Research Journal of

Medicinal Plant

ISSN 1819-3455

Academic
Journals Inc.

www.academicjournals.com

Research Journal of Medicinal Plant 9 (8): 395-405, 2015
ISSN 1819-3455 / DOI: 10.3923/rjmp.2015.395.405

Comparative Study of Biochemical Changes in Alloxan Induced Diabetic Mice Treated with Extracts of *Spathodea campanulata* Flowering Branch and Barks

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ABSTRACT

On the light of the antihyperglycemic effect of bark extract of *S. campanulata* (SC) that was detected by few previous studies, we aimed to investigate the glucose lowering effect of flowering branch and bark extracts of SC and its possible mechanistic background. The crude methanolic extracts of the bark and the flowering branch were tested for their anti-hyperglycemic effect in forty mice divided into 5 groups: group 1 received only 1% carboxymethyl cellulose (CMC) as negative control, all the other groups were subjected to alloxan induction of diabetes mellitus from which second group left without any treatment as positive control, the third and fourth groups were administrated the methanolic extracts of the flowering branch and bark respectively in dose of 500 mg kg^{-1}. b.wt. and the fifth group received metformin in dose of 150 mg kg^{-1} b.wt. At the end of treatment period blood levels of glucose, insulin, triacylglycerols and cholesterol were estimated; in addition to the evaluation of muscle tissue concentration of insulin receptors. A significant reduction of blood glucose was noticed after 2 h of treatment with bark extract but not with flowering branch extract ($p<0.05$), moreover a significant rise in concentration of insulin receptors was found in bark extract and metformin treated groups as compared to control group ($p<0.05$). Consequently, we concluded that bark extract of *S. campanulata* may have a prospective anti-hyperglycemic therapeutic effect in diabetes; this influence could be through modifying the tissue expression of insulin receptors.

Key words: *S. campanulata*, flowering branch, barks, alloxan induced diabetes, insulin receptors

INTRODUCTION

Diabetes mellitus is a major metabolic disease that affects all human races and it is associated with life threatening complications as renal failure, cerebrovascular disorders, coronary insufficiency and limb amputation (Lyra *et al*., 2006; Tielmans *et al*., 2007). The exploration of hypoglycemic effect of medicinal plants is elicited by the desire to obviate various side effects of traditional oral hypoglycemic drugs regarding the cost effectiveness, incidence of toxicity and limited availability in rural areas which may affect the management of diabetes.

Despite the presence of known anti-diabetic medicines in the pharmaceutical market, screening for new anti-diabetic source from natural plants is still attractive as they may contain substances that have an alternative and safe effect on diabetes mellitus (Ju *et al*., 2008).

According to reports of World Health Organization (WHO., 2010) about 80% of world's population uses herbal medicine in healing different illnesses, about 15% of estimated 400,000 plant species have been investigated phytochemically (Cragg *et al*., 1997; Srinivasan, 2005). There is progressive need for deliberate phytochemical evaluation of herbal drugs (Grover *et al*., 2002), as plant products are frequently considered to be less toxic and more devoid of side effects in comparison to the currently used synthetic oral antihypoglycemic drugs (Brinker, 1998). There is a continuous activity of searching for more effective and safer hypoglycemic agents to replace.

Spathodea campanulata (SC) was studied by many previous researches for its hypoglycemic (Niyonzima *et al*., 1999), anti-malarial and anti-schistosomiasis effect (Makinde *et al*., 1988).

Spathodea campanulata P. Beauv. (African Tulip, Bignoniaceae), a medium-size tree (15-25 m high) carrying red garish flowers, is native to equatorial Africa and grown as ornamental in tropical and subtropical areas (Joly, 1985). The flowers are used as anti-inflammatory and diuretic; while the bark is used in treatment of herpes and as antifungal in skin ailments and anti-diarrhea (Mende *et al*., 1986). The leaves are used as remedial for animal poisoning and kidney diseases. The flowers and stem bark exerted a molluscicidal effect; the stem bark, in addition, exhibited hypoglycemic, anti-HIV, antibacterial and antimalarial activities (Niyonzima *et al*., 1999; Mbosso *et al*., 2008). The aerial parts and leaves acted as antioxidant and antimicrobial (Nazif, 2007; Dhanabalan *et al*., 2008).

