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In vitro and *In vivo* Antioxidant and Antidiabetic Efficacy of *Cassia auriculata* L. Flowers

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Abstract: The present study was designed to assess *in vitro* and *in vivo* antioxidant and antidiabetic efficiency of *Cassia auriculata* L. flowers and its phytochemical analysis. *In vitro* antioxidant competence was screened for scavenging DPPH, superoxide, nitric oxide, hydroxyl, H_2O_2 and lipid peroxides in addition to reducing power and metal ion chelating capabilities. The inhibitory effect on carbohydrate digestive enzymes α-amylase and α-glucosidase was studied with reference to acarbose. *In vivo*, parameters such as fasting blood glucose, glycosylated hemoglobin, plasma insulin, reduced glutathione and activities of antioxidant enzymes were studied with reference to glibenclamide. The methanolic extract showed higher antilipid peroxide and DPPH radical scavenging ability. The potent inhibitory effect was observed on activities of α-amylase and α-glucosidase. The marked decrease in the glucose level in the extract treated streptozotocin induced diabetic rats was due to elevated levels of insulin. The increased activity of antioxidant enzymes and glutathione, accounts the antiradical activity of the extract. RP-HPLC analysis indicated the presence of mixed catechins, caffeine and quercetin. These bioactive constituents validate antioxidative ability and provides scientific basis for the usage of *Cassia auriculata* L. flowers in ayurvedic formulations in the treatment of diabetes and other related inflammatory diseases.

Key words: Free Radicals · Oxidative Stress · Diabetes Mellitus · Phytoconstituents

INTRODUCTION

Free radicals are highly reactive chemical species regularly produced in the human system by normal biological reactions and also by various exogenous factors [1]. Some of the radicals are superoxide ($O_2^{\cdot-}$), nitric oxide ($NO^{\cdot}$), peroxyl ($ROO^{\cdot}$) and hydroxyl (OH^{*}). Accordingly, their effects on the organism are checked by defense system that includes antioxidant enzymes superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione-S-transferase (GST) and glutathione reductase (GR) and molecules like vit-E, vit-C, flavonoids etc., Low levels of antioxidants or inhibition of antioxidant enzymes may cause oxidative stress and damage cells [2]. Increased oxidative stress leads to diabetes mellitus, cancer, myocardial infraction, atherosclerosis, neurodegenerative diseases and aging [3].

Diabetes mellitus is a metabolic disorder characterized by chronic hyperglycemia owing to defects in secretion or action of insulin or both. Prolonged glucose toxicity may lead to complications such as retinal damage, chronic renal failure, cardiovascular and neurodegenarative diseases. The degradation of starch and oligosaccharides to glucose by α-amylase and α-glucosidase enzymes if suppressed would in turn delay the glucose absorption in the intestine. Eventually, the elevated postprandial blood sugar is controlled [4]. Although numerous drugs are in use to control diabetes and oxidative stress, many are found to have serious and adverse drug reactions [5].

In the recent past, herbal medicines have gained an unprecedented importance in view of their hypoglycemic applications and safety. The apparent management of diabetes and related complications by bioactive constituents having antioxidant properties have been reported [6]. Thus there has been greater importance to

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use natural antioxidants as remedial approach to manage the same. Consequently, the promotion of medicinal plants as therapeutic agents requires systematic investigation in evaluating their efficacy using various *in vitro* systems, besides pharmacological and toxicological properties involving *in vivo* studies [7].

Cassia auriculata L. species with vivid attractive yellow flowers are distributed in dry and arid parts of India and Asia. Different parts of the plant has been reported to possess ethnobotanical use for treating skin disorder, burns, body odour, fever, urinary system infections, conjunctivitis, gonorrhea, gout, rheumatism and constipation [8]. Also, use of flowers as the principle constituent of Avarai Panchaga choornam, Kalpa drug and Dia herbal tea have shown remedial effect on diabetes [9]. Different solvent extracts of flowers have shown anti-inflammatory, antimicrobial, antioxidant and antidiabetic properties [10-12]. Use of flowers in traditional medicine and as hypoglycemic means by way of infusion prompted us to investigate in detail, the antioxidant effectiveness and the antidiabetic efficiency by evaluating the regulatory effect on α-amylase and α-glucosidase activity, reducing power, chelating ability on ferrous ions, anti-lipid peroxidative and free radical scavenging capabilities *in vitro* conditions. The effects on activities of stress releasing enzymes, lipid peroxidation, glutathione content, levels of fasting blood glucose, glycosylated hemoglobin and plasma insulin were determined *in vivo*. Besides phytochemical analysis and quantification of total phenolics and flavonoids, extract was analyzed for major bioactive principles using RP-HPLC technique.

