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Pharmacological activity investigation of *Clotalaria pallida*

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Abstract
Clotalaria pallida plant extract used to assess its different biological activity. Extract was made by soaking the dried plant powder in methanol. After comparing with the standard we found that Methanol extract of the sample gave the activity against all the experimented microbes of ZI (zone of inhibition) against *E. coli* and *B. subtillis.* After performing the antioxidant, thrombolytic, antidiarrheal, hypoglycemic and cytotoxic activity assay of methanol extract of sample plant we saw that it has a good biological activity that can be used as a potential traditional medicine.

Keywords: *Clotalaria pallida,* antioxidant, antimicrobial, antidiarrheal, hypoglycemic, thrombolytic activity

Introduction
Thousands of years ago, the ancient physicians acquired the knowledge of drugs due to curiosity nature of people and today it is said that nature has contributed a complete warehouse of remedies that can cure many diseases. Not only in developing countries, the traditional medicine are also used in developed countries because the modern medicine are failed to give effective treatment for chronic diseases and exposure of multi drug resistant bacteria and parasites. Recently, medicinal plants are having more attention because of their safety, inexpensiveness and fewer side effects compare to synthetic drug. It is also believed that natural products in general and as medicinal plant, they are the important source of new chemical substance that having potential therapeutics efficacy.
Clotalaria pallida is an erect shrub of 1.5m or more tall. It is belongs to fabaceae family. It is native to tropical Africa. The whole plants has medicinal uses like flowers can be used vegetable, roots are chewed with betel nuts, plants are used to treat urinary problem, fever, extract of leaves are used to expel intestine worms.

Methods and Materials
Collection of plant materials
The leaf part of *Clotalaria pallida* plant was collected in May, 2017 from Chittagong hill tract. After collection, the National Herbarium Bangladesh (NHB), Mirpur, and Dhaka authenticated the plant material and provided a plant identification number, which was 47697.

Preparation of the extract
At first, the leaves part was washed with fresh water to remove the unwanted dust particles and plant scrap. After that, the cleaned leaves were dried under the sun for a day. Then the leaves were again dried for 1 hour at 30-40◦***C*** in hot air oven. By using a high capacity grinding machine, the dry and crusty leaves were ground. After that, at a normal ambient temperature (22-25◦C) around 900 g of ground powder was soaked in 2.5 L of methanol for a period of 2 days with occasional stirring. With the help of cotton filter (pore size: 110mm) filtration was done and rotary evaporator was used at 100 rpm at 30◦C to evaporate the maximum amount of solvent. For vaporizing the solvent completely from the extract, the leaf extract was kept under laminar airflow cabinet. Moreover, it was used to avoid any possibility of microbial growth in the extract while drying. Finally, 22.4 g of plant leaf extract was obtained and kept in dry and cool place and proper labeling was done. After that, this extract was used to conduct antioxidant, brine shrimp lethality assay, thrombolytic, antidiabetic, antimicrobial and hypoglycemic studies.

Chemicals

The chemicals were gallic acid [Sigma-Aldrich, USA], sodium chloride [Sigma-Aldrich, USA], Folin-Ciocalteu reagent [Sigma-Aldrich, USA], vincristine sulphate [Sigma-Aldrich, USA], 2,2-Diphenyl-1-Picrylhydrazyl (DPPH) [Sigma-Aldrich, USA]., sodium carbonate [Merck, India] and ascorbic acid (ASA) [Merck, India], dimethyl sulfoxide (DMSO) [Fisher Scientific, UK]. Castor oil (WELL's Heath Care, Spain), 0.9% sodium chloride solution (normal saline) (Orion Infusions Ltd., Bangladesh), charcoal meal (10% activated charcoal in 5% gum acacia), and loperamide (Square Pharmaceuticals Ltd., Bangladesh) were used for antidiarrheal activity test, and dimethyl sulfoxide (DMSO) (Sigma-Aldrich, USA) and sodium chloride (Sigma) were used for cytotoxic activity test. All the chemicals used in this study were of analytical grade.

