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Original article

Effect of xanthoxylin on melanin content and melanogenic protein expression in B16F10 melanoma

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Background: Reduced production of melanin and decreased or absence of melanocytes leads to various hypopigmentation disorders. Melanin synthesis is regulated by melanogenic proteins such as tyrosinase, tyrosinase-related protein 1 (TRP-1) and tyrosinase-related protein 2 (TRP -2), as well as their transcription factors.
Objectives: This study elucidated the effects of xanthoxylin on melanin content, dendriticity, melanogenic protein expression and its signal transduction pathways in mouse B16F10 melanoma cells (B16F10 cells).
Methods: Melanin production of B16F10 cells was measured by using a melanin content assay. The effect of xanthoxylin on the dendriticity of B16F10 cells was determined by a melanocyte dendricity assay. RT-PCR was used to investigate the effects of xanthoxylin on the melanogenic protein expression.
Results: We found that xanthoxylin increased melanin production, number of dendrites, tyrosinase, and microphthalmia-associated transcription factor (MITF) expression in cultured B16F10 cells. In addition, PKA and PKC inhibitor decreased melanin production, tyrosinase, and MITF expression in xanthoxylin-treated cells. However, xanthoxylin did not inhibit TRP-1 and TRP-2 expression.
Conclusion: These results indicated that xanthoxylin induces melanogenesis mainly via cAMP-mediated PKA activation. Other signaling pathways may also play a role in xanthoxylin-induced melanogenesis.

Keywords: Melanocyte, melanin, xanthoxylin

Skin pigmentation plays an important role in absorbing toxic drugs and chemicals [1] as well as protecting the skin against skin cancer and DNA damage [2]. The loss of melanin in the epidermis can increase a person's risk in acquiring skin cancers and result in hypopigmentation such as vitiligo [3]. Melanin synthesis is controlled by enzymatic reactions of tyrosinase, and tyrosinase-related protein 1 and 2 (TRP-1, TRP-2) [4]. Tyrosinase is the key enzyme in the initial and rate-limiting reaction of melanin synthesis. It catalyzes the hydroxylation of tyrosine to b-3, 4-dihydroxyphenylalanine (DOPA) and catalyzes oxidation of DOPA to DOPAquinone, which is a common step for both eumelanin and pheomelanin biosynthesis. MITF is a key transcription factor in melanin biosynthesis. It is a factor for the basic-helix–loop–helix-leucine-zipper family (bHLHzip) [7]. MITF positively regulates transcription of genes that encode key enzymes in melanogenesis such as tyrosinase, TRP-1 and TRP-2 [8, 9]. These enzymes are transcriptionally regulated by a tissue-restricted cis-acting promoter containing a canonical E-box or M-box. Phosphorylated MITF binds to either M-box or E-box consensus sequences to activate transcription of these proteins [10]. MITF also regulates proliferation and survival of melanocytes [11].

There are several signaling pathways for enhancing melanin synthesis. The cyclic adenosine monophosphate (cAMP) pathway is a well-known signaling cascade and the most important pathway for regulating melanin synthesis [12], cAMP-elevating agents (forskolin [13, 14], IBMX [15], α-MSH [16-18], glycyrrhizin [4]) can activate melanin synthesis by increasing cAMP levels. cAMP activates the protein kinase A (PKA) pathway. This leads to the phosphorylation of cAMP response binding protein (CREB) transcription factor that binds to cAMP

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response elements (CRE) on the MITF promoter and induces MITF expression. MITF then positively regulates the expression of tyrosinase, TRP-1, and TRP-2 involved in melanogenesis [19].

In addition, cAMP is associated with other signaling pathways involved in the melanin synthesis. It inhibits AKT activation through phosphatidylinositol 3-kinase (PI3K)-dependent mechanism.

Protein kinase C activation can also increase melanin production by 1-oleyl-2-acetyl-glycerol and ultraviolet B radiations [20]. It has been reported that activated PKC-β binds the receptor of activated C-Kinase-I (RACK-I), which is the protein that stabilizes the active form of PKC-β and translocates this enzyme to melanosomal membrane, so it can phosphorylate tyrosinase. Phosphorylated tyrosinase forms a complex with TRP-1, which leads to the stabilization of tyrosinase and increases its enzymatic activity [21]. Melanin synthesis can also be increased with the phosphorylation of p38 MAPK cascade [22-24] and activation of the cGMP/PKG pathway [25].

