



Toxicological and melanin synthesis effects of *Polygonum multiflorum* root extracts on zebrafish embryos and human melanocytes

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Abstract— Introduction: *Polygonum multiflorum* (PM) has been commonly used as folk medicine for treatment of various conditions, such as early graying of hair in humans. However, there have been limited studies which have evaluated the toxicological and biological effects of PM in vitro as well as in vivo. In this study, PM root extracts in ethyl acetate (PM-E) and in distilled water (PM-W) were examined for their effects on the development of teratogenic defects/deaths. Additionally, they were evaluated for their effects on melanin formation in human melanocytes and pigmentation in embryos/larvae of wild type strain AB zebrafish (*Danio rerio*). **Methods:** Phytochemicals in PM roots were extracted in several solvents with increasing in their polarities. Chemical exposure tests were performed to investigate the toxic effects of PM root extracts on Zebrafish embryos/larvae. Total melanins formed in zebrafish embryos/larvae as well as in human melanocytes were measured by spectrophotometer using dual wavelengths of 415 and 400 nm. Gene expressions were analyzed by realtime PCR. All experiments were independently repeated three times and statistical analyzed. **Results:** Our results showed that PM root extracts at concentrations of 40 mg/L and 105 mg/L induced the development of teratogenic defects, including yolk sac edema (or heart edema), hemovascular defects, necrosis and abnormal trunk in zebrafish embryos at 4 days post fertilization; teratogenic indexes (TIs) were 1.43 and 0.63 for ethyl acetate extract and distilled water extract, respectively. Our results also demonstrated that PM-W significantly increased the pigmentation level of embryos/larvae and induced melanin formation in human melanocytes. The amount of melanin in PM-W-exposed embryos/larvae was 2.2-fold and 1.71-fold greater than those in the control embryos/larvae and control melanocytes, respectively. **Conclusion:** Our study also showed that the increased level of pigmentation in PM-W embryos/larvae or melanin biosynthesis in melanocytes were both regulated by activation of tyrosinase. Conclusively, our study suggests that PM root extracts could be used as potential agents for treatment of early hair graying as well as various other diseases related to loss of pigmentation. However, these PM root extracts may also have some negative effects on embryos; therefore it should be careful when using for women during pregnancy.

Keywords: *Polygonum multiflorum*, folk medicine, melanin formation, zebrafish embryos, teratogenicity, toxicity

INTRODUCTION

For a long time, *Polygonum multiflorum* (PM) has been traditionally used to treat various systemic diseases and biological conditions, including antioxidation (Lv et al., 2007), radical scavenging activity (Chen et al., 1999), lipid regulation (Wang et

al., 2012). In particular, it has been used to induce hair follicle growth as treatment for early gray hair (Begum et al., 2015; Park et al., 2011; Sun et al., 2013). Nowadays, zebrafish is a popular model to study developmental and cellular biology (Frank and Lisanti, 2006; Gousuddin et al., 2015). Zebrafish has

been used as an ideal model for study of melanin formation and dispersion (Parichy et al., 2000; Rawls et al., 2001). So far, there have been limited studies focusing on the evaluation of toxicological and melanin synthesis effects of PM *in vitro* as well as *in vivo*. In this study, phytochemicals from PM roots were extracted into different solvents with gradually increasing polarity, including n-hexane, ethyl acetate and distilled water. The extracts were tested for their effects on teratogenic defects/deaths, on melanin formation in human melanocytes, and on pigmentation in zebrafish embryos/larvae.

MATERIALS AND METHODS

Ethics statement

For experiments in this study, we used zebrafish embryos and larvae at early periods of development, from 1 to 4 days after fertilization (no more than 5 days old). Therefore, no license was required by OECD guidelines (OECD, 1992 and 2013), or the Council of Europe (1986)- Directive 86/609/EEC (Louhimies, 2002), which regulates the use of animals in scientific experiments.

Preparation of root extracts

PM roots were obtained from Vietnam Pharmacy Institute. Fresh roots of PM were cleaned and washed thoroughly with water and re-washed with distilled water. The washed roots were dried in the shade, powdered mechanically, and sieved through a mesh. For preparation of organic solvent extracts, 5 g of powdered material was refluxed in 1/10 w/v in a soxhlet apparatus for 1 h. The resulting extract was filtered and pooled, and then the solvent was removed under reduced pressure at 40±5 °C using a rotary flash evaporator. The effectiveness of the extraction procedure is depicted in Table 1. Three types of organic solvents were used consecutively with gradually increasing polarity; these included n-hexane, ethyl acetate (EtAc) and distilled water (DW).

Cell culture

Normal Human Primary Epidermal Melanocytes (code: ATCC® PCS-200-013™) were purchased from ATCC (Manassas, VA). Melanocytes were cultured in Dermal Cell Basal Medium (code: PCS-200-030™; ATCC), supplemented with Melanocyte Growth Kit (code: PCS-200-041™; ATCC) and 1%

penicillin/streptomycin (code: ATCC®30-2300™, ATCC) at 37°C, 5% CO₂.

