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In vitro α-amylase inhibitory activity and *in vivo* hypoglycemic effect of methanol extract of *Citrus macroptera* Montr. fruit

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PEER REVIEW

Peer reviewer

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Comments

This research is an excellent piece of work where hypoglycemic effect of a fruit extract from Citrus macroptera observed and this is a unique work of this fruit in the world as far as I know. Author coherently discussed therapeutic properties of phytochemical constituents to support the hypoglycemic potential of the fruit extract. α-Amylase inhibition property and OGTT was performed for this research and the result was significant; also there was a set up of standard methodology.

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ABSTRACT

Objective: To investigate the therapeutic effects of methanol extract of *Citrus macroptera* Montr. fruit in α-amylase inhibitory activity (*in vitro*) and hypoglycemic activity in normal and glucose induced hyperglycemic rats (*in vivo*).

Methods: Fruits of *Citrus macroptera* without rind was extracted with pure methanol following cold extraction and tested for presence of phytochemical constituents, α-amylase inhibitory activity, and hypoglycemic effect in normal rats and glucose induced hyperglycemic rats.

Results: Presence of saponin, steroid and terpenoid were identified in the extract. The results showed that fruit extract had moderate α-amylase inhibitory activity [IC_{50} value=(3.638±0.190) mg/mL] as compared to acarbose. Moreover at 500 mg/kg and 1 000 mg/kg doses fruit extract significantly (P<0.05 and P<0.01 respectively) reduced fasting blood glucose level in normal rats as compared to glibenclamide (5 mg/kg). In oral glucose tolerance test, 500 mg/kg dose significantly reduced blood glucose level (P<0.05) at 2 h but 1 000 mg/kg dose significantly reduced blood glucose level at 2 h and 3 h (P<0.05 and P<0.01 respectively) whereas glibenclamide (5 mg/kg) significantly reduced glucose level at every hour after administration. Overall time effect is also considered extremely significant with F value=23.83 and P value=0.0001 in oral glucose tolerance test.

Conclusion: These findings suggest that the plant may be a potential source for the development of new oral hypoglycemic agent.

KEYWORDS

Diabetes mellitus, Hypoglycemic, *Citrus macroptera*, α-Amylase, OGTT, Glibenclamide

1. Introduction

Diabetes mellitus (DM) is a chronic metabolic disorder characterized by both postprandial and fasting hyperglycemia with disturbances in carbohydrate, fat and protein metabolism. Hyperglycemia in diabetes results either from an absolute deficiency in insulin secretion (type 1 DM) or insulin action (type 2 DM) or both. The incidence of diabetes has increased worldwide in recent years. The estimated number of people with diabetes was 30 million in 1985, 150 million in 2000 and then 246 million in 2007, according to the International Diabetes Federation. It

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expects this number to hit 380 million by 2025[1].

Treatment of diabetes include: enhancement of the action of insulin at the target tissues, with the use of sensitizers (biguanides, thiozolidinediones); stimulation of endogenous insulin secretion with the use of sulfonylureas (glibenclamide, glimepiride), and reduction of the demand for insulin using specific enzyme inhibitors (acarbose, miglitol)[2]. However, there is a burden of unwanted side effects like diarrhea, nausea, dyspepsia, myocardial infarction, peripheral edema and dizziness with the use of these drugs. Plants have been an exemplary source of drugs that have been derived directly or indirectly from them. It is reported that about 800 plants may possess anti-diabetic potential[3]. Hypoglycemic activity of medicinal plants is due to their ability to restore the function of pancreatic tissues by causing an increase in insulin output, inhibiting the intestinal absorption of glucose or facilitating metabolites in insulin dependent processes[4,5].

