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Chemical and pharmacological characterization of hypolipidemic compound from *Cajanus Cajan*

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Abstract

The study was carried out to identify the compound responsible for hypolipidemic and hypoglycemic effects of *Cajanus cajan* (redgram). The methanol extract of redgram seeds were fractionated into petroleum ether, chloroform, and methanol. The methanol fraction significantly decreased fasting blood glucose, and lipid profiles ($p<0.001$) on streptozotocin-induced mice compared to control. The methanol fraction was then subjected to chromatographic analysis and a compound (CCA1) has been isolated. The structure of the compound is considered to be substituted cyclopentene with glucose by analysis its ^{1}H and ^{13}C-NMR data. Biological studies of the isolated compound possessed prominent hypolipidemic activity. Although a number of hypoglycemic compounds are reported, yet not any hypolipidemic compound from redgram. The compound CCA1 seems to be the first report on hypolipidemic activity from methanol extract of redgram.

Introduction

Diabetes mellitus is heterogeneous disorder with an increased risk for premature arteriosclerosis due to increased in triglycerides and low density lipoprotein levels (American Diabetic Association, 1989). About 70-80% of deaths in diabetic patients are due to vascular disease. Glucose control is essential, but this provides only minimal benefit with respect to coronary heart disease prevention. An ideal treatment for diabetes would be a drug that is not only controls the glycemic levels but also prevents the development of arteriosclerosis and other complications of diabetes (Halliwell and Gutteridge, 1985). Now-a-days, the use of complementary and alternative medicine and especially the consumption of botanicals have been increasing rapidly worldwide (Hu et al., 2003). For the insulin deficient diabetic model, streptozotocin-induced diabetic rats have been used for the study of diabetic hyperlipidemia (Bloks et al., 2004). Hyperlipidemia is a common feature of diabetes and is related to cardiovascular disease (Iwasaki et al., 2005).

Several plants have shown antidiabetic effects (Bandara et al., 2009; Bhowmik et al., 2009; Khanam and Dewan, 2009; Pattanayak et al., 2009; Ravi et al., 2009; Saha et al., 2009; Prajapati et al., 2008; Moosa et al., 2006). *Cajanus cajan* Linn. (redgram) is commonly used as pulse. Saraswathy and Kurup et al. (1970) have shown the hypoglycemic and hypolipidemic effects of redgram in hypercholesteromic animals. Redgram was found to reduce blood glucose and lipid profile in human volunteers (Panlasigui et al., 1995).

The objective of this study was to make an analysis of the ethnobotanical information on redgram to control diabetes mellitus, and efforts to isolate active chemicals having hypolipidemic and hypoglycemic activity.

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Materials and Methods

Preparation of powder

The dried ripe seeds of redgram were collected from the local market of Rajshahi in the month of April-May. These samples were identified by Professor Abul Hossain, Department of Botany, Rajshahi University College, Rajshahi, Bangladesh. Voucher specimen # 78. After removing the extraneous matter, redgram seeds were washed and dried in an oven for 4-5 days at 45°C. Then they were crushed into fine powder by electric grinder.

Extraction of the crude redgram seeds powder

Two kilograms powdered redgram seeds were taken in a clean flat-bottomed glass container. The powder was then extracted three times with 4 L of 99.5% methanol for 72 hours with intermittent shaking and heating in a water bath at 60°C. The combined extracts were filtered and concentrated to dryness with rotary evaporator under reduced pressure at 65°C and finally yielding 10.6 g dried crude extract of redgram. The dried methanol extract was then successively fractionated with petroleum ether and chloroform to get the respective fractions (petroleum ether 3.0 g and chloroform fraction 1.8 g).

Animal experiments

A total number of 56 Swiss albino mice of both sexes weighing about 30-40 g, age 2 months were purchased from animal house of International Centre for Diarrheal Disease Research, Bangladesh (ICDDR, B). Prior to the commencement of the experiment, all the mice were acclimatized to the new environmental condition for a period of one week. During the experimental period, the mice were kept in a well-ventilated animal house at room temperature of 23 ± 2°C, maintaining relative humidity 50-60% and were fed with standard pellets supplied from ICDDR, B and fresh drinking water *ad libitum*. They were kept in cages and maintained in well -ventilated room under conditions of natural light and dark cycle. Animals were fasted for 16 hours prior to drug administration allowing access only to water and were deprived of both food and water during the experiment.