A literature preview has shown that decoction of stem bark showed hypoglycemia in mice but had no influence on insulin levels. Diabetes Mellitus (DM) has been orally treated by oral herbal medicines or their extracts (Akhtar and Ali, 1984; Pepato *et al*., 2003). Despite of these reports for the flowering branch and barks, their effects on insulin receptors and insulin level in alloxan induced diabetic rats have not yet been explored.

The present study was aimed to investigate the biochemical changes in the blood and tissue of alloxan induced diabetic mice treated with both extracts of *S. campanulata* flowering branch and barks.

MATERIALS AND METHODS

Chemicals, kits and animals: Enzyme Liked Immuno Sorbent Assay (ELISA) kit for measurement of serum insulin and muscle's insulin receptors in mice was supplied by Millipore Corporation, Billerica, MA, USA. Alloxan was purchased from Sigma Chemicals (St. Louis, MO, USA). Colorimetric kits for lipid profile and blood glucose were purchased from Diagnostic system-Germany. Methanol and carboxymethyl cellulose (CMC) were purchased from Merck, Darmstadt, Germany. One-touch ultra glucometer (Johnson and Johnson).

Plant material: Flowering branch (flowers, leaves and stems) and bark were collected during flowering stage, from trees cultivated in UAE gardens.Voucher samples was kept in Dubai Pharmacy College, Dubai, UAE.

Preparation of the methanolic extracts: The air-dried powdered flowering branch and bark of *S. campanulata* (500 g each) were exhaustively extracted by cold maceration in methanol (2L×2). The solvent in each was evaporated under reduced pressure at 50°C using rotary evaporater. The residues were suspened in 1% CMC and kept for the biological study (Cole *et al*., 1997).

Experimental animals: Male and female albino mice (weight, 25±5 g) were used for acute toxicity study as well for testing the hypoglycemic activity. Animals were kept under the same standard hygienic conditions (temperature 22±2°C, relative humidity 50-60%, with 12 h day/night lighting cycle) and fed with well-balanced normal diet and water supplied ad libitum. They were left for a period of one week for accommodation before performing the experiments. All animals' investigations were performed in accordance with the ethical standards for the proper care and use of laboratory animals and upon approval of the Research and Ethical Committee of the Dubai Pharmacy College, Dubai, UAE.

Determination of acute toxicity: The LD_{50s} of the methanolic extracts of both flowering branch and barks of *S. campanulata* were estimated to assess their safeties (Lorke, 1983).

Evaluation of acute anti-hyperglycemic activity: Diabetes mellitus was induced in male albino mice by intraperitoneally injecting a single dose of alloxan (150 mg kg^{-1} b.wt.) in sterile normal saline. Blood samples were obtained from tail tip vein of all experimental animals and Fasting Blood Glucose (FBG) concentration was determined using One-touch ultra glucometer and compatible glucose strips (Sharma and Garg, 2009). Hyperglycemia was assessed after 72 h of induction of diabetes and animals with FBG >140 mg dL^{-1} were selected for the study (Katsumata *et al*., 1993).

Alloxan-treated mice were divided into four groups, each of 6 animals and receiving the treatment orally. Group I (diabetic control- received only1% CMC), group II (diabetic treated with 500 mg kg^{-1} b.wt. methanolic extract of the flowering branch), group III (diabetic treated with 500 mg kg^{-1} b.wt. methanolic extract of the bark) and group IV (diabetic treated with 150 mg kg^{-1} b.wt. metformin).

Biochemical analysis

Sample preparation

Blood samples: At the end of the experiment, fasting mice were sacrificed after inducing anesthesia. Whole blood samples were withdrawn into centrifuge tube that contains no anti-coagulant, blood is left to clot for 30 min and blood serum was separated for each sample by centrifuge for 15 min at 3000 rpm. Serum was stored at -80°C till analyzed.

