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Antihyperglycemic, antioxidant and hypolipidemic effect of *Capparis aphylla* stem extract in streptozotocin induced diabetic rats

KS Dangi*, SN Mishra
Department of Biosciences, Maharshi Dayanand University, Rohtak, Haryana, India, 124001.

***Corresponding Author:** klion321@gmail.com

Abstract
Capparis aphylla, a xerophytic plant, has been used in traditional Indian medicine for treating diabetes and cardiovascular disease. The methanol extracts and active fraction from stem of *C. aphylla* administered to diabetic rats and blood glucose, antioxidant level, lipid, urea and creatinine level were assayed. The single oral dosing(300mg/kg b.wt) of methanol extract and active fraction(30mg/kg b.wt) from stem part of *C. aphylla* significantly reduced blood glucose levels in normal and diabetic rats ($p<0.01$) during oral glucose tolerance test. While total plasma cholesterol, triglycerides, VLDL and LDL levels were significantly ($p<0.01$) reduced, serum HDL was elevated by 116% after 7 days of active fraction oral administration in diabetic rats. Active fraction treatment lead to significant ($p<0.01$) increase in GSH and decreased MDA level in liver, heart and kidney of diabetic rat. Finding supported the traditional use of *C. aphylla* in the treatment of diabetes and cardiovascular disease.

Keywords: *Capparis aphylla*; Antidiabetic; Hypolipidemic; Antioxidant.

Introduction

The world is facing an explosive increase in the incidence of diabetes mellitus. According to the World Health Organization (WHO) estimates, the number of adults with diabetes in the world will rise from 135 million in 1995 to 300 million in the year 2025 (King et al. 1998). India is the diabetic capital of the world, predicted to have 57.2 million diabetic populations by the year 2025 (Pradeepa et al. 2002). In addition to elevated blood glucose levels, diabetes is generally accompanied with lipid metabolism abnormality communally known as diabetic dyslipidaemia (Goldberg 2001) increase the risk for coronary heart disease (Mironava et al. 2000). In addition, prolonged hyperglycemia causes increased protein glycation, which has been known to be a source of free radicals (Ceriello 1999). Reactive oxygen species (ROS) and the products of advanced glycosylation are significant in the onset and development of complications in chronic diabetes. In addition, it has been equally suggested that cell membrane lipid peroxidation is involved in the etiology of neurodegenerative disorder associated with diabetes; lipid peroxides may cause oxidative damage to the myelin sheath surrounding the nerve. Therefore, oxidative stress may predispose diabetic patients to the development of neuropathy by a mechanism involving increased lipid peroxidation (Dickinson et al. 2002). There are reports indicating that worldwide, over 1200 species of plants have been recorded as traditional medicine for diabetes, although most of these species have not undergone rigorous scientific evaluation (Marles and Farnsworth 1995).

However, the study of plant for hypoglycemic, antioxidant and hypolipidemic activities may give new pharmacological approach in the treatment of diabetes mellitus (Hooft 2003). *C.aphylla* belongs to family Capparidaceae commonly found in the dry regions of India, Pakistan, Egypt and other tropical parts of Africa. The different parts of *C.aphylla* are considered to be analgesic, diaphoretic, alexeteric, laxative, antihelminthes, antibacterial, antifungal, antiviral, and good in cough, asthma, ulcers, boils, vomiting, piles and all inflammations (Mishra et al. 2007; Singh and Mishra 2010, Dangi and Mishra 2010). In view of this, the present study was planned to evaluate the hypoglycemic, antioxidant and hypolipidemic (lipid-lowering) activity of *C. aphylla* stem extract in STZ induced diabetic rats.

Materials and Methods

Plant material

C. aphylla plant, wildly growing in low irrigated area, was collected from village Madina of district Rohtak, Haryana, India. The stem wood (with bark and without bark) of the plant was chopped into small pieces and air-dried over night for extraction purposes.

Extraction
Air-dried stem pieces (without bark and with bark) ground by hammer mill was used for extraction with water, followed by remainder material extraction with appropriate concentration of methanol by hot percolation for 6 h using sohxlet distillation and for conventional extraction stem pieces were heated in earthen pot under high temperature condition for 5 h. The different extracts obtained were then filtered with filter paper (Whatman Intl. Ltd, Maidstone, UK) and concentrated to dryness in oven. The concentrate was then layered on aluminum foil and freeze-dried. Methanol extract without bark was selected for all experimental studies on the basis of confirmation of active fraction by chromatographic screening. The yield range varied, which was obtained (up to 1.5%) with different methanol system. Extract obtained was stored in sterile glass containers at – 4C^{o} until used for study.

