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Determination of Lethal Concentrations and Anti-Angiogenic Potential of Some Herbs in Zebrafish Model

K. B. Tambe, V. V. Karande* and M. M. Gatne

Department of Veterinary Pharmacology and Toxicology, Bombay Veterinary College, Parel, Mumbai – 400012, Maharashtra, India: drvikas111081@gmail.com

Abstract

Plants have always been used in various traditional medicinal systems, VEGF inhibitors from plant sources are matter of great interest in recent times. The present study aimed to validate the model for anti-angiogenesis in zebrafish using some herbal compounds. Zebrafish larvae, 24 hpf were exposed to different concentrations of test compound, positive and negative controls were selected on basis of data from laboratory experiments. The positive controls included SU5416 and 0.1%DMSO taken as negative control. The test compounds were *Withania somnifera, Ocimum sanctum* and *Ananas sativus.* DMSO was used as a vehicle control (except in *Ananas sativus* it was water). The concentrations to be tested were determined on the basis of a prior assay carried out to determine the median lethal concentration (LC_{50}) of the drug by exposing 6 hpf embryos to different concentrations of the drugs over a significant range. The anti-angiogenic assay was run on zebrafish larvae, 72 hpf, after fixation with 4% paraformaldehyde and staining with o-Dianisidine. Both vessel inhibition and morphological structure were observed under 10X power of inverted microscope. During the LC_{50} and anti-angiogenic assays, gross morphological abnormalities, if any, were observed for and randomly selected larvae were processed for staining. *Withania somnifera*, *Ocimum sanctum* and *Ananas sativus* showed inhibition of ISV, DA and DLAV when compared with positive and negative control. The LC_{50} for *W. somnifera*, *O. sanctum* and *A. sativus* was 300 µg/ml, 408.06 µg/ml and 500 µg/ml respectively. *W. somnifera* extract showed reduced RBCs in DA, but not in the ISV and DLAV region at a concentration of 100 µg/ml, *whereas* embryos revealed slight inhibition of ISV at concentration 200 µg/ml. At concentration of 250 µg/ml there was yolk sac oedema. For O. sanctum there was inhibition of ISV, DA and DLAV at the dose rate of 300 µg/ml and at dose 200 µg/ml there was slight inhibition of ISVs showing anti-angiogenic effect. *A. sativus* showed vessel inhibition particularly in ISV region at dose 400 µg/ml. At concentration 500 µg/ml showed developmental disorder was evident. Zebrafish model was judged to be practical in a preclinical set up. However further studies are required to establish correlation between zebrafish and mammalian models of angiogenesis.

Keywords: Antiangiogenesis, Zebrafish, VEGF

1. Introduction

Angiogenesis is a process of neovascularization, a complex, regulated, and multi-step physiological process which initiates a protease-mediated degradation of the basement membrane, loss of endothelial cell adhesion, proliferation and migration of endothelial cells into the surrounding stroma and finally reassembly of endothelial cells to form the lumen of the new blood vessels[1]. Excessive angiogenesis is closely related to many human diseases, such as tumor growth and metastasis, retinopathy and inflammation[2,3]. One of the major molecules involved in the angiogenic process is the Vascular Endothelial Growth Factor (VEGF) family of proteins and their receptors. High expression of VEGF has been observed in many cancers and is associated with worse survival. VEGF and VEGF receptors have been implicated in the angiogenesis that occurs in many solid

**Author for correspondence*

tumours like breast cancer[4], colon cancer[5], hepatom[6], bladder cancer[7], gastric cancer[8] and prostate cancer[9]. Over expression of angiogenic factors in particular VEGF and bFGF in most hematologic malignancies result in increased angiogenesis and show poor prognosis as well as decreased overall survival[10]. Anti-angiogenic therapeutic approaches have recently been shown to be effective for the treatment of certain cancers.

Anti-angiogenesis agents are reported to cause reduction in the blood flow or vascular permeability, in many types of cancer. Anti-angiogenic drugs exert therapeutic effects by blocking certain specific receptors. The application of anti-angiogenic compounds in cancer treatment is relatively new.

