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Evidence for antiangiogenic potentials of Herniarin (7-methoxycoumarin)

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ABSTRACT

Coumarins are known for their diverse biological activities. In this study, antiangiogenic potentials of Herniarin (7-methoxy derivative of coumarin-7MC) were tested using embryonic angiogenesis of zebrafish (Danio rerio) as model. Laboratory raised developing embryos (in triplicate sets of 20 embryos/set) were exposed to 0, 1, 2, 3, 4, and 5mM concentrations of 7MC from 6hpf (hours post fertilization) until completion of embryogenesis. After hatching (72hpf) development trajectory of larva was traced for a week. At 72hpf, patterning of blood vessels, detection of blood flow by the presence of red blood cells (RBC) by globin expression, cardiac anomalies/heart rates, cellular apoptosis, morphological deformities and mortality rates were recorded. IC50 (50% ISV inhibitory concentration) and LC50 (50% lethal concentration) were determined for 7MC exposed larvae. Whole mount staining for alkaline phosphatase at 72 hpf indicated that formation of major blood vessels Viz. intersegmental vessels (ISV), dorsal aorta (DA), dorsal longitudinal anastomotic vessel (DLAV), posterior cardinal vein (PCV) and common cardinal veins (CCV) was perturbed and blood flow in them was disturbed in larvae of chemical exposed embryo sin comparison with corresponding controls. Exposure of embryos to higher concentrations (> 4mM 7MC) not only interfered blood vessel patterning but resulted in a decrease in heart rates, tail distortions, increased cellular apoptosis and elevated mortality rates. These results suggest that 7MC manifests antiangiogenic effects on zebrafish embryonic angiogenesis with IC50 value of 2.64mM. This biological property of 7MC may be explored in tumour suppressor therapeutics to enhance the antiangiogenic effects drugs in synergistic combination.

Keywords: *Zebrafish, Embryo cultures, Methoxy coumarin, Angiogenesis inhibitor, Apoptosis.*

INTRODUCTION

Coumarins are phytochemicals with powerful aroma found in fruits, leafy vegetables, and herbs/ plants [1]. These compounds exhibit biological activities such as, anti-bacterial, anti-fungal, anti-inflammatory, anti-proliferative etc., therefore, derivatives of coumarins have pharmacological significance and used in the treatment of many human diseases [2-4]. Addition of a methyl group at C7 position of the aromatic nucleus of coumarin results in 7-methoxycoumarin with antibacterial (against gram-negative bacteria) and anti-microbial activity [5, 6]. 7MC extracted from plant Tagetes lucida (commonly called as Mexican marigold) exhibits cytotoxic effects on arthropods [7]. 7MC derived from a flowering plant Santolina oblongifolia (native from Spain) manifests anti-inflammatory properties [8]. One of the 7-hydroxyderivative of coumarin with a methoxy group at C6 position (Scopoletin) is used in the treatment of angiogenesis-mediated diseases, hinting that synthetic analogues of this compound may have a pro or anti angiogenic potentials [9]. Angiogenesis (a process of sprouting of blood vessels from pre-existing ones) is a

routine process in normal development of organ/organism, but, in case of tumorigenesis it has a special significance as it promotes tumor growth from benign to malignant and augments the metastasis [10].Therefore, antiangiogenic compounds have medicinal value in cancer therapeutics. Zebrafish is a rapidly upcoming vertebrate model replacing the laboratory rodents in current biological/biomedical research is also widely employed in pharmaceutical industry in drug screening/designing for human diseases. Zebrafish embryos form ideal models to study angiogenesis and test angiogenic or antiangiogenic compounds [11-14]. Optical transparency, exutero development, direct intake of chemical compounds through skin are additional attractions of this model. Further, patterning of blood vessels in zebrafish embryo is unique and distinct. Angiogenesis (sprouting of blood vessels) begins between 24 hrs and by 72 hrs, all major blood vessels are formed [15, 16]. In the trunk region, a total of 26 pairs of ISV run parallel to each other in between adjacent somites, extending from trunk to tail, in addition to paired dorsal aorta (DA), dorsal longitudinal anastomotic vessel (DLAV) and posterior cardinal vein (PCV) are formed [13,15,17-21]. 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 [22]. The present study is an attempt to test the antiangiogenic and associated teratogenic potentials of 7MC if any, on the developing embryos of zebrafish.