MATERIALS AND METHODS

Extraction and Phytochemical Analysis: The fresh plants collected in the month of June, 2010 from the Western Ghat region of South India, were identified and authenticated at National Ayruveda Dietetics Research Institute (NADRI), Bangalore, India (RRCBI-Acc No. 4925). The flowers were separated, washed thoroughly with milli-Q water and shade dried for 5 days. 100 g of powdered flowers was subjected to soxhlet extraction with methanol (800ml) at 40°C for 8 hours. The extract thus obtained was concentrated using rotary vacuum evaporator under reduced pressure and yield obtained was 27.8%. The extract was analyzed for alkaloids, saponins, tannins, flavonoids, glycosides and terpenoids as per the standard procedures [13].

Quantitative Estimation of Total Phenolics and Flavonoids: Total phenolic content was determined using Folin's Ciocalteau reagent employing Bray and Thorpe method [14] and expressed as mg of gallic acid equivalents/g of extract. The flavonoid content was quantified following the method of Chang *et al*., [15] and expressed in terms of mg of quercetin equivalents/g of extract.

***In vitro* Assays:** The methanolic extract was subjected to range of assays and absorbance was noted using double beam Shimadzu UV-Visible spectrophotometer. Percentage inhibition in each assay was calculated using the formula

$$\text{Inhibition (\%)} = \frac{\text{(Absorbance of control-Absorbance of test sample)}}{\text{Absorbance of control}} \times 100$$

Anti-lipid peroxidative assay was performed using the method described by Prashanth *et al*. [16] total antioxidant activity by Cotelle *et al*. [17] method, Fe^{3+}-reducing power following Oyaizu [18] method, ferrous ion chelating potential by Yamaguchi *et al*. [19] protocol, superoxide anion radical scavenging assayed following Nishmiki *et al*. [20], Nitric oxide radicals were assayed employing Green *et al*. [21] protocol, hydrogen peroxide scavenging ability was determined according to the method described by Gulcin *et al*. [22] and hydroxyl radical scavenging activity was estimated according to Kunchandy and Rao [23]. All assays were performed with reference standards.

The effect of flower extract on activity of α-amylase was assayed using the chromogenic method [24, 25] and α-glucosidase by glucose oxidase peroxidase method [26]. Acarbose was used as standard reference in both the assays.

***In vivo* Assays**

Experimental Design: Male wistar albino rats weighing 150-200□g were selected for the study and were housed in acrylic cages in standard environmental conditions (temperature (22 ± 2°C) and humidity (45 ± 5°C)) with 12 hours day: 12 hours night cycle. They had free access to standard dry pellet diet (Hindustan Lever, Kolkata, India) and water *ad libitum*. All experiments were carried out as per the guidelines of the Institutional Animal Ethical Committee (1164/ac/08/CPCSEA). Five groups of 6 rats in each were maintained for the experiment.

Group 1 : Normal control rats
Group 2 : Diabetic control rats
Group 3 : Diabetic rats treated with *C. auriculata* flower extract (150mg/kg body weight)
Group 4 : Diabetic rats treated with *C. auriculata* flower extract (250mg/kg body weight)
Group 5 : Diabetic rats treated with glibenclamide (750µg/kg body weight)

Diabetes was induced in rats with a single intraperitoneal injection of freshly prepared solution of streptozotocin (55 mg/kg b. w) dissolved in 0.1M citrate buffer (pH 4.5) [27]. After 48 hours of streptozotocin administration, rats with blood glucose levels between 250-300 mg/dl were selected for the studies and treated with doses of flower extract and glibenclamide for 30 days.

Biochemical Analysis: The control and the treated rats were starved over night on the 30th day and sacrificed by decapitation. Blood was collected in tubes containing potassium oxalate and sodium fluoride. Plasma was separated by centrifugation at 2000 rpm for 10 min. Fasting blood glucose was estimated by Dubowski method [28], glycosylated hemoglobin by Parker *et al*. method [29] and plasma insulin levels was assayed employing Enzyme linked immunosorbant assay (ELISA) kit (Roche Diagnostics, Germany).