Several phenolic compounds from natural sources have been reported to stimulate melanin synthesis [26]. Xanthoxylin is a phenolic compound found in several plants such as *Zanthoxylum piperitum* and *Sebastiana schottiana*. Several activities of this compound have been demonstrated. It had an antagonistic effect against several neurotransmitter-mediated contractions in nonvascular smooth muscles [27]. It exhibited antifungal [28], antispasmodic [29], and antioedema [30]. It can also inhibit prostaglandin synthetase and 5-lipoxygenase, and had cytotoxic effect against Ehrlich ascites tumor cells [31]. Due to its phenolic structure, this study investigated effects of xanthoxylin on melanogenesis in mouse B16F10 melanoma cells.

Materials and methods

Cell culture

Mouse melanoma cells, B16F10, from the American Collection (ATCC) were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% (v/v) fetal bovine serum, 100 µg/ml streptomycin, and 100 units/ml penicillin in a humidified atmosphere containing 5% CO_2 at 37°C. The cells were sub-cultured or collected by using a 0.25% (v/v) trypsin-EDTA solution.

Determining cells' viability

The effect of xanthoxylin on the viability of B16F10 cells was determined by using a resazurin assay. Only viable cells can reduce resazurin dye to a deoxygenated product known as resorufin. After the cells were treated with xanthoxylin, they were incubated at 37°C in a 5% CO_2 for 72 hours. Resazurin solution (1 mg/ml in H_2O) was later added to each well. Cells continued to be incubated at 37°C for another four hours. The absorbance was then measured at 570 nm and 600 nm by using a microplate reader.

Measurement of melanin content and melanocyte dendritic assay

Melanin content was measured according to the modified method of Tsuboi et al. [32]. B16F10 cells were treated for three days with xanthoxylin (3.125-50 µg/ml) and α-MSH (10 nM). Untreated B16F10 cells served as controls. Cells were collected by trypsinizing them in 0.25% (v/v) trypsin-EDTA solution and centrifuged at 12,000 g for 10 minutes. After precipitation, the color of the cell pellet was evaluated visually. 8×10^4 cells were resuspended in 200 µl of 2 M NaOH stirring at 60 °C for five minutes. Spectrophotometric analysis of melanin content was performed at 405 nm absorbance. Dendrites of the cells were visible and photographed under the microscope.

Determining mechanisms of xanthoxylin's action by using a melanin content assay

Effects of xanthoxylin on the signaling pathways of melanogenesis in B16F10 cells were elucidated by using the following kinase inhibitors, protein kinase A (PKA) inhibitor (H89), PKC inhibitor (Ro-32-0432), phosphatidylinositol-3-kinase (PI3K) inhibitor (LY294002), and MEK1 inhibitor (PD98059). Cells were pretreated with 5 µM of kinase inhibitors at 37°C for one hour and then treated with 25 µg/ml of xanthoxylin for 72 hours at 37°C. After treatment with xanthoxylin, the cells were collected and analyzed by using a melanin content assay.

Measurements of mRNA expression of proteins involved in melanogenesis

The B16F10 cells were pretreated with and without kinase inhibitors at 37°C for one hour and then with xanthoxylin for 72 hours at 37°C. After treatment, total RNA was extracted from the cells. One µg of total RNA was reverse transcribed to cDNA synthesis using Improme II™ reverse transcription system

reagent with oligo (dT)15 primer following the manufacturer's protocol. The oligonucleotide primers used for the PCR were as follows: tyrosinase upstream 5'- GGC CAG CTT TCA GGC AGA GGT-3'; downstream 5'- TGG TGC TTC ATG GGC AAA ATC-3', TRP-1 upstream 5'-GCT GCA GGA GCC TTC TTT CTC-3'; downstream 5'-AAG ACG CTG CAC TGC TGG TCT-3', TRP-2 upstream 5'-GGA TGA CCG TGA GCA ATG GCC-3'; downstream 5'-CGG TTG TGA CCA ATG GGT GCC-3', MITF upstream 5'-GTA TGA ACA CGC ACT CTC TCG A-3'; downstream 5'-CTT CTG CGC TCA TAC TGC TC-3', β-actin upstream 5'-GTG GGC CGC CCT AGG CAC CAG-3'; downstream 5'-GGA GGA AGA GGA TGC GGC AGT-3'. The PCR conditions were 94 °C for four minutes, followed by 20 cycles for TRP-1, TRP-2, and β-actin, 25 cycles for tyrosinase and MITF for one minute. Denaturation was done at 94°C for one minute. Annealing was done at the appropriate T_m with a one-minute extension at 72°C and a final extension at 72°C for another four minutes.

