American Institute of Integrative Oncology
RESEARCH & EDUCATION

Foundations of Integrative Oncology
COURSE

GRAND ROUNDS CALL
With Dr. Nalini Chilkov
June 12th, 2019
Second Wednesday of Every Month
5:30 PM Pacific / 6:30 PM Mountain / 7:30 PM Central / 8:30 PM Eastern

Clinical Pearl: Botanicals that Influence Angiogenesis

See slides

Case Study: 50yo F Invasive Malignant Melanoma

Submitted by: Isabel Galiano, Health Coach, Singapore

Overview: N 50 yo Female Dx with Invasive Malignant Melanoma, post op (Immunotherapy not recommended/Observation only) Patient seeking lifestyle/dietary/supplement suggestions
Multiple breast cysts, cervical dysplasia
Divorced, Mother, School Teacher, High Stress, Poor Sleep Quality, Sedentary
Describes herself as exhausted, low energy with poor self esteem and poor self confidence

Core Questions:

- WHAT WOULD BE YOUR RECOMMENDATIONS?
- ANY INPUT ON THE BENEFITS OF IMMUNOTHERAPY FOR HER?
- WHAT IS YOUR VIEW ON COQ10 FOR SKIN CANCER?
- DOSAGE OF SUPPLEMENTS?
- ANY OTHER SUPPLEMENTS?

Recommendations:
See PDF Case Study Notes Title "Malignant Melanoma 59 yo female submitted by I. Galiano"

Questions & Answers

Kiran Sangha: **Dr Lise Alschuler mentioned in one of her course's that you should avoid Lion's mane or basically anything that increases BDNF during GBM treatment as these can increase the growth of CNS tumours. I noticed in one of your case summaries that you did prescribe Lion's Mane to a GBM patient. Would you have any thoughts regarding this precaution? or have you not found it to be an issue? Lion's Mane is something I would like to prescribe to my client's with GBM but have been reluctant to do so after hearing this. Do you have any thoughts on the concomitant use of memory enhancing herbs like Bacopa / Rosemary / Sage during active treatment? (Temador / radiation)**
She also expressed concern with any pharmaceuticals (SSRI's) and or nutraceuticals (5HTP / tryptophan) which increase serotonin as these may increase glial cell line derived neurotrophic factor which again, could promote growth and invasion of glioma cells. Do you stay away from these during active treatment? more so the nutraceuticals - 5HTP / tryptophan? if so, when would you look to include these in your protocol if depression is an issue?

Dr. Chilkov:

Yes, we do want to avoid promoting nerve growth factors with brain cancers as we do not want to promote neuronal proliferation.

The prognosis in GBM patients is poor. I always weigh QOL along with tumor control.

We CAN still use nutrients and botanicals that reduce neuroinflammation and reduce oxidative stress. Bacopa, Ginkgo, Centella are good choices. Also Boswellia (AKBA) and Curcuma longa (Curcuminoids) and Omega 3 Fatty acids, Vitamin D, Probiotics, Taurine can be included.

During Radiotherapy we would be cautious with superantioxidants. Generally phytophenol antioxidants do not disrupt the effiicacy of radiotherapy but Glutathione, Ascorbic Acid, N-Acetyl cysteine do interfere. Some phytochemical Nrf2 promoters may interfere.

I would not initiate SSRI therapies or 5HTP after a GBM diagnosis. If a patient has has been on anti-depressants and is well managed and would be vulnerable to a depressive episode, we must weigh in on that factor for an individual patient.
In service to QOL a thoughtful assessment of mental health and history of mood disorder and psychiatric history and medications and an assessment of coping capacity is in order.

A diagnosis of GBM is devastating to most patients and the treatments can be brutal. The loss of function and the need to be on multiple medications is challenging and extremely stressful increasing risk of depression and anxiety.

In my opinion, our job is to slow down disease progression, support healthy function and enhance and support oncology interventions and QOL. There are no black and white answers with GBM. Most patients are aware that they have a dramatically shortened life span and a poor prognosis. Therefore we must know what is most important to the patient and honor their wishes and values as well.

Dr. Stacy Andre is a functional medicine neuro oncologist who is part of our AIIORE community and I would like her to share her point of view and opinion with us as well.

References

MELANOMA
Canc Lett 2013 July 28: 335(2): 251-258 Emerging Phytochemicals for prevention of melanoma invasion. Virgina Jones, Santosh K. Katiyar

J Am Acad Dermatol. 2006 Feb;54(2):234-41. Epub 2005 Dec 27. Low plasma coenzyme Q10 levels as an independent prognostic factor for melanoma progression Rusciani L

CoQ10 levels were significantly lower in patients than in control subjects (t test: P < .0001) and in patients who developed metastases than in the metastasis-free subgroup (t test: P < .0001). Logistic regression analysis indicated that plasma CoQ10 levels were a significant predictor of metastasis (P = .0013). The odds ratio for metastatic disease in patients with CoQ10 levels that were less than 0.6 mg/L (the low-end value of the range measured in a normal population) was 7.9, and the metastasis-free interval was almost double in patients with CoQ10 levels 0.6 mg/L or higher (Kaplan-Meier analysis: P < .001).

Cancer. 1981 Apr 1;47(7):1838-44.
Serum copper and zinc levels in melanoma patients. Fisher GL, Spitler LE, McNeill KL, Rosenblatt LS. Serum copper levels (SCL) and serum zinc levels (SZL) were evaluated in malignant melanomapatients at various clinical stages. Copper levels were generally found to be elevated, reflecting the degree and extent of tumor activity. Zinc levels and, hence, SCL:SZL ratios did not reflect tumor activity. SCL appeared to prognosticate disease progression in that all patients whose values never declined below 150 micrograms/100 ml died during the course of the study. However, not all patients who died from tumor metastases displayed persistent elevations of SCL. Patients receiving BCG immunotherapy appeared to have higher SCL than untreated patients.

Int J Oncol. 2016 Feb;48(2):624-34. doi: 10.3892/ijo.2015.3286. Epub 2015 Dec 10.**Bioactive proanthocyanidins inhibit growth and induce apoptosis in human melanoma cells by decreasing the accumulation β-catenin.**Vaid M

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TARGETING TUMOR ANGIOGENESIS

Phytochemicals as Anti-Cancer Therapy

Dr. Nalini Chilkov, Founder

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ANGIOGENESIS

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ANGIOGENESIS: Blood vessel formation involving migration, growth and differentiation of endothelial cells

Tumor Angiogenesis is the growth of new blood vessels that tumors need to grow.

This process is caused by the release of chemicals by the tumor and by host cells near the tumor.

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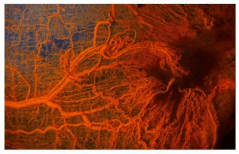
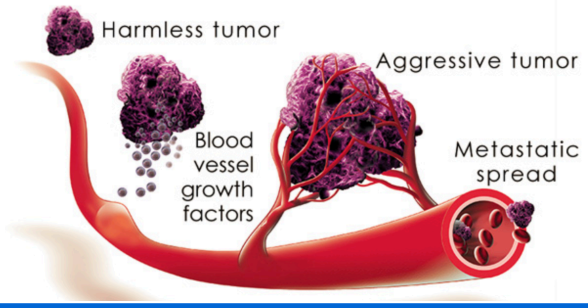
- Angiogenesis: growth of new blood vessels
- Tumors require blood supply to grow larger than 1-2 mm in diameter
- Tumors stimulate angiogenesis
- Angiogenesis inhibitors can be used to treat cancer

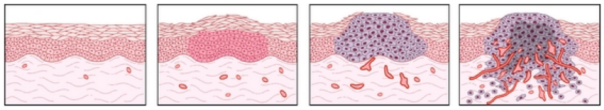


Sarcoma in rat
Normal blood vessels (left)
Tumor-induced vessels (right)
Courtesy of Dr. Robert D. Acland

Image from http://users.rcn.com/jkimball.ma.ultranet/BiologyPages/A/Angiogenesis2.jpg

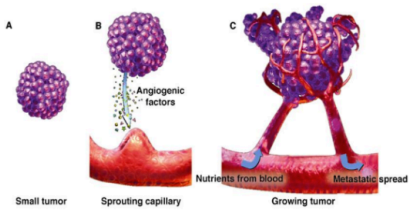


Switch to ANGIOGENIC Phenotype



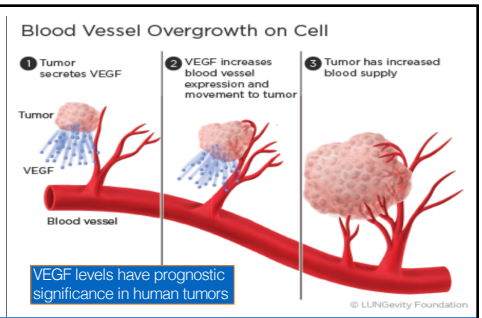
The switch to the angiogenic phenotype occurs during multistage tumorigenesis. As malignancy develops, cells progress from a prevascular stage (normal to early hyperplasia) to a vascular stage (late hyperplasia to dysplasia to invasive carcinoma). Angiogenesis becomes clearly evident during dysplasia and is critical for further growth

Angiogenesis Foundation 2011



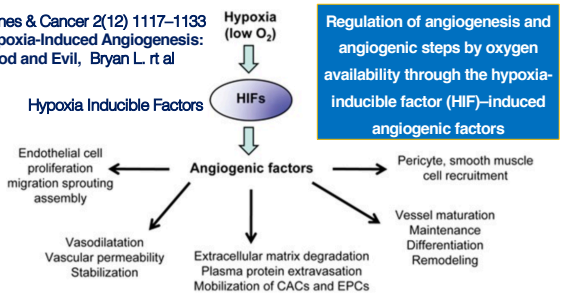
A, Tumors less than 1 mm³ receive oxygen and nutrients by diffusion from host vasculature. B, Larger tumors require new vessel network. Tumor secretes angiogenic factors that stimulate migration, proliferation, and neovessel formation by endothelial cells in adjacent established vessels. C, Newly vascularized tumor no longer relies solely on diffusion from host vasculature, facilitating progressive growth.

Tumor Angiogenesis Promotes Tumor Growth And Metastasis



VEGF levels have prognostic significance in human tumors

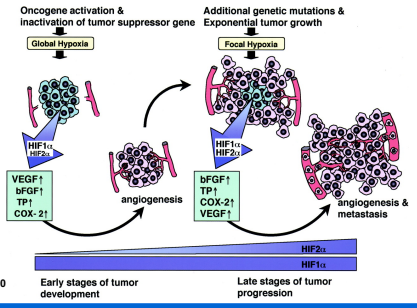
Genes & Cancer 2(12) 1117–1133
Hypoxia-Induced Angiogenesis: Good and Evil, Bryan L. rt al



Regulation of angiogenesis and angiogenic steps by oxygen availability through the hypoxia-inducible factor (HIF)–induced angiogenic factors

Clinical Cancer Research

Significantly worse survival rates were noticed in patients with HIF2 overexpression alone ($P < 0.05$) and in those with combined overexpression of HIF1 and HIF2 ($P < 0.01$).



Hiroshi Yoshimura et al.
Clin Cancer Res 2004;10:8554-8560

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TARGETING TUMOR ANGIOGENESIS for CANCER PREVENTION

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Angiogenesis is a critical, rate-limiting step in the development of all known cancers

Its inhibition suppresses tumor growth, progression, and metastases.

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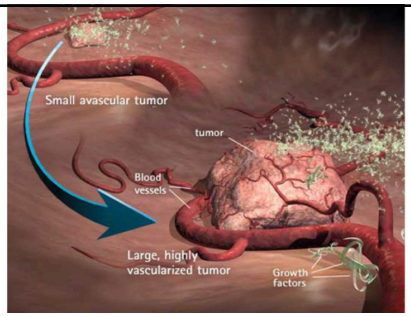
Angiogenesis Inhibitors

block the growth of new blood vessels in the tumor starving the cancer of the nutrition and oxygen it needs to survive



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TARGETING TUMOR ANGIOGENESIS for CANCER PREVENTION

Selective targeting of angiogenic blood vessels is possible as a result of differential proliferation rates between normal and tumor-associated endothelium.

The normal vasculature is highly quiescent, with only one in every 10,000 endothelial cells dividing at any given time, and a physiological doubling time ranging from 47 to 20,000 days.

In contrast, the doubling rate for tumor endothelium is 2–13 days.

Thus, antiangiogenic agents are selective in inhibiting proliferating tumor vasculature, but do not affect normal blood vessels.

J Oncol. 2011 Sep 29. doi: 10.1155/2012/879623. Tumor Angiogenesis as a Target for Dietary Cancer Prevention. William W. Li.

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INHIBITION of ANGIOGENESIS

Selected Nutriceuticals, Botanicals and Phytochemicals

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Selected Chemopreventive Agents That Possess Anti-Angiogenic Properties

1, 25 OH Vitamin D	Panax Ginseng	Genistein
Retinoids	Scutellaria baicalensis	Ellagic Acid
N-Acetylcysteine	Salvia milthiorrhiza	Lycopene
Selenium	Curcumin	Glucosinolates
Menaquinone (Vit K2)	Flavonoids, Polyphenols	Isothiocyanates , I3C
Resveratrol	Proanthocyanidins	Anthocyanidins
EGCG	Parthenolide	Honokiol

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(http://www.angio.org/). William Li, MD

www.AIIORE.com

Phytochemicals Targeting VEGF and VEGF-Related Multifactors as Anticancer Therapy. (2019)

- The role of vascular endothelial growth factor (VEGF) in cancer cells is not limited to angiogenesis;
- There are also multiple factors, such as neuropilins (non-tyrosine kinases receptors), tyrosine kinases receptors, immunodeficiencies, and integrins, that interact with VEGF signaling and cause cancer initiation.
- By combating these factors, tumor progression can be inhibited or limited.
- **Natural products are sources of several bioactive phytochemicals that can interact with VEGF-promoting factors and inhibit them through various signaling pathways, thereby inhibiting cancer growth.**

J Clin Med. 2019 Mar; 8(3): 350. Amma Parveen et al

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THE LINKS BETWEEN PHYTOCHEMICALS AND VEGF

- Emodin
- **Artemisinin**
- Artesunate
- **Berberine**
- **Curcumin**
- Daphnetin
- **Quercetin**
- Licoricidin
- Oxyresveratrol
- **Resveratrol**
- **Thymoquinone**
- **Ginsenoside Rd**
- **Ginsenoside Rg3**
- **Parthenolide**
- **Rosmarinic acid**
- **Tanshinone IIA**
- **Baicalein**
- **Wogonin**
- **Scutellarein**
- Lupeol
- 3-O-Acetyl-oleanolic acid
- **Sulforaphane**
- Luteolin

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J Clin Med. 2019 Mar; 8(3): 350. Amma Parveen et al

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Targeting VEGF/VEGFRs Pathway in the Antiangiogenic Treatment of Human Cancers by Traditional Chinese Medicine

Integr Cancer Ther. 2018 Sep; 17(3): 582–601.
Cheng Zhang.

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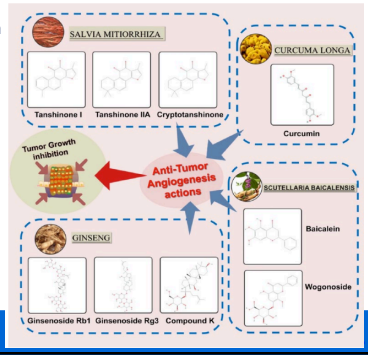
Salvia miltiorrhiza Dan Shen
(Tanshinones, Cryptotanshinione)

Panax Ginseng Ren Shen
(Ginsenosides)

Curcuma Longa
Yu Jin. Jiang Huang
(Curcuminoids)

Scutellaria baicalensis
Huang Qin
(Baicaelein, Wogonoside)

Zhang, et al



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Scutellaria baicalensis Root
Huang Qin

Baicaelein Wogonoside

Scutellaria Baicalensis and Inhibition of Angiogenesis

Baicalein (flavonoid)
Inhibits
- VEGF
- Fibroblast Growth Factor Receptor-2
- cJun
- cFos
- 12-lipoxegenase
- Endothelial cell migration and aggregation

Wogonoside/Wogonin (flavonoid)
Inhibits
- NFkB
- VEGF
- HIF1a
- IGF-1
- PI3K/Akt pathway
- B-catenin
- Wnt
- Endothelial cell migration

Integr Cancer Ther. 2018 Sep; 17(3): 582–601., Zhang, et al

Salvia miltiorrhiza

Red Sage Root

Dan Shen

Tanshinones
Cryptotanshinione

Salvia Miltiorrhiza and Inhibition of Angiogenesis

Tanshinone I
Inhibits – Down-regulates
NFkB, TNFa
Vascular Endothelial Growth Factor
Proliferation, Migration,
Differentiation of endothelial progenitor cells

Tanshinone IIA
- Inhibits HIF-1a
- Inhibits VEGF-VEGFR2
- Triggers apoptosis and cell cycle arrest
- Inhibits Matrix Metalloproteinase-2
- Down-regulates mTOR

Crytotanshinone
Inhibits-Downregulates
- TNFa. NFkB, STAT 3
- VEGF
- Cyclin D1
- B-catenin

Integr Cancer Ther. 2018 Sep; 17(3): 582–601., Zhang, et al

Panax Ginseng Root

Ren Shen

Ginsenosides

Ginsenosides and Inhibition of Angiogenesis

Ginsenoside Rb1
- Inhibits Endothelial cell migration, aggregation, tube formation
- Blocks Estrogen Receptor-beta

Ginsenoside Rg3
- Down-regulates angiogenesis related gene expression
- Enhances cytotoxic effect of oxaliplatin and 5FU >suppression angiogenesis
- Inhibits endothelial cell migration
- Induces apoptosis
- Reduces expression of Bcl-2
- Inhibits VEGF-A and VEGFR-2
- Inhibits basic Fibroblast Growth Factor
- Inhibits Matrix Metalloproteinase -2

Integr Cancer Ther. 2018 Sep; 17(3): 582–601., Zhang, et al

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Ginsenoside Rg3 targets cancer stem cells and tumor angiogenesis to inhibit colorectal cancer progression *in vivo*

- Anti-angiogenic therapy has been successfully applied to treat colorectal cancer (CRC). Ginsenoside Rg3, derived from the Chinese herb ginseng, has anti-vascularization effects and can inhibit tumor growth and metastasis, and can sensitize cancer cells to chemotherapy
- Rg3 strengthened the cytotoxicity of 5-Fluorouracil and oxaliplatin against orthotopic xenografts *in vivo*.
- Rg3 not only repressed the growth and stemness of CRC cells, but could also remodel the tumor microenvironment through repressing angiogenesis and promoting antitumor immunity.

Int J Oncol. 2018 Jan; 52(1): 127–138. YinChen Tang et al

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Ginsenosides and Angiogenesis references

Ginsenoside Rg3 targets cancer stem cells and tumor **angiogenesis** to inhibit colorectal cancer progression in vivo.Tang YC et al. Int J Oncol. (2018)

Ginsenoside Rd regulates the Akt/mTOR/p70S6K signaling cascade and suppresses **angiogenesis** and breast tumor growth.Zhang E et al. Oncol Rep. (2017)

MiR-23a targets RUNX2 and suppresses **ginsenoside** Rg1-induced **angiogenesis** in endothelial cells.Wu XD et al. Oncotarget. (2017)

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Curcuma Longa Rhizome

Yu Jin Jiang Huang

Curcuminoids

Curcumin and Angiogenesis

ADVANCES IN EXPERIMENTAL MEDICINE AND BIOLOGY The Molecular Targets and Therapeutic Uses of Curcumin in Health and Disease pp 185-195|

CURCUMIN AS AN INHIBITOR OF ANGIOGENESIS

Sulochana S. Bhandarkar Jack L. Arbiser

INHIBITS and DOWN-REGULATES

- Vascular Endothelial Growth Factor
- Basic Fibroblast Growth Factor
- Protein Kinase C
- NFkB and TNFa
- Matrix Metalloproteinases
- Urokinase Plasminogen Activator
- Cell Adhesion
- Endothelial Attachment
- Cell Motility

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Parthenolide can inhibit HIF-1α Signaling and Hypoxia-Induced Epithelial-Mesenchymal Transition, suggesting a novel molecular mechanism for HIF-1α mediated cancer progression and metastasis

Tanacetum parthenium

Feverfew

- **inhibits** hypoxia dependent **HIF-1α activity** and angiogenesis by preventing **NF-κB activation**
- **decreases the level of proteins associated with glucose metabolism, angiogenesis, development and survival** that are regulated by HIF-1α
- **protects the morphological change from epithelial to mesenchymal state (EMT)**
- **inhibits matrix metalloproteinase (MMP)** enzyme activity
- **decreases cell motility** involved in the regulation of the hypoxia-induced EMT markers
- **promotes apoptosis** of human colorectal cancer (CRC)

Parthenolide suppresses hypoxia-inducible factor-1α signaling and hypoxia induced epithelial-mesenchymal transition in colorectal cancer.
Int. J. Onc October 18, 2017 1809-1820, Se Lim Kim,et al https://doi.org/10.3892/ijo.2017.4166

Honokiol (Hou Po Cortex Magnolia spp.). 1-2g per day

Properties

❑Anti-inflammatory[1]

❑ Antioxidant[2]

❑Selective Pro-oxidant[3]

❑Anti-Microbial[4]

❑Anti-Tumor (Induces Apoptosis & Cell Cycle Arrest)[5]

❖Anti-Angiogenic[5]

❑Anti-Anxiety/Insomnia[6]

❑Neuro-protective[7]

❑Synergistic Anticancer Effect w/ Multiple Chemotherapy Drugs[8]

❑Crosses the Blood–Brain Barrier, Candidate for the Treatment of Central Nervous System Primary Tumors and Metastases[9]

❑No Appreciable Toxicity

Honokiol References

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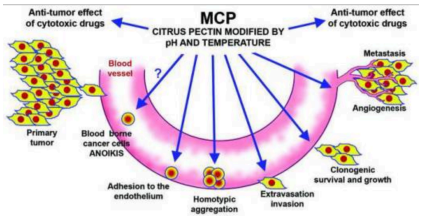
MODIFIED CITRUS PECTIN

A complex water soluble indigestible polysaccharide obtained from the peel and pulp of citrus fruits and modified by means of high pH and temperature treatment, to affect numerous rate-limiting steps in cancer metastasis.



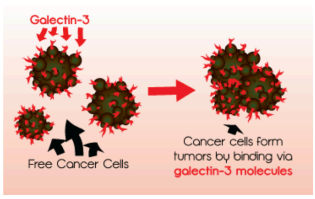
Carbohydr Res. 2009 September 28; 344(14): 1788–1791.
Modified citrus pectin anti-metastatic properties: one bullet, multiple targets Vladislav V. Glinsky

Modified Citrus Pectin
Inhibits Tumor Cell Adhesion, Tumor Growth, Metastasis and Angiogenesis

- Blocks tumor cell aggregation
- Blocks docking of cancer cells
- Blocks interactions with endothelium necessary for angiogenesis
- Inhibits Growth Factors/Oncogenes: EGFR, VEGF, bcl2
- Influences Cell Adhesion and Aggregation
- Apoptosis (Intracellular Inhibition/Extracellular Promotion)
- Decreases Immune Evasion/T-Cell Inactivation
- Inhibits Stromal/ECM Remodeling
- Increases apoptotic responses of tumor cells to chemotherapy
- Inhibits galectin-3 anti-apoptotic function
- Chelating agent for heavy metals promoting detoxification
- Radiosensitizer

Modified Citrus Pectin binds to Galectin-3

- Galectin-3 a soluble multi functional protein, a member of beta galactoside binding gene family (lectins)
- Regulatory roles in cancer tumorigenisis and metastasis, inflammation, fibrosis, and immune response
- Expressed in the nucleus, cytoplasm, cell surface, and extracellular
- microenvironment (stroma)



Kidney Int Suppl. 2000 Sep;77:S31- **Role of galectin-3 as a receptor for advanced glycosylation end products**
Flavia Pricci, Gaetano Leto, et al

NON FASTING BLOOD TEST for Galectin-3

Monitor every 3 months in patients with active disease
20% population will have changes within this time interval

Elevations reflect

- increased inflammation
- fibrosis and hyper-viscosity
- malignancy
- cardiovascular disease (CHF, endothelial dx)
- diabetes
- chronic hepatitis
- kidney disease

Approved as a CVD risk factor assessment
Both prognostic and diagnostic

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Modified Citrus Pectin Dosing Schedule

Must be taken 30+min before or after food, supplements, nutrients, medications

Dissolve in Hot Water

MAINTENANCE	NO ACTIVE DISEASE	
< 12-14.0 ng/ml	5 g	daily
12-14.0 -17.8 ng/ml	10g-15g	daily
>17.8 ng/ml.	15g-25g	daily

REMISSION		
< 17.8 ng/ml	15g	daily ‘

ACTIVE DISEASE		
>17.8 ng/ml	20-25g	daily

continue for 3 years at this dose
after 3 years follow maintenance dose schedule

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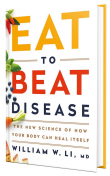
Dr. William Li, MD
Angiogenesis Foundation
www.angio.org



for patients

EAT TO BEAT DISEASE
gives readers a fascinating view of the body's health defense systems, which span angiogenesis, regeneration, microbiome, DNA protection, and immunity. Each system helps the body resist disease and can be activated by foods.
eattobeat.org

TED TALK: CAN WE EAT TO STARVE CANCER? https://www.ted.com/talks/william_li

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RESEARCH & EDUCATION

CASE STUDY SUBMISSION

Important: In observance of HIPAA and the sacred trust between care giver and patient, absolutely no patient names or identifying information is to be disclosed. Patient privacy is to be preserved. If you attach any medical records, pathology, surgical or laboratory reports, all names are to be removed.

Date	02/06/2019
Clinician Name & Credentials	Isabel Galiano, Health Coach & IFM AFMCP Graduate
Email	isabel@isabelgaliano.com

Describe Your Patient (Please SUMMARIZE and use economy of words. You will have 15 minutes to present)

Age, Gender & Ethnicity	50 years old, female, caucasian
Body Type	Endomorph
Values *What is most important to this patient? (Quality of Life, Decision Making, Side Effects?)*	do everything she can to avoid recurrency
Stress Resilience	Good at the moment
Other	
Primary Diagnosis & Date *(ex. Breast Cancer L, T3 N1 M0, BRCA1 positive, grade 3, Ki67 > 45%)*	Malignant Cutaneous Melanoma (left lower thigh), superficial spreading type, Clark level III, Breslow depth 0.8mm, non-ulcerated. Mitotic grade is 0. Tumor infiltrating lymphocytes are non-brisk, micro satellites not seen. Melan A positive cells were seen in lymph node favoring capsular/nodal nevus. Cells are negative for HMB45. No evidence of distant metastasis.
Secondary Diagnosis *(ex. Diabetes Type 2, Obesity)*	BRAF V600 mutation identified. CN1 cells on the cervix. Multiple cysts in both breasts.

Patient Status

☑ New Diagnosis ☐ Recurrence ☐ In Treatment ☐ In Recovery ☐ In Remission ☐ At Risk

Concomitant and/or Complicating Factors *(ex: poorly controlled diabetes, insomnia, poor support system)*	Poor support system, stressful job, financial struggles, poor sleep, chronic constipation, fatigue
Adverse Effects of Cancer or Cancer Treatments *(ex. anxiety-depression, diarrhea, peripheral neuropathy)*	n.a
Relevant Laboratory, Pathology & Medical Reports *(attach a PDF with patient identifying information removed or summarize)*	see attachment

Brief Summary of Recent History

After receiving all the results from biopsy, the oncologist was hesitant whether to prescribe immunotherapy or not. Her file was sent to Mayo Clinic for second opinion. They recommended close follow-up with dermatologist without further adjuvant medical treatment.

Apart from the skin cancer, she has been diagnosed with CN1 cells on the cervix and multiple cysts in both breasts. All these conditions have been left to close monitoring alone. Feeling unsettled with just going back to her "normal" life, she contacted me to help her on lifestyle changes and diet.

Brief Summary of Additional Relevant Health, Medical, Psycho-Social and/or Family History

Recently divorced mother and a teacher, she has been under a lot of stress during her divorce. When I first met her, she had very poor sleep quality, did not exercise and was a bit overweight; she described herself as exhausted all the time, very stressed, with very low energy levels and little self-esteem and self-confidence.

Other Relevant Information

Such as Chinese or Ayurvedic diagnosis, Naturopathic/Homeopathic Information, etc. *(ex. Liver Qi Stagnation, Dysbiosis)*

Functional Medicine Nutritionist diagnosed dysbiosis and adrenal fatigue.

Brief Summary of Relevant Past Oncology or Medical Treatments

(ex. surgery, radiotherapy, chemotherapy, immunotherapy, hormone therapy, drug therapy)

Surgery performed to remove melanoma and 5 sentinel lymph nodes. No additional treatment.

Summary of Recent and Current Treatments

Medical Oncology Care *(surgery, radiotherapy, chemotherapy, immunotherapy, hormone therapy, drug therapy)*

none

Integrative Oncology Care *(nutraceutical, botanical, phytochemical, acupuncture, energy medicine, other)*

none

Your 2 Core Questions (stated clearly and succinctly)

1. What would you add/change in my treatment plan?
2. is there any specific recommendations in terms of diet and supplements for SKIN cancer patients.

Attached Medical Records for Reference (with patient identifying information removed)

PROPOSED TREATMENT PLAN Your case will not be reviewed without a completed proposed treatment plan

Nutriceutical, Phytochemical and Botanical Supplements (name of supplement, dosing)

Foundation Nutrition Supplements:

- Omega 3 Fatty acids 2000 mg/day
- Vitamin C & flavonoids (Ester C from Pure Encapsulations) 2x /day (1250mg Vit C)
- Vitamin D3 5000 IU /day (monitor blood levels)
- B Complex (with Metafolin L-5 MTHF) 1/day
- Magnesium Citrate 300-600 mg/day (to help loose stool)

Targeted Supplements:

- Digestive enzymes
- Curcumin 3 gr/ day
- CoQ10 100mg/day
- Resveratrol 200mg/day
- Rhodiola 100mg/day

Functional Foods and/or Therapeutic Shake

Probiotic Foods (3x/day), Prebiotic Foods, Resistant starch - Bone Broth and Collagen Powder (daily) - Red and green powders 1 teaspoon (alternate) - Mushroom powders (Cordyceps, Reishi, Lion's Mane) - Coconut oil (1-2 tablespoons daily) - Cruciferous vegetables 2 servings/day - Green tea - Goji berries - Ginger

Basic Therapeutic Shake (High Protein, High antioxidant, Good fats, Low Glycemic) with digestive enzymes

Dietary Guidelines

Anti-inflammatory, immune supporting, low glycemic, gut supportive.
Mostly Plant based. High in phytonutrients. High in Fiber. High in antioxidants.
High in Healthy Fats (Omega 3)
Low in Iron
High in probiotic and prebiotic foods
Healthy Protein Intake (about 60 gr)
Remove processed foods. Chemical and Hormone free. Organic.

Lifestyle Guidelines

I have been working with her on: 1) stress management 2) healthy sleep habits 3) exercise: brisk walk every day for 30 minutes, 2 yoga classes a week 4) building a support system of health practitioners, family and friends 5) work on self-confidence and self-esteem 6) reduce exposure to toxins (in food, water, air, beauty products)

Recommended Diagnostics

Referrals to specialists

Functional Medicine Nutritionist, Yoga teacher.

Other Notes (please do not include additional notes in your email – notate them here within the case study)

Patient is still wondering if the decision NOT to do immunotherapy is the best option.

CASE STUDY Submitted by Isabel Galiano **05/30/19**

Overview: N 50 yo Female Dx with Invasive Malignant Melanoma L Lower Thigh (excision, clear margins), pT1b + nodal nevus (2 nodes with micrometastases)
Immunotherapy not recommended/Observation only
Patient is still wondering if the decision NOT to do immunotherapy is the best option.

PATHOLOGY Malignant Cutaneous Melanoma (left lower thigh), superficial spreading type, Clark level III, Breslow depth 0.8mm, non-ulcerated. **Mitotic grade is 0.** Tumor infiltrating lymphocytes are non- brisk, micro satellites not seen. Melan A positive cells were seen in lymph node favoring capsular/nodal nevus.
Cells are negative for HMB45. **BRAF V600 mutation identified.**
CRP 5.3
RADIOLOGY No evidence of distant metastasis.

Multiple breast cysts, cervical dysplasia CN1, chronic constipation, fatigue
Divorced, Mother, School Teacher, High Stress, Poor Sleep Quality, Sedentary
Describes herself as exhausted, low energy with poor self esteem and poor self confidence, poor support system

Considerations

- **Immunotherapy: Pembrolizumab (Keytruda)**
- **Oral Copper Chelation Tetrathiomolybdate**
- **High dose IV Vitamin C**
- **IV or SubQ Mistletoe Therapy**

PI3K-AKT
MAPK
BRAF mutations
Loss of Tumor Suppressor PTEN

Wnt/b-catenin signaling
Cell Adhesion- E Cadherin
COX2
NFkB

BIOMARKERS:
MONITOR SERUM
CoQ10,
Copper,
Zinc,
Ceruloplasmin,
hs CRP,
Neutrophil:Lymphcyte Ratio,
Fibrinogen activity,
D-Dimer,
Serum VEGF
Serum Vitamin A

Targeted Melanoma Support

DFH Curcumevail Curcumin	**1/2x/day**
DFH Resveratrol Supreme (+Quercetin)	1/2x/day
DFH Grapeseed Extract. Grapeseed proanthocyanidins	**1/2x/day**
DFH EGCG Green Tea Catachins	**1/2x/day**
DFH Milk Thistle Silymarin	**1/2x/day**
VN Berberine 500mg	1/2x/day
Clinical Synergy Mycoceutics Mushrooom Immune Max	3/2x/day.
Chinese Herbal Dan Shen Salvia Milthiiorrhiza extract (Target Melanoma)	
LIQUID Golden Lotus or Wise Woman Herbals extract	2 teaspoons daily
OR packets TCMZone Dan Shen granules	2 packets daily (20 day supply per box)
Clinical Synergy Pure Honokiol 2 at bedtime	
Clinical Synergy Pectasol C Professional 15 grams daily in two divided doses	

Foundation Nutrients for Cancer Control

ITI ProThriver Wellness Multi	1/2x/day
DFH Omegavail TG 1000	**1/2x/day**
DFH Buffered Magnesium Chelate (glycinate)	**1/2x/day**
DFH Vitamin D Supreme	**1/2x/day Cell Adhesion E Cadherin**
DFH Q-Evail 200mg	**1/2x/day (CoQ 10).** Low levels of CoQ10 linked to risk of Melanoma
DFH Osteoben	2/2x/day. Bone Health
DFH Zinc Supreme (30mg + Mb)	1/2x/day
VN Vitamin A 25,000iu	1 cap per week
DFH Annatto Tocotrienols 300mg	1/2x/day

Selected References

Canc Lett 2013 July 28: 335(2): 251-258 **Emerging Phytochemicals for prevention of melanoma invasion.** Virgina Jones, Santosh K. Katiyar

J Am Acad Dermatol. 2006 Feb;54(2):234-41. Epub 2005 Dec 27. **Low plasma coenzyme Q10 levels as an independent prognostic factor for melanoma progression** Rusciani L

CoQ10 levels were significantly lower in patients than in control subjects (t test: P < .0001) and in patients who developed metastases than in the metastasis-free subgroup (t test: P < .0001). Logistic regression analysis indicated that plasma CoQ10 levels were a significant predictor of metastasis (P = .0013). The odds ratio for metastatic disease in patients with CoQ10 levels that were less than 0.6 mg/L (the low-end value of the range measured in a normal population) was 7.9, and the metastasis-free interval was almost double in patients with CoQ10 levels 0.6 mg/L or higher (Kaplan-Meier analysis: P < .001).

Cancer. 1981 Apr 1;47(7):1838-44.

Serum copper and zinc levels in melanoma patients. Fisher GL, Spitler LE, McNeill KL, Rosenblatt LS. Serum copper levels (SCL) and serum zinc levels (SZL) were evaluated in malignant melanomapatients at various clinical stages. Copper levels were generally found to be elevated, reflecting the degree and extent of tumor activity. Zinc levels and, hence, SCL:SZL ratios did not reflect tumor activity. SCL appeared to prognosticate disease progression in that all patients whose values never declined below 150 micrograms/100 ml died during the course of the study. However, not

all patients who died from tumor metastases displayed persistent elevations of SCL. Patients receiving BCG immunotherapy appeared to have higher SCL than untreated patients.

Int J Oncol. 2016 Feb;48(2):624-34. doi: 10.3892/ijo.2015.3286. Epub 2015 Dec 10.**Bioactive proanthocyanidins inhibit growth and induce apoptosis in human melanoma cells by decreasing the accumulation β-catenin.**Vaid M

Nutr Metab (Lond). 2019 May 21;16:33. doi: 10.1186/s12986-019-0365-4. eCollection 2019. **Dietary compounds and cutaneous malignant melanoma: recent advances from a biological perspective. Ombra MN**

Notes from Isabel Galiano, Health Coach, Singapore

Proposed treatment plan

Nutriceutical, Phytochemical and Botanical Supplements (name of supplement, dosing) Foundation

Nutrition Supplements:

- Omega 3 Fatty acids 2000 mg/day
- Vitamin C & flavonoids (Ester C from Pure Encapsulations) 2x /day (1250mg Vit C)
- Vitamin D3 5000 IU /day (monitor blood levels)
- B Complex (with Metafolin L-5 MTHF) 1/day
- Magnesium Citrate 300-600 mg/day (to help loose stool)

Targeted Supplements:

- Digestive enzymes
- Curcumin 3 gr/ day
- CoQ10 100mg/day
- Resveratrol 200mg/day
- Rhodiola 100mg/day

Functional Foods and/or Therapeutic Shake

Probiotic Foods (3x/day), Prebiotic Foods, Resistant starch - Bone Broth and Collagen Powder (daily) - Red and green powders 1 teaspoon (alternate) - Mushroom powders (Cordyceps, Reishi, Lion's Mane) - Coconut oil (1-2 tablespoons daily) - Cruciferous vegetables 2 servings/day - Green tea - Goji berries - Ginger
Basic Therapeutic Shake (High Protein, High antioxidant, Good fats, Low Glycemic) with digestive enzymes

Dietary Guidelines Anti-inflammatory, immune supporting, low glycemic, gut supportive. Mostly Plant based. High in phytonutrients. High in Fiber. High in antioxidants. High in Healthy Fats (Omega 3) Low in Iron High in probiotic and prebiotic foods Healthy Protein Intake (about 60 gr) Remove processed foods. Chemical and Hormone free. Organic.

Lifestyle Guidelines I have been working with her on: 1) stress management 2) healthy sleep habits 3) exercise: brisk walk every day for 30 minutes, 2 yoga classes a week 4) building a support system of health practitioners, family and friends 5) work on self-confidence and self-esteem 6) reduce exposure to toxins (in food, water, air, beauty products)

Ombra et al. Nutrition & Metabolism (2019) 16:33
https://doi.org/10.1186/s12986-019-0365-4

Nutrition & Metabolism

REVIEW Open Access

Dietary compounds and cutaneous malignant melanoma: recent advances from a biological perspective

Maria Neve Ombra[1], Panagiotis Paliogiannis[2*], Luigia Stefania Stucci[3], Maria Colombino[4], Milena Casula[4], Maria Cristina Sini[4], Antonella Manca[4], Grazia Palomba[4], Ignazio Stanganelli[5], Mario Mandalà[6], Sara Gandini[7], Amelia Lissia[2], Valentina Doneddu[2], Antonio Cossu[2], Giuseppe Palmieri[4] and for the Italian Melanoma Intergroup (IMI)

Abstract

Cutaneous malignant melanoma is a heterogeneous disease, being the consequence of specific genetic alterations along several molecular pathways. Despite the increased knowledge about the biology and pathogenesis of melanoma, the incidence has grown markedly worldwide, making it extremely important to develop preventive measures. The beneficial role of correct nutrition and of some natural dietary compounds in preventing malignant melanoma has been widely demonstrated. This led to numerous studies investigating the role of several dietary attitudes, patterns, and supplements in the prevention of melanoma, and ongoing research investigates their impact in the clinical management and outcomes of patients diagnosed with the disease. This article is an overview of recent scientific advances regarding specific dietary compounds and their impact on melanoma development and treatment.

Keywords: Skin, Melanoma, Nutrition, Food, Dietary supplements

Introduction

Nutrition plays an important role in cancer. The American Institute for Cancer Research and the World Cancer Research Fund have estimated that 30–40% of all cancers can be prevented by a proper diet, physical activity, and the maintenance of correct body weight [1, 2]. Indeed, epidemiological evidence indicates that a poor quality diet, physical inactivity, and overweight and obesity are strong risk factors for multiple malignancies [3]. In this scenario, increasing numbers of foods and nutrients with a protective effect have been identified in recent years [4]. Despite the role of diet in cancer prevention, this evidence is widely perceived as inconsistent, underlining the need for greater research and communication clarity.

Cutaneous malignant melanoma (CMM) is the most dangerous form of skin cancer, having a growing incidence, high metastatic potential and affecting all age groups, which makes preventive measures particularly urgent. The incidence differs among countries but has increased markedly worldwide in recent years, especially in white-skinned populations [5]. CMM arises from melanocytes, the cells responsible for the production of the melanin pigment of the skin, hair, and eyes, and is the result of complex interactions between individual genetic factors and environmental risk factors. The scientific literature has provided direct evidence that sun exposure causes mutations in critical genes for melanoma [6]. Ultraviolet B (UVB) radiation is the most mutagenic component of the ultraviolet spectrum and promotes DNA damage more than ultraviolet A (UVA) radiation. UVB radiation is responsible for the production of DNA photoproducts such as cyclobutane pyrimidine dimers (CPDs). CPDs cause bulky lesions that distort the DNA helix, producing adducts that can suspend DNA replication and transcription. UVB can also damage DNA indirectly by causing oxidative stress resulting from lipid peroxidation and the formation of reactive oxygen and

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nitrogen intermediates [7, 8]. Moreover, exposure to UVB causes inflammation, including erythema and edema, and chronic inflammation is a recognized risk factor for tumor development [9].

Molecular pathways underlying melanoma genesis are complex; RAS-RAF-MEK-ERK mitogen-activated protein kinase (MAPK) and PI3K-PTEN-AKT (AKT) are the two major pathways constitutively activated through genetic abnormalities [10]. The two most common mutations occur in *BRAF* (40–55%) and *NRAS* (15–30%); clinically relevant *BRAF* mutations result in the substitution of valine at position 600 ($BRAF^{V600}$) in the gene encoding BRAF serine-threonine kinase in the MAPK pathway [10].

As mentioned above, the incidence of CMM has continued to rise in recent years despite public efforts to promote sun protection habits. Considering that the use of sunscreen does not entirely prevent skin cancer, additional chemo-preventive approaches are desirable. In this regard, attention has been focused on the possible role of diet in reducing the melanoma risk. Furthermore, dietary interventions may have systemic benefits, unlike purely topical methods of sun protection, and do not need constant reapplication. Numerous studies have suggested a protective role of some dietary elements, but relationships between dietary intake of certain foods and the cancer risk are still controversial. Dietary antioxidant phytochemicals have demonstrated protective effects and the presence of these compounds in the traditional Mediterranean diet may be partly responsible for the low incidence of CMM in this area, despite high levels of solar radiation; other studies showed a trend towards a reduced risk of CMM with a greater intake of vegetables and fruit, fish, as well as vitamins and beverages such as coffee or tea [11, 12]. The results appear encouraging and could reinforce nutritional prevention campaigns and the development of appropriate initiatives. Extrinsic factors thought to play a role in melanoma prevention are summarized in Fig. 1.

