

Full Length Research Paper

## Comparative study of antioxidant and free radical scavenging properties present in various parts of *Cassia auriculata* Linn.

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The increasing interest in powerful biological activity of plant phenolics and flavonoids outlined the necessity of determining their contents present in *Cassia auriculata* Linn. Antioxidant activities through biochemical assays [DPPH- radical scavenging activity, reducing power, inhibition of lipid peroxidation (TBARS)] were evaluated spectrophotometrically from 1 mg/ ml of crude extract. Interestingly, IC<sub>50</sub> values for scavenging activity (0.7 µg/mL) and reducing activity (0.51 µg/mL) and lipid peroxidation (0.6 µg/mL) of the flower extract had significant effect comparatively. Two known compounds (Kaempferol and Quercetin) identified by GC-MS in flower extract, indicating its preventive role against free radical effect.

**Key words:** Antioxidant activity, *Cassia auriculata* Linn, free radicals, peroxidation inhibition, phenolics, scavenging effects.

### INTRODUCTION

Plant derived medicines have been a part of traditional health care in most parts of the world for thousands of years and there is an increasing interest in plants as a source of agents to control free-radical mediated diseases. Free radicals contribute to more than one hundred disorders in humans including arthritis, atherosclerosis, ischemia and reperfusion injury of many tissues, central nervous system injury, gastritis, cancer and AIDS (Kumplainen and Salonen, 1999). Free radicals due to environmental pollutants, radiation, chemicals, toxins as well as physical stress, cause depletion of immune system antioxidants and induce abnormal proteins. Due to the depletion of immune system, natural antioxidants in different maladies, consuming antioxidants as free radical scavengers may be necessary (Cook and Samman, 1996).

Strong restrictions have been placed on synthetic antioxidants due to their negative health effects and there is a trend to substitute them with naturally occurring

antioxidants. Natural antioxidant food supplies the body with the essential antioxidant nutrients needed to enhance the immune system, eliminate excess free radicals and keep the oxidative stress state in balance. Recent studies have shown that many flavonoids and related polyphenols contribute significantly to the total antioxidant activities which are present in leaves, flowering tissues, pollens, fruits and vegetables (Halliwell, 1994; Luo et al., 2002). Human studies have found that flavonoids appear in blood plasma, at pharmacologically active levels after consuming flavonoid rich food which doesn't get accumulated in the plasma (Vinson et al., 1999). Also regular consumption of flavonoids in food or as supplement may increase longevity by reducing inflammation and contributing to a reduction in coronary heart diseases (CHD) (Cao et al., 1998). Known properties of the flavonoids include free radical scavenging, strong antioxidant activity, inhibition of hydrolytic and oxidative enzymes (phospholipase A<sub>2</sub>, cyclooxygenase,

lipoxygenase) and anti-inflammatory action (Frenkel et al., 1993).

*Cassia auriculata* Linn., an erect, annual or biennial shrub found throughout India, belongs to the family Caesalpiniaceae. It has been widely used in Ayurvedic medicine especially as an antidiabetic, in the treatments of rheumatism, dysentery, asthma, cough and renal disorders (Kirtikar and Basu, 1975; Rajagopal et al., 2002, 2003). All the parts of this plant are an excellent source of antioxidants which has proved their immense potency against degenerative diseases. The therapeutic property is that their root extracts were used to cure various skin diseases (Siva and Krishnamurthy 2005). Also known to contain various active principles of therapeutic value and possess biological activity against a number of diseases (Nandkarni, 1954). The leaves, seeds and flowers find a wide therapeutic value and used commonly against leprosy, jaundice, ulcers, toothache etc (Sharma and Malhotra, 1984). Effect of aqueous extract of the flowers was examined on antioxidants and lipid peroxidation in the brain of streptozotocin diabetic rats which showed the significant decrease in thiobarbituric reactive substances and hydroperoxide in brain (Latha and Pari, 2003). A survey of the literature revealed that the roots were reported to contain flavonoids, polysaccharides, tannins, and saponins, among other components (Rai and Dasundhi, 1990; Rao and Rao, 1998) which may contribute to its diverse uses in folklore medicine. As a part of our ongoing investigations about natural bioactive substances from local medicinal plants, the present study was conducted with the main objective of investigating the most effective technique for extracting potent antioxidant compounds, especially phenolics from different parts (flower, stem and root) of the plant, native to India. Some important parameters for antioxidant activities were also assayed to provide insight into the preventive effects.

