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Research Article

Phytochemical Investigation, Cytotoxic and Thrombolytic Activity of *Limonia acidissima* L. (Rutaceae) Fruit Peel Extracts

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ABSTRACT

The *Limonia acidissima* L is one of the useful traditional medicinal plants. In the rural area a lot of people use the *Limonia acidissima* L. for treating indigestion, flatulence, diarrhoea, dysentery and haemorrhoids. The present study was aimed to evaluate the various pharmacological efficacies of methanolic and acetonic fruit peels extracts of *Limonia acidissima* L. For cytotoxic activity, methanolic and acetonic extracts showed LC_{50} value 76.73 and 68.31µg/ml; respectively. On the other hand, the reference standard vincristine sulphate showed LC_{50} value was 2.63 µg/ml. In the thrombolytic activity the methanolic and acetonic extracts showed clot lysis (32.89%) and (32.43%); respectively. The standard of Streptokinase showed clot lysis (68.42%). This study will reveal the phytochemicals, cytotoxic and thrombolytic activity which may be used in future to open a new line of investigation.

INTRODUCTION

Plants are the giant source of medicines because they produce wide range of bioactive molecules, most of which as chemical defense against predation or infection [1]. *Limonia acidissima* L. is the family of Rutaceae (Citrus family) which belongs to the monotypic genus *Limonia*, confined to India, Pakistan, Sri Lanka and Southeast Asia [2]. It is also known as woodapple, elephant-apple, curd fruit, kath bel as well askaitha. This plant parts are used as a medicine for the treatment of several disorders [3]. Wood apple is an erect, slow-growing tree with a few upward-reaching branches bending outward near the summit. The bark is ridged, fissured and scaly. The deciduous, alternate leaves, 3 to 5 in long, dark-green, leathery, often minutely toothed. Yellowish green flowers, tinged with red, 1/2 in across, are borne in small, loose, terminal or lateral panicles. The tree is mostly known for its hard woody fruit, size of a tennis ball, round to oval in shape. The pulp is brown, mealy, odorous, resinous, astringent, acid or sweetish, with numerous small, white seeds scattered through it [4]. Especially it is used for treating indigestion, flatulence, diarrhoea, dysentery and haemorrhoids. The bark is chewed with that of Barringtonia and applied on venomous wounds [5].

Cancer is one of the major causes for humans especially in developing countries, in 2008 7.8 million peoplea were died cancer the entirely word [6] Cancer is an uncontrolled cell division nwhich has the ability to invade, metastasize as well as spread to distant sites [7]. Some of the anticancer agents such as vinblastine, irinotecan, topotecan, vincristine, taxanes are comes from plants. The searching of novice cytotoxic agents from natural—microbial, marine as well as plant sources continues all over the world [8].

Thrombolysis might be correlated with an expanded danger of intricacies in patients who are pregnant or at an expanded age, and in individuals with different conditions [9]. The breakdown or lysis of blood clump is named as thrombolysis is brought about by tissue plasminogen activator (tPA). Intravenous heparin utilized as the main line treatment while of its security profile just as movement [10-11]. A lot of drugs have been developed with the development of modern pharmaceutical science like anistreplase, alteplase, urokinase, streptokinase as well as tissue plasminogen (TPA) [12, 13]. The aim of this study was to assess the cytotoxic and thrombolytic properties of

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Limonia acidissima L. fruit peels extracts, the activities of which, to the best of our knowledge.

MATERIALS AND METHODS

Chemicals and Reagents

Vincristine sulphate, DMSO (Dimethyl sulfoxide) and Streptokinase were used.

Plant Materials

The peels part of the fruit of *Limonia acidissima* L. were collected from near Jahangirnagar University fields, Dhaka, Bangladesh. The identification of the plant material was confirmed by the experts of Bangladesh National Herbarium, Mirpur, Dhaka.

