International Journal of Science, Environment and Technology, Vol. 7, No 6, 2018, 1914 – 1924

ISSN 2278-3687 (O)
2277-663X (P)

ANTI - ANGIOGENIC POTENTIAL OF *TERMINALIA ARJUNA* ON ZEBRA FISH 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
E-mail: 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 *Terminalia arjuna*. Zebrafish larvae, 24hpf 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. DMSO was used as a vehicle control. 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 6hpf embryos to different concentrations of the drugs over a significant range. The anti-angiogenic assay was run on zebrafish larvae, 72hpf, 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. *Terminalia arjuna* did not show vessel inhibition. The LC_{50} for *Terminalia arjuna*, was 169.47 µg/ml. However further studies are required to establish correlation between zebrafish and mammalian models of angiogenesis.
Keywords: Zebra fish, Antiangiogenesis, VEGF.

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 tumours like breast cancer[4], colon cancer [5], hepatoma [6], bladder cancer[7], gastric cancer [8] and prostate cancer [9]. Over expression of angiogenic factors in particular VEGF and bFGF in most hematologic

*Received Oct 13, 2018 * Published Dec 2, 2018 * www.ijset.net*

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.

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 botanicals 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. Few authors had reported antiangiogenic potential of T. arjuna in different models such as chick embryo and mouse model[12,13] , however the reports on antiangiogenic activity of this plant in Zebra fish model seems scanty.

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 *Terminalia arjuna*. Both positive and negative controls were selected on basis of data from laboratory experiments.

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 zebra fish based assay are obvious: (i) hundreds of compounds can be tested simultaneously using a microplate format, (ii) the assay is relatively cost effective, fast, truly quantitative and suitable for large- scale screening, and (iii) embryo maintenance, compound addition and embryo assessment are technically simple[14,15,16]. It has a high genetic homology with humans over 85% as well as important parallels inorganogenesis and functional mechanisms[17]. 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 andendothelial cells [18].

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

MATERIALS AND METHODS

The anti-angiogenic potential of *Terminalia arjuna* 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^{o}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 [20] method.

Husbandry practices of Zebrafish

Housing and Feeding: 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^{0}C-31^{0}C. The water was oxygenated with commercially available aerators. The circadian cycle of 14 hours of light and 10 hours of darkness was strictly maintained. 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.

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.

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.

Embryo handling-

The embryos were maintained in embryo medium at 28.5^0C until the 21 somite stage(24hrs).Healthy embryos were dechorionated at 24hpf just prior to drug treatment by incubating with 1mg/ml Proteinase K at room temperature for three minutes. Dechorionated embryos were used for further analysis.

Embryo medium and controls

Embryo medium was prepared as per method suggested by Westerfield. DMSO (0.1%) was used as vehicle control and the same was used during dilution of test compound. 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. 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.

Design of experiment:

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

Determination of median lethal concentration (LC_{50})

LC_{50} of the test drug 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 6hpf were taken for the LC_{50} study. At least 12 embryos were subjected to treatment with each concentration 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 28^0C for 48 hours and readings were taken at 24hpf and 48hpf of the pharyngula stage in the development of the embryo, under the 4x objectives of the inverted microscope. While assessing mortality, the parameters were considered as post 24 hours – coagulation of eggs and 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.

1. Significant (greater than 50 %) mortality at 24 or 48 hours.
2. Precipitation of the drug in which case increasing or decreasing the test concentrations would not alter the readings.
3. 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.

Microscopic imaging-

Exposure of embryos to test substances -

For anti-angiogenesis assay, embryos at 24hpf (day1) were arrayed in 96-well plate, one embryo per well, and incubated with 100µl of embryo water containing various concentrations of drugs continuously for 48h. In all experiments, 0.1% dimethyl-sulfoxide (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 72hpf, using 10X objective of an Olympus inverted microscope.

b) 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 72hpf (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 (Appendix 1). Dechorionated embryos were stained for 30 minutes in the dark with o-Dianisidine 0.6mg/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.

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:

***Terminalia arjuna*:** Hundred percent mortality was evident at concentration of 300µg/ml whereas, highest concentration that produced zero percent mortality was 50µg/ml. The LC_{50} of the compound calculated by Reed & Muench (1938)[18] method was 169.47µg/ml. Fig.1 depicts the graph of concentration of compound versus percent mortality in zebrafish embryos. Ten embryos were exposed to each concentrations of *T.arjuna* (25, 50, 100, 150

µg/ml) stained at 72 hpf (Plate 3). There was no vessel inhibition in *T.arjuna* treated embryos when compared to control embryos (0.1% DMSO) and SU5416 treated embryos. Bharadwaj [23] had stated that *T.arjuna* has anti-angiogenic property which however was not evident through the present study.

From the present assay it can be concluded that the *T.arjuna,* did not show inhibition of ISV, DA and DLAV in 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 regardsto anti-angiogenic activity.

Acknowledgements

The authors thank Associate Dean, Bombay Veterinary college, Mumbai, for providing required facilities to carry out this research work.