MATERIALS AND METHODS

Zebrafish

Zebrafish (WT) were procured from Javed fisheries, Mumbai, India and maintained in the laboratory at 26 ± 1°C temperature and natural photo phase (11 hrs dark and 13 hrs light). Fish were fed twice a day on commercial pellets and Artemia nauplius ad libitum [23, 24]. 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

Gravid females and adult males in 1 : 2 ratio were placed in the breeding tank (containing a breeding trap made out of nylon mesh with a pore size of 3 mm) one hour after the last feeding of the day at 17.00 hrs. By next daybreak, numerous (200 - 300) fertilized eggs measuring 0.7 – 1mm were found collected at the bottom of the aquarium. Eggs were carefully siphoned off intopetri-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 in E3 embryo medium. Healthy and embryos under development were selected under a stereo zoom microscope (Leica MZ6) for experimentation.

Embryo cultures and exposure protocols

7MC (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 [24]. 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 was detected and presence of RBC by whole mount staining for expression of globin (O-dianisidine).Caridiac anomalies if any and heart rates (beats per minute), cellular apoptosis (acridine orange) were recorded.

Embryo (E3) medium and PFA Fixative

E3 embryo medium was prepared for 60X stock solution (17.2 g NaCl, 0.76 g KCl, 2.9g CaCl2.2H2O and 4.9 g MgSO4.7H2O were dissolved in 1 L double distilled water and stored at 5^0C). 16 ml of 60X stock solution was diluted in 984 ml of dd H2O to get 1X stock solution to which 3 ml of 0.01% methylene blue was added as fungicide [24, 25]. 4% paraformaldehyde (PFA) was prepared by heating 4 g PFA in 50 ml dd H2O at 60oC, 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 - 4oC [26]. 1X PBS

(Phosphate Buffer Saline) was prepared by dissolving 136.8 mM NaCl, 2.5 mM KCl, 0.8 mM Na2HPO4, 1.47 mM KH2PO4, 0.9 mM CaCl2 and 0.5 mM MgC`l2.6H2O in 800 ml dd H2O, the final volume was made to 1 L with dd H2O and pH was adjusted to 7.4 and autoclaved [26].

Whole-mount staining for endogenous alkaline phosphatase (ALP)

At 72 hpf, the zebrafish embryos were processed for endogenous alkaline phosphatase (ALP) staining [17, 19, 27]. Embryos were fixed in 4% PFA for 3.00 hrs, and incubated in 3% H2O2 and 0.5% KOH at room temperature for 30 - 60 minutes until pigmentation disappears completely[28]. 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 -20o 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 MgCl2, 100 mM NaCl and 0.1% Tween-20 for 45 mins followed by incubation in staining solution, 110µg/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.

Whole-mount staining for globin expression in RBC

Whole-mount staining for globin expression of red blood cells (RBC) was peformed at 72hpf using O-dianisidine staining method [29]. Larvae were stained in dark (30 mins) in O-dianisidine (0.6 mg/ml), 0.01 M sodium acetate (pH 4.5), 0.65% H2O2 and 40% ethanol. Stained larvae cleared in benzyl benzoate/benzyl alcohol (2:1) and examined under Olympus brightfield research microscope [30].

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 [31, 32]. They were then observed under a fluorescent microscope, the DNA intercalated AO fluoresces green at 525nm [31, 32].

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 [33].

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 this group underwent normal development. The patterning of all major blood vessels viz. DA, DLAV, ISV, CCV and PCV was complete by 72 hrs and blood flow in them was clearly observed (Figure 1). A total of 26 ± 0 ISVs were found distributed from trunk to tip of tail (Figure 1-2). The development of heart and patterning of CCVs was normal (Figure 1,3). The heartbeat ranged from 91 ± 1.02 to 154 ± 1.45 beats per minute (bpm) from day 1 - day 7 (Figure 3). All the 60 larvae survived until day 7 and no morphological abnormalities or cellular apoptosis were apparent (Figure 1,4).