Isolation and purification of active fraction
The thin layer chromatography and high performance liquid chromatography was performed to obtain desired compound undergone specific light/temperature and some chemical treatments during different steps of preparation. A variety of methanol systems were tested and tried until 'optimal' conditions (for the separation of the desired constituents) achieved. The active fraction obtained here denoted as test compound/ molecule/ bioactive fraction was further subjected for purification by column chromatography (silica gel 60-100) having 2 cm diameter and 20 cm length. The mobile phases (eluents) were comprised of chloroform and methanol in a particular ratio. The fraction of 4.0 ml (1mL/min) were collected and 20µL of sample was subjected to H.P.L.C. (Waters 600), fitted with auto sampler (Waters 717 plus) and a UV detector (Waters 996), Empower 2 software (Water, USA Ltd.) and C-18 column (300mm×3.9mm i.d. and particle size 10mm).The spectra obtained at 210 nm showed single peak at retention time 2 min was analyzed for hypoglycemic and hypolipidemic properties.

Determination of total phenols and total flavanoids
In order to check the level of contamination of phenols and flavanoids in sample, the phenols was assayed following Gao et al. (2000) and flavanoids quantification as a quercetin equivalent following method of Kosalec et al. (2004). The coefficient of quantification for phenols and flavanoids was r^2 = 0.9958 and 0.9961 respectively.

Experimental animals
Having approval of the Institutional Animal Ethical Committee (ACBT/2009/1043-51), Albino Wister rats, weighing about 150 to 200 g were obtained from Department of Pharmacy, M.D.U. Rohtak. They were housed in polypropylene cages measuring 12"x10"x8 under controlled temperature conditions (25 ± 2 C^{0}) with 12:12 h light and dark cycle. Animals were fed on balanced diet of soaked maize, wheat and chicken beans supplemented with multivitamins and water *ad libitum*.

Diabetes was induced in rats by the intraperitoneal injection of streptozotocin (STZ) at a dose of 55 mg/Kg b.w. dissolved in distilled water to raise the blood glucose concentration level above 250 mg/dL, considered to be diabetic after 7 days. To prevent the hypoglycemia, which occurred during the first 24 h following the STZ administration, 5% glucose solution was orally given to the diabetic rats. In all experiments, rats were fasted for 16 h prior to STZ injection (Aslan et al. 2007).

Experimental design
The rats were administered for 7 days with various extracts along with vehicle to control and experimental rat groups, consisting of 5 rats each:
Group 1: Normal control + vehicle (0.5% carboxy methyl cellulose (CMC).
Group 2: Diabetic control + vehicle (0.5 % CMC).
Group 3: Diabetic given methanol extract (without bark) treatment (300 mg/Kg).
Group 4: Diabetic given glibenclamide (600µg/Kg b. wt).
Group 5: Diabetic administrated with active fraction (30mg/Kg).
Group 6: Diabetic given conventional extract (without bark) treatment (300 mg/Kg).

Blood glucose estimations
The blood samples collected by puncture of tail tip method and blood glucose level was estimated using an electronic glucometer Accu Check (Bayers Diagnostic Pvt. Ltd., Germany) by glucose dehydrogenase method (Owiredu et al. 2009). Blood glucose levels were determined at 10:00 a.m. on 1st, 3rd, 5th and 8th days after the administration of test samples. The effect on body weight and water consumption under treatment was also monitored at the same days.

Oral glucose tolerance test
A methodology of Kato and Miura (1993) was followed for the activity assessment of extracts and compounds. After overnight fasting (16 h) the blood glucose level of rats were determined and then were given test samples orally by using a gastric gavage needle. The rats were loaded orally with 2 g/Kg glucose and simultaneously with test samples. The blood glucose concentrations were determined at 30, 60, 120 and 180 min after the dosing.

Serum lipids profile, creatinine and urea estimation
After 24 h of last administration, the blood serum was collected from heart puncture and centrifuged at 3000 rpm for 10 minutes and clear serum was aspirated, stored frozen and then used for desired analysis. Serum total cholesterol, triglyceride, High-density lipoprotein (HDL), creatinine and urea were measured by enzymatic colorimetric method using Mannheim diagnostic kits. The concentration of low-density lipoprotein (LDL), cholesterol and very low-density lipoprotein (VLDL) was calculated by the formula of Friedwald et al. (1972).