Statistical analysis

Mean plus or minus standard error of mean (mean±SEM) were calculated. Statistical analysis for comparing the results was performed by a one-way ANOVA followed by Tukey's post hoc test. All statistical analysis was performed according to the statistic program, SPSS version 16. P-value <0.05 and *p*-value <0.01 were considered significant.

Results

Effects of xanthoxylin on the cells' viability

The cytotoxic or proliferative effect of xanthoxylin detected from the melanin content assay was also confirmed by the resazurin assay. The B16F10 melanoma cells were incubated with xanthoxylin (3.125-50 μg/ml) for 3 days. Xanthoxylin at the concentration of 3.125 μg/ml significantly increased the cell's viability but significantly decreased when a concentration of 50 μg/ml was used as shown in **Figure 1**.

The effect of xanthoxylin on melanogenesis in mouse B16F10 melanoma cells

Alpha-MSH at the concentrations of 1, 10, 100 nM, 1, and 10 μM increased the melanin content in B16F10 cells by 2.46, 3.08, 3.14, 3.56, and 3.59 folds when compared to the untreated cells (**Figure 2A**). Ten nM of α -MSH was chosen as the positive control. Xanthoxylin at the concentrations of 3.125, 6.25, 12.5, 25, and 50 μg/ml increased melanin content by 1.27, 1.53, 2.69, 6.51, and 8.18 folds when compared to the untreated controls (**Figure 2B**). Xanthoxylin was found to significantly activate melanogenesis in a concentration dependent manner.

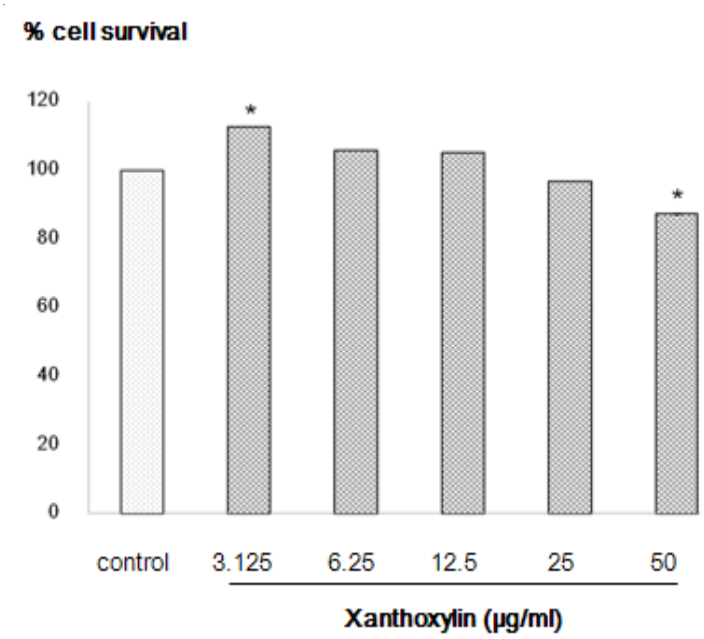

Figure 1. Effect of xanthoxylin on cell viability. The percentage of cell survival was calculated from the absorbance at 570 and 600 nm. The results are presented as the mean±SEM from 4 independent experiments. **p* <0.01 indicated that there was a significant difference when compared to the controls.

