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Research Article

Study on In-Vitro Cytotoxic and Thrombolytic Activity of Methanolic Extract of *Citrus macroptera*(Fruit)

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Abstract: The present study was designed to investigate the cytotoxic and thrombolytic activity of methanolic extract of fruits of *Citrus macroptera*. Methanolic extract of *Citrus macroptera*, was used to evaluate its cytotoxicity in Brine shrimp lethality bioassay where vincristinsulphate was used as standard drug. Thrombolytic effect of the fraction was investigated in clot lysis experiment. In Brine shrimp lethality bioassay, LC50 value of the extract was 30.90µg/ml and vincristinsulphate served as the positive control showed LC50 value 10.51 µg/ml. The extract exerted 48.47%lysis of the blood clot in thrombolytic activity test while 75.18% and 15.82% lysis were obtained for positive control (streptokinase) and negative control respectively. Compared to vincristinsulphate, it is evident that the methanolic extract of fruits of *Citrus macroptera*was cytotoxic. So, the extract possessed considerable thrombolytic activity. Which compounds is responsible for the present pharmacological actions and to know their mechanism of action, extensive pharmacological and phytochemical experiments are essential.
Keywords: *Citrus macroptera*, Cytotoxic Activity, Thrombolytic Activity, Vincristine, Streptokinase.

INTRODUCTION

Since ancient times plants have served as a natural source of treatments and therapies such as aspirin, quinine, and coffee. Today, scientists are using these renewable resources to produce a new generation of therapeutic solutions. Plants improved through the use of biotechnology can produce the essential proteins for innovative treatments for diseases such as cancer, HIV, heart disease, diabetes, Alzheimer's disease, kidney disease, Crohn's disease, cystic fibrosis, multiple sclerosis, spinal cord injuries, Hepatitis C, chronic obstructive pulmonary disorder (COPD), obesity, arthritis and iron deficiency.The use of natural products with therapeutic properties is as ancient as human civilization and, for a long time, mineral, plant and animal products were the main sources of drugs [1]. Vincristine and vinblastine from *Catharanthusroseus*, atropine from *Atropa belladonna* and morphine and codeine from *Papaver somniferum*. It is estimated that 60% of anti-tumour and anti-infectious drugs already on the market or under clinical trial are of natural origin [1]. The vast majority of these cannot yet be synthesized economically and are still obtained from wild or cultivated plants. Natural compounds can be lead compounds, allowing the design and rational planning of new drugs, biomimetic synthesis development and the discovery of new therapeutic properties not yet attributed to known compounds [2]. In addition, compounds such as muscarine, physostigmine, cannabinoids, yohimbine, forskolin, colchicine and phorbol esters, all obtained from plants, are important tools used in pharmacological, physiological and biochemical studies [3].

Cytotoxicity is the quality of being toxic to cells. Cells exposed to a cytotoxic compound can respond in a number of ways. The cells may undergo necrosis, in which they lose membrane integrity and die rapidly as a result of cell lysis; they can stop growing and dividing; or they can activate a genetic program of controlled cell death, termed apoptosis. Cells undergoing necrosis typically exhibit rapid swelling, lose membrane integrity, shut down metabolism, and release their contents into the environment upon lysis. Apoptosis is characterized by well-defined cytological and molecular events, including a change in the refractive index of the cell, cytoplasmic shrinkage, nuclear condensation, and cleavage of DNA. Cytotoxicity assays are used widely in drug discovery research to help predict which lead compounds might have safety concerns in humans before significant time and expense are incurred in their development. Other researchers study mechanisms of cytotoxicity as a way to gain a better understanding of the normal and abnormal biological processes that control cell growth, division, and death[4].

Thrombolysis is the breakdown (*lysis*) of blood clots by pharmacological means. It is colloquially referred to as *elot busting* for this reason. It works by stimulating fibrinolysis by plasmin through infusion of analogs of tissue plasminogen activator (tPA), the protein that normally activates plasmin. Thrombolytic therapy is the use of drugs to break up or dissolve blood clots, which are the main cause of both heart attacks and stroke. Thrombolytic medications are approved for the immediate treatment of stroke and heart attack. The most commonly used drug for thrombolytic therapy is tissue plasminogen activator (tPA), but other drugs can do the same thing[5].