Lipid peroxidation

Tissue preparation: The liver, kidney and heart of each rat were immediately excised and chilled in ice-cold 0.9% NaCl. After washing with 0.9% NaCl, 1.0 g of wet tissue was weighted exactly and homogenized in 9ml of 0.25M sucrose using a Teflon homogenizer to obtain a 10% suspension. The cytosolic compound was obtained by a two-step centrifugation first at 1000×*g* for 10 min and then at 2000×*g* for 30 min at 4 ◦C.

Assay: The method of Ohkawa et al. (1979) as modified by Jamall and Smith (1985) was used to determine lipid peroxidation in tissue samples. A volume of the homogenate (200µl) was transferred to a vial and was mixed with 0.2 ml of 8.1% (w/v) sodium dodecyl sulphate solution, 1.50 ml of a 0.8% (w/v) solution of TBA and the final volume was adjusted to 4.0 ml with distilled water. Each vial was tightly capped and heated in a boiling water bath for 60 min. The vials were then cooled under running water. Equal volumes of tissue blank or test sample and 10% TCA were transferred into a centrifuge tube and centrifuged at 1000×*g* for 10 min. The absorbance of the supernatant compound was measured at 532 nm. 1,1,3,3-Tetraethoxypropan was used as standard for calibration of the curve.

Reduced glutathione (GSH) level

Tissue preparation: Liver (200 mg), heart (400 mg), and kidney (400 mg) were homogenized in 8.0 ml of 0.02m EDTA in an ice bath.

Assay: Estimation of reduced glutathione content was done by using the method of Sedlak and Lindsay (1968). An aliquots of 5.0 ml of the homogenates were mixed in 15.0 ml test tubes with 4.0 ml distilled water and 1.0 ml of 50% trichloroacetic acid (TCA). The tubes were centrifuged for 15 min at approximately 3000×*g.* The supernatant 2.0 ml was mixed with 4.0 ml Tris buffer (0.4 M,pH 8.9), 0.1 ml Ellman's reagent (19.8 mg of 5,5'-dithiobisnitro benzoic acid (DTNB)) added, and the mixture shaked. The absorbance was read within 5 min of the addition of DTNB at 412 nm against a reagent blank with no homogenate. Results were expressed as µ mol GSH/g tissue by using the extinction coefficient (r^2 = 135.4µM^{-1} cm^{-1}).

Determination of acute toxicity
The acute toxicity test (LD_{50}) of the extract was determined according to the test guidelines of O.C.E.D (2002). Experiments were carried out on normal healthy rats. The behaviors of the treated rats appeared normal. No toxic effect was reported up to 5 and 10 times of the dose used here of the methanol extract including prepared biomolecule. There was no death in any of these groups.

Statistical analysis
The values are expressed as mean (n=4) ±SD. The results were analyzed statistically using one way ANOVA to find out the level of significance wherever required. The minimum level of significance was fixed at $p<0.01$.

Results
The results showed that blood glucose level reduced up to 75% as compared to diabetic control after 3 h of single dose of methanol extract (300mg/kg body wt.). Whereas, a dose of 600µg/kg body weight of glibenclamide reduced BGL by 20 and 28 % after 2 and 3 h. The active fraction hypoglycemic potential was found 300% higher than standard drug glibenclamide after 3 h of administration (fig.1). The fasting blood glucose (FBG) level at 1st, 3rd, 5th and 8th days elevated gradually. While the total reduction of blood glucose level by 28%, 64%, 45 % and 75% on 8th day by conventional, methanol extract, glibenclamide and active fraction respectively (fig.2). However, active fraction hypoglycemic

potential was found 129% higher than the standard drug glibenclamide induced level after 7 days of treatment (fig. 2).

The serum TG, TC, VLDL and LDL level increased in diabetic rats, while HDL decreased over normal rats (fig.3). The administration of the methanol extract at desired dose/day for 7 days brought down TG by 34%, VLDL by 45%, LDL by 71%, TC by 37% and subsequently raised serum HDL by 116% ($p< 0.01$). The glibenclamide (600µg/Kg body wt./day) 7 days treatment showed fall 24% in TC, 22 %TG, 33% VLDL, 44% LDL and 55 % rise in HDL. Alternatively, the active fraction (30 mg/kg body wt.) applied at specific dose caused decline in the all lipid components, 44% TC, 43% TG, 71% LDL, 45% VLDL, whereas HDL elevated by 108 % over diabetic control.

Fig. 4 shows decrease in serum urea 30% and 28% while, serum creatinine decreased by 34% and 39% after 7 days of treatment to diabetic rats with methanol extract and glibenclamide respectively over the control diabetic rats. The administration of active fraction for 7 days reduced the levels of urea and creatinine significantly ($p<0.01$).