Zebrafish culture and embryos selection

Adult zebrafish wild type strain AB (*Danio rerio*) (ZIRC, Eugene, OR) (Lawson and Weinstein, 2002) was maintained in the zebrafish facility of the Animal Laboratory. The fish were cultured in glass rectangular pools measuring 40 cm (wide) x 50 cm (long) x 30 cm (high). Several pools of adult fish were bred individually for each assay. 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 recirculating system under 14:10-h light/dark photoperiod. 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.

Chemical exposure and embryo observation

Zebrafish embryos were divided into five groups, which included: (1) a negative control group for toxicity testing, (2) a group for investigating the toxicity of EtAc on development of embryos, (3) a group for investigating the toxicity/teratogenicity of PM-E on development of embryos, (4) a group for investigating the toxicity/teratogenicity of PM-W on development of embryos, and (5) a group for investigating the effect of PM-W on melanin formation. Fish Embryo Acute Toxicity (FET) was determined according to OECD test guidelines (OECD, 1992 and 2013) or equivalent guidelines. Briefly, after sorting of embryos, E3 medium was replaced with test solution and incubated at 28°C. Test solutions were renewed daily until 4 dpf. Embryos/larvae were observed under a stereoscope

and all embryonic morphology and lethality were recorded. At 3 and 4 dpf, the embryos/larvae were photographed using an SZX10 stereomicroscope coupled with an XC50 camera (Olympus). Teratogenicity was assessed by determining the percentage of embryos/larvae with morphological defects over surviving ones. Phenotypes were compared with those described previously (Kimmel et al., 1995).

Typical defects occurring in the zebrafish embryos when exposed to chemicals are: (1) edema, (2) hemovascular defects, (3) necrosis, (4) abnormal trunk, and (5) retardation. Edema refers to the abnormal accumulation of fluid inside the embryo, causing the skin around the affected organ to swell and internal structures to be pressed into abnormal shape. The most common types of edema are heart and yolk sac edema. Hemovascular defects consist of two main types: hemostasis and hemorrhage; both appear as red dots due to local red blood cell accumulation. Hemostasis is the accumulation of blood inside the cardiovascular system due to obstruction by a blood clot (thrombosis) or by blood vessel narrowing (stenosis). Hemorrhaging refers to bleeding which causes blood to accumulate outside of the cardiovascular system. The third defect (necrosis) refers to cell and tissue death caused by external factors such as toxins. Lastly, abnormal trunk refers to a curved/short tail/body and retardation refers to delayed developmental processes (Lawson and Weinstein, 2002; Pruvot et al., 2012; Sylvain et al., 2010; Tsay et al., 2007).

All experiments were repeated in triplicate (n = 25 embryos for each test). Data was calculated to determine indices, including median lethal concentration (LC50), median effective concentration (EC50), and the teratogenic index (TI; defined as the ratio between LC50 and EC50). If the TI value is lower than 1.0 (< 1.0), it means the tested substance is an agent which induces death of embryos/larvae; if the TI value is greater than 1.0 (> 1.0), it means the tested substance is a teratogenic agent for embryos/larvae.

Gene analysis

Total RNA of zebrafish embryos/larvae were isolated using a High Pure RNA Kit (Thermo Fisher Scientific, Waltham, MA), according to the manufacturer's protocol. The cDNA was then synthesized by reverse transcription of total RNA using Super-scriptTM reverse transcriptase, according to a previously

published protocol (Dinh Thang et al., 2015; Nguyen et al., 2015). Real-time quantitative RT-PCR with SYBR green was performed using power SYBR1 Green PCR master mix (Applied Biosystems, i.e. Thermo Fisher Scientific) in an ABI Prism 7500 sequence detection system (Applied Biosystems, i.e. Thermo Fisher Scientific). The transcript level tyrosinase was adjusted through the transcript expression level of housekeeping genes *ef1 α* (for zebrafish) and GAPDH (for human melanocytes). Then, the PCR products were loaded on agarose gel for electrophoresis.

Sequences of primers for zebrafish were as follows:

Primers for tyrosinase, forward primer: 5'-CGCAGATGAACAATGGCTC-3' and reverse primer: 5'-AGCAGATACACCCGATGCC-3'

Primers for *ef1 α* , forward primer: 5'-CTGGAGGCCAGCTCAAACAT-3' and reverse primer: 5'-ATCAAGAAGAGTAGTACCGCTAGCATTAC-3'.

Sequences of primers for human were as follows:

Primers for tyrosinase, forward primer: 5'-TTGCCTGAGTTTGACCCAAT-3' and reverse primer: 5'-GCATCCGCTATCCCAGTAAG-3';

Primers for GAPDH, forward primer: 5'-CGGAGTCAACGGATTTGGTCGTAT-3' and reverse primer: 5'-AGCCTTCTCCATGGTGGTGAAGAC-3'.

Total melanin content

The melanin content was determined according to previous publications (Jiang et al., 2009; Meyer et al., 2009). Briefly, zebrafish embryos/larvae were homogenized in 1 M NaOH and incubated at 80°C for 30 min followed by centrifugation at 1,500 rpm for 5 min. The supernatant was collected for absorbance measurement using dual wavelengths at 415 and 400 nm. Melanin has a maximum absorbance at 415 nm; however, blood cells can also strongly absorb light at 400 and 415 nm. Therefore, to minimize the interference of blood cells when determining melanin concentration, we first measured absorbance at 400 and 415 nm; the real absorbance of melanin = absorbance at 415 nm – absorbance at 400 nm. When measuring melanin concentration in melanocytes *in vitro*, the absorbance was measured directly at the single wavelength of 415 nm, as there was no effect from blood cells.