Liver homogenates were assayed for lipid peroxidation following the method of Okhawa *et al*. [30], Catalase by Beer and Sizer [31], Superoxide dismutase by Misra and Fridovich [32], glutathione peroxidase by Wendel [33] protocol, reduced glutathione by Tietze *et al*. [34] and glutathione-S-transferase activity following Habig *et al*. [35].

HPLC Analysis of Bioactive Constituents: Determination of bioactive constituents was carried out using a Shimadzu HPLC system with RP C_{18} column (250 X 4.6 mm i.d., 5 µm) with an automated gradient controller, an in-line solvent degasser, temperature control module and photodiode array (PAD) detector. The identification was established by comparing retention time and spectra of the peaks in flower extract with those obtained with the standards.

Extract was analysed for mixed polyphenols according to ISO 14502-2 method [36] and following Xiao-qing chen and Jian-bo xiaoan [37] protocol, analyzed for quercetin.

Statistical Analysis: The experimental results were expressed as mean± SD of three parallel measurements. IC_{50} values were calculated by regression analysis quoting correlation coefficient. Data was evaluated by one way analysis of variance (ANOVA) followed by Dunnett's method of multiple comparisons using Graphpad Instat 3.0 software. $p<0.005$, $p<0.01$ and $p<0.05$ was considered to indicate the statistical significance.

RESULTS

The preliminary phytochemical analysis showed the presence of alkaloids, flavonoids, tannins, saponins and glycosides. The quantitative determination of total phenolics carried out with respect to standard curve of gallic acid (r^2= 0.994) showed 115.83 mg GAE/g of extract. Using quercetin standard curve (r^2= 0.9934), concentration of flavonoids observed was 114.3 mg QE/g of extract.

***In vitro* Studies:** The interface of flower extract with homogenate suppressed lipid peroxidation to a greater extent and was evident in all concentrations and showed correlation to doses. The antilipid peroxidative activity was more pronounced in flower extract with 98.84% inhibition compared to 31.58% of standard BHT at 100 µg/ml concentration. At the same concentration, 67.35% DPPH radicals were scavenged in the assay mixture by the extract compared to 82.3% by ascorbic acid. Initially, the extract (20 µg/ml) exerted 44.71% quenching ability compared to 41.28% of ascorbic acid.

The reducing potential of floral extract and ascorbic acid enhanced with increasing concentrations (Figure 1). Maximum absorbance of 1.76 nm was recorded for flowers (r^2=0.988) while ascorbic acid expressed 1.01 nm (r^2=0.994) absorbance at 100 µg/ml concentration.

Flower extract showed reasonably fair binding effect of 68.1% at 1000 µg/ml. EDTA showed superior binding power of 86.54% at 100 µg/ml. The superoxide radicals were effectively cleared by both extract and standard. Also it is noteworthy to mention that the inhibitory effect of flowers was 15% higher than ascorbic acid at 1000 µg/ml concentration. The floral extract well restrained nitroprusside from generating nitric oxide radicals and showed maximum inhibition of 50.2% indicating greater regulatory effect compared to gallic acid (40.97%). The inhibitory effect was directly proportional to concentration and was statistically significant ($p < 0.005$).

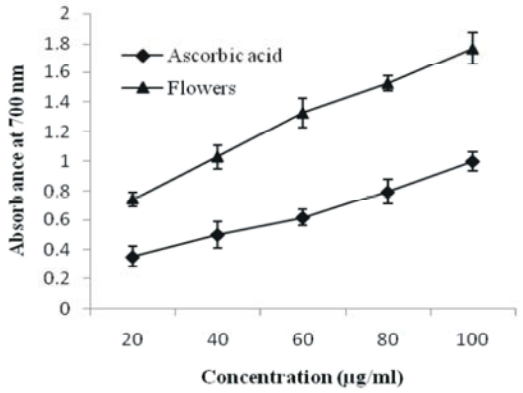

Fig. 1: Reductive capabilities of C.auriculata L. flower extract and standard ascorbic acid. Values are expressed as mean± SD of three parallel measurements.