References

[1] Auerbach, R., Lewis, R., Shinners, B., Kubai, N. Akhtar. Angiogenesis assays: A critical overview," *Clinical Chemistry 2003*; 49(1): 32–40.

[2] Folkman, J. Angiogenesis in cancer vascular, rheumatoid and other disease. *Nature Medicine* 1995; 1: 27-31.

[3] Kobayashi, H and Lin, P.C. Angiogenesis links chronic inflammation with cancer," Methodsin *Molecular Biology 2009*; 511:185-191.

[4] Kurebayashi, J. Expression of vascular endothelial growth factor (VEGF) family members in breast cancer, *Jpn. J. Cancer Research*1999; 90: 977–981.

[5] Shaheen, R.M. Anti-angiogenic therapy targeting the tyrosine kinase receptor for vascular endothelial growth factor receptor inhibits the growth of colon cancer liver metastasis and induces tumor and endothelial cell apoptosis. *Cancer Research.1999;* 59: 5412–5416.

[6] Yoshiji H. KDR/Flk-1 is a major regulator of vascular endothelial growth factor induced tumor development and angiogenesis in murine hepatocellular carcinoma cells. *Hepatology.* 1999; 30: 1179– 1186.

[7] Droller, M.J. Vascular endothelial growth factor is a predictor of relapse and stage progression in superficial bladder cancer. *J. Urology 1998:* 160-1932.

[8] Kitamura M. Concentrations of vascular endothelial growth factor in the sera of gastric cancer patients. *Oncology Receptor. 1998*; 5: 1419–1424.

[9] Balbay M. Highly metastatic human prostate cancer growing within the prostate of athymic mice over expresses vascular endothelial growth factor," Clinical Cancer Research, 1999; 5: 783– 789.

[10] Dong X, Han Z., Yang. Angiogenesis and anti-angiogenic therapy in hematologic malignancies," *Critical Reviews in oncology/ Hematology* 2007; 62: 105-118.

[11] Dubal Rs and Kamble K. Study of effects of bark extract in alcohol of Terminalia arjuna on angiogenesis by ChorioAllontoic Membrane assay (CAM) in chick embryo. International journal of biological research. 2013; 4(1), 47-52.

[12] Prabhu VV, Chidambaranathan N, Gopal V. Evaluation and quantification of angiogenesis activity of Terminalia belliricaroxb, by mice sponge implantation method. J Young Pharmacists 2012;4:22-7

[13] Quinn, T.P., Peters, K.G. De Vries, Ferrara N. and Williams L.T. Fetal liver kinase 1 is a receptor for vascular endothelial growth factor and is selectively expressed in vascular endothelium," *Proc. Natl. Acad. Sci. USA*, 1993; 90: 7533–7537.

[14] Serbedzija, G.N., Flynn, E. and Willett, C.E. Zebrafish angiogenesis: a new model for drug screening," *Angiogenesis*, 1999; 3: 353-359.

[15] Zon L.I., and Peterson R.T. In vivo drug discovery in the zebrafish. *Nature Reviews Drug Discovery*. 2005; 4:35-44.

[16] Norrby K. In vivo models of angiogenesis. *Journal of Cellular and Molecular medicine*, 2006; 10: 588-612.

[17] Vogel A. and Weinstein B., Studying vascular development in the Zebrafish. 2010;10: 847.

[18] Isogai S., Horiguchi M. and Weinstein B. M., The vascular anatomy of the developing zebrafish: an atlas of embryonic and early larval development," *Dev Biol* vol. 2001; 230: 278–301.

[19] Westerfield. M, The Zebrafish Book. Guide for the Laboratory Use of Zebrafish (Danio rerio) (University of Oregon Press, Eugene), 2000. 4th Edition 122-128.

[20] Reed, L.J.; Muench, H. (1938). "A simple method of estimating fifty percent endpoints". *The American Journal of Hygiene* 27: 493–497.

[21] Thamilarashi A.N, Mangalagowri. A, Gurumoorthi P(2013). Anti-angiogenic activity of myricetin through VEGF-A down regulation in zebrafish (*Danio rerio*)_*invivo.* International Journal of Scientific & Engineering Research, 4: 9.

[22] Arun Kumar, Mahapatra., Nisha Kumari, Ojha., and Abhimanyu, Kumar.(2013). "Prospective Role of Indian Medicinal Plants in Inhibiting Vascular Endothelial Growth Factor (VEGF) mediated Pathological Angiogenesis."J HomeopAyurv Med 2013, 2:2 http://dx.doi.org/10.4172/2167-1206.1000121.

[23] Makker, Nangia, Pratima, Larry, Tait., Malathy, P.V., Shekhar., Eduardo, Palomino., Victor Hogan., Marie P. Piechocki, Tatsuyoshi Funasaka and Avraham Raz. (2007) "Inhibition of breast tumor growth and angiogenesis by a medicinal herb: Ocimumgratissimum "Int. J. Cancer: 121, 884–894.

[24] Bhardwaj, L.K., Patil K.S., Juwatkar P.V., Shukla V.K. (2010). Plant products potential as anti- angiogenic and in cancer management. IJRAP 2010, 1(2), 339-349.

