evidence that the intestinal microbiome may serve as a potential therapeutic target for the treatment of PMO.

## THE INTESTINAL MICROBIOTA AND ITS REGULATORS

The intestinal microbiota is the collection of microorganisms that colonize the gastrointestinal tract, which consists of approximately 10 trillion bacteria.[16] Obligate anaerobes such as Bacteroidetes and Firmicutes are the predominant residents of the healthy gastrointestinal tract, outnumbering aerobes and facultative anaerobes.[16–17] On the basis of their roles in maintaining human health, intestinal microorganisms can be categorized into beneficial, harmful and neutral bacteria. Both host and environmental factors can shape intestinal microbial composition and structure (Figure 1). Animal experiments[18–21] and twin studies[22–23] revealed that the host genetic background had a significant impact on the abundance of the intestinal microbiota and the predisposition to the colonization of pathogens (for example, *Escherichia coli*). Though still disputed, gender may be another host factor affecting intestinal microbiome species diversity.[24–25] Environmental factors, including diet, lifestyle, hygiene, antibiotic treatment, and probiotics, also contribute to the alteration of the intestinal microbiota composition.[26–31] Notably, the effects of diet and antibiotics on the intestinal microbiota also depend on the host genetic background.[32–33]

Probiotics are defined as dietary or medical supplements consisting of live bacteria that can benefit the host if provided in adequate quantities.[34–36] Currently, ~20 types of beneficial bacteria are used in probiotic supplements. They are generally classified into five categories, including lactobacilli, bifidobacteria, streptococci, yeast, and others.[37] Lactobacilli and bifidobacteria are the most commonly used probiotics. Probiotics can selectively ferment prebiotics, which contain soluble dietary fibers such as oligosaccharides and inulin, facilitating the production of beneficial products conducive to the growth of certain probiotics such as bifidobacteria.[34,38–39] However, it is still disputed whether probiotics can alter the gut microbiota composition. Randomized controlled trials (RCTs) in healthy adults indicated that probiotic intervention or probiotics-fermented products resulted in changes in intestinal microbiota composition or diversity.[40–43] Although probiotics promoted the significant increase of certain bacteria, *Bacteroides* was the dominant genus under probiotics administration, while other bacteria such as Clostridiales were inhibited.[40–41] In addition, the effect of probiotics on Clostridiales genera may be associated with the initial status of the intestinal microbiome and butyrate concentrations.[41] RCTs in elder adults showed that the age-associated intestinal microbiota imbalance was restored by probiotic-based functional foods, with increased resident probiotic-related bacteria and decreased emergence of opportunistic pathogens.[44–45] Animal experimentation also showed that probiotic administration improved the intestinal microbiota composition in hyperlipidemic rats by recovering the abundance of *Bacteroidetes* and *Verrucomicrobia* and reducing *Firmicutes*.[46] However, another RCT in healthy adults demonstrated that *Lactobacillus rhamnosus* GG (LGG) supplement induced no alteration in gut microbiota composition or diversity stability, except for a transient increased fecal excretion of probiotic-associated bacteria during the intervention.[47] In addition, one RCT in healthy subjects and patients with irritable bowel syndrome (IBS) showed parallel, transient, and distinct increases in

probiotics but limited changes in other specific bacteria in fecal samples of both healthy and IBS-afflicted subjects with *Bifidobacterium infantis* intervention.[48]

## THE INTESTINAL MICROBIOTA REGULATES BONE METABOLISM

### Involvement of the intestinal microbiota in bone metabolism

The dynamic homeostasis of the gut microbiome is critical to health. Accumulating evidence has demonstrated that the gut microbiota is associated with physiological bone metabolism and a range of inflammatory or metabolic bone diseases.[12–15,49–50] In animal experimentation, germ-free mice showed higher trabecular volume bone mineral density (vBMD) and improved histomorphologic indices in trabecula compared with conventionally raised (CONV-R) mice.[12] However, both trabecular BMD and cortical cross-sectional area decreased when germ-free mice were recolonized by the gut microbiota, indicating that the gut microbiota is a major regulator of bone mass.[12] Microbial recolonization in germ-free mice induced an incipient acute decrease in bone mass but predominantly led to bone formation with a longer duration, leading to a new equilibrium in bone mass.[14] Furthermore, germ-free mice colonized with immature gut microbiota from donors of different ages or nutritional statuses showed varied femoral phenotypes, suggesting that the impact of the gut microbiota on bone morphologic properties is age/nutrition dependent.[13] Compromised bone biomechanical properties in mice was also induced by an altered gut microbiota resulting from immunodeficiency or long-term antibiotic intervention during growth.[15] In addition, through post-weaning exposure to low-dose penicillin (LDP) or by introducing LDP to their mother in pregnancy, adult offspring with a perturbed gut microbiota showed altered bone mineral content (BMC) and BMD.[51] In addition to physiological condition, inflammatory, or metabolic bone diseases, such as metabolic osteoarthritis, osteoporosis, autoinflammatory osteomyelitis, are also associated with gut microbial alteration.[49–50,52–53] The abundance of gut bacteria *Lactobacillus spp.* and *Methanobrevibacter spp.* was shown to have a significant relationship with the prediction of osteoarthritis assessed by the Modified Mankin Score in rats.[50] Gut microbiota modified by diet regulated the production of IL-1$\beta$ (Interleukin-1beta) and prevented the spontaneous development of osteomyelitis in Pstpip2cmo mice predisposed to autoinflammatory osteomyelitis.[52–53]

### Mechanisms by which the gut microbiota regulates bone metabolism

Gut microbiota can regulate bone metabolism, but the exact mechanisms are still unclear. Multiple approaches through which gut microbiota may regulate bone metabolism have been proposed, including actions on the immune system, endocrine system, and calcium absorption (Figure 1).

*(a) The gut microbiota regulates bone metabolism through the immune system*. Recent studies have revealed a close interrelationship between the immune system and bone metabolism, leading to the development of "osteoimmunology," which highlights the role of immune-related factors in modulating bone remodeling.[54–55] In immune-mediated bone metabolism, the RANKL (receptor activator NF kappa B ligand)-RANK-OPG axis and immunoreceptor tyrosine-based activation motif (ITAM) pathway play key roles in physiological bone turnover and bone diseases.[54,56] Recently, it has been widely recognized that the gut microbiota can interact with the host immune system and further influence host health.[57–59] One study showed that altered immune status in germ-free mice (for example, decreased pro-inflammatory cytokines, fewer $CD4^+$ T cells and reduced osteoclast/precursor cells in bone marrow) may account for the higher bone mass than in CONV-R mice.[12] Intestinal segmented filamentous bacteria in mice were shown to promote the production of IL-17 and IFN-$\gamma$ (Interferon-gamma), both of which played critical roles in the formation of osteoclasts and osteoblasts.[60–62] These studies suggest that the gut microbiota regulates bone metabolism by altering host immune status.

*(b) The gut microbiota regulates bone metabolism through the endocrine system*. In addition to the immune system, hormones are regarded as another important regulator of bone metabolism. As an autocrine or paracrine growth factor, insulin-like growth factor-1 (IGF-1) can promote the differentiation and growth of bone cells, including osteoblasts, osteoclasts, and chondrocytes, and enhance normal interactions among them.[63–65] Moreover, the IGF-1 signaling pathway is involved in the regulation of bone metabolism via both growth hormone and parathormone.[64] Intermittent administration of parathormone promoted bone formation by increasing local IGF-1 production and activating the IGF-1 signaling pathway in bone.[64] Growth hormone can directly or IGF-1-dependently target the growth plate to promote cartilage formation and longitudinal bone growth.[66–67] Moreover, gonadal steroids, including estrogen and androgen, play key roles in the regulation of bone mass and turnover in bone metabolism.[68–70] Furthermore, serum neurotransmitter 5-hydroxytryptamine, namely, circulating serotonin with a hormone-like effect, can stimulate or inhibit bone formation, and dual-directional effects may be gender/age dependent.[71–74]

The gut microbiota, which is currently considered a novel "endocrine organ" of the human body, can engage in an interplay with the endocrine system (for example, hypothalamic–pituitary–adrenal axis) and secrete hormones or hormone-like products to regulate host hormone levels, further influencing host health status.[75–76] In animal experimentation, gut microbial colonization in germ-free mice significantly increased the serum IGF-1 level, resulting in bone growth and normalized bone mass.[14] Isoflavones, the compounds classified as phytoestrogens and structurally similar to endogenous estrogen, were converted into more the estrogenic metabolite equol by specific gut microorganisms such as rod-shaped and gram-positive anaerobic bacteria in ~30%–50% of humans.[77–81] Polycyclic aromatic hydrocarbons—contaminants widely present in nature—can be biotransformed into products with estrogenic activity by the human colon microbiota.[82] A recent study showed that the gut microbiota, especially spore-forming bacteria, can enhance the biosynthesis of serotonin by colonic enterochromaffin cells.[83] Despite the lack of direct evidence, it has been suggested that gut microbiota-bone communication likely depends on the endocrine system or hormone-like substances.

*(c) The gut microbiota regulates bone metabolism by influencing calcium absorption*. Gut microbiota can affect the absorption of skeletal development-related nutrients such as calcium and vitamin D. Calcium, the dominant mineral component in bone, is essential for bone health. Calcium absorption can be facilitated by vitamin D. Either dietary calcium deprivation or vitamin D deficiency may induce osteoporosis.[84] Sufficient calcium consumption can be a prophylactic measure against osteoporosis and relevant fracture.[85] A clinical study in adolescent girls showed decreased bone resorption in the presence of high calcium consumption (47.4 mmol per day compared to the recommended 22.5 mmol per day).[86] Some studies showed that calcium metabolism differences among ethnic groups—in terms of dietary calcium intake, renal calcium excretion, and relevant regulatory hormone or factor—were associated with bone parameters related to osteoporosis/fracture risk.[87] In animal models, a low-calcium diet alone can lead to bone resorption, high bone turnover, and impaired bone trabecular microarchitecture in multiple bones, including the hard palate, mandible, vertebrae, femur, and proximal tibia.[88–91]

Calcium is absorbed by the active transcellular pathway (ion pumps) or passive paracellular diffusion (ion channels), depending on the level of 1,25-$(OH)_2$D (1,25-dihydroxy vitamin D).[92] The proteins involved in the transcellular pathway consist of transient receptor potential vanilloid type 6 (TRPV6/CaT1/ECaC2), which absorbs calcium from the gut lumen into cells; calbindin-D9k, which is responsible for intracellular calcium transportation; and plasma membrane calcium ATPase 1b (PMCA1b), which excretes calcium outside cells into the blood.[93] Passive paracellular calcium diffusion occurs as calcium ($Ca^{2+}$) flux across the intestinal epithelium and is based on tight junction (TJ) proteins between intestinal epithelial cells.[94] Normal calcium intake rates in adults are ~30%–35%;[95–96] these levels can be increased by probiotics, prebiotics, and synbiotics consisting of probiotics and their favorable prebiotics.[97] Specific probiotic bacteria, such as *Lactobacillus salivarius* rather than *Bifidobacterium infantis*, stimulated calcium uptake by enterocytes in a Caco-2 cell culture model.[98] Oligosaccharides (NDO), the dietary prebiotics containing fructooligosaccharides (FOS) and inulin, significantly facilitated intestinal calcium absorption and increased skeletal calcium content in growing and adult rats.[99–102] Prebiotic inulin produced an enhancement in calcium absorption compared to other oligosaccharides,[99–100] while the combination of both may act synergistically.[101–102] In addition, a study in healthy adolescent girls demonstrated that daily administration of GOS can increase calcium absorption.[103] Another clinical study reported the improvement of calcium absorption in young healthy women with long-term treatment with lactosucrose.[104]

As the fermentation substrates of gut microbiota, prebiotics affect bone metabolism by producing a variety of beneficial metabolites, such as short-chain fatty acids (SCFA). The potential mechanism by which SCFA regulate bone metabolism involves direct effects on proteins associated with calcium absorption. Experiments both *in vitro* and *in vivo* using animal models showed that an SCFA supplement could increase the transcriptional levels of TRPV6 and calbindin-D9k rather than PMCA1b in cultured Caco-2 human colonic epithelium and rat colorectal mucosa.[105–106] The *TRPV6* gene was shown to contain a segment characterized by a positive response to SCFA.[105] In addition, the response of calbindin-D9k to SCFA varied with time and SCFA dose.[106] The upregulation of calbindin-D9k by prebiotic diet specifically occurred in the colorectal segment regardless of dietary calcium uptake and serum 1,25-$(OH)_2$D level, and it was related to the transcription factors vitamin D receptor (VDR) and cdx-2.[107–109] The SCFA butyrate resulting from the prebiotic diet can upregulate VDR, activate the cdx-2 promoter, and facilitate cdx-2 mRNA expression.[110] Although direct evidence for the SCFA-related effect on intestinal paracellular calcium absorption is still absent, a ruminant model in which more than 50% of calcium absorption pre-intestinally occurs in the rumen manifested a dose-dependent promotion by SCFA on the ruminal

calcium ion flux rate from mucosa to serum in the paracellular pathway.[111–112] As stated above, both probiotics and prebiotics can influence intestinal epithelial permeability by regulating TJ protein expression and distribution, which possibly underlies the mechanism of prebiotic effects on paracellular calcium transport. In addition to direct action on the cellular structure involved in the calcium absorption process, prebiotics can also alter the intestinal microenvironment, thereby indirectly modulating bone metabolism. SCFA generated from prebiotics could lower the intestinal lumen pH and consequently inhibit the formation of calcium complexes, such as calcium phosphates, leading to increased calcium absorption.[113]

## RELATIONSHIP BETWEEN THE INTESTINAL MICROBIOTA AND PMO

### PMO animal models

Current data on the relationship between intestinal microbiota and PMO are primarily obtained from animal models. The most commonly used PMO animal models are rodents submitted to either surgery or medication. Ovariectomy is the most frequently used surgery to generate PMO rodent models. Bilateral ovariectomy is used to successfully set up morbid states of PMO in the proximal tibia, distal femur and lumbar vertebra according to the guidelines for the preclinical and clinical evaluation of PMO medication issued by the United States Food and Drug Administration (FDA).[114] Gonadotropin-releasing hormone (GnRH) agonists are frequently used to induce PMO in rodents. The long-term or high dose administration of GnRH agonists to rats typically housed under germ-free conditions [49] inhibits the secretion of endogenous GnRH, gonadotrophin and estrogen.[115–116] GnRH agonist-induced bone loss is reversible. Kurabayashi *et al* found that Sprague-Dawley (SD) rats submitted to long-term GnRH agonist treatment exhibited decreased bone mass, bone density, and bone turnover that could be partially recovered after treatment interruption.[115] Estrogen deficiency induced by either ovariectomy or GnRH agonist in murine models evidently increases bone turnover and bone loss and reduces bone mineral density and bone volume in lumbar vertebrae and long bones, thus recapitulating conditions in patients with PMO.[114–115,117]

Animal age can affect the final experimental results, as preadolescent mice undergo rapid bone growth and high bone turnover due to the presence of growth hormones.[118] In addition, mice are likely to undergo irreversible aging symptoms[119] and potentially develop senile osteoporosis as early as 5–6 months old. Therefore, 8- to 20-week-old rats or mice are usually used to establish PMO animal models.[49,115,118,120–128]

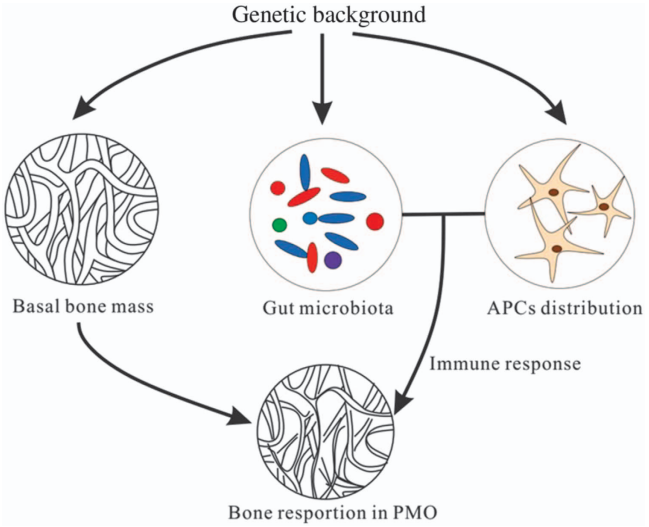


**Figure 2.** Genetic background acts on PMO bone loss. Genetic regulation affects bone loss in PMO by shaping the gut microbiota and determining basal bone mass as well as the distribution of APCs.

### PMO development depends on the intestinal microbiota and host genetic background

The intestinal microbiota is indispensable to PMO development. Compared to conventionally raised (Con-R) mice, germ-free (GF) mice showed no significant alteration in either pro-inflammatory cytokines in bone marrow or femoral trabecular parameters after PMO model establishment by the administration of GnRH agonists.[49] However, similar to Con-R mice, GF mice colonized with a normal gut microbiota exhibited increased pro-inflammatory cytokines and impaired bone properties due to estrogen deficiency.[49] Accordingly, intestinal microorganisms are involved in estrogen deficiency-associated trabecular bone resorption. These microorganisms may be correlated with certain trabecular bone parameters. In particular, trabecular number (Tb.N) and trabecular spacing (Tb.Sp) are influenced by the intestinal microbiota, whereas trabecular thickness (Tb.Th) is not.[49]

Bone resorption in PMO has also been shown to be closely related to genetic background (Figure 2). Previous studies have shown that estrogen deficiency-induced bone loss varies remarkably among different mouse strains.[124,126–127] Genetic regulation can act on PMO bone loss through multiple mechanisms. Genetic background determines basal bone mass[1,122] and the specific distribution of intestinal antigen-presenting cells (APCs) with different functions.[129] Intestinal APCs, especially dendritic cells (DCs), present pathogenic antigens from the gut microbiota and activate $CD4^+$ T cells to produce pro-inflammatory cytokines such as tumor necrosis factor-$\alpha$ (TNF-$\alpha$), which stimulates osteoclastogenesis and induces bone loss.[130–131] In addition, host genetic background can shape the intestinal microbiota,[20,22–23,33,132–133] which

can influence the development and activity of host immune systems [59,134] and thus may indirectly regulate bone loss in PMO.

Probiotics prevent bone loss in PMO murine models

Bone loss in PMO murine models can be prevented by probiotics. Several studies have shown that bone resorption of femur and vertebra in OVX mice could be completely inhibited by the administration of probiotics such as *Lactobacillus reuteri*, LGG and the commercial mixture VSL#3.[49,118] In addition, probiotics such as *Bifidobacterium longum*, *Lactobacillus paracasei* and a mixture of *Lactobacillus paracasei* and *Lactobacillus plantarum* alleviated femoral bone loss and increased bone mineral density in OVX rats or mice.[120–121] Furthermore, soy skim milk fermented by *Lactobacillus paracasei subsp. paracasei NTU 101* (NTU 101F) and *Lactobacillus plantarum NTU 102* (NTU 102F) mitigated bone loss and improved the trabecular microarchitecture in OVX mice.[125]

The effects of probiotics on bone tissues depend on the systemic conditions of the host. McCabe LR *et al*[123] showed that *L. reuteri* increased trabecular bone parameters of the femur and vertebra in healthy male mice (but not intact female mice), suggesting that estrogen level might affect the sensitivity of bone formation to *L. reuteri* in mice. *L. reuteri* may affect bone metabolism by activating the estrogen signaling pathway in male mice, whereas healthy adult female mice are impervious to *L. reuteri* due to sufficient estrogen. Notably, probiotics enhanced the trabecular bone parameters in intact female mice under inflammatory conditions after surgery.[49,135] These results indicate that inflammatory pathways may be potential targets of probiotics to normalize bone homeostasis.

## HOST AND MICROBIOTA INTERACTIONS IN THE PATHOGENESIS AND TREATMENT OF PMO

Immune responses mediated by antigens from the intestinal microbiota play a central role in the pathogenesis of PMO. Under healthy conditions, interplays between the intestinal microbiota, the intestinal epithelial barrier, and the host immune system maintain homeostasis, inhibiting the number of intestinal pathogens and maintaining musculoskeletal balance. If homeostasis is disturbed, intestinal pathogens intrude into the host through the epithelial barrier and provoke an immune response, ultimately promoting osteoclastic bone resorption and continual bone loss in PMO. Accordingly, probiotics ameliorate bone resorption and destruction by suppressing immune responses and restoring equilibrium between the intestinal microbiota and the host.

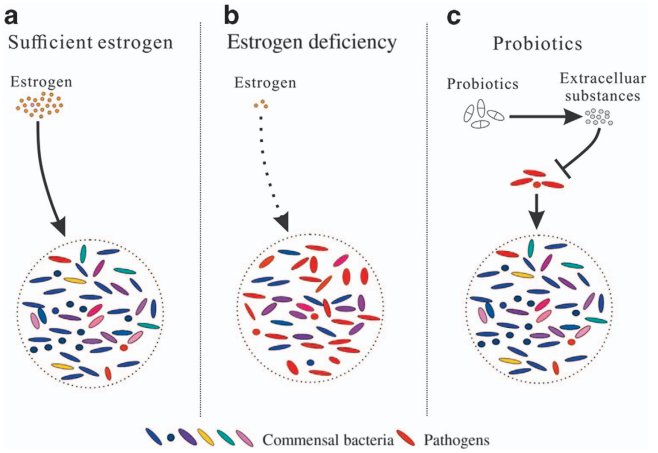


**Figure 3.** Intestinal microbial diversity in PMO is regulated by estrogen and probiotics. Healthy status can maintain gut microbial diversity and beneficial bacteria, which can activate Tregs to sustain immune homeostasis that is resistant to pathogens (**a**). Estrogen deficiency reduces gut microbial diversity and beneficial bacteria, while increased pathogens induce inflammation (**b**). Probiotics can prevent pathogens and increase gut microbial diversity by producing extracellular substances (**c**).

Intestinal microbial diversity in PMO is regulated by estrogen and probiotics

A healthy state and sufficient estrogen levels maintain intestinal microbial diversity (Figure 3a). Under these conditions, beneficial bacteria are predominant and stunt the growth of pathogenic species, preserving the stability of the intestinal microbiota composition. In postmenopausal women, the absence of estrogen alters intestinal microbial composition and structure, leading to decreased microbial diversity (Figure 3b). Clinical surveys of males and postmenopausal females have shown significant correlations between biodiversity (or *Clostridium* abundance) in feces and urinary levels of estrogen (or estrogen metabolites).[136–137] Estrogen deficiency destroys intestinal microbial diversity, which is reflected as a reduction in Firmicutes populations, including *Clostridium* species.[136–138] Firmicutes bacteria, especially *Clostridium* species, possess immune-regulatory effects that boost the formation of regulatory T cells (Tregs) and enhance their function, sustaining immune homeostasis.[139–140] Hence, estrogen deficiency undermines intestinal microbial diversity and reduces the abundance of intestinal bacteria that are conducive to immune homeostasis, consequently facilitating pathogen reproduction and initiating an immune response.

When used to treat PMO, probiotics improve intestinal microbial constitution and restore biodiversity. Probiotics halt pathogen growth and increase intestinal microbial diversity by synthesizing extracellular compounds (Figure 3c). A study by Preidis GA *et al*[141] showed that

*L. reuteri* increased microbial diversity and homogeneity in the feces of mice by producing reuterin. Reuterin, an antibiotic compound, promotes oxidative stress in cells by inducing the modification of thiols on proteins or small molecules, which in turn suppress the growth of pathogens such as *Bacteroides* while increasing the presence of *Clostridium* species.[118,142] Additionally, the *Lactococcus lactis* strain G50 prevented $H_2S$-producing bacteria from growing, while strain H61 had an inhibitory effect on *Staphylococcus* in a mouse model of senile osteoporosis.[119,143] However, it has not yet been demonstrated whether *L. lactis* has an equivalent role in PMO.

Intestinal epithelial barrier function in PMO is regulated by estrogen and probiotics

The intestinal epithelium is the first barrier to physically resist intestinal pathogens. This barrier not only absorbs water and nutrients but also limits the penetration of intestinal antigens. The ability of the barrier to function properly depends on transcellular and paracellular pathways. The fundamental paracellular pathway structure is the TJ, the integrity and selective permeability of which are of vital importance to intestinal epithelial barrier function. TJs are protein complexes consisting of claudin, occludin, and zonula occludens (ZO) proteins, which together allow selective passage of ions and small molecules.[144–150] TJ permeability can be represented by transepithelial electrical resistance (TER); higher TER usually indicates lower permeability.[151–152] Both physiological and pathological stimuli can affect the production and distribution of TJ proteins, thereby modulating intestinal epithelial permeability. TJ proteins are mainly regulated by phosphorylation through protein kinase A (PKA), protein kinase C (PKC), protein kinase G (PKG), serine/threonine (Ser/Thr) kinases, Rho, mitogen-activated protein kinase (MAPK), phosphatidylinositol-3-kinase/Akt (PI3K/Akt), and myosin light chain kinase (MLCK).[144,150]

Sufficient levels of estrogen activate the GTP-binding protein Ras and a series of kinases present in cytoplasm (Raf, MEK1/2, and ERK1/2) through estrogen receptors on the intestinal epithelium; they also maintain relatively high levels of occludin protein expression (Figure 4a).[144,153–155] As a result of this paracellular pathway, the intestinal epithelial barrier exhibits increased TER and can prevent pathogen invasion. Estrogen deficiency weakens the effect of the aforementioned estrogen-associated pathway, leading to increased intestinal epithelial permeability.[156] Antigens from intestinal pathogens initiate inflammatory cascades across the epithelial barrier, leading to the production of pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ). TNF-α and IFN-γ downregulate the TJ proteins occludin and ZO-1 via Raf-MEK1/2-ERK1/2 or MLKs-MKK3/6-p38 in the MAPK pathway and further compromise the intestinal epithelial barrier.[157] In addition, the pro-inflammatory factor interleukin-17 (IL-17) can increase claudin-1 protein expression and reinforce the intestinal epithelial barrier through Ras-Raf-MEK1/2-ERK1/2 in the MAPK pathway.[158] However, the positive action of IL-17 fails to completely compensate for the adverse effect of TNF-α and IFN-γ because TNF-α and IFN-γ may be central players in the immune responses elicited by intestinal bacteria. Hence, estrogen deficiency increases intestinal epithelial permeability (Figure 4b), facilitating the intrusion of intestinal pathogens and provoking immune reactions, and ultimately resulting in increased osteoclastic bone resorption and continual bone loss in PMO.

When used to treat PMO, probiotics fortify the intestinal epithelial barrier to protect the host against intestinal pathogen invasion (Figure 4c). Probiotics regulate the production and distribution of TJ proteins and reduce intestinal epithelial permeability by inducing changes in TJ-related gene expression. *In vitro* experiments have confirmed that *L. plantarum* can promote the production and rearrangement of claudin-1, occludin and ZO-1 proteins in the Caco-2 human colon adenocarcinoma cell line in a dose-dependent manner.[159–160] *Bifidobacteria infantis* was found to increase ZO-1 and occludin protein expression by inhibiting pro-inflammatory cytokines or through the secretion of polypeptide bioactive factors to augment Erk levels while decreasing p38 levels.[161] The probiotic mixture VSL#3 also promoted the expression and redistribution of occludin, ZO-1, and claudin-1 proteins in a mouse model of acute colitis.[162] The potential mechanism for the probiotic regulation of TJ proteins probably involves SCFAs as fermentation products, especially butyrate, which could stimulate the reorganization of TJ proteins and promote TJ assembly by up-regulating AMP-activated protein kinase (AMPK) activity in the Caco-2 cell model, resulting in increased TER and an enhanced intestinal epithelial barrier.[163] In addition, probiotics affected the growth and movement of intestinal epithelial cells by altering gene expression related to protein synthesis, metabolism, cell adhesion and apoptosis.[162,164] *L. reuteri* substantially promoted intestinal epithelial cell migration and proliferation and increased intestinal crypt depth, ultimately improving the absorptive function of the intestinal epithelial barrier.[141] Both LGG and *L. plantarum* can stimulate the intestinal epithelium to produce physiological levels of reactive oxygen species (ROS), which act as a second messenger to activate the Erk/MAPK pathway and consequently lead to intestinal epithelial proliferation.[165–166] Probiotics also offer resistance against the toxic effects produced by intestinal pathogens on the intestinal epithelium. Bifidobacteria reduce the

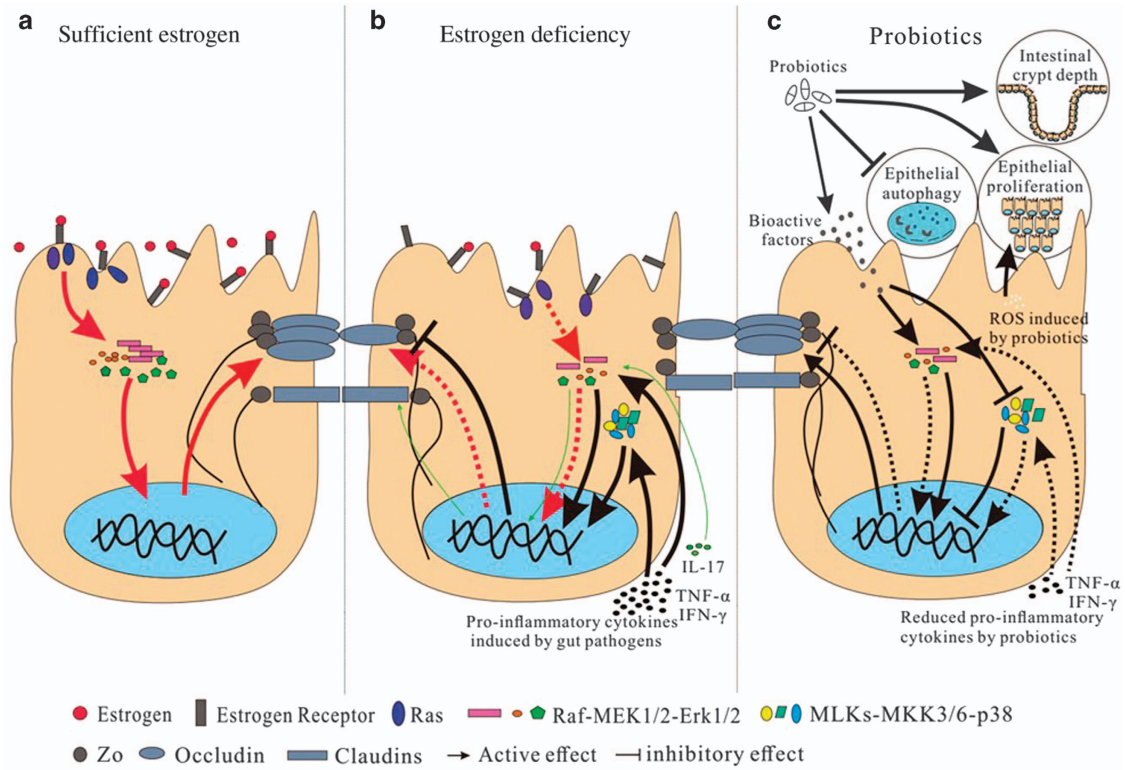


**Figure 4.** Intestinal epithelial barrier function in PMO is regulated by estrogen and probiotics. Sufficient estrogen can prompt the expression of tight junction (TJ) proteins through the Raf-MEK1/2-ERK1/2 pathway to enhance the gut epithelial barrier (**a**), while this active effect on TJ is weakened by estrogen deficiency (**b**). Under estrogen deficiency, pathogen-induced pro-inflammatory cytokines such as TNF-$\alpha$ and IFN-$\gamma$ reduce the production of TJ proteins through both the Raf-MEK1/2-ERK1/2 and MLKs-MKK3/6-p38 pathways and compromise the gut epithelial barrier (**b**). The positive action of IL-17 on TJ proteins (thin green arrows in **b**) fails to completely compensate for the adverse effect of TNF-α and IFN-γ. Probiotics can enhance the gut epithelial barrier by regulating the production and distribution of TJ proteins and affecting the growth and movement of intestinal epithelial cells (**c**).

production of autophagy-related proteins and further prevent intestinal epithelial autophagy triggered by endotoxins from gram-negative bacteria.[167]

Host immune responses in PMO are regulated by estrogen and the intestinal microbiota

The immune system is the final barrier to intestinal pathogen invasion and is also a critical target for PMO treatment. APCs in the intestinal lamina propria can be divided into dendritic cells (DCs) and macrophages.[129] Although all macrophages and DCs can induce Foxp3$^+$ Treg cell differentiation, macrophages with a higher T cell/APC ratio are more efficient than DCs.[129] By contrast, DCs only partially induce Th17 cell differentiation.[129] Treg cells are a subset of immunocytes with inhibitory effects on the differentiation and function of Th1, Th2, and Th17 cells.[130] In addition, Treg cells can inhibit osteoclast formation by cell-to-cell contact via the cytotoxic T lymphocyte antigen (CTLA-4) or by secreting anti-inflammatory cytokines such as IL-4, IL-10, and transforming growth factor-β (TGF-β).[168–171] Th17 cells, a subgroup of T cells, stimulate osteoclast formation and bone resorption by producing high levels of IL-17, RANKL, and TNF-$\alpha$.[172]

Both adequate estrogen levels and intestinal microbial diversity are needed to maintain immune homeostasis (Figure 5a). *Clostridium* improves the aggregation, quantity and function of Treg cells to create an environment abundant in TGF-β, which consequently prevents osteoclastogenesis.[139] Estrogen protects bone by down-regulating immune responses and modulating osteoblast/osteoclast equilibrium.[173] Estrogen not only activates the apoptosis-promoting Fas/FasL pathway through direct interaction with osteoclasts [174–177] but also indirectly increases TGF-β production by Treg cells and decreases the production of TNF-a and RANKL by Th17 cells, ultimately promoting osteoclast apoptosis.[131,168–169,171,178–179] Furthermore, estrogen exerts anti-apoptotic effects on osteoblasts and osteocytes through the ERK pathway.[177,180]

Estrogen deficiency and reduced intestinal biodiversity have negative effects on bone (Figure 5b). Pathogenic antigens cross the intestinal epithelium and trigger inflammatory immune responses that are mainly mediated by T cells. Estrogen deficiency boosts the antigen presentation

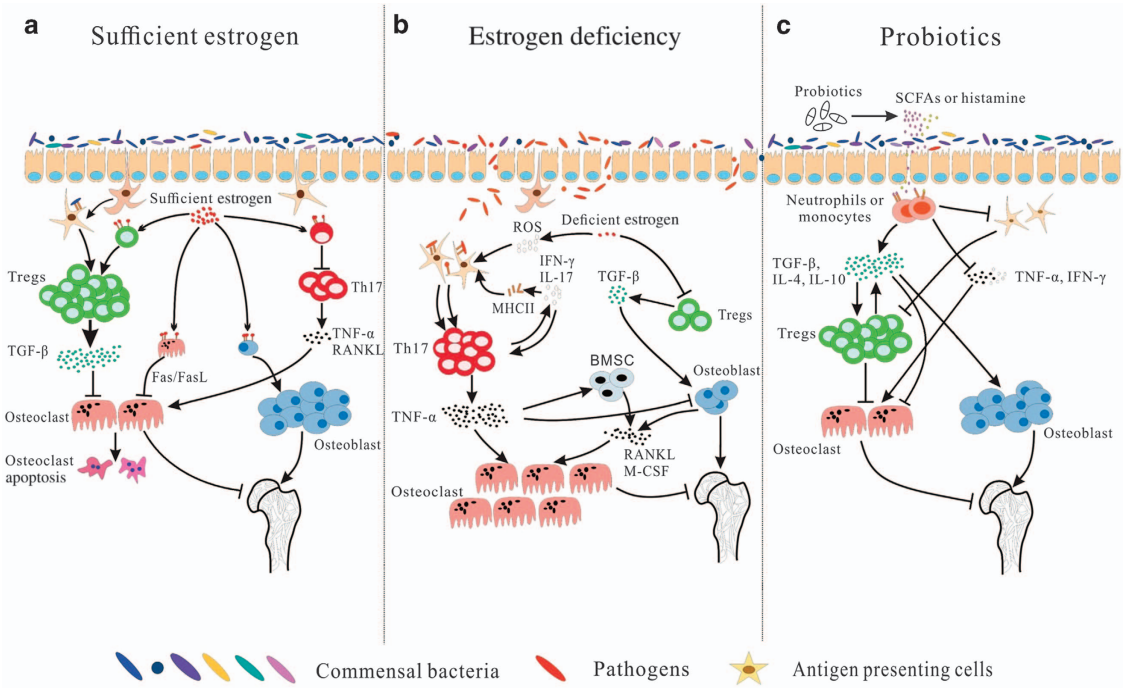


**Figure 5.** Host immune responses in PMO are regulated by estrogen and intestinal microbiota. Both beneficial gut bacteria and sufficient estrogen activate Tregs, which produce TGF-β to prevent osteoclastogenesis and induce osteoclast apoptosis; estrogen prompts osteoblast formation to improve bone mass and structure (**a**). Estrogen deficiency reduces osteoblast formation; the invasion of pathogens activates CD4+T cells including TH17, which mainly produce TNF-α to promote osteoclastogenesis, leading to bone loss and microstructural destruction (**b**). Probiotics can regulate immune responses by secreting small molecules such as SCFAs and histamine (**c**).

of DCs and macrophages through multiple pathways. Upon estrogen depletion, ROS excessively accumulate in bone marrow cells.[181–182] ROS enhance the antigen-presenting function of DCs, which further activates $CD4^+$ T cells to produce IFN-γ. The enhanced production of IFN-γ in turn improves the antigen-presenting ability of bone marrow macrophages (BMM) by up-regulating MHC II molecules.[183–187] In addition, estrogen deficiency upregulates co-stimulator CD80 to activate bone marrow DCs.[184] Increased antigen presentation motivates $CD4^+$ cells, including IL-17-producing Th17 cells, to mediate osteoclast formation and bone resorption.[130,188] In addition to antigen-dependent activation, increased levels of IFN-γ and IL-7, in combination with low levels of TGF-β, indirectly activate T cells in bone marrow.[130,188–189] Activated T cells generate a considerable quantity of TNF-α, which acts as a key pathogenic factor in PMO development.[131,190–193] TNF-α stimulates the production of RANKL and macrophage colony stimulatory factor (M-CSF); it also suppresses the production of osteoprotegerin (OPG) by inducing the expression of CD40L and the bone mass regulatory factor DLK1/FA-1.[130,194–195] In addition, TNF-α acts either directly on osteoclast precursors to promote their maturation[196] or indirectly on TNF-α receptor p55 to augment M-CSF- and RANKL-induced osteoclastogenesis.[131] Furthermore, estrogen deficiency increases levels of Act1 adaptor protein on the surfaces of osteoblasts and subsequently activates the IL-17 signal pathway to promote bone resorption.[197–198] These findings provide evidence that $CD4^+$ T cells (including Th17 cells) and the pro-inflammatory cytokine TNF-α are primary factors responsible for bone loss mediated by intestinal bacteria in PMO.

When used for PMO treatment, probiotics also suppress bone resorption by regulating immune responses to intestinal microorganisms. Probiotics secrete small molecules to regulate the host immune response (Figure 5c). Probiotics also produce SCFAs by utilizing prebiotics.[30,34,199–200] SCFA receptors contain GPR41 and GPR43, the latter of which is mainly found in immunocytes such as neutrophils and monocytes.[201] SCFAs, especially butyric acid, interact with GPR43 to reduce levels of monocyte chemotactic protein 1 (MCP-1) and LPS-induced cytokines such as TNF-α and IFN-γ. They also up-regulate the expression of TGF-β1, IL-4 and IL-10, ultimately activating Treg cells.[120,201–205] In addition, *L. reuteri* transforms dietary L-histidine to histamine, which inhibits the MEK1/2-ERK1/2 pathway via H2 receptors and further inhibits TNF-α production by monocytes.[206] *Lactobacillus* also impedes DC activation during inflammation and promotes Treg differentiation by inducing the expression of molecular ligands with inhibitory effects on pertinent DNA motifs.[207]

### The intestinal microbiota and estrogen orchestrate calcium absorption

As described above, both calcium content and estrogen level are critical to bone metabolism. In postmenopausal-osteoporotic rats, combined deficiencies of dietary calcium and estrogen had a more adverse effect on bone mass and microstructure than either single deficiency, with more bone loss and more severely impaired bone properties.[88,90,208] In addition, calcium balance can be regulated by estrogen. Under normal conditions, estrogen treatment can increase intestinal calcium absorption in rats.[209] Accumulating evidence suggests that estrogen deficiency could induce impaired calcium absorption, which was improved by estrogen supplementation.[210–212] The potential mechanisms of estrogen-associated regulation on calcium absorption are still disputed. Estrogen may indirectly promote vitamin D receptor (VDR) protein expression and enhance intestinal mucosal responsiveness to 1,25-$(OH)_2$D, resulting in increased intestinal calcium absorption.[213–214] However, estrogen deficiency-related calcium malabsorption may not depend on the serum 1,25-$(OH)_2$D pathway. Estrogen reversed the reduced calcium absorption by directly interacting with estrogen receptor alpha (ER-$\alpha$) on the intestine, up-regulating the calcium transport protein 1 (CaT 1) of the calcium influx channel without significantly altering serum 1,25-$(OH)_2$D level.[212,215–216] In addition, estrogen deficiency increased the urinary fractional excretion of calcium (FECa) in OVX rats.[120]

The imbalance in calcium metabolism induced by estrogen deficiency was also redressed by the application of probiotics and prebiotics for the treatment of PMO.[97] Probiotic supplements completely inhibited the increase in FECa due to estrogen deficiency in OVX rats.[120] Oligosaccharides (NDO), dietary prebiotics such as fructooligosaccharides (FOS), galactooligosaccharides (GOS), and inulin, can significantly promote intestinal calcium absorption and skeletal calcium retention in OVX rats, resulting in suppressed bone loss.[217–218]

### The gut microbiota produces estrogen-like metabolites with regulatory effects on bone metabolism

Estrogen plays a major role in promoting osteogenesis. The role of estrogen is not limited to the direct suppression of osteoclast activity and lifespan, facilitation of osteoblast lifespan and differentiation, or reduction of mature osteoblasts apoptosis to promote osteogenesis. It also inhibits the formation of both osteoblasts and osteoclasts from bone marrow precursors to prevent bone remodeling and regulate bone turnover.[69–70] In the absence of estrogen due to ovariectomy or post-menopause, estrogen-deficient women exhibit accelerated bone loss and increased bone turnover as well as impaired bone microarchitectural and mechanical properties.[49,177,219] Hormone replacement therapy (HRT), including supplementation with estrogen and progesterone, has been applied to postmenopausal women suffering from PMO and achieved favorable effects.[220] Instead of estrogen supplementation, the gut microbiota may act as another "endocrine organ" and potentiate novel access to replenish estrogen by utilizing exogenous nutrients and producing more estrogenic substances.

Phytoestrogens, which are predominantly present in natural foods such as soy, are exogenous nutrients with structures and bioactivity similar to human intrinsic estrogens. Various metabolites produced from phytoestrogens by the gut microbiota, including equol, urolithins, and enterolignans, are characterized by higher bioavailability and respectively more estrogenic, antiestrogenic and antioxidant bioactivities than their precursors in phytoestrogens, such as isoflavones, ellagitannins, and lignans.[221] Daidzein, the principle isoflavone in soy, has two metabolic patterns including equol and O-desmethylangolensin (O-DMA) production.[222] Equol shows much more estrogenic bioactivity or effects than O-DMA for bone metabolism in PMO.[223] Equol, which is mostly present as a glucuronide conjugate and binds to the estrogen receptor (ER), can suppress bone resorption, promote bone formation, and improve bone biomechanical and microstructural properties in subjects with PMO but has no impacts on bone in healthy early postmenopausal women.[224–229] The potential mechanism that involves equol may prevent osteoclast formation, stimulate the proliferation and differentiation of osteoblasts, and increase osteocalcin level by ER.[223,230] Additionally, equol can inhibit the expression of relevant inflammatory cytokines in bone marrow in a dose-dependent fashion due to estrogen deficiency or LPS from intestinal pathogens.[231–233] Although produced by gut microbiota, equol may modify gut microbiota diversity and composition in turn.[231] Isoflavone metabolism can promote the growth of *Clostridium* clusters XIVa and IV and suppress the genera *Bacteroides* and *Parabacteroides*.[234] Nevertheless, equol production from dietary phytoestrogens has significant interpersonal variations, predominantly depending on gut microbial composition and potential correlations among the three groups of phytoestrogen metabolism as well as dietary components.[77,221,235–236] At present, the key equol-producing gut bacteria have not yet been identified. Most studies target potential equol-producing bacteria by cultivation or sequence analysis of fecal samples. Two strains of *Eubacterium sp.* were isolated and considered the most likely equol-producing bacteria from pig feces.[237] Another intestinal bacteria, *Slackia TM-30*, a rod-shaped and gram-positive anaerobe isolated

**Table 1.** Current probiotics with beneficial effects on estrogen deficiency-induced bone loss

| Probiotics | Research models | Outcomes | References |
|---|---|---|---|
| *Lactobacillus* spp. | | | |
| *L. rhamnosus* GG | C57Bl/6 OVX mice | Attenuates intestinal and BM inflammation and completely inhibits bone loss | 49 |
| | C57Bl/6 OVX mice | Reduces TJ destruction and gut epithelial permeability | 49 |
| | C57Bl/6 OVX mice | Affects enterocyte proliferation and migration | 165 |
| *L. reuteri* | Balb/c OVX mice and healthy C57Bl/6 male mice or intact female mice with inflammation | Suppresses inflammation and bone loss in OVX mice and increases bone parameters in healthy male mice | 118,123,135,206 |
| | Outbred CD1 neonatal mice | Increases enterocyte migration, proliferation, and crypt height | 141 |
| | Outbred CD1 neonatal mice or Balb/c OVX mice | Increases intestinal microbial diversity and evenness and inhibits growth of pathogens | 118,141–142 |
| *L. paracasei* | C57Bl/6 OVX mice | Decreases inflammatory cytokines and bone loss | 120 |
| *L. plantarum* | Murine and drosophila intestine or Caco-2 cell monolayers | Induces enterocyte proliferation and modulates cellular processes e.g., metabolism, adhesion and apoptosis | 164,166 |
| | Caco-2 cell monolayers | Promotes production and rearrangement of TJ proteins and enhances TJ integrity | 159–160 |
| *Lactococcus lactis* | SAMP6 mice | Inhibits $H_2S$-producing bacteria and Staphylococcus | 119,143 |
| *Bifidobacterium spp.* | | | |
| *B. longum* | OVX SD rat | Reduces bone loss and enhances bone mineral density | 121 |
| *B. infantis* | IL-10-deficient mice | Induces rearrangement of TJ proteins and normalizes gut permeability | 161 |
| *Mixture* | | | |
| *L. paracasei* and *L. plantarum* | C57Bl/6 OVX mice | Decreases inflammatory cytokines and bone loss | 120 |
| VSL#3[a] | C57Bl/6 OVX mice | Attenuates intestinal and BM inflammation and completely inhibits bone loss | 49 |
| | C57Bl/6 OVX mice or BALB/c mice in acute colitis model | Promotes expression and redistribution of TJ proteins and reduces intestinal epithelial permeability | 49,162 |

[a]The mixture VSL#3 contains *Bifidobacterium breve*, *Bifidobacterium longum*, *Bifidobacterium infantis*, *Lactobacillus acidophilus*, *Lactobacillus plantarum*, *Lactobacillus paracasei*, *Lactobacillus bulgaricus*, and *Streptococcus thermophiles*.

from healthy human feces, also proved to be highly related to equol production.[78] The sequence information for fecal samples in postmenopausal women with dietary isoflavone uptake indicated obviously higher proportions of *Eubacterium* and *Bifidobacterium* in equol-producing subjects than in equol non-producers.[238] Other studies identified other bacteria that significantly increased in fecal samples of equol producers, including *Collinsella*, *Asaccharobacter*, *Dorea*, and *Finegoldia*.[234,239] In terms of function, sulfate-reducing bacteria were suggested to be involved in equol production.[235] In addition to specific gut bacteria, equol-producing capacity may inversely correlate with O-DMA production.[235] In addition, daidzein bioavailability and the equol/O-DMA production ratio could be elevated by the combined administration of isoflavones and prebiotic oligosaccharides or probiotic bacteria such as *Lactobacillus casei*.[240–242] However, another study showed that the combination of soy isoflavones and fructooligosaccharides had no synergistic effects on bone mineral density or bone mineral content but effectively improved bone microstructural properties, including trabecular number, thickness, and separation.[243] Overall, the beneficial effects of phytoestrogen supplementation on PMO mainly depend on individual metabolisms involving both the appropriate gut microbiome and dietary composition.[244]

## CONCLUSION

Bone resorption in PMO is the consequence of interactions among the estrogen level, the intestinal microbiota, and the host immune system. When estrogen levels are deficient, bacteria and intestinal antigens cross the compromised intestinal epithelium barrier and initiate the immune responses associated with bone loss in PMO. Probiotics prevent bone resorption by restoring intestinal microbial diversity, enhancing the intestinal epithelial barrier, and normalizing aberrant host immune responses, as well as facilitating intestinal calcium absorption and the potential production of estrogen-like metabolites, as summarized in Table 1. Hence, the intestinal microbiota serves as a key factor in the pathogenesis of PMO and will also serve as a new target in the treatment of PMO.

The application of probiotics may be a promising adjuvant to current therapies. However, current studies on probiotics for PMO treatment are limited to animal

studies. The translation from animal studies to clinical application faces many challenges, such as effective dosage and safety in humans. The safety and feasibility of probiotics application in humans have been demonstrated by clinical studies in specific groups, such as in healthy infants,[245] preterm infants, children with intractable diarrhea,[246] and children and adolescents undergoing HCT.[247] However, in patients with predicted severe acute pancreatitis, significant increases in bowel ischemia and mortality were related to probiotic prophylaxis, as reported in the study by Besselink *et al*[248] Hence, more studies are needed to validate the safety of probiotics and confirm the optimal dosage and the proper time and method of delivery for probiotics in the context of PMO treatment.

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## Competing interests

The authors declare no conflict of interest.

## References

1 Watts NB, Bilezikian JP, Camacho PM *et al.* American Association of Clinical Endocrinologists Medical Guidelines for clinical practice for the diagnosis and treatment of postmenopausal osteoporosis. *Endocr Pract* 2010; **16** (Suppl 3): 1–37.
2 Forsen L, Sogaard AJ, Meyer HE *et al.* Survival after hip fracture: short- and long-term excess mortality according to age and gender. *Osteoporos Int* 1999; **10**: 73–78.
3 Orwig DL, Chan J, Magaziner J. Hip fracture and its consequences: differences between men and women. *Orthop Clin North Am* 2006; **37**: 611–622.
4 Macari S, Duffles LF, Queiroz-Junior CM *et al.* Oestrogen regulates bone resorption and cytokine production in the maxillae of female mice. *Arch Oral Biol* 2015; **60**: 333–341.
5 Esfahanian V, Shamami MS, Shamami MS. Relationship between osteoporosis and periodontal disease: review of the literature. *J Dent (Tehran)* 2012; **9**: 256–264.
6 LaMonte MJ, Hovey KM, Genco RJ *et al.* Five-year changes in periodontal disease measures among postmenopausal females: the Buffalo OsteoPerio study. *J Periodontol* 2013; **84**: 572–584.
7 Passos JS, Vianna MI, Gomes-Filho IS *et al.* Osteoporosis/osteopenia as an independent factor associated with periodontitis in postmenopausal women: a case-control study. *Osteoporos Int* 2013; **24**: 1275–1283.
8 Calciolari E, Mardas N, Dereka X *et al.* The effect of experimental osteoporosis on bone regeneration: part 2, proteomics results. *Clin Oral Implants Res* 2017; **28**: e135–e145.
9 Durao SF, Gomes PS, Colaco BJ *et al.* The biomaterial-mediated healing of critical size bone defects in the ovariectomized rat. *Osteoporos Int* 2014; **25**: 1535–1545.
10 Black DM, Rosen CJ. Clinical Practice. Postmenopausal Osteoporosis. *N Engl J Med* 2016; **374**: 254–262.
11 Deal C. Potential new drug targets for osteoporosis. *Nat Clin Pract Rheumatol* 2009; **5**: 20–27.
12 Sjogren K, Engdahl C, Henning P *et al.* The gut microbiota regulates bone mass in mice. *J Bone Miner Res* 2012; **27**: 1357–1367.
13 Blanton LV, Charbonneau MR, Salih T *et al.* Gut bacteria that prevent growth impairments transmitted by microbiota from malnourished children. *Science* 2016; **351**. pii: aad3311.
14 Yan J, Herzog JW, Tsang K *et al.* Gut microbiota induce IGF-1 and promote bone formation and growth. *Proc Natl Acad Sci USA* 2016; **113**: E7554–e7563.
15 Guss JD, Horsfield MW, Fontenele FF *et al.* Alterations to the gut microbiome impair bone strength and tissue material properties. *J Bone Miner Res* 2017; **32**: 1343–1353.
16 Qin J, Li R, Raes J *et al.* A human gut microbial gene catalogue established by metagenomic sequencing. *Nature* 2010; **464**: 59–65.
17 Tojo R, Suarez A, Clemente MG *et al.* Intestinal microbiota in health and disease: role of bifidobacteria in gut homeostasis. *World J Gastroenterol* 2014; **20**: 15163–15176.
18 Org E, Parks BW, Joo JW *et al.* Genetic and environmental control of host-gut microbiota interactions. *Genome Res* 2015; **25**: 1558–1569.
19 Kovacs A, Ben-Jacob N, Tayem H *et al.* Genotype is a stronger determinant than sex of the mouse gut microbiota. *Microb Ecol* 2011; **61**: 423–428.
20 Benson AK, Kelly SA, Legge R *et al.* Individuality in gut microbiota composition is a complex polygenic trait shaped by multiple environmental and host genetic factors. *Proc Natl Acad Sci USA* 2010; **107**: 18933–18938.
21 Esworthy RS, Smith DD, Chu FF. A strong impact of genetic background on gut microflora in mice. *Int J Inflam* 2010; **2010**: 986046.
22 Goodrich JK, Waters JL, Poole AC *et al.* Human genetics shape the gut microbiome. *Cell* 2014; **159**: 789–799.
23 Turnbaugh PJ, Hamady M, Yatsunenko T *et al.* A core gut microbiome in obese and lean twins. *Nature* 2009; **457**: 480–484.
24 Zhao L, Wang G, Siegel P *et al.* Quantitative genetic background of the host influences gut microbiomes in chickens. *Sci Rep* 2013; **3**: 1163.
25 Dabrowska K, Witkiewicz W. Correlations of host genetics and gut microbiome composition. *Front Microbiol* 2016; **7**: 1357.
26 Kashtanova DA, Popenko AS, Tkacheva ON *et al.* Association between the gut microbiota and diet: Fetal life, early childhood, and further life. *Nutrition* 2016; **32**: 620–627.
27 Schmidt B, Mulder IE, Musk CC *et al.* Establishment of normal gut microbiota is compromised under excessive hygiene conditions. *PLoS One* 2011; **6**: e28284.
28 Panda S, El khader I, Casellas F *et al.* Short-term effect of antibiotics on human gut microbiota. *PLoS One* 2014; **9**: e95476.
29 Cho I, Yamanishi S, Cox L *et al.* Antibiotics in early life alter the murine colonic microbiome and adiposity. *Nature* 2012; **488**: 621–626.
30 Gibson GR, Roberfroid MB. Dietary modulation of the human colonic microbiota: introducing the concept of prebiotics. *J Nutr* 1995; **125**: 1401–1412.
31 Shin JH, Sim M, Lee JY *et al.* Lifestyle and geographic insights into the distinct gut microbiota in elderly women from two different geographic locations. *J Physiol Anthropol* 2016; **35**: 31.
32 Fujisaka S, Ussar S, Clish C *et al.* Antibiotic effects on gut microbiota and metabolism are host dependent. *J Clin Invest* 2016; **126**: 4430–4443.
33 Leamy LJ, Kelly SA, Nietfeldt J *et al.* Host genetics and diet, but not immunoglobulin A expression, converge to shape compositional features of the gut microbiome in an advanced intercross population of mice. *Genome Biol* 2014; **15**: 552.

