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Antidiabetic and antidiarrheal effects of the methanolic extract of *Phyllanthus reticulatus* leaves in mice

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ABSTRACT

Objective: To assess the anti-diabetic and antidiarrheal activity of methanolic extract of *Phyllanthus reticulates* (*P. reticulates*) leaves in an animal model. **Methods:** Phytochemical screening of methanolic extract of *P. reticulatus* leaves has been performed. Antidiabetic activity have been done by OGTT, normoglycemic hyperglycemia and alloxan induced diabetic mice. Plant extracts (150 mg/kg and 300 mg/kg, b.w.) were administered orally in fasting glucose loaded mice with regard to normal control and in alloxan induced (110 mg/kg body weight *i.p.*) diabetic mice in comparison with reference drug Metformin hydrochloride (100 mg/kg) during 7 day test period. Antidiarrheal test was conducted by castor oil and magnesium sulfate. **Results:** Findings confirmed that the continuous post-treatment for 7 days with both extracts showed significant ($P < 0.05$) hypoglycemic activity in OGTT, normoglycemic and alloxan induced mouse models. Castor oil and Magnesium sulfate induced diarrheal test of the extract (200 and 400 mg/kg) has given significant effect in compairing to control diarrheal group. **Conclusion:** Methanolic extract of *P. reticulatus* leaves have shown significant antidiabetic and antidiarrheal properties.

1. Introduction

Phyllanthus reticulatus (*P. reticulatus*) (Family: Euphorbiaceae) is usually a dense deciduous shrub or small tree with a distinct smell that is emitted by the minute flowers when they open towards the early evening. This is one of the fascinating characteristic smells of Africa. *P. reticulatus* is a many branched shrub, sometimes partially scrambling, usually 1-5 m high, or a small twiggy tree that grows up to 8 m in height. The bark is light reddish-brown or gray-brown with hairy stems when young, which become smooth with age. The leaves alternate along slender branches. They are up to 25 cm long and appear as the leaflets of large pinnate leaves. The leaves are thinly textured, usually hairless. They have a noticeable reddish net-veining which is more visible above than below. The plant grows throughout the tropical areas of India, Bangladesh, China and the Malay Islands[1]. Natural products are widely used to treat a plethora of acute and chronic diseases ranging from common cold to complex human diseases all over the world. The literature survey reveals that the whole plant is astringent, sweet, cooling, diuretic, alternate, stomachic, constipating and attendant. It is reported to be useful in vitiated condition of pita, burning sensation, strangury, gastropathy, ulemorrhagia, ophthalmodynia, sores, burns, suppuration, diarrhea, skin

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eruption and obesity[2-4].

The previous phytochemical investigations showed that the *P. reticulatus* contain lupeol, lupeol acetate, and stigmasterol(steroid)[5]. Dichloromethane extract of leaves of the plant have been reported the presence of three compounds, (5R*, 6R*)-4,6-dimethoxycarbonyl-5-[2 ,3 ,4 -trihydroxy-6 (methoxycarbonyl) phenyl] -5,6-dihydro-2H-pyran-2-one along with 3,4,3 -tri-O-methylellagic acid, and methyl gallate. The first compound reportedly demonstrated weak insecticidal activity against Spodopterafrugiperda[6]. Reticulatus contains Phytosterolo (sitesterol), descendants of friedelin, Olean and lupan type (butelin and glochidonol). It contains polyphenols, flavonoid glycosides11, tannic acid, friedelin, epifriedelinol, betulin, taraxerone, betasitosterol, glochidonol, Octacosanol, taraxeryl acetate and 21-alpha-hydroxyfriedelan-3- one12. The tannin of *P. reticulatus* are partly responsible for its medicinal and dyeing properties. A number of triterpenoids have been isolated from the stems and leaves, including sitosterol, friedelin and betulinicacid. Phytochemical experiments of the leaves revealed the presence of terpenoid glycosides, protein, carbohydrates but absence of alkaloids and steroids[7]. The biological investigation on *P. reticulatus* exhibited antibacterial[1], antinociceptive and antihyperglycemic[8], hypocholesterolemic[9]. The fruit is an astringent to the bowels and is used in the inflammation. The leaves are employed as a diuretic and cooling medicine[10,11]. Moreover the leaf juice is a remedy for spongy and bleeding gums[12]. An ethanolic extract of the plant has been revealed hepatoprotective activity against carbon tetrachloride-induced liver damages in rats[13]. Earlier reports also indicate the hypolipidemic action of methyanolic and alcoholic extract of the *P. reticulatus* in animals[14,15]. Recent studies also reveal the analgesic and anti-inflammatory properties of *P. reticulates* extracts (petroleum ether, ethyl acetate and methanol) in acetic-induced writhing and carrageenan induced mouse paw edema models respectively[16].