Citrus macroptera yield fragrant aromatic oil, which can be used in perfumery. Malays, Melanesians and Polynesians have used *Citrus macroptera* juice as a hair wash and added it to coconut oil for fragrance. Goldsmiths have used *Citrus macroptera* to clean gold objects. *Citrus macroptera* fruit are used in native medicines and *is* used in traditional Chinese medicine. Many *Citrus macroptera* occur only in the wild, but the Small-flowered *Citrus macroptera* is cultivated in the Bangladesh, Philippines, China, Japan and many countries of the Far-East. *Citrus macroptera* have many food uses and the plant is cultivated in most citrus growing regions around the world[6].

MATERIALS AND METHODS

Collection of Plant Materials

The fruits of *Citrus macroptera* were collected from Sylhet local vegetable market, The fruits of *Citrus macroptera* were collected at their fully mature form. After cleaning, the fruits were taken and splitting the peal, then air dried for 8 days, and then kept in an oven at 45°C at 72 hours. 250 gm of dried powder was cold extracted with Methanol. Dried powder soaked with methanol for 7 days. Then filtered to take the concentrated extract, extract containing beaker was placed on the water bath (at 40°C-45°C) to evaporate the solvent from the extract[7].

Preparation of Extraction:

The extract is prepared by cold extraction process. In this process the coarse powder was submerged in ethanol (95%) since ethanol is the most common solvent for extracting most of the constituents present in herbal materials. Amber glass bottle were used for this purpose, which were kept at room temperature and allowed to stand for 7 days with occasional shaking and stirring. When the solvent became concentrated the contents were first decanted by using cotton and then filtered through Whatmann No.1 filter paper. The filtrate so obtained was then concentrated to dryness through the evaporation of solvent using rotary evaporator. Finally we got the concentrated semi-solid extract. The concentrated were then used as crude extract of respective test experiments. In our present investigation, we used methanolic extract for cytotoxic and thrombolytic activity[8].

In-vitro Cytotoxic Study:

Brine shrimp lethality bioassay is widely used in the bioassay for the bioactive compounds. Here simple zoological organism (*Artemiasalina*) was used as a convenient monitor for the screening. The dried cyst of the brine shrimp were collected from an aquarium shop (Chittagong, Bangladesh) and hatched in artificial seawater (3.8% NaCl solution) with strong aeration for 48 hours day/dark cycles to mature shrimp called nauplii. The cytotoxicity assay was performed on brine shrimp nauplii using Meyer method[9].

Materials:

Artemiasalina Leach (brine shrimp eggs),Sea salt non ionized NaCl, Small tank with perforated dividing dam to hatch the shrimp, Lamp to attract the nauplii, Pipette (1 ml and 5 ml), Micropipette (1-10 micro liter), Glass vials (5ml), Magnifying glass, Test sample for experimental plants[9].

Hatching of Brine Shrimp Eggs:

Artemiasalina Leach (brine shrimp eggs) collected from the pet shop was used as the test organism. Simulated sea water was taken in the small tank and the shrimp eggs (1.5gm/L) were added to one side of the tank and this side was covered. The shrimps were allowed to one side of tank and this side was covered. The shrimp were allowed for two days to hatch and mature as nauplii (larvae). Constant oxygen supply was carried out during the hatching time. The hatched shrimps were attracted to the lamp on the other side of the divided tank through the perforated dam. These nauplii were taken for this bioassay[10].

Preparation of the Simulated Sea Water:

38 grams sea salt was weighted accurately, dissolved in 1 liter of sterilized distilled water and then filtered to get clear solution. The p^H of the sea water was maintained between 8-8.5 using 1N Na0H solution[11].

Preparation of Sample Solution:

At first take 19ml distilled water in beaker add 1ml DMSO (dimethyl sulfoxide)thus prepares stock solution. Clean test tubes were taken. These test tubes were used for different concentration (one test tube for each concentration) of test samples.4 mg methanolic extracts of *Citrus macroptera* were accurately weighed and dissolved in 4ml stock solution. Thus a concentration of 1000 µg/ml was obtained which used as an extract solution. Then taking 1ml extract solution from beaker & add 9ml stock solution In vials thus prepared final extract solution. From this extract solution 0.5 µg/ml, 2µg/ml, 4µg/ml, 8µ/ml, 10µg/ml,12µg/ml,20µg/ml,30µg/ml,40µg/ml and 50µg/ml were taken in ten test tubes respectively and

adjusted volume 5 ml sea water. Finally 10 nauplii are then applied in each test tubes[11].

Preparation of Control group:

Control groups are used in cytotoxicity study to validate the test method and ensure that the results obtained are only due to the activity of the test agent and the effects of the other possible factors are nullified. Usually two types of control groups are used-i) Positive controlii) Negative control[12].