In this review, we report the most recent advances on the comprehension of the biological mechanisms, which underlay the impact of foods and dietary compounds on the risk and prevention of melanoma. Considering the wideness of the topic, and in order to avoid redundancy, we chose to describe the main dietary compounds involved in active research with substantial advances in the last decade, excluding foods and compounds with well-known impact on the disease.

Foods and melanoma risk: recent advances

There has been growing interest in the role of nutrition for melanoma prevention in recent years, as demonstrated by the rising of the total number of articles published in PubMed on the topic (Fig. 2). Numerous

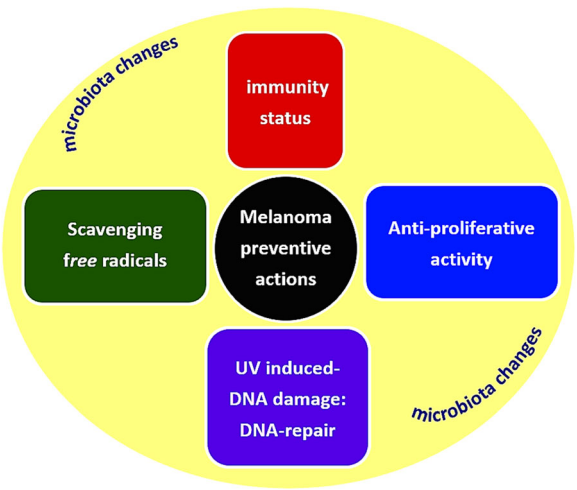

Fig. 1 Extrinsic factors involved in melanoma prevention

epidemiological studies have widely demonstrated that regular consumption of fruit and vegetables is associated with a reduced risk of cancer [13]. Modification of diet alone, by increasing vegetable and fruit intake, could even prevent cancer. This evidence has awakened interest in research on bioactive food components, and has led to the identification of compounds with a cancer preventive and therapeutic potential. Owing to their safety, low toxicity and antioxidant properties, fruits, vegetables and other dietary elements (phytochemicals and minerals) have being analysed as chemopreventive agents, intended to interrupt the carcinogenesis process, which includes the initiation, promotion, and progression of otherwise normal cells to cancer. Some evidence has also suggested that a variety of substances may enhance the therapeutic efficacy of drugs, reduce chemotherapy-induced side effects or overcome drug-resistance [14–16].

Bioactive food substances are identified on the basis of in vitro and in vivo studies. These compounds present tumor-suppressing properties in animal models of carcinogenesis, interfering with cellular processes of tumor formation. In phase II studies in humans, it has frequently been impossible to draw definite conclusions about the preventive or clinical efficacy because of the great variability and differences in study designs, patient numbers, study duration, as well as lack of a standardized formulation. Lastly, it is not always easy to reach a consensus due to discordant results obtained in similar studies.

In melanocytes, reactive oxygen species (ROS) accumulate - including singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2), and superoxide (O_2^-) - leading to oxidative stress-induced cell damage. In general, ROS may induce antioxidant defenses by enhancing the expression of

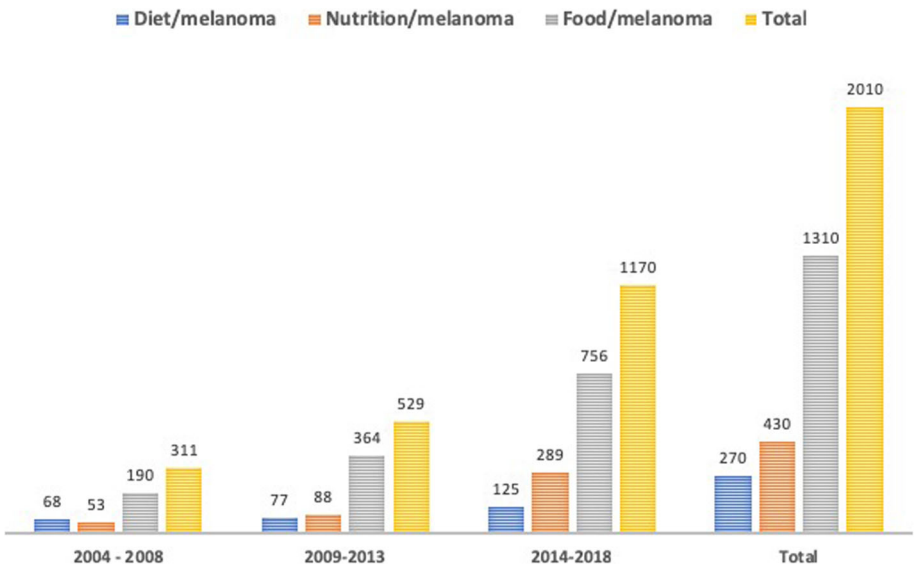

Fig. 2 Total amount of manuscripts on nutrition and melanoma published in recent years in PubMed retrieved using the following keywords: "diet" or "nutrition" or "food" and "melanoma" (until the 31st December 2018)

superoxide dismutase, catalase, glutathione peroxidase, and peroxiredoxins, which maintain the redox balance [17, 18]. However, when cellular ROS production overwhelms the antioxidant capacity, the ROS cause serious toxicity and damage in cells. Thus, ROS scavengers and inhibitors of ROS production may suppress melanomagenesis and protect against skin damage.

From the molecular point of view, ROS are reported to activate p21ras protein through increased phosphorylation [19]. Another signaling molecule which has been shown to act as a direct target of ROS and nitrogen species is ataxia-telangiectasia mutated (ATM) protein kinase. It has been shown that the ATM protein is activated after certain stresses, most notably after double-stranded DNA breaks, through oxidation at the C-terminal region of ATM [20]. Moreover, cells carrying inactivated ATM exhibit constitutively high levels of ROS [21]. The mechanism whereby ATM regulates the intracellular redox state is complex and may involve alterations of some mTOR-dependent mechanisms [22]. In recent years, studies have shown that ROS activate COX (three isoforms of cyclooxygenase, namely COX1, COX2, and COX3) and that COX and its products induce ROS generation. A diagram of the main molecular effects triggered by ROS is shown in Fig. 3.

Natural antioxidants are a focus of skin protection due to their potential to scavenge ROS and inhibit the UV-induced signal transduction pathway, thus offering a promising strategy for combating melanogenesis [23, 24]. Many dietary compounds have been identified: vitamins, minerals, carotenoids and a large class of phytochemicals (polyphenols, isothiocyanates, organosulfur compounds), as well as sulforaphane, anthocyanidins, lycopene, diallyl disulfide, rosmarinic acid, silymarins, oleuropein, etc. [25, 26]. Both in vitro and in vivo studies have elucidated various cellular and molecular mechanisms by which such compounds scavenge ROS and act against melanoma cell formation; we will focus initially on coffee, tea and pomegranate, and then in specific

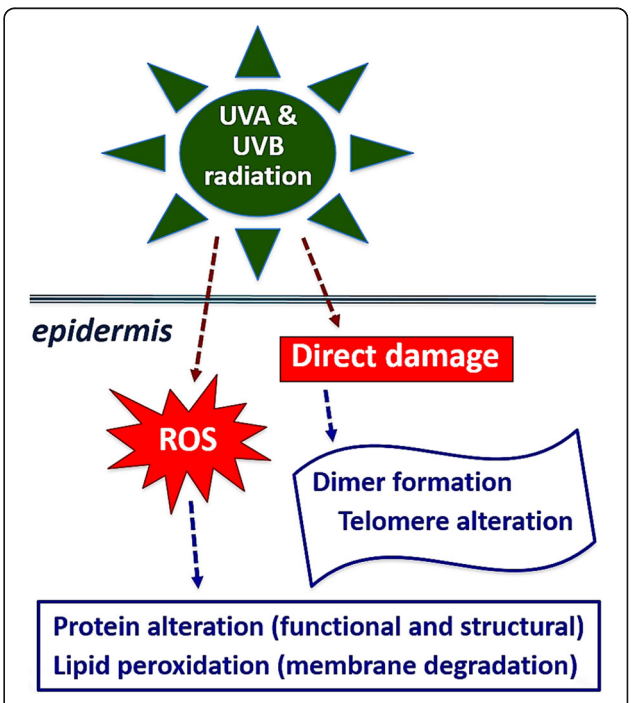

Fig. 3 Main functional effects of UVA/B radiation on skin

dietary compounds in which consistent advances were carried out in the last decade.

Coffee and tea are the most widely consumed beverages worldwide. They contain numerous phytochemicals, many of which are antioxidants, such as chlorogenic acids, quinic acid, caffeic acid, ferulic acid, and coumaric acid among the polyphenols and caffeine, diterpenes (coffee lipids). The quantities of these components depend on the brewing method [27, 28], and were object of active research in relation to melanoma in recent years.

Coffee

In vitro and animal studies suggest that bioactive constituents of coffee may have anti-carcinogenic effects against cutaneous melanoma; however, the epidemiological evidence is limited to date. Prospective studies on coffee consumption and malignant melanoma have shown conflicting results, ranging from no association to lower relative risk. Potential mechanisms of coffee phytochemicals include inhibition of oxidative stress and oxidative damage by ROS, regulation of DNA repair, phase II enzymatic activity, apoptosis, inflammation, as well as anti-proliferative, anti-angiogenetic effects, and antimetastatic effects.

According to Loftfield et al. [29], high coffee intake is associated with a lower risk of melanoma. The authors found a 20% lower risk for participants who drank 4 or more cups per day. The protective effect appeared to increase with a higher intake, increasing from 1 or fewer cups to 4 cups of coffee or more. The study on coffee consumption was performed on 447,357 white participants using a self-administered food frequency questionnaire in 1995 through 1996, and for a median follow-up of 10 years. The subjects were free of cancer at baseline and the authors adjusted for ultraviolet radiation exposure, body mass index, age, sex, physical activity level, alcohol intake, and smoking history. The preventive effect was found to be statistically significant only for caffeinated coffee, and only for protection against malignant melanoma and not melanoma in situ [29]. Their findings suggested that drinking four or more cups per day may decrease the risk of melanoma by 20%, but require replication also in other populations.

In another study, Wu et al. [30] reported that components in coffee and tea may have anti-carcinogenic properties. They prospectively analysed coffee, tea and CMM risk in the Women's Health Initiative: a cohort study of 66,484 postmenopausal women, followed for an average of 7.7 years. Coffee and tea intakes were measured through self-administered questionnaires at the beginning and at year 3 of follow-up. Daily coffee and tea intakes were not significantly associated with melanoma risk compared with no-daily intake of each beverage. No significant trends were observed between melanoma risk and increasing intakes of coffee or tea. Women who reported a daily coffee intake at both the starting point and year 3 had a significantly decreased risk compared with women who reported non-daily intake at both time points (HR = 0.68, 95% CI 0.48–0.97). Daily tea intake was not associated with a decreased melanoma risk. They concluded that there is no strong evidence that increasing coffee or tea consumption can lead to lower melanoma risk [30].

.In another large study, Wu et al. [31] used data from 163,886 women in the Nurses' Health Study II (NHS II, 1991–2009) and Nurses' Health Study (NHS, 1980–2008) and 39,424 men in the Health Professionals Follow-up Study (HPFS, 1986–2008). They documented 2254 melanoma cases over 4 million person-years of follow-up. After adjustment for other risk factors, higher total caffeine intake was associated with a lower risk for CMM (≥393 mg/d vs. < 60 mg/d: HR = 0.78, 95% CI = 0.64–0.96, P trend = 0.048). The association was more apparent in women (≥393 mg/d vs. < 60 mg/d: HR = 0.70, 95% CI = 0.58–0.85, P trend = 0.001) than in men (HR = 0.94, 95% CI = 0.75–1.18, P trend = 0.81), and more apparent for melanomas occurring on body sites with a higher continuous sun exposure (head, neck and extremities) (≥393 mg/d vs. < 60 mg/d: HR = 0.71, 95% CI = 0.59–0.86, P trend = 0.001) than for melanomas on other body sites (trunk including shoulders, back, hips, abdomen and chest) (HR = 0.90, 95% CI = 0.70–1.16, P trend = 0.60). No association was found between decaffeinated coffee consumption and CMM risk. They concluded that caffeinated coffee consumption may be protective against CMM [31].

A meta-analysis of cohort studies was conducted by Wang et al. [32] to investigate the association between coffee and the most common cancer types. This study evidenced an inverse association between coffee intake and oropharyngeal cancer, liver cancer, colon cancer, prostate cancer, endometrial cancer, and melanoma but an increased association for lung cancer. The reduction was found to be up to 31% for oropharyngeal cancer, 13% for colon cancer, 54% for liver cancer, 11% for prostate cancer, 27% for endometrial cancer, and 11% for melanoma, for the highest compared to the lowest coffee intake [32]. Simultaneously, Wang et al. [33] conducted another meta-analysis to study the associations between the consumption of total coffee, caffeinated or decaffeinated coffees, and melanoma risk, respectively. They selected 12 studies including 832,956 participants for total coffee consumption, 5 studies involving 717,151 participants for caffeinated coffee consumption and 6 studies for a total of 718,231 participants for decaffeinated coffee consumption. This meta-analysis suggests that coffee consumption may reduce the risk of CMM. A

dose-response analysis defined a decreased cutaneous melanoma risk by 3% [0.97 (0.93–1.00)] and 4% [0.96 (0.92–1.01)] per 1 cup/day increment of total coffee and caffeinated coffee consumption, respectively [33].

Also, Yew et al. [34] performed a meta-analysis of published studies to evaluate any association between coffee consumption and melanoma. Nine observational studies were identified, for a total of 927,173 participants, of which 3787 had melanoma. They calculated a 0.75 (95% CI 0.63–0.89, $p = 0.001$) relative risk (RR) for melanoma among regular coffee drinkers compared to controls. The pooled relative risk for melanoma among decaffeinated coffee drinkers was not, however, statistically significant, at 0.92 (95% CI 0.82–1.05, $p = 0.215$). The authors concluded that there is some evidence for a beneficial effect of regular coffee consumption on melanoma, but more studies would be necessary to confirm this association [34].

Liu et al. [35] identified and analyzed two case-control studies (846 CMM patients and 843 controls) and five cohort studies (including 844,246 participants and 5737 CMM cases). For caffeinated coffee, the RR for CMM was 0.81 (95% CI = 0.68–0.97; *P*-value for Q-test = 0.003; I2 = 63.5%) for those with the highest versus lowest quantity of coffee intake. In the dose-response analysis, the RR for CMM was 0.955 (95% CI = 0.912–0.999) per 1 cup/day increment of caffeinated coffee consumption, and a linear dose-response association was found (P-value = 0.326). Moreover, no significant association was found between the decaffeinated coffee intake and CMM risk (RR = 0.92; 95% CI = 0.81–1.05; P-value for Q-test = 0.967; I2 = 0%) for the highest versus lowest quantity of intake. This meta-analysis concluded that caffeinated coffee might have preventive actions against malignant melanoma but not decaffeinated coffee, in accordance with previous studies [35]. According to Lukic et al. [36] who performed the Norwegian Women and Cancer (NOWAC) study, moderate consumption of filtered coffee is associated with a decreased risk of malignant melanoma. Interestingly, the authors found no evidence of an association between instant, boiled, or total coffee consumption and the risk of CMM [35].

In a more recent study, Caini et al. [37] examined the relationships between coffee (total, caffeinated or decaffeinated) and tea consumption and the risk of melanoma in the European Prospective Investigation into Cancer and Nutrition (EPIC). EPIC was a multicentre prospective study that enrolled over 500,000 participants aged 25–70 years from ten European countries in the years 1992–2000. Information on coffee and tea drinking were collected at baseline using validated country-specific dietary questionnaires. In this study, 2712 melanoma cases were identified during a median follow-up of 14.9 years among 476,160 participants. Consumption of caffeinated coffee was inversely associated with melanoma risk among men (HR for the highest quartile of consumption versus non-consumers 0.31, 95% CI 0.14–0.69) but not among women (HR 0.96, 95% CI 0.62–1.47). There were no statistically significant associations between the consumption of decaffeinated coffee or tea and the melanoma risk among men or women. In this large cohort study, consumption of caffeinated coffee was inversely associated with melanoma risk, only among men [37].

A further study by Conney et al. [38] examined the effects of caffeine and the molecular mechanisms at the basis of its protective effect. They indicated that caffeine administration inhibits UVB-induced carcinogenesis by enhancing apoptosis in UVB-induced tumors. The stimulatory effect of caffeine on apoptosis occurs by p53-dependent and p53-independent mechanisms. Inhibition of the ATR/Chk1 pathway by caffeine is a major contributor to caffeine inhibition of UVB-induced carcinogenesis. In addition, a p53-independent effect indicated that caffeine enhanced UVB-induced apoptosis by inhibiting the increase in ATR-mediated formation of phospho-Chk1 (Ser345) and abolishing the decrease in cyclin B1, which resulted in caffeine-induced premature, lethal mitosis in mouse skin. In short, ATR-mediated phosphorylation of Chk1 is an important target for caffeine's inhibitory effect on UVB-induced carcinogenesis. Moreover, caffeic acid inhibited the activation of the IKK-NF-κB signaling pathway by scavenging intracellular ROS generated by oxidative stress (Fig. 4). Upon activation, NF-kB can undergo retention in the nucleus of the

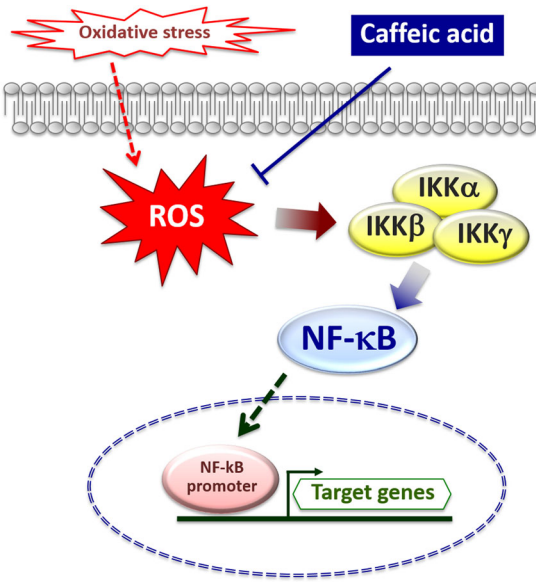

Fig. 4 The activity of caffeic acid on the molecular mechanism controlling cell survival

cells and regulate the transcription of a wide variety of genes, including those involved in cell proliferation [39].

Tea

Tea is also a popular beverage worldwide, derived from the infusion of leaves of *Camellia sinensis*, a species of the Theaceae family. The tea plant and its leaves have long been used for medicinal purposes. Several in vitro, in vivo, and epidemiological studies have reported that the consumption of green tea may decrease cancer risk. In particular, green tea and its major polyphenol constituents, such as epicatechin (EC), epicatechin-3-gallate (ECG), epigallocatechin (EGC), and epigallocatechin-3-gallate (EGCG), have been shown to possess many beneficial properties for health; furthermore, black tea polyphenols can induce apoptosis of melanoma cell lines in vitro [40]. Evidence is now accumulating that catechins and theaflavins, which are the main polyphenolic compounds of green and black tea, are responsible for further beneficial effects.

Regarding melanoma, large epidemiological studies did not evidence a strong association between the consumption of tea and risk prevention [30, 37]. Nevertheless, interesting results come from in vitro and in vivo studies. The anti-cancer properties of green tea are referred mainly to epigallocatechin-3-gallate (EGCG). Owing to its chemical properties, EGCG may act both as a sunscreen and as a quencher of free radicals [41]. Experiments in mouse models of melanoma indicated that EGCG inhibits the formation of lung metastases after tail vein injection of B16 melanoma cells [42], whereas topical application showed partial inhibition of skin papilloma growth in mice. Epigallocatechin-3-gallate helps to reverse damage caused by UV light, and drinking green tea has caused a decrease in UV-induced skin tumor incidence and size compared with controls. In mice, green tea polyphenols have also caused inhibition of UV-induced matrix metalloproteinase-2, − 3, − 7, and − 9 expression, involved in degradation of the basement membrane, preliminary to metastasis [43].

Animal studies have clearly demonstrated the anti-carcinogenic effects of EGCG through induction of melanoma cell apoptosis and cell cycle arrest by modulating B-cell lymphoma 2 (Bcl-2) and the CKI-Cyclin-CDK pathway [44–46]. In vitro studies evidenced that Green tea polyphenol epigallocatechin-3-O-gallate inhibits melanoma tumor growth by activating the 67-kDa laminin receptor (67LR) [44]. 67LR has been identified as a cell surface receptor of EGCG and plays a key role in the cancer preventive effects of EGCG. In melanoma, 67LR is expressed at a higher level than in normal skin cells. The authors have previously shown that EGCG suppresses melanoma tumor growth by activating the intercellular signaling pathway, cAMP/protein kinase A (PKA)/protein phosphatase 2A, as an agonist of 67LR. They assessed the involvement of 67LR signaling pathway in the miRNA regulation mechanism of EGCG. Tea polyphenols have also been implicated in multiple carcinogenesis pathways, including angiogenesis inhibition, immune system modulation, and activation of enzymatic systems involved in cellular detoxification through the glutathione S-transferase and quinone reductase pathways [45, 46]. Overall, EGCG, accounting for up to 80% of the total antioxidant polyphenols called catechins in tea, exert inhibitory effects on several components of the signaling cascades, which control proliferation and survival of cells of the melanocytic lineage (Fig. 5).

However, the concentrations of EGCG required to elicit the anticancer effects in a variety of cancer cell types are much higher than the peak plasma concentration registered after drinking an equivalent of 2–3 cups of green tea. Furthermore, the anti-cancer efficacy of EGCG can be due to or enhanced by combining it synergistically with other chemical compounds, mixtures of specific polyphenols or mixtures of polyphenols with vitamins, amino acids, and other micronutrients.

Previous human studies have demonstrated the topical effects of EGCG, which inhibits erythema, oxidative stress, and infiltration of inflammatory leukocytes and enhances pyrimidine dimer repair in DNA, in UV-irradiated human skin. Because of these properties, some skin-care products, including sunscreens, contain green tea extracts, although in many cases their quantitative polyphenol content is not standardized [47]. Still, the photoprotective bioactivities of orally administered polyphenols were validated in a 12-week, double-blind, placebo-controlled study [48]. Moreover, in a clinical study, topical use of 660 μM EGCG for 2 weeks during radiotherapy was non-toxic for patients with non-inflammatory breast cancer, effectively preventing radiation-induced dermatitis and significantly lowering the symptom scores of burning, pain, and itching [49]. Therefore, polyphenols and EGCG can relieve carcinogen-induced cutaneous damages and may then help to prevent cutaneous carcinogenesis.

The conflicting results obtained in the studies examined may be because of the various types of teas used, as well as variable tea preparations, unknown concentrations of different antioxidants, and also the bioavailability of many of these compounds after ingestion may be different across populations. Furthermore, many of these studies are often influenced by the intake of other protective or harmful substances, and it is difficult to distinguish these confounding variables [50]. Further preclinical and clinical studies on green tea compounds and, especially, polyphenols for the prevention of skin cancers including melanoma are required.

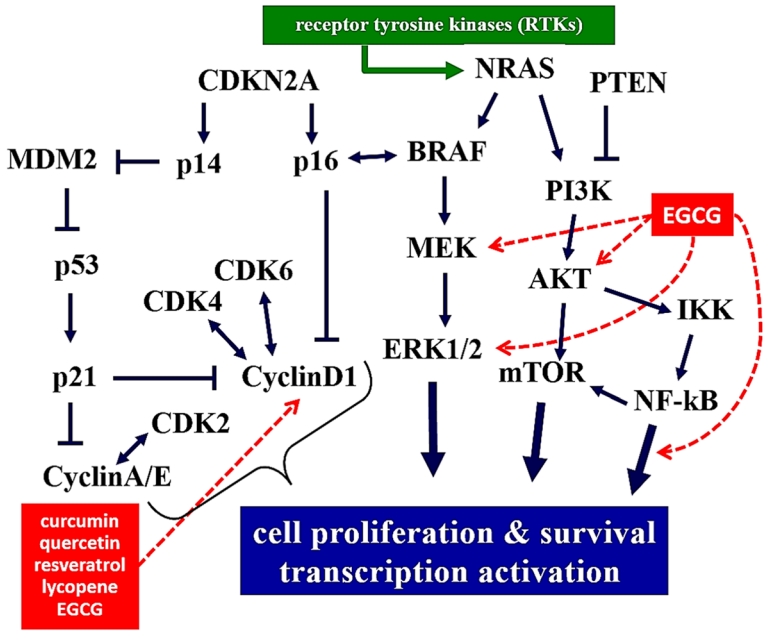

Fig. 5 Dietary components interfering with the main molecular pathways of melanomagenesis

Pomegranate

Several studies have demonstrated that pomegranates possess strong anti-oxidant actions due to their free radical scavenging capacity [51]. These fruits possess anti-proliferative, anti-inflammatory and anti-tumorigenic functions [52–54]. In preclinical animal studies, oral consumption of pomegranate extract inhibited the growth of lung, skin, colon and prostate tumors. Several clinical studies have been conducted on human volunteers. Pomegranate extract, given to 70 patients with diagnosed prostate adenocarcinoma for 4 weeks decreased 8-hydroxy-20-deoxyguanosine (8-OHdG), an oxidative stress biomarker. The presence of pomegranate metabolites was shown in benign and malignant prostate tissues [55]. Nevertheless, no epidemiological studies investigating their role in preventing melanoma are currently available.

Kang et al. [23] examined the underlying mechanisms of dried pomegranate concentrate powder (PCP) on melanin synthesis in B16F10 melanoma cells. Pomegranates are rich in ellagic acid and other polyphenols, such as flavonoids and hydrolyzable tannins. Recently, it was demonstrated that the skin-whitening effects of pomegranates are due to the inhibition of proliferation and melanin synthesis by tyrosinase in melanocytes. The results obtained in B16F10 cells suggest that pomegranate decreases tyrosinase activity and melanin production via inactivation of the p38 and PKA signaling pathways, and subsequently decreases phosphorylation of CREB, MITF, and melanogenic enzymes. Oral feeding of pomegranate fruit extract (PFE, 0.2%, wt/vol) was found to sustain protection from the adverse effects of single UVB radiation in mice. UVB-induced epidermal hyperplasia, infiltration of leukocytes, protein oxidation and lipid peroxidation were inhibited by pomegranate [56]. Pomegranate also elicited significant suppression of UVB-induced protein expression of COX-2, iNOS, PCNA, cyclin D1, and matrix metalloproteinases-2, – 3 and – 9. Moreover, the protection mechanism involved the inhibition of UVB-induced nuclear translocation and phosphorylation of NF-κB /p65, phosphorylation, and degradation of IκBα, activation of IKKα/IKKβ as well as phosphorylation of MAPK and c-Jun. [56]

In another study performed with HaCaT cell line, pomegranate seed oil nano-emulsion entrapping polyphenol-rich ethyl acetate fractions was able to protect the DNA against UVB-induced damage [57]. Studies in a mouse skin tumorigenesis model also showed that the combination of polyphenols and diallyl sulfide (DAS) synergistically reduced the tumor incidence by interfering with cell proliferation and by stimulating apoptosis, as shown by histological and cell death analysis [58]. In SKH-1 hairless mice, pomegranate fruit extract (PFE) reduced UVB-NF-κB activation and mitogen-stimulated protein kinase pathways. Per-oral administration of PFE (0.2%,wt/vol) for 14 days decreased the UVB-mediated skin edema, hyperplasia, infiltration of leukocytes, lipid peroxidation, hydrogen peroxide generation, ornithine decarboxylase (ODC) activity, expression of ODC, COX-2 and proliferating cell nuclear antigen protein. In addition, PFE increased the repair of UVB-stimulated production of cyclo-butane pyrimidine dimers and 8-oxodG. PFE increased the UVB-mediated rise of tumor suppressor p53 and cyclin kinase inhibitor p21.

Per-oral administration of PFE reduced the nuclear translocation of NF-κB, activation of IKKα and phosphorylation and degradation of IκBα mediated by UVB [59].

In a mouse model, topical application of PFE 2 mg prior to the application of 3.2 nmole 12-O-tetradecanoyl phorbol-13-acetate (TPA) time-dependently elicited a significant inhibition of the TPA-induced rise in skin edema and hyperplasia, epidermal ODC activity and protein expression of ODC and COX-2 [60]. In addition, PFE showed a decrease of TPA-mediated phosphorylation of ERK1/2, p38 and JNK1/2, as well as the activation of NF-κB and IKKα, and phosphorylation and degradation of IKBα. PFE-treated animals revealed a reduced tumor incidence and lower tumor volume burden. All these studies indicate that PFE is a strong antitumoral agent in animal models. More clinical trials are required to confirm the efficacy of pomegranate [60].

Nutrients/phytochemicals and melanoma

Resveratrol

Resveratrol is a natural polyphenol commonly found in fruits, grape skins, mulberries, and red wine. Early basic research studies identified anticancer effects of resveratrol against several different tumors and in multiple stages of tumor initiation and proliferation [61]. Specifically, resveratrol can induce cancer cell apoptosis by interfering with multiple signaling pathways of the transformed cells. Resveratrol may also promote immune-surveillance through the innate immune system, thereby influencing the elimination of spontaneous tumor cells prior to proliferation [62].

To delineate this role, a clinical trial focused on detecting differences in immune system profiles was performed in healthy subjects given 1 g of resveratrol daily for 2 weeks. Preclinical studies had confirmed that resveratrol can induce the expression of NKG2D ligands in transformed cells and thus render these cells more susceptible to NK cell lysis via NKG2D cytotoxic pathways [63]. Resveratrol may modulate this axis to allow for increased tumor surveillance by the innate immune system. Moreover, it has previously been shown to protect human skin from the effects of sun damage by decreasing sunburn cell formation [64, 65]. Pharmacokinetic evidence indicates that resveratrol has poor bioavailability due to its rapid and extensive liver metabolism, which severely impairs its therapeutic effects. Melanoma cells often rely on alpha-melanocyte-stimulating hormone signal transduction, a crucial process in the development and spread of melanoma cells, that is suppressed by resveratrol [66, 67]. The alpha-melanocyte-stimulating hormone has also demonstrated immunosuppressive properties and beneficial effects in modulating chronic inflammation, by down-regulating major histocompatibility complex (MHC) molecules, in addition to CD40, CD80, and CD86 co-stimulatory molecules [68, 69]. Resveratrol was shown to have other anticancer properties; in particular, it exerts anti-proliferative activity against melanoma A431 cells and induces apoptosis in A475 and SK-mel28 cells [70, 71].

Although human studies are limited, further data have shown that resveratrol is pharmacologically safe, making it a prime candidate for potential future cancer therapeutic agents. Resveratrol may also be an effective adjuvant treatment, as it prevents endothelial cell injury in high-dose interleukin 2 therapy for melanoma. A topical application of a formulation containing 1% resveratrol, 0.5% baicalin and 1% vitamin E for 12 weeks can mildly modulate photo-damaged skin, improving the chances of cutaneous rejuvenation [72, 73]. Given the low bioavailability of this compound when administered either orally or topically, novel formulation strategies have been attempted. Researchers have designed dermal resveratrol delivery into human skin by using formulation techniques such as micro-emulsions [74] or lipid-core nano-capsules [75]. In addition, Amiot et al. developed a soluble resveratrol formulation that had an 8.8-fold higher plasma concentration in healthy volunteers than that of powders [76]. Based on these pharmaceutical achievements in human subjects, it seems necessary to further verify the chemo-preventive activities of resveratrol.

Vitamins

Vitamin A (retinol) is a fat-soluble compound that is necessary for normal physiologic function and cannot be synthesized by humans, is therefore classified as an essential nutrient [77]. Vitamin A is obtained in the form of retinyl esters from the diet, mostly from animal sources such as eggs, milk, and liver. Also, plant-based pro-vitamin A carotenoids, such as α-carotene and β-cryptoxanthin, can be converted to vitamin A in the intestine but only < 10% of these carotenoids can undergo conversion [78, 79].

The effect of vitamin A on melanoma development is of particular interest. Results from epidemiologic studies concerning the association between vitamin A intake and melanoma risk are still controversial [80]. Older evidence suggests that retinoids have powerful effects in inhibiting cell growth, proliferation, inducing apoptosis and differentiation in human and murine melanoma cell lines. Dietary carotenoids have antioxidant properties, thus reducing the risk of UV-induced skin tumors in mice, and the administration of vitamin A has been proposed as a melanoma chemoprevention approach; pro-vitamin A carotenoids have also been proven to exert an anti-melanoma activity through alternate pathways including anti-angiogenic effects by altering cytokines profiles and nuclear translocation of transcription factors in melanoma cell lines [80–85].

Vitamin C may also have a potential role in melanoma chemoprevention [86, 87]. It is an essential water-soluble nutrient that acts as an antioxidant and a cofactor of various metabolic enzymes [88]. Moreover, vitamin C exerts effects on host defense mechanisms through the maintenance of immune homeostasis [89]. It has dual properties in oxidative processes, acting as an antioxidant and pro-oxidant in the presence of metal ions [90]. As an antioxidant, it protects cells and tissues from oxidative stress due to its conversion to the oxidized form, dehydroascorbic acid (DHA), that is reduced to ascorbic acid inside the cells, thereby decreasing intracellular ROS levels. On the other hand, it also accelerates oxidative metabolism by preventing the use of pyruvate for glycolysis. This feature helps to inhibit the proliferation of tumor cells, but not normal cells. Ascorbate decreases the mitochondrial membrane potential, activates caspase 3 which results in apoptosis in melanoma A375 cells. Ascorbate is even responsible for a decrease of HIF-1 levels, through the inhibition of COX-2 expression, through IGF-II production and caspase-independent autophagy [90–93].

Vitamin E and its various derivatives have demonstrated photo-protective and anti-oxidative properties against melanoma in animal studies. However, the results in epidemiological studies have been less convincing [94–96]. The studies have failed to demonstrate a clear relationship between the dietary intake of vitamin E and melanoma incidence. Accordingly, it has been suggested that oral supplementation may not have a clinically significant effect [62]. From a biological point of view, there are eight natural compounds that have been found to have vitamin E activity: D-α-, D-β-, D-γ- and D-δ-tocopherol, and D-α-, D-β-, D-γ- and D-δ-tocotrienol. α-Tocopherol may inhibit melanin synthesis both directly by inactivating tyrosinase, which is the key enzyme of melanogenesis in melanocytes, and by affecting the post-translation levels of tyrosinase-related protein 1 and 2 [97]. According to Kamei et al. [98], other forms of tocopherol (D-β-tocopherol and D-γ-tocopherol) have a promising anti-melanogenetic activity with less cytotoxicity at relatively high concentrations. Moreover, it has been reported that vitamin E succinate can inhibit the growth and survival of melanoma cells in vitro [99], while another study reported anti-melanoma effects of vitamin E succinate in vivo [100]. Vitamin E also reduces IL-6 and IFN-γ production by different leukocyte subsets and limits the toxic effects of ROS released during inflammation [101]. The translational value of these evidences remain to be clarified.

Vitamin D status has been widely suggested to affect cancer risk and play a role in cancer prevention (including melanoma) by exerting anti-proliferative effects [102, 103]. Solar radiation is critical for vitamin D synthesis in humans; however, uncontrolled and intensive sun exposure is dangerous to skin health and may contribute to the development of cutaneous malignant melanoma [104]. A correct balance between sun protection/exposure and vitamin D status is thus advocated. In recent years, there has been growing interest in understanding the link between vitamin D levels and melanoma**.** There are epidemiological studies to confirm the hypothesis that higher vitamin D levels might protect from melanoma, although a number of cohort studies have addressed a possible protective effect of vitamin D [103–108]. Nevertheless, there are insufficient indications to recommend vitamin D supplementation to decrease melanoma risk.

Vitamin D has a clear anti-proliferative activity on melanoma cell lines in vitro [109]. There is evidence of reduced expression of the vitamin D receptor during progression from nevi through primary to metastatic melanoma. These observations suggest that if vitamin D is anti-proliferative for melanoma cells in vivo, then those cells might be less likely to respond to the anti-proliferative effects of vitamin D as progression occurs. A high circulating vitamin D concentration has been found to be associated with reduced melanoma progression and improved survival. The reported effects of vitamin D on the immune system are extremely complex. If vitamin D supplements suppress adaptive immunity, then that would be a potentially harmful effect in melanoma patients. High doses of vitamin D are also to be avoided. The evidence that vitamin D levels might influence melanoma risk remains uncertain; however, it should also be pointed out that no studies of sufficient size to address this issue have been conducted [110]. In addition, patients with CMM who strictly avoid sun exposure might benefit from 25(OH) D supplements that are sufficient to maintain serum levels above 30 ng mL-1. Given the interest in using vitamin D to reduce cancer risk, more research is warranted to establish its role in the control and progression of melanoma, and whether vitamin D supplements can reduce cancer risk and progression and improve outcomes. Interestingly, it has been also shown that vitamin D could be used to control immune-related adverse events mediated by Th-17+ cell expansion occurring during immunotherapy for CMM [111–113].

Flavonoids

Flavonoids are a large group of polyphenolic compounds (more than 5000) found in vegetables, which exhibit anti-tumor activities that are attracting more and more attention in chemoprevention and cancer treatment. The molecular mechanisms of flavonoids and their activities in antioxidant, anti-inflammation and immune modulation, anti-proliferation, anti-angiogenesis, apoptosis induction, and epigenetic modifications have been studied

in vitro, or in mouse [114]. Large epidemiological studies (including melanoma) are currently lacking. The molecular mechanisms of flavonoids as antioxidants can be summarized in three major categories:

a. Reacting directly with free radicals via their free hydroxyl group(s) and quenching these activities.
b. As chelators for redox-potent transition metal ions, Cd2+,Fe2+, Co 2+, Ni 2+, Cu 2+, Cr 3+ and Zn2+ [46, 47]. These metals cause a ROS increase and the metal binding sites for flavonoids are usually adjacent to hydroxyl and/or ketone side groups.
c. Modulating multiple cellular anti-oxidant systems which re-establish the redox balance in cells after oxidative stress.

Flavonoids modulate inflammatory effects through a few key mediators in melanoma and skin tissues: AP-1 [115], NFkB [116], STAT3 [117] and nitric oxidases (mainly iNOS and nNOS) [118, 119]. Flavonoids exhibit also anti-proliferative and anti-apoptotic effects via HGF/SF-Met signaling, MAPK pathway and PI3K-Akt pathway [120].

Proanthocyanidins

Proanthocyanidins are effective antioxidants and anti-inflammatory agents found in particularly high concentrations in grapes (GSPs) [60]. GSPs have been found to reduce UV skin damage, like photo-aging, and to decrease melanin synthesis [121, 122]. .In humans, GSPs have been shown to reduce mutant p53-positive epidermal cells and prevent the depletion of Langerhans cells after sunburns. Mouse studies have also yielded strong evidence supporting the inhibition of UV-induced tumor incidence, growth, and size, as well as metastatic pulmonary nodules, after the administration of grape seed extract [123, 124]. GSPs were also shown to inhibit cell migration in highly metastasis-specific human A375 and Hs294t melanoma cell lines: 22 to 65%, ($P < 0.01$) and 29 to 69%, ($P < 0.01$), respectively. In addition, GSPs decreased tissue plasminogen activator-induced activation of extracellular-signal-regulated kinase 1/2 protein and nuclear factor-κB/p65. These proteins have been shown to enhance and mediate the migration of melanoma cells. The inhibitory effects of GSPs on NF-κB also helped to reverse the epithelial-to-mesenchymal transition occurring in both melanoma cell lines. This evidence suggests a potential utilization as an anti-melanoma agent, considering that no toxicity has been shown in vivo [125].

Luteolin

Luteolin is another flavonoid common to many plants. It protects against SSBs (single-strand breaks) induced by oxidative stress in PC12 rat pheochromocytoma cells [126]. It possessed apoptotic potential in human lung squamous carcinoma CH27 cells, showing greater DNA damage and "S" phase cell cycle arrest [127]. Luteolin activates intrinsic apoptotic pathways by inducing DNA damage and p53 in many cancer cells [128, 129]. It induced apoptosis by inhibiting fatty acid synthase, a key lipogenic enzyme overexpressed in many human cancers [130]. Moreover. a chemo-preventive effect of luteolin and associated mechanisms were reported in the JB6 P+ neoplastic mouse cell line and the SKH-1 hairless mouse models [131]. Luteolin has been shown to delay or block the development of cancer cells both in vitro and in vivo, to protect DNA and induce cell cycle arrest and apoptosis via intrinsic and extrinsic signaling pathways [132]. Additionally, luteolin induces apoptosis in multidrug-resistant cancer cells by ROS generation, DNA damage initiation, activation of ATR/Chk2/p53 signaling, inhibition of NF-kB signaling, activation of p38, and depletion of anti-apoptotic proteins [133]. In addition, luteolin inhibits the hypoxia-induced epithelial-mesenchymal transition (EMT) in malignant melanoma cells both in vitro and in vivo via the regulation of β3 integrin [134]. Another study demonstrated that luteolin 7-sulfate isolated from *P. iwatensis* (a marine plant) is a human TYR inhibitor with advantageous anti-melanogenic properties, and would be a useful agent for the control of unwanted skin pigmentation [135].

Apigenin

Like luteolin, apigenin is a natural dietary flavonoid with anti-inflammatory and anti-oxidant properties. Epidemiological evidence suggests that apigenin intake reduces the risk of cancers and it has been found that apigenin inhibited ultraviolet light-induced skin carcinogenesis in mice. Subsequent studies also suggested anti-melanoma effects of apigenin, including inhibition of melanoma metastasis [136, 137]. In Cao [138], the involvement of the STAT3 signaling pathway in the anti-metastatic effect of apigenin was examined. Two human melanoma cell lines, A375 and G361, with constitutive activation of STAT3, together with a murine melanoma cell line, B16F10, were employed, showing that inhibition of the STAT3 signaling pathway contributes to the anti-metastatic effect of apigenin. In view of the reported anti-proliferative activity and low toxicity property of this compound, apigenin may also have a potential role in melanoma treatment or prevention. In Table 1, the anti-melanoma effects of the main dietary compounds are synthesized.

Dietary lipids

Several studies suggest that high dietary fat intake is directly associated with the risk of colorectal, liver, breast, pancreatic, gastrointestinal and prostate cancer [139, 140]. An increased intake of certain fatty acids promotes cancer

Table 1 Dietary compounds and their effects against melanoma

Dietary source/ compounds	Anti-melanoma effect	References
Coffee/various phytochemicals	inhibition of oxidative stress and oxidative damage, regulation of DNA repair, phase II enzymatic activity, apoptosis, inflammation, antiproliferative, antiangiogenetic effects, and antimetastatic effects	29–39
Tea/catechins and theaflavins	reverse damage caused by UV light; decrease in UV-induced skin tumor incidence and size inhibiting angiogenesis, modulation of the immune system; activation of enzyme systems involved in cellular detoxification; EGCG inhibits erythema, enhances pyrimidine dimer repair in DNA, in UV-irradiated human skin	40–50
Pomegranate	decreases tyrosinase activity and melanin production; decreases phosphorylation of CREB, MITF, and melanogenic enzymes; strong antitumor agent in animal models	51–50
Resveratrol	antiproliferative activity against melanoma cells, induction of apoptosis; modulation of photodamaged skin	61–76
Vitamin A	Inhibition of growth, proliferation, apoptosis-induction, alteration of cytokines profiles	77–85
Vitamin C	to limit the toxic effects of ROS, immune homeostasis, apoptosis	86–93
Vitamin D	anti-proliferative activity, effects on the immune system	109–113
Vitamin E	reduction of IL-6 and IFN-γ production by different leukocyte subset, to limit the toxic effects of ROS, tyrosinase-inactivation	94–101
Flavonoids: GSPs, Luteolin, Apigenin, etc.	protection against UV damage; Induction of apoptosis Inhibition of cell growth in cell lines. Reversed epithelial-to-mesenchymal transition	114–138

growth while some other fatty acids have shown protective roles against cancer incidence. For example, palmitic acid and stearic acid seem to be potentially mutagenic to colonocytes [141], while the intake of arachidonic acid is not associated with colorectal cancer risk [142]. Dietary intake of linoleic acid increases the risk of prostate cancer; while consumption of ω-3 polyunsaturated fatty acids, docosahexaenoic and eicosapentaenoic acid, is associated with a decreased incidence of prostate cancer [143].

