Antioxidative and β cell regeneration effect of *Capparis aphylla* stem extract in streptozotocin induced diabetic rat

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

*Corresponding Author: klion321@gmail.com*

**Abstract**

The aim of the present study was to evaluate the possible effects of *C. aphylla* on pancreatic cells and antioxidant enzymes in experimental streptozotocin induced diabetes in rats. The single oral administration of methanol extract (300mg/kg b.wt) and active fraction (30mg/kg b.wt) from stem part of *C. aphylla* significantly (p<0.01) reduced blood glucose levels after 3h in diabetic rats. The treatment also resulted in a remarkable reduction in glutathione level, superoxide dismutase, catalase and glutathione peroxidase activity in the liver, heart and kidney of diabetic rats. The extract exerted rapid protective effects against lipid peroxidation by scavenging of free radicals thereby reducing the risk of diabetic complications. The results clearly suggested that *C. aphylla* stem extract treatment might effectively normalize the impaired pancreatic cells and antioxidant status in streptozotocin induced diabetes. Therefore, findings supported the traditional use of *C. aphylla* in the treatment of diabetes.

**Keywords:** *Capparis aphylla*; pancreatic β cells; antidiabetic; antioxidant.

**Introduction**

Diabetes mellitus is probably the fastest growing metabolic disorder in the world and it is a major source of morbidity in developed countries. Once regarded as a single disease entity, diabetes is now regarded as a heterogeneous group of diseases characterized by a state of chronic hyperglycemia, which causes a number of secondary complications like cardiovascular, renal, neurological and ocular (Thornally et al. 1996). There is increasing evidence that complications related to diabetes are associated with oxidative stress induced by the generation of free radicals (Garg et al. 1996). Thus, free radicals result in the consumption of antioxidant defenses which may lead to disruption of cellular functions and oxidative damage to membranes and enhance susceptibility to lipid peroxidation. Increased generation of reactive oxygen species (ROS) and lipid peroxidation has been found to be involved in the pathogenesis of many diseases of known and unknown etiology and in the toxic actions of many hypoglycemic drugs (Andallu and Varadacharyulu 2003). Antioxidants thus play an important role to protect the human body against damage caused by reactive oxygen species (Baynes 1991). The endogenous antioxidant enzymes (e.g. SOD, CAT, and GPx) are responsible for the detoxification of deleterious oxygen radicals (Jacob 1995).

In diabetes, oxidative stress has been found to be mainly due to an increased production of oxygen free radicals and a sharp reduction of antioxidant defenses (Oberley 1988). Hence, compounds with both hypoglycemic and antioxidative properties would be useful antidiabetic agents (Baynes 1995). Many plant extracts and plant products known to have significant antioxidant activity which may be an important property of plant medicines associated with the treatment of several ill fated diseases including diabetes (Anjali and Manoj 1995). Thus, herbal plants are considered useful means to prevent and/or ameliorate certain disorders, such as diabetes, atherosclerosis and other complications (Scartezzini and Speroni 2000).

Among these herbal resources, *C. aphylla* which belongs to family Capparidaceae commonly found in the dry regions of India, Pakistan, Egypt and other tropical parts of Africa was selected for the present study. The different parts of *C. aphylla* are considered to have analgesic, diaphoretic, alexeteric, laxative, anti-helminthic properties, good in cough and asthma, ulcers and boils, laxative, piles, and is anti-microbial, anti-diabetic and anti-inflammatory (Mishra et al. 2007, Singh and Mishra 2010, Dangi and Mishra 2010). In view of this, the present study was planned to evaluate the antidiabetic and antioxidant activity of *C. aphylla*.
aphylla stem extract in STZ induced diabetic rats.

Materials and Methods

Plant materials
C. aphylla plant wildly growing in low irrigated area was collected from village Madina of district Rohtak, Haryana, India. The stem wood of the plant was chopped into small pieces and air-dried overnight for extraction.

Extraction
Air-dried stem pieces ground by hammer mill was used for extraction with water, followed by methanol hot percolation for 6 h using Soxhlet distillation. The extracts obtained were then filtered with filter paper (Whatman, UK) and concentrated to dryness in oven. The concentrate was then layered on aluminum foil and freeze-dried. Extract obtained was stored in sterile glass containers at –4°C 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 solvent systems were tested and tried until optimal of chloroform: methanol (17:3) for the separation of the desired constituents. The fraction obtained here 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 (17:3). Fifty fractions of 4.0 ml each (1mL/min) were collected and 20μL of each was subjected to HPLC analysis (Waters 600), fitted with auto sampler (Waters 717 plus) and a UV detector (Waters 996), Empower 2 software (Water, USA) 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 termed here as active fraction and subjected to antioxidative and antidiabetic properties.

