7-Hydroxycoumarin Elicit Anti-Angiogenic Effects Through Cellular Apoptosis in Developing Embryos of Zebrafish (*Danio Rerio*)

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Abstract

Derivatives of coumarin have diverse biological activities and pharmacological significance. In this study we made an attempt to test one of the hydroxy derivative i.e. 7-hydroxycoumarin (7HC) for its anti-angiogenic potential using embryonic angiogenesis of zebrafish (*Danio rerio)* as biological model. Developing embryos of *D. rerio* were cultured in media containing one of the grade (1 mM or 2 mM or 3 mM or 4 mM or 5 mM) of concentration of 7HC from gastrula stage (~6 hrs post fertilization - hpf) until completion of embryonic development and hatching (72 hpf). Developmental trajectory of each post hatched larva was traced for two weeks. Angiogenesis and patterning of major blood vessels occurred in zebrafish embryos/ larvae between 24 - 72 hpf in controls. Exposure to 7HC resulted in an impairment in patterning of inter segmental vessels (ISVs) dorsal aorta (DA), dorsal longitudinal anastomotic vessel (DLAV), posterior cardinal vein (PCV), and common cardinal veins (CCVs) accompanied by increased site specific cellular apoptosis, declined heart rates in a dose-dependent manner. 7HC manifest anti-angiogenic effects mediated/ induced through cellular apoptosis, hence may deserve attention and consideration as tumour angiogenesis suppressors in malignancy therapeutics.

Keywords: Zebrafish, Development, Embryo cultures, Angiogenesis, 7-hydroxycoumarin

Introduction

Coumarins are phytochemicals found amply in fruits, vegetables, herbs and many plants. Derivatives of coumarin, are known to manifest variety of biological activities of medicinal value in treating human diseases and evolving therapeutics (Chen et al., 2004). For instance, many of the hydroxy derivative of coumarin, are used as an intermediate in the synthesis

of anti-microbial, anti-inflammatory, anti-coagulant, and anti-proliferative agents (Ghate et al., 2005; Basanagouda et al., 2009; Moshal et al., 2011). Umbelliferone or 7-hydroxycoumarin (7HC) is hydroxy coumarin derivative which is widely used in the synthesis of drugs, protective agents, fluorescent probes and dyes etc., exhibits anti-oxidant, anti-diabetic, anti-inflammatory, anti-fungal, anti-proliferative properties (Gok, 2013; Lacy and Kennedy, 2004; Timonen et al., 2011). Scopoletin, a 7HC with a methoxy group at C6 position is used for the treatment of angiogenesis-mediated diseases clueing that synthetic analogues of this compound may have a pro or anti angiogenic potential (Pan et al., 2009). Angiogenesis is a process of sprouting of new blood plexuses from pre-existing blood vessels is a routine process during normal development but, has a special significance in tumorigenesis, as it supports and nourishes the tumor to grow from benign to malignant thereby augmenting metastasis. While, testing protocols for medicinal properties such as, anti-proliferative, anti-inflammatory, anti-bacterial of a chemical compound could easily be studied using cell lines, assessing angiogenic or anti-angiogenic value of a compound requires a biological model in which angiogenesis is actively in progress.