In a recent epidemiological study performed by Donat – Vargas et al. the authors controlled for sun habits and skin type, including 20,785 women from the prospective population-based Swedish Mammography Cohort. Validated estimates of dietary PCB exposure and eicosapentaenoic acid-docosahexaenoic acid (EPA-DHA) intake were obtained via a food frequency questionnaire. They ascertained 67 cases of melanoma through register-linkage. After multivariable adjustments, exposure to dietary PCBs was associated with a four-fold increased risk of malignant melanoma (HR 4.0, 95% CI 1.2–13; P for trend = 0.02]), while EPA-DHA intake was associated with an 80% lower risk (HR 0.2, 95% CI 0.1–0.8; P for trend = 0.03), when comparing the highest exposure tertiles with the lowest. While a direct association between dietary PCB exposure and the risk of melanoma exists, EPA-DHA intake was shown to have a substantial protective association.

Although the effects of different dietary fatty acids on cancer pathogenicity are diverse, it is generally believed that an excessive intake of certain fatty acids or the development of obesity and complications caused by the excess calorie intake promotes cancer growth [144].

Another aspect to consider is metastasis. Recently, a small population of CD36+ cells, that are highly predisposed to promote metastasis and are predominantly defined by a lipid metabolism signature, has been identified [145]. Pascual et al. described a subpopulation of CD44 bright cells in human oral carcinomas that do not overexpress mesenchymal genes, are slow-cycling, express high levels of the fatty acid receptor CD36 and lipid metabolism genes, and are unique in their ability to initiate metastasis. Palmitic acid or a high-fat diet specifically boost the metastatic potential of CD36+ metastasis-initiating cells in a CD36-dependent manner [145]. Two recent studies evidenced that ω-3 polyunsaturated fatty acids exert antitumourigenic activities against melanoma metastasis, via autophagy-mediated p62 elimination, CXCR4 suppression, and anti-inflammatory properties [146, 147].

PCB and melanoma risk

Other than ultraviolet (UV) radiation risk factors may play a role in melanoma-genesis, such as environmental chemical exposures [148]. Polychlorinated biphenyls (PCBs) are synthetic organochlorine chemicals with well-described toxicity [149]. PCBs, which are widespread in the environment, accumulating in the food chain (they are classified in Group 1 as carcinogenic to humans by the International Agency for Research on Cancer) [150]. People are exposed to PCBs primarily through food, in particular when eating fatty fish. PCBs are absorbed and accumulated in adipose tissue, with a half-life ranging from 2 to 10 years [151].

The study of Donat-Vargas mentioned above is the only epidemiological study reporting results on interactions of PCBs and melanoma [152]. Gallagher et al. [153] conducted a case-control study of 80 CMM patients and 310 controls, collecting sun exposure

information, data on pigmentation and sun sensitivity, along with a blood sample from each. Cases and controls were assayed for plasma levels of 14 PCB congeners and 11 organochlorine pesticide residues using gas chromatography. Strong associations were seen between the risk of CMM and plasma levels of non-dioxin-like PCBs (adjusted OR = 7.02; 95% CI: 2.30–21.43) and several PCB congeners, organochlorine pesticides or metabolites. These associations persisted after controlling for sun sensitivity and sun exposure.

Conclusions

A great number of studies have been published recently investigating the roles of several dietary compounds in the prevention, development, and therapy of melanoma. Several foods and nutrients have been shown to have protective effects against melanoma-genesis or synergic effects with the medications used for CMM treatment. Recent literature reviews and data from the World Cancer Research Fund describe the epidemiological aspects of the interactions between diet and melanoma [154, 155]. In the present review, we focused mainly on recent advances regarding the biological mechanisms which lay under such interactions, involving specific compounds of current active research. Numerous encouraging results emerged, alone with conflicting outcomes, especially when basic research data are transferred to humans. This may depend on the heterogeneity of the compounds studied, their concentration, preparation, and administration, as well as on the heterogeneity of the methodological approaches and laboratory techniques employed. Further studies, at both the basic research and epidemiological level, performed with standardized approaches are needed to better comprehend the value of a wide range of nutrients in the prevention and clinical management of melanoma.

Abbreviations

8-OHdG: 8-hydroxy-20-deoxyguanosine; ATM: Ataxia-telangiectasia mutated; CI: Confidence intervals; CMM: Cutaneous malignant melanoima; COX: Cyclooxygenase; CPDs: Cyclobutane pyrimidine dimers; DAS: Diallyl sulphide; DHA: Dehydroascorbic acid; EC: Epicatechin; ECG: Epicatechin-3-gallate; EGC: Epigallocatechin; EGCG: Epigallocatechin-3-gallate; EMT: Epithelial-mesenchymal transition; EPA-DHA: Eicosapentaenoic acid-docosahexaenoic acid; EPIC: European prospective investigation into cancer and nutrition; GSPs: Grapes proanthocyanidins; HPFS: Health professionals' follow-up study; HR: Hazard ratio; MHC: Major histocompatibility complex; NHS: Nurses' health study; ODC: Ornithine decarboxylase; PCBs: Polychlorinated biphenyls; PCP: Pomegranate concentrate powder; PFE: Pomegranate fruit extract; PUFAs: Polyunsaturated fatty acids; ROS: Reactive oxygen species; RR: Relative risk; SSB: Single-strand break; TPA: O-tetradecanoyl phorbol-13-acetate; UVA: Ultraviolet A; UVB: Ultraviolet B

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Grape Seed Proanthocyanidins Inhibit Melanoma Cell Invasiveness by Reduction of PGE_2 Synthesis and Reversal of Epithelial-to-Mesenchymal Transition

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Abstract

Melanoma is the leading cause of death from skin disease due, in large part, to its propensity to metastasize. We have examined the effect of grape seed proanthocyanidins (GSPs) on melanoma cancer cell migration and the molecular mechanisms underlying these effects using highly metastasis-specific human melanoma cell lines, A375 and Hs294t. Using *in vitro* cell invasion assays, we observed that treatment of A375 and Hs294t cells with GSPs resulted in a concentration-dependent inhibition of invasion or cell migration of these cells, which was associated with a reduction in the levels of cyclooxygenase (COX)-2 expression and prostaglandin (PG) E_2 production. Treatment of cells with celecoxib, a COX-2 inhibitor, or transient transfection of melanoma cells with COX-2 small interfering RNA, also inhibited melanoma cell migration. Treatment of cells with 12-O-tetradecanoylphorbol-13-acetate, an inducer of COX-2, enhanced the phosphorylation of ERK1/2, a protein of mitogen-activated protein kinase family, and subsequently cell migration whereas both GSPs and celecoxib significantly inhibited 12-O-tetradecanoylphorbol-13-acetate -promoted cell migration as well as phosphorylation of ERK1/2. Treatment of cells with UO126, an inhibitor of MEK, also inhibited the migration of melanoma cells. Further, GSPs inhibited the activation of NF-κB/p65, an upstream regulator of COX-2, in melanoma cells, and treatment of cells with caffeic acid phenethyl ester, an inhibitor of NF-κB, also inhibited cell migration. Additionally, inhibition of melanoma cell migration by GSPs was associated with reversal of epithelial-mesenchymal transition process, which resulted in an increase in the levels of epithelial biomarkers (E-cadherin and cytokeratins) while loss of mesenchymal biomarkers (vimentin, fibronectin and N-cadherin) in melanoma cells. Together, these results indicate that GSPs have the ability to inhibit melanoma cell invasion/migration by targeting the endogenous expression of COX-2 and reversing the process of epithelial-to-mesenchymal transition.

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Introduction

Melanoma is the leading cause of death from skin disease due to its propensity to metastasis [1,2], and is increasing rapidly in children [3]. Although, melanoma is less common than other types of skin cancers, it causes the majority (75%) of skin cancer-related deaths [1,4]. The American Cancer Society estimated that in 2008, there were 8,420 melanoma-associated deaths in the U.S. and the number of new cases of invasive melanoma was estimated at 62,480 [1]. Solar ultraviolet (UV) radiation is a recognized risk factor for the development of skin cancers, including melanoma. Exposure of the skin to UV radiation induces an increase in the expression levels of cyclooxygenase -2 (COX-2), a rate-limiting enzyme that catalyzes the conversion of arachidonic acid to prostaglandins (PGs) [5,6]. These inflammatory mediators have been identified as a risk factor for the development of skin cancers [5,6], and thought to play a central role in orchestrating the multiple events involved in cancer invasion and metastasis [7,8]. Since, melanoma is a highly malignant cancer with a potent capacity to metastasize distantly, an approach that decreases its metastatic or invasive ability may facilitate the development of an effective strategy for its treatment or prevention.

Dietary phytochemicals offer promising new options for the development of more effective strategies for the prevention of cancer cell invasion, migration, or metastasis, and thus can be utilized as complementary and alternative medicine. Grape seed proanthocyanidins (GSPs) are promising phytochemicals that have shown anti-carcinogenic effects in some murine models and exhibit no apparent toxicity *in vivo* [9–11]. GSPs contain primarily proanthocyanidins (89%), which constitute dimers, trimers, tetramers, and oligomers of monomeric catechins and/or (-)-epicatechins, as described previously [10]. They are readily available as an extract of grape seeds and this extract, rather than the individual constituents, has been examined as an anti-carcinogenic agent against some forms of cancers [9]. It is believed that at least some of the constituents present in the extract may act synergistically and thus this product can be more effective than any single constituent. GSPs have been shown to inhibit UV radiation-induced skin cancer in mouse model [10] but its chemopreventive effect on the migration or invasive potential of melanoma cancer cells has not been explored.

In this study, we assessed the chemotherapeutic effects of GSPs on the migration potential of human melanoma cells, as the migration of cancer cells is a major event in the metastatic cascade. For this purpose, two highly metastasis-specific melanoma cancer cell lines were selected: one is A375 which is *BRAF* mutated and activating mutations of the protooncogene *BRAF* have been observed in approximately 50% of malignant melanomas. Second cell line is Hs294t, which is also highly metastatic but not *BRAF* mutated. In this study, we characterized the role of COX-2 and its metabolite PGE_2 on the migration of human melanoma cancer cells and ascertained whether GSPs have any suppressive effects on the COX-2-mediated migration of these cells. Epithelial-to-mesenchymal transition (EMT), the process whereby epithelial cells transform into mesenchymal cells, has recently been shown to be relevant for cancer and cancer metastasis. During EMT, cancer cells lose expression of proteins that promote cell-cell contact such as E-cadherin and acquire mesenchymal markers such as vimentin, fibronectin and N-cadherin, which promote cell invasion and metastasis [12]. The EMT has also been associated with higher levels of inflammation or inflammatory mediators, and therefore we have also checked whether inhibition of COX-2 expression by GSPs in melanoma cells is associated with reversal of EMT and that leads to inhibitory effect on melanoma cell migration. Here, we present evidence that GSPs inhibit the invasiveness or migratory behavior of melanoma cancer cells through inhibition or reversal of EMT in melanoma cells and that GSPs do so through a process that involves the reduction in COX-2 expression and PGE_2 production.

Materials and Methods

Source and composition of GSPs

GSPs were received from Kikkoman Corporation (Noda, Japan). Quality control of GSPs is maintained by the company on lot-to-lot basis. GSPs contain approximately 89% proanthocyanidins, with dimers (6.6%), trimers (5.0%), tetramers (2.9%) and oligomers (74.8%), as described earlier [10,11], and are stable for at least two years when refrigerated at 4°C.

Cell lines and cell culture conditions

The human melanoma cells lines, A375 and Hs294, were purchased from the American Type Culture Collection (Manassas, VA). The cell lines were cultured as monolayers in RPMI 1640 culture medium supplemented with 10% heat-inactivated fetal bovine serum (Hyclone, Logan, UT), 100 μg/ml penicillin, and 100 μg/ml streptomycin and maintained in an incubator with 5% CO_2 at 37°C. The GSPs were dissolved in a small amount of dimethylsulfoxide (DMSO), which was added to the complete cell culture medium [maximum concentration of DMSO, 0.1% (v/v) in media] prior to addition to sub-confluent cells (60–70% confluent). Cells treated with DMSO only served as a vehicle control. Normal human epidermal melanocytes (HEMa-LP, Cat. No. C-024-5C) were commercially obtained from Invitrogen (Carlsbad, CA), and were cultured in HMGS-2 medium supplemented with human melanocyte growth supplement provided by the supplier. To determine the effect of GSPs on 12-O-tetradecanoylphorbol-13-acetate (TPA)- or PGE_2-mediated effects, GSPs were added in cell culture medium at least 30 minutes before the treatment of the cells with TPA, PGE_2 or any other agent.

Antibodies, chemicals and reagents

Antibodies specific for COX-2 and an enzyme immunoassay kit for PGE_2 analysis were obtained from Cayman Chemicals (Ann Arbor, MI). Celecoxib, PGE_2 and 12-O-tetradecanoylphorbol-13-acetate (TPA) were purchased from Sigma Chemical Co. (St. Louis, MO). Boyden Chambers and polycarbonate membranes (8 μm pore size) for cell migration assays were obtained from Neuroprobe, Inc. (Gaithersburg, MD). The antibodies specific to N-cadherin, keratin-8, -18 and fibronectin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA), while antibodies for vimentin, E-cadherin, NF-κB, IKKα and IκBα were purchased from Cell Signaling Technology (Beverly, MA) while desmoglein-2 was obtained from Abcam (Cambridge, MA). The secondary antibodies conjugated with Alexa Fluor 488 or Alexa Fluor 594 were purchased from Invitrogen (Carlsbad, CA).

Cell migration or invasion assay

The migration capacity of melanoma cancer cells was determined *in vitro* using Boyden Chambers (Gaithersburg, MD) in which the two chambers were separated with matrigel coated Millipore membranes (6.5 mm diameter filters, 8 μM pore size), as detailed previously [13]. Briefly, melanoma cells (1.5×10^4 cells/100 μL serum-reduced medium) were placed in the upper chamber of Boyden chambers, test agents were added alone, or in combination, to the upper (200 μL) chamber, and the lower chamber contained the medium alone (150 μL). Chambers were assembled and kept in an incubator for 24 h. After incubation, cells from the upper surface of Millipore membranes were removed with gentle swabbing and the migrant cells on the lower surface of membranes were fixed and stained with either hematoxylin or crystal violet. Membranes were then washed with distilled water and mounted onto glass slides. The membranes were examined microscopically and cellular migration was determined by counting the number of stained cells on membranes in at least 4–5 randomly selected fields using an Olympus BX41 microscope. Representative photomicrographs were obtained using a Qcolor5 digital camera system fitted to an Olympus BX41 microscope. Each cell migration experiment was repeated at least three times.

PGE_2 immunoassay for quantitation of prostaglandin E2

The analysis of PGE_2 in cell homogenates was performed using the Cayman PGE_2 Enzyme Immunoassay Kit (Ann Arbor, MI) following the manufacturer's instructions. Briefly, at indicated time points, cells were harvested and homogenized in 100 mM phosphate buffer, pH 7.4 containing 1 mM ethylenediamine tetraacetic acid and 10 μM indomethacin using a homogenizer. Homogenates were centrifuged and the supernatants were collected and analyzed for PGE_2 concentration according to the manufacturer's instructions.

COX-2-siRNA transfection of A375 and Hs294t cells

Human-specific COX-2 siRNA was transfected into A375 and Hs294t cells using the siRNA Transfection Reagent Kit (Santa Cruz Biotechnology, Inc.; Santa Cruz, CA) according to the manufacturer's protocol. Briefly, 2×10^5 cells/well were seeded in a 6-well plate and allowed to grow to 70% confluency. The COX-2 siRNA mix with transfection reagents was overlaid on the cells for approximately 6 h at 37°C and transferred into 2× growth medium for about 18–20 h. At 24 h post-transfection, fresh medium was added to the cells and the cells were incubated for an additional 48 h. Thereafter, cells were harvested and subjected to the cell migration assay. The knockdown of COX-2 expression in cells after transfection was confirmed using western blot analysis.

NF-κB/p65 activity assay

For quantitative analysis of NF-κB/p65 activity, the NF-κB Trans^{AM} Activity Assay Kit (Active Motif, Carlsbad, CA) was used following the manufacturer's protocol. For this purpose, the

nuclear extracts of cells were prepared using the Nuclear Extraction Kit (Active Motif, Carlsbad, CA) following the manufacturer's instructions, and as performed previously [14]. Absorbance was recorded at 450 nm using absorbance at 650 nm as the reference. The results are expressed as the percentage of the optical density of the non-GSPs-treated control group.

Preparation of cell lysates and western blot analysis

Following treatment of melanoma cells for the indicated time periods with or without GSPs or any other agent, the cells were harvested, washed with cold PBS and lysed with ice-cold lysis buffer supplemented with protease inhibitors, as detailed previously [15]. Equal amounts of proteins were resolved on 10% Tris-Glycine gels and transferred onto a nitrocellulose membrane. After blocking the non-specific binding sites, the membrane was incubated with the primary antibody at 4°C overnight. The membrane was then incubated with the appropriate peroxidase-conjugated secondary antibody and the immunoreactive bands were visualized using the enhanced chemiluminescence reagents. To verify equal protein loading, the membrane was stripped and reprobed with anti-β actin antibody.

Immunofluorescent detection of EMT biomarkers in cells

In order to determine whether GSPs inhibit cell migration of melanoma cells is associated with reversal of epithelial-to-mesenchymal transition, the A375 melanoma cells were either treated with various concentrations of GSPs or celecoxib or TPA for 24 h. After 24 h, cells were harvested and cell lysates were prepared for western blotting for the analysis of epithelial and mesenchymal biomarkers. Cells were also used for cytostaining for the detection of EMT biomarkers such as vimentin, fibronectin and N-cadherin. Briefly, after harvesting the cells, cells were processed for cytospin (1×10^5 cells/slide). Cells were fixed with chilled methanol at −20°C for 10 minutes and non specific binding sites were blocked with 2% bovine serum albumin (Sigma, St. Louis, MO) in PBS for 30 min. Cells were then incubated with antibodies specific to EMT biomarkers for 2 h at room temperature. The cells were washed with PBS and antigen was detected by an Alexa Fluor-conjugated secondary antibody. Goat anti-rabbit IgG labeled with green-fluorescent Alexa Fluor 488 dye was used for detection of N-cadherin and vimentin, while donkey anti-mouse IgG labeled with red-fluorescent Alexa Fluor 594 was used for the detection of the expression of desmoglein 2. Cells were finally mounted with Vectashield mounting medium for fluorescence with DAPI (Vector Laboratories, Burlingame, CA) before they were observed under fluorescence microscope and photographed.

Statistical analysis

For migration assays, the control and GSPs-, TPA- or PGE_2-treatment groups or combined-treatment groups separately were compared using one-way analysis of variance (ANOVA) followed by *post hoc* Dunn's test using GraphPad Prism version 4.00 for Windows, GraphPad Software, San Diego, California, USA, www.graphpad.com. All quantitative data for cell migration are shown as mean ± SD/microscopic field. In each case $P<0.05$ was considered statistically significant.

Results

Comparative invasiveness of human melanoma cells and normal human epidermal melanocytes

First, we examined the migration capacity of melanoma cells and compared this capacity with normal human epidermal melanocytes under identical conditions. For this purpose, cells were incubated in Boyden chambers for 24 h to assess their migration capability. As shown in Figure 1A, the cell migration capacity of melanoma cells was significantly higher ($P<0.001$) than normal human epidermal melanocytes. The migration of A375 cells, which are *BRAF* mutated, was greater (390±14 cells/microscopic field) than Hs294t cells (340±12 cells/microscopic field), which are metastasis-specific but not *BRAF*-mutated. Under identical conditions, migration of normal human epidermal melanocytes was lower (19±4 cells/microscopic field) than melanoma cells.

GSPs inhibit human melanoma cancer cell migration

We determined whether treatment of A375 and Hs294t human melanoma cells with GSPs inhibited their invasiveness or migration using Boyden chamber cell migration assays. First, screening experiments were performed to determine the effects of lower concentrations of GSPs (μg/mL). The selection of the concentrations of GSPs was based on consideration of their relevance and achievability *in vivo*. As shown in Figure 1B, relative to untreated control cells, treatment of cells with GSPs at concentrations of 0, 10, 20 and 40 μg/mL reduced the invasive potential of A375 and Hs294t cells in a concentration-dependent manner. The density of the migrating cells on the membrane after staining with crystal violet is shown in Figure 1B, and the numbers of migrating cells/microscopic field are summarized in Figure 1C. The cell migration was inhibited by 22 to 64% ($P<0.01-0.001$) in A375 cells and by 29–69% ($P<0.01-0.001$) in Hs294t cells in a concentration-dependent manner after treatment with GSPs for 24 h. A similar but comparatively higher inhibitory effect on cell migration was observed at the 48 h time point (data not shown). To confirm that the inhibition of cancer cell migration by GSPs was a direct effect on migration ability, and that was not due to a reduction in cell viability, a trypan blue assay was performed using cells that were treated identically to those used in the migration assays. Treatment of A375 and Hs294t cells with various concentrations of GSPs (0, 10, 20 and 40 μg/mL) for 24 h had no significant effect on cell viability or cell death (data not shown).

The inhibitory effect of GSPs on invasiveness of melanoma cells is associated with the reduction of endogenous COX-2 expression and reduction of PGE_2 synthesis

To determine whether the inhibitory effect of GSPs on the migration of the melanoma cells is associated with inhibition of endogenous COX-2 expression, we determined the levels of COX-2 in lysates of cells from the various treatment groups using western blot analysis. As shown in Figure 2A, treatment of A375 and Hs294t cells with GSPs reduced the levels of COX-2 expression in a concentration-dependent manner as compared to the expression in untreated controls. As the COX-2 metabolite, PGE_2, has been implicated in COX-2-mediated effects including cancer cell metastasis; we determined the levels of PGE_2 in the GSPs-treated cells. Our results revealed that treatment with GSPs for 24 h resulted in significant reduction in the production or synthesis of PGE_2 in both A375 (19–76%, $P<0.001$) and Hs294t (18–71%, $P<0.001$) cells in a concentration-dependent manner (Figure 2B), suggesting that GSPs-induced reduction in PGE_2 production is associated with an inhibitory effect of the GSPs on COX-2 expression and inhibition of cell migration in these cells.

Selective COX-2 inhibitor inhibits melanoma cell migration

This experiment was performed to determine whether the inhibitory effect of GSPs on melanoma cell migration is mediated

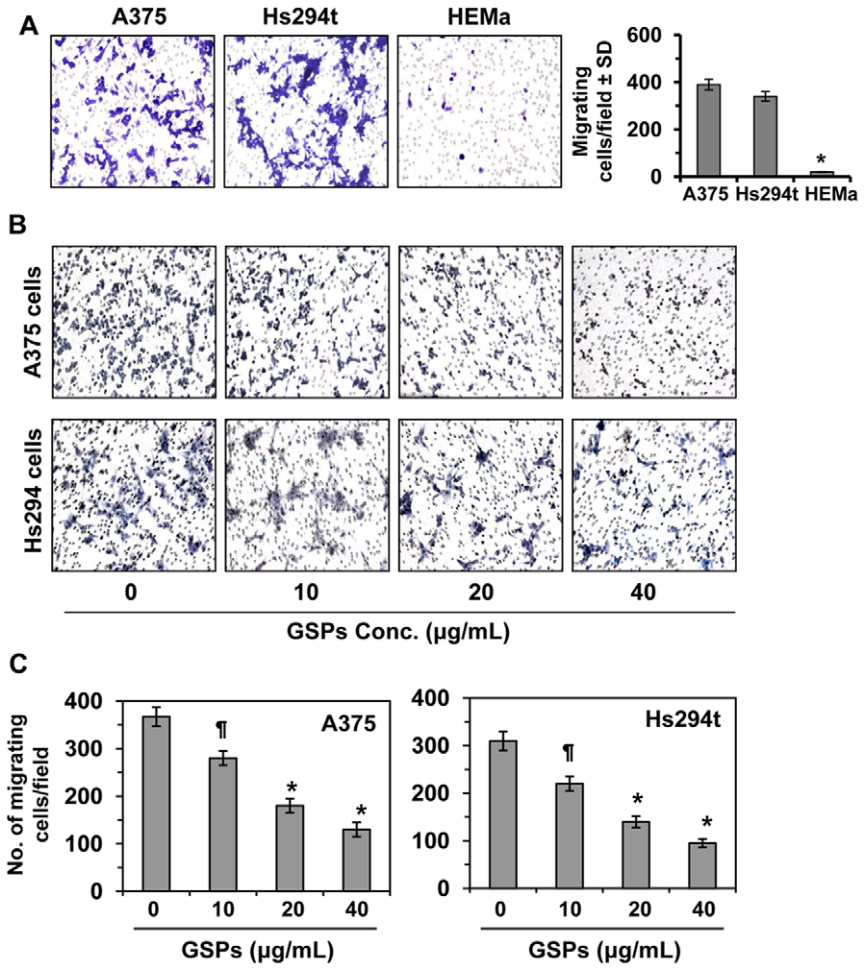

Figure 1. Effect of GSPs on melanoma cell migration. (**A**) Migration ability of human melanoma cells and comparison with normal human epidermal melanocytes (HEMa). Equal numbers of human melanoma cells (A375 and Hs294t) and HEMa were subjected to cell migration using standard Boyden chamber assay. Twenty four h later, migratory cells were detected on the membrane after staining with crystal violet. The migratory cells were counted and the results expressed as the mean number of migratory cells ± SD per microscopic field (n = 3). Significantly lower *versus* melanoma cells, $^{*}P<0.001$. (**B**) Treatment of human melanoma cancer cells (A375 and Hs294t) with GSPs for 24 h inhibit migration of cells in a concentration-dependent manner compared to non-GSPs-treated control cells. (**C**) The migratory cells were counted and the results expressed as the mean number of migratory cells ± SD/microscopic field. Significant inhibition *versus* non-GSPs-treated control, $^{*}P<0.001$.
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through its inhibitory effect on COX-2 expression. For this purpose, equal numbers of A375 and Hs294t cells were subjected to the cell migration assay after treatment with various concentrations of celecoxib (0, 5, 10, 20 µM), a well known inhibitor of COX-2, for 24 h. As shown in Figure 2C, treatment of the cells with celecoxib resulted in a dose-dependent reduction in the cell migration capacity of melanoma cells as compared with non-celecoxib-treated controls ($P<0.05-0.001$). These data suggested that the inhibition of constitutive levels of COX-2 expression is associated with the inhibition of melanoma cell migration.

siRNA knock-down of COX-2 leads to reduction of melanoma cell migration

We further verified the role of COX-2 in cell migration through siRNA knock-down of COX-2 in the melanoma cells and examined whether it would lead to the inhibition of the cell migration in these cells. The transfection of A375 and Hs294t cells with COX-2 siRNA resulted in significant reduction of cell migration in A375 (85%, $P<0.001$) and Hs294t (86%, $P<0.001$) cells after 24 h as compared to the migration of control siRNA-transfected A375 and Hs294t cells (Figure 2D).

GSPs inhibit PGE_2-induced cell migration of melanoma cells

As the effects of COX-2 are mediated through its metabolites, such as PGE_2, we examined whether GSPs inhibit PGE_2-induced cell migration in human melanoma cells. For this purpose, A375 and Hs294t cells were treated with PGE_2 (10 µM) with and without GSPs for 24 h and cell migration determined. We found that the treatment of melanoma cells with PGE_2 resulted in a significant increase in cell migration ($P<0.05$) compared to the cells which were not treated with PGE_2 (Figure 2E). Treatment of A375 and Hs294t cells with GSPs (20 or 40 µg/mL) resulted in a dose-dependent inhibition of PGE_2 (10 µM)-induced cell migration (Figure 2E). As the inhibitory effect of GSPs on the migration of A375 and Hs294t cell lines was very similar, the subsequent studies were performed only with A375 cells.

TPA, an inducer of COX-2, enhances melanoma cell migration, and GSPs inhibit TPA-induced cell migration

Treatment of skin with TPA stimulates the levels of COX-2 expression [11,16]; therefore, the melanoma cells were treated with

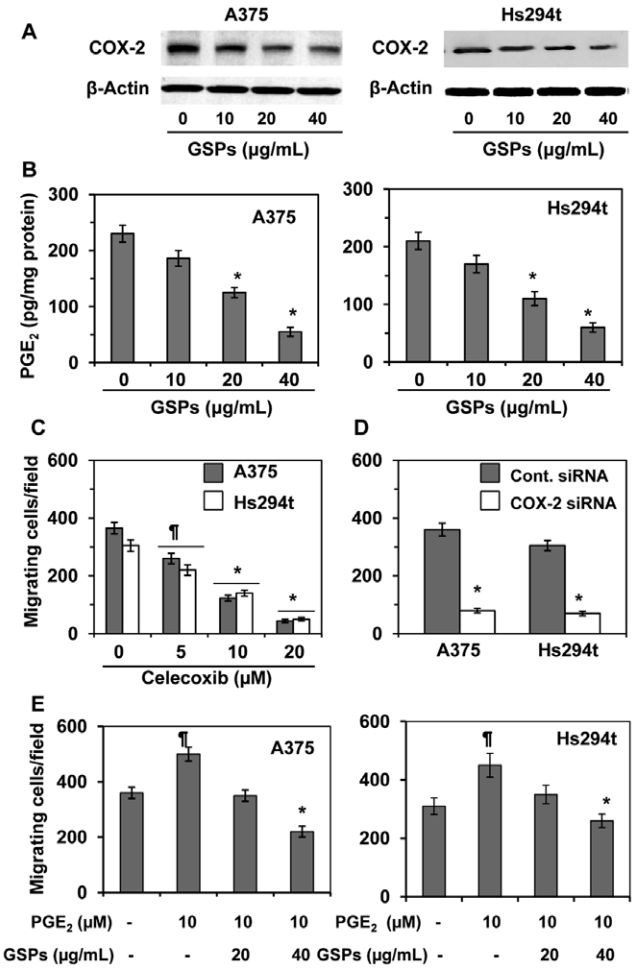

Figure 2. Effect of GSPs on COX-2 expression, PGE_2 production and cell migration in melanoma cells. (**A**) Effect of GSPs on the endogenous basal level of COX-2 in A375 and Hs294t cells. The levels of COX-2 were determined in cell lysates using western blot analysis. (**B**) Dose-dependent effect of GSPs on the levels of PGE_2 in melanoma cells. The levels of PGE_2 are expressed in terms of pg/mg protein± SD, n = 3 independent experiments. Significant inhibition of PGE_2 level by GSPs *versus* non-GSPs-treated controls, $^{*}P<0.001$. (**C**) Down-regulation of endogenous COX-2 reduces melanoma cell migration. Treatment of A375 and Hs294t cells with celecoxib, a COX-2 inhibitor, inhibits cell migration in a dose-dependent manner. Significant difference *versus* control (non-celecoxib-treated) cells, $^{\P}P<0.05$, $^{*}P<0.001$. (**D**) Transfection of cells, both A375 and Hs294t, with COX-2 siRNA significantly decreases cell migration. A375 and Hs294t cells were transfected with COX-2 siRNA to knockdown COX-2 expression. Significant reduction of cell migration *versus* control siRNA-treated cells: $^{*}P<0.001$. (**E**) Treatment of A375 and Hs294t cells with GSPs (20 and 40 μg/mL) inhibits PGE_2-enhanced cell migration. The data on cell migration are summarized as a mean number of migratory cells ±SD/microscopic field. In each case, the migrating cells were counted at 4–5 different microscopic fields, and data are expressed as the mean number of migratory cells± SD/microscopic field, n = 3.
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TPA for COX-2 stimulation, and thereafter determined the effect of TPA on the migration of melanoma cells. As shown in Figure 3A, treatment of A375 cells with TPA for 24 h resulted in significantly enhanced cell migration ($P<0.01$) compared to non-TPA-treated cells. To determine whether GSPs inhibit TPA-induced cell migration in human melanoma cells, A375 cells were treated with TPA (40 ng/mL) with and without the treatment of GSPs for 24 h. We found that the treatment of A375 cells with GSPs resulted in a dose-dependent inhibition of TPA-induced cell migration. A summary of the cell migration data for the various treatment groups is provided in Figure 3A. Treatment of cells with GSPs at the doses of 20 μg/mL and 40 μg/mL inhibited TPA-induced cell migration by 50% ($P<0.01$) and >100% ($P<0.001$) respectively.

GSPs and celecoxib inhibit TPA-induced activation of ERK1/2 protein in melanoma cells

As activation of MAPK proteins has been implicated in the enhancement of COX-2 expression or an upstream regulator of COX-2, we examined the effect of TPA on activation of ERK1/2 in melanoma cells, and simultaneously checked the effect of GSPs or celecoxib on TPA-induced activation of ERK1/2. Western blot analysis revealed that treatment of A375 cells with TPA enhanced the activation of ERK1/2, however, treatment of cells with GSPs or celecoxib inhibited TPA-induced activation of ERK1/2, as shown in Figure 3B. We further checked the effect of GSPs and celecoxib on TPA-induced cell migration. Cell migration assay analysis revealed that both GSPs and celecoxib significantly inhibited TPA-induced cell migration of melanoma cells (Figure 3C). We further verified the role of activated ERK1/2 in melanoma cell migration by using the inhibitor of MEK (UO126). Cell migration assay revealed that treatment of A375 cells with UO126 significantly inhibited ($P<0.001$) melanoma cell migration (Figure 3D). A summary of data related with cell migration are also shown. Additionally, western blot analysis revealed that the level of activated ERK1/2 was also decreased after the treatment of cells with MEK inhibitor UO126, as shown in Figure 3D.

GSPs decrease the activation of NF-κB/p65 in melanoma cells: NF-κB is an important mediator of melanoma cell migration

COX-2 is a downstream target of NF-κB, therefore we assessed whether GSPs also affect the proteins of NF-κB family in melanoma cells. For this purpose, A375 cells were treated with various concentrations of GSPs (0, 10, 20 and 40 μg/mL) for 24 h, and thereafter cells were harvested and whole cell lysates and nuclear lysates were prepared. The results of western blot analysis revealed that treatment of cells with GSPs reduce the nuclear translocation of NF-κB/p65 in a dose-dependent manner (Figure 4A). The activity of NF-κB also was significantly reduced (25–70%, $P<0.01$ and $P<0.001$) after the treatment of cells with GSPs in a concentration-dependent manner (Figure 4B). The western blot analysis also revealed that treatment of GSPs resulted in the down-regulation of IKKα, an enzyme responsible for NF-κB activation, and degradation of IκBα (Figure 4A), which leads to the inactivation of NF-κB. To check whether NF-κB has a role in melanoma cell migration, A375 melanoma cells were treated with caffeic acid phenethyl ester (0, 5, 10 and 20 μg/mL), a potent inhibitor of NF-κB, and cell migration was determined. As shown in Figure 4C, treatment of cells with caffeic acid phenethyl ester resulted in a dose-dependent reduction of cell migration (24–78%) relative to untreated control cells, and it was similar to that observed on treatment of the cells with GSPs (Figure 1B).

GSPs reverse epithelial-to-mesenchymal transition in melanoma cells

Activation of NF-κB has been implicated in inflammation-induced cancer development and progression, and has been identified as an important regulator of EMT in several cancer cell types [17–20]. As the inhibition of melanoma cell migration by GSPs is associated with the inactivation of NF-κB, we sought to determine whether GSPs also affect or reverse EMT in melanoma

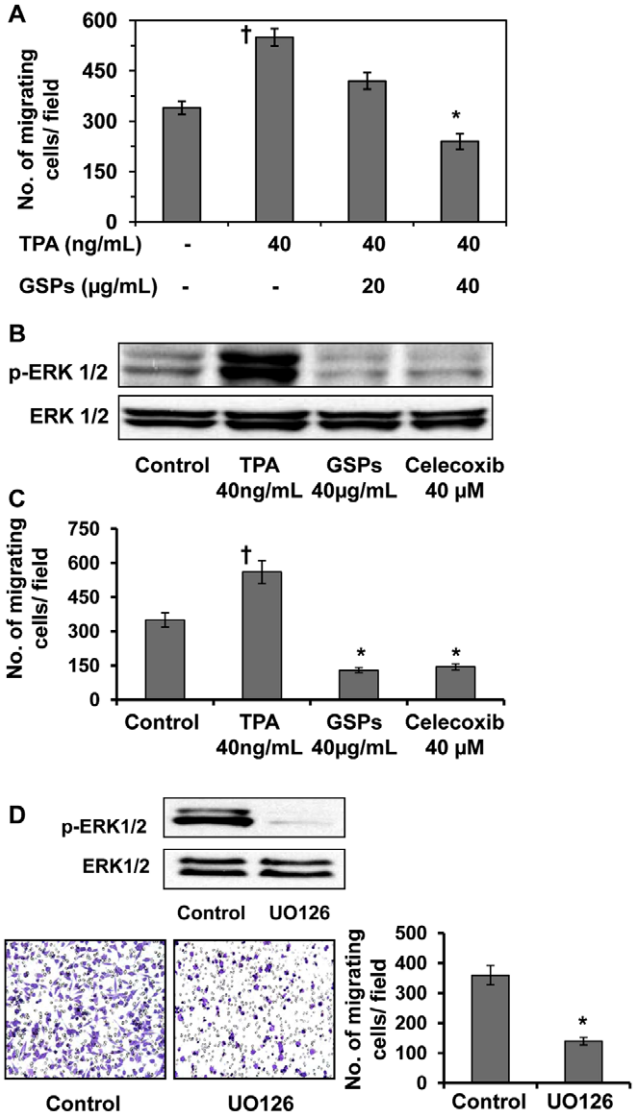

Figure 3. Effect of GSPs and celecoxib on TPA-induced activation of ERK1/2 and melanoma cell migration. (**A**) Effect of TPA or its combination with GSPs on melanoma cell migration. Treatment of A375 cells with TPA, a stimulator of COX-2, significantly enhances cell migration ($^{\dagger}P<0.001$) compared with non-TPA-treated control cells. (**B & C**) Treatment of A375 cells with TPA results in activation of ERK1/2. Treatment of cells with GSPs (40 μg/mL) or celecoxib (40 μM) inhibits TPA-induced activation of ERK1/2 protein, and simultaneously inhibits TPA-enhanced migration of melanoma cells. The data on cell migration capacity are summarized in Panel **C**. Significant inhibition versus TPA-treatment alone, $^{*}P<0.001$. (**D**) Treatment of melanoma cells with MEK inhibitor (UO126, 80 μM) resulted in inhibition of the activation of ERK1/2 as well as inhibition of cell migration compared to non-MEK inhibitor-treated control cells. The data are expressed as the mean number of migratory cells± SD/ microscopic field, n = 3. Significant difference versus controls $^{*}P<0.001$.
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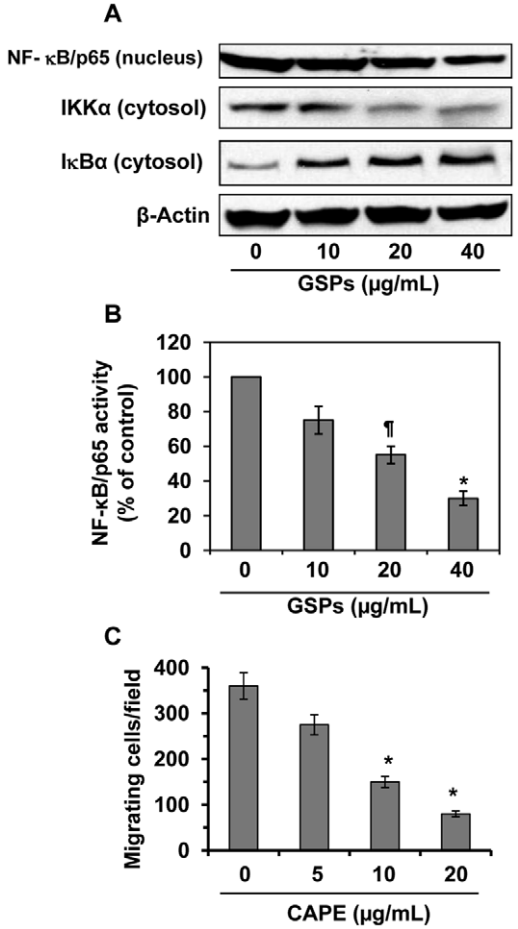

Figure 4. Effect of GSPs on NF-κB activation. (**A**) Treatment of A375 cells with GSPs decreases the basal levels of NF-κB/p65 and IKKα while inhibiting the degradation of IκBα. After treatment of cells for 24 h with various concentrations of GSPs the cells were harvested and cytosolic and nuclear fractions were prepared and subjected to the analysis of NF-κB/p65, IKKα and IκBα using western blot analysis. Representative blot is shown from three independent experiments with identical results. (**B**) The activity of NF-κB/p65 in the nuclear fraction of cells after treatment with and without GSPs for 24 h was measured using NF-κB/p65-specific activity assay kit, n = 3. Activity of NF-κB/p65 is expressed in terms of percent of control (non-GSPs-treated) group. Significant decrease versus control: $^{\P}P<0.01$, $^{*}P<0.001$. (**C**) Treatment of A375 cells with caffeic acid phenethyl ester (CAPE), an inhibitor of NF-κB, for 24 h inhibits cell migration in a concentration-dependent manner. Data on cell migration capacity are summarized as the mean number of migratory cells ± SD/microscopic field, n = 3. Significant inhibition versus non-CAPE-treated cells: $^{*}P<0.001$.
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cells and that is responsible for their inhibitory effect on melanoma invasiveness. For this purpose, A375 cells were treated with GSPs for 24 h, and cell lysates were prepared for the western blot analyses of various epithelial and mesenchymal biomarkers. Our western blot analyses revealed that GSPs restored or increased the levels of the epithelial biomarkers, such as E-cadherin, keratin-18, keratin-8 and desmoglein 2 in melanoma cells compared to untreated controls. In contrast, the levels of mesenchymal biomarkers, such as N-cadherin, vimentin, fibronectin and SLUG, were reduced in melanoma cells after treatment with GSPs in a dose-dependent manner, as shown in Figure 5A. GSPs-induced changes or effects on these epithelial and mesenchymal biomarkers were also detected and analyzed using immunofluorescence staining (Figure 5B). Immunofluorescnce staining data revealed that treatment of A375 cells with GSPs for 24 h resulted in reduction of mesenchymal biomarkers, such as vimentin, fibronectin and N-cadherin which is evident by the intensity of staining of the cells. In contrast, GSPs enhanced the levels of epithelial biomarker, such as desmoglein 2, in melanoma cells which is evident by the strong intensity of fluorescence staining compared to untreated controls. Similar

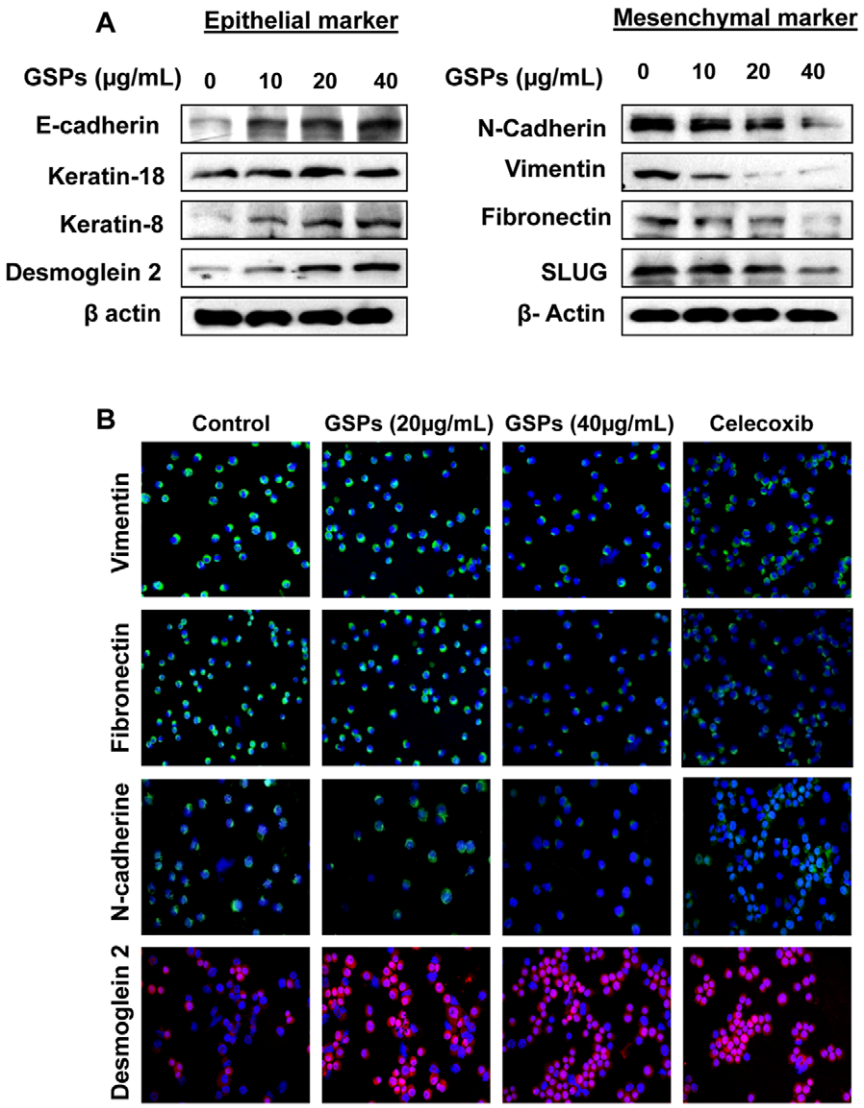

Figure 5. Treatment of melanoma cells with GSPs results in reversal of epithelial to mesenchymal transition. (**A**) Treatment of A375 cells with GSPs for 24 h enhances the levels of epithelial biomarkers in the cells, such as, the levels of E-cadherin, keratin-18, keratin-8 and desmoglein 2. Simultaneously the levels of mesenchymal biomarkers in melanoma cells, such as, vimentin, fibronectin, N-cadherin and SLUG were decreased dose-dependently. (**B**) Identification of the levels of epithelial and mesenchymal biomarkers in A375 cells after the treatment of cells with GSPs or celecoxib using immunocytostaining, as detailed in Materials and Methods. Treatment of A375 cells with GSPs (20 and 40 μg/mL) or celecoxib (20 μM) for 24 h resulted in reduced expression of vimentin, fibronectin and N-cadherine, while the level of desmoglein 2 was increased. Representative photomicrographs are representative of three independent experiments with similar results.
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observations were also noted when cells were treated with celecoxib, a COX-2-specific inhibitor, in identical manner. Representative photomicrographs are shown from three independent experiments.