34 Sekhon BS, Jairath S. Prebiotics, probiotics and synbiotics: an overview. *J Pharm Educ Res* 2010; **1**: 13–36.
35 Roberfroid MB. Prebiotics and probiotics: are they functional foods? *Am J Clin Nutr* 2000; **71**: 1682S–1687S.
36 Salminen S, Bouley C, Boutron-Ruault MC *et al.* Functional food science and gastrointestinal physiology and function. *Br J Nutr* 1998; **80** (Suppl 1): S147–S171.
37 Gupta V, Garg R. Probiotics. *Indian J Med Microbiol* 2009; **27**: 202–209.
38 Roberfroid MB. Prebiotics and synbiotics: concepts and nutritional properties. *Br J Nutr* 1998; **80**: S197–S202.
39 Roberfroid M, Gibson GR, Hoyles L *et al.* Prebiotic effects: metabolic and health benefits. *Br J Nutr* 2010; **104** (Suppl 2): S1–63.
40 Plaza-Diaz J, Fernandez-Caballero JA, Chueca N *et al.* Pyrosequencing analysis reveals changes in intestinal microbiota of healthy adults who received a daily dose of immunomodulatory probiotic strains. *Nutrients* 2015; **7**: 3999–4015.
41 Ferrario C, Taverniti V, Milani C *et al.* Modulation of fecal Clostridiales bacteria and butyrate by probiotic intervention with *Lactobacillus paracasei* DG varies among healthy adults. *J Nutr* 2014; **144**: 1787–1796.
42 Zhang J, Wang L, Guo Z *et al.* 454 pyrosequencing reveals changes in the faecal microbiota of adults consuming *Lactobacillus casei* Zhang. *FEMS Microbiol Ecol* 2014; **88**: 612–622.
43 Unno T, Choi JH, Hur HG *et al.* Changes in human gut microbiota influenced by probiotic fermented milk ingestion. *J Dairy Sci* 2015; **98**: 3568–3576.
44 Rampelli S, Candela M, Severgnini M *et al.* A probiotics-containing biscuit modulates the intestinal microbiota in the elderly. *J Nutr Health Aging* 2013; **17**: 166–172.
45 Ahmed M, Prasad J, Gill H *et al.* Impact of consumption of different levels of *Bifidobacterium lactis* HN019 on the intestinal microflora of elderly human subjects. *J Nutr Health Aging* 2007; **11**: 26–31.
46 Chen D, Yang Z, Chen X *et al.* The effect of *Lactobacillus rhamnosus* hsryfm 1301 on the intestinal microbiota of a hyperlipidemic rat model. *BMC Complement Altern Med* 2014; **14**: 386.
47 Lahti L, Salonen A, Kekkonen RA *et al.* Associations between the human intestinal microbiota, *Lactobacillus rhamnosus* GG and serum lipids indicated by integrated analysis of high-throughput profiling data. *Peer J* 2013; **1**: e32.
48 Charbonneau D, Gibb RD, Quigley EM. Fecal excretion of *Bifidobacterium infantis* 35624 and changes in fecal microbiota after eight weeks of oral supplementation with encapsulated probiotic. *Gut Microbes* 2013; **4**: 201–211.
49 Li JY, Chassaing B, Tyagi AM *et al.* Sex steroid deficiency-associated bone loss is microbiota dependent and prevented by probiotics. *J Clin Invest* 2016; **126**: 2049–2063.
50 Collins KH, Paul HA, Reimer RA *et al.* Relationship between inflammation, the gut microbiota, and metabolic osteoarthritis development: studies in a rat model. *Osteoarthritis Cartilage* 2015; **23**: 1989–1998.
51 Cox LM, Yamanishi S, Sohn J *et al.* Altering the intestinal microbiota during a critical developmental window has lasting metabolic consequences. *Cell* 2014; **158**: 705–721.
52 Phillips FC, Gurung P, Kanneganti TD. Microbiota and caspase-1/caspase-8 regulate IL-1beta-mediated bone disease. *Gut Microbes* 2016; **7**: 334–341.
53 Lukens JR, Gurung P, Vogel P *et al.* Dietary modulation of the microbiome affects autoinflammatory disease. *Nature* 2014; **516**: 246–249.
54 Crotti TN, Dharmapatni AA, Alias E *et al.* Osteoimmunology: major and costimulatory pathway expression associated with chronic inflammatory induced bone loss. *J Immunol Res* 2015;**2015**: 281287.
55 Zupan J, Jeras M, Marc J. Osteoimmunology and the influence of pro-inflammatory cytokines on osteoclasts. *Biochem Med (Zagreb)* 2013; **23**: 43–63.
56 Kim N, Takami M, Rho J *et al.* A novel member of the leukocyte receptor complex regulates osteoclast differentiation. *J Exp Med* 2002; **195**: 201–209.
57 Geuking MB, Koller Y, Rupp S *et al.* The interplay between the gut microbiota and the immune system. *Gut Microbes* 2014; **5**: 411–418.
58 Peterson CT, Sharma V, Elmen L *et al.* Immune homeostasis, dysbiosis and therapeutic modulation of the gut microbiota. *Clin Exp Immunol* 2015; **179**: 363–377.
59 Palm NW, de Zoete MR, Flavell RA. Immune-microbiota interactions in health and disease. *Clin Immunol* 2015; **159**: 122–127.
60 Ivanov II, Atarashi K, Manel N *et al.* Induction of intestinal Th17 cells by segmented filamentous bacteria. *Cell* 2009; **139**: 485–498.
61 Adamopoulos IE, Chao CC, Geissler R *et al.* Interleukin-17A upregulates receptor activator of NF-kappaB on osteoclast precursors. *Arthritis Res Ther* 2010; **12**: R29.
62 Duque G, Huang DC, Dion N *et al.* Interferon-gamma plays a role in bone formation *in vivo* and rescues osteoporosis in ovariectomized mice. *J Bone Miner Res* 2011; **26**: 1472–1483.
63 Wang Y, Nishida S, Elalieh HZ *et al.* Role of IGF-I signaling in regulating osteoclastogenesis. *J Bone Miner Res* 2006; **21**: 1350–1358.
64 Wang Y, Bikle DD, Chang W. Autocrine and paracrine actions of IGF-I signaling in skeletal development. *Bone Res* 2013; **1**: 249–259.
65 Wang Y, Nishida S, Sakata T *et al.* Insulin-like growth factor-I is essential for embryonic bone development. *Endocrinology* 2006; **147**: 4753–4761.
66 Wu S, Yang W, De Luca F. Insulin-like growth factor-independent effects of growth hormone on growth plate chondrogenesis and longitudinal bone growth. *Endocrinology* 2015; **156**: 2541–2551.
67 Giustina A, Mazziotti G, Canalis E. Growth hormone, insulin-like growth factors, and the skeleton. *Endocr Rev* 2008; **29**: 535–559.
68 Leder B. Gonadal steroids and bone metabolism in men. *Curr Opin Endocrinol Diabetes Obes* 2007; **14**: 241–246.
69 Imai Y, Youn MY, Kondoh S *et al.* Estrogens maintain bone mass by regulating expression of genes controlling function and life span in mature osteoclasts. *Ann NY Acad Sci* 2009; **1173** (Suppl 1): E31–E39.
70 Syed F, Khosla S. Mechanisms of sex steroid effects on bone. *Biochem Biophys Res Commun* 2005; **328**: 688–696.
71 Bliziotes M. Update in serotonin and bone. *J Clin Endocrinol Metab* 2010; **95**: 4124–4132.
72 Wang Q, Chen D, Nicholson P *et al.* The associations of serum serotonin with bone traits are age- and gender-specific. *PLoS One* 2014; **9**: e109028.
73 Gustafsson BI, Thommesen L, Stunes AK *et al.* Serotonin and fluoxetine modulate bone cell function *in vitro*. *J Cell Biochem* 2006; **98**: 139–151.
74 Battaglino R, Fu J, Spate U *et al.* Serotonin regulates osteoclast differentiation through its transporter. *J Bone Miner Res* 2004; **19**: 1420–1431.
75 Neuman H, Debelius JW, Knight R *et al.* Microbial endocrinology: the interplay between the microbiota and the endocrine system. *FEMS Microbiol Rev* 2015; **39**: 509–521.
76 Sudo N. Microbiome, HPA axis and production of endocrine hormones in the gut. *Adv Exp Med Biol* 2014; **817**: 177–194.
77 Landete JM, Arques J, Medina M *et al.* Bioactivation of phytoestrogens: intestinal bacteria and health. *Crit Rev Food Sci Nutr* 2016; **56**: 1826–1843.
78 Tamura M, Hori S, Nakagawa H *et al.* Effects of an equol-producing bacterium isolated from human faeces on isoflavone and lignan metabolism in mice. *J Sci Food Agric* 2016; **96**: 3126–3132.

79 Bowey E, Adlercreutz H, Rowland I. Metabolism of isoflavones and lignans by the gut microflora: a study in germ-free and human flora associated rats. *Food Chem Toxicol* 2003; **41**: 631–636.
80 Cassidy A, Brown JE, Hawdon A *et al.* Factors affecting the bioavailability of soy isoflavones in humans after ingestion of physiologically relevant levels from different soy foods. *J Nutr* 2006; **136**: 45–51.
81 Song KB, Atkinson C, Frankenfeld CL *et al.* Prevalence of daidzein-metabolizing phenotypes differs between Caucasian and Korean American women and girls. *J Nutr* 2006; **136**: 1347–1351.
82 Van de Wiele T, Vanhaecke L, Boeckaert C *et al.* Human colon microbiota transform polycyclic aromatic hydrocarbons to estrogenic metabolites. *Environ Health Perspect* 2005; **113**: 6–10.
83 Yano JM, Yu K, Donaldson GP *et al.* Indigenous bacteria from the gut microbiota regulate host serotonin biosynthesis. *Cell* 2015; **161**: 264–276.
84 Morris HA, O'Loughlin PD, Anderson PH. Experimental evidence for the effects of calcium and vitamin D on bone: a review. *Nutrients* 2010; **2**: 1026–1035.
85 Zhu K, Prince RL. Calcium and bone. *Clin Biochem* 2012; **45**: 936–942.
86 Wastney ME, Martin BR, Peacock M *et al.* Changes in calcium kinetics in adolescent girls induced by high calcium intake. *J Clin Endocrinol Metab* 2000; **85**: 4470–4475.
87 Redmond J, Jarjou LM, Zhou B *et al.* Ethnic differences in calcium, phosphate and bone metabolism. *Proc Nutr Soc* 2014; **73**: 340–351.
88 Hodgkinson A, Aaron JE, Horsman A *et al.* Effect of oophorectomy and calcium deprivation on bone mass in the rat. *Clin Sci Mol Med* 1978; **54**: 439–446.
89 Hara T, Sato T, Oka M *et al.* Effects of ovariectomy and/or dietary calcium deficiency on bone dynamics in the rat hard palate, mandible and proximal tibia. *Arch Oral Biol* 2001; **46**: 443–451.
90 Shen V, Birchman R, Xu R *et al.* Short-term changes in histomorphometric and biochemical turnover markers and bone mineral density in estrogen-and/or dietary calcium-deficient rats. *Bone* 1995; **16**: 149–156.
91 Goto S, Fujita Y, Hotta M *et al.* Influence of differences in the hardness and calcium content of diets on the growth of craniofacial bone in rats. *Angle Orthod* 2015; **85**: 969–979.
92 Fleet JC, Schoch RD. Molecular mechanisms for regulation of intestinal calcium absorption by vitamin D and other factors. *Crit Rev Clin Lab Sci* 2010; **47**: 181–195.
93 Hoenderop JG, Nilius B, Bindels RJ. Calcium absorption across epithelia. *Physiol Rev* 2005; **85**: 373–422.
94 Alexander RT, Rievaj J, Dimke H. Paracellular calcium transport across renal and intestinal epithelia. *Biochem Cell Biol* 2014; **92**: 467–480.
95 Kuwabara A, Tanaka K. The role of gastro-intestinal tract in the calcium absorption. *Clin Calcium* 2015; **25**: 1607–1612.
96 Sheikh MS, Schiller LR, Fordtran JS. *In vivo* intestinal absorption of calcium in humans. *Miner Electrolyte Metab* 1990; **16**: 130–146.
97 Scholz-Ahrens KE, Ade P, Marten B *et al.* Prebiotics, probiotics, and synbiotics affect mineral absorption, bone mineral content, and bone structure. *J Nutr* 2007; **137**: 838s–846s.
98 Gilman J, Cashman KD. The effect of probiotic bacteria on transepithelial calcium transport and calcium uptake in human intestinal-like Caco-2 cells. *Curr Issues Intest Microbiol* 2006; **7**: 1–5.
99 Kruger MC, Brown KE, Collett G *et al.* The effect of fructooligosaccharides with various degrees of polymerization on calcium bioavailability in the growing rat. *Exp Biol Med (Maywood)* 2003; **228**: 683–688.
100 Nzeusseu A, Dienst D, Haufroid V *et al.* Inulin and fructooligosaccharides differ in their ability to enhance the density of cancellous and cortical bone in the axial and peripheral skeleton of growing rats. *Bone* 2006; **38**: 394–399.
101 Coudray C, Tressol JC, Gueux E *et al.* Effects of inulin-type fructans of different chain length and type of branching on intestinal absorption and balance of calcium and magnesium in rats. *Eur J Nutr* 2003; **42**: 91–98.
102 Demigne C, Jacobs H, Moundras C *et al.* Comparison of native or reformulated chicory fructans, or non-purified chicory, on rat cecal fermentation and mineral metabolism. *Eur J Nutr* 2008; **47**: 366–374.
103 Whisner CM, Martin BR, Schoterman MH *et al.* Galacto-oligosaccharides increase calcium absorption and gut bifidobacteria in young girls: a double-blind cross-over trial. *Br J Nutr* 2013; **110**: 1292–1303.
104 Teramoto F, Rokutan K, Sugano Y *et al.* Long-term administration of 4G-beta-D-galactosylsucrose (lactosucrose) enhances intestinal calcium absorption in young women: a randomized, placebo-controlled 96-wk study. *J Nutr Sci Vitaminol (Tokyo)* 2006; **52**: 337–346.
105 Fukushima A, Aizaki Y, Sakuma K. Short-chain fatty acids induce intestinal transient receptor potential vanilloid type 6 expression in rats and Caco-2 cells. *J Nutr* 2009; **139**: 20–25.
106 Fukushima A, Aizaki Y, Sakuma K. Short-chain fatty acids increase the level of calbindin-D9k messenger RNA in Caco-2 cells. *J Nutr Sci Vitaminol (Tokyo)* 2012; **58**: 287–291.
107 Fukushima A, Ohta A, Sakai K *et al.* Expression of calbindin-D9k, VDR and Cdx-2 messenger RNA in the process by which fructooligosaccharides increase calcium absorption in rats. *J Nutr Sci Vitaminol (Tokyo)* 2005; **51**: 426–432.
108 Ohta A, Motohashi Y, Ohtsuki M *et al.* Dietary fructooligosaccharides change the concentration of calbindin-D9k differently in the mucosa of the small and large intestine of rats. *J Nutr* 1998; **128**: 934–939.
109 Takasaki M, Inaba H, Ohta A *et al.* Dietary short-chain fructooligosaccharides increase calbindin-D9k levels only in the large intestine in rats independent of dietary calcium deficiency or serum 1,25 dihydroxy vitamin D levels. *Int J Vitam Nutr Res* 2000; **70**: 206–213.
110 Domon-Dell C, Wang Q, Kim S *et al.* Stimulation of the intestinal Cdx2 homeobox gene by butyrate in colon cancer cells. *Gut* 2002; **50**: 525–529.
111 Leonhard-Marek S, Becker G, Breves G *et al.* Chloride, gluconate, sulfate, and short-chain fatty acids affect calcium flux rates across the sheep forestomach epithelium. *J Dairy Sci* 2007; **90**: 1516–1526.
112 Uppal SK, Wolf K, Martens H. The effect of short chain fatty acids on calcium flux rates across isolated rumen epithelium of hay-fed and concentrate-fed sheep. *J Anim Physiol Anim Nutr (Berl)* 2003; **87**: 12–20.
113 Weaver CM. Diet, gut microbiome, and bone health. *Curr Osteoporos Rep* 2015; **13**: 125–130.
114 Thompson DD, Simmons HA, Pirie CM *et al.* FDA Guidelines and animal models for osteoporosis. *Bone* 1995; **17**: 125s–133s.
115 Kurabayashi T, Fujimaki T, Yasuda M *et al.* Time-course of vertebral and femoral bone loss in rats administered gonadotrophin-releasing hormone agonist. *J Endocrinol* 1993; **138**: 115–125.
116 Wang Y, Yano T, Kikuchi A *et al.* Comparison of the effects of add-back therapy with various natural oestrogens on bone metabolism in rats administered a long-acting gonadotrophin-releasing hormone agonist. *J Endocrinol* 2000; **165**: 467–473.
117 Boyd SK, Davison P, Muller R *et al.* Monitoring individual morphological changes over time in ovariectomized rats by *in vivo* micro-computed tomography. *Bone* 2006; **39**: 854–862.
118 Britton RA, Irwin R, Quach D *et al.* Probiotic *L. reuteri* treatment prevents bone loss in a menopausal ovariectomized mouse model. *J Cell Physiol* 2014; **229**: 1822–1830.
119 Kimoto-Nira H, Mizumachi K, Okamoto T *et al.* Influence of long-term consumption of a *Lactococcus lactis* strain on the intestinal immunity

and intestinal flora of the senescence-accelerated mouse. *Br J Nutr* 2009; **102**: 181–185.

120 Ohlsson C, Engdahl C, Fåk F *et al.* Probiotics protect mice from ovariectomy-induced cortical bone loss. *PLoS One* 2014; **9**: e92368.

121 Parvaneh K, Ebrahimi M, Sabran MR *et al.* Probiotics (Bifidobacterium longum) increase bone mass density and upregulate sparc and Bmp-2 genes in rats with bone loss resulting from ovariectomy. *Biomed Res Int* 2015; **2015**: 897639.

122 Klinck J, Boyd SK. The magnitude and rate of bone loss in ovariectomized mice differs among inbred strains as determined by longitudinal *in vivo* micro-computed tomography. *Calcif Tissue Int* 2008; **83**: 70–79.

123 McCabe LR, Irwin R, Schaefer L *et al.* Probiotic use decreases intestinal inflammation and increases bone density in healthy male but not female mice. *J Cell Physiol* 2013; **228**: 1793–1798.

124 Bouxsein ML, Myers KS, Shultz KL *et al.* Ovariectomy-induced bone loss varies among inbred strains of mice. *J Bone Miner Res* 2005; **20**: 1085–1092.

125 Chiang S-S, Pan T-M. Antiosteoporotic effects of lactobacillus-fermented soy skim milk on bone mineral density and the microstructure of femoral bone in ovariectomized mice. *J Agric Food Chem* 2011; **59**: 7734–7742.

126 Li CY, Schaffler MB, Wolde-Semait HT *et al.* Genetic background influences cortical bone response to ovariectomy. *J Bone Miner Res* 2005; **20**: 2150–2158.

127 Iwaniec UT, Yuan D, Power RA *et al.* Strain-dependent variations in the response of cancellous bone to ovariectomy in mice. *J Bone Miner Res* 2006; **21**: 1068–1074.

128 Legette LL, Lee W-H, Martin BR *et al.* Genistein, a phytoestrogen, improves total cholesterol, and Synergy, a prebiotic, improves calcium utilization, but there were no synergistic effects. *Menopause* 2011; **18**: 923–931.

129 Denning TL, Norris BA, Medina-Contreras O *et al.* Functional specializations of intestinal dendritic cell and macrophage subsets that control Th17 and regulatory T cell responses are dependent on the T cell/APC ratio, source of mouse strain, and regional localization. *J Immunol* 2011; **187**: 733–747.

130 Pacifici R. Role of T cells in ovariectomy induced bone loss--revisited. *J Bone Miner Res* 2012; **27**: 231–239.

131 Cenci S, Weitzmann MN, Roggia C *et al.* Estrogen deficiency induces bone loss by enhancing T-cell production of TNF-alpha. *J Clin Invest* 2000; **106**: 1229–1237.

132 Alaish SM, Smith AD, Timmons J *et al.* Gut microbiota, tight junction protein expression, intestinal resistance, bacterial translocation and mortality following cholestasis depend on the genetic background of the host. *Gut Microbes* 2013; **4**: 292–305.

133 Davenport ER, Cusanovich DA, Michelini K *et al.* Genome-wide association studies of the human gut microbiota. *PLoS One* 2015; **10**: e0140301.

134 Wu HJ, Wu E. The role of gut microbiota in immune homeostasis and autoimmunity. *Gut Microbes* 2012; **3**: 4–14.

135 Collins FL, Irwin R, Bierhalter H *et al. Lactobacillus reuteri* 6475 increases bone density in intact females only under an inflammatory setting. *PLoS One* 2016; **11**: e0153180.

136 Fuhrman BJ, Feigelson HS, Flores R *et al.* Associations of the fecal microbiome with urinary estrogens and estrogen metabolites in postmenopausal women. *J Clin Endocrinol Metab* 2014; **99**: 4632–4640.

137 Flores R, Shi J, Fuhrman B *et al.* Fecal microbial determinants of fecal and systemic estrogens and estrogen metabolites: a cross-sectional study. *J Transl Med* 2012; **10**: 253.

138 Breban M. Gut microbiota and inflammatory joint diseases. *Joint Bone Spine* 2016; **83**: 645–649.

139 Atarashi K, Tanoue T, Shima T *et al.* Induction of colonic regulatory T cells by indigenous Clostridium species. *Science* 2011; **331**: 337–341.

140 Nagano Y, Itoh K, Honda K. The induction of Treg cells by gut-indigenous Clostridium. *Curr Opin Immunol* 2012; **24**: 392–397.

141 Preidis GA, Saulnier DM, Blutt SE *et al.* Probiotics stimulate enterocyte migration and microbial diversity in the neonatal mouse intestine. *FASEB J* 2012; **26**: 1960–1969.

142 Schaefer L, Auchtung TA, Hermans KE *et al.* The antimicrobial compound reuterin (3-hydroxypropionaldehyde) induces oxidative stress via interaction with thiol groups. *Microbiology* 2010; **156**: 1589–1599.

143 Kimoto-Nira H, Suzuki C, Kobayashi M *et al.* Anti-ageing effect of a lactococcal strain: analysis using senescence-accelerated mice. *Br J Nutr* 2007; **98**: 1178–1186.

144 Gonzalez-Mariscal L, Tapia R, Chamorro D. Crosstalk of tight junction components with signaling pathways. *Biochim Biophys Acta* 2008; **1778**: 729–756.

145 Muto S, Furuse M, Kusano E. Claudins and renal salt transport. *Clin Exp Nephrol* 2012; **16**: 61–67.

146 Muto S, Hata M, Taniguchi J *et al.* Claudin-2-deficient mice are defective in the leaky and cation-selective paracellular permeability properties of renal proximal tubules. *Proc Natl Acad Sci USA* 2010; **107**: 8011–8016.

147 Yu AS, Enck AH, Lencer WI *et al.* Claudin-8 expression in Madin-Darby canine kidney cells augments the paracellular barrier to cation permeation. *J Biol Chem* 2003; **278**: 17350–17359.

148 Angelow S, Kim KJ, Yu AS. Claudin-8 modulates paracellular permeability to acidic and basic ions in MDCK II cells. *J Physiol* 2006; **571**: 15–26.

149 Balda MS, Whitney JA, Flores C *et al.* Functional dissociation of paracellular permeability and transepithelial electrical resistance and disruption of the apical-basolateral intramembrane diffusion barrier by expression of a mutant tight junction membrane protein. *J Cell Biol* 1996; **134**: 1031–1049.

150 Ulluwishewa D, Anderson RC, McNabb WC *et al.* Regulation of tight junction permeability by intestinal bacteria and dietary components. *J Nutr* 2011; **141**: 769–776.

151 van der Helm MW, Odijk M, Frimat JP *et al.* Direct quantification of transendothelial electrical resistance in organs-on-chips. *Biosens Bioelectron* 2016; **85**: 924–929.

152 Srinivasan B, Kolli AR, Esch MB *et al.* TEER measurement techniques for *in vitro* barrier model systems. *J Lab Autom* 2015; **20**: 107–126.

153 Sumanasekera WK, Zhao L, Ivanova M *et al.* Effect of estradiol and dihydrotestosterone on hypergravity-induced MAPK signaling and occludin expression in human umbilical vein endothelial cells. *Cell Tissue Res* 2006; **324**: 243–253.

154 Sumanasekera WK, Sumanasekera GU, Mattingly KA *et al.* Estradiol and dihydrotestosterone regulate endothelial cell barrier function after hypergravity-induced alterations in MAPK activity. *Am J Physiol Cell Physiol* 2007; **293**: C566–C573.

155 Ye L, Martin TA, Parr C *et al.* Biphasic effects of 17-beta-estradiol on expression of occludin and transendothelial resistance and paracellular permeability in human vascular endothelial cells. *J Cell Physiol* 2003; **196**: 362–369.

156 Hass MA, Nichol P, Lee L *et al.* Estrogen modulates permeability and prostaglandin levels in the rabbit urinary bladder. *Prostaglandins Leukot Essent Fatty Acids* 2009; **80**: 125–129.

157 Patrick DM, Leone AK, Shellenberger JJ *et al.* Proinflammatory cytokines tumor necrosis factor-alpha and interferon-gamma modulate

epithelial barrier function in Madin-Darby canine kidney cells through mitogen activated protein kinase signaling. *BMC Physiol* 2006; **6**: 2.

158 Kinugasa T, Sakaguchi T, Gu X *et al.* Claudins regulate the intestinal barrier in response to immune mediators. *Gastroenterology* 2000; **118**: 1001–1011.

159 Anderson RC, Cookson AL, McNabb WC *et al. Lactobacillus plantarum* MB452 enhances the function of the intestinal barrier by increasing the expression levels of genes involved in tight junction formation. *BMC Microbiol* 2010; **10**: 316.

160 Qin H, Zhang Z, Hang X *et al. L. plantarum* prevents enteroinvasive Escherichia coli-induced tight junction proteins changes in intestinal epithelial cells. *BMC Microbiol* 2009; **9**: 63.

161 Ewaschuk JB, Diaz H, Meddings L *et al.* Secreted bioactive factors from *Bifidobacterium infantis* enhance epithelial cell barrier function. *Am J Physiol Gastrointest Liver Physiol* 2008; **295**: G1025–G1034.

162 Mennigen R, Nolte K, Rijcken E *et al.* Probiotic mixture VSL#3 protects the epithelial barrier by maintaining tight junction protein expression and preventing apoptosis in a murine model of colitis. *Am J Physiol Gastrointest Liver Physiol* 2009; **296**: G1140–G1149.

163 Peng L, Li ZR, Green RS *et al.* Butyrate enhances the intestinal barrier by facilitating tight junction assembly via activation of AMP-activated protein kinase in Caco-2 cell monolayers. *J Nutr* 2009; **139**: 1619–1625.

164 Panigrahi P, Braileanu GT, Chen H *et al.* Probiotic bacteria change *Escherichia coli*-induced gene expression in cultured colonocytes: Implications in intestinal pathophysiology. *World J Gastroenterol* 2007; **13**: 6370–6378.

165 Ardita CS, Mercante JW, Kwon YM *et al.* Epithelial adhesion mediated by pilin SpaC is required for *Lactobacillus rhamnosus* GG-induced cellular responses. *Appl Environ Microbiol* 2014; **80**: 5068–5077.

166 Jones RM, Luo L, Ardita CS *et al.* Symbiotic lactobacilli stimulate gut epithelial proliferation via Nox-mediated generation of reactive oxygen species. *EMBO J* 2013; **32**: 3017–3028.

167 Han C, Ding Z, Shi H *et al.* The role of probiotics in lipopolysaccharide-induced autophagy in intestinal epithelial cells. *Cell Physiol Biochem* 2016; **38**: 2464–2478.

168 Kim YG, Lee CK, Nah SS *et al.* Human $CD4^{+}CD25^{+}$ regulatory T cells inhibit the differentiation of osteoclasts from peripheral blood mononuclear cells. *Biochem Biophys Res Commun* 2007; **357**: 1046–1052.

169 Zaiss MM, Axmann R, Zwerina J *et al.* Treg cells suppress osteoclast formation: a new link between the immune system and bone. *Arthritis Rheum* 2007; **56**: 4104–4112.

170 Yuan FL, Li X, Lu WG *et al.* Regulatory T cells as a potent target for controlling bone loss. *Biochem Biophys Res Commun* 2010; **402**: 173–176.

171 Luo CY, Wang L, Sun C *et al.* Estrogen enhances the functions of CD4(+)CD25(+)Foxp3(+) regulatory T cells that suppress osteoclast differentiation and bone resorption *in vitro*. *Cell Mol Immunol* 2011; **8**: 50–58.

172 Sato K, Suematsu A, Okamoto K *et al.* Th17 functions as an osteoclastogenic helper T cell subset that links T cell activation and bone destruction. *J Exp Med* 2006; **203**: 2673–2682.

173 Carlsten H. Immune responses and bone loss: the estrogen connection. *Immunol Rev* 2005; **208**: 194–206.

174 Nakamura T, Imai Y, Matsumoto T *et al.* Estrogen prevents bone loss via estrogen receptor alpha and induction of Fas ligand in osteoclasts. *Cell* 2007; **130**: 811–823.

175 Krum SA, Miranda-Carboni GA, Hauschka PV *et al.* Estrogen protects bone by inducing Fas ligand in osteoblasts to regulate osteoclast survival. *EMBO J* 2008; **27**: 535–545.

176 Martin-Millan M, Almeida M, Ambrogini E *et al.* The estrogen receptor-alpha in osteoclasts mediates the protective effects of estrogens on cancellous but not cortical bone. *Mol Endocrinol* 2010; **24**: 323–334.

177 Manolagas SC, Kousteni S, Jilka RL. Sex steroids and bone. *Recent Prog Horm Res* 2002; **57**: 385–409.

178 Hughes DE, Dai A, Tiffee JC *et al.* Estrogen promotes apoptosis of murine osteoclasts mediated by TGF-beta. *Nat Med* 1996; **2**: 1132–1136.

179 Lelu K, Laffont S, Delpy L *et al.* Estrogen receptor alpha signaling in T lymphocytes is required for estradiol-mediated inhibition of Th1 and Th17 cell differentiation and protection against experimental autoimmune encephalomyelitis. *J Immunol* 2011; **187**: 2386–2393.

180 Chen JR, Plotkin LI, Aguirre JI *et al.* Transient versus sustained phosphorylation and nuclear accumulation of ERKs underlie anti-versus pro-apoptotic effects of estrogens. *J Biol Chem* 2005; **280**: 4632–4638.

181 Wood ZA, Poole LB, Karplus PA. Peroxiredoxin evolution and the regulation of hydrogen peroxide signaling. *Science* 2003; **300**: 650–653.

182 Ozaki M, Suzuki S, Irani K. Redox factor-1/APE suppresses oxidative stress by inhibiting the rac1 GTPase. *FASEB J* 2002; **16**: 889–890.

183 Kantengwa S, Jornot L, Devenoges C *et al.* Superoxide anions induce the maturation of human dendritic cells. *Am J Respir Crit Care Med* 2003; **167**: 431–437.

184 Grassi F, Tell G, Robbie-Ryan M *et al.* Oxidative stress causes bone loss in estrogen-deficient mice through enhanced bone marrow dendritic cell activation. *Proc Natl Acad Sci USA* 2007; **104**: 15087–15092.

185 Lean JM, Davies JT, Fuller K *et al.* A crucial role for thiol antioxidants in estrogen-deficiency bone loss. *J Clin Invest* 2003; **112**: 915–923.

186 Cenci S, Toraldo G, Weitzmann MN *et al.* Estrogen deficiency induces bone loss by increasing T cell proliferation and lifespan through IFN-gamma-induced class II transactivator. *Proc Natl Acad Sci USA* 2003; **100**: 10405–10410.

187 Gao Y, Grassi F, Ryan MR *et al.* IFN-gamma stimulates osteoclast formation and bone loss *in vivo* via antigen-driven T cell activation. *J Clin Invest* 2007; **117**: 122–132.

188 Ryan MR, Shepherd R, Leavey JK *et al.* An IL-7-dependent rebound in thymic T cell output contributes to the bone loss induced by estrogen deficiency. *Proc Natl Acad Sci USA* 2005; **102**: 16735–16740.

189 Yang NN, Venugopalan M, Hardikar S *et al.* Identification of an estrogen response element activated by metabolites of 17beta-estradiol and raloxifene. *Science* 1996; **273**: 1222–1225.

190 Adeel S, Singh K, Vydareny KH *et al.* Bone loss in surgically ovariectomized premenopausal women is associated with T lymphocyte activation and thymic hypertrophy. *J Investig Med* 2013; **61**: 1178–1183.

191 Ammann P, Rizzoli R, Bonjour JP *et al.* Transgenic mice expressing soluble tumor necrosis factor-receptor are protected against bone loss caused by estrogen deficiency. *J Clin Invest* 1997; **99**: 1699–1703.

192 Charatcharoenwitthaya N, Khosla S, Atkinson EJ *et al.* Effect of blockade of TNF-alpha and interleukin-1 action on bone resorption in early postmenopausal women. *J Bone Miner Res* 2007; **22**: 724–729.

193 D'Amelio P, Grimaldi A, Di Bella S *et al.* Estrogen deficiency increases osteoclastogenesis up-regulating T cells activity: a key mechanism in osteoporosis. *Bone* 2008; **43**: 92–100.

194 Sherman ML, Weber BL, Datta R *et al.* Transcriptional and post-transcriptional regulation of macrophage-specific colony stimulating factor gene expression by tumor necrosis factor. Involvement of arachidonic acid metabolites. *J Clin Invest* 1990; **85**: 442–447.

195 Hofbauer LC, Lacey DL, Dunstan CR *et al.* Interleukin-1beta and tumor necrosis factor-alpha, but not interleukin-6, stimulate osteoprotegerin ligand gene expression in human osteoblastic cells. *Bone* 1999; **25**: 255–259.

196 Lam J, Takeshita S, Barker JE *et al.* TNF-alpha induces osteoclastogenesis by direct stimulation of macrophages exposed to permissive levels of RANK ligand. *J Clin Invest* 2000; **106**: 1481–1488.

197 Molnar I, Bohaty I, Somogyine-Vari E. IL-17A-mediated sRANK ligand elevation involved in postmenopausal osteoporosis. *Osteoporos Int* 2014; **25**: 783–786.
198 Molnar I, Bohaty I, Somogyine-Vari E. High prevalence of increased interleukin-17A serum levels in postmenopausal estrogen deficiency. *Menopause* 2014; **21**: 749–752.
199 Roberfroid MB, Delzenne NM. Dietary fructans. *Annu Rev Nutr* 1998; **18**: 117–143.
200 Roberfroid MB, Van Loo JA, Gibson GR. The bifidogenic nature of chicory inulin and its hydrolysis products. *J Nutr* 1998; **128**: 11–19.
201 Cox MA, Jackson J, Stanton M *et al.* Short-chain fatty acids act as antiinflammatory mediators by regulating prostaglandin E(2) and cytokines. *World J Gastroenterol* 2009; **15**: 5549–5557.
202 Asarat M, Apostolopoulos V, Vasiljevic T *et al.* Short-chain fatty acids produced by synbiotic mixtures in skim milk differentially regulate proliferation and cytokine production in peripheral blood mononuclear cells. *Int J Food Sci Nutr* 2015; **66**: 755–765.
203 Asarat M, Apostolopoulos V, Vasiljevic T *et al.* Short-chain fatty acids regulate cytokines and Th17/Treg cells in human peripheral blood mononuclear cells *in vitro*. *Immunol Invest* 2016; **45**: 205–222.
204 Kwon HK, Lee CG, So JS *et al.* Generation of regulatory dendritic cells and CD4+Foxp3+ T cells by probiotics administration suppresses immune disorders. *Proc Natl Acad Sci USA* 2010; **107**: 2159–2164.
205 Lavasani S, Dzhambazov B, Nouri M *et al.* A novel probiotic mixture exerts a therapeutic effect on experimental autoimmune encephalomyelitis mediated by IL-10 producing regulatory T cells. *PLoS One* 2010; **5**: e9009.
206 Thomas CM, Hong T, van Pijkeren JP *et al.* Histamine derived from probiotic *Lactobacillus reuteri* suppresses TNF via modulation of PKA and ERK signaling. *PLoS One* 2012; **7**: e31951.
207 Bouladoux N, Hall JA, Grainger JR *et al.* Regulatory role of suppressive motifs from commensal DNA. *Mucosal Immunol* 2012; **5**: 623–634.
208 Agata U, Park JH, Hattori S *et al.* The effect of different amounts of calcium intake on bone metabolism and arterial calcification in ovariectomized rats. *J Nutr Sci Vitaminol (Tokyo)* 2013; **59**: 29–36.
209 Arjmandi BH, Hollis BW, Kalu DN. *In vivo* effect of 17 beta-estradiol on intestinal calcium absorption in rats. *Bone Miner* 1994; **26**: 181–189.
210 Gallagher JC, Riggs BL, DeLuca HF. Effect of estrogen on calcium absorption and serum vitamin D metabolites in postmenopausal osteoporosis. *J Clin Endocrinol Metab* 1980; **51**: 1359–1364.
211 Kalu DN, Chen C. Ovariectomized murine model of postmenopausal calcium malabsorption. *J Bone Miner Res* 1999; **14**: 593–601.
212 O'Loughlin PD, Morris HA. Oestrogen deficiency impairs intestinal calcium absorption in the rat. *J Physiol* 1998; **511** (Pt 1): 313–322.
213 Schwartz B, Smirnoff P, Shany S *et al.* Estrogen controls expression and bioresponse of 1,25-dihydroxyvitamin D receptors in the rat colon. *Mol Cell Biochem* 2000; **203**: 87–93.
214 Liel Y, Shany S, Smirnoff P *et al.*. Estrogen increases 1,25-dihydroxyvitamin D receptors expression and bioresponse in the rat duodenal mucosa. *Endocrinology* 1999; **140**: 280–285.
215 Ten Bolscher M, Netelenbos JC, Barto R *et al.* Estrogen regulation of intestinal calcium absorption in the intact and ovariectomized adult rat. *J Bone Miner Res* 1999; **14**: 1197–1202.
216 Van Cromphaut SJ, Rummens K, Stockmans I *et al.* Intestinal calcium transporter genes are upregulated by estrogens and the reproductive cycle through vitamin D receptor-independent mechanisms. *J Bone Miner Res* 2003; **18**: 1725–1736.
217 Chonan O, Matsumoto K, Watanuki M. Effect of galactooligosaccharides on calcium absorption and preventing bone loss in ovariectomized rats. *Biosci Biotechnol Biochem* 1995; **59**: 236–239.
218 Zafar TA, Weaver CM, Zhao Y *et al.* Nondigestible oligosaccharides increase calcium absorption and suppress bone resorption in ovariectomized rats. *J Nutr* 2004; **134**: 399–402.
219 Brennan O, Kuliwaba JS, Lee TC *et al.* Temporal changes in bone composition, architecture, and strength following estrogen deficiency in osteoporosis. *Calcif Tissue Int* 2012; **91**: 440–449.
220 Gambacciani M, Levancini M. Management of postmenopausal osteoporosis and the prevention of fractures. *Panminerva Med* 2014; **56**: 115–131.
221 Gaya P, Medina M, Sánchez-Jiménez A *et al.* Phytoestrogen metabolism by adult human gut microbiota. *Molecules* 2016; **21**. pii: E1034.
222 Uehara M. Isoflavone metabolism and bone-sparing effects of daidzein-metabolites. *J Clin Biochem Nutr* 2013; **52**: 193–201.
223 Ohtomo T, Uehara M, Penalvo JL *et al.* Comparative activities of daidzein metabolites, equol and O-desmethylangolensin, on bone mineral density and lipid metabolism in ovariectomized mice and in osteoclast cell cultures. *Eur J Nutr* 2008; **47**: 273–279.
224 Ishimi Y. Dietary equol and bone metabolism in postmenopausal Japanese women and osteoporotic mice. *J Nutr* 2010; **140**: 1373s–1376s.
225 Tezval M, Sehmisch S, Seidlova-Wuttke D *et al.* Changes in the histomorphometric and biomechanical properties of the proximal femur of ovariectomized rat after treatment with the phytoestrogens genistein and equol. *Planta Med* 2010; **76**: 235–240.
226 Legette LL, Prasain J, King J *et al.* Pharmacokinetics of equol, a soy isoflavone metabolite, changes with the form of equol (dietary versus intestinal production) in ovariectomized rats. *J Agric Food Chem* 2014; **62**: 1294–1300.
227 Tousen Y, Ezaki J, Fujii Y *et al.* Natural S-equol decreases bone resorption in postmenopausal, non-equol-producing Japanese women: a pilot randomized, placebo-controlled trial. *Menopause* 2011; **18**: 563–574.
228 Brink E, Coxam V, Robins S *et al.* Long-term consumption of isoflavone-enriched foods does not affect bone mineral density, bone metabolism, or hormonal status in early postmenopausal women: a randomized, double-blind, placebo controlled study. *Am J Clin Nutr* 2008; **87**: 761–770.
229 Fujioka M, Uehara M, Wu J *et al.* Equol, a metabolite of daidzein, inhibits bone loss in ovariectomized mice. *J Nutr* 2004; **134**: 2623–2627.
230 Wang J, Xu J, Wang B *et al.* Equol promotes rat osteoblast proliferation and differentiation through activating estrogen receptor. *Genet Mol Res* 2014; **13**: 5055–5063.
231 Tousen Y, Matsumoto Y, Matsumoto C *et al.* The combined effects of soya isoflavones and resistant starch on equol production and trabecular bone loss in ovariectomised mice. *Br J Nutr* 2016; **116**: 247–257.
232 Nishide Y, Tadaishi M, Kobori M *et al.* Possible role of S-equol on bone loss via amelioration of inflammatory indices in ovariectomized mice. *J Clin Biochem Nutr* 2013; **53**: 41–48.
233 Kang JS, Yoon YD, Han MH *et al.* Estrogen receptor-independent inhibition of tumor necrosis factor-alpha gene expression by phytoestrogen equol is mediated by blocking nuclear factor-kappaB activation in mouse macrophages. *Biochem Pharmacol* 2005; **71**: 136–143.
234 Guadamuro L, Dohrmann AB, Tebbe CC *et al.* Bacterial communities and metabolic activity of faecal cultures from equol producer and non-producer menopausal women under treatment with soy isoflavones. *BMC Microbiol* 2017; **17**: 93.
235 Possemiers S, Bolca S, Eeckhaut E *et al.* Metabolism of isoflavones, lignans and prenylflavonoids by intestinal bacteria: producer phenotyping and relation with intestinal community. *FEMS Microbiol Ecol* 2007; **61**: 372–383.

236 Bolca S, Wyns C, Possemiers S *et al.* Cosupplementation of isoflavones, prenylflavonoids, and lignans alters human exposure to phytoestrogen-derived 17beta-estradiol equivalents. *J Nutr* 2009; **139**: 2293–2300.
237 Yu ZT, Yao W, Zhu WY. Isolation and identification of equol-producing bacterial strains from cultures of pig faeces. *FEMS Microbiol Lett* 2008; **282**: 73–80.
238 Nakatsu CH, Armstrong A, Clavijo AP *et al.* Fecal bacterial community changes associated with isoflavone metabolites in postmenopausal women after soy bar consumption. *PLoS One* 2014; **9**: e108924.
239 Decroos K, Vanhemmens S, Cattoir S *et al.* Isolation and characterisation of an equol-producing mixed microbial culture from a human faecal sample and its activity under gastrointestinal conditions. *Arch Microbiol* 2005; **183**: 45–55.
240 Fujii S, Takahashi N, Inoue H *et al.* A combination of soy isoflavones and cello-oligosaccharides changes equol/O-desmethylangolensin production ratio and attenuates bone fragility in ovariectomized mice. *Biosci Biotechnol Biochem* 2016; **80**: 1632–1635.
241 Ohta A, Uehara M, Sakai K *et al.* A combination of dietary fructooligosaccharides and isoflavone conjugates increases femoral bone mineral density and equol production in ovariectomized mice. *J Nutr* 2002; **132**: 2048–2054.
242 Mathey J, Mardon J, Fokialakis N *et al.* Modulation of soy isoflavones bioavailability and subsequent effects on bone health in ovariectomized rats: the case for equol. *Osteoporos Int* 2007; **18**: 671–679.
243 Devareddy L, Khalil DA, Korlagunta K *et al.* The effects of fructo-oligosaccharides in combination with soy protein on bone in osteopenic ovariectomized rats. *Menopause* 2006; **13**: 692–699.
244 Wu J, Oka J, Ezaki J *et al.* Possible role of equol status in the effects of isoflavone on bone and fat mass in postmenopausal Japanese women: a double-blind, randomized, controlled trial. *Menopause* 2007; **14**: 866–874.
245 Hoy-Schulz YE, Jannat K, Roberts T *et al.* Safety and acceptability of *Lactobacillus reuteri* DSM 17938 and *Bifidobacterium longum* subspecies infantis 35624 in Bangladeshi infants: a phase I randomized clinical trial. *BMC Complement Altern Med* 2016; **16**: 44.
246 Kitajima H, Hirano S. Safety of *Bifidobacterium breve* (BBG-01) in preterm infants. *Pediatr Int* 2017; **59**: 328–333.
247 Ladas EJ, Bhatia M, Chen L *et al.* The safety and feasibility of probiotics in children and adolescents undergoing hematopoietic cell transplantation. *Bone Marrow Transplant* 2016; **51**: 262–266.
248 Besselink MG, van Santvoort HC, Buskens E *et al.* Probiotic prophylaxis in predicted severe acute pancreatitis: a randomised, double-blind, placebo-controlled trial. *Lancet* 2008; **371**: 651–659.

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# Phytonutrients for bone health during ageing

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Osteoporosis is a skeletal disease characterized by a decrease in bone mass and bone quality that predispose an individual to an increased risk of fragility fractures. Evidence demonstrating a positive link between certain dietary patterns (e.g. Mediterranean diet or high consumption of fruits and vegetables) and bone health highlights an opportunity to investigate their potential to protect against the deterioration of bone tissue during ageing. While the list of these phytonutrients is extensive, this review summarizes evidence on some which are commonly consumed and have gained increasing attention over recent years, including lycopene and various polyphenols (e.g. polyphenols from tea, grape seed, citrus fruit, olive and dried plum). Evidence to define a clear link between these phytonutrients and bone health is currently insufficient to generate precise dietary recommendations, owing to mixed findings or a scarcity in clinical data. Moreover, their consumption typically occurs within the context of a diet consisting of a mix of phytonutrients and other nutrients rather than in isolation. Future clinical trials that can apply a robust set of outcome measurements, including the determinants of bone strength, such as bone quantity (i.e. bone mineral density) and bone quality (i.e. bone turnover and bone microarchitecture), will help to provide a more comprehensive outlook on how bone responds to these various phytonutrients. Moreover, future trials that combine these phytonutrients with established bone nutrients (i.e. calcium and vitamin D) are needed to determine whether combined strategies can produce more robust effects on skeletal health.

## Introduction

Osteoporosis is a skeletal disease characterized by compromised bone strength, predisposing an individual to an increased risk of fractures [1]. A diagnosis of osteoporosis is reached when the bone mineral density (BMD) of an individual, as measured by dual energy X-ray absorptiometry, is 2.5 standard deviations below the mean value for young sex-matched adults [2]. Fractures associated with this disease affect one in three women and one in five men over the age of 50 years. Indeed, osteoporosis is responsible for consuming more hospital days than many other diseases, including diabetes, heart attack and breast cancer [3]. The direct annual costs of osteoporotic fractures are over €31 billion per year in Europe and $20 billion per year in the USA, and these costs are expected to rise substantially by the year 2050 [4, 5]. Thus, it is imperative to promote effective prevention and treatment strategies to counterbalance the significant morbidity, mortality and economic burden associated with this disease.

Several nutritional factors play a role in skeletal health during ageing. Macro- and micronutrients contribute to skeletal health by supporting bone matrix production and mineralization. Of these, calcium, vitamin D and proteins are the most important nutrients for supporting the skeleton and are reviewed extensively elsewhere [6–9]. However, in many developed countries, where the dietary intake of calcium is adequate for most individuals compared with recommended daily allowances, very high rates of osteoporosis are nevertheless observed. These observations suggest that dietary factors independent of calcium and/or vitamin D may influence bone and mineral homeostasis and may be important for long-term bone health. Indeed, dietary patterns consisting of a high consumption of fruits and vegetables, legumes, seafood, nuts, seeds, rice and/or rice dishes have been shown to be directly associated with BMD, independent of dietary calcium intake [10–13]. Albeit not causal, these data have led support to the hypothesis that there may be dietary factors (e.g. phytonutrients) independent from calcium and vitamin D that may be linked to skeletal health.

The primary focus of this review is to discuss the clinical and preclinical efficacy on bone health of novel nonvitamin phytonutrients (e.g. lycopene, polyphenols from tea, grape seed, citrus fruit, olive and dried plum) that are commonly consumed in the diet and that have gained increasing attention for skeletal health during ageing. Phytoestrogens, found in plants such as soy, are excluded from our current discussion because they have been extensively reviewed elsewhere [14–16]. Using PubMed/Medline databases, the present review focuses primarily on the clinical efficacy of phytonutrients commonly found in the daily diet; however, we also include some preclinical data because they provide valuable information on the efficacy of these ingredients when human data are sparse. Given that a significant number of the fractures that occur during ageing occur in individuals with BMD scores that do not meet the diagnostic criteria of osteoporosis [17–19], a secondary focus of this review is to identify other outcome measures of bone health (e.g. bone turnover and bone microarchitecture) that may complement standard BMD testing in future trials related to the skeletal efficacy of phytonutrients.

## Lycopene

Lycopene is a major carotenoid synthesized by many plants and micro-organisms, but not synthesized by animals or humans [20]. This lipid-soluble carotenoid is a highly stable molecule and is responsible for the red colour in many fruits (e.g. tomatoes) and vegetables (e.g. carrots). Lycopene exists in an all-*trans* configuration, which is the most thermodynamically stable form; however, in plasma and tissues, lycopene is present in large amounts as *cis* isomers [21]. The absorption of lycopene is greatest when it is processed into juice, tomato sauce or even ketchup. Unlike other carotenoids, such as α- and β-carotene and β-cryptoxanthin, lycopene has no vitamin A activity. Lycopene has gained attention for its strong antioxidative capabilities and for its potential to play a protective role against a number of chronic diseases, including osteoporosis [22].

### *Clinical evidence of lycopene for skeletal health*

Epidemiological data using various adult populations have demonstrated a positive relationship between the intake levels or serum levels of lycopene and bone mass, bone turnover and/or fracture risk [23–27] (Table 1). In the Framingham Osteoporosis Study, a higher intake of lycopene in elderly men and women was positively associated with a 4 year change in BMD at the lumbar vertebrae and a lower risk for hip and nonvertebral fractures [23, 24]. Other work [27] demonstrated that serum concentrations of lycopene were lower in postmenopausal osteoporotic women compared with their non-osteoporotic counterparts.

Clinical data have supported the epidemiological findings described above. For example, in postmenopausal women supplemented for 4 months with lycopene (as a juice or in a capsule), decreases in N-terminal telopeptide crosslinks of type I collagen and in oxidative parameters (i.e. lipid peroxidation and protein oxidation) were observed [28] (Table 1). In another study, the same research group demonstrated in postmenopausal women that a 1 month restriction in their dietary intake of lycopene-rich foods increased N-terminal telopeptide crosslinks of type I collagen and decreased the antioxidant enzymes catalase and superoxide dismutase [29]. These data, along with *in vitro* work showing that lycopene attenuates the production of osteoclast cells [30], suggest that lycopene protects bone mass during ageing by attenuating bone resorption.

### *Preclinical evidence of lycopene for skeletal health*

Our knowledge concerning the effects of lycopene on bone health stems mostly from epidemiological and clinical trials. Two rodent studies [31, 32] demonstrated that daily administration of lycopene protects against the loss of bone mass and bone strength induced by ovariectomy (Table 1). Given that preclinical research permits us directly to examine biomechanical strength indices of bone and mechanisms of action, as well as toxicological properties of novel ingredients, future studies using preclinical models are needed to characterize the physiological and toxicological effects of lycopene fully.

## Polyphenols and polyphenol-rich foods

Polyphenols are plant-based compounds that are present in our daily diet through fruit and vegetables, beans, grains and beverages, such as fruit juices, coffee and green or black tea. To date, around 5000 polyphenols have been identified in the food we consume. Polyphenols are classified according to the number of phenol rings they contain and on the structural elements bound to these rings. Thus, polyphenols have been classified as phenolic acids, flavonoids, stilbenes, tannins, coumarins and lignans [33].

