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In vitro α–amylase inhibitory activity and *in vivo* hypoglycemic effect of ethyl acetate extract of *Mallotus repandus* (Willd.) Muell. stem in rat model

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PEER REVIEW

Peer reviewer

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Comments

The article is a nice piece of work which can provide *in vitro* evidence for potential inhibition of α–amylase enzyme followed by an *in vivo* study on the effect of hypoglycemic activity in normal and glucose induced hyperglycemic rats. Authors followed the standard methodology for conducting the study. The results are pretty interesting and suggest that ethyl acetate extract of *M. repandus* stem may be exploited in the development of anti–diabetic therapeutics and can be considered as a potential candidate for the development of new oral hypoglycemic agent.
Details on Page 725

ABSTRACT

Objective: To investigate the therapeutic effects of ethyl acetate extract of *Mallotus repandus* stem in α–amylase inhibitory activity (*in vitro*) and hypoglycemic activity in normal and glucose induced hyperglycemic rats (*in vivo*).
Methods: Ethyl acetate extract of *Mallotus repandus* stem was tested for the presence of phytochemical constituents, α–amylase inhibitory activity and hypoglycemic effect in normal rats and glucose induced hyperglycemic rats.
Results: Presence of different types of phytochemicals was identified in the extract. The extract has moderate α–amylase inhibitory activity [IC_{50}=(2.038±0.033) mg/mL] as compared to acarbose. The does 1000 mg/kg significantly reduced (P<0.0100) fasting blood glucose level in normal rats. In oral glucose tolerance test, both 1000 and 2000 mg/kg doses showed good hypoglycemic activity (P<0.0001) like glibenclamide in each specific hour after administration. Overall time effect in oral glucose tolerance test was found extremely significant (P<0.0001) with F (3, 48) value=202.4.
Conclusions: These findings suggest that this plant may be a potential source for the development of new oral hypoglycemic agent.

KEYWORDS

Mallotus repandus, α–Amylase, Diabetes mellitus, Hypoglycemic, Oral glucose tolerance test

1. Introduction

Diabetes mellitus is a chronic metabolic disorder characterized by high level of blood glucose resulting from defects in insulin production (type 1 diabetes mellitus or insulin–dependent diabetes mellitus), insulin action (type 2 diabetes mellitus or non–insulin dependent diabetes mellitus) or both with disturbances in carbohydrate, fat and protein metabolism. Diabetes mellitus type 2 makes up about 90% of cases of diabetes with the other 10% primarily

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due to diabetes mellitus type 1 and gestational diabetes. In recent years, the incidence of diabetes has increased worldwide. Treatment of diabetes include: enhancement of the action of insulin at the target tissues with the use of sensitizers (biguanides and thiozolidinediones); stimulation of endogenous insulin secretion with the use of sulfonylureas (glibenclamide and glimipiride) and reduction of the demand for insulin using specific enzyme inhibitors (acarbose and meglitol). However, the use of these drugs causes some unwanted side effects like diarrhea, nausea, dyspepsia, myocardial infarction, peripheral edema and dizziness. Plants have been an excellent source of drugs that have been derived directly or indirectly from them.

Mallotus repandus (Willd.) Muell.-Arg. (Family: Euphorbiaceae) (*M. repandus*) commonly called Gunti or Jhante or Bon natai, is a wild species available in Bangladesh and it is used in traditional health practice for treating inflammation, liver-toxicity, ulcer and tumor. The plant also has anti-radical, anti-viral (HIV-1) and uterus muscle stimulant activity[1]. Hydro-ethanolic bark extract of *Mallotus philippinensis* (Family: Euphorbiaceae) showed good anti-diabetic activity in streptozotocin induced diabetic rats[2]. Investigation on *M. repandus* for hypoglycemic property has not been performed yet. That is why we have designed our research project to explore possible hypoglycemic activity of ethyl acetate extract of *M. repandus* stem. The current investigation is an attempt to study the hypoglycemic effect of the ethyl acetate extract of *M. repandus*.