GSPs and celecoxib inhibit TPA-induced EMT biomarkers in melanoma cells

As TPA induces COX-2 expression as well as enhances cell migration in melanoma cells, we next examined whether TPA promotes EMT in melanoma cells and whether GSPs and celecoxib inhibit TPA-induced EMT in these cells. For this purpose, A375 melanoma cells were treated with either TPA or celecoxib alone or TPA with the treatment of GSPs for 24 h, cell lysates prepared and subjected to western blot analysis. As shown in Figure 6, TPA decreased the level of desmoglein 2 (an epithelial biomarker), while enhanced the levels of mesenchymal biomarkers (N-cadherin and vimentin) compared with untreated control cells. Celecoxib enhanced the level of desmoglein 2 while decreased the levels of N-cadherin and vimentin compared with untreated control melanoma cells. Further, as shown in Figure 6, GSPs increased or restore the level of desmoglein 2 in TPA-treated melanoma cells, while reduced TPA-induced levels of N-cadherin and vimentin on A375 cells. These data further support the evidence that GSPs function as a COX-2 inhibitor and have the ability to reverse EMT in melanoma cancer cells and thus lead to reduce the invasiveness of melanoma cells.

Discussion

Melanoma cells can metastasize rapidly and that is the leading cause of death. According to a World Health Organization report, 48,000 melanoma-related deaths occur worldwide per year [21].

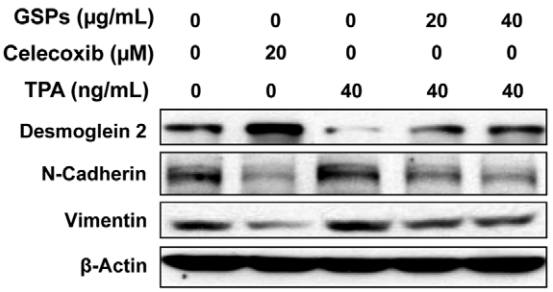

Figure 6. The effect of GSPs, TPA and celecoxib on the EMT biomarkers in melanoma cells. A375 cells were treated for 24 h and cell lysates were prepared for the analysis of N-cadherin, vimentin and desmoglein 2 using western blot analysis. Equal loading of proteins on the membranes were verified using β-actin antibody. Representative blots are shown from 3 independent experiments.
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Treatment is more difficult if it has spread beyond skin and lymph nodes [22]. Therefore, innovative strategies are required to be developed for the prevention of the invasive or the migratory potential of melanoma cells. Many human cancers express elevated levels of COX-2 and enhanced biosynthesis of PGs. COX-2 overexpression and abundant production of PGs, and particularly PGE_2, have been linked with tumor progression, invasion and metastasis [23]. Because of its important role in tumor invasion and metastasis, COX-2 is always a promising target for cancer therapy [8,24]; therefore, the search and development of potential COX-2 as well as PGE_2 inhibitors for the prevention or treatment of melanoma may prove to be an important and effective strategy.

The significant findings in the present study are that the treatment of melanoma cells with GSPs inhibits cell migration in a dose-dependent manner, and that is associated with the inhibition of COX-2 expression and PGE_2 production. The melanoma cells overexpress COX-2, and the inhibition of COX-2 by GSPs contributes to the inhibition of cell migration of these cells. This concept is supported by the evidence that treatment of the melanoma cells with celecoxib, a potent COX-2 inhibitor, resulted in a reduction in cell migration. Similar effects were also noted when the melanoma cancer cells, A375 and Hs294t, were transfected with COX-2 siRNA. It has been shown that TPA promotes COX-2 expression, and we found that treatment of melanoma cells with TPA enhances cell migration, and that this TPA-induced cell migration was blocked by the treatment of cells with GSPs. These observations support the evidence that inhibition of melanoma cell migration by GSPs requires the inhibition of COX-2 expression. It has been reported that COX-2 inhibitors can inhibit cell migration; however, they may also induce some form of toxicity. This possibility is not found in GSPs as these are dietary components and toxicity has not been observed in animal models [10,11].

It is well known that PGE_2 exerts its biologic functions by stimulating epithelial cell growth, invasion potential and cellular survival signals [25,26]. Singh et al. [27] have shown that PGE_2 treatment enhanced melanoma cell migration and that berberine, a phytochemical, inhibits PGE_2-induced migration of melanoma cells. Punathil and Katiyar [28] have examined the effect of GSPs on non-small cell lung cancer cell migration, and found that GSPs inhibit the migration of these cells by targeting nitric oxide, guanylate cyclase and ERK1/2 pathways. As COX-2 is a downstream target of NF-κB pathway, we further checked the effect of GSPs on the basal levels of NF-κB in melanoma cells, and found that treatment of melanoma cells with GSPs results in inactivation of NF-κB pathway in a dose-dependent manner. GSPs down-regulate the levels of IKKα which is responsible for NF-κB activation. Treatment of melanoma cells with caffeic acid phenethyl ester, an inhibitor of NF-κB, resulted in an inhibitory effect on melanoma cell migration. These observations support the concept that the inhibitory effect of GSPs on melanoma cell migration is mediated through the downregulation of COX-2 and PGE_2, which are the downstream targets of NF-κB. Our study also demonstrates the requirement of activated ERK1/2 in melanoma cancer cell migration. Our results show that inhibition of melanoma cell migration by GSPs is associated with the inhibition of ERK1/2 phosphorylation. The inhibition of MEK with UO126, a MEK inhibitor, blocked the migration capacity of melanoma cells which is similar to the action of GSPs. Treatment of A375 cells with TPA increased ERK1/2 phosphorylation and subsequently enhanced cell migration, while treatment of cells with celecoxib decreased ERK1/2 phosphorylation and subsequently decreased cell migration. These observations suggest a possible involvement of MAPK pathway (which is an upstream regulator of NF-κB) in inhibition of melanoma cell migration by GSPs.

The transcription factor NF-κB regulates a wide spectrum of biological processes, including inflammation, cell proliferation and apoptosis. Additional roles of NF-κB in cancer biology, such as in tissue invasion, cell migration and metastasis, have been investigated recently. Importantly, NF-κB is involved in inflammation-induced cancer development, and has been identified as an important regulator of EMT in several cancer cell types [17–20]. EMT has been observed to play a major role in invasion and metastasis of epithelial tumors. EMT can render tumor cells migratory and invasive through the involvement of all stages, invasion, intravasation and extravasation [12]. During the process of EMT, cells can change from an epithelial to a mesenchymal state. They lose their characteristic epithelial traits and instead gain properties of mesenchymal cells. This process is primarily coordinated by the disappearance or loss of epithelial biomarkers such as E-cadherin and certain cytokeratins with the concomitant appearance or gain of mesenchymal markers such as vimentin, fibronection and N-cadherin, etc. In the present study, GSPs treatment of melanoma cells showed the suppression of mesenchymal biomarkers, such as vimentin, fibronectin and N-cadherin while restored the levels of epithelial biomarkers such as, E-cadherin, desmoglein 2, keratin-8 and -18, etc, in melanoma cells which suggest that GSPs have the ability to reverse the EMT process in melanoma cells and this may also be one of the possible mechanisms through which GSPs reduce the invasiveness of melanoma cells and that lead to inhibition of melanoma cell migration in our system.

In summary, the results from this study have identified for the first time that GSPs inhibit the invasiveness of melanoma cells or inhibit the ability of melanoma cell migration and that involves: (i) the inhibitory effect of GSPs on endogenous COX-2 overexpression and successive down-regulation of PGE_2 synthesis, (ii) the inhibitory effect of GSPs on the activation of NF-κB and the proteins of MAPK family, which are the upstream regulators of COX-2 and PGE_2, and (iii) the reversal of EMT process. More detailed studies are needed to develop GSPs as a pharmacologically safe agent either alone or in combination with other anti-metastatic drugs for the treatment of metastatic melanoma in humans.

Author Contributions

Conceived and designed the experiments: SKK MV TS. Performed the experiments: MV TS. Analyzed the data: MV TS SKK. Contributed reagents/materials/analysis tools: SKK. Wrote the paper: SKK.

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Original article

Phenolic compounds from *Viscum album* tinctures enhanced antitumor activity in melanoma murine cancer cells

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ABSTRACT

Cancer is one of the biggest problems in public health worldwide. Plants have been shown important role in anticancer research. *Viscum album* L. (Santalaceae), commonly known as mistletoe, is a semi-parasitic plant that grows on different host trees. In complementary medicine, extracts from European mistletoe (*Viscum album* L.) have been used in the treatment of cancer. The study was conducted to identify chemical composition and antitumor potential of *Viscum album* tinctures. Chemical analysis performed by high resolution chromatography equipped with high resolution mass spectrometer identified caffeic acid, chlorogenic acid, sakuranetin, isosakuranetin, syringenin 4-O-glucoside, syringenin 4-O-apiosyl-glucoside, alangilignoside C and ligalbumoside A compounds. Some of these compounds are probably responsible for the reduction of tumoral cellular growth in a dose-dependent manner. It was observed that melanoma murine cells (B16F10) were more sensitive to *V. album* tinctures than human leukaemic cells (K562), besides non-tumoral cells (MA-104) had a much lower cytotoxicity to them. Apoptotic-like cells were observed under light microscopy and were confirmed by a typical DNA fragmentation pattern. Additionally, flow cytometry results using Annexin-V/FITC permitted to quantify increased expression of early and late apoptotic markers on tumoral cells, confirming augmented Sub G0 population, which was probably associated with a consistent decrease in G1, and an increase in S or G2/M populations. Results indicate the chemical composition of *V. album* tinctures influences the mechanisms of *in vitro* tumoral cell death, suggesting a potential use in cancer pharmacotherapy research.

Abbreviations: % v/v, % volume/volume; TA, tincture A; TB, tincture B; TLC, Thin Layer Chromatography; NP/PEG, Diphenylboriloxyethilamine/polyetileneglicol; HPLC, high performance liquid chromatography; PDA, photodiode array detector; UFLC, ultra fast liquid chromatography; UHPLC, ultra high performance liquid chromatography; HRMS, high resolution mass; DMEM, Dulbecco's Modified Eagle Medium.

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1. Introduction

Cancer is a group of diseases characterized by the abnormal growth and proliferation of cells that may or may not invade or spread to other parts of the body. According to data from World Health Organization, cancer is the second cause of death worldwide and caused more than 8.8 million deaths in 2015 (WHO, 2017).

The high rates of cancer incidence have encouraged the interest of researchers in the developing of new therapeutic modalities against it, and in the finding of new drugs with better activity and lower adverse effects. In this scenario, natural products have been considered useful to the anticancer activity. It is known that approximately 55% of the anticancer drugs approved between 1940 and 2014 had their origin from natural sources (Newman and Cragg, 2016).

Several studies suggest that metabolites derived from plants may have pro-apoptotic properties. *Viscum album* L. (Santalaceae), commonly known as mistletoe, is a semi-parasitic plant that grows on different host trees. This species has been commonly used for complementary cancer therapy, mainly in Central Europe (Tröger et al., 2013), and it is possible to find a multitude of studies in which the immunomodulatory (Jurin et al., 1993; Gardin, 2009; Weissenstein et al., 2014), cytotoxic and pro-apoptotic (Bussing and Schietzel, 1999; Urech et al., 2005; Facina et al., 2014) properties have been described. Aqueous preparations of *V. album* exert several immuno-stimulatory mechanisms, possibly by interacting with the cellular and humoral compartments of the immune system, increasing the antitumor immune response (Yoon et al., 2001; Stein et al., 2002; Heinzerling et al., 2006; Gardin, 2009).

The most studied active compounds in aqueous preparations of *V. album* are lectins and viscotoxins. These compounds induce macrophage cytotoxicity, stimulate phagocytosis of immune cells, increase cytokine secretion and enhance *in vitro* cytotoxic effects on various cell lines (Timoshenko et al., 1995; Estko et al., 2015).

Other compounds, such as phenolic acids, phenylpropanoids, flavonoids, triterpenes, phytosterols, oligo and polysaccharides, were also identified in the European mistletoe (Nazaruk and Orlikowski, 2016; Delebinski et al., 2015; Strüh et al., 2013; Cebović et al., 2008) and this variety of metabolites is probably involved with the antitumoral effects of *V. album* extracts.

Although *V. album* antitumor activity is mainly associated with the aqueous preparations, the use of different solvents as well as modifications in the extraction methodology influences *in vitro* and *in vivo* antitumor activity. It must be taken into account that the chemical composition of *V. album* extracts is directly related to the solvent used in the extraction process.

In this context, the cytotoxicity of *V. album* hydroalcoholic tincture associated or not with chemotherapeutic agents was detected in the Ehrlich ascites carcinoma (Stan et al., 2013), as well as in HeLa cancer cells proliferation (Sárpataki et al., 2015), indicating that ethanol soluble compounds are also related to the antitumoral *V. album* activity. Moreover, Cebović et al. (2008) showed the efficacy of non-polar supercritical CO_2 extract in the cytotoxicity of *V. album* towards Ehrlich carcinoma cells, confirming the importance of the optimization extraction methodology.

The purpose of the present study was to analyze the chemical profile of two *V. album* tinctures, as well as their *in vitro* effects in tumoral (murine melanoma cells, B16F10; human chronic myelogenic leukemia cell line, K562) and non-tumoral cells (monkey kidney cells, MA-104). The involvement of the identified chemical compounds with antitumoral *V. album* activity is also discussed in this paper.

2. Material and methods

2.1. Viscum album L. Tinctures

Tinctures of *Viscum album* L. used in this study were donated by two pharmaceutical laboratories, Homeopatia Almeida Prado (São Paulo, Brazil) and Boiron Laboratories (Lyon, France), for research purposes. Both tinctures were obtained by maceration extraction with ethanol (45% v/v) following *Viscum album* homeopathic monographs in pharmacopoeias (ANSM, 2010; ANVISA, 2011) and were labeled Tinctures TA and TB.

2.2. Identification of substances by thin layer chromatography

Thin layer chromatography (TLC) analyses were achieved by silica gel 60 F254 (250 μm thickness, SiliCycle, Quebec, Canada) using water/methanol/glacial acetic acid/methylene chloride (2:3:8:15) as mobile phases. The detections were done by spraying NP/PEG reagent (1% diphenylboriloxyethylamine in methanol p/v, followed by 5% polyethylene glycol 4000 in ethanol p/v). The plates were observed under ultraviolet light at 254 and 365 nm before and after spraying the reagent solution. Spots of non-diluted tinctures and standards were identified by Rf-values and color compared to the standard compounds caffeic and chlorogenic acids (ANSM, 2010) (MP Biomedicals, California, USA).

2.3. HPLC-PDA-MS conditions

Analyses were conducted using an HPLC Dionex Ultimate 3000, equipped with a photodiode array (PAD) detector (Thermo Fisher Scientific, USA) connected with LCQ Fleet Ion Trap Mass Spectrometer (Thermo Fisher Scientific, USA). The sample was prepared according to *V. album* monograph from French Pharmacopoeia (ANSM, 2010): in a 20.0 mL volumetric flask, 8.0 g of each tincture was diluted to 20.0 mL of a mixture of 10 volumes of acetonitrile and 90 volumes of trifluoroacetic acid (0.05 per cent v/v).

Separations were performed on a reverse-phase column (C-18, 250 mm × 4, 6 mm × 5.0 μm; Kromasil, Akzo Nobel). Water-formic acid 0.1% v/v (A) and acetonitrile (B) were used as mobile phases, as follows: (i) 0–20 min, 10% B, (ii) 20–25 min, 10–15% B, (iii) 25–45 min, 15% B, (iv) 45–50 min, 15–100% B, (v) 50–55 min 100% B, (vi) 55–57 min 100–10%, and (vii) 57–70 min 10% B. The flow rate was 1.0 mL/min and the injection volume was 20 μL. Absorption UV–VIS spectra were recorded on PDA-detector (with a total spectral range between 100 nm and 400 nm), set at detection wavelength 220 nm, simultaneously. Mass spectra were recorded in positive ion mode.

2.4. UFLC-PDA-MS/MS conditions

UFLC PDA MS/MS analyses were carried out on an ultra fast liquid chromatography (UFLC Shimadzu, model Nexera) equipped with a mass spectrometer (TOF Bruker, model Compact). The sample was prepared according to *V. album* monograph from French Pharmacopoeia (ANSM, 2010) as described in Section 2.3.

Separations were performed on a reverse-phase column (C-18, 100 mm × 3 mm × 2.6 μm; Kinetex, Phenomenex). A binary gradient system with water-formic acid 0.1% v/v (A) and acetonitrile (B) was used as follows: (i) 0–7.65 min, 5% B, (ii) 7.65–8.50 min, 5–15% B, (iii) 8.50–15.30 min, 15% B, (iv) 15.30–17 min, 15–100% B, (v) 17–18.70 100% B, (vi) 18.70–20.40 min 100–5% and (vii) 20.40–22 min 5% B. The flow rate was 0.5 mL/min and the injection volume was 2 μL. The detection wavelength was 220 nm. All analyses were done by electrospray ionization (ESI) in positive mode.

2.5. UHPLC-HRMS/MS conditions

Samples were prepared as described in Section 2.3 (ANSM, 2010) and analyzed on Dionex Ultimate 3000 system hyphenated to a QExactive Plus mass spectrometer (Thermo Fisher Scientific, USA) equipped with an electrospray ionization (ESI) probe. Separations were performed on a reverse-phase (C-18, 50 mm, 2, 1 mm × 1.7 μm; Syncronis, Thermo Fisher Scientific). Water-formic acid 0.1% v/v (A) and acetonitrile (B) were used as mobile phases as follows: (i) 0–2 min, 10% B, (ii) 2–3,5 min, 10–15% B, (iii) 3.5–6.4 min, 15% B, (iv) 6.4–7.0 min, 15–100% B, (v) 7.0–8.0 min 100% B, (vi) 8.0–9.2 min 100–10% and (vii) 9.2–10 min 10% B. The flow rate was 1.0 mL/min and the injection volume was 5 μL. Mass spectra were recorded in negative ion mode. The HRMS and HRMS/MS data were acquired in negative mode over a *m/z* range of 80–1000. The MS profile was performed in full scan mode and displayed in TIC (Total Ion Current) chromatogram. ESI conditions were as follows: capillary temperature 380 °C; spray voltage 3.9 kV; ESI voltage 2.9 kV. Nitrogen was used as sheath gas (60 Au) and auxiliary gas (20 Au). The raw data were acquired and processed with Xcalibur 2.0.7 software from Thermo Scientific.

2.6. Cell lines and culture

B16F10 cells (murine melanoma) was obtained from Laboratório de Oncobiologia Molecular (LabOMol), Federal University of Rio de Janeiro, the K562 (human chronic myelogenic leukemia cell line) and MA-104 (monkey kidney, non-tumoral) were obtained from Rio de Janeiro Cell Bank (Duque de Caxias, Rio de Janeiro, Brazil). Cells were grown in Dulbecco's Modified Eagle Medium (DMEM) in 25 cm^2 disposable plastic bottles (TPP Techno Plastic Products AG, Switzerland), at 37 °C. They were supplemented with 10% fetal bovine serum, penicillin (100 UI/mL) and streptomycin (100 μg/mL). These supplements were obtained from Invitrogen (California, USA),

2.7. Cell viability assay

Cell viability was measured by MTT colorimetric assay as described before (Mosmann, 1983; Meira et al., 2005). Briefly, for each experiment, 1×10^4 cells were seeded per well in 96-well plates. After 24 h, cells were treated with TA and TB in concentrations varying from 1 to 5% v/v. After incubation for 24 h, the cells were centrifuged for 8 min (500*g*) and the supernatant was discarded. In each well, 180 μL of DMEM and 20 μL of a 5 mg/mL solution of Thiazolyl Blue Tetrazolium Bromide (MTT - Sigma, USA) were added, and cells were incubated for further 3 h at 37 °C. After incubation, cells were centrifuged, the supernatant was discarded and 200 μL of Dimethyl Sulfoxide (DMSO) were added per well to dissolve the newly formed formazan salt. The absorbance was measured at 490 nm using an ELISA plate reader (TP Reader, Thermoplate). Mean values were calculated in 3 independent experiments using 5 wells per condition.

2.8. May-Grunwald-Giemsa assay

B16F10 cells (5×10^5 cells/ well) were cultivated in 6-well plate and incubated with TA, TB and their respective controls, at the concentrations of 3% and 5% v/v for 24 h. Subsequently, cells were washed and stained with May-Grunwald-Giemsa, as described before (Meira et al., 2005; Barbosa et al., 2017). Finally, morphological alterations were examined with a light microscopy (Axioplan 2, Carl Zeiss, Göttingen, Germany), and all images were obtained using a digital camera attached to the microscope.

2.9. DNA fragmentation analysis

DNA fragmentation was performed as follows: B16F10 cells were seeded to approximately 80% of confluence (3.5×10^5 cells) in 25 cm^2 disposable plastic bottles and incubated for 24 h, at 37 °C. Cells were treated with TA, TB and their respective hydroalcoholic controls, in the concentrations of 3% and 5% v/v, and then incubated for more 6 h or 24 h at 37 °C. After that, cells were trypsinized, counted and diluted to obtain 1×10^6 cells. Following, they were washed twice with PBS, and DNA extraction was performed according to the instructions from Wizard Genomic DNA Purification Kit (Promega, São Paulo, Brazil). For DNA ladder detection, the DNA samples extracted and the 1Kb DNA Ladder Marker (M1181, Sinapse, São Paulo, Brazil) were submitted to electrophoresis in 0.8% agarose gel. After 30 min, the gel was labeled with ethidium bromide, visualized in UV and photographed (Red®, Protein Simple, California, USA).

2.10. Apoptosis measurement

Apoptosis induction was measured as follows: B16F10 cells were seeded on 6-well plates at a concentration of 1×10^5 cell/ well. After 24 h, cells were treated with TB, TA and their respective controls in the concentrations of 3% and 5% v/v. After incubation for 6 h, the supernatants were collected, cells were trypsinized and added to the supernatants. Then, the suspensions were centrifuged and washed with cold PBS. The pellets were resuspended in 100 μL of FITC Annexin V Apoptosis Detection Kit I buffer (BD Biosciences, Pharmingen™), homogenized and transferred to flow cytometry tubes. Cells were then stained with annexin V-FITC and propidium iodide (PI) according to the manufacturer instructions for 15 min at room temperature, in the dark. After this time, cells were analyzed in FACSVerse (Beckton and Dickinson, USA). The percentages of apoptotic cells were evaluated using BD FACSuite software.

2.11. Cell cycle analysis

Cell cycle was evaluated by flow cytometry as follows: 24 h after cell incubation with 3% or 5% v/v TA or TB and their respective controls, cells were trypsinized, centrifuged and washed with PBS. The tubes were then centrifuged for 10 min (450*g*), the supernatant was discarded and the pellet was resuspended in 500 μL of propidium iodide solution (40 μg/mL) in the presence of 100 μg/ml RNase in PBS. The solution was homogenized and transferred to flow cytometry tubes. After incubation for 30 min at room temperature in the dark, the cell cycle was analyzed with a FACSVerse flow cytometer (Beckton and Dickinson, USA). The percentages of cells in the cell cycle phases G0/G1, S, G2/M and sub G0 were determined using BD FACSuite software.

2.12. Statistical analysis

All experiments were performed at least three different times, and results were analyzed by ANOVA (analysis of variance) with Dunnett post hoc test, using GraphPad 5 Software (California, USA). *P* values<.05 were considered statistically significant.

3. Results

3.1. Chemical profile of Viscum album tinctures

TLC plate of TA and TB showed blue zones, which are typical of phenolic acids at 365 nm UV light, after it was sprayed with NP/PEG reagent (Wagner and Bladt, 2001). TLC plate of TA sample showed one spot with Rf value (0.66) and similar color (fluorescent

blue) to the chlorogenic acid reference compound. TB chromatoplate exhibited two fluorescent blue spots, one with Rf value equal to caffeic acid (Rf of 0.91) and another similar to the chlorogenic acid standard (Rf of 0.66).

UFLC-PDA-MS/MS analysis of TA tincture revealed phenolic acids and flavonoids with typical UV absorption spectrum and mass fragmentation pattern as major metabolites. In addition, these results support the previous phenolic acid and flavonoids identification by TLC plate, which showed fluorescent blue spots, typical of phenolic acid, and yellow spots, characteristic of flavonoids. UFLC chromatogram of TA, detected at 220 nm, showed three main peaks (**1**, **2** and **3**), whose MS and MS/MS spectra were carefully analyzed and compared with literature data. Compound **1** presented a typical UV absorption spectrum for phenolic acids (Mabry et al., 1970) and protonated molecular ion at *m*/*z* 355.1036 $[M+H]^+$, with a mass fragmentation pattern similar to chlorogenic acid (Popova, 1991). Compounds **2** and **3** showed an UV spectra with absorption spectrum corresponding to a flavonoid (Mabry et al., 1970) and protonated molecular ions at *m*/*z* 287.0925 $[M+H]^+$ ($C_{16}H_{14}O_5$) and 287.0924 $[M+H]^+$ ($C_{16}H_{14}O_5$) (Urech and Baumgartner, 2015; Nazaruk and Orlikowski, 2016). According to the MS/MS spectra of **2** and **3**, *m*/*z* 285.0767, 242.0580, 167.0344, 147.0446, 119.0497 and 91.0548 $[M+H]^+$ ions had a typical ion fragmentation for sakuranetin and naringenin 5-methyl ether, when compared with previously reported data (Hammami et al., 2004; Portet et al., 2008).

TB tincture was analyzed initially by HPLC-PDA-MS, and the chromatogram at 220 nm exhibited six main peaks (**4**, **5**, **6**, **7**, **8** and **9**), as shown in Fig. 1. The UV spectra of those peaks showed absorption similar to phenolic acids and lignans (Mabry et al., 1970; Benković et al., 2014). Mass analysis of each peak exhibited the following protonated molecular ions at *m*/*z*: 354.91 $[M+H]^+$, 395.18 $[M+Na]^+$, 527.24 $[M+Na]^+$, 203.95 $[M+Na]^+$, 599.87 $[M+NH_4]^+$ and 599.88 $[M+NH_4]^+$, respectively. In addition, mass spectral fragmentation pattern and UV spectrum of compounds **4**–**9** were compared to literature data, especially to those phenolic acids and lignans described as chemical markers of *V. album* (Urech and Baumgartner, 2015). Afterwards, the identification of compounds 4–9 was followed by accurate mass measurements through UHPLC-HRMS/MS analysis that presented deprotonated molecular ion peaks for compounds **4**, **5**, **6**, **7**, **8** and **9** at *m*/*z*: 353.08844 $[M-H]^-$ ($C_{16}H_{18}O_9$), 371.13513 $[M-H]^-$ ($C_{17}H_{24}O_9$), 503.17807 $[M-H]^-$ ($C_{22}H_{32}O_{13}$), 179.03498 $[M-H]^-$ ($C_9H_8O_4$), 581.22498$[M-H]^-$ ($C_{28}H_{38}O_{13}$) and 581.22527 $[M-H]^-$ ($C_{28}H_{38}O_{13}$), respectively.

MS/MS spectra of compound **4**, *m*/*z* 191.05521, 179.03458 and 135.04408 $[M-H]^-$ ions, had a typical ion fragmentation for chlorogenic acid, according to the literature (Zhang et al., 2016). For some of the constituents, such as syringenin 4-*O*-glucoside (**5**) and syringenin 4-*O*-apiosyl-glucoside (**6**) fragment ions, it was observed that *m*/*z* 209, 194 and 175 is a syringenin fragmentation characteristic (Sun et al., 2016). Compound **7** showed MS/MS spectra with ions at *m*/*z* 161 and 135 $[M-H]^-$, similar to ion fragments of caffeic acid (Zhang et al., 2016).

In accordance with the accurate mass spectra of **8** and **9**, *m*/*z* 581.22498 $[M-H]^-$ and 581.22527 $[M-H]^-$ were typical for alangilignoside C and ligalbumoside A, as already described for *V. album* (Nhiem et al., 2012; Benković et al., 2014).

These results provided reliable information for confirming molecular weight and structure of these constituents. Therefore, compounds were identified as chlorogenic acid (**4**) (Ferracane et al., 2010; Mocan et al., 2016), syringenin 4-*O*-glucoside (Panossian et al., 1998) (eleutheroside B) (**5**), syringenin 4-*O*-apiosyl-glucoside (Panossian at al., 1998) (polygalatenoside E) (**6**), caffeic acid (Spagnol et al., 2015) (**7**), alangilignoside C (**8**) and ligalbumoside A (Nhiem et al., 2012; Benković et al., 2014) (**9**). Compounds identified in *V. album* tinctures are shown in the Fig. 2.

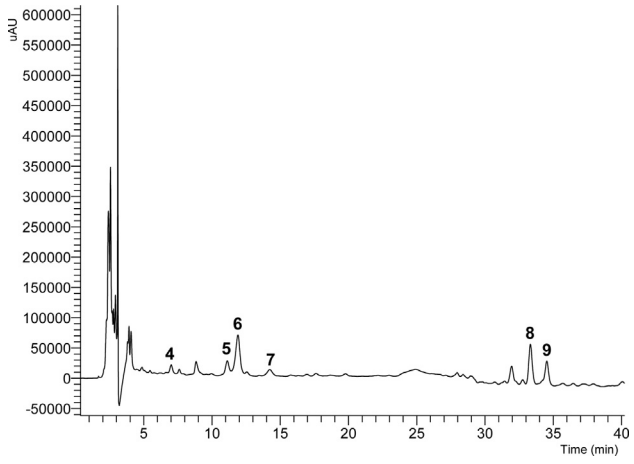

Fig. 1. UV Chromatogram at 220 nm of TB sample.

3.2. Cell viability is reduced by Viscum album L.

As shown in Fig. 3, control hydroalcoholic solutions decreased the viability of B16F10 and K562 cells in a dose-response way, having B16F10 presented a higher sensitivity. In contrast, MA-104 cell line was resistant to all hydroalcoholic concentrations used. Since the tumor cell lines were sensitive to the hydroalcoholic solutions, the same concentrations of these solutions were used as controls in the following experiments.

Both TA and TB were cytotoxic to the three cell lines, but this cytotoxicity was cell- and dose-dependent (Fig. 4). The MA-104 cell line was more sensitive to TA, which significantly diminished its viability in 50% ($p < .001$) in concentrations as low as 1% v/v, while TB reduced viable cells in 24% when compared to control (Fig. 4, panel A). However, for the concentration of 5% v/v, TA and TB were equally effective, reducing MA-104 cell viability in approximately 36%. For K562 cells (Fig. 4, panel B) and B16F10 cells, both TA and TB were almost equally efficient in reducing the cellular viability at any concentration tested. At 5% v/v, TA and TB reduced K562 and B16F10 viability in approximately 70% (Fig. 4, panels B and C). Since B16F10 cell line has a well-known high aggressive and drug-resistant phenotype and presented similar results to K562 with both tinctures treatments, the following experiments were performed only with B16F10.

3.3. Morphologic B16F10 alterations induced by TA and TB

Fig. 5 shows the morphology of B16F10 stained with Giemsa after 24 h of treatment, with TA and TB at concentrations of 3% and 5% v/v. Blebs, which were absent in the hydro-alcoholic control cells, can be seen in the plasma membrane (Fig. 5 d, e, g - black arrows), suggesting apoptosis. Moreover, cells treated with 3% v/v also showed fusiform pattern cells.

3.4. Viscum album tinctures induced DNA fragmentation in B16F10 cells

Fig. 6 shows B16F10 genomic DNA fragmentation induced by TA and TB. A DNA degradation pattern can be observed in lanes 2, 3 and 6, corresponding respectively to TB and TA at 5% v/v (6h), and TB at 3% v/v (24 h). Finally, the absence of DNA fragmentation was detected when hydro-alcoholic solvents (3% and 5% v/v) were incubated with B16F10 for 24 h (lane 1) and for 6 h (lane 4).

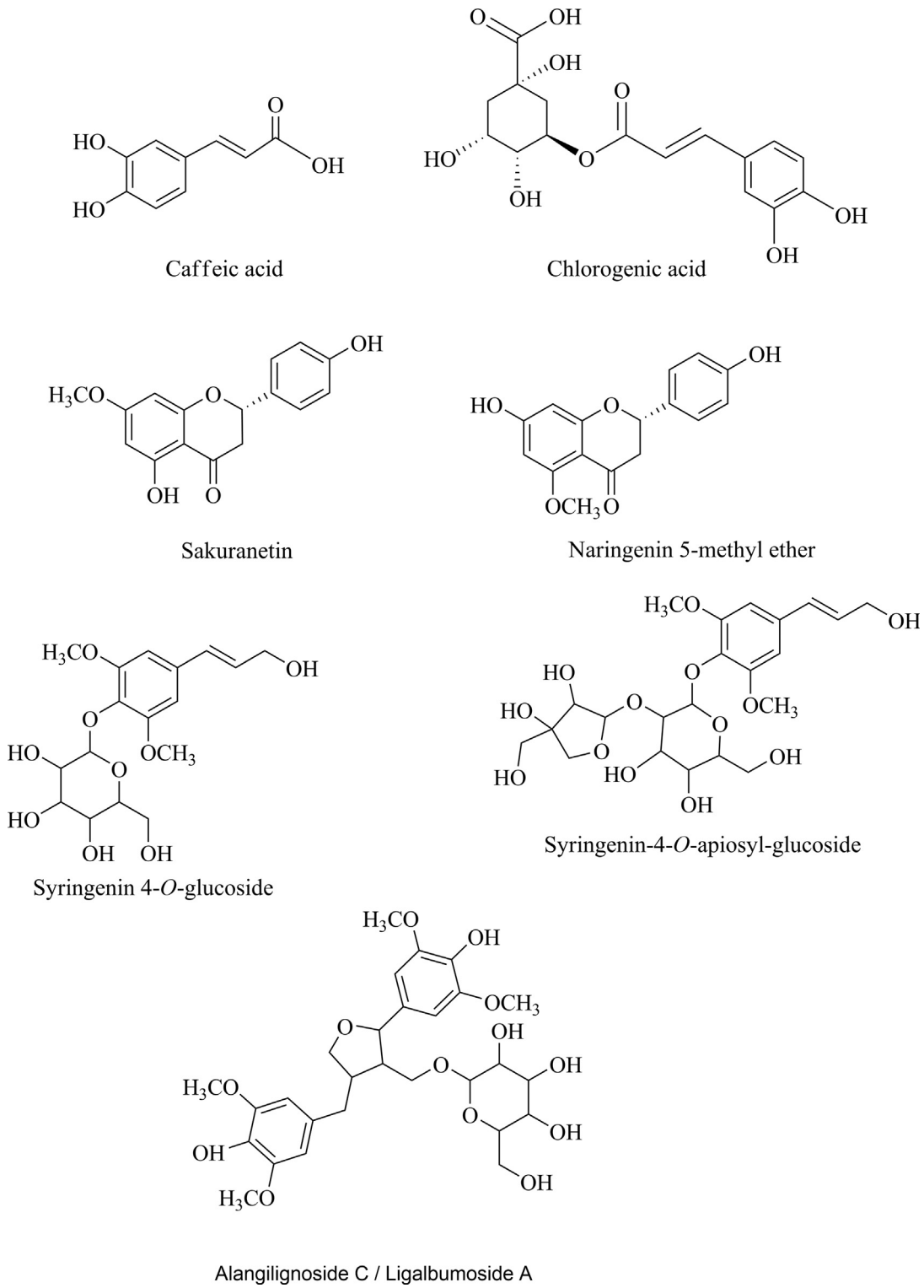

Fig. 2. Possible structures present in TA and TB samples.

3.5. Apoptosis evaluation by Annexin-V/FITC/PI

Since the results above suggested that *V. album* tinctures induce apoptosis in B16F10, Annexin V-FITC/PI V-PI methodology was also employed to confirm this data. As it can be seen in Fig. 8-panel A, the number of viable cells was reduced by 22.5%, in relation to its respective control, after treatment with 3% v/v of TB. This reduction was accompanied by a 21% increase in the number of early apoptotic cells (PI-/Annexin+) (Fig. 8, panel B, left). On the other hand, no statistically significant differences ($p < .05$), in relation to control, were detected after TA incubation.

Additionally, using higher tincture concentrations (5% v/v), statistically significant decreases in B16F10 viability were detected by 32% (TA) and 55% (TB), with $p < .0001$ (Fig. 8, panel A, right). Nevertheless, Fig. 8 (panel C, right) showed an increase of 18% (TA, $p < .05$) and 41% (TB, $p < .0001$) in late apoptotic cells (PI+/Annexin+).

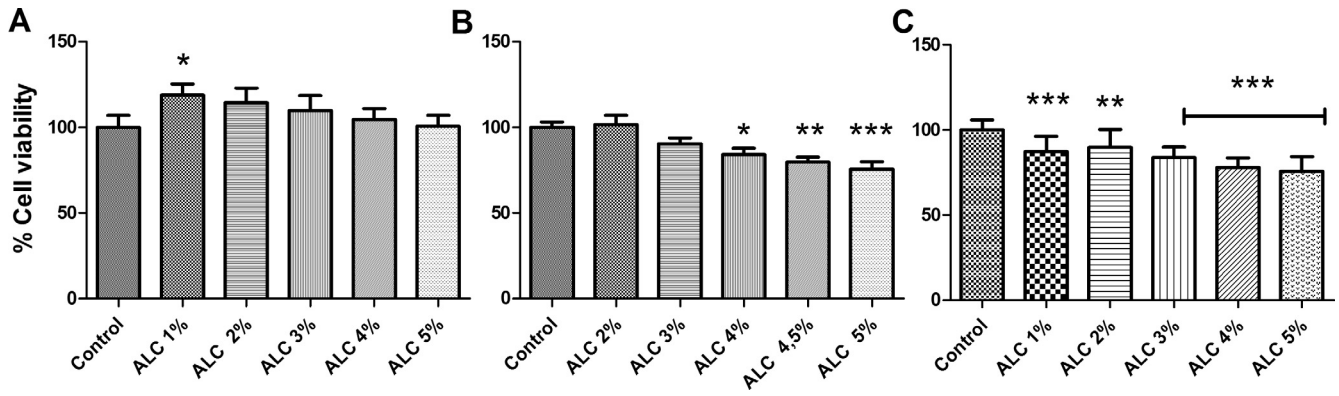

Fig. 3. MTT assay after 24 h of treatment with 45% v/v hydro-alcoholic solution. The final concentrations varied between 1 and 5% v/v. (A) MA-104, (B) K562, (C) B16F10. Data are shown as media ± SD from at least 3 experiments. $^{*}p < .05$, $^{**}p < .001$ and $^{***}p < .0001$, obtained with one way ANOVA with Dunnett post-test.

Finally, TA and TB led to an increase in PI labeled cells with percentages of 16.5% and 13.5% ($p < 0.05$), respectively, suggesting necrosis or other non-apoptotic cell death pathways (Fig. 8, panel D, right).

3.6. Viscum album tinctures modified B16F10 cell cycle

B16F10 cell cycles were evaluated after 24 h of treatment with TA and TB at 3% and 5% v/v. It can be seen in Fig. 7 that SubG0 had an increase of 43% after 24 h of incubation with TB (3% v/v), and 80% after 24 h of incubation with both tinctures at 5% v/v. The percentage of cells in G0/G1 was reduced by 22% (TA) and 36% (TB) at 3% v/v, while TA treatment (3% v/v) induced an increase in the number of cells of 13% and 8% in S and G2/M phases, respectively.

4. Discussion

Cancer is one of the main causes of death worldwide. Natural products and their secondary metabolites have a considerable importance as anticancer agents considering their major toxicity to cancer cells when compared with normal cells (Jiang et al., 2017).

