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ORIGINAL ARTICLE

Effect of Pelargonidin isolated from *Ficus benghalensis* L. on phenotypic changes in zebrafish (Danio rerio) embryos

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Abstract In the present study, the extraction and isolation of Pelargonidin, an anthocyanin compound from stem bark of *Ficus benghalensis* are described. The study also involves evaluation of the effect of Pelargonidin on phenotypic variations in zebra fish embryos. Extraction and isolation of Pelargonidin were carried out by employing liquid-liquid extraction technique, phytochemical tests, column chromatography, UV and FT-IR. In the zebra fish embryo model, Paclitaxel was employed as a negative control. A series of phenotypic changes in different stages of embryonic development were studied with treatment concentrations of Pelargonidin between 3.0 and 20 ppm at 0–72-hour post-fertilization (hpf).

The results of our studies indicate that, after exposure of zebra fish embryos to 3.3–20 ppm concentration of Pelargonidin for 72 h, a significant reduction in aortic development occurs.

At the dose level of 0.5 ppm Paclitaxel and Pelargonidin in the dose range between 3.3 and 20 ppm, the zebra fish embryos were found to have bent tail, malformed eyes and developmental delays in vasculature.

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Based on the results obtained, we infer that Pelargonidin can exhibit phenotypic anti-angiogenic variations in embryonic stage of fish embryos and it can be applied in future for exploration of its anti-angiogenic potential. Furthermore, Pelargonidin could serve as a candidate drug for *in vivo* inhibition of angiogenesis and can be applied for the treatment of neovascular diseases and tumor.

1. Introduction

Ficus benghalensis Linn. (Moraceae) is reported in literature, to be used as a traditional medicine in South Asia. The plant is found to be extensively distributed in India and other countries of South Asia (Deraniyagala and Wijesundera, 2002). The bark and the milky exudates from the tree are used as medicinal material in Traditional System of Medicine (TSM) of India (Mandal et al., 2010).

The presence of sterols, ketones, flavonoids, triterpenes and triterpenoids, furocoumarins and tiglic acid esters has been reported in several publications (Mandal et al., 2010). Bengalenosides such as 5,7-dimethyl ether of Leucoperalgonidin-3-0-α-L-rhamnoside, 5,3-dimethyl ether of leucocyanidin, 5,7,3-trimethoxy leucodelphinidin and 3-O-α-L-Rhamnoside are found in stem bark of *F. benghalensis* (Taur et al., 2007). Various parts of *F. benghalensis* are reported in several scientific publications to possess, antioxidant, immunomodulatory, hypoglycemic, anti-allergic, anthelmintic and hypoglycemic activities (Kong et al., 2003).

Anthocyanins and flavonoids occur in teas, honey, fruits, vegetables, nuts and cereals (Mazza and Miniati, 1993; Joseph et al., 1999; Lila, 2004; Jackman and Smith, 1996). Anthocyanidins have been reported to possess growth inhibitory properties against tumors and against epidermal growth-factor receptor (Asen et al., 1972; Meiers et al., 2001; Wang et al., 1999).

Angiogenesis is the process of formation of new blood vessels and in diseases such as cancer, diabetes, obesity and retinopathies, and it contributes to their progression. The essential role of angiogenesis in tumor growth was first proposed in 1971 by Judah Folkman. Since the past few decades, research identifying molecular mechanisms that regulate neovascularization has gained an upsurge (Yu et al., 2015; Lucioli, 2012).

Newer therapies involving angiogenesis have been targets for researchers worldwide. In comparison with various animal models, zebra fish provides a comprehensive and good vertebrate model, whose circulatory system is reported to be similar to mammals. In zebra fish, the formation of blood vessels can be easily visualized and evaluated (Fishman, 1999; Jensen et al., 2012).

In the present study, extraction and isolation of Pelargonidin from stem bark of *F. benghalensis* were carried out by application of chromatographic methods and its pharmacological effect on *in vivo* zebra fish model was studied. The pharmacological study was carried out to investigate the effect of Pelargonidin on inhibition of blood vessel formation and embryonic development.

2. Materials and methods

2.1. Plant material

The dried stem barks of *F. benghalensis* Linn. were collected from Uran region of Navi-Mumbai, Maharashtra, India. The collected plant material was authenticated from Agarkar Research Institute, Pune, India, and a voucher number S/B-110 was obtained.

