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Original Article

Isolation and 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 was found to decrease fasting blood glucose and lipid profile ($p<0.001$) on streptozotocin-induced mice compared to control. This activity- guided fraction led to the isolation of a compound, substituted benzene containing polyhydroxy functions fused with lactone (CCA3) by analysis of 1H and 13C-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 CCA3 seems to be the first report on hypolipidemic activity from methanol extract of redgram.

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Introduction

Diabetes mellitus is heterogeneous disorder with an increased risk for premature arteriosclerosis due to increased in triglycerides and low density lipoprotein levels[1]. 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 atherosclerosis and other complications of diabetes[2]. Now-a-days, the use of complementary and alternative medicine and especially the consumption of botanicals have been increasing rapidly worldwide[3]. For the insulin deficient diabetic model, streptozotocin-induced diabetic rats have been used for the study of diabetic hyperlipidemia[4]. Hyperlipidemia is a common feature of diabetes and is related to cardiovascular disease[5]. Several plants have shown antidiabetic effects[6 7 8 9 10 11 12 13].

Cajanus cajan Linn. (redgram) is commonly used as pulse. Saraswathy Devi have shown the hypoglycemic and hypolipidemic effects of redgram in hypercholesteromic animals[14]. Redgram was found to reduce blood glucose and lipid profile in human volunteers[15]. 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.

Material 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

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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 (2.0 kg) powdered redgram seeds were taken in a clean flat-bottomed glass container. The powder was then extracted three times with 4 liters 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 35 Swiss albino mice of both sexes weighing about 30-40 g age 2 months were purchased from animal house of International Centre for Diarrhoeal 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 supplied 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 5 groups, each containing 7 mice. After fasting 16 hours, mice of group (II-V) 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-1, after a base line glucose estimation was done[16]. 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 III were treated with the isolated compound, CCA3 at 120 mg kg-1 & atorvastatin 80 mg kg-1 were treated on group V and was used as reference for lipid profile. Group IV served as diabetic glibenclamide (60 mg kg-1) controlled group. The reference drugs (Glibenclamide, trade name-Daonil 5mg, Aventis Co. Bangladesh and Atorvastatin, trade name-Atova 10mg, Beximco Co. Bangladesh) & the compound were administered intraperitonially 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, Tokyo, 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 (TLC), column chromatography,

and preparative thin layer chromatography (PTLC)] 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 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 F5 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 CCA3. Although other fractions showed a number of spots on TLC but the amount of each fraction was too small to isolate the compounds. ^{1}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 (Graph Pad 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 ($p<0.05$).

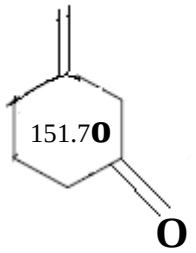
Results

The effects of the isolated compound, CCA3 on fasting blood sugar, serum TC and serum TG levels were investigated in streptozotocin-induced diabetic mice using glibenclamide and atorvastatin as standard for hypoglycemic and hypolipidemic agent respectively. The mean blood glucose concentration of controlled and CCA3 treated animals (after intraperitoneal administration of a single dose) on 0, 1, 2, 3, 6, 10, 16, and 24th hours (Table I). It was observed that the compound CCA3 have no effect on blood glucose level but reduced serum total cholesterol and triglyceride significantly in streptozotocin-induced diabetic mice (Table I). The lowering efficacy of the compound CCA3 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, CCA3 was checked by TLC using different solvent system. R_f values of the isolated compound in different solvent systems were as follows: 0.746 [(CHCl3:MeOH:H2O (8:4:0.5)], 0.572 [(EA:MeOH (1:1)] and 0.555 [(EA: nhexane (1:10)]. The isolated compound was characterized by its physical, chemical as well as spectrometric analysis. It is light yellow, semi-solid compound soluble in methanol but partially in chloroform. NMR spectra (500MHz for ^{1}H and 125MHz for ^{13}CNMR) were obtained on varian INOVA 500 spectrometer and the chemical shifts (δ) are reported in ppm relative to the residual nondeuterated solvent signals. The compound gave red colour on TLC with Dragendroff spray reagent.

