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RESEARCH ARTICLE

Antidiabetic Evaluation of Leaf Extracts of *Naringi crenulata* (Roxb.) Nicolson

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ABSTRACT

Naringi crenulata (Roxb.) Nicolson belongs to family Rutaceae is a widespread species of the genus-*Naringi*. It is commonly known as 'Bilvaparni' in Sanskrit and 'Benta' in Odia. The root extract is used in vomiting, dysentery and colic disorders. Fruit decoction is used as an antidote to insect poison. The bark juice is applied externally for getting speedy relief in sprain. The methanol extract is reported to have significant anthelmintic activity and ethanol extracts of leaf and bark showed anticancer, hepatoprotective, aphrodisiac, anti-inflammatory activities. *N. crenulata* leaves were collected from Talabira forest region in Sambalpur district, Odisha, in the month of February-March and extracted successively with petroleum ether (60-80^0 C) and methanol. Preliminary phytochemical screening of extracts indicated the presence of steroids, triterpenoids in petroleum ether extract and alkaloids, flavonoids, saponins, tannins, triterpenoids, steroids and glycosides in methanol extract. The methanol extract of *N. crenulata* was fractionated by column chromatography using a glass column packed with silica gel (100-200 mesh) and developed by gradient elution with n-hexane and combination of n-hexane : ethyl acetate in the increasing order of polarity (10%, 20% ethyl acetate in n-hexane) which resulted in the fractions NCMF-1, NCMF-2 and NCMF-3, respectively. The antihyperglycaemic activity was evaluated in normal, glucose-loaded and Streptozotocin-induced hyperglycaemic rats (single and multi dose treatment). In normoglycaemic rats, the test extracts showed progressive fall of blood glucose level till the end of 8 h. In glucose-loaded animals (OGTT), reduction in blood glucose level was observed after 60 minutes of administration of the test substances. The maximum reduction was observed at 4 h with methanol extract exhibiting maximum improvement in glucose tolerance. The extracts produced significant decrease in the blood glucose level in streptozotocin-induced hyperglycaemic rats when compared with the diabetic control group in the single dose treatment study at the tested dose level of 400 mg/kg of body weight. In multi-dose treated hyperglycaemic rats, both the extracts and fractions showed various degree of blood glucose reduction, among which NCMF-3 exhibited highest percentage of reduction in blood glucose level. Continuous administration of extracts and fractions for 14 days leads to significant decrease in serum total cholesterol, triglycerides, LDL and VLDL levels, while increase in total protein and HDL levels was recorded. The *in vitro* study showed an increased utilization of the glucose by α-amylase inhibition assay in presence of methanol extract which suggests that the test extract may inhibit the digestion and absorption of glucose through intestine. These findings suggest that the plant may be a potential source for the development of new oral antihyperglycaemic agent.

KEYWORDS

Naringi crenulata, Extract, Fraction, Antidiabetic, Streptozotocin, α- Amylase

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INTRODUCTION

Diabetes is a metabolic disorder which is affecting approximately more than 4% of the world population. The disease becomes a real problem of public health in developing countries, where its prevalence is increasing steadily and adequate treatment is often expensive or unavailable. Moreover, uncontrolled diabetes leads to many chronic complications such as blindness, heart failure, and renal failure. In order to prevent this alarming health problem, the development of research into new hypoglycaemic and potentially antidiabetic agents is of great interest. Currently available oral anti-diabetic drugs that are used clinically for glycaemic control include sulfonylureas, meglitinides, biguanides, α-glucosidase inhibitors, and thiazolidinediones (TZDs). Presently, there is growing interest in herbal remedies due to the side effects associated with the oral synthetic hypoglycaemic agents for the treatment of *Diabetes mellitus*. Herbal treatments for diabetes have been used in patients with insulin dependent and non-insulin dependent diabetes. Medicinal plant species such as *Cinnamomum verum* (cinnamon), *Brassica juncea* (mustard), *Allium sativum* (garlic), *Coriandrum sativum* (coriander), *Zingiber officinale* (ginger), etc. have been used as remedies for diabetes as they contain chemical components of therapeutic value[1] that produce physiological action on the human body. Type-1 diabetes is caused by the low level of insulin production by the pancreas. The main approach of treatment of this type of diabetes is through constant provision of synthesised insulin to patients. The type-2 diabetes is not due to low level of production of insulin by the pancreas. With type-2 diabetes, insulin is produced sufficiently but is not properly absorbed by the tissues in order to metabolise blood sugar. The gestational diabetes is similar to type-2 diabetes but occurs during pregnancy in women who are genetically predisposed to diabetes.

Diabetes mellitus is caused by the abnormality of carbohydrate metabolism which is linked to low blood insulin level or insensitivity of target organs to insulin.[2] Despite considerable progress in the treatment of diabetes by oral hypoglycaemic agents, search for newer drugs continues because the existing synthetic drugs have several limitations. The herbal drugs with antidiabetic activity are yet to be commercially formulated as modern medicines, even though they have been acclaimed for their therapeutic properties in the traditional systems of medicine.[3] Type-2 diabetes usually occurs in obese individuals and is associated with hypertension and dyslipidemia. Thus the treatment aims to reduce insulin resistance and to stimulate insulin secretion. Diabetes is a metabolic disorder where in human body does not produce or properly utilize insulin, a hormone that is required to convert sugar, starches, and other food into energy. '*Diabetes mellitus'* is characterized by constant high levels of blood glucose (sugar). Human body has to maintain the blood glucose levels at a very narrow range which is done with insulin and glucagon. The function of glucagon is causing the liver to release glucose from its cells into the blood for the production of energy.