Even though it is hard to quantify the dietary intake of polyphenols, it has been estimated that the average daily diet provides around 1.5 g [33]. Polyphenols, after consumption, are absorbed into the bloodstream as aglycone forms and are further metabolized by the organism and/or microflora enzymes into conjugates of glucuronate or sulfate and/or eventually eliminated [34]. Thus, circulating forms may possess different biological properties within cells and target tissues compared with polyphenol aglycones.

Despite the large number of molecules identified, most research to date on the potential benefits of polyphenols

## Table 1

Selected human and animal studies on the efficacy of selected phytonutrients on bone indices during ageing

| Study type | Subjects, mean age | Intervention, duration of study | Bone turnover | BMD, BMC | Bone structure | Bone strength | Other findings | Reference |
|---|---|---|---|---|---|---|---|---|
| **Lycopene** | | | | | | | | |
| EPI | Elderly men and women, 75 years old | Food frequency questionnaire completed and records of hip fractures obtained | Not assessed | Not assessed | Not assessed | Not assessed | Higher lycopene intake was associated with lower risk of hip fractures | Sahni *et al.* (2009) [23] |
| EPI | Postmenopausal women, 50–60 years old | 7 day dietary records completed and fasting blood samples taken from participants | Higher serum lycopene was associated with lower NTX levels | Not assessed | Not assessed | Not assessed | Higher serum lycopene was associated with lower protein oxidation | Rao & Rao (2007) [22] |
| RCT | Postmenopausal women, 50–60 years old | Tomato juice, lycopene-rich tomato juice, tomato Lyc-O-Mato lycopene capsules or placebo for 4 months | ↓ NTX in lycopene-supplemented participants (treatment groups pooled) | Not assessed | Not assessed | Not assessed | In pooled lycopene-supplemented groups, was associated with lower protein oxidation and lipid peroxidation | Mackinnon *et al.* (2011) [28] |
| Animal | OVX rats, 2 months old | Lycopene [0, 20, 30 or 40 mg (kg bodyweight)$^{-1}$ day$^{-1}$], 8 weeks duration | ↓ Alkaline phosphatase *vs.* OVX control with treatments | ↑ Femoral BMD and BMC *vs.* OVX control with 30 and 40 mg kg$^{-1}$ treatments | Not assessed | ↑ Femoral strength properties *vs.* OVX control with 30 and 40 mg kg$^{-1}$ treatments | ↓ Calcium and phosphate *vs.* OVX control with treatments.<br>↓ Interleukin-6 *vs.* OVX control with treatments | Liang *et al.* (2012) [32] |
| **Green tea and green tea polyphenols** | | | | | | | | |
| EPI | Men and women, ≥50 years old who sustained a fracture (cases) or did not (controls) | Questionnaire completed on health and lifestyle, including tea consumption | Not assessed | Not assessed | Not assessed | Not assessed | Tea consumption was inversely associated with hip fracture | Johnell *et al.* (1995) [36]<br>Kanis *et al.* (1999) [73] |
| RCT | Osteopenic women, mean age between 56.5 and 58.3 years old | Green tea polyphenols (500 mg day$^{-1}$ *vs.* placebo), both with or without tai chi (3 sessions week$^{-1}$), 6 months duration | ↑ BSALP *vs.* placebo in both green tea and tai chi groups.<br>↔ TRAP | Only assessed for screening purposes | Not assessed | Not assessed | ↔ Serum and urinary calcium, inorganic phosphate with any treatments | Shen *et al.* (2012) [39] |
| Animal | Sham and OVX virgin rats, 14 months old | Green tea polyphenols (0, 0.1 or 0.5% of diet in drinking water), 16 weeks duration | Not assessed | ↑ Femoral BMD with treatments compared with respective control groups | Not assessed | Not assessed | ↓ Urinary calcium with treatments compared with respective control groups | Shen *et al.* (2008) [41] |
| Animal | Sham and OVX virgin rats, 14 months old | Green tea polyphenols (0, 0.1 or 0.5% of diet in drinking water), 16 weeks duration | ↑ BFR/BS and ↓ ES/BS in tibial shaft with treatments compared with respective control groups | ↑ Femoral BMD with treatments compared with respective control groups | Improved bone structure at all skeletal sites with treatments | Not assessed | – | Shen *et al.* (2009) [74] |
| **Hesperidin** | | | | | | | | |
| RCT | Healthy postmenopausal women, 50–65 years old | Biscuits containing hesperidin (500 mg day$^{-1}$) *vs.* placebo biscuits, 2 years duration | ↑ PINP/CTX-1 ratio *vs.* placebo | ↔ BMD | Not assessed | Not assessed | – | Habauzit *et al.* (2011) [47] |
| Animal | Sham rats, 3 and 6 months old | 0.5% hesperidin diet or control diet, 90 days duration | ↓ DPY *vs.* control in younger rats.<br>↔ Osteocalcin | ↑ BMD *vs.* control in younger rats | Not assessed | Not assessed | – | Horcajada *et al.* (2008) [48] |
| Animal | OVX rats, 3 and 6 months old | 0.5% hesperidin diet or control diet, 90 days duration | ↓ DPY *vs.* control in both younger and older rats.<br>↔ Osteocalcin | ↑ BMD *vs.* control in younger and older rats | Not assessed | Not assessed | – | Horcajada *et al.* (2008) [48] |
| Animal | Senescent male rats, 20 months old | 0.5% hesperidin, 0.5% naringin, 0.25% hesperidin + 0.25% naringin in diet or control diet, 3 months duration | ↓ DPY *vs.* control with 0.5% hesperidin and 0.5% naringin treatments.<br>↔ Osteocalcin | ↑ BMD *vs.* control with 0.5% hesperidin and combination group | ↑ BV/TV *vs.* control with 0.5% hesperidin group.<br>↑ Tb.Th. *vs.* control with combination group | ↔ Strength parameters among any groups | ↑ BMP-2 and BMP-4 *vs.* control with 0.5% hesperidin | Habauzit *et al.* (2011) [47] |

## Table 1

Continued

| Study type | Subjects, mean age | Intervention, duration of study | Bone turnover | BMD, BMC | Bone structure | Bone strength | Other findings | Reference |
|---|---|---|---|---|---|---|---|---|
| **Olive oil and olive oil polyphenols** | | | | | | | | |
| EPI | Healthy pre-, peri- and postmenopausal women, 48 years old | 3 day food records completed and BMD at lumbar spine and total body BMC measured | Not assessed | High consumption of fish and olive oil and low intake of red meat was positively associated with BMC and BMD | Not assessed | Not assessed | – | Kontogianni *et al.* (2009) [57] |
| Animal | OVX rats, 6 months old | Oleuropein (0.15 g $kg^{-1}$ of diet), olive oil (50 g $kg^{-1}$ of diet), or control diet, 80 days duration, half of the rats induced with inflammation at day 59 | ↓ DPY *vs.* OVX control with oleuropein treatment in rats with inflammation.<br>↔ Osteocalcin *vs.* OVX control | ↑ Femur BMD *vs.* OVX control with oleuropein and olive oil treatment in rats with inflammation | Not assessed | ↑ Peak load at femoral midpoint *vs.* OVX control with oleuropein and olive oil in rats with inflammation.<br>↔ Peak load at femoral midpoint in rats without inflammation | ↓ α-1-Acid glycoprotein *vs.* OVX control with olive oil in rats with inflammation.<br>↓ Spleen weight *vs.* OVX control with oleuropein and olive oil in rats with inflammation | Puel *et al.* (2004) [59] |
| Animal | OVX rats, 6 months old | Oleuropein [0, 2.5, 5, 10 or 15 mg (kg bodyweight)$^{-1}$ $day^{-1}$], or control diet, 100 days duration, half of the rats induced with inflammation at day 79 | ↔ DPY<br>↓ Osteocalcin *vs.* OVX controls with 15 mg $kg^{-1}$ dose with inflammation | ↑ Femoral BMD *vs.* OVX control with oleuropein in rats with inflammation.<br>↔ Femoral BMD in rats without inflammation | Not assessed | ↔ Peak load at femur midpoint | ↓ Spleen weight *vs.* OVX control with 2.5 mg $kg^{-1}$ oleuropein.<br>↔ Ferric-reducing potential value.<br>↔ Fibrinongen *vs.* OVX controls | Puel *et al.* (2006) [58] |
| **Dried plums** | | | | | | | | |
| RCT | Osteopenic postmenopausal women, 55.6–57.5 years old | Dried plums (100 g $day^{-1}$) or a dried apple control group (75 g $day^{-1}$), 12 months duration | ↓ BSALP and osteocalcin *vs.* baseline and control group.<br>↓ TRAP with dried plum *vs.* baseline | ↑ Ulnar and spine BMD compared with control group | Not assessed | Not assessed | ↓ C-reactive protein levels compared with control group at 3 months | Hooshmand *et al.* (2011) [64] |
| Animal | OVX mice 3 months old | Dried plums (0, 5, 15 or 25% of diet), 4 weeks duration | ↓ PINP *vs.* OVX control group | ↑ Spine BMD and BMC compared with OVX control group with 25% dried plums | Improved structural properties at spine and tibia with 15 and 25% dried plums | Improved biomechanical properties at spine with 15 and 25% dried plums | ↑ Insulin-like growth factor-1 with 15% dried plums *vs.* OVX control group.<br>↓ Tumour necrosis factor-α levels with 15 and 25% dried plums | Rendina *et al.* (2012) [65] |
| Animal | Male intact mice, 6 and 12 months old | Dried plums (0, 15 or 25% of diet), 6 months duration | Diet × age interaction for BFR, trend ($P = 0.08$) for ↑ BFR with treatments in adult mice *vs.* 0% | ↔ Femoral BMD with treatments compared with 0% group | Improved structural properties at femur with treatments; effects more pronounced in adult mice | Not assessed | – | Halloran *et al.* (2010) [70] |

Abbreviations are as follows: BFR, bone formation rate; BFR/BS, bone formation rate per bone surface; BMC, bone mineral content; BMD, bone mineral density; BMP, bone morphogenic protein; BSALP, bone-specific alkaline phosphatase; BV/TV, bone volume fraction; DPY, deoxypyridinoline; EPI, epidemiology; ES/BS, eroded surface/bone surface; NTX, N-terminal telopeptide crosslinks of type I collagen; OVX, ovariectomized; PINP, procollagen I N-terminal propeptide; RCT, randomized controlled trial; Tb.Th., trabecular thickness; CTX-1, carboxy-terminal collagen crosslinks type 1; and TRAP, tartrate-resistant acid phosphatase. ↑ Significantly higher compared with other groups or a significant increase compared with baseline. ↓ Significantly lower compared with other groups or a significant decrease compared with baseline. ↔ No significant differences among groups or no significant increases/decreases within a group compared with baseline values.

for bone health has focused on the flavonoids subgroup, specifically on isoflavones from soybean, which are extensively reviewed elsewhere [14–16]. The flavonoids subgroup consists of six subclasses, which share a common structure of two aromatic rings. These are flavones, flavonols, flavanones, isoflavones, flavanols (catechins and proanthocyanidins) and anthocyanidins [35]. Summarized below are flavonoids of emerging research interest for their potential roles on protecting skeletal health during ageing. In addition, polyphenol-rich foods (olives and dried plums) that have increasingly gained attention for their implications in supporting bone health are also discussed below.

## Tea and grape flavanols

Flavanols exist in both the monomer form (e.g. catechins) and the polymer form (e.g. proanthocyanidins). The main flavanols include catechin, epicatechin, gallocatechin and epigallocatechin. Catechin and epicatechin are found in many types of fruit, but also in red wine, green tea (more than 80% of green tea polyphenols are catechins) and chocolate, whereas gallocatechin, epigallocatechin and epigallocatechin gallate occur in certain seeds of leguminous plants, in grapes and, above all, in tea. Tea, brewed from the dried leaves of the plant *Camellia sinensis*, is the most widely consumed beverage worldwide.

### *Clinical evidence for tea and grape flavanols*

Several epidemiological studies have reported reduced risk of hip fractures or higher bone BMD in habitual tea drinkers [36–38]. Despite these reports on the benefits of tea on human health, the osteoprotective effects of tea polyphenols and flavanols (including grape flavanols) using randomized control trials have been poorly investigated. Indeed, only a recent randomized control trial [39] has been published, in which 171 postmenopausal women with osteopenia received a supplement of green tea polyphenols (500 mg $day^{-1}$) and/or tai chi exercise for 6 months (Table 1). The findings of this short-term, 6 month clinical trial indicated that the consumption of green tea supplement provided higher values for serum bone-specific alkaline phosphatase (bone formation biomarker) after 4 weeks, while tai chi exercise provided higher values for bone-specific alkaline phosphatase after 12 and 24 weeks [39]. Neither green tea supplementation nor tai chi exercise had any effect on serum levels of tartrate-resistant acid phosphatase (bone resorption biomarker). Although the effects of green tea polyphenols on bone biomarkers are promising, a longer term clinical study assessing BMD is needed to confirm the bone-protective effects of green tea polyphenols in postmenopausal women [39].

### *Preclinical evidence for tea and grape flavanols*

Several lines of evidence concerning the osteoprotective effects of green tea on bone mass and microarchitecture in various induced bone loss models (by ageing, sex hormone deficiency and chronic inflammation) have been reported, as extensively reviewed elsewhere [40]. Green tea polyphenols provided daily in the drinking water of ovariectomized and sham-operated rats for 16 weeks resulted in higher femoral BMD and lower urinary levels of calcium compared with respective ovariectomized and sham control animals [41] (Table 1). These effects were accompanied by significant increases in urinary levels of epigallocatechin and epicatechin. Protective effects of green tea polyphenols on bone have also been observed in a model of bone loss induced by chronic inflammation [42, 43]. Grape seed proanthocyanidins could also have a potential role in skeletal protection. Grape seed proanthocyanidins extract was able to increase bone formation and bone strength at the mandibular bone in developing rats [44, 45]. Furthermore, grape seed proanthocyanidins extract supplementation was more effective in reversing debility of the mandibular condyle bone induced by a low-calcium diet than a standard diet or high-calcium diet alone. It would be of interest in the future to know whether grape seed proanthocyanidins extract supplementation could also protect skeletal mass and strength during ageing.

## Citrus flavanones

Hesperidin (hesperetin-7-*O*-rhamnoglucoside) represents one of the most abundant flavanones and is also the most studied flavanone with respect to bone health. The daily intake of hesperidin has not been precisely evaluated in different populations, but arguably it is relatively high for the flavanone class of polyphenols owing to worldwide consumption of citrus products, such as citrus fruits and juices (e.g. in Western countries, intakes of oranges range from 35 to 50 kg per person per year). Indeed, the content of hesperetin in oranges and in orange juice is considerable, ranging from 31 to 43.2 mg $(100\ g)^{-1}$ and from 200 to 700 mg $l^{-1}$, respectively [34, 46].

### *Clinical evidence for citrus flavanones*

Only one clinical study has been conducted on the ability of hesperidin to protect against postmenopausal bone loss [47] (Table 1). This study was a parallel, double-blind, placebo-controlled, 24 month randomized intervention trial assessing the effect of hesperidin on validated biomarkers of bone turnover and BMD. It was performed in healthy postmenopausal women (50–65 years old) not taking any hormone replacement therapy. Volunteers were assigned to either a hesperidin (500 mg hesperidin $day^{-1}$ in two biscuits) or placebo group (same biscuits without hesperidin). Subjects kept dietary records and minimized their citrus-rich food intake during the study period.

The yearly rates of bone loss (1–2%) were equivalent in the two groups. Evolution in BMD during the 2 years was not statistically different between the two groups.

However, the subjects consuming hesperidin presented a better balance in bone metabolism, as reflected by the bone turnover index (procollagen I N-terminal propeptide: carboxy-terminal collagen crosslinks type I ratio), during the second year of follow-up at the 18 and/or 21 month time points [47].

### *Preclinical evidence for citrus flavanones*

Dietary hesperidin at a level of 0.5% can improve bone mass in intact 3-month-old rats and protect against ovariectomy-induced bone loss in 6-month-old rats [48] (Table 1). These findings are in accordance with data obtained in ovariectomized mice fed with the same dose of hesperidin [49]. A further study examining hesperetin-7-glucoside, an intestinal metabolite of hesperidin which is more bioavailable than hesperidin itself, also demonstrated a greater efficiency than hesperidin in inhibiting bone loss resulting from ovariectomy in 6-month-old rats [50]. Positive effects of oranges as well as hesperidin on skeletal health have also been observed in growing [51] and older male rats [52]. These findings are in accordance with male orchidectomized rats consuming hesperidin through citrus juice [53]. Thus, hesperidin has the potential to play a protective role against the development of osteoporosis in both women and men.

The beneficial effects of hesperidin on bone mass have been mainly related to a slowing down in bone resorption (urinary free deoxypyridinoline). However, as first suggested by Chiba *et al*., hesperidin could not only modulate bone resorption but could also affect bone formation [49, 54]. Hesperidin may also exert protective effects on bone by modulating the production of inflammatory products [52].

## Olive polyphenols

Olives contain over 30 phenolic compounds, such as oleuropein, oleocanthin, tyrosol and hydroxytyrosol. The main phenolic compound of olive is oleuropein, and it is estimated that Mediterranean populations consume approximately 1.16 mg of oleuropein per day [55].

### *Clinical evidence for olive polyphenols*

Mediterranean populations are reported to have lower incidences of bone fractures compared with other European populations [56]. Epidemiological evidence suggests that adherence to certain dietary patterns of the Mediterranean diet (i.e. a high consumption of olive oil and fish and low consumption of red meat) and not to the Mediterranean diet *per se* (i.e. high consumption of plant foods and olive oil, low consumption of meat and dairy products, and moderate intake of alcohol) is associated with greater bone mass [57] (Table 1). Human data on the efficacy of olive polyphenols on bone health are still lacking; however, a number of preclinical data (described in the next subsection) demonstrate that polyphenolic compounds derived from olive may protect bone mass, especially in the presence of inflammation.

### *Preclinical evidence for olive polyphenols*

The effect of oleuropein was investigated by Puel *et al*. using a rat model of bone loss, associating ovariectomy and acute inflammation [58] (Table 1). All doses of oleuropein used [2.5, 5, 10 and 15 mg (kg bodyweight)$^{-1}$ day$^{-1}$] elicited protective effects on bone mass. It is interesting to note that no dose–response pattern was seen in this study, and the maximal bone effect was achieved at the lowest dose. Observations of a lower spleen weight *vs*. the respective control let to the hypothesis that oleuropein may exert its bone-sparing effect by modulating inflammation rather than by acting directly on bone metabolism. Neither oleuropein nor whole olive oil was able to affect BMD in ovariectomized rats when inflammation was not induced [59] (Table 1). Furthermore, a study to determine whether olive fruits might improve bone loss in ovariectomized rats and in ovariectomized rats with granulomatous inflammation was performed [60]. It was shown that black olive but not green olive was able to prevent bone loss in an experimental model of senile osteoporosis. Indeed, no protective effect was reported when rats were ovariectomized without induction of inflammation, as previously shown with pure oleuropein [59].

## Dried plum polyphenols

Dried plums, also known as prunes, the dried fruits of *Prunus domestica* L., are known to be rich in several polyphenols, including phenolic acid derivatives, flavonoids and coumarins. The total polyphenol content in dried plums has been reported to be 184 mg (100 g)$^{-1}$ [61]. The major components in these fruits are chlorogenic acid isomers (i.e. neochlorogenic acid, cryptochlorogenic acid and chlorogenic acid, which are esters of caffeic acid with quinic acid). The mean concentration of these chlorogenic acid isomers is as high as 174.2 mg (100 g)$^{-1}$, which represents more than 94% of total phenolics [61].

### *Clinical evidence for dried plum polyphenols*

Most evidence on the consumption of dried plums and skeletal health status stems from preclinical data [62]; however, two clinical trials conducted in postmenopausal women have been carried out. A short-term clinical study (3 months) with postmenopausal women demonstrated that dietary supplementation with 100 g of dried plums per day positively influenced the bone formation markers, bone-specific alkaline phosphatase and insulin-like growth factor-1 [63] (Table 1). A more recent clinical trial, 1 year in duration, using postmenopausal women fed 100 g of dried plums per day observed significantly increased BMD at the spine and ulna compared with baseline and

the dried apple control group [64] (Table 1), which also supports a beneficial effect linked to consumption of dried plums. A discrepancy was observed between these two trials, however, in relationship to the bone-specific alkaline phosphatase data. While the 3 month study observed an increase in bone-specific alkaline phosphatase, the 1 year study observed a decrease [64]. It is unknown why this discrepancy was found, but it may be due in part to differences in study designs, because the women in the short-term trial were advised to adjust their diets to account for the additional energy, protein and fat provided from the dried plums, while the long-term trial did not include this advice.

### *Preclinical evidence for dried plum polyphenols*

Most of the effects of dried plums on bone metabolism have been demonstrated using preclinical models of bone loss. In mice, consumption for 4 weeks of 25% dietary dried plums protected against ovariectomy-induced loss of BMD at the spine, while both 15 and 25% dietary dried plums protected against the deterioration of bone structure at both the spine and proximal tibial metaphysis [65] (Table 1). This study also demonstrated positive effects on bone strength at the spine using these doses. In addition, dried plums as both 15 and 25% of the diet restored some bone marrow myeloid and lymphoid populations and suppressed splenocyte activation, which occurs following ovarian hormone deficiency [65]. Thus, dried plums may protect ovariectomy-induced bone loss and deterioration of bone tissue and strength, in part by suppressing immune cell activation. In ovariectomized rats fed a standard diet for 40 days prior to treatments to establish bone loss, subsequent consumption of dried plums restored femoral and tibial bone density at doses as low as 5%. In addition, 5% dried plums resulted in higher trabecular microarchitecture in comparison with ovariectomized control animals at the end of the 60 day treatment [66]. Moreover, it was shown that the combination of 5% fructo-oligosacharride with 7.5% dried plums is capable of reversing ovariectomy-induced bone loss in 3-month-old female Sprague–Dawley rats, and this effect was enhanced when both compounds were added to soy-based diet [67]. Likewise, dried plums exert positive effects on bone mass, bone microarchitecture and bone strength in preclinical models of male osteoporosis [68–70], suggesting that this food may be an attractive strategy to explore further in both female and male clinical trials assessing skeletal health.

## Perspectives for future trials

Table 2 summarizes outcome measures that are commonly used in clinical and preclinical bone studies. The information obtained by each outcome measure offers insight into how the determinants of bone strength (i.e. bone quantity and bone quality) respond to various agents. Indeed, many preclinical studies, such as those described in the present review, are able to measure the mineral, material, structural and strength properties of bone directly, because *ex vivo* samples are easily obtained. In the clinical setting, however, the assessment of a comprehensive set of bone outcome measures, even in the most ideal conditions, may be limited by various factors, including the degree of invasiveness and costs associated with the outcome measure. Thus, BMD, the gold standard to determine skeletal responsiveness to various agents in clinical trials, and/or biochemical markers of bone turnover are commonly included as primary outcome measures to assess treatment response in clinical trials. Bone mineral density, however, is not always a reliable marker in predicting fracture risk because approximately half of all fractures that occur, at least in postmenopausal women, occur in women with BMD scores that do not meet the diagnostic criteria of osteoporosis [17–19]. It is, however, impossible to measure bone strength directly in humans because strength tests are invasive and destructive. In addition, measurement of bone turnover markers can be limited by biological and laboratory variations, as well as multiple methodologies used for the same analyte. Technological advances in quantitative computed tomography, which examines bone microarchitecture to predict the deformation of bone, can also predict fracture risk (Table 2) [71, 72]; however, it cannot be used to diagnose osteoporosis, and it is more expensive than measuring BMD by dual energy X-ray absorptiometry. Thus, BMD remains the gold standard in clinical bone studies and should be used when possible, along with biochemical markers of bone turnover, in future clinical trials that investigate the skeletal effects of phytonutrients or phytonutrient-rich foods. When feasible, other determinants of bone strength (e.g. bone structure) may provide valuable insight into the skeletal response of nutritional agents when BMD remains unchanged.

## Conclusion

The role of nutrition is of increasing interest for the support of skeletal health and for the prevention of osteoporosis, a disease which imposes significant health and financial burdens worldwide. While evidence to define a clear link between intakes of phytonutrients, in particular flavonoids, and bone health is currently insufficient to generate precise dietary recommendations, accumulating data suggest that the current public health guidance of 'five servings of fruit and vegetables each day' may also apply as a preventive strategy to slow down the development of osteoporosis. Indeed, the current guidance of five servings of fruit and vegetables each day highlights the possibility and probability that nutrition supports bone metabolism as a consortium of phytonutrients and other nutrients

## Table 2

Bone outcome measures used in clinical and preclinical trials

| Outcome measure | Technology | Species | Invasiveness | Description |
|---|---|---|---|---|
| BMD<br>BMC | Dual energy X-ray absorptiometry (DEXA) | Humans and animals | Not invasive; however, subjects are exposed to low doses of X-rays | Measures the BMC of a region of interest, after which the BMD can be calculated as follows: BMD = BMC (in grams)/area (in square centimetres).<br>A T-score is obtained and is compared with the BMD values of young healthy adults.<br>Most widely used technology for measuring BMD.<br>Up to 50% of fractures occur in postmenopausal women with normal BMD values, highlighting that BMD is not always a reliable marker for predicting fracture risk |
| BMD<br>Microarchitecture of cortical and trabecular bone<br>Prediction of bone strength using finite element analysis | Computed tomography (quantitative computed tomography for humans, mico- or nano-computed tomography for animals) | Humans and animals | Not invasive; however, subjects are exposed to low doses of X-rays | Measures the BMC of a region of interest, after which the volumetric BMD can be calculated as follows: BMD = BMC (in grams)/volume (in cubic centimetres).<br>Produces a three-dimensional image of bone, from which microarchitectural properties can be evaluated (e.g. trabecular number, trabecular separation, trabecular thickness, cortical surface area and cortical thickness).<br>Predictions of skeletal strength can be made using complex geometrical algorithms |
| Speed of sound (SOS)<br>Broadband ultrasound attenuation (BUA)<br>Stiffness Index (SI) | Quantitative ultrasound | Humans | Not invasive; no exposure to radiation | Provides an estimation of bone mass and skeletal quality.<br>Predictive ability of quantitative ultrasound is similar to that of DEXA .<br>-Low cost and portability of the instrument make quantitative ultrasound an attractive measure in various trials (e.g. children, remote locations where DEXA is not accssible or too costly to use) |
| Biochemical markers of bone turnover | Analytical instruments or kits (e.g. enzyme-linked immunosorbent assays and radioimmunoassays) | Humans and animals | Blood or urine collection required | Measures markers of bone formation (e.g. osteocalcin, alkaline phosphatase and type I collagen) and bone resorption (e.g. deoxypyridinoline, C-telopeptide of type I collagen, N-telopeptide of type I collagen and pyridinoline).<br>Some biochemical markers have a predictive value for fracture risk (e.g. C-telopeptide of type I collagen and procollagen I N-terminal propeptide) |
| Osteoblast number, osteoclast number, etc.<br>Bone formation rate, mineral apposition rate, etc. | Static and dynamic histomorphometry | Humans and animals | Invasive, because a bone biopsy is required | Static histomorphometry measures structural parameters of bone.<br>Dynamic histomorphometry measures rates of bone formation and bone resorption |
| Fracture | DEXA or radiography for confirmation.<br>Report of fracture to study investigators | Humans | Exposure to radiation if DEXA or radiography is used to confirm fracture | Used in long (≥2years) trials to determine whether interventions are effective in reducing the number of fractures at various skeletal sites (e.g. hip, spine and radius) |
| Bone strength parameters | Biomechanical strength-testing machine | Human cadavars and animals | None, because this destructive test is performed on bones excised from animals or human cadavers | Measures the amount of force a bone can withstand before it fractures.<br>Measures the elastic and plastic properties of bone |

Abbreviations are as follows: BMC, bone mineral content; and BMD, bone mineral density.

rather than in isolation. Thus, trials which examine the combined effects of various nutritional approaches, like those mentioned above, may provide more robust results regarding their effects on bone quantity and bone quality. In addition, human trials that include outcome measures related to bone quantity (BMD), bone quality (e.g. bone microarchitecture and bone turnover) and bone strength (using finite element analysis) should be implemented when possible to gain a more comprehensive outlook on how bone responds to these various nutritional factors.

## Competing Interests

All authors have completed the Unified Competing Interest form at www.icmje.org/coi_disclosure.pdf (available on request from the corresponding author) and declare; no support from any organisation for the submitted work, AS and CS have received funding from Soho Flordis International (SFI) in the previous 3 years; there are no other relationships or activities that could appear to have influenced the submitted work.

## REFERENCES


**1** NIH Consensus Development Panel on Osteoporosis Prevention Diagnosis and Therapy. Osteoporosis prevention diagnosis and therapy. JAMA 2001; 285: 785–95.

**2** WHO. Assessment of fracture risk and its application to screening for postmenopausal osteoporosis. Report of a WHO Study Group. World Health Organ Tech Rep Ser 1994; 843: 1–129.

**3** Kanis JA, Delmas P, Burckhardt P, Cooper C, Torgerson D. Guidelines for diagnosis and management of osteoporosis. The European Foundation for Osteoporosis and Bone Disease. Osteoporos Int 1997; 7: 390–406.

**4** Kanis JA, Johnell O. Requirements for DXA for the management of osteoporosis in Europe. Osteoporos Int 2005; 16: 229–38.

**5** Burge R, Dawson-Hughes B, Solomon DH, Wong JB, King A, Tosteson A. Incidence and economic burden of osteoporosis-related fractures in the United States, 2005–2025. J Bone Miner Res 2007; 22: 465–75.

**6** Heaney RP. Calcium, dairy products and osteoporosis. J Am Coll Nutr 2000; 19: 83S–99S.

**7** Heaney RP. Skeletal development and maintenance: the role of calcium and vitamin D. Adv Endocrinol Metab 1995; 6: 17–38.

**8** Bonjour JP. Protein intake and bone health. Int J Vitam Nutr Res 2011; 81: 134–42.

**9** Bonjour JP. Dietary protein: an essential nutrient for bone health. J Am Coll Nutr 2005; 24: 526S–36S.

**10** McNaughton SA, Wattanapenpaiboon N, Wark JD, Nowson CA. An energy-dense, nutrient-poor dietary pattern is inversely associated with bone health in women. J Nutr 2011; 141: 1516–23.

**11** Rivas A, Romero A, Mariscal-Arcas M, Monteagudo C, Feriche B, Lorenzo ML, Olea F. Mediterranean diet and bone mineral density in two age groups of women. Int J Food Sci Nutr 2012; [Epub ahead of print].

**12** New SA, Robins SP, Campbell MK, Martin JC, Garton MJ, Bolton-Smith C, Grubb DA, Lee SJ, Reid DM. Dietary influences on bone mass and bone metabolism: further evidence of a positive link between fruit and vegetable consumption and bone health? Am J Clin Nutr 2000; 71: 142–51.

**13** Okubo H, Sasaki S, Horiguchi H, Oguma E, Miyamoto K, Hosoi Y, Kim MK, Kayama F. Dietary patterns associated with bone mineral density in premenopausal Japanese farmwomen. Am J Clin Nutr 2006; 83: 1185–92.

**14** Lagari VS, Levis S. Phytoestrogens and bone health. Am J Clin Nutr 2000; 71: 142–51.

**15** Taku K, Melby MK, Kurzer MS, Mizuno S, Watanabe S, Ishimi Y. Effects of soy isoflavone supplements on bone turnover markers in menopausal women: systematic review and meta-analysis of randomized controlled trials. Bone 2010; 47: 413–23.

**16** Ishimi Y. Soybean isoflavones in bone health. Forum Nutr 2009; 61: 104–16.

**17** Delmas PD, Seeman E. Changes in bone mineral density explain little of the reduction in vertebral or nonvertebral fracture risk with anti-resorptive therapy. Bone 2004; 34: 599–604.

**18** Schuit SC, van der Klift M, Weel AE, de Laet CE, Burger H, Seeman E, Hofman A, Uitterlinden AG, van Leeuwen JP, Pols HA. Fracture incidence and association with bone mineral density in elderly men and women: the Rotterdam Study. Bone 2004; 34: 195–202.

**19** Stone KL, Seeley DG, Lui LY, Cauley JA, Ensrud K, Browner WS, Nevitt MC, Cummings SR. BMD at multiple sites and risk of fracture of multiple types: long-term results from the Study of Osteoporotic Fractures. J Bone Miner Res 2003; 18: 1947–54.

**20** Paiva SA, Russell RM. Beta-carotene and other carotenoids as antioxidants. J Am Coll Nutr 1999; 18: 426–33.

**21** Campbell JK, Engelmann NJ, Lila MA, Erdman JW Jr. Phytoene, phytofluene, and lycopene from tomato powder differentially accumulate in tissues of male fisher 344 rats. Nutr Res 2007; 27: 794–801.

**22** Rao AV, Rao LG. Carotenoids and human health. Pharmacol Res 2007; 55: 207–16.

**23** Sahni S, Hannan MT, Blumberg J, Cupples LA, Kiel DP, Tucker KL. Protective effect of total carotenoid and lycopene intake on the risk of hip fracture: a 17-year follow-up from the Framingham Osteoporosis Study. J Bone Miner Res 2009; 24: 1086–94.

**24** Sahni S, Hannan MT, Blumberg J, Cupples LA, Kiel DP, Tucker KL. Inverse association of carotenoid intakes with 4-y change in bone mineral density in elderly men and women: the Framingham Osteoporosis Study. Am J Clin Nutr 2009; 89: 416–24.

**25** Rao LG, Mackinnon ES, Josse RG, Murray TM, Strauss A, Rao AV. Lycopene consumption decreases oxidative stress and bone resorption markers in postmenopausal women. Osteoporos Int 2007; 18: 109–15.

**26** Wattanapenpaiboon N, Lukito W, Wahlqvist ML, Strauss BJ. Dietary carotenoid intake as a predictor of bone mineral density. Asia Pac J Clin Nutr 2003; 12: 467–73.

**27** Yang Z, Zhang Z, Penniston KL, Binkley N, Tanumihardjo SA. Serum carotenoid concentrations in postmenopausal women from the United States with and without osteoporosis. Int J Vitam Nutr Res 2008; 78: 105–11.

**28** Mackinnon ES, Rao AV, Josse RG, Rao LG. Supplementation with the antioxidant lycopene significantly decreases oxidative stress parameters and the bone resorption marker N-telopeptide of type I collagen in postmenopausal women. Osteoporos Int 2011; 22: 1091–101.

**29** Mackinnon ES, Rao AV, Rao LG. Dietary restriction of lycopene for a period of one month resulted in significantly increased biomarkers of oxidative stress and bone resorption in postmenopausal women. J Nutr Health Aging 2011; 15: 133–8.

**30** Rao LG, Krishnadev N, Banasikowska K, Rao AV. Lycopene I–effect on osteoclasts: lycopene inhibits basal and parathyroid hormone-stimulated osteoclast formation and mineral resorption mediated by reactive oxygen species in rat bone marrow cultures. J Med Food 2003; 6: 69–78.

**31** Xi-zhong Y, Cui-jie J, Xiao-liang C, Lei L. Effect of lycopene on bone mass and biomechanics in ovariectomized rats. J Clin Rehab Tissue Eng Res 2008; 12: 2811–14.

**32** Liang H, Yu F, Tong Z, Zeng W. Lycopene effects on serum mineral elements and bone strength in rats. Molecules 2012; 17: 7093–102.

**33** Cassidy A, Dalais FS. Phytochemicals. In: Nutrition and Metabolism, eds Gibney MJ, Macdonald IA, Roche HM. Oxford: Blackwell Publishing, 2003; 307–17.

**34** Manach C, Scalbert A, Morand C, Remesy C, Jimenez L. Polyphenols: food sources and bioavailability. Am J Clin Nutr 2004; 79: 727–47.

**35** Manach C, Williamson G, Morand C, Scalbert A, Remesy C. Bioavailability and bioefficacy of polyphenols in humans. I. Review of 97 bioavailability studies. Am J Clin Nutr 2005; 81: 230S–42S.

**36** Johnell O, Gullberg B, Kanis JA, Allander E, Elffors L, Dequeker J, Dilsen G, Gennari C, Lopes Vaz A, Lyritis G, Mazzuoli G, Miravet L, Passeri M, Perez Cano R, Rapado A, Ribot C. Risk factors for hip fracture in European women: the MEDOS Study. Mediterranean Osteoporosis Study. J Bone Miner Res 1995; 10: 1802–15.

**37** Kanis J, Johnell O, Gullberg B, Allander E, Elffors L, Ranstam J, Dequeker J, Dilsen G, Gennari C, Vaz AL, Lyritis G, Mazzuoli G, Miravet L, Passeri M, Perez Cano R, Rapado A, Ribot C. Risk factors for hip fracture in men from southern Europe: the MEDOS study. Mediterranean Osteoporosis Study. Osteoporos Int 1999; 9: 45–54.

**38** Hegarty VM, May HM, Khaw KT. Tea drinking and bone mineral density in older women. Am J Clin Nutr 2000; 71: 1003–07.

**39** Shen CL, Chyu MC, Yeh JK, Zhang Y, Pence BC, Felton CK, Brismee JM, Arjmandi BH, Doctolero S, Wang JS. Effect of green tea and Tai Chi on bone health in postmenopausal osteopenic women: a 6-month randomized placebo-controlled trial. Osteoporos Int 2012; 23: 1541–52.

**40** Shen CL, Yeh JK, Cao JJ, Chyu MC, Wang JS. Green tea and bone health: evidence from laboratory studies. Pharmacol Res 2011; 64: 155–61.

**41** Shen CL, Wang P, Guerrieri J, Yeh JK, Wang JS. Protective effect of green tea polyphenols on bone loss in middle-aged female rats. Osteoporos Int 2008; 19: 979–90.

**42** Shen CL, Yeh JK, Cao JJ, Tatum OL, Dagda RY, Wang JS. Green tea polyphenols mitigate bone loss of female rats in a chronic inflammation-induced bone loss model. J Nutr Biochem 2010; 21: 968–74.

**43** Shen CL, Yeh JK, Cao JJ, Tatum OL, Dagda RY, Wang JS. Synergistic effects of green tea polyphenols and alphacalcidol on chronic inflammation-induced bone loss in female rats. Osteoporos Int 2010; 21: 1841–52.

**44** Kamitani Y, Maki K, Tofani I, Nishikawa Y, Tsukamoto K, Kimura M. Effects of grape seed proanthocyanidins extract on mandibles in developing rats. Oral Dis 2004; 10: 27–31.

**45** Ishikawa M, Maki K, Tofani I, Kimura K, Kimura M. Grape seed proanthocyanidins extract promotes bone formation in rat's mandibular condyle. Eur J Oral Sci 2005; 113: 47–52.

**46** Kyle JAM, Duthie GG. Flavonoids in Foods. In: Flavonoids: Chemistry, Biochemistry and Applications, eds Andersen OM, Markham KR. Boca Raton, FL: CRC Press, 2006; 319–70.

**47** Habauzit V, Offord E, Chee W, Jafrelo L, Ameye L, Williamson G, Horcajada MN. Effect of two years dietary hesperidin supplementation on bone metabolism in postmenopausal women. Proceedings of the Annual Meeting of the American Society for Bone and Mineral Research. San Diego, CA, Sept. 16–29, 2011.

**48** Horcajada MN, Habauzit V, Trzeciakiewicz A, Morand C, Gil-Izquierdo A, Mardon J, Lebecque P, Davicco MJ, Chee WS, Coxam V, Offord E. Hesperidin inhibits ovariectomized-induced osteopenia and shows differential effects on bone mass and strength in young and adult intact rats. J Appl Physiol 2008; 104: 648–54.

**49** Chiba H, Uehara M, Wu J, Wang XX, Masuyama R, Suzuki K, Kanazawa K, Ishimi Y. Hesperidin, a citrus flavonoid, inhibits bone loss and decreases serum and hepatic lipids in ovariectomized mice. J Nutr 2003; 133: 1892–97.

**50** Habauzit V, Nielsen IL, Gil-Izquierdo A, Trzeciakiewicz A, Morand C, Chee W, Barron D, Lebecque P, Davicco MJ, Williamson G, Offord E, Coxam V, Horcajada MN. Increased bioavailability of hesperetin-7-glucoside compared with hesperidin results in more efficient prevention of bone loss in adult ovariectomised rats. Br J Nutr 2009; 102: 976–84.

**51** Muhlbauer RC, Lozano A, Reinli A, Wetli H. Various selected vegetables, fruits, mushrooms and red wine residue inhibit bone resorption in rats. J Nutr 2003; 133: 3592–97.

**52** Habauzit V, Sacco SM, Gil-Izquierdo A, Trzeciakiewicz A, Morand C, Barron D, Pinaud S, Offord E, Horcajada MN. Differential effects of two citrus flavanones on bone quality in senescent male rats in relation to their bioavailability and metabolism. Bone 2011; 49: 1108–16.

**53** Deyhim F, Garica K, Lopez E, Gonzalez J, Ino S, Garcia M, Patil BS. Citrus juice modulates bone strength in male senescent rat model of osteoporosis. Nutrition 2006; 22: 559–63.

**54** Uehara M. [Prevention of osteoporosis by foods and dietary supplements. Hesperidin and bone metabolism]. Clin Calcium 2006; 16: 1669–76.

**55** Vissers MN, Zock PL, Katan MB. Bioavailability and antioxidant effects of olive oil phenols in humans: a review. Eur J Clin Nutr 2004; 58: 955–65.

**56** Johnell O, Gullberg B, Allander E, Kanis JA. The apparent incidence of hip fracture in Europe: a study of national register sources. MEDOS Study Group. Osteoporos Int 1992; 2: 298–302.

**57** Kontogianni MD, Melistas L, Yannakoulia M, Malagaris I, Panagiotakos DB, Yiannakouris N. Association between dietary patterns and indices of bone mass in a sample of Mediterranean women. Nutrition 2009; 25: 165–71.

**58** Puel C, Mathey J, Agalias A, Kati-Coulibaly S, Mardon J, Obled C, Davicco MJ, Lebecque P, Horcajada MN, Skaltsounis AL, Coxam V. Dose-response study of effect of oleuropein, an olive oil polyphenol, in an ovariectomy/inflammation experimental model of bone loss in the rat. Clin Nutr 2006; 25: 859–68.

**59** Puel C, Quintin A, Agalias A, Mathey J, Obled C, Mazur A, Davicco MJ, Lebecque P, Skaltsounis AL, Coxam V. Olive oil and its main phenolic micronutrient (oleuropein) prevent inflammation-induced bone loss in the ovariectomised rat. Br J Nutr 2004; 92: 119–27.

**60** Puel C, Mardon J, Kati-Coulibaly S, Davicco MJ, Lebecque P, Obled C, Rock E, Horcajada MN, Agalias A, Skaltsounis LA, Coxam V. Black Lucques olives prevented bone loss caused by ovariectomy and talc granulomatosis in rats. Br J Nutr 2007; 97: 1012–20.

**61** Fang N, Yu S, Prior RL. LC/MS/MS characterization of phenolic constituents in dried plums. J Agric Food Chem 2002; 50: 3579–85.

**62** Hooshmand S, Arjmandi BH. Viewpoint: dried plum, an emerging functional food that may effectively improve bone health. Ageing Res Rev 2009; 8: 122–7.

**63** Arjmandi BH, Khalil DA, Lucas EA, Georgis A, Stoecker BJ, Hardin C, Payton ME, Wild RA. Dried plums improve indices of bone formation in postmenopausal women. J Wom. Health Gend Base Med 2002; 11: 61–8.

**64** Hooshmand S, Chai SC, Saadat RL, Payton ME, Brummel-Smith K, Arjmandi BH. Comparative effects of dried plum and dried apple on bone in postmenopausal women. Br J Nutr 2011; 106: 923–30.

**65** Rendina E, Lim YF, Marlow D, Wang Y, Clarke SL, Kuvibidila S, Lucas EA, Smith BJ. Dietary supplementation with dried plum prevents ovariectomy-induced bone loss while modulating the immune response in C57BL/6J mice. J Nutr Biochem 2012; 23: 60–8.

**66** Deyhim F, Stoecker BJ, Brusewitz GH, Devareddy L, Arjmandi BH. Dried plum reverses bone loss in an osteopenic rat model of osteoporosis. Menopause 2005; 12: 755–62.

**67** Arjmandi BH, Johnson CD, Campbell SC, Hooshmand S, Chai SC, Akhter MP. Combining fructooligosaccharide and dried plum has the greatest effect on restoring bone mineral density among select functional foods and bioactive compounds. J Med Food 2010; 13: 312–9.

**68** Franklin M, Bu SY, Lerner MR, Lancaster EA, Bellmer D, Marlow D, Lightfoot SA, Arjmandi BH, Brackett DJ, Lucas EA, Smith BJ. Dried plum prevents bone loss in a male osteoporosis model via IGF-I and the RANK pathway. Bone 2006; 39: 1331–42.

**69** Bu S, Lucas E, Franklin M, Marlow D, Brackett D, Boldrin E, Devareddy L, Arjmandi B, Smith B. Comparison of dried plum supplementation and intermittent PTH in restoring bone in osteopenic orchidectomized rats. Osteoporos Int 2007; 18: 931–42.

**70** Halloran BP, Wronski TJ, VonHerzen DC, Chu V, Xia X, Pingel JE, Williams AA, Smith BJ. Dietary dried plum increases bone mass in adult and aged male mice. J Nutr 2010; 140: 1781–7.

**71** Bouxsein ML, Seeman E. Quantifying the material and structural determinants of bone strength. Best Pract Res Clin Rheumatol 2009; 23: 741–53.

**72** Keaveny TM. Biomechanical computed tomography-noninvasive bone strength analysis using clinical computed tomography scans. Ann N Y Acad Sci 2010; 1192: 57–65.

**73** Kanis J, Johnell O, Gullberg B, Allander E, Elffors L, Ranstam J, Dequeker J, Dilsen G, Gennari C, Vaz AL, Lyritis G, Mazzuoli G, Miravet L, Passeri M, Perez Cano R, Rapado A, Ribot C. Risk factors for hip fracture in men from southern Europe: the MEDOS study. Mediterranean Osteoporosis Study. Osteoporos Int 1999; 9: 45–54.

**74** Shen CL, Yeh JK, Stoecker BJ, Chyu MC, Wang JS. Green tea polyphenols mitigate deterioration of bone microarchiteture in middle-aged female rats. Bone 2009; 44: 684–90.

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*Review Article*

# Potential Antiosteoporotic Agents from Plants: A Comprehensive Review

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Osteoporosis is a major health hazard and is a disease of old age; it is a silent epidemic affecting more than 200 million people worldwide in recent years. Based on a large number of chemical and pharmacological research many plants and their compounds have been shown to possess antiosteoporosis activity. This paper reviews the medicinal plants displaying antiosteoporosis properties including their origin, active constituents, and pharmacological data. The plants reported here are the ones which are commonly used in traditional medical systems and have demonstrated clinical effectiveness against osteoporosis. Although many plants have the potential to prevent and treat osteoporosis, so far, only a fraction of these plants have been thoroughly investigated for their physiological and pharmacological properties including their mechanism of action. An attempt should be made to highlight plant species with possible antiosteoporosis properties and they should be investigated further to help with future drug development for treating this disease.

## 1. Introduction

Osteoporosis is a systemic skeletal disease characterized by low bone mineral density (BMD) and microarchitectural deterioration of bone tissue, leading to a consequent increase in bone fragility and fracture risk. Hypogonadism is the most well-established cause of osteoporosis, which is usually thought to be an age-adjusted symptom [1]. In recent years, it has become a major health hazard afflicting more than 200 million people worldwide and has one of the highest incidence of all diseases in the elderly population [2]. The Health Departments in many countries are spending large amounts of money investigating new antiosteoporosis drugs every year.

Based on the principles of physiological bone regeneration and the role of osteoblasts and osteoclasts in the process, it is obvious that the rate of supply of new osteoblasts and osteoclasts, and the timing of the death of these cells by apoptosis are critical determinants of bone regeneration [3]. The activities of these cells are mainly associated with sex steroid deficiency, senescence, and glucocorticoid excess; furthermore, at menopause, the rate of bone remodeling increases precipitously. The loss of sex steroids upregulates the formation of osteoclasts and osteoblasts in the marrow by upregulating the production and action of cytokines, including IL-6, TNF, IL-1, and macrophage colony stimulating factors (M-CSF) which mediate osteoclastogenesis and osteoblastogenesis [4]. The imbalances between bone resorption and formation are due to an extension of the working lifespan of the osteoclasts and shortening of the working lifespan of the osteoblasts. The amount of bone formed during each remodeling cycle decreases with age in both sexes. In aging women, even in extreme old age, bone turnover is most likely increased by secondary hyperparathyroidism or by the continuing effect of estrogen deficiency [5]. Glucocorticoid excess decreases intestinal calcium absorption and hypercalciuria due to defective vitamin D metabolism. These changes result in increased bone resorption, decreased osteoblast proliferation and biosynthetic activity, and sex-steroid deficiency,

as well as hyperparathyroidism [6]. Glucocorticoid excess has a suppressive effect on osteoblastogenesis in the bone marrow and also promotes the apoptosis of osteoblasts and osteocytes. Glucocorticoids directly suppress BMP-2 (bone morphogenetic protein-2) and Cbf$\alpha$-1 (core binding factor$\alpha$1), two critical factors for osteoblastogenesis, and may also decrease the production of IGFs (insulin-like growth factors) while stimulate the transcriptional activity of PPAR-$\gamma$2 (peroxisome proliferator-activated receptor-$\gamma$2) [7].

There are certain risk factors which differ among individuals and are linked to the development of osteoporosis and contribute to the likelihood of developing the disease. These factors can be divided into two categories, the first being non-modifiable factors such as gender, age, body size, ethnicity, and family history, the other modifiable factors are sex hormones, anorexia nervosa, calcium and vitamin D intake, medication use, lifestyle, cigarette smoking, and alcohol intake, and so forth [2]. Physical exercise, dietary supplement, and pharmacotherapy are usually used for prevention and treatment of osteoporosis. The pharmacotherapy for osteoporosis is usually focused on accommodating the estrogen level or bone remodel. The mechanisms involves many aspects, such as stimulating parathyroid hormone (PTH) synthesizes; inducing the expression of OPG (osteoprotegerin); decreasing IL-1, 4, 6, and M-CSF; increasing estrogens or like-estrogens; supplementing Ca, P in bones; to inhibit the proliferation of osteoclast and induce osteoclast apoptosis; and to enhance the proliferation and differentiation of osteoblast. The drugs used mainly include estrogen, parathyroid hormone (PTH), various bisphosphonates, the selective oestrogen-receptor modulators (SERM) raloxifene, calcitonin, sodium fluoride, and calcium and vitamin D [8].

Calcium supplementation alone provides small beneficial effects on bone mineral density through postmenopausal life and may slightly reduce fracture rates, and vitamin D may be effective in deficient individuals [9]. The long-term hormone therapy for osteoporosis of postmenopausal women is controversial, because of increases in the risk of breast carcinoma, endometrial cancer, and cardiovascular disease. In postmenopausal women with osteoporosis and cardiovascular risk factors, combined oestrogen and progestagen or estrogen alone therapy should be avoided in favor of alternative antiresorptive agents. Hormone therapy remains an option only for short-term early use around the menopause in symptomatic women with high rates of risk fracture [10]. Bisphosphonates can reduce the risk of vertebral fractures and non-vertebral fractures including hip fractures. The dosing regimen (which require the patients to fast and remain upright for at least 30 min) and upper gastrointestinal side effects are often limiting factors in daily bisphosphonate therapy. Their duration of physiological effect is unclear, but bone turnover makers can remain suppressed for at least 5 years after their discontinuation [11]. Selective oestrogen-receptor modulators (including raloxifene, arzoxifene and lasofoxifene) are a chemically diverse set of compounds that do not have the steroid structure of oestrogen, but have a tertiary structure that allows binding to the oestrogen receptor to exert selective agonist or antagonist effects on different oestrogen target tissue. The most studied is raloxifene; its effects on markers of bone turnover and bone mineral density have generally been less than with biophosphonate therapy, so it should probably mainly be used in postmenopausal women with milder osteoporosis or in those with predominantly spinal osteoporosis. Potential side effects include an increase risk of venous thrombosis similar to that with hormone therapy and exacerbation of hot flushes [12]. The previously mentioned therapies act mainly to reduce bone resorption and the anabolic agent parathyroid hormone (PTH) mainly stimulates bone formation. The clinical trials in postmenopausal women showed PTH reduced the risk of fractures with 20 $\mu$g dose. However, the benefit in terms of bone mineral density seemed to wane after discontinuation unless followed by an antiresorptive agent [13]. In addition, strontium ranelate is a fairly new antiosteoporotic agent that has been approved in the European Union for the treatment of postmenopausal osteoporosis. It increase bone formation while reducing bone resorption, however its mechanism of action remains unclear. The clinical trials in postmenopausal women show that strontium ranelate reduces the risk of fractures and was well tolerated apart from a low rate of gastrointestinal side-effects and an increased risk of venous thrombosis [14].

Estrogen, bisphosphonates, calcitonin, calcium products, ipriflavone, and anabolic steroids are clinically used as effective medications [15]; however, each of them has established some side effects. Many medicinal plants have long been used to prevent and treat osteoporosis in many countries. These natural medicines derived from plants have fewer side effects and are more suitable for long-term use than synthesized drugs. These plant medicines containing numerous chemical constituents usually exert their therapeutic effects through multipathways and have multitargets, this property is parallel with the multiple factors of osteoporosis pathogenesis. In this paper, we summarize recent studies about antiosteoporotic medicinal plants with particular emphasis on the chemical constituents, mechanisms of action, and therapeutic applications. This will provide more information for the applications of medicinal plants in the prevention and treatment of osteoporosis.

## 2. Materials and Methods

The following computerized databases were searched from their inception to May 2012: MEDLINE (PUBMED), ALT HEALTH WATCH (EBSCO), and Google scholar. Text word search of titles and abstracts was conducted using the following entries in various conjunction or disjunction: osteoporosis, osteoblast, osteoclast, herbs, medicinal plant, natural product, herbal medicine, plant medicine, and phytomedicine. Each study included in this paper satisfies the following criteria: (i) the studies on antiosteoporotic activity were conducted on animal, or cultured osteoblast and osteoclast, and (ii) plant extracts, or compounds isolated from plant. The exclusion criteria consisted of (i) the herb studied was an herbal formula (i.e., neither a single herb nor a single herbal compound), (ii) the articles were not written in English or translated into English. Two reviewers

independently extracted the data and performed quality assessment.