Whereas, oral administration of methanol extract for 7 days reduced MDA level in liver, heart and kidney tissues to 41%, 31.6% and 34.5% respectively over diabetic control (table 1). The Glibenclamide administration caused 30 %, 26 % and 41% decrease in MDA level in liver, heart and kidney respectively as compared to diabetic control. The administration of active fraction under reference to diabetic rats caused the significant reduction of MDA level in liver (42%), heart (32.6%) and kidney (44.4%) compared with that of other treated groups after 7 days (table 1).

Oral administration of methanol extract for 7 days increased GSH level in liver, heart and kidney tissues to 17%, 10% and 13% respectively as compared to diabetic control. While the GSH level in tissues (liver, heart and kidney) of diabetic control decreased. The standard drug administration leads to increase in GSH level 6%, 7.3% and 13.7% in liver, heart and kidney respectively as compared to diabetic control (table 1). The significant increase of GSH level in liver (54%), heart (17%) and kidney (26%) of diabetic rat was observed after 7 days of oral administration of active fraction (table 1). However, active fraction led to increase in GSH level by 45, 9 and 11%, higher than standard drug glibenclamide treatment in liver, heart and kidney tissue of diabetic rat, respectively.

The total phenols and flavanoids content in methanol extract and in test compound, which revealed that the methanol extract with bark possessed higher concentration of total phenols (fig. 5), whereas extracts without bark contained low level of phenols and flavanoids, whereas, not found in active fraction.

Discussion

In fact, hyperglycemia a primary clinical manifestation of diabetes contributes to diabetic complications by altering vascular matrix and circulating lipoproteins (Chatopadhyay and Bandopadhyay 2005). Shirwaikar et al. (2004) have reported that increase in liver and kidney TBARS is an index of enhanced lipid peroxidation in diabetes, which may be due to enhance production of ROS. Griesmacher et al. (1995) reported that depletion of liver and kidney GSH levels represents enhanced oxidative stress. The decrease in the GSH level also represents increased utilization of GSH under oxidative stress (Anuradha and Selvam 1993). It was found that MDA level was considerably low in rat tissues after 7 days of treatment with active fraction. Therefore, the active fraction caused increase in GSH level, much higher than that standard drug glibenclamide after 7 days of treatment in liver, heart and kidney tissue of diabetic rat respectively (table 1) indicated its cumulative antioxidant potential.

Result showed better response of HDL in presence of the active fraction over glibenclamide suggesting the specificity of compound on lipid metabolism under diabetic condition (fig. 3). The increased level of serum total cholesterol and low-density lipoprotein (LDL) cholesterol have been employed as a primary risk factor for cardiovascular disease (Edijala et al. 2005). In fact, diabetes induced hyperlipidemia is implicated to excess mobilization of fat from the adipose due to the under utilization of glucose (Krishnakumar et al. 2000). However, methanol stem extract of *C. aphylla* reduced total plasma cholesterol and triglycerides levels in diabetic rats. Some studies have reported similar hypolipidemic activity of bark and fruit of *C. aphylla* in streptozotocin-induced diabetic rats (Neelkamal 2009). The observed hypolipidemic effect seems independent of insulin action and may involve the inhibition of the key enzymes on cholesterol and triglycerides synthesis (Zhang et al. 2002) or increasing cholesterol excretion throughout bile acid formation (Kim and Park 2003). However, it is not excluded that the observed hypolipidemic effect is mediated throughout

the control of glycemia since the glycemic control is the major determinant of total cholesterol and triglyceride levels, the evolution of glycemia was parallel to lipidemic parameters in both normal and diabetic rats (Markku 1995).

Since tissue damage associated with hyperglycemia has been related to oxidative stress, we evaluated the nonenzymic antioxidant status of liver, heart and kidney, since these organs have been object of targets in diabetic complications (Ratner 2001). With respect to antioxidants such as GSH observed to be significantly decreased and lipid peroxidation level increased in the liver, heart and kidney of diabetic rat and were restored by *C. aphylla* stem extract treatment (table 1) indicating that besides acting as an antioxidant, it may promote an increase in antioxidant system. It has been observed that reduced lipid peroxidation and improved antioxidant status may be one mechanism by which dietary treatment contributes to the prevention of diabetic complications (Armstrong et al. 1996). Improvement in antioxidant status in diabetes is likely to have a number of benefits. Evidence is accumulating to support the idea that there is a close relationship between the processes of oxidation, glycation, lipids content and antioxidants may specifically inhibit glycation of proteins (Davie et al. 1992). There are reports of presence of a number of alkaloids, terpenoids, glycosides and fatty acids in various parts of *C. aphylla* considered biomodulator (Mishra et al. 2007).

