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Original Research Article

A Study on *Litsea monopetala* for Evaluation of Pharmacological Activities

Md. Shohel Hossain[1], Mohammad Tarek Aziz[2], Khondoker Md. Akter-uz-zaman Sumon[1], Md. Ibrahim[3*], Abida Sultana[4], Nane Gopal Sarker[5], Palash Das[3], Md. Masudur Rahman[3], Md. Jahirul Islam[3], Mohammad Hasem Babu[3]

[1]Department of Pharmacy, Manarat International University, Bangladesh
[2]Department of Pharmacy, State University of Bangladesh, Bangladesh
[3]Department of Pharmacy, Atish Dipankar University of Science and Technology, Bangladesh
[4]Department of Applied Chemistry and Chemical Engeering, Noakhali Science and Technology University, Bangladesh
[5]Department of Pharmacy, University of Development Alternative, Bangladesh

***Corresponding Author:**
Md. Ibrahim
Email: md.ibrahim@adust.edu.bd

Abstract: The aim of this study was to evaluate the cytotoxicity, thrombolytic, anthelmintic and antioxidant activity of mehanolic extract of *Litsea monopetala* (Family: Lauraceae) leaves in laboratory using in vitro methods. Cytotoxicity test was done by brine shrimp lethality bioassay where the extract concentration was 400, 200, 100, 50, 25, 12.5, 6.25, 3.125, 1.5625, 0.78125 (µg/ml). *In vitro* thrombolytic activity was performed by clot lysis method using extract concentration 2.5, 5, 10 and 20 (mg/mL) in saline water. Anthelmintic activity test was done by using adult earthworms where 10, 20, 40, 60, 80 (mg/ml) extract concentration were used. Finally antioxidant activity was determined by total phenolic content determination using Folin-Ciocalteu reagent. The crude extract of *Litsea monopetala* showed cytotoxic activity against brine shrimp nauplii and LC50 value was 41.05(µg/ml) and the investigated thrombolytic activity in our research was 9.52,9.49, 13.64 and 17.50 % respectively as % of clot lysis. The paralysis time were at 76.75 min, 60min, 51.75 min, 44.5 min and 64.5 min and death were at 90.50min, 63.75min, 55.50min, 44.75min and 71min. respectively. The crude extract displayed significant antioxidant activity which was 20.75 (mg of GAE / gm) of extracts. The activities observed could be attributed to presence of some of the phytochemicals which have been related with cytotoxic, thrombolytic, anthelmintic and antioxidant property.

Keywords: *Litsea monopetala*, cytotoxic, thrombolytic, anthelmintic and antioxidant activity.

INTRODUCTION

Plant sources contain large amount of bioactive compounds that is beneficial for health as well as provide nutrition to human. For thousands of years people are using plant sources for treating human diseases. Different study suggests that taking diet filled with vegetables and fruits are beneficial for health [1]. According to world health organization 80 percentof world population relies on traditional therapies which are mainly produced from plant extracts or their active components [2, 3]. Approximately 10,000 to 15,000 of world's plants have been enumerated for medicinal value but in western medicine only 150-200 plants are in use [4]. Ninety percent of the medicinal plant are collected from wild source and its number is about 722 though South Asian Subcontinent carry only 2000 medicinal plant [5, 6].

Production of ROS and free radicals is caused due to aerobic metabolism and it is must for normal functioning of the human body [7]. From study it is found that excess level of free radical cause several diseases including: cancer, diabetes, cardiovascular and neurodegenerative diseases [8]. Plant extracts contain a large amount of Butylated hydroxyl anisole (BHA), Butylated hydroxyl toluene (BHT), Propyl Gallate (PG) and Tert-Butyl Hydro Quinone (TBHQ), Ascorbic acid (vitamin-c) that is operative against free radical and for cell survival [9,10]. Antioxidants, such as glutathione, vitamin C, vitamin A, vitamin E, total phenolic content are the major antioxidant for animal and plants [11]. Many studies suggest that plant encompasses many pharmacologically active compounds with limited toxicity to normal cell.

