

ANTIBACTERIAL & ANTIOXIDANT ACTIVITY OF FLAVANOID RICH FRACTION FROM THE PETALS OF *CASSIA AURICULATA* – AN *IN-VITRO* STUDY

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ABSTRACT

Cassia auriculata is a common plant and known for its various medicinal properties. The aim of the present study was to evaluate the invitro antimicrobial and antioxidant activity of flavonoid rich fraction of *C. auriculata* petals. The invitro antimicrobial activity was performed by disc diffusion and MIC method against *Staphylococcus aureus*, *Escherichia coli*, *Bacillus subtilis*, *Pseudomonas aeruginosa* and *Proteus vulgaris*. The antioxidant activity of rich flavonoid rich fraction from the petals of *C. auriculata* was tested using antioxidant assay techniques. The flavonoid rich fraction of *C. auriculata* petals exhibited antibacterial activity on *P. vulgaris* and *E. coli* significantly than the others. The flavonoid rich fraction of *C. auriculata* also showed effective antioxidant activity in all assay techniques. The present study indicated the petals of *C. auriculata* as a potential novel source with free radical scavenging compound and antibacterial properties.

Keywords: *Cassia auriculata*; Flavanoid; Antibacterial activity; Antioxidant activity.

INTRODUCTION

Medicinal plants are rich source of antimicrobial and antioxidant agents. The studies of medicinal plants attracted many researchers, and the plants have great potential in treating human diseases such as cancer, coronary heart diseases, diabetes and infectious diseases. About 25% of prescribed drugs in the world originate from plants [1] and over 3000 species of plants have been reported to have both antibacterial and anticancer properties [2]. Apart from being an antibacterial few medicinal plants also exhibit antiparasitic activity [3]. Antibacterial resistance has become a global problem. Approximately 20% of the plants have been submitted to pharmacological tests, and a number of new antibiotics introduced to the market are obtained from natural or semi-synthetic resources. According to World Health Organization (WHO), more than 80% of the world's population relies on medicinal plants for their primary health care needs and also for the treatment of infectious diseases [4].

Oxidative stress plays a major role in the development of chronic and degenerative disorders such as atherosclerosis, hypertension, diabetes mellitus, ischemic diseases, rheumatoid arthritis, cataract and malignancies [5, 6]. The antioxidants have been widely used as food additives. The most commonly used antioxidants are butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), tertiary butyl hydroquinone, propylgallate and ascorbic acid (ASA). However, BHA and BHT have been suspected of being responsible for liver damage and carcinogenesis [7]. Therefore, a need for identifying alternative non-toxic natural antioxidants could serve as good candidates for the development of standardized phytomedicine. Several compounds like flavanoids, alkaloids, terpenoids, glycosides, carotenoids & steroids have strong antioxidant or free radical scavenging properties which protect human body from chronic and neurodegenerative diseases. It is reported that phenolic compounds in plants possess strong antioxidant activity and may help to protect cells against the oxidative damage caused by free radicals.

The genus *Cassia auriculata* belongs to the family Caesalpiniaceae and are traditionally used for curing many diseases. Many therapeutic uses and medicinal properties are reported in whole plant. They are known for anti hyperglycemic activity [8], hepatoprotective activity [9], antihelmintic activity [10], antimicrobial activity [11] and antioxidant activity [12]. The dried flowers and flower buds are used as substitute for tea in case of diabetic patients. It is also believed to improve complexion in women. The powdered seed is also used in the treatment of leprosy,

skin and liver diseases [13]. The purpose of the study is to determine the antimicrobial and antioxidant potential of flavanoid rich fraction of petals of *Cassia auriculata*.

MATERIALS AND METHODS

Collection of plant material

The petals of *Cassia auriculata* were collected from the Sri Sairam Siddha Medical College and Research Centre, Herbal garden, Tamilnadu, India. The plant was identified with the help of available literature and authenticated by Dr. S. Sankaranarayanan, Head of the Department, Department of Medicinal Botany, Sri Sairam Siddha Medical College, West Tambaram, Chennai. The petals of the plant were washed with tap water followed by distilled water, dried in shade for 10 days prior to study and then stored in airtight glass jars, until in use.

Preparation of extract

The shade dried petals was ground to fine powder and sieved. Exactly 20g of the finely grounded petals were soaked in 70% methanol at room temperature for 24 hrs. The extract was filtered using Whatman filter paper No.1 and then concentrated in vacuum at 40°C- 50°C (overnight) using a rotary evaporator. The residue was mixed with various solvents such as Petroleum ether, ethyl acetate, chloroform and DMSO (Dimethyl sulphoxide), Methanol. These extracts were subjected to quantification of flavanoid.

Estimation of flavanoid

A 1ml aliquot of different extracts was placed into a 25 ml measuring flask. To this sample, 1ml of 2% aluminium chloride and 0.5 ml of 33% acetic acid was added, after which the flask is filled with 90% methanol to the mark and the content is thoroughly stirred. The obtained solution is allowed to stand for 30 minutes and the absorbance was measured at 414 nm using a UV-Visible Spectrophotometer. Rutin was used as a standard.

Total phenolic content of flavanoid rich fraction

The total phenolic content in the flavanoid rich fraction of *C. auriculata* was determined spectrophotometrically with Folin-Ciocalteu reagent using the modified method [14]. To the flavanoid extract, 1 ml of 50% Folin-Ciocalteu reagent and 1 ml of 2% sodium carbonate were added and mixed thoroughly. The mixture was centrifuged at 10,000 rpm for 10 minutes. The upper phase of the solution was incubated at room temperature for 30 minutes. The absorbance was measured at 750 nm using a UV-Visible

Spectrophotometer. Gallic acid was used as a standard. The maximum flavanoid and phenolic content yielding extract was observed and selected for further studies.