Anti-oxidant activity

Total phenolic content (TPC)

The phenols were oxidized by Folin-Ciocalteu in ionic phenolic solution. When the solution became yellow to dark blue, it is understood that the oxidation has been completed. After that, this color changed mixture measured in 760 nm in UV spectrophotometer. Finally, the value of the absorbance plotted in gallic acid calibration curve and data was evaluated as gallic acid equivalents (GAE).

Total flavonoid content

Aluminum chloride was used to determine the total amount of flavonoids. Firstly, 0.5 ml of plant extract was made the final volume of 1 ml for reaction medium ($MeOH/H_2O/CH_3COOH$=14:5:1) which was then mixed with Aluminum chloride reagent (4 ml, 133 mg of $AlCl_3 \times 6\ H_2O$ and 400 mg of CH_3COONa dissolved in 100 ml H_2O). After 5 minute, the absorbance was measured at 430 nm. Based on the calibration curve, total flavonoid content was calculated and it was expressed as gram equivalents.

DPPH free radical scavenging assay

The antioxidant activity of *Clotalaria pallida* was determined by performing DPPH free radical scavenging assay. To run this assay, different concentrations of plant extracts were mixed with 2, 2-diphenyl-1-picrylhydrazyl (DPHH) solution. In methanol or aqueous solution, free radicals were generated due to delocalization of the free electrons and a deep purple colored solution is produced. Then absorbance of different concentration solutions was measured at 517 nm in UV spectrophotometer. The decreasing value of DPHH at 517 nm is directly proportional to the radical scavenging activity.

Percentage of inhibition of DPHH free radical (1%) was calculated by using the following equation:

(1%) = (Absorbance of blank - Absorbance of sample)/Absorbance of blank ×100

50% of inhibition of the extract concentration was calculated from the graph and the percentage of inhibition was plotted against extract concentration.

Cytotoxic activity

Brine shrimp lethality assay

In this assay, *Artemia salina* shrimp was used. Its offspring was hatched in replicated seawater to cultivate nauplii. Here, calculated amount of dimethyl-sulfoxide (DMSO) was added with sample and desired concentration of sample was prepared by dilution. The counted nauplii were placed in vials that contained approximately 5 mL simulated seawater with visual inspection. With the help of micropipette, various concentrations of samples were added to tubes. Here, vincristine sulfate was used as standard. The sample containing tubes were then placed in a dry place for 24 hours at room temperature. At the last, after 24 hours, the survived nauplii were counted. Percentage (%) of mortality was calculated by using the following equation:

Percentage of mortality= (Number of nauplii taken - Number of nauplii alive)/ Number of nauplii taken ×100

50% of lethal concentration of extract concentration was calculated from the graph plotted percentage of mortality against concentration.

Thrombolytic activity

The normal blood flow to the cells and tissues can be hampered due to thrombus as it blocks the blood vessel which can lead to lack of blood and oxygen. There are some thrombolytic medications like utokinase, clopifogrel, and streptokinase remove this thrombus and cells and tissues are remained in normal conditions. For this assay, fresh human blood was collected. Then, they were taken in three different pre-weighed sterile microbes and incubated for 45 minutes at 37°C. The upper fluid was entirely dispensed from all micro-tube lines when the clot was appeared. As a standard streptokinase was used and as a negative control distilled, water was used. 100 microliter of plant extract was taken in each tube and incubated for 90 minutes at 37°C. Next, liquid that was released from the clot was removed and the tubes were weighted again to observe the weight difference when the clot disruption occurred.

