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Research Article
Anti-melanogenesis Efficacy of Ginkgolide B is Favored by Attenuating Oxidative Stress and Melanin Synthesis in B16f1 Melanoma Cell Model

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Abstract

Background and Objective: Ginkgolide B (GB) is a terpene lactone isolated from a popular ancient Chinese tree *Ginkgo biloba* leaves which possess numerous biological activity. The current study was intended to examine the anti-melanogenic property of GB in B16F1 melanoma cell model by assessing cell viability, free radical production (ROS), tyrosinase activity and melanin content (synthesis). **Materials and Methods:** B16F1 murine melanoma cells were cultures in DMEM and pre-treated with GB and later stimulated with H_2O_2 or α-MSH to mimic UV induced hyperpigmentation (hyper-melanogenesis). **Results:** B16F1 murine melanoma cells (stimulated with H_2O_2 or α-MSH) and pretreated with increased concentration of GB (5,10, 20 and 50 μg mL^{-1}- 1 h before H_2O_2 or α-MSH stimulation) showed reduced (p<0.001) melanoma cell count (proliferation), lesser (p<0.001) ROS production, inhibit (p<0.01; p<0.001) tyrosinase activity and thus lowered (p<0.001) the melanin content via down regulating (p<0.001) the proteins expression of tyrosinase-related proteins-1 and 2 (TYRP-1 and 2), microphthalmia-associated transcription factor (MITF). **Conclusion:** This result clearly endowed that GB from *Ginkgo biloba* leaves showed better anti-melanogenic property in a dose-dependent fashion by abolishing excessive ROS generation (oxidative stress) as well as effectively inhibited tyrosinase activity and thereby suppress the production of melanin which reflects in decreased melanin count. Hence, it recommended that GB could be used as a skin whitening agent or derma-protective agent in the cosmetic industry.

Key words: Ginkgolide B, melanoma, tyrosinase, MITF, TYPR-1 and 2

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Melanin is the main pigment secreted from melanosomes of melanocyte cells, which are highly expressed upon chronic sun exposure (UV light), oxidative stress or during other hyperpigmentation diseases such as melasma, leukomelanosis and melanoma which trigger the secretion of alpha melanin-stimulating hormone (α-MSH) and initiate melanin synthesis[1,2]. The main function of melanin pigment is to give color to skin and protect the skin from harmful ultraviolet (UV) radiation by abolishing the excessive production of Reactive Oxygen Species (ROS). Tyrosinase activity was directly controlled or stabilized by tyrosinase-related protein (TYRP-1 and 2) which is a copper containing enzyme that catalyzes the conversion of L-Tyrosine into 3,4-dihydroxyphenylalanine (DOPA) and ultimately into DOPAquinone[3,4].

Two types of melanin (pheomelanin and eumelanin) are synthesized by tyrosinase enzyme with the help of TYRP-1/2 and MITF. Tyrosinase is a rate-limiting enzyme for the *de novo* synthesis pathway of melanin (melanogenesis) as it catalyzes the first two steps of melanin synthesis[5,6]. Therefore, the quest for finding a potent natural tyrosinase inhibitor (anti-melanogenic agent) without adverse effect was in high demand[7,8]. Previously, many studies have hinted that polyphenols with anti-oxidant, anti-inflammatory, immune modulatory and anti-cancer activity would be the best contender for exploring anti-melanogenic agent against cell line model[9,10].