## EXPERIMENTAL

### Standards and reagents

Standards; BHA (2-tert-4-methoxyphenol), TBHQ (tert-butylhydroquinone), L-ascorbic acid,  $\alpha$ -tocopherol, gallic acid and (+)-catechin, 2,2-diphenyl-1-picrylhydrazyl (DPPH) were purchased from Loba Chemicals Pvt. Ltd. Solvents like petroleum ether, methanol was also obtained from Loba Chemicals Pvt. Ltd. Water treated in a Milli-Q purification system was used.

### Plant material

The fresh and healthy parts of *C. auriculata* were collected locally in open grounds in adjoining areas of the University Campus, Salem, Tamil Nadu, India. This plant sample was authenticated by a Botanist (Specimen voucher no.780), BSI-TNAU, Coimbatore. Plant was then separated into individual parts, washed well with dechlorinated water prior to distilled water deprived of dusts and insects, dried, powdered in a Willy Mill to 60-mesh size. It was then stored at 4 to 5°C until further use.

### Phytochemical screening

The methanolic extract of all the parts were tested for qualitative preliminary phytochemical screening to identify the presence of alkaloids, flavonoids, saponins / fatty acids, phenolics / tannins, carbohydrates, proteins / aminoacids, sterols, glycosides, terpenoids, resins (Trease and Evans, 1985).

### Soxhlet extraction

The powdered plant parts were individually subjected to soxhlet extraction at 40°C using hexane (to remove fats and lipids) and then with methanol. The extracted crude was dried in a vacuum dessicator, powdered, made upto the concentration of 1 mg/mL, from which the following parameters were estimated.

### Determination of antioxidant contents

Contents of total phenolics in the extracts were estimated by a colorimetric assay based on procedures described by Singleton and Rossi (1965) with some modifications. Basically, 1 mL of sample was mixed with 1 mL of Folin and Ciocalteu's phenol reagent. After 3 min, 1 mL of saturated sodium carbonate solution was added to the mixture and it was adjusted to 10 mL with distilled water. The reaction was kept in the dark for 90 min, after which the absorbance was read at 725 nm (Analytik Jena 200 to 2004 spectrophotometer). Gallic acid was used for constructing the standard curve (0.01 to 0.4 mM;  $y = 2.94848x - 0.09211$ ;  $R^2 = 0.99914$ ) and the results were expressed as mg of gallic acid equivalents /g of extract (GAEs). Flavonoid contents in the extracts were determined by a colorimetric method as described by Jia et al. (1999), with some modifications. The methanolic extract (250  $\mu$ L) was mixed with 1.25 mL of distilled water and 75  $\mu$ L of a 5% NaNO<sub>2</sub> solution. After 5 min, 150  $\mu$ L of 10% AlCl<sub>3</sub>.H<sub>2</sub>O solution was added. After 6 min, 500  $\mu$ L of 1 M NaOH and 275  $\mu$ L of distilled water were added to prepare the mixture. The solution was mixed well and the absorbance was read at 510 nm. (+)-Catechin was used to calculate the standard curve (0.250 to 2.500 mM;  $Y = 0.2903$ ;  $R^2 = 1.0000$ ) and the results were expressed as mg of (+)-catechin equivalents (CEs) per g of extract.

### DPPH radical-scavenging activity

Various concentrations of *C. auriculata* extracts (0.3 mL) were mixed with 2.7 mL of methanol solution containing DPPH radicals (6 x 10<sup>-5</sup> mol/L). The mixture was shaken vigorously and left to stand for 60 min. in the dark (until stable absorbance values were obtained). The reduction of the DPPH radical was determined by reading the absorbance at 517 nm. The radical scavenging activity (RSA) was calculated as a percentage of DPPH discoloration, using the equation: % RSA =  $[(A_{DPPH} - A_S)/A_{DPPH}] \times 100$ , where A<sub>S</sub> is the absorbance of the solution when the sample extract is added at a particular level, and A<sub>DPPH</sub> is the absorbance of the DPPH solution (Barros et al., 2007). BHA and  $\alpha$ -tocopherol were used as standards.