Cardiovascular disease (CVD) is a heart or blood vessel disease which may arise due to blood clot (thrombus) formation. It is responsible for 80% of CVD deaths in males and 75% of CVD deaths in females all over the world [12]. Myocardial infarction, anorexia, hypertension, stroke etc. occurs as a result of thrombus development. Reduction of blood supply to the liver

also enhances due to it. Now-a- daysplant sources for antiplatelet, anticoagulant, antithrombotic and thrombolytic activity are the major concern of research for the researcher. Healthcare providers are using tissue plasminogen activator (t-PA), Urokinase (UK), streptokinase (SK) etc. for clot lysis but sometimes they cause hemorrhage, anaphylactic reaction etc. [13,14].Herbal products are considered safer since ancient times and some studies show that they can exert momentous thrombolytic activity [15, 16]. Formerly it is testified about phytochemistry of herbs and their anti-thrombotic activity [17, 18].

Helminth infections are the common among a large proportion of the world's population. According to World Health Organization 2 million people are diseased by helminths and 100% school aged children become affected by these worms [19]. Nematodes (round worms), the major phyla of helminthes that mostly cause the intestinal infection, onchocerciasis and lymphatic filariasis are the result of filarial worms [20, 21]. Different parts of the body are targeted by worms such as *Ascaris*, *Trichuris*, *Enterobius*, *Strongyloides*, and tapeworms. In developing countries, people of the remote areas specially rural areas are affected mostly [20, 22].

Litsea monopetala (Roxb.) (*L. monopetala*) sometimes known as *Tetranthera monopetala* is under the family Lauraceae, known as meda in Bengali and Hindi language. It is mainly found in evergreen forest in Nepal. But outside Nepal it covers from Kumaon to Sikkim, Bangladesh, Burma and southwest China [23, 24]. In Bangladesh this plant is widely distributed in Chittagong hill tracts, Sylhet and Sal forests of Gazipur, Madhupur, Dinajpur. It is also found throughout the villages of Bangladesh. It is a medium sized tree having up to 18 meters height with a diameter of 60 cm, leaves are 7.5-23 cm long, elliptic-oblong, usually rounded at both ends, pubescent beneath [25]. The bark of this plant has traditional medicinal use as nerves and bone tonic, stomachache, stimulant, analgesic and antiseptic. Traditionally water extract of bark are used with sugar to treat diarrhea and dysentery in Pakistan and India. Pain arising from blows or bruises or from hard work may relief by the use of powder of bark [26]. Roots are also applicable for the management of pain, bruises and contusions as herbal medicine [27]. The leaves possesses several phytochemicals namely alkaloids, carbohydrates, tannins, flavonoids, steroids etc. having antihyperglycemic, antimicrobial, antidiarrheal and anti-inflammatory activities [26].

The present study was conducted to estimate the thrombolytic, cytotoxic, anthelmintic and antioxidant activity of crude methanolic extracts of leaves of *L. monopetala.*

MATERIALS AND METHODS

Chemicals

All of the chemicals used in this study were analytical grade.

Collection and proper identification of plants sample

The plant was collected from the village of Laxmipur district, Bangladesh. It was first identified by the botany department of Noakhali Government University College, Maijdee, Noakhali. Then taxonomical identification was done by Bangladesh National Herbarium Mirpur, Dhaka and 45413 were given as accession number.

Drying and grinding of plant materials

The collected plant parts (leaves) were separated from undesirable materials and washed with water to eradicate adhering dirt. They were sun-dried for one week and then dried in mechanical dryer at 50 – 60^0C. The plant parts were ground into a coarse powder by mechanical grinder and was stored in an airtight container and kept in a cool, dark and dry place until analysis commenced.

Extraction of Plant materials

Powered material having a weight of 400gm ware taken in a clean, flat-bottomed colored glass container and drenched in 2100 ml of methanol at 25^0C. To resist the entrance of air the container with its contents was closed properly and preserved for 7 days accompanying occasional shaking and stirring to get better extraction. Extract was filtered through cotton by decantation and finally through Whatman No. 1 filter paper. Final filtrates were concentrated at 40ºC by a rotary evaporator [28]. It rendered a gummy concentrate of greenish black color. The gummy concentrate was designated as crude extract of methanol.

Cytotoxic activity

Simple zoological organism *Artemia salina* (brine shrimp eggs) as a convenient means was used to determine the cytotoxic activity which is known as brine shrimp lethality bioassay. The brine shrimp eggs were allowed to hatch for two days in artificial sea water (3.8% NaCl solution) and to be matured as nauplii [29, 30]. Four (4mg) of sample was dissolved in 200µl of DMSO and diluted as 400, 200, 100, 50, 25, 12.5, 6.25, 3.125, 1.5625, 0.78125µg/ml by serial dilution in each vial containing 5 ml of saline water (3.8% NaCl solution). 100 µl DMSO diluted to 5 ml of saline water was used as a negative control. Standard vincristine sulphate was used as positive control. The matured nauplii were inoculated to all experimental and control vials. After 24 hours, each vial was inspected to count lived nauplii using a magnifying glass. Obtained data for each concentration were used to calculate the percent mortality by the following equation-

% mortality= (no. of dead nauplii/ initial no. of live nauplii) ×100

Where initial number of live nauplii is 10. The median lethal concentration (LC_{50}) was determined from the graph plotting log of concentration versus percent mortality.