Medicinal plants are vital sources of novel chemical compounds with potent pharmacological activities [17]. Diabetes mellitus is a chronic metabolic disorder distinguished by hyperglycemia, glycosuria, hyperlipidemia, negative nitrogen balance, and occasionally ketonemia[18]. Globally the prevalence of diabetes mellitus is increasing. Although research for diabetes has resulted in newer insulin preparations and medications, improved systems for insulin delivery and self-monitoring of blood glucose, and more aggressive treatment of diabetes complications and comorbid conditions[19], majority of the oral hypoglycemic agents, presently in use, produce severe side effects like hypoglycemic coma and hepato-renal disturbances. Therefore, there is a burning need to explore safer and more efficient hypoglycemic mediators. Since conventional medicine is an imperative source of potentially useful novel compounds for the development of chemotherapeutic agents; we have studied the hypoglycemic effect and antidiarrheal effect of the methanolic extracts of *P. reticulatus* leaves.

2. Materials and methods

2.1. Plant material

In this present investigation, the fresh leaves of *P. reticulatus* were collected from the area of Bandorban of Bangladesh and were identified by the experts of Bangladesh National Herbarium, Dhaka. The collected plant parts were dried for one week and pulverized into a coarse powder with the help of a suitable grinder. The powder was stored in an airtight container and kept in a cool, dark and dry place until analysis commenced.

2.2. Preparation of the extract

About 150 g of powdered material was taken in a clean, flat bottomed glass container and soaked in 200 mL of 85% methanol. The container with its contents was sealed and kept for a period of 7 days accompanying occasional shaking and stirring. The whole mixture then underwent a coarse filtration by a piece of clean, white cotton material. Then it was filtered through Whatman filter paper (Bibby RE200, SterilinLtd., UK). The filtrate (methanol extract) obtained was evaporated using a rotary evaporator. It rendered a gummy concentrate of reddish black color. The gummy concentrate was designated as a crude extract of methanol.

2.3. Animals

In the present study, Swiss albino mice (male), which weighed between 20-25 g were used. The animals were obtained from the International Centre for Diarrheal Disease Research, Bangladesh (ICDDRB). All animals were kept under ambient temperature with 12 h light followed by a 12 h dark cycle. The animals were acclimatized for one week prior to actual experiments. The study was conducted following approval by the Institutional Animal Ethical Committee of University of Development Alternative, Dhaka, Bangladesh.

2.4. Acute toxicity

The 50% lethal dose (LD_{50}) of the extract of *P. reticulatusin* mice was estimated by the up and down method. Doses were adjusted up or down by a constant multiplicative factor (1.5)

depending on the previous outcome.

2.5. Chemicals and drugs

Alloxan (Fluka, Germany), Tween-80, Castor oil (BDH Chemicals, UK), normal saline solution (Beximco Infusion Ltd., Bangladesh), Metformin (Square Pharmaceuticals Ltd., Bangladesh), Loperamide (Square Pharmaceuticals Ltd., Bangladesh), castor oil (WELL's Heath Care, Spain), Magnesium sulfate (Merc) were procured and used in the experiment. All chemicals in this investigation were of analytical reagent grade.

2.6. Phytochemical analysis

The methanolic extract of *P. reticulatus* leaves (MEPRL) was subjected to qualitative chemical screening for the identification of bioactive constituents (flavonoids, tannins, Glycoside, Terpene, Carbohydrate, alkaloids, etc.) using standard procedures[16].