Statistical analysis

Statistical analysis was performed according to methods previously described (Dinh Thang et al., 2015; Nguyen et al., 2015). For toxicity/teratogenicity tests on zebrafish embryos, all statistical analyses, including regressions and comparison tests, were carried out using GraphPad Prism v.5.04 for Windows (GraphPad Software, La Jolla, CA). 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:

$$Y = \text{Bottom} + \frac{(\text{Top} - \text{Bottom})}{1 + 10^{\left(\frac{\log_{10} X_{C50}}{X} - \text{HillSlope}\right)}}$$

“Top” and “bottom”, respectively, represent the lowest and highest y-value (% dead/defective); “XC50” is either LC50 or EC50 concentration, and “HillSlope” refers to the steepness of the curve at the inflection point. LC50 and EC50 values for each

substance were extracted from their corresponding equations. Results from 3 independent experiments in each group were statistically analyzed by Student’s t-test. The SPSS (version 18) software package (SPSS Japan Inc.) was used for statistical analyses, and the significance level was set at $p < 0.05$.

RESULTS

Extraction efficiencies of PM roots in different solvents

Phytochemicals in the PM roots were extracted into 3 different solvents with increasing of polar powers: n-hexane, EtAc and DW. The extraction efficiencies are depicted in Table 1. In general, the results showed that the percentage of phytochemicals dissolved in DW was dominant (29.6 %) compared to that in n-hexane (1.28 %) and ethyl acetate (0.98 %) (Table 1).

Table 1. PM roots were extracted with three different solvents, including n-hexane, ethyl acetate and distilled water.

Organic solvent	Dried weight of extracts (g/5g dried sample)	Efficiency (H %)
n-Hexane	0.064	1.28
Ethyl acetate	0.049	0.98
Distilled water	1.48	29.6

Table 2. Teratogenic effects of ethyl acetate (EtAc) on zebrafish embryos/larvae.

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

Toxicity of ethyl acetate (EtAc) on development of zebrafish embryos

Firstly, we performed experiments to evaluate the safe doses of EtAc for development of zebrafish embryos. EtAc at various concentrations of 0, 0.05, 0.075, 0.10, 0.125, 0.15, 0.20, and 0.25 % (w/v) in E3 medium were used. The summary of observed malformations of

zebrafish embryos/larvae with different exposure times to EtAc are presented in Table 2.

At concentrations below 0.1 %, EtAc had no effect on the development of zebrafish embryos/larvae. However, at 0.1 % concentration, EtAc started to cause abnormal development with slight yolk sac edema and haemovascular defects without any death of embryos/larvae. At a concentration of 0.25 %, EtAc

caused death of all embryos embryos at 4 dpf. Moreover, at concentrations of 0.125, 0.15 and 0.20 %, EtAc strongly induced toxicological effects on embryos/larvae, with a dose-dependent effect on embryo development (the higher the dose, the greater the defects). After 24 hpf, malformation of embryos/larvae started to appear, including development retardation, heart oedema, yolk sac edema. Additionally, hemovascular defects were seen with appearance of red dot accumulations and yolk/head/body necrosis (**Fig. 1A-D-G**); meanwhile the embryos/larvae in E3 medium developed normally without any defects (data not shown). After 3 dpf (**Fig. 1B-E-H**) or 4 dpf (**Fig. 1C-F-K**), many types of malformations occurred in the embryos/larvae and the number of embryos/larvae death rose with time exposure and increasing concentrations of EtAc. Statistical analysis of the data by GraphPad software yielded concentration-response

curves for lethality and developmental defects (**Fig. 1L**). LC50 and EC50 values at 4 dpf, calculated based on the respective curve equations, were 0.141 % (w/v) and 0.138 % (w/v), respectively. The teratogenic index (TI) was 1.02. The results suggest that EtAc at concentrations over 0.10 % are teratogenic for zebrafish larvae at 4 dpf.

Toxicity of PM root extracts in ethyl acetate (PM-E) and effect on development of zebrafish embryos
Next, we investigated the effect of PM-E (0.075 % ethyl acetate) on development of zebrafish embryos/larvae. The toxic effects of PM-E at various concentrations (0, 10, 20, 30, 40, 50, 80, and 100 mg/L) on embryos/larvae were investigated. Embryos/larvae were exposed to PM-E solution for 4 dpf. The observed malformations on embryos/larvae are summarized in **Table 3**.

Table 3. Teratogenic effects of PM-E on zebrafish embryos/larvae.