In conclusion, the methanol extract of *C. aphylla* stem exhibits potent antihyperglycemic, antioxidative and lipid lowering activity in STZ diabetic rats. These results support its traditional use in the treatment of diabetes and cardiovascular disease. Finally, the precise mechanism(s) and site(s) of action and the active constituent(s) involved are still to be determined in addition to toxicological studies.

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Table 1: Malondialdehyde (MDA) and reduced glutathione (GSH) level in liver, heart and kidney tissues of diabetic rats after 7 days of treatment with *C. aphylla* stem extract.

Treatment	MDA level (n mole/g tissue)			GSH level (µmole/g tissue)		
	Liver	Heart	Kidney	Liver	Heart	Kidney
Normal control	162.17±0.02	101.59±0.035	167.3±0.05	116.45±0.4	42.87±0.02	83.5±0.2
Diabetic control	283.64±0.25[a]	154.16±0.01	297.74±1[a]	76.28±0.02[a]	36.56±0.03	66.8±0.05
Diabetic +Methanol extract	167.31±.0.22[b]	105.44±0.05	194.86±0.04	89.37±0.01	40.17±0.06	75.37±0.2
Diabetic+ glibenclamide	198.71±0.41	114.09±0.08	174.99±0.038	80.79±0.25	39.26±0.01	75.97±0.1
Diabetic +active fraction	163.45±0.04[b]	103.84±0.06	165.37±0.05[b]	117.35±0.05[b]	42.87±0.1	84.4±0.08[b]

Data are the mean value (n = 4) with ± standard deviation shown.
[a]Significantly different from the normal control group ($p < 0.01$).
[b]Significantly different from the diabetic control group ($p < 0.01$).