Citrus macroptera Montr. (family-Rutaceae) (*C. macroptera*) commonly called Sat Kara (wild orange), is a semi-wild species of *Citrus* native in Malesia and Melanesia. The tree, which has thorns, can reach 5 m in height. Its fruit is about 6-7 cm in diameter, has a fairly smooth, moderately thick rind, and turns yellow when it is ripe. The pulp of the fruit is greenish yellow and dry (does not produce much juice). The juice is very sour, and somewhat bitter. In Bangladesh the rind of the *C. macroptera* is eaten as a vegetable. This plant is medicinally used locally in Assam. Physicians always suggest diabetic patient to eat *Citrus* fruits to control their blood glucose level. Researchers also concluded that *Citrus* fruit extracts represent an excellent alternative for nutraceuticals and functional foods geared towards the management of diabetes. Essential oil of fruit of *Citrus* maximally showed significant reduction of fasting blood glucose and hepatic glucose levels while hepatic glycogen significantly increased when compared to diabetic control animals[6]. According to a 2006 animal study, *Citrus* extracts not only slow glucose uptake, but also inhibit the movement or transport of glucose through the intestines and liver[7]. A 2009 study examining extracts from a Korean *Citrus* fruit called Dangyuja (*Citrus grandis*) found that it holds great potential for controlling blood glucose levels in diabetic patients[8]. *Citrus limetta* fruit peel demonstrated a potential anti-hyperglycemic effect in streptozotocin induced diabetic rats[9]. There are also many other research reports about this therapeutic effect of *Citrus* fruits. Investigation on *C. macroptera* for hypoglycemic property has not been performed yet. That's why we have designed our research work to explore possible mechanism of hypoglycemic activity of this fruit extract.

2. Materials and methods

2.1. Drugs, chemicals and apparatus

Methanol 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. Heparin injection was purchased from Rotex Medica, Germany. Amylase was obtained from Merck, Germany. All the chemicals and reagents were analytical grade. Match® glucometer with strips were purchased from Mohammadpur, Dhaka. Humalyzer 3500 was obtained from Human Inc., Germany.

2.2. Plant material

Fruits of *C. macroptera* were collected from Sylhet, Bangladesh and authenticated by Md. Abdur Rahim, Technical officer, Department of Botany, Jahangirnagar University. A voucher specimen (Acc. No. 38619) was deposited in the herbarium for future reference. The peels were removed and the fruits were dried and used for further processing.

2.3. Preparation of plant extract

Fruits without rind were treated with sufficient amount of pure methanol for one week at room temperature with occasional shaking. The extract was filtered through a cotton plug followed by Whatman No. 1 filter paper. The filtrate was then evaporated under reduced pressure to give a dark green viscous mass and stored at 4 °C until use.

2.4. Animals and experimental set-up

Sprague-Dawley female rats of 100-200 g were collected from Pharmacology Laboratory, Department of Pharmacy, Jahangirnagar University and were 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 (22±3) °C and light/darkness alternated 12 h apart. The animals were divided into four groups of five animals each.

2.5. Phytochemical screening

The crude methanol extract of *C. macroptera* fruits underwent phytochemical screening to detect presence of potential phytochemical constituents like alkaloid, flavonoid, saponin, tannin, carbohydrate, glycoside, glucoside, fat and fixed oil, steroid and terpenoid[10].

2.6. In vitro α-amylase inhibitory activity

This study was performed by a modified starch iodine protocol[11]. In short, 1 mL of plant extract or standard of different concentration (2, 1, 0.5 mg/mL) was taken in pre-labeled test tubes. A volume of 20 μL of α-amylase was added to each test tube and incubated for 10 min at 37 °C. After the incubation 200 μL of 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 by using regression analysis.

$$\%\ \alpha\text{-amylase inhibition} = \left[1 - \frac{(SA - SBB) - SMB}{AAB}\right] \times 100$$

SA=Sample absorbance, SMB=Sample blank, SBB=Substrate blank, AAB=α-Amylase blank

2.7. Acute toxicity study

According to the method of Walum, *et al*. rats were divided into four groups of five animals each[12]. Different doses (1 000 mg/kg, 2 000 mg/kg, 3 000 mg/kg and 4 000 mg/kg) of methanol extracts 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

Rats were kept fasting overnight with free access to water. Group I was treated as control group, Group II was treated with glibenclamide (5 mg/kg body weight), Group III and IV was treated with 500 mg/kg and 1 000 mg/kg body weight respectively. Before administration of drug and extract solutions fasting blood glucose levels were estimated by glucose oxidase method[13]. Then blood glucose levels were again estimated after 2 h of administration of drug and extract solutions. Glucose levels were measured by blood Humalyzer instrument. The maximum hypoglycemic effect of glibenclamide was found after 2 h of its administration.

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

Oral glucose tolerance test (OGTT) was performed according to the standard method[14]. Group I was treated as normal control group, Group II treated with glibenclamide (5 mg/kg body weight), Group III and IV treated with 500 mg/kg and 1 000 mg/kg body weight respectively. Glucose solution (1 g/kg body weight) was administered at first. Then drug and extract solutions were administered to the glucose fed. 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 with Dunnett's multiple comparison when compared against control in OGTT. Paired *t* test was performed to show significant variation between before and after blood glucose level. Student's *t* test was performed between IC_{50} values. Regression analysis was performed to calculate IC_{50} values. $P<0.05$, $P<0.01$ and $P<0.001$ were considered as statistically significant. Statistical programs used were GRAPHPAD PRISM® (version 6.00; 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; saponin, steroid and terpenoid. Results are shown in Table 1.