Induction of diabetes

The mice were randomly divided into 8 groups, each containing 7 mice. After fasting 16 hours, mice of group (II-VIII) were rendered diabetic by injecting intraperitoneally a freshly prepared solution of streptozotocin (50 mg kg^{-1} of body weight) in 0.1 M citrate buffer, pH 4.5, in volume 1 mL/kg (Siddique et al., 1987), after a base line glucose estimation was done. After 48 hours blood glucose content was measured by using BioLand G-423 Glucose Test Meter (BioLand, Germany) using Blood sample from the tail vein of the mice. When the condition of diabetes was established animals with blood glucose levels above 11.1 mM were selected for the study.

Effect on diabetic mice

Group I served as a non-diabetic control while group II for diabetic control group. Group VII served as diabetic glibenclamide (60 mg kg^{-1}) controlled group. Group III, IV, and V were treated with the pet-ether, chloroform and methanol fractions of methanolic extract of redgram, respectively at 150 mg kg^{-1} for 24 hours experiment. Group VI were treated with the isolated compound CCA1 at 120 mg kg^{-1} and atorvastatin (80 mg kg^{-1}) were treated on group VIII and was used as reference for lipid profile. The reference drug and the extracts were administered intraperitonialy to the mice.

Collection of blood and serum and determination of blood glucose, serum total cholesterol (TC) and serum triglycerides (TG)

Blood samples were collected from tail vein of each mouse of a group before and also at 0, 1, 2, 3, 6, 10, 16, and 24th hours of one day experiment. The samples were analyzed for blood glucose content by using BioLand G-423 glucose test meter (BioLand Germany). Then the mice were sacrificed and about 1-2 mL of blood was collected directly from the heart by syringes, centrifuged at 4,000 rpm for 10 min and the serum was obtained for the determination of TC and TG. Serum TC and TG concentrations were analyzed by measuring absorbance by UV spectrophotometer (Shimidzu UV-1200, Japan), using wet reagent diagnostic kits (Boehringer Mannheim, GmbH) according to manufacturer's protocol.

Fractionation, isolation, purification and characterization of compounds from the most effective methanol extract of redgram

Chromatographic techniques (thin layer chromatography, column chromatography, and preparative thin layer chromatography) were used for the isolation of compounds from the fractions. The column chromatographic technique most commonly used for separation of compounds into several fractions according to the affinity or solvating capacity of the components to the solvent used. The study involves in fractionation and isolation of compounds from the pharmacologically active methanol extract of redgram. The structures of the compounds were tried to establish by spectroscopic methods.

Study design

In order to carry out column chromatography, a solvent system was established by developing TLC technique. The silica gel (60-120 mesh size) slurry was made with the solvent system established earlier. The slurry was poured time to time into the column very carefully and

Table I

Effect of fractions and isolated compound from redgram

Parameters (mM)	Control (vehicle)	Diabetic control	Treatment after diabetes induced by streptozotocin					
			Pet. ether fraction	Chloroform fraction	Methanol fraction	CCA1	Gliben-clamide	Atorvas-tatin
Fasting blood glucose	4.5 ± 0.2	16.2 ± 0.3[a]	15.7 ± 0.3[a]	15.4 ± 0.3[a]	7.5 ± 0.1[b]	16.0 ± 0.4	8.9 ± 0.2	ND
Total cholesterol	1.4 ± 0.2	2.0 ± 0.04[a]	1.94 ± 0.1[a]	1.9 ± 0.1[a]	1.5 ± 0.02[b]	1.4 ± 0.01[b]	ND	1.35 ± .04[b]
Triglyceride	0.40 ± 0.3	0.96 ± .02[a]	0.94 ± .01[a]	0.94 ± 0.01[a]	0.67 ± .03[b]	0.57 ± 0.02[b]	ND	0.60 ± 0.02[b]

Data represent mean ± S.E.M. of 4-6 experiments. [a]Significantly different from the control; [b]Significant effect on STZ-induced diabetic mice; ND: Not determined

the silica gel was allowed to settle down to form a uniform packing. Then the stop-cock of the column was opened and the excess of solvent over the column head was allowed to run. The dry crude methanol extract of redgram was mixed with small amount of silica gel in a mortar to get a free flowing powder. The powdered sample was then applied carefully on the top of the prepared column and successfully eluted with solvent/solvent system.

