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Evaluation of antihyperglycemic activity of isolated phytosterols from fruits of *Lagenaria siceraria* in alloxan induced diabetic rats

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ABSTRACT

Lagenaria siceraria (Molina) Standl from cucurbitaceae family is a large, pubescent, climbing or trailing herb, cultivated throughout the India and the tropical regions of the world. Phytochemical investigations showed positive tests for phytosterols, these phytosterols were isolated from the methanol extract of fruits of plant *Lagenaria siceraria* and identified as Fucosterol, Stigmasterol, Racemosol and Stigmata-7, 22- dien - 3β, 4β - diol. The antihyperglycemic activity of isolated phytosterols and methanol extract was investigated in diabetic albino rats; diabetes was induced by intra-peritoneal administration of alloxan monohydrate at a dose of 125 mg/kg to albino rats. Albino rats were divided into various groups as follows: Group I received saline water, Group II received metformine 10 mg/kg served as diabetic control while group III, IV, V received phytosterols at a dose of 10, 20, 30 mg/kg respectively. While group VI, VII, VIII were treated with methanol extract at a dose of 100, 200, and 300 mg/kg of body weight, respectively. Blood samples were collected from tail vein and blood glucose was estimated in acute, sub-acute and oral glucose tolerance test (OGTT) study protocol. Oral administration of phytosterols at a dose of 30 mg/kg of body weight to alloxan induced diabetic rats resulted in significant ($p < 0.001$) decrease in blood glucose level in both acute and sub acute study protocol, it also significantly increase the glucose tolerance in OGTT test animals. These results indicate that the phytosterols exhibit significant anti-hyperglycemic activity.

KEYWORDS

Lagenaria siceraria;
Phytosterols;
Oral glucose tolerance test;
Alloxan.

INTRODUCTION

Diabetes is a clinical syndrome characterized by the hyperglycemia due to absolute or relative deficiency of Insulin. It is the third leading cause of death in many developed countries and common disorder among the Indian population. The incidence of both types of primary diabetes i.e., type 1 or insulin dependent diabetes mellitus (IDDM) and type 2 or non insulin dependent diabetes mellitus (NIDDM), is rising. Type 2 diabetes commonly occurs in subjects who are obese and insulin-resistant but these two factors alone are insufficient

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to cause diabetes unless accompanied by impaired β cell function. However, the prevalence of both varies considerable in different parts of the world and this is probably due to differences in genetic and environmental factors[1]. Currently, diabetes has been estimated to affect 177 million people worldwide and this figure is projected to increase to 300 million by 2025[2,3]. It causes number of complications like retinopathy, neuropathy, and peripheral vascular insufficiencies[4,5]. Diabetes is still not completely curable by the present anti-diabetic agents. Insulin therapy is the only satisfactory approach in diabetic mellitus; even though it has several drawbacks like insulin resistance[6], anorexia, brain atrophy and fatty liver in chronic treatment[7]. Herbal drugs are gaining popularity in the treatment of diabetic mellitus[8]. Despite the presence of known anti-diabetic medicine in the market, diabetes and the related complications continued to be a major medical problem. However, searching for new anti-diabetic drug from natural origin is still striking interest to find out the alternate and safe medicine. More than 400 plant species having hypoglycemic activity have been available in literature[9]. The major advantages of herbal medicine seem to be their efficacy, low incidence of side effects and low cost. The plant *Lagenaria siceraria* (Molina) Standl from a cucurbitaceae family is a large, pubescent, climbing or trailing herb, cultivated throughout the India and the tropical regions of the world. Phytochemical investigations showed positive tests for plant sterols (phytosterols), glycosides and tannins, the common chemical constituents of the plants, which show the anti-hyperglycemic activity. So the present paper deals with the isolation and identification of plant sterol and evaluation of anti-hyperglycemic activity of on alloxan-induced diabetic rats.

MATERIALS AND METHODS

With prior approval from the institutional animal ethical committee, the adult albino rats of Wistar strain weighing between 180-200 g were used for the study. They were procured from the national institute of nutrition, Hyderabad. Animal were housed in polypropylene cages at ambient temperature of 25±2ºC with a 12 h dark and light cycle and fed with standard pellet diet and water *ad libitum*. Drug metformin was received as gift sample from Zim laboratory Nagpur. Alloxan monohydrate was purchased from the Sigma Aldrich. Glucometer counter no coding machine was used to determine blood glucose level.