Table 1. Dose dependent mortality in LC_{50} assays for *Terminalia arjuna*

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	27	0	0	27	0
100	12	11	15	1	1	16	6.25
200	12	4	4	8	9	13	69.23
300	12	0	-	12	21	21	100
400	12	0	-	12	-	-	-
500	12	0	-	12	-	-	-
700	12	0	-	12	-	-	-
1000	12	0	-	12	-	-	-

Fig 1. Percent mortality in zebra fish embryos (6hpf) exposed to different concentrations of *Terminalia arjuna* extract

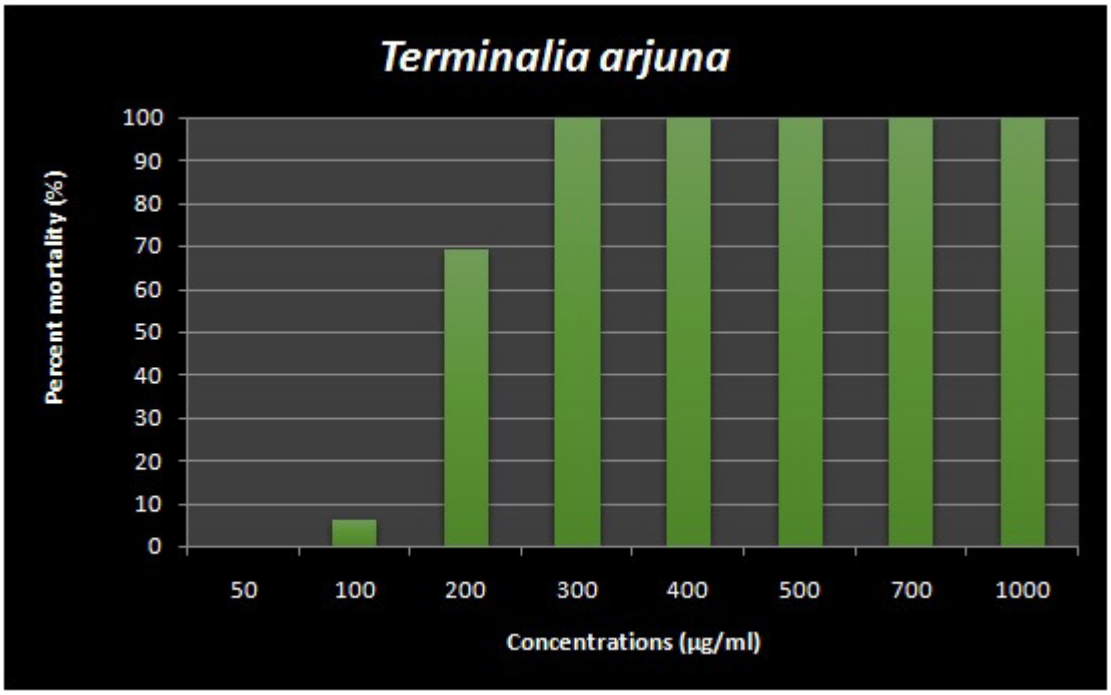

Plate 1a: Lateral view of RBC stained embryos at 72hpf with 0.1% DMSO had no effect on vessels formation as indicated by arrows.

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

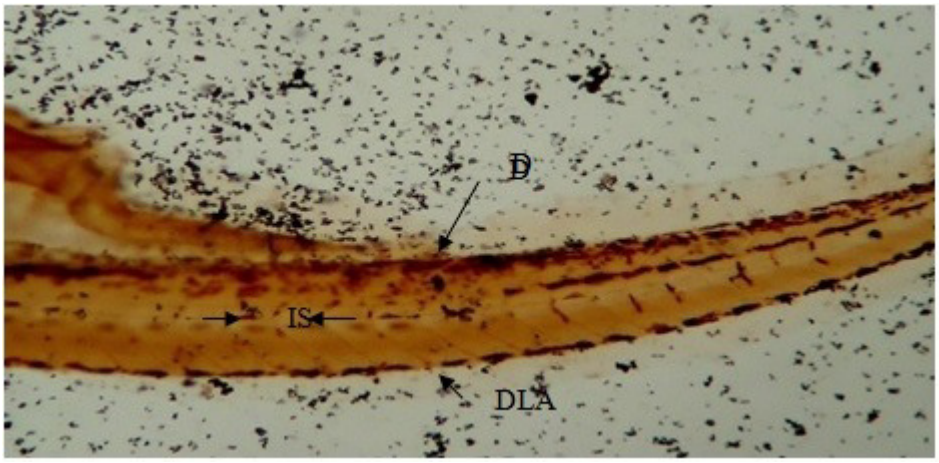

Plate 2: Absence of anti-angiogenic effect of *T.arjuna* in zebrafish embryo. Zebrafish embryos were treated with *T.arjuna* at concentration of 150 µg/ml. RBCs in DLAV, DA and ISV were clearly seen. There was no inhibition of blood vessels formation observed.