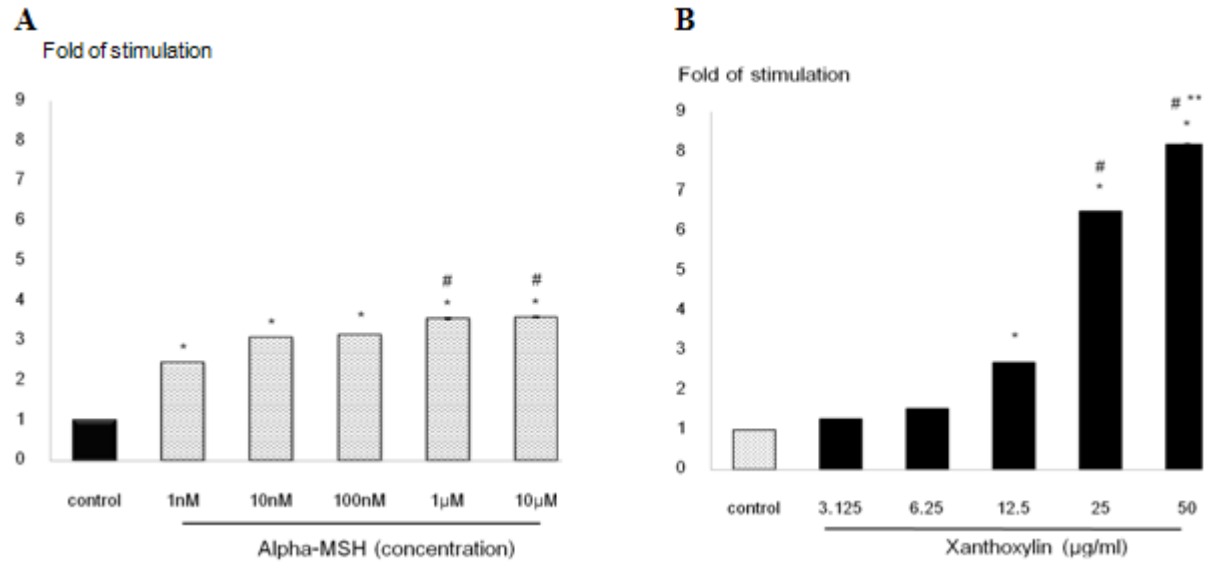

Figure 2. Effects of the tested compound on the synthesis of melanin. The data are presented as the mean□S.E.M of the folds of stimulation. **A:** Effect of α-MSH on melanin synthesis (n = 2). **p* <0.01 indicated that there was a significant difference when compared to the controls. #*p* <0.01 indicated that there was a significant difference when compared to α-MSH 10 nM. **B:** Effect of xanthoxylin on melanin synthesis (n = 4). **p* <0.01 indicated that there was a significant difference when compared to the controls. #*p* <0.01 indicated that there was a significant difference when compared to xanthoxylin at 12.5 μg/ml. ***p* <0.01 indicated that there was a significant difference when compared to xanthoxylin at 25 μg/ml.

Effects of xanthoxylin on dendricity

Melanocyte dendricity is important in transferring melanosome to keratinocytes. Xanthoxylin, at all concentrations, increased the number of B16F10 cells containing more than two dendrites in a concentration dependent manner (**Figure 3C-G**) whereas most of the untreated cells had only two dendrites (**Figure 3A**). Ten nM α-MSH also increased melanocyte dendricity (**Figure 3B**).

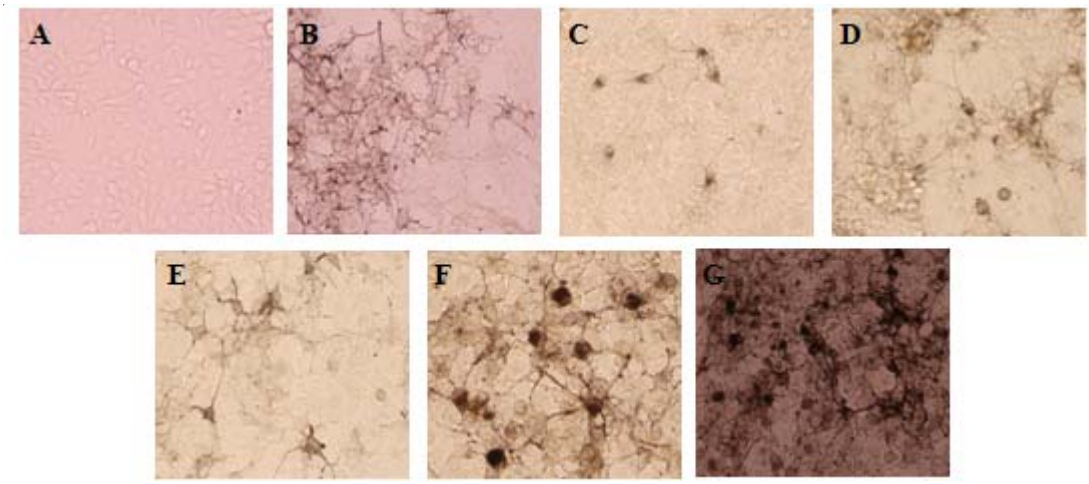

Figure 3. Effects of xanthoxylin on dendricity of mouse B16F10 melanoma cells under light microscope (10X); (n = 3). **A:** Untreated controls, **B:** 10 nM α-MSH, **C:** 3.125 μg/ml xanthoxylin, **D:** 6.25 μg/ml xanthoxylin, **E:** 12.5 μg/ml xanthoxylin, **F:** 25 μg/ml xanthoxylin, and **G:** 50 μg/ml xanthoxylin.