Malformations	Day 1	Day 2	Day 3	Day 4
Yolk sac edema		+	+	+
Heart edema		+	+	+
Hemovascular defect			+	+
Necrosis			+	+
Retardation	+	+	+	+
Abnormal trunk			+	+
Others			+	+

At concentrations below 40 mg/L, PM-E did not affect the development of embryos/larvae and there were no induced morphological defects. At the concentration of 40 mg/L, some morphological defects occurred; however, there was no death of embryos/larvae (**Fig. 2A**). At the concentrations ranging from 50 to 100 mg/L, both morphological defects and deaths occurred in embryos/larvae in a dose-dependent and time-dependent manner (**Fig. 2B-H**). Statistical analysis of the data yielded concentration-response curves for lethality and developmental defects (**Fig. 2K**). LC50 and EC50 values at 4 dpf, calculated based on the respective curve equations, were 70.94 mg/L and 49.75

mg/L, respectively; the TI was 1.43. The results suggest that PM-E at concentrations over 40 mg/L are teratogenic for zebrafish larvae/embryos at 4 dpf.

Toxicity of PM root extracts in distilled water (PM-W) on development of zebrafish embryos

We then examined the toxicity of PM-W at various concentrations (0, 70, 87.5, 105, 122.5, 140, 157.5, and 175 mg/L) on development of zebrafish embryos/larvae. Embryos/larvae were exposed to PM-W for 4 dpf. The observed malformations on zebrafish embryos/larvae are summarized in **Table 4**.

Table 4. Teratogenic effects of PM-W on zebrafish embryos/larvae.

Malformations	Day 1	Day 2	Day 3	Day 4
Yolk sac edema		+	+	+
Heart edema		+	+	+
Hemovascular defect			+	+
Necrosis				+
Retardation	+	+	+	+
Others				+

At the concentrations of 0% (negative control), 70 mg/L and 87.5 mg/L, embryos/larvae developed in a normal manner without any morphological defects (data not shown). At the concentration of 105 mg/L, some morphological defects started to occur; however, there was no death of embryos/larvae (Fig. 3A). At the concentrations ranging from 122.5 to 175 mg/L, both morphological defects and death of embryos/larvae occurred (Fig. 3B-E).

The results also revealed that at 1 dpf, no morphological defect was observed; at 2 dpf, only one morphological defect (yolk sac edema) was observed (at high concentrations of 157.5 and 175 mg/L but not at low concentrations, i.e. below 157.5 mg/L). However, at 4 dpf, many morphological defects were observed for all tested concentrations ranging from 105 to 175 mg/L. Statistical analysis of the data yielded concentration-response curves for lethality and developmental defects (Fig. 3F). LC50 and EC50 values at 4 dpf, calculated based on the respective curve equations, were 135.7 mg/L and 225.1 mg/L, respectively; the TI was 0.63. These results indicate that PM-W, at concentrations over 105 mg/L, is an agent which causes death of zebrafish embryos/larvae at 4 dpf.

PM-W induced melanin formation in zebrafish embryos/larvae

Based on the above toxicity tests, PM-W (at the safe doses) was evaluated for its effect on pigmentation of zebrafish embryos/larvae. The results (from observations under the microscope) showed that treatment with PM-W, at a concentration of 87.5 mg/L, significantly promoted pigment development of zebrafish embryos/larvae at 4 dpf (Fig. 4B), as compared to that for control embryos/larvae (Fig. 4A). The amount of melanin formed in the zebrafish embryos/larvae at 4 dpf were measured and demonstrated in Figure 4C; the embryos/larvae exposed to 87.5 mg/L PM-W had a 2.2-fold greater amount of melanin, compared to that of the control embryos/larvae (Fig. 4C).

PM-W promoted pigmentation of zebrafish embryos/larvae via activation of tyrosinase

We then investigated the effect of PM-W on transcript levels of tyrosinase (which plays a critical role in synthesis of melanin) and *ef1 α* (an internal control). Zebrafish embryos/larvae were exposed to E3 medium as a negative control (lane 1), and to PM-W at various concentrations (25 mg/L, 70 mg/L and 87.5 mg/L) for 4 dpf. After 4 dpf, embryos/larvae were collected for RNA isolation, cDNA synthesis and gene analysis. Our results indicated that 25 mg/L PM-W did not affect the transcription level of tyrosinase (lane 3, Fig. 5 A,B). However, at the concentrations of 70 mg/L (lane 4, Fig. 5 A,B) and 87.5 mg/L (lane 5, Fig. 5 A,B), PM-W significantly induced the transcription level of tyrosinase in embryos/larvae, up to 1.83- and 1.75-fold compared to the negative control (lane 2, Fig. 5 A,B). Meanwhile, PM-W at the tested concentrations did not change the transcription level of *ef1 α* (Fig. 5 C,D).

PM-W increased melanin synthesis of melanocytes via activation of tyrosinase

Next, we investigated the effect of PM-W on the biosynthesis of melanin in human melanocytes. The tested concentrations of PM-W were 0 (negative control), 50, 100, 250, 500, and 1000 μ g/mL. First, we examined the cytotoxicity of PM-W on melanocytes. The results showed that PM-W at high concentrations of 250, 500 and 1000 μ g/mL (but not at low concentrations of 50 and 100 μ g/mL) induced cytotoxicity in 36 %, 47 % and 52 % of the melanocytes, respectively (Fig. 6A,B). The ability of PM-W to stimulate melanin synthesis in melanocytes was examined next (Fig. 6C,D). The data showed that PM-W at 100 μ g/mL (the safe dose) significantly induced total melanin formation in melanocytes up to 179 %. Total melanin was measured and presented as a picture (Fig. 6C) and graph (Fig. 6D).

