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Effects of *Polygonum multiflorum* Root Extract in Methanol on Pigment Formation of Zebrafish Embryo

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Abstract: In this study, we investigated the effects of *Polygonum multiflorum* (PM) root extract in methanol on developments of teratogenic defects as well as the changing of transcript levels of molecules related to melanin formation in embryos of wild type strain AB zebrafish. Our results showed that PM root extract contributed an important role in melanin formation in zebrafish embryos via activation of MC1R/MITF/tyrosinase pathway. However we also found that, at the high concentration (above 225 mg/L), PM root extract acted as an agent for developments of teratogenic defects, including: heart/yolk-sac oedema, haemovascular defect with appearing of red dots accumulation; yolk/head/body necrosis; abnormal trunk with curved tail/body in zebrafish embryos. Taken together, we suggest that despite *Polygonum multiflorum* has been traditionally used as a traditional drug or an ingredient of drugs or cosmetics for early gray hair treatment and/or other diseases for a long time, it should be further carefully investigated the biological effects of PM root extract before using as a drug in clinic, especially for pregnant women.

Keywords: Polygonum multiflorum, graying hair, melanin, MITF, Tyrosinase, zebrafish.

1. Introduction

Melanin synthesis in melanocyte or melanoma cells are regulated by several signaling pathways including MC1R/MITF/ Tyrosinase and RAS/MEK/ ERK/MITF/ Tyrosinase pathways. MC1R or/and RAS localize in the plasma membrane and play important roles in activation of down-stream factors followed by sequential activation of MITF and Tyrosinase [1-4], an enzyme playing key role in melanin synthesis process with tyrosine substract via 3,4-dihydroxyphenylalanine (DOPA) reaction [1-3]. Nowadays, zebrafish was popular used as a model for developmental biology and cellular biology [5-7]. In particularly, zebrafish has been used as an ideal model for melanin formation and dispersion in previous studies [8, 9]. Differentiation to form melanocyte in zebrafish occurs very early; only 24 hours post fertilization (hpf), melanoblasts, which will be differentiated to become melanocyte start to produce melanin [10]. In generally, the regulation of melanin biosyntheis in melanocyte

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of zebrafish is quite similar with those of mammals, basically via activation of MC1R/MITF/tyrosinase signaling pathway [9, 11]. Currently, there are no medicines 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 [12], radical scavenging activity [13], lipid regulation [14] and hair follicle growing [15-17]. However, there is limited study focused on examination of the biological effects of PM *in vitro* as well *in vivo*. Therefore, in this study, we investigated the effects of PM root extract on melanin synthesis in zebrafish embryos.

2. Materials & Methods

2.1. Plant material

Polygonum multiflorum roots were obtained from Vietnam Pharmacy Institute in September 2013 and identified at the Department of Biochemistry and Plant physiology, Faculty of Biology, VNU University of Science, Vietnam National University, Hanoi, Vietnam. Three types of organic solvents including n-hexane, Ethylacetate (EtAc) and Methanol (MeOH) with gradually increasing in polarities were used to extract substances in roots of *Polygonum multiflorum*, and PM extract in methanol was used for this study.

2.2. Experimental zebrafish embryos

Adult zebrafish wild type strain AB (ZIRC, USA) [18] was maintained within the zebrafish facility in Animal Laboratory, VNU University of Science. Fish were cultured in glass rectangular pools with size of 40cm (wide size) x 50cm (length size) x 30cm (high size). Several pools of adult fishes were bred individually for each assay. After sorting, embryos from pools with high fertility (≥80%) were mixed and used for subsequent experiments. The experiment was validated only when the control survival rate was ≥90% at 4 day post fertilization (dpf). Zebrafish embryos were divided into five groups including: a negative control group for toxic test, a group for investigating the toxicity of solvent methanol on development of embryos, a group for investigating the toxicity of PM root extract in methanol on development of embryos, a negative control group for examining the expression level of molecules related to melanin synthesis, and a group for examining the effects of PM root extract on changing of these molecules. After testing of toxicities of methanol and PM extract in methanol on embryos, suitable concentrations of PM extract were used for examining the expression levels of melanin synthesis related molecules.

