ORIGINAL ARTICLE

Polygonum multiflorum root extract as a potential candidate for treatment of early graying hair

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J. Adv. Pharm. Technol. Res.

ABSTRACT

Despite *Polygonum multiflorum* (PM) has been experiencely used as a drug to treat early graying hair phenomenon in Asian countries for a long time, there is limited study examined the real biological effects of PM on hair graying *in vitro* and *in vivo*. In this study, we investigated the effects of PM root extract (PM-RE) on melanin synthesis in human melanoma SKMEL-28 cells and embryos/larvae of wild-type strain AB zebrafish. We also preliminary revealed the molecular mechanism of early hair graying phenomenon in both *in vitro* and *in vivo* models. Our results showed that PM-RE significantly induced melanin synthesis in melanin-producing SKMEL-28 melanoma cells and also in zebrafish embryos/larvae at 4-day postfertilization through activation of MC1R/MITF/tyrosinase-signaling pathway. We also investigated the differences in genotype between graying hair follicle and black hair follicle of young peoples and found that early hair graying phenomenon may be related to downregulation of MC1R/MITF/tyrosinase pathway. Taken together, we suggested that PM-RE at safe doses could be used as a potential agent for the treatment of early hair graying and other loss pigmentation-related diseases.

Key words: Graying hair, MC1R/MITF/tyrosinase signaling, melanin synthesis, *Polygonum multiflorum*, zebrafish

INTRODUCTION

Melanin synthesis in melanocyte or melanoma cells is mainly regulated by MC1R/MITF/tyrosinase-signaling pathway. MC1R localizes in the plasma membrane and plays an important role in the activation of downstream factors, followed by sequential activation of MITF and tyrosinase.[1]

Nowadays, zebrafish is popular used as a model for developmental biology and cellular biology.[2] In particular, zebrafish has been used as an ideal model for melanin formation and dispersion in previous studies.[3] Differentiation to form melanocyte in zebrafish occurs very early; only 24 h postfertilization (hpf), melanoblast, which will be differentiated to become melanocyte, starts to produce melanin.[3] Melanocytes are full developed within 48 hpf in the zebrafish embryos.[3] Therefore, normally, to study the formation of melanin in zebrafish embryos, they always used embryos in the period from 1 to 4 days postfertilization (dpf).[4] In generally, the regulation of melanin biosynthesis in melanocyte of zebrafish is quite similar with those of mammals, basically through activation MC1R/MITF/tyrosinase-signaling pathway.[3]

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Access this article online

Quick Response Code:

Website: www.japtr.org

DOI: 10.4103/2231-4040.197332

How to cite this article: Thang ND, Diep PN, Lien PT, Lien LT. *Polygonum multiflorum* root extract as a potential candidate for treatment of early graying hair. J Adv Pharm Technol Res 2017;8:8-13.

Currently, there is no medicine proven to prevent gray hair in humans. *Polygonum multiflorum* (PM) has been used traditionally to treat different systemic diseases and acclaimed for various biological activities including antioxidation,[5] radical scavenging activity,[6] lipid regulation,[7] and hair-follicle growing.[8] However, there is limited study focused on examination the ability of PM in treatment of early graying hair phenomenon *in vitro* and *in vivo*. Therefore, in this study, we investigated the effects of PM root extract (PM-RE) on melanin synthesis in human SKMEL-28 melanoma cells and zebrafish embryos and preliminary examined the molecular mechanism of this process.

MATERIALS AND METHODS

Ethics statement

In this study, we used zebrafish embryos and larvae at early periods, from 1 to 4 days after fertilization (no more than 5 days old) for experiments; therefore, no license is required by the OECD guidelines.[9] Human hair follicles were donated by the young students of VNU University of Science with their understanding about the purposes of the research.