Although H_2O_2 scavenging efficacy at 230 nm depicted dose dependent action, flower extract could not reach 50% inhibition at 20-100 µg/ml. However, ascorbic acid showed potential inhibitory activity exhibiting 68.74% scavenging effect. Flowers showed considerable hydroxyl radical scavenging effect and exhibited 42.84% inhibition while mannitol showed greater inhibitory activity of 68.01% at 100 µg/ml. IC_{50} values of flower extract and standards for various *in vitro* assays performed are summarized in Table 1.

The maximum inhibition of α-amylase activity by the flower extract was 78.49% whereas acarbose showed only 62.61% inhibition at 500 µg/ml. At the same concentration, α-glucosidase catalytic activity was regulated to 77.35% while standard acarbose inhibited the enzyme activity to 75.45%. IC_{50} values of the flower extract and standard acarbose on activity of carbohydrate metabolizing enzymes are shown in Table 2.

***In vivo* Studies:** The blood glucose levels of the rats in all the groups were found to be normal prior to streptozotocin administration. After 48 hours, blood glucose levels were significantly increased in experimental animals as compared to control sets.

Table 1: IC_{50} values of methanolic extract of *Cassia auriculata* L. flowers and standards for the antioxidant assays performed. Values are expressed as mean± SD of triplicates

Activity	Sample used	IC_{50} values (µg/ml)	r^2values
Antilipid peroxidation	BHT	260.907±4.67	0.982
	Flowers	21.744±1.88	0.975
DPPH radical scavenging	Ascorbic acid	35.29±4.18	0.988
	Flowers	40.176±3.09	0.996
Superoxide scavenging	Ascorbic acid	352.88l±1.57	0.988
	Flowers	101.4± 5.78	0.980
Metal ion chelating	EDTA	49.34±3.75	0.999
	Flowers	407.65±1.32	0.990
Nitric oxide radical scavenging	Gallic acid	122.93±4.85	0.991
	Flowers	97.51±2.86	0.991
Hydroxyl radical scavenging	Mannitol	7.72±4.24	0.984
	Flowers	110.01±1.87	0.989
Hydrogen peroxide scavenging	Ascorbic acid	46.60±2.83	0.988
	Flowers	105.76±5.60	0.992

Table 2: IC_{50} values of methanolic extract of *C. auriculata* L. flowers and reference standards on activity of carbohydrate metabolizing enzymes. Values are expressed as mean± SD of triplicates

Activity	Sample used	IC_{50} values (µg/ml)	r^2values
α-amylase	Acarbose	351.938±7.54	0.986
	Flowers	225. 157±2.49	0.990
α-glucosidase	Acarbose	243.636±1.88	0.980
	Flowers	238.59±3.12	0.987

Table 3: Effects of flower extract of *C. auriculata* and glibenclamide on fasting blood glucose, glycosylated hemoglobin and plasma insulin level in experimental rats. Values are expressed as mean± SD (n=7). *-represents statistical significance Vs diabetic control ($p<0.05$)

Group	Fasting blood glucose (mg/dl)	Glycosylated hemoglobin (%)	Plasma insulin (µU/ml)
Control	92.3±0.2	3.02±0.31	11.29±0.61
Diabetic control	281.2±0.02	9.42±0.27	03.99±0.49
Diabetic + flower extract (150mg/kg b w)	142.8±1.6*	6.90±0.19*	05.34±0.41*
Diabetic + flower extract (250mg/kg b w)	103.2±0.28*	3.92±0.47*	9.77±0.54*
Diabetic + glibenclamide (750µg/kg b w)	123.45±2.2*	4.69±0.52*	9.08±0.49*

Table 4: Effects of flower extract of *C. auriculata* and glibenclamide on antioxidant enzyme system in experimental rats. Values are expressed as mean± SD (n=7). *-represents statistical significance Vs normal control (p<0.05)

Groups	LPO nM MDA/ mg of tissue	GSH (µg/mg) of protein	CAT (µmolesH_2O_2 SOD (U/mg)	NADH decomposed /min/mg)	Gpx (µmoles /min/mg)	GSTµ moles of GSH- oxidized CDNB conjugate formed/mg/min)
Control	2.82±0.1	33.5±0.9	132±0.73	173±0.98	214±1.29	112±0.9
Diabetic control	7.5±0.3	18.81±0.3	109±0.15	131±2.46	162±1.38	58±1.2
Diabetic + flower extract (150mg/kg b w)	5.38±0.2*	22.71±0.52*	116.34±0.18*	146±3.05*	178±2.03*	81±0.47*
Diabetic + flower extract (250mg/kg b w)	3.42±0.2*	29.32±0.73*	129.53±1.02*	169±1.31*	204±1.23*	113±0.76*
Diabetic + gliblenclamide (750µg/kg b w)	4.79±1.6*	27.66±0.18*	126.4±0.8*	168±1.59*	199±1.56*	111±0.73*