2.3. Chemical exposure and embryo observation

Fish Embryo Acute Toxicity (FET) is determined according to OECD test guideline (OECD, 1992 and 2013) [19, 20] or equivalent guidelines. Briefly, Organic solvent methanol and PM root extract dissolved in methanol were assessed for lethality and developmental toxicity to zebrafish embryos. After sorting, embryos from pools with high fertility (≥80%) were mixed and used for subsequent experiments. Experiments were validated only when the survival rate of the controls was ≥90% at 4 days post fertilization (dpf). Fish were reared in a Tecniplast re-circulating system under 14:10-h light/dark photocycle. The day before breeding, males and females were placed in breeding chambers with a separator to prevent undesired spawning. The next morning, fish were placed in fresh water system and the separator was removed to allow mating. Eggs were collected after 2 h and placed in E3 medium (5 mM NaCl, 0.17 mM KCl, 0.4 mM CaCl2, and 0.16 mM MgSO4) containing 0.01% methylene blue. The point of divider removal and mating start was marked as 0 hours post-fertilization (0 hpf); the breeding date was marked as 0 dpf. At around 3-4 hpf, eggs were screened and sorted under a

stereoscope to remove the unfertilized and/or abnormal ones. Healthy embryos that showed normal cleavage were distributed into 6-well plates at 25 embryos/well for subsequent experiments. Teratogenicity was assessed by determining the percentage of embryos/larvae with any morphological defect over normal surviving ones. Phenotypes were compared with those described previously by Kimmel et al., [21]. All experiments were repeated triplicate (embryos with n = 25 for each test). Data was calculated to determine indices including median lethal concentrations (LC50), median effective concentration (EC50), and the teratogenic index (TI, defined as the ratio between LC50 and EC50).

2.4. Gene analysis

Total RNAs of zebrafish embryos at the 4-dpf were isolated using a High Pure RNA Kit (Thermo Scientific Kit) according to the protocol of the kit. The cDNA was then synthesized by reverse transcription of total RNA using reverse transcriptase. PCR reactions were performed to examine the expression levels of MC1R, MITFa, and tyrosinase genes. Ef1α gene were used as internal control. PCR products were loaded on agarose gel for electrophoresis.

Sequences of primers for PCR reaction are presented as follows:

ef1α (forward primer: 5'-CTGGAGGCCAGCTCAAACAT-3'

and reverse primer: 5'ATCAAGAAGAGTAGTACCGCTAGCATTAC-3');

MC1R (forward primer: 5-GACCACGGCCTCCTGGATGT-3

and reverse primer: 5-GTTGCAGAAGGGGCTGGTGG-3);

MITFa (forward primer: 5'-TGTACAGCAATCATGCTCTTCC-3'

and reverse primer:

5'-GTCCCCAGCTCCTTAATTCTGTC-3');

tyrosinase (forward primer: 5-CGCAGATGAACAATGGCTC-3

and reverse primer: 5-AGCAGATACACCCGATGCC-3).

2.5. Statistical analysis

Statistical analysis in this study was performed according to the method previously described [22, 23]. For toxicity tests on zebrafish embryos, all statistical analyses including regressions and comparison tests were carried out using Graphpad Prism v.5.04 for Windows. Percentages of dead/defective embryos were plotted against the log-transformed test concentrations of each substance. Sigmoidal concentration-response curves were obtained by fitting those data to the four-parameter equation:

$$Y = Bottom + \frac{(Top - Bottom)}{1 + 10^{\left(\frac{logXC50}{X}\right) - HillSlope}}$$

Where top and bottom respectively represents the lowest and highest y-value (%dead/defective), XC50 is either LC50 or EC50 concentration, and HillSlope describes the steepness of the curve at the inflection point. LC50, EC50 and EC10 values for each substance were extracted from their corresponding equation. Differences in gene expression data between treated and control groups were confirmed by parametric or non-parametric tests based on normality test results. 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 non-parametric tests were used. Significance was considered when P-values were lower than 0.05 for all analyses.