Zebrafish, has emerged as a powerful model system to understand human diseases and in evolving appropriate therapeutics. Developing embryos/ larvae of zebrafish are widely employed in testing the efficacy of a newly synthesized pharmaceutical or drug screening in medicine and cancer biology (Vascotto et al., 1997; Grunwald & Eisen, 2002; Rubinstein, 2003; Amsterdam & Hopkins, 2006; Tobia et al., 2011). *Ex utero* development, optical transparency and direct intake of chemicals/ drugs through skin have made zebrafish embryos/ larvae ideal for test protocols (Tobia et al., 2011). In this fish, formation of major blood vessels is initiated at 24 hpf extends till 72 hpf (Isogai et al., 2003; Ellertsdóttir et al., 2010). The patterning of blood vessels of the zebrafish is distinct, in addition to major blood vessels (dorsal aorta - DA, dorsal longitudinal anastomotic vessel - DLAV and posterior cardinal vein - PCV) 26 pairs of inter-segmental vessels (ISVs), originate from dorsal aorta, run vertically and parallel to each other in between adjacent segments from trunk to tail that merge into the DLAV (Childs et al., 2002; Isogai et al., 2003; Moshal et al., 2011; Tobia et al., 2011; Gore et al., 2012). The common cardinal veins (CCVs) are formed at the anterior region of the trunk, where they collect the venous blood and transport it to the heart (Helker et al., 2013). In newly hatched semitransparent larvae (72 hpf) blood vessels are detectable and blood flow in them easily observable therefore, form ideal models/ test system for assessing the pro-angiogenic/ anti-angiogenic property of a pharmaceutical (Tobia et al., 2011). The present study is an attempt to test whether 7HC exhibit anti-angiogenic activity in developing embryos of zebrafish. Live imaging, whole mount staining with

alkaline phosphatase, O-dianisidine and acridine orange were carried out to trace patterning of blood vessels, path of blood flow and cellular apoptosis respectively. Cardiac anomalies (variation in size and pericardial edema) heartbeat rate, morphological abnormalities and rate of mortality were also recorded.

Materials and Methods
Zebrafish

Zebrafish (WT) were procured from Javed fisheries, Mumbai, India and maintained in the laboratory at $26 \pm 1°C$ temperature and natural photo phase (11h dark and 13h light). Fish were fed twice a day on commercial pellets and *Artemia nauplius ad libitum* (Westerfield, 2007; Rajapurohit and Pancharatna, 2007). Dried tubifex worms were fed on alternate day. Permission to work on zebrafish was obtained from Animal Ethical Committee, CPCSEA, Government of India, under Institutional Registration # 639/GO/02/a/CPCSEA.

Breeding and raising embryos in Laboratory

Gravid females and adult males in 1 : 2 ratio were placed in the breeding tank (in which a breeding trap made out of nylon mesh with a pore size of 3 mm was immersed) one hour after the last feeding of the day at 17.00 hrs. By next daybreak, numerous (200 - 300) fertilized eggs measuring 0.7 – 1 mm were found collected at the bottom of the aquarium. Eggs were carefully siphoned off into petri-plates (20 mm diameter) using Pasteur pipettes and washed thoroughly (2 - 3 times) with distilled water to get rid of dirt/ waste particles and then rinsed with E3 embryo medium. Healthy and embryos under development were selected under a stereo zoom microscope (Leica MZ6) for experimentation.

Embryo cultures and exposure protocols

7-hydroxycoumarin (Sigma-Aldrich, St Louis, USA) was dissolved in 0.5% DMSO (Dimethyl sulfoxide) (Qualigens, India) in double distilled water to get a stock solution. Exposure media (graded concentrations of 1 mM, 2 mM, 3 mM, 4 mM and 5 mM) were prepared in 5 ml of E3 embryo medium with 0.12 ml of DMSO. A total of 60 embryos in triplicate sets (n=20/set) were used for exposure to each concentration. Corresponding controls (exposed to 5 ml of E3 medium with final concentration of 0.12 ml of DMSO) were maintained. The duration of exposure was for a total of 68 hours starting from 06 - 72 hpf. After 24 hrs, 0.003% 1-Pheny 2-thiourea (PTU) (Himedia, India) was added to the medium to minimize the development of pigmentation (Westerfield, 2007). At 72 hpf, embryos were hatched into larvae. The larvae were observed critically for patterning of

blood vessels using alkaline phophatase staining (ALP), direction of blood flow/ RBC by whole mount staining for expression of globulin (O-dianisidine) in RBC, caridiac structure and heart rates (beats per minute), cellular apoptosis (acridine orange) were recorded using Olympus bright field research microscope and images were captured (ProgRess 3 camera).