Ginkgolide B (GB) is a terpene lactone (diterpenoid) isolated from a popular ancient Chinese tree *Ginkgo biloba* leaves which possess numerous biological activity like antioxidant, anti-inflammatory and anti-cancer properties[11]. Studies have shown that GB also possesses neuroprotective activity against amyloid (Aβ) induced oxidative stress for Alzheimer disease model in SH-SY5Y cell line owing to potent antioxidant and free radical scavenging activity[12]. Ample amount of studies has demonstrated that GB exhibit protective effect against various abnormalities like ischemic brain/cardiac damage[13,14]. Previously, GB has been reported as anti-atherogenic and anti-inflammatory agent owing to platelet activating factor antagonist property[15]. Moreover, GB is well known for its anti-tumor or cancer property particularly against urinary bladder, colitis and pancreatic cancer[16,17]. Based on the above beneficial properties (anti-oxidant, anti-inflammatory and anti-cancer properties), this study choosed GB for current investigation to explore the anti-melanogenic property against B16F1 melanoma cell model by assessing cell viability, free radical production (ROS), tyrosinase activity and melanin synthesis.

MATERIALS AND METHODS

B16F1 cell line studies: B16F1 melanoma cells (from murine) was provided by the American Type-Culture Collection (ATCC) center (MD, USA). B16F1 melanoma cells were grown (cultured) in Dulbecco's Modified Eagles Medium (DMEM) containing 1% Penicillin and Streptomycin, 10% Fetal Bovine Serum (FBS), 1mM Glutathione (Invitrogen, CA, USA) in 5% CO_2 at 37°C. Generally, the media will be changed thrice a week and cells were sub-cultured when it reached the confluence with 0.25% Trypsin/0.55 mM Ethylenediamine tetra-acetic acid (EDTA).

Sample preparation procedure: The GB (99% HPLC grade) were bought from the National Institute for Food and Drug Control (Beijing, China). The GB was dissolved in di-methyl-sulfoxide (DMSO) solvent at a concentration of 10 g dL^{-1}. The protein content was determined by Pierce BCA Protein Assay Kit (MA, USA).

Cell viability assay or anti-proliferative activity: The viability of B16F1 melanoma cells were assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) method. Next day media was discarded and substituted with new media and mixed with increasing concentrations of GB (5, 10, 20 and 50 μg mL^{-1}), DMSO (alone) at 37°C for 24 h. To the above mixture add 100 μL of MTT reagent and incubated at 37°C for 40 min and the absorbance (optical density-OD) was measured at 560 nm using microplate reader (ELx808, BioTek Instruments; VT, USA).

Morphological analysis: For morphological analysis, B16F1 melanoma cells were co-cultured with GB (5, 10, 20 and 50 μg mL^{-1}) and DMSO (control) and then the cells were observed under phase contract light microscope (Nikon Eclipse T 100, Tokyo, Japan) and pictured (photographed) using a digital camera (Nikon-300, Tokyo, Japan).

Determination of free radical generation (ROS): The intracellular ROS (free radical) generation was quantified by 2',7'-dichlorofluorescein diacetate (DCFH-DA) by the Bai and Cederbaum[18] method and the absorbance was read using a fluorescence spectrophotometer (λ_{ex} = 488 nm and λ_{em} = 525 nm).

Cellular tyrosinase activity: The cellular tyrosinase activity was assayed by Huang *et al.*[19] method. The tyrosinase activity was calculated by the equation:

$$\text{Tyrosinase activity (\%)} = \frac{\text{OD Sample (GB)}}{\text{OD Control (DMSO)}} \times 100$$

Melanin contents: The B16F1 melanoma cells melanin content was measured by the method as described previously with minor changes[20]. The amount of melanin (cellular) was corrected according to the protein content of the samples and expressed as µg melanin/mg protein.