## 3. Results

*3.1. Medicinal Herbs.* As shown in Table 1, literature survey showed that 76 medicinal plants were reported in ethnopharmacological studies for their potential benefits in osteoporosis treatment. These plants were distributed among 44 families, including Amaranthaceae (1 spp), Amaryllidaceae (1 spp), Apiaceae (6 spp), Berberidaceae (5 spp), Brassicaceae (1 spp), Campanulaceae (1 spp), Caprifoliaceae (1 spp), Compositae (4 spp), Convolvulaceae (1 spp), Davalliaceae (1 spp), Dicksoniaceae (1 spp), Dioscoreaceae (2 spp), Dipsacaceae (1 spp), Ericaceae (1 spp), Eucommiaceae (1 spp), Euphorbiaceae (1 spp), Fabaceae (11 spp), Ginkgoaceae (1 spp), Juglandaceae (1 spp), Labiatae (2 spp), Lauraceae (1 spp), Liliaceae (5 spp), Lythraceae (1 spp), Malvaceae (1 spp), Menispermaceae (1 spp), Myrsinaceae (1 spp), Oleaceae (1 spp), Orchidaceae (1 spp), Orobanchaceae (2 spp), Pleurotaceae (1 spp), Polypodiaceae (1 spp), Punicaceae (1 spp), Ranunculaceae (2 spp), Rosaceae (2 spp), Rubiaceae (1 spp), Rutaceae (2 spp), Scrophulariaceae (1 spp), Solanaceae (1 spp), Taxaceae (1 spp), Theaceae (1 spp), Ulmaceae (2 spp), Verbenaceae (1 spp), Vitaceae (1 spp), and Zingiberaceae (1 spp). The evaluations of antiosteoporotic activity of these plants are based on the animal experiment (58 spp), cultured osteoblast, and osteoclast in vitro (18 spp). The more highly represented botanic families were: Fabaceae (11 spp), Apiaceae (6 spp), Liliaceae (5 spp), and Compositae (4 spp). Among plant parts, root and rhizome (28 spp) were maximally utilized for antiosteoporosis. Among various parts of plants used in bone metabolism regulation, are root and rhizome (28 spp), fruit and seed (21 spp), stem and bark (13 spp), leaf (7 spp), whole plant and aerial parts (6 spp), and flower (1 spp). Multiple references were consulted for detailed information on research status of 10 plant species which are discussed below.

*3.1.1. Epimedium Plants. Epimedium* (Berberidaceae) is a low-growing, deciduous, perennial plant. The leaves of *E. brevicornum* Maxim., *E. sagittatum* (Sieb.et Zucc.) Maxim., *E. pubescens* Maxim., *E. wushanense* T. S. Ying, and *E. koreanum* Nakai have long been used to prevent and treat osteoporosis and other menopause diseases in China. These are the most frequently used herb drugs in antiosteoporotic Chinese traditional medicine formula [16]. Flavonoids including icariin, epimedin B, and epimedin C (Figure 1) are the main antiosteoporotic constituents, which inhibit bone resorption, stimulate bone formation, suppress urinary calcium excretion, and accordingly prevent osteoporosis without hyperplastic effects on the uterus in the ovariectomized (OVX) rat model [17]. The flavonoids from *Epimedium* plants possess an estrogen-like activity and modulate the bone metabolism through estrogen receptor pathway, and may improve the development of osteoblasts by promoting the ALP (alkaline phosphatase) activity through regulating the expression of IL-6, OPG, RANKL (receptor activator of nuclear factor-$\kappa$B ligand), M-CSF, Cbf$\alpha$1 (core binding factor$\alpha$1), BMP-2 and SMAD4 involved in the bone remodel and modulate proliferation and activity of osteoblasts and osteoclasts [18, 19]. *Epimedium* flavonoids enhance the mRNA expression of BMP-2, BMP-4, Runx2 (Runt-related transcription factor 2), and cyclinD1, all of which are BMP or Wnt-signaling pathway related regulators, indicating that *Epimedium* flavonoids exerts promoting effects on osteogenic differentiation, which plausibly functions via the BMP and Wnt/$\beta$-catenin signaling pathways [20].

Icariin (Figure 1), the main active flavonoid glucoside isolated from *Epimedium* plant, is found to have a therapeutic effect on osteoporosis in ovariectomy rat models and postmenopausal women and has been shown to suppress the loss of bone mass and strength in distal femur in tibia following OVX through increasing the mRNA expression ratio of OPG/RANKL [21, 22]. Icariin increases estrogen receptor (ER) dependent cell proliferation, ALP activity, and the OPG/RANKL ratio in UMR 106 cells, and increases ER$\alpha$ phosphorylation, showing that icariin exerts anabolic effects in bone possibly by activating ER [23]. In addition, icariin decreases the TRAP activity of osteoclasts, reduces the size of LPS-induced osteoclasts formation without inhibition of cell viability, inhibits LPS-induced bone resorption and the expression of IL-6 and TNF-$\alpha$. The synthesis of cyclooxygenase type-2 (COX-2) and prostaglandin $E_2$ ($PGE_2$), and expression of LPS-induced hypoxia inducible factor-1$\alpha$ (HIF-1$\alpha$) in osteoclasts, LPS-mediated activation of the p38 and JNK on osteoclasts is also inhibited. It also reduces the LPS-induced activation of ERK1/2 and I$\kappa$-B$\alpha$, indicating that icariin has an in vitro inhibitory effect on osteoclasts differentiation that can prevent inflammatory bone loss by suppressing activation of the p38 and JNK pathway [24].

Ikarisoside A (Figure 1), a natural flavonoid isolated from *E. koreanum,* exerts antioxidant potential and anti-inflammatory effects in LPS-stimulated bone marrow-derived macrophage precursor cells and RAW 264.7 cells, also inhibits osteoclastogenesis in RANKL-stimulated RAW 264.7 cells as well as in bone marrow-derived macrophages [25]. Ikarisoside A has been found to decrease the osteoclast-specific genes, like matrix metalloproteinase 9 (MMP-9), tartrate-resistant acid phosphatase (TRAP), receptor activator of NF-$\kappa$B (RANK), and cathepsin K, and blocks the resorbing capacity of RAW 264.7 cells on calcium phosphate-coated plates, and inhibits the RANKL-mediated activation of NF-$\kappa$B, JNK, and Akt. This indicates that Ikarisoside A has potential for use in treatment of diseases involving abnormal bone lysis such as osteoporosis, rheumatoid arthritis, and periodontal bone erosion [26].

*3.1.2. Glycine max* L. *Glycine max* L. (Fabaceae) originally grows in the southwest of Asia, and is now widely planted in warm areas. Its seed, also called soybeanis a common dietary supplement, and contains plenty of nutritional substances, such as proteins and flavonoids including genistein, daidzein and biochanin A (Figure 2). The soy flavonoids which are structurally and functionally related to 17-beta-estradiol have strong effects on bone metabolism in postmenopausal

Table 1: Antiosteoporotic medicinal plants.

| Family | Scientific name | Plant parts used | Reported relevant ethnomedical uses | Pharmacological study/chemical constituents | Reference |
|---|---|---|---|---|---|
| Amaranthaceae | *Achyranthes bidentata* Blume | Root | Bone related diseases | Decrease bone loss in OVX rats by inhibiting osteoclast formation/oleanolic acid glycosides, ecdysone and allantoin | [123, 124] |
| Amaryllidaceae | *Curculigo orchioides* Gaertn. | Rhizome | Impotence, tinnitus | Decrease bone loss by inhibiting bone resorption/phenolic glycosides | [106, 125] |
| Apiaceae | *Cnidium monnieri* (L.) Cuss. | Fruit | Impotence, lumbar pain | Reverse prednisone-induced bone mass loss, inhibit the high bone turnover; enhance osteoblastic proliferation and differentiation, inhibit formation and maturation of osteoclast/coumarins | [87, 126, 127] |
| Apiaceae | *Cuminum cyminum* L. | Fruit | Toothache, diarrhea, epilepsy | Prevent ovariectomy—induced bone loss/$\beta$-sitosterol, stigmasterol, luteolin and apigenin | [128] |
| Apiaceae | *Ferula hermonis* Boiss | Root | Frigidity, impotence | Prevent bone loss caused by severe estrogen deficiency by regulating calcium mobilization and mitochondrial permeability/daucane sesquiterpenes, ferutinin | [129] |
| Apiaceae | *Angelica sinensis* (Oliv.) Diels | Root | Hematopoietic, abnormal or painful menstruation, other women's diseases | Increase ALP activity and synthesis of collagenase type I of osteoblast/ligustilide, butylidene phihalide, ferulic acid | [130] |
| Araliaceae | *Panax notoginseng* (Burk.) F. H. Chen | Root | Trauma, injury of muscles, bone fracture | Prevent bone loss and deterioration of trabecular microarchitecture, stimulate proliferation and differentiation of osteoblast/triterpene saponins | [131, 132] |
| Araliaceae | *Acanthopanax senticosus* (Rupr. et Maxim.) Harms | Stem | Hypertension, rheumatism, ischemic heart disease, diabetes | Decrease bone loss in postmenopausal women/acanthosides, eleutherosides, senticoside, triterpen saponin, flavones | [133] |
| Berberidaceae | *Epimedium brevicornu* Maxim | Leaf | Impotence, prospermia, hyperdiuresis, osteoporosis, menopause syndrome, rheumatic arthritis, hypertension and chronic tracheitis | See section 3.1.1 | [16–26] |
| Berberidaceae | *Epimedium koreanum* Nakai | Leaf | | | |
| Berberidaceae | *Epimedium pubescens* Maxim | Leaf | | | |
| Berberidaceae | *Epimedium sagittatum* (Sieb. et Zucc.) | Leaf | | | |
| Berberidaceae | *Berberis aristata* DC | Stem bark | Menopausal disorders, osteoporosis | Decrease bone loss/berberine chloride, palmatine chloride, magnoflavine, canadine, berberastine, obaberine, columbavine and talifendine | [134, 135] |
| Brassicaceae | *Lepidium meyenii* Walp. | Root | Hot flushes, tender breast, vaginal dryness, osteoporosis | Improve the bone mass in OVX rats/macaridine, macaene, macamides, and maca alkaloids | [136, 137] |
| Campanulaceae | *Platycodon grandiflorum* (Jacq.) A. DC. | Root | Cough, chronic diseases | Stimulate osteoblast differentiation through p38 MAPK and ERK signaling pathways/saponin | [138] |
| Caprifoliaceae | *Sambucus williamsii* Hance | Stem and ramulus | Inflammation, bone fractures, joint diseases | Suppress the OVX-induced increase in bone turnover, inhibit bone resorption, stimulate bone formation/lignans | [139] |
| Compositae | *Carthamus tinctorius* L. | Seed | Ankyloenteron, rheumatism, and chronic nephritis | Prevent bone loss through modulation ALP and IGF-1/lignans, flavones, serotonins | [140, 141] |
| Compositae | *Silybum marianun* (L.) Gaertn | Seed | Liver disease | Prevent bone loss in rats induced by OVX with mild proliferative effects in uterus/silibinin, isosilibinin, silydianin and silychristin | [142, 143] |

Table 1: Continued.

| Family | Scientific name | Plant parts used | Reported relevant ethnomedical uses | Pharmacological study/chemical constituents | Reference |
|---|---|---|---|---|---|
| Compositae | *Wedelia calendulacea* Less. | Flower | Liver disorders, jaundice, uterine hemorrhage, menorrhagia | Promote bone formation, decrease bone loss/isoflavones and wedelolactone | [144] |
| Compositae | *Artemisia iwayomogi* Kitamura | Aerial parts | Diabetes and hepatitis | Stimulate bone formation/phenolic compounds | [145] |
| Convolvulaceae | *Cuscuta chinensis* Lam. | Seed | Sexual dysfunction, osteoporosis, senescence | Enhance osteoblast differentiation and mineralization/quercetin, kaempferol, isorhamnetin, hyperoside and astragalin | [146, 147] |
| Davalliaceae | *Davallia formosana* Hayata | Rhizome | Bone disease, osteoporosis | Prevent bone loss, enhance bone strength, inhibit the deterioration of trabecular microarchitecture via inhibition of bone resorption/(−)-epicatechin 3-O-$\beta$-D-allopyranoside | [148] |
| Dicksoniaceae | *Cibotium barometz* (L.) J. Sm. | Rhizome | Lumbago, rheumatism, polyuria, leucorrhoea | Prevent bone loss induced by ovariectomy, inhibit osteoclast formation | [149] |
| Dioscoreaceae | *Dioscorea alata* L. | Rhizome | Dyspnea, spermatorrhea, leucorrhagia, diabetes | Increase bone formation by inducing mesenchymal stem cells differentiation into osteoblasts | [150] |
| Dioscoreaceae | *Dioscorea spongiosa* J. Q. Xi et al. | Rhizome | Rheumatoid arthritis, bone disorder | Inhibit the decrease in bone mineral density, stimulate proliferation and mineralization of osteoblast, inhibit formation and bone resorption of osteoclast/seroidal saponins | [151, 152] |
| Dipsacaceae | *Dipsacus asperoides* C. Y. Cheng et T. M. Ai | Root | Traumatic ecchymoma, injury of muscles, bone fractures | Inhibit bone loss induced by ovariectomy, enhance osteoblast maturation and differentiation by increasing BMP-2 synthesis and activating p38 and ERK1/2/asperosaponin VI | [108, 153] |
| Ericaceae | *Vaccinium angustifolium* Aiton | Fruit | Cardiovascular disease | Prevent bone loss in ovarian hormone deficiency, stimulate osteoblast differentiation and reduce mesenchymal stromal cell senescence/phenolic acids (gallic acid, p-hydroxybenzoic acid, chlorogenic, p-coumaric, caffeic, ferulic and ellagic acids), flavonoids (anthocyanins, catechin, epichatechin, quercetin, kaempferol and myrecetin) | [154] |
| Eucommiaceae | *Eucommia ulmoides* Oliv. | Bark | Hypertension, renal injury | Prevent estrogen deficiency-induced bone loss, increase osteoblast proliferation and inhibit differentiation of osteoclast/lignans, iridoids, flavonoids and terpenoids | [155–157] |
| Euphorbiaceae | *Emblica officinalis* Gaertn. | Fruit | Dyslipidemia, atherosclerosis | Induce osteoclast apoptosis through downregulating the expression of IL-6 and NF-$\kappa$B | [158] |
| Fabaceae | *Erythrina variegata* Linn | Stem bark | Stomachache, rheumatism, eye ailments, swellings | Suppress the bone loss by inhibiting osteoclast differentiation and maturation/genistein derivatives | [159, 160] |
| Fabaceae | *Glycine max* (Linn.) Merr. | Seed | Cardiovascular disease, cancer, osteoporosis, renal function | See section 3.1.2 | [27–33] |
| Fabaceae | *Onobrychis ebenoides* Boiss. et Spruner | Whole plant | Estrogenic activity | Decrease bone loss without affecting body and uterine weight/isoflavones (ebenosin, afrormosin, formononetin and daidzein), benzofurans and benzoypyrans (ebenfuran I, ebenfuran II and ebenfuran III ) | [161–163] |

Table 1: Continued.

| Family | Scientific name | Plant parts used | Reported relevant ethnomedical uses | Pharmacological study/chemical constituents | Reference |
|---|---|---|---|---|---|
| Fabaceae | *Psoralea corylifolia* L. | Fruit | Bone fracture, osteomalacia and osteoporosis | See section 3.1.3 | [34–37] |
| Fabaceae | *Pueraria lobate* (Willd.) Ohwi | Root | Influenza, hypertension, angina pectoris | See section 3.1.4 | [38–41] |
| Fabaceae | *Pueraria mirifica* Airy Shaw et Suvatabandhu | Root | Reproductive organs, cardiovascular diseases, climacteric related symptoms | See section 3.1.4 | [42, 43] |
| Fabaceae | *Rhynchosia volubilis* Lour. | Seed | Toothache, rheumatic arthritis, snake bite | Facilitate osteoblastic MG-63 cell proliferation/genistein and daidzein | [164] |
| Fabaceae | *Sophora japonica* L. | Fruit | Hematochezia, bleeding hemorrhoids | Suppress formation and differentiation of osteoclast/isoflavonoids | [165, 166] |
| Fabaceae | *Butea monosperma* (L.) Kuntze | Stem bark | Bone fracture | Prevent OVX-induced bone loss by stimulating bone formation/methoxyisoflavones (cajanin, isoformononetin, cladrin and medicarpin) | [167] |
| Fabaceae | *Phaseolus vulgaris* L | Seed | Estrogenic activity | Prevent estrogen deficiency-induced osteopenia without affecting the uterine mass | [168] |
| Fabaceae | *Trifolium pratense* L. | Aerial parts | Menopause symptoms, cardiovascular disease | See section 3.1.5 | [44–47] |
| Ginkgoaceae | *Ginkgo biloba* Linn. | Leaf | Cardiovascular disease | Reverse bone loss in glucocorticoid-induced osteoporosis and mandibular osteoporosis/kaempferol, quercetin, isorhamnetin, and terpenoids (ginkgolides and bilobalides) | [169, 170] |
| Juglandaceae | *Juglans regia* L. | Fuit | Heart disease, prostate cancer, hyperlipidemic | Induce nodule formation of osteoblast/ellagic acid, $\alpha$-tocopherol, fatty acids, flavonoids and phenolic acids | [171] |
| Labiatae | *Ajuga decumbens* Thunb. | Whole plant | Hypertension, hemoptysis, carbuncles and joint pain | Downregulate the differentiation of osteoclast, upregulate mineralization of osteoblast-like MC3T3-E1 cells | [172] |
| Labiatae | *Salvia miltiorrhiza* Bge | Root | Cardiovascular diseases | See section 3.1.6 | [48–52] |
| Lauraceae | *Cinnamomum cassia* (L.) C. Presl | Bark | Dyspepsia, gastritis, blood circulation disturbances, inflammatory diseases | Stimulate bone formation in vitro and may contribute to the prevention of osteoporosis and inflammatory bone diseases/cinnamic aldehyde, cinnamic alcohol, cinnamic acid, and coumarin | [173] |
| Liliaceae | *Allium cepa* L. | Bulb | Insomnia, hyperglycemic, Hyperlipidemic | Decrease the ovariectomy-induced bone resorption via attenuation of RANKL—induced ERK, p38, and NF-$\kappa$B activation | [174] |
| Liliaceae | *Allium sativum* L. | Bulb | Influenza, dysentery, tuberculosis | Prevent bone loss, reverse the low BMD and low tensile strength caused by ovariectomy/allicin, allylmethyltrisulphide, diallyldisulphide, ajoene, monoterpenes (citral, geraniol and linalool), and flavonoids (quercetin and rutin) | [175] |
| Liliaceae | *Anemarrhena asphodeloides* Bge. | Rhizome | Lung disease, fever, diabetes and constipation | Prevent OVX-induced bone loss in rats through the promotion of bone formation but not the inhibition of bone resorption/steroidal saponins | [176] |

Table 1: Continued.

| Family | Scientific name | Plant parts used | Reported relevant ethnomedical uses | Pharmacological study/chemical constituents | Reference |
|---|---|---|---|---|---|
| Liliaceae | *Polygonatum sibiricum* Red. | Rhizome | Hypotension, Hyperglycemic, Hyperlipidemic | Prevent bone loss/polysaccharide | [177] |
| Linaceae | *Linum usitatissimum* L. | Seed | Postmenopausal osteoporosis | See section 3.1.7 | [53–56] |
| Lythraceae | *Heimia myrtifolia* Cham. | Leaf | Osteoporosis | Stimulate formation and mineralization of osteoblastic cell lines HOS58 and saos-2/vertine (cryogenine), lythrine, lythridine, polyphenols | [178] |
| Malvaceae | *Abelmoschus manihot* (L.) Medik. | Leaf | Chronic glomerulonephritis | Reduce bone loss in conditions of estrogen deficiency/calcium | [179] |
| Menispermaceae | *Tinospora cordifolia* (Willd.) Miers | Stem | Dyspepsia, fever, urinary diseases | Estrogenic activity, prevent bone loss in ovariectomized rats/alkaloids, terpenoids, glycosides, sterols, lactones and fatty acids | [180] |
| Myrsinaceae | *Labisia pumila* var. *alata* (Scheff.) Mez. | Root | Menstrual irregularities, painful menstruation | Prevent the changes in bone biochemical markers but failed to prevent the bone calcium loss induced by ovariectomy/C15 monoene resorcinols, phenolic compounds, flavonoids | [181] |
| Oleaceae | *Ligustrum lucidum* Ait. | Fruit | Menopausal problems, tinnitus, rheumatic pains, palpitations, insomnia symptoms | Improve bone properties in aged rats via increasing osteoblast formation and mineralization/oleanolic acid, ursolic acid, acetyloleanolic acid | [182, 183] |
| Orchidaceae | *Anoectochilus formosanus* Hayata | Whole plants | Lung disease, pleurodynia, abdominal pain, fever, hypertension and snake bites | Suppress the bone loss caused by estrogen deficiency through suppression of RANKL expression required for osteoclast formation. | [184] |
| Orobanchaceae | *Cistanche deserticola* Y. C. Ma | Stem | Forgetfulness, loss of hearing, chronic constipation. | Enhanced bone mineral density and bone mineral content/harmine | [101] |
| Orobanchaceae | *Cistanche salsa* (C. A. Mey.) G. Beck | Stem | Kidney deficiency, neurasthenia | Suppress bone loss in ovariectomized mice/(2E, 6R)-8-hydroxy-2, 6-dimethyl-2-octenoic acid | [185] |
| Pleurotaceae | *Pleurotus eryngii* (De Candolle: Fr.) Quel. | Fruiting body | Liver, kidney and gastrointestinal disorders | Alleviate the decrease in the trabecular bond mineral density in ovariectomized rats, increase the ALP activity and secretion of osteoprotegerin, improve the osteocalcin mRNA and Runx2 gene expression in osteoblasts; Decrease the number of tartrate-resistant acid phosphatase (TRAP)-positive multinucleated cells and resorption areas of osteoclast | [186] |
| Polypodiaceae | *Drynaria fortunei* (Kunze) J. Sm. | Rhizome | Bone fractures and joint diseases | See section 3.1.8 | [57–61] |
| Punicaceae | *Punica granatum* Linn. | Fruit | Parasitic infections, ulcers, diarrhea, dysentery, hemorrhage, respiratory pathologies | Increase bone volume and trabecular number, and decrease trabecular separation in OVX rats/genistein, daidzein, ellagitannins and ellagic acid | [187] |
| Ranunculaceae | *Cimicifuga foetida* L. | Rhizome | Cooling and detoxification agent | Inhibit osteoclastic bone resorption, increase BMD in OVX mice/oxidized cycloartane-type triterpenoids and phenol type derivatives | [188] |
| Ranunculaceae | *Cimicifuga racemosa* (L.) Nuttall | Rhizome | Dysmenorrhea, labor pains, menopausal symptoms | See section 3.1.9 | [62–66] |

Table 1: Continued.

| Family | Scientific name | Plant parts used | Reported relevant ethnomedical uses | Pharmacological study/chemical constituents | Reference |
|---|---|---|---|---|---|
| Rosaceae | *Prunus mume* Sieb et ZUCC. | Fruit | Chronic gastritis | Increase alkaline phosphatase activity, cell proliferation and mineralization, enhance the expression of BMP-2 of osteoblast/citric acid, malic acid, chlorogenic acid and 5-hydroxymethy-furfural | [189, 190] |
| Rosaceae | *Rubus coreanus* Miq. | Fruit | Impotence, spermatorrhoea, and back pain | Prevent bone loss caused by estrogen deficiency by dual regulation of the enhancement of osteoblast function and induction of osteoclast apoptosis/ellagic acid, fupenzic acid, $\beta$-sitosterol | [191, 192] |
| Rubiaceae | *Morinda officinalis* How | Root | Rheumatism | See Section 3.1.10 | [67–71] |
| Rutaceae | *Poncirus trifoliata* (L.) Raf. | Fruit | Gastritis, dysentery, digestive tract ulcers, uterine contraction, and cardiovascular diseases | Inhibit glucocorticoid-induced bone loss by decreasing expression of anxA6/flavone (poncirin, hesperidin, rhoifolin, naringin, neohesperidin) | [193] |
| Rutaceae | *Citrus paradisi* Macf. | Fruit | Digestion system, lose weight | Improve bone quality by enhancing bone mineral deposition in ORX rats/vitamin C, hesperidin and limonoids | [194, 195] |
| Scrophulariaceae | *Rehmannia glutinosa* Libosch | Root | Haemostatic, cardiotonic, and diuretic agent | Increase ALP activity and the expression of the OPG of osteoblast, decrease the number of TRAP-positive MNCs and the resorption areas of osteoclast, alleviate the decrease in the trabecular BMD, and increase the cortical bone thickness ovariectomy-induced osteoporotic rats/luteolin, mannitol, stigmasterol, campesterol, catalpol, rehmannin. | [196] |
| Solanaceae | *Withania somnifera* Dunn. | Root | Nerve diseases and anxiety | Inhibit bone loss in ovariectomized rats/withanolides | [197] |
| Taxaceae | *Taxus yunnanensis* cheng et L.K. | Seed, bark | Cancer | Increase bone mineral content and bone mineral density in ovariectomized rats/isotaxiresinol, taxol, harringtonine | [90] |
| Theaceae | *Stewartia koreana* Nakai ex Rehd. | Leaf | Inflammatory diseases | Inhibit osteoclast differentiation and prevent inflammatory bone loss/spinasterol glycoside | [198] |
| Ulmaceae | *Ulmus davidiana* Planch. | Bark | Oedema, mastitis, gastric cancer and inflammation | Promote osteoblastic differentiation by increasing bone morphogenic protein-2 as well as ALP mRNA expression in MC3T3-E1 cells, inhibit bone resorption/davidianones A, B, and C, mansonones E, F, H, and I | [199] |
| Ulmaceae | *Ulmus wallichiana* Planch. | Bark | Bone fracture | Mitigate ovariectomy-induced osteoporosis in rats, stimulate osteoblast function and inhibit osteoclast differentiation/quercetin-6-C-$\beta$-D-glucopyranoside | [200–203] |
| Verbenaceae | *Vitex agnus-castus* L. | Fruit | Premenstrual symptoms, climacteric complaints | Protect bone in orchidectomized rats/apigenin, cascitin, and dopaminergic compounds | [204] |
| Vitaceae | *Cissus quadrangularis* L. | Aerial parts, root | Hemorrhoids, menstrual disorders, scurvy, flatulence, bone fractures, bone diseases | Prevent bone loss in ovariectomized rats, stimulate osteoblastogenesis through up-regulation of MAPK-dependent alkaline phosphatase activity/$\beta$-sitosterol, $\delta$-amyrin, $\delta$-amyrone, favanoids (quercetin), 6′-O-trans-cinnamoyl-catalpol | [205–207] |

Table 1: Continued.

| Family | Scientific name | Plant parts used | Reported relevant ethnomedical uses | Pharmacological study/chemical constituents | Reference |
|---|---|---|---|---|---|
| Zingiberaceae | *Curcuma comosa* Roxb. | Rhizome | Postpartum, uterine bleeding, inflammation | Prevent bone loss induced by estrogen deficiency/diarylheptanoids, curcumin | [208, 209] |

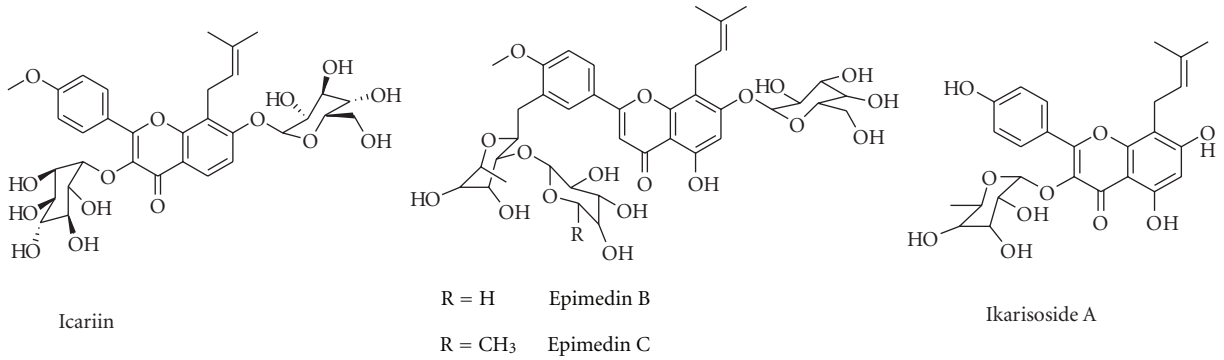
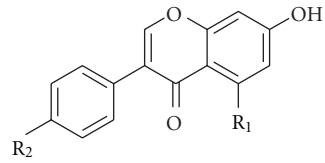



Figure 1: Chemical structure of compounds from *Epimedium* plants.

women and have a role in the prevention and treatment of postmenopausal osteoporosis [27]. Epidemiological studies and clinical trials suggest that soy isoflavones have beneficial effects on bone mineral density, bone turnover markers, and bone mechanical strength in postmenopausal women. The diet containing 22% soybean protein can be just as effective as daily estrogen administration in suppressing bone loss induced by ovariectomy. However, unlike estrogen, a soybean protein diet does not have uterotrophic side effects, and does not decrease the markers of bone turnover. The modulation of soybean protein and flavonoids on nuclear receptors focuses especially on the expression of receptors for estrogens, progesterone, androgen, vitamin D, retinoic acid, and thyroid hormones as well as the potential impact on physiological functions [28]. Soy flavonoids can modulate trabecular microstructural properties, inhibit bone loss in both osteoporotic animal models and postmenopausal women by regulating bone metabolism-related gene expression, including calciotropic receptor, cytokines, growth factors, ALP, collagen type I (COL I), and osteocalcin. In addition, soy flavonoids, phytoestrogen, in chemical structure, are antiestrogenic on both ER alpha and ER beta-dependent gene expression in the brain and estrogen-dependent behavior [29, 30].

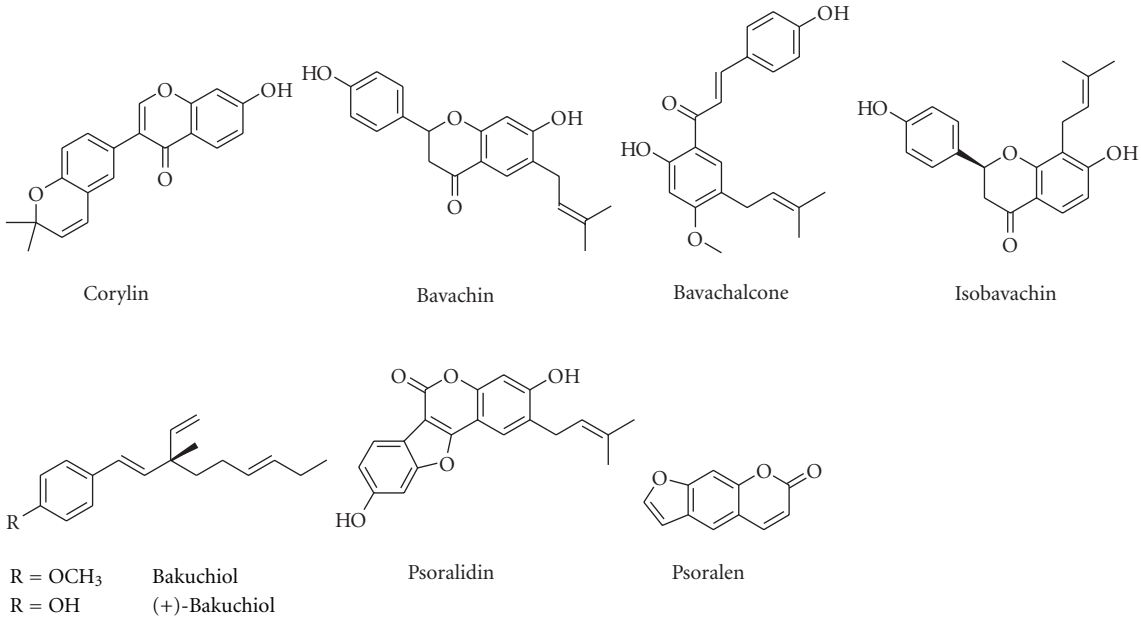
Genistein (Figure 2) exhibits estrogenic action in bone and bone marrow to regulate B-lymphopoiesis and prevent bone loss without exhibiting estrogenic action in the uterus. The mechanism through which flavonoids may exert antiosteoporotic effects seems to depend, at least in part, on their mixed estrogen agonist-antagonist properties. An alternative hypothetical mechanism could derive from other biochemical actions of flavonoids such as inhibition of enzymatic activity, in particular protein kinases, or activation of an "orphan" receptor distinct from the estrogen type I receptor [31]. The results from intervention studies are still controversial. One of the potential reasons for these inconsistencies could be due to the individual differences in the flavonoids metabolism. Recently, it has been suggested that the clinical effectiveness of flavonoids might partly depend on the ability to produce equol, a gut bacterial metabolite of daidzein showing stronger estrogenic activity than the predominant flavonoids [32, 33].

*3.1.3. Psoralea corylifolia* L. *Psoralea corylifolia* L. belongs to Fabaceae, the fruit is one of the commonly used herbs in formulas that are prescribed for the treatment of fractures, bone and joint diseases. Recent research suggests that *P. corylifolia* has potent oestrogenic effects and that its fruits may be a useful remedy for bone fractures, osteomalacia and osteoporosis [34]. The extract of *P. corylifolia* fruits cannot only significantly increase the concentration of inorganic phosphorus in serum, but also evidently promote bone calcification in rats. Both the extracts of its fruits and seeds and two isoflavones (corylin and bavachin, Figure 3) isolated from this plant can stimulate bone formation and have potential antiosteoporotic activity [35]. Bavachalcone (Figure 3) inhibits osteoclastogenesis by interfering with the ERK and Akt signaling pathways and the induction of c-Fos and NFATc1 during differentiation. Components derived from *P. corylifolia*, including bakuchiol, corylin, psoralidin, and isobavachin (Figure 3), have strong antioxidant activities, and corylin and bavachin have been shown to stimulate osteoblastic proliferation. Bakuchiol has a three-fold higher binding affinity for ER$\alpha$ than for ER$\beta$. Bakuchiol and extracts treatments had no uterotrophic activity even though they demonstrated oestrogenic activity in the in vitro assays, and reduced postmenopausal bone loss by increasing ALP, Ca concentrations, serum $E_2$ concentration, and bone mineral density [36]. Psoralen (Figure 3), a coumarin-like derivative extracted from fruits of *P. corylifolia* L., has been



| | $R_1$ | $R_2$ |
|---|---|---|
| Genistein | OH | OH |
| Daidzein | H | OH |
| Biochanin A | OH | $OCH_3$ |

Figure 2: Chemical structure of compounds from *Glycine max* L.



Corylin | Bavachin | Bavachalcone | Isobavachin

| | |
|---|---|
| R = $OCH_3$ | Bakuchiol |
| R = OH | (+)-Bakuchiol |

Psoralidin | Psoralen

Figure 3: Chemical structure of compounds from *Psoralea corylifolia* L.

reported to posses stimulatory effect on local new bone formation in vivo, and promote osteoblast differentiation in primary mouse calvarial osteoblasts in a dose-dependent manner by upregulation of expressions of osteoblast-specific marker genes including type I collagen, osteocalcin and bone sialoprotein and enhancement of ALP activity. It also upregulates the expression of BMP-2 and BMP-4 genes, increases the protein level of phospho-Smad1/5/8, and activates BMP reporter (12xSBE-OC-Luc) activity in a dose-dependent manner, as well as enhancing the expression of Osx, the direct target gene of BMP signaling. This suggests that psoralen acts through the activation of BMP signaling to promote osteoblast differentiation and demonstrates that psoralen could be a potential anabolic agent to treat patients with bone loss-associated diseases such as osteoporosis [37].

*3.1.4. Pueraria lobata* (Willd.) Ohwi *and P. mirifica* Airy Shaw et Suvatabandhu. *Pueraria lobata* (Willd.) Ohwi is a wild creeper plant of family Fabaceae. Its root, which is one of the earliest and most important crude herbs used in Chinese medicine for various medicinal purposes has a high content of isoflavonoids such as daidzein and genistein (Figure 2). The root of *P. lobata* shows a preventive effect on bone loss by increasing the BMD (bone mineral density) and BMC (bone mineral content) in the rats and mice of ovariectomy and orchidectomy without exhibiting estrogenic action in the uterus [38–40]. Puerarin (Figure 4), a natural isoflavonoid found in *P. lobata*, caused a significant increase in cell viability, ALP activity and mineral nodules formation in osteoblasts through activation of the PI3K/Akt pathway [41].

In Thailand, another species of the genus *Pueraria* plant, *P. mirifica* Airy Shaw et Suvatabandhu has been thoroughly examined for its estrogenic effects on female reproductive organs, which exhibited a higher estrogenic activity on reproductive organs than that of *P. lobata*. The long-term administration of *P. mirifica* prolongs the menstrual cycle length, suppresses folliculogenesis and ovulation in adult female monkeys, and decreases serum luteinizing hormone and follicle stimulating hormone levels, indicating that *P. mirifica* has an estrogenic effect on female reproductive systems. Phytoestrogens found in *P. mirifica* can be categorized

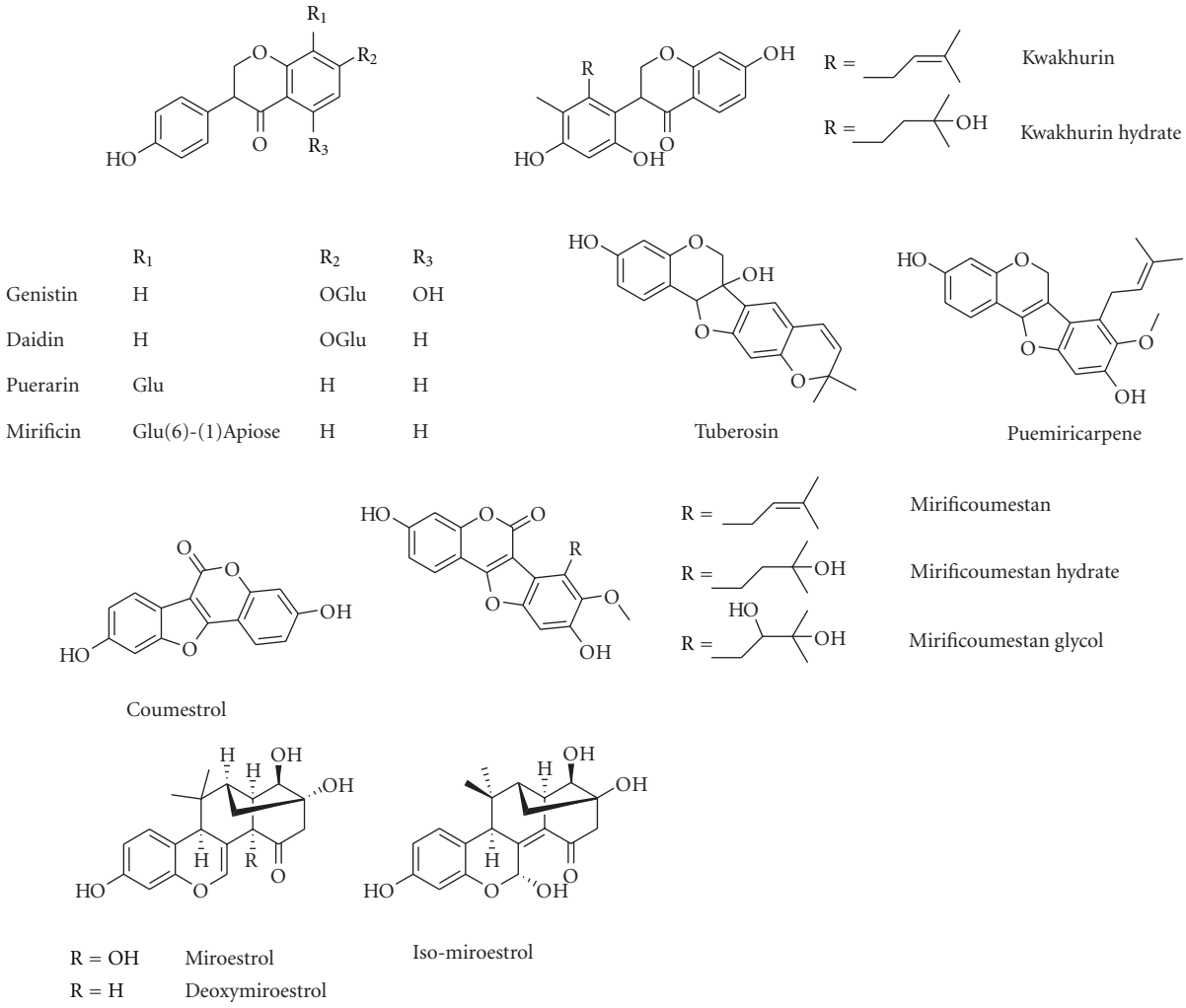
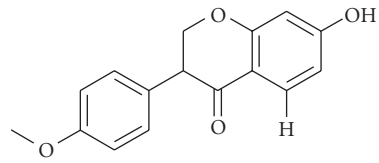



Figure 4: Chemical structure of compounds from *Pueraria lobata* (Willd.) Ohwi and *P. mirica* Airy Shaw et Suvatabandhu.

into three groups as (i) ten isoflavonoids, comprised of daidzein, daidzin, genistin, genistein, kwakhurin, kwakhurin hydrate, tuberosin, puerarin, mirificin and puemiricarpene (Figure 4); (ii) four coumestrans, comprised of coumestrol, mirificoumestan, mirificoumestan glycol and mirificoumestan hydrate (Figure 4); and (iii) three chromenes, comprised of miroestrol, deoxymiroestrol, and isomiroestrol (Figure 4), which are rich in the plant and are known for preventing bone loss induced by estrogen deficiency [42]. *P. mirifica* dose-dependently prevents bone loss induced by orchidectomy and ovariectomy, and can be used a preventative medicine or as a therapeutic agent for the symptoms related to estrogen deficiency in menopausal women as well as in andropausal men [43].

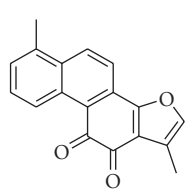
*3.1.5. Trifolium pratense* L. *Trifolium pratense* (red clover) is one of the 250 species of the genus *Trifolium* belonging to Fabaceae. Red clover has been cultivated in Europe since the third or fourth century and contains four detectable estrogenic isoflavones: daidzein (Figure 2), genistein (Figure 2), formononetin (Figure 5), and biochanin A (Figure 2). Its isoflavones are effective in decreasing bone loss induced by ovariectomy, probably by reduction of the bone turnover via inhibition of bone resorption [44, 45]. Daidzein can inhibit the proliferation and differentiation of osteoclasts; this is possibly due to increasing apoptosis of osteoclast progenitors mediated by ERs. The mechanism of action of isoflavones is evidently different from that of estrogens, which have a phytoestrogen-mediated stimulation in osteoblasts rather than an inhibition in osteoclasts [46, 47].

*3.1.6. Salvia miltiorrhiza Bunge. Salvia miltiorrhiza* Bunge (Labiatae), a traditional Chinese medicine, widely used in clinical practice for the prevention and treatment of cardiocerebral vascular diseases. Pharmacological testing showed that *S. miltiorrhiza* has anticoagulant, vasodilatory, increased blood flow, anti-inflammatory, free radical scavenging, mitochondrial protective activities. Phytochemical studies revealed multiple groups of compounds from *S. miltiorrhiza* Bunge extract, the main constituents of which include

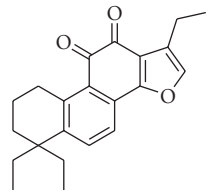


Formononetin

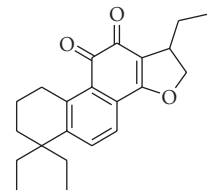
Figure 5: Chemical structure of formononetin.



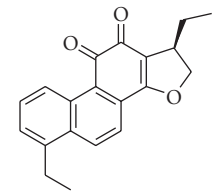
Tanshinone I



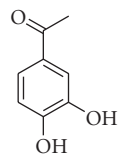
Tanshinone II A



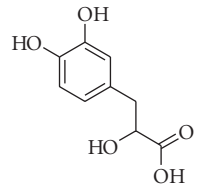
Cryptotanshinone



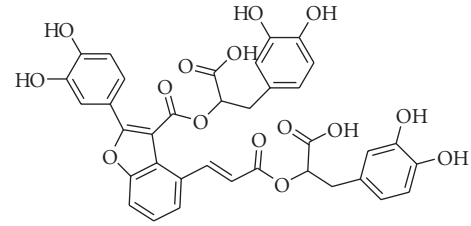
15,16-dihydrotanshinone I



Protocatechuicaldehyde



Salvianolic acid A



Salvianolic acid B

Figure 6: Chemical structure of compounds from *Salvia miltiorrhiza* Bunge.

tanshinones (tanshinone I, tanshinone IIA, cryptotanshinone, 15, 16-dihydrotanshinone I) and phenolics (protocatechuic aldehyde, salvianolic acid A, and salvianolic acid B) (Figure 6) [48]. *S. miltirrhiza* treatment significantly ameliorate the decrease in BMD and trabecular bone mass, decreases the TRAP activity and oxidative stress parameters including MDA (malondialdehyde) and NO (nitric oxide) induced by OVX in castrated male mice [49]. The tanshinones can reduce the formation of TRAP-positive multinuclear osteoclasts; Tanshinone IIA (Figure 6) can partially prevent ovariectomy-induced bone loss by suppressing bone turnover in vivo without stimulating osteoblast ALP activity, suppress osteoclast formation by inhibiting the expression of c-fos and NFATc1 induced by RANKL [50]. Salvianolic acid A, the aqueous bioactive component from *S. miltiorrhiza* Bunge, effectively prevents bone loss from long-term administration of prednisone in rats, protects bone from glucocorticoid induced bone marrow impairment by stimulating osteogenesis and depressing adipogenesis in bone marrow stromal cells [51]. Salvianolic acid B, another aqueous bioactive component, prevents glucocorticoid induced cancellous bone loss and decreases adipogenesis. Salvianolic acid B stimulates bone marrow stromal cell (MSC) differentiation to osteoblast and increases osteoblast activities, whilst decreasing glucocorticoid associated adipogenic differentiation through regulating the mRNA expression of PPAR-$\gamma$, Runx2, Dickkopf-1, and $\beta$-catenin in MSC [52].

*3.1.7. Linum usitatissimum* L. *Linum usitatissimum* L. originally grows in Europe and warm areas of Asia, and is now widely cultivated in warm areas including America, Canada and North Europe. Its seed, also called linseed or flaxseed, can potentially exert positive effects on bone of postmenopausal women. Flaxseed is the richest source of lignans including enterodiol, enterolactone, secoisolariciresinol, and matairesinol (Figure 7), all of which are reported to have both weak estrogenic and anti-estrogenic activities [53]. Lignans are structurally similar to tamoxifen, which has beneficial effects on bone [54]. Flaxseed is also a rich source of polyunsaturated fatty acids (PUFA), especially $\alpha$-linolenic acid. Alpha-linolenic acid may decrease the rate of bone resorption by inhibiting the biosynthesis of prostaglandins. Lignans present in flaxseed may also possess antioxidant properties. Oxygen-derived free radicals, which are formed by a number of phagocytes including monocytes, macrophages, and neutrophils, have been reported to increase chronic inflammatory diseases, aging and osteoporosis. In vivo and in vitro findings indicate that free radicals generated in the bone environment enhance osteoclast formation and bone resorption. Hence, flaxseed may reduce the rapid rate of bone loss experienced by

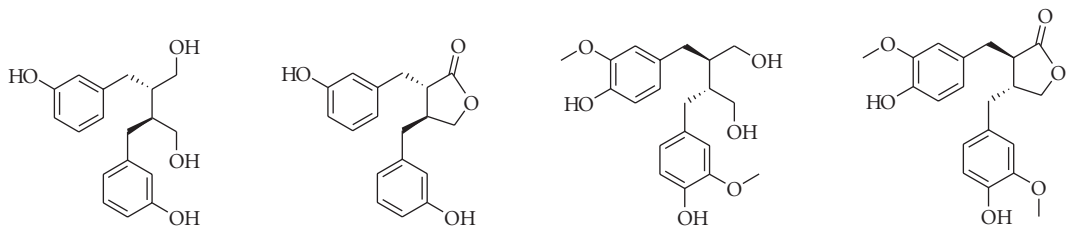
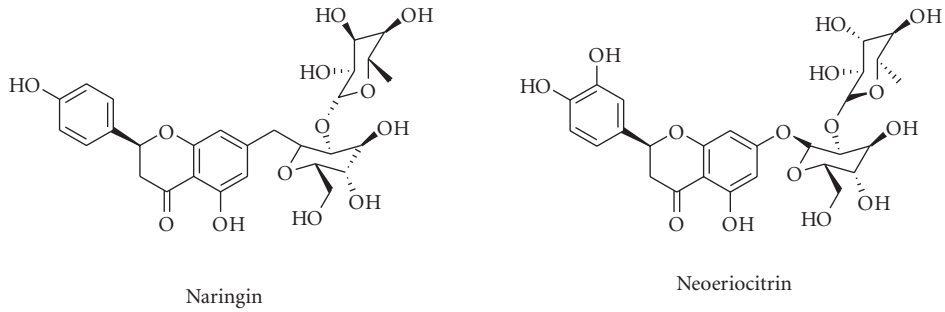



Figure 7: Chemical structure of compounds from *Linum usitatissimum* L.



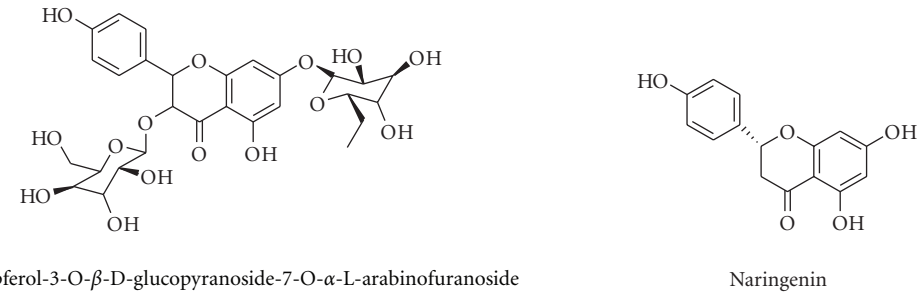
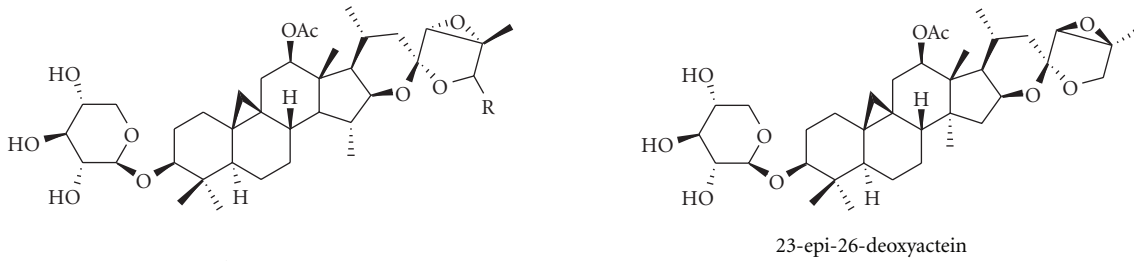




Figure 8: Chemical structure of compounds from *Drynaria fortunei* (Kunze) J. Sm.

postmenopausal women, in part, by enhancing antioxidant status [55, 56].

*3.1.8. Drynaria fortunei* (Kunze) J. Sm. The rhizome of *Drynaria fortunei* (Kunze) J. Sm., family Polypodiaceae, has a long medicinal history in the eastern Asia and is effective for the treatment of inflammation, hyperlipemia, arteriosclerosis, and gynecological diseases such as osteoporosis. The traditional Chinese and Korean prescription drugs to treat osteoporosis usually contain the rhizome of *Drynaria fortune*. In recent study, it has been found that *Drynaria fortune* has therapeutic effects on osteoporosis and bone fracture in the ovariectomized rat model, and can enhance bone formation through induction of BMP-2 and ALP, accumulation of bone matrix proteins such as type I collagen, up-regulated Runx2 and osteocalcin expression [57]. The flavonoids in *Drynaria* rhizome, including naringin, neoeriocitrin, kaempferol-3-O-$\beta$-D-glucopyranoside-7-O-$\alpha$-L-arabinofuranoside (Figure 8), are antiosteoporotic chemical constituents which can activate the estrogen receptors (ERs), and replace estrogen which can be of clinical use [58]. Naringin (Figure 8) is the main active ingredients of drynariae flavonoids, which could inhibit the retinoic acid-induced osteoporosis in rats, increase BMP-2 expression and induce the bone formation, enhance the proliferation and osteogenic differentiation of human bone mesenchymal stem cells (BMSCs) in osteoporosis diseases [59, 60]. Naringin and its metabolite naringenin revealed a double directional adjusting function of estrogenic and anti-estrogenic activities primarily through selectively binding with ER, which could prevent and treat osteoporosis with the mechanism of estrogenic receptor agitation [61].

*3.1.9. Cimicifuga racemosa* (L.) Nuttall. *Cimicifuga racemosa* (Black cohosh), botanically a member of Ranunculaceae, has been widely used in native American therapy for a variety of ailments including dysmenorrheal and labor pains as well as for the treatment of menopausal symptoms. Black cohosh contains a number of compounds with potential bioactivity such as triterpene, glycosides, resin, salycilates, isoferulic acid, sterols, and alkaloids [62]. Black cohosh does not appear to alter the hormonal pattern associated

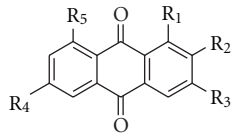



R = H 26-deoxyactein

R = OH Actein

23-epi-26-deoxyactein

Figure 9: Chemical structure of compounds from *Cimicifuga racemosa* (L.) Nuttall.

