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Md. Ashrafudoulla
Manarat International University, Dhaka, Bangladesh

Nazneen Ahmed Luna
Manarat International University, Dhaka, Bangladesh

Fahmida Hoque Jyothy
Manarat International University, Dhaka, Bangladesh

Taslima Khatun
Manarat International University, Dhaka, Bangladesh

Tabia Fatima Rafa
Manarat International University, Dhaka, Bangladesh

Salma Parvin
Assistant Professor, Manarat International University, Dhaka, Bangladesh

Md. Siddiqul Islam
Assistant Professor, Department of Pharmacy Southeast University, Dhaka, Bangladesh

Correspondence
Md. Siddiqul Islam
Assistant Professor, Department of Pharmacy Southeast University, Dhaka, Bangladesh

Phytochemical and Pharmacological investigation of *Calotropis gigantea*

Md. Ashrafudoulla, Nazneen Ahmed Luna, Fahmida Hoque Jyothy, Taslima Khatun, Tabia Fatima Rafa, Salma Parvin, Md. Siddiqul Islam

Abstract
Calotropis gigantea belonging to the family Asclepiadaceae has been investigated for the presence of its secondary metabolites and evaluation of biological activities of the crude extracts with special emphasis to the brine shrimp lethality bioassay, thrombolytic activity and antimicrobial activity.
Approximately 850 gm of the powder of whole plant of *Calotropis gigantea* was soaked with ethanol and kept for 7 days and then filtered. For the evaporation of the ethanol, the obtained filtrate was kept in an open air. The concentrated ethanol extract was then stored for further uses.
The preliminary phytochemical studies are represented by the testing of different chemical groups which are present in the extract. The chemical group tests are performed by 10% (w/v) solution of *Calotropis gigantea* extract in ethanol. Alkaloid group, reducing sugar group and tannin group was founded in the group 1. In Brine Shrimp Lethality Bioassay, after 18hrs later the LC_{50} and LC_{90} of n-hexane extract of the *Calotropis gigantea* were 106.09µg/ml and 248.54µg/ml, after 21hrs later 77.37 µg/ml and 225.02µg/ml and after 24hrs later 31.75 µg/ml and 175.17µg/ml. After 18hrs later the LC_{50} and LC_{90} of CCl4 extract were 80.76 µg/ml and 216.35µg/ml, after 21 hrs later 41.44 µg/ml and 173.23µg/ml, after 24hrs later 6.841 µg/ml and 135.37µg/ml. After 18hrs later the LC_{50} and LC_{90} of crude extract were 43.92µg/ml and 136.02 µg/ml, after 21hrs later 20.59 µg/ml and 128.00 µg/ml and after 24hrs later 2.08 µg/ml and 122.35 µg/ml respectively.
The percentages found in thrombolytic test are 41.81%, 22.47%, 51.60%, 42.74% and standard: 93.29%. So, in comparison with standard *Calotropis gigantea* can be further used as a mild thrombolytic agent. The extract of the plant also showed activity against a wide variety of microorganism tests. All the activities were compared by measuring the zone of inhibition with the standard antibiotic. Based on the findings of Antimicrobial, thrombolytic and toxicological activity, it can be said from the obtained results that the plant can be used as traditional medicine.

Keywords: Phytochemical, Pharmacological, *Calotropis gigantean*, Thrombolytic Effect, Brine Shrimp Lethality.

Introduction
Phytochemicals are chemical compounds such as reducing sugar (carbohydrate), tannins, saponins, and alkaloids etc. that occur naturally in plants. Generally, those chemicals which may affect the health is referred as phytochemical. Due to the increase of use rate of herbal medicine, it is being popular gradually in the different parts of the world (Habibur *et al*, 2016) [23]. To explain the medicinal and pharmaceutical capability of plant extract phytochemical and pharmacological examination is very important (Zarrin Tasnim Rafa *et al*, 2016) [26]
There is not adequate proof to suggest these affects are due to some specific phytochemicals, while there is adequate evidence to support the health benefits of a diet rich in vegetable and fruits.
The ethanol extract of *Calotropis gigantea* was subjected to be solvent Partition, a method based on the relative solubility of an analyte in two immiscible liquids. The method is performed to remove interferences, concentrate species prior analysis and to produce measurable form of a species.
Formation of a blood clot or the presence of blood clot in the blood vessel (i.e. vain or artery) is called thrombosis. The clot itself referred as thrombus. When these blood clots are break loose and travel throughout the blood stream is called thromboembolism. Thrombosais, thrombus, thrombo comes from the Greek word “thrombos” which means “clot of milk” or “a clump or lump.”

Pharmacologically thrombolysis means breakdown or lyses of blood clots. The term thrombosis comes from two Greek words which mean “clot” and “loosening”. For this reason colloquially it is referred as clot busting. It works by stimulating fibrinolysis by plasmin.

The term "thrombolytic" comes from two Greek words which mean "clot" and "loosening." Dissolving blood clot by using drugs is called thrombolytic therapy. First the plasminogen enzyme is activated through the thrombolytic agents and clears the cross linked fibrin mesh (the backbone of a clot). Thus the clots become soluble and through other enzyme further proteolysis is occurring which restore blood flow over blocked blood vessels. (Reynolds, J. E. F., 1996).

In the assay procedure of the extraction of natural product and bioactive compound, brine shrimp lethality bioassay is a recent development. Brine shrimp lethality bioassay indicates cytotoxicity and wide range of pharmacological activity for e.g. antiviral, anticancer, and pharmacological activities of natural product. (Anderon *et al*, 1988)

In high dose biologically toxic compounds are always lethal. Pharmacology is simply toxicology at a lower dose or toxicology is simply pharmacology at a higher dose. Thus (*in-vivo*) lethality, a simple zoological organism (brine shrimp napulii-Artemia salina) can be used as a convenient monitoring for screening and fractionation in the discover of new bioactive natural products (Hui *et al*, 1990).

In English the genus Artemia of aquatic crustaceans is known as brine shrimp. Artemia is only the genus in the family Artemiidae which have manifested little since the Triassic period. The historic evidence of existence of Artemia more than one thousand years ago, which was dated back to 982, from Iran, Lake Urmia, while Schlösser only the person who scratched drawings of Artemia in 1756. Artemia is found worldwide generally in inland lakes of saltwater, but not in the oceans. (Alireza Asem May 6, 2008).

Brine shrimp eggs are not active metabolically and for two years can remain in total stasis in dry oxygen-free conditions, even below freezing temperature. This characteristic is called “cryptobiosis” which means "hidden life" and also known as diapauses. In cryptobiosis, eggs of brine shrimp can survive in liquid air temperatures of (−190 °C, −310 °F) and only a very few eggs can survive above boiling temperature for up to two hours which is about 105 °C, 221 °F.