Preparation of extract

The plant of *Lagenaria siceraria* was collected from the local farm of Nagpur District and authenticated from Department of Botany Rashtrasanta Tukadoji Maharaj Nagpur University, Nagpur. The plant specimen voucher no. 9012 was available in the institute's herbarium department for future reference. The fruits were collected and cut into small pieces, sun dried and powdered in a grinder. The powder was extracted successively with increasing polarity of solvents using Soxhlet apparatus.

Study design

Diabetic was induced experimentally by single intra peritoneal administration of alloxan monohydrate dissolved in normal saline at a dose of 125 mg/kg of body weight in albino rats. The induction of diabetes mellitus was confirmed after the 5th day of alloxan administration.

Albino rats showing elevated fasting blood glucose level ≥150 mg/dL were selected for the study. These rats were divided into various groups as follows: Group I received saline water, Group II received metformine 10 mg/kg of body weight served as diabetic control while group III, IV, V received phytosterols at a dose of 10, 20 and 30 mg/kg of body weight, respectively. While group VI, VII, VIII were treated with methanol extract at a dose of 100, 200, and 300 mg/kg of body weight, respectively. All the drugs were orally administered once daily using intra gastric tube in the morning hours. Blood glucose was measured after induction of diabetic and weekly thereafter up to the end of the treatment period. Blood was collected from the tail vein and blood glucose was estimated using the glucometer.

In acute study protocol blood samples were withdrawn at 0, 2, 4, 6, and 24th h. by tail vein puncture method and glucose level was analyzed.

Sub acute protocol involves 28 days of study with once daily administration of doses to alloxan induced diabetic rats and blood was withdrawn on 0, 7, 14, 21 and 28th day from tail vein. The oral glucose tolerance

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TABLE 1 : Blood glucose level in alloxan induced diabetic rats (acute study)

Groups	Time in hours				
	0	2	4	6	24
I Saline water	472.66±36.1	473.33±37.05	509.16±39.9	536.66±34.12	570±66.16
II Metformin (10 mg/kg)	513.88±17.36	485.16±20.28	457.00±33.46	385.83±23.43	518.16±36.16
III Phytosterols (10 mg/kg)	553.83±33.07	525.66±24.86	513.66±18.84	472.33±13.01	542.00±32.79
IV Phytosterols (20 mg/kg)	561.83±19.01	548.33±16.75	477.00±23.47	456.16±30.15	547.50±35.14
V Phytosterols (30 mg/kg)	570.83±20.63	539.83±26.08	470.83±26.69	389.66±33.27	519.33±30.47
VI Methanol extract (100 mg/kg)	569.33±31.81	529.16±33.92	493.66±20.42	464.66±28.23	565.16±35.78
VII Methanol extract (200 mg/kg)	584.16±25.69	543.33±15.07	502.66±24.62	460.33±30.63	513.16±33.81
VIII Methanol extract (300 mg/kg)	560.5±31.05	546.5±15.19	460.0±32.17	444.5±29.65	462.0±13.48

Each value is ± SEM n=6 in each group. p < 0.001 extremely significant, Two way ANOVA followed by post Bonferroni test

TABLE 2 : Blood serum glucose level in alloxan induced diabetic rats (sub acute study)

Groups	Days				
	0	7	14	21	28
I Saline water	472.66±36.1	526.83±32.76	539.16±25.91	545.5±24.01	592.66±8.57
II Metformin (10 mg/kg)	513.83±17.36	490.66±28.18	366.5±22.06	339.0±15.56	286.83±17.03
III Phytosterols (10 mg/kg)	553.83±33.07	493.83±15.79	488.5±27.4	440.66±19.35	390.33±21.67
IV Phytosterols (20 mg/kg)	561.83±19.01	464.5±15.53	441.5±18.87	380.66±21.2	364.5±15.34
V Phytosterols (30 mg/kg)	570.83±20.63	516.5±20.046	408.83±11.18	337.5±12.233	310.83±18.01
VI Methanol extract (100 mg/kg)	569.33±31.81	508.00±29.47	472.16±21.8	446.5±20.05	438.16±17.8
VII Methanol extract (200 mg/kg)	584.16±25.69	543.33±15.07	502.66±24.62	460.33±30.63	513.16±33.81
VIII Methanol extract (300 mg/kg)	560.7±31.05	493.33±5.42	479.5±16.82	392.33±9.797	336.16±20.15

Each value is ± SEM n=6 in each group. p < 0.001 extremely significant. Two way ANOVA followed by post Bonferroni test

test (OGTT) was performed by dividing the normal albino rat in four groups (n = 6). Group I was treated with only glucose (2.5 g/kg p. o.). Group II received metformin (10 mg/kg p. o.). Group III was treated with phytosterols (30 mg/kg p. o.) and Group IV with the methanol extract (300 mg/kg p. o). The animals were fasted overnight before instigate the experiment. All albino rats were loaded with 2.5 g/kg p. o., of *d*-glucose solution after 30 min of drug administration. Blood samples were collected from tail vein method prior to drug administration and 30, 60, 90, 120, 150, 180th min. after glucose loading.