Embryo (E3) medium and PFA Fixative

E3 embryo medium was prepared for 60X stock solution (17.2 g NaCl, 0.76 g KCl, 2.9 g $CaCl_2.2H_2O$ and 4.9 g $MgSO_4.7H_2O$ were dissolved in 1 L double distilled water and stored at 5^oC). 16 ml of 60X stock solution was diluted in 984 ml of dd H_2O to get 1X stock solution to which 3 ml of 0.01% methylene blue was added as fungicide (Westerfield, 2007; Nusslein-Volhard and Dahm, 2002).

4% paraformaldehyde (PFA) was prepared by heating 4 g PFA in 50 ml dd H_2O at 60^oC, to which 2 drops of 2N NaOH (to dissolve PFA), and 10 ml 10X PBS were added to make up the final volume (100 ml) the pH of which was then adjusted 7.2 and stored at 2 - 4^oC (Ausubel et al., 2003).

1X PBS (Phosphate Buffer Saline) was prepared by dissolving 136.8 mM NaCl, 2.5 mM KCl, 0.8 mM Na_2HPO_4, 1.47 mM KH_2PO_4, 0.9 mM $CaCl_2$ and 0.5 mM $MgCl_2.6H_2O$ in 800 ml dd H_2O, the final volume was made to 1 L with dd H_2O and pH was adjusted to 7.4 and autoclaved (Ausubel et al., 2003).

Whole-mount staining for endogenous alkaline phosphatase (ALP)

At 72 hpf, the zebrafish embryos were processed for endogenous alkaline phosphatase (ALP) staining (Serbedzija et al., 1999; Moshal et al., 2011; Yeh et al., 2012). Embryos were fixed in 4% PFA for 3.00 hrs, and incubated in 3% H_2O_2 and 0.5% KOH at room temperature for 30 - 60 minutes until pigmentation disappears completely (Thisse and Thisse, 2008). Embryos were then washed 5-6 times in PBS (pH 7.4) with 0.1% Tween-20 (Sigma-Aldrich, St Louis, USA) and subjected for dehydration and rehydration process (15 mins) in methanol (Himedia, India) - PBST gradation (25%, 50%, 75%, 100%, 75%, 50%, 25%) and acetone for 30 min at -20° C and finally suspended in PBST for 10 – 15 min. These embryos were then equilibrated in alkaline phosphate (ALP) buffer with 100 mM Tris HCl (pH 9.5) (Himedia, India), 50 mM $MgCl_2$, 100 mM NaCl and 0.1% Tween-20 for 45 mins followed by incubation in staining solution, 110 µg/mL NBT (Nitro Blue Tetrazolium, Sigma-Aldrich, St Louis, USA) and 55 µg/mL BCIP (5-bromo-4-chloro-3-indolyl phosphate, Sigma-Aldrich, St Louis, USA) in alkaline phosphatase buffer at room temperature for 15 – 20 min. The staining was terminated by using stop buffer which was prepared with 0.25 mM EDTA (ethylene diamine tetra acetic acid) (Himedia, India) in

PBST, pH 5.5 for 5 min. After completion of staining, embryos were fixed in 4% PFA overnight at 4ºC, stored in 80% glycerol and were observed and photographed.

Whole-mount staining for globin expression in RBC

Whole-mount staining for globin expression in red blood cells (RBC) was peformed at 72 hpf using O-dianisidine staining method (Iuchi and Yamamoto, 1983). Larvae were stained in dark (30 mins) in o-dianisidine (0.6 mg/ml), 0.01 M sodium acetate (pH 4.5), 0.65 % H_2O_2 and 40 % ethanol. Stained larvae cleared in benzyl benzoate/ benzyl alcohol (2:1) and examined under Olympus brightfield research microscope and photographed (Detrich et al., 1995).

Acridine Orange (AO) staining

In order to check the cellular apoptosis induced if any, owing to drug exposure, larvae of both control and chemical exposed groups were stained at 72 hpf with 5 µg/mL acridine orange dissolved in E3 medium for 15 min followed by 3 washings in the medium (David and Pancharatna 2009ab). They were then observed under a fluorescent microscope, the DNA intercalated AO fluoresces green at 525 nm (David and Pancharatna 2009ab).