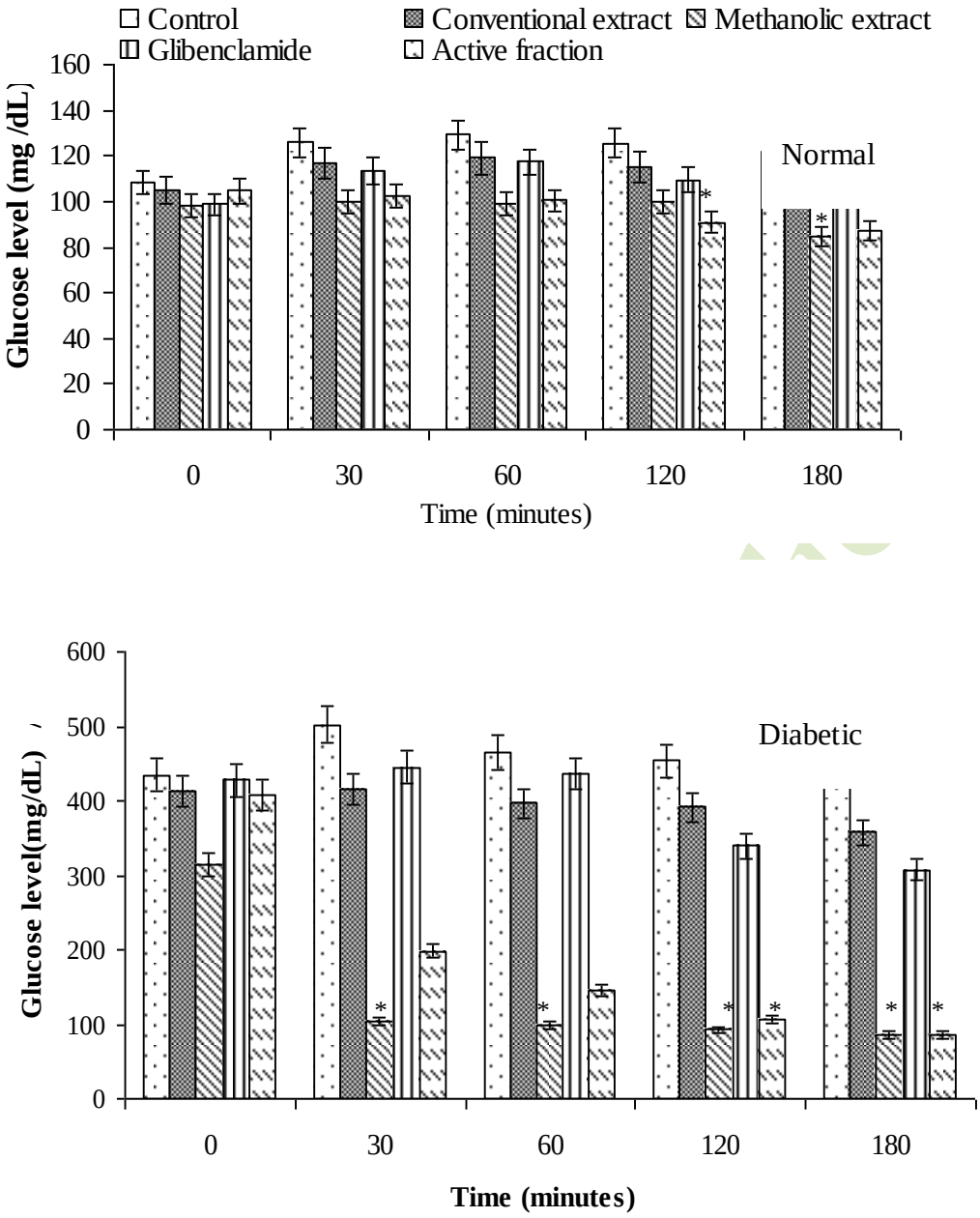

Fig. 1: The effect of various preparations of *C. aphylla* on blood glucose level in normal and diabetic hyperglycaemic rat by oral glucose tolerance test (OGTT). Data are the mean value (n = 4) with ± SD. Data are significant (*) at $p < 0.01$ compared to diabetic control.

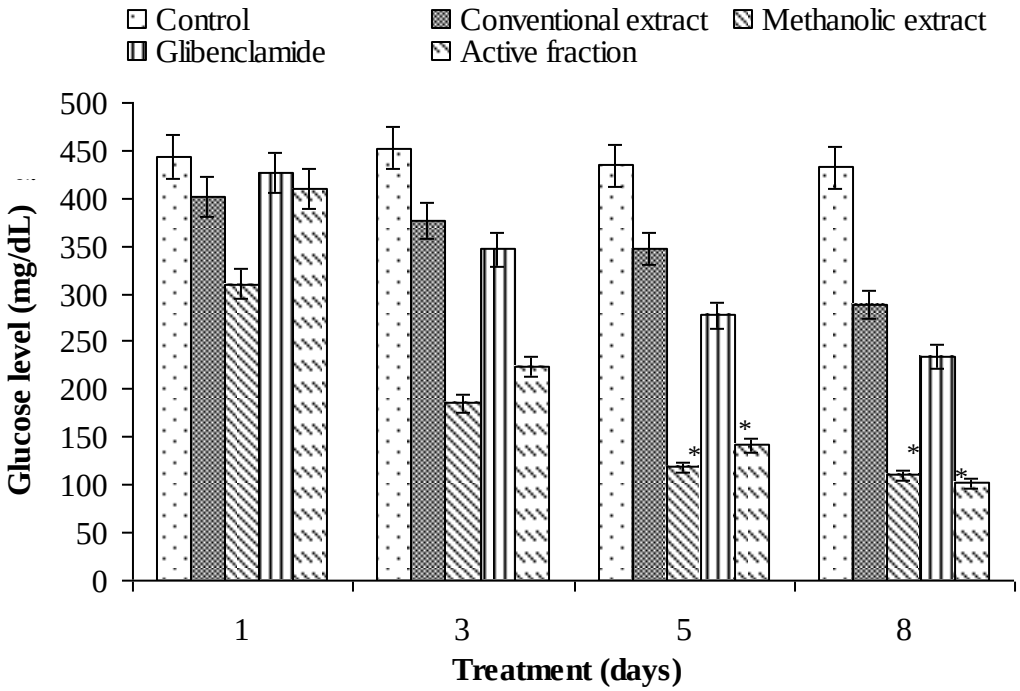

Fig. 2: Hypoglycemic effect of *C. aphylla* stem extracts in diabetic rats after 7 days treatment. Data are the mean value (n = 4) with ± SD. Data are significant (*) at $p < 0.01$ compared to diabetic control.