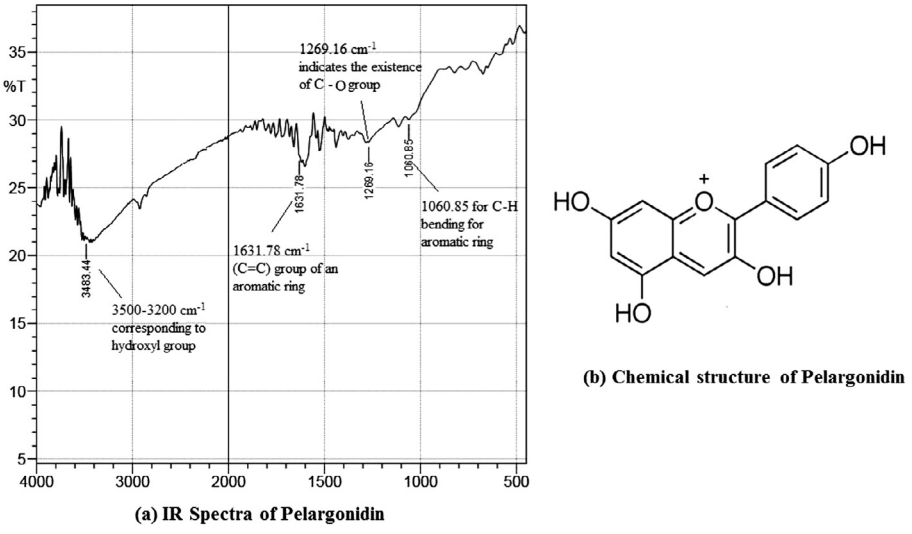

Figure 1 IR spectra and structure of Pelargonidin.

2.2. Extraction, isolation and preliminary phytochemical investigation

The drug material was powdered to coarse size using a stainless steel blender and used for extraction. For extraction of anthocyanins, 70% v/v aqueous acetone was used as the extraction solvent. A ratio of 1:5 (drug:solvent) was used for extraction. The resultant extract was then subjected to partitioning with chloroform. A liquid-liquid extraction was performed in a separatory funnel with chloroform. The aqueous phase was collected for further processing and the organic phase was discarded. The aqueous phase was used for isolation and purification of anthocyanins (Rodriguez-Saona and Wrolstad, 2001). The aqueous phase was concentrated using a rota-evaporator and was passed through a column containing silica gel 60, previously activated with 0.01% aqueous HCl. Activation was done to retain anthocyanins and other phenolics on the column. The retained pigments were washed with ethyl acetate to wash away all pigments, except Pelargonidin. Methanol containing 0.01% HCl (v/v) was used for recovery of Pelargonidin. The obtained crude pelargonidin was then purified with 0.01% HCL. The obtained Pelargonidin was analyzed for its UV λ_{max}, FTIR and chemical tests (Kokate, 1994; Giusti and Wrolstad, 2001).

2.3. Fish and embryo maintenance

The experiments on the zebra fish embryo (Danio rerio) were carried out as per the OECD test guidelines 236 (OECD, 2013). The zebra fish were obtained from the animal house facility of L.H. Hiranandani College of Pharmacy and maintained in an animal holding room. The experimental protocol was approved by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) and protocol registration no. 879/ac/05/CPCSEA was obtained. For the study, the procured zebra fish (wild type AB strain) were maintained at 28 °C with a 14 h light and 10 h dark cycle. The zebra fish were maintained in glass tanks of 40 l capacity. For the study, a 1:2 ratio of female: male was maintained and 4 females and 8 males were kept in separate tanks. For breeding, 5.5 gallon tanks were used with 25 watt submersible heaters. Air stones were supplied with the help of vibrator pumps and sufficient quantities of marbles were placed on the bottom of the tanks covering a height up to 2.5 in. across the tank bed (Westerfield, 1995). The temperature was set to 78–82 °F and pH 6.6–6.8 was maintained. Embryos were collected by natural spawning method by maintaining a 2:1 male to female ratio. The zebra fish were fed with various kinds of frozen and flake food about 3–5 times a day, until it was observed that female zebra fish were loaded up with eggs and the eggs were fertilized by males (Lawrence, 2007). The stages of embryo were denoted as hours post-fertilization (hpf). The study was conducted with 70 embryos for each concentration of the selected group of treatment drug and control (Kimmel et al., 1995).

2.4. Dose determination

The dose determination for treatment was carried out to ascertain the active safe dose of Pelargonidin for the zebra fish embryos. This survival rate count of embryos was used in determination of the dose of Pelargonidin. The various groups used for Pelargonidin treatment are as follows: *group A (Control group)* - in 150 ml of fresh water, *group B (PAC - 0.5 ppm - Positive control)* - treated with 0.5 ppm of Paclitaxel, group *C* (PAC – 3.3 ppm - *Positive control*) - treated with 3.3 ppm of Paclitaxel, *group D (ANT – 3.3 ppm treatment group)* – treated with 3.3 ppm concentration of Pelargonidin, *group E (ANT – 6.6 ppm treatment group)* – treated with 6.6 ppm concentration of Pelargonidin, *group E (ANT – 15 ppm treatment group)* - treated with 15 ppm concentration of Pelargonidin, *group F (ANT – 20 ppm treatment Group)* - treated with 20 ppm concentration of Pelargonidin. No visual heart beat was considered as the indication of death of embryos. The abbreviations PAC and ANT were used to denote Paclitaxel and Pelargonidin. Paclitaxel (PAC) is reported to be a potent anti-angiogenic drug and it interferes with the adenosine A3 receptor, inhibits VEGF signaling and prolongs cell viability (Merighi et al., 2007). Thus it was used as positive control for the study.