Type-1 diabetes leads to inability to release insulin results in low rates of glucose uptake into muscles and adipose tissue.[4] Traditional medicine or herbal medicine is used for treatment of diabetes in developing countries where the cost of conventional medicines is a burden to the population.[5] Despite the introduction of hypoglycaemic agents from natural and synthetic sources, diabetes and its secondary complications continue to be a major medical problem. Many indigenous Indian medicinal plants have been found to be useful to successfully manage diabetes. One of the great advantages of medicinal plants is that these are readily available and have very low side effects. Plants have always been an exemplary source of drugs and many of the currently available drugs have been derived directly or indirectly from them. The ethnobotanical information reports about 800 plants that may possess antidiabetic potential.[6] Several herbs have shown antidiabetic activity when assessed using presently available experimental techniques.[7]

The involved cost and lack of infrastructure, to produce allopathic drugs prevailing in rural India

gives a scope to incline to its traditional source of healthcare through 'Ayurveda' particularly in the use of medicinal plants. Mankind has a long history in the use of herbal medicines. Rigveda and 'Atharvaveda' (4500-1600 BC) reveal that ancient Indians had a rich knowledge of the use of medicinal plants. India unquestionably occupies the topmost position in the use of herbal drugs since ancient times utilizing nearly 600 plant species in different formulations. Great majorities of people in India have been depending on crude drugs for the treatment of various diseases as evidenced from well-documented indigenous system of medicines, 'Ayurveda' and 'Unani'. The Materia Medica of these systems contains a rich heritage of indigenous herbal drugs.[8] The role of traditional medicines in the solution of health problems is invaluable on a global level. This is more striking when we consider the fact that approximately 80% of the people living in less developed countries rely exclusively on traditional medicine for their health care needs. Traditional Indian and Ayurvedic medical system for example, have been evolved during thousands of years and have left for posterity a well documented literary legacy which permits us to recognize immediately a theoretical base whose conceptual framework even if were more or less archaic is found to be logical. In other countries the ethnomedical heritage has not reached a high status. However, many useful points can still be found in them. The sources, which support the popular Pharmacopoeia, are raw materials of vegetable, animal and mineral origin. However, the most important therapeutic resource is that of vegetable origin. They are qualitatively and quantitatively superior to the other two. Often impregnated with contradictory religious creeds we find plants used as proper medicines, food and no less importantly plants used in religious rituals acknowledged as 'placebo' or those used in 'baths'.

As diabetes is a multifactorial disease leading to several complications, and therefore demands a multiple therapeutic approach. Patients of diabetes either do not make enough insulin or their cells do not respond to insulin. In case of total lack of insulin, patients are given insulin injections. Whereas in case of those where cells do not respond to insulin, many different drugs are developed taking into consideration possible disturbances in carbohydrate-metabolism. For example, to manage post-prandial hyperglycaemia at digestive level, glucosidase inhibitors such as acarbose, miglitol and voglibose are used. These inhibit degradation of carbohydrates, thereby reducing the glucose absorption by the cells. Sulphonylureas like glibenclamide is insulinotropic and works as secretagogue for pancreatic cells. Although several therapies are in use for treatment, there are certain limitations due to high cost and side effects such as development of hypoglycaemia, weight gain, gastrointestinal disturbances, liver toxicity etc.[9] Based on recent advances and involvement of oxidative stress in complicating *Diabetes mellitus*, efforts are on to find suitable antidiabetic and antioxidant therapy.

N. crenulata (Roxb.) Nicolson, belongs to family Rutaceae is a widespread species of the genus *"Naringi"*. It is commonly known as 'Bilvaparni' in Sanskrit and 'Benta' in Odia. It has been used as folk medicine. The root extract is used in vomiting, dysentery and colic disorders. Fruit decoction is used as an antidote to insect poison. The bark juice is applied externally for getting speedy relief in sprain.[10] It is reported that its methanol extract showed significant anthelmintic activity.[11] Pectic polysaccharides have been isolated from the fruits of *N. crenulata* by extraction with water.[12] Biological activities such as anticancer,[13] hepatoprotectivity,[14] aphrodisiac,[15] anti-inflammatory activities[16] of ethanol extracts of leaf and bark of *N. crenulata* have also been reported. The present research work is concerned with the leaves of the above mentioned medicinal plant, which has reported folklore uses but yet not thoroughly explored so far for their exploitation in medicinal use. Present investigation deals with the evaluation of the extracts and fractions of *N. crenulata* leaves for their possible *in vivo* and *in vitro* anti-diabetic activity. A preliminary phytochemical screening of leaf extracts has also been carried out.