2.7. Animals

Young Long-Evans rats of either sex weighing about 80-120 g were used to conduct the research. The rats were procured from the animal Research Branch of the International Centre for Diarrhoeal Disease and Research, Bangladesh (ICDDRB). They were kept in standard environmental condition (at 24.0 ℃ temperature & 55%-65% relative humidity and 12 hours light/dark cycle) for two weeks for acclimation and fed ICDDRB formulated rodent food and tap water *ad libitum*. All animals were fasted overnight before tests while providing tap water ad libitum.

2.8. Ethical approval

The guidelines followed for animal experiment were accepted by the institutional animal ethics committee[17].

2.9. Oral toxicity studies

An acute oral toxicity study was followed according to "Organization for Environmental Control Development" guidelines (OECD: Guidelines 420; Fixed Dose Method) for oral administration of methanol extract. Long Evan mice (n=5, 150-200 g) overnight fasted for 18h were used for the study. Different doses of plant extracts up to 1 600 mg/kg, p.o. was administered and the animals were observed for the first 3 hours of administration and mortality recorded within 48 hours.

2.10. Induction of diabetes in mice

Diabetes was created by a single dose subcutaneous injection of freshly preparedalloxanmonohydrate dissolved in normal saline to overnight fasted mice. Blood glucose level (BGL) was measured by using one-touch glucometer and diabetes was confirmed after 72 hours of alloxanisation. Micewhich showed hyperglycemia (BGL> 10 mm/L) were chosen for research.

2.11. Experimental design

2.11.1. Oral glucose tolerance test (OGTT)

In the beginning, hypoglycemic activity of plant extracts was checked in overnight fasted normal mice, which were uniformly alienated into five groups of each. Normal control group (I) received only vehicle (1 normal saline *p.o.*), Group II received only glucose (2 g/kg) and standard group (III) received 1 mL of reference drug Metformin in the vehicle (100 mg/kg, *p.o.*), while a group from IV and V were administered with 1 mL of MEPRL at 150 and 300 mg/kg b.w. *p.o.* doses respectively. Subsequent 30 min post extract administration all the mice were fed with glucose (2 g/kg). Blood samples were taken from the tail vein prior to dosing and then at 0, 30, 60, 90 and 120 min after glucose administration. The fasting blood glucose level was analyzed using glucose-oxidase-peroxide reactive strips (Accu-Chek, Roche Diagnostics, GmbH, Germany).

2.11.2. Effect of methanolic extracts in normoglycemic mice

The mice were divided into four groups of 5 animals (n=5) each. Group I served as control and received saline water. Group II served as standard control, received Metformin in the vehicle (100 mg/kg *p.o.*). Group III and IV received 150 and 300 mg/kg MEPRL extracts orally respectively. Blood glucose levels were determined at 0 min, 30 min, 60 min, 90 min and 120 min following treatment from the tail vein.

2.11.3. Study on alloxan-induced diabetic mice

The experimental mice were randomly divided into five groups consisting of 5 mice in each group. The groups were denoted as a group 1, group 2, group 3, group 4 and group 5 which indicated normal control, diabetic control, standardization and test extract (150 and 300 mg/kg b.w.). Each group of mice received a specific treatment. Test samples at a dose of 150 mg/kg and 300 mg/kg body weight of mice were used to evaluate the hypoglycemic activity. Standard Metformin was used at a dose of 100 mg/kg b.w. Before administering the drugs, each mouse was weighed properly and the doses were adjusted accordingly. In the evaluation of the hypoglycemic effect, the blood glucose level of the experimental animals was measured at 0 hours

by tail tipping method[18] using a glucometer (Bioland G-423S). Then the control, standard and *P. reticulatus* extracts were administered orally to the experimental animals with the help of feeding needle. Blood samples were collected from the tail vein prior to dosing (day 0) and then at regular intervals of 1st, 3rd and 7th day respectively.

2.12. In vivo antidiarrheal activity

2.12.1. Castor oil-induced diarrhea

The experiment was performed according to the method described by Shoba& Thomas[19]. Briefly, mice fasted for 24 hardware randomly allocated to four groups of five animals each. The animals were all screened initially by giving 0.5 mL of castor oil. Only those showing diarrhea was selected for the final experiment. The group I received 1% carboxy-methyl cellulose (CMC) (10 mL/kg *p.o.*), Group II was given antidiarrheal drug loperamide (3 mg/kg, *p.o.*) in suspension and groups III and IV received *p.o.* the drug extract (100 and 200 mg/kg) respectively. After 60 min, each animal was given 0.5 ml of castor oil, each animal was placed in an individual cage, the floor of which was lined with blotting paper which was changed every hour, observed for 3 h and the characteristic diarrheal droppings were recorded.