### Reducing power

Various concentrations of *C. auriculata* extracts (2.5 mL) were mixed with 2.5 mL of 200 mM sodium phosphate buffer (pH 6.6) and 2.5 mL of 1% potassium ferricyanide. The mixture was incubated at 50°C for 20 min. After 2.5 mL of 10% trichloroacetic acid (w/v) was added, the mixture was centrifuged at 1000 rpm for 8 min (Centorion K24OR-2003 refrigerated centrifuge). The upper

**Table 1.** Phytochemical screening of *Cassia auriculata* in methanol extract.

Phytochemical constituents	Presence or absence in methanol extract		
	Flower	Root	Stem
Alkaloids	+	+	+
Flavones / Isoflavones (Alkaline) (Mg-Hcl)	-	-	-
Flavanones (Alkaline) (Mg-Hcl)	+	+	+
Flavonoids (Ferric chloride) (Shinoda's)	+	+	+
Flavanols (Alkaline) (Mg-Hcl)	-	-	-
Saponins / Fatty acids	+	-	+
Phenolics / Tannins	+	+	+
Carbohydrates	+	-	-
Proteins / Aminoacids	+	+	+
Oils / Steroids	-	-	-
Glycosides	+	+	-
Terpenoids	-	-	-
Leucoanthocyanins	+	-	-
Resins	-	-	-

+ presence ; - absence

layer (5 mL) was mixed with 5 mL of deionised water and 1 mL of 0.1% of ferric chloride, and the absorbance was measured spectrophotometrically at 700 nm [Barros et al., 2007]. BHA and  $\alpha$ -tocopherol were used as standards.

#### Inhibition of lipid peroxidation using thiobarbituric acid reactive substances (TBARS)

Brains were obtained from hen of body weight ~150 kg, dissected and homogenized with a Polytron in ice-cold Tris-HCl buffer (20 mM, pH 7.4) to produce a 1:2 (w/v) brain tissue homogenate which was centrifuged at 3000 g for 10 min. An aliquot (0.1 mL) of the supernatant was incubated with the *Cassia auriculata* extracts (0.2 mL) in the presence of FeSO<sub>4</sub> (10  $\mu$ M; 0.1 mL) and ascorbic acid (0.1 mM; 0.1 mL) at 37°C for 1 h. The reaction was stopped by the addition of trichloroacetic acid (28% w/v, 0.5 mL), followed by thiobarbituric acid (TBA, 2%, w/v, 0.38 mL), and the mixture was then heated at 80°C for 20 min. After centrifugation at 3000 g for 10 min to remove the precipitated protein, the colour intensity of TBARS in the supernatant was measured by its absorbance at 532 nm. The inhibition ratio (%) was calculated using the following formula: Inhibition ratio (%) = [(A-B)/A] x 100%, where A and B are the absorbances of the control and the extract solution, respectively. BHA was used as standard.

#### Gas chromatography - mass spectrometry (GC-MS)

GC-MS is established as a routine technique for the analysis of flavonoids aglycones and is carried out with either EI or CI sources. Because of limited volatility, analysis of flavonoids glycosides by GC-MS was not generally found favour; so after hydrolysis, the sample was subjected to GC-MS analysis (Fiamegos et al., 2004).

#### Sample preparation for gas chromatography - mass spectrometry (GC-MS)

Hydrolysates were extracted with ethyl acetate (1:1, v/v) after acid hydrolysis. The ethyl acetate extract was treated with 0.5 M

NaHCO<sub>3</sub> (1:1, v/v) three times, to eliminate free phenolic acids. The ethyl acetate extract was evaporated to dryness under a flow of nitrogen and the flavonols were re-dissolved in ethyl acetate. This solution was dried with Na<sub>2</sub>SO<sub>4</sub> for 5 min. The dried solution (400  $\mu$ L) was transferred to vial, 100  $\mu$ L bis (trimethylsilyl) trifluoroacetamide (BSTFA) was added, and the vial was heated at 70°C for 30 min (Molnar-Perl et al., 1988).

#### Statistical analysis

For all the experiments three parts were analyzed and all the assays were carried out in triplets. The results were expressed as mean values and standard error or standard deviation (SD).