MATERIAL AND METHODS

Chemicals and Instruments

Streptozotocin (STZ) was procured from Sigma life science (Mumbai), α-amylase was procured from Hi-media laboratories and Glibenclamide tablet (Daonil; Emcure- Sanofi India Ltd.) was purchased from local market. Total cholesterol, HDL cholesterol, LDL cholesterol, VLDL, total protein, triglycerides, total bilirubin and creatinin were assayed by using the kits procured from Span Diagnostics Ltd. (India). Blood glucose level was measured by using Dr. Morpen Gluco-meter (Model No-BG-03)

Plant Materials and Preparation of Extracts

N. crenulata leaves were collected from Talabira forest region in Sambalpur district, Odisha, in the month of February-March, 2014 and authenticated by Dr. K. B. Satapathy, P.G. Department of Botany, Utkal University, Bhubaneswar, Odisha, India. After authentification, the leaf part of the plant was collected in bulk quantity, washed under running tap water to remove the adhering dirt and shade dried at room temperature. The dried plant material was crushed to course powder by using mechanical grinder. The powdered plant material was extracted successively with petroleum ether (60-80 ^{0}C) and methanol. The extracts were concentrated by evaporating the solvent under reduced pressure using Rotary evaporator (IKA Rv 10 V digital). The yield of the petroleum ether and methanol extracts were found to be 2.83 and 19.64 % w/w respectively.

Preliminary Phytochemical Screening of Extracts

The preliminary phytochemical screening of petroleum ether and methanol extracts of *N. crenulata* was performed according to the method described by Gupta *et al*.[17] The tests were based on the visual observation of colour change or formation of a precipitate after the addition of specific reagents.

Fractionation of the Methanol Extract of *N. crenulata*

The methanol extract of *N. crenulata* was further fractionated by column chromatography since it exhibited promising blood glucose lowering effect. The dried methanol extract (5 g) was reconstituted in a minimum amount of chloroform and heated to dissolve. Then it was adsorbed onto small amount of silica gel (100-200 mesh; in dry form) and heated in a water bath by continuous stirring for evaporation of the solvent.

Then it was air dried. A glass column (60 cm height and 2.5 cm diameter) was packed with silica gel (100-200 mesh, 250 g) by suspending it in n-hexane and allowed to settle. The dried silica gel impregnated with extract was loaded into the column. The column was developed by gradient elution with n-hexane and combination of n-hexane : ethyl acetate in the increasing order of polarity (10%, 20% ethyl acetate in n-hexane) which resulted in the fractions NCMF-1, NCMF-2 & NCMF-3, respectively. 30 eluates of 50 ml each were collected with each solvent system (i.e. a total of 90 eluates).

The fractions of each solvent system were concentrated separately by evaporating the solvent under reduced pressure and used for further studies.

Animals and Approval Status

Albino rats of either sex weighing between 150-200 g were used for the experiment. Animals were housed in a group of six in polypropylene cages at controlled room temperature 25 ± 2 ^{0}C, relative humidity 55% and 12 h light: dark cycle. They were fed with standard chow diet and water *ad libitum* during the experiment.

The experimental protocol of the animal experiments was approved by the IAEC of P.G Departments of Zoology and Biotechnology, Utkal University, Bhubanwswar (Regd. No: 192/ CPCSEA, 22-05-2000).

Preparation of Test Extracts and Fractions for Animal Experiments

The suspension of petroleum ether and methanol extracts and fractions of the methanol extract was prepared by using distilled water and Tween-40 which was used for animal experiments.

Acute Oral Toxicity Study of Extracts and Fractions

Swiss albino mice of either sex were fasted overnight prior to the experiment. The study was carried out as per OECD guidelines, 2000. The different doses of the extracts and fractions were administered to mice by oral route in different dose levels of 1000, 2000, 3000 and 4000 mg/kg of body weight (b.w.). The food was withheld for further 3-4 h after drug administration to avoid any complications relating to absorption of test extracts and fractions arising from food. Animals were critically observed individually at least once during the first 30 minutes of dosing followed by occasional observation for first 24 h and continued for 72 h for the recording of mortality, if any. The LD_{50} was calculated according to Miller and Tainter.[18] One-tenth ($1/10^{th}$) of the lethal dose was taken as a screening dose.[19] The rats were observed continuously and the following profiles were observed.

I. Behavioural profile: Alertness, restlessness, irritability, and fearfulness

II. Neurological profile: Spontaneous activities, reactivity, touch response, pain response, and gait

III. Autonomic profile: Defecation and urination.

After a period of 72 h the rats were observed for any lethality or death. Since no mortality was observed up to the dose level of 4000 mg/kg b.w., so the dose of 400 mg/kg was fixed for screening of anti-diabetic activity.

Evaluation of Antidiabetic Activity of Extracts

The antidiabetic activity of the extracts (petroleum ether and methanol) of *N. crenulata* leaves was assessed in normoglycaemic, glucose-loaded, streptozotocin-induced single dose and multi dose treated hyperglycaemic animals, including the *in-vitro* antidiabetic potentials using α- amylase assay method.

Evaluation of Activity on Normoglycaemic Animals[20]

Healthy Wistar albino rats of either sex weighing 150-200 g deprived of food for 12 h before the experiment were divided into five groups of six rats each. At the end of the fasting period, taken as zero time (0 h), blood was withdrawn from the tip of tail and the fasting blood glucose level was estimated.