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

Effects of exposure to graded concentration of 7MC on blood vessel patterning, cardiac output, and morphological anomalies are shown in (Figure. 1-4). In 1 mM concentration exposed embryos, 3 out of 26 ISVs were found defective in 9 out of 60 larvae while in 5 mM drug exposed embryos, the effects were more pronounced i.e. in51 out of 60 larvae, 22/26 ISVs were found defective (Figure. 1,2). Average 50% of ISVs patterning inhibition concentration (IC50) at 72 hpf recorded a value of 2.64 mM ISVs (Figure 2). CCVs were underdeveloped and blood flow in them was blocked evidenced by the absence of RBC in CCVs (Figure 1). Heart rate elevated from day 1 today 3 followed by a sharp decline until day 7 in all concentrations (Figure 3). Rate of mortality varied from 3 ± 0.12 (day 1) to 57 ± 0.87 (day 7) in 1 mM concentration and 18 ± 0.98 to 60 ± 0 from day 1 to 4 in 5 mM concentration with a LC50 value of 3.07 at 72 hpf (Figure 4). The embryos in three lower concentrations (1 mM - 3 mM), were comparable to controls while, in 4 - 5 mM concentrations tail distortions and increased cellular apoptosis near pericardial region and along DA, ISV and DLAV were observed (Figure1).