Western blot: The B16F1 melanoma cells stimulated with α-MSH (1 µM) and pre-treated with different concentration of GB (5,10, 20 and 50 µg mL^{-1}-1 h before α-MSH stimulation) and harvested using RIPA cell lysis buffer solution (100 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 2% Triton X-100 and 0.2% SDS at pH 7.4) and homogenized to yield the lysate. The lysate was then centrifuged at 12000 g for 15 min at 4°C and the supernatant was used to determine the protein content using Pierce BCA Protein Assay Kit. For protein separation, the equal concentration of protein (50 µg/lane) was resolved on 12% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) and then electro-transferred onto polyvinylidene (PVDF) membrane. The primary anti-bodies includes rabbit monoclonal anti-MITF (1:1200; Abcam, Cambridge, UK), rabbit polyclonal anti-TYRP-1 (1:800; Abcam, Cambridge, UK) and anti-TYRP-2 (1:800; Abcam, Cambridge, UK) and rabbit monoclonal anti-β-actin (control; 1;800; Zhongshan Biotechnology, Beijing, China). Then the membrane was washed with TBS to remove unbound primary antibodies and finally the secondary antibody-like anti-goat HRP antibody; (1:10000; Santa Cruz Biotechnology, CA, USA) were probed with the membrane for 1 h at 37°C and again washed with TBS. The bounded antibodies with specific protein on the membrane were detected by the enhanced chemiluminescence (Pierce biotechnology; MA, USA) system and the protein expression (band) are quantified using Bio-Rad Laboratories Quality one D analytic software (V 4.6; Bio-Rad; CA, USA).

Data analysis: The results are expressed as the mean±Standard Error mean (SEM). All the experiments were tested in triplicates (n = 3). The difference between each group (control vs. H_2O_2 or α-MSH; H_2O_2 or α-MSH Vs GB) was explored using Student t-test with the help of SPSS software (V 21, SPSS Inc; NY, USA). Th p-value less than 5 (p<0.05) was deemed as statistically significant.

RESULTS

Effect of GB on B16F1 melanoma cells viability or anti-proliferative activity: The MTT assay was used to evaluate the cytotoxicity (anti-proliferative) activity of GB (Fig. 1). B16F1
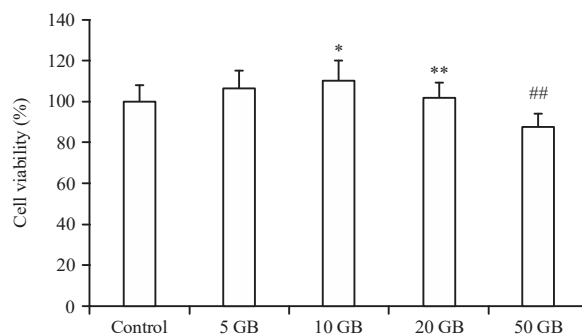

Fig. 1: Effect of GB on the B16F1 melanoma cell viability
Data are expressed as Mean±SEM (n = 3), p-value (*p<0.05, **p<0.01, #p<0.001): Control vs. GB (5, 10, 20 and 50 µg mL^{-1})

melanoma cells were co-cultured with DMSO (control) or different concentration of GB for 24 h. The results of this MTT assay clearly (Fig. 1) showed that as the concentration increases the cell count started to increase at the beginning (5-10 µg; (p<0.05)) and later as the GB dose increased the cell count start to decline considerably (10-50 µg; p<0.01 and p<0.001). Particularly at the dose of 50 µg mL^{-1} (p<0.001), GB showed highest cytotoxic or anti-proliferative activity (87%) by decreasing almost 13% of cell count as compared to control cell (100%).

Effect of GB on B16F1 melanoma cells morphological changes: For morphological analysis, B16F1 melanoma cells were treated with DMSO and with GB (5, 10, 20 and 50 µg mL^{-1}) for 24 h and were observed under a normal phase contrast microscope, which was portraited in Fig. 2. The morphology of control (DMSO; 2A) treated cell displayed normal architecture with spherical shaped c ells. While, B16F10 cells treated with different concentration [5 (2B), 10 (2C), 20 (2D) and 50 (2E) µg mL^{-1}] of GB for 24 h also appeared normal but slight increase in cell count was noted at lesser dosage of GB (5 and 10 µg mL^{-1}). However, the cell count started to decrease as the dose increased (20 and 50 µg mL^{-1}) and thus prove its anti-proliferative activity. The morphological analysis results were in concordance with the results of the MTT assay.