Phytochemical investigation (Test for Sterols)

Liebermann-Burchard test: methanol extract 1 mg was dissolved in chloroform and few drops of acetic anhydride were added to it, followed by concentrated sulphuric acid from the side of the tube. A transient color development from red to blue and finally green indicated the presence of sterol.

Salkowaski reaction: methanol extract 1 mg was dissolved in 2 ml of chloroform and 2 ml of concentrated sulphuric acid was added from the side of the test tube. The test tube was shaken for few minutes. The development of red color in the chloroform layer indicated the presence of sterol.

Isolation of phytosterols[10]

Three hundred grams of unsaponifiable methanol extract was admixed with the 500 mL of methyl cyanide. The mixture was heated to a temperature of approximately the boiling point of the methyl cyanide. This temperature was maintained for 10 to 15 minutes at which time it was visually evident that the insoluble portion of unsaponifiables, which comprises the undesirable gummy material, formed a layer at the bottom of the vessel whereas the sterols and methyl cyanide resolved in a clear solution. This sterol-methyl cyanide solution was then decanted off. The solution was then immediately allowed to cool in ice bath which resulted in the formation of white sterol crystals at the bottom of the beaker. The resulting white sterol crystals were collected and investigated.

Statistical analysis

Statistical analysis was performed using graph pad prism 5.0 software. The results were expressed as mean ±SEM. Two way ANOVA followed by post Bonferroni test was used for the analysis of significant difference among the collected data and p < 0.001was considered significant.

RESULTS

In acute study protocol, as depicted in TABLE 1. administration of phytosterols at a dose of 10, 20, 30 mg/kg, p. o. in diabetic rats showed reduction in serum glucose level after 2, 4, 6 h. interval. Significant reduction was observed in 30 mg/kg on 6th h. [$F_{time\ x\ treatment}$ = 1.31; DFn = 28; DFd = 160; $F_{treatment}$ = 1.71; DFn = 7

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TABLE 3 : Body weight in grams of alloxan induced diabetic rats in sub acute study protocol

Group	Days				
	0	7	14	21	28
I Saline water	180.33± 1.222	169± 2.463	159.16± 1.222	150± 1.713	141.166± 2.431
II Metformin (10 mg/kg)	181± 0.8563	174.33± 3.127	164.16± 2.845	154.83± 2.056	155.5± 2.078
III Phytosterols (10 mg/kg)	177.83± 1.621	164.33± 2.692	157.83± 4.438	140.33± 1.994	131.66± 2.319
IV Phytosterols (20 mg/kg)	180.5± 1.335	168.66± 2.629	159.66± 2.459	150.16± 2.136	141± 1.673
V Phytosterols (30 mg/kg)	180.16± 1.887	172.5± 1.91	163.16± 1.778	153.66± 1.892	144± 3.327
VI Methanol extract (100 mg/kg)	179.66± 1.745	169.83± 3.341	156.16± 1.558	140.33± 1.994	130.66± 1.961
VII Methanol extract (200 mg/kg)	179.66± 1.498	167.83± 3.535	157.83± 1.956	136.83± 3.497	131.33± 3.313
VIII Methanol extract (300 mg/kg)	180.16± 1.138	170.5± 3.128	156.33± 4.485	138.33± 4.883	133.5± 3.948

Each value is ± SEM n=6 in each group. p < 0.001 extremely significant. Two way ANOVA followed by post Bonferroni test

