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α-amylase and α-glucosidase Inhibitory effect by the aerial parts of *C.auriculata* L. and HPTLC analysis for ferulic acid and cinnamic acid .

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Abstract

The antidiabetic activity of the methanolic extracts from leaves, twigs and flower buds of *C.auriculata* L. evaluated was based on the inhibitory effects on the activity of key carbohydrate metabolizing enzymes α-amylase and α-glucosidase. HPTLC analysis was performed for the presence of specific phytochemicals like ferulic acid and cinnamic acid and tannin content was also determined. The leaves extract exhibited a higher antidiabetic activity with IC_{50} value of 324.99±1.09 µg mL^{-1} and 331.61±2.78 µg mL^{-1} for α-amylase and α-glucosidase inhibitory activity respectively and was found comparable with that of acarbose, a standard drug. Incidentally, ferulic acid content was found more in flower buds (0.819%) while leaves showed maximum cinnamic acid content (0.505%). Extract of leaves also expressed greater total tannin content (284.38±0.005 TAE g^{-1} of the extract). The results emphasize that *C.auriculata* L. is not only a potential hypoglycemic source but also of selective phytochemicals possessing health beneficiary effects.

Keywords: Antidiabetic, Carbohydrate metabolizing enzymes, Tannins, Phenolic acids.

INTRODUCTION

Glucose metabolism plays a pivotal role in maintaining the physiological condition of the animal system. Carbohydrates are broken down by the digestive enzymes in the biological system releasing glucose molecules which stimulate the β-islets of pancreatic cells to secrete the hormone insulin. This key hormone stimulates glucose transporter (GLUT-4) in the target tissues like skeletal muscle (myocytes), liver (hepatocytes) and adipose tissues (adipocytes) for glucose uptake. Prolonged exposure of β-cells to elevated glucose levels (as a consequence of hyperglycemia referred to as glucose toxicity) in turn leads to chronic oxidative stress, resulting in generation of reactive oxygen species (ROS) **[Tiwari and Rao, 2002]**. Both hyperglycemia and glucose toxicity hampers β-cell function mediated by oxidative stress which may in turn results in the development of diabetes **[Robertson, 2004]**. Pathological changes associated with diabetes leads to risk factors such as obesity, high blood pressure, abnormal cholesterol level, high triglycerides, acceleration of kidney failure, nerve damage, blindness, heart disease, stroke etc., **[Vijayaraj *et al.*, 2011]**.

The therapeutic strategies for the treatment of type 2 diabetes mellitus (T2DM) include the stimulation of endogenous insulin secretion, enhancement of action of insulin at the target tissues and controlling the post prandial hyperglycemia (PPHG) by regulating the carbohydrate metabolizing enzymes like α-amylase and α-glucosidase, in order to have slow degradation of starch and oligosaccharides **[Funke and Melzing, 2006]**. In such cases, the most commonly used drugs are insulin, sulfonylureas, biguanides, α-glucosidase inhibitors and insulin mimetics. However, their utility is restricted owing to limited action and accompanying side effects like weight gain, hypoglycemia, cardiovascular defects and gastrointestinal disturbances **[Tahrani *et al.*, 2011]**. Also it may be noted that these pharmaceutical drugs fail to address the complications arising due to oxidative stress **[Payne *et al.*, 2014]**.

Herbal and alternative systems of medicine like Ayurveda, Siddha and Unani are in use to treat 'Madhumeha' (diabetes) since time immemorial in view of its effective management in treating and controlling T2DM and also its associated complications. According to ethnobotanical survey there are more than 1200 medicinal plants with hypoglycemic potential activity being used for the treatment and management of type 2 diabetes mellitus **[Sudha *et al.*, 2011]**. The ethnopharmacological and biochemical studies have confirmed the potentially diverse activity of phytoconstituents such as polyphenols, flavonoids, tannins, saponins, alkaloids, steroids, polysaccharides, terpenoids, glycosides, amino acids, essential oils etc., **[Middleton *et al.*, 2000]**. Therefore, there is a curiosity and keen interest in searching for the plants and herbs based on the pharmacological evidences having multiple beneficial health effects in rejuvenating pancreatic β-cells, improving insulin sensitivity, controlling PPHG and protecting the tissues from oxidative damage.