Table1
Phytochemical constituents identified in methanol extract of *C. macroptera* fruit.

Phytochemical constituents	Specific tests	Result
Carbohydrate	Molisch 's test	−
	Barfoed's test	−
	Benedict's test	−
	Fehling test	−
Glycoside	General test	−
	Bromine water test	−
Glucoside	General test	−
Fat and fixed oils	Stain test	−
	Saponification test	−
Alkaloid	Mayer's test	−
	Hager's test	−
	Dragendorff's test	−
	Wagner test	−
	Tannic acid test	−
Tannin	Lead acetate test	−
	Ferric chloride test	−
	Alkaline reagent test	−
Steroid	Liebermann-Burchard's test	+
Terpenoid	Salkowski test	+
Saponin	Frothing test	+ +
Flavonoid	General test	−
	Shinoda test	+/−

+: Presence, −: Absence, +/−: Presence/absence not ascertained.

3.2. In vitro α-amylase inhibitory activity

Methanol extract showed IC_{50} value (3.638±0.190) mg/mL whereas standard acarbose showed (0.912±0.015) mg/mL. Methanol extract significantly inhibited α-amylase activity in a dose dependent manner like acarbose. Therefore we can conclude that this fruit extract have moderate α-amylase inhibitory activity. All results are shown in Table 2.

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 for rats.

Table 2

IC_{50} values (mg/mL) for *C. macroptera* methanolic fruit extract and acarbose in α-amylase inhibitory assay.

Extract/ Standard	Concentrations in mg/mL with (% Inhibition)			IC_{50} value (mg/mL)
Methanol extract	0.50 (8.974±0.256)	1.00 (13.846±0.444)	2.00 (28.718±1.282)	3.638±0.190[b]
Acarbose	0.25 (15.897±1.117)	0.50 (26.051±0.438)	1.00 (55.385±0.888)	0.912± 0.015[a]

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

3.4. In vivo hypoglycemic activity

3.4.1. Hypoglycemic effect in normal rats

Both doses of methanol extract and glibenclamide significantly reduced fasting blood glucose level. Glibenclamide showed significant reduction at level of P<0.01. Dose of 500 mg/kg and 1 000mg/kg methanolic fruit extract showed significant reduction at level of P<0.05 and P<0.01 respectively. These results suggest that hypoglycemic activity of 1 000 mg/kg dose and glibenclamide has similar significance level. All results are presented in Table 3.

Table 3

Effect of *C. macroptera* methanolic fruit extract on fasting blood glucose level (mmol/L) in normal rats.

Group	Dose (oral)	Before administration	After administration
Control	10 mL/kg	4.851±1.188[a]	5.764±0.668[a]
Glibenclamide	5 mg/kg	3.952±0.334[a]	2.016±0.113[b]
500 mg/kg	500 mg/kg	4.280±0.195[a]	3.473±0.102[b]
1000 mg/kg	1000 mg/kg	6.089±0.397[a]	4.055±0.317[b]

Values are presented in mean±SEM (n=4). Values in the same row with different superscripts are significantly different. For control (a, b) P>0.05, for glibenclamide (a, b) P<0.01, for Fruit-500 mg/kg (a, b) P<0.05 and for Fruit-1 000 mg/kg (a, b) P<0.01. Paired t test was performed to analyze this data set.

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

Experimental induction of hyperglycemia resulted in increased glucose level in blood (comparing the bar diagram of control of 0 h and 1 hour, Figure 1). Both dose of fruit extract did not manifest any significant reduction in 1st hour after administration. Most significant reduction (P<0.05) was observed for 500 mg/kg dose of methanolic fruit extract at 2 h but maximum reduction of 1 000 mg/kg dose occurred at 3 h showing a significance level of P<0.01. At 2 h this dose also showed significant reduction (P<0.05). Standard glibenclamide (5 mg/kg) showed significant reduction in 1, 2 and 3 h. These findings suggest that evidently 1 000 mg/kg dose is more potent than 500 mg/kg dose. Time interaction with each specific hour in this experiment was also found extremely significant (P<0.000 1) with an F value 23.83 (Table 4).