3. Results

3.1. Toxicity of methanol on development of zebrafish embryos

Because PM root extract was dissolved in methanol solvent and then to be used to

investigate its effects on melanin synthesis of zebrafish embryos, we firstly examined the toxicity of methanol on zebrafish embryos to find out the safe dose used for dissolving PM root extract. Methanol at various concentrations of 0, 0.75, 0.95, 1.25, 1.50, 1.95, 2.45, 3.0 % (v/v) in E3 medium were used. The summary the malformations of zebrafish embryos with different exposure times to methanol at various concentrations were presented in the table 1.

At the concentrations of 0% (negative control) and 0.75% methanol embryos developed in a normal manner without any morphological defect; at the concentrations of 0.95% and 1.25% methanol some morphological defects occurred however there was no death of embryos (data not shown) and at the concentration of 3%, at the 4-dpf, all embryos dead (table 1). It was also indicated that at the 1-dpf only a morphological defect (yolk sac oedema) was obseverd; however, at the 2-, 3- 4-dpf many morphological defects were observed.

Table 1. Teratogenic effects of methanol (3.0 % v/v) on zebrafish embryos

	Day 1	Day 2	Day 3	Day 4
Malformation				
Yolk sac oedema	+	+	+	+
Heart oedema		+	+	+
Haemovascular defect		+	+	+
Necrosis		+	+	+

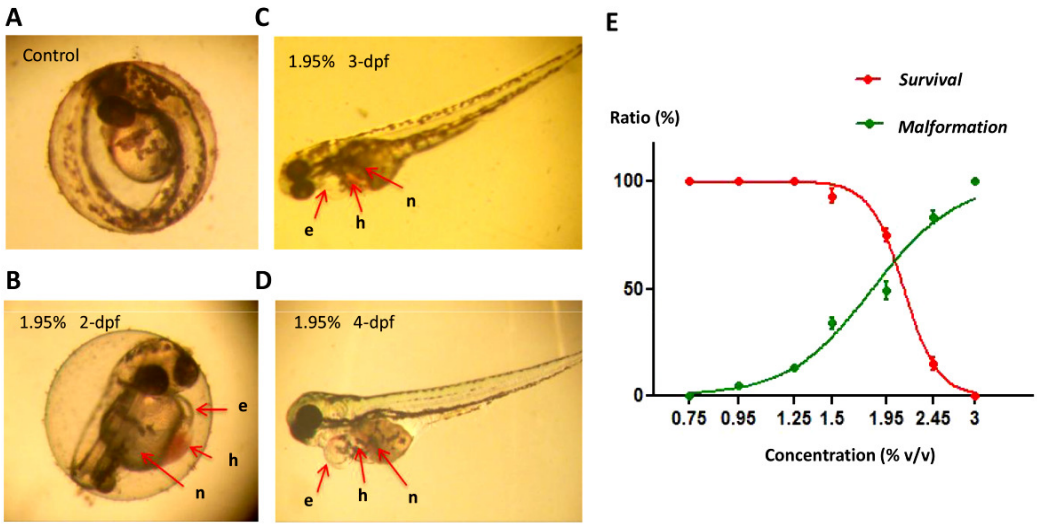

Figure 1. Toxicity of methanol on zebrafish lavae/embryos. A: Zebrafish embryos at 2-dpf without any treatment used as negative control (NC); B, C and D: Zebrafish embryos treated with 1.95% methanol for 2-, 3- and 4-dpf, respectively. E: the EC50 and LC50 of zebrafish embryos exposed to methanol. Typical defects: e - yolk sac oedema (or heart oedema), h - haemovascular defect; n – necrosis. Arrows indicate positions/sites of defects occurred on zebrafish lavae/embryos.

