

Full Length Research Paper

Isolation of β -sitosterol and evaluation of antidiabetic activity of *Aristolochia indica* in alloxan-induced diabetic mice with a reference to *in-vitro* antioxidant activity

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Stigmast-5-en-3 β -ol (β -sitosterol) was isolated from the chloroform extract of the aerial parts of *Aristolochia indica* (CEAI). The structure and relative configuration of Stigmast-5-en-3 β -ol (β -sitosterol) was determined by spectroscopic methods (1H- and 13C-NMR, IR, and MS). CEAI was found to produce good antidiabetic activity in treated mice. It reversed the weight loss of diabetic mice and restored to normal blood sugar level. The efficacy of the extract was compared with standard drug, Glibenclamide (10 mg/kg p.o.) in diabetic mice. CEAI also showed significant antioxidant activity in the level of 1,1-diphenyl-2-picrylhydrazyl (DPPH) and superoxide radicals with IC₅₀ value being 7.325 and 8.498 μ g/ml, respectively. The anti-hyperglycemic effect of the extract might be due to an increase in peripheral glucose consumption as well as protection against oxidative damaged in alloxanised diabetes.

Key words: *Aristolochia indica*, antioxidant, antidiabetic activity, alloxan, glibenclamide, diabetes, 1,1-diphenyl-2-picrylhydrazyl (DPPH), superoxide anion radical, β -sitosterol.

INTRODUCTION

Diabetes mellitus is the most common metabolic disorder characterized by hyperglycemic, glucoseurea and negative nitrogen balance, and it is mainly due to lack of insulin secretion in beta cells of pancreas and desensitization of insulin receptors for insulin. It is the most prevalent disease in the world affecting 25% of population and afflicts 150 million people and, which is expected to touch 300 million mark by 2025. In diabetes, hyperglycaemia generates reactive oxygen species (ROS), which in turn cause lipid peroxidation and membrane damage and these free radicals play an important role in the production of secondary complications in diabetes mellitus (kidney, eye, blood

vessel, and nerve damage) (Aslan et al., 2010). In this context, herbal drugs are gaining increasing popularity for the treatment of diabetic mellitus due to their efficacy, low incidence of side effects, and low cost and easy availability.

Aristolochia indica commonly known as Indian birthwort (family- Aristolochiaceae) is an important medicinal plant which is a perennial climber with greenish white woody stems found in low hills and plains of India from Nepal and lower Bengal to Chittagong in Bangladesh and the hilly region of Odisha, India. This plant has a beneficiary effect, especially anti-venom (Meenatchisundaram et al., 2009), anti-inflammatory (Das et al., 2010), antibacterial activity (Shafi et al., 2002), anti fertility activity in experimental animals (Che et al., 1984). However, there is no scientific report of this plant having anti-diabetic activity, and so it has been selected to evaluate its anti-diabetic activity to support the folklore claim made by the

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tribal people of Odisha, India. As anti-diabetic activity is closely related with antioxidant activity, in this communication, we have assessed the antioxidant activity of the same plant. Experiments were also designed to isolate active constituent(s) from the chloroform extract of *A. indica*.

MATERIALS AND METHODS

Preparation of extract

Aerial parts of *A. indica* were collected in the month of November, from the Simlipal biosphere in the district of Mayurbhanj, Odisha, India. The collected plant was authenticated at Botanical survey of India, Central National Herbarium, Botanical garden, Kolkata, India, vide letter no CNH/1-1/34/2010/Tech. II/205. The voucher specimen has been submitted to our laboratory for further references.

After collection, plant parts were dried under the shade and powdered to coarse particles. Two kilogram of powdered plant material were defatted with petroleum ether (40 to 60°C) in a Soxhlet apparatus, and further the same plant materials were extracted successively with chloroform. The yield of the chloroform extract was found to be 1.47% w/w with respect to dry starting materials. The chloroform extract of *A. indica* (CEAI) on pharmacological screening gave the best result and hence was selected for the present study.

Isolation of Stigmast-5-en-3 β -ol (β -sitosterol) from *A. indica* extracts

The chloroform soluble fraction (5 g) was fractionated over the silica gel column eluted with Pet ether: acetone with gradual increasing of the acetone content and seventy six fractions were collected. Fraction 6 to 10 are mixed together, which shows a single spot having similar R_f and was rechromatographed over the silica gel column eluted with 2% acetone: pet. ether to afford 70 mg of Stigmast-5-en-3 β -ol (β -sitosterol).

Animals

Swiss albino mice of either sex weighing 20 \pm 2 g were used for the present investigation. They were housed in clean polypropylene cages and were fed with standard pellet diet (Hindustan Lever, Kolkata, India) and water *ad libitum*. The animals were acclimatized to laboratory condition for one week before proceeding of the experiment. The experiment was performed under the guidance of the Institutional Ethical Committee. The animals were handled as per the guidance of the Committee for the Purpose of Control and Supervision on Experimental animals (CPCSEA), New Delhi, India.