Preparation of Positive Control group:

Positive control in cytotoxicity study is a widely accepted cytotoxic agent and the result of the test agent is compared with the result obtained for the positive control. In the present study, vincristine sulphate was used as the positive control. 3 mg of vincristine sulphate was dissolved in 1.8 ml of distilled water to get a concentration of 5 mg/ml. This was used as stock solution of vincristine sulphate. With the help of a micropipette 500,300,100, 50 and 10 µl of the stock solution were transferred in 6 different vials. NaCl solution (brine water) was added to each vial making the volume up to 5 ml. The final concentration of vincristine sulphate in the vials became 500µg/ml, 300µg/ml, 100µg/ml, 50µg/ml and 10µg/ml respectively. The experiment was repeated three times[12].

Preparation of negative control:

100 µl of distilled water, DMSO and ethanol was added to each of the three remarked glass vials containing 5 ml of simulated sea water and 10 shrimp nauplii to use as control groups. If the brine shrimp nauplii in these vials show a rapid mortality rate, then the test is considered as in valid as the nauplii died due to some reason other than the cytotoxicity of the samples[12].

Application of Brine shrimp Nauplii:

With the help of the Pasteur pipette 15 living nauplii were added to each of the vials containing 5 ml of simulated sea water. A magnifying glass was used for convenient count of nauplii. If the counting of the 15 nauplii was not be possible accurately[13].

Counting of the Nauplii:

After 24 hours, the vials are observed using a magnifying glass and the number of survival nauplii in each vial were counted and recorded. From this data, the percentage of mortality of nauplii was calculated for each concentration of the sample. The median lethal concentration (LC_{50}) of the test samples was obtained by a plot of percentage of the shrimps killed against the logarithm of the sample concentration[13].

In-Vitro Thrombolytic Study:

Thrombolysis is the breakdown (*lysis*) of blood clots by pharmacological means. It is colloquially referred to as *clot busting* for this reason. It works by stimulating fibrinolysis by plasmin through infusion of analogs of tissue plasminogen activator (tPA), the protein that normally activates plasmin.

Preparation of Extract Solution for Thrombolytic Test:

10 mg of the extract was suspended in 10ml distilled water and shaken vigorously on a vortex mixer. Then the suspension was kept overnight and decanted to remove the soluble supernatant, which was filtered through a filter paper (Whatman No. 1).The solution was then ready for *in vitro* evaluation of clot lysis activity[8].

Preparation of Streptokinase (SK) Solution:

To the commercially available lyophilized SK vial (PolaminWerk GmbH, Herdecke, Germany) of 1,500,000 I.U., 5 ml sterile distilled water was added and mixed properly. This suspension was used as a stock from which 100 µl (30,000 I.U) was used for in vitro thrombolysis [8].

Specimen of Thrombolytic Test:

3ml blood was drawn from healthy human volunteers without a history of oral contraceptive or anticoagulant therapy (using a protocol approved by the Institutional Ethics Committee of Central India Institute of Medical Sciences, Nagpur). 500 µl of blood was transferred to each of the ten previously weighed alpine tubes to form clots [8].

Test Procedure for Thrombolytic test:

Experiments for clot lysis were carried as reported earlier [8]. Venous blood drawn from healthy volunteers was transferred in different pre-weighed sterile Epen drop tube (500µl/tube) and incubated at 37°C for 45 minutes. After clot formation, serum was completely removed (aspirated out without disturbing the clot formed).Each tube having clot was again weighed to determine the clot weight (Clot weight = weight of clot containing tube – weight of tube alone).Each Epen drop tube containing clot was properly labeled and 100 µl of plant extract was added to the tubes. All the tubes were then incubated at 37°C for 90 minutes and observed for clot lysis. After incubation, fluid obtained was removed and tubes were again weighed to observe the difference in weight after clot disruption. Difference obtained in weight taken before and after clot lysis was expressed as percentage. Thrombolytic Activity of methanolic extract of *Citrus macroptera* fruits clot lysis. Streptokinase and water were used as a positive and negative (non-thrombolytic) control respectively. The experiment was repeated several times with the blood samples of different volunteers.

RESULT AND DISCUSSION

Brine Shrimp Lethality Bioassay:

Brine shrimp lethality results of the fraction of *Citrus macroptera* fruits is shown in Fi-1 and LC_{50} calculated value is recorded in Table 1. The fraction showed potential cytotoxic activity with LC_{50} value of 30.90µg/ml. Vincristin sulphate served as the positive control for this brine shrimp lethality assay and its LC_{50} value was 10.51µg/ml.