Drying and Grinding

The collected fruit peels were separated from undesirable materials. Then these were dried in for one week in the sunlight and these were cutting into small pieces. The fruit peels were converted into coarse powder by using a suitable grinder. The powder was stored in an airtight container and kept in a cool, dark and dry place until analysis commenced.

Preparation of Methanol and Acetone Extracts

At first, two clean flat flat-bottomed glass containers was taken and added about 400 and 450gm of powdered sample into the container, respectively. Then 1500 ml of 90% methanol and 1800ml acetone were added into the two containers as well as soaked the powder into the methanol and acetone, respectively. Afterwards, containers were sealed with their contents and kept for a period of 10 days accompanying occasional shaking and stirring. After that, the coarse parts of the fruits were separated from the mixture by using white cotton. Then the liquid portion was also filtered three times with the help of white cotton. Then again, these were filtered through whatman filter paper. Then the filtrates were kept in Rotary evaporator machine which separates solvent and desirable crude extracts was obtained.

Phytochemical Screening

Phytochemical screening of *Limonia acidissima* L. was carried out to identify the functional groups as described [14-16].

Cytotoxic Activity

For cytotoxicity screening, DMSO (Dimethyl sulfoxide) solutions of the methanol and fruit peels extracts were applied to *Artemia salina* in a one day in vivo assay. For the experiment, 4 mg of each of the extracts were dissolved in DMSO and solutions of varying concentrations (640, 320, 160, 80, 40, 20, 10, 5 µg/ml) were obtained by serial dilution technique. The solutions were then added to the pre-marked vials containing ten live brine shrimp nauplii in 5 ml simulated sea water. After 24 hours, the vials were inspected using a magnifying glass and the number of survived nauplii in each vial was counted. From this data, the percent of lethality of the brine shrimp nauplii was calculated for each concentration. The lethal concentration (LC50 and LC90) of the test samples were obtained by plotting percentage of the shrimp killed against the logarithm of the sample concentration [17].

Thrombolytic Activity

Preparation of Extract Dose: Extract Concentration, Stock solution = 100mg /10ml. Standard: Streptokinase 1500000, IU/5ml, Dose: 30000 IU in 100µl.

Procedure: In vitro clot lysis activity of the fruit peels was carried out according to the method with minor modifications. With ethical considerations, and aseptic precaution, 5 ml of venous blood was drawn from healthy volunteers (n = 3) having no history of smoking, taking lipid lowering drugs, oral contraceptive or anticoagulant therapy and transferred to different pre weighed sterile micro-centrifuge tube (1 ml/tube). The micro-centrifuged tubes were subjected to incubation at 37°C for 45 min. After the formation of clot, serum was completely removed from the tubes (carried out without disturbing the clot formed) and each tube having clot was again weighed to determine the weight of the clot.

(Clot weight = weight of clot containing tube – weight of tube alone).

To each micro-centrifuge tube containing pre-weighed clot, 100 µl solutions of different extracts, concentration 1 mg/mL, were added accordingly. As a positive control, 100 µl of streptokinase and as a negative non thrombolytic control, 100 µl of sterilized distilled water were separately added to the control tubes numbered. Then all the tubes were incubated again at 37°C for 90 min and observed for clot lysis. After incubation, the obtained fluid was removed from the tubes and they were again weighed to observe the difference in weight after clot

disruption. At last, difference obtained in weight was calculated and the result was expressed as percentage of clot lysis following the underneath equation.

% of clot lysis = (wt. of lysis clot /initial clot wt.)×100 [18, 19].

RESULT AND DISCUSSION

Table1: Result of phytochemical investigation

Tested groups	Methanol extract	Acetone extract
Tannins	+	+
Phenols	-	+
Flavonoids	+	-
Saponins	-	+
Terpenoids	+	+
Gum	+	+
Alkaloids	+	+
Glycosides	+	+

(+) Indicates presence, (-) Indicates absence.

