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ORIGINAL ARTICLE

Antihyperglycemic Activity and Brine Shrimp Lethality Studies on Methanol Extract of *Cajanus Cajan* (L.) Millsp. Leaves and Roots

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Md. Mazharul Anwar, Marjina Akhter Kalpana, Bithika Bhadra, Shahnaz Rahman, Sanjoy Sarker, Majeedul H. Chowdhury, Mohammed Rahmatullah: Antihyperglycemic Activity and Brine Shrimp Lethality Studies on Methanol Extract of *Cajanus Cajan* (L.) Millsp. Leaves and Roots

ABSTRACT

Cajanus cajan (L.) Millsp. (Fabaceae, local name: orhor, English name: pigeon pea) is widely cultivated in Bangladesh for its edible seeds. The leaves are also consumed during times of food scarcity. In the folk medicinal system of Bangladesh, the leaves and stems of the plant are predominantly used for treatment of jaundice and diabetes. The objective of the present study was to evaluate the antihyperglycemic and cytotoxic potential of methanol extract of leaves and roots of the plant. Antihyperglycemic activity was studied by oral glucose tolerance tests in glucose-loaded Swiss albino mice. The methanol extract of leaves showed dose-dependent and significant reductions of serum glucose levels in mice. At doses of 200 and 400 mg leaf extract/kg body weight, the reductions in glucose levels were comparable to that of the standard antihyperglycemic drug, glibenclamide, administered at a dose of 10 mg/kg body weight. In contrast, the methanol extract of roots showed a dose-dependent increase in serum glucose levels, which was statistically significant at the highest dose of 400 mg extract/kg body weight. The cytotoxic potential of methanol extracts of leaves and roots were measured with the brine shrimp lethality bioassay. Both leaf and root extracts showed a high degree of toxicity to brine shrimp with LC_{50} values, respectively, of 0.0264 and 1.849 mg/ml. The results obtained with antihyperglycemic tests with leaves of the plant validate its folk medicinal use for diabetes. The brine shrimp lethality assay results suggest that the plant can be a promising source of anti-cancer compounds.

Key words: *Cajanus cajan*, antihyperglycemic, brine shrimp, cytotoxicity

Introduction

Cajanus cajan (L.) Millsp. belongs to the Fabaceae family and is an important legume crop in the Indian sub-continent, Eastern Africa, and Central America. It is widely cultivated in Bangladesh for its edible seeds and is locally known as orhor or arhar. In English, the plant is known as pigeon pea. In the folk medicinal system of Bangladesh, the leaves and stems of the plant are predominantly used for treatment, primarily of jaundice, and secondarily, of diabetes. The seeds of the plant are also claimed to cure leprosy, tumors, and heart diseases (Ghani, 2003).

Various pharmacological activities have been attributed to extracts of parts of the plant, as well as their

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phytochemical constituents. The aqueous extract of leaves reportedly had protective effects on bones in mice, and which effects have been hypothesized to be mediated by decreasing adipocytic cell formation from bone marrow stromal cells (Zhang *et al*., 2010). Stilbene extracts obtained from the plant has been shown to exert a protective effect on ovariectomy-induced bone loss in rats (Zheng *et al*., 2007). Cajanol, isolated from roots of the plant, has been described as a novel anticancer agent, which induced apoptosis in human breast cancer cells (Luo *et al*., 2010). Methanol extract of the plant has been shown to demonstrate cytotoxicity against three cancer cell lines, namely human breast adenocarcinoma cell line MCF-7, human large cell lung carcinoma cell line COR-L23 and human amelanotic melanoma C32. The stilbene compounds in the extract, longistylins A and C appeared to be responsible for the cytotoxic effects (Ashidi *et al*., 2010). Antimicrobial activity has been reported for supercritical fluid extraction extracts of the plant and its various constituents, namely the flavonoids – orientin, vitexin, isovitexin and pinostrobin, and the stilbene compound – cajaninstilbene acid (Zu *et al*., 2010a). Aqueous and ethanolic extracts of the plant have been shown to inhibit type 1 and type 2 herpes simplex virus in vitro (Zu *et al*., 2010b). Antioxidant effects have been reported for aqueous and ethanol extracts of leaves, as well as constituents of the ethanol extract, namely cajaninstilbene acid (3-hydroxy-4-prenylmethoxystilbene-2-carboxylic acid), pinostrobin, vitexin and orientin (Wu *et al*., 2009).