Heart rate

In order to assess the changes if any in the appearance of heart and heart rate of experimental embryos/larvae associated with drug exposure, heartbeats per minute were (bpm) recorded at (26 ± 1ºC) once in every 24 hour till day 7 using an inverted microscope Olympus IX71S8F. The Mean ± S.E / group were calculated. Tricaine (100 µg/ml in E3 media) (Sigma-Aldrich, St Louis, USA) was used to immobilize larvae (Schwerte et al., 2003).

Statistical Analysis

The data were analyzed using one-way analysis of variance (ANOVA) using SPSS (version 16.0), followed by Tukey's multiple comparison test. Statistical significance was defined at $P< 0.05$ level of significance. Percent inhibition of formation of blood vessels especially inter segmental blood vessels (ISVs) were determined for each drug by linear regression analysis of dose-response curve plotted for percent of inhibition of ISVs development against concentrations of drugs tested and IC50 (50 % inhibition) values were calculated.

Results
Controls

The larvae of control group, underwent normal time-line development. Patterning of all major blood vessels i.e. DA, DLAV, ISVs and PCV was complete by 72 hpf. In newly hatched larvae of this group, a total of 26 ± 0 ISVs were found distributed from trunk to the tip of tail (Figs. 1A–2). The development of heart and patterning of CCVs was normal (Fig. 3A). And RBCs were seen in all major blood vessels including ISVs (Fig. 3B). Heart rate varied from 91 ± 1.02 bpm to 160 ± 1.45 bpm from day 1 to day 7 (Fig. 4). No morphological abnormalities or cellular apoptosis were evident and all the 60 larvae survived up to 15 days (Fig. 5A, B and 6).
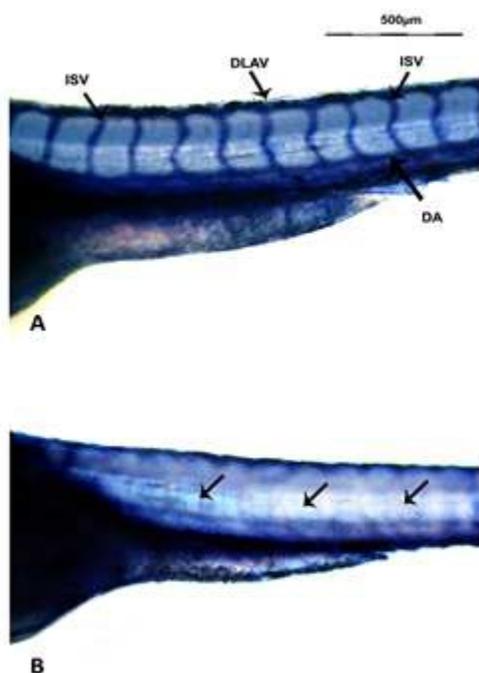

Fig. 1A-B. Trunk region of whole-mount stained *D. rerio* larvae (at 72 hpf) for endogenous alkaline phosphatase (ALP). (Scale bar = 500 µm).
Larva belonging to control group showing inter segmental vessels (ISV) and other major blood vessels (arrows) (A). Larva of embryo exposed to 5 mM 7HC (B). Showing formation of incomplete ISVs (arrows).
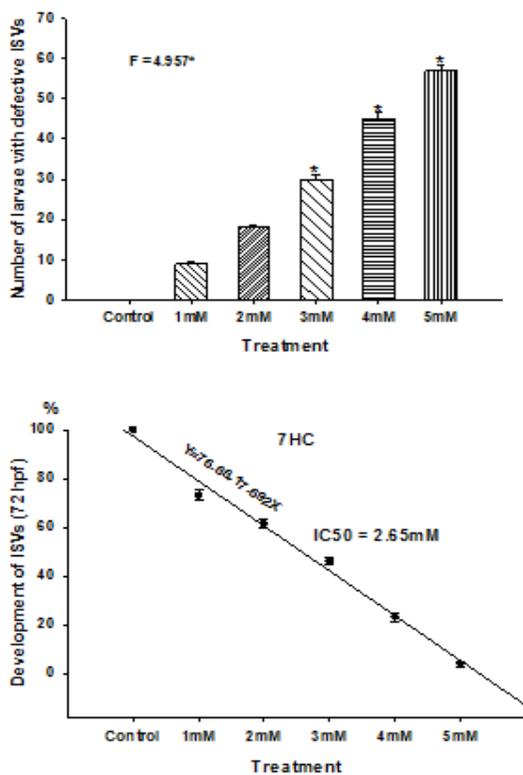(DA -Dorsal aorta, DLAV-Dorsal longitudinal anastomotic vessel, ISVs-Inter segmental vessels).