Percentage of clot lysis was calculated by following equation:

(%) of clot lysis = (released clot weighted) / (clot weight after clot disruption) ×100

Antimicrobial assay

Disc diffusion assay method

In recent years, different studies are developing as antimicrobial agents to fight antibiotics resistance from different sources and highest concentration has given to screen and evaluate the antimicrobial activity. By using disc diffusion assay method, antimicrobial activity of *Clotalaria pallida* was evaluated. *E. coli* bacteria (gram negative) and *Bacillus Subtilis* bacteria (gram positive) were used in this study. Mular Hinton agar (MHA) was used as media in this assay. Firstly, every petri dish was autoclaved for sterilization and 20 ml of MHA was poured in every petri dish. After that, the plates were kept for a time being to be settled. With the help of cotton swab, the nutrient broth of bacterial strains was incubated in MHA. Small disc of filter paper was made by using paper punch machine and then different concentrations of plant extract (200 mg/mL and 400 mg/mL) were used to swallow that filter paper. When the discs become dry, they were transferred to the petri dishes and kept in incubator for 24 hours at 37ºC. After 24 hours the zone of inhibition were calculated and for keeping the contamination limited, whole experiment was done under laminar flow.

Hypoglycemia activity

The anti-diabetic activity of the plant leaves was evaluated with glucose tolerance test. The test was done in two different ways like orally and intraperitoneally.

Oral glucose tolerance test

In Oral glucose tolerance test, 24 healthy mice were fasted for 18 hrs. Then they were divided into four groups that contained six mice in each group. Here, 0.9% (w/v) normal saline was given to group I. Group II was received Glibenclamide (250 mg/kg). In addition, group IV and V was received methanol plant extract of 200 mg/kg and 400 mg/kg respectively. After 30 minutes, glucose (3g/kg) was fed. After that at 0, 30, 90, and 120 minutes of glucose administration blood sample were taken from retro-orbital sinus and glucose level was estimated by glucose oxidase-peroxidase method.

Intraperitoneal glucose tolerance test

Initially 24 mice were fasted for 18 hours and then they were divided into four groups that contain six rats each. The group of negative control received only 0.9percentage (w/v) normal saline and standard group received Glibenclamide (250 mg/kg) while the samples were administered the plant extract (200 mg/kg and 400 mg/kg respectively). After 30 minutes, glucose solution (3g/kg) was injected intraperitoneally. At different time after giving glucose solution like t=0, t=30 minutes, t=90 minutes and t=120 minutes, blood sampling was taken and glucose level was determined by using glucose oxidase peroxidase method.

Antidiarrheal activity

Two different tests were conducted to evaluate the antidiarrheal activity of the experimented plant.

Castor oil-induced diarrhea in rats

Normal healthy 24 rats were fasted for 18 hours. The rates were divided into 4 groups (n=6). Group I was given normal saline (0.9% w/v) orally and Group II received Loperamide (5 mg/kg) as standard group. Groups III-IV received plant extract (200 and 400 mg/kg b. wt. respectively). After 1 hour, all groups received castor oil 1 mL each orally. Next, all the rats were placed in cages with adsorbent papers and observed for 4 hours for the presence of characteristic diarrheal droppings. 100% was considered as the total number of feces of control group. The activity was expressed as % of inhibition of diarrhea. The % of inhibition was measured by using following formula:

$$\text{Percent (\%) inhibition of defecation} = [(A-B)/A] \times 100$$

Where A is mean number of defecation time caused by castor oil and B is mean number of defecation time caused by drug or extract.

Magnesium sulfate induced diarrhea

In the similar protocol as for castor oil induced diarrhea was followed for magnesium sulfate induced diarrhea. Initially, 24 healthy rats were fasted for 18 hours. The rats were divided into four groups that contained 6 rats each group. Normal saline (0.9% w/v) was given to group I. Loperamide (5 mg/kg) was given to group II and methanol plant extract (200 mg/kg and 400 mg/kg) was given to group III and IV respectively. After 60 minutes, 1 mL of magnesium sulfate solution was administrated orally and placed in cages lined with adsorbent papers and observed for 4 hours to see the presence of characteristic diarrheal dropping. 100% was considered as total number of feces of control group and % of inhibition was calculated.

Result and Discussion

Antioxidant activity

Total phenolic content (TPC)

In total phenolic content test, Gallic acid was used ad standard and methanol extract of leaves which was used as a sample. The absorbance of the sample plotted in Gallic acid calibration curve. The absorbance of the plant extract was found 0.575 and TPC value was 78.71 GAE/g against that absorbance which indicates that the plant has antioxidant activity.