Thrombolytic activity

In vitro thrombolytic activity of *L. monopetala* was performed by clot lysis method using methanolic extract [31]. A standard clot lysis agent known as streptokinase (SK) was used as a positive control and normal saline (0.9% NaCl solution) was used as a negative control. To a commercially available lyophilised streptokinase vial (S-kinase, Popular Pharmaceuticals Ltd, and Bangladesh) of 15, 00,000 IU. 5 ml phosphate buffered saline (PBS) was added and mixed properly. This suspension was used as a stock solution and diluted to 30000IU and 15000IU conc. which was used as the reference standard for thrombolytic activity. 600 mg of crude methanolic extract of leaves of the *L. monopetala* was dissolved in 0.9% NaCl solution to get a concentration 20mg/ml. The prepared stock solution was used to make different concentrations of extract in isotonic saline: 2.5, 5, 10 and 20 mg/mL. Venous blood (10 ml) without a history of oral contraceptive or anticoagulant therapy was drawn from healthy human volunteer (n=10) and was transferred to different pre weighed appendorf tube (0.5 ml/tube) and incubated at 37°C for 45 minutes for clot formation. After clot formation serum was removed without agitating the clot and each tube with clot was weighed again to define the clot weight (clot weight = weight of clot containing tube – weight of tube alone). Different concentrations of the plant extract, 2.5 mg/mL (n = 10), 5 mg/mL(n = 10), 10 mg/mL (n = 10) and 20 mg/mL (n = 10) about 500 µl was added to each eppendorf tube containing pre weighed clot. As a positive control 500 µl streptokinase (300000IU and 15000IU) was used and 500 µl saline water was used as a negative control. All the tubes were then incubated at 37 °C for 90 min and observed for clot lysis. After incubation fluid produced was removed and weighed to observe the difference in weight after clot distraction. Difference obtained in weight taken before and after clot lysis was expressed as percentage of clotlysis [32].

Anthelmintic test

Adult earthworms were used to study anthelmintic activity due to resemblance of them with the intestinal roundworm parasites of human being and availability [33, 34]. The earthworms belonging to species *Pheritima posthuma* (Annelida), about 3-5 cm in length and 0.1- 0.2 cm in width weighing about 0.8-3.04 g, were collected from the moist soil of Noakhali Science and Technology University, Sonapur, Noakhali and thoroughly washed with saline water. Methanolic extracts of leaves of *L. monopetala* were used to prepare 10,20,40,60,80 mg/ml concentration as test sample. Piperazine citrate (10 mg/ml) was used as reference standard solution and saline water for control study. Four earthworms were used in each test sample concentration, reference and control solution to observe the physical change of them and counted their paralysis timeand death time.

Antioxidant activity

In-vitro antioxidant activity of *L. monopetala* extract was determined by total phenolic content determination using Folin-Ciocalteu reagent as oxidizing agent and gallic acid as standard [35,36]. Gallic acid solution were prepared having a concentration ranging from 100 µg / ml to 0 µg / ml. 2.5 ml of Folin-Ciocalteu reagent (diluted 10 times with water) and 2.0 ml of Na_2CO_3 (7.5 % w/v) solution was added to 0.5 ml of gallic acid solution. The mixture was incubated for 20 minutes and absorbance was measured at 760 nm to prepare a standard curve. To 2 mg / ml extract concentration 2.5 ml of Folin-Ciocalteu reagent (diluted 10 times with water) and 2.0 ml of Na_2CO_3 (7.5 % w/v) solution was added, incubated for 20 minutes and absorbance was measured at 760 nm. Standard curve prepared from gallic acid solution with different concentration and the total phenol content of the sample was measured as mg of GAE (gallic acid equivalent) / gm of the extract.

Statistical Analysis

The data are expressed as the mean ± SEM analyzed by one-way analysis of variance(ANOVA) and Dunnett's t-test was used as the test of significance. P value <0.05 was considered as the minimum level of significance. All statistical tests were carried out using SPSS (version 16) statistical software.