Preparation of root extract

Fresh roots of PM were cleaned and washed thoroughly with water and rewashed with distilled water. Washed fresh roots were shade dried, powdered mechanically, and sieved using a mesh. In the preparation of organic solvent extracts, 5 g of powdered material was refluxed with 1/10 w/v in a Soxhlet apparatus for an hour. The resulting extract was filtered, pooled, and the solvent removed under reduced pressure at 40°C ± 5°C using a rotary flash evaporator. We used consecutively three types of organic solvents including n-hexane, EtOAc, and MeOH with gradually increasing in polarities to extract substances in roots of PM, and finally, the extract in methanol was used for the further experiments.

Cell culture

Human melanoma SKMEL-28 cells obtained from Riken BioResource Center.[10] This cell line was cultured in RPMI-1640 supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin at 37°C in 5% CO_2.

Total melanin content

The melanin content was determined according to previous publications with modifications.[11] Briefly, the content of total melanin was measured directly from cell culture medium by spectrophotometer at 415 nm using microplate reader.

Chemical exposure and embryo observation

Fish embryo acute toxicity is determined according to the OECD test guideline (OECD, 1992 and 2013)[9] or equivalent guidelines. All experiments were repeated triplicate (embryos with n = 25 for each test). Data were calculated to determine indices including median lethal concentrations (LC50), median effective concentration (EC50), and teratogenic index (TI, defined as the ratio between LC50 and EC50).

Gene analysis

The expression levels of MC1R, MITF, tyrosinase transcripts measured by quantitative real-time polymerase chain reaction (PCR) were adjusted through the transcript expression level of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or ef1α. Then, PCR products were loaded for electrophoresis running.

Sequences of primers for human

- GAPDH (forward primer: 5′-CGGAGTCAA CGGATTTGGTCGTAT-3′ and reverse primer: 5′-AGCCTTCTCCATGGTGGTGAAGAC-3′)
- MC1R (forward primer: 5′-ACTCCGTCTGC TCCAATGAC-3′ and reverse primer: 5′-GCTGTGGGA GTAGCTCTTGG-3′)
- MITF (forward primer: 5′-CCGTCTCTCA CTGGATTGGT-3′ and reverse primer: 5′-TGGGCTT GCTGTATGTGGTA-3′)
- Tyrosinase (forward primer: 5′-TTGCCTGA GTTTGACCCAAT-3′ and reverse primer: 5′-GCATCCG CTATCCCAGTAAG-3′).

Sequences of primers for zebrafish

- ef1α (forward primer: 5′-CTGGAG GCCAGCTCAAACAT-3′ and reverse primer: 5′-ATCAAGAAGAGTAGTACCGCTAGCATTAC-3′)
- MC1R (forward primer: 5-GACCACG GCCTCCTGGATGT-3 and reverse primer: 5-GTTGCAGAAGGGGCTGGTGG-3)
- MITFa (forward primer: 5′-TGTACAGC AATCATGCTCTTCC-3′ and reverse primer: 5′-GTCCCCAGCTCCTTAATTCTGTC-3′)
- Tyrosinase (forward primer: 5-CGCAGATGA ACAATGGCTC-3 and reverse primer: 5-AGCAGATAC ACCCGATGCC-3).

Statistical analysis

Statistical analysis in this study was performed according to the method previously described.[10] When Gaussian requirement was met, one-way ANOVA analysis was employed, followed by individual *t*-test between each treated group and the control group, otherwise nonparametric tests were used. Significance was considered when $P < 0.05$ for all analyses, significantly different ($^*P < 0.05$ and $^{**}P < 0.01$, respectively) from the control.

RESULTS

Expression levels of MC1R/MITF/tyrosinase transcripts in human hair follicles

We examined the transcript levels of molecules which play important roles in regulating the melanin synthesis

in pigment cells SKMEL-28, including MC1R, MITF, and tyrosinase, and GAPDH was used as internal control. Hair follicles of immature graying hair volunteers were collected for analysis. The differences in transcript levels of these molecules in black (B) and graying (G) hair follicles are showed in Figure 1. Our results showed that the transcript levels of MC1R, MITF, and tyrosinase in the graying hair follicles were 36%, 48%, and 77% lower than those in black hair follicles, respectively. This indicated the key role of MC1R/MITF/tyrosinase-signaling pathway in hair graying phenomenon.