Table -1: Cytotoxic activity of *Citrus macroptera*

Conc	Nauplii	Live nauplii	Log C	% of Mortality	LC_{50} µg/ml
10	20	13	1	35	**30.90**
50	20	11	1.69897	45	
100	20	4	2	80	
300	20	3	2.477121	85	
500	20	2	2.69897	90	
800	20	0	2.90309	100	

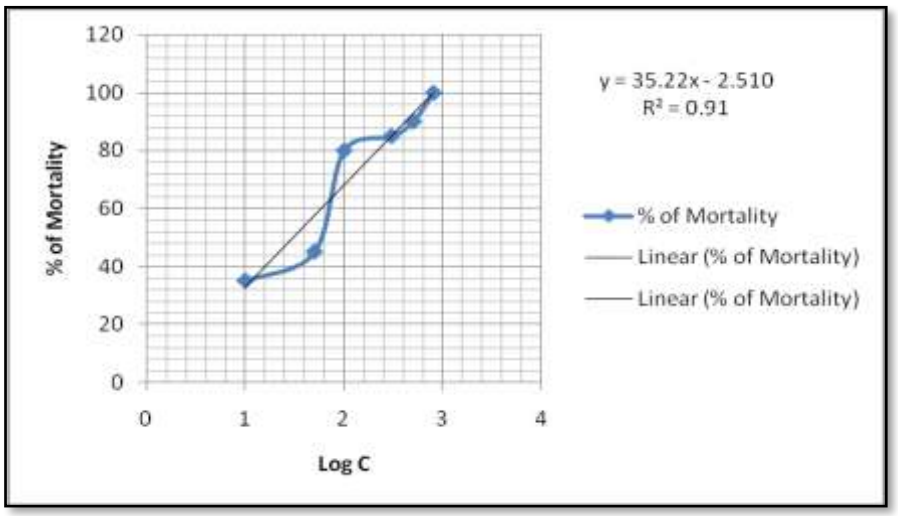


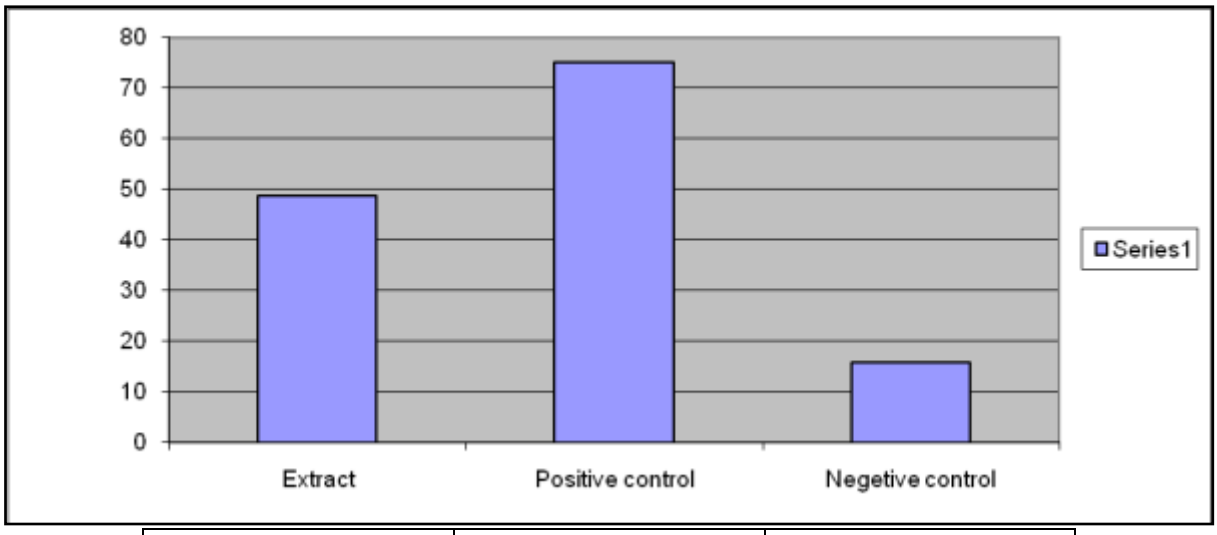
Fig-1: Determination of LC_{50} value for fraction of *Citrus macroptera* fruits from linear correlation between log concentrations versus percentage of mortality.