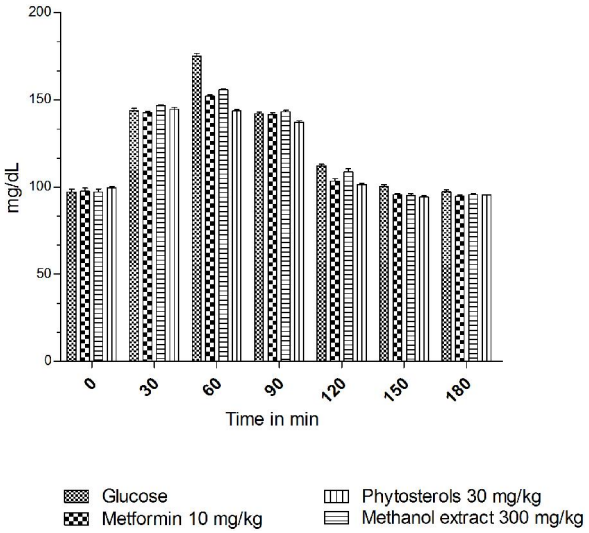

Figure 1 : Oral glucose tolerance test (OGTT) in normal albino rats, Each value is ± SEM n=6 in each group. Values are statistically significant p < 0.01 Two way ANOVA

DFd = 160; F_{time} = 13.98; DFn = 4; DFd = 160] p < 0.001 extremely significant.

In sub acute study the repeated administration of phytosterols at a dose of 10, 20, 30 mg/kg, p. o. ones daily for 28 days showed significant reduction in blood glucose level. Maximum activity was seen with a significant decrease in serum glucose level at the dose of 30 mg/kg which is shown in TABLE 2. [$F_{time\ x\ treatment}$ = 5.58; DFn = 28; DFd = 160; $F_{treatment}$ = 17.77; DFn = 7; DFd = 160; F_{time} = 60.07; DFn = 4; DFd = 160] p < 0.001 extremely significant.

The oral glucose tolerance test (OGTT) study protocol on normal albino rats shows that phytosterols significantly depresses the peak of serum glucose level at 120 min after glucose loading, depicted in figure 1 [$F_{time\ x\ treatment}$ = 21.61 DFn = 18; DFd = 120; $F_{treatment}$ = 75.29; DFn = 3; DFd = 120; F_{time} = 2425.11; DFn = 6; DFd = 120] p < 0.0001 considered extremely significant.

The isolated phytosterols showed positive Liebermann-Burchard and Salkowaski test and were identified using FTIR, ^{1}H NMR and EIMS analysis. Compound I was identified by its IR (KBr) cm^{-1} : 3439 (OH), 2936, 1626 (ethylidine sterol). The ^{1}H NMR (TMS) δppm: 5.366 (1H, H-6), 3.535 (1H, H-3), 1.595 (3H, H-29), 0.973. EI-MS spectrum m/z: 411(M^+), 397, 394, 379, 311.5, 297.5, 280, 270, 255, 230, 213 and 145. This corresponded to the molecular formula of $C_{29}H_{48}O$. Which led to a proposal that the compound I is Fucosterol. Compound II was identified by its IR (KBr) cm^{-1} : 3409 and 1053 (OH). The ^{1}H NMR (TMS) δppm: 0.58(3H H-18), 0.824 (3H H-19), 0.847 (3H H-26), 0.876 (3H H-27), 5.08 (1H H-23). EI-MS spectrum, which showed a molecular ion peak at m/z: 426 (M^+), 411, 396, 367, 300, 271, 255, 213, 187, 159, 147, 133, 119, 107. This corresponded to the molecular formula of $C_{30}H_{50}O$. This above data led to propose that compound II is Racemosol. Compound III was identified by its IR spectrum showed peaks at IR (KBr) cm^{-1} : 3494 (OH), 1053 and 1020 (OH). The ^{1}H NMR (TMS) δppm: 0.824, 0.876, 0.94, 0.973 (each 3H Me-6), 3.531 (1H, H-3), 5.366 (1H, H-6), 5.120 (1H H-22), 5.08 (1H H-23). EIMS spectrum molecular ion peak at m/z 412 (M^+). This above FTIR, H-HMR and EIMS data led to propose the molecular formula of $C_{29}H_{48}O$ which is closely resembles to Stigmasterol. Compound IV was identified by its IR spectrum showed major peaks at IR (KBr) cm^{-1}: 3400 (OH), 1670 (olefins). The ^{1}H NMR(TMS) δppm: 5.35 (1H, H-7), 5.08 (1H, H-23), 4.135 (1H, H-4), 3.848 (1H, H-3), 2.210 (1H, H-2ax), 2.041 (1H, H-20), 2.001 (1H, H-12), 1.876 (1H, H-14), 1.595 (1H, H-24), 1.286 (1H, H-17), 0.899 (3H, H-29), 0,847 (3H, H-27). EIMS spectrum, molecular ion peak at m/z: 428 (M^+), 417, 410, 377. This above data led to propose the molecular formula of $C_{29}H_{48}O_2$ which is closely resembles to Stigmasta-7, 22-dien-3β, 4β –diol. The probable structures of isolated phytosterols are given in figure 2.