Effect of *Polygonum multiflorum* root extract on cellular toxicity

PM roots were extracted in consecutively three types of organic solvents including n-hexane, ethyl acetate, and methanol. Results of extraction process are given in Table 1. Normally, substances which could be dissolved in methanol have high biological and pharmaceutical activities; therefore, we decided to focus on investigating the effect of PM root extracted in methanol on the synthesis of melanin in human melanin-producing SKMEL-28 melanoma cells. Because PM-RE has been traditional used by oral administration for gray hair treatment, we decided to tested the toxic effect of this extract at quite high range of concentrations (312–5000 μg/ml). The result showed that the PM-RE only expressed its toxicity toward SKMEL-28 cells at the concentrations of 2500 and 5000 μg/ml [Figure 2] with cause of 16% and 22% cell death, respectively.

Table 1: *Polygonum multiflorum* was extracted in consecutive three types of organic solvents including n-Hexane, EtOAc, and MeOH

Organic solvent	Dried weight of extracts (g/5 g dried sample)
n-Hexane	0.0536
Ethyl acetate	0.0292
Methanol	1.2864

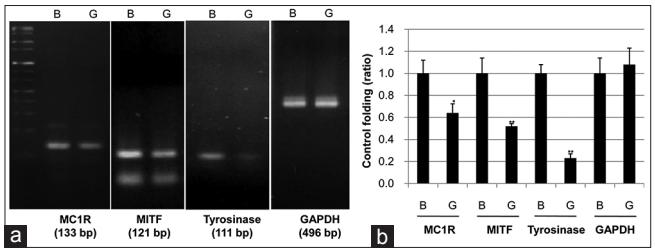

Figure 1: Transcript expression levels of MC1R, MITF, and tyrosinase in black (b) and graying (g) hair follicles are presented in a picture (a) and a graph (b). Expression levels of glyceraldehyde-3-phosphate dehydrogenase are presented as an internal control

Polygonum multiflorum root extract induced melanin synthesis in melanin-producing cells

We next investigated the capability of PM-RE in stimulating of melanin synthesis in SKMEL-28 cells. PM-RE at various concentrations of 0, 312.5, 625, and 2500 μg/ml was used. The results showed that the PM-RE at tested concentration induced melanin formation in SKMEL-28 cells with dose-dependent manner [Figure 3a and b]. Total melanin was also measured and presented in a graph [Figure 3c]. PM-RE at concentrations of 312.5, 625, and 2500 μg/ml increased total melanin formed in SKMEL-28 cells up to 186%, 223%, and 247% compared with those in control SKMEL-28 cells, respectively.

Polygonum multiflorum root extract stimulated melanin synthesis through activation of plasma membrane receptors MC1R

We then examined the effects of PM-RE on the transcript levels of molecules in well-known MC1R/MITF/tyrosinase pathway, which plays important roles in regulation of melanin synthesis in melanocytes. Our results revealed that 1250 mg/L PM-RE increased transcript expression levels of MC1R, MITF, and tyrosinase in SKMEL-28 cells up to 1.19, 2.06, and 1.69 folds compared with those in nontreated SKMEL-28 cells, respectively [Figure 4]. We recognized the fact that, although the increase of level of MC1R was not so impressive, the levels of MITF and tyrosinase were significantly high. This fact may be caused by magnification property of the signaling pathway. These results suggested that PM-RE might promote melanin synthesis through activation of MC1R/MITF/tyrosinase.