Several studies have reported cytotoxic activity of *V. album* aqueous extracts in different cell lines, such as MOLT-4 and Yoshida (Urech et al., 1995), cells of tongue squamous cell carcinoma (Klingbeil et al., 2013), lymphoma human cells (Seifert et al., 2008), human T cell lines CEM and monocyte cell lines HL-60 (Singh et al., 2016). The most important compounds involved with the antitumoral activity of *V. album* aqueous extracts are mistletoe lectins (I-III) (Valentiner et al., 2002), viscotoxins and polysaccharides (Urech et al., 1995; Tröger et al., 2013). However, its antitumoral potential is controversial because there are studies confirming that non-aqueous extracts contain compounds, such as epi-oleanolic acid (Jung et al., 2004), viscothionin (Kim et al., 2014), and alkaloids (Khwaja et al., 1980), which are also directly involved with antioxidative (Kim et al., 2016) and, consequently, antitumoral properties. Recently, Sárpataki et al. (2015) showed that *V. album* alcoholic extract reduces Hela cells proliferation with no significant effects on normal fibroblasts, confirming the involvement of other bioactive molecules.

In the present study, we observed that hydroalcoholic *V. album* tinctures (TA and TB) were cytotoxic to tumoral cell lines (K562 and B16F10). Since mistletoe lectins are not stable in hydroalcoholic solvents, the anticancer activity of these extracts should be also attributed to the presence of different compounds from the ones in aqueous *V. album* preparations. Our results showed the presence of phenolic compounds in both TA and TB tinctures, confirming previous analyses done by other authors (Pfüller, 2000; Luczkiewicz et al., 2001).

According to the literature, caffeic and chlorogenic acids presented inhibitory activities against tumoral cells, evidencing the effects in the adhesion, proliferation, migration, and invasion of tumoral cells (Yagasaki et al., 2000; Weng and Yen, 2012). Caffeic acid phenethyl ester showed a decrease of melanoma cell migration due to a nuclear factor kappa B inhibition (NF-κB) (Jones and Katiyar, 2013). Chlorogenic acid did not have cytotoxic effects on cellular viability when Hep3B cells were treated with different concentrations of this compound (Jin et al., 2005). However, the same study demonstrated that chlorogenic acid inhibited the activity of the metalloproteinase-9 (MMP-9), related with tumor progression.

Flavanone sakuranetin present in TA is well-known in *V. album* (Urech and Baumgartner, 2015). However, the flavanone naringenin 5-methyl ether was described for the first time in this work. Drira and Sakamoto (2016) demonstrated by MTT assay the cytotoxic effects of sakuranetin (0–75 μmol/L) in B16BL6 melanoma cells after 72 h of treatment.

Phenylpropanoids and lignan are other classes of secondary metabolites with important anticancer activity. Etoposide and teniposide, two important anticancer drugs, were derived from lignan podophyllotoxin presented in genus *Podophyllum* (Brandão et al., 2010). In the present work, two phenylpropanoids and two lignans were identified in TB sample, syringenin 4-*O*-glucoside (eleutheroside B), syringenin 4-*O*-apiosyl- glucoside (polygalatenoside E), alangilignoside C and ligalbumoside A, in accordance with previous works described with *V. album* species (Panossian et al., 1998; Popova, 1991; Nhiem et al., 2012; Urech and Baumgartner, 2015).

Eleutheroside B and polygalatenoside E presented cytotoxic activity against HL-60 and Hell-299 cell lines (Thao et al., 2015). Additionally, Zhu et al. (2012) demonstrated eleutheroside B cytotoxic activity against MCG-803 cell line with IC50 50.9 μM. Based on the literature data, the presence of phenylpropanoids and lignans in TB and flavanones in TA could be also related to their cytotoxic and apoptotic activities.

Giemsa staining revealed the occurrence of morphological B16F10 changes, induced by TA and TB treatments, mainly characterized by blebs and cell shrinkage, which are suggestive of apoptotic cell death (Zainal Ariffin et al., 2009). In fact, biochemical features of apoptosis were detected after gel electrophoresis analysis, confirming DNA ladder fragmentation into segments of 180–200 multiple base pairs, which is a typical apoptosis pattern (Veiga et al., 2005). Additionally, Annexin V-FITC/PI assay confirmed the cell death in a dose-dependent manner since the lower

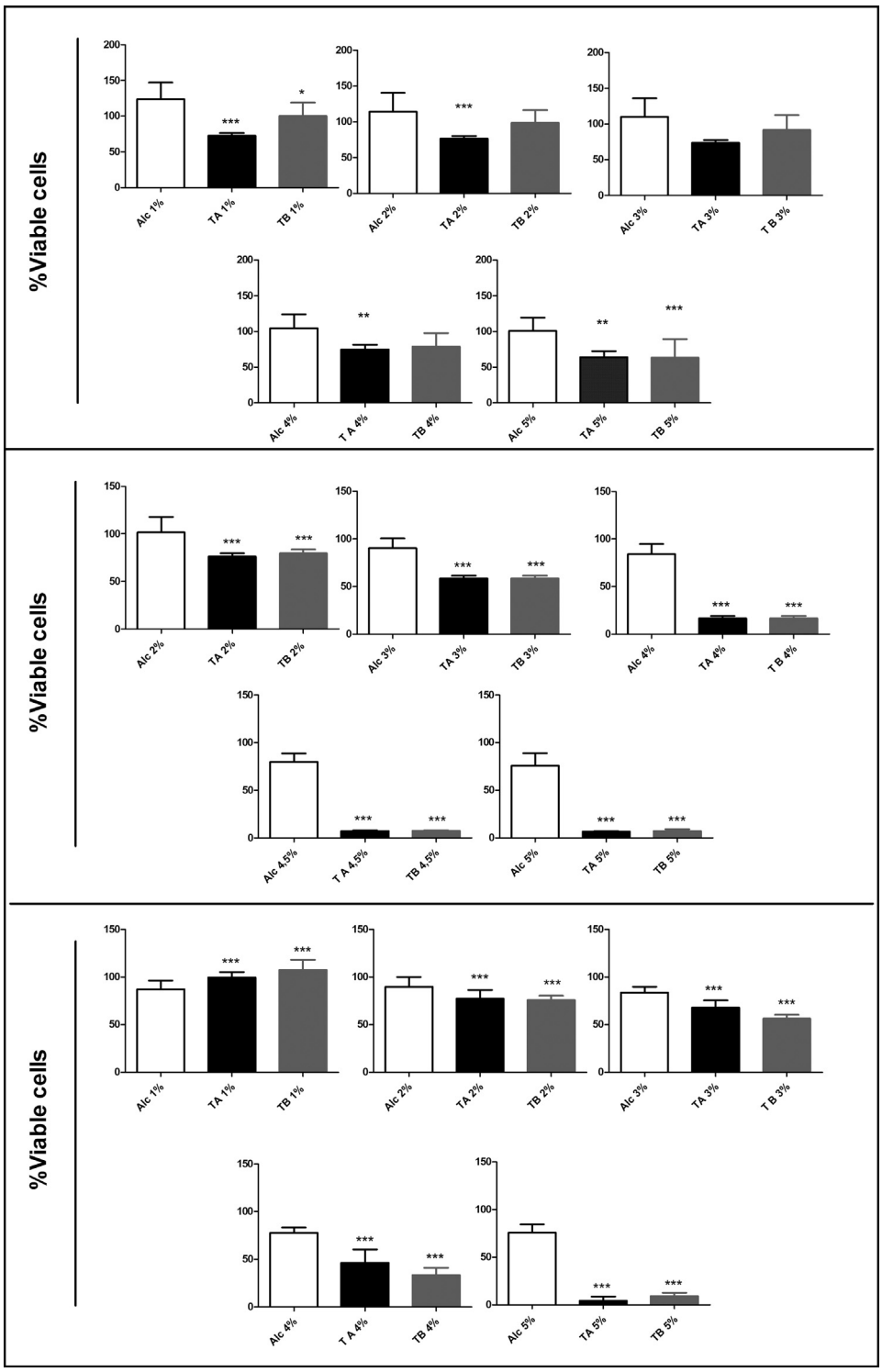

Fig. 4. MTT assay in MA-104 (panel A), K562 (panel B) and B16F10 (panel C) cells after 24 h of treatment with TA and TB solutions. The final concentrations varied between 1 and 5% v/v. Data are shown as media ± SD from at least 3 experiments. $^{*}p < .05$, $^{**}p < .001$ and $^{***}p < .0001$, obtained with one way ANOVA with Dunnett post-test.

TB concentration (3% v/v) induced a higher percentage of early apoptotic cells. Moreover, using 5% (v/v) of TB and TA, the flow cytometry analyses showed an increase in late apoptosis or other cell death mechanisms.

Finally, the proportion of hypodiploid B16F10 cells in the total cell population was significantly higher after incubation with both tinctures at 5% (v/v), with around 80% of cells in Sub G0 (Agrawal et al., 2011) (Fig. 7), confirming the increase of late apoptotic cells detected by Annexin V-FITC/PI-FITC/PI assay (Fig. 8). The observed increase in apoptotic cells, confirmed by the augmented Sub G0 population was associated with a consistent decrease in G1, without alterations in S or G2/M populations (Fig. 7, panel B). These evidences suggest that the death-inducing mechanisms promoted by *V. album* tinctures 5% v/v did not impact cell cycle progression. On

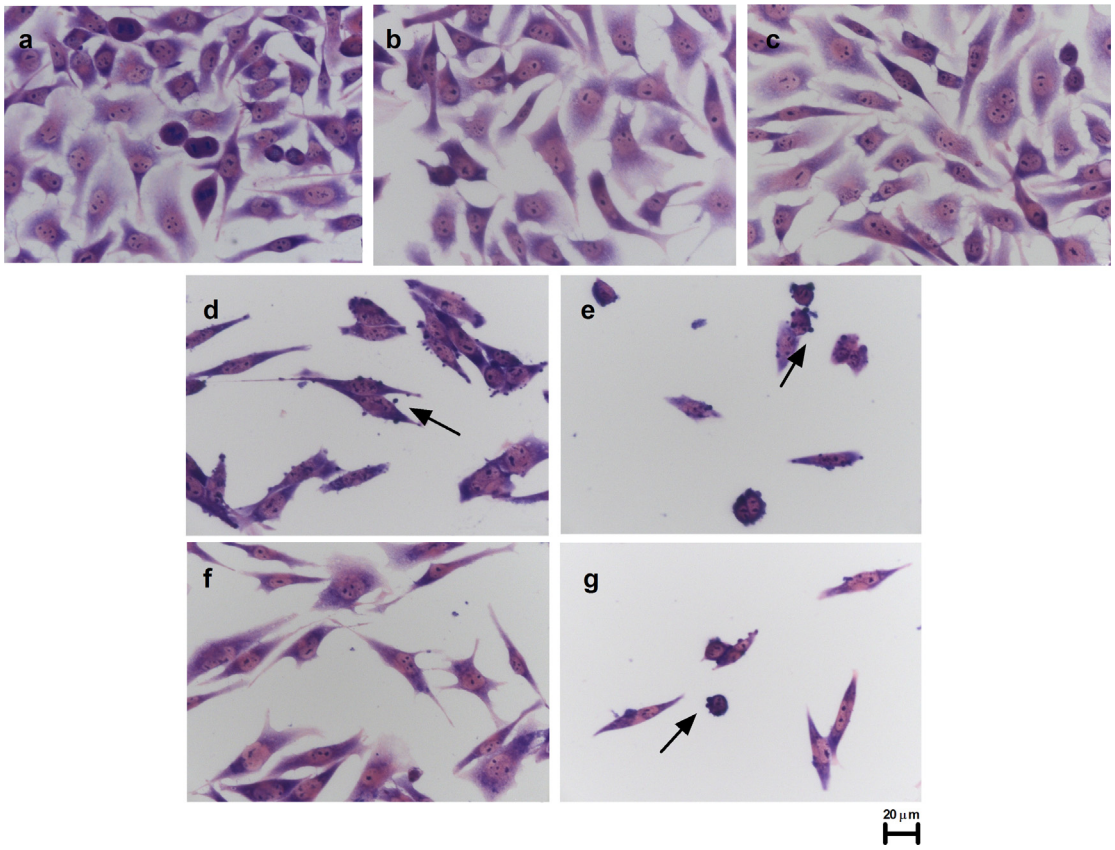

Fig. 5. Representative photography of B16F10 Giemsa staining after 24 h of treatment. Cells were treated as stated in Section 2.8. (a) Control, non-treated cells. (b) Cells treated with 3% v/v hydro-alcoholic solution. (c) Cells treated with 5% v/v hydro-alcoholic solution. (d) Cells treated with TA 3% v/v. (e) Cells treated with TA 5% v/v. (f) Cells treated with TB 3% v/v. (g) Cells treated with TB 5% v/v. The black arrows indicate possible blebs in the plasma membrane.

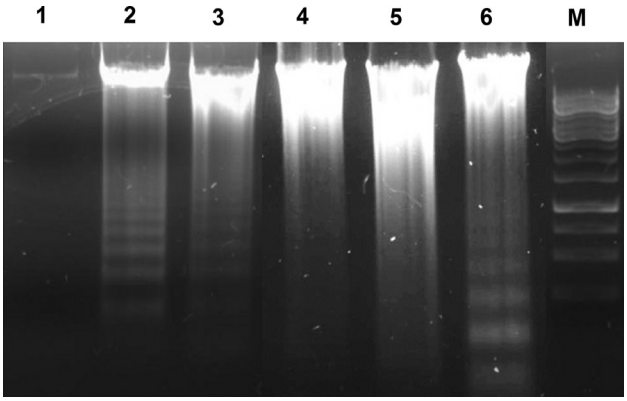

Fig. 6. Genomic DNA fragmentation of B16F10 cells by *Viscum album* tinctures. Lane M: DNA ladder marker; Lane 1: hydro-alcoholic solution 5% v/v 6 h; Lane 2: TB 5% v/v 6 h; Lane 3: TA 5% v/v 6 h; Lane 4: hydro-alcoholic solution 3% v/v 24 h; lane 5: TA 3% v/v 24 h; lane 6: TB 3% v/v 24 h. The figure is representative of 3 (three) independent experiments.

the other hand, cells treated with TA 3% v/v exhibited enriched populations in S and G2/M phases in association with a decline in G0/G1 after 24 h of incubation. Similar effects using plant extracts on cell cycle progression of cancer cells were previously observed. Karimian et al. (2017) reported an arrest of breast cancer cell line LA7 in S phase when treated with *Kelussia odoratissima* methanol extracts. Additionally, the population in G1 phase was decreased and the expression of p21 and p27 were augmented (Karimian et al., 2017). The arrest in S phase was also observed in Caco-2 and HEPG2 cells treated with *Olea europaea* L. ethanol extracts. This effect was accompanied by DNA fragmentation and subsequent cell death induction (Maalej et al., 2017).

Previously, Han et al. (2015) showed a G0/G1 arrest in both B16BL6 and B16F10 melanoma cells treated with Korean mistletoe lectin (*V. album* var. *coloratum agglutinin*) and its extract *in vitro*. They also observed an increase in both early and late apoptosis, and that this was probably caused by an increase in the activation of caspases-1, 3, 4, 5, 6, 7, 8, and 9, in a dose-dependently way, accompanied by a significant decrease in the expression of procaspase-3 and 8 (Han et al., 2015).

Corroborating these and our results, Korean mistletoe lectin (*V. album* var. *coloratum agglutinin*-VCA) increased the survival of mice inoculated with B16-BL6 melanoma cells and inhibited lung metastasis by VCA (Park et al., 2001).

They also showed that treatment of cells with VCA resulted in growth suppression, nuclear morphological changes, DNA fragmentation, and an increased fraction of cells in sub-G1 consistent with apoptosis, confirming our results. When analyzing anti-angiogenesis of VCA, they observed that vessel growth induced by fat emulsion was decreased. These results suggest that VCA has different mechanisms for inhibiting tumor growth and metastasis by increasing apoptosis and also by inhibiting angiogenesis (Park et al., 2001).

In the late decade of 1990, Antony et al. (1997) had yet observed the effects of *V. album* on the inhibition of lung metastatic colony formation induced by B16F10 melanoma cells in mice, and

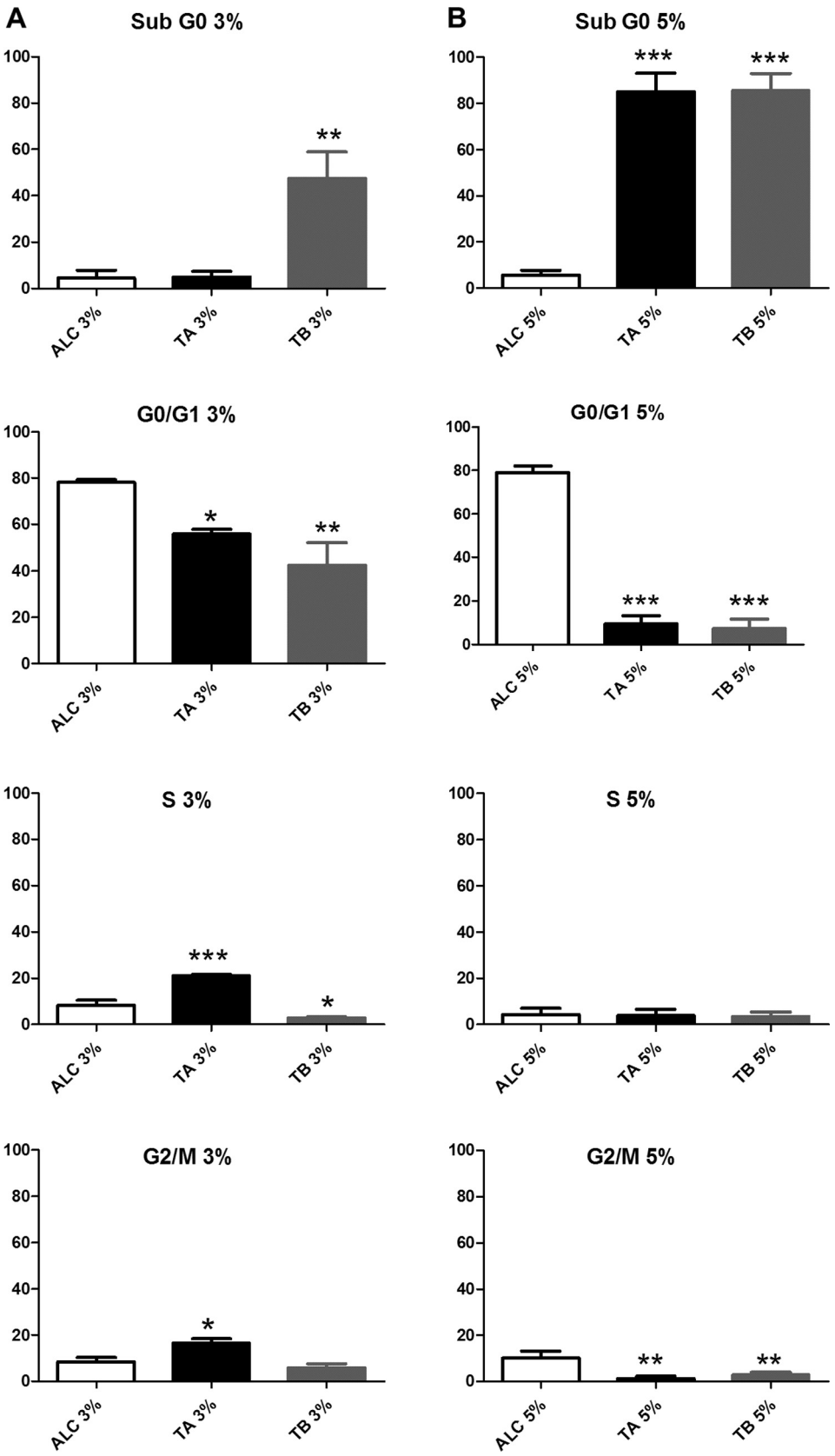

Fig. 7. Effect of *Viscum album* tinctures on B16F10 cell cycle. Cells were treated with TA or TB at concentrations of 3% v/v (Panel A) or 5% v/v (panel B) for 24 h. Cells were incubated with PI and the DNA content was evaluated by flow cytometry, as described in Section 2.11. Data represent the mean ± SD of at least three independent experiments. $^{*}p < .05$; $^{**}p < .001$ and $^{***}p < .0001$, as measured be one-way ANOVA with Dunnet post-test.

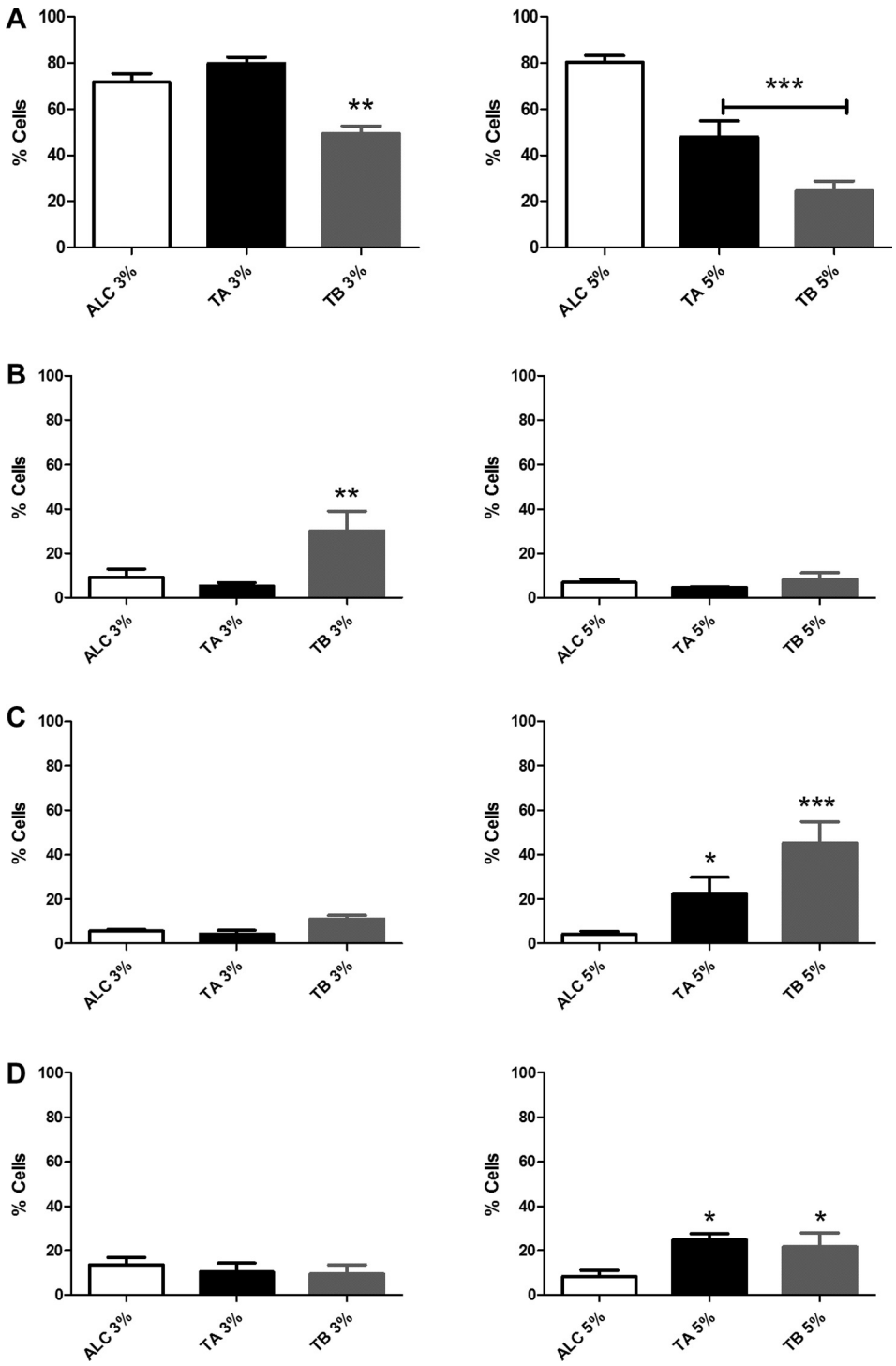

Fig. 8. Apoptosis induced by treatment with TA and TB (3% and 5% v/v) for 6 h. Panel A – cells not labeled with Annexin V-FITC or PI (viable cells). Panel B – cells labeled with Annexin-V/FITC (early apoptosis). Panel C – cells labeled with both Annexin-V/FITC and PI (late apoptosis). Panel D – cells labeled only with PI (necrotic cells). Data are Mean ± SD of at least three independent experiments. $^{*}p < .05$; $^{**}p < .001$ and $^{***}p < .0001$, as obtained by one way ANOVA with Dunnet post-test.

Kuttan et al. (1997) showed the anticarcinogenic and antimetastatic effects of Iscador on methylcholanthrene-induced sarcoma formation in mice, although the mechanisms in both studies were not identified at that time.

Moreover, Zarković et al. (1998) showed that low concentrations of *V. album* commercial extracts (Isorel) inhibited B16F10 and HeLa tumor cell lines more strongly than purified lectin-1. For the authors, the therapeutic effect of Isorel is a result of the association of low and high molecular weight components present in the extracts.

Although the above commented researches observed the effects of lectins in the antitumoral effects of *V. album*, in the present work, we evidenced the effects of *V. album* tinctures in a metastatic melanoma murine (B16F10) model for the first time, confirming the wide variety of chemical compounds in the ethanolic *V. album* and their promising antitumoral potential.

5. Conclusion

V. album tinctures presented anticancer activity against B16F10 and K562 tumor cell lines. Our results showed a selective tumoral cytotoxicity with apoptosis induction and cell cycle effect. Furthermore, the identified compounds chlorogenic acid, caffeic acid, sakuranetin, naringenin 5-methyl ether, syringenin 4-*O*-glucoside (eleutheroside B), syringenin 4-*O*-apiosyl-glucoside (polygalatenoside E), alangilignoside C and ligalbumoside A are the possible contributors to the antiproliferative and apoptotic effects of *V. album* tinctures, suggesting an interesting potential for the pharmacotherapy of cancer. Based on these results, the present authors suggest further toxicologic investigations with *V. album* tinctures as well as preclinical assays in order to evaluate the clinical potential of these preparations.

Conflict of interest

The authors confirm that there are no conflicts of interest associated with this publication.

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Phytochemicals for the Management of Melanoma

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Abstract

Melanoma claims approximately 80% of skin cancer-related deaths. Its life-threatening nature is primarily due to a propensity to metastasize. The prognosis for melanoma patients with distal metastasis is bleak, with median survival of six months even with the latest available treatments. The most commonly mutated oncogenes in melanoma are BRAF and NRAS accounting approximately 60% and 20% of cases, respectively. In malignant melanoma, accumulating evidence suggests that multiple signaling pathways are constitutively activated and play an important role in cell proliferation, cell survival, epithelial to mesenchymal transition, metastasis and resistance to therapeutic regimens. Phytochemicals are gaining considerable attention because of their low toxicity, low cost, and public acceptance as dietary supplements. Cell culture and animals studies have elucidated several cellular and molecular mechanisms by which phytochemicals act in the prevention and treatment of metastatic melanoma. Several promising phytochemicals, such as, fisetin, epigallocatechin-3-gallate, resveratrol, curcumin, proanthocyanidins, silymarin, apigenin, capsaicin, genistein, indole-3-carbinol, and luteolin are gaining considerable attention and found in a variety of fresh fruits, vegetables, roots, and herbs. In this review, we will discuss the preventive potential, therapeutic effects, bioavailability and structure activity relationship of these selected phytochemicals for the management of melanoma.

Keywords

Apoptosis; Cell proliferation; Invasion; Metastasis; Melanoma; Phytochemicals; Signaling pathways

1. INTRODUCTION

Skin cancer is the most common malignancy worldwide with particularly high incidence among fair-skinned populations [1]. Skin cancer poses a major threat to public health as incidence and mortality rates of skin cancers are dramatically increasing [2–4]. Skin cancers

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CONFLICTS OF INTEREST

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are divided into two major groups according to cellular origin as either melanoma (melanocytic) or non-melanoma (epithelial) skin cancers (NMSCs) [5]. The NMSCs are comprised of basal cell carcinomas (BCCs) and squamous cell carcinomas (SSCs) and accounts for 80 and 16 percent of all skin cancer cases respectively. Although both BCCs and SCCs arise from the epidermal basal layer, they have different characteristics [5]. Though BCCs exhibit slow growth and rarely metastasize, their SCCs counterparts metastasize 2 to 5 of the time and carry a poor prognosis if metastasis has occurred [6]. Melanoma, which accounts for only 4 percent of all skin cancers, is a potentially life-threatening skin cancer due to its propensity to metastasize. It claims approximately 80 percent of skin cancer related deaths. The prognosis for melanoma patients with distal metastasis is minimal, with median survival of only six months [7, 8]. Moreover, the incidence of melanoma is rapidly increasing worldwide. The majority of melanoma cancers occur in developed countries such as Europe, Australia, New Zealand and the United States [8, 9]. The World Health Organization reports the annual incidence of melanoma to be approximately 160,000 with an associated 48,000 deaths worldwide each year [10–12]. In recent years, melanoma is the only common cancer with an increasing incidence and death rate. More importantly, the incidence of melanoma in children is also increasing rapidly. Reports estimate that 1 in 50 Americans will be diagnosed with melanoma during their lifetime. According to an estimate from the American Cancer Society, one person dies every hour from melanoma [13, 14]. Furthermore, about 9,940 melanoma-related deaths are projected to occur in the United States in 2015 [15, 16]. A gender-based analysis predicted that approximately 42,670 men and 31,200 women are expected to be diagnosed with melanoma and about 6,640 men and 3,300 women will die from melanoma in the United States in 2015.

Melanoma develops when melanocytes proliferate abnormally and become unresponsive to the regulatory signals from fibroblasts and keratinocytes. The signaling pathways that regulate melanocyte proliferation become aberrantly activated, and thus these cells divide to develop melanoma [17–20]. Melanoma development is characterized by two distinct growth phases. The radial growth phase (RGP) is characterized by the growth of single cells or small clusters of cells confined to the epidermis and extend to the papillary dermis. Alternatively, the invasion of melanoma cells into the dermis and development of tumor-like-nodules or plaques is referred to as vertical growth phase (VGP) [17, 18, 21, 22]. Depending upon the frequency and location, melanoma of the skin can be divided into various types according to clinical and histological growth patterns. However, they all pose the same risk according to their tumor depth, mitotic index (dividing melanoma cells), ulceration and, more importantly, level of spread in the regional lymph nodes [23–25].

Depending upon the clinical and pathological growth patterns melanoma can be divided into four major subtypes [26, 27]. Superficial spreading melanoma is the most common type that accounts for approximately half to three-quarters of all diagnosed melanoma and is most common in fair skinned persons of all ages. Superficial spreading melanoma usually derived from a preexisting benign melanocytic nevus and remain confined to the epidermis (RGP) for an extended time before beginning to VGP [28]. Areas of the skin with the highest nevus density (most commonly found on the back and trunk in men and on the back and legs in women) and with intermittent sun exposure are at high risk for superficial spreading

melanoma. Nodular melanoma is the second most common type that accounts for approximately 15 to 35% of all diagnosed melanomas and is notoriously rapidly-growing and invasive. It usually develops as uniform dark blue-black, blue-red or sometimes colorless bump at the trunk, head and neck areas [29, 30]. Lentigo malignant melanoma is less common subtype of melanoma that accounts approximately 5 to 15% of cases. It occurs on sun-exposed skin of faces of middle aged to elderly adults with a history of photodamage. Lentigo maligna melanoma is slow growing and has a prolonged RGP and is less invasive [31, 32]. Acral lentiginous melanoma is the least common form that accounts for approximately 5 to 10% of all diagnosed melanomas. It develops most commonly in dark-skinned individuals such as in people of Japanese, African American, Latin American, and Native American descents [33]. The most common sites for occurrence of acryl lentiginous melanoma are the soles of the feet, palms and beneath the nail plates [34, 35]. Acryl lentiginous melanomas appear clinically as tan to brown-black, macules and patches with irregular borders and an average size of 3 centimeters [36]. Melanoma can develop anywhere in the body, including the internal organs. Some less common types of melanoma include ocular and mucosal melanoma. Ocular melanoma, which represents 3–5% of all melanoma cases, occurs inside the eyes when melanocytes of the iris or choroid layer begin to proliferate abnormally. Uveal melanoma is the most common form of ocular melanoma and primarily affects light-skinned populations [37]. Mucosal melanoma represents less than 2% of all cases of melanoma and can develop in the nose, mouth, throat, and in the genital areas [38]. This review begins with a review of the gene mutations and dysregulated signaling pathways in melanoma. Beyond this, we explain the preventative and therapeutic effects of key phytochemicals for the management of melanoma in the context of their bioavailability and structure-activity relationship.

2. GENE MUTATIONS AND SIGNALING PATHWAYS ACTIVATED IN MELANOMA

The use of advanced technology in the analysis of the human genome has helped to shed light on specific gene mutations that occur frequently in melanomas and the impact of these mutations on the regulation of signaling pathways. More importantly, studies have confirmed that these mutations control the progression and malignancy of melanomas. Some of the most frequently mutated genes found in in melanoma are described below.

2.1. BRAF Mutations

BRAF, a serine/threonine protein kinase of RAF family (ARAF, BRAF and CRAF), is generally expressed in neuronal tissues and melanocytes [39–41]. It is encoded on chromosome 7q34 and is a component of the mitogen-activated protein kinase (MAPK) pathway. MAPK a pro-growth signaling pathway is activated by cell surface growth factors receptors [42]. BRAF signaling is mutated in approximately 60% of melanomas resulting in induction of genomic instability, driving the proliferation of melanoma cells. The Sanger Institute first described activating mutations in BRAF signaling in 2002 [43]. The most common BRAF mutation (> 90% of known BRAF mutations) results from substitution of glutamic acid (E) in place of valine (V) at codon 600 (a single nucleotide mutation resulting in $BRAF^{V600E}$, nucleotide 1799 T>A; codon GTG>GAG). Mutated $BRAF^{V600E}$ leads to

hyper-activation of MAPK signaling. Another common BRAF mutation ($BRAF^{V600K}$) is the substitution of lysine (K) for valine (V) at position 600, which has been reported more common in some populations [44]. Activating mutation $BRAF^{V600E}$ has been implicated in melanoma progression by activating the downstream MEK/ERK signaling pathway and thus enhancing replicative potential, reducing senescence and apoptosis, and promoting angiogenesis [45, 46]. Activation of this pathway also stimulates cell invasion and metastasis by upregulating proteins involved in migration, cell contractility, as well as evasion of the immune response [47, 48].

2.2. NRAS Mutations

NRAS, another crucial signaling molecule of RAS family (HRAS, KRAS and NRAS), is located upstream of the BRAF/MEK/ERK (MAPK) and PI3K/AKT pathways [49–51]. After BRAF, NRAS is the second most commonly activated oncogene found mutated in 15–25% of all melanomas. Although KRAS mutations are the most common among all human malignancies, NRAS mutations are most frequently found in melanoma [52]. The substitution of an arginine (R) for a glutamine (Q) at position 61 within the NRAS protein is $NRAS^{Q61R}$. The NRAS protein is a small GTP binding proteins (guanosine-5-triphosphate; GTPase) that cleaves bound GTP and thus regulates cellular responses to many extracellular stimuli, including soluble growth factors [49]. Signal transduction through this pathway begins when extracellular growth factors interact with cell surface receptors [53]. This creates intracellular docking sites that then interact with specific adaptor molecules and signal transducing proteins leading to activation of guanine nucleotide-exchange factors (GNEFs). GNEFs remove guanine nucleotides from NRAS and allow passive binding to GTP, which is abundantly present in the cytosol. GTP bound NRAS then interacts with downstream effector molecules including BRAF, PI3K and others to regulate proliferation, survival and differentiation. Although NRAS mutations are distinct from BRAF, they both cause the constitutive activation of MAPK and PI3K/AKT signaling pathways [54, 55]. Melanomas with NRAS mutations develop higher-grade tumors with higher mitotic and invasive potentials than the BRAF mutated melanomas [56, 57]. BRAF and NRAS mutations can be detected at early stages of melanoma progression and remains activated throughout the progression of disease [58, 59]. It is well established that these mutations are almost never simultaneously present and, alone, are insufficient to initiate melanoma development [60].

2.3. PTEN Mutations

PTEN (phosphatase and tensin homologue), a tumor suppressor gene located at 10q23–24, is mutated in 30–50% of melanoma cell lines and in 5–20% primary melanomas [61–63]. PTEN functions as a dual specificity phosphatase with the ability to dephosphorylate both proteins and lipids. It can dephosphorylate phosphoserine and phosphotyrosine residues in proteins, and convert the lipid phosphatidylinositol 3,4,5-trisphosphate (PIP3) to phosphatidylinositol 4,5-biphosphate (PIP2) via dephosphorylation. As a protein phosphatase, PTEN suppress MAPK signaling through dephosphorylation of focal adhesion kinase (FAK), and regulates cell-to-cell adhesion and thus inhibits cell survival, proliferation, migration and invasion [64–66]. PTEN's lipid phosphatase activity is considered as its primary and major function. PTEN converts PIP3 to PIP2 and thus

negatively regulates the PI3K/AKT signaling pathway that is important for cell proliferation, survival, and apoptosis [67–69].

2.4. p53 Mutations

The tumor suppressor, p53, is regarded as the guardian of the genome. It responds to variety of stress stimuli, including DNA damage and hypoxia [70, 71]. p53 acts as a transcription factor for a variety of genes related to DNA repair, cell cycle progression, apoptosis and the maintenance of cellular homeostasis [72]. Mutations in the p53 gene are associated with more aggressive phenotypes in various human cancers. p53 gene mutation analysis in melanoma established that approximately 10% of all melanomas harbor p53 mutations [73, 74]. Most of these mutations are ultraviolet radiation-induced mutations [75–77]. Furthermore, higher frequency of p53 inactivation in wild type BRAF and NRAS melanoma exhibited low ERK activity, suggesting MAPK independent melanoma progression [78, 79]. Although the frequency of p53 mutations in melanomas is low, various studies have demonstrated p53's major role in the suppression of progression from nevus to melanoma [80, 81]. Several studies of superficial spreading melanoma have also found a longer relapse-free-survival rate among patients whose tumors expressed wild type p53 [82, 83].

2.5. CDKN2A/p16 Mutations

CDKN2A/p16 (also known as CDK4I or INK4a), a tumor suppressor gene associated with red hair and freckles, encodes a cyclin-dependent kinase (CDK) inhibitor p16. CDKN2A/p16 is responsible for cell cycle arrest at G1 checkpoint prior to the DNA synthesis (S) phase [84–86]. The CDKN2A/p16 protein inhibits phosphorylation and activation of retinoblastoma (Rb) proteins by CDK4 and CDK6. Since non-functional or inactivated CDKN2A/p16 shows reduced binding with CDK4, phosphorylation and activation of Rb occurs, resulting in abnormal cell proliferation [87, 88]. Cytogenetic, linkage and molecular analyses of the 9p21 region in familial and sporadic melanoma with multiple primary lesions has implicated CDKN2A/p16 as melanoma susceptibility gene [89]. CDKN2A/p16 mutations have been detected in variety of tumors including melanomas, providing evidence for CDKN2A/p16 involvement in the development of these malignancies [90, 91]. A study of 60 melanoma cell lines demonstrated that 92% of melanoma cell lines had aberrantly activated CDKN2A/p16 or CDK4. In the same study, 80% cell lines carried either non-function p16 or absent in p16 [92]. More importantly, the presence of CDKN2A/p16 germline mutations are not only associated with melanoma but also increase the risk of other malignancies such as pancreatic and breast cancer [54]. According to an estimate 5–10% of melanomas are known to be hereditary, and among those only 20–40% are associated with a pathogenic mutation in CDKN2A/p16 [93]. Frequency of CDKN2A/p16 mutation in familial melanoma ranged from 8–50% established after screening of 230 melanoma families worldwide for germline mutation in CDKN2A/p16 [94]. Furthermore, Americans who carry a CDKN2A/p16 mutation have an estimated 76% lifetime risk of developing melanoma [95]. Due to the exceptionally high probability of developing melanoma in the CDKN2A/p16 mutated population with poor prognosis of late-stage disease, it may be useful to institute a screening process to identify and warn CDKN2A/p16 mutation carriers of their high-risk status before the development of

melanoma [96–98]. Recent studies have implicated that CDKN2B mutation is also involved in the promotion and progression of benign melanocytic nevi to melanoma [99].

3. PHYTOCHEMICALS FOR THE PREVENTION/TREATMENT OF MELANOMA

The role of diet and nutrition in the prevention of disease has gained public attention recently. A growing body of scientific evidence has established the protective effect of dietary manipulation, especially the use of dietary supplements to protect the skin from various diseases such as cancer. Epidemiologic studies have also addressed the role of dietary factors in melanoma prevention. Since these dietary phytochemicals are safe and carry minimal toxicity, they hold promising potential as complementary therapies for the treatment of melanoma as well [100–103]. In the present review, we have explored the protective and therapeutic potential of dietary phytochemicals against cell proliferation, apoptosis, invasion and metastasis by targeting signal transduction pathways (Table 1 and Fig. 1). These phytochemicals includes fisetin, epigallocatechin-3-gallate, resveratrol, curcumin, proanthocyanidin, silymarin, apigenin, capsaicin, genistein, indole-3-carbinol, and luteolin.

3.1. Fisetin

Fisetin (3,3′,4′,7-Tetrahydroxyflavone) (Fig. 2A), a naturally occurring flavonoid, is commonly found in various fruits and vegetables such as strawberries, mangoes, kiwis, apples, grapes, persimmons, cucumbers and onions [104, 105]. Fisetin has shown to possess antioxidant, anti-inflammatory, and anti-proliferative properties against various cancers including melanoma and non-melanoma skin cancers [106–110]. Treatment of various malignant melanoma cell lines carrying different genetic characteristics (BRAF-mutant, NRAS-mutant, BRAF-NRAS wild type) with fisetin inhibited their invasion. Strengthening this finding, fisetin also decreased the invasive potential of melanoma cells in experiments using three-dimensional human skin equivalents. The anti-invasive effects of fisetin were associated with a decrease in the phosphorylation of MEK1/2 and ERK1/2 as well as inhibition of nuclear factor kappaB (NFκB) signaling pathway. Fisetin treatment also promoted mesenchymal to epithelial transition (MET) by decreasing mesenchymal marker proteins and increasing epithelial marker proteins [108]. Syed *et al.* [111, 112] observed downregulation of Wnt/β-catenin, PI3K/AKT, mTOR, and microphthalmia-associated transcription factor (MITF) signaling proteins in melanoma cell lines and in a three-dimensional human skin equivalent melanoma model. These findings show that fisetin is a phytochemical with promising anti-melanoma activities.

3.1.1. Bioavailability of Fisetin—Murine investigations have not been able to demonstrate any measurable toxicity of the phytochemical, fisetin [113, 114]. Bioavailability studies have demonstrated that fisetin was readily absorbed with detectable levels in the serum of mice [113, 115–117]. Pharmacokinetics studies involved conversion of fisetin-loaded dimyristoylphosphatidylcholine liposomal vesicles into nanocochleates by the action of Ca^{2+} ions. Analysis following intraperitoneal injection of nanocochleates showed a 141-fold higher relative bioavailability in mice [115]. In another study, liposomal encapsulation

of fisetin increased bioavailability by 47-fold and enhanced the anti-tumor potential when compared to free fisetin [116]. Furthermore, intraperitoneal administration of the fisetin nano-emulsion resulted in a 24-fold increase of relative fisetin bioavailability [113].

