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Study of *in vitro* antioxidant activity and HPTLC fingerprint of quercetin in *Cassia auriculata* L.

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ABSTRACT

The objective of the present study was to investigate the antioxidant activity of various parts of Cassia auriculata L., a species rich in antioxidants. The antioxidant competence of methanolic extracts of twigs, leaves and flower buds were screened using in vitro scavenging assays of 1,1-diphenyl-2-picryl hydrazyl (DPPH), superoxide, nitric oxide, hydroxyl, H_2O_2, lipid peroxides as well as reducing power and metal chelating abilities with reference to standards. The leaf extract showed significant higher lipid peroxide and DPPH radical scavenging activity exhibiting IC_{50} value of 8.902 µg mL^{-1} and 16.93 µg mL^{-1} as opposed to 3.623 µg mL^{-1} of ascorbic acid and 260.907 µg mL^{-1} of butylated hydroxyl toluene (BHT) respectively. Twigs exhibited maximum inhibitory activity on nitric oxide with IC_{50} 26.60 µg mL^{-1} compared to 122.93 µg mL^{-1} of gallic acid. Preliminary phytochemical analysis revealed the presence of alkaloids, saponins, flavonoids, phenolics, glycosides, tannins and steroids. Flower buds exhibited higher phenolic content of 237.83 mg gallic acid equivalents g^{-1} of extract and twigs showed maximum flavonoid content of 136.23 mg quercetin equivalents g^{-1} of extract. HPTLC analysis of plant parts indicated the presence of the most abundant dietary flavonol, Quercetin.

Key words: *Cassia auriculata* L., Antioxidant activity, Free radicals, Phenolics, Flavonoids, Quercetin.

INTRODUCTION

In human system reactive oxygen species (ROS) are regularly produced by normal biological reactions along with various exogenous factors. ROS are free radicals that cause damage to cells and tissues during infectious and degenerative disorders such as cardiovascular diseases, aging and neurodegenarative diseases like Alzheimer's disease, mutations and cancer [1-2]. The most widely used synthetic antioxidants BHA and BHT have been restricted because they are carcinogenically potential. Hence, there has been much attention to use natural antioxidants to defend against oxidative stress. *C.auriculata* L., is one such plant which possess strong antioxidant properties attributed to various secondary metabolites present in them [3].

Cassia auriculata L., is a shrub found throughout in open areas in India. In Indian ethnomedicine it is used as antidiuretic, astringent, antirheumatic, antihelmentic and antiemetic. Various scientific studies have reported antidiabetic, antimicrobial, antioxidant, antihelmentic, anticancer, antihepatoprotective immunomodulatory, antihyperlipidemic, laxative, nephroprotective properties of this plant [4]. Use of various parts of this plant in traditional medicine and *in vivo* antioxidant activity as reported by several researchers prompted us to investigate the antioxidant activity of various parts of the plant by employing a range of *in vitro* designs. An attempt to evaluate the antioxidant activity of the flower buds has been made for the first time. In the present study, quantification of

quercetin by HPTLC technique has been made in relation to its antioxidant activity which was not investigated previously.

MATERIALS AND METHODS

Chemicals:
DPPH, 2-deoxy-2-ribose, napthyl ethylene diamine dihydrochloride, NADH, NBT, PMS, 2,2'-bipyridyl, TBA, BHT, H_2O_2, EDTA, TCA, potassium ferricyanide, ferric chloride, Folin Ciocalteau reagent, quercetin were purchased from Sigma Aldrich, USA. All other chemicals and solvents used were of HPLC andanalytical grade.

