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Antiangiogenetic Effect of Neem Root Extract on the Fin of Zebrafish (Fin Regeneration Method)

Authors

L. P. B. Lavanya Uppuluri, Ms. V. N. Garge, Dr.V.J.Kadam

Bharati Vidyapeeth College of Pharmacy, Navi Mumbai, Maharashtra, India
Email: *roses4lavanya@gmail.com*
Corresponding Author
L. P. B. Lavanya Uppuluri
M. Pharm Final Year
Bharati Vidyapeeth College of Pharmacy, Sector 8, CBD Belapur,
Navi Mumbai, Maharashtra, India, 400614
Email Id: *roses4lavanya@gmail.com*

Abstract

Cancer is a serious cause of death globally. Cancerous cells cannot be distinguished easily from the normal cells. Rapid development of cancerous cells depends on the process of Angiogenesis. Inhibition of the angiogenesis is a novel strategy in cancer treatment. Currently, herbal medicines are also being used, along with chemotherapy to reduce the side effects. For the present study, Neem was selected as it is a versatile tree in India. From the ancient times it has been considered as a mighty tree which gives good health. Roots of that tree were taken to study anti-angiogenesis activity on Zebra fish. The different parts of Neem tree were proven for their cytotoxic activity; hence water soluble fractions of crude methanolic extract of neem roots were taken as a test drug. Fin Regeneration in Zebra fish was selected as an experimental model. Fish were divided into groups, each with six fish. Fins were amputated, allowed to grow for 15 days. They were treated with Different concentrations of the test drug and standard drug. Length of the regenerated fin was measured frequently using digital microscope, and different software. Dose response was calculated for the test drug in comparison with standard drug and control groups. A graph of dose response vs. concentration was plotted accordingly. The test drug has shown desirable effect in angiogenesis inhibition.

Keywords- *Neem root. Water soluble extractives, Zebrafish, Angiogenesis, fin regeneration, cancer.*

1. INTRODUCTION

Angiogenesis is the process that leads to the neo-vascularisation in normal or cancerous cells. The growth of malignant tumors depends on the angiogenesis process. The angiogenesis provides oxygen and nutrition for the cell growth, and also helps the cancerous cells to spread into other tissues of the body. This process is controlled by angiogenesis inhibitors as well as inducers. Both natural and synthetic inhibitors were identified. Researchers have found that inhibitors of angiogenesis can helps in stopping the tumour growth. The tumors treated with inhibitors did not develop resistance to the anti-cancer drugs. Hence, it can be a promising therapy in cancer management [1, 2]. However, along with synthetic drugs, many herbal drugs are also available into the market. Herbal drugs possess better compatibility than synthetic drugs. Chemical constituents present in the plants are also a part of physiological cycle. Thus, they are more efficacious and show less harmful side effects than synthetic drugs [3]. Therefore, secondary metabolites that are present in plants which are having anti-angiogenesis potential are being discovered.

In the present study, Neem root has been taken as a test drug. It is a very efficacious and potent medicinal tree from the ancient times. The Neem tree (*Azadirachta indica*) belongs to the family Meliaceae [4]. Continuous research is being done on neem tree worldwide [5]. This tree is reported for anti infective, antipyretic, antiseptic, antiparalytic and many other pharmacological actions [6]. Chemical constituents present in Leaves, bark, wood and seeds were reported for cytotoxic activity [7]. Researchers have found that neem root is having antioxidant activity [8]. Based on this fact root extract was taken to study Antiangiogenetic activity.

Fin Regeneration method using Zebrafish is selected as an experimental model. It is an emerging drug screening model in animal study. The zebra fish possess circular system which is similar to vertebrates. When compared to the conventional disease models they grow faster, easy to handle, reliable and economic [9]. They are able to regenerate their organs like fin, optical nerve, retina etc. when injured or amputated. Surgery of the fin is a simple procedure. They are outstanding models for the study of angiogenesis, apoptosis and many other complex biological studies [10]. In this study, caudal fin of Adult Zebrafish had been used to investigate the anti-angiogenesis activity of Water soluble fractions of crude methanolic extract of Neem root.

2. PROCEDURE

2.1 Preparation of extract:

Dried neem roots were taken, powdered, extracted with methanol as a solvent using soxhlet apparatus [8] for 52hrs. Extractive was concentrated under reduced pressure. Then that concentrated extractive (4.4% w/w) is treated

with distilled water to separate water soluble fractions. These water soluble fractions were used as test drug.

2.2 Fin Regeneration Method:

2.2.1 Fish husbandry and general methods

Wild type fish stocks were reared at a constant temperature of 25°C and maintained on a 13L: 11D photoperiod. Fish were fed three times daily with both micro-pellets and flake food [11, 12]. They were kept for acclimatization up to two weeks.

Stock solutions of Standard and test drugs were prepared using DMSO as a solvent in volumetric flasks and stored in cool place.