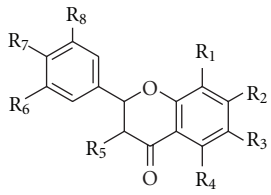



| | $R_1$ | $R_2$ | $R_3$ | $R_4$ | $R_5$ |
|---|---|---|---|---|---|
| 1,3,8-trihydroxy-2-methoxy-anthraquinone | OH | $OCH_3$ | OH | H | OH |
| 2-hydroxy-1-methoxy-anthraquinone | $OCH_3$ | OH | H | H | H |
| Rubiadin | OH | $CH_3$ | OH | H | H |

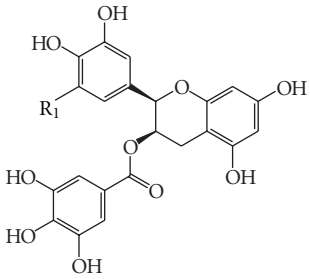
Figure 10: Chemical structure of compounds from *Morinda officinalis* How.

with menopause, lower estrogen accompanied by elevated luteinizing hormone (LH), and follicle-stimulating hormone (FSH). Black cohosh results in a significant increase in trabecular bone mineral density of the proximal metaphysis of the tibia, enhances differentiation and increases the OPG-to-RANKL ratio of normal human osteoblasts [63]. Deoxyactein, including 26-deoxyactein, acetin, and 23-epi-26-deoxyactein (Figure 9), active component from black cohosh causes a significant elevation of cell growth, alkaline phosphatase activity, collagen content, and mineralization in the cells. Moreover, deoxyactein significantly decreases the production of reactive oxygen species (ROS) and osteoclast differentiation-inducing factors such as TNF-$\alpha$, IL-6, and receptor activator of nuclear factor-$\kappa$B ligand in the presence of antimycin A [64, 65]. In an ovariectomized rat model of osteoporosis, extracts of black cohosh decreased urinary excretion of cross-links; however, the positive effect on trabecular BMD and on bone quality as assessed by mechanical testing was weaker than that of raloxifene. In a similar study on orchidectomized rats, extracts of black cohosh mitigated bone loss at the tibial metaphysis after 3 months. The analysis of skeletal and uterine effects by black cohosh in an ovariectomized rat model revealed weak protective effects on bone loss and on reduction of serum levels of osteocalcin and cross-laps, but no increase in uterine weight. In a small randomized controlled trial of 62 women, black cohosh alleviated menopause symptoms without affecting endometrial thickness of the uterus. However, the supplementation with black cohosh did not exhibit positive effects in severe (senile) osteopenic fracture healing as seen in early osteoporosis in rats [66].

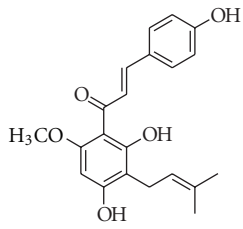
*3.1.10. Morinda officinalis* How. *Morinda officinalis* How belongs to family of Rubiaceae and grows in the south of China. In Chinese traditional medicine, it has been used as a kidney tonic and for strengthening bones. In a sciatic neurectomized mice model, the root extracts significantly and dose-dependently suppressed the decrease in hind limb thickness, tibia failure load, BMD, tibia Ca and P contents with an increase in serum osteocalcin levels. In addition, the root extract also significantly and dose-dependently suppressed the decrease in histomorphometric parameters of the tibia such as volume, length and thickness of trabecular bone and thickness of cortical bone in ovariectomized rats. They may act as both a suppressor of bone resorption and an enhancer of bone formation in vivo and may have some favorable effects for preventing and treating the osteoporosis induced by sciatic neurectomy and ovariectomy [67, 68]. The polysaccharides from *Morinda officinalis* can exert an increase in bone mineral density and mineral element concentration, a decrease in serum cytokines level in OVX rats [69]. The anthraquinones isolated from *M. officinalis*, have been proved to have inhibitory effects on osteoclastic bone resorption. 1,3,8-trihydroxy-2-methoxy-anthraquinone, 2-hydroxy-1-methoxy-anthraquinone and rubiadin (Figure 10) decrease the formation of bone resorption pits, the number of multinucleated osteoclasts, and the activity of tartrate resistant acid phosphates (TRAP) and cathepsin K in the coculture system of osteoblasts and bone marrow cells in the presence of 1,25-dihydroxyvitamin D3 and dexamethasone. They also enhance the apoptosis of osteoclasts induced from bone marrow cells with M-CSF and RANKL. In addition, these compounds improve the ratio of OPG and RANKL in osteoblasts, interfere with the JNK and NF-$\kappa$B signal pathway, and reduce the expression of calcitonin receptor (CTR) and carbonic anhydrase/II (CA II) in osteoclasts induced from bone marrow cells with M-CSF and RANKL. These findings indicate that the anthraquinone compounds from *M. officinalis* are potential inhibitors of bone resorption, and may also serve as evidence to explain the mechanism of the inhibitory effects of some other reported anthraquinones on bone loss [70, 71].



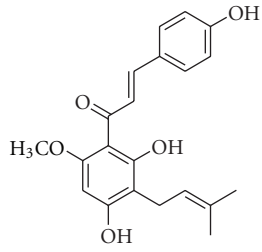
| | $R_1$ | $R_2$ | $R_3$ | $R_4$ | $R_5$ | $R_6$ | $R_7$ | $R_8$ |
|---|---|---|---|---|---|---|---|---|
| Kaempferol | H | OH | H | OH | OH | H | OH | H |
| Quercetin | H | OH | H | OH | OH | H | OH | OH |
| Nobiletin | $OCH_3$ | $OCH_3$ | $OCH_3$ | $OCH_3$ | H | $OCH_3$ | $OCH_3$ | H |
| Luteolin | H | OH | H | OH | H | H | OH | OH |
| Baicalein | H | OH | OH | OH | H | H | H | H |



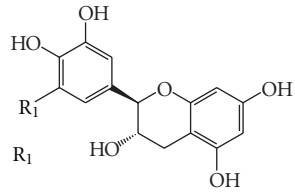
Epigallocatechin-3-gallat, $R_1$ = OH
Epigallocatechin, $R_1$ = H



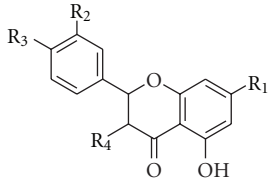
Xanthohumol



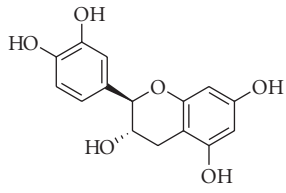
Honokiol, $R_1$ = OH, $R_2$ = H
Magnolol, $R_1$ = H, $R_2$ = OH



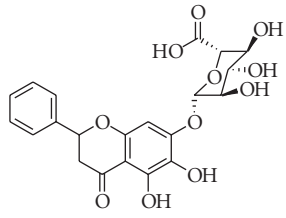
Epicatechin-3-gallate, $R_1$ = OH
Epi-catechin, $R_1$ = H



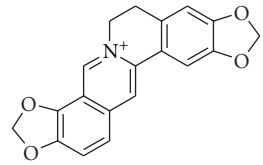
| | $R_1$ | $R_2$ | $R_3$ | $R_4$ |
|---|---|---|---|---|
| Linarin | Rha (6-1)Rha | H | $OCH_3$ | H |
| Rutin | OH | OH | OH | Rha (6-1)Rha |



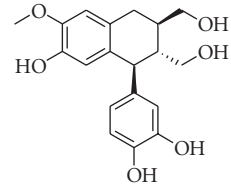
(+)-Catechin



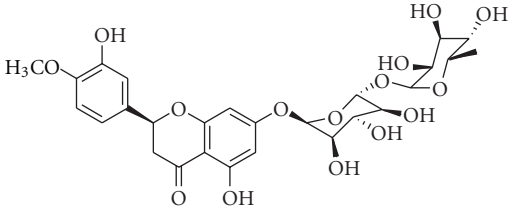
Baicalin



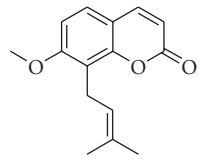
Coptisine



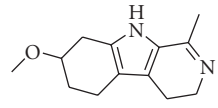
Isotaxiresinol



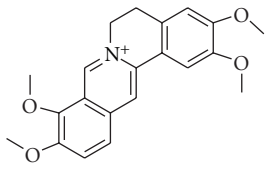
Hesperidin



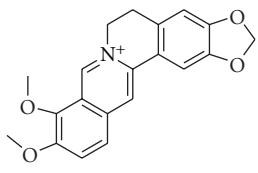
Osthole



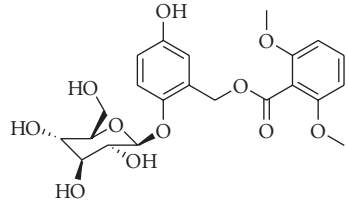
Harmine



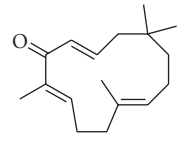
Palmatine



Berberine



Curculigoside



Zerumbone

Figure 11: Continued.

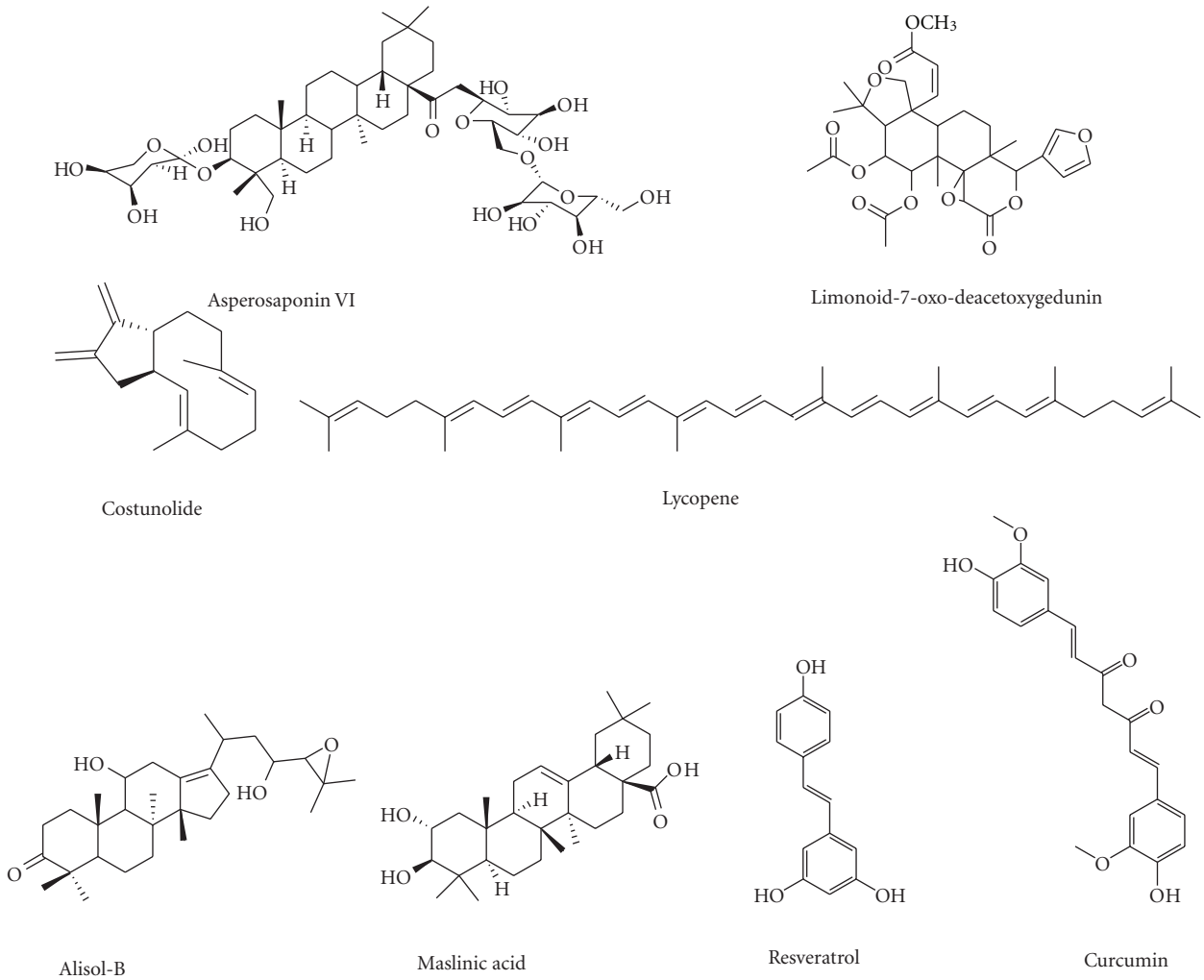



Figure 11: Chemical structure of compounds with antiosteoporotic activity.

*3.2. Antiosteoporotic Compounds Isolated from Medicinal Plants.* A wealth of information indicates numerous bioactive components isolated from plants with antiosteoporotic potential (Table 2, Figures 1–11). These compounds can be divided into 6 categories, including flavonoids: icariin (Figure 1) [23–26], genistein (Figure 2) [29], daidzein (Figure 2) [31, 32], kaempferol (Figure 11) [72], quercetin (Figure 11) [73, 74], naringin (Figure 8) [75–77], hesperidin (Figure 11) [78], linarin (Figure 11) [79], bavachalcone (Figure 3) [36], rutin (Figure 11) [80], (+)-catechin (Figure 11) [81], nobiletin (Figure 11) [82], luteolin (Figure 11) [83], baicalein (Figure 11) [84], baicalin (Figure 1) [85], xanthohumol (Figure 11) [86]; coumarins: psoralen (Figure 3) [35], osthole (Figure 11) [87]; lignans: honokiol (Figure 11) [88, 89], isotaxiresinol (Figure 11) [90], magnolol (Figure 11) [91]; polyphenol: resveratrol (Figure 11) [92–95], curcumin (Figure 11) [96–98], tea polyphenols (including epigallocatechin-3-gallate, epigallocatechin, epi-catechin, epicatechin-3-gallate, Figure 11) [99, 100]; anthraquinones: rubiadin (Figure 10), 2-hydroxy-1-methoxy-anthraquinone (Figure 10), 1,3,8-trihydroxy-2-methoxy-anthraquinone (Figure 10) [71]; alkaloids: harmine (Figure 11) [101], coptisine (Figure 11) [102], palmatine (Figure 11) [103], berberine (Figure 11) [104, 105]; and other compounds: curculigoside (Figure 11) [106, 107], asperosaponin VI (Figure 11) [108], limonoid 7-oxo-deacetoxygedunin (Figure 11) [109], zerumbone (Figure 11) [110], costunolide (Figure 11) [111], lycopene (Figure 11) [112, 113], tanshinone IIA (Figure 6) [49, 50], salvianolic acid A (Figure 6) [51], salvianolic acid B (Figure 6) [52], alisol-B (Figure 11) [114], and maslinic acid (Figure 11) [115].

Postmenopausal bone loss appears to be associated with the estrogen deficiency that leads to excessive osteoclastic and depressed osteoblastic activity [116], and possibly also impairs intestinal absorption of calcium [117]. In recent years, evidence has been provided linking bone loss to reactive oxygen species. Estrogen deficiency induces oxidative stress, impairs bone antioxidant system in adult rats, induces increase of lipid peroxidation and $H_2O_2$, and reduction of enzymatic antioxidants like SOD (super oxygen dehydrogenises) and GSH-Px (glutathione peroxidase) in rats [118]. Some phytochemicals, which have estrogen-like and/or antioxidative activity, produce bone protective effects,

Table 2: Antiosteoporotic compounds isolated from medicinal plants.

| Compound | Pharmacological activity | reference |
|---|---|---|
| *Flavonoids* | | |
| Icariin | See Section 3.1.1 | [23–26] |
| Genistein | See Section 3.1.2 | [29] |
| Daidzein | prevent bone loss in ovariectomized rats and orchidectomized rats; inhibit osteoclastic differentiation and bone resorption by increasing the activity of mature osteoblasts via ER$\beta$, regulating RUNX 2/Cbf$\alpha$1 production, and stimulating the secretion osteoprotegerin. | [31, 32] |
| Kaempferol | increase ALP activity in cultured human MG-63 osteoblasts through ERK and ER pathway; prevent antimycin A-induced cell damage in mitochondrial membrane potential dissipation, complex IV inactivation, ROS production through activation of PI3K (phosphoinositide 3-kinase), Akt (protein kinase B), CREB (cAMP-response element-binding protein) in MC3T3-E1. | [72] |
| Quercetin | reverse the decreased biomechanical quality and the impaired microarchitecture of the femurs in diabetic rats through improving antioxidant capacity; inhibit osteoclastic differentiation and bone resorption via inducing apoptosis and involving NF-$\kappa$B and AP-1. | [73, 74] |
| Naringin | protect against retinoic acid-induced osteoporosis and improve bone quality in rats; perturb osteoclast formation and bone resorption by inhibiting RANK-mediated NF-$\kappa$B and ERK signaling; induce bone morphogenetic protein-2 expression via PI3K, Akt, c-Fos/c-Jun and AP-1 pathway in osteoblasts; prevent hydrogen peroxide-induced dysfunction in osteoblastic MC3T3-E1 cells. | [75–77] |
| Hesperidin | protect bone loss in OVX rats, improve BMD and femoral load in intact rats | [78] |
| Linarin | protect osteoblasts against hydrogen peroxide-induced osteoblastic dysfunction, exert antiresorptive actions via the reduction of RANKL and oxidative damage | [79] |
| Bavachalcone | inhibit osteoclastogenesis by interfering with the ERK and Akt signaling pathways and the induction of c-Fos and NFATc1. | [36] |
| Rutin | inhibit ovariectomy—induced trabecular bone loss in rats by slowing down resorption and increasing osteoblastic activity. | [80] |
| (+)-Catechin | enhance cell survival, alkaline phosphatase activity, decrease bone-resorbing cytokines (TNF-$\alpha$ and IL-6) production and apoptosis in osteoblasts. | [81] |
| Nobiletin | prevent bone loss in ovariectomized rats; suppress formation and bone resorption of osteoclast induced by interleukin-1; suppress the expression of cyclooxygenase-2, NF-$\kappa$B-dependent transcription, and prostaglandin E production in osteoblasts. | [82] |
| Luteolin | increase bone mineral density and bone mineral content of trabecular and cortical bones in the femur of OVX rats; inhibit the differentiation of both bone marrow mononuclear cells and RAW 264.7 cells into osteoclasts and the bone resorptive activity of osteoclasts. | [83] |

Table 2: Continued.

| Compound | Pharmacological activity | reference |
|---|---|---|
| Baicalein | inhibit the differentiation and bone resorptive activity of osteoclasts by inhibiting RANKL-induced activation of signaling molecules (Akt, ERK/MAP kinase and NF-$\kappa$B) and mRNA expression of osteoclast-associated genes TRAP, matrix metalloproteinase 9 and c-Src, c-Fos, Fra-2 and NFATc1. | [84] |
| Baicalin | promote osteoblastic differentiation via Wnt/$\beta$-catenin signaling and enhance the mRNA expression of osteoprotegerin | [85] |
| Xanthohumol | upregulate ALP activity and expression of osteogenic marker genes by activation of RUNX2 via mechanisms related to the p38 MAPK and ERK signaling pathway | [86] |
| *Coumarins* | | |
| Psoralen | promote osteoblast differentiation by up-regulation of expressions of osteoblast-specific marker through the activation of BMP signaling | [35] |
| Osthole | prevent bone loss and improve bone microarchitecture, histomorphometric parameters, and biomechanical properties in OVX rats;<br>stimulate osteoblast proliferation and differentiation through $\beta$-catenin/BMP signaling. | [87] |
| *Lignan* | | |
| Honokiol | increase cell growth, alkaline phosphatase activity, collagen synthesis, mineralization, glutathione content, and osteoprotegerin release in the osteoblast;<br>decrease the production of TNF-$\alpha$, IL-6, and RANKL in the presence of antimycin A;<br>stimulate osteoblastogenesis by suppressing NF-$\kappa$B activation. | [88, 89] |
| Isotaxiresinol | improve bone mineral content, bone mineral density, and bone strength indexes in OVX control rats;<br>slightly increase bone formation and significantly inhibit bone resorption | [90] |
| Magnolol | cause a significant elevation of cell growth, alkaline phosphatase activity, collagen synthesis, mineralization, and glutathione content in osteoblast;<br>decrease the production of osteoclast differentiation inducing factors such as RANKL, TNF-$\alpha$, and IL-6 in the presence of antimycin A | [91] |
| *Polyphenol* | | |
| Resveratrol | prevent osteoporosis induced by cyclosporin A;<br>inhibit the differentiation and bone resorbing activity of osteoclasts through inhibition of ROS production;<br>promote the formation of osteoblasts by induction of bone morphogenetic protein-2 through Src kinase-dependent estrogen receptor activation;<br>promote osteogenesis of human mesenchymal stem cells by upregulating RUNX2 gene expression via the SIRT1/FOXO3A axis. | [92–95] |
| Curcumin | improve bone microarchitecture and mineral density in APP/PS1 transgenic mice;<br>improve bone strength and biochemical marker in ovariectomized mature rat model;<br>inhibit OVX-induced bone loss by reducing osteoclastogenesis through increasing antioxidant activity and impairing RANKL signaling. | [96–98] |

Table 2: Continued.

| Compound | Pharmacological activity | reference |
|---|---|---|
| Tea polyphenols (including epigallocatechin-3-gallate, epigallocatechin epi-catechin epicatechin-3-gallate) | attenuate trabecular and cortical bone loss through increasing bone formation while suppressing bone resorption due to its antioxidant capacity; inhibit the formation and differentiation of osteoclasts via inhibition of matrix metalloproteinases. | [99, 100] |
| *Anthraquinones* | | |
| Rubiadin; 2-hydroxy-1-methoxy-anthraquinone; 1,3,8-trihydroxy-2-methoxy-anthraquinone | decrease bone resorption, the number of multinucleated osteoclasts, and the activity TRAP and cathepsin K of osteoclast; induce the apoptosis of osteoclasts through improving the ratio of OPG and RANKL in osteoblasts, interfering with the JNK and NF-$\kappa$B signal pathway, and reducing the expression of calcitonin receptor and carbonic anhydrase/II in osteoclasts. | [71] |
| *Alkaloids* | | |
| Harmine | prevent bone loss in ovariectomized osteoporosis model mice; inhibit osteoclast formation and bone resorption via downregulation of c-Fos and NFATc1 induced by RANKL. | [101] |
| Coptisine | inhibit RANKL-induced NF-$\kappa$B phosphorylation in osteoclast precursors; suppress the formation, differentiation and bone resorption of osteoclast through regulation of RANKL and OPG gene expression in osteoblastic cells | [102] |
| Palmatine | inhibit osteoclast formation and bone resorption in the co-culture system with mouse bone marrow cells (BMC) and osteoblasts; induce disruption of actin ring formation in mature osteoclasts with an impact on cell viability | [103] |
| Berberine | prevent bone loss in SAMP6 senile osteoporosis model and ovariectomized rats; inhibit formation and differentiation of osteoclast; promote osteoblast differentiation through activation of Runx2 by p38 MAPK. | [104, 105] |
| *Other compounds* | | |
| Curculigoside | inhibit bone loss in ovariectomized mice; promote the proliferation and differentiation of osteoblast; prevent hydrogen peroxide-induced dysfunction and oxidative damage in calvarial osteoblasts; inhibit the formation, differentiation and bone resorption of osteoclast. | [106, 107] |
| Asperosaponin VI | induce osteoblast maturation and differentiation, and bone formation via increasing BMP-2 synthesis and activating p38 and ERK1/2 pathway | [108] |
| Limonoid 7-oxo-deacetoxygedunin | inhibit RANKL-induced osteoclastogenesis by suppressing activation of the NF-$\kappa$B and MAPK pathways | [109] |
| Zerumbone | abolish RANKL-induced NF-$\kappa$B activation, inhibit osteoclastogenesis, and suppress human breast cancer—induced bone loss in athymic nude mice | [110] |
| Costunolide | stimulate the growth and differentiation of osteoblastic MC3T3-E1 cells, which may be associated with ER, PI3K, PKC, and MAPK signaling pathway | [111] |

Table 2: Continued.

| Compound | Pharmacological activity | reference |
|---|---|---|
| Lycopene | reduce oxidative stress and the levels of bone turnover markers in postmenopausal women; stimulate proliferation and alkaline phosphatase activity of osteoblasts; inhibit osteoclasts formation and bone resorption activity. | [112, 113] |
| Tanshinone IIA | inhibit osteoclast differentiation and bone resorption through disruption of the actin ring by inhibiting c-Fos and NFATc1 expression. | [49, 50] |
| Salvianic acid A | prevent bone loss from long-term administration of prednisone in rats; protect bone from glucocorticoid—induced bone marrow impairment by stimulating osteogenesis and depressing adipogenesis in bone marrow stromal cells. | [51] |
| Salvianolic acid B | prevent glucocorticoid—induced cancellous bone loss and decrease adipogenesis; stimulate bone marrow stromal cell differentiation to osteoblast and increase osteoblast activities; decrease glucocorticoid—induced associated adipogenic differentiation through regulating the mRNA expression of PPAR-$\gamma$, Runx2, Dickkopf-1 and $\beta$-catenin in MSC | [52] |
| Alisol-B | prevent bone loss in mice; inhibit osteoclastogenesis by inhibiting the phosphorylation of JNK, and expression of NFATc1 and c-Fos; suppresses 2-methylene-19-nor-(20S)-1$\alpha$, 25$(OH)_2D_3$—induced hypercalcemia as resulting from the inhibition of osteoclastogenesis | [114] |
| Maslinic acid | suppress osteoclastogenesis and prevent ovariectomy-induced bone loss by regulating RANKL-mediated NF-$\kappa$B and MAPK signaling pathway | [115] |

via estrogen receptor and/or improving antioxidative capacity, and some may directly regulate the proliferation and activity of osteoblast and osteoclast [119].

Flavonoids, lignans, and coumarins, which are phytoestrogenic constituents, modulate the bone metabolism through estrogen receptor. Icariin, genistein, daidzein, kaempferol, and costunolide have been reported to decrease bone loss through increasing osteoblast proliferation and activity, via estrogen receptor. The phytochemicals with antioxidative capacity, such as kaempferol, quercetin, linarin, naringin, resveratrol, curcumin, tea polyphenols, curculigoside, and lycopene regulate bone metabolism through reducing the production of ROS and improving antioxidative capacity. Other compounds such as bavachalcone, (+)-catechin, nobiletin, luteolin, baicalein, baicalin, harmine, berberine, honokiol, osthole and tanshinone IIA, salvianolic acid B, alisol-B, and maslinic acid and so forth directly exert effects on osteoblst and osteoclast through modulating cytokines, and regulating pathway, such as MAPK, NF-$\kappa$B, Wnt/$\beta$-catenin, and RANKL/RANK/OPG pathway.

## 4. Discussions and Conclusion

Although chemical and biochemical agents such as bisphosphonates, estrogen, and calcitonin are the mainstay in the treatment of osteoporosis and controlling fracture, they have many side effects and fail to significantly alter the course of bone fracture complications. Plants are always an exemplary source of many currently available drugs. Clinical practice and folk experience have shown the possibility of obtaining natural products to recover osteoporosis and its complications. Chinese herbs, all of which come from natural products, are thought to treat osteoporosis mainly through, tonifying kidney and improving bone quality. Numerous medicinal plants can modulate bone metabolism to reduce bone loss [120]. Therefore, biological, chemical, and pharmacological methods should be applied to screen and obtain active lead compounds from natural medicinal plants for the treatment of osteoporosis and its complications.

There are two primary types of drugs used in the treatment of osteoporosis. One is antiresorptive agents which mainly inhibit bone resorption and the other is anabolic agents which mainly build bone. Most drugs act as agents against bone resorption, such as bisphosphonates, estrogen, selective estrogen receptor modulators (SERMs), and calcitonin which could reduce bone loss, stabilize the microarchitecture of the bone, and decrease bone turnover. However, the anabolic drugs increasing bone formation are relatively rare [5]. Teriparatide, a synthetic form of

parathyroid hormone, is the only anabolic agent currently approved by the US Food and Drug Administration (FDA) for the treatment of osteoporosis. The anabolic therapy is now available for those individuals who continue to fracture or lose bone on an adequate program of general prevention and antiresorptive therapy [121]. Some medicinal plants not only inhibit bone resorption, but also increase new bone formation. So these plant medicines which can increase osteoblast proliferation activity and improve bone formation should be developed to satisfy patient needs. According to the clinical needs of the patients, doctors can select antiresorptive therapy or anabolic therapy or their combination.

There is good evidence that proper nutrition and lifestyle can promote bone health and pharmacotherapy can slow bone loss or even build new bone. However, there is still no "cure" for osteoporosis or for most other bone disorders. Those drugs that do exist, moreover, are still not ideal in terms of their expense, ease of administration, and/or side effects. When medicinal plants are being researched and developed for the treatment of osteoporosis, some questions should be considered. These questions include: (1) controllability: the effective chemical components of the drug should be clear and controllable. (2) Selectivity: the action of the drug should be specifically targeted to bone and to the molecule or rate-limiting process that is the cause of the disease. (3) Therapeutic index: the developed therapy should optimize the benefit-to-risk ratio of the drug. (4) Convenience: a more optimal drug should be the one that can be administered orally rather than parentally.

The safety of herbal remedies should also be considered. Although the popular view that herbals are natural and harmless, some herbal toxic effects have also be reported out of which, the hepatotoxicity is the most frequently reported toxic effect [122]. The investigation of compounds and composite formula regarding safety and toxicity is needed before definitive clinical guidelines can be made. On the other hand, the medicinal plants lack standardization; this makes it difficult to validate the plant use, and may discourage further studies. However, the chances of finding an active compound in a plant traced from ethnobotanical information are significantly higher than random chance in conventional techniques. Plants which are utilized often should be investigated for pharmacological and therapeutic effects in patients suffering from osteoporosis.

It is obvious that many plants have the potential to prevent and treat osteoporosis however, only a fraction of these plants have been thoroughly investigated so far. More efficient and reliable bioassays should be developed as a matter of urgency to systematically evaluate the antiosteoporotic efficacy of plant extracts, to identify the bioactive compounds responsible for the bone protective manifestation, and to elucidate antiosteoporotic mechanisms. In addition, as most antiosteoporotic agents from medicinal plants are prophylactic in nature rather than therapeutic and clinical trials have not yet been undertaken, the application of herbal agents are restricted. If such studies are encouraged and performed more herbal drugs for human use may soon be available.

## Author's Contribution

M. Jia and Y. Nie contributed equally to this paper.

## Conflict of Interests

The authors report no Conflict of interests.

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## References

[1] P. Sambrook and C. Cooper, "Osteoporosis," *Lancet*, vol. 367, no. 9527, pp. 2010–2018, 2006.

[2] T. D. Rachner, S. Khosla, and L. C. Hofbauer, "Osteoporosis: now and the future," *The Lancet*, vol. 377, no. 9773, pp. 1276–1287, 2011.

[3] P. J. Enriori and C. L. Enriori, "The pathogenesis of osteoporosis in older women and men: a review," *Journal of Steroid Biochemistry and Molecular Biology*, vol. 82, no. 1, pp. 1–6, 2002.

[4] F. Tremollieres and C. Ribot, "Bone mineral density and prediction of non-osteoporotic disease," *Maturitas*, vol. 65, no. 4, pp. 348–351, 2010.

[5] J. C. Gallagher, "Advances in bone biology and new treatments for bone loss," *Maturitas*, vol. 60, no. 1, pp. 65–69, 2008.

[6] S. L. Teitelbaum, "Bone: the conundrum of glucocorticoid-induced of osteoporosis," *Nature Reviews Endocrinology*, vol. 8, no. 8, pp. 451–452, 2012.

[7] P. Moutsatsou, E. Kassi, and A. G. Papavassiliou, "Glucocorticoid receptor signaling in bone cells," *Trends in Molecular Medicine*, vol. 18, no. 6, pp. 348–359, 2012.

[8] D. Müller, J. Pulm, and A. Gandjour, "Cost-effectiveness of different strategies for selecting and treating individuals at increased risk of osteoporosis or osteopenia, a systematic review," *Value in Health*, vol. 15, no. 2, pp. 284–298, 2012.

[9] J. J. Body, P. Bergmann, S. Boonen et al., "Extraskeletal benefits and risks of calcium, Vitamin D and anti-osteoporosis medications," *Osteoporosis International*, vol. 23, supplement 1, pp. 1–23, 2012.

[10] D. A. Davey, "Update: estrogen and estrogen plus progestin therapy in the care of women at and after the menopause," *Womens Health*, vol. 8, no. 2, pp. 169–189, 2012.

[11] T. I. annitti, S. Rosini, D. Lodi, B. Frediani, V. Rottigni, and B. Palmieri, "Bisphosphonates: focus on inflammation and bone loss," *American Journal of Therapeutics*, vol. 19, no. 3, pp. 228–246, 2012.

[12] B. S. Komm and A. A. Chines, "An update on selective estrogen receptor modulators for the prevention and treatment of osteoporosis," *Maturitas*, vol. 71, no. 3, pp. 221–226, 2012.

[13] M. Saito and K. Marumo, "The effects of parathyroid hormone (teriparatide) on bone quality in osteoporosis," *Clinical Calcium*, vol. 22, no. 3, pp. 343–355, 2012.

[14] J. Y. Reginster and A. Neuprez, "Strontium ranelate: a look back at its use for osteoporosis," *Expert Opinion on Pharmacotherapy*, vol. 11, no. 17, pp. 2915–2927, 2010.

[15] J. Compston, "The use of combination therapy in the treatment of postmenopausal osteoporosis," *Endocrine*, vol. 41, no. 1, pp. 11–18, 2012.

[16] G. Zhang, L. Qin, W. Y. Hung et al., "Flavonoids derived from herbal *Epimedium brevicornum* Maxim prevent OVX-induced osteoporosis in rats independent of its enhancement in intestinal calcium absorption," *Bone*, vol. 38, no. 6, pp. 818–825, 2006.

[17] D. W. Zhang, Y. Cheng, N. L. Wang, J. C. Zhang, M. S. Yang, and X. S. Yao, "Effects of total flavonoids and flavonol glycosides from *Epimedium koreanum* Nakai on the proliferation and differentiation of primary osteoblasts," *Phytomedicine*, vol. 15, no. 1-2, pp. 55–61, 2008.

[18] G. Qian, X. Zhang, L. Lu, X. Wu, S. Li, and J. Meng, "Regulation of Cbfa1 expression by total flavonoids of Herba Epimedii," *Endocrine Journal*, vol. 53, no. 1, pp. 87–94, 2006.

[19] P. Songlin, Z. Ge, H. Yixin et al., "Epimedium-derived flavonoids promote osteoblastogenesis and suppress adipogenesis in bone marrow stromal cells while exerting an anabolic effect on osteoporotic bone," *Bone*, vol. 45, no. 3, pp. 534–544, 2009.

[20] J. F. Zhang, G. Li, C. Y. Chan et al., "Flavonoids of Herba Epimedii regulate osteogenesis of human mesenchymal stem cells through BMP and Wnt/$\beta$-catenin signaling pathway," *Molecular and Cellular Endocrinology*, vol. 314, no. 1, pp. 70–74, 2010.

[21] H. Nian, M. H. Ma, S. S. Nian, and L. L. Xu, "Antiosteoporotic activity of icariin in ovariectomized rats," *Phytomedicine*, vol. 16, no. 4, pp. 320–326, 2009.

[22] T. P. Hsieh, S. Y. Sheu, J. S. Sun, and M. H. Chen, "Icariin inhibits osteoclast differentiation and bone resorption by suppression of MAPKs/NF-$\kappa$B regulated HIF-1$\alpha$ and PGE2 synthesis," *Phytomedicine*, vol. 18, no. 2-3, pp. 176–185, 2011.

[23] M. S. Wong, S. K. Mok, W. F. Chen et al., "Icariin protects against bone loss induced by oestrogen deficiency and activates oestrogen receptor-dependent osteoblastic functions in UMR 106 cells," *British Journal of Pharmacology*, vol. 159, no. 4, pp. 939–949, 2010.

[24] T. P. Hsieh, S. Y. Sheu, J. S. Sun, M. H. Chen, and M. H. Liu, "Icariin isolated from *Epimedium pubescens* regulates osteoblasts anabolism through BMP-2, SMAD4, and Cbfa1 expression," *Phytomedicine*, vol. 17, no. 6, pp. 414–423, 2010.

[25] H. J. Choi, J. S. Eun, Y. R. Park et al., "Ikarisoside A inhibits inducible nitric oxide synthase in lipopolysaccharide-stimulated RAW 264.7 cells via p38 kinase and nuclear factor-kappaB signaling pathways," *European Journal of Pharmacology*, vol. 601, no. 1–3, pp. 171–178, 2008.

[26] H. J. Choi, Y. R. Park, M. Nepal et al., "Inhibition of osteoclastogenic differentiation by Ikarisoside A in RAW 264.7 cells via JNK and NF-kappa B signaling pathways," *European Journal of Pharmacology*, vol. 636, no. 1–3, pp. 28–35, 2010.

[27] S. M. Potter, J. A. Baum, H. Teng, R. J. Stillman, N. F. Shay, and J. W. Erdman, "Soy protein and isoflavones: their effects on blood lipids and bone density in postmenopausal women," *American Journal of Clinical Nutrition*, vol. 68, no. 6, pp. 1375S–1379S, 1998.

[28] K. Taku, M. K. Melby, N. Nishi, T. Omori, and M. S. Kurzer, "Soy isoflavones for osteoporosis: an evidence-based approach," *Maturitas*, vol. 70, no. 4, pp. 333–338, 2011.

[29] A. Bitto, F. Polito, B. Burnett et al., "Protective effect of genistein aglycone on the development of osteonecrosis of the femoral head and secondary osteoporosis induced by methylprednisolone in rats," *Journal of Endocrinology*, vol. 201, no. 3, pp. 321–328, 2009.

[30] A. Bitto, B. P. Burnett, F. Polito et al., "Genistein aglycone reverses glucocorticoid-induced osteoporosis and increases bone breaking strength in rats: a comparative study with alendronate," *British Journal of Pharmacology*, vol. 156, no. 8, pp. 1287–1295, 2009.

[31] M. Atteritano, S. Mazzaferro, A. Frisina et al., "Genistein effects on quantitative ultrasound parameters and bone mineral density in osteopenic postmenopausal women," *Osteoporosis International*, vol. 20, no. 11, pp. 1947–1954, 2009.

[32] B. Filipović, B. Šošić-Jurjević, V. Ajdžanović et al., "Daidzein administration positively affects thyroid C cells and bone structure in orchidectomized middle-aged rats," *Osteoporosis International*, vol. 21, no. 9, pp. 1609–1616, 2010.

[33] M. Komrakova, S. Sehmisch, M. Tezval et al., "Impact of 4-methylbenzylidene camphor, daidzein, and estrogen on intact and osteotomized bone in osteopenic rats," *Journal of Endocrinology*, vol. 211, no. 2, pp. 157–168, 2011.

[34] D. Wang, F. Li, and Z. Jiang, "Osteoblastic proliferation stimulating activity of *Psoralea corylifolia* extracts and two of its flavonoids," *Planta Medica*, vol. 67, no. 8, pp. 748–749, 2001.

[35] D. Xin, H. Wang, J. Yang et al., "Phytoestrogens from *Psoralea corylifolia* reveal estrogen receptor-subtype selectivity," *Phytomedicine*, vol. 17, no. 2, pp. 126–131, 2010.

[36] C. K. Park, Y. Lee, E. J. Chang et al., "Bavachalcone inhibits osteoclast differentiation through suppression of NFATc1 induction by RANKL," *Biochemical Pharmacology*, vol. 75, no. 11, pp. 2175–2182, 2008.

[37] D. Z. Tang, F. Yang, Z. Yang et al., "Psoralen stimulates osteoblast differentiation through activation of BMP signaling," *Biochemical and Biophysical Research Communications*, vol. 405, no. 2, pp. 256–261, 2011.

[38] X. Wang, J. Wu, H. Chiba, K. Yamada, and Y. Ishimi, "Puerariae radix prevents bone loss in castrated male mice," *Metabolism*, vol. 54, no. 11, pp. 1536–1541, 2005.

[39] X. Wang, J. Wu, H. Chiba, K. Umegaki, K. Yamada, and Y. Ishimi, "Puerariae radix prevents bone loss in ovariectomized mice," *Journal of Bone and Mineral Metabolism*, vol. 21, no. 5, pp. 268–275, 2003.

[40] J. Woo, E. Lau, S. C. Ho et al., "Comparison of *Pueraria lobata* with hormone replacement therapy in treating the adverse health consequences of menopause," *Menopause*, vol. 10, no. 4, pp. 352–361, 2003.

[41] Y. Zhang, X. Zeng, L. Zhang, and X. Zheng, "Stimulatory effect of puerarin on bone formation through activation of PI3K/Akt pathway in rat calvaria osteoblasts," *Planta Medica*, vol. 73, no. 4, pp. 341–347, 2007.

[42] N. Urasopon, Y. Hamada, K. Asaoka, W. Cherdshewasart, and S. Malaivijitnond, "*Pueraria mirifica*, a phytoestrogen-rich herb, prevents bone loss in orchidectomized rats," *Maturitas*, vol. 56, no. 3, pp. 322–331, 2007.

[43] N. Urasopon, Y. Hamada, W. Cherdshewasart, and S. Malaivijitnond, "Preventive effects of *Pueraria mirifica* on bone loss in ovariectomized rats," *Maturitas*, vol. 59, no. 2, pp. 137–148, 2008.

[44] C. Circosta, R. De Pasquale, D. R. Palumbo, S. Samperi, and F. Occhiuto, "Effects of isoflavones from red clover (*Trifolium pratense*) on skin changes induced by ovariectomy in rats," *Phytotherapy Research*, vol. 20, no. 12, pp. 1096–1099, 2006.

[45] V. Beck, U. Rohr, and A. Jungbauer, "Phytoestrogens derived from red clover: an alternative to estrogen replacement

therapy?" *Journal of Steroid Biochemistry and Molecular Biology*, vol. 94, no. 5, pp. 499–518, 2005.

[46] S. Geller and L. Studee, "Soy and red clover for mid-life and aging," *Climacteric*, vol. 9, no. 4, pp. 245–263, 2006.

[47] S. Kawakita, F. Marotta, Y. Naito et al., "Effect of an isoflavones-containing red clover preparation and alkaline supplementation on bone metabolism in ovariectomized rats," *Clinical Interventions in Aging*, vol. 4, no. 1, pp. 91–100, 2009.

[48] H. K. Kim, E. R. Woo, H. W. Lee et al., "The correlation of *Salvia miltiorrhiza* extract-induced regulation of osteoclastogenesis with the amount of components tanshinone I, tanshinone IIA, cryptotanshinone, and dihydrotanshinone," *Immunopharmacology and Immunotoxicology*, vol. 30, no. 2, pp. 347–364, 2008.

[49] H. J. Chae, S. W. Chae, D. H. Yun, K. S. Keum, S. K. Yoo, and H. R. Kim, "Prevention of bone loss in ovariectomized rats: the effect of *Salvia miltiorrhiza* extracts," *Immunopharmacology and Immunotoxicology*, vol. 26, no. 1, pp. 135–144, 2004.

[50] B. K. Han, D. Yang, H. Ha et al., "Tanshinone IIA inhibits osteoclast differentiation through down-regulation of c-Fos and NFATc1," *Experimental and Molecular Medicine*, vol. 38, no. 3, pp. 256–264, 2006.

[51] L. Cui, Y. Y. Liu, T. Wu, C. M. Ai, and H. Q. Chen, "Osteogenic effects of D(+)$\beta$-3, 4-dihydroxyphenyl lactic acid (salvianic acid A, SAA) on osteoblasts and bone marrow stromal cells of intact and prednisone-treated rats," *Acta Pharmacologica Sinica*, vol. 30, no. 3, pp. 321–332, 2009.

[52] L. Cui, T. Li, Y. Liu et al., "Salvianolic acid B prevents bone loss in prednisone-treated rats through stimulation osteogenensis and bone marrow angiogenesis," *PLoS One*, vol. 7, no. 4, Article ID e34647, 2012.

[53] S. M. Sacco, J. M. Y. Jiang, S. Reza-López, D. W. L. Ma, L. U. Thompson, and W. E. Ward, "Flaxseed combined with low-dose estrogen therapy preserves bone tissue in ovariectomized rats," *Menopause*, vol. 16, no. 3, pp. 545–554, 2009.

[54] S. Boulbaroud, A. Mesfioui, A. Arfaoui, A. Ouichou, and A. El Hessni, "Preventive effects of flaxseed and sesame oil on bone loss in ovariectomized rats," *Pakistan Journal of Biological Sciences*, vol. 11, no. 13, pp. 1696–1701, 2008.

[55] D. B. Kettler, "Can manipulation of the ratios of essential fatty acids slow the rapid rate of postmenopausal bone loss?" *Alternative Medicine Review*, vol. 6, no. 1, pp. 61–77, 2001.

[56] B. H. Arjmandi, "The role of phytoestrogens in the prevention and treatment of osteoporosis in ovarian hormone deficiency," *Journal of the American College of Nutrition*, vol. 20, no. 5, supplement, pp. 398S–402S, 2001.

[57] J. C. Jeong, J. W. Lee, C. H. Yoon, H. M. Kim, and C. H. Kim, "Drynariae Rhizoma promotes osteoblast differentiation and mineralization in MC3T3-E1 cells through regulation of bone morphogenetic protein-2, alkaline phosphatase, type I collagen and collagenase-1," *Toxicology* in vitro, vol. 18, no. 6, pp. 829–834, 2004.

[58] X. L. Wang, N. L. Wang, Y. Zhang et al., "Effects of eleven flavonoids from the osteoprotective fraction of *Drynaria fortunei* (Kunze) J. Sm. on osteoblastic proliferation using an osteoblast-like cell line," *Chemical and Pharmaceutical Bulletin*, vol. 56, no. 1, pp. 46–51, 2008.

[59] X. Wang, L. Zhen, G. Zhang, M. S. Wong, L. Qin, and X. Yao, "Osteogenic effects of flavonoid aglycones from an osteoprotective fraction of *Drynaria fortunei*—an *in vitro* efficacy study," *Phytomedicine*, vol. 18, no. 10, pp. 868–872, 2011.

[60] J. C. Eun, J. L. Won, H. C. Sung, and W. C. Sang, "Proliferative effects of flavan-3-ols and propelargonidins from rhizomes of *Drynaria fortunei* on MCF-7 and osteoblastic cells," *Archives of Pharmacal Research*, vol. 26, no. 8, pp. 620–630, 2003.

[61] D. Guo, J. Wang, X. Wang et al., "Double directional adjusting estrogenic effect of naringin from *Rhizoma drynariae* (Gusuibu)," *Journal of Ethnopharmacology*, vol. 138, pp. 451–457, 2011.

[62] D. Seidlová-Wuttke, H. Jarry, T. Becker, V. Christoffel, and W. Wuttke, "Pharmacology of *Cimicifuga racemosa* extract BNO 1055 in rats: bone, fat and uterus," *Maturitas*, vol. 44, supplement, pp. S39–S50, 2003.

[63] W. Wuttke, D. Seidlová-Wuttke, and C. Gorkow, "The Cimicifuga preparation BNO 1055 versus conjugated estrogens in a double-blind placebo-controlled study: effects on menopause symptoms and bone markers," *Maturitas*, vol. 44, supplement, pp. S67–S77, 2003.

[64] E. M. Choi, "Deoxyactein stimulates osteoblast function and inhibits bone-resorbing mediators in MC3T3-E1 cells," *Journal of Applied Toxicology*. In press.

[65] V. Viereck, C. Gründker, S. C. Friess et al., "Isopropanolic extract of black cohosh stimulates osteoprotegerin production by human osteoblasts," *Journal of Bone and Mineral Research*, vol. 20, no. 11, pp. 2036–2043, 2005.

[66] L. Kolios, J. Schumann, S. Sehmisch et al., "Effects of black cohosh (*Cimicifuga racemosa*) and estrogen on metaphyseal fracture healing in the early stage of osteoporosis in ovariectomized rats," *Planta Medica*, vol. 76, no. 9, pp. 850–857, 2010.

[67] B. I. Seo, S. K. Ku, E. M. Cha et al., "Effect of *Mornidae radix* extracts on experimental osteoporosis in sciatic neurectomized mice," *Phytotherapy Research*, vol. 19, no. 3, pp. 231–238, 2005.

[68] N. Li, L. P. Qin, T. Han, Y. B. Wu, Q. Y. Zhang, and H. Zhang, "Inhibitory effects of *Morinda officinalis* extract on bone loss in ovariectomized rats," *Molecules*, vol. 14, no. 6, pp. 2049–2061, 2009.

[69] Z. MengYong, W. CaiJiao, Z. HuSheng, P. XianWu, and F. JianMin, "Protective effect of polysaccharides from *Morinda officinalis* on bone loss in ovariectomized rats," *International Journal of Biological Macromolecules*, vol. 43, no. 3, pp. 276–278, 2008.

[70] Y. B. Wu, C. J. Zheng, L. P. Qin et al., "Antiosteoporotic activity of anthraquinones from *Morinda officinalis* on osteoblasts and osteoclasts," *Molecules*, vol. 14, no. 1, pp. 573–583, 2009.

[71] L. Bao, L. Qin, L. Liu et al., "Anthraquinone compounds from *Morinda officinalis* inhibit osteoclastic bone resorption *in vitro*," *Chemico-Biological Interactions*, vol. 194, no. 2-3, pp. 97–105, 2011.

[72] E. M. Choi, "Kaempferol protects MC3T3-E1 cells through antioxidant effect and regulation of mitochondrial function," *Food and Chemical Toxicology*, vol. 49, no. 8, pp. 1800–1805, 2011.

[73] W. Liang, Z. Luo, S. Ge et al., "Oral administration of quercetin inhibits bone loss in rat model of diabetic osteopenia," *European Journal of Pharmacology*, vol. 670, no. 1, pp. 317–324, 2011.

[74] J. Inoue, J. M. Choi, T. Yoshidomi, T. Yashiro, and R. Sato, "Quercetin enhances VDR activity, leading to stimulation of its target gene expression in Caco-2 Cells," *Journal of Nutritional Science and Vitaminology*, vol. 56, no. 5, pp. 326–330, 2010.

[75] P. Zhang, K. R. Dai, S. G. Yan et al., "Effects of naringin on the proliferation and osteogenic differentiation of human bone mesenchymal stem cell," *European Journal of Pharmacology*, vol. 607, no. 1–3, pp. 11–15, 2009.

[76] J. B. Wu, Y. C. Fong, H. Y. Tsai, Y. F. Chen, M. Tsuzuki, and C. H. Tang, "Naringin-induced bone morphogenetic protein-2 expression via PI3K, Akt, c-Fos/c-Jun and AP-1 pathway in osteoblasts," *European Journal of Pharmacology*, vol. 588, no. 2-3, pp. 333–341, 2008.

[77] E. S. Ang, X. Yang, H. Chen, Q. Liu, M. H. Zheng, and J. Xu, "Naringin abrogates osteoclastogenesis and bone resorption via the inhibition of RANKL-induced NF-kappaB and ERK activation," *FEBS Letters*, vol. 585, no. 17, pp. 2755–2762, 2011.

[78] M. N. Horcajada, V. Habauzit, A. Trzeciakiewicz et al., "Hesperidin inhibits ovariectomized-induced osteopenia and shows differential effects on bone mass and strength in young and adult intact rats," *Journal of Applied Physiology*, vol. 104, no. 3, pp. 648–654, 2008.

[79] Y. H. Kim, Y. S. Lee, and E. M. Choi, "Linarin isolated from Buddleja officinalis prevents hydrogen peroxide-induced dysfunction in osteoblastic MC3T3-E1 cells," *Cellular Immunology*, vol. 268, no. 2, pp. 112–116, 2011.

[80] M. N. Horcajada-Molteni, V. Crespy, V. Coxam, M. J. Davicco, C. Remesy, and J. P. Barlet, "Rutin inhibits ovariectomy-induced osteopenia in rats," *Journal of Bone and Mineral Research*, vol. 15, no. 11, pp. 2251–2258, 2000.

[81] E. M. Choi and J. K. Hwang, "Effects of (+)-catechin on the function of osteoblastic cells," *Biological and Pharmaceutical Bulletin*, vol. 26, no. 4, pp. 523–526, 2003.

[82] S. Harada, T. Tominari, C. Matsumoto et al., "Nobiletin, a polymethoxy flavonoid, suppresses bone resorption by inhibiting NF$\kappa$B-dependent prostaglandin E synthesis in osteoblasts and prevents bone loss due to estrogen deficiency," *Journal of Pharmacological Sciences*, vol. 115, no. 1, pp. 89–93, 2011.

[83] T. H. Kim, J. W. Jung, B. G. Ha et al., "The effects of luteolin on osteoclast differentiation, function *in vitro* and ovariectomy-induced bone loss," *Journal of Nutritional Biochemistry*, vol. 22, no. 1, pp. 8–15, 2011.

[84] M. H. Kim, S. Y. Ryu, M. A. Bae, J. S. Choi, Y. K. Min, and S. H. Kim, "Baicalein inhibits osteoclast differentiation and induces mature osteoclast apoptosis," *Food and Chemical Toxicology*, vol. 46, no. 11, pp. 3375–3382, 2008.

[85] A. J. Guo, R. C. Choi, A. W. Cheung et al., "Baicalin, a flavone, induces the differentiation of cultured osteoblasts: an action via the Wnt/beta-catenin signaling pathway," *Journal of Biological Chemistry*, vol. 286, no. 32, pp. 27882–27893, 2011.

[86] H. M. Jeong, E. H. Han, Y. H. Jin, Y. H. Choi, K. Y. Lee, and H. G. Jeong, "Xanthohumol from the hop plant stimulates osteoblast differentiation by RUNX2 activation," *Biochemical and Biophysical Research Communications*, vol. 409, no. 1, pp. 82–89, 2011.

[87] Q. Zhang, L. Qin, W. He et al., "Coumarins from *Cnidium monnieri* and their antiosteoporotic activity," *Planta Medica*, vol. 73, no. 1, pp. 13–19, 2007.

[88] E. M. Choi, "Honokiol isolated from *Magnolia officinalis* stimulates osteoblast function and inhibits the release of bone-resorbing mediators," *International Immunopharmacology*, vol. 11, no. 10, pp. 1541–1545, 2011.

[89] E. M. Choi, "Honokiol protects osteoblastic MC3T3-E1 cells against antimycin A-induced cytotoxicity," *Inflammation Research*, vol. 60, no. 11, pp. 1005–1012, 2011.

[90] J. Yin, Y. Tezuka, Subehan et al., "*In vivo* anti-osteoporotic activity of isotaxiresinol, a lignan from wood of *Taxus yunnanensis*," *Phytomedicine*, vol. 13, no. 1-2, pp. 37–42, 2006.

[91] E. M. Choi, "Magnolol protects osteoblastic MC3T3-E1 cells against antimycin A-induced cytotoxicity through activation of mitochondrial function," *Inflammation*, vol. 35, no. 3, pp. 1204–1212, 2012.