The cyst-like eggs within a few hours get hatch after putting in brine (salt) water. Brine shrimp can grow to a mature length of all most one centimeter on average through their biological life cycle which is about one year. This short life span, they become able to remain dormant for long periods, and for those characteristics they are invaluable in scientific research and space experiments. (Whitey Hitchcock, March 13, 2010).

Antimicrobial drugs are drugs designed to kill, or prevent the growth of microorganisms (bacteria, fungi, and viruses). Bacteria, fungi, and viruses are responsible for almost all of the common infectious diseases. Plants are the natural reservoir of many antimicrobial agents. In recent times, traditional medicine as an alternative form of health care and to overcome microbial resistance has led the researchers to investigate the antimicrobial activity of medicinal plants (Austin *et al*., 1999).

Methods

Plant collection and Identification

Leaves *Calotropis gigantea* were collected from Dhaka District of Bangladesh in May 2010 and were identified by the Bangladesh National Herbarium, Mirpur, Dhaka. One voucher specimen was deposited in Bangladesh National Herbarium.

Preparation of Extract

Collected leave of *Calotropis gigantea* was segregated from all undesirable materials and then dried under shade in open air for 15 to 20 days. Then the dried leaves were crushed by a suitable grinder. In an airtight container the crushed powder was stored and kept in a dry, dark and cool place until analysis commenced. In a clean-glass container about 850 gm of dried leaf powder were taken and socked with about 4 litters of ethanol. Then the glass container was sealed for 15 days accompanying with casual stirring and shaking. After the 15 days the whole mixture was filtrate by using a piece of white and clean cotton material. Then the filtrate was again filter by using Whatman filter paper. The final filtrate which was obtained was evaporated by using ceiling fan and water bath. After a complete evaporation the filtrate became dried and a dark greenish color was observed. That extract of dark greenish color was designated as a crude extract of ethanol.

Percentage of Yielding

850sgm of powdered *Calotropis gigantea* was taken and after evaporation it yields 16gm of *Calotropis gigantea*

So, percent yield is {(16/850) X 100%} =1.88%

Preparation of Mother Solution

10 ml distilled water was added to 80 ml of ethanol and this solution was used to triturate 7gm of ethanol extract. Then the extract was completely dissolved and this is the mother solution. By using three solvent of different polarity this mother solution was partitioned off successively. Then each fraction was analyzed separately in the posterior stages for the identification and detection of compounds having cytotoxic and antibacterial property.

Partitioning with n-Hexane

In a separating funnel the mother solution was taken. Then 100 ml of the n-hexane was added to the mother solution and shaken the funnel and after then kept undisturbed. The organic portion was collected. The process was repeated thrice; n-hexane fractions were collected together and evaporated into the air until dried.

Partitioning with Carbon tetrachloride

The mother solution which was left after washing with n-hexane was mixed with 12.5 ml of distilled water and then taken in a separating funnel which was extracted with CCl4 (100 ml). The CCl4 fractions were collected together and evaporated. The aqueous fraction was preserved for the next step.

Partitioning with chloroform

The mother solution which was left after washing with n- hexane and CCl4 was diluted by adding 16 ml of distilled water and mixed uniformly. Then in a separating funnel the mother solution was taken and extracted with Chloroform (100 ml X 3). The chloroform soluble fractions were collected together and evaporated. The aqueous ethanolic fraction was preserved as aqueous fraction.

Plant	Fraction	Weight
Calotropis gigantea	n-hexane soluble fraction	3.20gm
	Carbon tetrachloride soluble fraction	1.86gm
	Chloroform soluble fraction	0.77gm
	Aqueous fraction	1.10gm

The percentages (%) of the yielding are given bellow:

- n-hexane 45.71%
- Carbon tetrachloride 26.57%
- Chloroform soluble fraction 11%
- Aqueous 15.71%

Chemical Group Tests for the Extracts

Preliminary phytochemical studies were completed by testing different chemical groups present in the plant extract. The chemical group tests, which were performed as follows in each particular test 10% (w/v) solution of plant extract in ethanol was taken.

Tests procedure for identifying different chemical groups

Tests for Reducing Sugar

Benedict's test: 5 ml Benedict's solution was added to 0.5 ml of solution of plant extract in a test tube and boiled for 5minutes and then cooled spontaneously. A red color precipitate of cuprous oxide should form in the presence of a reducing sugar. According to this test reducing sugar was absent.

Fehling's Test (Standard Test): Fehling's solution A and B were mixed together in equal volume and boiled for a few minutes. From this mixed Fehling's solution 1 ml was added with 2 ml of aqueous plant extract. A red or brick red color precipitate was formed in the presence of a reducing sugar. According to this test reducing sugar was Present.

Alpha Napthol Solution Test

2 drops of 5% Alpha-Napthol solution (Freshly prepared) mixed with 5 ml of aqueous plant extract in a test tube and 1ml of sulfuric acid was added to it. The junction of two liquids a violet color ring was formed in the presence of reducing sugars. According to this test reducing sugar was Present.

Tests for Tannins

Ferric Chloride Test

1ml of 5% Ferric chloride solution was added with 5 ml solution of the aqueous plant extract in a test tube. Greenish black precipitate was formed and indicated the presence of tannins. According to this test tannin was present.

Potassium dichromate Test

1ml of 10% potassium dichromate solution was added with 5 ml solution of the extract in a test tube. A yellow precipitate was formed in the presence of tannins. According to this test Tannin was Present.

Test for Flavonoids

In the alcoholic extract of plant material two to three drops of concentrated HCl acid were added. Immediate development of a red color indicates the presence of flavonoids. According to this test flavonoid was absent.

Test for Saponins: in a cylinder 1ml solution of plant extract was diluted with distilled water up to 20 ml and graduated it for 15 minutes. One centimeter layer of foam indicates the presence of saponins. According to this test Saponin was present.

Test for Gums: in a test tube 5ml solution of plant extract was taken and Molisch reagent and sulphuric acid were added to it. Red violet ring produced at the junction of two liquids indicate the presence of gums and carbohydrate. According to this test Gum was absent.

Test for Steroids

Libermann-Burchard Test: 2 ml Libermann-Burchard reagent was added to 1 ml solution of chloroform extract. Reddish purple color indicates the presence of steroids. According to this test Steroid was absent.

Sulphuric acid Test: 1 ml of sulphuric acid was added to 1 ml solution of chloroform extract. Red color indicates the presence of steroid. According to this test Steroid was absent.