2.12.2. Magnesium sulfate-induced diarrhea

Diarrhea was induced by oral administration of magnesium sulfate at the dose of 2 g/kg to the animals 30 min after pre-treatment with vehicle (1% Tween 80 in water, 10 mL/kg, *p.o.*) to the control group, Loperamide (3 mg/kg) to the positive control group, and the methanol extract at the doses of 100 and 200 mg/kg to the test groups[20].

2.13. Statistical analysis

All the values in the test are expressed as mean ± standard error mean (SEM). The data were statistically analyzed by ANOVA (Analysis of variance) and post-hoc Dunnett's tests with the Statistical Package for Social Sciences (SPSS) program (SPSS 16.0, USA). Dissimilarity between the means of the various groups were measured significant at $P< 0.05$.

3. Results

3.1. Acute toxicity

The 50% lethal dose (LD_{50}) of the extract of *P. reticulatus* in mice was estimated from the dose of the petroleum ether, ethyl acetate and methanol up and down method. Doses were adjusted up or down by a constant multiplicative factor (1.5) depending on the previous outcome.

3.2. Oral glucose tolerance test

In oral glucose tolerance test, we have observed that both extracts were active and comparable to that of the glucose treated control group at both doses (Figure 1). A good anti-hyperglycemic effect of MEPR was observed at 150 and 300 mg/kg dose in the 60 min after glucose loading in mice under OGTT. This effect was still present 120 min after the oral administration of glucose.

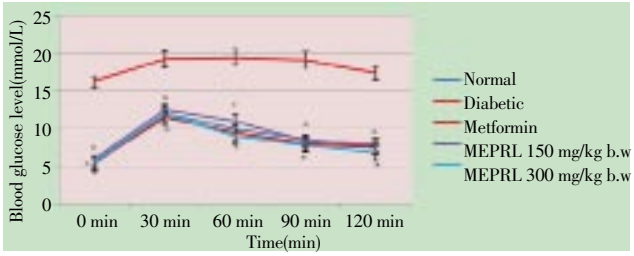

Figure 1. Oral glucose tolerance test in mice for MEPRL.
All values are expressed as mean SEM (n=5); One-Way Analysis of Variance (ANOVA) followed by Dunnet's test. *P<0.05 significant compared to control.

3.3. Effect of methanolic extracts in normoglycemic mice

The administration of both doses (150 and 300 mg/kg) in normal mice showed reduction in blood glucose levels at different time intervals compared to control group. In Figure 2, oral administration of MEPRL (300 mg/kg) caused better significant (P<0.05) reduction at 30, 60, 90, 120 min in glucose levels compared to normal control where 150 mg/kg was significant only at 90 and 120 min.

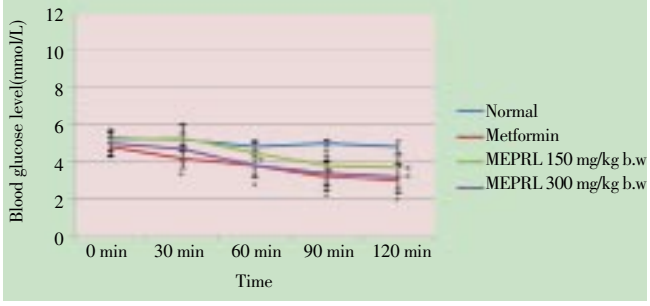

Figure 2. Effect of MEPRL on blood glucose in normoglycemic mice.
All values are expressed as mean±SEM (n=5); One-way analysis of variance (ANOVA) followed by Dunnet's test. *P<0.05 significant compared to control.

3.4. Study on alloxan-induced diabetic mice

The effect of graded doses of MEPRL on blood glucose level of hyperglycemia mice has been shown in Figure 3. There was a significant decrease in the blood glucose levels at 1st, 3rd and 7th day (P<0.05) with 300 mg/kg but 150 mg/kg doses was significant only at 3rd and 7th day.