[92] L. H. Song, W. Pan, Y. H. Yu, L. D. Quarles, H. H. Zhou, and Z. S. Xiao, "Resveratrol prevents CsA inhibition of proliferation and osteoblastic differentiation of mouse bone marrow-derived mesenchymal stem cells through an ER/NO/cGMP pathway," *Toxicology in Vitro*, vol. 20, no. 6, pp. 915–922, 2006.

[93] X. He, G. Andersson, U. Lindgren, and Y. Li, "Resveratrol prevents RANKL-induced osteoclast differentiation of murine osteoclast progenitor RAW 264.7 cells through inhibition of ROS production," *Biochemical and Biophysical Research Communications*, vol. 401, no. 3, pp. 356–362, 2010.

[94] M. Shakibaei, C. Buhrmann, and A. Mobasheri, "Resveratrol-mediated SIRT-1 interactions with p300 modulate receptor activator of NF-$\kappa$B ligand (RANKL) activation of NF-$\kappa$B Signaling and inhibit osteoclastogenesis in bone-derived cells," *Journal of Biological Chemistry*, vol. 286, no. 13, pp. 11492–11505, 2011.

[95] P. C. Tseng, S. M. Hou, R. J. Chen et al., "Resveratrol promotes osteogenesis of human mesenchymal stem cells by upregulating RUNX2 gene expression via the SIRT1/FOXO3A axis," *Journal of Bone and Mineral Research*, vol. 26, no. 10, pp. 2552–2563, 2011.

[96] M. W. Yang, T. H. Wang, P. P. Yan et al., "Curcumin improves bone microarchitecture and enhances mineral density in APP/PS1 transgenic mice," *Phytomedicine*, vol. 18, no. 2-3, pp. 205–213, 2011.

[97] D. L. French, J. M. Muir, and C. E. Webber, "The ovariectomized, mature rat model of postmenopausal osteoporosis: an assessment of the bone sparing effects of curcumin," *Phytomedicine*, vol. 15, no. 12, pp. 1069–1078, 2008.

[98] W. K. Kim, K. Ke, O. J. Sul et al., "Curcumin protects against ovariectomy-induced bone loss and decreases osteoclastogenesis," *Journal of Cellular Biochemistry*, vol. 112, no. 11, pp. 3159–3166, 2011.

[99] M. Kamon, R. Zhao, and K. Sakamoto, "Green tea polyphenol (−)-epigallocatechin gallate suppressed the differentiation of murine osteoblastic MC3T3-E1 cells," *Cell Biology International*, vol. 34, no. 1, pp. 109–116, 2010.

[100] C. L. Shen, P. Wang, J. Guerrieri, J. K. Yeh, and J. S. Wang, "Protective effect of green tea polyphenols on bone loss in middle-aged female rats," *Osteoporosis International*, vol. 19, no. 7, pp. 979–990, 2008.

[101] T. Yonezawa, S. I. Hasegawa, M. Asai et al., "Harmine, a $\beta$-carboline alkaloid, inhibits osteoclast differentiation and bone resorption *in vitro* and *in vivo*," *European Journal of Pharmacology*, vol. 650, no. 2-3, pp. 511–518, 2011.

[102] J. W. Lee, A. Iwahashi, S. I. Hasegawa et al., "Coptisine inhibits RANKL-induced NF-$\kappa$B phosphorylation in osteoclast precursors and suppresses function through the regulation of RANKL and OPG gene expression in osteoblastic cells," *Journal of Natural Medicines*, vol. 66, no. 1, pp. 8–16, 2011.

[103] J. W. Lee, N. Mase, T. Yonezawa et al., "Palmatine attenuates osteoclast differentiation and function through inhibition of receptor activator of nuclear factor-$\kappa$B ligand expression in

osteoblast cells," *Biological and Pharmaceutical Bulletin*, vol. 33, no. 10, pp. 1733–1739, 2010.

[104] H. Li, T. Miyahara, Y. Tezuka, Q. Le Tran, H. Seto, and S. Kadota, "Effect of berberine on bone mineral density in SAMP6 as a senile osteoporosis model," *Biological and Pharmaceutical Bulletin*, vol. 26, no. 1, pp. 110–111, 2003.

[105] W. L. Hyun, H. S. Jung, H. N. Kim et al., "Berberine promotes osteoblast differentiation by Runx2 activation with p38 MAPK," *Journal of Bone and Mineral Research*, vol. 23, no. 8, pp. 1227–1237, 2008.

[106] L. Jiao, D. P. Cao, L. P. Qin et al., "Antiosteoporotic activity of phenolic compounds from *Curculigo orchioides*," *Phytomedicine*, vol. 16, no. 9, pp. 874–881, 2009.

[107] Y. Wang, L. Zhao, Y. Wang et al., "Curculigoside isolated from *Curculigo orchioides* prevents hydrogen peroxide-induced dysfunction and oxidative damage in calvarial osteoblasts," *Acta Biochimica et Biophysica Sinica*, vol. 44, no. 5, pp. 431–441, 2012.

[108] Y. Niu, Y. Li, H. Huang et al., "Asperosaponin VI, a saponin component from *Dipsacus asper* wall, induces osteoblast differentiation through bone morphogenetic protein-2/p38 and extracellular signal-regulated kinase 1/2 pathway," *Phytotherapy Research*, vol. 25, no. 11, pp. 1700–1706, 2011.

[109] C. Wisutsitthiwong, C. Buranaruk, K. Pudhom, and T. Palaga, "The plant limonoid 7-oxo-deacetoxygedunin inhibits RANKL-induced osteoclastogenesis by suppressing activation of the NF-kappaB and MAPK pathways," *Biochemical and Biophysical Research Communications*, vol. 415, no. 2, pp. 361–366, 2011.

[110] B. Sung, A. Murakami, B. O. Oyajobi, and B. B. Aggarwal, "Zerumbone abolishes RaNKL-induced NF-$\kappa$B activation, inhibits osteoclastogenesis, and suppresses human breast cancer-induced bone loss in athymic nude mice," *Cancer Research*, vol. 69, no. 4, pp. 1477–1484, 2009.

[111] Y. S. Lee and E. M. Choi, "Costunolide stimulates the function of osteoblastic MC3T3-E1 cells," *International Immunopharmacology*, vol. 11, no. 6, pp. 712–718, 2011.

[112] S. Sahni, M. T. Hannan, J. Blumberg, L. A. Cupples, D. P. Kiel, and K. L. Tucker, "Protective effect of total carotenoid and lycopene intake on the risk of hip fracture: a 17-year follow-up from the Framingham osteoporosis atudy," *Journal of Bone and Mineral Research*, vol. 24, no. 6, pp. 1086–1094, 2009.

[113] E. S. MacKinnon, A. V. Rao, R. G. Josse, and L. G. Rao, "Supplementation with the antioxidant lycopene significantly decreases oxidative stress parameters and the bone resorption marker N-telopeptide of type i collagen in postmenopausal women," *Osteoporosis International*, vol. 22, no. 4, pp. 1091–1101, 2011.

[114] J. W. Lee, Y. Kobayashi, Y. Nakamichi et al., "Alisol-B, a novel phyto-steroid, suppresses the RANKL-induced osteoclast formation and prevents bone loss in mice," *Biochemical Pharmacology*, vol. 80, no. 3, pp. 352–361, 2010.

[115] C. Li, Z. Yang, Z. Li et al., "Maslinic acid suppresses osteoclastogenesis and prevents ovariectomy-induced bone loss by regulating RANKL-mediated NF-$\kappa$B and MAPK signaling pathways," *Journal of Bone and Mineral Research*, vol. 26, no. 3, pp. 644–656, 2011.

[116] L. G. Raisz, "The osteoporosis revolution," *Annals of Internal Medicine*, vol. 126, no. 6, pp. 458–462, 1997.

[117] P. D. O'Loughlin and H. A. Morris, "Oestrogen deficiency impairs intestinal calcium absorption in the rat," *Journal of Physiology*, vol. 511, no. 1, pp. 313–322, 1998.

[118] J. M. Lean, J. T. Davies, K. Fuller et al., "A crucial role for thiol antioxidants in estrogen-deficiency bone loss," *Journal of Clinical Investigation*, vol. 112, no. 6, pp. 915–923, 2003.

[119] C. Puel, A. Quintin, A. Agalias et al., "Olive oil and its main phenolic micronutrient (oleuropein) prevent inflammation-induced bone loss in the ovariectomised rat," *British Journal of Nutrition*, vol. 92, no. 1, pp. 119–127, 2004.

[120] M. Xu, C. Qi, B. Deng, P. X. Deng, and C. W. Mo, "Phytotherapy versus hormonal therapy for postmenopausal bone loss: a meta-analysis," *Osteoporosis International*, vol. 20, no. 4, pp. 519–526, 2009.

[121] G. Resmini and G. Iolascon, "New insights into the teriparatide," *Aging Clinical and Experimental Research*, vol. 23, no. 2, supplement, pp. 30–32, 2011.

[122] S. C. Man, K. W. Chan, J. H. Lu et al., "Systematic review on the efficacy and safety of herbal medicines for vascular dementia," *Evidence-Based Complementary and Alternative Medicine*, vol. 2012, Article ID 426215, 22 pages, 2012.

[123] C. C. He, R. R. Hui, Y. Tezuka, S. Kadota, and J. X. Li, "Osteoprotective effect of extract from Achyranthes bidentata in ovariectomized rats," *Journal of Ethnopharmacology*, vol. 127, no. 2, pp. 229–234, 2010.

[124] R. Zhang, S. J. Hu, C. Li, F. Zhang, H. Q. Gan, and Q. B. Mei, "Achyranthes bidentata root extract prevent OVX-induced osteoporosis in rats," *Journal of Ethnopharmacology*, vol. 139, no. 1, pp. 12–18, 2010.

[125] D. P. Cao, Y. N. Zheng, L. P. Qin et al., "*Curculigo orchioides*, a traditional Chinese medicinal plant, prevents bone loss in ovariectomized rats," *Maturitas*, vol. 59, no. 4, pp. 373–380, 2008.

[126] J. M. Liao, Q. A. Zhu, H. J. Lu, Q. N. Li, T. Wu, and L. F. Huang, "Effects of total coumarins of *Cnidium monnieri* on bone density and biomechanics of glucocorticoids-induced osteoporosis in rats," *Acta Pharmacologica Sinica*, vol. 18, no. 6, pp. 519–521, 1997.

[127] C. Y. Li, T. Wu, Q. N. Li et al., "Effects of fructus *Cnidii coumarins* compared with nilestriol on osteoporosis in ovariectomized rats," *Zhongguo Yao Li Xue Bao*, vol. 18, no. 3, pp. 286–288, 1997.

[128] S. S. Shirke, S. R. Jadhav, and A. G. Jagtap, "Methanolic extract of cuminum cyminum inhibits ovariectomy-induced bone loss in rats," *Experimental Biology and Medicine*, vol. 233, no. 11, pp. 1403–1410, 2008.

[129] M. Ferretti, L. Bertoni, F. Cavani et al., "Influence of ferutinin on bone metabolism in ovariectomized rats. II: role in recovering osteoporosis," *Journal of Anatomy*, vol. 217, no. 1, pp. 48–56, 2010.

[130] Q. Yang, S. M. Populo, J. Zhang, G. Yang, and H. Kodama, "Effect of *Angelica sinensis* on the proliferation of human bone cells," *Clinica Chimica Acta*, vol. 324, no. 1-2, pp. 89–97, 2002.

[131] Y. Shen, Y. Q. Li, S. P. Li, L. Ma, L. J. Ding, and H. Ji, "Alleviation of ovariectomy-induced osteoporosis in rats by *Panax notoginseng* saponins," *Journal of Natural Medicines*, vol. 64, no. 3, pp. 336–345, 2010.

[132] X. D. Li, J. S. Wang, B. Chang et al., "*Panax notoginseng* saponins promotes proliferation and osteogenic differentiation of rat bone marrow stromal cells," *Journal of Ethnopharmacology*, vol. 134, no. 2, pp. 268–274, 2011.

[133] Y. C. Hwang, I. K. Jeong, K. J. Ahn, and H. Y. Chung, "The effects of *Acanthopanax senticosus* extract on bone turnover and bone mineral density in Korean postmenopausal women," *Journal of Bone and Mineral Metabolism*, vol. 27, no. 5, pp. 584–590, 2009.

[134] H. S. Yogesh, V. M. Chandrashekhar, H. R. Katti et al., "Anti-osteoporotic activity of aqueous-methanol extract of *Berberis aristata* in ovariectomized rats," *Journal of Ethnopharmacology*, vol. 134, no. 2, pp. 334–338, 2011.

[135] V. M. Pattell, *Berberis Aristata Plants Extracts for Treating Osteoporosis and the Extraction Process Thereof*, Avestha Gengraine Technologies PVT, 2008, http://patentscope.wipo.int/search/en/WO2008007215.

[136] Y. Zhang, L. Yu, M. Ao, and W. Jin, "Effect of ethanol extract of *Lepidium meyenii* Walp. on osteoporosis in ovariectomized rat," *Journal of Ethnopharmacology*, vol. 105, no. 1-2, pp. 274–279, 2006.

[137] G. F. Gonzales, "Ethnobiology and ethnopharmacology of *Lepidium meyenii* (Maca), a plant from the Peruvian Highlands," *Evidence-Based Complementary and Alternative Medicine*, vol. 2012, Article ID 193496, 10 pages, 2012.

[138] H. M. Jeong, E. H. Han, Y. H. Jin et al., "Saponins from the roots of *Platycodon grandiflorum* stimulate osteoblast differentiation via p38 MAPK- and ERK-dependent RUNX2 activation," *Food and Chemical Toxicology*, vol. 48, no. 12, pp. 3362–3368, 2010.

[139] Y. Zhang, Q. Li, H. Y. Wan et al., "Study of the mechanisms by which *Sambucus williamsii* HANCE extract exert protective effects against ovariectomy-induced osteoporosis *in vivo*," *Osteoporosis International*, vol. 22, no. 2, pp. 703–709, 2011.

[140] H. J. Kim, Y. C. Bae, R. W. Park et al., "Bone-protecting effect of safflower seeds in ovariectomized rats," *Calcified Tissue International*, vol. 71, no. 1, pp. 88–94, 2002.

[141] Y. S. Lee, C. W. Choi, J. J. Kim, A. Ganapathi, R. Udayakumar, and S. C. Kim, "Determination of mineral content in methanolic safflower (*Carthamus tinctorius* L.) seed extract and its effect on osteoblast markers," *International Journal of Molecular Sciences*, vol. 10, no. 1, pp. 292–305, 2009.

[142] J. L. Kim, S. W. Kang, M. K. Kang et al., "Osteoblastogenesis and osteoprotection enhanced by flavonolignan silibinin in osteoblasts and osteoclasts," *Journal of Cellular Biochemistry*, vol. 113, no. 1, pp. 247–259, 2012.

[143] N. A. El-Shitany, S. Hegazy, and K. El-desoky, "Evidences for antiosteoporotic and selective estrogen receptor modulator activity of silymarin compared with ethinylestradiol in ovariectomized rats," *Phytomedicine*, vol. 17, no. 2, pp. 116–125, 2010.

[144] S. Annie, R. G. Prabhu, and S. Malini, "Activity of *Wedelia calendulacea* Less. in post-menopausal osteoporosis," *Phytomedicine*, vol. 13, no. 1-2, pp. 43–48, 2006.

[145] Y. Ding, C. Liang, S. Y. Yang et al., "Phenolic compounds from *Artemisia iwayomogi* and their effects on osteoblastic MC3T3-E1 cells," *Biological and Pharmaceutical Bulletin*, vol. 33, no. 8, pp. 1448–1453, 2010.

[146] L. Yang, Q. Chen, F. Wang, and G. Zhang, "Antiosteoporotic compounds from seeds of *Cuscuta chinensis*," *Journal of Ethnopharmacology*, vol. 135, no. 2, pp. 553–560, 2011.

[147] H. M. Yang, H. K. Shin, Y. H. Kang, and J. K. Kim, "*Cuscuta chinensis* extract promotes osteoblast differentiation and mineralization in human osteoblast-like MG-63 cells," *Journal of Medicinal Food*, vol. 12, no. 1, pp. 85–92, 2009.

[148] Y. J. Ko, J. B. Wu, H. Y. Ho, and W. C. Lin, "Antiosteoporotic activity of *Davallia formosana*," *Journal of Ethnopharmacology*, vol. 139, pp. 558–565, 2012.

[149] X. Zhao, Z. X. Wu, Y. Zhang et al., "Anti-osteoporosis activity of *Cibotium barometz* extract on ovariectomy-induced bone loss in rats," *Journal of Ethnopharmacology*, vol. 137, no. 3, pp. 1083–1088, 2011.

[150] K. Y. Peng, L. Y. Horng, H. C. Sung, H. C. Huang, and R. T. Wu, "Antiosteoporotic activity of *Dioscorea alata* L. cv. phyto through driving mesenchymal stem cells differentiation for bone formation," *Evidence-Based Complementary and Alternative Medicine*, vol. 2011, Article ID 712892, 12 pages, 2011.

[151] J. Yin, Y. Tezuka, K. Kouda et al., "Antiosteoporotic activity of the water extract of *Dioscorea spongiosa*," *Biological and Pharmaceutical Bulletin*, vol. 27, no. 4, pp. 583–586, 2004.

[152] J. Yin, Y. Tezuka, K. Kouda et al., "*In vivo* antiosteoporotic activity of a fraction of *Dioscorea spongiosa* and its constituent, 22-O-methylprotodioscin," *Planta Medica*, vol. 70, no. 3, pp. 220–226, 2004.

[153] Z. G. Liu, R. Zhang, C. Li et al., "The osteoprotective effect of Radix Dipsaci extract in ovariectomized rats," *Journal of Ethnopharmacology*, vol. 123, no. 1, pp. 74–81, 2009.

[154] L. Devareddy, S. Hooshmand, J. K. Collins, E. A. Lucas, S. C. Chai, and B. H. Arjmandi, "Blueberry prevents bone loss in ovariectomized rat model of postmenopausal osteoporosis," *Journal of Nutritional Biochemistry*, vol. 19, no. 10, pp. 694–699, 2008.

[155] H. Ha, J. Ho, S. Shin et al., "Effects of *Eucommiae cortex* on osteoblast-like cell proliferation and osteoclast inhibition," *Archives of Pharmacal Research*, vol. 26, no. 11, pp. 929–936, 2003.

[156] R. Zhang, Z. G. Liu, C. Li et al., "Du-Zhong (*Eucommia ulmoides* Oliv.) cortex extract prevent OVX-induced osteoporosis in rats," *Bone*, vol. 45, no. 3, pp. 553–559, 2009.

[157] Y. Li, M. J. Wang, S. Li et al., "Effect of total glycosides from *Eucommia ulmoides* seed on bone microarchitecture in rats," *Phytotherapy Research*, vol. 25, no. 12, pp. 1895–1897, 2011.

[158] L. Penolazzi, I. Lampronti, M. Borgatti et al., "Induction of apoptosis of human primary osteoclasts treated with extracts from the medicinal plant *Emblica officinalis*," *BMC Complementary and Alternative Medicine*, vol. 8, article no. 59, pp. 59–71, 2008.

[159] Y. Zhang, Q. Li, X. Li, H. Y. Wan, and M. S. Wong, "*Erythrina variegata* extract exerts osteoprotective effects by suppression of the process of bone resorption," *British Journal of Nutrition*, vol. 104, no. 7, pp. 965–971, 2010.

[160] Y. Zhang, X. L. Li, W. P. Lai et al., "Anti-osteoporotic effect of *Erythrina variegata* L. in ovariectomized rats," *Journal of Ethnopharmacology*, vol. 109, no. 1, pp. 165–169, 2007.

[161] I. Dontas, M. Halabalaki, P. Moutsatsou et al., "Protective effect of plant extract from *Onobrychis ebenoides* on ovariectomy-induced bone loss in rats," *Maturitas*, vol. 53, no. 2, pp. 234–242, 2006.

[162] M. Halabalaki, X. Alexi, N. Aligiannis et al., "Estrogenic activity of isoflavonoids from *Onobrychis ebenoides*," *Planta Medica*, vol. 72, no. 6, pp. 488–493, 2006.

[163] Z. Papoutsi, E. Kassi, D. Papaevangeliou et al., "Plant 2-arylobenzofurans demonstrate a selective estrogen receptor modulator profile," *Steroids*, vol. 69, no. 11-12, pp. 727–734, 2004.

[164] J. Kim, S. J. Um, J. Woo et al., "Comparative effect of seeds of *Rhynchosia volubilis* and soybean on MG-63 human osteoblastic cell proliferation and estrogenicity," *Life Sciences*, vol. 78, no. 1, pp. 30–40, 2005.

[165] S. S. Joo, T. J. Won, H. C. Kang, and D. I. Lee, "Isoflavones extracted from *Sophorae fructus* upregulate IGF-I and TGF-$\beta$ and inhibit osteoclastogenesis in rat born marrow cells," *Archives of Pharmacal Research*, vol. 27, no. 1, pp. 99–105, 2004.

[166] S. S. Joo, H. C. Kang, M. W. Lee, Y. W. Choi, and D. I. Lee, "Inhibition of IL-1beta and IL-6 in osteoblast-like cell by isoflavones extracted from *Sophorae fructus*," *Archives of Pharmacal Research*, vol. 26, no. 12, pp. 1029–1035, 2003.

[167] R. Pandey, A. K. Gautam, B. Bhargavan et al., "Total extract and standardized fraction from the stem bark of Butea monosperma have osteoprotective action: evidence for the nonestrogenic osteogenic effect of the standardized fraction," *Menopause*, vol. 17, no. 3, pp. 602–610, 2010.

[168] S. S. Shirke, S. R. Jadhav, and A. G. Jagtap, "Osteoprotective effect of *Phaseolus vulgaris* L in ovariectomy-induced osteopenia in rats," *Menopause*, vol. 16, no. 3, pp. 589–596, 2009.

[169] L. M. F. Lucinda, B. J. Vieira, T. T. Oliveira et al., "Evidences of osteoporosis improvement in Wistar rats treated with *Ginkgo biloba* extract: a histomorphometric study of mandible and femur," *Fitoterapia*, vol. 81, no. 8, pp. 982–987, 2010.

[170] S. M. Oh, H. R. Kim, and K. H. Chung, "Effects of *Ginkgo biloba* on *in vitro* osteoblast cells and ovariectomized rat osteoclast cells," *Archives of Pharmacal Research*, vol. 31, no. 2, pp. 216–224, 2008.

[171] Z. Papoutsi, E. Kassi, I. Chinou, M. Halabalaki, L. A. Skaltsounis, and P. Moutsatsou, "Walnut extract (*Juglans regia* L.) and its component ellagic acid exhibit anti-inflammatory activity in human aorta endothelial cells and osteoblastic activity in the cell line KS483," *British Journal of Nutrition*, vol. 99, no. 4, pp. 715–722, 2008.

[172] Y. Ono, Y. Fukaya, S. Imai, and T. Yamakuni, "Beneficial effects of *Ajuga decumbens* on osteoporosis and arthritis," *Biological and Pharmaceutical Bulletin*, vol. 31, no. 6, pp. 1199–1204, 2008.

[173] K. H. Lee and E. M. Choi, "Stimulatory effects of extract prepared from the bark of *Cinnamomum cassia* blume on the function of osteoblastic MC3T3-E1 cells," *Phytotherapy Research*, vol. 20, no. 11, pp. 952–960, 2006.

[174] C. H. Tang, T. H. Huang, C. S. Chang, W. M. Fu, and R. S. Yang, "Water solution of onion crude powder inhibits RANKL-induced osteoclastogenesis through ERK, p38 and NF-$\kappa$B pathways," *Osteoporosis International*, vol. 20, no. 1, pp. 93–103, 2009.

[175] M. Mukherjee, A. S. Das, S. Mitra, and C. Mitra, "Prevention of bone loss by oil extract of garlic (*Allium sativum* Linn.) in an ovariectomized rat model of osteoporosis," *Phytotherapy Research*, vol. 18, no. 5, pp. 389–394, 2004.

[176] H. Nian, L. P. Qin, W. S. Chen, Q. Y. Zhang, H. C. Zheng, and Y. Wang, "Protective effect of steroidal saponins from rhizome of *Anemarrhena asphodeloides* on ovariectomy-induced bone loss in rats," *Acta Pharmacologica Sinica*, vol. 27, no. 6, pp. 728–734, 2006.

[177] G. F. Zeng, Z. Y. Zhang, L. Lu et al., "Protective effects of *Polygonatum sibiricum* polysaccharide on ovariectomy-induced bone loss in rats," *Journal of Ethnopharmacology*, vol. 136, no. 1, pp. 224–229, 2011.

[178] N. Ayoub, A. N. Singab, M. El-Naggar, and U. Lindequist, "Investigation of phenolic leaf extract of *Heimia myrtifolia* (Lythraceae): pharmacological properties (stimulation of mineralization of SaOS-2 osteosarcoma cells) and identification of polyphenols," *Drug Discoveries and Therapeutics*, vol. 4, no. 5, pp. 341–348, 2010.

[179] C. Puel, J. Mathey, S. Kati-Coulibaly et al., "Preventive effect of *Abelmoschus manihot* (L.) Medik. on bone loss in the ovariectomised rats," *Journal of Ethnopharmacology*, vol. 99, no. 1, pp. 55–60, 2005.

[180] P. Kapur, H. Jarry, W. Wuttke, B. M. J. Pereira, and D. Seidlova-Wuttke, "Evaluation of the antiosteoporotic potential of *Tinospora cordifolia* in female rats," *Maturitas*, vol. 59, no. 4, pp. 329–338, 2008.

[181] A. N. Shuid, L. L. Ping, N. Muhammad, N. Mohamed, and I. N. Soelaiman, "The effects of *Labisia pumila* var. alata on bone markers and bone calcium in a rat model of postmenopausal osteoporosis," *Journal of Ethnopharmacology*, vol. 133, no. 2, pp. 538–542, 2011.

[182] Y. Zhang, W. P. Lai, P. C. Leung, C. F. Wu, X. S. Yao, and M. S. Wong, "Effects of Fructus Ligustri Lucidi extract on bone turnover and calcium balance in ovariectomized rats," *Biological and Pharmaceutical Bulletin*, vol. 29, no. 2, pp. 291–296, 2006.

[183] X. L. Dong, Y. Zhang, M. J. Favus, C. T. Che, and M. S. Wong, "Ethanol extract of Fructus Ligustri Lucidi increases circulating 1,25-dihydroxyvitamin D3 by inducing renal 25-hydroxyvitamin D-1$\alpha$ hydroxylase activity," *Menopause*, vol. 17, no. 6, pp. 1174–1181, 2010.

[184] K. Masuda, M. Ikeuchi, T. Koyama et al., "Suppressive effects of *Anoectochilus formosanus* extract on osteoclast formation *in vitro* and bone resorption *in vivo*," *Journal of Bone and Mineral Metabolism*, vol. 26, no. 2, pp. 123–129, 2008.

[185] H. Liang, F. Yu, Z. Tong, and Z. Huang, "Effect of cistanches herba aqueous extract on bone loss in ovariectomized rat," *International Journal of Molecular Sciences*, vol. 12, no. 8, pp. 5060–5069, 2011.

[186] S. W. Kim, H. G. Kim, B. E. Lee, H. H. Hwang, D. H. Baek, and S. Y. Ko, "Effects of mushroom, *Pleurotus eryngii*, extracts on bone metabolism," *Clinical Nutrition*, vol. 25, no. 1, pp. 166–170, 2006.

[187] J. Mori-Okamoto, Y. Otawara-Hamamoto, H. Yamato, and H. Yoshimura, "Pomegranate extract improves a depressive state and bone properties in menopausal syndrome model ovariectomized mice," *Journal of Ethnopharmacology*, vol. 92, no. 1, pp. 93–101, 2004.

[188] J. X. Li, J. Liu, C. C. He et al., "Triterpenoids from *Cimicifugae rhizoma*, a novel class of inhibitors on bone resorption and ovariectomy-induced bone loss," *Maturitas*, vol. 58, no. 1, pp. 59–69, 2007.

[189] S. Hooshmand, S. C. Chai, R. L. Saadat, M. E. Payton, K. Brummel-Smith, and B. H. Arjmandi, "Comparative effects of dried plum and dried apple on bone in postmenopausal women," *British Journal of Nutrition*, vol. 106, no. 6, pp. 923–930, 2011.

[190] B. H. Arjmandi, C. D. Johnson, S. C. Campbell, S. Hooshmand, S. C. Chai, and M. P. Akhter, "Combining fructooligosaccharide and dried plum has the greatest effect on restoring bone mineral density among select functional foods and bioactive compounds," *Journal of Medicinal Food*, vol. 13, no. 2, pp. 312–319, 2010.

[191] K. H. Lee and E. M. Choi, "*Rubus coreanus* Miq. extract promotes osteoblast differentiation and inhibits bone-resorbing mediators in MC3T3-E1 cells," *American Journal of Chinese Medicine*, vol. 34, no. 4, pp. 643–654, 2006.

[192] S. H. Do, J. W. Lee, W. I. Jeong et al., "Bone-protecting effect of *Rubus coreanus* by dual regulation of osteoblasts and osteoclasts," *Menopause*, vol. 15, no. 4, pp. 676–683, 2008.

[193] B. Y. Kim, H. Y. Yoon, S. I. Yun et al., "*In vitro* and *in vivo* inhibition of glucocorticoid-induced osteoporosis by the hexane extract of *Poncirus trifoliata*," *Phytotherapy Research*, vol. 25, no. 7, pp. 1000–1010, 2011.

[194] F. Deyhim, K. Mandadi, B. S. Patil, and B. Faraji, "Grapefruit pulp increases antioxidant status and improves bone quality

in orchidectomized rats," *Nutrition*, vol. 24, no. 10, pp. 1039–1044, 2008.

[195] F. Deyhim, K. Mandadi, B. Faraji, and B. S. Patil, "Grapefruit juice modulates bone quality in rats," *Journal of Medicinal Food*, vol. 11, no. 1, pp. 99–104, 2008.

[196] K. O. Oh, S. W. Kim, J. Y. Kim et al., "Effect of *Rehmannia glutinosa* Libosch extracts on bone metabolism," *Clinica Chimica Acta*, vol. 334, no. 1-2, pp. 185–195, 2003.

[197] P. R. Nagareddy and M. Lakshmana, "Withania somnifera improves bone calcification in calcium-deficient ovariectomized rats," *Journal of Pharmacy and Pharmacology*, vol. 58, no. 4, pp. 513–519, 2006.

[198] C. K. Park, H. J. Kim, H. B. Kwak et al., "Inhibitory effects of *Stewartia koreana* on osteoclast differentiation and bone resorption," *International Immunopharmacology*, vol. 7, no. 12, pp. 1507–1516, 2007.

[199] S. K. Kang, K. S. Kim, Y. S. Byun et al., "Effects of *Ulmus davidiana* planch on mineralization, bone morphogenetic protein-2, alkaline phosphatase, type I collagen, and collagenase-1 in bone cells," *In Vitro Cellular and Developmental Biology*, vol. 42, no. 7, pp. 225–229, 2006.

[200] J. A. Siddiqui, K. Sharan, G. Swarnkar et al., "Quercetin-6-C-$\beta$-d-glucopyranoside isolated from *Ulmus wallichiana* planchon is more potent than quercetin in inhibiting osteoclastogenesis and mitigating ovariectomy-induced bone loss in rats," *Menopause*, vol. 18, no. 2, pp. 198–207, 2011.

[201] K. Sharan, G. Swarnkar, J. A. Siddiqui et al., "A novel flavonoid, 6-C-$\beta$-d-glucopyranosyl-(2S,3S)-(+)-3′, 4′,5,7-tetrahydroxyflavanone, isolated from *Ulmus wallichiana* Planchon mitigates ovariectomy-induced osteoporosis in rats," *Menopause*, vol. 17, no. 3, pp. 577–586, 2010.

[202] G. Swarnkar, K. Sharan, J. A. Siddiqui et al., "A novel flavonoid isolated from the steam-bark of *Ulmus wallichiana* Planchon stimulates osteoblast function and inhibits osteoclast and adipocyte differentiation," *European Journal of Pharmacology*, vol. 658, no. 2-3, pp. 65–73, 2011.

[203] K. Sharan, J. A. Siddiqui, G. Swarnkar et al., "Extract and fraction from *Ulmus wallichiana* Planchon promote peak bone achievement and have a nonestrogenic osteoprotective effect," *Menopause*, vol. 17, no. 2, pp. 393–402, 2010.

[204] S. Sehmisch, J. Boeckhoff, J. Wille et al., "Vitex agnus castus as prophylaxis for osteopenia after orchidectomy in rats compared with estradiol and testosterone supplementation," *Phytotherapy Research*, vol. 23, no. 6, pp. 851–858, 2009.

[205] A. Shirwaikar, S. Khan, and S. Malini, "Antiosteoporotic effect of ethanol extract of Cissus quadrangularis Linn. on ovariectomized rat," *Journal of Ethnopharmacology*, vol. 89, no. 2-3, pp. 245–250, 2003.

[206] S. Muthusami, K. Senthilkumar, C. Vignesh et al., "Effects of Cissus quadrangularis on the proliferation, differentiation and matrix mineralization of human osteoblast like SaOS-2 cells," *Journal of Cellular Biochemistry*, vol. 112, no. 4, pp. 1035–1045, 2011.

[207] S. Muthusami, I. Ramachandran, S. Krishnamoorthy, R. Govindan, and S. Narasimhan, "*Cissus quadrangularis* augments IGF system components in human osteoblast like SaOS-2 cells," *Growth Hormone and IGF Research*, vol. 21, no. 6, pp. 343–348, 2011.

[208] J. Weerachayaphorn, A. Chuncharunee, C. Mahagita, B. Lewchalermwongse, A. Suksamrarn, and P. Piyachaturawat, "A protective effect of *Curcuma comosa* Roxb. on bone loss in estrogen deficient mice," *Journal of Ethnopharmacology*, vol. 137, no. 2, pp. 956–962, 2011.

[209] S. Oh, T. W. Kyung, and H. S. Choi, "Curcumin inhibits osteoclastogenesis by decreasing receptor activator of nuclear factor-$\kappa$B ligand (RANKL) in bone marrow stromal cells," *Molecules and Cells*, vol. 26, no. 5, pp. 486–489, 2008.

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# Protective Effects of Selected Botanical Agents on Bone

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**Abstract:** Osteoporosis is a serious health problem affecting more than 200 million elderly people worldwide. The early symptoms of this disease are hardly detectable. It causes progressive bone loss, which ultimately renders the patients susceptible to fractures. Osteoporosis must be prevented because the associated fragility fractures result in high morbidity, mortality, and healthcare costs. Many plants used in herbal medicine contain bioactive compounds possessing skeletal protective effects. This paper explores the anti-osteoporotic properties of selected herbal plants, including their actions on osteoblasts (bone forming cells), osteoclasts (bone resorbing cells), and bone remodelling. Some of the herbal plant families included in this review are Berberidaceae, Fabaceae, Arecaceae, Labiatae, Simaroubaceaea, and Myrsinaceae. Their active constituents, mechanisms of action, and pharmaceutical applications were discussed. The literature shows that very few herbal plants have undergone human clinical trials to evaluate their pharmacological effects on bone to date. Therefore, more intensive research should be performed on these plants to validate their anti-osteoporotic properties so that they can complement the currently available conventional drugs in the battle against osteoporosis.

**Keywords:** bone remodelling; complementary therapies; herbal medicine; osteoblast; osteoclast

## 1. Introduction

Osteoporosis is a metabolic bone disorder resulting from an imbalance of bone remodelling, in which the rate of bone resorption is higher than the rate of bone formation [1,2]. In turn, this gives rise to low bone mass, microarchitectural deterioration, and eventually an increased risk for fragility fractures [1–3]. Osteoporosis can be classified into primary (Type I and II) and secondary osteoporosis. Primary type I osteoporosis occurs in women soon after menopause (postmenopausal osteoporosis) and in men during and after middle-age [4]. On the other hand, primary type II or senile osteoporosis is due to old age. Both sexes may develop primary type II osteoporosis over the age of 70, whereby both trabecular and cortical bones degenerate, thus causing proximal femora, vertebrae, and radii fractures. Women have a two-fold higher risk than men to suffer from primary type II osteoporosis due to their low peak bone mass [2,4–6]. Secondary osteoporosis is due to medications or certain medical conditions, such as hypogonadism, hyperparathyroidism, or leukemia [7]. Prolonged

use of some medications can lead to bone loss, such as oral or high-dose inhaled corticosteroids, thyroid hormone replacement, and aromatase inhibitors [7–9]. Osteoporosis is closely associated with increased mortality due to complications of osteoporotic fractures, particularly at the vertebrae and hips [2,10,11].

Most current therapies for osteoporosis focus on inhibiting bone resorption and reducing bone remodelling [12,13]. Parathyroid hormone, and its analogue teriparatide, are the only anabolic therapies available to treat severe osteoporosis [14]. The current drug therapies have been proven to improve bone mineral density and reduce fracture risk, but prolonged use has been associated with various side effects [15,16]. Therefore, the search for new drugs is ongoing [17,18]. In addition, the prophylactic agents for osteoporosis are limited to calcium and vitamin D. Recent advancement in phytomedicine has stimulated interests to transform herbal plants into treatment for chronic diseases, like osteoporosis [2,12,19]. Some vigorously studied herbal plants have demonstrated antiosteoporotic effects in cellular and animal studies [13,19,20]. These include *Rhizoma alismatis* [21], *Curculiginis rhizoma* [22], *Hemidesmus indicus* (L). R. Br [23], *Passiflora foetida* [24], *Cissus quadrangularis* [25], and *Dalbergia sissoo* [26].

In this paper, selected herbal plants which have demonstrated skeletal protecting effects in scientific studies were reviewed. Their geographical origin, active chemical components, and mechanism of action were discussed. The herbal plants included in this review were tested at least in animal or cellular (cultured osteoblasts and osteoclasts) studies, and their bioactive constituents had been identified. Six plant families originating from the Asian continent were discussed, namely Berberidaceae (East Asia), Fabaceae (East Asia), Arecaceae (Southeast Asia), Labiatae (Southeast Asia), Simaroubaceaea (Southeast Asia), and Myrsinaceae (Southeast Asia).

## 2. Antiosteoporotic Constituents Extracted from Natural Plants

### 2.1. The Berberidaceae Family

Epimedium plants (a genus of flowering plants from the Berberidaceae family) are low-growing and deciduous perennial plants [27–29]. They are also known as barrenwort, fairy wings, and bishop's hat. The leaves of other species such as *Epimedium brevicornum* Maxim, *Epimedium sagittatum* Maxim, *Epimedium pubescens* Maxim, and *Epimedium koreanum* Nakai have been used traditionally to combat osteoporosis and menopause-related diseases in China [27,30–32]. These herbal medicinal plants are used throughout the ages as an antiosteoporotic agent in Chinese traditional medicine [27,30–32]. The crude extract of Epimedium flavonoids contain icariin, epimedin B, and epimedin C. These compounds have been identified as the main antiosteoporotic constituents of Epimedium plants by inhibiting bone resorption, triggering bone formation, and blocking urinary calcium excretion [27,30–32]. They have also been shown to prevent osteoporosis without causing uterine hyperplasia in the ovariectomized rat model [20,27,30,31].

The Epimedium flavonoids possess estrogenic activity and improve the maturation of osteoblasts by inducing the expression of alkaline phosphatase (ALP), bone morphogenetic protein-2 (BMP-2) and core binding factor α1 (Cbfα1). They also increase expression of osteoprotegerin (OPG) but reduce the expression of receptor activator of nuclear factor-κB ligand (RANKL), thereby inhibiting the formation of osteoclasts [27,30–33]. Several studies also showed that Epimedium flavonoids upregulated expressions of BMP or Wingless-type signalling (Wnt-signaling) pathway related regulators, like cyclin D [20,27,30,31].

Icariin has been identified as the most active flavonoid glucoside extract of Epimedium plant [27,31]. Icariin inhibits bone loss in the distal femur and tibia in ovariectomized rat models [20,27,30,31]. It is suggested that icariin activates estrogen receptor (ER) and induces ER-dependent bone activity [20,27,30,31]. Icariin also decreases the tartrate-resistant acid phosphate activity (TRAP) activity of osteoclasts, their size and bone resorption activity. This is achieved by lowering IL-6 and TNF-α expression [20,27,30,31]. Icariin can inhibit cyclooxygenase type-2 (COX-2)

activity, expression of LPS-induced hypoxia inducible factor-1α (HIF-1α), and activation of the p38 and c-Jun N-terminal kinase (JNK) in osteoclasts [20,27,30,31]. It also inhibits osteoclasts differentiation by reducing ERK1/2 and Iκ-Bα LPS-induced activation [20,27,30,31].

Ikarisoside A is a natural flavonoid extracted from Epimedium species of *E. koreanum*. It possesses antioxidant and anti-inflammatory properties in LPS-stimulated bone marrow-derived macrophage precursor cells and in RAW264.7 cells [20,30,31]. It also inhibits the formation of osteoclasts and bone resorption activity from these precursor cells [20,31]. Moreover, Ikarisoside A reduces the expression of osteoclastic genes, such as TRAP, matrix metalloproteinase 9 (MMP-9), cathepsin K, and receptor activator of NF-κB (RANK) [20,30,31]. This is achieved by suppressing the activation of the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), JNK, and protein kinase B (Akt)-RANKL [20,30,31]. Thus, it can be concluded that Ikarisoside A has the potential to be used as a remedy to treat diseases involving rheumatoid arthritis and osteoporosis [20,30,34]

### 2.2. The Fabaceae Family

The soybean, scientifically known as *Glycine max* L. (Fabaceae), is mainly grown in Southwest Asia [27]. It is a rich source of proteins and flavonoids, such as daidzein, biochanin A, and genistein [27]. Supplementing soybean protein in the diet is effective in reducing the loss of bone mineral density in ovariectomized rats [27,35,36]. In animal models of bone loss, isoflavones can preserve trabecular microstructure [27,37]. They act by modulating gene expression of collagen type I (COL I), osteocalcin, calciotropic receptor, ALP, cytokines, and growth factors [27,38]. The phytoestrogens in soybean have been shown to exert significant effects on bone metabolism in postmenopausal women. It could be used as a dietary supplement to prevent postmenopausal osteoporosis since isoflavones can improve bone turnover markers, bone mineral density, and bone strength among postmenopausal women [27,36–38]. However, the skeletal effects of soy isoflavones supplementation in humans remain debatable because several meta-analyses reported that the effects were minimal [39,40]. Nevertheless, further studies are necessary to verify the magnitude of the skeletal effects of soy isoflavones in humans.

Genistein is an isoflavone exhibiting estrogenic effect on bone. It modulates B-lymphopoiesis in bone marrow and inhibits bone degradation without any estrogenic effect in the uterus [27,41]. The antiosteoporotic effects of flavonoids depend on the mixture of their estrogenic agonist–antagonist properties [27,41]. Other studies suggest that the antiosteoporotic effects may be derived from other biochemical properties of flavonoids, including enzymatic inhibition of certain protein kinases or activation of estrogen type I receptors [27]. The clinical effectiveness of the flavonoids may be dependent on their ability to produce equol, an isoflavandiol metabolized by gut microflora from daidzein [27,42]. It shows a higher estrogenic activity than the predominant flavonoids [27,42].

Herbal plants of the species *Psoralea corylifolia* L. (commonly known as Malay Tea, Cot Chu, or Ku Tzu locally) belongs to the family Fabaceae [27]. The fruit of this plant is used traditionally to treat bone fractures, osteomalacia, osteoporosis, and joint disorders [13,43]. The fruit extract of *P. corylifolia* significantly increases the serum concentration of inorganic phosphorus and induces bone calcification in rats [27,43]. The crude extracts of its fruit and seed, as well as two of its dominant isoflavones (corylin and bavachin), have been found to stimulate bone formation [27,43]. Extracts of *P. corylifolia* from different parts of the plants also contain bakuchalcone, psoralen, bakuchiol, psoralidin, bavachinin, isopsoralen, and flavones [44].

Some bioactive compounds isolated from *P. corylifolia* have been found to exert bone-protective effects. Bavachalcone can inhibit osteoclastogenesis by hindering the ERK and Akt signaling, as well as Chromosome-Fos (c-Fos) and nuclear factor of activated T cells c1 (NFATc1) induction during differentiation [27,43]. Psoralidin, bakuchiol, isobavachin, and corylin have been found to have strong antioxidant activities, whereas other compounds, such as bavachin and corylin, have been shown to stimulate osteoblastic proliferation [13,27]. Bakuchiol has a three-fold higher binding affinity for estrogen receptor alpha (ERα) than for estrogen receptor beta (ERβ) [13,27]. It does not have significant uterotrophic activity, although demonstrating in vitro estrogenic activity [27,45]. It

can reduce postmenopausal bone loss by increasing ALP, calcium concentrations, serum estrogen concentration, and bone mineral density [27,45]. Psoralen, a coumarin-like derivative extracted from the fruit of *P. corylifolia* L., has stimulatory effects on new bone formation [27,46,47]. It also modulates differentiation of osteoblasts in a dose-dependent manner in primary mouse calvariae by upregulating osteoblast-specific genes expression of osteocalcin, type I collagen, and sialoprotein [46,47]. Psoralen affects BMP signalling activation in order to promote differentiation of osteoblasts [46–48]. It stimulates BMP-2 and BMP-4 gene expression, as well as increases phospho-Smad1/5/8protein level [46–48]. This evidence suggests that psoralen is a potent anabolic agent in treating osteoporosis [46–48].

### 2.3. The Arecaceae Family

Oil palm in the palm family (Arecaceae) is mostly cultivated as a source of oil [49]. Oil palm is grown extensively in the equator region of native West and Central Africa, as well as in Asian countries including Malaysia and Indonesia [50]. The most planted species of Arecaceae Family is *Elaeis guineensis* (African oil palm) and other species such as *Elaeis oleifera* (American oil palm) and *Attalea maripa* (Maripa palm) are lesser known [51]. Palm oil is an edible vegetable oil derived from the mesocarp (orange-red pulp) of the oil palm fruits [49]. It is naturally reddish in colour due to the presence of high beta-carotene content [52,53].

Palm oil of *Elaeis guineensis* is well known to have high content of vitamin E [49]. Vitamin E is a conjoint term for tocopherol and tocotrienol isoforms which are well-known for their antioxidant and anti-inflammatory properties as well as other beneficial effects on the body [54,55]. Both isoforms of tocopherols and tocotrienols exist in four different forms in nature: namely; α-, β-, γ-, and δ- [55,56]. In nature, these isomers are normally present as a mixture of varying composition [57]. For example, vitamin E extracted from crude palm oil consists of around 36% α-tocopherol, and the rest are made up by the four tocotrienol isomers [58]. On the other hand, vitamin E from annatto extract comprises of approximately 90% δ-tocotrienol and the rest is γ-tocotrienol [59].

The anti-oxidative and anti-inflammatory properties of tocotrienol make it a suitable anti-osteoporotic agent [60,61]. Both oxidative stress and inflammation are known to be involved in the pathogenesis of osteoporosis [62,63]. Oxidative stress has been shown to harm osteoblasts by affecting their differentiation and survival rate [64]. Additionally, oxidative stress also enhances the signalling of osteoclasts and simultaneously promotes their differentiation [65]. Proinflammatory cytokines—such as interleukin-1, interleukin-6, and tumour necrosis factor α—are also increased by oxidative stress, and they are also harmful to the bone [66].

A study by Hermizi et al. (2009) has shown that, both tocotrienol-rich fraction and gamma-tocotrienol supplementations were effective in retaining trabecular bone structure in nicotine-induced bone loss model [67]. Also, Aktifanus et al. (2012) and Soelaiman et al. (2012) have reported that, supplementation with tocotrienol reduced single-labelled surface and increased double-labelled surface in the ovariectomized rats [68,69]. In addition, ovariectomized rats supplemented with 30 and 60 mg/kg body weight of palm vitamin E had shown significantly higher bone mineral density at the femur and vertebrae as compared to the control untreated group [70]. Similar findings were reported in the testosterone deficiency, buserelin, and glucocorticoid-induced bone loss model [71–76]. Studies also have shown that palm vitamin E was able to restore bone calcium levels in the femur and vertebra of orchidectomized and ovariectomized rats [70,71].

The skeletal effects of vitamin E have been tested in many human studies but in most cases synthetic alpha-tocopherol was used (reviewed in [77,78]). The efficacy of palm vitamin E mixture rich in tocotrienol in preventing osteoporosis has not been studied so far. A similar vitamin E mixture, also rich in tocotrienol, from annatto beans has been tested by Shen et al. (2018) [79]. The results showed that tocotrienol decreased bone resorption markers and oxidative stress in post-menopausal osteopenic women after 12 weeks [79].

### 2.4. The Labiatae Family

A Chinese herb known as *Salvia miltiorrhiza* Bunge (commonly known as 'dan shen' or 'red sage root') from the family of Labiatae is traditionally used to treat diseases related to cardio-cerebral disorders [48,80,81]. *S. miltiorrhiza* has been shown pharmacologically to possess anticoagulation, blood flow improvement, anti-inflammatory, free radical scavenging, and mitochondrial protective properties [48,81,82]. Phytochemical studies of *S. miltiorrhiza* Bunge have revealed multiple groups of compounds, including tanshinones (tanshinone I, tanshinone IIA, 16-dihydrotanshinone I, cryptotanshinone) and phenolics (salvianolic acid A, protocatechuicaldehyde, and salvianolic acid B) [27,83,84]. Treatment with *S. miltiorrhiza* significantly prevents the decrease in trabecular bone mass and bone mineral density, reduces TRAP activity and parameters of oxidative stress, which includes malondialdehyde (MDA) and nitric oxide (NO) induced by sex hormones deficiency in rodents [20,27,82]. Tanshinones are reported to reduce the TRAP-positive multinucleated osteoclast formation [85]. Tanshinone IIA is proven to partially inhibit ovariectomy-induced bone loss by reducing bone turnover in vivo [27,85,86]. It inhibits osteoclast formation by suppressing the c-fos and NFATc1 expression induced by RANKL [27,85,86].

Salvianolic acid A from *S. miltiorrhiza* Bunge can inhibit bone loss in rats given long-term prednisone [20,87]. This is achieved by regulating osteogenesis and suppressing adipogenesis in bone marrow stromal cells [20,87]. Similarly, Salvianolic acid B has been used to inhibit glucocorticoid-induced cancellous bone loss and suppress adipogenesis [20]. It modulates the differentiation of bone marrow stromal cell (MSC) to osteoblasts and upregulates osteoblastic activities. It decreases the differentiation of glucocorticoid-associated adipogenesis through modulating the expression of Dickkopf-1, RUNX2, peroxisome proliferator-activated receptor-gamma (PPAR-$\gamma$), and $\beta$-catenin in MSC [20,88].

### 2.5. The Simaroubaceae Family

Tongkat Ali, also known as *Eurycoma longifolia*, from the family Simaroubaceae, is a traditional herbal plant found in Malaysia [89,90]. The root extract of Tongkat Ali is a well-known folk remedy among the Malaysians used to enhance fertility and sexuality, and delay ageing [89]. The bioactive compounds of these plants contain quassinoid alkaloids which are believed to cure allergies, relieve fevers, reduce tumours, and treat malaria [89,91]. Other bioactive compounds found in this plant are tannins and high-molecular-weight glycoproteins, polysaccharides and mucopolysaccharides [89].

Eurycomalactone, eurycomanone, and eurycomanol of *E. longifolia* have been shown to increase testosterone level in the blood and are capable of inhibiting the sex hormone-binding globulin [89,92,93]. Testosterone is known to enhance bone formation and prevent osteoporosis [89,94,95]. Testosterone and 5-$\alpha$-dihydrotestosterone suppress RANKL and the number of colony-forming unit-macrophages, thereby reducing osteoclast numbers [96]. Consequently, the bone degradation process will be halted and bone density will be maintained [92,93]. Testosterone replacement increases bone density and mass and is an effective treatment for male osteoporosis due to hypogonadism [89,93–95,97]. However, it comes with some side effects, such as increased risk for prostate cancer, polycythemia, and cardiovascular events [98]. *E. longifolia*, as an androgenic compound, may act as an alternative to prevent osteoporosis associated with low testosterone level [89,92,93]. It has a good safety profile and convenient oral administration [89,92,93].

### 2.6. The Myrsinaceae Family

The herbal plant traditionally known as Kacip Fatimah (*Labisia pumila*) belongs to the family *Myrsinaceae* [99,100]. *Labisia pumila* water extract is traditionally used by Malay women to treat menstrual irregularities and dysmenorrhoea [99,100]. It is also used to improve uterine contraction post-delivery and to promote sexual function [99,100]. Its water extract is also being consumed to treat diseases such as gonorrhoea, rheumatism, dysentery, and bone disorders [101]. The plant

*L. pumila* is capable of inducing the production of estrogen. Post-menopausal women are prone to have osteoporosis due to decreased circulating estrogen [100,102]. Estrogen induces osteoclast apoptosis and inhibits osteoblast apoptosis [99,100]. This reduces bone degradation and increases bone formation activity [99,100].

Pro-inflammatory cytokines, such as IL-1 and IL-6, are capable of influencing osteoclastogenesis by self-renewal stimulation [101]. These pro-inflammatory cytokines are inhibited by the presence of estrogen [99,101,102]. According to recent studies, *L. pumila* is capable of inducing the production of estrogen. Therefore, *L. pumila* can be regarded as an alternative to estrogen replacement therapy (ERT) [99,101,102].

Also, *L. pumila* exerts anti-oxidant properties due to the presence of active compounds, such as ascorbic acid, anthocyanin, beta-carotene, flavonoids, and phenolic compounds [101,102]. Other active constituents of *L. pumila*, such as anthocyanin and phenolics, also play a role as anti-oxidant and anti-inflammatory agents [101,102]. These effective free radical scavengers can help to improve chronic diseases related to oxidative stress [102].

## 3. Perspectives

Several important issues should be considered when using natural herbal plants to treat osteoporosis. These issues are (i) selectivity: the mechanism of action, selective binding to sites of action and any possible resistance of the compound towards bioactive site action; (ii) therapeutic/pharmaceutical index: the benefit-to-risk ratio of the applied bioactive compound and clinical trials before being used as a standard therapy or along with standard therapy; (iii) controllability: the rate of targeted bioactive compound must be clear, reproducible and controllable; and lastly, (iv) convenience: preferably, the drug should be orally administered; therefore the liquid or tablet dosage form must be initially formulated and stabilized, making it easier to be taken orally [22].