Group-I animals served as control received only solvent (distilled water + Tween 40, 2 ml/kg, b.w.), group-II standard group received Glibenclamide (10 mg/kg b.w.) and group-III and IV served as test group animals were treated with the suspensions of petroleum ether and methanol extracts of *N. crenulata* (400 mg/kg, b.w.).

All the treatments were made by oral route. The blood glucose level was determined at 0, 1, 2, 4, 6 and 8 h after administration of test extracts and standard. The blood samples were collected from tail vein of the animals and blood glucose level was measured by using glucose oxidase-peroxidase reactive strips and glucometer.

Evaluation of Activity on Glucose-Loaded Animals (OGTT)

The oral glucose tolerance test was performed as per the method of Shirwaikar *et al.*[21] In this method, rats were fasted for 16 h before and during the experiment. Rats were divided into four groups of six rats each. Group-I solvent treated group received (distilled water + Tween 40, 2 ml/kg b.w.); Group-II standard group was treated with Glibenclamide (10 mg/kg, b.w.); and test groups Group III and IV received suspensions of petroleum ether and methanol extracts of *N. crenulata* (400 mg/kg, b.w.) respectively.

Glucose (3 g/kg) was fed 30 minutes after the administration of vehicle, standard and test extracts. Blood was withdrawn from tail vein of the animal at 0, 1, 2, and 4 h of glucose administration. The blood glucose level was estimated by using glucose oxidase-peroxidase reactive strips and glucometer.

Evaluation of Activity on Streptozotocin-Induced Diabetic Animals (Single dose)[20]

The effect of extracts on blood glucose level was studied in STZ-induced diabetic rats. The rats were divided into five groups of six rats each and

fasted for 12 h with free access of water. Six normal rats were treated only with solvent and served as solvent control.

The treatments were made orally as: Group-I Solvent control (distilled water + Tween 40, 2 ml/kg, b.w.); Group-II Diabetic control (distilled water + Tween 40, 2 ml/kg, b.w.); Group-III Glibenclamide (10 mg/kg b.w.); Group-IV Petroleum ether extract (400 mg/kg); Group-V Methanol extract (400 mg/kg). The blood glucose level was estimated at 0, 1, 2, 4, 8, and 10 h following the treatment.

Evaluation of Antihyperglycaemic Activity of Extracts and Fractions on STZ-induced Diabetic Animals (Multi-dose)

The Wistar albino rats of either sex of body weight of 150-200 g were divided into eight groups, six animal each (n=6) and kept fasting for 24 h. Diabetes was induced by intra-peritoneal injection of STZ freshly dissolved in citrate buffer (pH 4.5) immediately before use at a dose of 65 mg/kg body weight.[22] Six normal rats were treated only with solvent and served as solvent control. In order to avoid STZ induced hypoglycaemic mortality, 5% glucose solution was given for 24 h to STZ treated rats.[23] After 72 h of STZ administration, the blood glucose levels were measured and the rats showing blood glucose level greater than 220 mg/dl were considered to be diabetic and were used for the present study. Group-I served as solvent control received only solvent (distilled water + Tween 40, 2 ml/kg b.w.); Group-II Diabetic control (distilled water + Tween 40, 2ml/kg b.w.); group-III served as standard group received standard drug, Glibanclamide (10 mg/kg b.w.) by oral route once daily for 14 days. Test group animals received various extracts and fractions. Group-IV and V received petroleum ether and methanol extracts and Group-VI to VIII received the fractions, NCMF-1, NCMF-2 & NCMF-3 at the dose of 400 mg/kg body weight, respectively. The blood samples were collected from tail vein and blood glucose level was measured. The blood glucose levels were determined on 1st, 2nd, 4th, 7th and 14th day after administration of solvent, standard drug and test extracts and fractions.[24]

Serum Lipid Profile[25]

The lipid profile was done on 14th day after induction of diabetes. The serum lipid parameters such as total cholesterol, triglycerides, high density lipoproteins, low density lipoproteins and very low density lipoproteins, total bilirubin and creatinin were estimated using commercial kits (Span diagnostics, Mumbai).

***In-vitro* Antidiabetic Activity**

Inhibition Assay for α-Amylase Activity

α-Amylase was premixed with the methanol extract at various concentrations (25-250 µg/ml) and starch (0.5% solution) was added as a substrate to start the reaction. This was carried out at 37 ^{0}C for 5 minutes and terminated by addition of 2 ml of DNS (3,5-dinitrosalicylic acid) reagent. The reaction mixture was heated for 15 min at 100 ^{0}C and diluted with 10 ml of distilled water in an ice bath. α-Amylase activity was determined by measuring the spectrum at 540 nm using colorimeter.[26] The IC_{50} value is defined as the concentration of α-amylase inhibitor to inhibit 50% of its activity under the assay conditions.

$$\% \text{ inhibition} = \frac{\text{Abs 540 control - Abs 540 test}}{\text{Abs 540 control}} \times 100$$

Statistical analysis

All the results are expressed as mean ±SEM. Comparison was made between the test groups and diabetic control. The data were statistically analysed by one way analysis of variance (ANOVA) followed by Dunnet's t-test and p value less than 0.05 was considered significant.