SU5416, a selective inhibitor of the tyrosine kinase activity of the Vascular Endothelial Growth Factor (VEGF) receptor Flk-1/KDR, is currently in Phase III clinical trials for the treatment of advanced malignancies, used as a positive control. SU5416 is a small molecule drug prevents formation of new blood vessels required for tumor growth by blocking the Flk1/KDR[11].

Plants have always been used in various traditional medicinal systems like Ayurveda, Unani, Chinese, Egyptian and Greek to treat or prevent various diseases. An alternative to chemotherapy, which is the most common means by which doctors and specialists treat cancer, organically based treatments may not have severe side effects that radial treatments and chemotherapy has. The harsh side effects of cancer treatments are one motivating factor to finding alternative methods. The use of botanical when treating cancer patients is considered a natural alternative, because some plants may contain properties that naturally have the ability to prevent the spread or risk of developing various forms of cancer.

VEGF inhibitors from plant sources are matter of great interest in recent times. Studies have shown that treatment of zebrafish embryos with clinical stage anti-angiogenic compounds inhibits growth of angiogenic blood vessels, suggesting that larger-scale anti-angiogenic compound screening in zebrafish is possible. There are many drugs which affect the angiogenesis in cancer therapy, and so many *in vivo* and *in vitro* models used for the anti-angiogenesis. It was therefore decided to validate the model for anti-angiogenesis in zebrafish using some herbal drugs. Both positive and negative controls were selected on basis of data from laboratory experiments. The positive controls included SU5416 and 0.1% DMSO taken as negative control. Literature showed that few researches were carried out for anti-angiogenicity in zebrafish. Therefore test compound selected were *Withania somnifera*, *Ocimum sanctum*, and *Ananas sativus.*

Zebrafish (Danio rerio) has emerged as a valuable model organism in drug discovery processes, including target identification, disease modelling, lead discovery and compound toxicology. The major advantages of a zebrafish based assay are obvious:

- Hundreds of compounds can be tested simultaneously using a microplate format.
- The assay is relatively cost effective, fast, truly quantitative and suitable for large- scale screening and Embryo maintenance, compound addition and embryo assessment are technically simple[12–14].

It has a high genetic homology with humans over 85% as well as important parallels in organogenesis and functional mechanisms[15].

During the zebrafish embryonic development, the Intersegmental Vessels (ISVs) are formed from the main artery, the Dorsal Aorta (DA), through an angiogenesis mechanism between 16–19 h postfertilization (hpf) [12,16]. Vasculogenesis in zebrafish, as in other vertebrates, involves the differentiation of hemangioblasts from mesoderm, with subsequent differentiation of angioblasts and endothelial cells[16].

In the present study employs developing zebrafish (Danio rerio) embryos as an in-vivo vertebrate model for studying inhibitors of angiogenesis.

2. Materials and Methods

The anti-angiogenic potential of different herbal compounds was screened by using Zebrafish model in 96 well plates as per method described by Westerfield[17]. The embryos were produced by natural pair wise mating and maintained in embryo medium at 28°C until the somite stage. Different concentrations of test compounds, positive control and vehicle control were prepared after testing solubility in embryo medium. LC_{50} of the test compounds was determined to predetermine the drug concentrations for the anti-angiogenic assay. The assay was performed under controlled temperature in zebrafish laboratory. The changes in vessel development were captured under 10X objective of inverted microscope at different concentrations of test substances. LC_{50} were determined by Reed & Muench[18] method.

3. Test Material

Herbal compounds to be tested were prepared by extracting with either methanol or water:chloroform. The detailed information of these compounds are as given in Table 1.