Hypocholesterolemic effects of stilbene-containing extract fraction from the plant has been demonstrated in diet-induced hyperlipidemic and hypercholesterolemic mice (Luo *et al*., 2008 a,b). Various scientific studies have reported on the hepatoprotective, nephroprotective and protection against oxidative stresses activities of the plant. Methanol-aqueous fraction of leaf extract reportedly prevented chronically treated alcohol-induced rat liver damage (Kundu *et al*., 2008). A 43 kDa protein isolated from the leaves of the plant has been shown to have protective effects in mice against acetaminophen-induced hepato-nephro toxicity (Ghosh and Sil, 2007), ameliorative effects against galactosamine-induced nephrotoxicity (Sinha *et al*., 2007a), protective effects against fluoride-induced oxidative stress in mice erythrocytes (Sinha *et al.*, 2007b), attenuation effects against cadmium chloride induced cytotoxicity in murine hepatocytes (Sinha *et al*., 2007c), attenuation effects against acetaminophen-induced hepatotoxicity (Sarkar and Sil, 2007), hepatoprotective effects against carbon tetrachloride-induced hepatotoxicity (Sarkar *et al*., 2006), protective effects against chloroform-induced hepatic disorder (Ghosh *et al*., 2006), and protective effects against thioacetamide-induced cytotoxicity in hepatocytes (Sarkar and Sil, 2006).

Extract of the plant has been reported to cause a reduction of painful crises and amelioration of the adverse effects of sickle cell anemia in liver (Akinsulie *et al*., 2005). Antiplasmodial constituents like the stilbenes, longistylin A and C, and betulinic acid has been reported from extracts of roots and leaves of the plant (Duker-Eshun *et al*., 2004). The plant is considered as antidiabetic in Indian traditional medicine like Ayurveda (Grover *et al*., 2002).

Considering the folk medicinal uses of the plant in Bangladesh as well as various scientific reports, it was the objective of the present study to evaluate the antihyperglycemic potential of methanol extracts of leaves and roots of the plant in oral glucose tolerance tests conducted with glucose-loaded mice. The cytotoxic potential of the methanol extracts of leaves and roots were also analyzed in the present study through brine shrimp lethality bioassays.

Materials and Methods

Plant material and extraction

The leaves and roots of *Cajanus cajan* were collected, respectively, from Sayedpur in Narayanganj district of Bangladesh in April, 2010 and Eklashpur in Chandpur district of Bangladesh in October, 2009. The plant was taxonomically identified by Bangladesh National Herbarium at Dhaka (Accession Number 34,416). The leaves and roots of *Cajanus cajan* were air-dried in the shade for 120 hours, grounded into a fine powder, and were extracted with methanol at a ratio of 1:8 (w/v). After 24 hrs, the mixture was filtered; filtrate was collected and the residue was again extracted with methanol at a ratio of 1:4 (w/v) for 24 hrs. Filtrates were combined and evaporated to dryness. The initial weight of dried leaf powder or root powder used for extraction was 100g; the final weight of the extract was 12.2g for leaves and 8g for roots.

Chemicals and Drugs

Glacial acetic acid was obtained from Sigma Chemicals, USA; aspirin, glibenclamide and glucose were obtained from Square Pharmaceuticals Ltd., Bangladesh. All other chemicals were of analytical grade.

Animals

In the present study, Swiss albino mice (male), which weighed between 20-25g were used for studies with leaf extract, and Swiss albino mice (male), which weighed between 15-20g were used for studies with root extract. The animals were obtained from International Centre for Diarrheal Disease Research, Bangladesh (ICDDR,B). All animals were kept under ambient temperature with 12h light followed by a 12h dark cycle. The animals were acclimatized for one week prior to actual experiments. The study was conducted following approval by the Institutional Animal Ethical Committee of University of Development Alternative, Dhaka, Bangladesh.