Mechanisms of xanthoxylin in inducing melanogenesis

Five μM of PKA (H-89), PKB (LY294002), PKC (Ro-32-0432), and MEK1 (PD98059) were used to inhibit signaling pathways involved in melanogenesis in mouse B16F10 melanoma cells. PKA, PKC, PKB, and MEK1 inhibitors decreased the stimulatory effects of xanthoxylin from 8.43 folds to 3.77, 5.67, 7.12, and 7.28 folds, respectively. They reduced the percentage of xanthoxylin-stimulated melanin content from 100% in non-pretreated cells to 44.66%, 67.25%, 84.39%, and 86.28% in PKA, PKC, PKB, and MEK1 inhibitor-pretreated cells, respectively (**Figure 4**). These findings suggest that protein kinase was the major signaling pathway mechanisms of xanthoxylin-induced melanogenesis in mouse B16F10 melanoma cells. However, the results also show that xanthoxylin may partially induce melanin synthesis via PKC, PKB, and MAPK signaling pathways.

Effects of xanthoxylin on the mRNA expression of proteins involved in melanogenesis

Xanthoxylin significantly increased tyrosinase expression in a dose dependent manner (**Figure 5**). It increased the ratio of tyrosinase to β-actin expression from 1.06 in the untreated control to 1.47, 1.78, and 2.01 in 6.25, 12.5, and 25 μg/ml xanthoxylin-treated cells, respectively. It also significantly induced MITF expression at all concentrations used in the study. It increased the ratio of MITF to beta-actin expression from 0.26 in the untreated controls to 0.93, 1.11, and 1.13 in 6.25, 12.5, and 25 μg/ml xanthoxylin-treated cells, respectively. However, it did not have any effect on TRP-1 and TRP-2 expressions.

Effects of protein kinase inhibitors on xanthoxylin-induced tyrosinase and MITF expression

The PKA inhibitor, H-89, significantly reduced xanthoxylin-induced mRNA expression of tyrosinase at all concentrations of xanthoxylin (6.25, 12.5, and 25 μg/ml) used in the study. It also significantly decreased the effect of xanthoxylin (25 μg/ml) on MITF expression as can be seen in **Figure 6**.

Effects of the other protein kinase inhibitors were studied on 25 μg/ml xanthoxylin-treated cells. The PKC inhibitor, Ro-32-0432, significantly reduced the xanthoxylin-induced tyrosinase expression but did not significantly decrease the MITF expression. The PKB and MEK1 inhibitors, LY294002 and PD98059, did not reduce tyrosinase or MITF expression in xanthoxylin-treated cells.

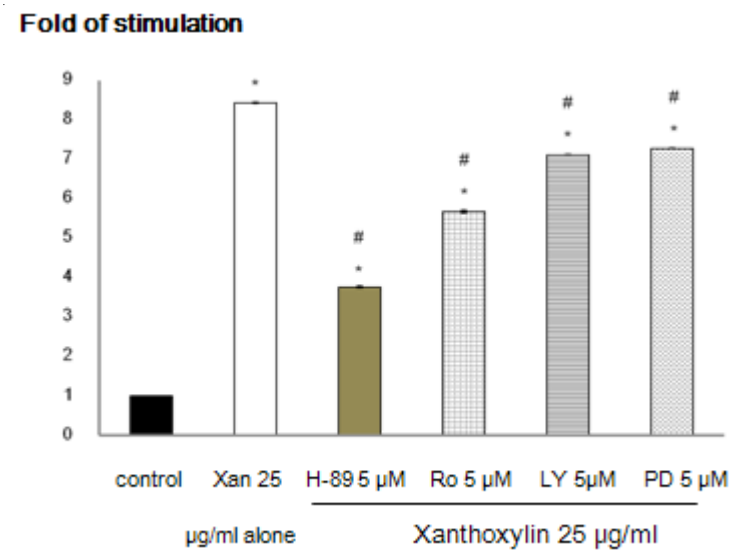

Figure 4. Effects of protein kinase inhibitors on xanthoxylin-induced melanogenesis. Melanin content in treated cells was measured after 72 hours, with or without inhibitors. The data was presented as the mean±S.E.M from 4 independent experiments. *p <0.01 indicated that there was a significant difference when compared to the controls. #p <0.01 indicating that there was a significant difference when compared only to xanthoxylin at 25 μg/ml.