Moreover, we examined the effect of PM-W on the transcript levels of tyrosinase and GAPDH (another internal control). Our results revealed that PM-W at concentrations of 50 and 100 μ g/mL increased transcript expression of tyrosinase up to 1.63- and 1.71-fold, respectively (Fig. 6F-G). Our results show that PM-W has a low toxicity toward melanocytes but that it can promote melanin synthesis via activation of tyrosinase.

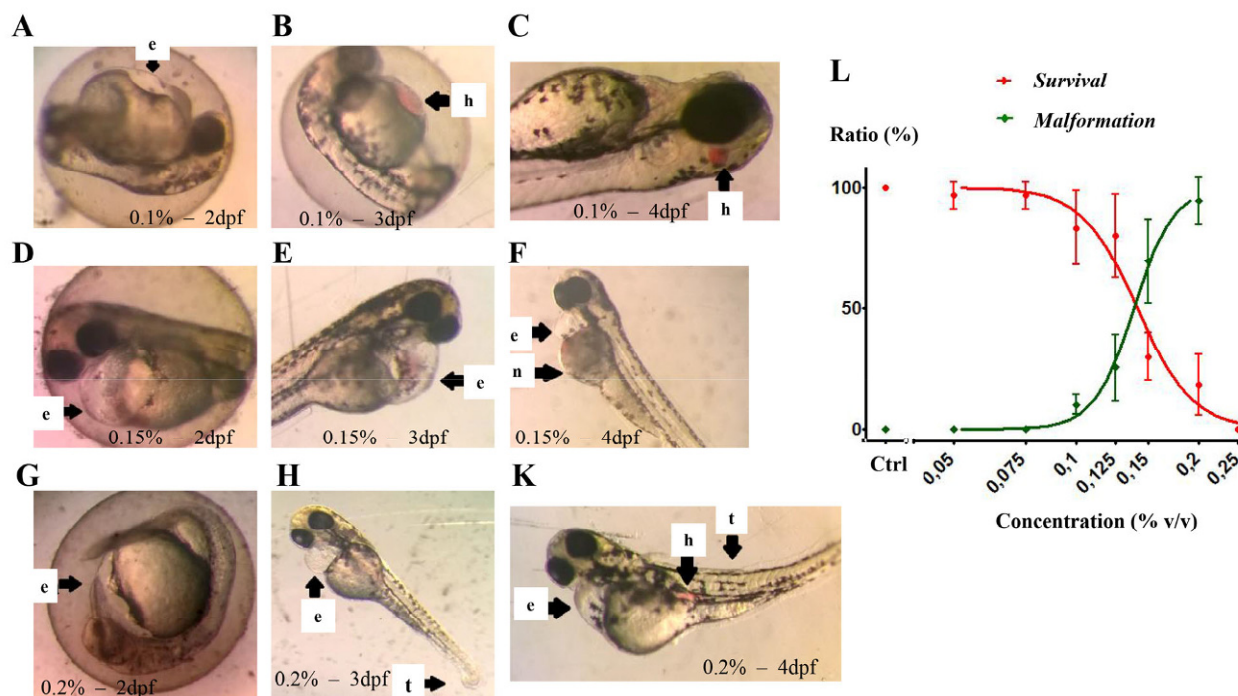


Figure 1. Toxicity of ethyl acetate on zebrafish embryos/larvae. A-B-C: Zebrafish larvae/embryos at 2-, 3-, 4 days post fertilization (dpf) treated with 0.1 % ethyl acetate; D-E-F: Zebrafish embryos at 2-, 3-, 4-dpf treated with 0.15 % ethyl acetate; G-H-K: Zebrafish embryos at 2-, 3-, 4-dpf treated with 0.2 % ethyl acetate; L: Graph indicates the EC50 and LC50 of zebrafish embryos exposed to ethyl acetate. Typical defects: e - yolk sac edema (or heart edema), h - hemovascular defect; n – necrosis. Arrows indicate types of defects occurring in the zebrafish larvae/embryos.