2.2.2 Toxicity test:

Toxicity test was conducted to calculate the maximum tolerable dose for standard and test drugs using Up and Down method [14]. Those determined values are useful in selecting the range of dose for administration.

Fish water: Sodium thiosulphate and rock salt has been dissolved in normal tap water to prepare fish water.

Test solution: 1mg/ml of test solution (water soluble fractions of methanolic extract of *Azadirachta indica* roots) has been prepared by using 1.5% DMSO as a co-solvent to dissolve the drug along with water.

Standard solution: 1mg/ml of standard drug, (Imatinib) solution has been prepared by using 1.5% DMSO as a co-solvent to dissolve the drug along with water.

One fish/per dose has taken. They were kept in a250ml beaker containing 150ml fish water [15] along with drug and observed for lethality for 24hrs. Based on the mortality, dose was increased or decreased by the factor log0.5 or 3.2.

2.2.3 Experiment:

Adult fish were obtained from the local suppliers and kept in large tanks for acclimatization. On the day of experiment, fish were taken out and they were divided into 9 treatment groups: 6 fish in each group. They were anesthetized using 0.1% 2-phenoxyethanol [16]. Their fin was cut up to 50% by using sterilized straight razor blade.

They were again recovered using fish water. Pre and post amputated images were collected and length was measured using scale. 250ml capacity beakers were taken. Upto 150ml filled with fish water which is suspended with the drug, fish were kept in them, one fish per beaker with continuous aeration. Test drug, standard drug (Imatinib) and control were given to each group respectively. Dose for test and standard were determined from the toxicity value [17]. On every alternate day, water was refreshed with the dose up to 15 days.

The images of fin were collected to check the growth using digital microscope (4X lens) and dissection microscope (10X) which is attached

with webcam. On day 15th, final images were collected. Length was measured using Motic image plus 2.0 and ImageJ softwares. Percent of inhibition and percent of regeneration was calculated.

In dissection microscope, up to day 8 images were captured and the length of the 3rd fin ray of every fish was calculated using the software Motic image plus2.0.

On the day1 and day 15 images were captured using dissection microscope and webcam. Area of the fin was measured using an external software image J. percentage of area regeneration was calculated.

Table 1: Dose table

Groups	**Treatment**
Control	Water
Standard group1	0.2mg/150ml imatinib
Standard group2	0.4mg/150ml imatinib
Standard group3	0.6mg/150ml imatinib
Standard group4	0.8mg/150ml imatinib
Test group1	1mg/150ml water soluble fractions of methanolic extract of *Azadirachta indica* roots
Test group2	1.5mg/150ml water soluble fractions of methanolic extract of *Azadirachta indica* roots
Test group3	2mg/150ml water soluble fractions of methanolic extract of *Azadirachta indica* roots
Test group4	2.5mg/150ml water soluble fractions of methanolic extract of *Azadirachta indica* roots

2.3 RESULTS AND DISCUSSION:

Toxicity:

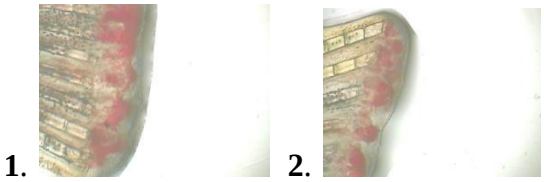
Toxicity determined for test and standard drugs is given table 2:

Table 2 Toxicity results

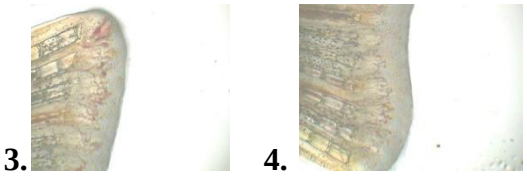
Group	Maximum tolerable dose (mg/150ml)
Standard	2.6
Test	2.6

Day 3:

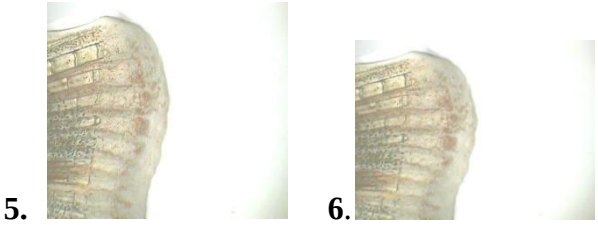
Images were collected on this day to observe the blastemma formation.