Test for Alkaloids

Mayer's Test: In a test tube 0.2 ml of dilute hydrochloric acid was added to 2 ml solution of the extract. Then 1ml of Mayer's reagent was added. Yellow precipitate was formed and that was indicated as the presence of alkaloids. According to this test Alkaloids were Present.

Wagner's Test: 2 ml solution of the extract and 0.2 ml of dilute hydrochloric acid were taken in a test tube. Then 1 ml of iodine solution (Wagner's solution) was added. Reddish brown precipitate indicates the presence of alkaloids. According to this test Alkaloids were absent.

Dragendroff's Test: 2 ml of the extract and 0.2 ml of dilute hydrochloric acid were taken in a test tube. Then 1 ml of Dragendroff's reagent was added. Orange brown color precipitation indicates the presence of alkaloids. According to this Alkaloids were Present.

Hager's Test: 2 ml solution of the extract was taken in a test tube and 0.2 ml of dilute hydrochloric acid was added with the solution. Than 1 ml picric acid solution which is also known as Hager's reagent was added to it. A yellowish precipitate was observed, which indicated the presence of alkaloid. According to this Alkaloids were Present.

Thrombolytic Effect of *Calotropis gigantean*

Streptokinase (SK): human plasminogen can be bind and activated through a protein which is secreted by several species of streptococci. Streptokinase is considered as inexpensive and effective clot-dissolving medication and is also used in case of pulmonary embolism and myocardial infarction (heart attack). Streptokinase belongs to a group of medications known as fibrinolytics and complexes of streptokinase with human plasminogen can hydrolytically activate other unbound plasminogen by activating through bond cleavage to produce plasmin. α (residues 1–150), β (residues 151–287), and γ (residues 288–414) are granted as the three domains to Streptokinase and each domain has the binding ability with plasminogen, although independently none of these can activate plasminogen.

Herbal/Plant Sample Preparation

10 ml of distilled water was mixed with 100 mg of extract and in a vortex mixer the mixture was shaken. Then it was kept for overnight. The soluble supernatant was decanted and filtered. 100 µl of this aqueous preparation was added to each micro centrifuge tubes.

Blood Specimen Preparation

(n = no. of plant /herb extract = *Calotropis gigantea)* 5 micro centrifuge tubes were taken, sterilized, weighed. (Let n= 1).5 ml blood was drawn from volunteer. Pre weighed (W_1) 5 different micro centrifuge tubes were taken and in each tube the blood was distributed by 1 ml. The blood specimen was centrifuged at 2500 rpm for 5 minutes. Incubated the blood for 45 minutes a 37 ºC. After clot formation i.e. incubation; after incubation the serum of the blood was removed through capillary absorption or decantation. After withdrawal of the serum from the inner surface of the tube, again confirmed the complete withdrawal of serum by use of cotton bound without disrupting the clot. This process ensures complete removal of serum otherwise the result will be erroneous. Then on a tray for 6 minutes kept the tubes at lying position after the first removal of serum and then by using a cotton rod liquids were removed from the tube surface. Each tube was weighed (W_2) again. Weight of the clot was found as, weight of clot = weight of clot containing tube (W_2) -weight of the tube alone (W_1). The weight of the test tubes was taken very carefully so that the result can't vary because of inappropriate weighing. To each micro centrifuge tube containing pre-weighed clot, 100µl of aqueous extract of the plant (*Calotropis gigantea*) was added separately. As a positive control, 100µl of streptokinase was added to clot of tube no. 5 (Standard). As a negative control, 100µl water is added to clot of tube no.4 (Blank)

Brine Shrimp Lethality Bioassay of *Calotropis gigantea*

Test Organism

Artemia saline Leach (brine shrimp). The egg of the shrimp was collected from the Katabon University Market.

The study was accomplished consequent to the Brine shrimp lethality bioassay method. (Meyer, et al, 1982)

Preparation of Stock Solution

4 mg of the dried crude extract was taken in 10 ml volumetric flask and volume was adjusted by 5ml water and then the concentration of that solution was 400 µg /µl. Once this stage was completed 100 µl DMSO solution was added to it. Likewise, we made concentration of 400 µg/µl like as n-hexane, CCl_4, and Fr-1.

Preparation of Simulated Sea Water

20 g of NaCl and 18 g of table salt were weighed accurately, dissolved in distilled water to make one liter and then filtered off to get a clear solution.

Hatching of Brine Shrimp

In a small tank of two deviations the prepared see water was taken and on the one side of the tank shrimp age were added. It had been taken about 24 hours to hatch or mature these shrimp egg as nuplii (larvae). Through the piercing in dam the hatched shrimps were attracted to the lamp and then they were taken for bioassay. The time of hatching was about 24 hours.

Application of Test Solution and Brine Shrimp Nauplii to the Test Tubes

For crude extract

Seven (07) clean test tubes were taken, sample of different concentration was taken six (06) test tubes and one was for the negative control test. Then in the first test tube ½ (half) volume of the stock solution was taken and 100 µl DMSO solution was added to it and thirdly 2.5ml of sea water was added to that test tube. The same process was followed for the next test tube and negative control test tube and these test tube contain 5ml distill water. The concentration of those six test tubes content, respectively 200 µg/µl, 100 µg/µl, 50 µg/µl, 25 µg/µl, 12.5 µg/µl, 6.75 µg/µl, 3.125 µg/µl. Finally, with the help of a Pasteur pipette 15 living shrimps were kept for each of the test tubes (Meyer et al. 1982).

For n-hexane, CCl_4, and Fr-1 solution was prepared by the following above this procedure.

Counting of Nauplii

After 18hrs each test tube was observed and the number of total survived nauplii in each test tube was counted and the results were noted. Form this, for each sample the percentage of brine shrimp nauplii lethality was calculated at each concentration. After 24 hours the same process was again followed for calculating the percentage of brine shrimp nauplii lethality.

Antimicrobial Screening of *Calotropis gigantea*

For preliminary screening of test agents, disc diffusion process (Bauer et al., 1966) is an *in-vitro* investigation that may reflect antimicrobial activity and it is a widely accepted process. It is considered as essential qualitative and quantitative test which indicate the resistance or sensitivity of the microorganisms to the test materials. However, through this method no distinction was made between bactericidal and bacteriostatic activity (Roland, R., 1982).

Principle of Disc Diffusion Method

Disk diffusion method is a classical method. In this method, antibiotics diffuse through the nutrient agar gel from a limited source and create a concentration gradient. Sterilized and dried filter paper discs (6 mm diameter) which contain the test samples of known and specific amounts are kept on nutrient agar medium indiscriminately seeded with the test microorganisms. Standard antibiotic (Kanamycin) discs and blank discs are used as positive and negative control. To allow maximum diffusion of the test these plates are kept for 24 hours at low temperature which is about 4 °C.