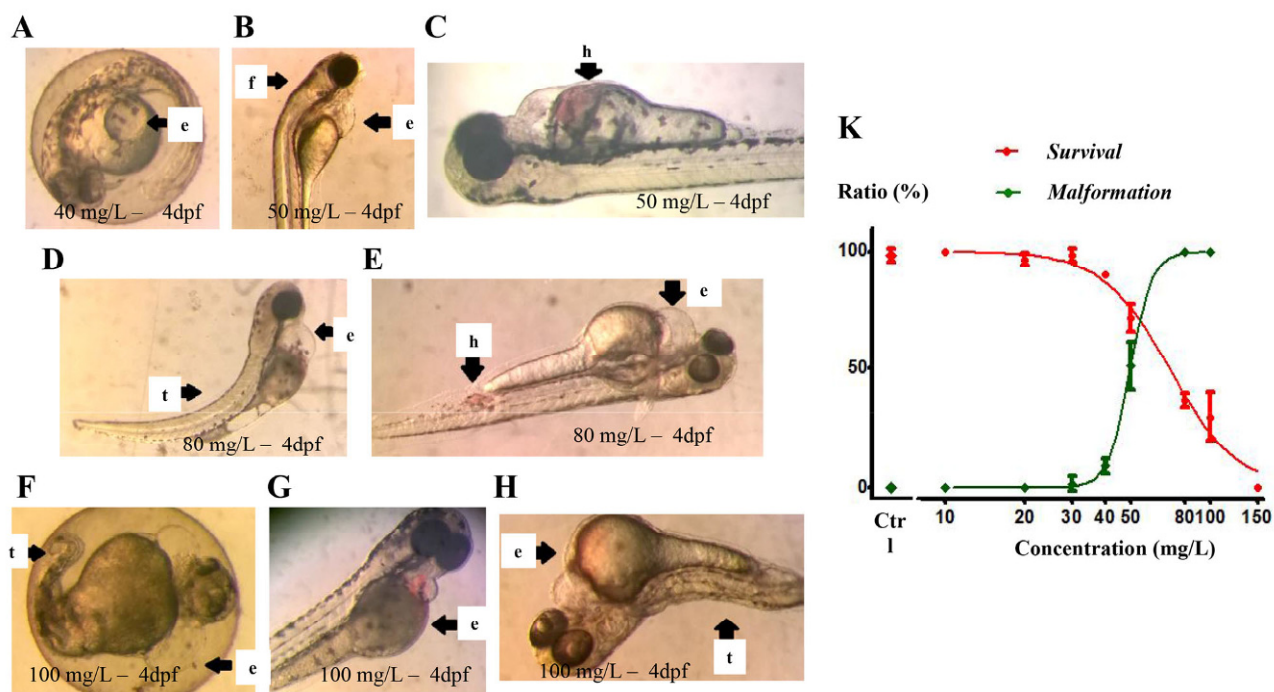


Figure 2. Toxicity of PM-E on zebrafish larvae/embryos. A: Zebrafish embryos at 4-dpf treated with 40 mg/L extract; B-C: Zebrafish embryos at 4-dpf treated with 50 mg/L extract; D-E: Zebrafish embryos at 4-dpf treated with 80 mg/L extract; F-G-H: Zebrafish embryos at 4-dpf treated with 100 mg/L extract; K: Graph indicates the EC50 and LC50 of zebrafish embryos exposed to PM root extract in ethyl acetate. Typical defects: e - yolk sac edema (or heart edema), h - hemovascular defect; n – necrosis; t - abnormal trunk. Arrows indicate types of defects occurring in the zebrafish larvae/embryos.

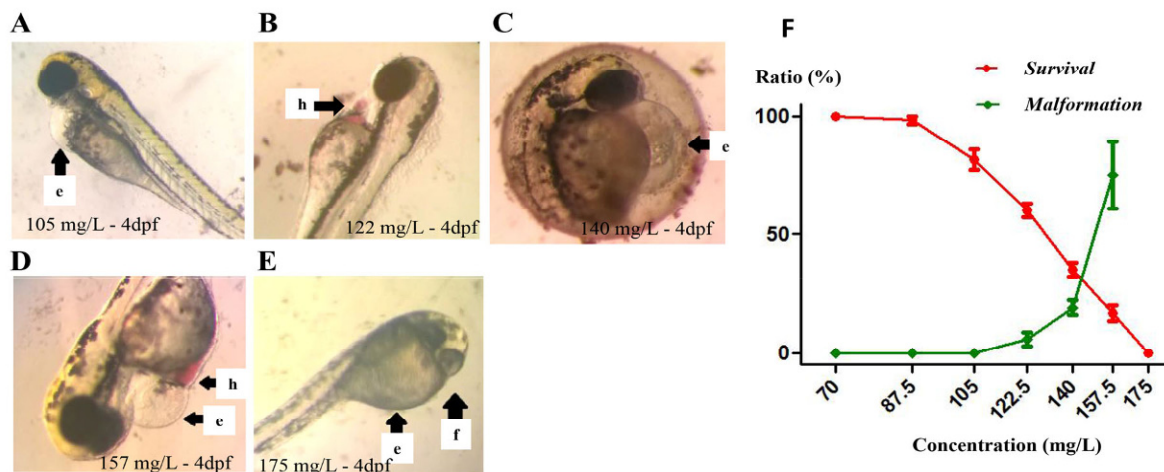


Figure 3. Toxicity of PM-W on zebrafish larvae/embryos. A-B-C-D-E: Zebrafish embryos at 4-dpf treated with 105 mg/L, 122 mg/L, 140 mg/L, 157 mg/L and 175 mg/L extract, respectively; F: Graph indicates the EC50 and LC50 of zebrafish embryos exposed to PM root extract in distilled water. Typical defects: e - yolk sac edema (or heart edema), h - hemovascular defect; n - necrosis; t - abnormal trunk. Arrows indicate types of defects occurring in the zebrafish larvae/embryos.