Tissue homogenate: Muscle tissues were dissected out, frozen at -80°C. Soluble extracts were prepared from frozen muscle tissue to measure the tissue content of Insulin Receptors (IR). Approximately 50 mg of frozen tissue was weighed and homogenized using DAIHAN digital homogenizer in 0.5 mL ice cold buffer (Pierce® IP lysis buffer (AEBSF 1 mM, Aprotinin 800 nM, Bestatin 50 uM, E64 15 uM, Leupeptin 20 uM prpstatin A, EDTA 5 mM, TrisHCl mM 25, 150 mM NaCl, 1% NP-40, 1 mM EDTA, 5% glycerol, pH 7.4). Homogenates were centrifuged at 15,000 rpm for 1 h; the supernatants were collected in separate aliquots which were stored at -80°C for later analysis of beta subunit of insulin receptors (Pender *et al*., 2004; Yaspelkis *et al*., 2009).

Determination of blood glucose levels: The samples for glucose estimation were collected by cutting the tail artery of mice, FBG levels were measured at zero time, after 1 and 2 h following oral administration of extracts and metformin. Determination of blood glucose was done by the glucose oxidase principle using one touch glucometer strips and reported in mg dL^{-1} (Beach and Turner, 1958; Barham and Trinder, 1972).

Estimation of serum lipids: Total serum cholesterol, HDL cholesterol and triglycerides were estimated colorimetrically using commercial diagnostic kits. The LDL cholesterol was calculated using Friedewald formula (Rifai *et al*., 1997):

$$LDL = TC\text{-}HDL\text{-}TG/5.0 \ (mg \ dL^{-1})$$

Serum insulin level and IR (β-subunit): Following instructions of manufacturer, serum insulin level and muscle content of IR (β) were assayed using solid phase colorimetric signal Rat/Mouse ELISA kits. The concentration of captured molecules (insulin and insulin receptors) of unknown samples was derived by interpolation from reference standard curve.

Statistical analysis: The collected data was subjected to statistical analysis using one way analysis of variance (ANOVA) followed by fisher's Least Significance Difference (LSD) *post-hoc* test and t-student test, using SPSS 17 for windows (SPSS, Cary, NC, USA). All data were expressed as Mean+Standard Error of Mean (SEM). The statistical significance level was set at $p<0.05$ (Duncan *et al*., 1977).

RESULTS

Determination of acute toxicity: Oral administration of the methanolic extracts of the flowering branch and the bark of *S. campanulata* in doses up to about 5 g kg^{-1} b.wt. produced neither mortality nor signs of morbidity or behavioral changes in any of the treated animal groups under examination.

Alloxan induced diabetes mellitus: Although fasting insulin levels were not significantly different, fasting glucose levels were significantly increased in diabetic groups as compared to normal control group. Blood glucose level was significantly elevated ($p<0.05$) (Table 1).

Effect of *Spathodea campanulata* extracts on blood glucose level: The administration of flowering branch and bark extracts of SC have shown marked decline in the level of blood glucose, but it was statistically significant after 1 h of treatment with bark extract (44.5% decline), which was comparable to the hypoglycemic effect of metformin (56.6% decline) $p<0.05$ (Table 1). When the level of blood glucose was compared in different groups, the only significant reduction of blood glucose was noticed with bark extract and metformin in comparison to diabetic control group $p<0.05$ (Fig. 1 and 2).