Then the plates are inverted and incubated for about 24 hours at 37 °C for optimum growth of the organisms. Microbial growth in the media surrounding the discs is inhibited through the test materials containing antimicrobial activity and thereby a clear, distinct area is observed which is defined as a zone of inhibition. The test agent's antimicrobial activity was determined by using a measuring diameter of zone of inhibition and the zone of inhibition is expressed in millimeter (Lester, 1972; Bary, 1976; Bauer et al, 1966)

In the present study the crude extracts, fractions as well as some pure compounds were tested for antimicrobial activity by disc diffusion method. In order to calculate the mean reading, the experiment is carried out more than once (Bayer et al., 1966).

Results

The result of Chemical Group Tests for the Extracts has shown in table 1.

Table 1: Different Chemical group tests results

Test for Reducing sugar	Result
1. Benedict's Test	-
2. Fehling's Test	+
3. Alpha Napthol Solution Test	+

Test for Tannins	Result
1.Ferric Chloride Test	+
2. Potassium dichromate Test	+

Test	Result
1. Test for Flavonoids	-
Test	Result
1. Test for Saponins	+
Test	Result
1. Test for Gums	-
Test for Steroids	Result
1. Libermann-Burchard Test	-
2. Sulphuric acid Test	-
Test for Alkaloids	Result
1. Mayer's Test	+
2. Wagner's Test	-
3. Dragendroff's Test	+
4. Hager's Test	+

The result showed that the extract of leaves contain, saponins, alkaloids and tannin. So the plant is rich of polar compound.

Approximately all the samples of *Calotropis gigantea* had been found to contain alkaloids, Saponins and tannin. Alkaloids being bitter substances exert notable antimicrobial actions. So it is quite reasonable that the plant containing alkaloids exert beneficial therapeutic effects against infectious diseases for which it is used. Tannins are not only remarkable for their antiseptic property, but also for their astringent actions. This astringent property affords them the therapeutic value in arresting hemorrhage by constricting blood vessels and in protecting wounds, inflammations and ulcers form external irritations by precipitating surface protein which forms an impervious coating on them. Thus, it is evident that the constituents (alkaloids, saponins) are sufficient to cure infections tannins are also responsible to cure inflammatory disease.

Thrombolytic Effect of *Calotropis gigantean*

Thrombolytic Effect of *Calotropis gigantean* has represented in figure 1.

Data analysis: Experiment

No of sample	W_1 (gm)	W_2 (gm)	W_3 (gm)
Sample 1	5.9321g	6.5022g	6.2638g
Sample 2	6.0840g	6.5743g	6.4641g
Sample 3	6.0545g	6.5004g	6.2703g
Standard	6.0711g	6.2351g	6.0820g
Blank	6.0792g	6.5571g	6.3528g

Calculation

% of clot lysis = (wt of released clot /clot wt) × 100

$= (W_2 - W_3 / W_2 - W_1) \times 100$

Sample 1 (ethanol extract)

% of clot lysis = $(W_2 - W_3 / W_2 - W_1) \times 100$

= 41.81%

Sample 2 (n-hexane)

% of clot lysis = $(W_2 - W_3 / W_2 - W_1) \times 100$

= 22.47%

Sample 3 (CCl_4)

% of clot lysis = $(W_2 - W_3 / W_2 - W_1) \times 100$

= 51.60%

Standard (Streptokinase)

% of clot lysis = $(W_2 - W_3 / W_2 - W_1) \times 100$

= 93.29%

Blank:

% of clot lysis = $(W_2 - W_3 / W_2 - W_1) \times 100$

= 42.74%

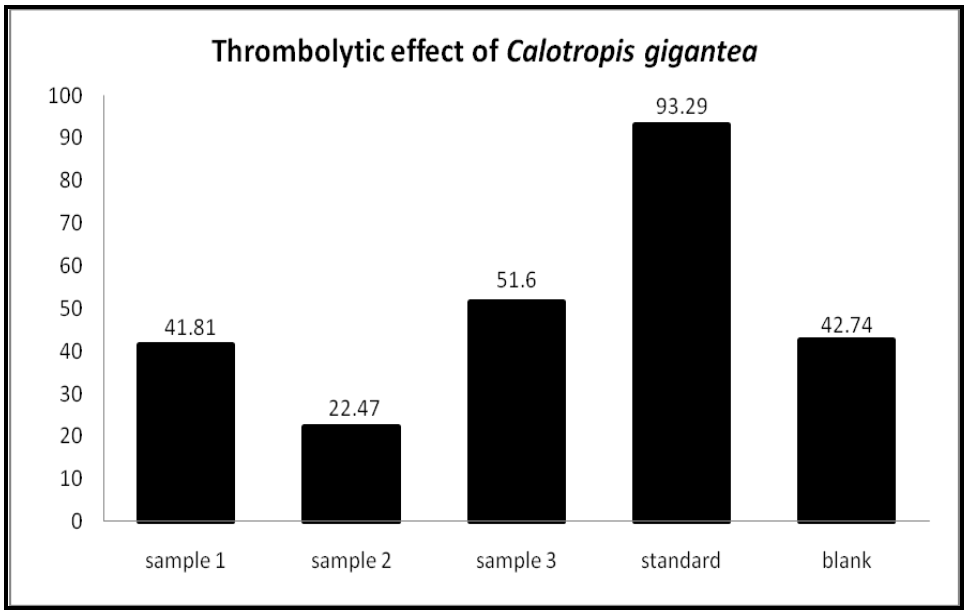

Fig 1: Graphical representation of thrombolytic effect of *Calotropis gigantea*

The percentages found in thrombolytic test were- sample1**:** 41.81%, sample2**:** 22.47%, sample3**:** 51.60%, blank**:** 42.74% and standard (streptokinase)**:** 93.29%.

The thrombolytic activity of standard found 93.29% and the ethanol, n-hexane and CCl_4 extracts of *Calotropis gigantea* were found 41.81%, 22.47%, and 51.60% respectively, which indicates mild thrombolytic activity. So, in comparison with standard *Calotropis gigantea* can be further use as mild thrombolytic agent.