Acute toxicity test

Acute toxicity study was performed for the extract according to the acute toxic classic method as per the method of Litchfield and Wilcoxon (1949). The animals were divided into six groups containing 10 animals each. The CEAI suspension was administered orally in increasing dose up to 1500 mg/kg, b.w. These animals were observed for mortality and toxicity for 72 h.

Preparation of the drug solution

CEAI was dissolved in normal saline to prepare dose level of 100, 250, 500 and 750 mg/kg, body weight for administration into mice.

Induction of experimental diabetes

Diabetes was induced in Swiss albino mice of either sex by a single intravenous injection of aqueous alloxan monohydrate (150 mg/kg, i.v.) (Badole et al., 2006). After 48 h, animals with the serum glucose level above 200 mg/dl (diabetic) were selected for the study.

Collection of blood and determination of serum glucose

Blood samples were collected by cutting the tail vein of mice and blood glucose levels are checked by glucometer (Dr. Morepen, 9F, 31 code).

Experimental protocol

The method described by Dunn et al. (1943) was adopted to determine the serum glucose in alloxan-induced diabetic mice. The diabetic mice were fasted overnight and divide into six groups of six mice each.

- Group I - Vehicle (Distilled water, 10 ml/kg p.o.)
- Group II - Glibenclamide (10 mg/kg p.o.)
- Group III - CEAI (100 mg/kg p.o.)
- Group IV - CEAI (250 mg/kg p.o.)
- Group V - CEAI (500 mg/kg p.o.)
- Group VI - CEAI (750 mg/kg p.o.)

For acute antihyperglycaemic study, blood samples are collected at 0, 2, 4, 6, 8 and 24 h, after administration of vehicle, glibenclamide and CEAI. Subacute study involved administration of vehicle, glibenclamide and chloroform extract of *A. indica* (CEAI). At different concentrations, the blood glucose levels were estimated on the day 1, 7, 14, 21 and 28th day. Mean change in blood glucose levels was calculated.

In vitro antioxidant study

DPPH radical scavenging effect

DPPH radical scavenging effects of CEAI were performed according to the method of Blois (1958). 0.1 mM solution of DPPH in methanol was prepared and 1 ml of this solution was added to 3 ml of extract's solution at different concentrations (5, 10, 25 and 50 μ g/ml). After 30 min, absorbance was measured at 517 nm. Vitamin C (ascorbic acid) was used as a reference drug. The percentage inhibition was evaluated by comparing the absorbance values for the control and experimental samples (Suksomtrip et al., 2010).

Superoxide anion radical scavenging effect

Superoxide anion scavenging activities of CEAI were done as per the method of Nishimiki et al. (1972) with slight modification. About 1 ml of nitroblue tetrazolium (NBT) solution (156 μ M NBT in 100 mM phosphate buffer, pH 7.4), 1 ml of NADH solution (468 μ M in 100 mM phosphate buffer, pH 7.4) and 0.1 ml of sample solution of CEAI in different concentrations (5, 10, 25 and 50 μ g/ml) were mixed and the reaction started by adding 100 μ l of phenazine methosulphate (PMS) solution (60 μ M PMS in 100 mM phosphate buffer, pH 7.4).

The reaction mixture was incubated at 25°C for 5 min and the absorbance at 560 nm was measured against blank samples. Decreased absorbance of the reaction mixture indicated increased superoxide anion scavenging activity. Curcumin was used as



Figure 1. Chromatogram of β -sitosterol.

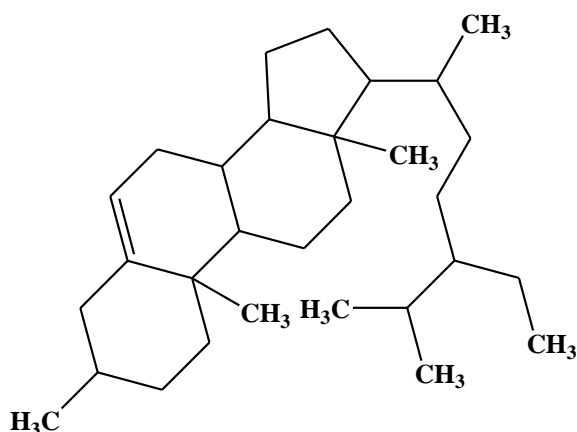


Figure 2. Structure of Stigmast-5-en-3 β -ol (β -sitosterol).

reference standard. The percentage of inhibition was determined by comparing the results of control and test samples.