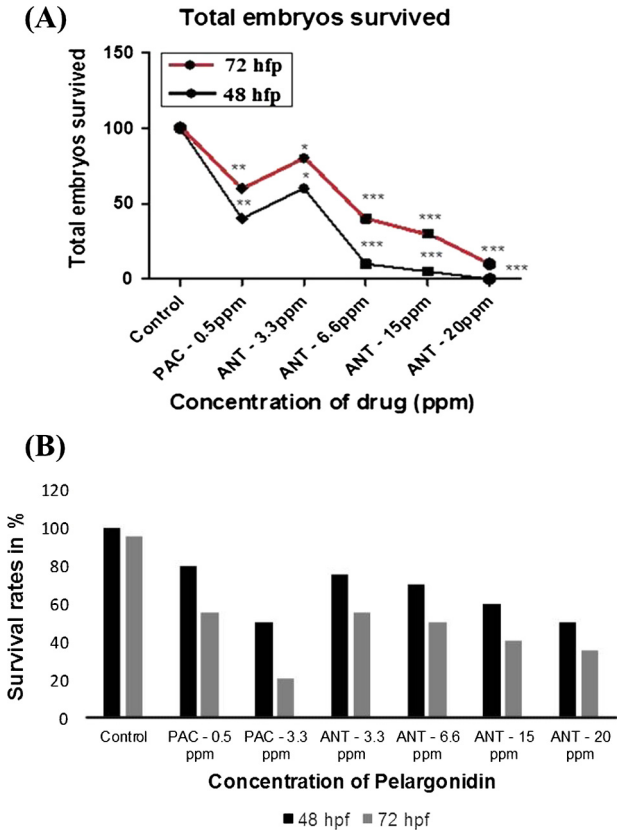

Figure 2 Graphical representation of doses selected for the study and their effects on survival rate on zebra fish embryos. (A) Total numbers of embryos survived. (B) Survival rates of embryos. Data expressed as mean ± SEM. $n = 20$. $^{***}P < 0.05$ for rate of abnormal elongation of yolk sac when compared with control group using one-way ANOVA followed by Dunnett's test as a post-ANOVA test. For ANOVA the P value < 0.0004. Pelargonidin is denoted as (ANT) and Paclitaxel is represented as (PAC) in the graph.

2.5. Anti-angiogenic effects of Pelargonidin on developing zebra fish embryos

After treatment with the drug (Pelargonidin) groups, the embryos were maintained in individual wells of culture plates at 28 °C until 72 hpf. The treated embryos were visually inspected for viability, gross morphological defects, hatching rate and circulation. Recording of the phenotypes exhibited by each embryo was carried out at 72 hpf. All the phenotypic variation stages were recorded with the help of a stage microscope (Bakkiyanathan et al., 2012).

3. Results

3.1. Preliminary phytochemical investigation of isolated Pelargonidin

The lead acetate test and Shinoda test were found to be positive for the isolated Pelargonidin and these indicated the chemical nature of the isolated Pelargonidin as a flavonoid. The IR spectrum of the isolated Pelargonidin indicates a broad band at 3500–3200 cm^{-1} corresponding to hydroxyl group (bonded). The second band indicated signal band at 1650 cm^{-1}. The signal obtained at 1570–1465 cm^{-1} indicated the existence of (C═C) group of an aromatic ring and 1300–1000 cm^{-1} indicated the existence of C—O group. The band below 900 cm^{-1} for C—H bending confirmed the presence of aromatic ring and a distinct bend at 829 cm^{-1} confirmed the presence of α-D-glucoside linkage. Signals at 3483.44, 1631.78, 1269.16, and 1060.85 cm^{-1} were the major absorption bands in the isolated sample of Pelargonidin (see Fig. 1). The UV visible spectrum of isolated Pelargonidin recorded by using methanol as a solvent, exhibited a UV λ_{max} at 482 nm. Thus we confirmed that the isolated constituent from the aqueous ethyl acetate extract of *F. benghalensis* was Pelargonidin.

3.2. Dose determination study and survival rates

With the treatment of 15 and 20 ppm dose levels of Pelargonidin, a death rate of 80–90% was found in the zebra fish embryos, till 72 hpf. Similar death rates were found with treatment concentrations of 3.3 ppm and 6.6 ppm of Pelargonidin, for 24 h. Dose below 3.3 ppm concentration produced less toxic effect on embryos. After 80 h of exposure, none of the embryos survived with 15 ppm concentration of Pelargonidin. The survival rate of embryos treated with 6.6 ppm of Pelargonidin also decreased significantly. Mentioned below are the results obtained for various phenotypic changes studied in Pelargonidin (3.3 ppm dose level) and Paclitaxel (0.5 ppm dose) treated embryos (see Fig. 2). Pelargonidin is denoted as (ANT) and Paclitaxel is represented as (PAC) in all the figures.