Fig. 2. Effects of 7HC on patterning of ISVs. IC50 Inhibitory concentration of chemical for 50% of ISV development. * Significant at 5% level.
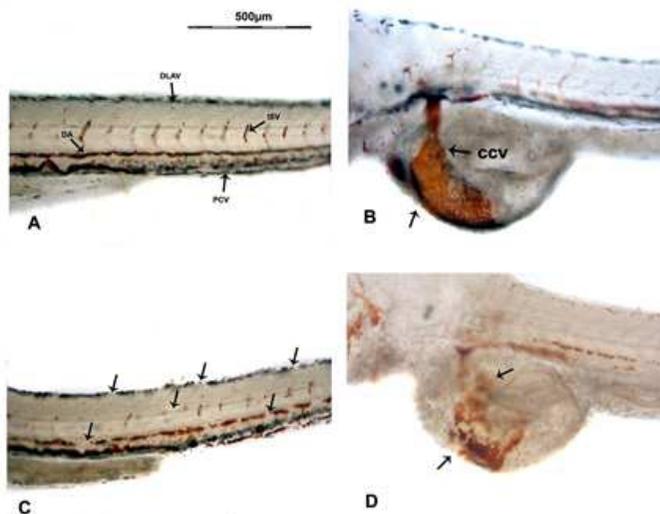

Fig. 3A-D. Trunk and heart regions of *D. rerio* larvae at 72hpf for globin expression in RBC (whole-mount staining for O-dianisidne). (Scale bar = 500 µm).

Larva belonging to control group (A-B) showing ISVs, PCV, DA and DLAV (A) and Cardiac region and CCVs (arrows) (B). Larva of embryo exposed to 7HC (C- D) showing malformed ISVs and blood flow restriction in CCVs (arrows).

DA - Dorsal Aorta, DLAV-Dorsal longitudinal anastomotic vessel, ISVs-Inter segmental vessels and PCV-Posterior cardinal vein, CCVs-Common cardinal veins.
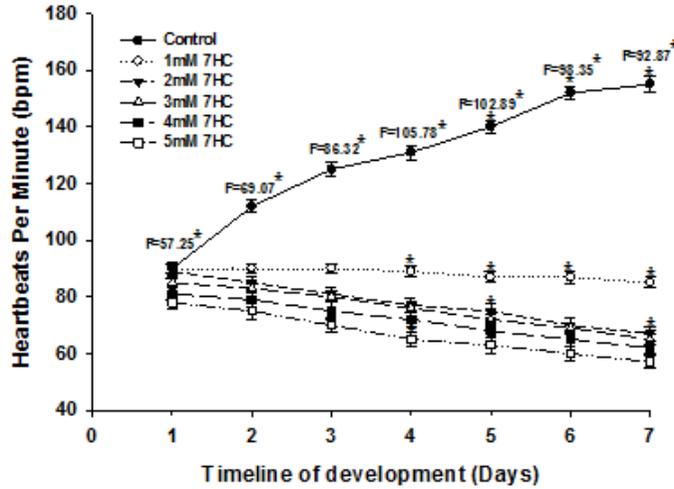