Brine Shrimp Lethality Bioassay of *Calotropis gigantean*
Results have shown in table 2, 3, 4, 5, 6, 7, 8, 9 and 10 with figure

Table 2: After 18 hours later result of Brine shrimp lethality bioassay of distilled crude extracts of the leave *Calotropis gigantea*

Conc. of Extract µg/ml	No of Brine shrimp inserted	No of Alive Brine Shrimp	No of death Brine shrimp	% Mortality	Log Conc. µg/ml	LC_{50} µg/ml	LC_{90} µg/ml
0(blank)	15	14	1	6.66	∞	43.92	136.02
200	15	0	15	100	2.30		
100	15	0	15	100	2		
50	15	5	10	66.66	1.698		
25	15	7	8	53.33	1.397		
12.5	15	9	6	40	1.096		
6.75	15	10	5	33.33	0.829		
3.125	15	12	3	20	0.495		

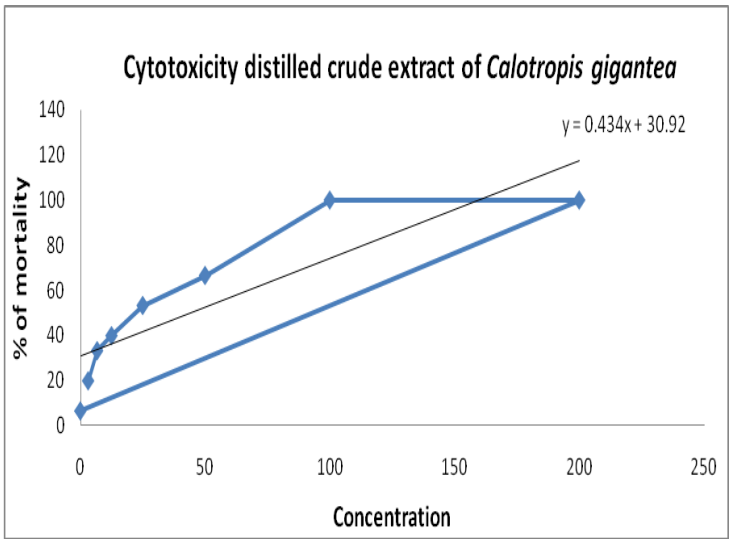

Table 3: After 18 hours later result of Brine shrimp lethality bioassay of distilled n-hexane extracts of the leave *Calotropis gigantea*

Conc. of Extract µg/ml	No of Brine shrimp inserted	No of Alive Brine Shrimp	No of death Brine shrimp	% Mortality	Log Conc. µg/ml	LC50 µg/ml	LC_{90} µg/ml
0(blank)	15	14	1	6.66	∞	106.09	248.54
200	15	5	10	66.66	2.30		
100	15	6	9	60	2		
50	15	8	7	46.66	1.698		
25	15	10	5	33.33	1.397		
12.5	15	11	4	26.60	1.096		
6.75	15	12	3	20	0.829		
3.125	15	13	2	13.34	0.495		

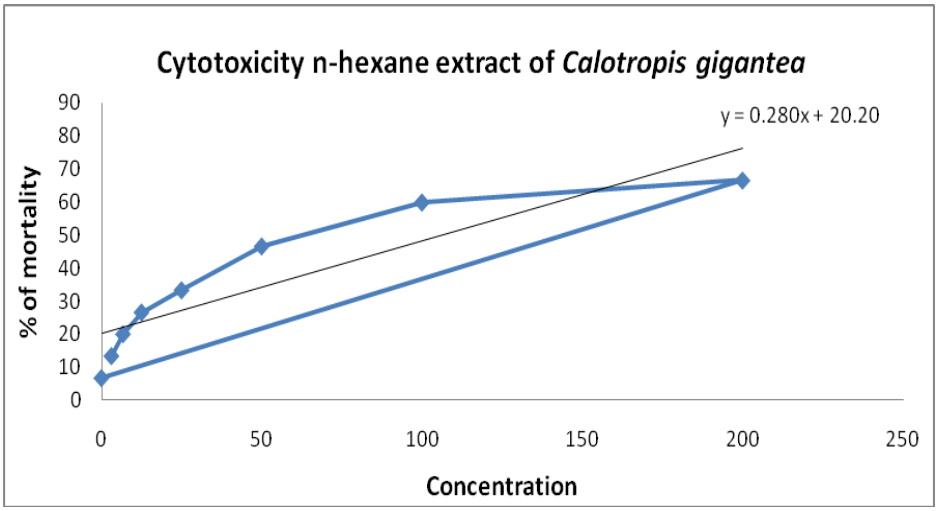

Table 4: After 18 hours later result of Brine shrimp lethality bioassay of distilled **CCl_4** extracts of the leave *Calotropis gigantea*

Conc. of Extract µg/ml	No of Brine shrimp inserted	No of Alive Brine Shrimp	No of death Brine shrimp	% Mortality	Log Conc. µg/ml	LC_{50} µg/ml	LC_{90} µg/ml
0(blank)	15	14	1	6.66	∞	80.76	216.35
200	15	4	11	73.33	2.30		
100	15	5	10	66.66	2		
50	15	6	9	60	1.698		
25	15	8	7	46.66	1.397		
12.5	15	10	5	33.33	1.096		
6.75	15	11	4	26.66	0.829		
3.125	15	13	2	13.34	0.495		

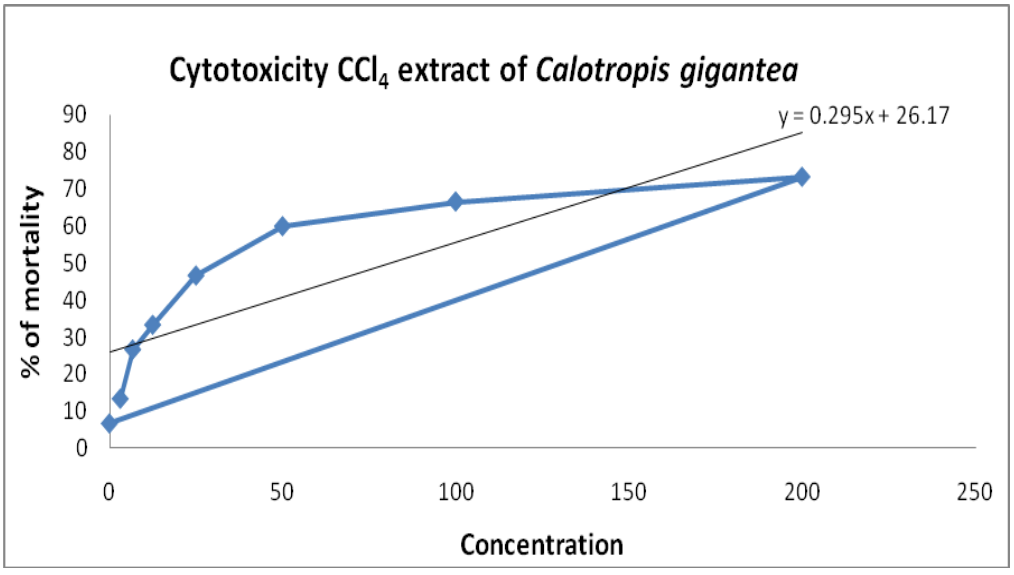

Table 5: After 21 hour later result of Brine shrimp lethality bioassay of distilled crude extracts of the leave *Calotropis gigantea*