The elutes were collected in a number of conical flasks marked from fractions 1-53. The elutes were spotted successfully on TLC plate and the flasks having similar spots were combined together.

Analysis of fraction F5

The fraction F_5 containing 10-22 conical flasks having similar spots on TLC plate were combined. Then the fractions were subjected to PTLC by using chloroform:methanol:water (8:4:0.5) as a solvent system. The expected bands were separated off and eluted with chloroform 100%, chloroform:methanol (1:1) using cotton plug and the solvent were evaporated off to afford compound CCA1. Although other fractions showed a number of spots on TLC but the amount of each fraction was too small to isolate the compounds. H -(500 MHz) and ^{13}C-NMR (125 MHz) spectra were acquired on a JEOL JNM alpha spectrometer using TMS as internal standard.

Statistics

Data were analyzed by Prism (GraphPad Software, San Diego, CA, USA). The results were compared using one -way ANOVA followed by Scheffe's post-hoc test. Results were considered significant when p values were less than 0.05 ($p<0.05$).

Results

The effects of different fractions of the methanolic extracts of redgram on the fasting blood sugar, serum TC and serum TG levels were investigated in the streptozotocin-induced diabetic mice using glibenclamide and atorvastatin as standard for hypoglycemic and hypolipidemic agent, respectively. The mean blood glucose concentration of controlled and fractions of redgram treated animals (after intraperitoneal administration of a single dose) on 0, 1, 2, 3, 6, 10, 16, and 24th hours (Table I). Hypoglycemia was observed in animals treated with methanol fraction. The significant reduction ($p<0.01$) observed at 10th hour of the experiment. The petroleum ether and chloroform fractions of redgram and the compound CCA1, have no effect on the blood glucose level on streptozotocin-induced mice.

The methanol fraction reduced the serum total cholesterol and triglyceride significantly in streptozotocin-induced mice (Table I). The lowering efficiency of the compound CCA1 was found comparable with the reference standard, atorvastatin. This is the first compound to our knowledge as lipid lowering from redgram.

The purity of the isolated compound, CCA1 was checked by TLC using different solvent system. Rf values of the isolated compounds in different solvent systems were as follows: 0.677 [(CHCl3:MeOH:H2O (8:4:0.5)], 0.8 [(EA:MeOH (1:1)] and 0.701 [(EA: n-hexane (1:10)].

The isolated compound was characterized by its physical, chemical as well as spectrometric analysis. It is brown crystalline compound soluble in methanol. The melting point is 73°C.

NMR spectra (500MHz for ^{1}H and 125MHz for ^{13}C-NMR) were obtained on varian INOVA 500 spectrometer and the chemical shifts (8) are reported in ppm relative to the residual nondeuterated solvent signals. The compound gave pink color on TLC with vanillin sulfuric acid spray reagent.

The ^{13}C-NMR spectrum together with DEPT, revealed signals for 20 carbons including three (3) downward-CH2- peak and eleven (11) -CH-CH3 peak. In ^{J}H-NMR spectrum, the compound exhibited signals for methyl