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 (12”x10”x8) under controlled temperature conditions (25 ± 2°C) 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 diabetic after 7 days. 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 test samples along with vehicle to control and experimental rat groups consisted of 5 rats each as follows:

Group 1: Normal control + vehicle (0.5% carboxymethylcellulose i.e. CMC).
Group 2: Diabetic control + vehicle (0.5 % CMC).
Group 3: Diabetic given methanol extract treatment (300 mg/Kg b wt).
Group 4: Diabetic given glibenclamide (600µg/Kg b.wt).
Group 5: Diabetic administered with active fraction (30mg/Kg b.wt).

Oral glucose tolerance test
A methodology of Kato and Miura (1993) was followed for the activity assessment of test samples. 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. Blood glucose level was estimated by electronic glucometer AccuCheck (Bayers, Germany) using glucose dehydrogenase method by Owiredu et al. (2009). The blood glucose concentrations were determined at 30, 60, 120 and 180 min after the dosing.

Lipid peroxidation

a) 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 and homogenized in 9ml of 0.25M sucrose using a Teflon homogenizer. 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.

b) 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) SDS 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 volume test sample and 10% TCA were transferred into a centrifuge tube and centrifuged at 1000×g for 5 minutes. The absorbance was taken within 5 min of the addition of DTNB at 412 nm against a reagent blank with no homogenate. Results were expressed as μmol GSH/g tissue by using the extinction coefficient (ε = 135.4 μM⁻¹ cm⁻¹).

Reduced glutathione (GSH) level

a) 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.

b) Assay: Estimation of reduced glutathione content was done by using the method of Sedlak and Lindsay (1968). An aliquot of 5.0 ml of the homogenates was 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'-dithiobisnitrobenzoic acid, DTNB) added and shaken. The absorbance was taken within 5 min of the addition of DTNB at 412 nm against a reagent blank with no homogenate. Results were expressed as μmol GSH/g tissue by using the extinction coefficient (ε = 135.4 μM⁻¹ cm⁻¹).

Superoxide Dismutase (SOD)
The rat liver, heart and kidney (200 mg) were homogenized (1:10 w/v) in 0.1M sodium phosphate buffer (pH 7.4) and centrifuged at 5000 rpm for 10 minutes at 4 °C. The supernatant was used for estimation. Superoxide Dismutase activity was assayed by the method described by Kakkar et al. (1984) using nitroblue tetrazolium as the indicator reagent. The reagents contained sodium pyrophosphate buffer 1.2 ml (0.52 M, pH 8.3), 0.1 ml phenazine methosulphate (186 μM), 0.3 ml nitro blue tetrazolium (300 μM), 0.2 ml NADH (780 μM) and 0.1 ml of supernatant. The mixture was then incubated for 90 min at 30°C then 4 ml of n-butanol and 1 ml of acetic acid were added. The mixture was shaken vigorously followed by centrifugation at 4000 rpm for 10 min and absorbance at 560 nm.

Catalase

a) Preparation of reagents: Solution 1. Phosphate buffer (0.15 M, pH 7.0).
Solution 2. H₂O₂-phosphate buffer (0.15 M, pH 7.0) diluted by 0.16 ml hydrogen peroxide (30% w/v) to 100 ml with buffer (solution 1).

b) Assay: Catalase activity was assayed by method of Luck (1971) with some modifications. Homogenized rat liver, heart and kidney tissue (600 mg) in 10 ml buffer solution phosphate buffer (solution 1, diluted 1:10) at 4°C and centrifuged at 5000 rpm for 5 minutes. Supernatant was used for assay and 100μl of sample was added to 3 ml of H₂O₂ buffer (solution 2) and optical density was taken immediately at λ 240 nm. The decrease in optical density was observed for 3 minutes at interval of 30 s and activity was expressed in μmole of H₂O₂ consumed/minute/g tissue.

Glutathione peroxidase (GPx)

Rat tissues (1g) were homogenized using glass homogenizer in 0.4 M Tris hydrochloric acid buffer (10ml) containing pH 7.0 and 0.2ml of tissue homogenate was used for experiment. GPx activity was measured by the method described by Rotruck et al. (1973). The reaction mixture contained 0.2 ml of 0.4 M Tris-HCl buffer pH 7.0, 0.1 ml of 10 mM sodium azide, 0.2 ml of tissue homogenate (homogenized in 0.4 M, Tris-HCl buffer, pH 7.0), 0.2 ml glutathione, 0.1 ml of 0.2 mM hydrogen peroxide and incubated at 37°C for 10 min. The reaction was arrested by 0.4 ml of 10% TCA, and centrifuged. Supernatant was assayed for glutathione content by using 50μl Ellman’s reagent (19.8 mg of 5,5'-dithiobisnitrobenzoic acid (DTNB) in 100 ml of 0.1% sodium nitrate). Absorbance was measured at 412 nm and activity expressed in μmole of GSSG formed/minute at 30°C and pH 7 by using extinction coefficient of 9.6mM⁻¹ cm⁻¹.