Conc. of Extract µg/ml	No of Brine shrimp inserted	No of Alive Brine Shrimp	No of death Brine shrimp	% Mortality	Log Conc. µg/ml	LC_{50} µg/ml	LC_{90} µg/ml
0(blank)	15	12	3	20	∞	20.59	128.00
200	15	0	15	100	2.30		
100	15	0	15	100	2		
50	15	3	12	80	1.698		
25	15	5	10	66.66	1.397		
12.5	15	7	8	53.33	1.096		
6.75	15	9	6	40	0.829		
3.125	15	11	4	26.66	0.495		

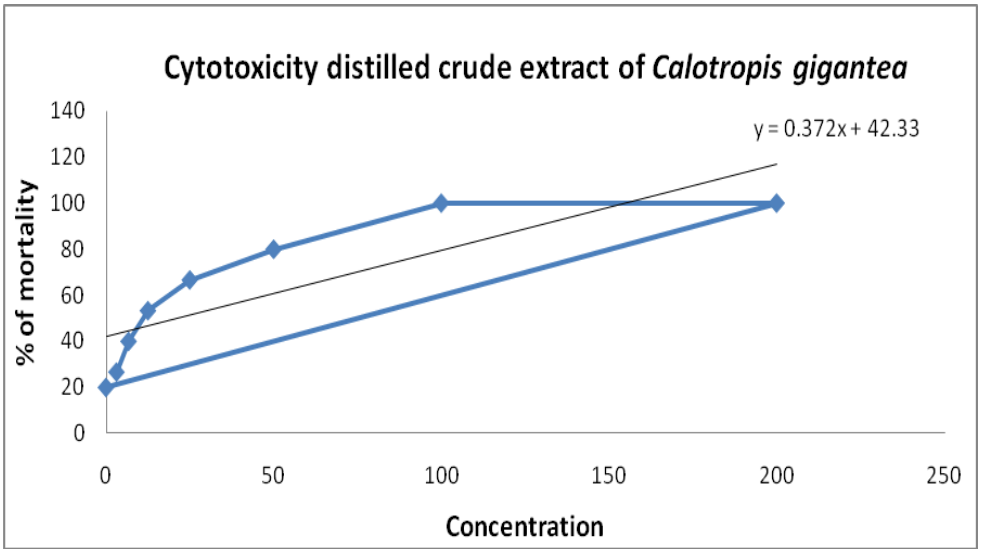

Table 6: After 21 hours later result of Brine shrimp lethality bioassay of distilled n-hexane extracts of the leave *Calotropis gigantea*

3	**No of Brine shrimp inserted**	**No of Alive Brine Shrimp**	**No of death Brine shrimp**	**% Mortality**	**Log Conc. µg/ml**	**LC_{50} µg/ml**	**LC_{90} µg/ml**
0(blank)	15	12	3	20	∞	77.37	225.02
200	15	4	11	73.33	2.30		
100	15	5	10	66.66	2		
50	15	6	9	60	1.698		
25	15	9	6	40	1.397		
12.5	15	10	5	33.33	1.096		
6.75	15	11	4	26.67	0.829		
3.125	15	12	3	20	0.495		

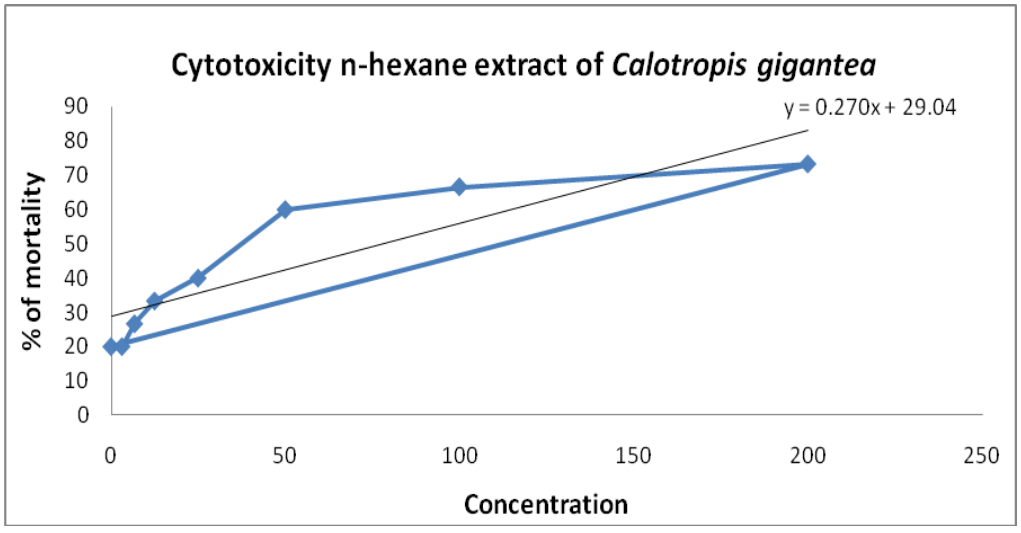

Table 7: After 21 hours later result of Brine shrimp lethality bioassay of distilled CCl_4 extracts of the leave *Calotropis gigantea*

Conc. of Extract µg/ml	No of Brine shrimp inserted	No of Alive Brine Shrimp	No of death Brine shrimp	% Mortality	Log Conc. µg/ml	LC_{50} µg/ml	LC_{90} µg/ml
0(blank)	15	12	3	20	∞	41.44	173.23
200	15	2	13	86.66	2.30		
100	15	3	12	80	2		
50	15	5	10	66.66	1.698		
25	15	6	8	60	1.397		
12.5	15	8	7	46.66	1.096		
6.75	15	10	5	33.33	0.829		
3.125	15	11	4	26.66	0.495		

Table 8: After 24 hours later result of Brine shrimp lethality bioassay of distilled crude extracts of the leave *Calotropis gigantea*

Conc. of Extract µg/ml	**No of Brine shrimp inserted**	**No of Alive Brine Shrimp**	**No of death Brine shrimp**	**% Mortality**	**Log Conc. µg/ml**	**LC_{50} µg/ml**	**LC_{90} µg/ml**
0(blank)	15	11	4	26.66	∞	2.08	122.35
200	15	0	15	100	2.30		
100	15	0	15	100	2		
50	15	2	13	86.66	1.698		
25	15	4	11	73.33	1.397		
12.5	15	5	9	60	1.096		
6.75	15	8	7	53.33	0.829		
3.125	15	10	5	33.33	0.495		