2. Materials and methods

2.1. Drugs, chemicals and apparatus

Ethyl acetate was bought from SIGMA® (Sigma-Aldrich®, St Louis, USA), while acarbose tablet was purchased from local market, manufactured by Pacific Pharmaceuticals Ltd., Bangladesh. Starch was purchased from local scientific market, Motijheel, Dhaka. Glibenclamide was obtained from Square Pharmaceuticals Ltd., Bangladesh. Amylase was obtained from Merck, Germany. All the chemicals and reagents were of analytical grade. Match® glucometer with strips were purchased from OK Biotech Co. Ltd, Taiwan.

2.2. Plant material

The stems of *M. repandus* (Willd.) Muell.-Arg. were collected from Savar, Dhaka, Bangladesh during the dry season and authenticated by Md. Abdur Rahim, Technical Officer, Department of Botany, Jahangirnagar University. A voucher specimen (DACB Accession No. 38733) was deposited in the herbarium of Bangladesh National Herbarium for future reference.

2.3. Preparation of plant extract

The collected plant parts of stem were cleaned and washed well with water. The cleansed stems were then partially dried by fan aeration and then fully dried in the oven at below 40 °C for 4 d. The fully dried stems were then grinded to a powdered form and stored in suitable condition for few days. The powdered plant materials of stems (500 g) were used for extraction by Soxhlet apparatus at elevated temperature (65 °C) using petroleum ether, ethyl acetate and methanol consecutively (500 mL of each solvent). After each extraction the powder was dried and used again for the next extraction. Extraction was considered to be complete when the plant materials become exhausted of their constituents that were confirmed from cycles of colorless liquid siphoning in the Soxhlet apparatus. All three extracts of stem were filtered individually through fresh cotton bed. The filtrates obtained were dried at temperature of (40±2) °C to have gummy concentrate of the crude extracts. Each extract was kept in suitable container with proper labeling and stored in cold and dry place. The yield value for ethyl acetate extract was 2.5%.

2.4. Animals and experimental set-up

Sprague-Dawley female rats of 80-100 g and Swiss albino female mice (Body weight 25-30 g, non-pregnant) were collected from Focused Research on Ayurvedic Medicine and Education Laboratory, Department of Pharmacy, Jahangirnagar University, acclimatized to normal laboratory conditions for one week prior to study and were assessed to pellet diet and water *ad libitum*. Temperature of facility was (25±3) °C and light/darkness alternated 12 h apart. The animals were divided into five groups of five animals each. In these experiments, principles of laboratory animal care were followed and the study was conducted following the approval by the Institutional Animal Ethical Committee of Jahangirnagar University, Savar, Dhaka, Bangladesh-1342.

2.5. Phytochemical screening

The ethyl acetate extract of *M. repandus* stem underwent phytochemical screening to detect presence of potential phytochemical constituents like alkaloids, carbohydrates, glycosides, flavonoids, saponins, steroids, tannins and terpenoids[3].

2.6. *In vitro* α-amylase inhibitory activity

This study was performed by a modified starch iodine protocol[4]. In short, 1 mL of stem extract or standard acarbose of different concentrations (2, 1, 0.5, 0.25 mg/mL) was taken in pre-labeled test tubes. About 20 μL of α-amylase was added to each test tube and incubated for 10 min at 37 °C. After the incubation 200 μL 1% starch solution was added to each test tube and the mixture was re-incubated for 1 h at 37 °C. Then 200 μL of 1% iodine solution was added to each test tube and after that 10 mL distilled water was added. Absorbance of the mixture was taken at 565 nm. Sample, substrate and α-amylase blank were undertaken under the same conditions. Each experiment was done in triplicate. IC_{50} value was calculated using linear regression analysis.

2.7. Acute toxicity study

For acute toxicity study, forty Swiss albino non-pregnant female mice were used. According to the method of Walum *et al.*, mice were divided into four groups of ten animals each[5]. Different doses (1 000 mg/kg, 2 000 mg/kg, 3 000 mg/kg and 4 000 mg/kg) of ethyl acetate extract of stem were administered by stomach tube. Then the animals were observed for general toxicity signs.