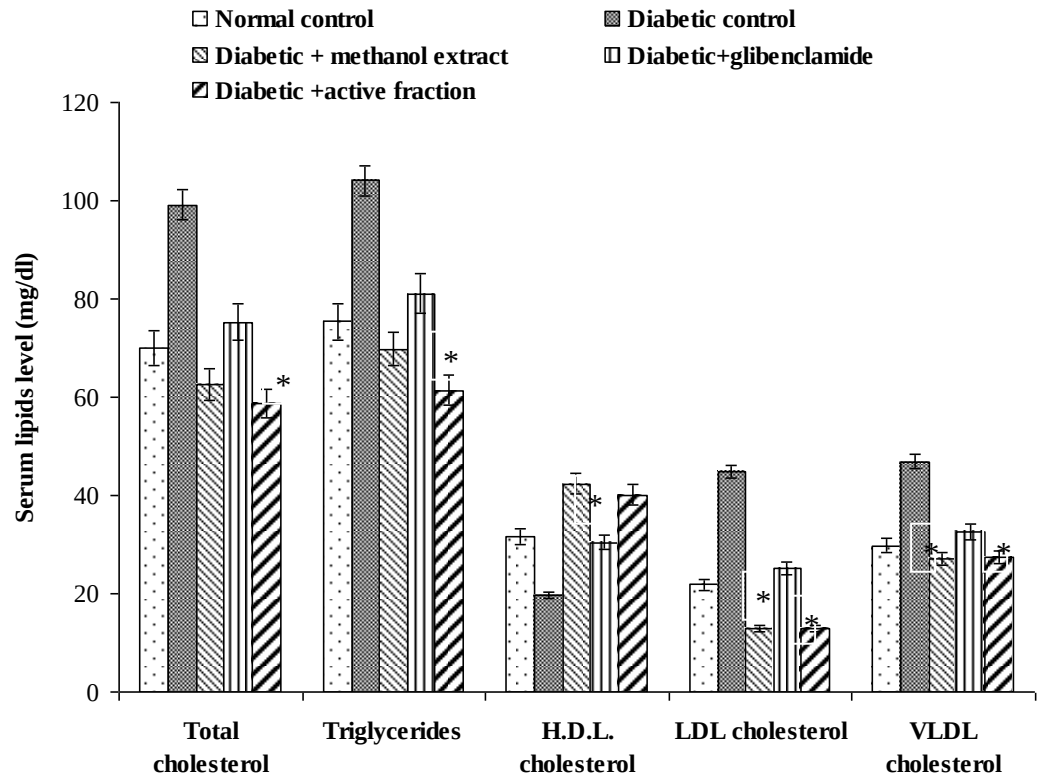

Fig. 3: Effect of *C. aphylla* stem extract on lipid profile in STZ induced diabetic rat after 7 days of administration. Data are the mean value (n = 4) with ± SD. Data are significant (*) at $p < 0.01$ compared to diabetic control. HDL= high density lipoproteins, LDL= low density lipoproteins, VLDL= very low density lipoproteins.