Teratogenic effects of methanol on zebrafish embryos with various typical morphological defects including: Oedema (edema) with the most common types of heart oedema and yolk sac oedema; Haemovascular defect with appearing of red dots accumulation and Yolk/head/body necrosis were showed (figure 1 B-D) while embryos in E3 medium normally developed without any defect (figure 1A). The concentration-response curves for

lethality and developmental defects were analyzed by GraphPad software and shown in figure 1 E. LC50 and EC50 values at the 4-dpf were 2,128 %v/v and 1,821 %v/v, respectively. Teratogenicity (TI = LC50/EC50) was 1.17. A substance is considered to be teratogenic when TI > 1, otherwise it would be considered as producing embryo lethal effects only [19, 20]. This result suggested that methanol at concentration over 0.95% was teratogenic agent for zebrafish embryos at the 4-dpf.

3.2. Toxicity of PM root extract in methanol on development of zebrafish embryos.

Based on the above result we decided to use methanol at concentration of 0.75% as solvent to dissolve PM root extract for further experiments. We then investigated the toxicity of PM root extract at various concentrations: 0, 135, 175, 225, 295, 385, 500, 625 and 845 mg/L on development of zebrafish embryos. Zebrafish embryos were exposured with PM root extract sulution for 4 day post fertilization. The appeared malformations of zebrafish embryos were summaried in the table 2.

Table 2. Teratogenic effects of PM extract (845 mg/L) on zebrafish embryos

	Day 1	Day 2	Day 3	Day 4
Malformation				
Yolk sac oedema		+	+	+
Heart oedema			+	+
Haemovascular defect			+	+
Necrosis			+	+
Abnormal trunk			+	+

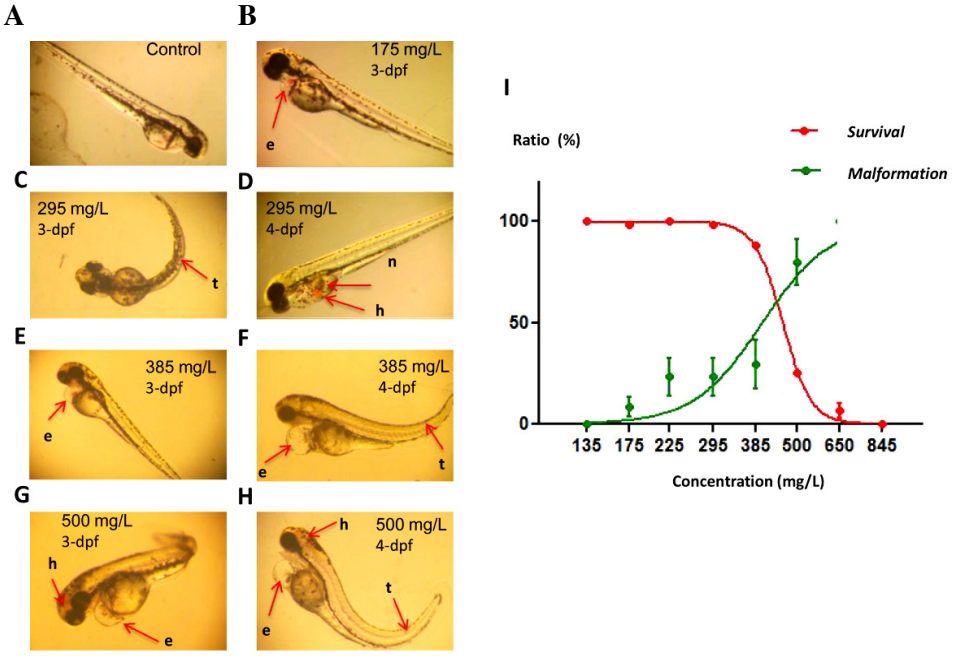

Figure 2. Toxicity of PM root extract dissolved in 0.75% methanol on zebrafish lavaae/embryos. A: Zebrafish embryos at 4-dpf without any treatment used as negative control (NC); Zebrafish embryos treated with PM root extract at 175 mg/L at 3-dpf (B); 295 mg/L at 3-dpf (C); 295 mg/L at 4-dpf (D); 385 mg/L at 3-dpf (E); 385 mg/L at 4-dpf (F); 500 mg/L at 3-dpf (G) and 500 mg/L at 4-dpf (H), respectively. I: the EC50 and LC50 of zebrafish embryos exposed to PM root extract in methanol. Typical defects: e - yolk sac oedema (or heart oedema), h - haemovascular defect; n – necrosis; t - abnormal trunk. Arrows indicate positions of defects occurred on zebrafish lavae/embryos.