Effect of GB on intracellular ROS production: Figure 3 represented the efficacy of GB on intracellular ROS production in B16F1 melanoma cells. The levels of intracellular ROS were significantly increased (p<0.001) in B16F1 melanoma cells after exposed or stimulated to H_2O_2 alone (positive control) as compared to control (non-GB treated cells). Where, melanoma cells pre-treated
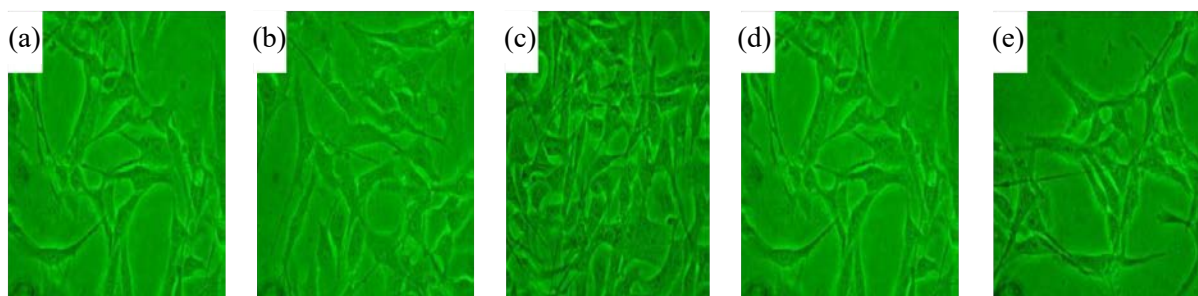
Fig. 2(a-e): Effect of GB on B16F1 melanoma cells morphological changes

Cells treated with DMSO (control) displayed normal architecture with spherical shaped cells. Whereas, cells treated with different concentration of GB [5 (2B), 10 (2C), 20 (2D) and 50 (2E) µg mL^{-1}] also appeared normal but the count increased (5/10 µg mL^{-1}) and gradually decreased as the dose increases (20/50 µg mL^{-1})
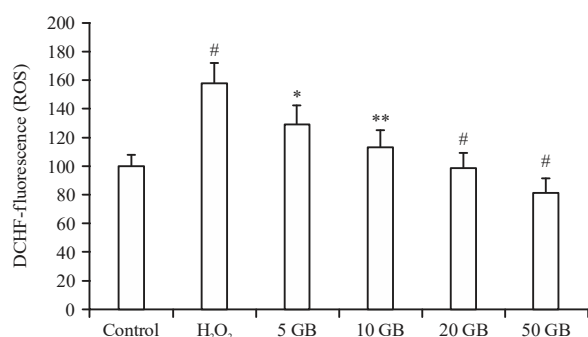

Fig. 3: Effect of GB on intracellular ROS generation in B16F1 melanoma cells after stimulated with H_2O_2

Data are expressed as Mean±SEM (n = 3), p-value (*p<0.05, **p<0.01, #p<0.001): Control vs. H_2O_2; H_2O_2 vs. GB (5, 10, 20 and 50 µg mL^{-1})
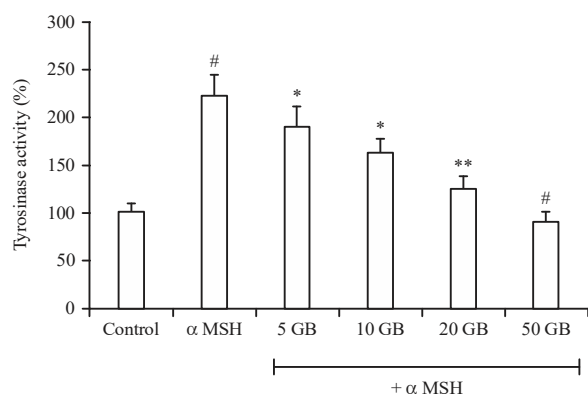

Fig. 4: Effect of GB on cellular tyrosinase activity in B16F1 melanoma cells

Data are expressed as Mean±SEM (n = 3), p-value (*p<0.05, **p<0.01, #p<0.001): Control vs. α-MSH; α-MSH vs. GB (5, 10, 20 and 50 µg mL^{-1})

with increased concentration of GB (1 h before stimulated with H_2O_2) showed marked reduction (p<0.001) in ROS production than H_2O_2 stimulated melanoma cells. The above data endowed that GB would abolish ROS production in a dose-dependent manner.