1&2 are 0.8mg/150ml of standard drug



3&4 are 2.5mg/150ml of test

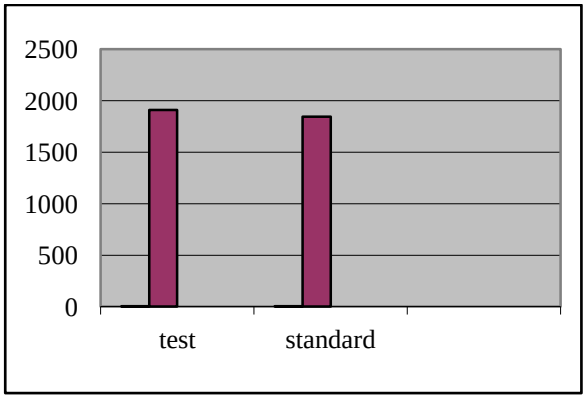


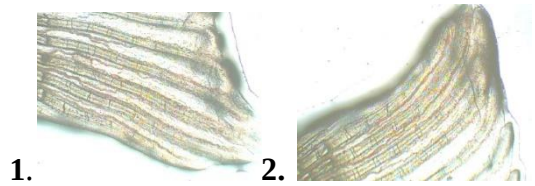
5 &6 are blank

Day 8:

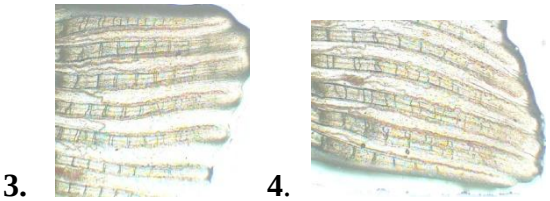
On day8, length was measured using Motic image plus2.0, both standard and test drugs have shown inhibition in dose dependant manner. Somehow the test drug at 2mg/150ml was showing similar regeneration capacity to that of 0.8mg/150ml dose of standard drug. As shown in the Figure 1.

Figure 1

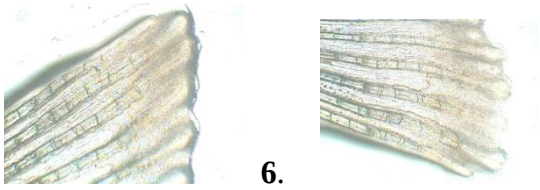




1&2 are blank group



3 & 4 are 2.5mg/150ml of test drug

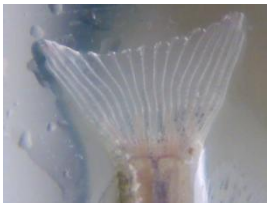


5& 6 are 0.8mg/150ml of standard drug

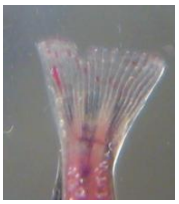
Day 15:

On day 15, images were taken in dissection microscope attached with a web cam, regenerated length was measured using imageJ

and indicated as percentage area of regeneration in the graphs figure 2and 3.

1, 

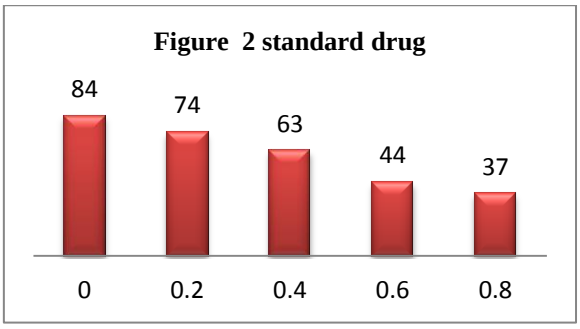
(2.5mg/150ml) Test

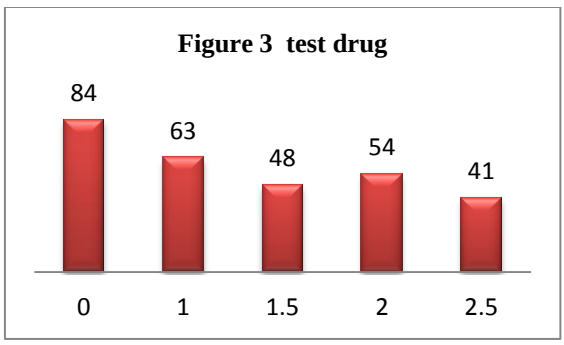
2. 

(0.8mg/150ml) Standard

3. 

Blank





Minimum regeneration was shown by 0.8mg/150ml of standard drug and 2.5mg/150ml of test drug. All the responses of standard drug are in dose dependant manner. While in the case of test, results are somewhat deviated from the dose response curve. Percentage regeneration can be implied as percentage inhibition by subtracting the regeneration value from 100. i.e. percentage inhibition of 0.8mg/150ml of standard drug is 63% as well as for test drug is 59%.

A Part of crude material was used as test material in this paper. That may cause the deviation of activity in comparison with a pure standard drug.

3. CONCLUSION

Adult Zebrafish was used to assess the anti angiogenesis activity of the study drug. Percent of regeneration was calculated hence the percent of inhibition for every fish. This percentage inhibition can also be extrapolated as Water soluble fractions of crude methanolic neem root extract has shown considerable antiangiogenesis activity when compared with standard in 15days. Chemical constituents present in that crude material should be further isolated from the mixture. And each chemical constituent should further investigated for the Antiangiogenetic property. This may aid the cancer research in future. However, herbal drug therapy in cancer management is growing now.

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