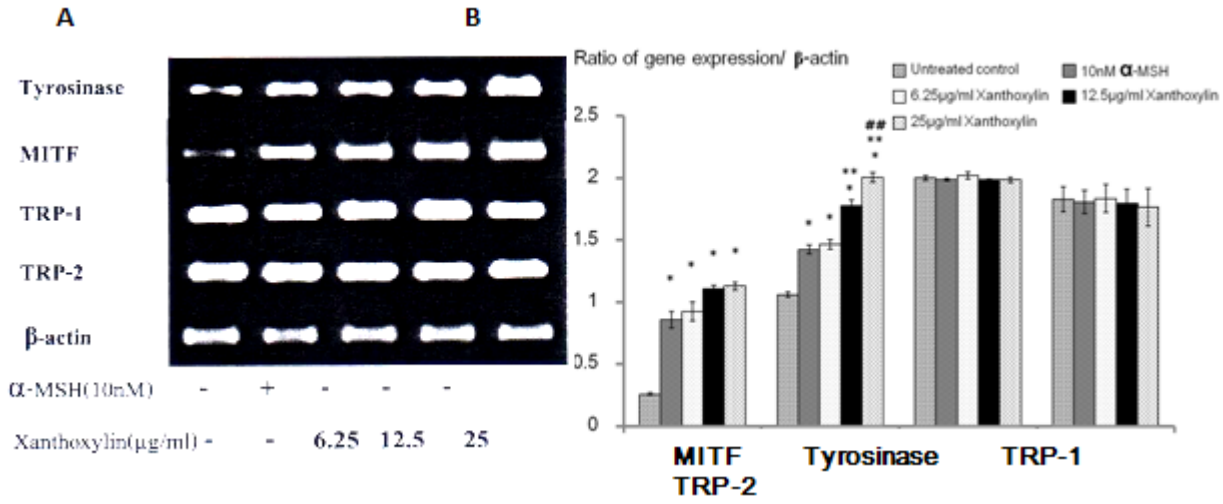

Figure 5. Effects of xanthoxylin on tyrosinase, MITF, TRP-1, and TRP-2 expressions, as described in Section 2. **A:** Representative PCR products. **B:** Densitometric analysis of the PCR products. The data are represented as the density ratio of gene to β-actin. The values are shown as the mean±SEM (n = 3). **p* <0.05 indicated that there was a significant difference when compared to the controls. #*p* <0.05 indicated that there was a significant difference when compared to α-MSH at 10 nM. ***p* <0.05 indicated that there was a significant difference when compared to xanthoxylin at 6.25 μg/ml. ##*p* <0.05 indicated that there was a significant difference when compared to xanthoxylin at 12.5 μg/ml.

Discussion

Melanogenesis is the complex process of melanin production. It includes the process of melanin synthesis in melanosome of melanocytes as well as the process of transport and transfer of these melanosomes through dendrites of melanocytes to keratinocytes. Melanin plays an important role in protecting the skin against UV radiation. Stimulation of melanogenesis will act as a protective mechanism to prevent DNA damage and skin cancers from UV radiation. Melanin synthesis is enhanced by numerous agents such as UV radiation, □-MSH, forskolin, and IBMX. Very few of these are used as skin enhancers because of their ineffectiveness or undesirable side effects. Facultative pigmentation, more commonly known as skin tanning, occurs when the skin is exposed to UV radiation [33]. This radiation increases number of melanocytes, number and sizes of melanosomes, melanin content in melanosomes, and number of melanosomal dendrites. It should be noted that UV-induced hyperpigmentation could lead to DNA damage and skin cancer. Finding a highly effective and UV-less skin enhancer without skin damage may be desirable. We demonstrated in this study that xanthoxylin purified from fruits such as *Zanthoxylum piperitum* or Japanese pepper may be one of those agents that can be used to effectively enhance skin without any unwanted side effects. Mouse B16F19 melanoma cells were used in this study because they are widely available as a cell model system to study the effects of various compounds on melanogenesis [34]. These cells have a short population doubling time. They are easy to culture and have better survival rates than human melanocyte cells [35].

Xanthoxylin significantly increased melanin content in mouse B16F10 melanoma cells in a concentration dependent manner with little effect on the cells' viability. It increased not only melanin content in B16F10 cells but also dendricity of the cells, which is important in transporting melanosomes to keratinocytes during melanogenesis. These findings were similar to a previous study that demonstrated that quercetin (3,5,7,3',4'-pentahydroxyflavone) induced melanogenesis by increasing the melanin content and stimulating dendrites of melanocytes extended towards the adjacent keratinocytes in human epidermis culture model [36].