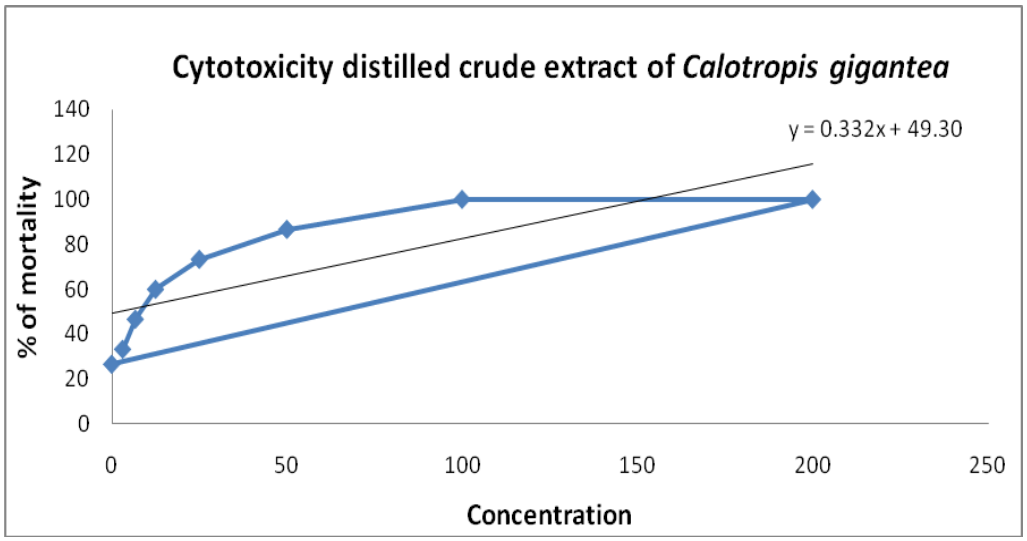

Table 9: After 24 hours later result of Brine shrimp lethality bioassay of distilled n-hexane extracts of the leave *Calotropis gigantea*

Conc. of Extract µg/ml	**No of Brine shrimp inserted**	**No of Alive Brine Shrimp**	**No of death Brine shrimp**	**% Mortality**	**Log Conc. µg/ml**	**LC_{50} µg/ml**	**LC_{90} µg/ml**
0(blank)	15	9	6	40	∞	31.75	175.17
200	15	2	13	86.66	2.30		
100	15	3	12	80	2		
50	15	4	11	73.33	1.698		
25	15	7	8	53.33	1.397		
12.5	15	8	7	46.66	1.096		
6.75	15	10	5	33.33	0.829		
3.125	15	11	4	26.66	0.495		

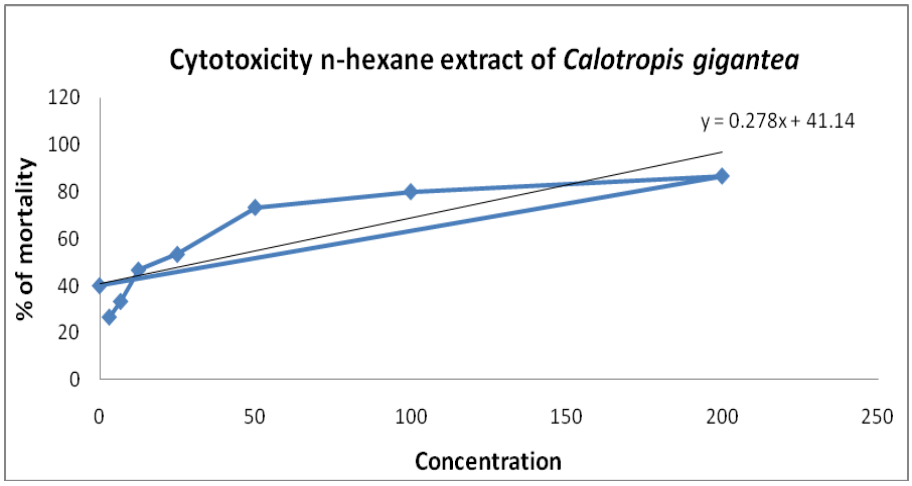

Table 10: After 24 hours later result of Brine shrimp lethality bioassay of distilled CCl_4 extracts of the leave *Calotropis gigantea*

Conc. of Extract µg/ml	No of Brine shrimp inserted	No of Alive Brine Shrimp	No of death Brine shrimp	% Mortality	Log Conc. µg/ml	LC_{50} µg/ml	LC_{90} µg/ml
0(blank)	15	10	5	33.33	∞	6.841	135.37
200	15	0	15	100	2.30		
100	15	2	13	86.66	2		
50	15	3	12	80	1.698		
25	15	4	11	73.33	1.397		
12.5	15	6	9	60	1.096		
6.75	15	9	6	40	0.829		
3.125	15	10	5	33.33	0.495		

Antimicrobial Screening of *Calotropis gigantea*
In table 11 has shown the Antimicrobial activity result

Table 11: Antimicrobial activity of test samples of *Calotropis gigantea*

Microbial antibiotic sensitivity test	Standard	CTCSF	NHSF	EELP
Gram positive bacteria				
Bacillus cereus	40	9	-	9
Staphylococcus aureus	41	9	-	9
Gram negative bacteria				
Escherichia coli	40	9	-	8
Pseudomonas aureus	40	10	-	8
Salmonella typhi	41	10	7	8
Vibrio mimicus	40	9	-	8
Shigella boydii	40	10	-	8
Shigella dysenteriae	40	10	-	8

NHSF: N-Hexane soluble fractions of the ethanolic extract (400 µg/disc)

CTCSF: Carbon tetrachloride soluble fractions of the ethanolic extract (400 µg/disc)

EELP: Ethanolic extract of the leaves of plant (400 µg/disc)
8 mg dissolved in (200µl ethanol) per disc 10µl concentration 400 µg
The n-hexane, carbon tetrachloride soluble fraction of the ethanolic extract exhibited antimicrobial activity against most of the test organisms. The zones of inhibition produced by carbon tetrachloride, n-hexane soluble fraction of the ethanolic extract showed average zones of inhibition (9.5mm), (7mm) and (8.25 mm) respectively at a concentration of 400 µg/disc.