2.8. Experimental protocol for in vivo hypoglycemic activity

2.8.1. Hypoglycemic effect in normal rats

This experiment was performed with slight modification[6]. Twenty Sprague-Dawley female rats were used and they were divided into four groups of five animals each. Rats were kept fasting overnight with free access to water. Group I was treated as control group, Group II was treated with glibenclamide (10 mg/kg body weight), Group III and Group IV was treated with 1 000 mg/kg and 2 000 mg/Kg body weight plant extract respectively. Before administration of drug and extract solutions, fasting blood glucose levels were estimated. Then blood glucose levels were again estimated after 2 h of administration of drug and extract solutions. Glucose levels were measured by glucometer. The maximum hypoglycemic effect of glibenclamide was found after 2 h of its administration.

2.8.2. Hypoglycemic effect in glucose induced hyperglycemic rats [oral glucose tolerance test (OGTT)]

OGTT was performed according to the standard method[7]. Twenty Sprague-Dawley female rats were used and they were divided into four groups of five animals each. Group I was treated as normal control group, Group II was treated with glibenclamide (10 mg/kg body weight), Group III and Group IV were treated with 1 000 mg/kg and 2 000 mg/kg body weight plant extract respectively. Glucose solution (1 g/kg body weight) was administered at first. Then drug and extract solutions were administered to the glucose fed rats. Serum glucose level of blood sample from tail vein was estimated by using glucometer at 0, 1, 2 and 3 h.

2.9. Statistical analysis

The results were expressed as the mean±SEM. The results were statistically analyzed using repeated measures analysis of variance (RM-ANOVA) with Dunnett's and Bonferroni multiple comparisons in OGTT. Paired t-test and One-way ANOVA followed by Dunnett's and Bonferroni multiple comparisons were performed to show significant variation in fasting glucose test. Student's t-test was performed between IC_{50} values. Linear regression analysis was performed to calculate IC_{50} values. $P<0.05$, $P<0.01$, $P<0.001$ and $P<0.0001$ were considered as statistically significant. Statistical programs used were GraphPAD Prism® (version 6.02; GraphPad Software Inc., San Diego, CA, USA), SigmaPlot (version 12.0, Systat Software Inc., San Jose, California, USA), and Microsoft Excel 2007.

3. Results

3.1. Phytochemical screening

The active components found in the extract include glycosides, flavonoids, tannins and terpenoids. Results are further summarized in Table 1.

Table 1

Phytochemical constituents identified in ethyl acetate extract of *M. repandus* stem.

Phytochemicals	Name of the test	Observed changes	Result
Alkaloids	Mayer's test	Creamy white precipitate	−
	Hager's test	Yellow crystalline precipitate	−
	Wagner's test	Brown or deep brown precipitate	−
	Dragendorff's test	Orange or orange-red precipitate	+
	Tannic acid test	Buff color precipitate	−
Carbohydrates	Molisch's test	A red or reddish violet ring is formed at the junction of two layer and on shaking a dark purple solution is formed	−
	Barfoed's test	Red precipitate	−
	Fehling's test	A red or brick-red precipitate	−
	Test for combined reducing sugar	A brick-red precipitate	−
Glycosides	General test	Yellow color	+
	Bromine water test	Yellow precipitate	+
	Test for glucoside	Production of brick-red precipitation	−
Flavonoids	General test	Red color	+
	Shinoda test (magnesium hydrochloride reduction test)	Green to blue color	+
	Zinc hydrochloride reduction test	Red color after few minutes	−
Saponins	Frothing test	Formation of stable foam	−
Steroids	Libermann-Burchard's test	Greenish color	−
Tannins	Lead acetate test	A yellow or red precipitate	+
	Ferric chloride test	Blue green color	+
	Alkaline reagent test	Yellow to red precipitate	+
Terpenoids	Salkowski test	Yellow color appears at the lower layer	+

+: presence, −: absence.