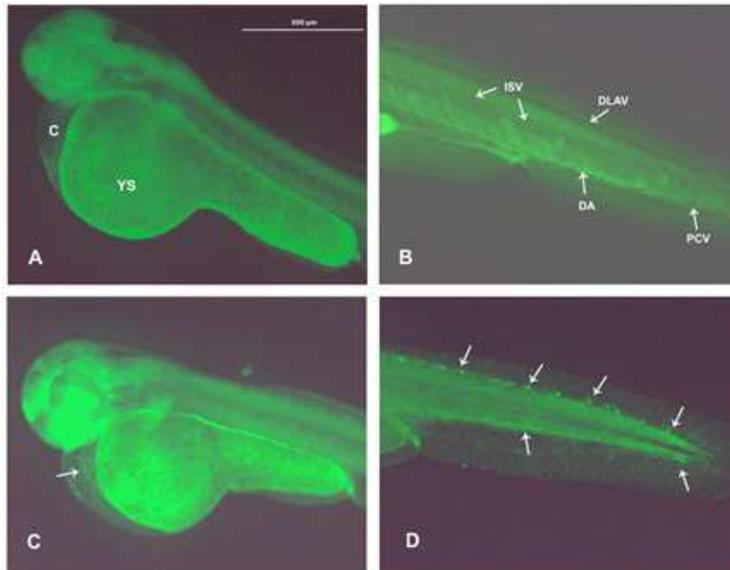**Fig. 4.** Effects of 7HC on heart rate.   * significant at 5% level.

Fig. 5. Trunk and heart regions of zebrafish larvae (72 hpf) showing accumulation of - positive cells (arrows) (Scale bar = 500 µm)

(A-B) Larva belonging to control group.
(C-D) Larva of embryo exposed to 5 mM 7HC.
Arrows show AO - positive cells.
C - Cardiac region, YS - Yolk Sac, ISV - Inter segmental vessels, DLAV - Dorsal longitudinal anastomotic vessel, DA - Dorsal Aorta
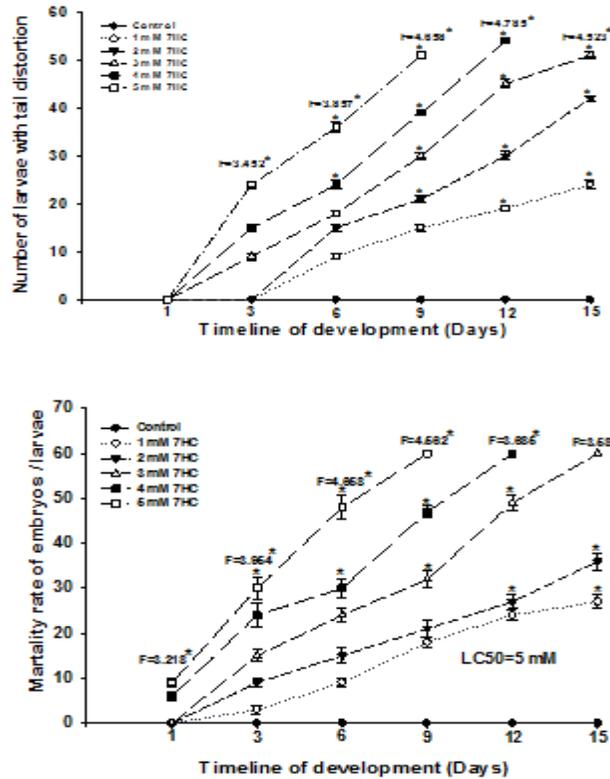
Fig. 6. Tail distortion and rate of mortality in larvae of embryo exposed to 7HC. * significant at 5% level.

Effects of exposure to graded concentrations of 7HC on the development

Figures 1 - 2 show the suppression effects of 7HC on the formation of ISVs, number of larvae showing malformed ISVs and the impairment of blood flow in the larvae of treated groups as compared to controls. Twenty one out of 26 ISVs were defective in 9/60 larvae in 1 mM 7HC exposure group, while, in 5 mM concentration, 24/26 ISVs were found defective in 57/60 as compared to all 26 well developed ISVs in all 60 control larvae (Figs. 1B-2). Overall, 7HC interfered ISVs formation with an average of 50% inhibition (IC50) value of 2.65 mM (Fig. 2). RBC were observed in DA, DLAV, CCV, PCV and ISVs of control larvae but remained absent in ISVs or CCVs of 5 mM concentration drug exposed larvae (Fig. 3C and D). Heart rates declined in all graded 7HC exposure groups in a dose dependent manner compared to those of controls (Figs. 4). Accumulation of AO positive cells was clearly visible in cardiac region and along DA, ISVs and DLAV of larvae of chemical exposed groups as compared to corresponding controls (Figs. 5C and D). The tail distortion was noticed in greater number

of larvae and survival rate decreased as concentration of 7 HC increased with LC50 value of 5 mM (Fig. 6).