Antihyperglycemic activity

Glucose tolerance property of methanol extract of C*ajanus cajan* leaves and roots was determined as per the procedure previously described by Joy and Kuttan (1999) with minor modifications. In brief, fasted mice were grouped into six groups of six mice each. The various groups received different treatments like Group-I received vehicle (1% Tween 80 in water, 10 ml/kg body weight) and served as control, group-II received standard drug (glibenclamide, 10 mg/kg body weight) and the other four groups (III-VI) received the methanol extract of *Cajanus cajan* leaves or roots at four different doses of 50, 100, 200 and 400 mg/kg body weight. Each mouse was weighed and doses adjusted accordingly prior to administration of vehicle, standard drug, and test samples. All substances were orally administered. Following a period of one hour, all mice were orally administered 2 g glucose/kg of body weight. Blood samples were collected two hours after the glucose administration through puncturing heart. Serum glucose levels were measured by glucose oxidase method (Venkatesh *et al*., 2004). Antihyperglycemic activity experiments with leaf and root extracts were conducted on different days.

Toxicity testing against brine shrimp

Hatching shrimp. Brine shrimp eggs, *Artemia salina* leach were hatched in artificial seawater prepared by dissolving 38g of sea salt in 1L of distilled water. The pH of the solution was adjusted to 8.5. After 48h incubation at room temperature (26-30^{o}C) under constant aeration, the larvae (nauplii) were attracted to one side of the vessel with a light source and collected with a pipette. Nauplii were separated from eggs by aliquoting them three times in small beakers containing seawater.

Brine shrimp assay. The bioactivity of the extracts was monitored by the brine shrimp lethality test (Meyer *et al*., 1982). Samples were dissolved in dimethylsulfoxide (DMSO) and diluted with artificial sea salt water so that final DMSO concentration did not exceed 0.05%. 50 ml of 2000 mg of the plant extract was placed in one sample tube and a two-fold dilution carried out down the column of sample tubes. The last sample tube was left with sea salt water and DMSO only to serve as the drug-free control. The total volume was adjusted to 5 ml with sea salt water. 100 ml of suspension of nauplii containing 10 larvae was added into each tube and incubated for 24h. The tubes were then examined under a magnifying glass and the number of dead nauplii in each tube counted. Experiments were conducted with control (vehicle treated), and different concentrations of the test substances in a set of three tubes per dose. Lethality assays were evaluated by Finney computer statistical program to determine the LC_{50} values, as described before (Rahmatullah *et al*., 2010).

Statistical analysis

Experimental values for antihyperglycemic activity tests are expressed as mean ± SEM. Independent Sample t-test was carried out for statistical comparison. Statistical significance was considered to be indicated by a p value < 0.05 in all cases.

Results and Discussion

Antihyperglycemic effect

The results from the present study showed that the methanol extract of leaves of *Cajanus cajan* had significant antihyperglycemic effects at the two highest doses of the extract, i.e. when administered at doses of 200 and 400 mg/kg body weight. At the lowest dose of the extract tested (50 mg/kg body weight), the extract did not have any effect at all on reduction of serum glucose levels; the levels of serum glucose were nearly similar to control animals. There was a 7.7% reduction in serum glucose levels when the extract was

administered at a dose of 100 mg/kg body weight; however, the reduction was not statistically significant. In contrast, serum glucose levels were dose-dependently and significantly reduced when the extract was administered at doses of 200 and 400 mg/kg body weight. The reductions of serum glucose at these two doses of leaf extract were 20.2% and 36.3%, respectively. The highest dose of 400 mg extract/kg body weight compares favorably with the standard antihyperglycemic drug, glibenclamide in the reduction of serum glucose levels. Notably, glibenclamide, when administered at a dose of 10 mg/kg body weight was found to reduce serum glucose levels by 26.8%. The results are shown in Table 1.

Reduction of serum glucose levels by a plant extract can stem from several factors. The extract may influence in a positive manner the pancreatic secretion of insulin, or the extract may increase the glucose uptake (Nyunai *et al*., 2009; Farjou *et al*., 1987). It is also possible that the extract may inhibit glucose absorption in gut, thus reducing the presence of glucose in serum (Bhowmik *et al*., 2009). The exact mechanism through which the methanol extract of *Cajanas cajan* leaves caused reductions in serum glucose levels in glucose-loaded mice has not been elucidated in the present study and is currently under investigation.