Statistical analysis

Results are expressed as mean \pm SD. Comparison between the test groups with control was made by one way analysis of variance (ANOVA), followed by Dunnett's 't' test. The values of $P < 0.01$ and $P < 0.05$ were considered as statistically significant (Naveen and Khanum, 2010).

RESULTS AND DISCUSSION

Acute toxicity study

In the acute toxicity assay it was found that no mortality was observed up to doses 1500 mg/kg, orally and were

considered as safe and no lethality or any toxic reaction were found up to the end of the study period.

Isolation and characterization of β -sitosterol

In order to investigate the purity of the β -sitosterol, it was analyzed by thin layer chromatography (TLC) (Figure 1). The spot on the TLC chromatogram developed with petroleum ether and acetone gave a positive result in Libermann- Burchard test indicating a steroidal substance. Preliminary identification of β -sitosterol was based on the comparison of its R_f value and retention time with those of authentic standard compound. Finally, the structure assignment of β -sitosterol was based on its spectral data IR, MS, and ^1H and ^{13}C NMR and was identified as β -sitosterol (Figure 2).

It has the characteristics of white colored; TLC (2% acetone: pet ether) R_f value 0.54; UV λ_{max} (ethanol) nm: 206; IR (KBr) m cm^{-1} : 3426.89, 2924.52, 2855.1, 1738.51, 1057.76; HRMS (ESI) m/z 414.4 (calcd. for $\text{C}_{29}\text{H}_{50}\text{O}$, 414). The carbons of alkenes conjugated are at 140.74 ppm (C5) and 121.73 ppm (C6) (confirmed from ^{13}C NMR). The compound is also having six methyl, eleven methylene and three quaternary carbons with a hydroxyl group (Dryer, 1994).

Effect of CEAI on serum glucose level in alloxan-induced diabetic mice

Administration of CEAI (100, 250, 500, 750 mg/kg, p.o) in diabetic Swiss albino mice showed reduction in serum glucose level after 2, 4, 6 and 8 h interval. Maximum reduction in serum glucose level was found at the doses of (100, 250, 500, 750 mg/kg, p.o) from 226.3 ± 4.502 to 198.7 ± 2.16 mg/dl, 244.2 ± 3.76 to 206.5 ± 1.871 mg/dl, 414.2 ± 3.869 to 187.2 ± 2.312 and 273 ± 3.742 to 184.7 ± 3.141 mg/dl respectively, after 4 h of administration of CEAI. Glibenclamide (10 mg/kg, p.o) showed maximum reduction from 208.3 ± 8.710 to 192 ± 2.898 mg/dl in the same experimental design as shown in (Table 1). On sub acute treatment (repeated administration) of glibenclamide and CEAI for 28 days, a significant ($p < 0.01$) decrease in serum glucose level in diabetic mice was seen at the doses of 100, 250, 500 and 750 mg/kg, p.o. in the dose dependent manner as compared to the vehicle control group (Table 2). Of course, glibenclamide (standard) showed a significant ($p < 0.01$) decrease in serum glucose level at a dose of 10 mg/kg, p.o. as compared with that of vehicle control.

In vitro antioxidant study

CEAI was found to scavenge DPPH and superoxide

Table 1. Effect of chloroform extract of *A. indica* on serum glucose level in alloxan induced diabetic mice (acute study).

Drug	0 h (mg/dl)	2 h (mg/dl)	4 h (mg/dl)	6 h (mg/dl)	8 h (mg/dl)	24 h (mg/dl)
Control	203.3±2.160	190.5±1.871	181.5±1.871	176.5±1.871	194.2±3.488	227.3±3.327
Standard (Glibenclamide)	208.3±8.710**	177.8±2.317*	192±2.898*	227.5±1.871*	180.8±3.92*	211.8±2.858*
Test 1 (100 mg/kg)	226.3±4.502*	209±3.742*	198.7±2.16*	232.7±2.805*	216.8±2.317*	235±3.742*
Test 2 (250 mg/kg)	244.2±3.764*	197±2.366*	206.5±1.871*	219.8±2.858*	203.5±3.082*	253.3±2.16*
Test 3 (500 mg/kg)	414.2±3.869*	329±3.225*	187.2±2.317*	160.8±2.639*	256.5±1.871*	274.7±2.160*
Test 4 (750 mg/kg)	273±3.742*	218.3±2.582*	184.7±3.141**	170.7±2.160*	204.8±3.488*	241.7±4.633*

Values are mean ± SD. (n = 6), *P<0.01 and **P<0.05 were considered significant in comparison with control group.

Table 2. Effect of chloroform extract of *A. indica* on serum glucose level in Alloxan induced diabetic mice (subacute study).