Cassia auriculata L. is one of the well known and widely used plants in indigenous system of medicine in South Asian countries like India and Sri Lanka for treating diabetes. The pharmacological evaluation studies of different parts of *Cassia auriculata* L. conducted till date has established scientific evidence for its ethnomedicinal usage. Investigation on antihyperglycemic, antipyretic, hypolipidemic and *in vitro* anticancerous activity of leaf extract, nephroprotective activity of root extract, antidiabetic, antioxidant, antimicrobial, hepatoprotective and α-glucosidase inhibitory **[Abesundara *et al.*, 2004; Juan-Badaturuge et al., 2011; Kumaran and Karunakaran, 2007]** action of flower extract are well

documented. The influence of the methanolic extract of flowers on hyperglycemia and oxidative stress by simultaneously performing the experiments *in vitro* and *in vivo* conditions has been reported by the authors **[Jyothi *et al.*, 2012]**. However, the regulatory effect on the activities of α-amylase and α-glucosidase enzymes *in vitro* condition by the methanolic extracts from leaves, twigs and flower buds of *C.auriculata* L. is lacking. Thus, the present study evaluated the inhibitory action of the different parts of *C.auriculata* L. plant on key carbohydrate metabolizing enzymes and HPTLC analysis was performed for the presence of important phenolic acids such as ferulic and cinnamic acid.

MATERIALS AND METHODS

Collection and extraction of plant materials

The fresh plants collected from Chikmagalur region of South India, were identified and authenticated at National Ayruveda Dietetics Research Institute [NADRI], Bangalore, India [RRCBI-Acc No. 4925]. Twigs, leaves and flower buds were separated, washed, shade dried, powdered and subjected to soxhlet extraction at 40°C using methanol, for 10 hours. The extracts were concentrated using rotary vacuum evaporator over water bath and used for the studies.

Chemicals

All the chemicals used were of analytical grade and acarbose, porcine pancreatic α-amylase and yeast α-glucosidase were purchased from Sigma chemicals (St.Louis, USA).

In vitro ANTIDIABETIC ASSAY

α-glucosidase inhibitory assay

The assay mixture containing various concentrations of the extract (100-500 µg mL^{-1}), 2% maltose and 1 mL 0.2M Tris buffer (pH 8.0) was incubated at 37°C for 5 min. The reaction was initiated by adding 1 mL yeast α-glucosidase (1 U mL^{-1}) followed by incubation at 37°C for 10 min and reaction was stopped by heating in boiling water bath for 2 min. α-glucosidase activity was determined by measuring the liberated glucose using glucose oxidase peroxidase method **[Andrade-Cetto *et al.*, 2008]**. Acarbose was used as reference standard. The inhibitory effect in terms of percentage was calculated using the following equation:

$$\text{Inhibition (\%)} = \frac{(\text{Absorbance of Control} - \text{Absorbance of Test}) \times 100}{\text{Absorbance of Control}} \quad (1)$$

α-amylase inhibitory assay

Effect of the extract on activity of α-amylase was assessed using the chromogenic method **[Thalapaneni *et al.*, 2008]**. To 500 µL extracts (100-500 µg mL^{-1}) and acarbose, 500 µL porcine pancreatic α-amylase (0.5 mg mL^{-1} prepared in 0.20 mM phosphate buffer (pH 6.9) was added. To this mixture 500 µL 1% starch solution was added and incubated for 10 min at 25°C. The reaction was stopped by adding 3,5 di-nitro salicylic acid reagent. The test tubes were incubated in boiling water bath for 5 min, cooled and 10 mL milli Q water was added. Absorbance was measured at 540 nm. The percentage inhibition of the amylase enzyme by the plant extracts was determined using equation (1).