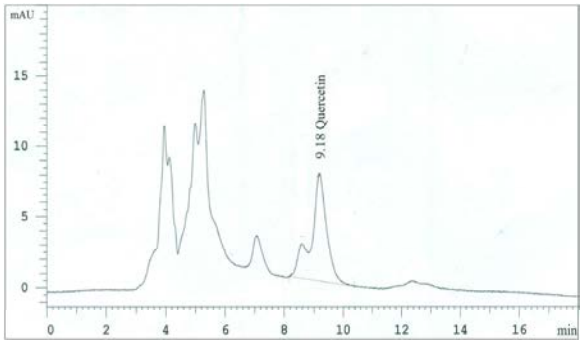

Fig. 2: Chromatogram of *C.auriculata* L. flower extract monitored at 352nm indicating quercetin (R_t-9.18).

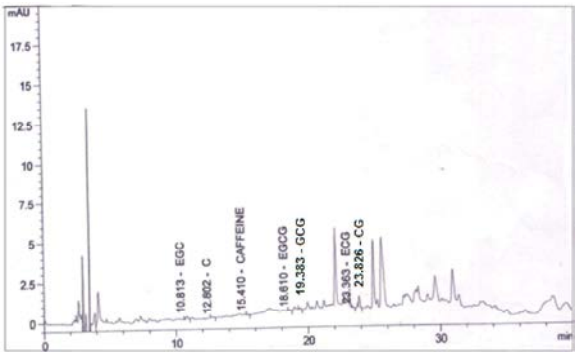

Fig. 3: Chromatogram of *C.auriculata* L. flower extract monitored at 278nm indicating (+)catechin (R_t-12.802), (-) epigallo catechin (R_t-10.813), (-) epigallocatechin gallate (R_t-18.610), (-) epicatechin gallate (R_t-23.363), (-) gallocatechin gallate (R_t-19.383), (-) catechin gallate (R_t-23.824) and caffeine (R_t-15.410).

Table 3 illustrates the antihyperglycaemic effect of flower extract on streptozotocin induced diabetic rats. Blood glucose and glycosylated hemoglobin levels in groups administered with 250 mg/kg extract was decreased significantly (p<0.05) to a greater extent compared to groups treated with glibenclamide. The effect of flower extract was found to increase with concentration. Plasma insulin recorded for extract treated group and glibenclamide was significant (p<0.05) with 79.19% and 69.73% increase compared to diabetic control.

Elevated lipid peroxidation level and decreased activities of antioxidant enzymes and GSH content, post streptozotocin injection is represented in Table 4. Floral extract could cause 8% higher decrease in LPO levels compared to glibenclamide. Treatment of diabetic rats with different doses of flower extract produced enhancement in the activities of antioxidant enzymes. This was evident from the normalization of activity of glutathione-S-transferase (78.28%) and reduced glutathione content (71.55%). The SOD, CAT and GPx activities were significantly (p<0.05) increased to the extent of 86.54%, 89.7% and 75.86% compared to glibenclamide which produced 73.25%, 87.37% and 71.57% enhancement respectively.

RP-HPLC analysis of flowers for quercetin and mixed catechin fingerprint revealed the presence of 1.17% quercetin, 0.06% (+)catechin, 1.65% (-)epigallo catechin, 7.58% (-)epigallocatechin gallate, 1.95% (-)epicatechin gallate, 0.23% (-)gallocatechin gallate, 0.003% (-)catechin gallate and 4.8% caffeine. Chromatograms with retention time (R_t) are represented in Figures 2 and 3.

DISCUSSION

The present results indicate the potential antihyperglycemic and antioxidant effects of the flower extract that may be due to numerous phytoconstituents present in the flowers. Abundant presence of flavonoids and phenolics substantiates the profound therapeutic applications of *C.auriculata* L. flowers. The current findings are in well agreement with the earlier observations [38] in *C.auriculata* as well in other medicinal plants [39, 40].