RESULT

Brine shrimp lethality bioassay

The brine shrimp test (BST) represents a rapid, inexpensive and simple bioassay for testing plant extract lethality which in most cases correlates reasonably well with cytotoxic and anti-tumor properties. The summary of the result was given below (Table 1, 2). Here the $\mathbf{LC_{50}}$ for standard Vincristine Sulphate is 0.839 (µg/ml) and for methanolic extract of *L. monopetala* is 41.05 (µg/ml) (Table 1, 2).

Table 1: Effect of Methanolic Extract of *L. monopetala* Leaves on Brine Shrimp Nauplii.

Methanol Extract				**Vincristine Sulphate**			
Conc (C) (µg/ml)	**Log C**	**% Mort-ality**	**LC_{50} (µg/ml) Based on LogC**	**Conc (C) (µg/ml)**	**Log C**	**% Mort-ality**	**LC_{50} (µg/ml) Based on LogC**
400	2.602059991	100	41.05	40	1.602059991	100	0.839
200	2.301029996	90		20	1.301029996	90	
100	2.00000000	60		10	1.00000000	90	
50	1.698970004	50		5	0.698970004	80	
25	1.397940009	20		2.5	0.397940009	70	
12.5	1.096910013	20		1.25	0.096910013	70	
6.25	0.795880017	10		0.625	-0.20411998	50	
3.125	0.494850022	10		0.3125	-0.50514997	30	
1.5625	0.193820026	00		0.15625	-0.80617997	20	
0.78125	-0.10720997	00		0.07812	-1.10720997	10	

Table 2: Results of the test sample of *L. monopetala* leaves

Sample	**LC_{50} (µg/ml)**	**Regression Equation**	**R^2**
Vincristine Sulphate (Positive control)	0.839	y=34.02x+52.58	0.952
Methanol Extract	41.05	Y=38.25-11.71	0.893

In-Vitro thrombolytic activity

The clots were treated by four different concentrations of leaves extracts i.e., 2.5, 5, 10 and 20 mg/ml and clot lysis % was 9.52, 9.49, 13.64 and 17.50 % respectively (Table 3, Figure 1). Standard streptokinase 30000 IU and 15000 IU evoked a significant ($p<0.001$) clot lysis 47.22 and 24.73 % respectively (Table 3, Figure 1). This effect showed a dose related trend.

Table 3: Effect of methanolic crude extracts of stem of *L. monopetala* on blood clot lysis of human blood in vitro (mean± SEM)

Concentrations of plantextracts, control andstandard	**n**	**Mean % of Blood clot lysis**
0.9% NaCl solutions	10	5.3540±1.01*
Streptokinase (30,000 I.U.)	10	47.2189±1.15*
Streptokinase (15,000 I.U.)	10	24.7321±1.12*
Leaves extract 2.5 mg/mL	10	9.5231±1.24
Leaves extract 5 mg/mL	10	9.4987±1.20
Leaves extract 10 mg/mL	10	13.6436±1.22
Leaves extract 20 mg/mL	10	17.5013±1.29*

***** Determines significance level

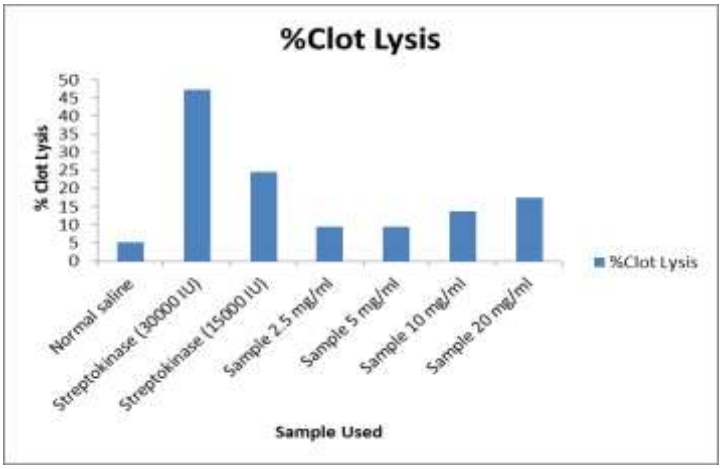

Fig-1: Clot lysis of blood samples of normal subjects by different concentrations of crude methanolic extracts of leaves of *L. monopetala*

Anthelmintic activity

From the study it was observed that the extract of L. monopetala showed not only paralysis but also death of earthworms. Whereas methanol extract at different concentrations showed paralysis at 76.75 min, 60min, 51.75 min, 44.5 min and 64.5 min and death at 90.50min, 63.75 min, 55.50 min, 44.75 min and 71 min. for 10 mg, 20 mg, 40 mg, 60 mg and 80 mg respectively. The standard drug piperazine at 10 mg/ml concentration shows paralysis at 56.2 and death at 71 min of earthworms respectively (Table 4, Figure 2).