Table 1. Details of herbal compounds tested

Sr. No.	Scientific name of herbs	Local name	Plant parts used	Extract used
1	*Ocimum sanctum*	Tulsi	Fresh and dried leaves	Water: Chloroform extract
2	*Withania somnifera*	Ashwagandha	Roots	Methanolic extract
3	*Ananas sativus*	Pineapple	Stem	Water extract

4. Husbandry Practices of Zebrafish:

4.1 Housing

Adult fish were maintained in regular fish tanks or beakers of 1-2 litre capacity, depending on the number of fish to be maintained in each group. Fish of the same age were grouped together. The temperature was maintained between 25^0C-31^0C. The water was oxygenated with commercially available aerators. The circadian cycle of 14 hours of light and 10 hours of darkness was strictly maintained.

4.2 Feeding

Adult fish (fifteen days or older) were fed twice a day on larvae of Artemia nauplii (brine shrimp) which is salt water zooplankton and ground dry trout pellet to maintain good breeding condition. Fish were fed with enough food so that all food was consumed within five minutes.

4.3 Breeding

Separate tanks were maintained for spawning of fish. Breeding tanks were procured from the local market. Males and females (aged 4-12 months) were introduced into these tanks in the ratio of 1:2. Eggs that were deposited passed through the net and settled at the bottom of the tank. This system prevented the predation of eggs by adults and eggs collected at the bottom of the tank were siphoned out with a glass pipette.

4.4 Care and Maintenance of Eggs

Eggs collected from the tank were cleaned of debris manually. If not used directly, the eggs were counted and put in the embryo medium. Maintaining the developing embryos in the embryo medium ensured that all the necessary minerals were supplemented. Quality of each batch of eggs was verified by directly observing under the inverted microscope for the stage of development. The normal embryo may be either single celled or a multicellular structure (blastula) depending on the time after fertilization. On gross and microscopic examination the live and dead embryos were distinguished. Live embryos appeared transparent while dead were milky crescent shaped bodies within the egg. The dead embryos were separated out from the viable ones and the batches of viable embryos were transferred to the BOD incubator. The temperature of the incubator was maintained at 28^0 C. Embryos/larvae were maintained in the incubator for not beyond 7 days.

4.5 Embryo Handling

The embryos were maintained in embryo medium at 28.5°C until the 21 somite stage (24 hrs). Healthy embryos were dechorionated at 24 hpf just prior to drug treatment by incubating with 1 mg/ml Proteinase K at room temperature for three minutes. Dechorionated embryos were used for further analysis.

4.6 Embryo Medium and Controls

Embryo medium was prepared as per Westerfield, the detailed procedure is as in Table 2. DMSO (0.1%) was used as vehicle control and the same was used during dilution of test compounds. The final solution of 0.1% DMSO was prepared by diluting 1 µl DMSO to 999 µl embryo medium. SU5416 (1, 3-Dihydro-3-[(3, 5-dimethyl-1 H-pyrrol-2-yl) methylene]-2 H-indol-2-one, Sigma-Aldrich) was used as positive control (Quinn et al., 1993). It is a selective Vascular Endothelial Growth Factor (VEGF) receptor-2 inhibitor. It was weighed and dissolved in DMSO for getting final stock solution of 10 mM (Appendix 1). From this stock solution, concentrations of 0.1 µM, 0.3 µM, 0.6 µM, 1 µM and 2 µM were prepared to which zebrafish embryos were exposed during anti-angiogenic assay.

Table 2. Composition of Hank's stock solutions

Hank's Stock solutions	Composition
Hank's Stock solution#1	NaCl 8 g KCl 0.4 g Distilled water to make volume 100 ml
Hank's Stock solution#2	Na_2HPO_4 $7H_2O$ 0.67 g KH_2PO_4 0.6 g Distilled water to make volume 100 ml.
Hank's Stock solution#4	$CaCl_2$ $2H_2O$ 1.92 g Distilled water to make volume 100 ml.
Hank's Stock solution#5	$MgSO_4$ $7H_2O$ 2.46 g Distilled water to make volume 100 ml.
Hank's Stock solution#6	$NaHCO_3$ 0.35 g Distilled water to make volume 10 ml

4.7 Design of Experiment

The experiment was carried out in two stages as determination of median LC_{50} (Lethal concentration 50) and anti-angiogenic assay through 1. Microscopic imaging and 2. RBC staining for visual inspection determination of median lethal concentration (LC_{50}).