Drug	Day 0 (mg/dl)	Day 1 (mg/dl)	Day 7 (mg/dl)	Day 14 (mg/dl)	Day 21 (mg/dl)	Day 28 (mg/dl)
Control	203.5±1.160	227.5±1.877	271±3.742	277±3.742	292.7±2.16	297.5±1.871
Glibenclamide	207.5±1.871**	211.5±1.871*	164.3±2.16*	139±2.366*	120.5±1.871*	111.3±3.077*
Test 1 (100 mg/kg)	215.8±14.50*	235±3.742*	215±3.742*	182.2±2.317*	160.5±1.871*	171.8±2.858*
Test 2 (250 mg/kg)	244.8±3.656*	252.5±1.871*	211.2±4.579*	197.8±2.858*	178.7±4.457*	142.2±4.021*
Test3 (500 mg/kg)	414±2.366*	274.2±2.858*	239.2±3.488*	198.5±1.871*	162.7±2.16*	131.5±4.889*
Test 4 (750 mg/kg)	274.5±2.429*	241±3.742*	232±2.608*	201.5±1.871*	184.8±1.722*	156.7±3.777*

Values are mean ± SD. (n = 6), *P<0.01 and **P<0.05 were considered significant in comparison with control group

radicals *in vitro* in a concentration dependent manner.

DPPH radical scavenging effect

DPPH radical scavenging activity of CEAI is presented in (Figure 3). DPPH, a nitrogen centered free radical with a characteristic absorbance at 517 nm and convert to 1,1-diphenyl 2-picryl hydrazine due to its hydrogen accepting ability at a rapid rate. 1,1-diphenyl-2-picrylhydrazyl (DPPH) has been used extensively as a free radical to evaluate reducing substances (Schimada

et al., 1992). Stable free radical, DPPH was effectively scavenged by CEAI and the IC₅₀ value of CEAI was found to be 7.325 µg/ml. The standard antioxidant, vitamin C was used to compare the antioxidant potential, which exhibited 43.33% inhibition at a concentration of 25 µg/ml (Pal and Nimse, 2006).

Superoxide anion radical scavenging effect

In the PMS/ NADH-NBT system, superoxide radicals

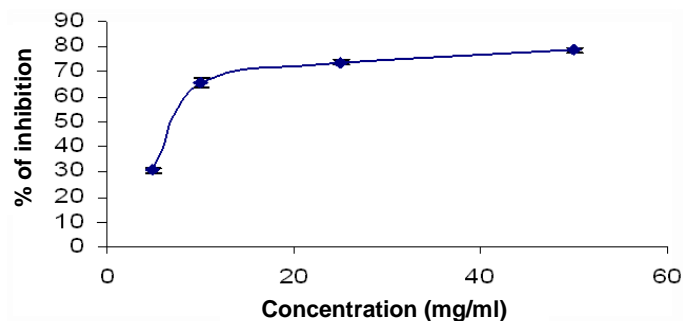


Figure 3. Radical scavenging activity of the studied *A. indica* (CEAI) extract using DPPH.

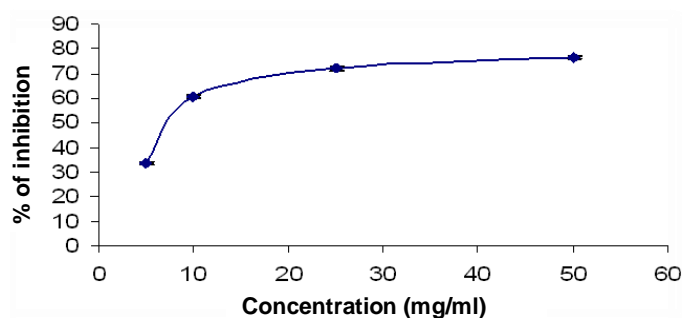


Figure 4. Radical scavenging activity of the studied *A. indica* (CEAI) extracts using superoxide.

generated from a non enzymatic reaction of PMS in the presence NADH and molecular oxygen reduce NBT to formazan at pH 7.8 (Robak and Gryglewski, 1988). The superoxide scavenging effect of CEAI is exhibited in (Figure 4). The IC₅₀ value of CEAI was found to be 8.498 µg/ml. Curcumin used as reference standard, which showed 40.11% inhibition at a concentration of 10 µg/ml.

Conclusion

The active component Stigmast-5-en-3β-ol (β-sitosterol) was isolated from the chloroform extract of *A. indica* and characterized in the present investigation. Results showed that CEAI has excellent antihyperglycemic activity as it lowers the serum glucose level in diabetic mice. CEAI also exhibits greatest antioxidant activity estimated through the scavenging of free radicals such as DPPH and superoxide radical. So, the chloroform extract of the aerial parts of *A. indica* is the rich source of natural antioxidants, which can be accounted for the traditional uses in prevention of diabetes and conservation of good health.

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