Histological studies

Whole pancreas from each animal was removed after sacrificing, collected in 10% formalin.
solution and immediately processed by the paraffin technique. Sections of 5μm thickness were cut and stained by hematoxylin and eosin (H & E) for histological examination. Photomicrograph were taken under binocular light microscope at 100 x optical zoom by using camera of 10 mega pixels with 4x optical zoom (Nagappa et al. 2003).

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 active fraction. 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
Figure 1 shows that blood glucose level was reduced significantly (p<0.01) as compared to diabetic control after 3 h of single dose of methanol extract (300mg/kg body wt.) and active fraction (30mg/kg body wt.). MDA level in liver, heart and kidney tissues was decreased by 41%, 31.6% and 34.5% respectively over diabetic control by oral administration of methanol extract for 7 days. 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 glibenclamide treated groups after 7 days (fig. 2).

The significant (p<0.01) 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 (fig. 3). 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 rats respectively. The decrease in SOD activity in liver, heart and kidney observed in tissues of diabetic rat was enhanced in all tissues approximately 50% after 7 days treatment of methanol extract as compared to diabetic control. The active fraction (30mg/kg b.wt) led to increase in SOD activity by 39.5, 4 and 12.2% higher than standard drug glibenclamide after 7 days of treatment of diabetic rat (fig. 4). Catalase (CAT) activity was remarkably increased in liver (100%), heart (104%) and kidney (69%) after 7 days treatment of active fraction (30mg/kg b. wt) compared to glibenclamide treated group (fig. 5). Methanol extract treatment enhanced the GPx activities in liver, heart and kidney tissues by 51.5%, 16.9% and 55.5% respectively and significantly (p<0.01) increased in active fraction treated diabetic rat tissues (liver, heart, kidney) compared to diabetic control (fig.6).

Histological studies revealed that the islets of Langerhans of normal control rat were evenly scattered in the pancreatic tissue with well delineated islets cells which were completely enmeshed in the surrounding acinar cells as compared to diabetic control pancreas acinar cells which were compactly arranged, with negligible intercellular space (fig. 7a,b,d). Pancreatic islets of diabetic control rats revealed hyperplasia and acinar cells with disarray (fig. 7c). Photomicrographs (fig. 7e) showed the significant restoration of normal cellular population and enlarged size of cells of islets of Langerhans without hyperplasia by methanol extract treatment indicated the β cell proliferation effect of C. aphylla. Pancreatic acinar cells of methanol extract treated rat showed without architectural disarray as compared to induced diabetic rats (fig. 7f).

Discussion
The present study was conducted to evaluate the beneficial effects of C. aphylla stem extracts on antioxidant status in STZ induced diabetic rats. It has been stated that STZ diabetic animals may exhibit most of the diabetic complications mediated through oxidative stress (Ozturia et al. 1996). Studies also suggest free radical involvement in pancreatic cell destruction (Tomlinson et al. 1992). Glibenclamide is often used as an insulin stimulant in many studies and used as a standard antidiabetic drug in STZ-induced moderate diabetes to compare the antidiabetic properties of a variety of hypoglycemic compounds (Andrade-Cetto et al. 2000). The possible mechanism by which C. aphylla brings about decrease in blood glucose may be of the insulin effect by increasing either the pancreatic secretion of insulin from β cells of islets of langerhans or its responsiveness (Padmini and Chakrabati 1982). Hyperglycemia results in free radical formation through various biochemical reactions. Free radicals may also
be formed via the auto-oxidation of unsaturated lipids in plasma and membrane lipids. The free radical produced may react with polyunsaturated fatty acids in cell membranes leading to lipid peroxidation and results in increased production of free radicals (Lery et al. 1999). The increased lipid peroxidation in the diabetic animals may be due to the observed remarkable increase in the concentration of TBARS and hydroperoxides (lipid peroxidative markers) in the liver and kidney of diabetic rats (Stanely et al. 2001).