Table 4

Effect of *C. macroptera* methanolic fruit extract on glucose induced hyperglycemia (mmol/L) in normal rats.

Treatment groups	0 hour	1 hour	2 hour	3 hour	Time effect (F value)	P value for time effect
Control	6.850±0.222	7.850±0.569	7.150±0.272	6.475±0.175		
Glibenclamide	6.950±0.301	6.250±0.589[b]	4.125±0.239[c]	4.275±0.476[c]		
500 mg/kg	6.500±0.267	6.875±0.095	5.900±0.303[a]	5.500±0.450	$F_{(3, 36)}$=23.83	P<0.000 1
1000 mg/kg	6.600±0.168	6.750±0.189	5.700±0.470[a]	4.675±0.459[b]		

Values are presented in mean±SEM (n=4). Values with different superscripts in the same column are significantly different from control at each specific hour. [a]: P<0.05, [b]: P<0.01 and [c]: P<0.001. Overall time effect is considered extremely significant and its F and P value are given in the table. Repeated measures Anova with Dunnett's multiple comparison were performed to analyze this data set.

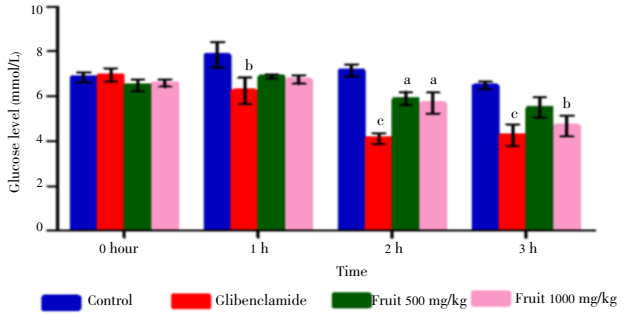

Figure 1. Effect of different doses of fruit extract of *C. macroptera* and glibenclamide on oral glucose tolerance test. Results are expressed as mean± SEM. Values with different superscripts are significantly different from control.

4. Discussion

4.1. α-Amylase inhibitory activity

α-Amylase is one of the main enzymes in human body that is responsible for the breakdown of starch to more simple

sugars. α-Amylases hydrolyze complex polysaccharides to produce oligosaccharides and disaccharides which are then hydrolyzed by α-glycosidase to monosaccharide which are absorbed through the small intestines into the hepatic portal vein and increase postprandial glucose levels[15,16]. Amylase inhibitors are also known as starch blockers because they prevent dietary starch from being absorbed by the body and thereby lower postprandial glucose levels. Slowing the digestion and breakdown of starch may have beneficial effects on insulin resistance and glycemic index control in people with diabetes[17-19]. In our investigation we found that methanolic fruit extract moderately inhibited α-amylase. From phytochemical screening we can see that presence of saponin, steroid and terpenoid which may be responsible for this therapeutic activity. Natural polyphenols have been reported to inhibit the activity of carbohydrate hydrolyzing enzymes like α-amylase, α-glucosidase[18]. Terpenoids represent a promising source for biologically active natural compounds which have potential for research and development of new substances with pharmacologic activity. α-Amylase inhibitory activity was related only for oleanane, ursane and lupane type terpenoids[20]. In a previous study saponins have also been found to be a probable α-amylase inhibitor[21,22]. Lupeol, a terpenoid compound has been isolated from stem bark of *C. macroptera* which could be α-amylase inhibitor[23]. There are previous findings about the potentiality of this compound. Lupeol have been shown to inhibit α-amylase reported by Hasenah Ali, *et al*[24]. The mechanism by which this fruit extract exerted this effect 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 and other related polysaccharides have also been targeted for the suppression of postprandial hyperglycemia. Therefore, this study buttress the claim that natural inhibitors from dietary plants have α-amylase inhibitory activity and could be used as effective therapy for the management of postprandial hyperglycemia with minimal side effects.