Total flavonoid content

The content of total flavonoid of the plant extracts was measured spectrophotometric ally by using the aluminium chloride colorimetric assay. The flavonoid content of the extracts was expressed as mg quercetin equivalent per gram of the extract and it is 282.90 QE/g against the absorbance of 0.291 that indicates the present of flavonoid content.

DPPH free radical scavenging assay

It is known that DPPH free radical scavenging activity is increasing along with increasing concentration of the methanol extract. As the reference standard, ascorbic acid was used in this experiment for which IC_{50} value was 72.521 µg/ml. on the other hand, the IC_{50} value of the methanol extract of the sample plant was 231.25 µg/ml. this result indicates the presence of antioxidant activity which is less significant.

Table 1: Evaluation of DPPH free radical scavenging activity of methanol extract of *Clotalaria pallida.*

	R^2 value	Ic_{50}
Standard	0.6277	72.521
Sample (Methanol extract)	0.4975	231.25

Cytotoxic activity

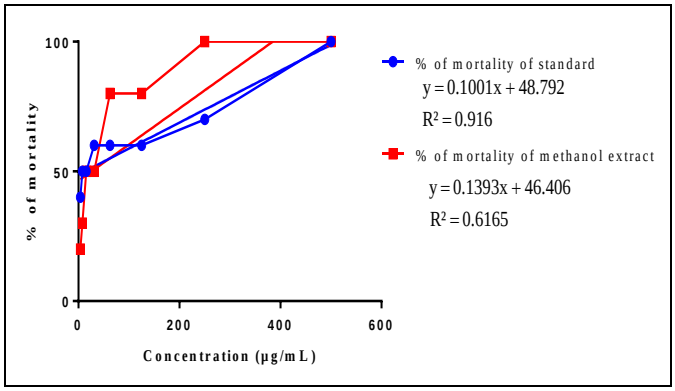

Fig 1: Graph between % of mortality and concentration (µg/mL)

This brine shrimp lethality assay was used to assess the cytotoxic property of methanol extract of plant material. Here, different concentrations standard and sample were plotted that provided different percentages of mortality. Percentage of mortality was found to increase along with the increasing concentrations of standard and methanol extract. This study indicates the methanol extract of plant material has cytotoxic activity.

Thrombolytic activity

Table 2: Evaluation and results of the thrombolytic activity.

Name of the sample	W1	W2	W3	W4	W5	% of clot lysis
Plant extract	0.742	1.558	1.079	0.347	0.372	75.46
Standard	0.795	1.519	1.272	0.578	0.05	15.90
Blank	0.795	1.478	1.163	0. 468	0.115	23

Here, W1 = Micro-tube weight, W2 = Clot with micro-tube weight, W3 = Clot with micro-tube weight after clot disruption, W4 = Clot weight after clot disruption, W5 = Released clot weight.

Plasminogen enzyme is usually activated by thrombolytic agents and it also removes fibrin bonds in blood, as a result, the clot becomes soluble and blood flow is restored. Here, methanol extract showed much lower level of thrombolytic activity than standard. Standard gave 15.90% clot lysis, distilled water was used as a negative control, which provided 23% clot lysis and methanol extract of plant leaves showed 75.46% clot lysis. After comparing the clots lysis value of plant extract with the positive control value, it was observed that plant material revealed thrombolytic activity but less than standard.

Antimicrobial assay

Table 3: Antimicrobial activity of the leaves of *Clotalaria pallida.*

Group	Inhibition zone (mm)	
	Gram (-ve) bacteria *(E. coli)*	Gram (+ve) bacteria *(B. subtilis)*
Control	0.00	0.00
Standard	23.657 ± 2.082	19.3±1.58
Plant extract (200mg/mL)	0.00	0.00
Plant extract (400mg/mL)	12.333±1.17	24±1

The plant extract showed antimicrobial activity at all concentrations tested with a broad spectrum of activity, inhibiting against the growth of both Gram positive and Gram-negative bacteria. The antimicrobial potential was especially showed against *E. coli* and *B. subtilis*, when exposed to 400 mg/mL of methanol extract of plant and made it impossible when exposed to 200 mg/mL of methanol extract of dried leaves as shown in the table. These results indicate that the antimicrobial activity of the plant extract is not as significant as standard.