Extraction and phytochemical analysis of plant:
The fresh plants collected from Chikmagalur, India was identified and authenticated at National Ayruveda Dietetics Research Institute (NADRI), Bangalore, India (RRCBI-Acc No. 4925). Twigs, leaves and flower buds were separated, washed thoroughly with milli-Q water and shade dried for 5 days. They were powdered and subjected to soxhlet extraction at 40 ^{0}C using methanol for 10 hours. The extracts were concentrated using rotary vacuum evaporator and stored at -4 ^{0}C for further studies. The methanolic extracts of twigs, leaves, and flower buds of *Cassia auriculata* L., were subjected to preliminary phytochemical screening following the methodology of Horborne [5] for the presence of alkaloids, saponins, tannins, flavonoids, glycosides, steroids and terpenoids.

Quantitative estimation of total phenolics and flavonoids:
Total phenolic content of different parts of the plant was determined using Folin's Ciocalteau reagent [6] and estimated using calibration curve of gallic acid. The flavonoid content was quantified following Chang *et al* [7] method and expressed on the basis of standard curve of quercetin. The results were expressed as mg of standard equivalents g^{-1} of the extract.

Lipid peroxidation inhibition assay:
Anti-lipid peroxidative potential was measured according to Kumar *et al* [8] using double beam Shimadzu UV-Visible spectrophotometer. Freshly excised goat liver was processed in cold 40 mmol L^{-1} Tris-HCl buffer (pH 7.0) using glass teflon homogenizer and centrifuged at 3000 rpm for 10 min at 4 ^{0}C to get a clear homogenate. The reaction mixture containing 0.5 mL of homogenate, 1 mL extract (20-100 µg mL^{-1}), 0.1 mL (0.15 mol L^{-1}) KCl, 0.1 mL (15 mmol L^{-1}) $FeSO_4$, and 0.1 mL (6 mmol L^{-1}) ascorbic acid was incubated for 1 hour at 37 ^{0}C. 1 mL of 10% trichloro acetic acid (TCA) was added to the mixture and centrifuged at 3000 rpm for 20 min at 4 ^{0}C. 1 ml of 0.8% TBA was added to the supernatant followed by heating at 90 ^{0}C for 20 minutes. Absorbance was measured at 532 nm.

DPPH radical scavenging assay:
Total antioxidant activity of plant extracts were estimated using stable DPPH radical scavenging assay described by Cotelle *et al* [9]. To 200 µL of DPPH (100 µ mol L^{-1} in methanol), 2.8 mL methanolic extract (20-100 µg mL^{-1}) were added. After 20 minutes the absorbance of assay mixture was read at 517 nm.

Reducing power:
The Fe^{3+}-reducing power of extracts was determined based on the method described by Oyaizu [10] using ascorbic acid as reference standard. 1 mL methanolic extract (20-100 µg mL^{-1}) was mixed with 2.5 mL of 1% potassium ferricyanide and 2.5 mL of phosphate buffer pH 6.6. The mixture was incubated at 50 ^{0}C for 20 minutes. 10% TCA was added to the mixture and centrifuged at 3000 rpm for 10 minutes. The supernatant was mixed with distilled water and 0.1% $FeCl_3$. Absorbance was measured at 700 nm.

Metal ion chelating activity:
The chelating activity of the extracts for Fe^{2+} was determined by Yamaguchi *et al* [11] with minor modification. 0.25 mL (1 m mol L^{-1}) $FeSO_4$ solution was mixed with equal volume of extract (200-1000 µg mL^{-1}). 1 mL Tris-HCl buffer (pH 7.4) and 0.25 mL (0.1%) 2,2'-bipyridyl solution were added along with 0.4 mL hydroxyl amine-HCl and 2.5 mL ethanol. The reaction mixture was adjusted to a final volume of 5 mL with distilled water and incubated for 10 minutes at room temperature. The absorbance was measured at 522 nm with Ethylene diamine tetra acetic acid (EDTA) as reference standard.