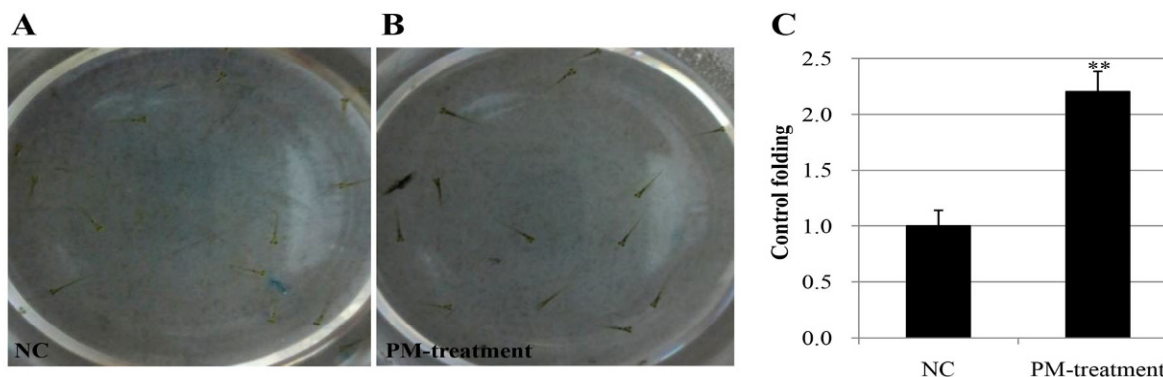


Figure 4. Melanin formation in zebrafish embryos/larvae. A: Pigmentation of control zebrafish embryos/larvae; B: Pigmentation of zebrafish embryos/larvae exposed with PM-W at concentration of 87.5 mg/L; C: Total melanin synthesis in zebrafish embryos/larvae with or without PM-W treatment. **, significant difference from the control with $P < 0.01$ by Student's t-test.

DISCUSSION

The use of plant extracts as drugs is increasing in Asia. However, there are concerns that hazardous substances exist in these extracts, which may have serious effects but have been understudied. Therefore, it is necessary to have toxicity testing before use. The zebrafish embryo is an emerging and useful model system for toxicity testing in the laboratory. Its advantages are rapid development (at the rate of about 50-300 embryos/pair/week), high availability and easy observation of transparent embryos (Kimmel et al., 1995). More importantly, because the developmental process of a zebrafish embryo represents that of other vertebrates with highly similar

embryogenesis and organogenesis, it is considered a complex and independent organism with a "non-animal" status. In addition, the well-characterized zebrafish genome shows many similarities with higher vertebrates, including humans (Howe et al., 2013), especially with regard to structure and function of genes, such as those of the CYP family involved in drug metabolism in mammals (Rubinstein, 2006). Therefore, zebrafish embryos are an ideal vertebrate model for melanin formation and dispersion tests, as well as for toxicity testing without raising any ethical issues (Lessman, 2011; Lieschke and Currie, 2007; OECD).

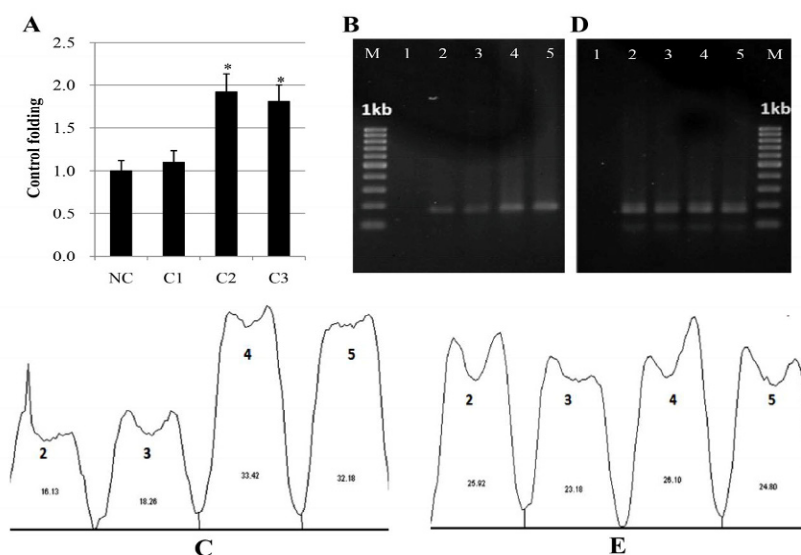


Figure 5. Transcript expression levels of tyrosinase and *e1f1α* (internal control) in zebrafish embryos/larvae. Transcript expression levels of tyrosinase (A and B) and *e1f1α* (C and D) in control zebrafish embryos/larvae (lane 2), treated with PM-W at 25 mg/L (lane 3), 70 mg/L (lane 4) and 87.5 mg/L (lane 5) were presented. Lane 1 was loaded with only E medium. *, significant difference from the control with $P < 0.05$ by Student's t-test. The sequences of primers are presented here.