Cytotoxicity Evaluation

The lethal concentration (LC50) of the test both extracts after 24 hours was found by a plot of percentage of the shrimps died against the logarithm of the extracts concentration as well as the best fit line was found from the curve data by means of regression analysis. Vincristine sulphate is used as a standard positive control and the LC50 compared with negative control.

The LC50 of the methanol, acetone extract and standard are 76.73, 68.31 and 2.63 µg/ml; respectively whereas The LC90 of the methanol, acetone extract and standard are 523.16, 651.40 and 5.73 µg/ml; respectively. The positive control (vincristine sulphate), the cytotoxicity exhibited by the methanolic and acetonic extracts has been shown activity. This evidently introduces the presence of bioactive principles in these both extracts which may be very necessary as antiproliferative, antitumor, pesticidal as well as other bioactive agents [20].

Table 2: Test result of the cytotoxic activity of different methanol and acetone extracts.

Treatment	Conc.(µg/ml)	No. of nauplii taken	No. of dead nauplii	% Mortality	LC50 (µg/ml)	LC90 (µg/ml)
Methanol extract	640	10	9	90	76.73	523.16
	320	10	8	80		
	160	10	8	80		
	80	10	6	60		
	40	10	5	50		
	20	10	4	40		
	10	10	3	30		
	5	10	3	30		
Acetone extract	640	10	8	80	68.31	651.40
	320	10	7	70		
	160	10	8	80		
	80	10	6	60		
	40	10	5	50		
	20	10	4	40		
	10	10	3	30		
	5	10	3	30		
Vincristine Sulphate	5	10	10	100	2.63	5.73
	2.5	10	9	90		
	1.25	10	8	80		
	0.625	10	6	60		
	0.315	10	5	50		
	0.156	10	4	40		
	0.078	10	3	30		

Table 3: Thrombolytic activity test

Sample	Wt. of Blank tube (g)	1st clot + tube (g)	1st clot	2nd clot + tube (g)	2nd clot	Lysis weight (g)	% of lysis
SK	0.83±0.01	1.79±0.006	0.95±0.02	1.26±0.05	0.32±0.01	0.65±0.02	68.42
DW	0.83±0.01	1.48±0.01	0.66±0.01	1.43±0.01	0.62±0.01	0.05±0.06	7.58
ME	0.82±0.01	1.64±0.01	0.76±0.06	1.33±0.01	0.51±0.02	0.25±0.06	32.89
AC	0.82±0.01	1.60±0.047	0.74±0.02	1.34±0.01	0.53±0.02	0.24±0.06	32.43

SK=Streptokinase as a standard reference, DW=Distill Water as control, ME= Methanol extract, AC= Acetone extract. Values are represented as Mean±SD