Quantitative estimation of Tannins

Tannin content of different parts of the plant was estimated using Folin-Denis reagent, as per the method given by **Anonymous [1980]**. Accurately Weighed 0.5 g of the powdered sample was transferred to a 250 mL conical flask and mixed with 75mL water. The flask was boiled for 30 min, cooled and centrifuged at 2,000 rpm for 20 min. The supernatant was transferred to 100 mL volumetric flask and made up to the volume. 1mL of the sample extract was taken in 100 mL volumetric flask containing 75 mL water. 5 mL of Folin-Denis reagent and 10 mL of sodium carbonate solution was added and diluted to 100mL with water and Shaken well. Absorbance was noted at 700 nm after 30 min. An aliquot of the sample extract containing not more than 0.5mg of tannic acid was used and the percentage of tannin was determined. The tannin content was determined using standard curve of tannic acid (20-100 µg mL^{-1}) in triplicates and expressed in terms of mg tannic acid equivalents (TAE) g^{-1} of the extract.

HPTLC analysis

The acid hydrolyzed methanolic extracts of *C.auriculata* (500 mg each) twigs, leaves, and flower buds (with 2M HCl heated for 30 min in boiling water bath at 100°C) were cooled, extracted twice into ethyl acetate and concentrated to dryness. The residues were re-dissolved in 1mL ethanol and subjected to HPTLC analysis for ferulic acid and cinnamic acid. Standards were prepared in methanol (1mg mL^{-1}).

TLC plate pre-coated with silica gel 60 F_{254} of thickness 0.2 mm (E.Merck) of size 10×10 cm was used to perform HPTLC. Standard (2.0 µL) and samples (4.0 µL) were applied on the plate as band of 8.0 mm width using Hamilton syringe and CAMAG Linomat V sample applicator. The plate was developed to a distance of 8.0 cm in a CAMAG twin trough chamber previously saturated with mobile phase toluene: acetone: formic acid in the ratio 9:9:2 for cinnamic acid **[Harborne, 1984]** and toluene, ethyl acetate, formic acid (7:4:0.5 v/v) for ferulic acid **[Tandon and Sharma, 2010]**. After the development, the plate was dried at room temperature and densitometric evaluation was performed at 254nm in CAMAG TLC scanner 3 linked to WINCATS software.

The quantification of the phytochemicals in all the samples were determined by comparing the total peak area observed for standard bands with that in sample solutions and calculated using the formula **(CRCL, 2007)** –

$$\text{Amount of phytochemical}_{sample} = \frac{\text{Concentration}_{std} \times \text{Volume}_{std} \times \text{Area}_{sample}}{\text{Area}_{std}}$$

$$\text{Percentage of phytochemical}_{sample} = \frac{\text{Amount of phytochemical}_{sample} \times 100}{\text{Concentration}_{sample} \times \text{Volume}_{sample}}$$

Statistical analysis

The results of the experiments were expressed as mean±SD of three parallel trials. Regression analysis was used to measure the IC_{50} values and the data was measured by one way ANOVA test ($p<0.05$ was taken as statistically significant).

RESULTS AND DISCUSSION

***in vitro* Antidiabetic assay**

α-amylase and α-glucosidase inhibition activity

One way of controlling the blood glucose index in diabetic condition is by regulating some carbohydrate metabolizing enzymes like α-amylase and α-glucosidase. α-amylase enzyme primarily acts on starch molecules and hydrolyze them to oligosaccharides like maltose while α-glucosidase are responsible for absorption of glucose in the intestine. Delayed hydrolysis of carbohydrate in the early stages of metabolism and retarded absorption of glucose into the blood by the intestine, has been a strategy to manage PPHG **[Rammohan *et al*., 2008]**.

A wide range of plant derived principles, mainly alkaloids, glycosides, galactomannan gum, polysaccharides, hypoglycans, peptidoglycans, guanidine, steroids, glycopeptides and terpenoids, have demonstrated bioactivity against hyperglycemia and are found to be potent α-amylase and α-glucosidase inhibitors. These phytochemicals have been found to slowdown the hydrolysis of starch to oligosaccharides thereby retarding the glucose absorption in the small intestine **[Mentreddy, 2007]**. Inhibition of glucosidase enzymes also reduces the glycogenolytic rate in the liver thereby preventing further release of glucose into the blood (maintains the glycogen storage level in the liver) **[Meenakshi *et al*., 2011]**. The inhibition of these enzymes reduces the glucose level in the blood (as a short term effect) and facilitates decline in glycosylated haemoglobin level (HbA1C) as a long term effect **[Venable and Aschenbrenner, 2005]**.