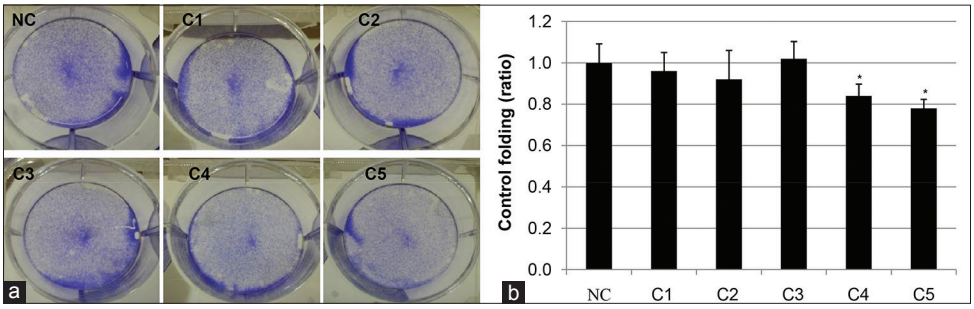

Figure 2: Toxicological effect of *Polygonum multiflorum* extract on SMEL-28 cells is presented in a picture (a) and a graph (b). Tested concentrations of *Polygonum multiflorum* extract were 0 (negative control), 312 (C1), 625 (C2), 1250 (C3), 2500 (C4), and 5000 (C5) μg/ml

Toxicological effects of *Polygonum multiflorum* root extract on development of zebrafish embryos

We then investigated the toxicity of PM-RE at various concentrations: 0, 135, 175, 225, 295, 385, 500, 625, and 845 mg/L on development of zebrafish embryos/larvae. Zebrafish embryos/larvae were exposed with PM-RE solution for 4 dpf. The appeared malformations of zebrafish embryos/larvae are summarized in Table 2. At the concentrations of 0% (negative control [NC]) and 135 mg/L, embryos/larvae developed in a normal manner without any morphological defect; at the concentrations of 175 mg/L, some morphological defects started to occur; however, there was no death of embryos/larvae. At the concentrations in the range of 385–845 mg/L, both morphological defects and death of embryos/larvae occurred. Our results also revealed that at the 1-dpf no morphological defect was observed; at the 2-dpf, only a morphological defect (yolk sac edema) was observed; however, at the 3- and 4-dpf, many morphological defects were observed. Teratogenic effects of methanol on zebrafish embryos/larvae with various typical morphological defects including edema (edema) with the most common types being heart edema and yolk sac edema, hemovascular defect with appearing of red dots accumulation, and yolk/head/body necrosis. Abnormal trunk with curved tail/body were showed [Figure 5b-h] while embryos in E3 medium normally developed without any defect [Figure 5a]. Data statistical analysis by GraphPad software (GraphPad Software, Inc., La Jolla, CA 92037 USA) gave the concentration-response curves for lethality and developmental defects [Figure 5i]. LC50, EC50, and EC10 values at the 4-dpf calculated based on the respective curve equation were 456 mg/L, 400 mg/L, and 245 mg/l, respectively. Teratogenicity TI was 1.14. This result suggested that PM-RE at concentration over 175 mg/L was teratogenic agent for zebrafish larvae at the 4-dpf (typical defects: e-yolk sac edema [or heart edema], h-hemovascular defect; n-necrosis; t-abnormal trunk. Red arrows indicate types of defects occurred on zebrafish embryos/larvae).

Table 2: Teratogenic effects of *Polygonum multiflorum* root extract on zebrafish embryos

Malformation	Day 1	Day 2	Day 3	Day 4
Yolk sac edema		+	+	+
Heart edema			+	+
Hemovascular defect			+	+
Necrosis			+	+
Abnormal trunk			+	+

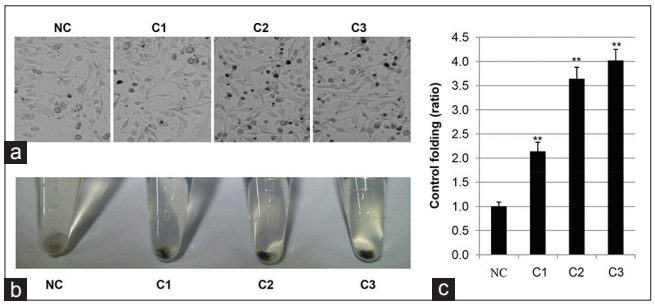

Figure 3: Melanins formed by SMEL-28 cells are presented in pictures (a and b). Total melanin synthesis by SKMEL-28 cells is presented in a graph (c). Tested concentrations of *Polygonum multiflorum* extract were 0 (negative control), 312, 625, and 2500 μg/ml