LC_{50} of the test drugs was determined in order to predetermine the drug concentrations at which anti-angiogenic assay could be carried out. Stock solutions of each test drug with different concentrations were prepared in DMSO (dimethylsulphoxide). Concentrations chosen for LC_{50} determinations were expressed in micrograms per ml of solvent. Zebrafish embryos at 6 hpf were taken for the LC_{50} study. At least 12 embryos were subjected to treatment with each concentration of each of test compounds. Using a micropipette, zebrafish embryos were laid at the bottom of the wells of a 96 well plate, such that each well contained 180 microlitres of distilled water and one zebrafish egg. Since 20 microlitres of each dilution had to be added per well, serial solutions that were prepared from the stock solution were 10 times more concentrated than the final desired concentration. The test concentrations that were used for the LC_{50} assay were 50,100, 200, 250, 500, 750 and 1000 µg/ml. A control group containing a concentration of DMSO equal to its concentration in the highest concentration group was also maintained during each assay. The plate was incubated in the BOD incubator at 28^{0}C for 48 hours and readings were taken at 24 hpf and 48 hpf of the pharyngula stage in the development of the embryo, under the 4x objectives of the inverted microscope. While assessing mortality, the following parameters were considered as a. post 24 hours – coagulation of eggs b. post 48 hours – failure of tail detachment, absence of heart beats or circulation and derangement of somites.

Other abnormalities such as oedema of the yolk sac, failure of organ development and necrosis were also noted, in order to observe the Lowest Observable Effect Concentration (LOEC). If significant mortality (greater than 50%) was observed at the highest dilution, the test concentrations were further reduced. If no significant mortality (that is greater than 50%) was observed the test concentrations were further increased. However, in no case did the concentrations exceed 1000 micrograms per ml of the compound as greater than 1% solutions of DMSO adversely affect growth and development of the larvae. Thus the following end points have been determined for the assay.

- Significant (greater than 50%) mortality at 24 or 48 hours.
- Precipitation of the drug in which case increasing or decreasing the test concentrations would not alter the readings.
- No mortality at a concentration of 1000 micrograms per ml of the drugs.

Based on these readings LC_{50} for the test compounds were determined by plotting a graph of concentration versus mortality and determining the concentration at which median mortality was observed on the graph.

4.8 Microscopic Imaging

4.8.1 Exposure of Embryos to Test Substances

For anti-angiogenesis assay, embryos at 24 hpf (day1) were arrayed in 96-well plate, one embryo per well and incubated with 100 µl of embryo water containing various concentrations of drugs continuously for 48 h. In all experiments, 0.1% dimethylsulfoxide (DMSO) was added as a carrier and vehicle control with 0.1% DMSO was performed. Each sample group contained 10 embryos.

The embryos at 24 hpf were incubated with embryo water containing 0.003% PTU (1-phenyl-2-thiourea, Sigma-Aldrich) before exposing larvae to test drugs, to inhibit pigment formation. The enhanced green fluorescent proteins -expressing endothelial cells of vasculature in Intersegmental Blood Vessels (ISVs),

Dorsal Aorta (DA) and Dorsal Longitudinal Anastomotic Vessel (DLAV) were observed and recorded at 72 hpf, using 10X objective of an Olympus inverted microscope.

4.8.2 RBC Staining

RBC staining was performed to test the ISV formation which could form by angiogenic process and sprouting from dorsal longitudinal anastomotic vessel. In the present study RBCs were stained as described by Thamilarashi[19]. At 72 hpf (on 3rd day) during the development, embryos were fixed with 4% paraformaldehyde for half an hour and washed with PBS (Phosphate Buffer Saline) 3-4 times. Dechorionated embryos were stained for 30 minutes in the dark with o-Dianisidine 0.6 mg/ml, 0.01 M sodium acetate (pH 4.5), 0.65% Hydrogen peroxide and 40% (v/v) ethanol. After 30 min it was washed with PBS for 3-4 times and examined under the 10X objective of inverted microscope.