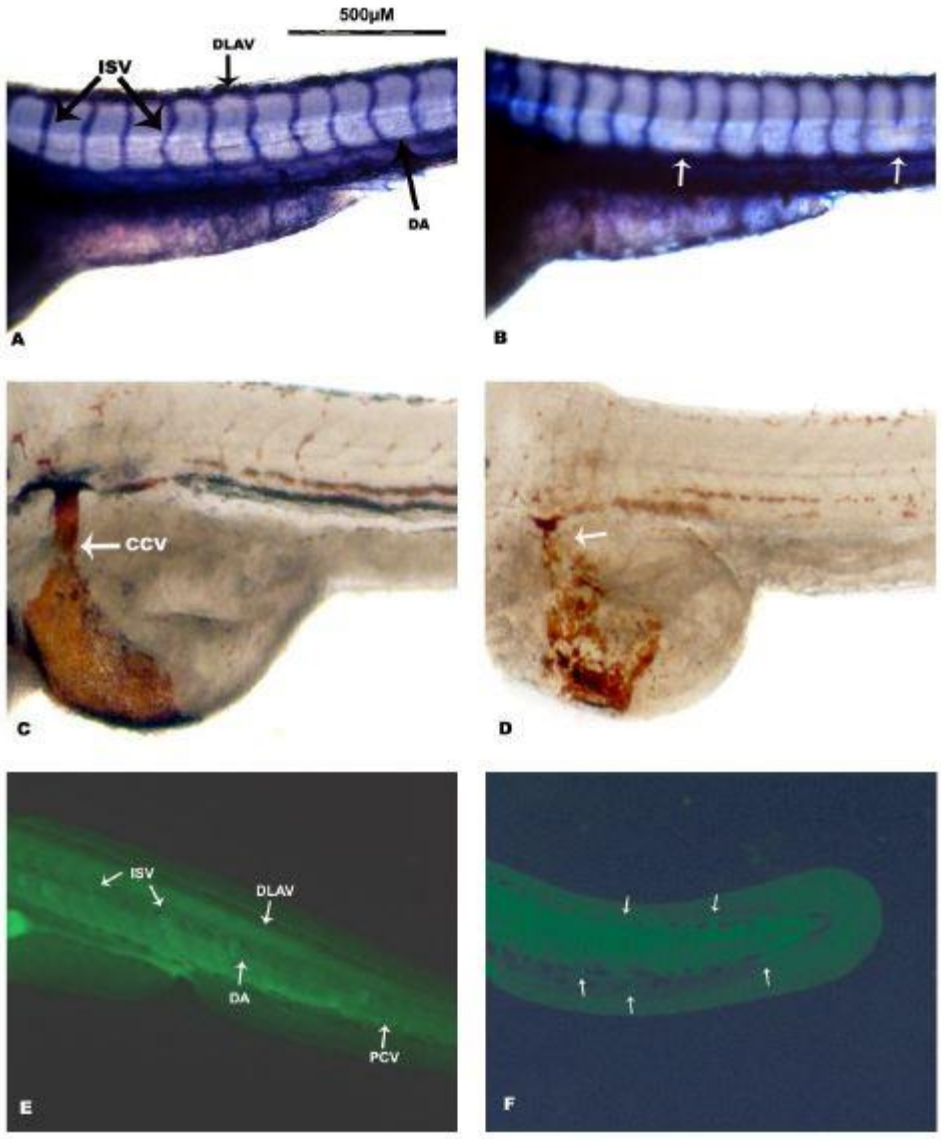

Figure 1. A-B. Trunk region of D. rerio larvae (at 72 hpf) whole-mount stained for endogenous alkaline phosphatase (ALP). (Scale bar = 500 µm). Larva belonging to control group showing inter segmental vessels (ISV) and other major blood vessels (arrows).

(A). Larva of embryo exposed to 1 mM 7MC.

(B). (DA-Dorsal aorta, DLAV-Dorsal longitudinal anastomotic vessel, ISVs-Inter segmental vessels).

(C-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 showing ISVs, DA and DLAV and Cardiac region and CCVs (arrows).

(C). Larva of embryo exposed to 7MC.

(D). Showing malformed ISVs and blood flow restriction in CCVs (arrows).

(E-F). Trunk region of zebrafish larvae (72 hpf) showing accumulation of AO-positive cells (arrows) (Scale bar = 500 μm) Larva belonging to control group.

(E). Larva of embryo exposed to 5 mM 7MC.

(F). Arrows show AO - positive cells.

(DA-Dorsal aorta, DLAV-Dorsal longitudinal anastomotic vessel, ISVs-Inter segmental vessels, PCV-Posterior cardinal vein).

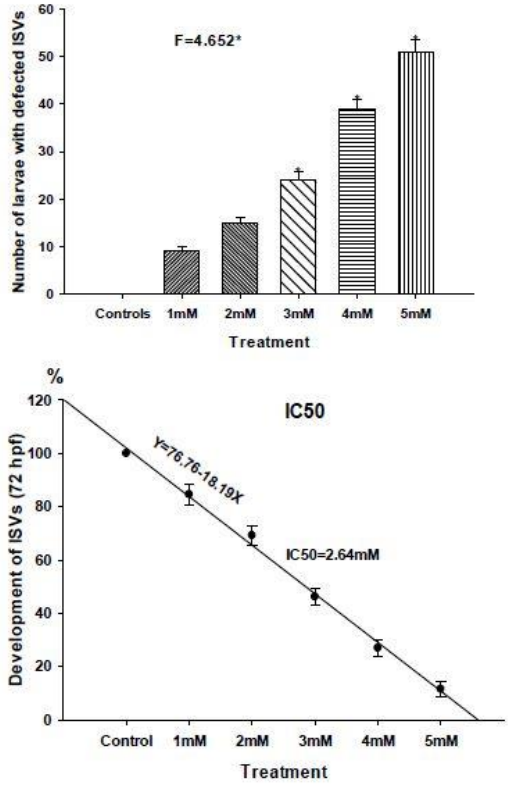

Figure 2. Effects of 7MC on patterning of ISVs. IC50 Inhibitory concentration of chemical for 50% of ISV development. * Significant at 5% level.

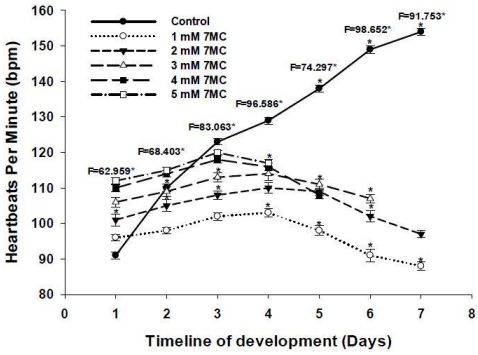

Figure 3. Effects of 7MC on heart rate. * significant at 5% level.