## RESULTS

### Phytochemical screening

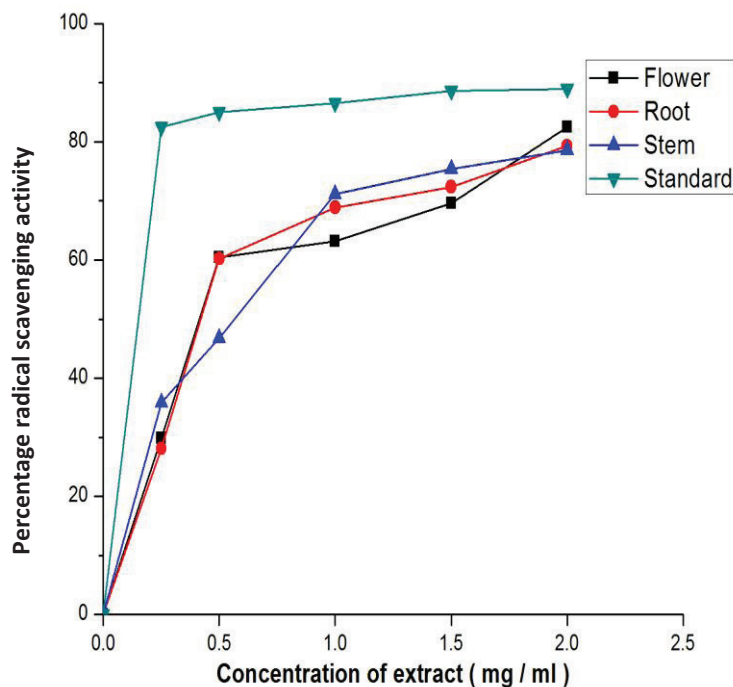
Phytochemical screening revealed the presence of phenolics and flavonoids in high levels, which could be mainly responsible for the remarkable antioxidant effect of this plant. Also, the presence of other phytochemical constituents like alkaloids, carbohydrates, proteins, glycosides, leucoanthocyanins etc., were found to be present in the methanolic extracts of all the parts listed in Table 1.

### Total flavonoids and polyphenolic estimation

The results of the Folin-Ciocalteu's total phenolic assay and Aluminium chloride total flavonoid assay clearly outlined the richest sources of flavonoids in flower and phenolics in root. It can be estimated that about 5% of total flavonoids are present in the crude extract of flower,

**Table 2.** Identification of flavonoids.

Retention time (min)	Compound	Characteristic MS data m/z
25.36	Kaempferol standard	<sup>+</sup> M = 207; 73 (100), 281, 200, 55
25.01	Plant extract	<sup>+</sup> M = 207; 74.1 (100), 281, 87, 97
29.96	Quercetin standard	<sup>+</sup> M = 281; 207 (100), 95, 273, 81
29.08	Plant extract	<sup>+</sup> M = 281; 207 (100), 95, 267, 73



**Figure 1.** DPPH radical scavenging activity of *Cassia auriculata* in various concentrations of methanol.

simultaneously 3 and 1% in stem and root. On the otherhand, the total phenolics present in flower were about 6 and 7% of stem and 9% of root in their respective methanolic crude extracts.

### DPPH scavenging activity

Table 2 lists out the percentage RSA for the studied extracts. For all the parts there was an increase in the percentage RSA values with the increase in the concentration of crude sample. Moreover, the IC<sub>50</sub> value calculated from Figure 1 was found to be high in flower (0.7 µg/mL) compared with stem (0.80 µg/mL) and root (0.76 µg/mL).

### Reducing power

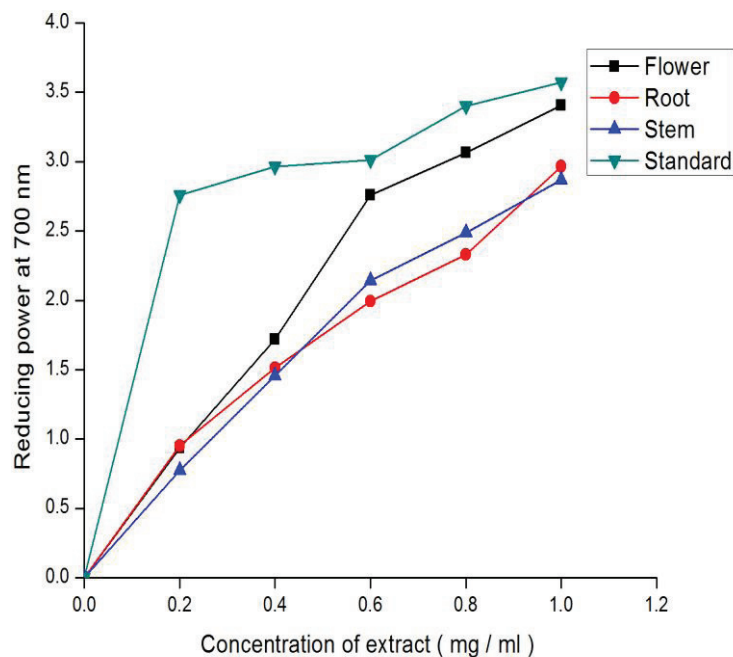
A gradual increase in the activity with the concentration

was observed for all the extracts (Figure 2) comparing with the activity of standard BHA. In this method ferric ions are reduced to ferrous ions and with colour change depending on the selected plant extracts. Higher IC<sub>50</sub> value was observed in flower (0.51 µg/mL) in contrast with root (0.49 µg/mL) and stem (0.50 µg/mL).