Hatching rate: The hatching rates of embryos treated with Pelargonidin (3.3 ppm dose level) and Paclitaxel (0.5 ppm dose

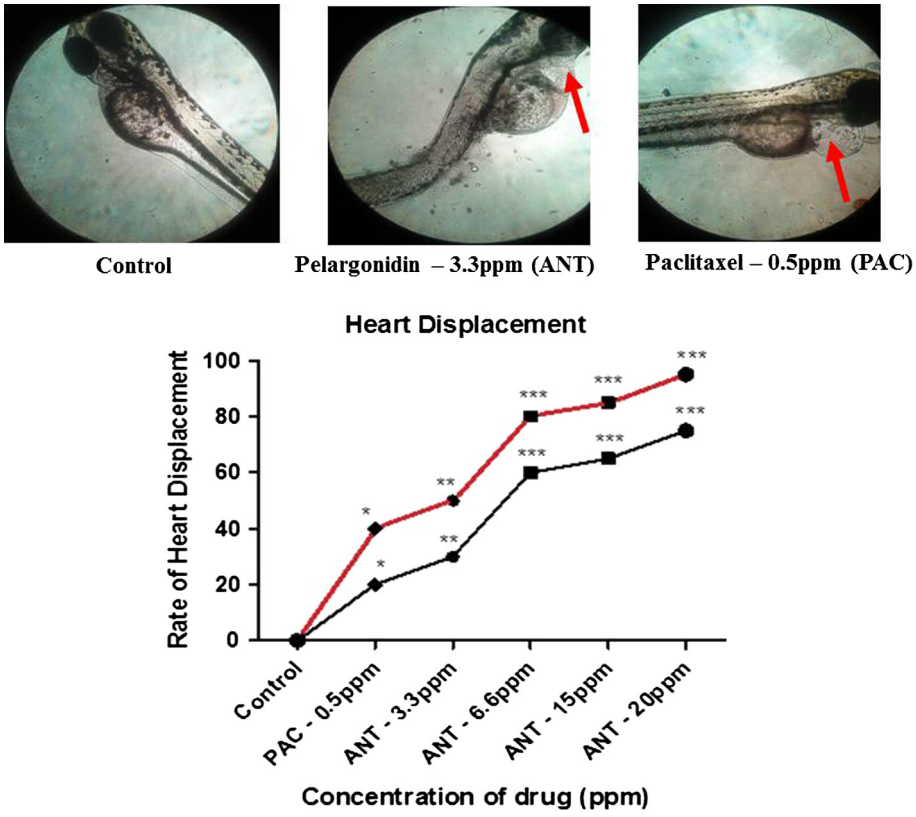

Figure 3 Heart displacement characteristics after treatment with Pelargonidin (3.3 ppm dose level) and Paclitaxel (0.5 ppm dose level). Data expressed as mean ± SEM. $n = 20$. $^{***}P < 0.05$ for rate of abnormal elongation of yolk sac when compared with control group using one-way ANOVA followed by Dunnett's test as a post-ANOVA test. For ANOVA the P value < 0.0002. Pelargonidin is denoted as (ANT) and Paclitaxel is represented as (PAC) in the figure.