The safety of herbal remedies should also be studied intensively. There is a widespread belief that herbals are natural and harmless. However, studies have shown that hepatotoxicity is the most frequently reported toxic effect of herbal remedies [22,100]. Therefore, precise investigation of the bioactive compounds and scientific data regarding the safety and toxicity are needed before definite clinical trials are conducted.

In addition, standardization of medical herbal plants should also be emphasized. The lack of standardization has contributed to difficulties in validating the efficacy of the plants, which is important for further study of targeted bioactive compounds. Plants that are commonly used in laboratory experiments should be investigated thoroughly in terms of their pharmacology and therapeutic effect before being tested in patients suffering from osteoporosis and other bone-related diseases.

Many natural herbal plants have the potential to be developed as anti-osteoporotic agents. However, only a fraction of these plants has been thoroughly investigated by researchers. More reliable, efficient, and rapid bioassays should be developed to examine the antiosteoporotic efficacy of these botanical extracts, as well as to identify the compounds responsible for the bone-protective effects and mechanism involved. Most anti-osteoporotic agents derived from herbal medicinal plants can be used as prophylactic rather than therapeutic agents. If no clinical trials are done, the application and development of these herbal plants will remain restricted and undiscovered. It is important to translate laboratory findings to clinical outcomes to enable drugs from natural plants to be used for human therapy.

There are some limitations pertaining to the discussion of this review. Quality assessment was not performed on the studies included in this review. Therefore, some studies quoted might be subjected to biases and errors. The readers should interpret the studies with caution. Most botanical agents cited do not have a complete safety profile, either in animal or in humans. In most animal studies, the efficacy data of these botanical agents are not complemented with safety data. Therefore, the therapeutic index of these agents remains elusive to the readers.

## 4. Conclusions

Herbal plants are a rich source of medicinal compounds that can be used to prevent osteoporosis. Many animal and cellular studies have been conducted to demonstrate the antiosteoporotic effects of these botanical extracts and their bioactive compounds (Table 1). They modulate bone remodelling by acting directly on the bone cells or through lowering oxidative stress and inflammation or increasing sex hormone levels (Figure 1). Through enhancing bone formation and suppressing bone reabsorption, these agents can improve bone mass and reduce the risk of fragility fracture. Fracture prevention also relies on improvements in muscle strength, coordination, and cognitive function. Botanical agents may affect these bodily functions, but they are outside the scope of this review. A proper human clinical trial to validate their bone-protective effects needs to be conducted. The use of botanical compounds as an intervention for osteoporosis also faces issues of standardization, selectivity, and safety. These issues should be overcome to promote their use in preventing osteoporosis.

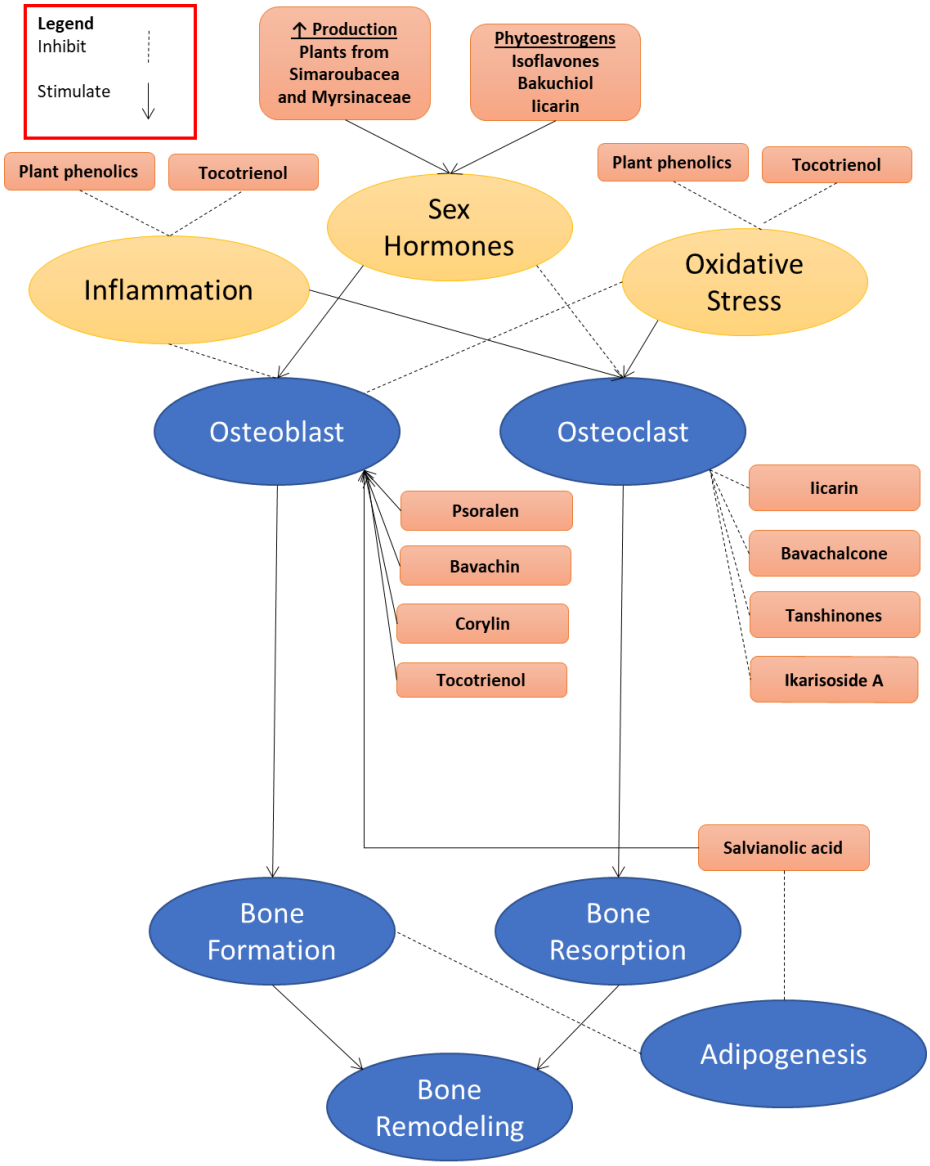


**Figure 1.** The role of botanical bioactive compounds in regulating bone metabolism. They may act directly on the bone cells, or through reducing inflammation and oxidative stress, or indirectly via increasing the level of sex hormones and interacting with sex hormone receptors on bone cells.

**Table 1.** Summary of anti-osteoporotic properties of medicinal plants.

| Family | Scientific Name | Compound | Pharmacological study |
| --- | --- | --- | --- |
| *Berberidaceae* | *E. brevicornum* Maxim<br>*E. sagittatum* Maxim<br>*E. pubescens* Maxim<br>*E. koreanum* Nakai<br>*E. koreanum* | | ➢ Prevents osteoporosis without causing uterine hyperplasia in ovariectomized rats.<br>➢ Inhibits bone resorption, triggers bone formation, and blocks urinary calcium excretion.<br>➢ Increases the messenger ribonucleic acid expressions of bone morphogenetic protein and wingless-type signaling pathway related regulators such as bone morphogenetic protein-2 and cyclin D.<br>➢ Stimulates osteoblast proliferation via estrogen receptor-dependent mechanism.<br>➢ Possesses estrogenic activity and is able to regulate bone metabolism and improve the maturation of osteoblasts by inducing alkaline phosphatase, bone morphogenetic protein-2, macrophage colony stimulating factor, osteoprotegerin, receptor activator of nuclear factor-κB ligand, core binding factor α1, and interliukin-6 and signaling effectors against decapentaplegic protein 4. |
| | | Iicarin | ➢ Inhibits bone loss in the distal femur and tibia of the rat model and postmenopausal women.<br>➢ Decreases tartrate-resistant acid phosphatase activity of osteoclasts, decreases the size of lipopolysaccharide-induced osteoclasts formation, prevents lipopolysaccharide-induced bone resorption and interleukin-6 and tumor necrosis factor-α expression.<br>➢ Inhibits cyclooxygenasetype-2 synthesis, expression of lipopolysaccharide-induced hypoxia inducible factor-1α, and lipopolysaccharide-mediated activation of the p38 and Jun N-terminal kinase involved in osteoclasts differentiation.<br>➢ Reduces extracellular regulated-kinases 1/2 and lipopolysaccharide-induced activation.<br>➢ Reduces specific genes of osteoclasts: tartrate-resistant acid phosphatase, matrix metalloproteinase-9, cathepsin K and receptor activator of nuclear factor-kappa-B ligand. |
| | | Ikarisoside A | ➢ Shows antioxidant and anti-inflammatory properties in lipopolysaccharide-stimulated bone marrow-derived macrophage precursor cells and in RAW264.7 cells.<br>➢ Inhibits activation of nuclear factor kappa-light-chain-enhancer of activated B cells, Jun N-terminal kinase, protein kinase B-receptor activator of nuclear factor-κB ligand pathway in osteoclasts and their resorbing activity. |
| *Fabaceae* | *Glycine max* L.<br>*Psoralea corylifolia* L. | | ➢ Dietary soybean protein supplementation is effective in reducing loss of bone mineral density in ovariectomized rats.<br>➢ Improves bone turnover markers, bone mineral density, and bone strength among postmenopausal women.<br>➢ Modulates bone metabolism-related gene expression of collagen type I, osteocalcin, calciotropic receptor, alkaline phosphatase, cytokines, and growth factors.<br>➢ Induces bone calcification in rats.<br>➢ Increases the concentration of inorganic phosphorus in serum.<br>➢ Regulates the trabecular microstructure and prevent bone loss in postmenopausal women and animal models. |
| | | Genistein | ➢ Shows estrogenic effects in the bone but not in the uterus.<br>➢ Modulates B-lymphopoiesis.<br>➢ Inhibits bone degradation. |

**Table 1.** *Cont.*

| Family | Scientific Name | Compound | Pharmacological Study |
| --- | --- | --- | --- |
| *Fabaceae* | *Glycine max* L. *Psoralea corylifolia* L. | Bavachalcone | ➢ Inhibits osteoclastogenesis.<br>➢ Inhibits the extracellular regulated-kinases and protein kinase B signalling and chromosome-Fos and nuclear factor of activated T cells c1 induction during differentiation. |
| | | Psoralidin, Isobavachin | ➢ Strong antioxidant. |
| | | Bavachin Corylin | ➢ Stimulates osteoblastic proliferation. |
| | | Bakuchiol | ➢ Has high binding affinity for ERα.<br>➢ Shows no significant uterotrophic activity.<br>➢ Stimulates estrogenic activity in vitro.<br>➢ Reduces postmenopausal bone loss by increasing alkaline phosphatase, calcium concentrations, serum estrogen concentration, and bone mineral density. |
| | | Psoralen | ➢ Stimulates new bone formation.<br>➢ Stimulates differentiation of osteoblasts in a dose-dependent manner in primary mouse calvariae.<br>➢ Upregulates osteoblast-specific genes expression of osteocalcin, type I collagen and sialoprotein.<br>➢ Stimulates bone morphogenetic protein-2 and bone morphogenetic protein-4 gene expression. |
| *Arecaceae* | *Elaeis guineensis* | Tocotrienol | ➢ Well-known for their antioxidant, anti-oxidative stress, anti-inflammatory properties and anti-osteoporotic agent.<br>➢ Suppresses the proinflammatory cytokines expression.<br>➢ Effective in retaining trabecular bone structure in the nicotine-induced bone loss model.<br>➢ Reduces of single-labelled surface and increased in double-labelled surface in the ovariectomized rats.<br>➢ Increases bone mineral density at the femur and vertebrae of the rats in the testosterone deficiency and the glucocorticoid bone loss model.<br>➢ Restores bone calcium level at the femur and vertebra of orchidectomized and ovariectomized rats.<br>➢ Improves biomechanical strength of the femur in normal male rats. |
| *Labiatae* | *Salvia miltiorrhiza* Bunge | | In ovariectomized rats:<br>➢ Prevents the decrease in trabecular bone mass and bone mineral density.<br>➢ Reduces the tartrate-resistant acid phosphatase activity.<br>➢ Decreases oxidative stress. |
| | | Tanshinones | ➢ Reduces the tartrate-resistant acid phosphatase-positive multinucleated osteoclast formation |
| | | Tanshinones IIA | ➢ Partially inhibits ovariectomy-induced bone loss by reducing bone turnover. |
| | | Salvianolic acid A | ➢ Inhibits bone loss in rats given long-term prednisone.<br>➢ Stimulates osteogenesis.<br>➢ Suppresses adipogenesis in bone marrow stromal cells. |

**Table 1.** *Cont.*

| Family | Scientific Name | Compound | Pharmacological Study |
| --- | --- | --- | --- |
| *Labiatae* | *Salvia miltiorrhiza* Bunge | Salvianolic acid B | ➢ Inhibits glucocorticoid-induced cancellous bone loss.<br>➢ Suppresses adipogenesis.<br>➢ Stimulates bone marrow stromal cell differentiation to osteoblasts.<br>➢ Upregulates osteoblastic activities.<br>➢ Modulates the expression of messenger of ribonucleic acid of dickkopf-1, runt-related transcription factor 2, peroxisome proliferator-activated receptor gamma, and β-catenin in mesenchymal stem cell. |
| *Simaroubaceaea* | *Eurycoma longifolia* | | ➢ Androgenic substance with a good safety profile. |
| | | Eurycomalactone<br>Eurycomanol | ➢ Increases testosterone level in the blood.<br>➢ Inhibits sex hormone-binding globulin. |
| | | Eurycomanone | ➢ Increases testosterone level in the blood. |
| *Myrsinaceae* | *Labisia pumila* | | ➢ Used traditionally to treat female sexual problems.<br>➢ Stimulates the production of estrogen.<br>➢ Stimulates the production of estrogen. |
| | | Ascorbic acid<br>Anthocyanin<br>Beta-carotene,<br>Flavonoids phenolic compounds | ➢ Anti-oxidant and free radical scavengers-effective free radical scavengers in conditions, such as osteoporosis and rheumatism, which are related to ageing and oxidative stress.<br>➢ Anti-inflammatory agents. |

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## Abbreviations

| | |
| --- | --- |
| ALP | Alkaline phosphatase |
| BMP-2/4 | Bone morphogenetic protein-2/4 |
| M-CSF | Macrophage colony stimulating factor |
| OPG | Osteoprotegerin |
| RANKL | Receptor activator of nuclear factor-κB ligand |
| Cbfα1 | Core binding factorα1 |
| SMAD4 | Signaling effectors mothers against decapentaplegic protein 4 |
| Wnt-signaling | Wingless-type signaling |
| cyclinD | Cyclin dependent |
| OVX | Ovariectomized |
| ER | Estrogen receptor |
| TRAP | Tartrate-resistant acid phosphatase |
| LPS | Lipopolysaccharides |
| IL-6/1 | Interleukin-6/1 |
| TNF-α | Tumor necrosis factor |
| COX-2 | Cyclooxygenasetype-2 |
| HIF-1α | Hypoxia inducible factor-1α |
| p38 | Protein 38 |
| JNK | Jun N-terminal kinase |

| | |
|---|---|
| ERK1/2 | Extracellular regulated-kinases 1/2 |
| Iκ-BαLPS | ikappa-Balpha lipopolysaccharide |
| MMP-9 | Matrix metalloproteinase-9 |
| Akt | Protein Kinase B |
| NF-κB | nuclear factor kappa-light-chain-enhancer of activated B cells |
| RANK | receptor activator of NF-κB |
| COL I | collagen type I |
| NFATc1 | nuclear factor of activated T cells c1 |
| c-Fos | Chromosome-Fos |
| B-lymphopoiesis | Bone marrow-lymphopoiesis |
| ERα/ERβ | Estrogen receptor alpha/beta |
| BMD | Bone mineral density |
| Osx | Osteoblast-specific transcription factor osterix |
| MDA | malondialdehyde |
| NO | nitric oxide |
| mRNA | Messenger ribonucleic acid |
| Dickkopf-1 | DKK-1 |
| Runx2 | Runt-related transcription factor 2 |
| PPAR-γ | Peroxisome proliferator-activated receptor gamma |
| β-catenin | Beta-cateni |
| MSC | Mesenchymal stem cell |
| EL | *Eurycoma longifolia* |
| ERT | estrogen replacement therapy |

## References


1. Lama, A.; Santoro, A.; Corrado, B.; Pirozzi, C.; Paciello, O.; Pagano, T.B.; Russo, S.; Calignano, A.; Mattace Raso, G.; Meli, R. Extracorporeal shock waves alone or combined with raloxifene promote bone formation and suppress resorption in ovariectomized rats. *PLoS ONE* **2017**, *12*, e0171276. [CrossRef] [PubMed]
2. Sucuoglu, H.; Koyuncu, H. Distribution of male osteoporosis patients according to age, classification, and fracture. *Istanb. Med. J.* **2017**, *18*, 13–17. [CrossRef]
3. Poole, K.E.S.; Skingle, L.; Gee, A.H.; Turmezei, T.D.; Johannesdottir, F.; Blesic, K.; Rose, C.; Vindlacheruvu, M.; Donell, S.; Vaculik, J.; et al. Focal osteoporosis defects play a key role in hip fracture. *Bone* **2017**, *94*, 124–134. [CrossRef] [PubMed]
4. Iseme, R.A.; Mcevoy, M.; Kelly, B.; Agnew, L.; Walker, F.R.; Attia, J. Is osteoporosis an autoimmune mediated disorder? *Bone Rep.* **2017**, 121–131. [CrossRef] [PubMed]
5. Eastell, R.; Christiansen, C.; Grauer, A.; Kutilek, S.; Libanati, C.; McClung, M.R.; Reid, I.R.; Resch, H.; Siris, E.; Uebelhart, D. Effects of denosumab on bone turnover markers in postmenopausal osteoporosis. *J. Bone Miner. Res.* **2011**, *26*, 530–537. [CrossRef] [PubMed]
6. Szulc, P.; Delmas, P. Bone loss in elderly men: Increased endosteal bone loss and stable periosteal apposition. The prospective minos study. *Osteoporos. Int.* **2007**, *18*, 495–503. [CrossRef] [PubMed]
7. Misiorowski, W. Osteoporosis in men. *Prz. Menopauzalny* **2017**, *16*, 70–73. [CrossRef] [PubMed]
8. Moreira-Marconi, E.; Dionello, C.F.; Morel, D.S.; Sá-Caputo, D.C.; Souza-Gonçalves, C.R.; Paineiras-Domingos, L.L.; Guedes-Aguiar, E.O.; Marin, P.J.; del Pozo Cruz, B.; Bernardo-Filho, M. Could whole body vibration exercises influence the risk factors for fractures in women with osteoporosis? *Osteoporos. Sarcopenia* **2016**, *2*, 214–220. [CrossRef]
9. Rachner, T.D.; Khosla, S.; Hofbauer, L.C. Osteoporosis: Now and the future. *Lancet* **2011**, *377*, 1276–1287. [CrossRef]
10. Orcel, P.; Funck-Brentano, T. Medical management following an osteoporotic fracture. *Orthop. Traumatol. Surg. Res.* **2011**, *97*, 860–869. [CrossRef] [PubMed]
11. Khosla, S.; Hofbauer, L.C. Osteoporosis treatment: Recent developments and ongoing challenges. *Lancet Diabetes Endocrinol.* **2017**, *5*, 898–907. [CrossRef]

12. Wu, L.; Ling, Z.; Feng, X.; Mao, C.; Xu, Z. Herb medicines against osteoporosis: Active compounds & relevant biological mechanisms. *Curr. Top. Med. Chem.* **2017**, *17*, 1670–1691. [PubMed]
13. Augustine, M.; Horwitz, M.J. Parathyroid hormone and parathyroid hormone-related protein analogs as therapies for osteoporosis. *Curr. Osteoporos. Rep.* **2013**, *11*, 400–406. [CrossRef] [PubMed]
14. Reginster, J.Y.; Pelousse, F.; Bruyere, O. Safety concerns with the long-term management of osteoporosis. *Expert Opin. Drug Saf.* **2013**, *12*, 507–522. [CrossRef] [PubMed]
15. Hough, F.S.; Brown, S.L.; Cassim, B.; Davey, M.R.; de Lange, W.; de Villiers, T.J.; Ellis, G.C.; Lipschitz, S.; Lukhele, M.; Pettifor, J.M. The safety of osteoporosis medication. *S. Afr. Med. J.* **2014**, *104*, 279–282. [CrossRef] [PubMed]
16. Deal, C. Potential new drug targets for osteoporosis. *Nat. Rev. Rheumatol.* **2009**, *5*, 20. [CrossRef] [PubMed]
17. Kenakin, T.; Christopoulos, A. Signalling bias in new drug discovery: Detection, quantification and therapeutic impact. *Nat. Rev. Drug Discov.* **2013**, *12*, 205. [CrossRef] [PubMed]
18. Fouda, A.-M.; Youssef, A.R. Antiosteoporotic activity of salvadora persica sticks extract in an estrogen deficient model of osteoporosis. *Osteoporos. Sarcopenia* **2017**, *3*, 132–137. [CrossRef]
19. Zhang, N.-D.; Han, T.; Huang, B.-K.; Rahman, K.; Jiang, Y.-P.; Xu, H.-T.; Qin, L.-P.; Xin, H.-L.; Zhang, Q.-Y.; Li, Y.-M. Traditional chinese medicine formulas for the treatment of osteoporosis: Implication for antiosteoporotic drug discovery. *J. Ethnopharmacol.* **2016**, *189*, 61–80. [CrossRef] [PubMed]
20. Zhang, L.L.; Xu, W.; Xu, Y.L.; Chen, X.; Huang, M.; Lu, J.J. Therapeutic potential of rhizoma alismatis: A review on ethnomedicinal application, phytochemistry, pharmacology, and toxicology. *Ann. N. Y. Acad. Sci.* **2017**, *1401*, 90–101. [CrossRef] [PubMed]
21. Wang, L.; He, Y.J.; Han, T.; Zhao, L.; Lv, L.; He, Y.Q.; Zhang, Q.Y.; Xin, H.L. Metabolites of curculigoside in rats and their antiosteoporotic activities in osteoblastic mc3t3-e1 cells. *Fitoterapia* **2017**, *117*, 109–117. [CrossRef] [PubMed]
22. Desai, S.; Babaria, P.; Nakarani, M.; Shah, K.; Paranjape, A. Antiosteoporotic effect of hemidesmus indicus linn. On ovariectomised rats. *J. Ethnopharmacol.* **2017**, *199*, 1–8. [CrossRef] [PubMed]
23. Ahmad, N.; Chillara, R.; Kushwaha, P.; Khedgikar, V.; Karvande, A.; Choudhary, D.; Adhikary, S.; Maurya, R.; Trivedi, R. Evaluation of anti-osteoporotic activity of butanolic fraction from passiflora foetida in ovariectomy-induced bone loss in mice. *Biomed. Pharmacother.* **2017**, *88*, 804–813. [CrossRef] [PubMed]
24. Tasadduq, R.; Gordon, J.; Al-Ghanim, K.A.; Lian, J.B.; Van Wijnen, A.J.; Stein, J.L.; Stein, G.S.; Shakoori, A.R. Ethanol extract of cissus quadrangularis enhances osteoblast differentiation and mineralization of murine pre-osteoblastic mc3t3-e1 cells. *J. Cell. Physiol.* **2017**, *232*, 540–547. [CrossRef] [PubMed]
25. Karvande, A.; Khedgikar, V.; Kushwaha, P.; Ahmad, N.; Kothari, P.; Verma, A.; Kumar, P.; Nagar, G.K.; Mishra, P.R.; Maurya, R. Heartwood extract from dalbergia sissoo promotes fracture healing and its application in ovariectomy-induced osteoporotic rats. *J. Pharm. Pharmacol.* **2017**, *69*, 1381–1397. [CrossRef] [PubMed]
26. Jia, M.; Nie, Y.; Cao, D.-P.; Xue, Y.-Y.; Wang, J.-S.; Zhao, L.; Rahman, K.; Zhang, Q.-Y.; Qin, L.-P. Potential antiosteoporotic agents from plants: A comprehensive review. *Evid.-Based Complement. Altern. Med.* **2012**, *2012*, 364604. [CrossRef] [PubMed]
27. Liu, X.L.; Li, J.H.; Yang, Y.F.; Zhu, J.Y. Floral development of gymnospermium microrrhynchum (berberidaceae) and its systematic significance in the nandinoideae. *Flora* **2017**, *228*, 10–16. [CrossRef]
28. Sheng, M.; Chen, Q.; Wang, L.; Tian, X. Hybridization among epimedium (berberidaceae) species native to china. *Sci. Hortic.* **2011**, *128*, 342–351. [CrossRef]
29. Indran, I.R.; Liang, R.L.Z.; Min, T.E.; Yong, E.-L. Preclinical studies and clinical evaluation of compounds from the genus epimedium for osteoporosis and bone health. *Pharmacol. Ther.* **2016**, *162*, 188–205. [CrossRef] [PubMed]
30. Ma, H.; He, X.; Yang, Y.; Li, M.; Hao, D.; Jia, Z. The genus epimedium: An ethnopharmacological and phytochemical review. *J. Ethnopharmacol.* **2011**, *134*, 519–541. [CrossRef] [PubMed]
31. Hsieh, T.P.; Sheu, S.Y.; Sun, J.S.; Chen, M.H.; Liu, M.H. Icariin isolated from epimedium pubescens regulates osteoblasts anabolism through bmp-2, smad4, and cbfa1 expression. *Phytomedicine* **2010**, *17*, 414–423. [CrossRef] [PubMed]
32. Tantry, M.A.; Dar, J.A.; Idris, A.; Akbar, S.; Shawl, A.S. Acylated flavonol glycosides from epimedium elatum, a plant endemic to the western himalayas. *Fitoterapia* **2012**, *83*, 665–670. [CrossRef] [PubMed]

33. Hidaka, S.; Okamoto, Y.; Miyazaki, K.; Uesugi, T. Evaluation of a soybean product fujiflavone p40 as an antiosteoporotic agent in rats. *Phytother. Res.* **2003**, *17*, 112–119. [CrossRef] [PubMed]
34. Ye, S.F.; Saga, I.; Ichimura, K.; Nagai, T.; Shinoda, M.; Matsuzaki, S. Coumestrol as well as isoflavones in soybean extract prevent bone resorption in ovariectomized rats. *Endocr. Regul.* **2003**, *37*, 145–152. [PubMed]
35. Chin, K.Y.; Ima-Nirwana, S. Can soy prevent male osteoporosis? A review of the current evidence. *Curr. Drug Targets* **2013**, *14*, 1632–1641. [CrossRef] [PubMed]
36. Lambert, M.N.T.; Thybo, C.B.; Lykkeboe, S.; Rasmussen, L.M.; Frette, X.; Christensen, L.P.; Jeppesen, P.B. Combined bioavailable isoflavones and probiotics improve bone status and estrogen metabolism in postmenopausal osteopenic women: A randomized controlled trial. *Am. J. Clin. Nutr.* **2017**, *106*, 909–920. [CrossRef] [PubMed]
37. Ricci, E.; Cipriani, S.; Chiaffarino, F.; Malvezzi, M.; Parazzini, F. Soy isoflavones and bone mineral density in perimenopausal and postmenopausal western women: A systematic review and meta-analysis of randomized controlled trials. *J. Womens Health (Larchmt)* **2010**, *19*, 1609–1617. [CrossRef] [PubMed]
38. Taku, K.; Melby, M.K.; Kurzer, M.S.; Mizuno, S.; Watanabe, S.; Ishimi, Y. Effects of soy isoflavone supplements on bone turnover markers in menopausal women: Systematic review and meta-analysis of randomized controlled trials. *Bone* **2010**, *47*, 413–423. [CrossRef] [PubMed]
39. Arcoraci, V.; Atteritano, M.; Squadrito, F.; D'Anna, R.; Marini, H.; Santoro, D.; Minutoli, L.; Messina, S.; Altavilla, D.; Bitto, A. Antiosteoporotic activity of genistein aglycone in postmenopausal women: Evidence from a post-hoc analysis of a multicenter randomized controlled trial. *Nutrients* **2017**, *9*, 179. [CrossRef] [PubMed]
40. Jin, X.; Sun, J.; Yu, B.; Wang, Y.; Sun, W.J.; Yang, J.; Huang, S.H.; Xie, W.L. Daidzein stimulates osteogenesis facilitating proliferation, differentiation, and antiapoptosis in human osteoblast-like mg-63 cells via estrogen receptor–dependent mek/erk and pi3k/akt activation. *Nutr. Res.* **2017**, *42*, 20–30. [CrossRef] [PubMed]
41. Zhai, Y.; Li, Y.; Wang, Y.; Cui, J.; Feng, K.; Kong, X.; Chen, L. Psoralidin, a prenylated coumestan, as a novel anti-osteoporosis candidate to enhance bone formation of osteoblasts and decrease bone resorption of osteoclasts. *Eur. J. Pharmacol.* **2017**, *801*, 62–71. [CrossRef] [PubMed]
42. Chopra, B.; Dhingra, A.K.; Dhar, K.L. Psoralea corylifolia l. (buguchi)—Folklore to modern evidence: Review. *Fitoterapia* **2013**, *90*, 44–56. [CrossRef] [PubMed]
43. Weng, Z.-B.; Gao, Q.-Q.; Wang, F.; Zhao, G.-H.; Yin, F.-Z.; Cai, B.-C.; Chen, Z.-P.; Li, W.-D. Positive skeletal effect of two ingredients of *Psoralea corylifolia* L. On estrogen deficiency-induced osteoporosis and the possible mechanisms of action. *Mol. Cell. Endocrinol.* **2015**, *417*, 103–113. [CrossRef] [PubMed]
44. Tang, D.-Z.; Yang, F.; Yang, Z.; Huang, J.; Shi, Q.; Chen, D.; Wang, Y.-J. Psoralen stimulates osteoblast differentiation through activation of bmp signaling. *Biochem. Biophys. Res. Commun.* **2011**, *405*, 256–261. [CrossRef] [PubMed]
45. Li, F.; Li, Q.; Huang, X.; Wang, Y.; Ge, C.; Qi, Y.; Guo, W.; Sun, H. Psoralen stimulates osteoblast proliferation through the activation of nuclear factor-κb-mitogen-activated protein kinase signaling. *Exp. Ther. Med.* **2017**, *14*, 2385–2391. [CrossRef] [PubMed]
46. Sen, C.K.; Rink, C.; Khanna, S.; Palm o Chen, R.; Zhang, Y.; Dong, L.; Huang, J.; Hua, S.; Fu, X. Current Persperctive in the Discovery of Anti-aging Agents from Natural Products. *Int. J. Curr. Adv. Res.* **2017**, *27*, 335–404.
47. Sen, C.K.; Rink, C.; Khanna, S. Palm oil–derived natural vitamin e α-tocotrienol in brain health and disease. *J. Am. Coll. Nutr.* **2010**, *29*, 314S–323S. [CrossRef] [PubMed]
48. Sundram, K.; Sambanthamurthi, R.; Tan, Y.-A. Palm fruit chemistry and nutrition. *Asia Pac. J. Clin. Nutr.* **2003**, *12*, 355–362. [PubMed]
49. Reeves, J., III; Weihrauch, J.L. Composition of Foods. Fats and Oils, Raw-Processed-Prepared; Chigago, IL, USA, 1979. Available online: https://www.cabdirect.org/cabdirect/abstract/19811420761 (accessed on 10 May 2018).
50. Mustapa, A.; Manan, Z.; Azizi, C.M.; Setianto, W.; Omar, A.M. Extraction of β-carotenes from palm oil mesocarp using sub-critical r134a. *Food Chem.* **2011**, *125*, 262–267. [CrossRef]
51. Poku, K. *Small-Scale Palm Oil Processing in Africa*; Food & Agriculture Org.: Roma, Italy, 2002; Volume 148.
52. Peh, H.Y.; Tan, W.D.; Liao, W.; Wong, W.F. Vitamin e therapy beyond cancer: Tocopherol versus tocotrienol. *Pharmacol. Ther.* **2016**, *162*, 152–169. [CrossRef] [PubMed]

53. Ahsan, H.; Ahad, A.; Iqbal, J.; Siddiqui, W.A. Pharmacological potential of tocotrienols: A review. *Nutr. Metab.* **2014**, *11*, 52. [CrossRef] [PubMed]
54. Zhao, L.; Fang, X.; Marshall, M.R.; Chung, S. Regulation of obesity and metabolic complications by gamma and delta tocotrienols. *Molecules* **2016**, *21*, 344. [CrossRef] [PubMed]
55. Shen, C.L.; Klein, A.; Chin, K.Y.; Mo, H.; Tsai, P.; Yang, R.S.; Chyu, M.C.; Ima-Nirwana, S. Tocotrienols for bone health: A translational approach. *Ann. N. Y. Acad. Sci.* **2017**, *1401*, 150–165. [CrossRef] [PubMed]
56. Ng, M.H.; Choo, Y.M.; Ma, A.N.; Chuah, C.H.; Hashim, M.A. Separation of vitamin e (tocopherol, tocotrienol, and tocomonoenol) in palm oil. *Lipids* **2004**, *39*, 1031–1035. [CrossRef] [PubMed]
57. Frega, N.; Mozzon, M.; Bocci, F. Identification and estimation of tocotrienols in the annatto lipid fraction by gas chromatography-mass spectrometry. *J. Am. Oil Chem. Soc.* **1998**, *75*, 1723–1727. [CrossRef]
58. Chin, K.-Y.; Ima-Nirwana, S. The biological effects of tocotrienol on bone: A review on evidence from rodent models. *Drug Des. Dev. Ther.* **2015**, *9*, 2049. [CrossRef] [PubMed]
59. Wong, S.K.; Chin, K.-Y.; Suhaimi, F.H.; Ahmad, F.; Ima-Nirwana, S. The effects of palm tocotrienol on metabolic syndrome and bone loss in male rats induced by high-carbohydrate high-fat diet. *J. Funct. Foods* **2018**, *44*, 246–254. [CrossRef]
60. Ginaldi, L.; Di Benedetto, M.C.; De Martinis, M. Osteoporosis, inflammation and ageing. *Immun. Ageing* **2005**, *2*, 14. [CrossRef] [PubMed]
61. Manolagas, S.C. From estrogen-centric to aging and oxidative stress: A revised perspective of the pathogenesis of osteoporosis. *Endocr. Rev.* **2010**, *31*, 266–300. [CrossRef] [PubMed]
62. Fatokun, A.A.; Stone, T.W.; Smith, R.A. Responses of differentiated mc3t3-e1 osteoblast-like cells to reactive oxygen species. *Eur. J. Pharmacol.* **2008**, *587*, 35–41. [CrossRef] [PubMed]
63. Ha, H.; Kwak, H.B.; Lee, S.W.; Jin, H.M.; Kim, H.-M.; Kim, H.-H.; Lee, Z.H. Reactive oxygen species mediate rank signaling in osteoclasts. *Exp. Cell Res.* **2004**, *301*, 119–127. [CrossRef] [PubMed]
64. McLean, R.R. Proinflammatory cytokines and osteoporosis. *Curr. Osteoporos. Rep.* **2009**, *7*, 134–139. [CrossRef] [PubMed]
65. Hermizi, H.; Faizah, O.; Ima-Nirwana, S.; Nazrun, S.A.; Norazlina, M. Beneficial effects of tocotrienol and tocopherol on bone histomorphometric parameters in sprague–dawley male rats after nicotine cessation. *Calcif. Tissue Int.* **2009**, *84*, 65–74. [CrossRef] [PubMed]
66. Aktifanus, A.T.; Shuid, A.N.; Rashid, N.H.A.; Ling, T.H.; Loong, C.Y.; Saat, N.M.; Muhammad, N.; Mohamed, N.; Soelaiman, I.N. Comparison of the effects of tocotrienol and estrogen on the bone markers and dynamic changes in postmenopausal osteoporosis rat model. *Asian J. Anim. Vet. Adv.* **2012**, *7*, 225–234.
67. Soelaiman, I.N.; Ming, W.; Abu Bakar, R.; Hashnan, N.A.; Mohd Ali, H.; Mohamed, N.; Muhammad, N.; Shuid, A.N. Palm tocotrienol supplementation enhanced bone formation in oestrogen-deficient rats. *Int. J. Endocrinol.* **2012**, *2012*, 532862. [CrossRef] [PubMed]
68. Norazlina, M.; Ima-Nirwana, S.; Gapor, M.; Khalid, B. Palm vitamin e is comparable to α-tocopherol in maintaining bone mineral density in ovariectomised female rats. *Exp. Clin. Endocrinol. Diabetes* **2000**, *108*, 305–310. [CrossRef] [PubMed]
69. Ima-Nirwana, S.; Kiftiah, A.; Zainal, A.; Norazlina, M.; Gapor, M.; Khalid, B. Palm vitamin e prevents osteoporosis in orchidectomized growing male rats. *Nat. Prod. Sci.* **2000**, *6*, 155–160.
70. Ima, S.N.; Fakhrurazi, H. Palm vitamin eprotects bone against dexamethasone-induced osteoporosis in male rats. *Med. J. Malaysia* **2002**, *57*, 136–144.
71. Chin, K.-Y.; Gengatharan, D.; Mohd Nasru, F.S.; Khairussam, R.A.; Ern, S.L.H.; Aminuddin, S.A.W.; Ima-Nirwana, S. The effects of annatto tocotrienol on bone biomechanical strength and bone calcium content in an animal model of osteoporosis due to testosterone deficiency. *Nutrients* **2016**, *8*, 808. [CrossRef] [PubMed]
72. Mohamad, N.-V.; Ima-Nirwana, S.; Chin, K.-Y. Effect of tocotrienol from bixa orellana (annatto) on bone microstructure, calcium content, and biomechanical strength in a model of male osteoporosis induced by buserelin. *Drug Des. Dev. Ther.* **2018**, *12*, 555. [CrossRef] [PubMed]
73. Mohamad, N.V.; Soelaiman, I.N.; Chin, K.Y. Effects of tocotrienol from bixa orellana (annatto) on bone histomorphometry in a male osteoporosis model induced by buserelin. *Biomed. Pharmacother.* **2018**, *103*, 453–462. [CrossRef] [PubMed]
74. Chin, K.Y.; Ima-Nirwana, S. Effects of annatto-derived tocotrienol supplementation on osteoporosis induced by testosterone deficiency in rats. *Clin. Interv. Aging* **2014**, *9*, 1247–1259. [CrossRef] [PubMed]

75. Chin, K.Y.; Ima-Nirwana, S. The effects of alpha-tocopherol on bone: A double-edged sword? *Nutrients* **2014**, *6*, 1424–1441. [CrossRef] [PubMed]
76. Guralp, O. Effects of vitamin e on bone remodeling in perimenopausal women: Mini review. *Maturitas* **2014**, *79*, 476–480. [CrossRef] [PubMed]
77. Shen, C.-L.; Yang, S.; Tomison, M.D.; Romero, A.W.; Felton, C.K.; Mo, H. Tocotrienol supplementation suppressed bone resorption and oxidative stress in postmenopausal osteopenic women: A 12-week randomized double-blinded placebo-controlled trial. *Osteoporos. Int.* **2018**, *29*, 881–891. [CrossRef] [PubMed]
78. Li, X.; Xu, X.; Wang, J.; Yu, H.; Wang, X.; Yang, H.; Xu, H.; Tang, S.; Li, Y.; Yang, L. A system-level investigation into the mechanisms of chinese traditional medicine: Compound danshen formula for cardiovascular disease treatment. *PLoS ONE* **2012**, *7*, e43918. [CrossRef] [PubMed]
79. Wu, W.-Y.; Wang, Y.-P. Pharmacological actions and therapeutic applications of salvia miltiorrhiza depside salt and its active components. *Acta Pharmacol. Sin.* **2012**, *33*, 1119. [CrossRef] [PubMed]
80. Guo, Y.; Li, Y.; Xue, L.; Severino, R.P.; Gao, S.; Niu, J.; Qin, L.-P.; Zhang, D.; Brömme, D. Salvia miltiorrhiza: An ancient chinese herbal medicine as a source for anti-osteoporotic drugs. *J. Ethnopharmacol.* **2014**, *155*, 1401–1416. [CrossRef] [PubMed]
81. Baricevic, D.; Bartol, T.V. Pharmacology 11. The biological/pharmacological activity of the salvia genus. In *The Genus Salvia*; Kintzios, S.E., Ed.; Harwood Academic Publishers: Amsterdam, The Netherland, 2000; pp. 143–184.
82. Kim, H.-K.; Woo, E.-R.; Lee, H.-W.; Park, H.-R.; Kim, H.-N.; Jung, Y.-K.; Choi, J.-Y.; Chae, S.-W.; Kim, H.-R.; Chae, H.-J. The correlation of salvia miltiorrhiza extract–induced regulation of osteoclastogenesis with the amount of components tanshinone i, tanshinone iia, cryptotanshinone, and dihydrotanshinone. *Immunopharmacol. Immunotoxicol.* **2008**, *30*, 347–364. [CrossRef] [PubMed]
83. Lee, S.-Y.; Choi, D.-Y.; Woo, E.-R. Inhibition of osteoclast differentiation by tanshinones from the root ofsalvia miltiorrhiza bunge. *Arch. Pharm. Res.* **2005**, *28*, 909–913. [CrossRef] [PubMed]
84. Kwak, H.B.; Yang, D.; Ha, H.; Lee, J.-H.; Kim, H.-N.; Woo, E.-R.; Lee, S.; Kim, H.-H.; Lee, Z.H. Tanshinone iia inhibits osteoclast differentiation through down-regulation of c-fos and nfatc1. *Exp. Mol. Med.* **2006**, *38*, 256. [CrossRef] [PubMed]
85. Cui, L.; Liu, Y.-Y.; Wu, T.; Ai, C.-M.; Chen, H.-Q. Osteogenic effects of d (+) β-3, 4-dihydroxyphenyl lactic acid (salvianic acid a, saa) on osteoblasts and bone marrow stromal cells of intact and prednisone-treated rats. *Acta Pharmacol. Sin.* **2009**, *30*, 321. [CrossRef] [PubMed]
86. Cui, L.; Li, T.; Liu, Y.; Zhou, L.; Li, P.; Xu, B.; Huang, L.; Chen, Y.; Liu, Y.; Tian, X. Salvianolic acid b prevents bone loss in prednisone-treated rats through stimulation of osteogenesis and bone marrow angiogenesis. *PLoS ONE* **2012**, *7*, e34647. [CrossRef] [PubMed]
87. Thu, H.E.; Mohamed, I.N.; Hussain, Z.; Jayusman, P.A.; Shuid, A.N. Eurycoma longifolia as a potential adoptogen of male sexual health: A systematic review on clinical studies. *Chin. J. Nat. Med.* **2017**, *15*, 71–80. [CrossRef]
88. Edwards, S.E.; da Costa Rocha, I.; Williamson, E.M.; Heinrich, M. Tongkat ali eurycoma longifolia jack. In *Phytopharmacy: An Evidence-Based Guide to Herbal Medicinal Products*; John Wiley & Sons: Hoboken, NJ, USA, 2015; p. 375.
89. Faisal, G.G.; Zakaria, S.M.; Najmuldeen, G.F.; Al-Ani, I.M. Antifungal activity of eurycoma longifolia jack (tongkat ali) root extract. *J. Int. Dent. Med. Res.* **2016**, *9*, 70–74.
90. Thu, H.E.; Mohamed, I.N.; Hussain, Z.; Shuid, A.N. Eurycoma longifolia as a potential alternative to testosterone for the treatment of osteoporosis: Exploring time-mannered proliferative, differentiative and morphogenic modulation in osteoblasts. *J. Ethnopharmacol.* **2017**, *195*, 143–158. [CrossRef] [PubMed]
91. Low, B.-S.; Choi, S.-B.; Abdul Wahab, H.; Kumar Das, P.; Chan, K.-L. Eurycomanone, the major quassinoid in eurycoma longifolia root extract increases spermatogenesis by inhibiting the activity of phosphodiesterase and aromatase in steroidogenesis. *J. Ethnopharmacol.* **2013**, *149*, 201–207. [CrossRef] [PubMed]
92. Chin, K.-Y.; Ima-Nirwana, S. Sex steroids and bone health status in men. *Int. J. Endocrinol.* **2012**, *2012*, 7. [CrossRef] [PubMed]
93. Mohamad, N.-V.; Soelaiman, I.-N.; Chin, K.-Y. A concise review of testosterone and bone health. *Clin. Interv. Aging* **2016**, *11*, 1317. [CrossRef] [PubMed]

94. Huber, D.M.; Bendixen, A.C.; Pathrose, P.; Srivastava, S.; Dienger, K.M.; Shevde, N.K.; Pike, J.W. Androgens suppress osteoclast formation induced by rankl and macrophage-colony stimulating factor. *Endocrinology* **2001**, *142*, 3800–3808. [CrossRef] [PubMed]
95. Chin, K.Y.; Ima-Nirwana, S. The effects of orchidectomy and supraphysiological testosterone administration on trabecular bone structure and gene expression in rats. *Aging Male* **2015**, *18*, 60–66. [CrossRef] [PubMed]
96. Corona, G.; Sforza, A.; Maggi, M. Testosterone replacement therapy: Long-term safety and efficacy. *World J. Men's Health* **2017**, *35*, 65–76. [CrossRef] [PubMed]
97. Shuid, A.N.; Ping, L.L.; Muhammad, N.; Mohamed, N.; Soelaiman, I.N. The effects of *Labisia pumila* var. Alata on bone markers and bone calcium in a rat model of post-menopausal osteoporosis. *J. Ethnopharmacol.* **2011**, *133*, 538–542. [CrossRef] [PubMed]
98. Fathilah, S.N.; Nazrun Shuid, A.; Mohamed, N.; Muhammad, N.; Nirwana Soelaiman, I. *Labisia pumila* protects the bone of estrogen-deficient rat model: A histomorphometric study. *J. Ethnopharmacol.* **2012**, *142*, 294–299. [CrossRef] [PubMed]
99. Nadia, M.; Nazrun, A.; Norazlina, M.; Isa, N.; Norliza, M.; Ima Nirwana, S. The anti-inflammatory, phytoestrogenic, and antioxidative role of *Labisia pumila* in prevention of postmenopausal osteoporosis. *Adv. Pharmacol. Sci.* **2012**, *2012*, 706905. [PubMed]
100. Mohd Effendy, N.; Abdullah, S.; Yunoh, M.F.; Shuid, A.N. Time and dose-dependent effects of *Labisia pumila* on the bone strength of postmenopausal osteoporosis rat model. *BMC Complement. Altern. Med.* **2015**, *15*, 58. [CrossRef] [PubMed]
101. Fathilah, S.N.; Mohamed, N.; Muhammad, N.; Mohamed, I.N.; Soelaiman, I.N.; Shuid, A.N. *Labisia pumila* regulates bone-related genes expressions in postmenopausal osteoporosis model. *BMC Complement. Altern. Med.* **2013**, *13*, 217. [CrossRef] [PubMed]
102. Effendy, N.M.; Shuid, A.N. Time and dose-dependent effects of *Labisia pumila* on bone oxidative status of postmenopausal osteoporosis rat model. *Nutrients* **2014**, *6*, 3288–3302. [CrossRef] [PubMed]


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REVIEW

# The biological effects of tocotrienol on bone: a review on evidence from rodent models

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**Abstract:** Osteoporosis causes significant health care and economic burden to society, leading to a relentless search for effective preventive agents. Tocotrienol, a member of the vitamin E family, has demonstrated promising potential as an osteoporosis-preventing agent. This review summarizes evidence on the effects of tocotrienol on bone in animal models. Techniques used to examine the effects of tocotrienol on bone in animals included bone histomorphometry, X-ray microtomography, dual-energy X-ray absorptiometry, bone turnover markers, bone calcium content, and biomechanical strength. Tocotrienol was shown to improve osteoblast number, bone formation, mineral deposition, and bone microarchitecture in osteopenic rats. It also decreased osteoclast number and bone erosion in the rats. Tocotrienol supplementation resulted in an improvement in bone mineral density, although biomechanical strength was not significantly altered in the rats. The beneficial effects of tocotrienol on bone can be attributed to its role as an antioxidant, anti-inflammatory agent, suppressor of the mevalonate pathway, and modulator of genes favorable to bone formation.

**Keywords:** bone, osteoporosis, tocotrienol, vitamin E

## Introduction

The skeletal system undergoes a constant remodeling process governed by bone cells, ie, osteoblasts for bone formation, osteoclasts for bone resorption, and osteocytes for mechanosensing and mediation of bone remodeling.[1] An imbalance in bone remodeling, whereby the rate of bone resorption is faster than bone formation, will result in osteoporosis.[2] Osteoporosis is a metabolic bone disease suffered by both men and women.[3] The hallmark of osteoporosis is the degeneration of bone density and microarchitecture, leading to bone fragility and fracture.[4] The prevalence of osteoporosis as reflected by fragility fracture is higher in women than in men, with a 6:1 ratio of women to men.[3] However, the mortality rate post-fracture is higher in men compared to their female counterparts.[5,6] The major cause of osteoporosis in women is estrogen deficiency due to menopause, while in men it is late-onset testosterone deficiency.[7,8] Other causes of osteoporosis include prolonged use of glucocorticoid, chronic smoking, alcohol abuse, inflammatory bowel syndrome, celiac disease, immobility, and the use of drugs affecting the skeletal system.[9] Osteoporosis causes a significant economic burden to society due to loss of productivity and the high cost of treatment.[3,10]

The current therapies for osteoporosis, such as bisphosphonates, teriparatide, and strontium ranelate, are effective in increasing bone mineral density of the patients and reducing fractures, with rare cases of adverse side effects.[11] They are indicated for patients over 50 years old with a hip or vertebral fracture, osteoporosis, or osteopenia determined by bone mineral density and a high fracture probability.[9] The commonly prescribed preventive agents, ie, calcium and vitamin D, have been found to be effective

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in preventing fracture among the institutionalized elderly only. According to a meta-analysis, the relative fracture risk for supplemented institutionalized elderly was 0.71 (confidence interval [CI]: 0.57 to 0.89) as compared to 0.89 (CI: 0.76 to 1.04) in the community-dwelling elderly.[12] Calcium supplementation alone or in combination with vitamin D has also been linked to a modest but significant increase in the risk of myocardial infarction (relative risk: 1.24 [CI: 1.07 to 1.45]) in a meta-analysis involving 28,072 participants.[13] There are limited alternatives for those who wish to prevent osteoporosis even before onset of bone loss.

Many studies aiming to develop alternative osteoporosis-preventing agents using natural products have been performed. One natural product that has received much attention is tocotrienol. Tocotrienol, along with tocopherol, belongs to the lipid-soluble vitamin E family. The molecular structure of tocotrienol consists of a chromanol ring and a long carbon tail with three double bonds, whereas the long carbon tail of tocopherol consists solely of single bonds.[14,15] Tocotrienol can be further divided into four different homologues, which are alpha-, beta-, gamma-, and delta-tocotrienol, based on the position of side chains on the chromanol ring.[14,15] Vitamin E from natural sources is usually a mixture of tocotrienols and tocopherols.[16] The predominant tocotrienol homologue in palm oil is gamma-tocotrienol,[17] whereas in annatto bean it is delta-tocotrienol.[18]

The antioxidative and anti-inflammatory activities of tocotrienol make it a suitable osteoporosis-preventing agent. Both oxidative stress and inflammation are implicated in the pathogenesis of osteoporosis.[19,20] Oxidative stress damages osteoblasts and affects their differentiation and survival.[21] Increased oxidative stress also enhances the signaling of osteoclasts and promotes their differentiation.[22] Proinflammatory cytokines, such as interleukin-1, interleukin-6, and tumor necrosis factor alpha, promote the differentiation of osteoclasts.[23] Thus, increased oxidative stress and inflammation will lead to an imbalance in bone remodeling favoring resorption, subsequently resulting in osteoporosis. Tocotrienol exhibits superior antioxidant activity compared to tocopherol due to its uniform distribution in cell membrane, high efficacy in radical recycling, and interaction with lipid radicals.[24] Tocotrienol also suppresses the expression of proinflammatory cytokines induced by nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB).[25] Hence, it is reasonable to hypothesize that tocotrienol can prevent osteoporosis induced by oxidative stress and inflammation. In fact, several in vitro studies have revealed that tocotrienol homologues suppress the formation of osteoclasts,[26,27] promote the expression of bone formation genes,[28] and promote the survival of osteoblasts challenged with oxidative stress.[29]

This review aims to summarize the evidence on the effects of tocotrienol on bone in various rodent models. The effects of tocotrienol on several aspects of bone health, such as bone mineral density, bone microstructure, mineral deposition, and bone strength, are discussed. The review concludes with an overview of the mechanism of action of tocotrienol on bone.

## General study design

Tocotrienol has been tested in various animal models, such as animals that are gonadectomized or treated with glucocorticoid, nicotine, or oxidizing agent (Table 1). The indices of bone health examined include bone microarchitecture determined using histomorphometry and X-ray microtomography, bone turnover markers, bone calcium level, bone mineral density, and biomechanical strength. The treatment period and composition of the tocotrienols used varied from study to study. The general observation was that tocotrienol at the dose of 60 mg/kg body weight administered orally for 8 weeks was effective in preventing bone loss in rats. A lower dose of tocotrienol (30 mg/kg body weight) had been used, but it took a longer time to show effects. Thus, in the following discussion, tocotrienol administered to the animal was 60 mg/kg unless mentioned otherwise. Tocotrienol was administered via force-feeding to mimic its consumption as a supplement in humans.