Superoxide anion radical scavenging activity:
Superoxide anion radical scavenging activity was determined by Nitro blue tetrazolium (NBT) reduction method of Nishikimi *et al* [12] with slight modifications. The assay mixture in a final volume of 2.5 mL contained 1 mL (156 µmol L^{-1}) NBT (in phosphate buffer pH 7.4), 1 mL of 468 µ mol L^{-1} β-nicotine amide adenine dinucleotide (NADH) solution and 0.1 mL of plant extracts (200-1000 µg mL^{-1}). The reaction was initiated by the addition of 100 µL (60 µmol L^{-1}) phenazine methosulphate (PMS). The reaction mixture was incubated at 30 ^{0}C for 15 minutes and the absorbance was measured at 560 nm using appropriate blank to quantify the formazan developed. Ascorbic acid was used as reference compound.

Nitric oxide radical scavenging activity:
Nitric oxide radical scavenging abilities were assayed using Griess reaction according to method described by Green *et al* [13]. 1 mL of 10 mmol L^{-1} sodium nitroprusside was mixed with 1 mL of flower extracts of *Cassia auriculata* L. (20-100 µg mL^{-1}). The mixture was incubated at 25 ^{0}C for 150 minutes. To 1 mL of incubated solution, 1 mL Griess reagent (1% sulphanilamide, 2% ortho phosphoric acid and 0.1% naphthyl ethylene diamine dihydrochloride) was added. Absorbance of the chromophore formed was measured at 546 nm and percent inhibition was calculated. Gallic acid was used as reference compound.

Hydrogen peroxide scavenging activity:
Hydrogen peroxide scavenging ability was determined following the method described by Gulcin *et al* [14]. 1 mL extract dissolved in phosphate buffer (0.1 mmol L^{-1}, pH 7.4) at various concentrations were mixed with 2 mL (100 mmol L^{-1}) hydrogen peroxide solution. The concentration of the hydrogen peroxide was measured by reading the absorbance at 230 nm after 10 minutes against blank solution. For each concentration, a separate blank sample was used.

Hydroxyl radical scavenging activity:
Hydroxyl radical scavenging activity of the plant extracts were estimated by the method given by Kunchandy and Rao [15]. The reaction mixtures containing plant extracts (20-100 µg mL^{-1}), 2-deoxy-2-ribose (28 mmol L^{-1}), EDTA (1.04 mmol L^{-1}), $FeCl_3$ (0.2 mmol L^{-1}) and ascorbic acid (1 mmol L^{-1}) were incubated at 37 ^{0}C, for 1 hour. The preventive effects of extracts on deoxyribose damage, imposed by hydroxyl radicals were determined colorimetrically at 532 nm against separate blank for each concentration. Mannitol was used as the reference compound.

The percentage inhibition of the extracts was calculated for all the above parameters using the formula:

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

HPTLC analysis for quercetin:
500mg each methanolic extracts of *C.auriculata* twigs, leaves and flower buds were immersed in 2 mol L^{-1} HCl and heated for 30min in boiling water bath at 100 ^{0}C. After cooling, the solutions were extracted twice with ethyl acetate and combined extracts were pooled and concentrated to dryness. The residues were re-dissolved in ethanol (10mg mL^{-1}) and subjected to HPTLC analysis for Quercetin. Standard quercetin was prepared in methanol (1mg mL^{-1}).

HPTLC was performed on TLC plate pre-coated with silica gel 60 F_{254} of thickness 0.2 mm (E.Merck) of size 10 × 10 cm. Quercetin (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 chloroform: ethyl acetate: formic acid in the ratio 5:4:1 for 30 min. After development, the plate was dried at room temperature and densitometric evaluation was performed at 254 nm in CAMAG TLC scanner 3 linked to WINCATS software. The image of the plate was captured at short range in UV chamber. Quercetin in the extracts was quantified by comparing the total peak area of quercetin band in standard solution with that of the sample solutions.

STATISTICAL ANALYSIS
The experimental results were expressed as mean ± SD of three parallel measurements. IC_{50} values were calculated by regression analysis quoting r^2 values (regression co-efficient). Two parameters were related by mentioning

correlation co-efficient. Data was evaluated by one way ANOVA test. $p<0.05$ was considered to be statistically significant.