Nakakimura and Mizuno (1980) have reported that the concentration of lipid peroxides increases in the kidney of diabetic rats. In the present study, Lipid peroxidation levels in liver, heart and kidney were significantly lower in the methanolic extract and active fraction treated group compared to the diabetic control group (fig. 2). The above result suggests that the methanolic extract may exert antioxidant activities and protect the tissues from lipid peroxidation. Loven et al. (1986) suggested that the decrease in tissue GSH could be the result of decreased synthesis or increased degradation of GSH by oxidative stress in diabetes. In the present study, the elevation of GSH levels in liver, heart and kidney was observed in C. aphylla extract (methanolic extract and active fraction) and glibenclamide treated diabetic rats. This indicates that the C. aphylla stem extract and glibenclamide can either increase the biosynthesis of GSH or reduce the oxidative stress leading to less degradation of GSH, or have both effects. SOD has been postulated as one of the most important enzymes in the enzymatic antioxidant defense system which catalyses the dismutation of superoxide radicals to produce H₂O₂ and molecular oxygen hence diminishing the toxic effects caused by their radical. The observed decrease in SOD activity could result from inactivation by H₂O₂ or by glycation of enzymes (Sozmen et al. 2001). Thus, the increase in SOD activity may indirectly play an important role in the activity of catalase. Reduced activities of SOD and CAT in the liver and kidney have been observed during diabetes and this may result in a number of deleterious effects due to the accumulation of superoxide radicals and hydrogen peroxides (Wohaieb and Godin 1987). Whereas, the extract treated groups showed a significant increase in SOD and CAT activities in all tissues (liver, heart and kidney) of the diabetic rats. This means that the extracts can reduce the potential glycation of enzymes or they may reduce reactive oxygen free radicals and improve the activities of antioxidant enzymes. Glutathione peroxidase (GPx), an enzyme with selenium, works together with glutathione in the decomposition of H₂O₂ or other organic hydroperoxides to non-toxic products at the expense of reduced glutathione (Bruce et al. 1982). Reduced activities of GPx in the liver, heart and kidney have been observed during diabetes and this may result in a number of deleterious effects due to the accumulation of toxic products. In this context, other workers also reported a decrease in the activity of these antioxidant enzymes (SOD, CAT and GPx) in the liver, heart and kidneys of diabetic rats (Anuradha and Selvam 1993). Administration of C. aphylla extracts and glibenclamide increased the activities of GPx in the tissues of diabetic rats.

In conclusion, the present study showed that C. aphylla stem possesses potent β-cell regeneration, hypoglycemic and antioxidant activity, which may be directly or indirectly responsible for its antidiabetic property. Further studies are in progress to identify the active components in C. aphylla and their role in controlling diabetes.

Acknowledgement

We are grateful to University Grants Commission (UGC), New Delhi, India for providing Meritorious Research Fellowship to K.S. Dangi.

References


Sozmen BY, Sozmen B, Delen Y, Onat T, 2001. Catalase/superoxide dismutase (SOD) and catalase/paraoxonase (PON) ratios may implicate poor glycemic control. Arabian Medical Research, 32: 283.


Wohaieb SA, Godin DV, 1987. Alterations in free radical tissue - defense mechanisms in streptozotocin...

Figure 1: Glucose tolerance curves of normoglycemic and streptozotocin induced diabetic rats with oral administration of C. aphylla stem extract. Data are the mean value (n = 4) with ± SD. Data are significant (*) at p < 0.01 compared to diabetic control.

Figure 2: Change in Malondialdehyde (MDA) level in liver, heart and kidney tissues of diabetic rats after 7 days of treatment with C. aphylla stem extract. Data are the mean value (n = 4) with ± SD. Data are significant (*) at p < 0.01 compared to diabetic control. Unit = nmole.
Figure 3: Change in Reduced Glutathione (GSH) level in liver, heart and kidney tissues of diabetic rats after 7 days of treatment with *C. aphylla* stem extract. Data are the mean value (n = 4) with ± SD. Data are significant (*) at p < 0.01 compared to diabetic control. Unit = µmol.

Figure 4: Change in superoxide dismutase (SOD) activity in liver, heart and kidney tissues of diabetic rats after 7 days of treatment with *C. aphylla* stem extract. Data are the mean value (n = 4) with ± SD. Data are significant (*) at p < 0.01 compared to diabetic control. Unit = 50% inhibition of NBT reduction/minute.
Figure 5: Change in catalase (CAT) activity in liver, heart and kidney tissues of diabetic rats after 7 days of treatment with *C. aphylla* stem extract. Data are the mean value (n = 4) with ± SD. Data are significant (*) at p < 0.01 compared to diabetic control. Unit = μ mole of H₂O₂ consumed/minute.

Figure 6: Change in glutathione peroxidase (GPx) activity in liver, heart and kidney tissues of diabetic rats after 7 days of treatment with *C. aphylla* stem extract. Data are the mean value (n = 4) with ± SD. Data are significant (*) at p < 0.01 compared to diabetic control. Unit = μg of GSH consumed/minute.
Figure 7: Photomicrograph of rat pancreas treated with *C. aphylla* stem methanolic extract (300mg/kg) stained with hematoxylin and eosin. 

- a = islets of Langerhans of normal control;
- b = acinar cells with normal intercellular space of normal control;
- c = islets of Langerhans of diabetic control with hyperplasia;
- d = acinar cells of pancreas of diabetic control without intercellular space;
- e = normal appearance of islets of Langerhans without hyperplasia of diabetic + treatment;
- f = acinar cells with normal appearance of intercellular space of pancreas of diabetic + treatment. 400x.