5. Results and Discussion

The results for LC_{50} assay as well as anti-angiogenesis assay for each of the test compound under study are described below

5.1 *Ocimum Sanctum*

Hundred percent mortality was evident at concentration of 700 µg/ml whereas, highest concentration that produced zero percent mortality was 100 µg/ml. The LC_{50} of the compound calculated by Reed and Muench (1938) method was 408.06 µg/ml. Figure 1 and Table 3 depicts the graph of concentration of Ocimum sanctum versus percent mortality in zebrafish embryos. Anti-angiogenic assay of O sanctum (300 µg/ml) revealed that there were very few RBCs observed in DA but not in SIV and DLAV region when compared with negative and positive control (Plate 1a and 1b respectively). Depending on LC_{50} value, the concentrations chosen for anti-angiogenic assay were 25, 50, 100, 200, 300 µg/ml. For each concentration ten embryos were taken, out of that nine embryo showed inhibition of ISV at a concentration of 300 µg/ml (Plate 2).

Arun kumar informed that administration of O. sanctum leaf extract in a dose of 300 mg/kg body weight three times per week resulted in decreased expression of VEGF and over expression of Bax, cytochrome C and caspase 3 in N-methyl-N'-nitro-N-nitrosoguanidine (MNNG)-induced gastric carcinogenesis in mice20. The anti-angiogenic action of water extract of O. sanctum in mice has been reported by21.

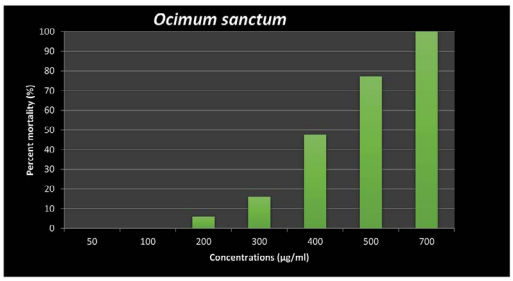


Figure 1. Percent mortality in zebrafish embryos (6 hpf) exposed to different concentrations of *Ocimum sanctum* extract.



Plate 1a. Lateral view of RBC stained embryos at 72 hpf with 0.1% DMSO had no effect on vessels formation as indicated by arrows.



Plate 1b. SU5416 blocks angiogenic vessel formation in zebrafish embryos. Lateral view of RBC stained embryos at 72 hpf, Treatment with 2 µM concentration of SU5416 caused a reduction of the ISV formation.

Table 3. Dose dependent mortality in LC_{50} assays for *Ocimum sanctum*

Concentration of the drug (µg/ml)	Number of eggs utilized	Number of live embryos	Cumulative survival	Number of dead embryos	Cumulative death	Total	Percent mortality (%)
50	12	12	-	0		-	0
100	12	12	43	0	0	43	0
200	12	10	31	2	2	33	6.06
300	12	10	21	2	4	25	16
400	12	6	11	6	10	21	47.61
500	12	5	5	7	17	22	77.27
700	12	0	0	12	29	29	100



Plate 2. Anti-angiogenic effect of *O. sanctum* in zebrafish embryo.

5.2 *Withania somnifera*

Hundred percent mortality was evident at concentration of 500 µg/ml whereas, highest concentration that produced zero percent mortality was 100 µg/ml. The LC_{50} of the compound calculated by Reed and Muench (1938) method was 300 µg/ml. Figure 2 and Table 4 depicts the graph of concentration of compound versus percent mortality in zebrafish embryos. Anti-angiogenic assay of *W. somnifera* revealed that there was inhibition of vessels formation in ISV, DA and DLAV at concentration of 200 µg/ml (Plate 3). Ten embryos were subjected to each concentration, all embryos showed significant inhibition at concentration 200 µg/ml. The concentrations (10 per group) from 50, 100, 200, 250 µg/ml were selected after calculating $LC_{50.}$ At a concentration of 250 µg/ml there was yolk sac oedema and absence of eye (Plate 4).