Table 1: Effect of bark and flowering branch extracts of *Spathodea campanulata* on the level of blood glucose in mice with alloxan induced diabetes

	Blood glucose (mg dL^{-1})			Decreases (%)	
Groups	G0	G1	G2	1 h	2 h
Normal (-ve) control	80±4	61±4.9	66.5±2.1	23.8*	16.9*
Diabetic (+ve) control	154±16.5[a] (193%)	142±16.4[a] (233%)	134.3±12.9[a] (202%)	7.8	12.8
Diabetic+bark extract	168.8±21.9[a] (211%)	114.7±8.7[b] (188%)	93.7±8.2[a,b] (141%)	32.1*	44.5*
Diabetic+flowering branch extract	213±31.8[a] (266%)	154.8±21.5[a] (254%)	138.2±25.6[a] (208%)	27.3	35.1
Metformin	206±45.4[a] (258%)	89.5±5.2[a,b] (147%)	87±7.7[a,b] (131%)	56.6*	57.8*

Data is expressed as Mean±SEM (% of control) of 6 animals, Statistical significance is considered at p<0.05.G0: Glucose level before administration of drug or saline, G1: Glucose level after 1 h of administration, G2: Glucose level after 2 h of administration, Decrease%: Decrease in blood glucose level relative to G0, expressed in % value, *Significant difference as compared to G0, [a]Significant difference as compared to the -ve control group, [b]Significant difference as compared to the +ve control group

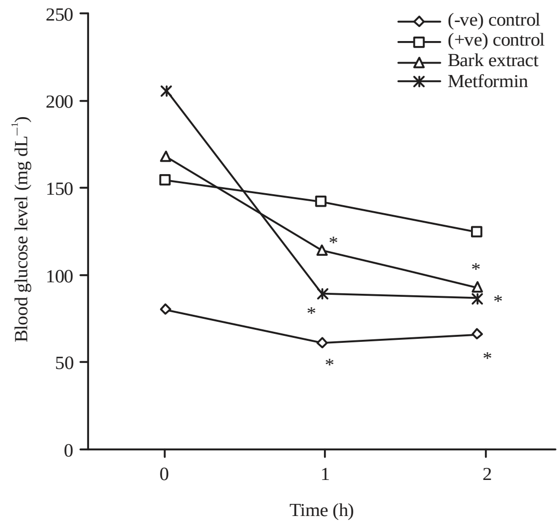

Fig. 1: Effect of bark extract of *Spathodea campanulata* on the level of blood glucose, *Significant as compared to the +ve control group

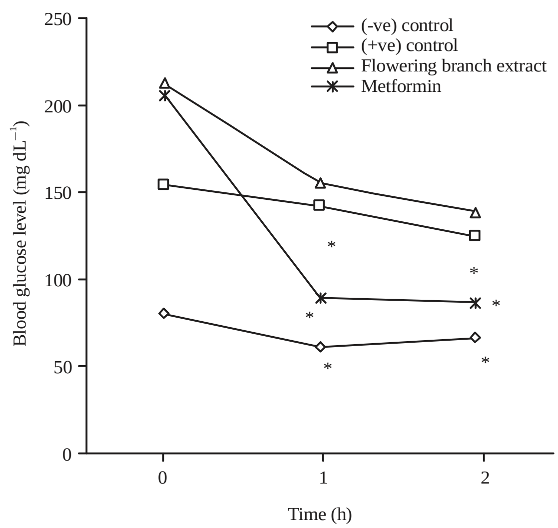

Fig. 2: Effect of flowering branch extract of *Spathodea campanulata* on the level of blood glucose, *Significant as compared to the +ve control group

Effect on serum lipid profile: As presented in Table 2, the only significant change was noticed in triglyceride which was increased 28% of normal in the positive control group, after treatment the only significant decline of triglyceride serum level was noticed in metformin group (26% less than positive control).

Effect on blood insulin: No significant change was noticed in fasting serum insulin level between different groups (Fig. 3).

Effect on insulin receptors in muscle tissue: In alloxan induced DM positive control group marked decline occurred in the level of insulin receptors (60% decline) ($p<0.05$).

