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EVALUATION OF ANTI- ANGIOGENESIS ACTIVITY OF NEEM ROOT USING ZEBRA FISH MODEL

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ABSTRACT: Introduction: Angiogenesis is the formation of new blood vessels which is a process involved in development of tumors and other neovascular diseases. Developmental angiogenesis in zebra fish embryos is a relevant and emergent model to evaluate the anti-angiogenesis activity which was used in present study. With this model, blood vessel formation and the blood flow can be clearly observed. Various parts of Neem tree were proved to have potential anticancer activity. Based on this fact, neem root has been selected for this study. **Method:** Zebra fish embryos were treated with different concentrations of Water soluble fractions of crude methanolic extract of neem root, Imatinib (standard) and control. Then, they were observed for 3 days within 24 hour interval. **Results:** Various phenotypical abnormalities that were observed during the development of larvae had been captured using digital microscope. Toxicity and survival rates were noted. **Conclusion:** Based on the results obtained, the water soluble fractions of methanolic extract of neem root was found to have the ability to inhibit in -vivo angiogenesis. Therefore, it can be concluded that, the study drug have the tumor growth inhibition properties.

INTRODUCTION: Angiogenesis is the process of formation of the new capillaries from the existing small blood vessels which is associated with cell proliferation. This proliferation accounts for the normal physiological and pathological changes that involve growth, wound healing, repair, hypertrophy and hyperplasia. This is an important event in the tumor growth [1]. In tumor Angiogenesis, blood vessels penetrate into the cancerous cells and provide nutrients, oxygen and remove waste products. The cell proliferation initiate with cancerous cells sending signals to the surrounding normal tissues.

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These signals stimulate certain genes in the tissue cells, which in turn activate respective proteins that encourage the growth of new blood vessels[2]. Treating tumor angiogenesis can provide new targets for cancer therapy, since the inhibition of angiogenesis suppresses the tumor growth by inhibiting the supply of oxygen and nutrients. This therapy is safer compared to the traditional treatment because it causes tumor regression by inhibiting only angiogenetic events [3].

Many synthetic drugs are available in the market for anti-angiogenesis therapy. But these drugs cause troublesome adverse effects like severe emesis, myelosuppression, alopecia and many more[4]. Hence, to reduce these toxic effects, researchers are developing alternative or complementary remedies. In this respect, many herbal drugs have been developed with the potential anti- angiogenic activity. As the chemical constituents present in the herbal medicines are a

part of the physiological function of the living flora, those are considered as more compatible drugs with the human body [5]. In the present study Neem root has been used as a test compound.

Neem tree is known to have well- proven medicinal properties. It is wildly grown and perennial tree. Benefits of this tree were even discussed in charaka samhita and sushruta samhita. Various parts of the neem tree have been studied for their medicinal properties like anti-cancer, anti-ulcer, anti-infective, and anti-allergic activities [6]. The phyto-constituents present in neem were found to cause apoptosis in tumor cells and can make the immune system strong [7, 8]. Based on the antioxidant activity of the neem root, the present study has been carried out using water soluble fractions of methanolic extract of neem root [9].

Developmental angiogenesis in Zebrafish embryos is an emergent model for the study of genetics and many human diseases. The basic vascular structure of the embryos is similar to that of vertebrate animals. Blood vessels of the embryos develop immediately after the angiogenesis development[10]. To this respect, Zebrafish embryos are more advantageous for angiogenesis model rather than usual vertebrate model. During embryonic development they fertilize externally as well as they can be visible directly which enables researchers to observe the phenotypical changes and drug effects easily [11, 12]. This transparency in development is useful to assess the activity of anti angiogenic compounds. This Angiogenesis is mainly characterized by the sprouting of intersegmental vessels which are located at the trunk in 24 hpf embryos and as well as the development of sub intestinal veins in 48 hpf embryos[11].

MATERIALS AND METHODS:

Preparation of extract:

Roots were shade dried. Dried roots were crushed and powdered. The coarse powder was subjected to extraction using methanol as a solvent in Soxhlet apparatus. Then the crude extract was further dried and evaporated in Rotavac evaporator. Dried extract was divided into water soluble and water insoluble fractions. The yield, colour and consistency of extracts were noted [9].

Procedure:

Embryo medium (E3): [13]

Nacl - 2.94gm
Kcl - 0.13gm
$Cacl_2$ - 0.49gm
$MgSo_4$ - 0.81gm
Methylene blue - 10gm
Fish system water - 10 litres

Embryo medium (2litres) of was prepared by using above composition. Zebra fish embryos were obtained from local suppliers. They were washed with embryo medium (E3) and again recovered into the beaker containing embryo medium. Each well in microtitre plate is filled with 100µl of embryo medium suspended with drug solutions (blank, test and standard) in different range of concentrations (10-100 µg/ml). Embryos were transferred using a dropper to the microtitre plate, one embryo per well [14, 15].

Different phenotypical changes like tail bending, abdominal elongation of yolk sac, haemorrhages, delayed hatching, stunted growth, and pericardial edema had been observed with a frequency of 24 hrs for 3 days (24hrs, 48hrs, and 72hrs)[16]. Images of corresponding observations were collected using digital microscope.

RESULTS AND DISCUSSION: Zebrafish embryos were observed for 24, 48 and 72 hpf and their develpomental teratogenic effects were observed as shown. From zero hours to 24 hours all the 3 groups (blank, test and standard) of embryos hadn't shown any difference in their characteristics. Maximum embryos were hatched by 40 hpf but after 48 hpf both test and satandard drug induced embryos had been started showing phenotypical deformalities. After 48 hrs, pericardial edema, heamorrahage and tail bending were started developing.