4.2. In vivo hypoglycemic activity

In this study methanolic fruit extract of *C. macroptera* exerted significant hypoglycemic activity in both fasting glucose level reduction in normal rats and oral glucose tolerance test in glucose induced hyperglycemic rats. To our best knowledge this is the first study about hypoglycemic activity of *C. macroptera*. That's why the precise mode of action is not determined yet. However this fruit extract contains some potent phytochemical constituents like saponin, steroid and terpenoid which may be responsible for this action. Earlier investigations found saponins to be bioactive against diabetes[25,26]. Saponins can influence transport systems that are situated in the brush-border. For example, Sidhu, *et al.* gave rats soyabean saponins (2 g/L) directly into the small intestine and this led to a reduced glucose uptake[27]. Glycemic decrease that may also occur due to the stimulatory effect on beta cells to promote insulin release and intracellular glycogen deposition. Total saponins identified from traditional medicinal plant in China dramatically reduced fasted blood glucose and serum insulin levels and alleviated hyperglycemia associated oxidative stress in experimental type 2 DM rats[28]. Terpenoids identified in primary screening may contribute to this property. Terpenoids isolated from the some anti-diabetic medicinal plants has been found to stimulate secretion or possess an insulin like-effect[29]. Glycogen concentration is directly proportional to insulin level[30]. Insulin promotes intracellular glycogen deposition by stimulating glycogen synthesis and inhibiting glycogen phosphorylase[30]. Such as insulin, terpenoids type component or monoterpenes may cause a restoration to normal glycogen metabolism when hepatic glycogen concentration is decreased[31]. Lupeol and stigmasterol isolated from stem bark of *C. macroptera* may have hypoglycemic potential[23]. Lupeol is a pharmacologically active terpenoid. One study has also found some activity as a dipeptidyl peptidase-4 inhibitor. Dipeptidyl peptidase-4 plays a major role in glucose metabolism. It is responsible for the degradation of incretins such as glucagon-like peptide-1[32]. Lupeol may also have amylase inhibition property which we told in previous section. We found the presence of steroid in phytochemical screening test which could also contribute to hypoglycemic action. In an earlier investigation Daisy, *et al.* isolated a novel steroid demonstrated a significant anti-diabetic activity by reducing the elevated blood glucose levels and restoring the insulin levels in streptozotocin-induced diabetic rats[33]. Stigmasterol, an steroid molecule, also possess hypoglycemic property[34]. Lemonene isolated from peel extract of *C. macroptera* may be a potential alternative[35]. In a previous study its anti-hyperglycemic activity has been confirmed in streptozotocin-induced diabetic rats by increasing glucokinase activity along with liver glycogen synthesis and decreasing plasma glucose and glycosylated hemoglobin levels[36]. Besides phytochemical constituents found in this extract may also have anti-α-amylase activity (discussed in previous section) and contribute to this *in vivo* hypoglycemic action.

We are still not sure about how this fruit extract can exert

potent hypoglycemic activity. It is a logical inference that this extract may decrease the activity of α-amylase in the digestive canal, improve the metabolism of glucose and increase insulin secretion by stimulating beta cells. It is possibe to suggest that the bioactive compounds present in the fruit extract may be responsible for multifaceted effects. However further co-ordinated and well-structured studies would be required to isolate the bioactive compounds and determine their underlying molecular mechanism of action on diabetes induced rat model.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgements

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Comments

Background

C. macroptera is commonly called 'wild orange'. It is so-called name because of the large 'wings' (-ptera) on the petiole, which is as large as the blade of the leaf. The juice is very sour, and somewhat bitter. It has a unique taste and aroma. According to the literature search, there is no work that has been done on the fruit of this plant for hypoglycemic activity.

Research frontiers

The hypoglycemic effect in OGTT and α-amylase inhibitory activity was evaluated. The results support significant activity of this fruit extract which was the cutting age in the field of the research in this paper.

Related reports

Lupeol and stigmasterol were isolated from the crude extracts of the stem bark of *C. macroptera* (family: Rutaceae) by Chowdhury, *et al* (2008). The essential oil of *C. macroptera* Montr. contained limonene, beta-caryophyllene and geranial as main compounds reported by Rana, *et al* (2012). Traditionally this fruit is used as vegetable and curries cooked with Satkara and beef or mutton is now served in many restaurants in Bangladesh.

Innovations and breakthroughs

This is the first research work on *C. macroptera* fruit for hypoglycemic effect. In future these findings will help researchers to find out potential antidiabetic agents in this plant.

Applications

C. macroptera fruit is safe to eat. This study supports the claim that diabetes patients can eat this fruit because there is no restriction from physicians to take *Citrus* fruits. So this plant could be further studied for both drug development and establishment of the ethno medicinal use from this.

Peer review

This research is an excellent piece of work where hypoglycemic effect of a fruit extract from *C. macroptera* observed and this is a unique work of this fruit in the world as far as I know. Author coherently discussed therapeutic properties of phytochemical constituents to support the hypoglycemic potential of the fruit extract. α-Amylase inhibition property and OGTT was performed for this research and the result was significant; also there was a set up of standard methodology.

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