## Validity of the animal model

The gonadectomized young rats generally showed a reduction in bone volume, trabecular number, and trabecular thickness, and an increase in trabecular separation as compared to the sham group after 2 months of surgery.[30–32] Osteoblast surface, osteoid surface, and osteoid volume were reduced, and osteoclast surface and eroded surface were elevated in the castrated animals compared to the sham group.[33,34] In an experiment by Ima-Nirwana et al,[35] bone mineral density of the orchidectomized young rats was significantly reduced as compared to the sham group after 8 months, but Norazlina et al[36] failed to demonstrate similar effects of ovariectomy on female rats. There were no significant changes in the bone dynamic parameters between the sham and the gonadectomized animals 2 months post-surgery.[30,37,38] This might indicate that although bone loss transpired in the castrated growing animals, the changes in bone turnover were brief and undetectable at sacrifice. There were significant changes

**Table 1** Osteopenia models used to evaluate the bone-protective effects of tocotrienol

| Researchers (year) | Mode of bone loss | Method of inducing bone loss | Treatment (dose in mg/kg body weight) | Composition of tocotrienol (%) | | | | Age when bone loss is induced (months) | Age when treatment starts (months) | Treatment period |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | ATF | ATT | GTT | DTT | | | |
| Hermizi et al[40] (2009) | Smoking | Nicotine injection (7 mg/kg body weight; intraperitoneal) | Palm tocotrienol (60) | | 43 | 31 | 14 | 3 | 5 | 2 months |
| | | | Gamma-tocotrienol (60) | | | 100 | | | | |
| Ima-Nirwana et al[35] (2000) | Testosterone deficiency | Bilateral orchidectomy | Palm vitamin E (30) | 24.4 | 21.6 | 27.7 | 11 | 3 | 3 | 8 months |
| Chin and Ima Nirwana[30] (2014) | Testosterone deficiency | Bilateral orchidectomy | Annatto tocotrienol (60) | | | 10 | 90 | 3 | 3 | 8 weeks |
| Chin et al[34] (2014) | Testosterone deficiency | Bilateral orchidectomy | Annatto tocotrienol (60) | | | 10 | 90 | 3 | 3 | 8 weeks |
| Ahmad et al[39] (2005) | Free radical | Ferric nitrilotriacetate injection (2 mg/kg; intraperitoneal) | Palm tocotrienol (100) | | 30.7 | 55.2 | 14.1 | 1 | 1 | 8 weeks |
| Nazrun et al[41] (2005) | Free radical | Ferric nitrilotriacetate injection (2 mg/kg; intraperitoneal) | Palm tocotrienol (100) | | 30.7 | 55.2 | 14.1 | 1 | 1 | 8 weeks |
| Ima Nirwana and Fakhrurazi[44] (2002) | Glucocorticoid | Dexamethasone injection (120 μg/kg body weight; oral) | Palm vitamin E (60) | 24.83 | 20.73 | 26.68 | 13.32 | 3 | 3 | 8 weeks |
| Norazlina et al[36] (2000) | Estrogen deficiency | Bilateral ovariectomy | Palm vitamin E (30 and 60) | 24.83 | 20.73 | 26.68 | 13.32 | 3 | 3 | 8 months |
| Nazrun et al[64] (2008) | Estrogen deficiency | Bilateral ovariectomy | Palm tocotrienol (60) | | 30.7 | 55.2 | 14.1 | 3 | 3 | 3 weeks |
| Aktifanus et al[37] (2012) | Estrogen deficiency | Bilateral ovariectomy | Tocotrienol-enriched fraction (60) | 20.11 | 24.67 | 38.95 | 4.55 | 4 | 4 | 8 weeks |
| Soelaiman et al[38] (2012) | Estrogen deficiency | Bilateral ovariectomy | Palm tocotrienol (60) | 20.11 | 24.67 | 38.95 | 4.55 | 4 | 4 | 8 weeks |
| Muhammad et al[31] (2012) | Estrogen deficiency | Bilateral ovariectomy | Palm tocotrienol (60) | | 37.2 | 39.1 | 22.6 | 3 | 3 | 8 weeks |
| Abdul-Majeed et al[33] (2012) | Estrogen deficiency | Bilateral ovariectomy | Annatto tocotrienol (60) | | | 10 | 90 | 3 | 3 | 8 weeks |
| Muhammad et al[32] (2013) | Estrogen deficiency | Bilateral ovariectomy | Tocotrienol-enriched fraction (60) | 20.11 | 24.67 | 38.95 | 4.55 | Not mentioned | Not mentioned | 8 weeks |

**Abbreviations:** ATF, alpha-tocopherol; ATT, alpha-tocotrienol; GTT, gamma-tocotrienol; DTT, delta-tocotrienol.

in the bone volume and cellular parameters of the rats treated with nicotine and ferric nitrilotriacetate indicative of bone loss.[39–41] However, other studies using bone mineral density showed that bone loss did not occur in young[42] or aged animals[43] treated with nicotine. The underlying reason for this discrepancy is not known. In the glucocorticoid-induced model, bone loss was not observed because bone mineral density of the rats continued to increase with time.[44] However, the increase in bone mineral density within the study period was not significant in the glucocorticoid-treated group, whereas it was significant for the other groups. This might indicate an inhibition of growth with glucocorticoid administration.[44] Nevertheless, other studies have shown that bone mineral density continued to increase with time in young rats (3 months old) treated with glucocorticoid.[45]

With the exception of a few studies,[35–37] most of the studies had a baseline group. The bone histomorphometric indices of all female rats showed no significant differences between the baseline (3 months old) and the sham group (5 months old).[31–33,38] Although there were no significant changes in structural histomorphometry, significant differences in dynamic and cellular histomorphometry were observed between the baseline (3 months old) and the sham group (5 months old) in a study using male rats.[30] Double-labeled surface, mineral apposition rate, and bone formation rate were higher in the baseline compared to the sham group. This might indicate active bone modeling in the baseline group, which had slowed down after 2 months.

## The effects of tocotrienol on bone health

### The effects of tocotrienol on bone histomorphometry

Bone histomorphometric examination provides direct information on changes in bone microarchitecture, remodeling/modeling, and cellular properties, which could not otherwise be assessed using bone densitometry and serum bone turnover markers. Bone histomorphometry is guided with computer-aided analysis and stereological technique to provide an accurate depiction of the skeletal changes due to osteoporosis and drug intervention.[46,47] Nomenclature and definition of indices of bone histomorphometry have been standardized and discussed elsewhere.[48] Three aspects of bone histomorphometry were given emphasis in previous studies on the effects of tocotrienol on bone, namely bone structural, dynamic, and static/cellular histomorphometry.[30,31,33–35,37–41] The preferred site of measurement is the trabecular bone at the metaphysis of the distal femur. The trabecular bone is metabolically more active and offers a large surface-to-volume ratio for maximal exposure of stimuli.[49] Thus, it responds faster to internal or external stimuli compared to the cortical bone.

Indices of bone structural histomorphometry describe the bone amount (bone volume and trabecular number), size (trabecular thickness), and trabecular separation. Two separate studies using an estrogen deficiency model showed that palm tocotrienol preserved trabecular bone structure, especially bone volume and trabecular separation in the ovariectomized rats.[31,32] In a study by Muhammad et al, the bone-sparing effects of tocotrienol were found to be equivalent to calcium supplementation and estrogen replacement.[32] Using a testosterone-deficient model, Chin and Ima-Nirwana demonstrated that annatto tocotrienol improved all bone structural indices at the distal femur except trabecular thickness in the orchidectomized rats.[30] In the same study, bone volume was found to be higher in the testosterone-treated group compared to the tocotrienol-supplemented group, implying that testosterone was more effective than tocotrienol in preventing bone loss due to testosterone deficiency.[30] Hermizi et al showed that both tocotrienol-rich fraction and gamma-tocotrienol were effective in preserving trabecular bone structure in the nicotine-induced bone loss model.[40] The effects of tocotrienol on bone structural histomorphometric indices were less pronounced in the ferric nitrilotriacetate-induced osteopenia in rats, whereby only trabecular thickness was maintained in the supplemented group.[41]

Bone dynamic histomorphometry visualizes the mineralization process using fluorescent calcein labeling.[50] Aktifanus et al[37] and Soelaiman et al[38] indicated that single-labeled surface was reduced and double-labeled surface was increased in the ovariectomized rats supplemented with tocotrienol. Furthermore, mineral apposition rate and bone formation rate were increased in the supplemented group in both studies. Improvements in mineral apposition rate and bone formation rate were observed in the osteopenic rats supplemented with either tocotrienol-rich fraction or gamma-tocotrienol homologue in the nicotine-induced bone loss model.[40] Annatto tocotrienol was shown to increase double-labeled surface and reduce single-labeled surface significantly in orchidectomized male rats.[30] However, mineral apposition rate and bone formation rate were not affected by annatto tocotrienol in the testosterone-deficient model.[30]

Proliferation of bone cells, trabecular erosion, and osteoid deposition were characterized by bone static/cellular histomorphometry indices.[47] Palm tocotrienol was shown to increase osteoblast surface and decrease osteoclast surface

in estrogen-deficient rats.[31] Similarly, Abdul-Majeed et al indicated that osteoblast surface was elevated and osteoclast surface was reduced in ovariectomized rats supplemented with annatto tocotrienol alone or in combination with low-dose lovastatin.[33] In the same study, they also discovered that both treatment groups had lower eroded surface and higher osteoid surface and volume compared to the unsupplemented ovariectomized group.[33] In the testosterone deficiency model, annatto tocotrienol increased osteoblast number, osteoid surface, and osteoid volume, and decreased osteoclast and eroded surface in orchidectomized rats.[34] In the nicotine-treated osteopenic rats, tocotrienol mixture and gamma-tocotrienol prevented the increase in osteoclast surface and eroded surface.[40] Ahmad et al demonstrated that, in the ferric nitrilotriacetate-induced bone loss model, palm tocotrienol decreased eroded surface and increased osteoblast number, osteoid surface, and osteoid volume of the supplemented rats compared to the unsupplemented rats.[39]

## The effects of tocotrienol on bone microarchitecture assessed by X-ray microtomography

X-ray microtomography provides a more accurate estimation of bone microarchitecture compared to two-dimensional bone histomorphometry. It provides a high-resolution three-dimensional reconstruction of the bone.[51] A study on the effects of annatto tocotrienol on bone microarchitecture at the proximal tibia in orchidectomized rats was performed by Chin and Ima-Nirwana using X-ray microtomography.[30] There were trends of improvement in the structural indices such as bone volume, trabecular number, and connectivity density. However, only the difference in trabecular separation reached statistical significance.[30]

## The effects of tocotrienol on bone turnover markers

Bone turnover can also be determined with the circulating level of bone turnover markers, which can be classified into formation and resorption markers. Bone formation markers are proteins secreted by osteoblasts during the fabrication of bone matrix, such as osteocalcin, alkaline phosphatase (ALP), and procollagen type 1 N-terminal propeptide (P1NP). Bone resorption markers are the degradation products of bone matrix, such as carboxyl terminal telopeptide of type 1 collagen crosslinks (CTX-1), pyridinoline crosslinks (PYD), and deoxypyridinoline crosslinks (DYP). Osteoclast-specific proteins like tartrate resistant phosphatase (TRAP) are also used as bone resorption markers. Bone turnover markers are useful for providing continuous monitoring of bone turnover throughout the antiosteoporotic drug intervention.[52,53]

Most of the studies revealed insignificant changes in both bone formation and resorption markers in the ovariectomized rats supplemented with tocotrienol.[30,37,38,54] In a study by Norazlina et al, supplementation of palm vitamin E at 30 mg/kg body weight showed an increase in serum ALP level but a negligible effect on TRAP level in the ovariectomized rats.[36] This observation could be incidental because supplementation of tocotrienol at a higher dose (60 mg/kg body weight) did not produce a significant effect.[36] A study by Abdul-Majeed et al indicated a significant lowering of CTX-1 level and an increase in osteocalcin level in the annatto tocotrienol-supplemented rats compared to the unsupplemented ovariectomized rats.[33] In a testosterone deficiency model, supplementation of annatto tocotrienol did not produce significant changes in either bone formation (osteocalcin and P1NP) or resorption markers (CTX-1 and TRAP5b) in the orchidectomized rats.[30,34]

Norazlina et al administered nicotine (7 mg/kg body weight) in male rats for 3 months and initiated tocotrienol treatment in the second and third month. The levels of both osteocalcin and DYP did not differ before (week 0) and after treatment (week 12).[54] Due to the lack of a proper negative control, the authors could not conclude whether this lack of change was due to the beneficial effect of tocotrienol in suppressing bone turnover or the failure of nicotine in inducing adverse changes in bone turnover.[54] In a later experiment, Norazlina et al administered nicotine (7 mg/kg body weight) in male rats for 2 months to induce bone loss. Immediately after nicotine cessation, they supplemented the rats with tocotrienol for 2 months.[55] The elevation of PYD and the decrease of osteocalcin due to nicotine administration were averted by tocotrienol supplementation.[55] Ahmad et al showed that tocotrienol lowered DYP level in the ferric nitrilotriacetate-treated rats compared to the unsupplemented rats.[39] However, tocotrienol had no effects on the osteocalcin level in this study.

## The effects of tocotrienol on bone mineral density

Dual-energy X-ray absorptiometry is the gold standard in the diagnosis of osteoporosis, as the World Health Organization defines the disease based on bone mineral density.[56] The recommended skeletal sites for the assessment of bone mineral density are the proximal femur, femoral neck, trochanter, and spine.[57] Changes in bone histomorphometry precede changes in bone mineral density. In our studies using dual-energy

X-ray absorptiometry, rats needed to undergo tocotrienol treatment for a longer period of time (9–10 months) as compared to studies employing bone histomorphometry (4–8 weeks) for a significant difference to be observed.[35,36,44] The ovariectomized rats treated with palm vitamin E at 30 and 60 mg/kg body weight had significantly higher bone mineral density at the femur and vertebrae compared to the untreated group.[36] Similar findings were obtained in the testosterone deficiency and the glucocorticoid bone loss model.[35,44]

## The effects of tocotrienol on bone calcium level

Calcium in the form of hydroxyapatite is the principal inorganic component of bone.[58] Vitamin D deficiency (<50 nmol/L) will cause an increase in the parathyroid hormone, which in turn mobilizes calcium from bone to the circulation, subsequently causing osteoporosis.[59,60] The level of calcium in the bone can be measured using an atomic absorption spectrophotometer. Palm vitamin E was found to restore bone calcium level at the femur and vertebra of orchidectomized and ovariectomized rats.[35,36] It was also found to preserve bone calcium level in rats receiving dexamethasone.[44] A study by Muhammad et al showed that tocotrienol did not improve bone calcium level at the vertebrae of ovariectomized rats.[32] This discrepancy might stem from the fact that the rats were treated in a shorter period of time (8 weeks) compared to former studies[35,36] (9–10 months). Hence, the beneficial effects of tocotrienol on bone calcium level, as in the case of bone mineral density, might take a longer time to manifest.

## The effects of tocotrienol on biomechanical strength of bone

Biomechanical strength of the bone is determined by its material properties and geometric properties (architectural design).[61] Since tocotrienol has been proved to improve bone mineral density and microarchitecture, it is logical to postulate that it will enhance bone biomechanical strength as well. The biomechanical strength of the bone can be tested using a destructive mechanical test.[62] A load is applied to a part of the bone to induce strain and fracture, so that its ability to resist deformation (stiffness) and fracture (strength) can be determined.[62] Shuid et al showed supplementation of gamma-tocotrienol at 60 mg/kg body weight significantly improved biomechanical strength of the femur in normal male rats.[63] However, there are limited studies on the effects of tocotrienol on bone biomechanical strength in osteopenia models. In studies by both Nazrun et al and Muhammad et al, palm tocotrienol supplementation did not produce significant improvements in bone biomechanical strength in ovariectomized rats .[32,64]

## Overview on the effects of tocotrienol on bone properties

The studies discussed so far confirmed that tocotrienol increased osteoblast number and decreased osteoclast number in osteopenic rats. This resulted in an increase in bone matrix deposition and a decrease in eroded surface on the trabecular bone. The increase in osteoblast activity and number led to an elevation of mineralizing surface, mineral apposition rate, and bone formation rate. Thus, bone calcium content was raised. Increased bone formation and decreased bone resorption brought about an accumulation of bone volume and a reduction in bone porosity, as shown in structural histomorphometry. The improved material content (calcium) and microarchitecture should have subsequently preserved the biomechanical strength of the bone in the animal, but this might take a longer time to manifest. Thus, bone health was preserved in the tocotrienol-supplemented group (Figure 1). The effects of tocotrienol on bone histomorphometry, bone mineral density, and bone calcium content are summarized in Table 2.

# The mechanism of action of tocotrienol

## Antioxidant activity of tocotrienol

Clinical and experimental studies have demonstrated that oxidative stress is implicated in the development of osteoporosis.[65,66] An increase in oxidative stress leads to decreased differentiation and survival of osteoblasts,[67] and also increased differentiation of osteoclasts and bone resorption activity,[68] thus impairing the skeletal system.

Maniam et al showed that supplementation of palm tocotrienol at 100 mg/kg body weight reduced malondialdehyde, a product of lipid peroxidation, and increased glutathione peroxidase activity, an antioxidant enzyme in the bone of normal male rats.[69] Nazrun et al indicated that the ovariectomized rats treated with palm tocotrienol showed increased erythrocyte superoxide dismutase and plasma glutathione peroxidase activities and lower malondialdehyde level.[64] These in vivo studies showed that supplementation of tocotrienol reduced oxidative stress products and antioxidant enzyme activities, subsequently decreasing oxidative stress. In an in vitro study, Nizar et al showed that gamma-tocotrienol homologue decreased oxidative damage on primary osteoblast culture.[29] A further study indicated that tocotrienol achieved

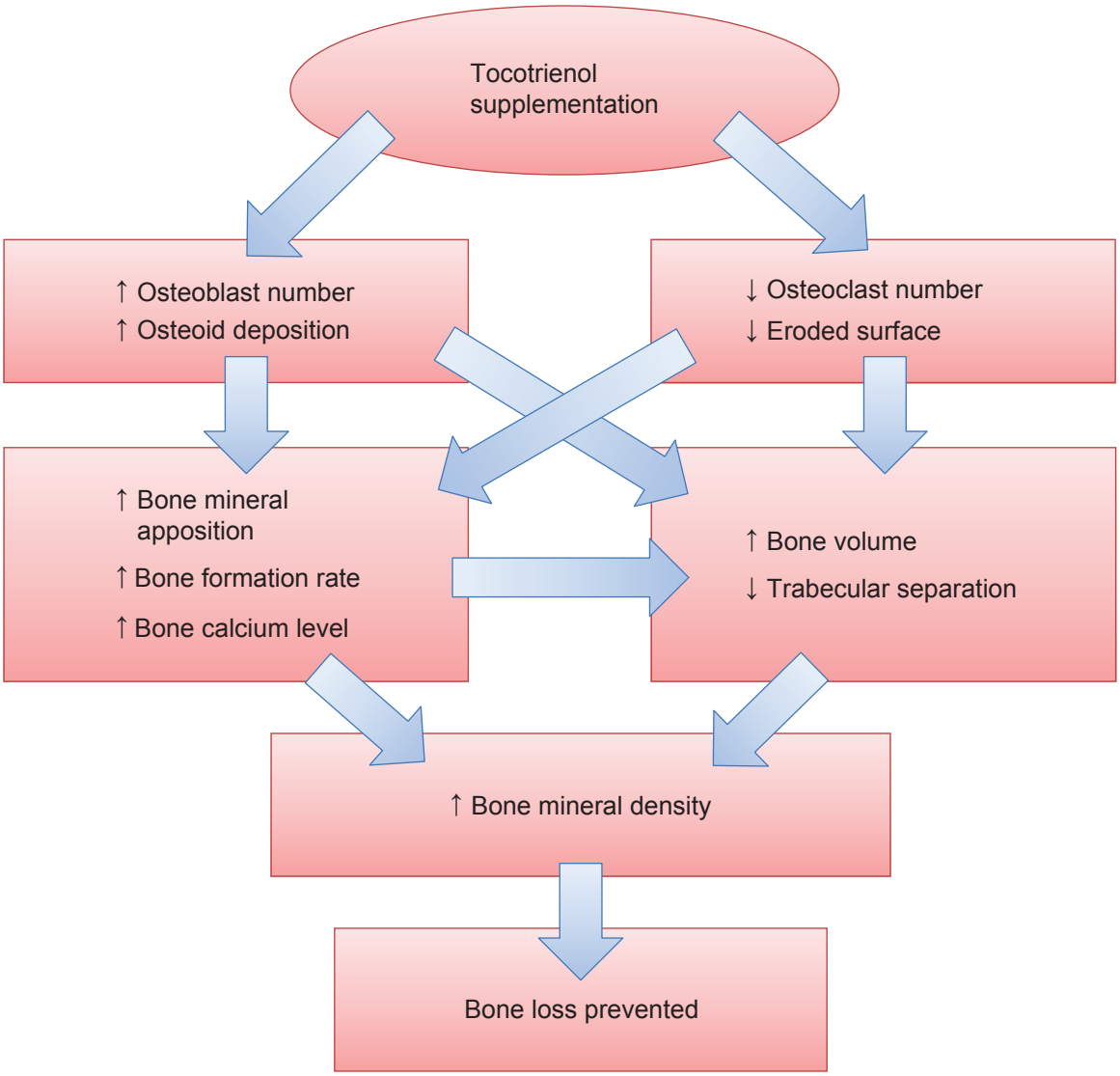


**Figure 1** The effects of tocotrienol on bone.
**Notes:** ↑, increases; ↓, decreases.

its protective effects by preserving the antioxidant enzyme activities in osteoblasts challenged with oxidative stress.[70]

## The effects of tocotrienol on the mevalonate pathway

The mevalonate pathway regulates osteoblastogenesis and osteoclastogenesis through prenylation of small guanosine triphosphate-binding proteins (GTPases), whereby activation of GTPase enhances bone loss.[71] Similar to statins, tocotrienol can suppress the mevalonate pathway as indicated in a previous study on the hypocholesterolemic effects of tocotrienol.[72] Tocotrienol achieves this effect by downregulating the activity of hydroxy-methyl-glutaryl-coenzyme A (HMG-CoA) reductase, a key enzyme involved in cholesterol synthesis.[73]

A recent study by Deng et al found that gamma-tocotrienol (100 mg/kg body weight, subcutaneous injection, once monthly for 3 months) improved bone mineral density, bone microarchitecture determined using X-ray microtomography, and bone static and dynamic histomorphometry in the ovariectomized mice.[74] These effects were brought about by an increased gene expression of bone formation transcription factors (Runx2 and Osterix) and osteoprotegerin, and a decreased expression of gene coding for RANKL in the supplemented mice.[74] Daily supplementation of mevalonate in the tocotrienol-treated ovariectomized mice reverted these beneficial changes.[74] This implies that the bone-protective effects of tocotrienol were mediated through the mevalonate pathway. In another study by Abdul-Majeed et al, the combination of tocotrienol together with statins

**Table 2** The effects of tocotrienol on bone histomorphometry and BMD in rat osteopenia models

| Researchers (year) | Model | Treatment (dose in mg/kg body weight) | BV/TV | TbN | TbSp | TbTh | sLS/BS | dLS/BS | MS | MAR | BFR/BS | ObS/BS or ObN | OcS/BS or OcN | ES/BS | OS/BS | OV/BV | Femoral BMD | Vertebral BMD | Femoral calcium | Vertebral calcium |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Hermizi et al[40] (2009) | N | TRF (60) | ↑ | ↔ | na | ↑ | ↓ | na | na | ↑ | ↑ | na | ↓ | ↓ | na | na | na | na | na | na |
| | | GTT (60) | ↑ | ↑ | na | ↑ | ↓ | na | na | ↑ | ↑ | na | ↓ | ↓ | na | na | na | na | na | na |
| Ima-Nirwana et al[35] (2000) | T | PVE (30) | na | na | na | na | na | na | na | na | na | na | na | na | na | na | ↔ with sham | ↔ with sham | ↔ with sham | ↔ with sham |
| Chin and Ima Nirwana[30] (2014) | T | AnTT (60) | ↑ | ↑ | ↑ | ↔ | ↓ | ↑ | ↔ | ↔ | ↔ | na | na | na | na | na | na | na | na | na |
| Chin et al[34] (2014) | T | AnTT (60) | na | na | na | na | na | na | na | na | na | ↑ | ↓ | ↓ | ↑ | ↑ | na | na | na | na |
| Ahmad et al[39] (2005) | FR | PTT (100) | ↔ | ↔ | na | ↑ | na | na | na | na | ↑ | ↑ | ↔ | ↓ | na | na | na | na | na | na |
| Nazrun et al[41] (2005) | FR | PTT (100) | ↔ | ↔ | ↔ | ↑ | na | na | na | na | na | ↑ | ↔ | ↓ | ↓ | ↓ | na | na | na | na |
| Norazlina et al[36] (2000) | E | PVE (30) | na | na | na | na | na | na | na | na | na | na | na | na | na | na | ↔ with sham | ↔ with sham | ↔ | ↔ |
| | | PVE (60) | na | na | na | na | na | na | na | na | na | na | na | na | na | na | ↔ with sham | ↔ with sham | ↔ | ↔ |
| Aktifanus et al[37] (2012) | E | TRF (60) | na | na | na | na | ↓ | ↑ | ↔ | ↑ | ↑ | na | na | na | na | na | na | na | na | na |
| Soelaiman et al[38] (2012) | E | PTT (60) | na | na | na | na | ↓ | ↑ | ↔ | ↑ | ↑ | na | na | na | na | na | na | na | na | na |
| Muhammad et al[31] (2012) | E | PTT (60) | ↑ | ↑ | ↓ | ↔ | na | na | na | na | na | ↔ | ↓ | na | na | na | na | na | na | na |
| Muhammad et al[32] (2013) | E | TRF (60) | ↑ | ↔ | ↓ | ↑ | na | na | na | na | na | na | na | na | na | na | na | na | na | ↔ |
| Abdul-Majeed[33] et al (2012) | E | AnTT (60) | na | na | na | na | na | na | na | na | na | ↑ | ↓ | ↓ | ↑ | ↑ | na | na | na | na |
| Ima Nirwana and Fakhrurazi[44] (2002) | G | PVE (60) | na | na | na | na | na | na | na | na | na | na | na | na | na | na | ↔ | ↔ | ↔ | ↔ |

**Notes:** ↑, increased; ↓, decreased; ↔, no significant change in the treatment group compared to osteopenic control animal; ↔ with sham, no significant change compared to sham group given the same treatment. Comparison with osteopenic control was not performed.

**Abbreviations:** BFR/BS, bone formation rate; BMD, bone mineral density; BV/TV, bone volume; dLS/BS, double-labeled surface; E, estrogen deficiency; ES/BS, eroded surface; FR, free radical; G, glucocorticoid; MAR, mineral apposition rate; MS, mineralizing surface; N, nicotine; na, not applicable; ObN, osteoblast number; ObS/BS, osteoblast surface; OcN, osteoclast number; OcS/BS, osteoclast surface; OS/BS, osteoid surface; OV/BV, osteoid volume; T, testosterone deficiency; TbN, trabecular number; TbSp, trabecular separation; TbTh, trabecular thickness; sLS/BS, single-labeled surface; TRF, tocotrienol-rich fraction; GTT, gamma-tocotrienol; PVE, palm tocotrienol; AnTT, annatto tocotrienol; PTT, palm tocotrienol.

enhanced the effects of tocotrienol in improving bone static histomorphometry and remodeling markers in the ovariectomized rats.[33] However, there was no confirmation as to whether this effect was produced by the mevalonate pathway per se or by other pathways as well.

## The anti-inflammatory effects of tocotrienol

Proinflammatory cytokines such as interleukin-1, interleukin-6, and tumor necrosis factor alpha are important mediators of bone resorption.[23] They are also implicated in the pathogenesis of postmenopausal osteoporosis.[75] Previous studies showed that tocotrienol could prevent ferric nitrilotriacetate- or nicotine-induced elevation of proinflammatory cytokines such as interleukin-1 and interleukin-6 and concurrently preserve the bone health of rats.[39,54,55] In an in vitro study, Ha et al demonstrated that alpha-tocotrienol could suppress the formation of osteoclasts from co-culture of bone marrow macrophages and osteoblasts induced by interleukin-1 or vitamin D and prostaglandin $E_2$.[22] Concurrently, it was observed that RANKL production by osteoblasts was suppressed and RANKL signaling in the osteoclasts was disrupted.[22]

## Gene-modulating effects of tocotrienol

Differentiation and activity of osteoblasts and osteoclasts are governed by a cascade of genes.[76,77] Abukhadir et al showed that supplementation of palm vitamin E significantly enhanced the gene expression of Runx2, Osterix, and bone morphogenetic protein-2 in a nicotine cessation osteopenia model.[78] Chin and Ima-Nirwana showed that annatto tocotrienol could enhance the expression of genes related to bone formation and osteoblast activity such as alkaline phosphatase, beta-catenin, collagen type I alpha 1, and osteopontin.[30] Gene expression of RANKL was also decreased in the supplemented group.[30] However, annatto tocotrienol did not affect bone resorption genes in the supplemented rats.[30]

A detailed description of the possible bone-protective mechanism of tocotrienol has been published elsewhere.[79]

# The difference in the effects on bone between tocotrienol and alpha-tocopherol

While most studies revealed beneficial effects of tocotrienol on bone, the effects of alpha-tocopherol supplementation on bone in animals are heterogenous.[80] Some studies revealed beneficial effects of alpha-tocopherol on bone while others did not.[81,82] Several studies showed that high-dose alpha-tocopherol supplementation might exert negative effects on bone in normal animals but was protective in stressed animals.[83,84] A study by Fujita et al showed that there was increased bone resorption in mice fed with high-dose alpha-tocopherol, probably due to increased differentiation of osteoclasts.[85] However, this study could not be replicated successfully by other researchers.[86] When alpha-tocopherol and tocotrienol were compared, most studies showed that the effects of the former was either lesser to[40,87] or on par with tocotrienol in protecting bone in rats.[31,36] The effects of alpha-tocopherols on bone have been summarized previously in a review.[80]

# The safety of tocotrienol

Few studies have been performed to assess the safety of tocotrienol. Ima-Nirwana et al showed that treatment with palm tocotrienol at the doses of 500 and 1,000 mg/kg body weight (oral) increased the bleeding and clotting time of mice in subacute (14 days treatment) and subchronic (42 days treatment) studies.[88] After conversion,[89] these are equivalent to 250 and 500 mg/kg body weight in rats. In another study in which rats were fed with palm tocotrienol for 13 weeks, Nakamura et al observed some changes in hematological and serum enzyme biochemical indices and organ histology.[90] They concluded that the no-observed-adverse-effect level was 120 mg/kg body weight for male rats and 130 mg/kg body weight for female rats.[90]

According to the existing toxicological reports,[88,89] the therapeutic index for tocotrienol is relatively low for a noncritical agent. There is a two- to fivefold difference between the effective dose and the toxic dose. The toxicological profile of tocotrienol is not as well established as that of alpha-tocopherol, thus more studies are needed. The available evidence shows that it may lower platelet count and prolong bleeding and clotting time.[88,90] This suggests that it may contraindicate with anticoagulants like warfarin. Apart from that, the pharmacokinetics and disposition of tocotrienol in skeletal tissue is not known. Previous studies revealed that the bioavailability of tocotrienol is relatively low compared to alpha-tocopherol due to the selective binding of tocopherol transport protein with the latter.[91,92] This may be the reason a higher dose of tocotrienol is needed to achieve its bone-protective effects.

# Limitations

Several limitations should be considered when interpreting the studies presented in the current review. Significant publication bias was noted during the literature search, whereby a majority of the studies on the effects of tocotrienol on bone were published by one research group. While many other researchers also study the effects of vitamin E on bone, most

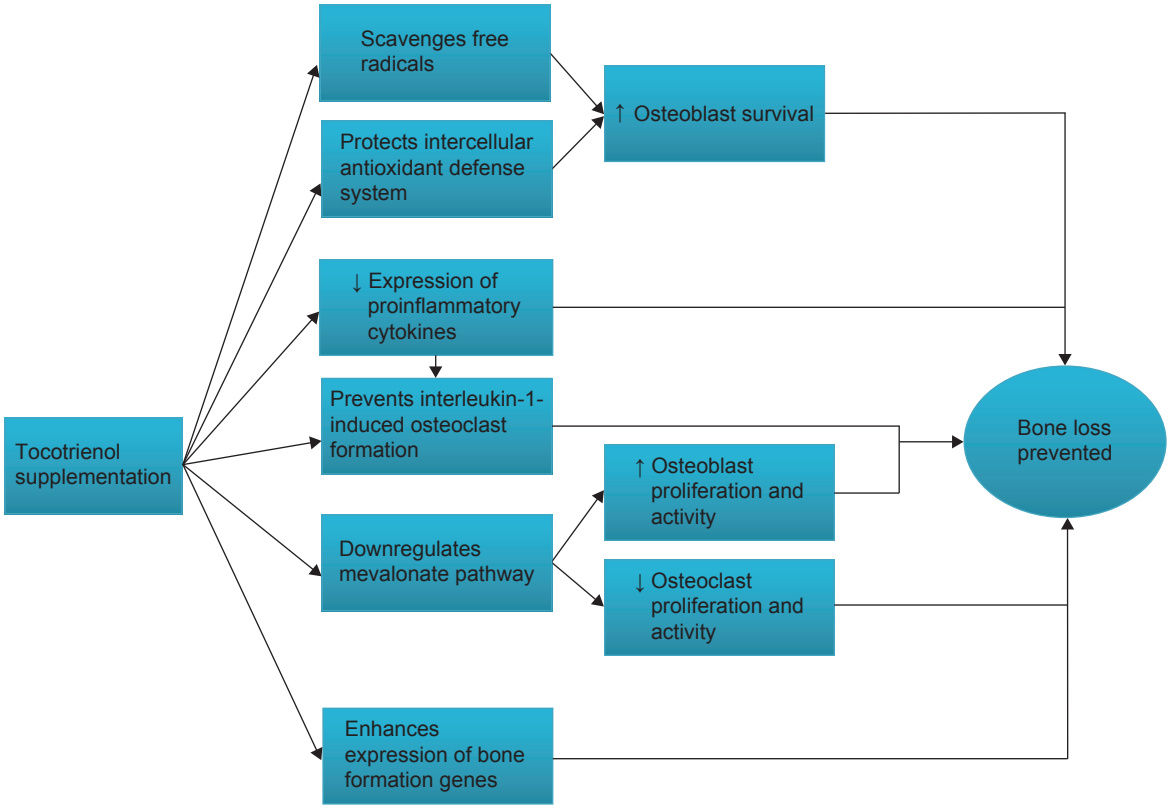


**Figure 2** The bone-protective mechanism of tocotrienol.
**Notes:** ↑, increases; ↓, decreases.

of them focus on alpha-tocopherol,[83,84] which is the predominant vitamin E homologue in our body and in nature.[14]

## Conclusion

The studies on tocotrienol have confirmed that it possesses promising bone-protective effects in various rat models subjected to estrogen deficiency, testosterone deficiency, glucocorticoid, nicotine, and free radicals. Tocotrienol increases osteoblast number, mineral deposition, and bone formation activity and decreases osteoclast number, erosion on bone, and bone resorption activity, thus preventing the degeneration of bone mineral density and bone microarchitecture in osteopenic animals. These effects could be attributed to the antioxidative, anti-inflammatory, gene-modulating activities of tocotrienol. Tocotrienol may also suppress the mevalonate pathway and prevent the activation of GTPase to achieve its bone-protective effects (Figure 2). More studies may be needed to establish the safety profile of tocotrienol. There is also a need to study the effects of tocotrienol in the aged animal model. The data obtained will serve as a basis for future clinical trials to validate the protective effects of tocotrienol in the elderly who are at risk for osteoporosis.

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## Disclosure

The authors report no conflicts of interest in this work.

## References

1. Jilka RL. Biology of the basic multicellular unit and the pathophysiology of osteoporosis. *Med Pediatr Oncol.* 2003;41(3):182–185.
2. Feng X, McDonald JM. Disorders of bone remodeling. *Annu Rev Pathol.* 2011;6:121–145.
3. Johnell O, Kanis JA. An estimate of the worldwide prevalence and disability associated with osteoporotic fractures. *Osteoporos Int.* 2006;17(12):1726–1733.
4. Kanis JA, Melton LJ 3rd, Christiansen C, Johnston CC, Khaltaev N. The diagnosis of osteoporosis. *J Bone Miner Res.* 1994;9(8):1137–1141.
5. Koh GC, Tai BC, Ang LW, Heng D, Yuan JM, Koh WP. All-cause and cause-specific mortality after hip fracture among Chinese women and men: the Singapore Chinese Health Study. *Osteoporos Int.* 2013;24(7):1981–1989.
6. Kannegaard PN, van der Mark S, Eiken P, Abrahamsen B. Excess mortality in men compared with women following a hip fracture. National analysis of comedications, comorbidity and survival. *Age Ageing.* 2010;39(2):203–209.
7. Riggs BL, Khosla S, Melton LJ 3rd. Sex steroids and the construction and conservation of the adult skeleton. *Endocr Rev.* 2002;23(3):279–302.

8. Chin KY, Ima-Nirwana S. Sex steroids and bone health status in men. *Int J Endocrinol.* 2012;2012:208719.
9. National Osteoporosis Foundation. *Clinician's Guide to Prevention and Treatment of Osteoporosis.* Washington, DC: National Osteoporosis Foundation; 2010. Available from: http://nof.org/files/nof/public/content/file/344/upload/159.pdf. Accessed March 24, 2015.
10. Burge R, Dawson-Hughes B, Solomon DH, Wong JB, King A, Tosteson A. Incidence and economic burden of osteoporosis-related fractures in the United States, 2005–2025. *J Bone Miner Res.* 2007;22(3):465–475.
11. Hough FS, Brown SL, Cassim B, et al; National Osteoporosis Foundation of South Africa. The safety of osteoporosis medication. *S Afr Med J.* 2014;104(4):279–282.
12. Chung M, Lee J, Terasawa T, Lau J, Trikalinos TA. Vitamin D with or without calcium supplementation for prevention of cancer and fractures: an updated meta-analysis for the U.S. Preventive Services Task Force. *Ann Intern Med.* 2011;155(12):827–838.
13. Bolland MJ, Grey A, Avenell A, Gamble GD, Reid IR. Calcium supplements with or without vitamin D and risk of cardiovascular events: reanalysis of the Women's Health Initiative limited access dataset and meta-analysis. *BMJ.* 2011;342:d2040.
14. Aggarwal B, Sundaram C, Prasad S, Kannappan R. Tocotrienols, the vitamin E of the 21st century: its potential against cancer and other chronic diseases. *Biochem Pharmacol.* 2010;80(11):1613–1631.
15. Colombo ML. An update on vitamin E, tocopherol and tocotrienol-perspectives. *Molecules.* 2010;15(4):2103–2113.
16. Chun J, Lee J, Ye L, Exler J, Eitenmiller RR. Tocopherol and tocotrienol contents of raw and processed fruits and vegetables in the United States diet. *J Food Compost Anal.* 2006;19(2–3):196–204.
17. Ng MH, Choo YM, Ma AN, Chuah CH, Hashim MA. Separation of vitamin E (tocopherol, tocotrienol, and tocomonoenol) in palm oil. *Lipids.* 2004;39(10):1031–1035.
18. Frega N, Mozzon M, Bocci F. Identification and estimation of tocotrienols in the annatto lipid fraction by gas chromatography-mass spectrometry. *J Am Oil Chem Soc.* 1998;75(12):1723–1727.
19. Ginaldi L, Di Benedetto MC, De Martinis M. Osteoporosis, inflammation and ageing. *Immun Ageing.* 2005;2:14.
20. Manolagas SC. From estrogen-centric to aging and oxidative stress: a revised perspective of the pathogenesis of osteoporosis. *Endocr Rev.* 2010;31(3):266–300.
21. Fatokun AA, Stone TW, Smith RA. Responses of differentiated MC3T3-E1 osteoblast-like cells to reactive oxygen species. *Eur J Pharmacol.* 2008;587(1–3):35–41.
22. Ha H, Kwak HB, Lee SW, et al. Reactive oxygen species mediate RANK signaling in osteoclasts. *Exp Cell Res.* 2004;301(2):119–127.
23. McLean RR. Proinflammatory cytokines and osteoporosis. *Curr Osteoporos Rep.* 2009;7(4):134–139.
24. Serbinova E, Kagan V, Han D, Packer L. Free radical recycling and intramembrane mobility in the antioxidant properties of alpha-tocopherol and alpha-tocotrienol. *Free Radic Biol Med.* 1991;10(5):263–275.
25. Kaileh M, Sen R. Role of NF-kappaB in the anti-inflammatory effects of tocotrienols. *J Am Coll Nutr.* 2010;29(3 Suppl):334S–339S.
26. Brooks R, Kalia P, Ireland DC, Beeton C, Rushton N. Direct inhibition of osteoclast formation and activity by the vitamin E isomer gamma-tocotrienol. *Int J Vitam Nutr Res.* 2011;81(6):358–367.
27. Ha H, Lee JH, Kim HN, Lee ZH. α-Tocotrienol inhibits osteoclastic bone resorption by suppressing RANKL expression and signaling and bone resorbing activity. *Biochem Biophys Res Commun.* 2011;406(4):546–551.
28. Ahn KH, Jung HK, Jung SE, et al. Microarray analysis of gene expression during differentiation of human mesenchymal stem cells treated with vitamin E in vitro into osteoblasts. *Korean Journal of Bone Metabolism.* 2011;18(1):23–32.
29. Nizar AM, Nazrun AS, Norazlina M, Norliza M, Ima Nirwana S. Low dose of tocotrienols protects osteoblasts against oxidative stress. *Clin Ter.* 2011;162(6):533–538.
30. Chin KY, Ima Nirwana S. Effects of annatto-derived tocotrienol supplementation in osteoporosis induced by testosterone deficiency in rats. *Clin Interv Aging.* 2014;9:1247–1259.
31. Muhammad N, Luke DA, Shuid AN, Mohamed N, Soelaiman IN. Two different isomers of vitamin e prevent bone loss in postmenopausal osteoporosis rat model. *Evid Based Complement Alternat Med.* 2012;2012:161527.
32. Muhammad N, Razali S, Shuid AN, Mohamed N, Soelaiman IN. Membandingkan kesan antara fraksi-kaya tokotrienol, kalsium dan estrogen terhadap metabolisme tulang tikus terovariektomi. *Sains Malaysiana.* 2013;42(11):1591–1597. Malay.
33. Abdul-Majeed S, Mohamed N, Soelaiman IN. Effects of tocotrienol and lovastatin combination on osteoblast and osteoclast activity in estrogen-deficient osteoporosis. *Evid Based Complement Alternat Med.* 2012;2012:960742.
34. Chin KY, Abdul-Majeed S, Fozi NF, Ima-Nirwana S. Annatto tocotrienol improves indices of bone static histomorphometry in osteoporosis due to testosterone deficiency in rats. *Nutrients.* 2014;6(11):4974–4983.
35. Ima-Nirwana S, Kiftiah A, Zainal AG, Norazlina M, Gapor MT, Khalid BAK. Palm vitamin E prevents osteoporosis in orchidectomized growing male rats. *Natural Product Sciences.* 2000;6(4):155–160.
36. Norazlina M, Ima-Nirwana S, Gapor MT, Khalid BA. Palm vitamin E is comparable to alpha-tocopherol in maintaining bone mineral density in ovariectomised female rats. *Exp Clin Endocrinol Diabetes.* 2000;108:305–310.
37. Aktifanus AT, Shuid AN, Rashid NH, et al. Comparison of the effects of tocotrienol and estrogen on the bone markers and dynamic changes in postmenopausal osteoporosis rat model. *Asian J Anim Vet Adv.* 2012;7(3):225–234.
38. Soelaiman IN, Ming W, Abu Bakar R, et al. Palm tocotrienol supplementation enhanced bone formation in oestrogen-deficient rats. *Int J Endocrinol.* 2012;2012:532862.
39. Ahmad NS, Khalid BA, Luke DA, Ima Nirwana S. Tocotrienol offers better protection than tocopherol from free radical-induced damage of rat bone. *Clin Exp Pharmacol Physiol.* 2005;32(9):761–770.
40. Hermizi H, Faizah O, Ima-Nirwana S, Ahmad Nazrun S, Norazlina M. Beneficial effects of tocotrienol and tocopherol on bone histomorphometric parameters in sprague-dawley male rats after nicotine cessation. *Calcif Tissue Int.* 2009;84(1):65–74.
41. Khalid BAK, Luke DA, Ima-Nirwana S, Nazrun AS. Vitamin E protects from free-radical damage on femur of rats treated with ferric nitrilotriacetate. *Curr Top Pharmacol.* 2005;9(2):107–115.
42. Turan V, Mizrak S, Yurekli B, Yilmaz C, Ercan G. The effect of long-term nicotine exposure on bone mineral density and oxidative stress in female Swiss Albino rats. *Arch Gynecol Obstet.* 2013;287(2):281–287.
43. Iwaniec UT, Fung YK, Akhter MP, et al. Effects of nicotine on bone mass, turnover, and strength in adult female rats. *Calcif Tissue Int.* 2001;68(6):358–364.
44. Ima Nirwana S, Fakhrurazi H. Palm vitamin E protects bone against dexamethasone-induced osteoporosis in male rats. *Med J Malaysia.* 2002;57(2):136–144.
45. Ogoshi T, Hagino H, Fukata S, Tanishima S, Okano T, Teshima R. Influence of glucocorticoid on bone in 3-, 6-, and 12-month-old rats as determined by bone mass and histomorphometry. *Mod Rheumatol.* 2008;18(6):552–561.
46. Vedi S, Compston J. Bone histomorphometry. *Methods Mol Med.* 2003;80:283–298.
47. Kulak CA, Dempster DW. Bone histomorphometry: a concise review for endocrinologists and clinicians. *Arq Bras Endocrinol Metabol.* 2010;54:87–98.
48. Dempster DW, Compston JE, Drezner MK, et al. Standardized nomenclature, symbols, and units for bone histomorphometry: a 2012 update of the report of the ASBMR Histomorphometry Nomenclature Committee. *J Bone Miner Res.* 2013;28(1):2–17.
49. Jacobs CR. The mechanobiology of cancellous bone structural adaptation. *J Rehabil Res Dev.* 2000;37(2):209–216.

50. Erben R. Bone-labeling techniques. In: An YH, Martin KL, editors. *Handbook of Histology Methods for Bone and Cartilage*. Totowa, NJ: Humana Press; 2003:99–117.
51. Effendy NM, Khamis MF, Shuid AN. Micro-CT assessments of potential anti-osteoporotic agents. *Curr Drug Targets.* 2013;14(13): 1542–1551.
52. Naylor K, Eastell R. Bone turnover markers: use in osteoporosis. *Nat Rev Rheumatol.* 2012;8(7):379–389.
53. Wheater G, Elshahaly M, Tuck SP, Datta HK, van Laar JM. The clinical utility of bone marker measurements in osteoporosis. *J Transl Med.* 2013;11:201.
54. Norazlina M, Lee PL, Lukman HI, Nazrun AS, Ima-Nirwana S. Effects of vitamin E supplementation on bone metabolism in nicotine-treated rats. *Singapore Med J.* 2007;48(3):195–199.
55. Norazlina M, Hermizi H, Faizah O, Nazrun AS, Norliza M, Ima-Nirwana S. Vitamin E reversed nicotine-induced toxic effects on bone biochemical markers in male rats. *Arch Med Sci.* 2010;6(4): 505–512.
56. World Health Organization. *Assessment of Fracture Risk and its Application to Screening for Postmenopausal Osteoporosis: Report of a World Health Organization Study Group*. Geneva: World Health Organization; 1994. Available from: http://apps.who.int/iris/bitstream/10665/39142/1/WHO_TRS_843_eng.pdf?ua=1. Accessed March 24, 2015.
57. Hans D, Downs RW Jr, Duboeuf F, et al; International Society for Clinical Densitometry. Skeletal sites for osteoporosis diagnosis: the 2005 ISCD Official Positions. *J Clin Densitom.* 2006;9(1):15–21.
58. Legros R, Balmain N, Bonel G. Age-related changes in mineral of rat and bovine cortical bone. *Calcif Tissue Int.* 1987;41(3):137–144.
59. Saliba W, Barnett O, Rennert HS, Lavi I, Rennert G. The relationship between serum 25(OH)D and parathyroid hormone levels. *Am J Med.* 2011;124(12):1165–1170.
60. Lips P, Duong T, Oleksik A, et al. A global study of vitamin D status and parathyroid function in postmenopausal women with osteoporosis: baseline data from the multiple outcomes of raloxifene evaluation clinical trial. *J Clin Endocrinol Metab.* 2001;86(3):1212–1221.
61. Winter W. Bone strength in pure bending: bearing of geometric and material properties. *Stud Health Technol Inform.* 2008;133:230–237.
62. Ferretti JL, Cointry GR, Capozza RF, Capiglioni R, Chiappe MA. Analysis of biomechanical effects on bone and on the muscle-bone interactions in small animal models. *J Musculoskelet Neuronal Interact.* 2001;1(3):263–274.
63. Shuid A, Mehat Z, Mohamed N, Muhammad N, Soelaiman IN. Vitamin E exhibits bone anabolic actions in normal male rats. *J Bone Miner Metab.* 2010;28(2):149–156.
64. Nazrun A, Khairunnur A, Norliza M, Norazlina M, Ima Nirwana S. Effects of palm tocotrienol on oxidative stress and bone strength in ovariectomised rats. *Med Health.* 2008;3(2):83–90.
65. Cervellati C, Bonaccorsi G, Cremonini E, et al. Oxidative stress and bone resorption interplay as a possible trigger for postmenopausal osteoporosis. *Biomed Res Int.* 2014;2014:569563.
66. Ibáñez L, Ferrándiz ML, Brines R, Guede D, Cuadrado A, Alcaraz MJ. Effects of Nrf2 deficiency on bone microarchitecture in an experimental model of osteoporosis. *Oxid Med Cell Longev.* 2014;2014:726590.
67. Yang Y, Su Y, Wang D, et al. Tanshinol attenuates the deleterious effects of oxidative stress on osteoblastic differentiation via Wnt/FoxO3a signaling. *Oxid Med Cell Longev.* 2013;2013:351895.
68. Baek KH, Oh KW, Lee WY, et al. Association of oxidative stress with postmenopausal osteoporosis and the effects of hydrogen peroxide on osteoclast formation in human bone marrow cell cultures. *Calcif Tissue Int.* 2010;87(3):226–235.
69. Maniam S, Mohamed N, Shuid AN, Soelaiman IN. Palm tocotrienol exerted better antioxidant activities in bone than alpha-tocopherol. *Basic Clin Pharmacol Toxicol.* 2008;103(1):55–60.
70. Abd Manan N, Mohamed N, Shuid AN. Effects of low-dose versus high-dose γ-tocotrienol on the bone cells exposed to the hydrogen peroxide-induced oxidative stress and apoptosis. *Evid Based Complement Alternat Med.* 2012;2012:680834.
71. Mo H, Yeganehjoo H, Shah A, Mo WK, Soelaiman IN, Shen CL. Mevalonate-suppressive dietary isoprenoids for bone health. *J Nutr Biochem.* 2012;23(12):1543–1551.
72. Salman Khan M, Akhtar S, Al-Sagair OA, Arif JM. Protective effect of dietary tocotrienols against infection and inflammation-induced hyperlipidemia: an in vivo and in silico study. *Phytother Res.* 2011;25(11): 1586–1595.
73. Abdul-Majeed S, Mohamed N, Soelaiman IN. A review on the use of statins and tocotrienols, individually or in combination for the treatment of osteoporosis. *Curr Drug Targets.* 2013;14(13):1579–1590.
74. Deng L, Ding Y, Peng Y, et al. γ-Tocotrienol protects against ovariectomy-induced bone loss via mevalonate pathway as HMG-CoA reductase inhibitor. *Bone.* 2014;67:200–207.
75. Brincat SD, Borg M, Camilleri G, Calleja-Agius J. The role of cytokines in postmenopausal osteoporosis. *Minerva Ginecol.* 2014;66(4): 391–407.
76. Bruzzaniti A, Baron R. Molecular regulation of osteoclast activity. *Rev Endocr Metab Disord.* 2006;7(1–2):123–139.
77. Kirkham GR, Cartmell SH. Genes and proteins involved in the regulation of osteogenesis. In: Ashammakhi N, Reis RL, Chiellini E, editors. *Topics in Tissue Engineering.* Vol 3. 2007. Available from: http://www.oulu.fi/spareparts/ebook_topics_in_t_e_vol3/abstracts/kirkham_chapter_01.pdf. Accessed March 31, 2015.
78. Abukhadir SS, Mohamed N, Makpol S, Muhammad N. Effects of palm vitamin e on bone-formation-related gene expression in nicotine-treated rats. *Evid Based Complement Alternat Med.* 2012;2012:656025.
79. Chin KY, Mo H, Soelaiman IN. A review of the possible mechanisms of action of tocotrienol – a potential antiosteoporotic agent. *Curr Drug Targets.* 2013;14(13):1533–1541.
80. Chin KY, Ima-Nirwana S. The effects of α-tocopherol on bone: a double-edged sword? *Nutrients.* 2014;6(4):1424–1441.
81. Chai SC, Wei CI, Brummel-Smith K, Arjmandi BH. The role of vitamin E in reversing bone loss. *Aging Clin Exp Res.* 2008;20(6):521–527.
82. Feresin RG, Johnson SA, Elam ML, et al. Effects of vitamin e on bone biomechanical and histomorphometric parameters in ovariectomized rats. *J Osteoporos.* 2013;2013:1–9.
83. Arjmandi B, Juma S, Beharka A, Bapna MS, Akhter M, Meydani SN. Vitamin E improves bone quality in the aged but not in young adult male mice. *J Nutr Biochem.* 2002;13(9):543.
84. Smith BJ, Lucas EA, Turner RT, et al. Vitamin E provides protection for bone in mature hindlimb unloaded male rats. *Calcif Tissue Int.* 2005;76(4):272–279.
85. Fujita K, Iwasaki M, Ochi H, et al. Vitamin E decreases bone mass by stimulating osteoclast fusion. *Nat Med.* 2012;18:589–594.
86. Iwaniec UT, Turner RT, Smith BJ, et al. Evaluation of long-term vitamin E insufficiency or excess on bone mass, density, and microarchitecture in rodents. *Free Radic Biol Med.* 2013;65:1209–1214.
87. Mehat MZ, Shuid AN, Mohamed N, Muhammad N, Soelaiman IN. Beneficial effects of vitamin E isomer supplementation on static and dynamic bone histomorphometry parameters in normal male rats. *J Bone Miner Metab.* 2010;28(5):503–509.
88. Ima-Nirwana S, Nurshazwani Y, Nazrun AS, Norliza M, Norazlina M. Subacute and subchronic toxicity studies of palm vitamin E in mice. *Journal of Pharmacology and Toxicology.* 2011;6:166–173.
89. Reagan-Shaw S, Nihal M, Ahmad N. Dose translation from animal to human studies revisited. *FASEB J.* 2008;22(3):659–661.
90. Nakamura H, Furukawa F, Nishikawa A, et al. Oral toxicity of a tocotrienol preparation in rats. *Food Chem Toxicol.* 2001;39(8):799–805.
91. Herrera E, Barbas C. Vitamin E: action, metabolism and perspectives. *J Physiol Biochem.* 2001;57(1):43–56.
92. Stocker A. Molecular mechanisms of vitamin E transport. *Ann N Y Acad Sci.* 2004;1031:44–59.