Polygonum multiflorum root extract promoted melanin formation in zebrafish embryos/larvae

Based on the above toxic test of PM-RE on zebrafish embryos/larvae, we chose PM-RE at the safe doses for investigating its effect on changing of phenotype (pigmentation) and genotype (transcription levels of MC1R, MITF, and tyrosinase) of zebrafish embryos/larvae. Our result showed that treatment with PM-RE at concentration 135 mg/L and 225 mg/L enhanced pigmentation [Figure 6a] and melanin formation [Figure 6b] in zebrafish embryos/larvae at 4-dpf. Moreover, it was also showed that PM-RE at concentrations of 135 mg/L (C1) and 225 mg/L (C2) significantly induced transcript levels of MC1R, MITF, and especially tyrosinase in zebrafish embryos/larvae at 4-dpf compared with those of NC ones [Figure 6c and d]. This result again confirmed that PM-RE promoted melanin formation in zebrafish through activation MC1R/MITF/tyrosinase-signaling pathway.

DISCUSSION

PM has been used in folk medicine for treatments of various diseases including hair aging. Recently, there are

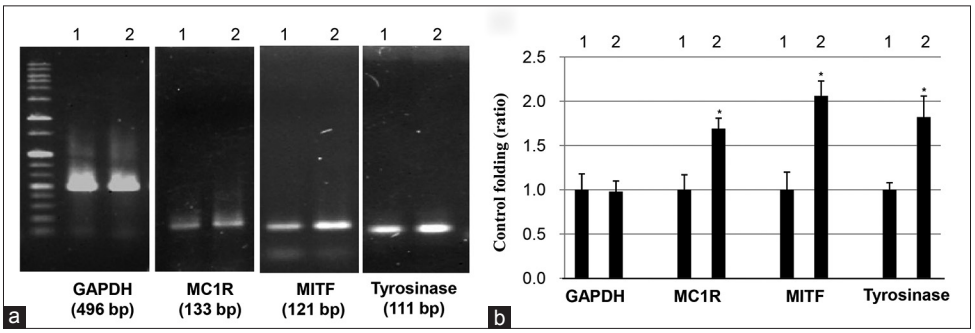

Figure 4: Transcript expression levels of MC1R/MITF/tyrosinase in SKMEL-28 cells are presented in a picture (a) and a graph (b). Glyceraldehyde-3-phosphate dehydrogenase is used as an internal control

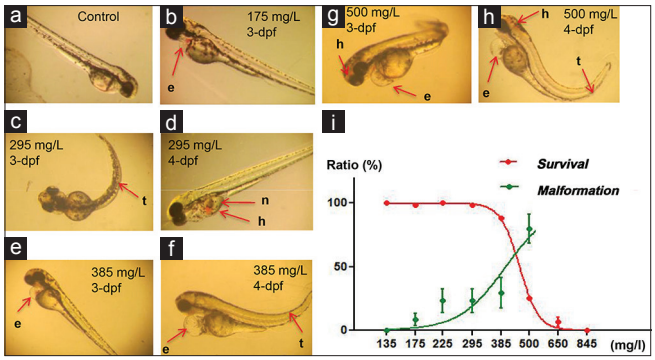

Figure 5: Toxicity of *Polygonum multiflorum* root extract on zebrafish embryos/larvae. (a) Zebrafish embryos at 4-day postfertilization without any treatment used as negative control; zebrafish embryos treated with *Polygonum multiflorum* root extract at 175 mg/L at 3-day postfertilization (b); 295 mg/L at 3-day postfertilization (c); 295 mg/L at 4-day postfertilization (d); 385 mg/L at 3-day postfertilization (e); 385 mg/L at 4-day postfertilization (f); 500 mg/L at 3-day postfertilization (g), and 500 mg/L at 4-day postfertilization (h). (i) Graph indicates the EC50 and LC50 of zebrafish embryos exposed to *Polygonum multiflorum* root extract