RESULTS

The preliminary phytochemical analysis showed the presence of alkaloids, flavonoids, tannins, saponins, glycosides and steroids in twigs, leaves and flower buds. The total phenolic content determined using standard curve of gallic acid (r^2= 0.994) was found to be higher in flower buds (237.83 mg GAE g^{-1} of the extract). Leaf and twig extracts showed similar phenolic content of 135.17 and 144.42 mg GAE g^{-1} of the extract respectively. Total flavonoids estimated in the extracts with respect to quercetin calibration curve (r^2= 0.9934) was found to be twigs (136.23 mg QE g^{-1}) > flower buds (133.92 mg QE g^{-1}) > leaves (131.77 mg QE g^{-1}).

The inhibitory effects of the plant extracts on lipid peroxidation were significant as compared to standard BHT and showed direct correlation to concentrations used. Antiperoxidative effect of leaves extract was found to be highest with 98.19% while lower scavenging effect of 57.88% was exhibited by twigs at 100 µg mL^{-1}. BHT was able to eliminate only 31.58% peroxide radicals at the same concentration.

Leaves showed excellent DPPH radical scavenging activity (94.44%) over twigs (62.96%) and flower buds (59.62%) at 100 µg mL^{-1} against 95.84% inhibitory response of ascorbic acid at 10 µg mL^{-1}. Leaf extract was significant ($p<0.005$) in a concentration dependent manner than standard ascorbic acid.

Reducing potential of the extracts showed maximum absorbance of 1.30 nm (r^2=0.991) for leaves, twigs 1.21 nm (r^2=0.965) and flower buds 0.573 nm (r^2=0.935) respectively at 100 µg mL^{-1} concentration monitored at 700 nm. At similar condition ascorbic acid expressed 1.14 nm (r^2=0.941) absorbance (Figure 1).

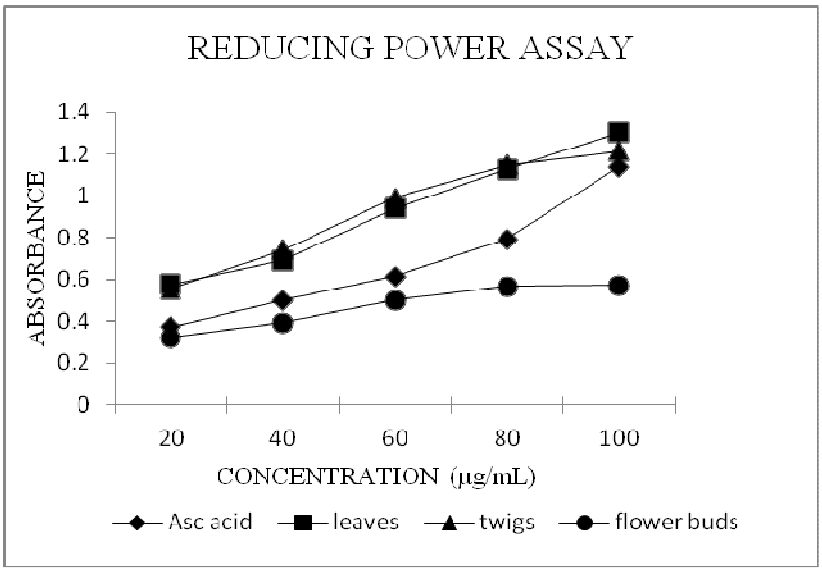

Figure1. Reductive capability of methanolic extracts of *Cassia auriculata* L., and standard ascorbic acid
Values are expressed as mean± SD of three parallel measurements

The correlation co-efficient calculated between the DPPH radical scavenging assay and reducing power showed strong relation in the plant extracts than in ascorbic acid. Leaf extract expressed maximum correlation co-efficient (r^2=0.981), followed by twigs 0.9764 and flower buds 0.9660, compared to r^2=0.9625 of Ascorbic acid.