Yeast α-glucosidase **[Jung-Hum *et al*., 2008]** and porcine pancreatic α-amylase **[Harold, 1992]** has been used frequently to evaluate the inhibitory potency of medicinal plant extracts. In the present study, the inhibitory activity of the methanolic extracts on α-amylase and α-glucosidase enzyme activity was investigated and the results are represented in Fig.1 and 2. It was observed that the leaves extract showed significant inhibitory activity ($p<0.05$) on both α-amylase and α-glucosidase enzymes compared to twigs and flower bud extracts. Leaves extract exhibited 70.87% α-amylase inhibitory activity followed by twigs and flower buds which showed 62.9% and 60.14% inhibition respectively. The extract of leaves, twigs and flower buds showed 67.67% 62.21%, and 61.11% decrease in the activity of α-glucosidase enzyme respectively and all the above inhibitory activities were expressed at 500 µg mL^{-1} concentrations and found to be dose dependent. Acarbose drug expressed 62.61% and 75.45% inhibition respectively for α-amylase and α-glucosidase enzyme activity. The inhibition shown by different extracts and standard acarbose, on the activity of carbohydrate hydrolyzing enzymes are expressed in terms of IC_{50} values and are shown in Table.1 and 2 respectively.

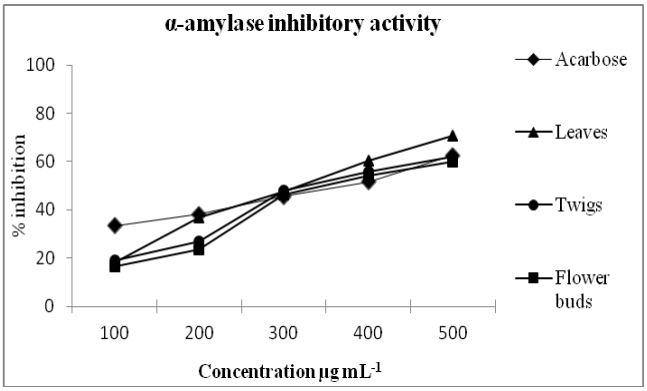

Fig.1 α-amylase inhibitory activity of the extracts of different parts of *C.auriculata* and standard acarbose.

Table.1 IC_{50} and regression co-efficient values of methanolic extracts of different parts of *C. auriculata* L. and acarbose on inhibitory activity of α-amylase enzyme.

Activity	Sample used	IC_{50} values (µg mL^{-1})	r^2 values
α- amylase inhibition	Acarbose	351.938±7.54	0.9862
	Leaves	324.999±1.09	0.9883
	Twigs	364.346±3.11	0.9915
	Buds	383.622±4.51	0.9941

(Values are expressed as mean± SD of triplicates).

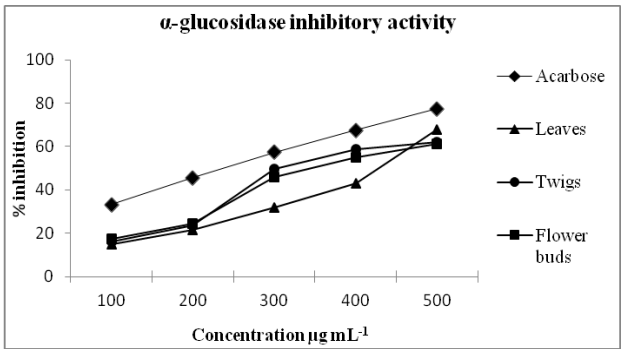

Fig.2 α-glucosidase inhibitory activity of different parts of *C.auriculata* and standard acarbose.

Table.2 IC_{50} and regression co-efficient values of methanolic extracts of different parts of *C.auriculata* L. and acarbose on inhibitory activity of α-glucosidase enzyme.

Activity	Sample used	IC_{50} values (µg mL^{-1})	r^2 values
α-glucosidase inhibition	Acarbose	243.636±1.88	0.9805
	Leaves	331.61±2.78	0.9624
	Twigs	349.682±4.16	0.9950
	Buds	378.261±3.17	0.9892

(Values are expressed as mean± SD of triplicates).