3.1.2. Structure Activity Relationship of Fisetin—The basic flavonoid structure is two benzene rings (A- and B-ring) linked through a central heterocyclic pyrane (also known as pyrone or C-ring). The 2-position of the pyrone ring is generally with B-ring. The presence of an oxy group at position 4, a double bond between carbon atoms 2 and 3 (C2=C3 double bond), and a hydroxyl group at position 3 (3-OH) of the C-ring determine the type of flavonoid compound. Flavonols (e.g., quercetin, myricetin, quercetagetin, fisetin) all have an oxygen group at position 4, a C2=C3 double bond, and a 3-hydroxyl (3-OH) group and are known to possess anti-cancer properties [118]. Furthermore, fisetin, has 3′ - and 4' -OH on the B-ring, and elimination of 3' -OH on the B-ring, 4',5,7 trihydroxyflavone (THF) (Fig. 2B), has been shown to reduce the anti-cancer potency of fisetin. The important structural features of flavonoids (3'- and 4' -OH on the B-ring, 3-OH on the C-ring, the C2=C3 double bond in the C-ring and the phenylchromone C6–C5–C6) promote inhibition of epidermal growth factor (EGF)-induced cell transformation [118]. In another study, the structure activity relationship of fisetin was examined using different derivatives of the flavonoid. Sagara *et al.* [119] evaluated four trihydroxyflavones (THF), lacking one hydroxyl group, and three dihydroxyflavones (DHF), lacking two hydroxyl groups. All the derivatives tested promoted nerve cell differentiation and protected nerve cells from oxidative stress induced death, although there was a significant difference in both potency and efficacy. The 3,3',4' THF (Fig. 2C) most effectively induced differentiation, accomplishing this in >80% cells. Furthermore, Akaishi *et al.* [120] recently reported that the 3',4'-dihydroxyl (Fig. 2D) group is essential for the inhibitory effect of fisetin on amyloid beta protein fibril formation.

3.2. Epigallocatechin Gallate (EGCG)

Green tea (*Camellia sinensis*) leaves contain many polyphenols such as flavanols (catechins), flavandiols, and phenolic acid. Among them, the four main catechins present in green tea leaves are (−)-epigallocatechin gallate (EGCG), (−)-epicatechin gallate (ECG), (−)-epigallocatechin (EGC) and (−)-epicatechin (EC) (Fig. 3A–D). Epidemiological studies suggested that regular consumption of green tea attenuates the risk of many cancers. Green tea polyphenols and EGCG are known to have antioxidant, anti-mutagenic, anti-inflammatory, and anti-carcinogenic activities. [121–123]. Evidence shows that EGCG is more potent than other catechins in reducing the human melanoma cell growth [124]. Most importantly, neither EGCG nor other catechins have any effect on growth of normal melanocytes [125]. EGCG inhibits growth, promotes cell cycle arrest, and induces apoptosis of melanoma cells by modulating cyclin-dependent kinase network and Bcl-2 family proteins [125, 126]. In addition, combining EGCG with interferon/vorinostat therapy enhanced its efficacy against melanoma by targeting NFκB signaling pathways. Combination of EGCG/vorinostat significantly inhibited melanoma cell proliferation and increased apoptosis through activation of cell cycle inhibitory proteins, modulation of Bcl-2 family proteins, and NFκB signaling pathway. A recent study established that treatment of melanoma cells with EGCG at physiological dose reduced melanoma growth by inhibiting NFκB activity [127]. This was correlated with decreased IL-1β secretion. In addition,

numerous investigations have demonstrated that EGCG inhibited many pro-inflammatory enzymes and cytokines such as iNOS, COX-2, MMPs, IL-6, IL-8, IL-12 and TNFα [128, 129]. EGCG-induced IL-1β suppression was mediated by downregulation of the inflammasome, decreased nuclear localization leucine-rich-repeat protein 1 (NLRP1), and reduced caspase-1 activation.

In a recent study, Singh and Katiyar [130] demonstrated the anti-invasive potential of various green tea catechins on human melanoma cell invasion. They showed that EGCG carried the greatest inhibitory effect of the green tea catechins, with lesser effects from EGC > ECG, GC and EC. EGCG was found to inhibit melanoma cell invasion by decreasing EMT through reduced COX-2 expression, PGE_2, and PGE_2 receptors in melanoma cells [130]. Overall these findings revealed signaling pathways by which EGCG may inhibit invasion of melanoma cells. Thus this non-toxic, dietary component of green tea, EGCG, possesses antioxidant, anti-inflammatory, anti-carcinogenic potential, which makes it a logical candidate molecule for melanoma prevention and therapeutics.

3.2.1. Bioavailability of EGCG—Centuries of tea consumption provide evidence of EGCG's safety in humans. Phase I clinical investigations have demonstrated that catechins and polyphenon E in doses ranging from 200 to 1200 mg were well-tolerated. Murine investigations found a broad range of bioavailability from 26.5% in mice to 1.6% in rats [131–133]. HPLC studies have revealed levels ranging from 5 to 150 ng/ml of EGCG, EC, and ECG in serum after oral tea consumption [134, 135]. Some bioavailability investigations have postulated that the low bioavailability may be because of the large size of the compounds [136]. The bioavailability of tea catechins in humans is relatively low at a range of only 0.2 to 2% [133, 137–139]. Oral administration of 1.5 mM of EGC, ECG and EGCG resulted in an average plasma concentration of 5 μM, 3.1 μM, and 1.3 μM respectively [140]. Oral consumption of 100 to 1600 mg of pure EGCG resulted in 0.26 to 6.35 μM of EGCG detectable in plasma after 2 to 3 hrs [141]. These studies show that tea catechins reach human plasma at the micromolar level (less than 1–2%) while the metabolites are present to a greater extent in human plasma after oral consumption of green tea [136, 142].

3.2.2. Structure Activity Relationship of EGCG—The catechins are the major components of green tea. These catechins contain a benzopyran skeleton with a hydroxyl or ester moiety at the 3-position and a phenyl group substituted at the 2-position. Catechins are classified by 2,3 subsituents and by the number of hydroxyl groups in the B- and D-ring [143]. In a recent study, using 10 different polyphenols demonstrated that anti-cancer potential of polyphenols such as catechin and epicatechin increases significantly if they were esterified with gallic acid to produce catechin gallate and ECG [144]. Among the 10 polyphenols, EGCG was most potent in inhibiting cell growth and inducing apoptosis. Although epicatechin has not demonstrated anti-proliferative effects, the catechin and epicatechin were found to significantly inhibit proliferation after esterification with gallic acid to form gallate and ECG. Similarly, gallic acid group of EGCG significantly enhanced catechin's anti-cancer potential. This property may be used to synthesize flavonoid derivatives to develop novel anti-cancer agents in the future [144]. Khandelwal *et al.* established that EGCG functions as an Hsp90 inhibitor [145]. They found that the prenyl-

substituted aryl ester of 3,5-dihydroxychroman-3-ol ring system act as a novel scaffold that exhibits greater Hsp90 inhibition than EGCG. The abilities of various green tea polyphenols to inhibit cell growth, RAS signaling, and activator protein-1 (AP-1) activity were compared. With the exception of epicatechin, all of the tea polyphenols showed strong inhibition of cell growth and AP-1 activity. Among these compounds, both the galloyl structure on the B-ring and the gallate moiety inhibited growth and AP-1 signaling with the galloyl structure contributing the strongest effects. Catechin epimers such as theaflavin-3,3'-digallate, inhibited the phosphorylation of p38. EGCG decreased levels of c-Jun, while theaflavin-3,3'-digallate decreased the level of fra-1. Based on the results they suggested that catechins and theaflavins inhibited AP-1 activity and the MAPK pathway through different mechanisms [146]. EGCG inhibited the chymotrypsin-like activity of the proteasome organelle both *in vitro* and *in vivo* at concentrations equivalent to that found in the serum of green tea drinkers. Atomic orbital energy analyses and HPLC demonstrated that the carbon of the polyphenol ester bond is necessary for inhibition of proteasome activity in cancer cells [147, 148]. In an effort to discover more stable polyphenol proteasome inhibitors, Landis-Piwowar *et al.* [149] synthesized several novel EGCG analogs and observed that elimination of hydroxyl groups from either the B- or D-ring decreased proteasome inhibition *in vitro*. Another group developed alkyl gallate and gallamide derivatives with strong anti-proliferative and apoptotic activity against human cancer cells by using the EGCG-derived galloyl group as a core structure [150]. Dodo *et al.* [150] the pioneers of the aforementioned technique, subsequently added a galloyl group into alkyl gallates and gallamides to synthesize various bisgallate and bisgallamide derivatives. This enhanced the anti-proliferative effects of gallamides but not alkyl gallates.

3.3. Resveratrol

It is a polyphenolic phytoalexin stilbenoid derivative of stilbene (Fig. 4A), produced naturally by a wide variety of plants such as grapes, peanuts, mulberries, cranberries, and eucalyptus. Resveratrol is known to possess cardio-protective, antioxidant, neuroprotective, immunomodulatory, anti-inflammatory, metabolism-regulating, and anti-cancer effects [151]. An accumulating body of evidence has shown that resveratrol inhibits cell growth and induces apoptosis in various melanoma cells by S-phase cell cycle arrest and down-regulation of cyclins [152, 153]. In addition, Caltagirone *et al.* [154] found that intraperitoneal administration of resveratrol delayed the tumor growth in mice. Fang *et al.* [155] recently found that resveratrol enhances the sensitivity of melanoma cells to radiation by inhibiting proliferation and inducing apoptosis. The anti-proliferative effects of resveratrol following radiation were associated with decreased expression of the proliferative molecules. Decreased expression of the anti-apoptotic molecules cFLIP, Bcl-2 and survivin correlated with the apoptotic effects of resveratrol after radiation. In a recent study, Gatouillat *et al.* [156] showed that resveratrol inhibited the growth of a doxorubicin-resistant B16 melanoma cells through inhibition of the G1/S phase transition, down-regulation of cyclin D1/cdk4, and increased expression of p53, which ultimately induced apoptosis. Furthermore, resveratrol reduced the growth of doxorubicin-resistant B16 melanoma cells in mice and prolonged survival of mice compared to untreated controls. In studies of DR5-positive melanoma cells, Ivanov *et al.* [157] found that resveratrol decreased signal transducer and activator of transcription 3 (STAT3) and NFκB activation, suppressed Bcl-xL

and cFLIP proteins, and enhanced cellular sensitivity to exogenous TRAIL. Furthermore, upregulation of the α-melanocyte-stimulating hormone (α-MSH) signal transduction pathway involving Wnt/β-catenin, c-kit, and MITF were suppressed by resveratrol [158]. These signaling pathways have well-established roles in the immortality, viability, and invasiveness of melanoma cells [159]. In addition, Lee *et al.* [160] found that resveratrol inhibited STAT3 acetylation, which is markedly increased in melanoma cells when compared to normal melanocytes. Inactivation of the estrogen receptor alpha (ERα) gene via methylation strongly correlates with poor prognosis as well as an aggressive phenotype of melanoma [159]. Resveratrol reactivates gene expression in tumors by reducing STAT3 acetylation [158]. In addition, it triggers protective autophagy by increasing signaling through the ceramide/AKT/mTOR pathway in B16 melanoma cells [161]. In a recent study, Trapp *et al.* [162] found that resveratrol stimulated isolated human umbilical vein cell (HUVEC) proliferation, however it resulted in growth inhibition of HUVECs grown with melanoma cells in three-dimensional co-culture and in three-dimensional spheroids. This selective effect of the compound on melanoma cells was associated with increased p53 expression and matrix protein thrombospondin-1. Resveratrol inhibited vascular endothelial growth factor (VEGF) production through enhanced expression of hypoxia inducible factor-1α. Furthermore, resveratrol reduced hepatic metastatic invasion of B16M melanoma cells inoculated intra-splenically by decreasing vascular adhesion molecule 1 expression in the hepatic sinusoidal endothelium. This consequently decreased B16M cell adhesion to endothelial cells through late activation of antigen 4. Resveratrol was found to inhibit the nuclear translocation and transcriptional activity of NFκBp65, an important inducer of EMT. This lead to significantly prolonged animal survival time and reduced melanoma tumor sizes *in vivo*. This was associated with inhibition of lipopolysaccharide induced tumor migration and decreased markers of EMT [159, 163]. These data support a potential use of mono or combination resveratrol therapy for the management of melanoma.

3.3.1. Bioavailability of Resveratrol—The bioavailability and pharmacological activity of resveratrol in rabbits, rats, and mice models have been reported after intravenous and oral administration [151]. Oral treatment of mice with 20 mg/kg resveratrol yielded the highest plasma concentration (2.6 ±1.0μM) after 5 min. In another study, Asensi *et al.* [164] reported that intravenous administration of resveratrol (20 mg/kg) to rabbits yielded its maximum concentration in plasma (42.8 ± 4.4 μM) 5 min after administration, but reduced rapidly to 0.9 ± 0.2 μM (0.2 mg/l) at 1 hr. More importantly, when the same quantity of resveratrol is administered orally, the maximum concentration reached in plasma (2–3 μM in mice and about 1 μM in rabbits or rats) within the first 5 min after administration followed by a decrease in its level to less than 0.1 μM at 1 hr [165–167]. Compounds such as glucuronides or sulfates are the most common resveratrol metabolites found in human plasma or urine following oral consumption [168, 169]. In another studies, administration of single dose of 0.5 g to 5 g resveratrol in 40 healthy volunteers showed that free resveratrol is rapidly absorbed with a comparatively low mean plasma concentration from 73 ng/mL (50.3 mmol/L) to 539 ng/mL (52.4 mmol/L) respectively [168–170]. After 5–6 hrs, the concentration of resveratrol metabolites [resveratrol-3-O-sulfate (with a maximum concentration of 1135–4294 ng/mL (3.7–14 mmol/L) and monoglucuronides] was 20 fold high than the free resveratrol with plasma half-life of 2.9 – 11.5 hrs.

3.3.2. Structure Activity Relationship of Resveratrol—Resveratrol has demonstrated its potential to inhibit melanoma growth by targeting various signaling pathways [153–157, 159, 171]. Murias *et al.* [172, 173] synthesized resveratrol derivatives by increasing the number of hydroxyl groups on the phenol rings of the stilbene structure and evaluated their anti-tumor potential against human cancer cell lines. The cytostatic activity exhibited by hydroxystilbenes with ortho-hydroxyl groups was three-fold higher as compared to hydroxystilbenes with other moieties. Hydroxylated resveratrol analogs exhibited COX-2 inhibition, while methoxylated resveratrol did not exhibit these effects. In addition, resveratrol derivatives such as 3',4′,3,5-tetrahydroxy-trans-stilbene (piceatannol), 3,4,4′,5-tetrahydroxy-transstilbene and 3,4,5,3′,4′,5′-hexahydroxy-trans-stilbene (Fig. 4B,C,E) demonstrated a 6,600-fold anti-radical activity above resveratrol. In another study, Lee *et al.* [174] found that the hydroxyl group at the meta position of the B-ring is crucial for MEK/ERK inhibition in the resveratrol analogue, 3,5,3′,4′,5′-pentahydroxy-trans-stilbene (Fig. 4D), which acts by inhibiting cell transformation. An accumulating body of evidence has demonstrated that increasing the number of hydroxyl groups on the ring-structure further enhances the cytotoxic and free-radical-scavenging effects of resveratrol [172–175]. Furthermore, Murias *et al.* [172, 173] reported that hexahydroxystilbene (M8) (Fig. 4E) was the most effective resveratrol analogue against various cancer cell lines including melanoma through inhibition of deoxynucleosidetriphosphate formation (dATP and dTTP) by inhibition of enzyme ribonucleotide reductase. Moreover, hexahydroxystilbene (M8) was active in inhibiting the melanoma growth in two different melanoma animal models. Wachek *et al.* [176] demonstrated that hexahydroxystilbene (M8) was effective in sensitizing malignant melanoma *in vivo* as a single compound and in combination with dacarbacine. In this study, animals that were treated with a combination of hexahydroxystilbene (M8) and decarbacine were found to be tumor free due to synergistic action of these compounds. Furthermore, hexahydroxystilbene (M8) inhibited cell migration in matrigel assays. In addition, hexahydroxystilbene (M8) inhibited melanoma tumor growth and prevented metastasis of melanoma cells to distant lymph nodes in the SCID mouse model [177–179]. Monohydroxylated resveratrol (also, piceatannol) possesses better anti-tumor activity than resveratrol itself and is a potent COX-2 inhibitor. Piceatannol has also been found to induce apoptosis of SK-MEL-28 melanoma cells by downregulating cyclins A, E and B1 (causing cell cycle arrest at the G2 phase) and by inhibiting NFκB signaling [152]. In fact, Ashikawa *et al.* [180] demonstrated that the hydroxyl groups of stilbenes are critical for suppression of NFκB activation. Studies using human melanoma cells demonstrated that 3,4,5,4′-tetramethoxystilbene (DMU-212) (Fig. 4F) exerts it anti-mitotic effect by ERK1/2 activation [181]. Moreover studies have demonstrated that methylation at key positions of the cis-resveratrol (Fig. 4G) form results in more potent methylated analogs of cis-resveratrol (cis-3,5,4′-trimethoxystilbene) (Fig. 4H) with enhanced anti-cancer properties [182].

3.4. Curcumin

Curcumin (Fig. 5A) is a hydrophobic, polyphenolic, bioactive compound extracted from the rhizome of the dietary spice turmeric (*Curcuma longa*). Turmeric, commonly found in curry powder, has been used for centuries in indigenous medicine as it possesses wide-range of anti-inflammatory, antioxidant, and anti-cancer properties [183]. The chemotherapeutic potential of curcumin is evident by its known abilities to induce apoptosis and cell adhesion

as well as inhibit angiogenesis in cancer cells. Phase I and phase II clinical trials have established its safety and therapeutic efficacy in cancer patients [184]. The compound was found to exert its anti-cancer effects by targeting AKT [185], NFκB [186], AP-1 [187], and c-Jun N-terminal kinase [188].

In melanoma cells, curcumin has been found to induce apoptosis through several mechanisms including upregulation of p53, p21(Cip1), p27(Kip1), and checkpoint kinase 2. It also decreases cancer cell survival by downregulating NFκB, iNOS, and DNA-dependent protein kinase catalytic subunit expression [189]. Furthermore, curcumin treatment of melanoma cells attenuated NFκB binding activity without suppressing the BRAF kinase pathway or AKT phosphorylation. Evidence suggests that curcumin decreases tumor growth by inhibiting glutathione S-transferase, inducing apoptosis through the Fas receptor/caspase-8 pathway, inhibiting COX enzymes, and by downregulating NFκB signaling [190–192]. Endoplasmic reticulum (ER) stress triggers curcumin to activate death pathways through p23 cleavage and downregulation of the anti-apoptotic Mcl-1 protein in melanoma cells [193]. Furthermore, the anti-metastatic potential of curcumin worked by downregulating collagenase activity, FAK expression, and MMP-2 function. It also modulated integrin receptors and upregulated the expression of E-cadherin [194]. Curcumin also has the potential to reverse melanoma cell multi-drug resistance by inhibiting glutathione-S-transferases [195, 196]. Elevated expression of the phosphatase of regenerating liver 3 (PRL-3), a member of tyrosine phosphatase family, has been found in highly metastatic melanomas. PRL-3, an oncogene that promotes tumor cell motility and invasion [197, 198], was downregulated by curcumin [199], thus raising its profile as a possible anti-cancer agent.

Curcumin suppresses osteopontin (OPN)-induced IκBα phosphorylation and degradation by inhibiting the IKK activity in B16F10 murine melanoma cells. Furthermore, curcumin inhibited the OPN-induced translocation of NFκBp65, NFκB-DNA binding, and NFκB transcriptional activity along with reduction of MMP activities. As a consequence of reduction in the NFκB signaling pathway, it synergistically induced apoptosis by inhibiting OPN-induced cell proliferation, migration and invasion. More importantly, curcumin suppressed the OPN-induced tumor growth in nude mice [200, 201]. Oral administration of curcumin in melanoma-tumor-bearing mice has been found to inhibit the lung metastasis of melanoma by as much as 80%, thus lengthening the survival of mice by 144% [202, 203].

3.4.1. Bioavailability of Curcumin—Pharmacological studies have shown that curcumin is non-toxic and effective in the treatment and prevention of many diseases. Epidemiological and scientific evidence have shown its beneficial health effects as antioxidant, anti-inflammatory and anti-cancer agent [204]. In one study, oral administration of curcumin 2g/kg in experimental rats resulted in 1.35±0.23 μg/ml peak serum concentration after 0.83 hr, though the same dose in humans resulted in very low serum levels (0.006±0.005 μg/ml) after 1 hr [205]. In another study, Cheng *et al.* [206] reported that 4–8 g/kg oral administration of curcumin to humans resulted in 0.41–1.75μM/ml peak plasma concentration after 1 hr. Clinical trials demonstrated a 11.1 nmol/L plasma concentration in participants one hour after 3.6 g/kg oral administration of curcumin [207]. However,

intravenous administration of curcumin in rats showed a 50-fold higher serum concentration when compared to oral administration [208].

3.4.2. Structure Activity Relationship of Curcumin—Curcumin, a natural diaryl heptanoid, can be obtained from substituted aryl aldehydes and acetylacetone, which enables the synthesis of a diverse set of curcumin analogs. Numerous analogs have been synthesized and are being tested against known biological targets to improve upon the pharmacological, absorption, distribution, metabolism and secretion profile by modifying substitutions on its aromatic rings, the β-diketone moiety, and the two flanking double bonds that are conjugated to the β-diketone moiety. This has led the way for researchers to further study the structure-activity relationship of curcumin in medicinal chemistry [209, 210]. Interestingly, Pisano *et al.* [211] recently showed that a new curcumin analog alpha,beta-unsaturated ketone D6 [(3E,3'E)-4,4'-(5,5',6,6'-tetramethoxy-[1,1'-biphenyl]-3,3'-diyl)bis(but-3-en-2-one)] (Fig. 5E) was more effective at inhibiting melanoma cell growth and inducing apoptosis *in vitro* and *in vivo* when compared to curcumin [211, 212]. Other curcumin analogs (FLLL32 and FLLL62) (Fig. 5C–D) reduced STAT3 phosphorylation resulting in apoptosis induction at micromolar concentrations in human melanoma cell lines. Furthermore, treatment with these analogs inhibited STAT3 target genes expression, uncoupled mitochondrial membrane potential and induced caspase-mediated apoptosis [213, 214]. The DM-1 compound (Sodium 4-[5-(4-hydroxy-3-methoxyphenyl)-3- oxo-penta-1,4-dienyl]-2-methoxy-phenolate) (Fig. 5B) is a curcumin analog that possesses curcumin's anti-proliferative, anti-tumor, and anti-metastatic characteristics [215]. A recent study by Zhang et al. [216] demonstrated that EF24 (diphenyl difluoroketone) (Fig. 5F) suppressed melanoma cell migration and EMT by targeting HMGA2.

3.5. Proanthocyanidins

Proanthocyanidins, the secondary metabolites of plants are present in various fruits and plant-derived beverages such as cocoa, grapes, apple, tea and red wine [183, 217, 218]. Proanthocyanidins belong to a class of condensed tannins made of (+)-catechin, (−)-epicatechin and other flavonoid oligomers and polymers. Linkages between these oligomers and polymers typically consist of B type (C4→C6 or C8) or A-type bonds (C2→O7). Grape seed extract proanthocyanidins (GSPs), the most common proanthocyanidin consumed by humans, contain B-type linkages. Grape seeds are considered as a good source of polyphenolic proanthocyanidins and procyanidins. GSPs exhibit antioxidant and anti-inflammatory properties [183, 219, 220]. GSPs are predominantly composed of proanthocyanidin, catechin or epicatechin oligomers [219]. Although GSPs have been shown to have antioxidant, photo-protective, and anti-tumor effects, their chemotherapeutic/preventive effects on melanoma are yet to be explored. In a recent study, Vaid *et al.* [221] demonstrated the effect of GSPs on melanoma cell invasion and delineated the molecular mechanism underlying these effects. GSPs inhibited melanoma cell invasion through decreased COX-2 expression and PGE_2 production. It also decreased cellular migration and phosphorylation of ERK1/2 induced by 12-O-tetradecanoylphorbol-13-acetate. GSPs also inhibited the activation of NFκBp65. Inhibition of melanoma cell invasion by GSPs increased the levels of epithelial biomarkers with a concomitant loss of mesenchymal biomarkers in melanoma cells, indicating that inhibition of invasion was related with

reversal of the EMT process [221]. In addition, a murine study found grape seed extract administration to be associated with significantly fewer metastatic pulmonary melanoma nodules (a 26% decrease) when compared to controls [222].

3.5.1. Bioavailability of Proanthocyanidins—In the United States, the average dietary intake of proanthocyanidins has been estimated to be 58 mg/day [223, 224]. Absorption of monomeric flavonoids including (+)-catechin and (−)-epicatechin following the consumption of dietary proanthocyanidins has been reported in humans and animals, either as parent compounds or conjugated metabolites. There have been limited and conflicting bioavailability studies of larger proanthocyanidins to-date [225, 226]. In a study, Deprez *et al.* [227] demonstrated that human microbiota degrade proanthocyanidins to low molecular weight phenolic compounds. Moreover, appearance of simpler compounds such as benzoic acids, phenylpropionic acids, phenylacetic acids, phenylvaleric acids, phenylpropionic acids, and phenylacetic acids in the plasma and urine have been reported after proanthocyanidin melabolism [228, 229]. Animal and human studies have demonstrated the detection of unconjugated procyanidin B2 in plasma 30 min following consumption of proanthocyanidin extracts or proanthocyanidin-rich seeds. Levels of unconjugated procyanidin B2 were found to peak approximately 2 hrs after high-dose oral proanthocyanidin administration with an associated 63% excreted in the urine within 4 days of administration [224–226]. However, the bioavailability of larger proanthocyanidins is still unclear [226, 230].

3.5.2. Structure Activity Relationship of Proanthocyanidins—Structural analogs of proanthocynidins have been synthesized and evaluated for anti-cancer potential due to their unique structures and significant biological activities. Kashiwada *et al.* [231] reported on the cytotoxic potential of proanthocyanidins in an analysis of 57 tannin-related compounds such as gallotannins, ellagitannins and condensed/complex tannins. These compounds exhibited weak cytotoxicity against RPMI-7951 human melanoma cells while being less active against several other cell lines [231, 232]. Synthesis and evaluation of anti-tumor activity of proanthocynidin was first reported by Kozikowski *et al.* [233]. Numerous compounds were synthesized and their efficacy was tested against various human cancer cell lines. Kozikowski *et al.* [234] synthesized procyanidin epicatechin oligomers and evaluated for anti-tumor potential. Cytotoxic effects were observed based on the induction of cell cycle arrest in the G0/G1 phase was associated with high degree of oligomerization and activity was observed for the epicatechin dimer, trimer and tetramer. Prodelphinidin B1, B2 and B4 showed significantly better cytotoxic activity than EGCG and prodelphinidin B3 (Fig. 6A–D). Prodelphinidin B3 and C2 were almost the same as EGCG (Fig. 6D,E) [235].

3.6. Silymarin

Silymarin, a flavanolignan extracted from the milk thistle plant (*Silybum marianum* L. Gaertn) [236, 237], possesses important therapeutic potential. Silymarin exists as a mixture of three flavonolignans, silybin, silydianin and silychristin with silybin serving as the most abundant (70–80%) and most biologically active component [238]. Pharmacological studies have shown that silymarin is safe even at higher physiological doses, which may translate to safety for human use. Silymarin possesses antioxidant, anti-inflammatory, cytoprotective,

and anti-carcinogenic properties [239, 240]. An accumulating evidence suggests that both silymarin and silybin exhibit similar chemopreventive properties [241, 242].

Silymarin has been found to inhibit migration and invasion of melanoma cells by targeting the β-catenin signaling pathway. It inhibited nuclear translocation of β-catenin, increased levels of casein kinase 1α, upregulated glycogen synthase kinase-3β (GSK-3β), enhanced β-catenin phosphorylation and thus increased binding of phosphorylated β-catenin with β-transducin repeat-containing proteins (β-TrCP) [102]. Furthermore, silymarin reduced MMP-2 and MMP-9 levels, which are down-stream effectors of β-catenin signaling pathway. In another study, Lee *et al.* [243] reported that silybin induces cell cycle arrest at G1 phase and inhibits MEK1/2, ribosomal S6 Kinase 2 signaling and phosphorylation of ERK1/2 in melanoma cells. Furthermore, it reduced NFκB, AP-1 and STAT3 activation in melanoma cells *in vitro* and *in vivo*. In addition, silymarin increased apoptosis of melanoma cells by upregulating Fas-associated proteins with death domain (FADD) expression and enhancing procaspase-8 cleavage [244].

3.6.1. Bioavailability of Silymarin—In a study Schandalik *et al.* [245] demonstrated that, after single dose of oral administration of 120 mg silybin in 14 patients demonstrated that it was quickly absorbed from the gastrointestinal tract and levels of free drug peaked within 3 hrs. Total (free + conjugated) silybin levels (≥400 ng/ml) reached a peak at about 3 to 4 hrs were >40 fold higher than the free silybin and maintained up to 24 hrs. After reaching peak concentration at about 3 to 4 hrs, free silybin levels were declined and at 12 hrs reaches to limit of quantification (2 ng/ml). Administration of single dose of silipide and silymarin (120 mg, as silybin) demonstrated a several-fold higher serum concentration of silipide after 4 hrs versus silymarin. Forty-eight hours after oral silipide and silymarin administration, 11% and 3% silybin was observed in bile, respectively [246]. This study confirmed that silybin (derived from silipide) has superior bioavalability as compared to silymarin. Furthermore, Weyhenmeyer *et al.* [247] demonstrated a linear dose-response relationship in a human investigation of oral silybin administration. Approximately 10% of total silybin in plasma was found to be unconjugated within 4–6 hrs. Moreover, elimination half-life for total silybin was ~6 hrs and about 5% of the silybin administered was excreted into the urine indicating the good renal clearance. More importantly, silymarin is very well tolerated in humans without any adverse health effects [247, 248].

3.6.2. Structure Activity Relationship of Silymarin—Since silymarin is a mixture of three isomers with silybin (Fig. 7A) acting as the most active as hepato-protective agent, Ahmed *et al.* [249] synthesized different analogous and evaluated them for structure activity relationship. The most hepato-protective analog contained a hydroxymethyl group at position 2 of the dioxanes ring. In a recent study, Agarwal *et al.* [250] synthesized and characterized a series of silybin derivatives namely 2,3-dehydrosilybin (DHS), 7-O-methylsilybin (7OM), 7-Ogalloylsilybin (7OG), 7,23-disulphatesilybin (DSS), 7-O-palmitoylsilybin (7OP), and 23-O-palmitoylsilybin (23OP). In an investigation of the anti-cancer activity of these compounds, 2,3-dehydrosilybin (DHS), 7-O-methylsilybin (7OM), 7-Ogalloylsilybin (7OG) (Fig. 7B–D) exhibited improved growth inhibitory effects compared to silybin. Results from their study clearly suggest that structural modifications

can improved the anti-cancer efficacy of silybin. Furthermore, many water-soluble semi-synthetic analogs of silybin were prepared by various laboratories but increased in water solubility led to decrease in the activity.

3.7. Apigenin

Apigenin (4',5,7-trihydroxyflavone) (Fig. 8A), a flavonoid belonging to the flavone structural class, is found in plants such as parsley, celery, artichokes and chamomile. A growing body of evidence has shown that apigenin possess antioxidant, anti-mutagenic, anti-carcinogenic, anti-inflammatory, and anti-proliferative properties [251]. In addition, preparations from chamomile have been historically used to treat cutaneous inflammation and other dermatological diseases [251]. Apigenin has been identified as a cancer chemopreventive agent owing to its potent antioxidant, anti-melanoma and anti-inflammatory activities with low toxicities [252, 253]. In two separate studies, Caltagirone *et al.* [154] and Piantelli *et al.* [254] found that apigenin inhibited melanoma tumor growth (B16-BL6 melanoma cells) *in vivo* and inhibited VEGF secretion by downregulating ERK1/2 and PI3K/AKT signaling *in vitro* [252]. Moreover, apigenin significantly decreased the invasion of melanoma cells *in vitro* and inhibited lung metastasis *in vivo* by reducing lung colonization of B16-BL6 cells in mice [154, 254]. In a recent study, Das *et al.* [255] observed that apigenin inhibits cell growth and induced apoptosis in A375 melanoma cells with no concomitant toxicity to peripheral blood mononuclear cells. Further, apigenin's therapeutic efficacy was enhanced by the poly-lactic-co-glycolide formulation, since this vehicle readily enters cancer cells, intercalates with dsDNA, and induces conformational change [256].

3.7.1. Bioavailability of Apigenin—Apigenin's low intrinsic toxicity and anti-cancer potential have gained attention in recent years. The pure form of apigenin is unstable and least soluble in water and organic solvents. In nature, apigenin exists in the form of water-soluble glycoside conjugates and acylated derivatives [257, 258]. The absorption and bioavailability of apigenin is determined by conjugated moiety, since the compound undergoes enzymatic cleavage by mammalian or microbial glucosidases before absorption. It has been found that apigenin remains bound to β-galactoside in its natural form, which provides its best bioavailability [259–261]. In the gut, apigenin is metabolized via uridine 5'-diphospho-glucuronosyltransferase UGT1A1 as glucoroside and sulfate conjugates, which are more easily excreted in bile or urine. Gradolatto *et al.* [259] have shown that apigenin appeared in the blood 24 hrs after oral intake of a single dose of radio labeled apigenin. In rats, oral intake after a single dose of radio-labeled apigenin resulted in 51% detection of 51, 12, 1.2, 0.4, 9.4, 1.2, and 25% of the radioactive compound in urine, feces, blood, kidneys, intestine, liver, and the remaining tissues, respectively, within 10 days. Furthermore, kinetics of apigenin in blood exhibited a relatively high elimination half-life of 91.8 hrs (slow excretion) compared to other dietary flavonoids [258, 261]. These results clearly demonstrated the limited bioavailability of apigenin, though slow pharmacokinetics may lead to possible accumulation of this flavonoid in the tissues to effectively impart its chemopreventive effects [259–262].

3.7.2. Structure Activity Relationship of Apigenin—Protoapigenone, a natural apigenin derivative, has demonstrated a 10-fold greater anti-tumor activity than apigenin *in vitro* and *in vivo* [143, 263, 264]. Several apigenin derivatives have been synthesized and studied for their anti-cancer potential [265, 266]. Derivatives such as 1'-O-alkyl-protoapigenone and protoapigenone 1'-O-butyl ether (Fig. 8B,C) exerted significantly stronger activity than the non-substituted analog protoapigenone. On the contrary, β-naphthoflavone derivatives containing same pharmacophore when substituted with an O-alkyl side-chain at position 1 showed decreased cytotoxic activities. Furthermore, Liu *et al.* [267] demonstrated that nitrogen-containing apigenin analogs (R= ethylamino, propylamino, isopropylamino etc.) showed better anti-cancer and antioxidant potential (Fig. 8D).

3.8. Capsaicin

Capsaicin (Fig. 9A), a pungent component of chili peppers, is one of the most commonly used spices in the world [268]. Though capsaicin has historically been used for its anti-inflammatory and analgesic abilities [268], it is receiving increasing attention for its anti-tumor properties [269]. Evidence suggests that capsaicin induces A375 melanoma cell apoptosis by activating caspases 3, 8, and 9, which is accelerated by its downregulation of Bcl-2 [270]. It also reduces melanoma cell survival by decreasing NFκB activity [271, 272]. The compound was found to inhibit growth and increase apoptosis of A375 and SK-MEL-28 melanoma cells by inhibiting plasma membrane NADH oxidase activity [273]. It also inhibited the migration of B16-F10 melanoma cells through inhibition of the PI3K/AKT/Rac-1 pathway [268]. The compound's effects on VEGF are currently under debate, however. While Patel *et al.* [271, 272] found that capsaicin decreased cell proliferation through enhanced VEGF production in A375 melanoma cells. Min and colleagues [274] observed its ability to decrease angiogenesis in cultured human endothelial cells. Despite the current uncertainty of capsaicin's effects on VEGF production, the body of evidence suggests that capsaicin promotes apoptosis, decreases survival, and inhibits migration of melanoma cells.

3.8.1. Bioavailability of Capsaicin—Capsaicin is a lipophilic compound that has demonstrated fast absorption and a short half-life in humans. In addition, the compound resists diffusion and *in vitro* studies have shown that it exhibits a slow cutaneous biotransformation in humans [275, 276]. After intragastric administration of 15 mg/kg body weight capsaicin in rats, the plasma concentrations peaked to approximately 10 ng/ml after the first hr and then declined rapidly, with an absolute bioavailability of orally administered capsaicin to be 0.106% [277, 278]. According to Chaiyasit *et al.* [279] oral administration of capsaicin (26.6 mg) in humans resulted in maximum plasma concentration of 47.1 (2.0) min with $T_{1/2}$ of 24.9 (5.0) min, and 2.5 (0.1) ng ml^{-1} of C_{max}. In another study conducted by Suresh and Srinivasan [280], oral administration of 30 mg/kg body weight capsaicin in rats demonstrated 94% bioavailability. In a recent study, Rollyson *et al.* [281] demonstrated that orally administered capsaicin resulted in detectable levels in the liver, lungs, kidney and serum of the mice within 15 min after oral administration. The maximum concentration was observed at 60 min and the lungs exhibited the greatest absorption.

3.8.2. Structure Activity Relationship of Capsaicin—The cytotoxic potential of vanilloid compounds was evaluated by modifying vanilloid ring pharmacophores and nonivamide analogs of capsaicin versus the transient receptor potential vanilloid-1 (TRPV1). Specific regions of the compound including the aromatic "A-ring", the hydrogen bond-donating group in the "B-region" and the hydrophobic hydrocarbon "C-region" tail are required for maximum potency at TRPV1 [282–285]. Analogs with "A-ring" modification such as 6-iodo-nordihydrocapsaicin (Fig. 9B) and 3-methoxy-4-hydroxybenzyl have been identified as the most potent. Removal of the 4-OH or 3-MeO moieties or modification of positions 2, 5 and 6 of the "A-ring" has been found to significantly reduce its activity [277, 286, 287].

3.9. Genistein

Genistein (4',5,7-trihydroxyisoflavone) (Fig. 10A), a phytoestrogen and isoflavone found in soybeans with structural and functional similarities to estrogen, has anti-inflammatory and antioxidant effects with ongoing investigation into its ability to prevent and treat cancer, metabolic syndrome, diabetes, and chronic inflammatory diseases. Genistein has been found to inhibit angiogenesis, promote apoptosis, reduce tumor metastasis, and decrease proliferation in various malignancies ranging from neuroblastoma to breast cancer [100, 288, 289]. It has been found, however, to promote melanoma cell differentiation through stabilization of protein-linked DNA strand breakage in SK-MEL-131 cells [290, 291]. Genistein has been shown to have beneficial effects against various melanoma cells by inhibiting cell cycle progression and inducing apoptosis while inhibiting tumor growth and metastasis potential of B164A5 melanoma cells in C57BL/6J mouse model [289, 292, 293]. Genistein inhibited cell cycle progression in melanoma cells by targeting cellular p53, p21, checkpoint kinase 2 (Chk2) [294–297]. Furthermore, it inhibited invasion, cell adhesion and lung metastasis of melanoma cells in mice [298–300]. It also inhibited angiogenesis in mouse model of melanoma [288, 301]. Moreover, it induced differentiation of mouse and human melanoma cells [302–304].

3.9.1. Bioavailability of Genistein—Genistein's high lipid solubility, significant first-pass metabolism and fast gut transit time strongly limit its bioavailability [305, 306]. However, Coldham *et al.* [307] observed a peak serum concentration 30 min after an oral administration of 4 mg/kg. In another study, Steensma *et al.* [308] were able to detect serum genistein 15 min after portal vein administration. Oral treatment yielded a 5.49 μM maximum concentration after 15 min. In another study, Kwon *et al.* [309] found that 4, 20 and 40 mg/kg genistein oral administration resulted in 39, 24 and 31% bioavailabilities, respectively. Oral genistein treatment (40 mg/kg) yielded a T_{max}, C_{max} and $AUC_{(0-\infty)}$ of 2 hrs, 4880 ng/ml and 0.03 mg hr/ml, respectively. It is suggested that in infants, consumption of 4–7 mg/kg per day of total genistein will result in 1–5 μM of total circulating genistein. Adults can safely consume 1mg/kg of genistein per day resulting in ~0.5 μM serum concentration. An accumulating body of evidence has shown that genistein is safe and very well tolerated in humans [310–312].

3.9.2. Structure Activity Relationship of Genistein—Several synthetic analogs of genistein have been developed to explore its potential beneficial effects [313]. Ullah *et al.*

[314] found that genistein possessed greater antioxidant and DNA-protective effects than its methylated structural analogue biochanin A (Fig. 10B). Genistein derivatives with substitutions at the C7 hydroxyl group of the A-ring exhibited anti-mitotic activity while analogs substituted at the 4′-position of the B-ring (Fig. 10C) induced p53-independent G1 cell-cycle arrest and autophagy [315]. In addition, genistein derivatives have been found to be non-genotoxic [316]. Several studies have shown that genistein glycoconjugates (Fig. 10D) exhibit anti-proliferative potential [313, 317, 318] and even induce cell cycle arrest [319, 320]. Interestingly, some of these analogs also inhibited microtubule assembly [318, 319, 321].

3.10. Indole-3-Carbinol

Indole-3-carbinol (I3C) (Fig. 11A) is a bioactive metabolite of glucosinolate glucobrassicin, a substance found at high concentrations in vegetables from the family *Cruciferae* including broccoli, cauliflower, and Brussels sprouts [322, 323]. The compound has been found to inhibit proliferation through various mechanisms in various cancer cell lines [322]. Animal models have also demonstrated the promise of I3C in the prevention of chemical-induced tumorigenesis of breast, liver, lung, cervical, and gastrointestinal tract tissues [324]. Thus far, I3C has only been found to promote apoptosis in melanoma cell lines. In addition, I3C sensitized G361 melanoma cells to UVB radiation through decreased anti-apoptotic Bcl-2 expression [325]. Another study found that I3C induces apoptosis in SK-MEL-5 melanoma cells by down-regulation of MITF [326]. Furthermore, I3C-mediated anti-proliferative effect was through interaction with neuronal precursor cell-expressed developmentally down regulated 4 and wild-type PTEN degradation in human melanoma cells [327]. I3C consumption was also associated with increased sensitivity to chemotherapy in a study of mice with B16 melanoma [328]. I3C is a promising compound that may be effective in melanoma therapy, especially since evidence suggests that it decreases the expression of MITF, a signaling molecule known to be over-activated in resistant cases of melanoma [326].

3.10.1 Bioavailability of Indole-3-Carbinol—Consumption of vegetables from the cruciferous family serves as a good source of the I3C precursor, glucobrassicin. Mechanical damage to cells from these plants (e.g., during chewing or chopping) results in the formation of I3C from the interaction between myrosinase and glucobrassicin [186, 323]. After oral ingestion, I3C combines with gastric HCl to form a complex mixture of biologically active compounds such as a 3,3'-diindolylmethane dimer and a cyclic trimer. However, acid condensation products are less likely to form in the more alkaline environment of the intestine. To date, there have been few animal studies investigating the bioavailability of I3C [329, 330]. A phase 1 clinical trial demonstrated undetectable plasma concentrations of I3C in women who received 400 to 1200 mg oral doses. However, DIM levels (Fig. 11B) were detectable with a Cmax of 61 ng/mL and 607 ng/mL after 400 and 1000 mg doses, respectively [329, 330].