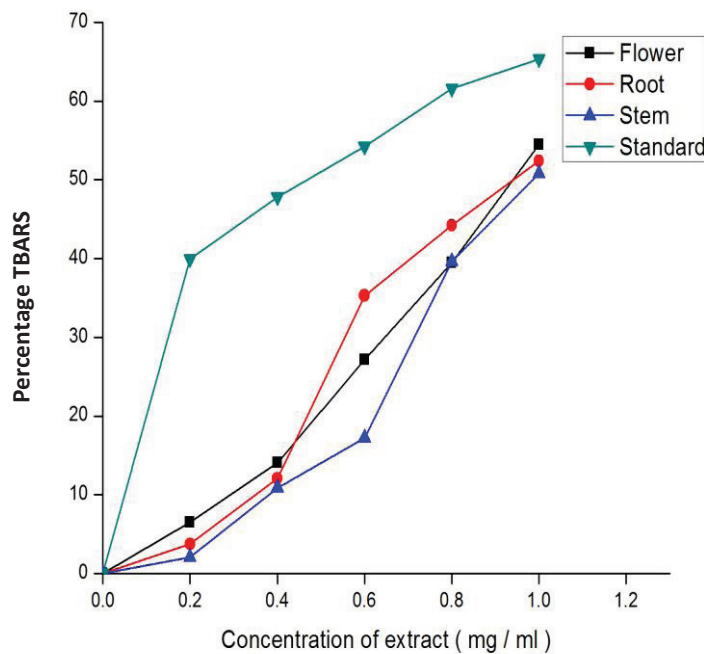
### Inhibition of lipid peroxidation (TBARS)

Various concentrations of the methanolic extracts of the investigated parts lowered the lipid peroxidation induced by hydroxyl radical generated by an iron / ascorbate system.

The 50% inhibition (IC<sub>50</sub>) was 0.6 µg/mL in flowers, 0.59 µg/mL in stem and 0.56 µg/mL in root. For comparison, the same concentration of BHA inhibited the lipid peroxidation by 0.6 µg/mL. Figure 3 presents a clear idea of the lipid peroxidation inhibition.