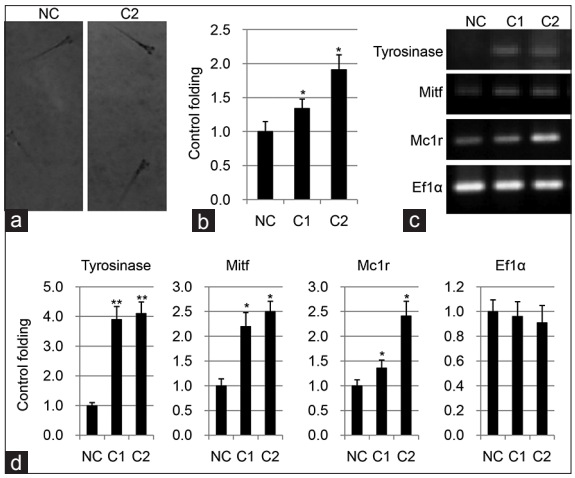

Figure 6: Effect of *Polygonum multiflorum* root extract on melanin formation in zebrafish embryos/larvae. Morphology of zebrafish embryos at 4-day postfertilization without (negative control) or with 225 mg/L (C2) *Polygonum multiflorum* root extract treatment (a). Total melanin formed in zebrafish embryos at 4-day postfertilization without (negative control) or with 135 mg/L (C1) and 225 mg/L (C2) *Polygonum multiflorum* root extract treatments (b). Transcript expression levels of MC1R, MITF, tyrosinase, and internal control Ef1α in zebrafish embryos exposed to *Polygonum multiflorum* root extract at 0 mg/L (negative control), 135 mg/L (C1), and 225 mg/L (C2), respectively, in a picture (c) and in graphs (d)

several studies showed that PM had potential effects on melanin synthesis *in vitro* (in B16 cells) model[12] as well as *in vivo* (mouse) models.[8] However, molecular mechanisms of these effects of PM are not fully understood. Recently, Han *et al.* and Li *et al.* demonstrated that PM-RE could help to recover black pigment for the hairs of hair-fading 57BL/6 mice through reactivation of MC1R and tyrosinase.[13,14] In accompany with previous researches,[8,12-14] in this study, using different tested models of human melanoma cells and zebrafish embryos, we also found out that PM extracted in methanol promoted melanin synthesis in melanin-producing human SKMEL-28 melanoma cells through activation MC1R/MITF/tyrosinase-signaling pathway. Moreover, we for the first time indicated that PM extract significantly induced transcript levels of MC1R, MITF, and tyrosinase in zebrafish embryos/larvae at the 4-dpf through activation MC1R/MITF/tyrosinase-signaling pathway. It was questioned that whether or not this results may be caused by developmental delay of the zebrafish due to toxicity of the PM-RE or specific action of PM-RE. However, by performing the toxic test, we could confirm that at the used concentrations (135 mg/L and 225 mg/L), there was no effect of PM-RE on retardation of zebrafish development. This result supported the hypothesis that PM-RE induced pigmentation of zebrafish through upregulation of MC1R/MITF/tyrosinase pathway. In addition, our results also revealed that the PM-RE at the concentrations above 225 mg/L might act as teratogenic agent for zebrafish larvae at the 4-dpf.

CONCLUSION

Conclusively, we suggested that PM-RE might be a potential candidate in using as an ingredient in drugs or cosmetics for early graying hair treatment or other related diseases. However, it should be also carried further studies to examine the toxicity of PM-RE before using especially for pregnant women because, at high concentration (above 225 mg/L), it caused teratogenicity/death of zebrafish embryos/larvae.

Financial support and sponsorship

This work was supported by Grants-in-Aid for Scientific Research under grant number KLEPT-14-02.

Conflicts of interest

There are no conflicts of interest.

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