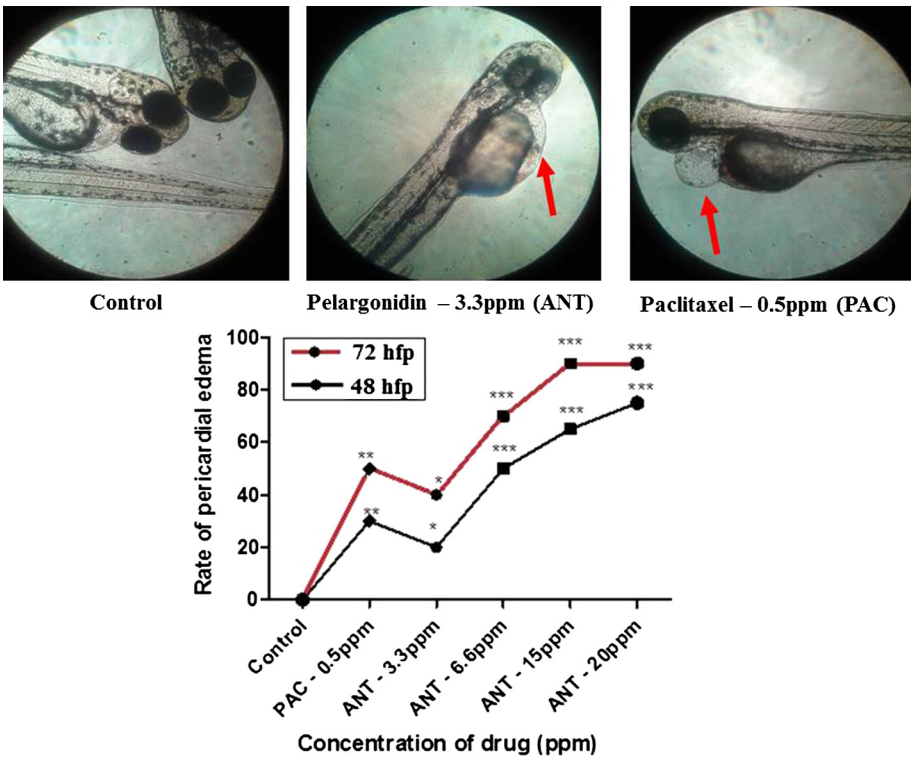

Figure 4 Pericardial edema observed after treatment with Pelargonidin (3.3 ppm dose level) and Paclitaxel (0.5 ppm dose level). Data expressed as mean ± SEM. $n = 20$. $^{***}P < 0.05$ for rate of abnormal elongation of yolk sac when compared with control group using one-way ANOVA followed by Dunnett's test as a post-ANOVA test. For ANOVA the P value < 0.0003. Pelargonidin is denoted as (ANT) and Paclitaxel is represented as (PAC) in the figure.

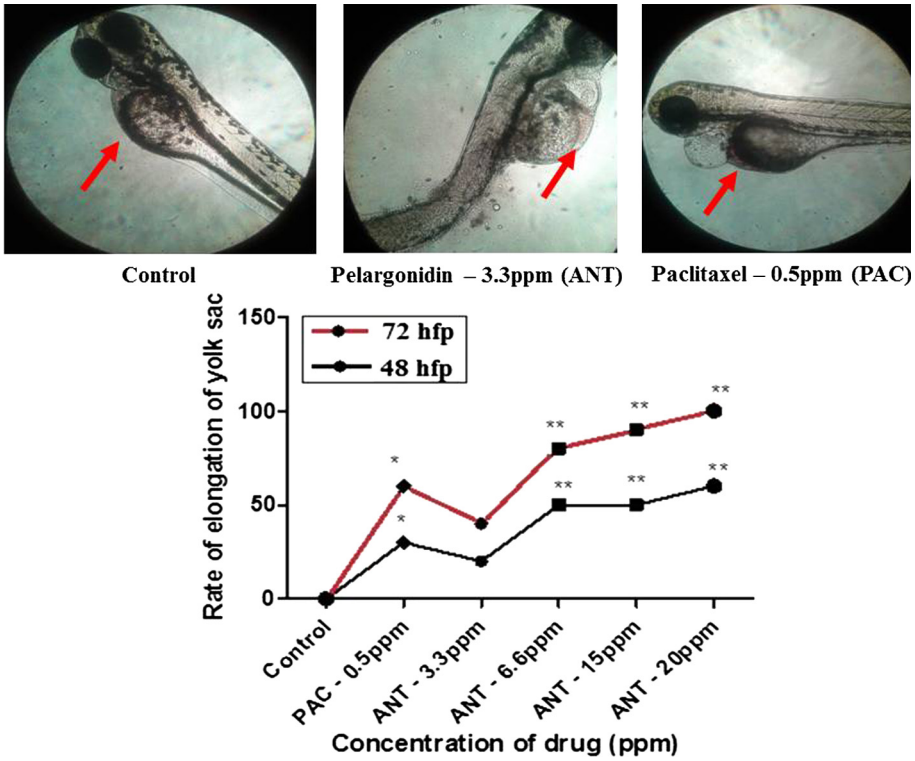

Figure 5 Abnormal elongation of yolk sac observed after treatment with Pelargonidin (3.3 ppm dose level) and Paclitaxel (0.5 ppm dose level). Data expressed as mean ± SEM. $n = 20$. $^{***}P < 0.05$ for rate of abnormal elongation of yolk sac when compared with control group using one-way ANOVA followed by Dunnett's test as a post-ANOVA test. For ANOVA the P value < 0.0045. Pelargonidin is denoted as (ANT) and Paclitaxel is represented as (PAC) in the figure.

level) were found to be significantly lower than those of the control. In pelargonidin group, embryos hatched after 64 hpf. In Paclitaxel group embryos hatched after 72 hpf, whereas in the positive control group embryos hatched after 40–44 hpf.

Heart displacement: In Paclitaxel (0.5 ppm dose level) the heart was found to be displaced toward mandibular arch and in Pelargonidin (3.3 ppm dose level) the heart was found to be displaced toward the duct of cuvier at 72 hpf (see Fig. 3).

Pericardial edema: Embryos developed with moderate to severe phenotypic changes during development stage. At 72 hpf, pericardial edema was observed in the surviving embryos treated with Pelargonidin (3.3 ppm dose level) and Paclitaxel (0.5 ppm dose level). The pericardial edema results due to the leakage of blood from common cardinal vein. There were no deformities observed in the positive control group (see Fig. 4).