Within 72- 80hpf Yolk sac was separated from the abdominal region because of the edema development. Heart rate became low. Blood circulation had stopped but still heart beat was seen. At higher concentrations stunted growth was observed in some embryos. 100µg/ml dose had become toxic to the embryos they died within 24hpf and the remaining had shown 90% mortality

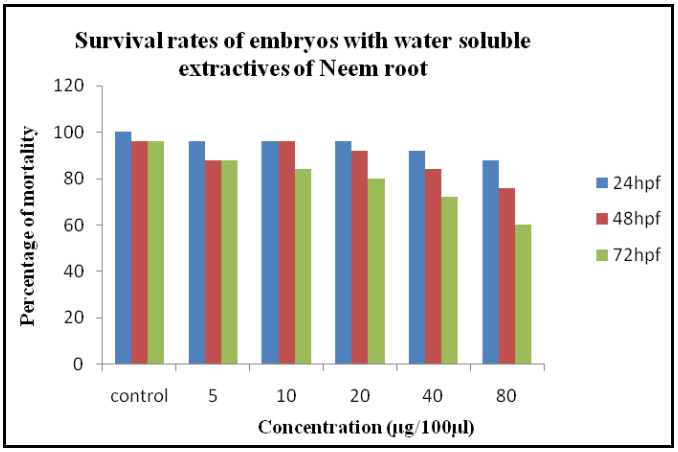
at their respective concentartions upto 72hpf. Survival rate was decreased with the increase in exposure time of the drug which has shown in the below **Fig.1**. After 5-6 dpf, all the embryos were died at higher concentrations.

TABLE 1: PERCENTAGE OF MORTALITY FOR EMBRYOS TREATED WITH WATER SOLUBLE EXTRACTIVES OF CRUDE METHANOLIC EXTRACT OF NEEM ROOT

Water soluble extractive of crude methanolic extracts of Neem root			
Sr.no	**24hpf**	**48hpf**	**72hpf**
control	100	96	96
5	96	88	88
10	96	96	84
20	96	92	80
40	92	84	72
80	88	76	60

TABLE 2: PERCENTAGE OF SURVIVAL RATES FOR THE EMBRYOS TREATED WITH IMATINIB

Imatinib			
Sr.no	**24hpf**	**48hpf**	**72hpf**
control	100	96	96
5	96	92	88
10	96	88	80
20	84	76	68
40	84	76	64
80	80	68	48



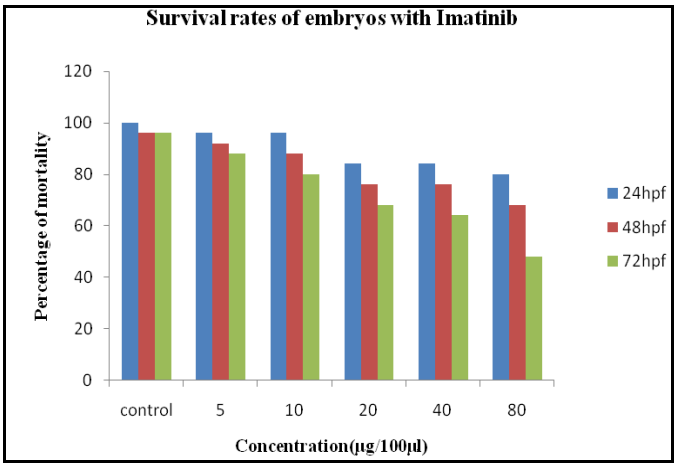
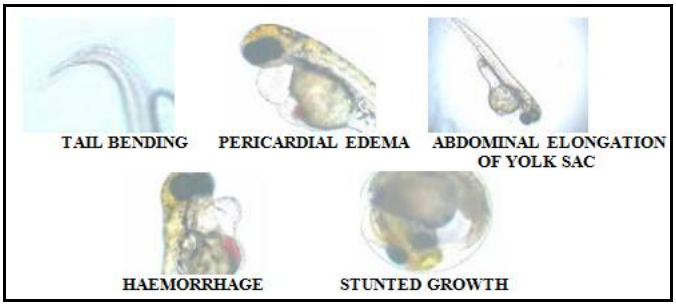


FIG.1: X-AXIS=CONCENTRATION (µg/100µl), Y-AXIS = SURVIVAL RATE PERCENTAGES

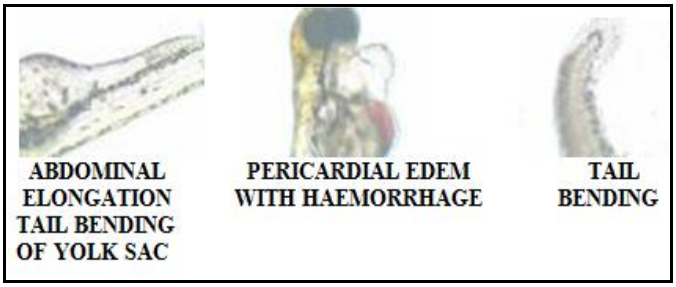
From zero hpf to 24hpf:

After 48hpf:

Test:



Standard drug:



Blank:

After 72hrs:
Test:

Standard:

Blank:

CONCLUSION: Zebra fish embryos and larvae were used to evaluate the embryonic and teratogenic effects of drugs that have been developed. They are helpful to predict the anti-angiogenesis potential value of compounds. In the current study zebrafish embryos were observed for 24, 48 and 72 hpf to screen the anti-angiogenesis potential of Neem root extract, when compared with standard. Survival rate of the embryos were decreased with time. The study drug has shown several developmental abnormalities in the above mentioned embryos. Based on these observations it can be concluded that the test compound can be used for the anti-angiogenesis treatment at higher doses.

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