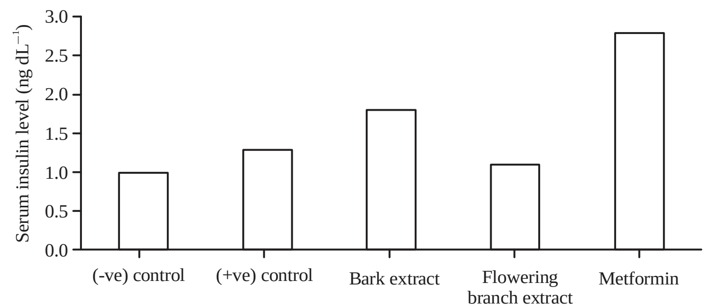

Fig. 3: Effect of bark and flowering branch extracts of *Spathodea campanulata* on serum insulin level, No significant difference could be detected

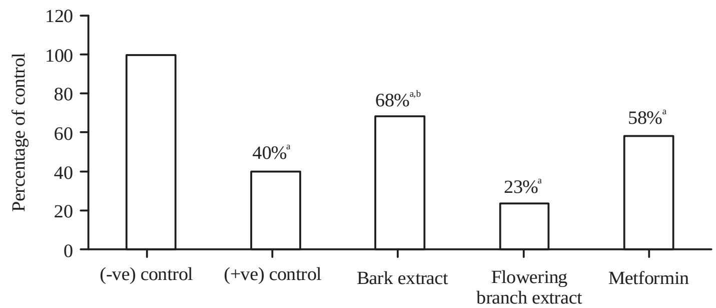

Fig. 4: Effect of bark and flowering branch extracts of *Spathodea campanulata* on insulin receptor (β-subunit), [a]Significant difference as compared to the -ve control group, [b]Significant difference as compared to the +ve control group

Table 2: Effect of bark and flowering branch extracts of *Spathodea* campanulata and metformin treatment on lipid profile in mice with alloxan induced diabetes mellitus

Groups	TC (mg dL^{-1})	TG (mg dL^{-1})	HDL-c (mg dL^{-1})	LDL-c (mg dL^{-1})
Normal control	116.3±13.4	90.0±0.6	32.0±2.1	70.2±12.4
Diabetic control	124.0±12.3	115.6±6.5[a]	32.0±0.6	66.4±13.4
Diabetic+bark extract	106.0±6	104.8±5.4[a]	31.5±1.3	53.8±6.5
Diabetic+flowering branch extract	102.5±5.4	106.5±3.2[a]	33.0±0.8	48.1±5.1
Metformin	109.0±11.1	85.5±3.1[b,c,d]	29.8±0.85	62.1±10.6

Data is expressed as Mean±SEM of 6 animals, Statistical significance is considered at $p<0.05$, [a]Significant difference as compared to the -ve control group, [b]Significant difference as compared to the +ve control group, [c]Significant difference as compared to bark extract treated group, [d]Significant difference as compared to flowering branch extract -treated group, TC: Total cholesterol, TG: Triglyceride cholesterol, HDL-c: High density lipoprotein-cholesterol, LDL-c: Low density lipoprotein cholesterol

After treatment with bark extract of SC the level of insulin receptors has been significantly retrieved (28% increment, $p<0.05$) in comparison to positive control but no noticeable significant elevation was found in other groups (Fig. 4).

DISCUSSION

Diabetes mellitus is a worldwide metabolic disease which has been progressively propagating. Diabetic population is expected to increase about 300 million or more by the year 2025 (BMA, 2004). Egypt is counted as the 9th out of the top 10 countries for number of diabetic people with 7.5 million diabetic at 2013 which is expected to increase to 13.1 million by 2035 (IDF, 2013). Currently, the synthetic agents and insulin are used effectively for the treatment of diabetes, but they have noticeable side effects such as hypoglycemia, drug resistance and weight gain.

(Inzucchi, 2002; Tahrani *et al*., 2010). Accordingly, the interest is growing in the direction of alternative lines of therapy as medicinal plants with anti-hyperglycemic effects (Grover *et al*., 2002). A large number of anti-hyperglycemic plants have been demonstrated and used as traditional remedies for treatment of diabetes (Li *et al*., 2004; Cazarolli *et al*., 2008) but few of these plants have been subjected to scientific investigation.

In the present study we elucidated the hypoglycemic effect of flowering branch (flowers, leaves and stem) and bark extracts of SC on alloxan induced diabetic mice by measuring blood glucose level. There was significant elevation of the blood glucose after induction of diabetes in comparison to normal control group, which was agreeable with previous experimental studies used alloxan (Khan and Schechter, 1991; Katsumata *et al*., 1993; Sun *et al*., 2008).