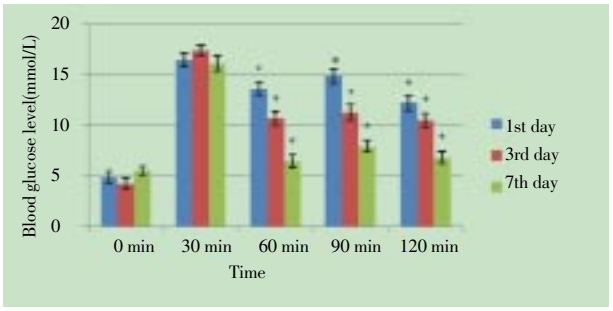

Figure 3. Blood glucose level of alloxan induced diabetic mice after treatment with MEPRL.
All values are expressed as mean±SEM (n=5); One-Way analysis of variance (ANOVA) followed by Dunnet's test. *P<0.05 significant compared to control.

3.5. Castor oil-induced diarrhea

In the castor oil-induced diarrhea, the methanol extract of leaves of *P. reticulates* (200 and 400 mg/kg) reduced the number of faeces in a dose dependent manner (Figure 4). These results of both doses were statistically significant (P<0.05).

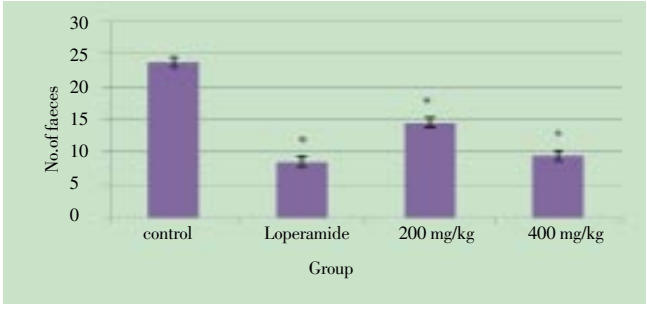

Figure 4. Effect of MEPRL on castor oil-induced diarrhea in mice.
Values are mean±SEM, (n=5); One-Way Analysis of Variance (ANOVA) followed by Dunnet's test. *P<0.05 significant compared to control.

3.6. Magnesium sulfate-induced diarrhea

P. reticulatusleaves extract reduced the number of diarrhea induced by magnesium sulfate (Figure 5). The extract of both doses has shown significant (P<0.05) reduces the number of diarrhea in a dose dependent manner.

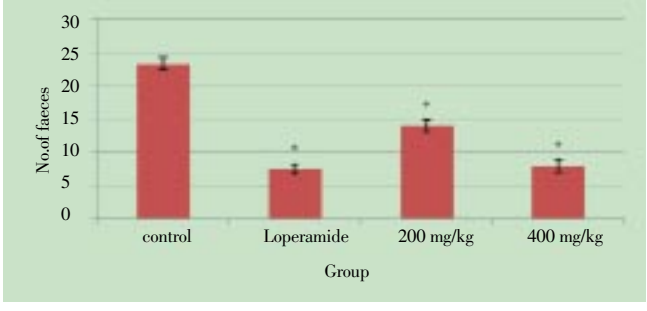

Figure 5. Effect of MEPRL extract on castor oil-induced diarrhea in mice.
Values are mean±SEM, (n=5); One-Way Analysis of Variance (ANOVA) followed by Dunnet's test. *P<0.05 significant compared to control.