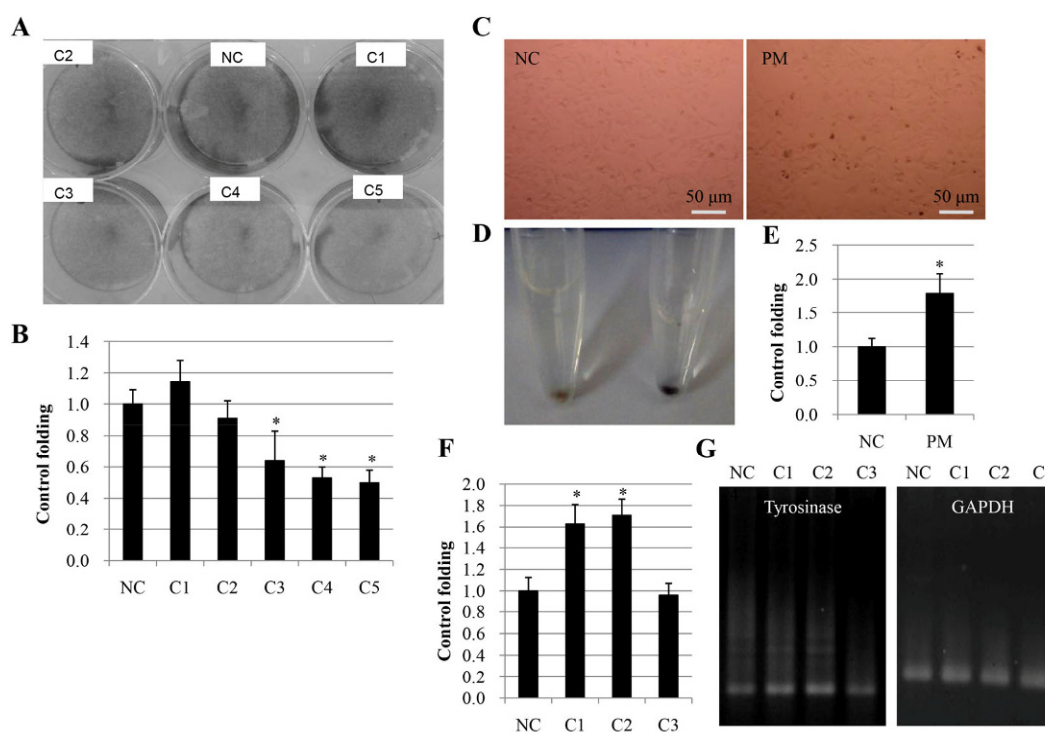


Figure 6. Effects of PM-W on human melanocytes. Cytotoxicity of PM-W on melanocytes is presented as a picture (A) and in graph form (B), with tested concentrations of 0 (negative control), 50 (C1), 100 (C2), 250 (C3), 500 (C4) and 1000 μg/mL (C5). Melanin biosynthesis in melanocytes with or without 100 μg/mL PM-W treatment are presented as pictures (C and D) and as graphs (E). Transcript expression levels of tyrosinase and GAPDH in control zebrafish embryos/larvae (NC), or treated with PM-W at 50 μg/mL (C1), 100 μg/mL (C2) and 250 μg/mL (C3), are presented in a graph by real-time-PCR analysis (F) and in pictures by electrophoresis analysis (G). *, significant difference from the control with $P < 0.05$ by Student's t-test.

PM root extracts have been used in folk medicine for treatment of various diseases, such as those related to pigmentation disorders including early graying of hair. Recently, there have been several studies showing that PM has potential effects on melanin synthesis in vitro (e.g. in B16 cells) (Hu, 2008), as well as in vivo (e.g. mouse models) (Sun, et al., 2013). However, so far the toxicity of PM root extracts have not been well elucidated. In our study, we extracted phytochemicals in the roots of PM using different solvents, including n-hexane, ethyl acetate and distilled water, and examined the toxicological and melanin biosynthesis effects of these extracts on development of zebrafish larvae/embryos. Our results showed that all these extracts have toxic properties and cause abnormal development as well as teratogenic defects of zebrafish larvae/embryos. Among the two extracts, PM-W had a lower toxicity effect than PM-E on the embryos/larvae. Importantly, our results showed that PM-W induced melanin formation in embryos/larvae and human melanocytes via activation of tyrosinase.

There are many signaling pathways which contribute to the regulation of biosynthesis of melanin in melanocytes; these include MC1R/cAMP/MITF/Tyrosinase, β -catenin/MITF/Tyrosinase, and RTK/ERK/MITF/Tyrosinase (Amsen, 2009; Bae-Harboe and Park, 2012; Schiaffino, 2010). Among these signaling pathways, tyrosinase is the most important since it catalyzes the metabolism of DOPA into melanin as the final product (Gray-Schopfer et al., 2007; Millington, 2013; Peters et al., 2011; Slominski, 2004). In this study, we examined the expression level of tyrosinase to understand the effects of PM root extracts on melanin formation in the zebrafish embryos/larvae model and melanocyte cell model. Our data revealed that PM-W enhances the transcript expression level of tyrosinase, not only in zebrafish embryos/larvae but also in human melanocytes. Our results lend support to the traditional use of PM-W for treating early gray hair development in humans.

CONCLUSIONS

In summary, results from our study indicate that PM root extracts (especially in water) is a very potent agent for treatment of diseases related to loss of pigmentation. Due to the toxicological and biological

effects of PM-W on embryos of zebrafish, however, more studies are warranted before its use as folk medicine for humans, especially use in pregnant women, to avoid possible negative effects of PM root extracts on the fetus.

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Competing Interests

The authors declare they have no competing interests.

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