The elevation of blood glucose was recovered on administration of the bark extract of SC with significant reduction of blood glucose level, the values were decreased by 32.1% after 1 h of treatment (G1) and 44.5% after 2 h (G2), in comparison to 7.8% reduction at G1and 12.8% at G2 in diabetic control ($p<0.05$). This was convenient with findings of a similar previous study of Tanayen *et al*. (2014).

The mechanism of antihyperglycemic action at the cellular level SC bark extract is not yet clear, in trial of its elucidation; we analyzed serum insulin level and the concentration of beta subunit of insulin receptors in skeletal muscle homogenate. Regarding serum insulin level no significant change could be noticed which may be explained by the massive destruction of β-cells of pancreas induced by alloxan (Sharma *et al*., 2003; Ju *et al*., 2008).

Consequently, SC bark extract may not work through stimulating β-cells to release insulin and it could be effective in insulin independent diabetes. However, this remains to be confirmed by further evaluation at a study with longer time span to eliminate the possible stimulatory effect on insulin secretion after partial regeneration of pancreatic cells.

Insulin hormone binds to the special high affinity insulin receptors which have been demonstrated in cells of a large variety of tissues from different animal species including liver, muscles and adipose tissues, then cascade of reactions lead to diverse array of biologic actions (Pari *et al*., 2007). Many studies have shown decreased insulin binding in diabetes mellitus (Olefsky and Kolterman, 1981; Kolterman *et al*., 1981).

In the current study the increased level of insulin receptors which was found in skeletal muscle homogenate it may indicate the potential effect of SC bark extract on insulin sensitivity at tissue level.

The only significant change detected in the level of serum lipids, was the abnormal increase of triacylglycerols in diabetic mice which is mainly due to increase in the mobilization of free fatty acids from adipose tissue, as the lack of insulin has a stimulatory effect on hormone sensitive lipase (Marshall *et al*., 2011). The level of triacylglycerols was significantly decreased in diabetic rats treated with metformin but not with any of SC extracts, which agrees with recorded lipid lowering activity of the metformin through increased fatty acid oxidation (Lord *et al*., 1983a). The lack of significant change of serum cholesterol in all studied groups may owe to the short time of experimental procedure, as cholesterol genesis needs longer time to manifest.

The effect of SC bark extract was comparable to the effect of metformin which reduces hyperglycemia by increasing peripheral glucose uptake and reducing hepatic gluconeogenesis, without increase in plasma insulin concentration (Lord *et al*., 1983b).

Phytochemical analysis of SC revealed the presence of tannins, saponins, anthraquinone glycosides and flavonoids. Saponins and polyphenolic compounds are widely distributed plant metabolites (Coman *et al*., 2012). Saponins have proved to have antihyperglycemic effect by many researchers (Li *et al*., 2002; Oishi *et al*., 2007; Elekofehinti *et al*., 2013; Smith *et al*., 2012). Several phenolic compounds have the potential to serve as a remedy against hyperglycemia-induced diseases (Coman *et al*., 2012; Asgar, 2013). The bioactive antioxidant principles as well as the saponin compounds detected in the *S. campanulata* plant are probably responsible for the antihyperglycemic activity. Relying on our findings, bark extract of *S. campanulata* may has a significant antihyperglycemic activity which could be through induction of insulin receptors, rather than stimulation of pancreatic excretion of insulin. This effect may be attributed to the previously isolated phenolic and saponin compounds. Nevertheless, more detailed pharmacological, toxicological and clinical studies on both the extracts and the isolated compounds should be performed before suggesting its use as herbal medicine.

ACKNOWLEDGMENTS

The authors would like to express gratitude to Dubai Medical College for the financial support through the program of Medical Research Fund (MRF). Moreover, we are grateful for technical support provided by laboratory technicians of Biochemistry and Pharmacognosy Labs.

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