4. Discussion

Huge numbers of anti-diabetic herbs are identified through folklore, however pharmacological assessment of scientific methods is obligatory to establish the antidiabetic activity. The study of such medicines might propose natural key to unlock a diabetologist's pharmacy for the future[21]. Although huge number of antidiabetic medicines is now available in the pharmaceutical market, still remedies from medicinal plant are used with success to treat this disease. According to WHO, hypoglycemic agents of plant source used in traditional medicine are essential. The attributed anti-hyperglycemic property of these plants is due to their capability to repair the role of pancreatic tissues by causing a boost in insulin output or diminish in the intestinal absorption of glucose. Healing with herbal drugs to protect β-cells and smoothing out oscillation in glucose levels. Generally there is very tiny biological knowledge of the specific modes of action in the treatment of diabetes, but most of the plants have been found to contain substances like glycosides, terpenoids, flavonoids etc. that are frequently implicated as having antidiabetic effects. The research for alternate remedies (from the plant kingdom) for diabetes mellitus will continue all over the world as the disease possess many challenges not only to the physician but also to the researcher. The current research is an earliest estimation of hypoglycemic and antihyperglycemic activity of *P. reticulatus* methanolic extracts at 150 and 300 mg/kg b.w. in mice. It has been proved that most medicinal plants contain phenolic compounds and bioflavonoids that have outstanding antioxidant and antidiabetic properties. Generally diabetes is of two categories out of which one is genetically based and other as a result of dietary indiscretion[22]. Alloxan leads to a fall in insulin release there by a drastic diminution in plasma insulin concentration leading to stable hyperglycemic states[23]. It induces diabetes by dose dependent destruction of β-cells of islets of langrhans[24,25]. In OGTT test results indicated that 150 and 300 mg/kg b.w. extract exhibited significant effect in 0, 30, 60, 90, 120 min compared to control. The test of normmoglycemic study revealed that 300 mg/kg of *P. reticulatus* has shown better antihyperglycemic activity in 30, 60, 90, 120 min than 150 mg/kg dose extract. In alloxane induced diabetic test, alloxan was chosen to create diabetic condition in mice and significant hyperglycemia was achieved within 48 hours after alloxan (110 g/kg b.w. *i.p.*) injection. The results proved that after a single administration of glucose

2 g/kg in mice, extracts (150 mg/kg) showed significant reduction (P<0.05) of fasting blood glucose level during the 3rd and 7th day but 300 mg/kg doses of extract significantly reduced blood glucose level at 1st, 3rd and 7th day. So, In this study, administration of MEPR leaves up to 7 days was able to correct abnormality of glucose level to normal range. These observations suggest that the experimental extract might acquire insulin like effect on peripheral tissues either by promoting glucose consumption metabolism or inhibiting hepatic gluconeogenes is since alloxan treatment causes permanent destruction of β-cells[26]. Several reports suggested that flavonoids, sterols/terpenoids, tannins etcpossesses antidiabetic activity[27, 28]. According to this reference the presence of alkaloids, flavonoids, tannin *etc*. which were present in this plant might be involved for antidiabetic action.

Numerous reports confirmed that castor oil induced diarrhoeal action[29]. These diarrheal action involves reduction of normal fluid absorption by inhibition of intestinal Na^+, K^+ ATPase activity[30], activation of adenylatecyclase or mucosal cAMP mediated active secretion[31, 32] stimulation of prostaglandin formation[33], plateletactivating factor[34] and nitric oxide has been reported to contribution of the diarrhoeal effect by castoroil[35]. The activity of castoroil which produces diarrhoea due to its most active component recinoleic acid by a hypersecretory response was proved[36]. In this trial, the extract of *P. reticulates* leaves (200 and 400 mg/kg) reduced the no. of diarrhea induced by castor oil and this reducing capacity might be its antisecretory mechanism. On the other hand, magnesium sulphate which induces diarrhoea by increasing the volume of intestinal content through prevention of reabsorption of water has been established. Moreover it promotes the liberation of cholecystokinin from the duodenal mucosa, which increases the secretion and motility of small intestine and thereby prevents the reabsorption of sodium chloride and water[37]. In Magnesiunsulphate diarrhea test, the extract alleviated the diarrheal condition and both 200 and 400 mg/kg dose extract showed significant action compaired to control group. The extract might have increased the absorption of water and electrolyte from the gastrointestinal tract, since it delayed the gastrointestinal transit in mice as compared to the control. *P. reticulatus* contains flavonoids, tannins, glycoside, carbohydrate, terpene *etc*.[16]. Flavonoids which possess a wide range of activities *in vitro* including antidiarrhoealactivity[37] may have contributed to this antidiarrheal activity, but further studies are required.In our study, the methanolic extract of *P. reticulates* leaves exhibited antidiarrhoeal activity in a number of models of diarrheic conditions in test animals. However, further bioassay guided phytochemical and pharmacological studies are required to identify the active principles and exact mechanisms of action.

Conflict of interest statement

We don't have potential conflicts of interest.

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