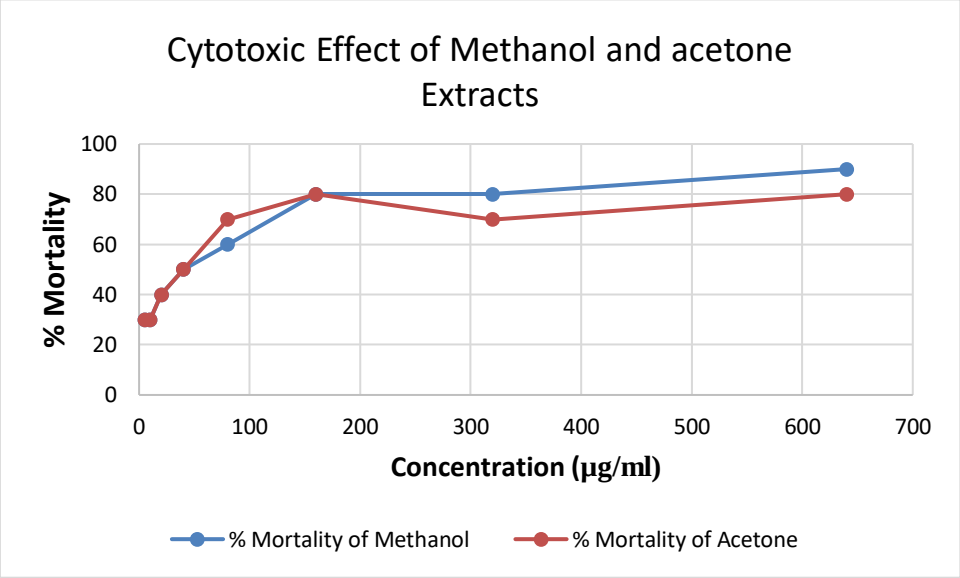

Figure 1: Linear equation: y= 0.0896x+43.125; R^2=0.6975 [Methanol extract]
Linear equation: y= 0.0686x+45.314; R^2=0.5066 [Acetone extract]

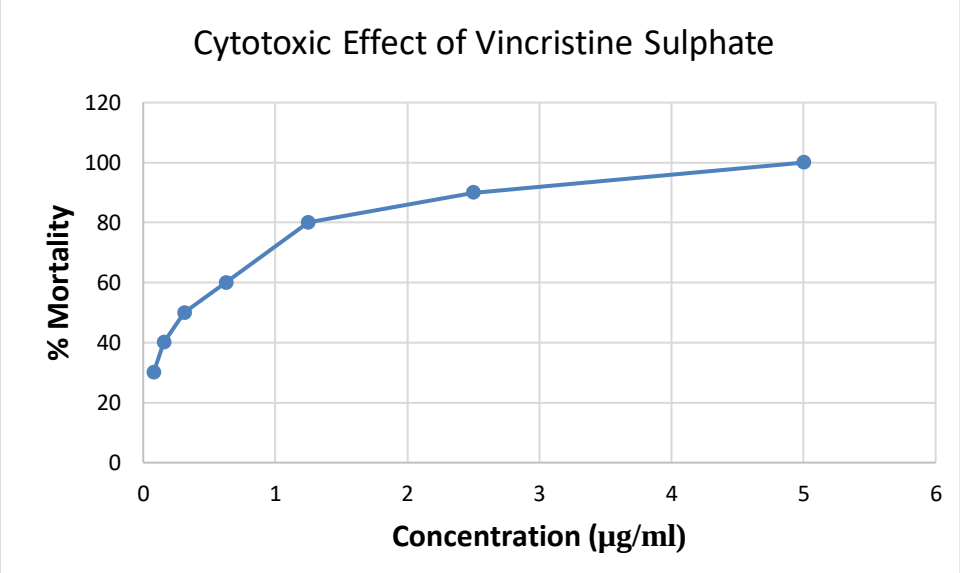

Figure 2: Linear equation: y= 12.917x+45.979; R^2= 0.7707

Both figures are comparison between thecytotoxic effect of samples and standard.

Thrombolytic Activity Assay

Table-3 indicates the results in which 100 µl SK, a positive control (30,000 I.U.), was used for comparison methanolic and acetonic extract of*Limonia acidissima* L. was shown 32.89 and 32.43% clot lysis, compared to control; respectively. Streptokinase used as the standard was shown 68.42 % clot lysis as well. Platelets plays a essential role in the process of formation

of thrombus by adhering to be damaged regions of the endothelial surface. The activated platelets form platelets to platelets bonds and apart from bind to the leucocytes and bring off them into a perplex method of plaque formation as well as growth [21]. Streptokinase forms a 1:1 stochiometric complex with plasminogen which is cpable converting additional plasminogen to plasmin [22].

CONCLUSION

The ethnopharmacological study is beneficial to guidence the search for plants with potential cytotoxic activity. This study was revealed that extracts*Limonia acidissima* L. of that could be used as a source for cytotoxic activity as well as clot lysis activities.

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