Discussion

Zebrafish cell lines, embryo cultures, and larvae are widely employed in drug screening research by pharmaceutical industries (Rubinstein, 2003;Parng, 2004; Pancharatna and David, 2008). The efficacy of a newly synthesized pharmaceutical or drug can be easily tested on zebrafish embryos/ larvae owing to their ready uptake of test compounds through chorion/ skin without involving complicated procedures for mode of administration (Sussman, 2001; Spitsbergen and Kent 2003; Rubinstein, 2003; Parng et al., 2004). Experimental studies on zebrafish embryos have demonstrated that many naturally occurring plant products/ metabolites promote or interfere important biological/ developmental pathways, for instance, the anti-angiogenic effects of cinnamon (Bansode et al., 2013), effects of caffeine on angiogenesis (Yeh et al., 2012) and protective effects of silymarin on curcumin intoxication (Shiau et al., 2011) etc. Earlier studies from our laboratory have reported that over the counter (OTC) pharmaceuticals (COX inhibitors, non steroidal anti-inflammatory drugs and anti ulcer drugs) have teratological implications and induce developmental anomalies zebrafish embryos/ larvae (Pancharatna and David, 2008; David and Pancharatna 2009ab).

The findings of our present study indicate that hydroxy derivative of coumarin (7HC) tested interfered with the patterning of major blood vessels thereby interrupting the blood flow in them. Standardized whole mount staining protocols for ALP as a marker for endothelial cells of sprouting blood vessels (Serbedzija et al., 1999; Moshal et al., 2011; Yeh et al., 2012), O-dionisidine for detecting RBC thereby the detection blood flow (Detrich et al., 1995) and AO staining for cells under apoptosis (Darzynkiewicz, 1990; David and Pancharatna 2009ab) were used. The angiogenesis suppressing activity of the drug 7 HC was clearly evident by IC 50 value calculated (Fig. 2) and the mode of action was through increased cellular apoptosis was clear by the presence of AO positive cells at the regions of blood vessel sprouting suggesting explicitly that the cellular of action of drugs is via the enzymes that initiate apoptosis (Fig. 5). Earlier studies conducted in laboratory on malignant cell lines report that, coumarins especially 7HC is known to interfere cell cycle through apoptosis in human lung carcinoma cells (Lopez-Gonzaleza et al., 2004) and HeLa cells (Chuang et al., 2007; Bronikowska et al., 2012). Osthole, a natural coumarin inhibits growth of hepatocellular carcinoma by the induction of apoptosis (Zhang et al., 2012). In this investigation, 7HC induced cellular apoptosis at cardiac region also as evidenced by the accumulation of AO positive apoptotic cells accompanied

by decline in heart rates (Fig. 4-5). In addition, 7HC caused tail distortion and increased mortality at higher dose levels (with LC50 value of 5 mM).

Angiogenesis is known to play an important role in the growth and spread of tumour (Folkman and Shing, 1992; Folkman, 1995). Many naturally occurring and laboratory synthesized chemical compounds are demonstrated to interfere/ inhibit the angiogenesis and have therapeutic value in suppression of the tumour growth (Yeh et al., 2012; Bansode et al., 2013; Shiau et al., 2011). In our present study, we have demonstrated that in fish embryos 7HC manifest anti-angiogenic potential. This coumarin derivative have been already shown to exhibit properties such as, anti-tumour and anti-cancer on cell lines, and the present study is the first report to demonstrate anti-angiogenic property of this compound in embryonic angiogenic model. The results lead us to conclude that 7 hydroxy derivative of coumarin have anti-angiogenic potential and may form effective combination with other anti-neoplastic drugs in early cancer therapy.

Acknowledgements

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