At the concentrations of 0% (negative control) and 135 mg/L PM extract, embryos developed in a normal manner without any morphological defect; at the concentrations of 175 mg/L PM extract some morphological defects started to occur however there was no death of embryos (data not shown). At the concentrations in the range of 385 to 845 mg/L, both morphological defects and death of embryos occurred (table 2). Our results also revealed that at the 1-dpf no morphological defect was obseverd; at the 2-dpf only a morphological defect (yolk sac oedema) was obseverd; however, at the 3-, 4-dpf many morphological defects were observed. Teratogenic effects of methanol on zebrafish embryos with various typical morphological defects including: Oedema (edema) with the most common types of heart oedema and yolk sac oedema; Haemovascular defect with appearing of red dots accumulation; Yolk/head/body necrosis; Abnormal trunk with curved tail/body were showed (figure 2B-H) while embryos in E3 medium normally developed without any defect (figure 2A). Statistical data analysis by GraphPad software gave the concentration-response curves for lethality and developmental defects as shown infigure 2I. 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 root extract at concentration over 175 mg/L was teratogenic agent for zebrafish embryos at the 4-dpf.

3.3. PM induced transcription levels of MC1R, MITF and Tyrosinase genes

Based on the above toxic test of PM on zebrafish embryos, we chose PM root extract at the safe doses for investigating its effect on changing of morphology of zebrafish embryos and transcription levels of MC1R, MITF and Tyrosinase genes in zebrafish embryos. Ef1α gene was used as an internal control. It showed that the pigment of zebrafish embryos treated with 135 mg/mL PM root extract (figure 3B) were promoted comparing with that of control zebrafish embryos (figure 3A).

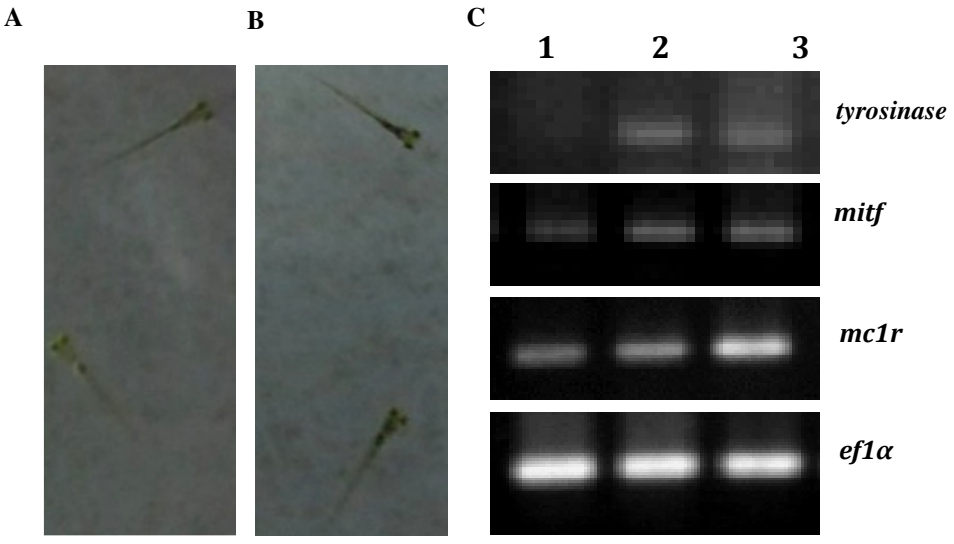

Figure 3. Morphologies of control zebrafish embryos (A) and PM-extract treated zebrafish embryos (B) and expression levels of MC1R, MITF, Tyrosinase genes and internal control ef1α in zebrafish embryos exposed to PM root extract at 0 mg/L (lane 1), 135 mg/L (lane 2) and 225 mg/L (lane 3), respectively (C).