RESULTS

Preliminary phytochemical screening

The study indicates the presence of steroids, triterpenoids in petroleum ether extract and alkaloids, flavonoids, saponins, tannins, triterpenoids, steroids and glycosides in methanol extract (Table 1).

Table 1: Preliminary phytochemical screening of different extracts of *Naringi crenulata* (Roxb.) Nicolson

Test	Petroleum ether extract	Methanol extract
Alkaloids	-	+
Glycosides	-	+
Flavonoids	-	+
Carbohydrates	-	-
Phytosterols	-	-
Fixed Oils & fats	-	-
Saponins	-	+
Phenolic compounds & Tannins	-	+
Proteins & Amino acids	-	-
Steroids	+	+
Triterpenoids	+	+
Gums & mucilage	-	-

Acute Oral Toxicity Study

The gross observational results revealed that the extracts and fractions did not show any sign of toxicity and mortality up to 72 h of the study at the dose level of 4000 mg/kg b.w. Hence, 400 mg/kg b.w. was fixed as the screening dose during antidiabetic evaluation.

Effect of Extracts on Normoglycaemic Rats

The effect of extracts on blood glucose level of normal rats is presented in Table 2. The test extracts at 400 mg/kg body weight showed a significant fall of blood glucose level when compared with solvent control group at the end of 8 h ($p<0.01$). Methanol extract exhibited the highest reduction of blood glucose level with the percentage reduction of 7.14 followed by petroleum ether extract (6.07%).

Effect of Extracts on Glucose Loaded Hyperglycaemic Rats

As per the results depicted in Table 3, petroleum ether and methanol extracts showed significant fall of blood glucose level with $p<0.01$ & $p<0.001$, respectively at 4 h following the administration of test substances. Methanol extract exhibited maximum reduction of blood glucose and better glucose tolerability (23.86%) as compared to petroleum ether extract (13.53%).

Effect of Extracts on STZ-induced Diabetic Animals (Single dose)

The results revealed that methanol extract exhibited highest reduction of blood glucose level with the percentage reduction of 56.91 followed by petroleum ether extract (45.01%) at 10 h after administration of test substances when compared with the diabetic control group (Table 4).

Effect of Extracts and Fractions on STZ-induced Diabetic Animals (Multi-dose)

The effect of extracts and fractions on STZ-induced diabetic rats in multi-dose treatment is presented in Table 5. The results revealed that the fraction, NCMF-3 exhibited highest reduction of blood glucose level with the percentage reduction of 65.49 followed by the fractions, NCMF-1 (62.33%) and NCMF-2 (60.26%). However, methanol and petroleum ether extracts respectively showed 65.25 and 44.86% reduction of blood glucose level at the end of 14th day when compared with the diabetic control group.

Biochemical Parameters of STZ-induced Diabetic Animals after 14th day

The results of extracts and fractions on biochemical parameters of STZ-induced diabetic animals after 14th day treatment are depicted in Table 6. Overall, the fractions of methanol extract showed significantly more activity as compared to the crude methanol and petroleum ether extracts. The fractions at a dose of 400 mg/kg b.w. was found to exhibit highest degree of action in reducing the triglyceride, total cholesterol, total bilirubin, creatinine, LDL and VLDL levels followed by methanol and petroleum ether extracts.

Table 2: Evaluation of different extracts of *Naringi crenulata* (Roxb.) Nicolson on normoglycemic animals

Groups and treatment	Blood glucose level (mg/dl)						
	0h	1h	2h	4h	6h	8h	% decrease at the end of 8 hrs
Solvent control (2ml/kg)	98.16 ± 0.60	97.66 ± 0.61	97.50 ± 0.76	96.83 ± 0.60	85.83 ± 0.60	93.33 ± 0.49	--
Glibenclamide (10 mg/kg)	92.83 ± 0.60	85.66 ± 0.66*	79.66 ± 0.88**	67.83 ± 0.79**	63.50 ± 0.76**	52.50 ± 0.84***	43.74
Pet. ether extract (400 mg/kg)	93.83 ± 0.70	92.83 ± 0.79**	92.16 ± 0.79**	90.16 ± 0.94*	88.83 ± 0.79**	87.66 ± 0.71**	6.07
Methanolic extract (400 mg/kg)	93.16 ± 0.74	91.50 ± 0.76*	90.33 ± 0.71**	89.33 ± 0. 66**	87.83 ± 0.47**	86.66 ± 0.71**	7.14

Values expressed as mean ± SD (n=6). The data were statistically analysed by one- way ANOVA, followed by Dunnet's t-test. p values less than 0.05 were considered significant. *: p <0.05; **: p <0.01; ***: p <0.001.