3.2. In vitro α-amylase inhibitory activity

From Table 2, we can see that ethyl acetate extract significantly inhibited α-amylase activity in a dose dependent manner like acarbose. When strength of dose was increased, % inhibition also increased. At 2 mg/mL concentration, ethyl acetate extract of *M. repandus* stem and acarbose exhibited highest % inhibition of α-amylase [(49.670±0.865)% and (79.890±1.170)% respectively]. Moreover, ethyl acetate extract showed IC_{50} value (2.038±0.033) mg/mL whereas standard acarbose showed (0.950±0.000) mg/mL (Table 2). Therefore we can conclude that this stem extract has moderate α-amylase inhibitory activity.

Table 2

IC_{50} values for ethyl acetate extract of *M. repandus* stem and acarbose in α-amylase inhibitory assay.

Extract/ standard	Concentrations with % inhibition				IC_{50} value (mg/mL)
	0.25 mg/mL	0.5 mg/mL	1 mg/mL	2 mg/mL	
MSEA	(2.940±0.566)%	(14.050±1.729)%	(21.900±0.654)%	(49.670±0.865)%	2.038±0.033^b
Acarbose	(16.240±0.670)%	(32.940±0.490)%	(68.290±0.610)%	(79.890±1.170)%	0.950±0.000^a

Values are the mean of triplicate experiments and represented as mean±SEM (n=3). Values in same column with different superscripts are significantly different ($P<0.05$). Student's t test was performed to analyze this data set. MSEA: ethyl acetate extract of *M. repandus* stem.

3.3. Acute toxicity study

The extract administered up to high dose (4 000 mg/kg) produced no mortality. The animals did not manifest any sign of restlessness, respiratory distress, general irritation, coma or convulsion. Hence this extract was considered safe.

3.4. *In vivo* hypoglycemic activity

3.4.1. Hypoglycemic effect in normal rats

A total of 1000 mg/kg of ethyl acetate stem extract and glibenclamide significantly reduced fasting blood glucose level compared to control. Glibenclamide showed significant reduction ($P<0.001$). About 1000 mg/kg of ethyl acetate extract of *M. repandus* stem showed significant reduction ($P<0.01$) but 2000 mg/kg didn't show significant reduction of glucose level. These results suggest that 1000 mg/kg dose may have good blood glucose control capacity like glibenclamide. All results are presented in Table 3.

Table 3

Effect of ethyl acetate extract of *M. repandus* stem on fasting blood glucose level in normal rats.

Group (dose, oral)	Fasting blood glucose level (mmol/L)	
	Before administration	After administration
Control (10 mL/kg)	6.700 ± 0.134^{a}	6.724 ± 0.139^{a}
Glibenclamide (10 mg/kg)	6.040 ± 0.172^{a}	$5.600\pm0.192^{b,x,\beta}$
MSEA (1000 mg/kg)	5.9400 ± 0.0678^{a}	$5.7800\pm0.0583^{b,x,\alpha}$
MSEA (2000 mg/kg)	7.300 ± 0.249^{a}	$6.960\pm0.254^{b,y}$

Values are presented in mean±SEM (n=5). MSEA: ethyl acetate extract of *M. repandus* stem. A) Values in same row with different superscripts are significantly different. For $^{a,a}P>0.05$ and for $^{a,b}P<0.05$. Paired t-test was performed to analyze before and after relationship. B) Values with different superscripts in same column are significantly different from control after the administration of standard and different doses of the extract. For $^{\alpha}P<0.01$ and $^{\beta}P<0.001$. One-way ANOVA followed by Dunnett's multiple comparison was performed to analyze this comparison. C) Values with different superscripts $^{(x,y)}$ are significantly different from each other in the same column among standard and different doses of the extract after administration. One-way ANOVA followed by Bonferroni multiple comparison was performed to analyze this inter relationship.

3.4.2. Hypoglycemic effect in glucose induced hyperglycemic rats (OGTT)

Doses 1000 mg/kg and 2000 mg/kg of ethyl acetate stem extract manifested good hypoglycemic activity in each specific hour ($P<0.0001$). Standard glibenclamide (10 mg/kg) showed well hypoglycemic activity in each specific hour after administration. The highest hypoglycemic activity for glibenclamide was observed at 3 h while in case of plant extract 2000 mg/kg dose showed maximum hypoglycemic activity at 3 h. Relation between different timing and hypoglycemic activity was also found extremely significant ($P<0.0001$) with an F (3, 48) value 202.4 in this experiment (Table 4).