Abnormal elongation of yolk sac: It was found that development of yolk sac was not normal in Paclitaxel 0.5 ppm and in Pelargonidin 3.3 ppm treated group. In normal embryo growth, nutrients are supplied to the developing embryo from yolk sac. An abnormal development of yolk sac results in abnormal growth of blood vessels (see Fig. 5).

Hemorrhages at yolk sac: The leakage of blood from common cardinal vein causes hemorrhage at yolk sac. Hemorrhage was found in yolk sac in both Pelargonidin (3.3 ppm dose level) and Paclitaxel (0.5 ppm dose level) treated groups (see Fig. 6).

Development of eyes: The eye development was not distinct in Pelargonidin (3.3 ppm dose level) treated group compared to the control. Deformation of eye was clearly observed in embryos treated with Paclitaxel (0.5 ppm dose level).

Tail bending: Tail bending and whole body curvature were the most marked abnormal phenotype observed in the treated embryos. Bending of tail indicates the process of deformation of blood vessels that include Dorsal Aorta (DA) and Cardinal Vein (CV), indicating anti-angiogenic effect. Tail bending was clearly observed in embryos treated with Pelargonidin (3.3 ppm dose level) and Paclitaxel (0.5 ppm dose level). This phenotypic change was observed in greater than 70% of embryos at 48 and 72 hpf during the study (see Fig. 7).

Vessel intensity: Embryos exposed to Paclitaxel had no blood flow in trunk vessels at 72 hpf but were found to demonstrate the presence of heart beats. The absence of dorsal aorta (DA) and posterior cardinal vein (PCV) and reduction in the aortic development were observed in developing zebra fish embryos treated with Pelargonidin (3.3 ppm dose level) and Paclitaxel (0.5 ppm dose level) at 24, 48 and 72 hpf. It was observed that when treated with Paclitaxel (0.5 ppm dose level) and Pelargonidin (3.3 ppm dose level), embryos developed abnormal vasculature (see Fig. 8).

Intersegmental vessels (ISV) development defect: Observations revealed the absence of major angiogenic vessels, namely the Intersegmental vessels (ISVs) in Pelargonidin (3.3 ppm dose level) and Paclitaxel (0.5 ppm dose level) treated groups. In control group embryos, a normal development of

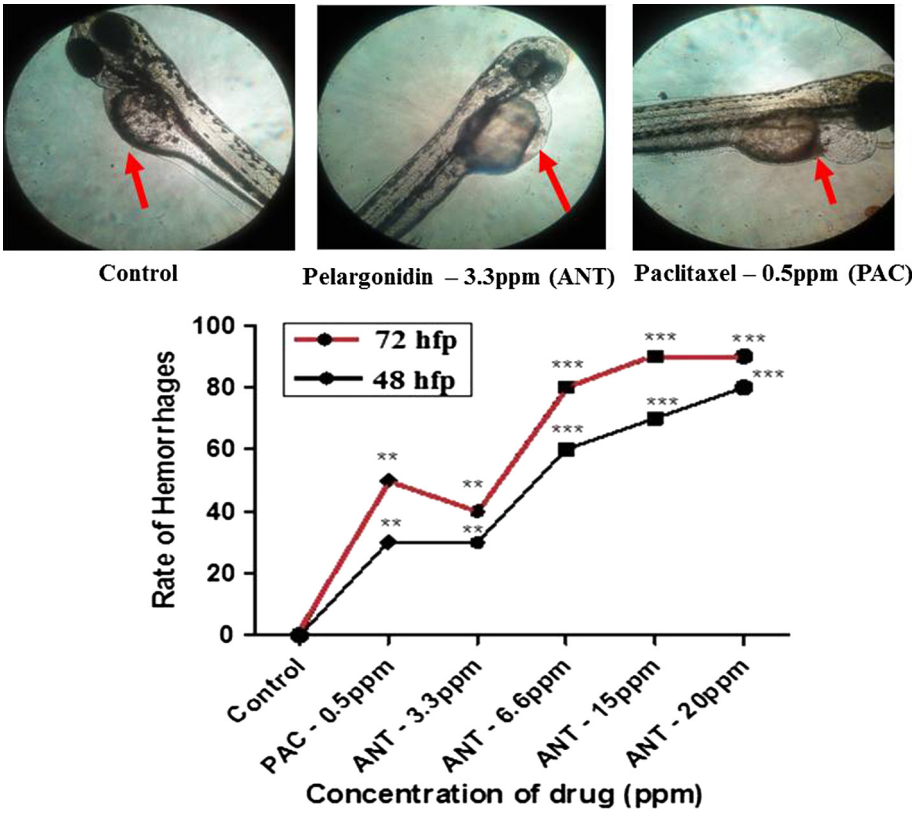

Figure 6 Hemorrhages at yolk sac observed after treatment with Pelargonidin (3.3 ppm dose level) and Paclitaxel (0.5 ppm dose level). Data expressed as mean ± SEM. $n = 20$. $^{***}P < 0.05$ for rate of hemorrhage when compared with control group using one-way ANOVA followed by Dunnett's test as a post-ANOVA test. For ANOVA the P value < 0.0002. Pelargonidin is denoted as (ANT) and Paclitaxel is represented as (PAC) in the figure.