Effect of GB on cellular tyrosinase activity: Effect of GB on cellular tyrosinase activity in B16F1 melanoma cells after stimulated with α-MSH (Fig. 4). A pronounced increase (p<0.001) in the tyrosinase activity were observed in α-MSH (alone) stimulated B16F1 melanoma cells on equivalence with DMSO (control) alone treated B16F1 melanoma cells. However, a substantial decline (p<0.05-10; p<0.01-20; p<0.001-50 µg) in the tyrosinase activity was noted in GB pre-treated B16F1 melanoma cells after stimulated with α-MSH.

Effect of GB on melanin count or synthesis: Figure 5 showed the effect of GB on melanin count (synthesis) in melanoma cells after stimulated with α-MSH. Comparison with control melanoma cells, the melanin count was significantly elevated (p<0.001) in α-MSH stimulated B16F1 melanoma cells. B16F1 melanoma cells pre-treated with GB after stimulated with α-MSH showed a considerable decline in melanin count (dose-dependent manner) as compared with α-MSH alone stimulated B16F1 melanoma cells.

Effect of GB on proteins expression of MITF, TYRP-1 and 2: An immune blot technique was used to assess the protein (involved in melanogenesis) expression of MITF; TYRP-1 and 2 in B16F1 melanoma cells after stimulated with α-MSH. All the protein expression were notably up-regulated (p<0.001) in α-MSH stimulated melanoma cells than control melanoma cells (Fig. 6). In case of melanoma cells stimulated with α-MSH and pre-treated with increased concentration of GB (5, 10, 20 and 50 µg mL^{-1}) displayed marked down-regulation (p<0.05-10; p<0.01-20 and p<0.001-50 µg) in the protein
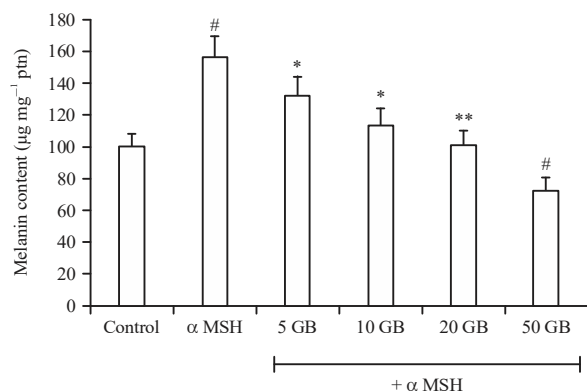
Fig. 5: Effect of GB on melanin count (synthesis) in B16F1 melanoma cells

Data are expressed as Mean±SEM (n = 3), p-value (*p<0.05, **p<0.01, #p<0.001): Control vs. α-MSH; α-MSH vs. GB (5, 10, 20 and 50 μg mL^{-1})
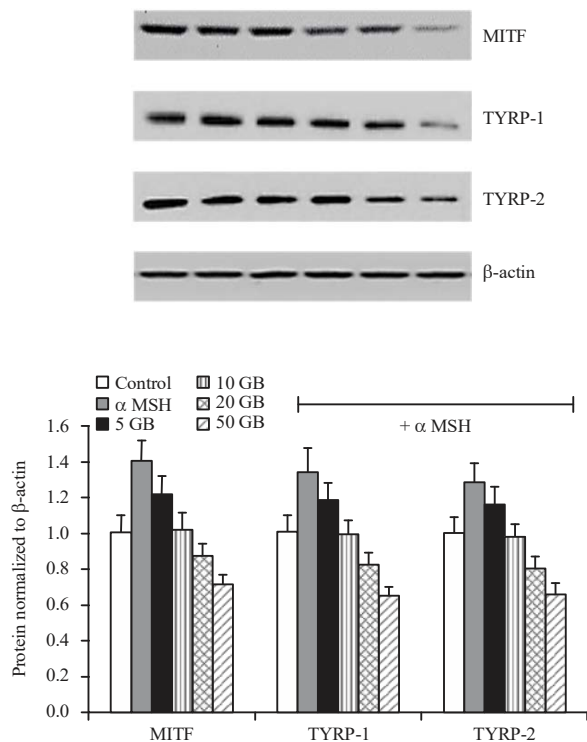