The study indicated significant (p<0.005) inhibitory efficacy on different kinds of free radicals produced *in vitro*. Antilipid peroxidative effect of the flower extract *in vitro* was three-fold greater than the reference standard

inferring the possible role in resistance to cellular damages [41] and is in agreement with the results in the experimental animals. The effective inhibition of DPPH free radicals indicated the antiradical activity of secondary metabolites present in the flowers. Here, the radical quenching ability is well correlated with the results of reducing power assay. The result of our study supports the earlier reports on the influence of reducing power on radical scavenging potential in medicinal plants [42]. Metal chelating activity affords protection against oxidative damages [43] and hence observed ability indicated the presence of compounds capable of chelating metal ions.

The ability of the flower extract to forage superoxide radicals revealed the ability to inhibit the formation of hydroxyl radical *in vivo* [44] and could be a sign of possible role in protection towards macromolecules. The inhibition of nitric oxide radicals by the extract may offer scientific evidence for the use of flowers to treat inflammatory diseases [45]. The hydrogen peroxide and hydroxyl clearance effects were evident only at higher concentration. However, the positive correlation between concentration and activity apparent from the data suggests that the extract may defend the biological system against deleterious effects of hydroxyl radicals and hydrogen peroxide. Nevertheless, variation observed in the levels of inhibition towards different radicals indicated the differential response towards the type of radical. The varying inhibitory effects of plants with the nature of radicals are already reported [46, 47].

The significant ($p<0.01$) suppression of the α-amylase and α-glucosidase indicated the hypoglycemic feature of the components present in the extract. The regulated α-glucosidase enzyme activity was analogous to commercial drug acarbose, further reflecting the presence of potent carbohydrate metabolizing enzyme inhibitors. Plant extracts controlling the elevated blood glucose by binding the active site of these enzymes and non-competitively inhibiting their activity has been reported earlier [48]. Thus effective regulation of enzymes implies the influence of hypoglycemic components of the infusion of flowers, which enables the post prondial hyperglycemic (PPHG) control in diabetics.

The elevated blood sugar and decreased insulin level in diabetic rats were normalized by the floral extract and may be attributed to stimulatory effect of bioconstituents on pancreatic â-cells to enhance insulin secretion or its protective and restorative effect against the damage caused by streptozotocin or due to augmented glucose transport to the peripheral tissues. The invigorating property of flower constituents on damaged pancreatic cells recorded in *C.auriculata* has been previously substantiated by histological studies [49].

The increased activity of SOD and CAT in flower extract administered diabetic rats indicated the antiradical properties of flower components on diabetic induced lipid peroxidation. The restored activities of antioxidant enzymes shown in extract treated animals were correlated to increased glutathione content and decreased LPO levels [50]. Similar results were also observed in *Citrullus colocynthis* pulp extract [51].

Decreased blood glucose levels observed in the extract treated rats was an indication of restorative action of the extract and marked increase in insulin level supports the hypoglycemic effect [52].

The catechins, polyphenols, flavonoids are well known superoxide, hydroxyl and hydrogen peroxide scavengers [53] and presence of these along with other phytoconstituents suggests synergistic/antagonistic and antihyperglycaemic action of the flowers. Polyphenolics, known antioxidants were also reported to inhibit carbohydrate hydrolyzing enzymes. Furthermore, they also regulate glucose transport across the intestine by modulating sodium glucose co-transporter-1(S-GLUT-1), altering PPHG to normalcy. Occurrence of polyphenolics in the methanolic extract typical of tea extract like (+) catechin, (-) epigallo catechin, (-) epicatechin gallate, (-) epicatechin have been reported to have antihyperglycemic activity and potent S-GLUT-1 mediated glucose inhibitory action [54]. Presence of abundant polyphenolics not only explains the relaxing effect on oxidative stress but also antihyperglycemic potential and hence supports the multiple therapeutic benefits of *C. auriculata* L. flowers in herbal formulations.

CONCLUSION

The result of the present study provides scientific evidence for antioxidative and antidiabetic activities of flowers in various *in vitro* and *in vivo* models and hence supports the therapeutic usage of flowers in traditional medicines for treating diabetes, inflammation, burns, urinary disorder and skin diseases.

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