In[23] reported that roots of W. somnifera possess anti-angiogenic activity as evident by in vitro and in vivo assays.

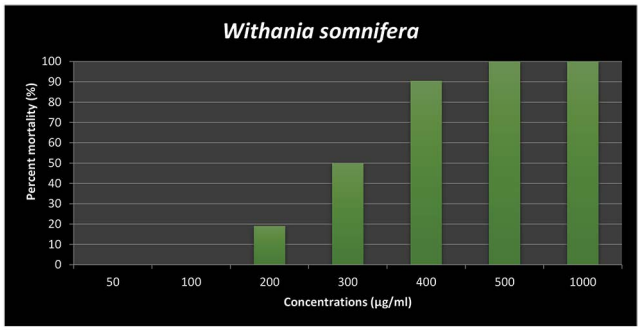


Figure 2. Percent mortality in zebrafish embryos (6hpf) exposed to different concentrations of *Withania somnifera* extract

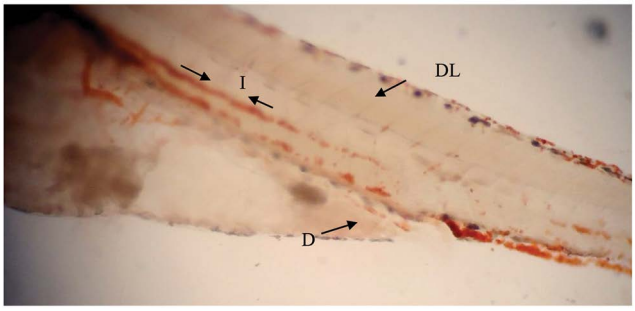


Plate 3. Anti-angiogenic effect of *W. somnifera* in zebrafish embryo.

VEGF mediated neovascularization was significantly inhibited in chickchorio-allantoic membrane assay at 10ng as well as in mouse sponge implantation at 100 ng dose.

5.3 *Ananas sativus*

Hundred percent mortality was evident at concentration of 1000 µg/ml whereas, highest concentration that produced zero percent mortality was 100 µg/ml. The LC_{50} of the compound calculated by Reed and Muench (1938) method

Table 4. Dose dependent mortality in LC_{50} assays for *Withania somnifera*

Concentration of the drug (µg/ml)	Number of eggs utilised	Number of live embryo	Cumulative survival	Number of dead embryos	Cumulative death	Total	Percent mortality(%)
50	12	12	-	0	-	-	0
100	12	12	29	0	0	29	0
200	12	8	17	4	4	21	19.04
300	12	7	9	5	9	18	50
400	12	2	2	10	19	21	90.47
500	12	0	0	12	31	31	100
100	12	0	0	12	31	31	100

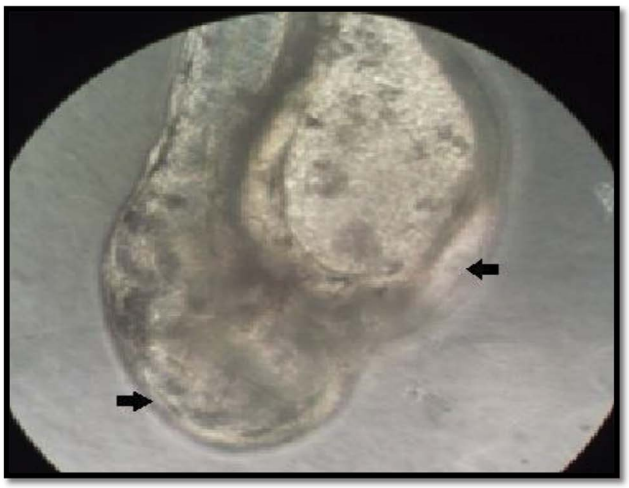


Plate 4. Yolk sac oedema and absence of eye. At concentration 250 µg/ml of *W. somnifera* (at 72 hpf, indicated by arrow).