Table 3: Evaluation of different extracts of *Naringi crenulata* (Roxb.) Nicolson on blood glucose levels in glucose loaded animals (Oral Glucose Tolerance Test)

Groups and treatment	Blood glucose level (mg/dl)				
	Pre-treatment	Post-treatment			
	0 hr	1hr	2 hr	4 hr	% decrease at the end of 4 hrs
Solvent control (2 ml/kg)	85.83 ± 0.30	140.50± 0.76	133.00± 0.36	124.33± 0.49	--
Glibenclamide (10 mg/kg)	79.66 ± 0.55	126.17± 0.44*	99.83 ± 0.54**	76.66 ± 0.76***	38.34
Pet. ether extract (400 mg/kg)	93.66 ± 0.76	135.83±0.94*	122.50±0.76*	107.50±0.91**	13.53
Methanolic extract (400 mg/kg)	80.16±0.10	130.67±0.76*	114.83±0.79**	94.66±0.33***	23.86

Values expressed as mean ± SD (n=6). The data were statistically analysed by one- way ANOVA, followed by Dunnet's *t*-test. p values less than 0.05 were considered significant. Rats of all groups were loaded with glucose (2 g/kg p.o.) 30 min after extracts, Glibenclamide and water * p < 0.05; ** p < 0.01; ***: p <0.001.

Table 4: Effect of different extracts of *Naringi crenulata* (Roxb.) Nicolson on blood glucose levels in single dose treated streptozotocin-induced diabetic animals

Groups and treatment	Blood glucose level (mg/dl)						
	0h	1h	2h	4h	8h	10 hr	% decrease at the end of 10 hrs
Solvent control (2ml/kg)	87.66 ± 0.88	88.50 ± 0.99	89.83 ± 1.04	92.50 ± 1.08	89.33 ± 0.95	87.66 ± 1.02	--
Diabetic control	279.17 ± 0.70 ^{b}	277.67 ± 0.76^{a}	278.17 ± 0.70 ^{b}	285.67 ± 0.88 ^{a}	276.17 ± 0.60 ^{c}	275.83 ± 0.74 ^{a}	--
Glibenclamide (10 mg/kg)	266.83 ± 0.60**	218.67 ± 0.88*	190.33 ± 0.88**	147.17 ± 0.60**	121.33 ± 0.71*	106.33 ± 1.14***	61.45
Pet. Ether extract (400 mg/kg)	268.17 ± 0.19*	265.67 ± 0.11**	244.17 ± 0.94*	231.83 ± 0.19*	195.83 ± 0.94*	151.67 ± 0.70**	45.01
Methanolic extract (400 mg/kg)	267.33 ± 0.22**	262.17 ± 0.87**	234.17 ± 0.35*	195.50 ± 0.76*	162.83 ± 0.24*	118.83 ± 0.87***	56.91

Values expressed as mean ± SD (n=6). The data were statistically analysed by one- way ANOVA, followed by Dunnet's *t*-test. p values less than 0.05 were considered significant. Solvent control vs Diabetic control ^{a} $p < 0.05$; ^{b} $p < 0.01$; ^{c} $p < 0.001$. Diabetic control vs all other groups * $p < 0.05$; ** $p < 0.01$; ***: $p < 0.001$.

Table 5: Effect of different extracts and fractions of *Naringi crenulata* (Roxb.) Nicolson on blood glucose levels in multi dose treated streptozotocin-induced diabetic animals

Groups and treatment	Blood glucose level (mg/dl)					
	day 1	day 2	day 4	day 7	day 14	% decrease at the end of 14th Day
Solvent control (2ml/kg)	91.16 ± 0.60	93.33 ± 0.95	90.83 ± 0.60	93.66 ± 0.66	89.33 ± 0.95	--
Diabetic control	280.17 ± 0.47 ^{b}	282.50 ± 0.76 ^{a}	302.50 ± 0.76 ^{c}	317.33 ± 0.88 ^{b}	337.67 ± 0.88 ^{b}	--
Glibenclamide (10 mg/kg)	267.67 ± 0.66*	253.33 ± 0.88*	236.33 ± 0.88**	172.33 ± 0.66**	106.50 ± 0.76***	68.46
Pet. Ether extract (400 mg/kg)	264.67 ± 0.88	268.50 ± 0.76*	264.83 ± 0.56*	253.33 ± 0.28*	186.17 ± 0.13*	44.86
Methanolic extract (400 mg/kg)	266.50 ± 0.76	259.17 ± 0.70	247.33 ± 1.05	208.67 ± 0.66	117.33 ± 0.88**	65.25
NCMF-1 (400 mg/kg)	271.17 ± 0.60*	273.33 ± 0.88*	250.33 ± 0.88**	229.17 ± 0.60*	127.17 ± 0.94**	62.33
NCMF-2 (400 mg/kg)	270.50 ± 0.76*	267.83 ± 1.01**	251.16 ± 1.16*	232.17 ± 1.35**	134.17 ± 0.70*	60.26
NCMF-3 (400 mg/kg)	273.67 ± 0.88*	271.50 ± 0.76**	258.50 ± 0.76*	226.67 ± 0.88***	116.50 ± 0.76***	65.49

Values expressed as mean ± SD (n=6). The data were statistically analysed by one-way ANOVA, followed by Dunnet's *t*-test. p values less than 0.05 were considered significant. Solvent control vs Diabetic control ^{a} $p < 0.05$; ^{b} $p < 0.01$; ^{c} $p < 0.001$. Diabetic control vs all other groups * $p < 0.05$; ** $p < 0.01$; ***: $p < 0.001$.