Thrombolytic Activity

The methanolic extract of *Citrus macroptera fruits* is exerted 48.87% lysis of the blood clot in thrombolytic activity test while 75.18% were obtained for positive control (streptokinase) and 15.82% were obtained for negative control respectively which shown in table 2. So, the extract possessed considerable thrombolytic activity.

Table-2: Thrombolytic Activity of *Citrus macroptera*

Volunteer	Empty weight of tube (gm)	Clot weight with tube (gm)	Clot weight after lysis (gm)	Percentage	Average(%) of lysis
A	0.8092	1.3939	1.0897	48.48	**48.87**
B	0.8025	1.2484	1.1001	49.81	
C	0.8139	1.4475	1.1183	48.34	



Exract	Positive control	Negetive control
48.81%	75.81%	15.82%

Fig-2: Methanolic extract of *Citrus macroptera* thrombolytic effect compared with standard (Streptokinase) and negative control.

DISCUSSIONS

Plant-derived medicines have a long history of use for the prevention and treatment of human diseases. Today, many pharmaceuticals currently approved by the Food and Drug Administration (FDA) have origins to plant sources. A major role for plant-derived compounds based on the reported immunemodulatory effects has emerged in recent times and has led to the rigorous scientific examination to determine efficacy and safety. A number of plants source especially several fruits and vegetables have been studied for their supplements having anticoagulant, antiplatelet and fibrinolytic activity and there is evidence that consuming such food leads to prevention of coronary events and stroke. Some of these plant products are modified further with recombinant technology to make them more effective and site specific.

In our thrombolytic assay, the comparison of positive control with negative control clearly demonstrated that clot dissolution does not occur when water was added to the clot. When compared with the clot lysis percentage obtained through SK and water, an extremely significant thrombolytic activity was observed after treating the clots with *C. arborea*, chloroform fraction. Cell surface bound plasminogen is easily activated to plasmin, which could lead to fibrinolysis. Bacterial plasminogen activator: staphylokinase, streptokinase, act as cofactor molecules that contribute to exosite formation and enhance the substrate presentation to the enzyme. Staphylokinase activates plasminogen to dissolve clots, also destroys the extracellular matrix and fibrin fibers that hold cells together.

Toxicity of plant materials is a major concern to scientists and medical practitioners and therefore cytotoxic assay was conducted in this study to determine the toxicity profile of the plant extracts through the Brine Shrimp Lethality (LC_{50}, 24 h) test. Lagar to demonstrated a good correlation (r^2 = 0.91) between the LC_{50} of the brine shrimp lethality test and the acute oral toxicity assay in mice. Based on that correlation, brine shrimp lethality LC_{50}< 10 µg/ml (LD_{50} between 100 and 1000 mg/kg) is considered as the cut off value of cytotoxicity. According to the measured LC_{50} values of the extracts no one was found severely lethal or toxic to be processed as pharmaceutical products in thrombolytic uses. However, the extremely significant effect of *Citrus macroptera* demonstrates it to be the best thrombolytic component for further processing.

CONCLUSION

From the above study it can be concluded that the methanolic extract of *Citrus macroptera* may be a potential candidate for future thrombolytic agent. Furthermore study and isolation is needed to obtain site specific and more potent agent that causing this effect. The test was made under full concentration to develop a new compound. I found that the extract I choose, was quite good in use. At the conclusion I can recommend that this plant part is useful for further use and isolation. The thrombolytic and cytotoxic study was close to the standard used. The thrombolytic potency of *Citrus macroptera* is found 48.87% and the standard have 75.18%. It seems good result or may be said significant as the extract was the mixture of many phytochemical, it shows nearby percent of clot lysis. The cytotoxic result obtained 30.90µg/ml (LC_{50}) it so good, but proper isolation can make it more potent and useful. So, it could be suggested to modify for site specific use.

At present scientist give their best regard in developing a more potent and site specific drug in the treatment of cancer. Nature could be a great source in

this purpose. Most of potent drugs are using came from nature, either directly or in their derived form. In this regard my study can help to find a new lead compound for future drug discovery.

Here experimental studies of leaves extract exhibited considerable thrombolytic and cytotoxic activity and moderate activity. So, further comprehensive pharmacological and phytochemical investigations are needed to elucidate the specific chemical compounds responsible for cytotoxic and thrombolytic activities and their mode of actions. The long term toxic effect and its protective effects should also be elucidated.

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