Table 4: Paralysis and death time of *Pheritima posthuma*

Test Substance	Concentration (mg/ml)	Time taken for paralysis (Mean±SEM)	Time taken for death (Mean±SEM)
Control (Distilled water)	10	0.00	0.00
Standard (Piperazine)	10	56.2±.20000	71±.244
Methanolic extract	10	76.75±1.65	*90.50±0.64*
	20	60.00±0.91	63.75±0.85
	40	51.75±0.62	55.50±1.25
	60	44.50±2.10	44.75±1.49
	80	64.50±0.20	71±0.24

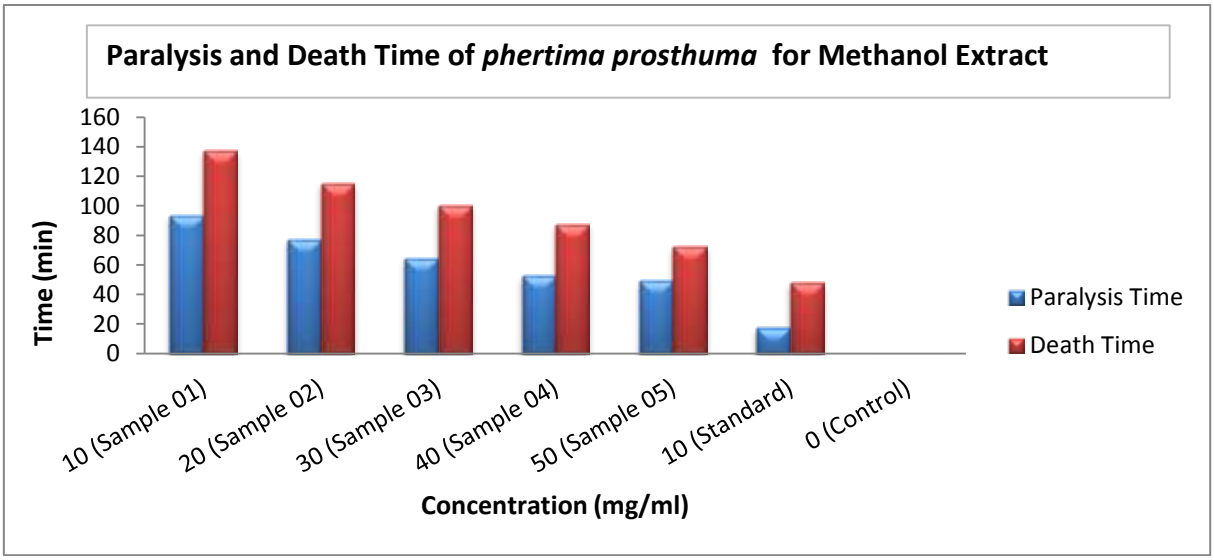

Fig-2: Paralysis and Death Time of *phertima prosthuma* for Methanol Extract

Total phenolic content determination

Total phenolic content of the samples are expressed as mg of GAE (gallic acid equivalent)/ gm which was determined by Folin-Ciocalteu reagent and gallic acid as standard (Table 5, Figure 3). The amount of total phenolic content of methanol extract of plant of *L. monopetala* is 20.75 mg of GAE / gm of extract (Table 6).

Table 5: Total Phenolic Content of Gallic Acid (Standard)

Conc. of the Standard (µg/ml)	Avg. Absorbance at 760 nm	Regression line	R^2
100	0.373	y = 0.002x + 0.107	0.889
50	0.323		
25	0.159		
12.5	0.135		
6.5	0.115		