Fig. 6: Effect of GB on protein (involved in melanogenesis) expression of MITF, TYRP-1 and 2 in B16F1 melanoma cells after stimulated with α-MSH using immunoblot technique

Data are expressed as Mean±SEM (n = 3), p-value (*p<0.05, **p<0.01, #p<0.001): Control vs. α-MSH; α-MSH vs. GB (5, 10, 20 and 50 μg mL^{-1})

expression levels of all proteins (MITF; TYRP-1 and 2) in comparison with α-MSH alone stimulated melanoma cells. Overall, GB showed better anti-melanogenesis activity in dose-concentration fashion.

DISCUSSION

Ginkgolide B (GB) is a diterpenoid from a popular Chinese tree *Ginkgo biloba* leaves which possess numerous biological activity like anti-oxidant, anti-inflammatory and anti-cancer properties[11]. However, no studies were conducted to explore the hypopigmentation or anti-melanogenic property of GB. Hence, the present study was conducted to check the anti-melanogenic property of GB in B16F1 melanoma cell model (stimulated to H_2O_2 or α-MSH- to mimic UV induced melanogenesis) by assessing the morphological and biochemical changes like cell viability, free radical production (ROS), tyrosinase activity and melanin synthesis. The outcome of current cell line study shows that pretreated with increased concentration of GB could significantly reduce the melanoma cell count, ROS production and inhibit tyrosinase activity and thus lowered the melanin synthesis (content) via downregulating the protein expression of MITF, TYRP-1 and 2.

The anti-proliferative activity was determined using MTT assay, where co-culturing of B16F1 melanoma cells with different concentration of GB showed a significant change in cell count owing to its apoptotic and necrotic property[21]. Previously, Lou and his colleagues[22] demonstrated that treatment with increasing concentration of Ginkgolide B in pancreatic cancer cell lines like PT45-P1 and T3M4 showed a significant decline in the cell count (viability) to endorse its anti-proliferative activity. Similar kind of results is also observed during morphological analysis, where the cell count starts to decrease as the GB dose increases (20 and 50 μg mL^{-1}) and thus prove its anti-proliferative activity.

A growing body of evidence has demonstrated that free radicals especially ROS generation are highly implicated during melanogenesis in melanocytes. Hence, several natural anti-oxidants are recommended for delaying hyperpigmentation or anti-melanogenesis process[5,23]. The levels of intracellular ROS were significantly decreased in B16F1 melanoma cells pre-treated with increased concentration of GB (1 h before stimulated with H_2O_2) showed decreased ROS production as compared to H_2O_2 stimulated melanoma cells due to anti-oxidant and free radical scavenging property of GB[24]. Earlier, Gill and his co-workers[12] indicated that pre-treatment with different concentration of Ginkgolide B could significantly attenuate ROS production in SH-SY5Y cells. The author has indicated that due to the presence of many free hydroxyl group present in Ginkgolide B might act as an electron donor which contribute to its potent free radical scavenging property.

