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Original Article

Melaleuca quinquenervia essential oil inhibits α-melanocyte-stimulating hormone-induced melanin production and oxidative stress in B16 melanoma cells

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ABSTRACT

Background: Essential oils are odorous, volatile products of plant secondary metabolism, which are found in many leaves and stems. They show important biological activities, which account for the development of aromatherapy used in complementary and alternative medicine. The essential oil extracted from *Melaleuca quinquenervia* (Cav.) S.T. Blake (paperbark) (MQ-EO) has various functional properties.
Purpose: The aim of this study is to investigate the chemical composition of MQ-EO by using gas chromatography-mass spectrometry (GC-MS) and evaluate its tyrosinase inhibitory activity.
Methods: Gas chromatography-mass spectrometry (GC-MS)-based metabolomics was used to identify 18 components in MQ-EO. The main components identified were 1,8-cineole (21.60%), α-pinene (15.93%), viridiflorol (14.55%), and α-terpineol (13.73%). B16 melanoma cells were treated with α-melanocyte-stimulating hormone (α-MSH) in the presence of various concentrations of MQ-EO or its major compounds. Cell viability was accessed by MTT assay and cellular tyrosinase activity and melanin content were determined by using spectrophotographic methods. The antioxidant mechanism of MQ-EO in α-MSH stimulated B16 cells was also investigated.
Results: In α-melanocyte-stimulating hormone (α-MSH)-stimulated murine B16 melanoma cells, MQ-EO, 1,8-cineole, α-pinene, and α-terpineol significantly reduced melanin content and tyrosinase activity. Moreover, MQ-EO, 1,8-cineole, α-pinene, and α-terpineol decreased malondialdehyde (MDA) levels. In addition, restored glutathione (GSH) levels, glutathione peroxidase (GPx), superoxide dismutase (SOD), and catalase activities were increased in α-MSH-stimulated B16 cells. MQ-EO not only decreased apoptosis but also reduced DNA damage in α-MSH stimulated B16 cells. These results showed that MQ-EO and its main components, 1,8-cineole, α-pinene, and α-terpineol, possessed potent anti-tyrosinase and anti-melanogenic activities besides the antioxidant properties.
Conclusions: The active functional components of MQ-EO were found to be 1,8-cineole, α-pinene, and α-terpineol. Consequently, the results of present study suggest that MQ-EO is non-cytotoxic and can be used as a skin-whitening agent, both medically and cosmetically.

Introduction

Tyrosinase (polyphenol oxidase, EC 1.14.18.1) is a multifunctional copper-containing enzyme, which is widely distributed in plants, microorganisms, fungi, and animals. Tyrosinase, the key enzyme in melanin biosynthesis, has been the popular target for melanogenesis inhibitors; many compounds have been found to inhibit tyrosinase activity. Tyrosinase inhibition is the most common approach used for skin whitening because it is the key enzyme that catalyzes the rate-limiting step in melanin biosynthesis (Ando et al., 2007; Gillbro and Olsson, 2011). Tyrosinase catalyzes the hydroxylation of L-tyrosine to yield 3,4-dihydroxyphenylalanine (L-DOPA), followed by oxidation of L-DOPA to

Abbreviations: MQ-EO, *Melaleuca quinquenervia* essential oil; GC-MS, Gas chromatography-mass spectrometry; α-MSH, α-melanocyte-stimulating hormone; L-DOPA, 3,4-dihydroxyphenylalanine; TRPs, tyrosinase-related proteins; ROS, reactive oxygen species; H_2O_2, hydrogen peroxide; MDA, malondialdehyde; GSH, glutathione; GPx, glutathione peroxidase; SOD, superoxide dismutase; CAT, catalase

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dopaquinone. Oxidative polymerization of several dopaquinone derivatives results in formation of melanin. Hyperpigmentation, hypermelanosis, skin darkening, or tanning can result from ultraviolet (UV) exposure, drugs, or post-inflammatory conditions (Ando et al., 2007; Gillbro and Olsson, 2011; Tang and Chen, 2015).