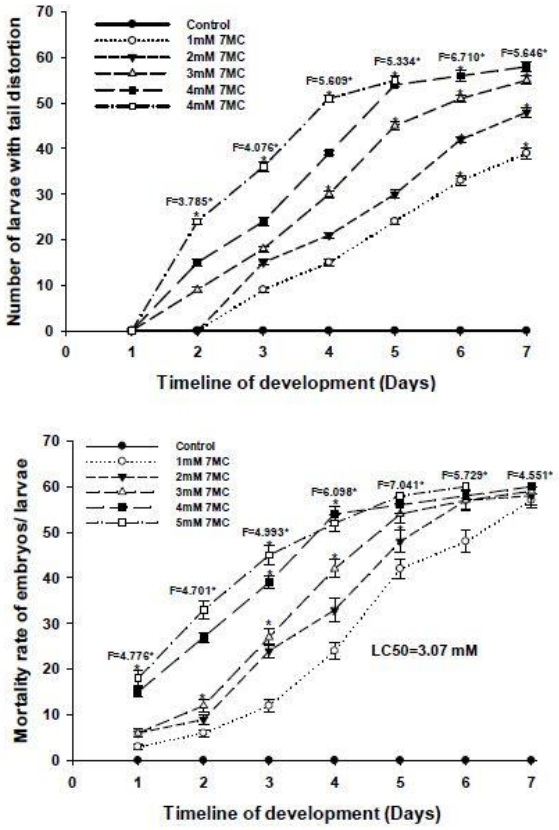

Figure 4. Tail distortion and rate of mortality in larvae of embryo exposed to 7MC. * significant at 5% level.

DISCUSSION AND CONCLUSION

Angiogenesis is known to play an important role in the growth and spread of tumour [34,35]. Presently, several strategies are in practice to achieve tumour suppression by regulating angiogenic signaling pathways [10,36,37]. To date, a number of antiangiogenic agents, single or in combination, are being tried [38]. 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 [27,39,40]. The present investigation is the first of its kind to report the antiangiogenic potentials of coumarin derivative, 7MC, and using zebrafish embryonic angiogenesis as biological model. Derivatives of coumarin are documented as antiproliferative, and antiangiogenic on cancer cell lines [9,41-43]. We made an attempt to elucidate the antiangiogenic nature of 7MC using embryonic angiogenesis as model and found that at an effective dose of > 2.5 mM, 7MC is able to perturb angiogenesis in zebrafish embryos, especially the patterning of ISV, DA, DLAV, PCV, CCV using whole mount staining methods. For example, ALP staining is one of the most proven marker for endogenous alkaline phosphatase activity is encountered in lymphoid tissues and endothelial cell surfaces [17, 19]. As endothelial cells are the source for sprouting of new blood vessels the staining serves as a sensitive marker for angiogenesis [37]. Likewise, the standard protocols for O-dianisidne stain for the expression of globin in RBC and acridine orange a vital marker that intercalates between DNA of apoptotic cells were used as markers to detect RBC/blood flow and cellular apoptosis [30-32, 44]. 7MC manifested antiangiogenic effects in a dose dependent manner, inhibited the growth of ISVs with a 50% ISV inhibitory concentration (IC50) of 2.64mM and in higher concentrations, growth of DA, DLAV, PCV and CCV was also disturbed along with teratogenic effects of the compound. Further, the antiangiogenic effects of 7MC seems to be mediated through increased cellular apoptosis as evident by the accumulation of cluster of AO positive cells at the point of origin of blood plexus such as ISVs. Thus the study emphasizes on the development/ patterning of the ISVs as the most sensitive and consistent parameter for quantification of angiogenic activity.

Importantly, the observed effects were specific and dose related indicating zebrafish embryos/larvae are reliable and sensitive tool to define and quantitate the angiogenic activity. We conclude that this biological property of 7MC may be explored to enhance the antiangiogenic effects of other drugs already in use in tumour suppressor therapeutics.

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