Table 4

Effect of ethyl acetate extract of *M. repandus* stem on glucose induced hyperglycemia in normal rats.

Treatment groups	Blood glucose level (mmol/L)			
	0 h	1 h	2 h	3 h
Control (10 mL/kg)	6.560 ± 0.175	7.820 ± 0.245	7.420 ± 0.180	7.220 ± 0.116
Glibenclamide (10 mg/kg)	7.060 ± 0.133	$3.440\pm0.117^{\alpha,x}$	$3.160\pm0.087^{\alpha,x}$	$2.440\pm0.098^{\alpha,x}$
MSEA (1000 mg/kg)	6.600 ± 0.152	$6.320\pm0.146^{\alpha,y}$	$6.180\pm0.111^{\alpha,y}$	$5.580\pm0.136^{\alpha,z}$
MSEA (2000 mg/kg)	6.860 ± 0.112	$6.600\pm0.109^{\alpha,y}$	$5.620\pm0.256^{\alpha,y}$	$4.880\pm0.250^{\alpha,y}$

Values are presented in mean±SEM (n=5). MSEA: ethyl acetate extract of *M. repandus* stem. Repeated measures ANOVA with Dunnett's and Bonferroni multiple comparisons were performed to analyze this data set. Overall time effect [$F(3, 48)=202.4$, $P<0.0001$] is considered extremely significant. A) Values with different superscripts in same column are significantly different from control at each specific hour. For $^{\alpha}P<0.0001$. Dunnett's multiple comparison was performed. B) Values with different superscripts $^{(x,y,z)}$ in same column are significantly different from each other ($P<0.05$) at each specific hour when compared among different doses of the extract and standard. Bonferroni multiple comparison was performed.

4. Discussion

4.1. α-Amylase inhibitory activity

In human body, α-amylase is one of the key enzymes that breaks down starch to more simple sugars and increase the absorption rate of glucose. As a consequence, postprandial blood glucose level is increased[8,9]. Slowing the digestion and breakdown of starch may have promising effects on insulin resistance and glycemic index control in people with diabetes mellitus[10-12]. In our study, we found that ethyl acetate extract of stem moderately inhibit α-amylase. From preliminary phytochemical screening, we can report the presence of flavonoids, tannins, terpenoids and glycosides. These natural compounds may attribute to this activity. Natural polyphenols have been described to have potential to hinder the activity of carbohydrate hydrolyzing enzymes like α-amylase and α-glucosidase[13]. Flavonoids are recognized as selective candidate for amylase enzyme inhibition. Several types of flavonoids like flavone, amentoflavone, isoflavone, flavanonol, luteolin *etc*. were identified and tested for amylase inhibitory property previously[14,15]. α-Amylase inhibitory activity was associated with many types of terpenoids, for example, lupeol, ursolic acid, oleanolic acid *etc*[16]. Hui and Li identified lupeol and ursolic acid in this plant which strongly support the claim of α-amylase inhibition property[17]. The main α-amylase inhibitory activity of the tannin is associated with its capability to strongly bind to carbohydrates and proteins[18]. But tannins are not always an effective inhibitor of α-amylase[19-21]. Inhibitory activities of cyanidin and its glycosides and synergistic effect with acarbose against intestinal α-glucosidase and pancreatic α-amylase have been proven successfully[22]. The mechanism by which this plant extract exerted this activity may be due to its action on carbohydrate binding regions of α-amylase enzymes that catalyze hydrolysis of the internal α-1, 4 glucosidic linkages in starch. Natural inhibitors from this dietary plant have α-amylase inhibitory activity and could be used as effective treatment for the management of postprandial hyperglycemia. A drug-development program should be undertaken to develop modern drugs with the compounds isolated from this plant.