At lower concentrations (20-100 µg mL^{-1}), plant extracts did not exhibit promising chelating activity compared to EDTA, synthetic metal chelator which exhibited maximum binding power of 86.54% at 100 µg mL^{-1}. When tested at higher concentration range (200-1000 µg mL^{-1}), 93.44% binding efficacy was shown by flower buds and 87.2% by leaves. Twigs expressed 34.51% binding activity at 1000 µg mL^{-1} concentration.

The plant extracts significantly ($p<0.005$) inhibited superoxide anions generated *in vitro*. At 1000 µg mL^{-1}, the percentage scavenging of the plant extracts were in the order flower buds 98.9%> twigs 98.7%> leaves 97.95%. Ascorbic acid showed 82.3% inhibitory activity.

Twig extract inhibited (77.48%) nitroprusside from generating nitric oxide radicals while flower buds and leaves showed almost similar effect with 57.5% and 55.35% inhibition at 100 µg mL^{-1} concentration. Gallic acid showed 40.97% scavenging effect. The inhibitory effect was found to rise with extract concentration giving statistically significant ($P < 0.005$) results.

Flower buds and twigs indicated 52.01% and 50.33% H_2O_2 scavenging activity whereas leaf extract showed 50% inhibition at 100 µg mL^{-1}. However, 68.74 % potential inhibitory activity was shown by ascorbic acid.

Flowers buds and leaves showed moderate competence with deoxy ribose for hydroxyl radical (43.07 and 39.72%) compared to twigs (26.19%) at 100 µg mL^{-1} concentration. Mannitol showed greater inhibitory activity of 68.01% at similar condition. In all the above parameters the plant extracts consistently exhibited the inhibitory activities which were concentration dependent.

Table 1. represents the antiperoxidative, metal chelating and radical scavenging activities of leaves, flowers buds and twigs against the standards used. IC_{50} values and respective r^2 values are mentioned with equations.

Table 1. IC_{50} (µg mL^{-1}) values of *Cassia auriculata* L., plant extracts and standards on antioxidant activities

Activity	Plant part used	r^2 values	Equation	IC_{50}
Antilipid peroxidative activity	BHT	0.982	y=0.152x+17.03	216.91
	Leaves	0.951	y=52.37x+0.273*	8.90
	Buds	0.993	y=26.55x+9.004*	35.00
	Twigs	0.999	y=35.76x-13.62*	60.13
DPPH radical scavenging activity	Ascorbic acid	0.982	y=7.278x+23.63	3.623
	Leaves	0.973	y=59.39x-22.98*	16.93
	Twigs	0.994	y=28.22x+5.976*	36.31
	Buds	0.996	y=41.48x-23.81*	60.17
Metal ion chelating power	EDTA	0.999	y=0.680x+16.45	49.34
	Buds	0.992	y=0.048x+46.03	82.71
	Leaves	0.989	y=0.042x+45.41	109.29
	Twigs	0.998	y=0.024x+15.29	1446.2
Superoxide scavenging activity	Ascorbic acid	0.988	y=0.052+31.65	352.88l
	Buds	0.950	y=41.40x-20.09*	49.32
	Twigs	0.954	y=44.76x-29.55*	59.87
	Leaves	0.924	y=51.12x-47.55*	80.95
Nitric oxide radical scavenging activity	Gallic acid	0.991	y=0.354x+6.483	122.93
	Twigs	0.999	y=0.371x+40.13	26.60
	Buds	0.994	y=0.342x+23.82	76.55
	Leaves	0.991	y=0.267x+29.22	77.83
Hydrogen peroxide scavenging activity	Ascorbic acid	0.988	y=0.326x+34.81	46.60
	Buds	0.996	y=0.339x+17.33	96.37
	Twigs	0.997	y=.264x+24.00	98.48
	Leaves	0.993	y=0.392x+9.755	102.67
Hydroxyl radical scavenging activity	Mannitol	0.984	y=0.193x+48.51	7.72
	Buds	0.989	y=0.456x-1.847	113.70
	Leaves	0.996	y=0.439x-4.811	124.85
	Twigs	0.992	y=0.282x-2.494	186.15

*Values are expressed for n=3. *antilog of the values are taken as IC_{50}.*

Well defined spots of quercetin in the extracts matching the retention factor of standard quercetin were visualized under UV light at 254nm. The amount of quercetin found in *C.auriculata* parts ranged from 0.109 to 1.466%. The result of the HPTLC analysis for the extracts is shown in Table 2.