**Figure 2.** Reducing power of *Cassia auriculata* in various concentrations of methanol.

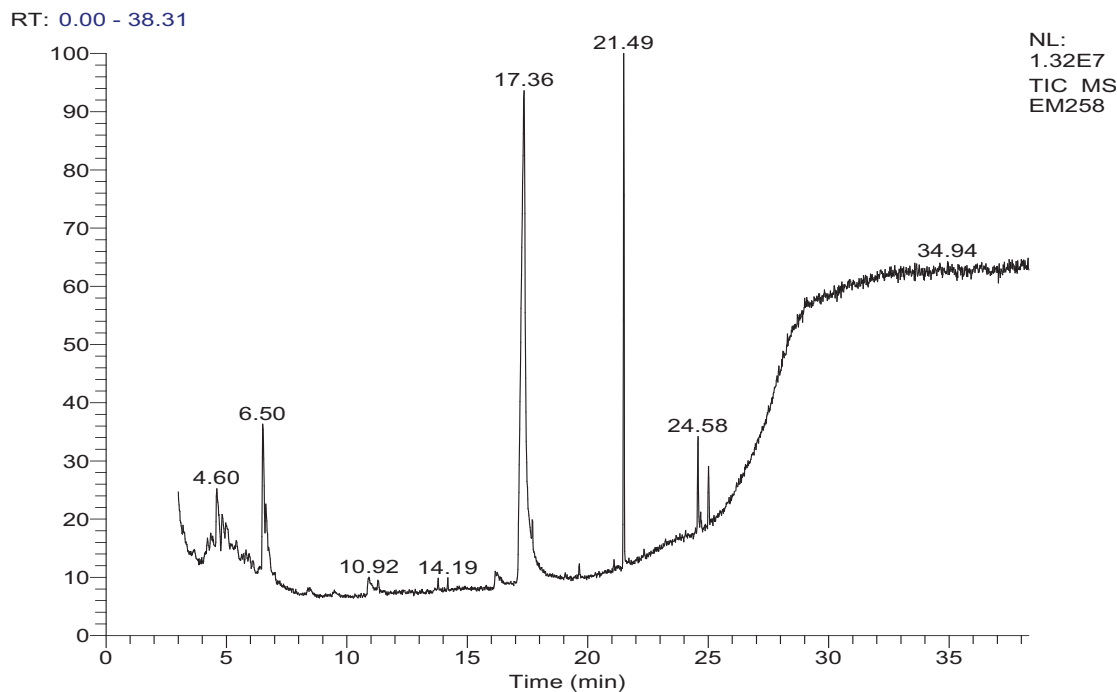


**Figure 3.** Inhibition of lipid peroxidation of *Cassia auriculata* in various concentrations of methanol.

### GC-MS analysis

The foregoing results reported the presence of rich flavonoid contents in the flower extracts. Hence, they were subjected for GC-MS for the identification and analysis of

flavonol compounds. The GC-MS for the methanolic extract of *C. auriculata* Linn. was quantitatively analyzed for obtained 10 chromatograms and the molecular ions, fragmentation patterns for all the samples were studied by mass at various retention time (min), peaks eluting



**Figure 4.** The total ion chromatogram (TIC) of *Cassia auriculata* flower extract obtained by GC-MS.

after 25.10 and 29.08 min (Figure 4) were tentatively identified as bis (trimethylsilyl) trifluoroacetamide (BSTFA) derivatives of kaempferol ( $m/z$  207) (Figure 5), and quercetin ( $m/z$  281) (Figure 6). GC retention times of reference compounds, given to two decimal places, to indicate the elution sequence of peaks were very close to the retention times of the flavonol aglycones in samples as shown in Table 2.

## DISCUSSION

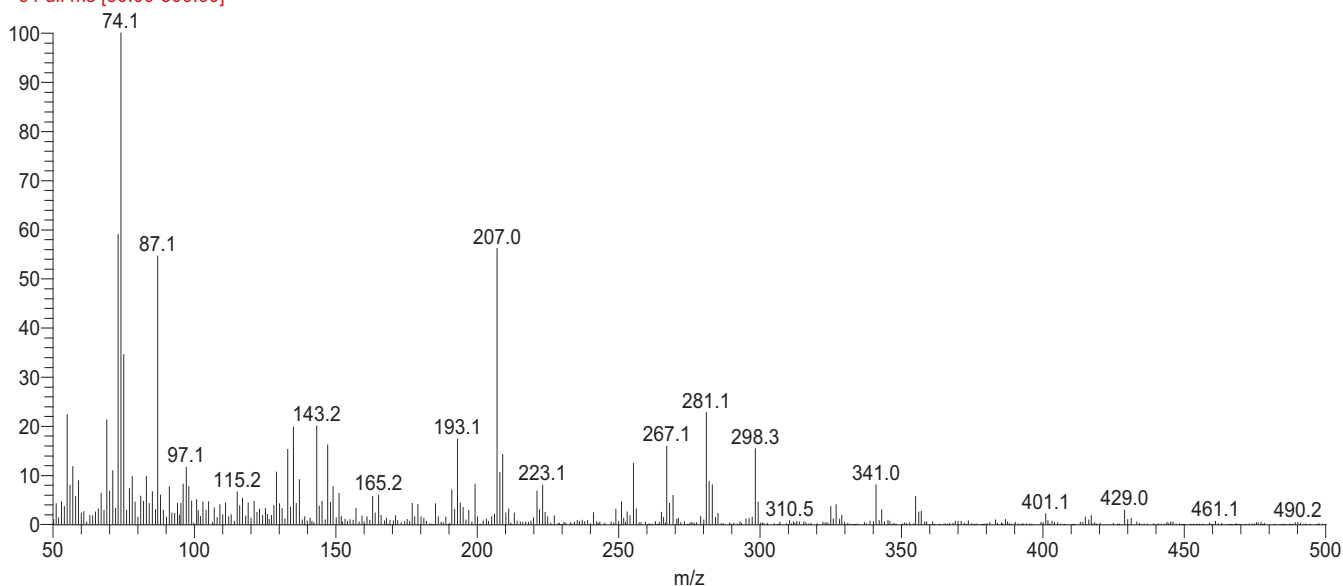
The successive extracts of flower, root and stem of *C. auriculata* have revealed the presence of alkaloids, flavonoids, phenolics, carbohydrates, proteins, glycosides and leucoanthocyanins. Thus the preliminary screening tests may be useful in the detection of the bioactive principles and subsequently may lead to the drug discovery and development. These classes (such as alkaloids, flavonoids, phenolics and anthocyanins) of compounds are known to have curative activity against several pathogens and therefore suggest the use traditionally for the treatment of various illness (Hassan et al., 2004; Usman and Osuji, 2007).