Melanogenesis is primarily stimulated by the UV induced α-melanin-stimulating hormone (α-MSH) in melanocytes (Roulin et al., 2011). Melanogenesis is a complex process regulated by tyrosinase and tyrosinase-related proteins (TRPs). Tyrosinase plays a critical role in melanin production *via* hydroxylation of tyrosine into DOPA followed by further oxidation of DOPA into dopaquinone (Munoz-Munoz et al., 2009). Therefore, inhibition of tyrosinase is the simplest approach used to achieve skin hypopigmentation because it is the key enzyme that catalyzes the rate-limiting step in melanogenesis. Tyrosinase inhibitors are widely used in agriculture and food industry, as well as in medicinal and cosmetic products owing to their antioxidant effects and their ability to reduce excessive pigmentation. Melanin, a pigment produced by the melanocytes, is responsible for the skin color as well as protection of the skin from UV-induced injury. Melanogenesis is reported to result in the production of hydrogen peroxide (H_2O_2) and other reactive oxygen species (ROS), which subject the melanocytes to high-grade oxidative stress. ROS are considered to play a significant role in the regulation of melanogenesis, whereas ROS scavengers and inhibitors of ROS generation may down-regulate UV-induced melanogenesis (Perluigi et al., 2003; Yamakoshi et al., 2003). Therefore, several antioxidants, such as ascorbic acid derivatives and reduced glutathione (GSH), have been applied as inhibitors of melanogenesis (Imokawa, 1989; Kumano et al., 1998).

Arbutin, a *β*-D-glucopyranoside derivative of hydroquinone, was found to be a competitive inhibitor of tyrosinase activity in cultured melanocytes at non-cytotoxic concentrations (Ando et al., 2007). Depigmenting agents, such as hydroquinone, kojic acid, arbutin, ascorbic acid, and sulfhydryl compounds, are effective in interfering with tyrosinase activity (Chang, 2009). Tyrosinase inhibitors can chelate copper to prevent the substrate binding. Commercially available skin-whitening agents often irritate the skin. For example, kojic acid has been associated with dermatitis and erythema (Nakagawa et al., 1995). In addition, well-known whitening agents, such as kojic acid and hydroquinone, may induce adverse reactions, such as skin irritation, dermatitis, depigmentation, and even cancer (Tang and Chen, 2015).

Essential oils (EOs) are odorous, volatile products of plant secondary metabolism, are which found in many leaves and stems. They show important biological activities, which account for the development of aromatherapy used in complementary and alternative medicine. Analysis of the oil isolated from the leaves of *Melaleuca quinquenervia* (Cav) S.T. Blake (broadleaf paperbark) over its geographical range (Australia and Papua New Guinea) has shown wide variation in its chemical composition with two major chemotypes. Chemotype 1 is comprised of *E*-nerolidol and linalool, whereas chemotype 2 contains 1,8-cineole (monoterpene), viridiflorol (sesquiterpene), α-terpineol (monoterpene), and β-caryophyllene (Ireland et al., 2002). The leaves of *M. quinquenervia* are considered a source of 1,8-cineole-rich essential oil, called Niaouli oil, which is used in pharmaceutical preparations for the relief from cough and cold, rheumatism, and neuralgia as well as in aromatherapy (Elliot and Jones, 1993).

In this study, we aimed to investigate the chemical composition of MQ-EO by using gas chromatography-mass spectrometry (GC-MS) and evaluate its tyrosinase inhibitory activity. For this purpose, the effects of MQ-EO and its chemical components on α-MSH-stimulated B16 melanoma cells were assessed.