Discussion

Chemical group test

By using the reagents named Mayer's Reagent, Dragendorff's Reagent, Fehling's Solution A, Fehling's Solution B, Benedict's reagent, Molish Reagent, and Lieberman-Burchard Reagent it was found the reducing sugar group, tannin group and alkaloid groups. "The possibility of the presence of phytochemical constituents like tannins, proteins, alkaloids, flavonoides and saponins may increase or decrease according to the condition of areas such as dry and shady areas" (Md. Ashrafudoulla *et al.*, 2016) [20]

Brine shrimp lethality bioassay

The brine shrimp lethality bioassay is a convenient and rapid method for screening of natural products for finding leads for anticancer, antimicrobial, anti HIV drugs and was reasonably

included in the present study. Here the ethanolic extracts of *Calotropis gigantea* have shown to have some lethal effect against the brine shrimp nauplii. In Brine Shrimp Lethality Bioassay, after 18 hrs later the LC_{50} and LC_{90} of n-hexane extract of the *Calotropis gigantea* was 106.09 µg/ml and 248.54 µg/ml, after 21hrs later 77.37 µg/ml and 225.02 µg/ml and after 24hrs later 31.75 µg/ml and 175.17 µg/ml. After 18hrs later the LC_{50} and LC_{90} of CCl4 extract were80.76 µg/ml and 216.35 µg/ml, after 21hrs later 41.44 µg/ml and 173.23 µg/ml, after 24hrs later 6.841 µg/ml and 135.37 µg/ml. After 18hrs later the LC_{50} and LC_{90} of crude extract were 43.92 µg /ml and 136.02 µg/ml, after 21hrs later 20.59 µg/ml and 128.00 µg/ml and after 24hrs later 2.08 µg/ml and 122.35 µg/ml respectively.

Thrombolytic effect of *Calotropis gigantea*

Calotropis gigantea extract has mild thrombolytic activity. The percentages found in thrombolytic test are 41.81%, 22.47%, 51.60%, 42.74% and standard: 93.29%. So, in comparison with standard *Calotropis gigantea* can be further use as mild thrombolytic agent. The extract of the plant also showed activity against a wide variety of microorganism tests. All the activities were compared by measuring the zone of inhibition with the standard antibiotic. Based on the findings of Antimicrobial, thrombolytic and toxicological activity we can firmly say that the results which obtained extremely support for the uses of this plant as traditional medicine.

Antimicrobial screening of *Calotropis gigantea*

The n-hexane, carbon tetrachloride, dichloromethane soluble fraction of the ethanolic extract and crude ethanolic extract exhibited antimicrobial activity against most of the test organisms. The zones of inhibition produced by carbon tetrachloride, n-hexane soluble fraction of the ethanolic extract showed average zones of inhibition (9.5mm), (7mm), and (8.25mm) respectively at a concentration of 400 µg/disc.

Conclusion

The result showed that the extract of leaves contain, saponins, alkaloids and tannin. Successive chromatographic separation and the *n*-hexane, carbon tetrachloride, chloroform and aqueous soluble fractions of ethanolic extract of *Calotropis gigantea* showed significant antimicrobial, cytotoxic activities, chemical group and thrombolytic test which supports the traditional use of this plant in various diseases.

The plant can be further screened against various diseases in order to find out its unexplored efficacy and can be a potential source of chemically interesting and biologically important drug candidates.

Competing interests

The authors declare that they have no competing interests.

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References

1. Farnsworth NR, Akerelo O, Bingel AS. Bull. World Health Organ. 1985; 63(6):965-981.
2. Furie B, Furie BC. Mechanisms of thrombus formation. New England Journal of Medicine. 2008; 359(9):938-949.
3. Ghani A. Medicinal Plants of Bangladesh, published by Asiatic Society, 1st edition, 1998, 13.
4. Goodman, Gillman. The Pharmacological Basis of Therapeutics, McGraw-Hill, 9th edition, USA, 1996, 925.
5. Ghani A. Traditional Medicine, Jahangirnagar University, Savar, Dhaka. 1990; 26-27:39-40.
6. Ghani A. Practical Phytochemistry, Jahangirnagar University, Savar, Dhaka, 1997, 84-89.
7. Graves G. Bracken Book Publisher, London Medicinal Plants, 1990, 6.
8. George M *et al.* J Sci Indust Res. 1947; 63(3):42.
9. Gopalanchari R, Dhar ML. J Sci Indust Res. 1985; 17(B):276.
10. Ghosh A, Sengupta P, Chatterjee S, Ghoshal KK. Probe, 1980; 19(4):270.
11. Gupta SS, Bhagwat AW, Ram AK.
12. PMID: 4144812 [PubMed - indexed for MEDLINE]
13. Handin RI. Chapter 53: bleeding and thrombosis. in Kasper DL, Braunwald E, Fauci AS *et al*. Harrison's Principles of Internal Medicine (16th ed.), 2005.
14. Hasan F. Pakistan J Sci Res. 1962; 14(1):4.
15. Haerdi F, Eingerborenon D. Acta Tropic Suppl. 1964; 8(1):278.
16. Hariharan V, Rangaswami S. Phytochem 1970; 9(2):379.
17. Hiroshi H, Hisanon J, Takemota T. Chem Pharm Bull. 1971; 19(12):438.
18. http://utahreach.org/cache/govt/weedept/pics/fieldguide/ Lannea coromandelica (Houtt.) Mer
19. Tripathi KD. Essentials of Medical Pharmacology, 3rd ed, 2001, 432.
20. Md. Ashrafudoulla, Sm Faysal Bellah, Feroz Alam, Sayed Sakib Faisal, Md. Abdullah Hil Kafi, Farhan Fuad. Phytochemical screening of *Solanum nigrum* L, *S. myriacanthus* Dunal, *Solanum melongena* and *Averrhoa bilimbi* in Bangladesh. Journal of Medicinal Plants Studies. 2016; 4(1):35-38.
21. Tayler VE, Brady LR, Robbers JE. Pharmacognosy, Leu and Febiger, Philadelphia, 9th edition, 1985, 159.
22. Tyeler VD, Brady LR, Robbers JE. Pharmacognosy, Lea and Febiger Publisher, Philadelphia, 9th edition, 1981, 1-3.
23. sMd. Habibur Rahman, Samira Yesmin, Md. Arifur Rahman Chowdhury, Md. Ashrafudoulla, Mehedee Hasan. Pharmacological And Toxicological Evaluation of *Mimosa pudica*. International Journal of Innovative Pharmaceutical Sciences and Research. 2016; 4(2):75-86.
24. Ghani A. Medicinal Plants of Bangladesh: Chemical constituents & uses. Asiatic Society of Bangladesh. 2nd edition, 2003; 381:1-16.
25. Goldstein, Principles of drug action-the basis of pharmacology, 2nd edition, 1974, 736.
26. Zarrin Tasnim Rafa, Md. Ashrafudoulla, Farhan Fuad, Rafiqul Islam, Mehedee Hasan, Md. Abdullah Hil Kafi *et al*. Phytochemical and pharmacological investigation of *Plumbago indica* L. Journal of Medicinal Plants Studies. 2016; 4(2):115-118.