There are numerous proteins involved in melanogenesis. Among these, tyrosinase, TRP-1, and TRP-2 are essential for melanin synthesis. Expressions of these three proteins are mainly regulated by a key transcription factor MITF and by several other transcription factors and regulatory proteins or DNA elements [6]. MITF expression is also regulated by

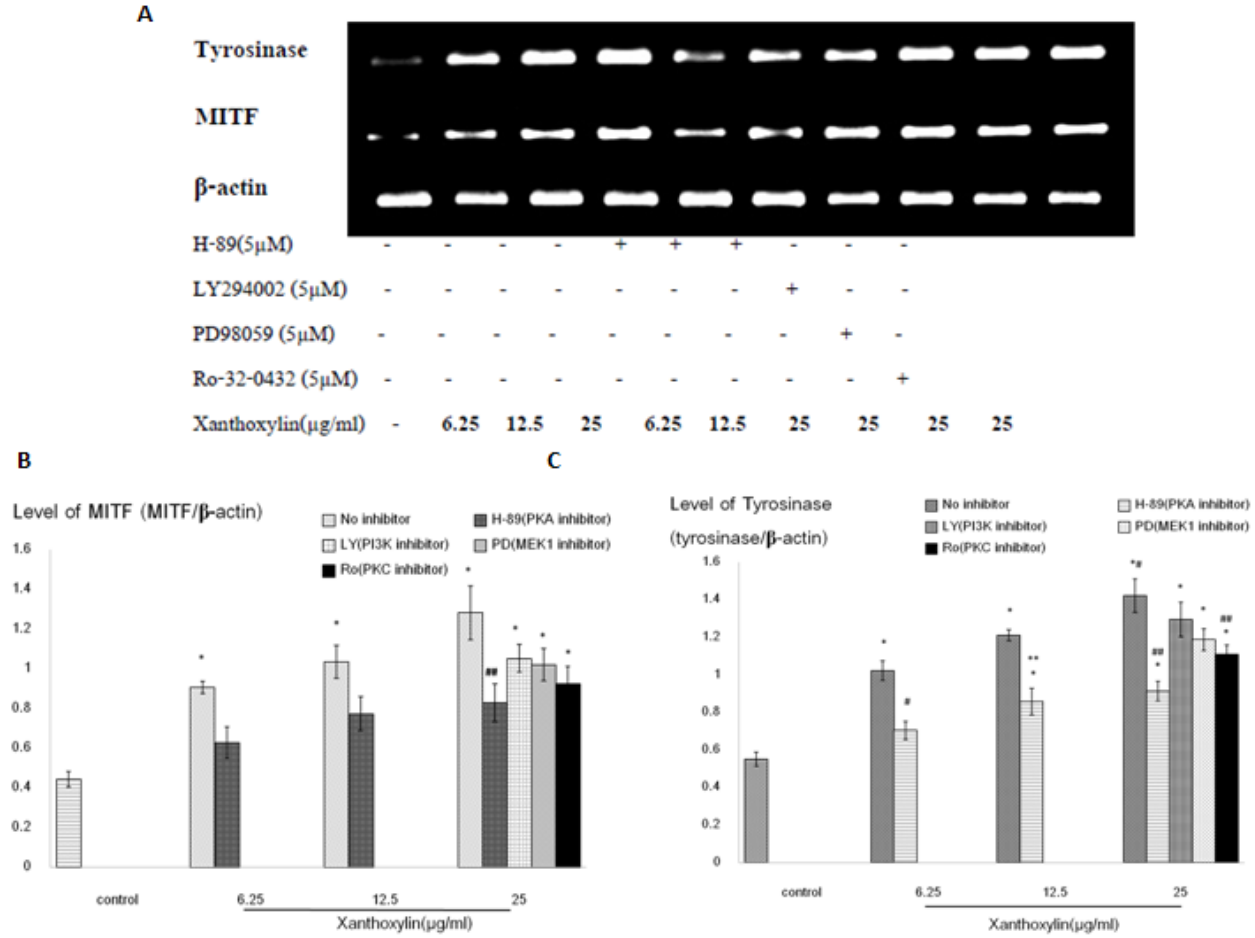

Figure 6. Effects of protein kinase inhibitors on xanthoxylin-induced tyrosinase and MITF expression, as described in section 2. **A:** Representative PCR products. **B** and **C:** Densitometric analysis of the PCR products. The data are represented as the density ratio of gene to β-actin (mean±SEM) of three independent experiments. **p* <0.05 indicated that there was a significant difference when compared to the controls. #*p* <0.05 indicated that there was a significant difference when compared only to xanthoxylin at 6.25 μg/ml. ***p* <0.05 indicated that there was a significant difference when compared only to xanthoxylin at 12.5 μg/ml. ##*p* <0.05 indicated that there was a significant difference when compared only to xanthoxylin at 25 μg/ml.