Table 2. Results of HPTLC analysis for *C.auriculata* L., plant parts

Sample solution	R_f values	Area of the peak	Amoun of quercetin µg	% of Quercetin
Leaves	0.66	6250.2	2.346	1.466
Flower buds	0.62	2957.6	1.1100	0.6938
Twigs	0.62	467.1	0.1751	0.1095

DISCUSSION

The preliminary phytochemical analysis of plant extracts showed the presence of active phytoconstituents like alkaloids, phenolics, flavonoids, saponoins, tannins etc., which are known for their ability to scavenge free radicals and prevent lipid peroxidation [16]. The antilipid peroxidative effect of leaves was better than the reference standard (BHT) inferring its role in combating cellular damages [17]. The inhibition differed with the type of plant part used, indicating that the antioxidant present in the twigs, leaves and flower buds could react differently with the different lipid groups [18]. The total antioxidant activity (DPPH) exhibited by the extracts represents the reducing abilities of the secondary metabolites. Here, the radical scavenging ability is well correlated with the results of reducing power assay carried out using the same extracts. The results are consistent with Chanda *et al* [19] who observed the influence of reducing power on radical scavenging potential in medicinal plants. The curative efficiency of *C.auriculata* on diabetes and related complications stems from metal binding capability of the bioactive constituents present which inhibits Fe^{3+}-Fe^{2+} transformation thereby preventing the metal dependent oxidative processes responsible for ROS generation.

The most significant findings of the present study are that the flower buds showed greatest inhibitory activity on hydroxyl radicals, a novel work reported for the first time, twigs expressed maximum inhibition on nitric oxide radicals and both extracts presented higher effect on hydrogen peroxide as compared to leaves. ROS like superoxide, hydroxyl and hydrogen peroxide scavenging potential of the extracts supports the plants usage in treating ulcers as these are implicated in oxidative damages causing lesions in stomach and duodenum [20]. Quercetin, a bioflavonoid and a dietary substance, is a known antioxidant which brings about the formation of considerably less reactive species from free radicals by its reactivity [21]. Occurrence of quercetin in the different extracts supplements the bioactivity of the plant in regulating the diverse factors responsible for aging and cellular damages.

The effective inhibitory activity of the different parts of *C.auriculata* may be on account of the presence of active compounds in the plant extracts. Many researchers have also reported the antioxidant and hypoglycemic effects of *C.auriculata* to various bioactive compounds present in the plant extracts [22-23]. The inhibitory effects of the plant parts on different radicals varied in response to the nature of the radicals exposed to. Difference in the degree of inhibition exhibited by the extract towards different radicals was also observed in *C.auriculata* flower extract by the authors [24]. As reported by Cheel *et al* [25], the differential response could also be due to types of phytoconstituents acting as antioxidants which support our findings.

Figure 1. HPTLC of *C.auriculata* leaves, flower buds and twigs with quercetin

Figure 2. HPTLC chromatograms of Standard quercetin, Flower bud, Leaf and Twig extracts of *C.auriculata* at 254nm

CONCLUSION

The present study provides evidence for antioxidative and free radical scavenging capacities of twigs, leaves and flower buds in different *in vitro* models. It is apparent from the above findings that the extracts not only inhibited lipid peroxidation, scavenged free radicals but also suppressed the generation of reactive species. The presence of quercetin enhances the effective antioxidant potential of the plant. The result supports the traditional remedy of *C.auriculata* for treating chronic diseases.

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