Hypoglycemia activity

Table 4: Oral glucose tolerance test in rats as a part of hypoglycemic activity of leaves of *Clotalaria pallida.*

Group	Dose (mg/kg)	Oral glucose tolerance test(OGTT)			
		Initial (mmol/L)	30 min. (mmol/L)	90 min. (mmol/L)	120 min. (mmol/L)
Standard	_	5.2333333±0.7704977	19.85±0.7704977	9.6±0.7704977	3.1166667±0.7704977
Control		5.2833333±1.0033278	21.3833333±1.0033278	11.733333±1.0033278	4.733333±1.0033278
MEPG 200	200	3.6333333±1.267149	19.1±1.267149	11.766667±1.267149	4.4833333±1.267149
MEPG 400	400	4.5666667±0.6470446	19±0.6470446	11.15±0.6470446	2.9333333±0.6470446

Table 5: Intra peritoneal glucose tolerance test in rats as a part of hypoglycemic activity of the leaves of *Clotalaria pallida.*

Group	Dose (mg/kg)	Intraperitoneal glucose tolerance test (IPGTT)			
		Initial (mmol/L)	30 min. (mmol/L)	90 min. (mmol/L)	120 min. (mmol/L)
Standrd	_	20.333333±0.825631	11.633333±0.825631	4.95±0.825631	2.883333±0.825631
Control		21.366667±1.256583	15.416667±1.256583	10.08333±1.256583	5.85±1.256583
MEPG 200	200	22.2±0.750333	14.666667±0.750333	10.46667±0.750333	4.25±0.750333
MEPG 400	400	22.366667±1.263329	16.15±1.263329	7.716667±1.263329	4.5±1.263329

From the Table 3 and 4 we can say that our sample plant has the ability to act as a potential hypoglycemic medicine. Here MEPG denotes methanol extract of *Clotalaria pallida*. In both the cases which means in oral and intraperitoneal we saw that the administered glucose level go low as the time increases. If we compare them the intraperitoneal administration of glucose got a high blood glucose level at a short time and it went to low level at a short period of time.

Antidiarrheal activity

Table 6: anti-diarrheal activity (Castor oil induced diarrhea and $MgSO_4$ induced diarrhea) methanol extract of the leaves of *Clotalaria pallida.*

Group	Dose (mg/kg)	Castrol oil induced diarrhea		$MgSO_4$ induced diarrhea	
		Total number of faeces in 4 hours	Percentage of inhibition	Total number of faeces in 4 hours	Percentage of inhibition
Control	_	21.333333±3.5023801		20.83333±1.7224014	
Standard		8.8333333±1.8348479	58.59	11±1.4142136	47.19
MEPG200	200	15.333333±2.9439203	28.13	19.5±1.7606817	6.3998
MEPG400	400	7.8333333±1.7224014	65.28	9.166667±1.7224014	58.99

A significant reduction in the number of defecation instances was observed with all the test doses of the extract compared with the control group and standard group. There was graded reduction in intestinal fluid volume in graded MEPG extracts. MEPG (400 mg/kg) showed the reduction in the intestinal fluid volume with significant difference as compared with control group and standard group and % inhibition was 65.28% and 58.99% for castor oil induced diarrhea and magnesium sulfate induced diarrhea.

Conclusion

The plant has been brought into effective action in various traditional uses of which some have been proved clinically. Further studies can be conducted on secondary metabolites to explore more activities. This review can be helpful in promoting research that can help to develop new agents for therapeutic applications based on bioactive chemical compounds. Therefore, this plant is significantly used for the treatment and prevention of diseases.

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