Zebrafish embryos were exposed to 0.75% methanol (negative control), 135 mg/L and 225 mg/L for 4-dpf. Then, embryos were collected for RNA isolation, cDNA synthesis and gene expression analysis. The results showed that PM root extract at concentrations of 135 mg/L (lane 2, figure 3C) and 225 mg/L (lane 3, figure 3C) induced transcript levels of MC1R, MITF and especially Tyrosinase genes in zebrafish embryos at 4-dpf compared with those of negative control ones (lane 1) (figure 3C).

4. Conclusions

Polygonum multiflorum (PM) has been used in folk medicine for treatments of various diseases including hair aging. Recently, there are several studies showed that PM had potential effects on melanin synthesis *in-vitro* (in B16 cells) [24] as well as *in-vivo* (mouse) [15-17]. However, molecular mechanisms of these effects of *Polygonum multiflorum* are not fully understood. In this study, we found out that *Polygonum multiflorum* extracted in methanol promoted melanin synthesis in zebrafish embryos at the 4-dpf via enhancing the transcript levels of MC1R, MITF and Tyrosinase genes. In addition, our results revealed the fact that the *Polygonum multiflorum* extract at the concentrations above 225 mg/L might act as teratogenic agent for zebrafish embryos at the 4-dpf. These results suggested that *Polygonum multiflorum* might not only be a potential source for drug ingredient or cosmetics for early graying hair treatment but also be a teratogenic agent that causes the development of teratogenic defects in the zebrafish embryos. Conclusively, we suggested that it should be further carefully investigated the biological effects of *Polygonum multiflorum,* especially in mouse which has higher genetic similarity with human than that of zebrafish embryos, before using it as an ingredient in drug or cosmetic products for treatment of early hair graying or other diseases.

Conflict of Interests: The authors declare that they have no competing interests.

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Ảnh hưởng của dịch chiết xuất từ rễ Hà thủ ô đỏ (*Polygonum multiflorum*) trong methanol lên sự hình thành sắc tố của phôi cá ngựa vằn

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Tóm tắt: Trong nghiên cứu này, chúng tôi khảo sát sự ảnh hưởng của dịch chiết xuất rễ hà thủ ô đỏ trong methanol lên sự hình thành các loại dị dạng (quái thai) cũng như sự thay đổi mức độ biểu hiện của các gene liên quan đến sự hình thành sắc tố melanin trên phôi cá ngựa vằn thuần chủng AB.

Các kết nghiên cứu cho thấy dịch chiết rễ hà thủ ô đỏ tác động quan trọng vào sự hình thành sắc tố melanin ở phôi cá ngựa vằn thông qua khả năng hoạt hóa con đường tín hiệu MC1R/MITF/Tyrosinase. Tuy nhiên, kết quả cũng cho thấy rằng, ở các nồng độ cao (lớn hơn 225 mg/L), dịch chiết rễ hà thủ ô đỏ cũng có tác dụng như một tác nhân gây ra sự phát triển của các loại dị dạng trên phôi cá ngựa vằn, chẳng hạn như: phù nề noãn hoàng/phù nề bao tim, sự tụ máu ở mạch, sự hoại tử noãn hoàng/đầu/thân, sự cong đuôi/thân bất thường,… Trên cơ sở đó, mặc dù từ lâu dịch chiết rễ hà thủ ô đỏ đã được sử dụng trong dân gian như các thành phần của thuốc hay thành phần của các sản phẩm làm đẹp để điều trị chứng bạc tóc sớm, hay các bệnh liên quan đến sự mất sắc tố khác; chúng tôi khuyến cáo rằng cần phải có các nghiên cứu sâu hơn để đánh giá những tác động sinh học của dịch chiết rễ hà thủ ô đỏ (đặc biệt chú ý liều dùng) trên các mô hình phù hợp trước khi sử dụng trong lâm sàng, đặc biệt là sử dụng cho các bà mẹ đang mang thai.

Từ khóa: Hà thủ ô đỏ (*Polygonum multiflorum*), tóc bạc sớm, melanin, MITF, Tyrosinase, cá ngựa vằn.