Table 6: Effect of different extracts and fractions of *Naringi crenulata* (Roxb.) Nicolson on Lipid profile and other biochemical parameters in multi dose treated streptozotocin-induced diabetic animals

Groups and treatment	Lipid profile and other biochemical parameters							
	TC (mg/dl)	**TG** (mg/dl)	**HDL** (mg/dl)	**LDL** (mg/dl)	**VLDL** (mg/dl)	**TP** (g/dl)	**TB** (mg/dl)	**Creatinine** (mg/dl)
Solvent control (2ml/kg)	123.17 ± 1.19	87.16 ± 0.70	61.33 ± 0.88	73.16 ± 1.01	17.66 ± 0.88	07.66 ± 0.66	0.58 ± 0.11	0.66 ± 0.08
Diabetic control (STZ-65 mg/kg)	245.83 ± 0.94	132.83 ± 0.94	33.66 ± 0.66	135.67 ± 0.55	38.66 ± 0.66	3.83 ± 0.70	2.33 ± 0.49	2.21 ± 0.41
Glibenclamide (10 mg/kg)	119.83 ± 1.01	67.33 ± 1.05	57.33 ± 1.14	83.83 ± 0.94	18.16 ± 0.60	6.50 ± 0.76	0.88 ± 0.23	0.66 ± 0.15
Pet. Ether extract (400 mg/kg)	139.83 ± 0.94	94.33 ± 0.71	38.16 ± 1.13	107.83 ± 0.94	24.66 ± 0.66	4.50 ± 0.76	1.17 ± 0.16	0.94 ± 0.02
Methanolic extract (400 mg/kg)	132.67 ± 1.05	84.33 ± 0.66	39.66 ± 0.88	100.67 ± 1.14	23.66 ± 1.05	5.83 ± 0.60	0.99 ± 0.04	0.94 ± 0.10
NCMF-1 (400 mg/kg)	133.33 ± 0.88	81.16 ± 0.87	40.83 ± 0.87	98.66 ± 0.88	21.33 ± 1.28	5.66 ± 0.88	1.01 ± 0.04	0.92 ± 0.05
NCMF-2 (400 mg/kg)	135.67 ± 0.76	79.83 ± 0.07	44.66 ± 0.76	101.83 ± 0.60	23.33 ± 0.88	6.66 ± 0.88	0.98 ± 0.07	0.90 ± 0.04
NCMF-3 (400 mg/kg)	128.50 ± 0.76	83.66 ± 0.88	45.50 ± 0.76	92.66 ± 0.88	20.83 ± 0.94	6.33 ± 0.88	0.95 ± 0.02	0.89 ± 0.02

Values expressed as mean ± SD (n=6). The data were statistically analysed by one-way ANOVA, followed by Dunnet's *t*-test. p values less than 0.05 were considered significant. TC-Total cholesterol, HDL cholesterol, LDL cholesterol, VLDL, TP-Total protein, TG-Triglycerides, TB-Total bilirubin.

Table 7: α-amylase inhibition assay of methanol extract of *Naringi crenulata* (Roxb.) Nicolson

Sample	Concentration (µg/ml)	% inhibition	IC_{50} (µg/ml)
Acarbose (standard)	10	39.53±0.17	21.30
	20	45.58±0.71	
	30	60.61±0.80	
	40	69.30±0.55	
	50	74.10±0.94	
	60	76.39±0.66	
***N. crenulata* Methanol extract**	25	42.87±0.88	75.65
	50	47.13±0.71	
	100	51.68±0.93	
	150	60.93±0.76	
	200	69.86±0.60	
	250	76.92±0.69	

All determinations were carried out in triplicate manner and values are expressed as mean ± SEM. The IC_{50} value is defined as the concentration of inhibitor to inhibit 50% of its activity under the assayed conditions.

Furthermore, the fraction, NCMF-3 and methanol extract significantly increase the total protein and HDL levels after 14th day treatment.

In-vitro α-Amylase Inhibition Assay

Acarbose is a standard drug for α-amylase inhibition. Acarbose at a concentration of (10-60 µg/ml) showed α-amylase inhibitory activity from 39.53 ± 0.17 to 76.39 ± 0.66% with an IC_{50} value of 21.30 µg/ml (Table 7). Methanol extract (25-250 µg/ml) of *N. crenulata* exhibited potent α-amylase inhibitory activity in a dose dependent manner. Methanol extract showed highest inhibitory activity from 42.87±0.88 to 76.92±0.69 with an IC_{50} value of 75.65 µg/ml.

DISCUSSION

Streptozotocin induced hyperglycaemia has been described as a useful experimental model to study the activity of hypoglycaemic agents.[27] Streptozotocin selectively destroys the pancreatic insulin secreting β-cells, leaving less active cells and resulting in a diabetic state.[27-28] Glibenclamide treatment (5 mg/kg) was not as effective in reducing blood glucose in STZ-diabetic rats as in normoglycaemic rats. It has been reported that glibenclamide was not effective when destruction of β-cells has occurred and hence more effective in moderate diabetic rats than in severe diabetic animals.[29-31] The acute hypoglycaemic effect of glibenclamide results has been shown from the stimulation of insulin release from the residual β-cells and inhibition of glucagon secretion.[32] The extract might possess insulin like effect on peripheral tissues either by promoting glucose uptake and metabolism or inhibiting hepatic gluconeogenesis. In this study, it was observed that administration of *N. crenulata* extract to diabetic rats reversed their blood glucose at lower doses.