HPTLC analysis

Well defined spots of ferulic acid and cinnamic acid in the extracts matching the retention factor (R_f) of standards were visualized under UV light and the result of the analysis for the extracts are shown in Table.3 and 4. *C.auriculata* flower buds have shown to possess higher percentage of ferulic acid compared to leaves and twigs

respectively. Antioxidant activity of ferulic acid is well recognized and the antilipid peroxidative effect and inhibition of ROS production by ferulic acid reported in rat liver microsomal membranes and cultured fibroblasts, respectively **[Trombino *et al.*, 2004]** indicates the potential antiradical nature of ferulic acid. It has also reported to show, significant superoxide radical scavenging and chain-breaking activity **[Itagaki *et al.*, 2009]** and thus it is of significance in identifying and quantifying ferulic acid in different parts of *C.auriculata* that may play a contributory role in the protective effect of the extracts on oxidative injury associated with diabetes.

Comparable cinnamic acid content was observed in leaves, flower buds and twigs of *C.auriculata* in HPTLC analysis for cinnamic acid (Table.4). Cinnamic acid and its derivatives are known to possess a variety of pharmacological properties **[Adisakwattana *et al.*, 2012]**. A greater inhibitory effect of cinnamic acid on advanced glycated end products (AGE) compared to its derivatives along with shielding effect on proteins from oxidative damages has been reported when evaluated for protein glycation inhibitory activity in a bovine serum albumin (BSA)/fructose system and therefore, considered playing an important role in preventing the AGE mediated diabetic complications **[Kasetti *et al.*, 201]**.

Thus the presence of ferulic acid and cinnamic acid in twigs and floral buds of *C.auriculata* provides evidence for the antioxidative and hypoglycemic properties and results of the present study is first of its kind in identifying ferulic acid and cinnamic acid using HPTLC analysis.There are no reports mentioning the presence of ferulic and cinnamic acid in twigs and flower buds of *C.auriculata* in the literature except for the presence of cinnamic acid and ferulic acid content in the leaf extract of *Cassia auriculata* [**Usha et al 2019].** Previous report on *C.auriculata* flowers has mentioned the presence of quercetin **[Jyothi *et al.*, 2012]**, and the present evaluation also indicated the presence of ferulic acid (1.358) and cinnamic acid (0.511%) in the flowers The tannin content determined with respect to standard curve of tannic acid (r^2=0.9951), was found higher in twigs (302.79±0.005) compared to leaves (284.59±0.005) and flower buds (186.21±0.005) (mg TAE g^{-1} of extract) respectively. Thus potent hypoglycemic and antioxidant activity of the *C.auriculata* L. plant may be due to the cumulative effect of these phytoconstituents. Variation in the regulatory effect with respect to different parts of the plant observed may be on account of the presence of diversified phytoconstituents and their varied amounts as evident from the phytochemical results and due to different mode of interaction with the active or regulatory site of the enzymes **[Kazeem *et al.*, 201]**.

Table.3 Results of HPTLC analysis for *C.auriculata* L. plant parts for ferulic acid

Sample	R_f values	Area of the peak	% of ferulic acid
Leaves	0.29	793.18	0.649
Buds	0.30	1001.63	0.819
Twigs	0.28	373.31	0.305

Table.4 Results of HPTLC analysis for *C.auriculata* L. plant parts for cinnamic acid

Sample	R_f values	Area of the peak	% of cinnamic acid
Leaves	0.47	2725.97	0.5049
Buds	0.46	1581.47	0.2930
Twigs	0.45	1477.29	0.2736

CONCLUSION

The present investigation establishes the therapeutic functionalities of the *C.auriculata* L. plant constituents in treating hyperglycemic condition and its multidimensional usage in ethnomedicine for curing diabetes. The presence of unique phenolic compounds, ferulic acid and cinnamic acid substantiates the effective and pronounced antidiabetic activity of the different parts of the plant and can be considered as a potential source of pharmaceutics. Further systematic examinations to isolate the bioactive phytochemicals in *Cassia auriculata* that are responsible for notable inhibitory effect are validated.

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