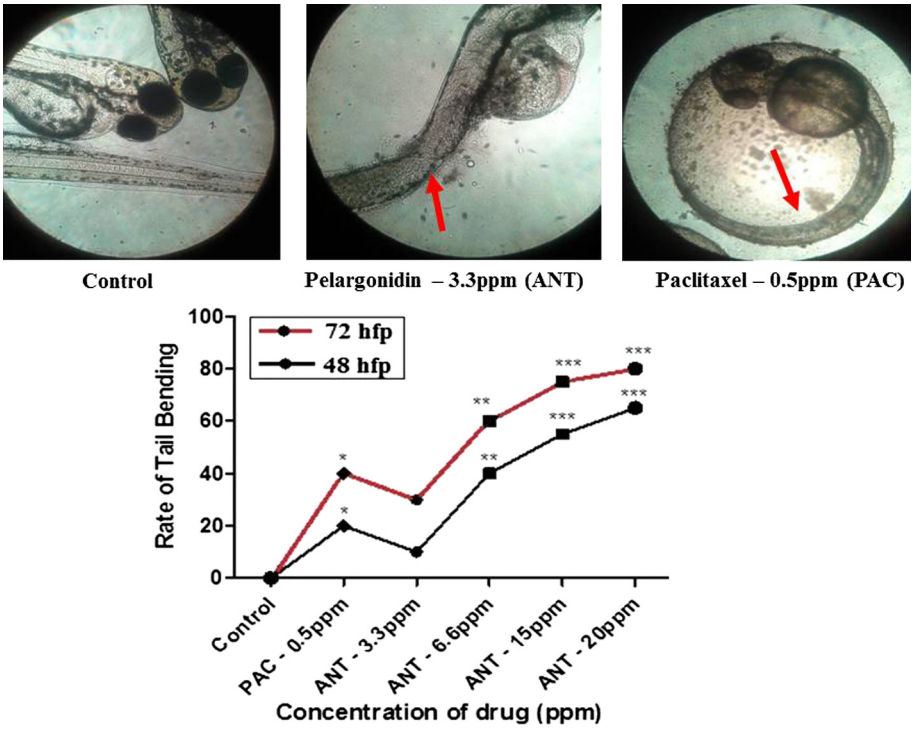

Figure 7 Tail bending observed after treatment with Pelargonidin (3.3 ppm dose level) and Paclitaxel (0.5 ppm dose level). Data expressed as mean ± SEM. $n = 20$. $^{***}P < 0.05$ for rate of abnormal elongation of yolk sac when compared with control group using one-way ANOVA followed by Dunnett's test as a post-ANOVA test. For ANOVA the P value < 0.0003. Pelargonidin is denoted as (ANT) and Paclitaxel is represented as (PAC) in the figure.

vasculature was found. Embryos treated with Pelargonidin (3.3 ppm dose level) and Paclitaxel (0.5 ppm dose level), showed reduction in formation of ISV's and at 72 hpf a complete arrest in the formation of ISVs was found.

4. Discussion

The zebra fish embryo has been extensively used by researchers and it has been adopted for assessment of drug effects. The zebra fish model can be applied in genetics, cell biology and embryology (Bakkiyanathan et al., 2012). There have been several studies developed to assess embryonic and teratogenic effects of drugs in zebra fish. These studies support the application of zebra fish embryos or larvae in prediction of the anti-angiogenic or angiogenic effects of new investigational compounds. Flavonoids are reported to inhibit adenosine receptors. They have the most inhibitory activity against A3 receptors and comparatively lower inhibitory effect against A1 and A2 receptors (Karton et al., 1996). Anthocyanidins such as cyanidin, peonidin, petunidin, delphinidin, pelargonidin and malvidin are reported to have potent anti-angiogenic activity. *In vitro* studies have also reported the inhibition of VEGF-induced activation and its up-regulation in Erk1/2 signaling by flavonoids. Studies that report the inhibitory effect of flavonoids on chemotactic motility of human endothelial cells (ECs) and their differentiation, have also been published (Lamy et al., 2006). Studies also report the anti-angiogenic effects of flavonoids against epithelial cancers (Jiang et al., 2000). In previous studies carried out by authors, it was found that anthocyanins inhibit vessel formation in cotton plug implant model in rats and fin regeneration and developmental defects in adult zebra fish model (Kundap et al., 2014, 2015).

In the present study, zebra fish embryos at 24, 48 and 72 hpf were screened to evaluate the anti-angiogenic property of Pelargonidin (3.3–20 ppm dose). Pelargonidin was found to inhibit major blood vessel formation in the developing embryos. Some natural compounds have been reported to exhibit strong anti-angiogenic activity by inhibiting the growth of intersegmental vessels and arresting the blood flow in zebra fish embryos (Atalay et al., 2003). The results of this study demonstrate that 72 h exposures of zebra fish embryos to 3.3–20 ppm concentration of Pelargonidin, resulted in significant reduction in aortic development and overall growth. Thus we infer that, there may be no blood flow in trunk vessels and it thus leads to anti-angiogenic activity of Pelargonidin.