N. crenulata (Roxb.) Nicolson is a medicinally important plant indigenous to tropical and subtropical regions of the world. The present study has been undertaken to evaluate the antihyperglycaemic activity of *N. crenulata* in normal, glucose-loaded and STZ-induced hyperglycaemic rats (single and multi-dose treatment). Sulfonylureas like glibenclamide are commonly used as a standard antidiabetic drug in STZ-induced diabetes to compare the efficacy of a variety of antihyperglycaemic compounds.[30] In normoglycaemic rats, the test extracts showed progressive fall of blood glucose level at a single dose of 400 mg/kg of both the extracts. However, the methanol extract produced significant reduction ($P < 0.01$) in the blood glucose concentration of fasted normal rats after 8 h. A similar observation was reported by Kameswara *et al.*[33] on the effect of bark extract of *Pterocarpus santalinus* on blood glucose in experimental animals. Among the extracts, methanol extract showed maximum activity. In glucose loaded animals (OGTT), reduction in blood glucose levels was observed after 60 minutes of administration of the test substances. The maximum reduction was observed at 4 h where both methanol and petroleum ether extracts showed a significant reduction in blood glucose level and methanol extract exhibited maximum improvement in glucose tolerance.

The extracts produced significant decrease in the blood glucose level in STZ-induced hyperglycaemic rats when compared with the diabetic control group in the single dose treatment study at the tested dose levels. In multi-dose treated hyperglycaemic rats, both the extracts and fractions of the methanol extract showed various degree of blood glucose reduction, among which OCMF-3 exhibited highest percentage of reduction in blood glucose level. This might suggest that the said effect is due to extra intestinal action of the test substances.[34] From the results of the biochemical parameter study, it was observed that there was an increase in total bilirubin and serum creatinine levels in streptozotocin induced diabetic rats. However, 14 days of administration of extracts and fractions lead to a significant fall in total bilirubin and serum creatinine levels when compared with the diabetic control group. It was also observed that there was an increase in serum total cholesterol, triglycerides, LDL and VLDL levels and decrease in total protein and HDL levels in STZ-induced hyperglycaemic rats. Continuous administration of petroleum ether

and methanol extracts and fractions of methanol extract for 14 days leads to significant decrease in serum total cholesterol, triglycerides, LDL and VLDL levels, while increase in total protein and HDL levels was recorded. The results indicate that, the treatment of diabetic rats with *N. crenulata* prevents the alteration in serum biochemistry values and returns nearer to their normal values, which supports its anti-diabetic activity.

Medicinal plants that exhibit anti-diabetic activity usually possess active substances which are able to mimic the action of insulin or which exert similar effect on the β-cells of the pancreas, causing them to synthesize and secrete insulin.[35] The blood glucose lowering ability of the test substances showed encouraging results in our study among which methanol extract and fractions of methanol extract showed maximum potency. Lack of insulin affects the metabolism of carbohydrates, proteins, fat and causes significance disturbance of water and electrolyte homeostasis.[35] Recent advances in understanding the activity of intestinal enzymes have lead to the development of newer pharmacological agents. One therapeutic approach for treating diabetes is to decrease the postprandial hyperglycaemia by retarding the absorption of glucose through the inhibition of the carbohydrate hydrolyzing enzymes, *α*-amylase, *α*-glucosidase, and *β*-galactosidase, in the digestive tract. Inhibition of these enzymes delays carbohydrate digestion and prolongs overall carbohydrate digestion time, causing a reduction in the rate of glucose absorption and consequently blunting the postprandial plasma glucose rise.[36] α-amylase is important in carbohydrate digestion and glucose absorption.[37] The *in vitro* study showed an increased utilization of the glucose by α-amylase inhibition assay in presence of methanol extract which suggests that the test extract may inhibit the digestion and absorption of glucose through intestine. The possible mechanism might be the potentiation of pancreatic secretion of insulin from existing β-cells of islets, and inhibition of digestion and absorption of glucose through intestine as evidenced by the significant increase in glucose utilization by α-amylase inhibition assay.

CONCLUSION

The experimental results of the present investigation conclude that the extracts and fractions of *N. crenulata* (Roxb.) Nicolson leaf showed various degree of antihyperglycaemic effect, among which methanol extract and fractions of methanol extract especially NCMF-3 showed potent antihyperglycaemic activity in streptozotocin induced multi dose treated diabetic animals. The fraction, NCMF-3 exhibited highest activity among the test substances which suggests the presence of higher concentration of active constituents in it. The methanol extract also showed persuasive inhibition of glucose digestion and absorption in α-amylase inhibition assay. The antihyperglycaemic activity of the test substances was comparable with the standard drug. These findings suggest that the plant may be a potential source for the development of new oral antihyperglycaemic agent. The results also indicated that the leaf extract of *N. crenulata* was effective in decreasing the blood glucose level in normal, glucose loaded, STZ-induced diabetic animals and *in vitro* α-amylase inhibition assay. However, the molecule(s) responsible for such an effect requires further investigation. The possible mode of action of the plant extract might be by potentiation of the insulin effect by increasing the pancreatic secretion of insulin from β-cells of islet of Langerhans.

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