In total contrast to the results obtained with the methanol extract of leaves, the methanol extract of roots of *Cajanus cajan* was observed to cause a dose-dependent increase in serum glucose levels in experimental mice. At the four administered doses of 50, 100, 200 and 400 mg extract/kg body weight, serum glucose levels rose above control values by 14.2, 15.2, 17.8 and 29.4%, respectively. The results behind the contrasting results obtained with two different parts of the same plant are not clear but may reflect a difference in the nature of phytochemical constituents present in the parts. To a certain extent, the results obtained with roots are surprising. Roots of *Cajanus cajan*, among other constituents are known to contain a-amyrin, genistein, and lupeol (Duke, 1992). The antihyperglycemic activity of a-amyrin has been described in rats and db/db mice (Singh *et al*., 2009). Genistein is also known to induce proliferation of pancreatic b-cells (Fu *et al*., 2010), as well as lower blood glucose levels of diabetic Wistar rats (Rauter *et al*., 2010). A synthetic lupeol derivative has also been shown to lower blood glucose levels in sucrose-challenged streptozotocin diabetic rats (Papi Reddy *et al*., 2009). Under the circumstances, the only conclusion to be drawn from the results is that possible pro-hyperglycemic constituents present in the roots are exerting greater activity than the antihyperglycemic constituents. It is to be noted in this context, that the folk medicinal practitioners of Bangladesh use the leaves and not the roots of the plant for treatment of diabetes. Thus the results obtained in the present study validate the folk medicinal uses of the plant.

Table 1: Effect of methanol extract of *Cajanus cajan* leaves on serum glucose level in hyperglycemic mice.

Treatment	Dose (mg/kg body weight)	Serum glucose level (mg/dl)	% lowering of serum glucose level
Control	10 ml	68.29 ± 4.03	-
Glibenclamide	10 mg	49.99 ± 2.33	26.8*
Cajanus cajan	50 mg	70.73 ± 2.95	-
Cajanus cajan	100 mg	63.00 ± 6.31	7.7
Cajanus cajan	200 mg	54.47 ± 2.65	20.2*
Cajanus cajan	400 mg	43.49 ± 2.63	36.3*

All administrations were made orally. Values represented as mean ± SEM, (n=6); $^{*}P < 0.05$; significant compared to hyperglycemic control animals.

Table 2: Effect of methanol extract of *Cajanus cajan* roots on serum glucose level in hyperglycemic mice.

Treatment	Dose (mg/kg body weight)	Serum glucose level (mg/dl)	% lowering of serum glucose level
Control	10 ml	86.40 ± 7.33	-
Glibenclamide	10 mg	38.60 ± 5.67	55.3*
Cajanus cajan	50 mg	98.68 ± 12.51	-
Cajanus cajan	100 mg	99.56 ± 8.13	-
Cajanus cajan	200 mg	101.76 ± 12.49	-
Cajanus cajan	400 mg	111.84 ± 6.92	-

All administrations were made orally. Values represented as mean ± SEM, (n=6); $^{*}P < 0.05$; significant compared to hyperglycemic control animals.

Brine shrimp lethality assay

The LC_{50} values obtained with methanolic extract of leaves and roots of *Cajanus cajan* in brine shrimp lethality assay were, respectively, 0.0264 and 1.8492 mg/ml. McLaughlin (1991) has reported that the results obtained with *Artemia salina* (brine shrimp) are quantitative and reproducible, and the activities parallel cytotoxicities. The results obtained in the present study indicate the considerable cytotoxic activities present in both leaves and roots of the plant, and considerably more so in the leaves (any LC_{50} value of less than 30 mg/ml is considered to be cytotoxic). It is to be noted that in earlier studies, methanol extract of the plant demonstrated cytotoxicity against three cancer cell lines (Ashidi *et al*., 2010), and that cajanol, a compound isolated from roots of the plant induced apoptosis in human breast cancer cells (Luo *et al*., 2010). As such,

further clinical studies need to be carried out for isolation of potential chemicals, which can augment the cancer fighting ability of modern medicines.

The antihyperglycemic studies conducted with the plant validate its use for treatment of diabetes by folk medicinal practitioners of Bangladesh. Studies are ongoing in our laboratory for isolation of active antidiabetic components that are present in the leaves of the plant.

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