3.10.2. Structure Activity Relationship of Indole-3-Carbinol—Due to its significant anti-cancer potential but weak acid stability, several successful attempts have been made to synthesize structural analogs of I3C such as (p-substituted phenyl)-diindoylmethanes

peroxisome proliferator-activated receptor γ agonists [331–335], SR13668 (Fig.11H) as an AKT inhibitor [336, 337], and an I3C tetrameric derivative (Fig. 11C) as a CDK6 inhibitor [338]. Acid-catalyzed condensation resistant analog OSU-A9 ([1-(4-chloro-3-nitrobenzenesulfonyl)- 1H-indol-3-yl]-methanol) (Fig. 11G) exhibited significantly improved pro-apoptotic (100-fold higher) and anti-tumor properties than I3C *in vitro* and *in vivo* [324, 339]. In addition, I3C-based N-alkoxy derivatives produced a marked increase in cell cycle arrest and apoptosis. Furthermore, the inhibitory potential of 3-methoxymethylindole and 3-ethoxymethylindole (Fig. 11D–E) was similar to I3C. The hydroxymethyl group at the C-3 position of the indole ring is likely important in I3C's ability to induce cell cycle arrest since substitutions at that position was found to inactivate the compound [340]. Another I3C analog, 1-benzyl-I3C (Fig. 11F), displayed an approximate 1000-fold stronger abilities to inhibit proliferation, induce cell cycle arrest and down-regulate the production of ERα protein in estrogen responsive cancer cells [341].

3.11. Luteolin

Luteolin (Fig. 12A) is a flavanoid found in a wide variety of dietary sources such as carrots, peppers, celery, olives, peppermint, thyme, rosemary, and oregano with known antioxidant, anti-inflammatory, and anti-tumor activities. It has been found to inhibit angiogenesis, promote apoptosis, and sensitize cells to anti-cancer therapy in a variety of malignancies [342]. The compound was found to induce melanogenesis and reduce invasive potential of B16F10 melanoma cells by decreasing EMT through inhibition of the β3 integrin/FAK signal pathway [343–345]. Luteolin protected DNA, inhibited cell cycle progression and promoted apoptosis in A375 melanoma cells in a different investigation [346–348]. In addition, the compound was found to inhibit cell growth and increase apoptosis (B16 and 4A5 melanoma cells) via Bcl-2 downregulation and Bax upregulation [349]. An *in vitro* study of B16 melanoma cells showed that the flavanoid decreased ERK1/2 signaling [350]. This compound exhibits promising preliminary activity against melanoma, though further investigation will be necessary to confirm its efficacy in the management of the disease.

3.11.1. Bioavailability of Luteolin—Shimoi *et al.* [351] observed free luteolin and its conjugates present in plasma after administration of the compound to rats and humans. Plasma concentrations of luteolin and its conjugates peaked between 15 and 30 min after treatment with luteolin. Luteolin was absorbed more rapidly when administered with propyleneglycol versus 0.5% carboxymethyl cellulose. Furthermore, Zohu *et al.* [306] reported that the absorption rate constant of pure luteolin (5.0 microg/mL) was markedly higher in the duodenum and jejunum than the colon and ileum. Luteolin was likely passively absorbed given that it was taken up in an ATP-independent manner. Furthermore, oral administration in rats resulted a peak concentration and AUC of 1.97 ± 0.15 microg/mL and 10.7 ± 2.2 microg/mL/hr, respectively [306].

3.11.2. Structure Activity Relationship of Luteolin—Though luteolin is known for its antioxidant, anti-inflammatory, and anti-tumor activities, limited studies on its structure-activity relationship have been conducted. In one study, Cheng *et al.* [352] synthesized luteolin analogs by replacing hydroxy groups at C-3' and C-4' with alkynyl groups using neutral, electron-deficient, electron-rich and heteroaromatic (4-pyridine, imidazole alkynes)

at the C-4' position. This luteolin analog LA-12 (Fig. 12B), more efficiently sensitized cells to TNFα-induced cell death than did luteolin.

4. CONCLUSIONS AND FUTURE DIRECTIONS

Melanoma, with its unique ability to metastasize early, is the most deadly skin cancer affecting humans. Patients with metastatic melanoma face a median survival time of only six months and have very few targeted chemotherapies available to them. Although personalized chemotherapies targeting common mutations such as BRAF and MEK have been developed recently, these drugs have only been found to extend progression free survival by a few months. Patients who attempt these treatments frequently develop resistance and succumb to their disease. Recent studies of combination chemotherapies for the prevention and treatment of melanoma give us hope that achieving a cure is indeed possible. If ongoing investigations continue their current trajectory, the anti-cancer properties of phytochemicals may yield complimentary chemotherapies to augment today's treatments. Accumulating evidence has recently begun to illuminate the various pathways by which these bioactive molecules promote apoptosis, inhibit proliferation and suppress EMT of melanoma cells. By targeting multiple disrupted signaling molecules critical to the pathogenesis of melanoma, phytochemicals such as fisetin, EGCG, silymarin, proanthocyanidin, resveratrol, curcumin, apigenin, capsaicin, lupeol, genistein, indole-3-carbinol and luteolin may one-day serve as necessary adjuvant chemotherapies to successfully treat metastatic melanoma with minimal toxicity. This review highlights the largely untapped anti-cancer potential that phytochemicals possess. The obvious need for effective combination therapies to combat melanoma and the therapeutic promise that phytochemicals offer grant us a glimpse at a cure for melanoma. Ongoing studies are needed to evaluate various combinations of phytochemicals with existing drugs such as BRAF and MEK inhibitors. Numerous combinations of phytochemicals and targeted chemotherapies will need to be evaluated to determine which pairings yield the greatest synergy so that translational studies may then be attempted.

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LIST OF ABBREVIATIONS

AP-1	Activator protein-1
CDKN2A	Cyclin-dependent kinase inhibitor 2A
COX	Cyclooxygenase
EMT	Epithelial-mesenchymal transition
FAK	Focal adhesion kinase
FLIP	Cellular FLICE-like inhibitory protein
GSK-3β	Glycogen synthase kinase-3β

GTP	Guanosine-5-triphosphate
MAPK	Mitogen-activated protein kinase
MITF	Microphthalmia-associated transcription factor
MMP	Matrix metallopeptidase
mTOR	Mammalian target of rapamycin
NFκB	Nuclear factor kappa B
NLRP1	Nuclear localization leucine-rich-repeat protein 1
PGE_2	Prostaglandin E_2
PI3K	Phosphatidylinositol 3-kinase
PTEN	Phosphatase and tensin homolog
Rb	Retinoblastoma
RGP	Radial growth phase
STAT3	Signal transducer and activator of transcription 3
VGP	Vertical growth phase
α-MSH	Alpha-melanocyte-stimulating hormone

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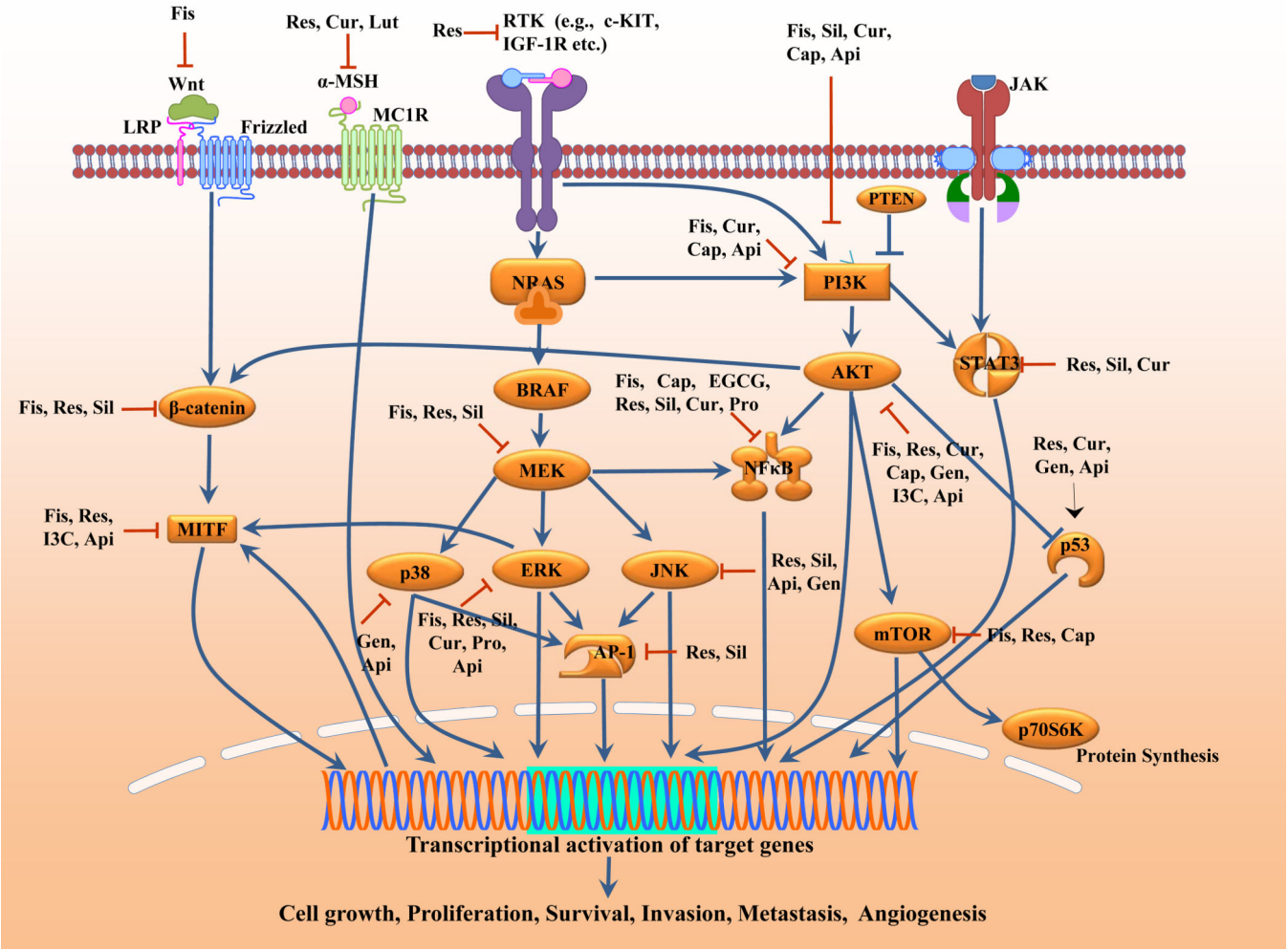


Fig. 1.

Signal transduction pathways altered by selected phytochemicals. Fis inhibits PI3K, AKT, mTOR, NFκB, MEK, ERK, Wnt, β-catenin, MITF; EGCG inhibits NFκB; Res inhibits AKT, mTOR, NFκB, MEK, ERK, β-catenin, MITF, STAT3, c-kit, c-Jun, α-MSH; Sil inhibits NFκB, MEK, ERK, β-catenin, STAT3; Cur inhibits PI3K, AKT, NFκB, ERK, STAT3; Pro inhibits NFκB, ERK; Cap inhibits PI3K, AKT, mTOR, NFκB; Gen inhibits AKT, p38; I3C inhibits AKT, MITF; Lut inhibits α-MSH; Api inhibits PI3K, AKT, ERK, MITF, p38. p53 is upregulated by Res, Cur, Gen, Api.

Abbreviations: Fis=Fisetin, EGCG=Epigallocatechin gallate, Res=Resveratrol, Sil=Silymarin, Cur=Curcumin, Pro=Proanthocyanidin, Cap=Capsaicin, Gen=Genistein, I3C=Indole-3-carbinol, Lut=Luteolin, Api=Apigenin

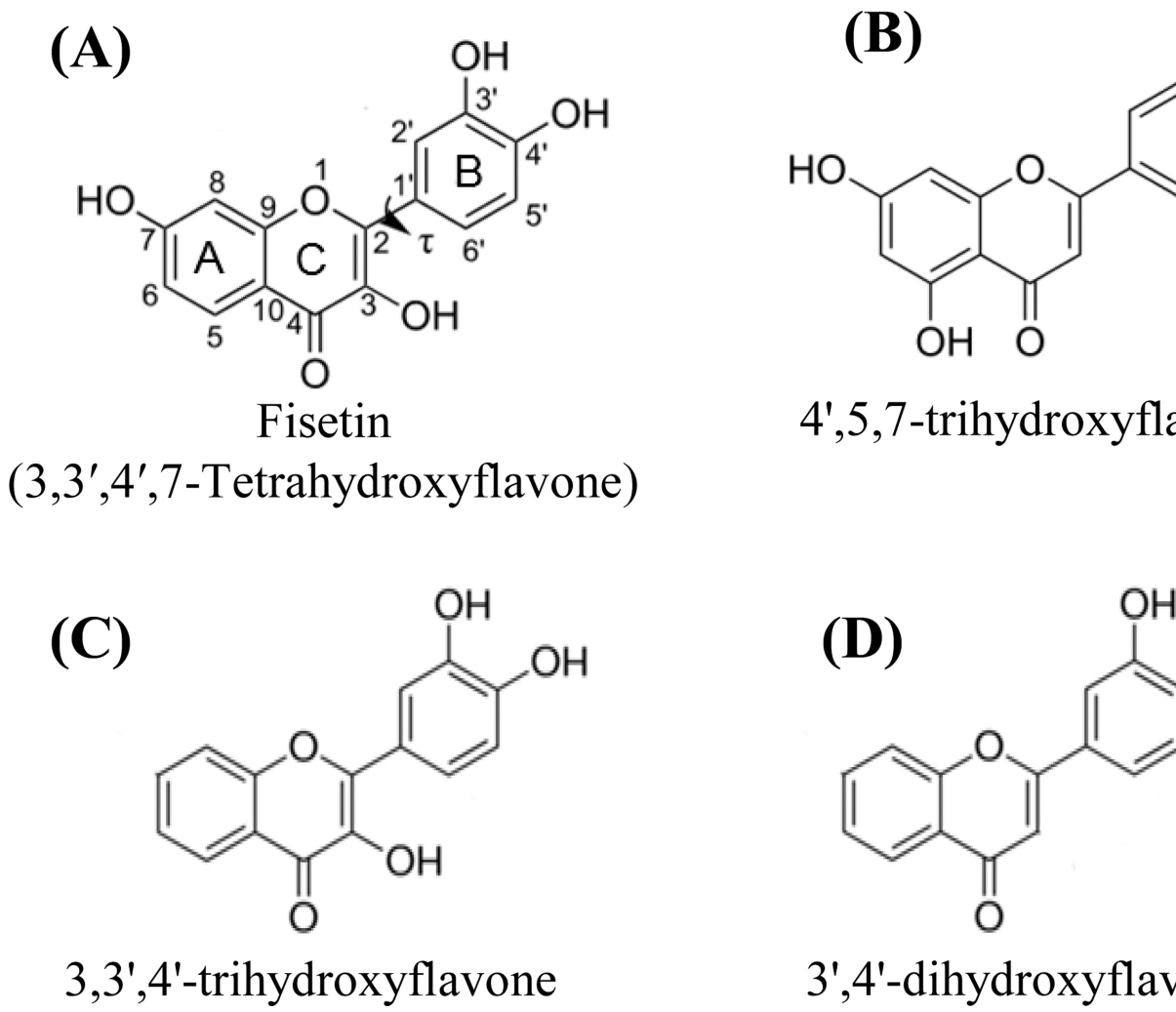
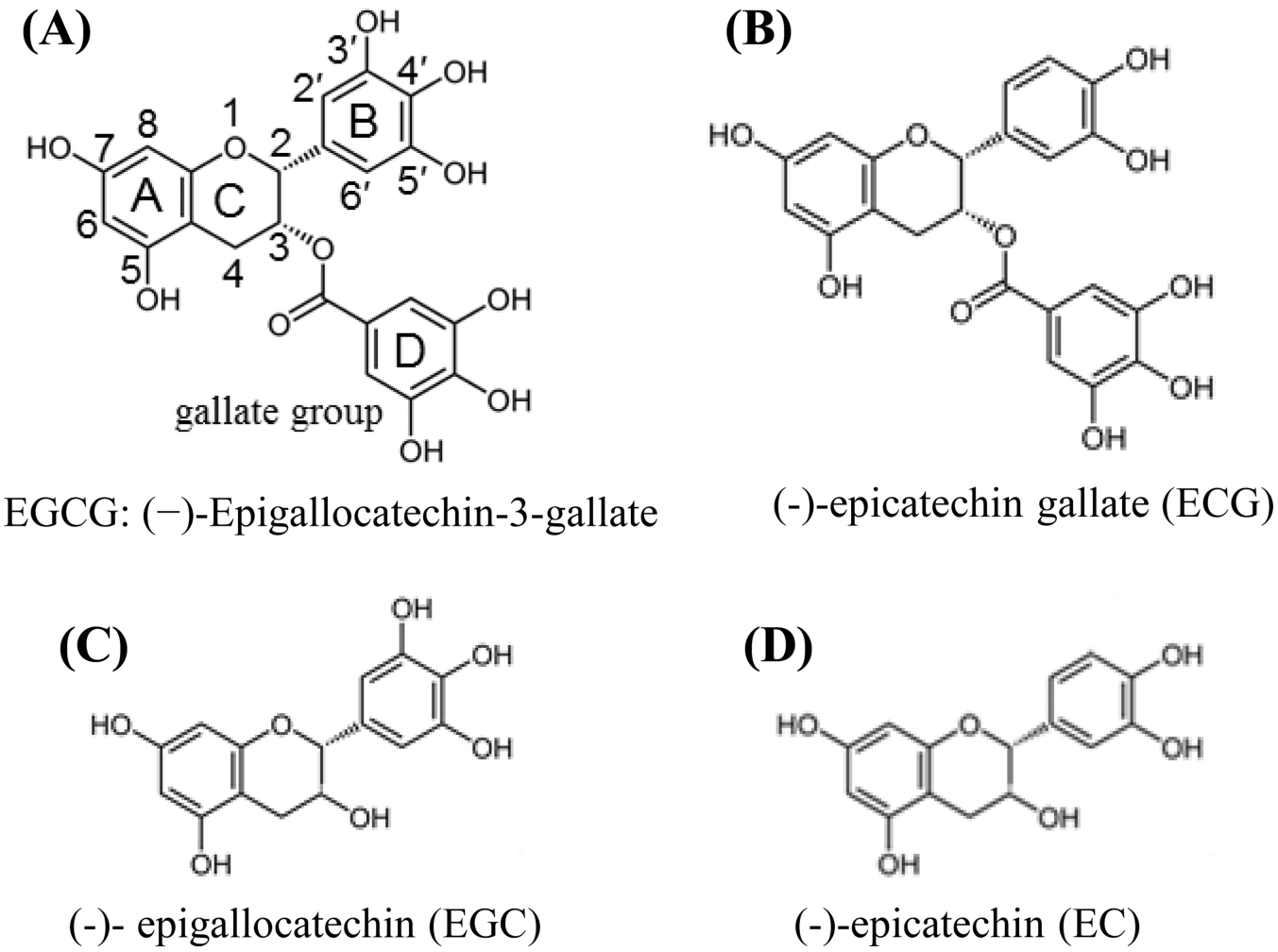


Fig. 2.
Fisetin and its structural analogs.



EGCG: (−)-Epigallocatechin-3-gallate

(-)-epicatechin gallate (ECG)

(-)- epigallocatechin (EGC)

(-)-epicatechin (EC)

Fig. 3.
EGCG and its structural analogs.

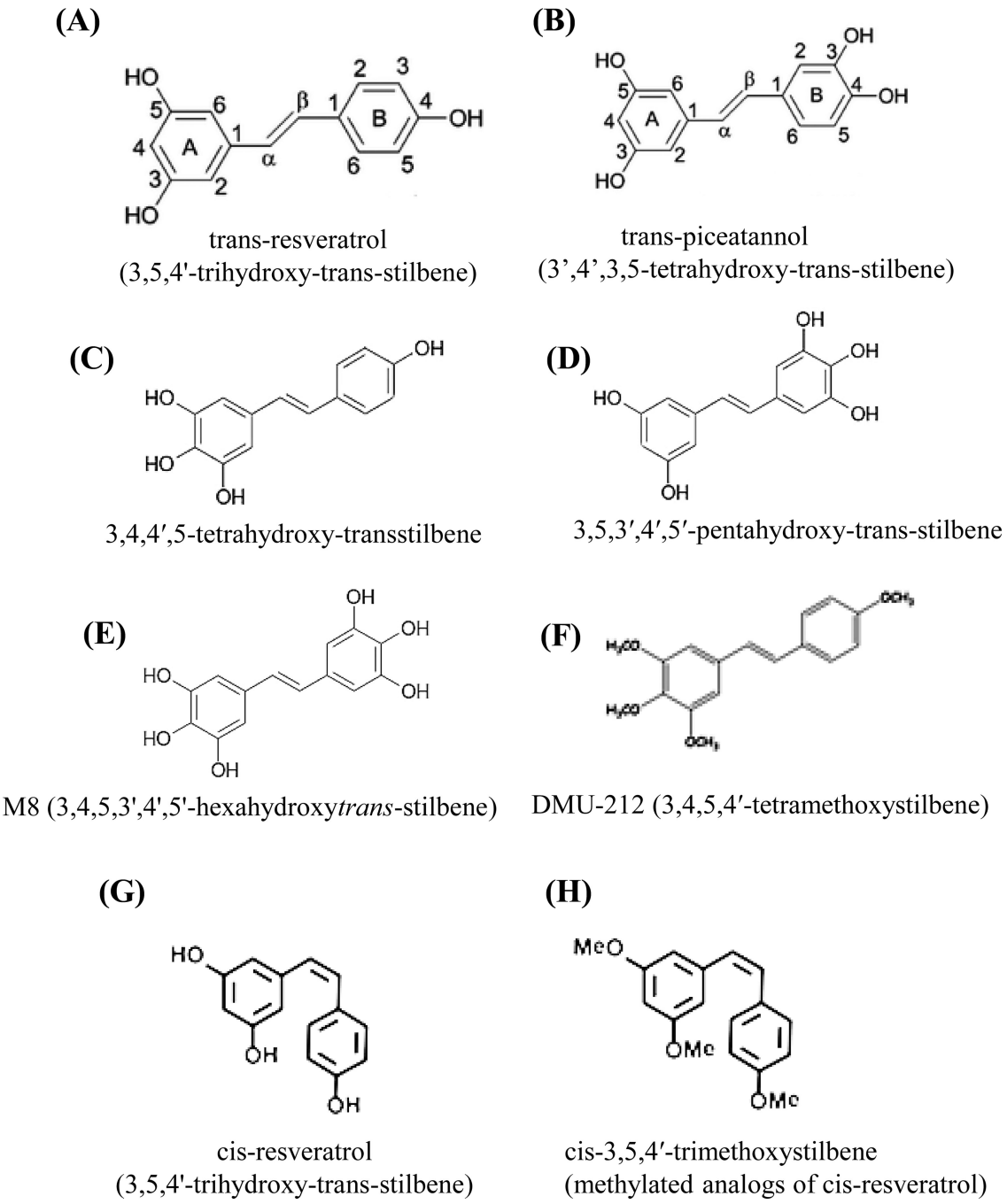


Fig. 4.
Resveratrol and its structural analogs.

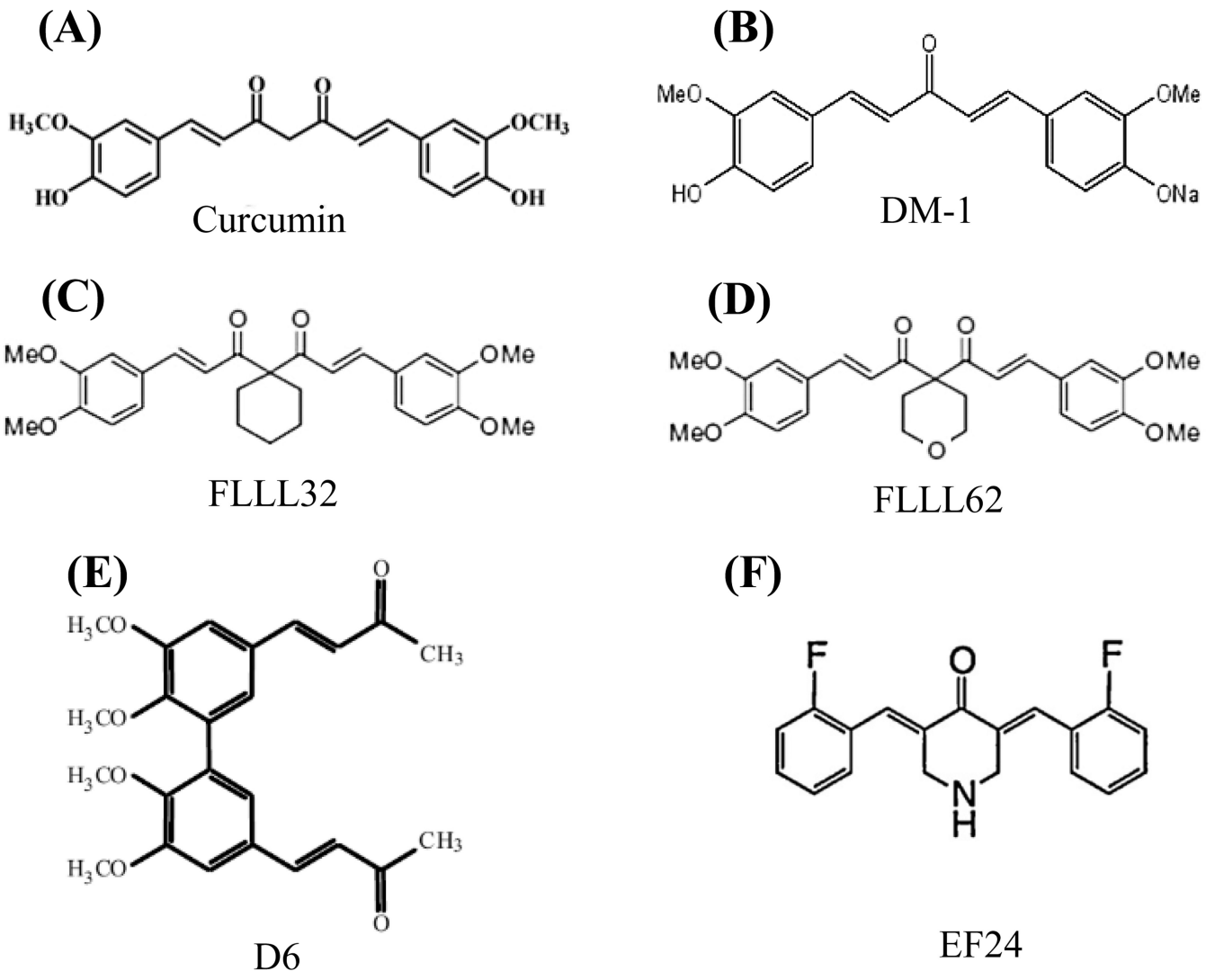


Fig. 5.
Curcumin and its structural analogs.

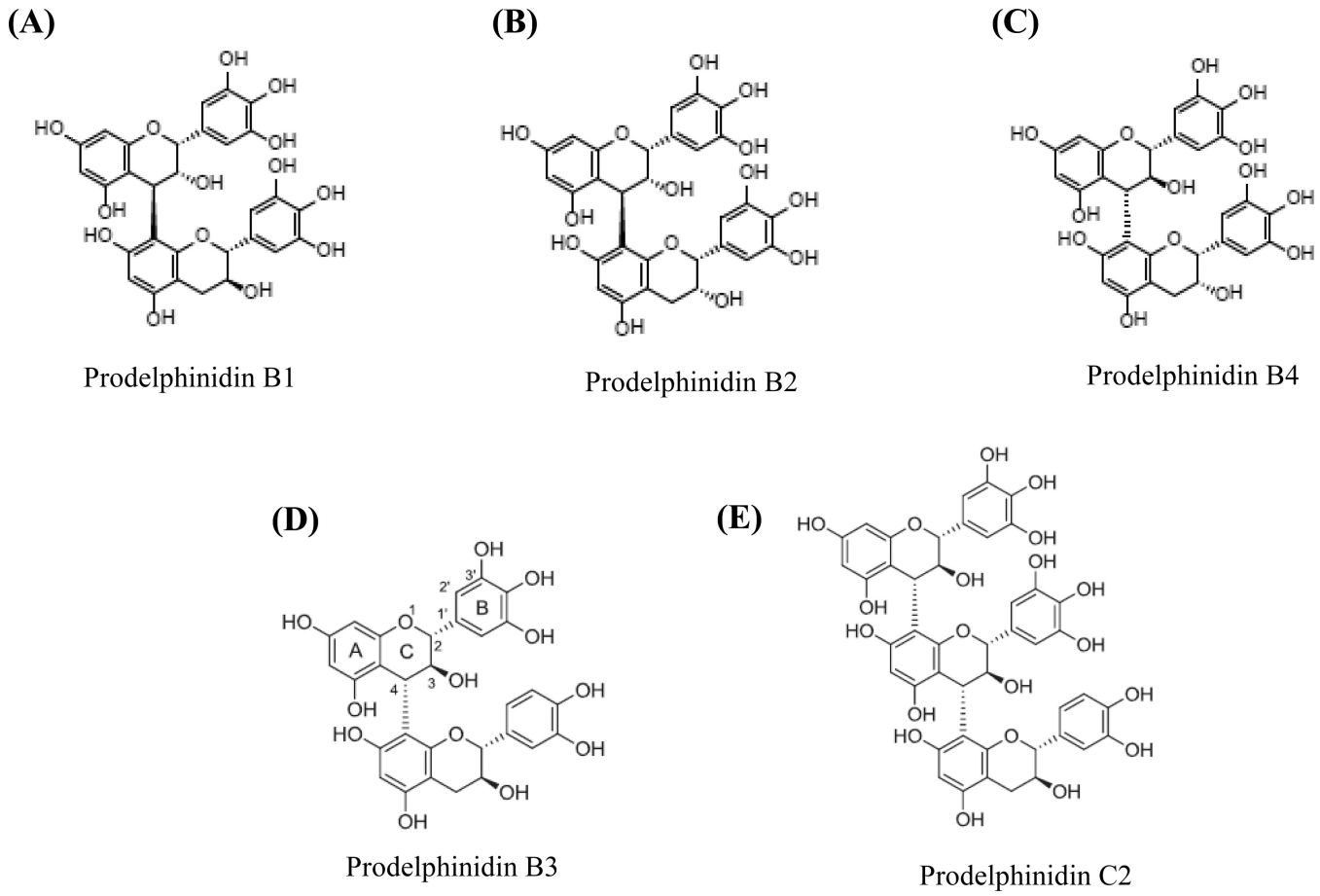


Fig. 6.
Proanthocyanidin and its structural analogs.

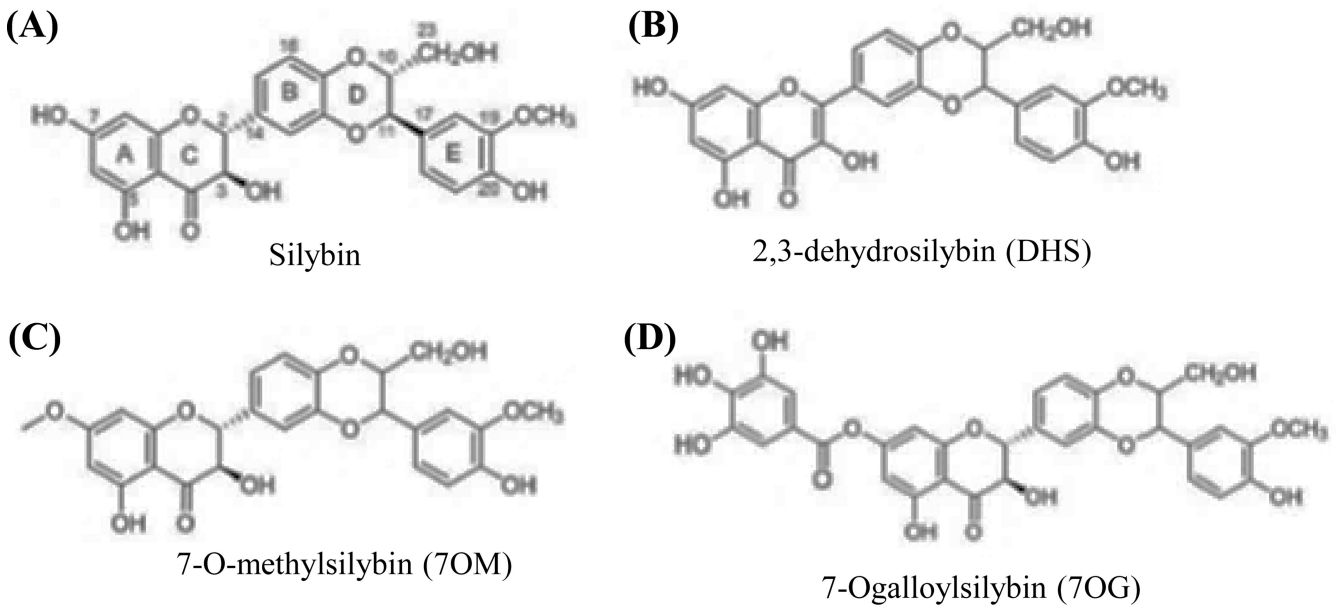


Fig. 7.
Silybin and its structural analogs.

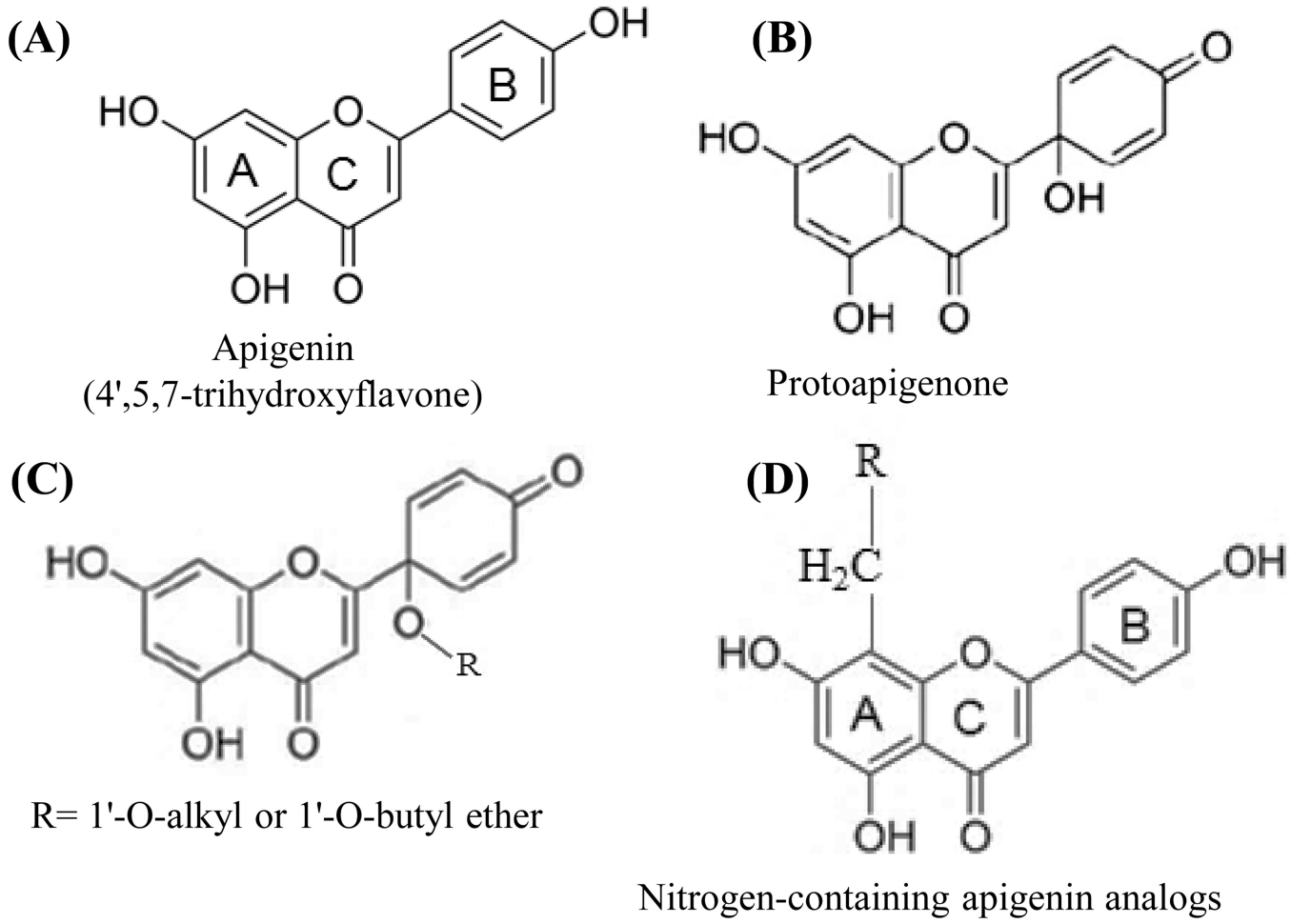


Fig. 8.
Apigenin and its structural analogs.

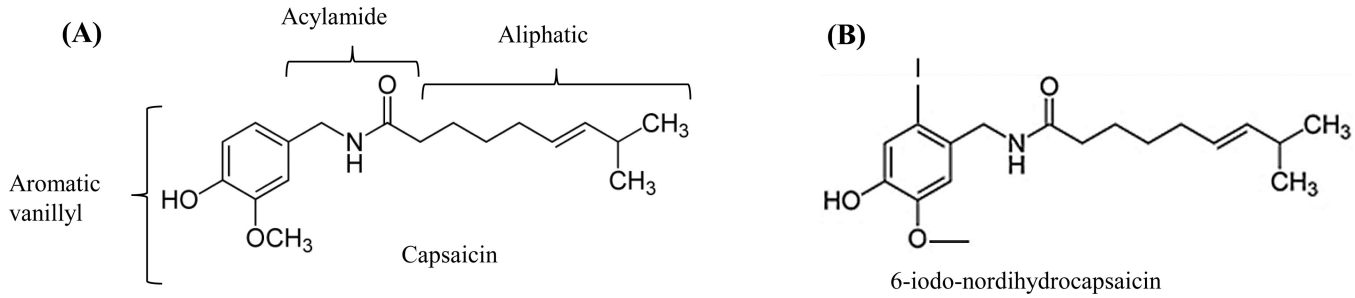


Fig. 9.
Capsaicin and its structural analogs.

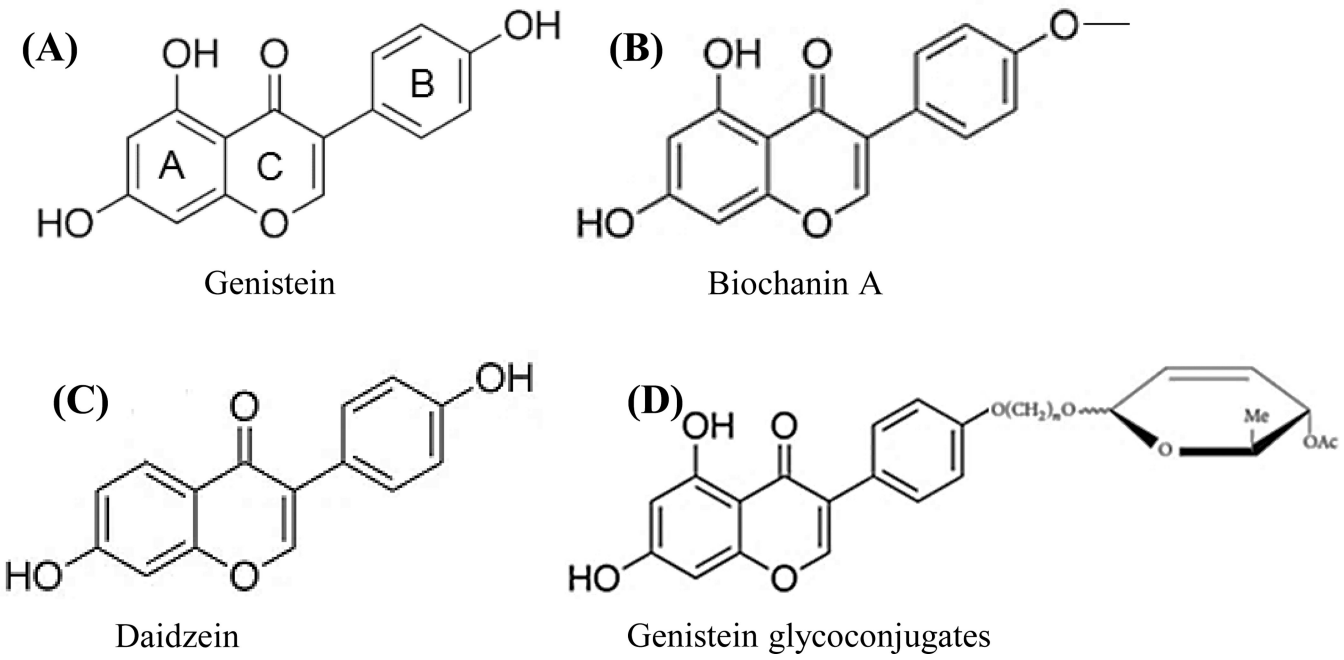


Fig. 10.
Genistein and its structural analogs.

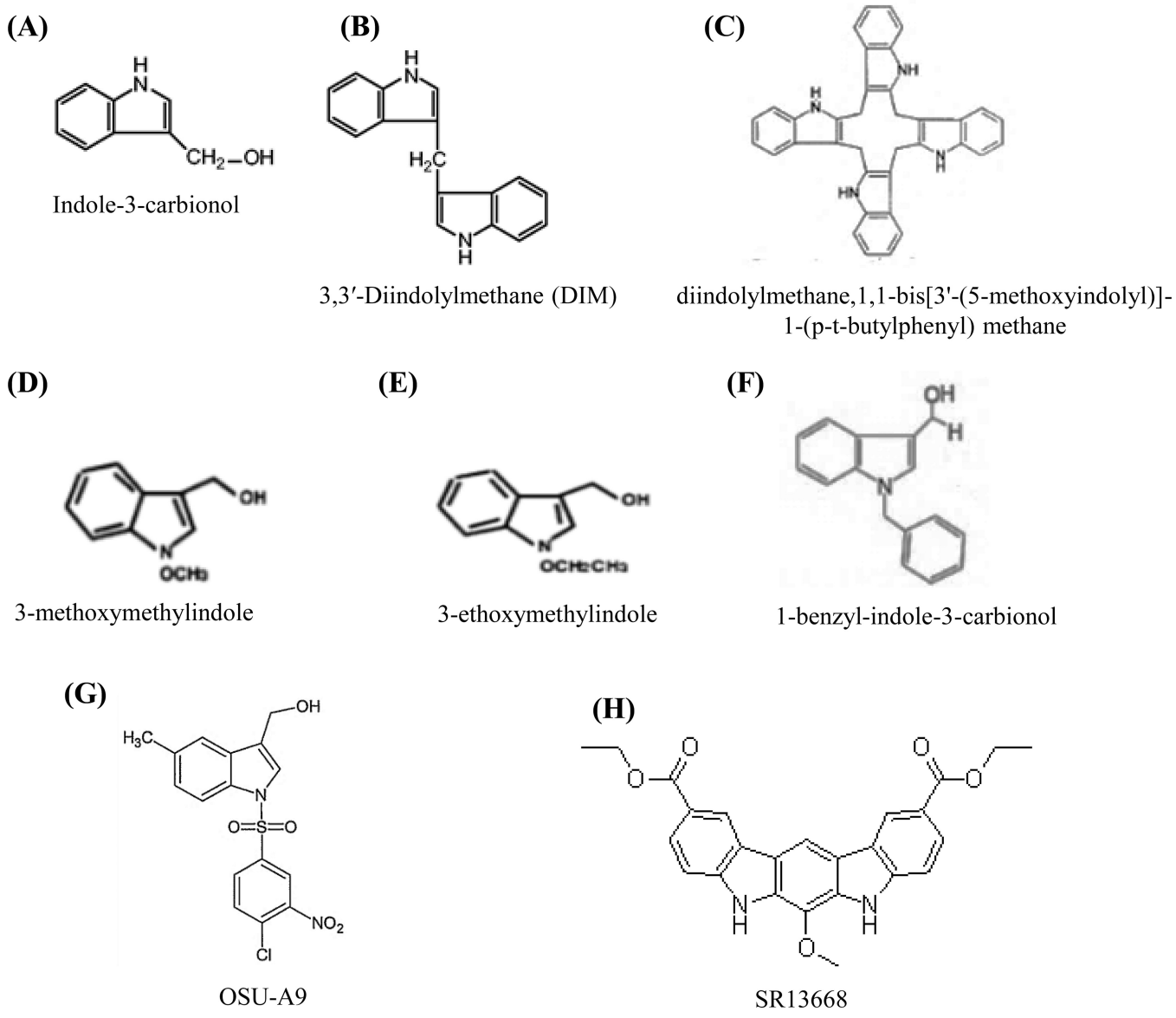


Fig. 11.
Indol-3-carbionol and its structural analogs.