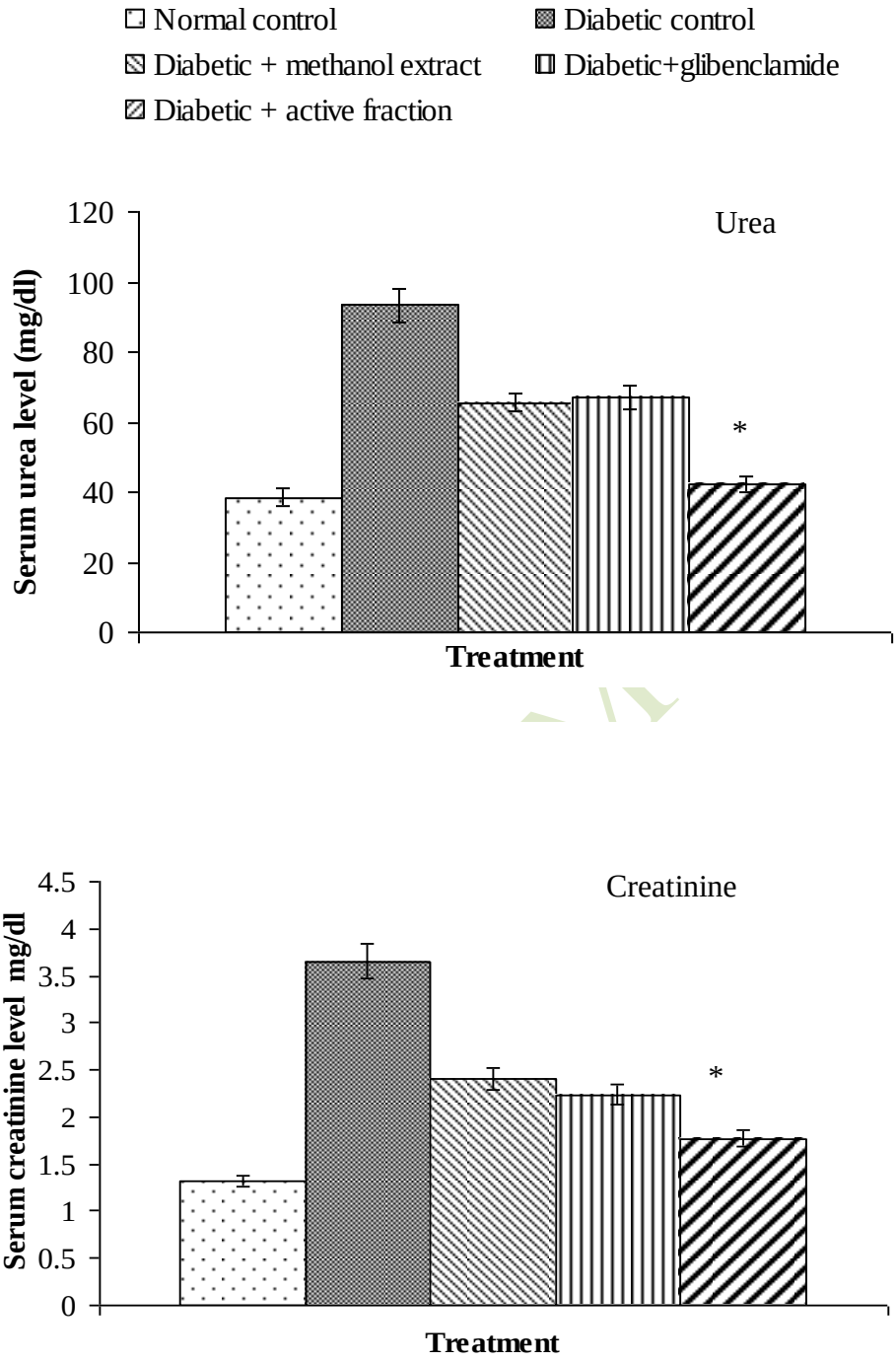

Fig. 4: Effect of methanolic extract and active fraction from stem of *C. aphylla* on serum urea and creatinine level. The estimation was done after 7 days of treatment. Data are the mean value (n = 4) with ± SD. Data are significant (*) at $p < 0.01$ compared to diabetic control.

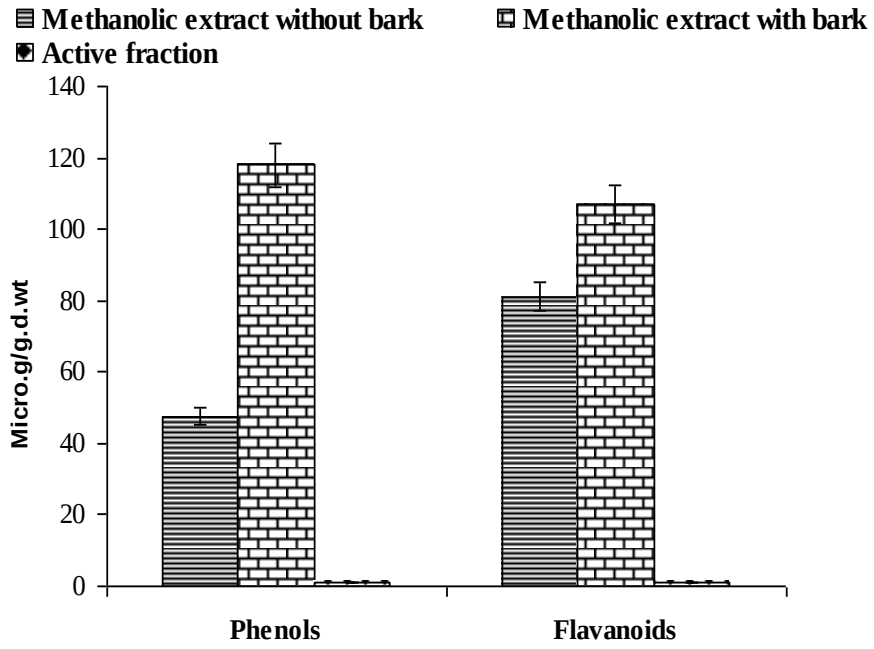

Fig. 5: Total phenols and flavanoids contents of *C. aphylla* stem methanolic extract and active fraction. Data are the mean value (n = 4) with ± SD.