Vascular epithelial growth factor (VEGF), is an angiogenic cytokine released by cancer cells. Inhibition of VEGF has thus become a target for various cancer researchers in evaluation of anti-angiogenic property (Wu et al., 2006). Anti-mutagenic and anti-carcinogenic effect of anthocyanins is attributed to the antioxidant properties of phenolic compounds (Seng et al., 2004). The double bonds in the ring and the hydroxyl side chains are responsible for the antioxidant effect of the phenolic compounds and they enhance their metal chelation and protein binding properties (Wilchek and Bayer, 1990).

Zebra fish embryos when subjected to Paclitaxel at the dose level of 0.5 ppm and Pelargonidin at the dose range between 3.3 and 20 ppm, were found to have bent tail, malformed eyes

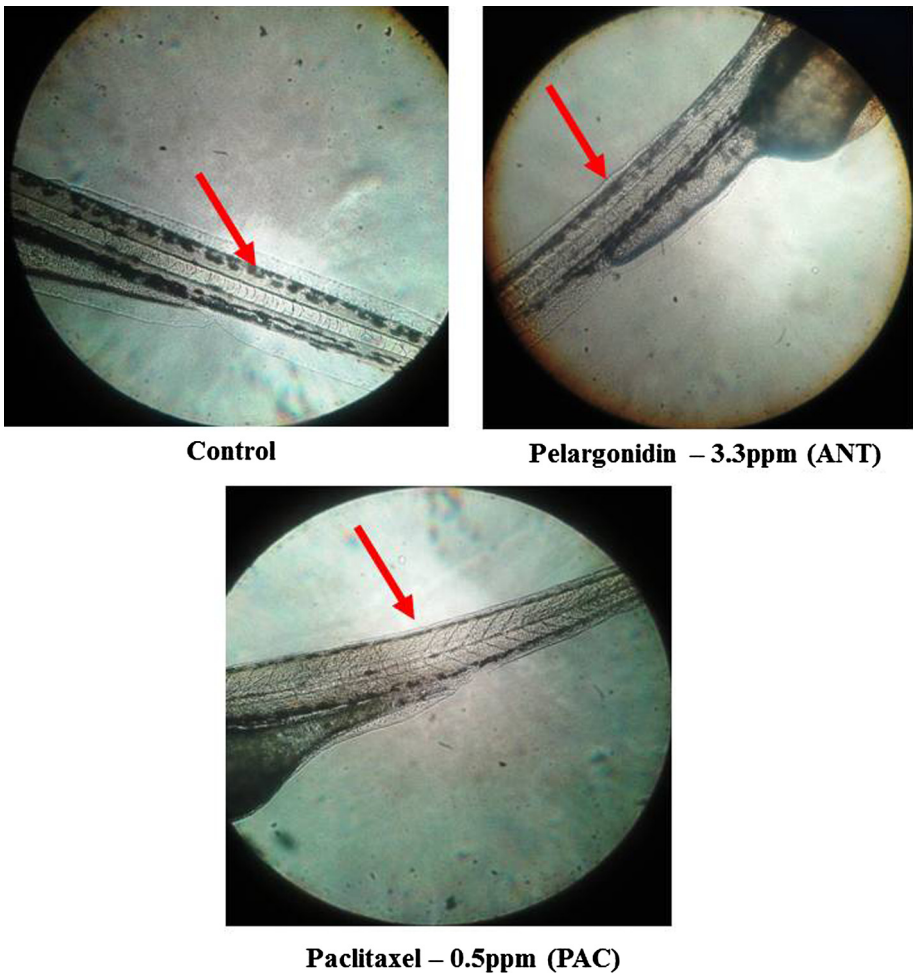

Figure 8 Vessel intensity changes observed after treatment with Pelargonidin (3.3 ppm dose level) and Paclitaxel (0.5 ppm dose level). Pelargonidin is denoted as (ANT) and Paclitaxel is represented as (PAC) in the figure.

or no eye development and developmental delay, with the complete absence of vasculature. The survival rates and hatching rates of embryos were found to be significantly lower. The results of this study correlate with the findings in the literature. Based on the results obtained in this study, we infer that anthocyanins such as Pelargonidin can suppress angiogenesis by the inhibition of hydrogen peroxide radicals and inhibit tumor necrosis factor alpha (TNF-a)-induced VEGF expression. However, these conclusions need further exploration of mechanism of action for Pelargonidin.

5. Conclusion

This study suggests that Pelargonidin can be an effective drug for *in vivo* inhibition of angiogenesis and can be explored for application in future therapeutics. The study presented in this article demonstrates that the zebra fish is a viable model for screening antiangiogenic properties of small molecules.

Acknowledgments

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