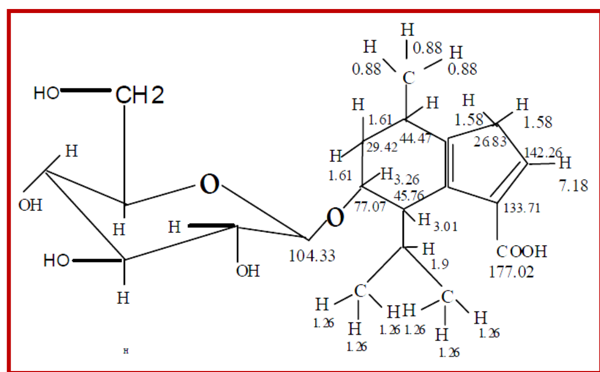

Figure 1: Proposed structure of CCA1

doublet at δ 0.92 and 1.26, and a methine multiplet at 1.9 (for an isopropyl moiety) together with an anomeric proton peak at δ 4.68 (doublet) indicating the presence of a sugar moiety which corresponds to a carbon peak at 104.33. In 'H-NMR spectrum the proton at δ 4.37, 4.49, 4.53, 4.55 and 3.33 are seem to be polyhydroxy proton corresponding to the carbon peaks at δ 76.96, 79.76, 70.75, 77.07 and 63.46, respectively are almost identical to those of reported P-D-glucopyranosyl unit indicating the presence of P-D-glucopyranosyl unit in the molecule. In ^{1}H-NMR spectrum the compound also showed the signals at δ 0.88(s) ascribed for one tertiary methyl peak, two peaks at δ 7.18(s) and 6.5(s) may be due to olefinic methines, at δ 3.01, 3.26 for two methines and at δ 1.61 and 1.58 for two methylenes. In ^{13}C-NMR spectrum, the compound exhibited peaks at 177.18 may be ascrible for carbonyl acid, at δ 133.71, 142.26, 146.37 and 147.18 four olefenic carbon; at δ 32.96, 44.47, 45.76 and 77.07 for four methiens; at δ 26.83 and 29.42 for two methylenes and at δ 13.02, 18.64 and 20.57 may be due to three methyl. The carbon peak shifted to the down field from 71.00 to 77.07 due to the presence of glucose moiety in the molecule attached with the methiene carbon and the carbon peak is shifted from 141.00 to 133.71 may be due to the attachment of acid at carbon δ 133.71. In combination of 'H-NMR and ^{13}C-NMR spectra the structure of the compound may be assumed as structure having substituted cyclopentene with glucose moiety and the structure as follows: ^{13}C-NMR (Pyridine): C-1 (142.26), C-2 (26.83), C-3 (147.68), C-4 (44.47), C-5 (29.42), C-6 (77.07), C-7 (45.76), C-8 (146.3), C-9 (133.71), C-10 (45.76), C-11 (18.64), C-12 (20.57), C-13 (177.02), C-14 (13.02), C-1/ (104.33), C-2/ (76.96), C-3/ (79.76), C-4/ (70.75), C-5/ (74.03), C-6/ (63.46).

iH-NMR (MeOH): H-1 (7.18), H-2 (1.58), H-4 (1.58), H-5 (1.61), H-6 (3.26), H-7 (3.01), H-10 (1.9), H-11 (1.26), H-12 (1.26), H-1/ (4.37), H-2/ (4.49), H-3/ (4.53), H-4/ (4.55), H-5/ (4.68), H-6/ (3.33).

Discussion

The methanol extract of redgram powder was fractionated with petroleum ether, chloroform and methanol which ultimately yielded respective dry crude extracts of redgram. A single intraperitoneal injection of streptozotocin (50 mg kg^{-1}) produced severe hyperglycemia in experimental group of mice in comparison to control ($p<0.001$). The fractions of redgram were evaluated for their effects on blood glucose and lipid levels in streptozotocin-induced diabetic mice in a dose 150 mg kg^{-1}. The methanol fraction produced significant reduction of blood glucose ($p<0.001$) and lipid profile ($p<0.001$) as compared to control. The reduction of blood glucose and lipid levels was not significant by petroleum ether and chloroform fractions of redgram. On the basis of results, it is indicated that the methanol extract of redgram contains bioactive component responsible for producing hypoglycaemic and hypolipidemic activities.

The compound, CCA1 was isolated from the fraction F5 using column chromatography and PTLC. This compound was studied for its effects on blood glucose level and lipid profile in streptozotocin-induced diabetic mice and reducing capacity of total cholesterol ($p<0.001$) and triglyceride ($p<0.001$) levels. It has insignificant blood glucose lowering effect. The other fractions of the column (except F5) were not investigated due to insufficient amount of constituents.

The structure of the compound may be considered from ^{1}H and ^{13}C-NMR data as substituted cyclopentene with glucose (Figure 1). This report seems to be the first on the hypolipidemic effect of CCA1 from the methanol extract of redgram seeds.

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