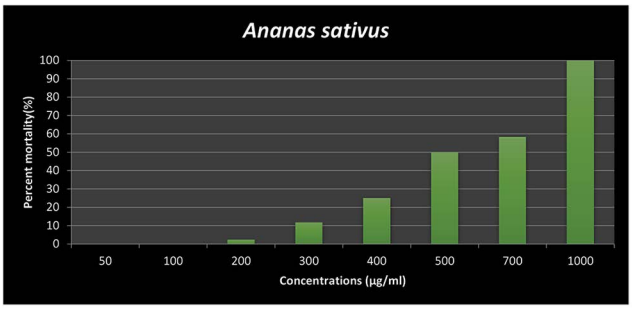


Figure 3. Percent mortality in zebrafish embryos (6 hpf) exposed to different concentrations of *Ananas sativus* extract.

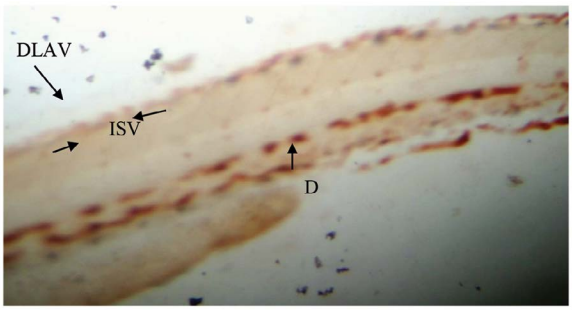


Plate 5. At 500 µg/ml *A. sativus* treated zebrafish embryos showing developmental disorders - a) curved body and b) oedema of yolk sac.

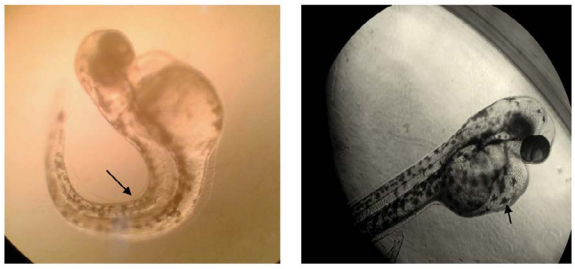


Plate 5a. Anti-angiogenic effect of *A. sativus* in zebrafish embryo. Zebrafish embryos were treated with A. sativus at concentrations of 400 µg/ml showing inhibition of blood vessels formation. (indicated by arrow).

was 500 µg/ml. Figure 3 and Table 5 depicts the graph of concentration of compound versus percent mortality in zebrafish embryos. Anti-angiogenic assay of A. sativus revealed that there were very few RBCs observed in DA,

Table 5. Dose dependent mortality in LC_{50} assays for *Ananas sativus*

Concentration of the drug (µg/ml)	Number of eggs utilised	Number of live embryos	Cumulative survival	Number of dead embryos	Cumulative death	Total	Percent mortality (%)
50	12	12	-	0	-	-	0
100	12	12	53	0	0	53	0
200	12	11	41	1	1	42	2.38
300	12	9	30	3	4	34	11.76
400	12	9	21	3	7	28	25
500	12	7	12	5	12	24	50
700	12	5	5	7	19	24	79.16
1000	12	0	0	12	31	31	100

not in ISV and DLAV. The concentrations used for anti-angiogenesis assay were 50, 100, 200, 300, 400, 500 µg/ml. Ten embryos were subjected for each concentration. All larvae from concentration of 400 µg/ml showed inhibition of vessels in ISV and DLAV. At concentration of 500 µg/ml it showed morphological abnormalities (Plate 5a and 5b).

Chobotova[26] opined that aqueous extract of pineapple stem has the capacity to modulate key pathways that support malignancy. Anti-angiogenesis might be the mechanism involved behind it.

6. Conclusion

From the present assay it can be concluded that the *O. sanctum*, *W. somnifera* and *A. sativus* have anti-angiogenic effect on zebrafish larvae. Given the characteristics of the zebrafish model, its role in pharmacological studies and research institute would be to shortlist compounds that shall reach the subsequent drug development stages, in the initial phases itself.

Further studies would be required to establish correlation between outcome in zebrafish model and mammalian models as regards anti-angiogenic activity.

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