Evidences suggest that the pharmacological effects of flavonoids and polyphenols are correlated with their antioxidant activity and their effects on human nutrition and health are considerable. The determination of their content seems to be much important to explore the phytochemical properties / activities of this species. The results revealed the highest flavonoid content in flower of

about  $51 \pm 0.18$  and  $10 \pm 0.63$ ,  $30 \pm 0.27$  mg/mL for root and stem, respectively. The rich abundance of flavonoids in flower in contrast with other parts of this species can be compared with the concept reported by Matkowski (2006). Root were found to contain the accumulation of high amounts of phenolics ( $90 \pm 1.08$  mg/mL) compared to flower ( $61 \pm 0.94$  mg/mL) and stem ( $70 \pm 0.79$  mg/mL) indicating the presence of number of hydroxylic groups in phenolic compounds. The increasing order of their contents is: flower > stem > root for flavonoids, and root > stem > flower for phenolics. The wide distribution of these antioxidant constituents in those parts reveals their therapeutic benefits.

Reducing power and DPPH- radical scavenging assay are well- established methods to assess the antioxidant properties. The radical scavenging activity (RSA) values were expressed as the ratio percentage of sample absorbance decrease and the absorbance of DPPH solution in the absence of extract at 517 nm. From the Figure 1 it can be analyzed that the scavenging effects of all the extracts on DPPH radicals increased with the increase in concentration and were excellent, especially in the case of flower (82.5% at 1 mg/mL) comparing with the standard (89% at the 1 mg/mL). The RSA values were also remarkably good for root (78.6%) and stem (79.35%) at 1 mg/mL. Moreover, the  $IC_{50}$  value calculated from the Figure 1 were 0.70  $\mu$ g/mL for flower, 0.72  $\mu$ g/mL for root and 0.80  $\mu$ g/mL for stem comparing with the standard (0.72  $\mu$ g/mL). From these values it can be inferred that the flower extracts blend with that of the standard activity to scavenge the DPPH radical, thereby

EM258 #1433 RT: 25.01 AV: 1 NL: 3.15E5  
F: + c Full ms [50.00-500.00]



**Figure 5.** Mass spectral data of Kaempferol.

showing its significant potency. Therefore, antioxidants, which can neutralize free radicals, may be of central importance in the prevention of diseases.

The principle behind the reducing power estimation is based on breaking the free radical chain by electron donating activity (reductones) which is an important mechanism of phenolic antioxidant action (Shimada et al., 1992). Measurement of reducing potential can reflect some aspects of antioxidant activity in the extracts.  $IC_{50}$  value obtained from Figure 2, again showed a striking correlation between the parts with the standard. 0.51  $\mu\text{g/mL}$  for flower, 0.49  $\mu\text{g/mL}$  for root, 0.50  $\mu\text{g/mL}$  for stem and 0.50  $\mu\text{g/mL}$  for standard. Also, there has been an increase in the absorbance for reducing power with the increase in the concentration. Even at the lower extract concentration, very good results were obtained. Very close  $IC_{50}$  values of the plant extracts with the standard BHA, shows their very strong efficiency of donating hydrogen atoms to terminate the free radicals.

Iron-induced lipid peroxidation is a well-validated system for generating reactive oxygen species (Gutteridge, 1995). It is a highly sensitive method, the results being fully dependent on efficient centrifugation to remove the precipitated protein, else it would lead to erroneous absorbance results. It can be easily understood from the Figure 3 that the capacity of inhibition of lipid peroxidation is proportional to the extract concentration. An equal  $IC_{50}$  values were obtained for the flower and standard, next stands the stem. Comparatively little lower  $IC_{50}$  value was obtained for root. This method permitted the achievement of very high inhibition percentages at extremely low concentrations. Hence, the

inhibition effect of the different concentration of the extracts revealed, indicated the ability of TBARS to retard the oxidation of lipids.