Melanogenesis (melanin synthesis) were triggered by various factors especially UV irradiation, which also initiate excess free radical generation and enhance the production of α-MSH, which bind to melanocortin-1 receptor (MC1R) and leads to CREB (cAMP-response element binding protein), PKA (Protein Kinase A) activation and subsequently upregulate MITF, TYRP-1 and 2 and tyrosinase[25,26]. As indicated previously that tyrosinase is a rate-limiting enzyme for the *de novo* synthesis pathway of melanin (melanogenesis) as it catalyzes the first two steps of melanin synthesis[5,6]. Hence for the present study, author would like to check the tyrosinase activity and melanin content to testify the anti-melanogenic activity of GB on B16F1 melanoma cells. A significant decline in the tyrosinase activity and melanin count were noted in GB pre-treated B16F1 melanoma cells after stimulated with α-MSH. It was hypothesize that GB might abolish the oxidative stress and thereby down-regulate the tyrosinase activity and which eventually results in decreased melanin count. Recently, α-terpineol a monoterpene present in the *Melaleuca quinquenervia* essential oil could effectively inhibit α-MSH induced melanogenesis and oxidative stress in B16 melanoma cells by significantly improving the antioxidant status and abolish tyrosinase activity and thereby reduce the melanin content[4].

Based on the above evidence it was cleared that GB possesses strong anti-melanogenesis property. However, the underlying mechanism is still unknown. Hence, author would like to explore GBs hypopigmentation or anti-melanogenesis activity by quantifying some major protein involved in melanogenesis including MITF, TYRP-1 and 2. A western blot technique was used to assess the protein (involved in melanogenesis) expression of MITF; TYRP-1 and 2 in B16F1 melanoma cells after stimulated with α-MSH. Present results showed that the protein expression of MITF, TYRP-1 and 2 was exponentially up-regulated in α-MSH stimulated melanoma cells. Whereas, melanoma cells stimulated with α-MSH and pre-treated with increased concentration of GB (5,10, 20 and 50 µg mL^{-1}) displayed marked down-regulation in the protein expression of MITF; TYRP-1 and 2. The above results are due to the antioxidant, free radical scavenging activity and anti-tyrosinase activity of GB. Momilactone B (MB) a terpenoid isolated from rice bran have demonstrated potent anti-melanogenesis property by notably suppressing the mRNA and protein expression of MITF, tyrosinase, TYRP-1 and 2 in B16 melanoma cells attributing to its potent anti-oxidant activity[27]. Similarly, Azam *et al.*[28] also demonstrated that Sargaquinoic acid a meroterpenoid from *Sargassum serratifolium* act as a potent anti-melanogenic compound through downregulating MITF and other melanogenic enzymes like TYRP-1 and 2 via ERK signaling pathway in

B16F10 melanoma cells. Hence, we speculate that GB would also lower the ROS production as well as inhibit the activation of CREB and PKA and thereby downregulate the protein expression and MITF. Down-regulated MITF subsequently suppress the activation of downstream protein like tyrosinase, TYRP-1 and 2 and thereby halt the melanin synthesis (melanogenesis). The major limitation of this study is the avoidance of assessment of apoptotic markers as well as a reference or standard drug for comparison.

CONCLUSION

Taking together, this *in vitro* study clearly endowed that GB from *Ginkgo biloba* leaves showed better anti-melanogenic property in a dose-dependent fashion by abolishing excessive ROS generation (oxidative stress) as well as effectively inhibited tyrosinase activity and thereby suppress the production of melanin via downregulating various proteins (MITF. TYRP-1 and 2) involved in melanogenesis. Further studies are required to ensure which signaling pathway (PI3K/Akt or ½ ERK pathway) did GB depends to exhibits its anti-melanogenesis property.

SIGNIFICANCE STATEMENT

This is the very first study (novel study) conducted to check the anti-melanogenic property of Ginkgolide B in B16F1 melanoma cell model. The results showed a potent anti-melanogenic property which influence in the development of novel anti-melanogenic agent against hyperpigmentation or melanoma condition. However, further clinical trials are needed to confirm the similar kind of results in human as well.

ACKNOWLEDGMENT

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