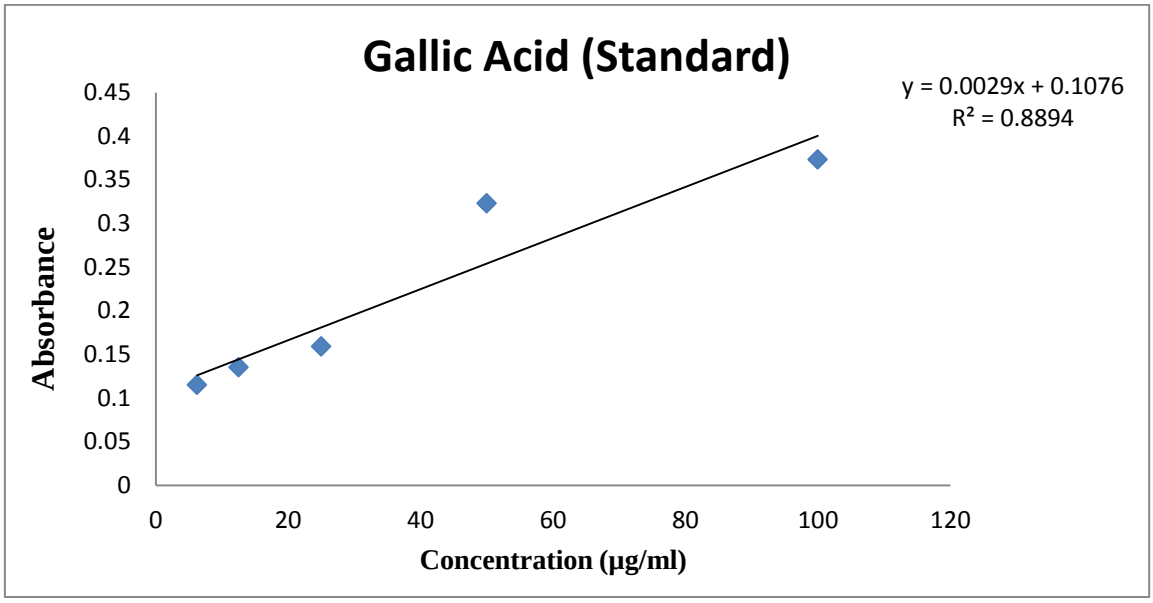

Fig-3: Total Phenolic Content of Gallic Acid (Standard)

Table 6: Total Phenolic Content of *L. monopetala*

Extract	Absorbance of the sample	Total Phenolic Content (mg of GAE / gm) of Extracts
Methanol Extract	0.190	20.75

DISCUSSION

For drug development cytotoxicity should be taken into consideration. The leaves extract of *L. monopetala* shows minimal amount of cytotoxicity which is 41.05 (µg/ml) because the biological activities of plants may be due to the presence of diverse group of chemical compounds like glycosides, alkaloids, flavonoids and saponin [37.38].

Most thrombolytic agent activates the enzyme plasminogen which clears the cross-linked fibrin mesh, makes the blood soluble and refurbishes blood flow over occluded blood vessels. That's why thrombolytic agents are beneficial for the treatment of myocardial infarction, thromboembolic strokes, deep vein thrombosis and PE to clear a blocked artery and avoid permanent damage to the perfused tissue (e.g. myocardium, brain, and leg). All four concentrations of crude methanolic extracts of stem of *L. monopetala* induced significant ($p < 0.001$) clot lysis activity *in vitro*, compared to control which shows a dose-related trend ($r2 = 0.7565$; $p < 0.001$). Study shows that *L. monopetala* contains phytosterols which is responsible for clot lysis [39].

From the study it was observed that the methanolic extracts of L. monopetala showed not only paralysis but also death of earthworms which shows a dose related trend. Preliminary phytochemical screening of *L. monopetala* observed in few studies showed the presence of saponins, tannins and alkaloids which interfere with helmintic parasites [40, 41]. Therefore, the anthelmintic activity of methanol extract as described herein against earthworms suggests that it could be effective against parasitic infections of humans.

Phenolic compounds allow them to act as antioxidants due to their redox properties. The total phenolic concentration could be used as a basis for rapid screening of antioxidant activity as their free radical scavenging ability is facilitated by their hydroxyl groups [42]. The amount of total phenolic content was significant in compared standard Gallic acid. As this study was conducted by crude extract, further advanced studies should be carried out for compound isolation and it is necessary to observe which compounds are actually responsible for specific effects.

CONCLUSION

Results of our study suggest the great value of the species *L. monopetala* for use in pharmacy and phytotherapy. Based on this information, it could be concluded that this plant is natural sources of antioxidant substances of high importance. The methanol extract shows a limited amount of cytotoxic activity. In case of thrombolytic and anthelmintic activity the extract was an appreciable effect. This is only a preliminary study. Further phytochemical analysis is required to isolate the elements of the plant to ensure the use in human health remedy like cancer, cardio vascular disease, ageing problem, and anthelmintic effect etc.

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