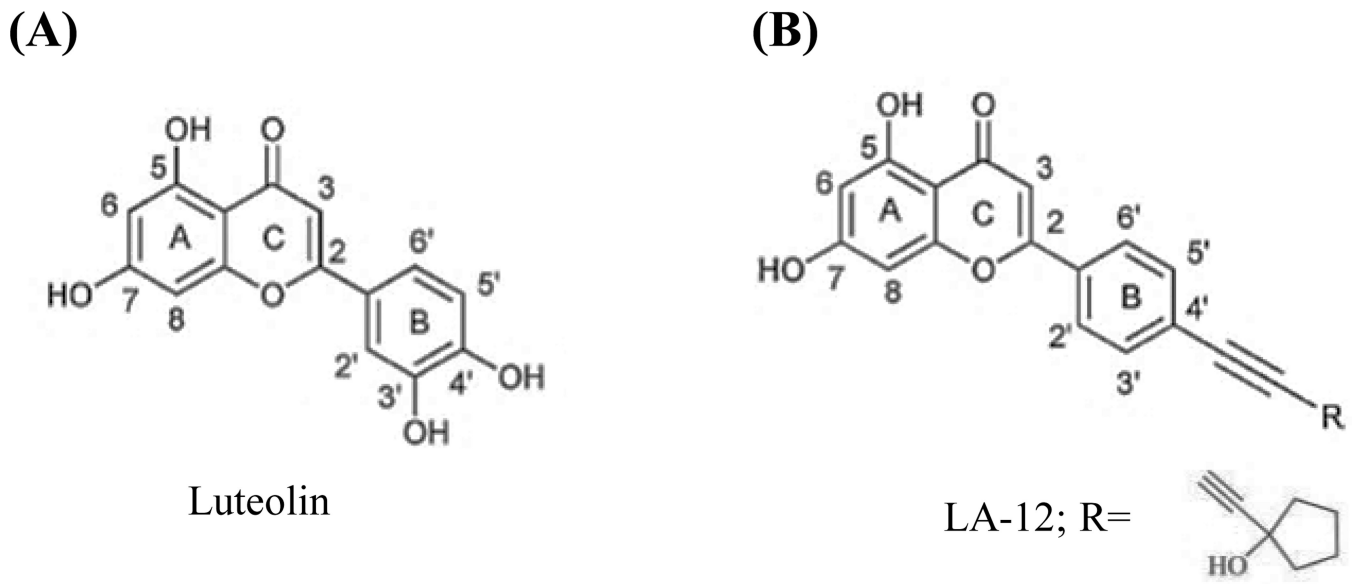


Fig. 12.
Luteolin and its structural analogs.

Table 1

Phytochemicals and their cellular targets

Name	Sources	Targets	References
Fisetin	Onion, cucumber, apple, persimmon, strawberry	• Inhibits cell growth, EMT and invasion • Induces cell cycle arrest and apoptosis • Inhibits PI3K, AKT, mTOR, NFκB, MEK1/2, ERK1/2, Wnt, β-catenin, and MITF	[106, 108, 110–112]
EGCG	Green tea	• Inhibits cell growth, EMT and invasion • Induces cell cycle arrest and apoptosis • Inhibits NFκB signaling pathway	[124–127, 129, 130]
Resveratrol	Peanut, grape skin, mulberry	• Inhibits cell growth, EMT and invasion • Induces cell cycle arrest and apoptosis • Inhibits AKT, mTOR, NFκB, MEK1/2, ERK1/2, β-catenin, MITF, STAT3, c-kit, AP-1/JunD, c-Jun and α-MSH	[152,153,155–163,171]
Curcumin	Turmeric	• Inhibits cell growth • Induces cell cycle arrest and apoptosis • Inhibits PI3K, AKT, NFκB, ERK1/2 and STAT3	[185–203]
Proanthocyanidins	Cocoa, grape, apple, tea, red wine	• Inhibits cell growth • Inhibits NFκB and ERK1/2	[220–222]
Silymarin	Milk thistle	• Inhibits cell growth • Induces cell cycle arrest and apoptosis • Inhibits NFκB, MEK1/2, ERK1/2, β-catenin, STAT3, AP-1/JunD and c-Jun	[102,243,244]
Apigenin	Chicory, clove, apple, cherry, grape, beans, broccoli, celery, leeks, onion, barley, parsley, tomato, tea	• Inhibits cell growth • Induces cell cycle arrest and apoptosis • Inhibits PI3K, AKT, ERK1/2, MITF, MAPK p38 and JNK	[154,252–256]
Capsaicin	Chili pepper	• Inhibits cell growth, invasion and angiogenesis • Induces cell cycle arrest and apoptosis • Inhibits PI3K, AKT, mTOR and NFκB	[268, 270–273]
Genistein	Soybean	• Inhibits cell growth and invasion • Induces cell cycle arrest and apoptosis • Inhibits signal transduction: AKT, MAPK p38 and JNK	[289–302]
Indole-3-carbinol	Broccoli, cauliflower, Brussels sprouts	• Inhibits cell growth • Induces cell cycle arrest and apoptosis • Inhibits AKT and MITF	[325–327]

Name	Sources	Targets		References
Luteolin	Carrot, pepper, celery, olive, peppermint, thyme, rosemary, oregano	•	Inhibits cell growth	[343–350]
		•	Induces cell cycle arrest and apoptosis	
		•	Inhibits α-MSH	

Hericium erinaceus (Lion's Mane Yambushitake) an edible culinary mushroom from Japan

Therapeutic dose of dried mycelia and fruiting body 3-5 grams/day

Support for Healthy Cognitive Function

Mushroom-nutrition based disease modifying therapy for treatment of patients with mild cognitive impairment, neurodegenerative diseases, neuronal injury

- Supports Control of Neuroinflammation
- Strengthens Memory and Concentration
- Enhances Cognition
- Stimulates the synthesis of Nerve Growth Factor (NGF) (Neuroregenerative)
- Promotes and Accelerates Myelination
- Promotes functional recovery and enhances nerve regeneration
- Provides Neuroprotection against toxicity, inflammation, oxidative stress, hypoxia
- Peripheral Neuropathy
- Neuropathic Pain

Active constituents

Erinacines (mycelia) are groups of cyathin diterpenoids that show biological activities as stimulators of NGF synthesis and could be useful as a treatment for neurodegenerative disorders and peripheral neuropathy

Hericenones (fruiting body) Studies on Mild Cognitive Impairment

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Review Article

Neurohealth Properties of *Hericium erinaceus* Mycelia Enriched with Erinacines

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Hericium erinaceus, an ideal culinary-medicinal mushroom, has become a well-established candidate in promoting positive brain and nerve health-related activities by inducing the nerve growth factor from its bioactive ingredient. Among its active compounds, only erinacine A has confirmed pharmacological actions in the central nervous system in rats. Hence, this review has summarized the available information on the neurohealth properties of *H. erinaceus* mycelia enriched with erinacines, which may contribute to further research on the therapeutic roles of these mycelia. The safety of this mushroom has also been discussed. Although it has been difficult to extrapolate the *in vivo* studies to clinical situations, preclinical studies have shown that there can be improvements in ischemic stroke, Parkinson's disease, Alzheimer's disease, and depression if *H. erinaceus* mycelia enriched with erinacines are included in daily meals.

1. Introduction

Diseases of the aging nervous system, such as Parkinson's disease, Alzheimer's disease, and stroke, are serious global public health crises as there is no cure for them currently. These lucrative markets have thus attracted the interest of a majority of large pharmaceutical companies which have put a tremendous effort into seeking medications to relieve the symptoms. However, despite successful preclinical testing, clinical trials for novel drugs have a poor track record of success.

In stroke and traumatic brain injuries, a variety of N-methyl-D-aspartate receptor antagonists have halted the progression of secondary damages in rodent models [1, 2], yet they have failed in human clinical trials due to unwanted side effects of the drugs [3, 4]. Likewise, levodopa is the primary treatment for Parkinson's disease that passes through the blood-brain barrier and gets converted into dopamine, but its long-term use can elicit additional clinical symptoms such as psychosis, mood fluctuations, increased cognitive impairment, or drug-induced dyskinesias [5]. Similarly, despite one new drug out of 244 compounds tested in 413 Alzheimer's disease clinical trials between 2002 and 2012 being approved for use, it cannot stop Alzheimer's from progressing [6]. Even though several other studies are underway, huge disappointment from the largest pharmaceutical companies, such as Axovant Sciences Ltd., Merck & Co Inc., Biogen Inc., Prana Biotechnology Ltd., and Pfizer Inc., was observed during recent times [7]. With a significant number of failed clinical trials and without a clear understanding of the potential mechanism of these diseases, dementia specialists have therefore turned their focus from treatment to prevention to stop further disease progression [8].

It is time to stop dementia before it starts. Recently, the search for small preventative neurotrophic compounds that

can cross the brain-blood and are responsible for the maintenance, survival, and regeneration of neurons has attracted much attention [9]. In particular, compounds derived from natural sources with fewer side effects that can be part of everyday nutrition may help with dementia prevention. Mushrooms, which are considered nutritionally functional foods and sources of physiologically beneficial medicines, can be excellent candidates for this cause.

Among all culinary mushrooms, *Hericium erinaceus* (most commonly known as lion's mane) has been widely reported to have therapeutic activities related to the promotion of nerve and brain health. Different compounds isolated from this mushroom inducing the expression of neurotrophic factors such as nerve growth factors (NGF) have been actively studied and reported [10–15]. Hericenones were typically found in the fruiting bodies while erinacines were derived from the mycelia of the mushroom (Figure 1).

A previous double-blinded clinical study has shown that oral administration of *H. erinaceus* fruiting body was effective in improving mild cognitive impairment in 50- to 80-year-old Japanese patients [16]. However, when examining the constituents of this effect, hericenones failed to stimulate NGF gene expression in primary cultured rat astroglial cells and 1321N1 human astrocytoma cells [17], suggesting that hericenones were not the key components responsible for the neuroprotective activities of this mushroom. On the other hand, the prominent beneficial effect of erinacine A was confirmed in the central nervous system in rats [18]. It is essential to know the concentrations of the bioactive compounds present in the functional ingredients to better assess their effects on the quality and bioactivity. For food industries, it is even critical that strict specifications of their ingredients are complied with. Therefore, this review will summarize the recent advances on the neurohealth properties of *H. erinaceus* mycelia enriched with erinacines (≥3 mg/g) and discuss the potential mechanisms of action responsible for these medicinal properties.

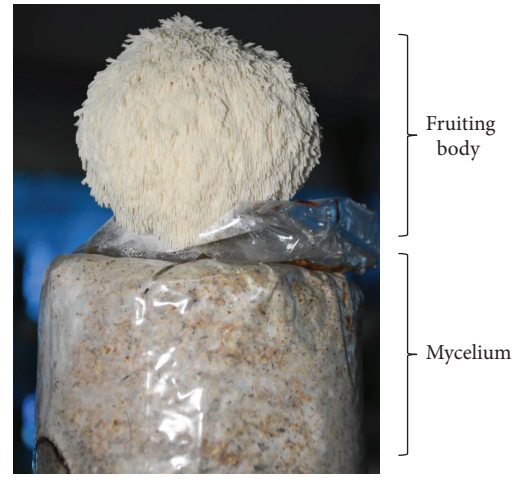

Figure 1: Fruiting body and mycelium of *H. erinaceus.*

2. Erinacines

Erinacines are groups of cyathin diterpenoids that show biological activities as stimulators of NGF synthesis and could be useful as a treatment for neurodegenerative disorders and peripheral neuropathy [19]. To date, 15 erinacines (erinacines A–K and P–S) have been identified (Figure 2) and further investigations have demonstrated that eight of them have various neuroprotective properties, such as enhancing NGF release (erinacines A–I), reducing amyloid-β deposition, increasing insulin-degrading enzyme (IDE) expression (erinacines A and S), or managing neuropathic pain (erinacine E), while others are either being currently discovered or have other pharmacological activities (Table 1). However, no direct evidence has yet shown that these compounds could pass through the blood-brain barrier. While other bioactive agents are still being explored, erinacine A has currently been the only one designed specifically to correlate results from *in vitro* studies with outcomes observed from *in vivo* studies [18], which could bring scientists a step closer to developing a better treatment option for neurodegenerative disorders.

2.1. Erinacine A. Erinacine A, the main representative of the erinacine group, not only has an enhancing effect on NGF synthesis *in vitro* [12] but also can increase NGF and catecholamine content in the locus coeruleus and hippocampus of rats after administration (8 mg/kg body weight) [18]. This enhanced amount of NGF appears to markedly increase neuronal survival in different brain areas and substantially improve behavioral outcomes in various animal models. In the experimental model of stroke, 1 mg/kg erinacine A administered intraperitoneally in rats for 90 min significantly increased cell survival, attenuated the expression of proinflammatory mediators, and reduced infarct volume after transient focal cerebral ischemia [24]. In another study, it was shown that oral treatment with erinacine A could reduce amyloid-β plaque burden by increasing Aβ degradation by elevating the level of IDE in 5-month-old APPswe/PS1ΔE9 double transgenic mice [20]. These preclinical studies are very encouraging and suggest that erinacine A is effective in reducing neurodegenerative disease-induced cell death. However, no studies have shown that erinacine A could be absorbed into the blood capillaries, cross the blood-brain barrier, and be localized in the brain. Hence, future studies measuring the concentration of erinacine A in the brain and blood could be performed to clarify these mechanisms in detail.

Interestingly, neuroprotective compounds may also be effective in cancer therapy. Given the increasing evidence showing that genes are upregulated in central nervous system disorders and downregulated in cancers and *vice versa* [25], it suggests a bright future for developing common therapeutic approaches in the treatment of these diseases. In line with this finding, treatments with erinacine A have been found to inhibit the proliferation of DLD-1 colorectal adenocarcinoma cells *in vitro* as well as the growth of DLD-1 tumors *in vivo* [21] (Table 1). Despite the promising results, erinacines in *H. erinaceus* mycelia are usually present in microquantities and minor variations in the environment can

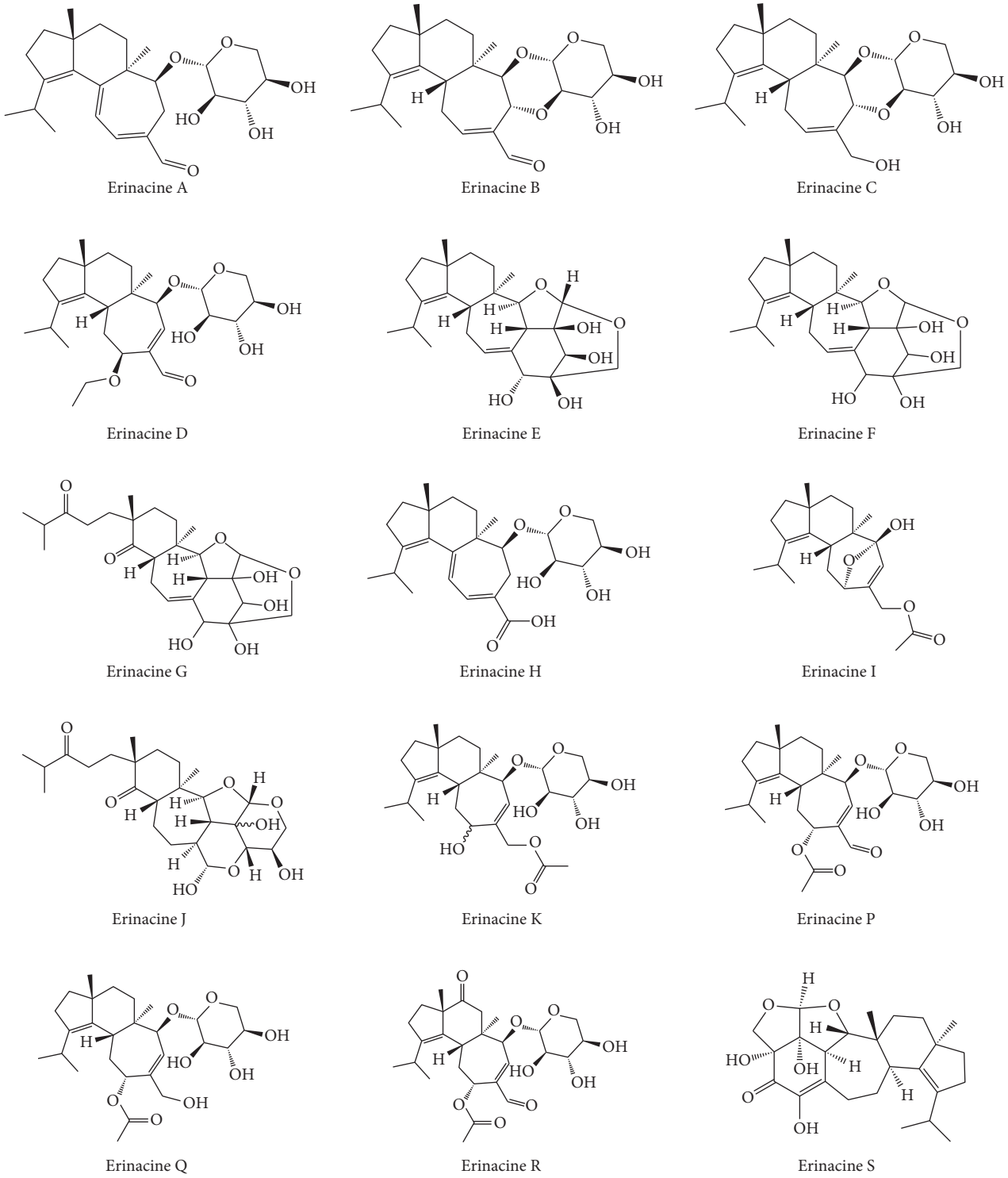

Figure 2: Chemical structures of 15 erinacines.

have huge impacts on the quantity, quality, and diversity of the metabolic products.

3. Production of Erinacines

As the fruiting body was reported to contain no erinacines [26], the best option would be to enhance erinacine production in *H. erinaceus* mycelia via submerged fermentation under constantly controlled culture parameters. Although chemical syntheses of cyathane-type diterpenoids are not impossible, they are complex, multistep processes that result in low yields and low purity levels [27]. Therefore, it seems highly desirable to biosynthesize erinacines using bioreactors to obtain a high yield of mycelia with high

Table 1: Erinacines with biological activities demonstrated *in vitro* and *in vivo*.

Erinacines	Tests	Concentration	Biological activities	Reference
Erinacine A	*In vitro*	1 mM	Induced 250.1 ± 36.2 pg/ml NGF synthesis	[12]
	In vivo	30 mg/kg body weight/day	(1) Reduced amyloid burden by 38.1 ± 19.7% (2) Increased IDE levels by 141.1 ± 63.7%	[20]
	In vivo	1 mg/kg body weight/day	Inhibited DLD-1 tumor growth by 66%	[21]
	In vivo	30 mg/kg body weight/day	(1) Reduced both the size and number of amyloid plaques (2) Increased IDE levels by 303.5% (3) Recovered from impairments in burrowing, nesting, and Morris water maze tasks	[22]
Erinacine B	*In vitro*	1 mM	Induced 129.7 ± 6.5 pg/ml NGF synthesis	[12]
Erinacine C	*In vitro*	1 mM	Induced 299.1 ± 59.6 pg/ml NGF synthesis	[12]
Erinacine D	*In vitro*	1.67 mM	Induced 141.5 ± 18.2 pg/ml NGF synthesis	[14]
Erinacine E	*In vitro*	5 mM	Induced 105.0 ± 5.2 pg/ml NGF synthesis	[13]
	In vitro	IC_{50}	Binding inhibitor for κ-opioid receptor at 0.8 μM	[23]
Erinacine F	*In vitro*	5 mM	Induced 175.0 ± 5.2 pg/ml NGF synthesis	[13]
Erinacine H	*In vitro*	70.8 mM	Induced 31.5 ± 1.7 pg/ml NGF synthesis	[15]
Erinacine S	*In vivo*	30 mg/kg body weight/day	(1) Reduced amyloid burden by 40.2 ± 15.2% (2) Increased IDE levels by 130.5 ± 68.9%	[20]
	In vivo	30 mg/kg body weight/day	(1) Reduced the size of amyloid plaques (2) Increased IDE levels by 269.8% (3) Recovered from impairments in burrowing, nesting, and Morris water maze tasks	[22]

concentrations of bioactive metabolites, which can expand mushroom potentialities for the development of functional foods, nutraceuticals, and novel drugs [28].

While there may have been various strategies developed over the past few decades for erinacine accumulation, it appeared, however, that only three reports concerning erinacines A and C have been published. In a 10 l bioreactor, a medium comprised of glucose 69.87 g/l, casein peptone 11.17 g/l, NaCl 1.45 g/l, $ZnSO_4$ 55.24 mg/l, and KH_2PO_4 1.0 g/l with a pH of 4.5 has produced 192 ± 42 mg/l of erinacine A after 8 days of cultivation [29]. With the monitoring of the temperature and ventilation during the processing, the highest yield of 206 ± 7 mg/l (17.34 mg/g) of erinacine A could be obtained after 14 days of cultivation using a 100 l bioreactor with the medium containing 0.5% yeast extract, 4% glucose, 0.5% soybean powder, 0.25% peptone, 1% oat, and 0.05% KH_2PO_4 at pH 5 [30]. These results suggest that a carbon-to-nitrogen (C/N) ratio of 6 and a pH value of 4 to 5 in a medium may be important parameters in promoting the biosynthesis of erinacine A in *H. erinaceus* mycelia.

Scale-up of pilot plant fermentors to large-scale bioreactors to enhance the biomass as well as erinacine production could also be an attractive proposal. Although various factors such as improper distribution of oxygen, uneven distribution of the media, or insufficient agitation environment could cause negative impacts on product formation and quality at a higher scale of operation [31], there has been one successful example of commercial exploitation. In this case, the medium was optimized for a C/N ratio of 10, temperature of 26°C, pH of 4.5, and agitation of 120 rpm. The highest accumulation of erinacine A (5 mg/g) was observed with 20-ton fermentors after 12 days [32]. This preliminary result was satisfactory, showing that implementation and successful commercial exploitation of research results in large-scale bioreactors are possible.

For erinacine C production, the optimal medium was found to include 5 g/l oatmeal, 1.5 g/l calcium carbonate, and 0.5 g/l Edamin® K at pH 7.5, which can generate concentrations up to 2.73 g/l after six days of cultivation [33]. However, it is noteworthy that this process was accomplished in a two-step course. The fungal pellets were concentrated by centrifugation to remove preculture medium components before inoculation of the main culture. Although an inoculation ratio of 5 : 10 volume/volume (*v*/*v*) is beneficial in producing erinacine C, it is only reproducible at a small laboratory scale and not feasible in industrial operations as the concentrated biomass is not easily adapted for the aseptic handling of large volumes.

These findings are extremely important as they could be used as references to enhance the production of useful secondary metabolites for industrial applications. Moreover, it should be noted that the presence of erinacines in *H. erinaceus* mycelia can also achieve pharmacological benefits. In this regard, isolation of erinacines from *H. erinaceus* mycelia is particularly important, as they could serve as quality controls in assuring the efficacy, quality, and safety of this mushroom in future markets.

4. *In Vivo* Preclinical Studies of *Hericium erinaceus* Mycelia Enriched with Erinacines

While 1/5 of dementia cases can be reversible in some cases when caused by drugs, alcohol, hormone imbalances, or depression, a significant proportion of individuals suffer

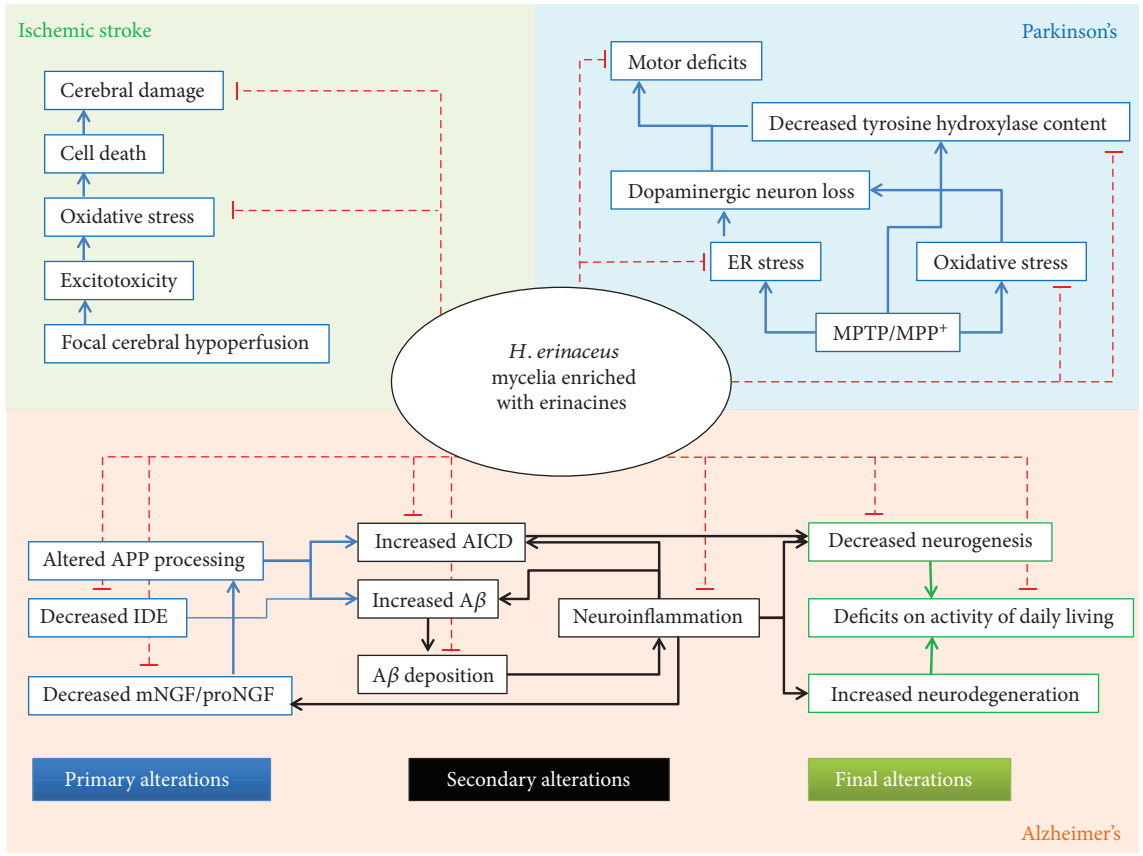

Figure 3: Summary of mechanisms of action of *H. erinaceus* mycelia enriched with erinacines in dementia. Primary alterations are possible contributors and drivers in the pathogenesis of Alzheimer's disease. Secondary alterations include increased amyloid precursor protein intracellular domain (AICD) and accumulation of Aβ, leading to neuroinflammation. Finally, decreased neurogenesis and increased neurodegeneration can cause deficits in activities of daily living. The red dashed lines indicate potential mechanisms of *H. erinaceus* mycelia-attenuated pathological and behavioral changes in stroke, Parkinson's disease, and Alzheimer's disease.

from dementias that are irreversible [34]. The most common irreversible dementia types include Alzheimer's disease, vascular dementia, Lewy body dementia, Parkinson's disease, and frontotemporal dementia [35]. Luckily, growing preclinical studies have demonstrated that the risk of dementia and cognitive impairment could be reduced in the early stages by erinacine-enriched *H. erinaceus* mycelium consumption. Figure 3 illustrates the overall therapeutic mechanism of action of *H. erinaceus* mycelia enriched with erinacine in dementia.

4.1. Protection against Ischemic Stroke. In a rat model of transient focal cerebral ischemia via the middle cerebral artery occlusion method, pretreatment with 3 mg/g erinacine A-enriched *H. erinaceus* mycelia orally at concentrations of 50 and 300 mg/kg for 5 days could reduce the total infarcted volumes by 22% and 44%, respectively [24]. Moreover, immunohistochemistry for neuronal nuclei (NeuN) revealed the presence of significantly more neurons after brain injuries in rats which were treated with erinacine A-enriched *H. erinaceus* mycelia. Excessive reactive oxygen species and oxidative stress have been strongly implicated in the pathogenesis of ischemic brain injury [36]. Decreased levels of proinflammatory cytokines and inducible NO synthase (iNOS), however, have been detected in ischemic neurons after mycelia exposure. These findings suggested that erinacine A-enriched *H. erinaceus* mycelia may be a promising agent for stroke injury as these have the ability to decrease neuronal apoptosis and reduce stroke cavity size in the rat brains by targeting iNOS/reactive nitrogen species (RNS) and p38 mitogen-activated protein kinase (MAPK)/CCAAT enhancer-binding protein homologous protein (CHOP) pathways.

4.2. Protection against Parkinson's Disease. Parkinson's disease (PD) is the second most common neurodegenerative disorder that is characterized by the progressive loss of dopaminergic cells in the substantia nigra pars compacta region of the brain, which results in motor problems including resting tremor, rigidity, bradykinesia, and postural instability [37]. Among models of PD, the involvement of the drug 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) is most widely used. Once inside the brain, MPTP is metabolized into the toxic cation 1-methyl-4-phenylpyridinium (MPP^+) by the enzyme monoamine oxidase B, resulting in nigral dopaminergic neuronal death and mitochondrial damage, which can mimic the clinical and pathological features of PD [38]. In one study, the neuroprotective effect of erinacine

A-enriched *H. erinaceus* mycelia was assessed in the MPTP-induced PD model. Results showed that dopaminergic lesions and oxidative stress in the stratum and substantia nigra were significantly improved after pretreatment with 3 mg/g erinacine A-enriched *H. erinaceus* mycelia for 25 days [39]. Furthermore, the mycelia could reverse MPTP-associated motor deficits, as revealed by the analysis of the rotarod assessment. The mechanisms underlying the neuroprotective effect of erinacine A-enriched *H. erinaceus* mycelia were associated with the inhibition on the endoplasmic reticulum stress by lowering the expression of Fas and Bax via inositol-requiring enzyme 1α (IRE1α)/tumor necrosis factor receptor-associated factor 2 (TRAF2) complex formation and phosphorylation of c-Jun N-terminal protein kinase (JNK) 1/2, p38 and nuclear factor kappa light chain enhancer of activated B cell (NF-κB) pathways. Taken together, these results have demonstrated that erinacine A-enriched *H. erinaceus* mycelia have the potential to be a new therapeutic agent for the prevention and treatment of PD.

4.3. Protection against Alzheimer's Disease. There has been growing evidence which suggested that Alzheimer's disease progression becomes a runaway chain reaction after a certain point. In the presence of amyloid-β plaques, secondary injuries such as inflammation, excitotoxicity, and apoptosis may trigger the deposition of hyperphosphorylated tau proteins [40]. Once the process starts, the tau tangles are unabated even after the removal of amyloid-β plaques. Moreover, studies in transgenic amyloid precursor protein (APP) mice have shown that therapies are most effective when administered before plaque formation [41, 42]. Therefore, amyloid-β has become an ideal therapeutic target for primary prevention.

In one study, APPswe/PS1dE9 transgenic mice were utilized to evaluate the therapeutic effect of *H. erinaceus* mycelia containing 19 mg/g erinacine A on Alzheimer's disease. After 30 days of oral administration to 5-month-old transgenic mice, these mycelia were able to attenuate cerebral Aβ plaque burden, prevent recruitment and activation of plaque-associated microglia and astrocytes, promote the expression of IDE, increase the NGF-to-NGF precursor (proNGF) ratio, and enhance the proliferation of neuron progenitors and the number of newly born neurons in the dentate gyrus region [43]. Additionally, improvements in the impairment of other multiple brain regions were also shown when APP/PS1 transgenic mice treated with *H. erinaceus* mycelia could recover behavioral deficits after 81 days of administration. Collectively, these findings raise the possibility that prevention with erinacine A-enriched *H. erinaceus* mycelia could be an effective therapeutic strategy for managing Alzheimer's disease.

4.4. Protection against Depressive Symptoms. Depression is the most frequently occurring psychiatric comorbidity, with prevalence in Alzheimer's, Parkinson's, and stroke as high as 87%, 75%, and 79%, respectively [44]. Prior data has shown that levels of NGF are significantly lower in patients with major depressive disorder than in healthy subjects [45]. *H. erinaceus* mycelia enriched with erinacines, which are involved in the creation of the neurotrophic factors, are thereby hypothesized to play a role in depression.

In animal models, chronic restraint stress is known to cause decreased BDNF expression in the hippocampus and depression-like behaviors [46]. Hence, alleviation of *H. erinaceus* mycelia enriched with erinacines in animals subjected to repeated chronic stress was examined [47]. Two weeks of treatment with *H. erinaceus* mycelia have reduced the immobility time in the tail suspension test and forced swimming test as well as decreased the number of entries and the time spent in the open arm. In addition, restraint-induced low levels of norepinephrine, dopamine, serotonin, high interleukin-6, and tumor necrosis factor-α in the hippocampus were completely reversed by *H. erinaceus* mycelium administration. Furthermore, *H. erinaceus* mycelium was shown to activate the BDNF pathways and block NF-κB signals in mice. Hence, these results indicate that *H. erinaceus* mycelia could be an attractive agent for the treatment of depressive disorders through the modulation of monoamine neurotransmitters and proinflammatory cytokines as well as the regulation of brain-derived neurotrophic factor (BDNF) pathways.

4.5. Protection against Neuropathic Pain. Currently, there is a growing realization that lesions to the peripheral or central nervous system could lead to neuropathic pain [48]. Currently, both ionotropic P2X receptors and metabotropic P2Y receptors have been identified as key receptors in mediating neuropathic pain [49]. As *H. erinaceus* mycelium has a crucial role in nerve regeneration via the stimulation of neurotrophic factors, the analgesic potential of this mycelium using both a P2 purinergic receptor-coupled Ca^{2+} signaling platform and an *in vivo* model was investigated. The results indicated that the extracts of *H. erinaceus* mycelium could completely block ATP-induced Ca^{2+} signaling in human HOS cells, suggesting its inhibitory potential as a modulator of pain-related P2X receptors [50]. In addition, administration of the extracts of *H. erinaceus* mycelium in heat-induced mice could significantly postpone the tail-flick response to heat stimulation as well as the paw-lifting response to a hot plate, indicating that it has an excellent potential for pain relief.

4.6. Protection against Presbycusis. Recent research has highlighted that presbycusis may precede the onset of clinical dementia and may present as an early manifestation of probable Alzheimer's disease [51]. Exogenous application of NGF has been the first to promote nerve fiber regrowth or sprouting in deafened guinea pigs caused by neomycin [52]. Moreover, clinical studies in patients with sensorineural hearing defects have revealed that the amount of circulating NGF is relatively lower compared to the level found in normal patients [53]. Therefore, the otoprotective effect of *H. erinaceus* mycelia enriched with erinacines in rapidly aging mice has been observed [54]. The results indicated that the *H. erinaceus* mycelium-treated group had significantly lower hearing thresholds according to auditory brainstem responses measured using click sounds and 8 kHz and 16 kHz tone burst sound stimulation when compared with the control group. These findings suggested that *H. erinaceus*

Table 2: The beneficial activities of *H. erinaceus* mycelium and its active components on age-associated cognitive change and early dementia.

Material studied (dose used)	*In vivo* models	Effects	Reference
Erinacine A	Normal Wistar rats	Enhanced NGF and catecholamine secretion in the LC and hippocampus after intragastric dosing erinacine A at 8 mg/kg body weight	[18]
Erinacine A-enriched mycelia and erinacine A	Ischemic stroke in Sprague-Dawley rats	(1) Mycelia at 50 and 300 mg/kg body weight reduced infarcted volume in cortex and subcortex of transient stroke rats (2) Erinacine A at 1, 5, and 10 mg/kg body weight reduced levels of proinflammatory cytokines such as iNOS, IL-1β, IL-6, and TNF-α in the serum of transient stroke rats	[24]
Erinacine A-enriched mycelia	APPswe/PS1dE9 transgenic mice	(1) Mycelia at 300 mg/kg body weight reduced amyloid plaque burden in the area including the cerebral cortex and hippocampus (2) Increased NGF/proNGF ratio and promoted hippocampal neurogenesis (3) Restored nesting behavior	[43]
Erinacine A Erinacine S	APPswe/PS1dE9 transgenic mice	(1) Both compounds at 30 mg/kg body weight reduced amyloid plaque burden in the cerebral cortex (2) Increased the level of IDE in the cortex by $130.5 \pm 68.9\%$ and $141.1 \pm 63.7\%$, respectively	[20]
Erinacine A-enriched mycelia	MPTP-induced neurotoxicity in C57BL/6 mice	(1) Treatment at 10.76 and 21.52 mg/day elevated dopamine, NGF, and GSH levels (2) Reduced motor dysfunction (3) Reduced dopaminergic neurons apoptosis in the striatum and substantia nigra	[39]
Mycelia ethanolic extract	C57BL/6 mice	(1) Treatment at 2000 mg/kg body weight blocked the rise in $[Ca^{2+}]$ induced by ATP (2) Increased the latency in tail-flick and paw-lifting times exposed to a thermal stimulus	[50]
Erinacine A-enriched mycelium	Restraint stress induced depression in ICR mice	(1) Treatment at 200 and 400 mg/kg body weight increased dopamine and serotonin levels (2) Increased BDNF, TrκB, and PI3K expressions in the hippocampus (3) Reduced IL-6 and TNF-α levels (4) Reduced the immobility time in the tail suspension test and forced swimming test, as well as decreased the number of entries and the time spent in the open arm	[47]

mycelium diet supplementation was effective in slowing hearing threshold deterioration.

The beneficial activities of *H. erinaceus* mycelia on age-associated cognitive change and early dementia are summarized in Table 2. Given the fact that all seven of these studies have provided very encouraging findings, it is also of paramount importance that the daily intake of *H. erinaceus* mycelia in the context of the entire diet is established before the treatment is administered.

5. Toxicology Studies

To date, all experimental studies have suggested that *H. erinaceus* mycelium is safe and devoid of adverse effects (Table 3). In an animal study, the acute oral LD_{50} of *H. erinaceus* mycelia enriched with its active compounds was found to be higher than 5 g/kg in rats [55], indicating that the mycelium is reasonably safe in cases of overdose. Repeated daily doses of *H. erinaceus* mycelium enriched with its active compounds up to 3 g/kg have also been used without any adverse effects in rats [32]. Moreover, *H. erinaceus* mycelium was found not to be mutagenic in the bacterial reverse mutation test (Ames test), *in vitro* chromosome aberration test, and *in vivo* erythrocyte micronucleus test, with and without metabolic activation [56]. Further investigations also showed that erinacine-enriched *H. erinaceus* mycelium was not teratogenic in Sprague-Dawley rats with doses up to 2625 mg/kg [55]. In a well-designed clinical trial, erinacine-enriched *H. erinaceus* mycelia demonstrated significant clinical efficacy and had good safety and tolerability in 36 patients with Alzheimer's disease (unpublished data).

6. Conclusion

The evidence so far has shown that *H. erinaceus* mycelium enriched with its active compounds is capable of delaying neuronal cell death in rats with neurodegenerative diseases, such as ischemic stroke, Parkinson's disease, Alzheimer's disease, and depression. Moreover, results have indicated that administration of *H. erinaceus* mycelia enriched with its active compounds can promote functional recovery and enhance nerve regeneration in rats with neuropathic pain or presbycusis. Despite that more clinical research is needed to fully understand the potential applications of erinacine-

Table 3: The safety of *H. erinaceus* mycelia.

Material studied (dose used)	*In vivo* models	Effects	Reference
Erinacine A-enriched mycelia	Normal ICR mice	No adverse effects in (1) Bacterial reverse mutation test (Ames test) up to 5 mg/plate (2) *In vitro* chromosome aberration test up to 2.5 mg/ml (3) *In vivo* erythrocyte micronucleus test up to 5 mg/kg body weight	[56]
Erinacine A-enriched mycelia	Normal Sprague-Dawley rats	(1) Ethanolic extract induced neuritogenesis in postnatal cortical neurons (2) No adverse effect up to 5 g/kg body weight/day after acute exposure (3) No adverse effect up to 2625 mg/kg body weight/day for prenatal developmental study	[55]
Erinacine A-enriched mycelia	Normal Sprague-Dawley rats	No adverse effect up to 3 g/kg body weight/day for 28 days	[32]

Based on these results, the toxicity profile of *H. erinaceus* mycelium enriched with its active compound is extremely low and therefore has the potential to be developed into a functional ingredient or food associated with improved brain and nerve health. With this idea in mind, the first erinacine A-enriched *H. erinaceus* mycelium product was introduced to the market in 2015 in Taiwan [57].

enriched *Hericium erinaceus* mycelium, the majority of preclinical data strongly suggests that it is safe and offers much-needed neuroprotective applications.

Abbreviations

AICD: Amyloid precursor protein intracellular domain
APP: Amyloid precursor protein
BDNF: Brain-derived neurotrophic factor
CHOP: CCAAT enhancer-binding protein homologous protein
C/N: Carbon to nitrogen
ERK: Extracellular-signal-regulated kinase
IDE: Insulin-degrading enzyme
iNOS: Inducible NO synthase
IRE1α: Inositol-requiring enzyme 1α
JNK: c-Jun N-terminal protein kinase
MAPK: Mitogen-activated protein kinase
MPTP: 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MPP^+: 1-Methyl-4-phenylpyridinium
NeuN: Neuronal nuclei
NF-κB: Nuclear factor kappa light chain enhancer of activated B cells
NGF: Nerve growth factor
PD: Parkinson's disease
proNGF: NGF precursor
RNS: Reactive nitrogen species
TRAF2: Tumor necrosis factor receptor-associated factor 2
v/*v*: Volume/volume.

Conflicts of Interest

The authors declare that they have no conflict of interest.

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