Materials and methods

Chemicals and reagents

Steam-distilled essential oil of *M. quinquenervia* (MQ-EO) was purchased from Lorien Vana Biotech, Inc., Taiwan. α-Melanocyte-stimulating hormone (α-MSH), dimethyl sulfoxide (DMSO), monobromobimane (MbBr), phenylmethylsulfonyl fluoride (PMSF), 4′,6-diamidino-2-phenylindole (DAPI), formaldehyde, and arbutin were purchased from Sigma-Aldrich Chemicals Co. (St. Louis, MO, USA). L-3,4-dihydroxyphenylalanine (L-DOPA) was purchased from Merck (Darmstadt, Germany). Dulbecco's modified eagle medium (DMEM), fetal bovine serum (FBS), L-glutamine, and penicillin-streptomycin were purchased from Invitrogen Life Technologies (Carlsbad, CA, USA). 1,8-cineole and α-terpineol were purchased from Alfa Aesar. α-pinene was purchased from Sigma-Aldrich. All reagents used were of at least reagent grade. The purity of α-terpineol, α-pinene and 1,8-cineole were 96%, 98%, 99% respectively. Deionized distilled water (ddH_2O), used for preparation of solutions and buffers, was purified using a Milli-Q system (Millipore, Bedford, MA, USA).

Table 1
Main constituents of *Melaleuca quinquenervia* essential oil.

Peak no.	Compound	Rt	KI	Area%
1	α-Pinene (monoterpene)	6.64	934	15.93
2	β-Pinene (monoterpene)	7.40	980	4.12
3	*o*-Cymene (monoterpene)	8.21	1029	7.61
4	Limonene (monoterpene)	8.28	1034	5.38
5	1,8-Cineole (monoterpene)	8.42	1042	21.60
6	γ-Terpinene	10.57	1181	1.30
7	Terpinolene (monoterpene)	10.61	1184	1.37
8	4-Terpineol	10.68	1188	1.82
9	α-Terpineol (monoterpene)	10.92	1205	13.73
10	α-Terpinyl acetate	12.98	1353	2.29
11	Caryophyllene (sesquiterpene)	13.97	1429	2.07
12	α-Humulene (sesquiterpene)	14.43	1466	0.55
13	β-Humulene	14.49	1471	1.13
14	Varidiflorene	14.87	1501	2.59
15	α-Selinene	14.94	1507	0.53
16	Nerolidol	15.66	1567	1.66
17	Viridiflorol (sesquiterpene)	16.19	1612	14.55
18	Ledol	16.29	1621	1.76

Rt: Retention time; KI: Kovat's Index.

Gas chromatograph-mass spectrometry analysis

The essential oil was analyzed by gas chromatography-mass spectrometry (GC-MS) to identify its constituents. GC/MS with a TSQ Quantum GC system (Thermo Scientific, San Jose, CA, USA) was equipped with a DB-5MS capillary column (30 m × 0.25 mm i.d., 0.25 μm film thickness, Agilent Technologies, Santa Clara, CA, USA). The *Melaleuca quinquenervia* essential oil was diluted to 0.1% (v/v) in hexane. The injector temperature was maintained at 250 °C. Helium (He) was used as the carrier gas at a flow rate of 1.5 ml/min, and the split ratio was set at 1:30. The initial oven temperature was maintained at 35 °C for 2 min and then was programmed to increase at a rate of 10 °C/min to 300 °C and held at 300 °C for 10 min. Electron ionization (EI) was used as the ionization method and the ion source temperature was set at 200 °C. The mass spectrometry conditions were as follows: scan range, 40–350 amu; ion source temperature, 230 °C; and interface temperature, 260 °C; solvent delay time, 6 min. The essential oil constituents were identified by comparing the retention times and retention indices of the chromatographic peaks with a standard library from the National Institute of Standards and Technology (NIST) MS spectral database (version, 2005). In addition, the measured Kovats index (KI) was compared to a homologous series of *n*-alkanes (C_7–C_{40}).

Cell cultures

B16 murine melanoma cell line was purchased from the Bioresource Collection and Research Center (BCRC; Hsinchu, Taiwan). The B16 cells were cultured in DMEM supplemented with 10% FBS, 2 mM glutamine,