4.2. *In vivo* hypoglycemic activity

In fasting glucose test and OGTT, *M. repandus* stem extract showed significant hypoglycemic potential. We observed the presence of some potential phytochemicals in preliminary phytochemical screening test which may be liable for this activity. Flavonoids have been found to stimulate insulin secretion from β-cells and have an insulin like effect isolated from the other anti-diabetic medicinal plants[23-26]. Effect of the flavonoids such as quercetin and ferulic acid on pancreatic β-cells leading to their production and secretion of more insulin have been proposed by Mahesh and Menon[27]. Terpenoids identified in primary phytochemical screening may contribute to this promising hypoglycemic activity[28,29]. Terpenes have been found to stimulate the secretion of insulin or possess insulin like effect isolated from anti-diabetic medicinal plants. Terpenoids type components mono-terpenes cause a restoration to normal glycogen metabolism when hepatic glycogen concentration is reduced[30]. The presence of a pharmacologically active terpenoid called lupeol has been confirmed in this plant[17]. A research group has also found its activity as a dipeptidyl peptidase–4 inhibitor. Dipeptidyl peptidase–4 plays a key role in glucose metabolism. It is responsible for the degradation of incretins such as glucagon–like peptide–1[31]. Tannins may also possess hypoglycemic property[32,33]. Tannic acid, a major component of tannin stimulates glucose transport and inhibits adipocyte differentiation in 3T3–L1 cells and thus may be useful for the prevention and treatment of type 2 diabetes mellitus and its associated obesity[34]. Hypoglycemic effect of glycoside is also proved[35,36]. Biological evaluation exhibited that isolated cucurbitane type triterpene glycosides from *Momordica charantia* showed potent hypoglycemic effect through glucose uptake assay[37]. We are still not sure about how this plant extract exert potent hypoglycemic effect. In the future, detecting the active biological compounds responsible for this hypoglycemic action may offer novel and safe anti–diabetic compound.

We are still not sure about how ethyl acetate extract of *M. repandus* stem can exert multifaceted medicinal property. It may be suggested that the bioactive compounds present in the ethyl acetate stem extract may be responsible for multifaceted effects. Further co–ordinated and well–structured studies would be required to isolate the bioactive compounds and determine their underlying molecular mechanism of action, pharmaco–therapeutics and toxicity. After proper standardization and clinical trials, development of modern drugs from this plant should be emphasized for the control of diabetes.

Conflict of interest statement

We declare that we have no conflict of interest.

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Comments

Background

M. repandus (Family: Euphorbiaceae), commonly called Gunti or Jhante or Bon natai, is a wild species available in Bangladesh. This plant has some medicinal properties such as anti–radical, anti–viral (HIV–1) and uterus muscle stimulant activity. Also, it can be used to treat inflammation, liver–toxicity, ulcer and tumor. According to the literature search, there is no work has been done on the stem of this plant for hypoglycemic and amylase enzyme inhibition activity.

Research frontiers

The hypoglycemic effect in OGTT and α–amylase inhibitory activity was evaluated. The results support significant activity of the stem extract.

Related reports

Hui and Li (1977) identified lupeol and ursolic acid in this plant which strongly support the claim of α–amylase inhibition property. Nandhini *et al*. (2013) proved the antidiabetic effect of *Mallotus philippinensis* in the same model. This plant is another species of the same genus.

Innovations & breakthroughs

This is the first research work on *M. repandus* stem for hypoglycemic effect. In future these findings will help researchers to find out potential anti–diabetic agents in this plant.

Applications

This plant could be further studied for both drug development and establishment of the ethno–medicinal use of this plant. Finding out potential phytochemicals may contribute to research on other sector such as inflammation, liver toxicity, tumor, antimicrobial and muscle stimulant activity studies.

Peer review

The article is a nice piece of work which can provide *in vitro* evidence for potential inhibition of α–amylase enzyme followed by an *in vivo* study on the effect of hypoglycemic activity in normal and glucose induced hyperglycemic rats. Authors followed the standard methodology for conducting the study. The results are pretty interesting and suggest that ethyl acetate extract of *M. repandus* stem may be exploited in the development of anti–diabetic therapeutics and can be considered as a potential candidate for the development of new oral hypoglycemic agent.

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