Identification of the peaks from GC spectrum was achieved by comparison of the retention times, UV spectra, as well as MS data of the separated compounds with the respective standards. The GC retention time sequence of the two flavonol aglycones kaempferol and quercetin was also the same as reported by Greenaway et al. (1990) and Sabatier et al. (1992). Kaempferol and Quercetin groups identified here may also be present in the form of glycosidic linkage. During hydrolysis, these high molecular glycosidic linkages breakup normally which would not be generally identified in GC-MS (Tokusoglu et al., 2003). Thus the isolated kaempferol and quercetin are converted into aglycones and then subjected to GC-MS to avoid the complications. The ions at  $m/z$  207 and 281 observed in the mass spectrum of kaempferol and quercetin are possibly due to fragmentation of the molecular ion resulting in  $[M-CH_2OH]^-$  and  $[M-OH]^-$  respectively.

The work herein indicates that the flowers presented the highest antioxidant activity values. Despite, it is not mentioned or concluded that the other parts that is, stem and root, were poor in their activities, but they showed a less good significant values comparatively.

## Conclusion

In conclusion, the results of the present work on *C. auriculata* gave the beneficial outcome of its therapeutic

EM258 #1698 RT: 29.08 AV: 1 NL: 7.40E5  
F: + c Full ms [50.00-500.00]

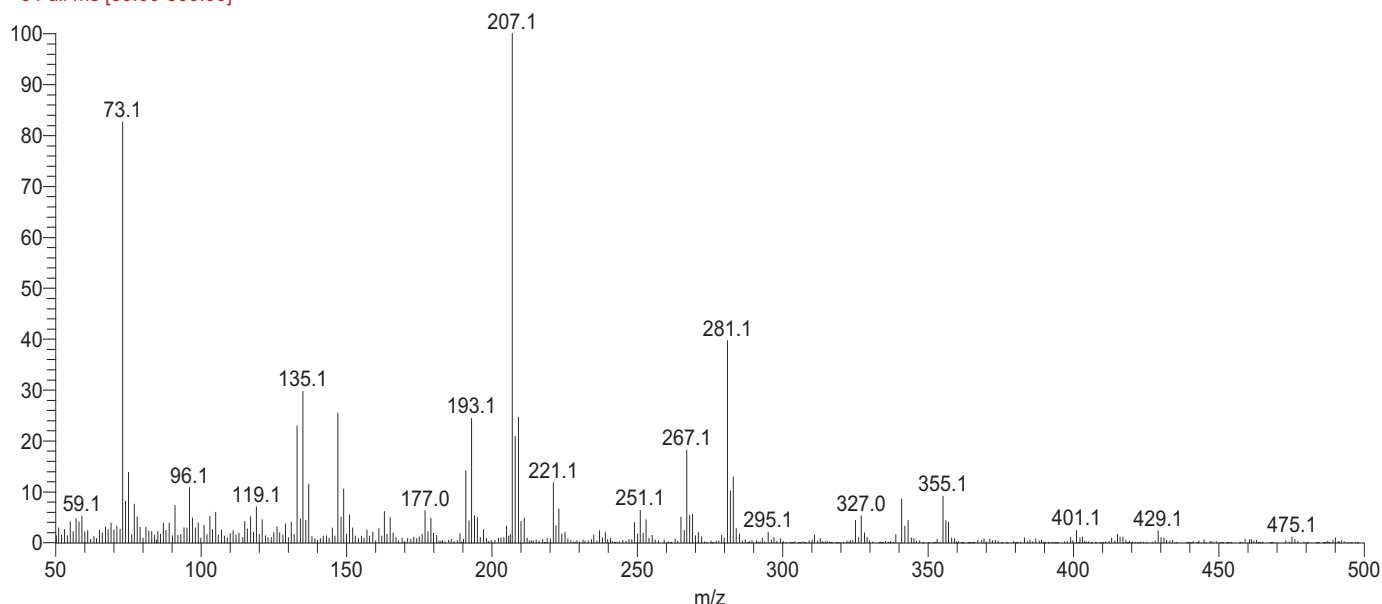


Figure 6. Mass spectral data of Quercetin.

applications and could be additionally ascribed to the strong antioxidant properties of its parts. On the other hand, the accumulation of high amount of phenolic compounds such as flavonoids in flowers would suggest the adaptive strategy of this species using antioxidants and the necessity to support this fact, several assays evaluated also showed a detailed activity / property profile. Kaempferol and Quercetin identified as a new source from the flower extracts would show its potential health benefits if administered as an antioxidant without any undesirable side effects. Thus *C. auriculata* would represent one of the best solution to prevent diseases being safe and economic, also would give an impact on more directed search of new natural sources of powerful antioxidants for therapeutic use.

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