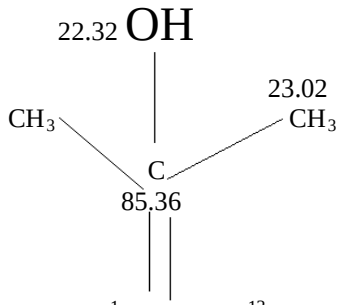
In ^{1}H-NMR spectrum, the compound showed the signals at 1.26 (3H, s) & 1.36 (3H,s) indicating the

presence of tertiary methyl, which corresponds to the carbon peck at δ 22.23 & 23.02 respectively.

The proton peaks from 3.59 to 4.21 indicates the presence of polyhydroxy proton which corresponds to the carbon peak at δ 61.41, 63.46, 70.32, 74.52 & 89.53. In ^{13}C-NMR spectrum, the peak at 165.40 is an indicative of carbonyl group of six membered lactone ring & the carbon peak at δ 151.70 may ascribe for the double bonded carbon attached with oxygen.



The appearance of proton peak at δ 7.98 & 5.9 indicative for the olefenic proton of the substituted benzene ring that may be corresponds to the carbon peak at 110.50 & 137.42 respectively. The carbon peak at δ 85.36 together with the carbon peak at 22.32 & 23.02 may be due to the following function



In combination with ^{1}H-NMR & ^{13}C-NMR spectra the structure of the compound may be assumed as structure having six membered lactone ring fused with substituted benzene with polyhydroxy functions.

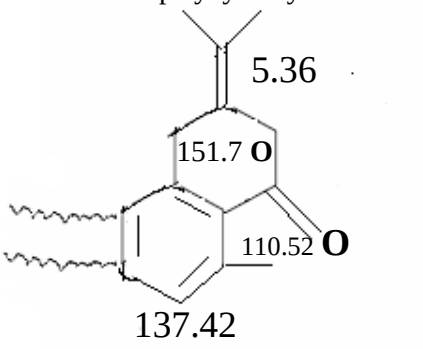


Figure :1 Proposed structure of CCA3

Table I: Effect of isolated compound from redgram

Parameters (mM)	Control (vehicle)	Diabetic control	Treatment after diabetes induced by streptozotocin		
			CCA3	Glibenclamide	Atorvastatin
Fasting blood glucose	4.5 ± 0.2	16.2 ± 0.3#	16.5 ± 0.19	8.9 ± 0.2	ND
Total cholesterol	1.4 ± 0.2	2.0 ± 0.04#	1.39 ± 0.01*	ND	1.35 ± 0.04*
Triglyceride	0.40 ± 0.3	0.96 ± .02#	0.60 ± 0.02*	ND	0.60 ± 0.02*
Data represent mean ± S.E.M of experiments. #: Significantly different from the control;*: significant effect on STZ-induced diabetic mice; ND: Not determined.					

Discussion

We have reported earlier that among the three extracts of redgram (petroleum ether, chloroform and methanol), only methanol extract decreased fasting blood glucose & lipid profile level in streptozotocin- induced diabetic mice significantly[17]. Therefore, it is indicated that the methanol extract contains pharmacologically active principles responsible for hypoglycaemic and / or hypolipidemic activity. This activity guided fraction led to isolate the compound, CCA3 from the fraction F5 using column chromatography and PTLC. This compound was studied for its effect on blood glucose level and lipid profile in streptozotocin-induced diabetic mice and found 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 F_5) 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 benzene containing polyhydroxy functions fused with

lactone (figure I). This report seems to be the first on the hypolipidemic effect of CCA3 from the methanol extract of redgram seeds.

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