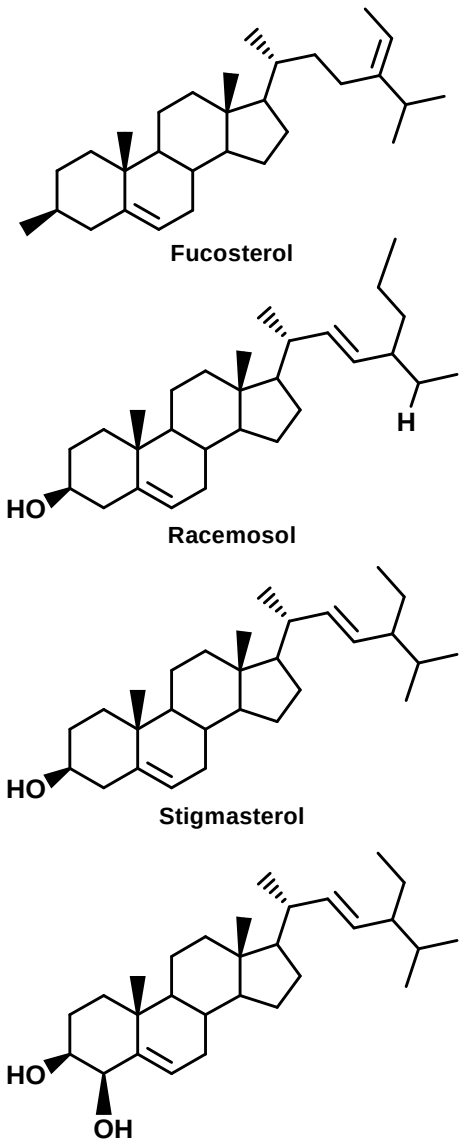

Figure 2 : Structures of phytosterols

DISCUSSION

Blood sugar level increased as expected in alloxan-administered animals, since alloxan causes a massive reduction in insulin release, by the destruction of the β cells of the islets of Langerhans and inducing hyperglycemia[11]. The cytotoxic action of alloxan is mediated by reactive oxygen species, with a simultaneous massive increase in cytosolic calcium concentration leading to a rapid destruction of *β* cells[12]. The present study demonstrated that the phytosterols at the dose of 30 mg/kg of body weight shows the anti-hyperglycemic effect when studied on both the acute and sub-acute study protocol. Also it significantly increases glucose tolerance in OGTT[13] study protocol in normal albinos. Oral administration of metformin 10 mg/kg of body weight showed the maximum reduction in blood glucose level. There was a significant weight loss observed in the saline as well as in treatment groups as depicted in TABLE 3.

Decrease in uptake of glucose, free fatty acids from circulation and accelerated oxidation in adipose tissue leads to weight loss in diabetes. Methanol extract at a dose of 300 mg/kg of body weight showed significant decrease in blood glucose level but phytosterols are significantly effective. Plants are inexhaustible sources of biological active compounds which include phytosterols, triterpenoids, flavonoids, tannins, alkaloids and glycosides. Vegetables and fruits contain substantial amounts of terpenoids, particularly C29 and C28 sterols (phytosterols)[14]. Chemically phytosterols are steroid alcohols with chemical structures similar to that of cholesterol, except for an extra methyl or ethyl group. They form a group of triterpenes with tetracyclic cyclopenta [a] phenanthrene structure and a side chain at C-17. The four rings have *trans* ring junctions, the side chain and two methyl groups at C-18 and C-19 are at an angle to the rings above the plane with β stereochemistry and double bond in position 5. The Liebermann-Burchard[15] color reactions for cholesterol yields oxidation products, a homologous series of conjugated cholestapolyenes. According to this mechanism the common initial step is the protonation of the hydroxyl group of the steroid and subsequent loss of water to give the carbonium ion of 3,5-cholestadiene and serial oxidation gives the blue-green color product of pentaenylic cation. This is the typical reaction for the steroid and phytosterols which have the structural similarity with the cholesterol. Research over the last 15 years indicates phytosterols and sterolins have important anti-diabetic, anti-cancer anti-inflammatory, anti-viral, antiulcer and immune T-cell proliferative activities[16,17]. In animal study the phytosterols protects the animal from an excessive rise in serum glucose level due to glucose loading[18]. So it has been concluded that the phytosterols isolated from the fruits of plant *Lagenaria siceraria* could be useful for the treatment or prevention of type 2 diabetes mellitus.

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