several transcription factors, regulatory proteins, and DNA elements. An increase in melanin content by xanthoxylin correlated with an increase in the mRNA expression of MITF and tyrosinase. It has also been reported that glycyrrhizin (GR) stimulates melanogenesis in B16F10 cells by increasing the expression of MITF and tyrosinase [4]. These results strongly suggest that xanthoxylin induces an increase in melanin synthesis by MITF-dependent activation of tyrosinase expression. However, xanthoxylin did not have any change in the mRNA expression of TRP-1 and TRP-2 were genes are regulated with the tyrosinase gene. It has been known that tyrosinase, TRP-1, and TRP-2 genes are regulated by MITF transcription factor, which binds to M-box sequences of these genes with different affinity [37, 38]. There are also several reports indicating that these genes are regulated independently of each other as well as independently of MITF [39, 40].

There are several signal pathways in regulating melanogenesis. Recent studies revealed that cAMP and PKC signaling pathways are critical for melanin synthesis [41]. PI3K-PKB pathway and p38-MAPK signaling cascades have also been reported to be involved in the induction of melanogenesis [3]. The cAMP signaling pathway plays a key role in melanogenesis by increasing preexisting tyrosinase enzyme activity as well as inducing the mRNA expression level of tyrosinase. The main mechanism of cAMP is to induce melanogenesis, which also

involves the activation of PKA and CREB transcription factors by phosphorylation. Phosphorylated CREB then interacts with CBP to activate the expression of MITF, which results in the activation of tyrosinase gene transcription. Increased melanin content as well as MITF and tyrosinase mRNA expressions in B16F10 melanoma cells by xanthoxylin were inhibited by a PKA inhibitor H-89 in this study. These results suggest that the cAMP signaling pathway, through PKA, activation is involved in xanthoxylin-induced stimulation of melanogenesis. Rosmarinic acid (a-o-caffeoyl-3, 4-dihydroxyphenyl lactic acid) has also been reported to induce melanogenesis through PKA activation. Its activity was inhibited by a PKA inhibitor H-89 [3]. However, H-89 could not completely block the xanthoxylin effect. This raises the possibility that other signaling mediators, aside from PKA, may be associated with an increased production of melanin induced by xanthoxylin. PKA activation may be the main signaling pathway of xanthoxylin-increased melanin content as well as the expression of crucial proteins involved in melanin synthesis.

Some extracellular signals such as endothelin-1 (ET-1) have been shown to increase melanogenesis by activating PKC [42]. Active PKC activates tyrosinase by directly phosphorylating tyrosinase into melanosome. Similar to H-89 PKA inhibitor, an inhibitor of PKC Ro-32-0432 has been shown to decrease xanthoxylin-stimulated melanogenesis and mRNA expressions of MITF and tyrosinase genes but at a lesser extent. These results suggest that xanthoxylin may also enhance melanin synthesis through PKC signaling. Future studies are needed to identify whether this is associated with cAMP/PKA pathway or not.

Inhibitors of PKB (LY294002) and MEK1 (PD98069) also slightly decreased the xanthoxylin-increased melanin content and the mRNA expressions of MITF and tyrosinase genes. It has been reported that cAMP activates not only PKA activation but also other signaling pathways. These pathways include PI3K/AKT and PKB/GSK-3β pathways [6, 20]. Xanthoxylin may act through cAMP upstream of PKA, PKB, and MEK1 activation. Regulation of melanogenesis at the level of transcription is only the initial step; the other important step is posttranslational modification such as phosphorylation. Effects of xanthoxylin on proteins involved in melanogenesis and signaling molecules at the protein level should be investigated.

In conclusion, this report is the first of its kind to reveal the melanogenic effect of xanthoxylin as well as its molecular mechanisms in melanogenesis. The results in this study demonstrate that xanthoxylin was able to increase the melanin content and number of dendrites in B16F10 melanoma cells without affecting the cells' viability. It is possible that xanthoxylin induced melanin synthesis mainly via PKA activation to stimulate the expression of MITF transcription factor and tyrosinase.

Xanthoxylin may be a potential therapeutic agent useful in hypopigmentation-related diseases and in UV-less skin tanning.

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