Bacterial strains

Bacteria used for the determination of antibacterial activities of isolated compounds were Gram positive; *Staphylococcus aureus* MTCC 29213, *Bacillus subtilis* MTCC441, Gram negative; *Escherichia coli* MTCC 25922, *Pseudomonas aeruginosa* MTCC 2488, *Proteus vulgaris* MTCC 1771. All bacterial strains were obtained from Microbial Type Culture Collection and Gene Bank, Institute of Microbial Technology Sector 39-A, Chandigarh-160036, India. All bacterial strains were sub cultured on nutrient agar medium, incubated at 37°C for 24 hrs and stored at 4°C in refrigerator to maintain stock culture.

Antibacterial assay

Antibacterial activity was carried out using disc diffusion method [15]. Petriplates were prepared with 20 ml of sterile nutrient agar (HIMEDIA). The tested cultures were swabbed on top of the solidified media and allowed to dry for 10 minutes. The crude extract impregnated discs (Whatman No.1 filter paper was used to prepare discs) were prepared and air dried well. The test was conducted at four different concentrations of the crude extract (5, 10, 15 & 20 µl/ml) with 3 replicates. The loaded discs were placed on the surface of the medium and incubated at room temperature for 24 hrs. The relative susceptibility of the organisms to the crude extract indicated by the clear zone of inhibition around the discs, were observed, measured and recorded in millimeters.

Minimum inhibitory concentration

The minimum inhibitory concentration was determined according to method [16]. The different concentration of the flavanoid rich fraction (5-20µl/ml) was mixed with 0.5 ml bacterial cultures were incubated at 37°C for 18 hrs and OD was measured spectrophotometrically at 580 nm.

Reducing power determination of flavanoid rich fraction:

The reducing power of the flavanoid rich fraction was determined by spectrophotometric method [17]. The flavanoid rich fraction (5-20 µl) was mixed with 2.5 ml of 0.2 M Potassium phosphate buffer (pH-6.6) and 2.5 ml of 15 Potassium ferricyanide [K₃Fe(CN)₆]. The mixture was incubated at 50°C for 20 minutes, then rapidly cooled, mixed with 2.5 ml of trichloroacetic acid and centrifuged at 5000 rpm for 3 minutes. An aliquot (2.5ml) of supernatant was diluted with distilled water (2.5ml) and 0.5 ml of 1% Ferric chloride was added and allowed to stand for 10 minutes. The absorbance was read spectrophotometrically at 700 nm. Increased absorbance indicates increased reducing power. Vitamin C was used as positive control.

Metal chelating activity of flavanoid rich fraction

The chelation of ferrous ions of flavanoid rich fraction was estimated by the method [18]. The different concentration of flavanoid rich fraction (5-20 µl) was mixed with 0.05 ml of 2 mM FeCl₃ followed by the addition of 0.2 ml of 5 mM Ferrozine. The mixture was then shaken vigorously and left standing at room temperature for 10 minutes. Absorbance levels of the solutions were measured using spectrophotometer at 562 nm. The percentage of inhibition of ferrozine- Fe²⁺ complex formation was calculated using the formula given below:

$$\% \text{ Inhibition} = \frac{[A_0 - A_s]}{A_s} \times 100$$

Antioxidant activity in hemoglobin induced linoleic acid of flavanoid rich fraction

The antioxidant activity of flavanoid rich fraction was carried out by following the procedure [19]. The flavanoid rich fraction (5-20 µl) was mixed with 1 ml of 1 mmol/l of Potassium phosphate buffer

(pH-6.5) followed by the addition of 20 µl of 0.0016% hemoglobin was shaken vigorously. The mixed solution was incubated at 37°C for 45 minutes. After incubation, 2.5 ml of 0.6% HCl in ethanol was added and mixed thoroughly to stop the lipid peroxidation. Then, 100 µl of 0.02 mol/l FeCl₃ and 100 µl of ammonium thiocyanate (15g/50ml) was added and vortexed thoroughly. The total antioxidant activity determination was performed in triplicate using the thiocyanate method by reading the absorbance at 480 nm.

ABTS assay

ABTS radical scavenging activity of flavanoid rich fraction was determined according to method [20]. ABTS radical was freshly prepared by adding 5 ml of a 4.9 mM potassium persulfate solution to 5 ml of a 14 mM ABTS solution and kept for 16 h in dark. This solution was diluted with distilled water to yield an absorbance of 0.70 at 734 nm and the same was used for the antioxidant assay. The final reaction mixture of standard group was made up to 1 ml with 950 µl of ABTS solution and 50 µl of Vitamin C. Similarly, in the test group 1 ml reaction mixture comprised 950 µl of ABTS solution and 50 µl of the extract solutions. The reaction mixture was vortexed for 10 sec and after 6 min absorbance was recorded at 734 nm against distilled water by UV-Vis Spectrophotometer and compared with the control ABTS solution. Ascorbic acid was used as reference antioxidant compound.

Scavenging of hydrogen peroxide activity of flavanoid rich fraction

The hydrogen peroxide scavenging assay was carried out by using the method [21]. The hydrogen peroxide (40mM) was prepared in 0.1 M Potassium phosphate buffer (pH-7.4). The different concentration of flavanoid rich fraction (5-20 µl) was mixed with 0.6 ml of Hydrogen peroxide solution (40mM). The absorbance value of the reaction mixture was recorded at 230 nm. Blank solution contains Potassium phosphate buffer without H₂O₂. The percentage of H₂O₂ scavenging of crude extract and standard compounds was calculated as;

$$\text{H}_2\text{O}_2 \text{ Scavenging effect (\%)} = \frac{[1 - A_s/A_c]}{A_c} \times 100$$

RESULT AND DISCUSSION

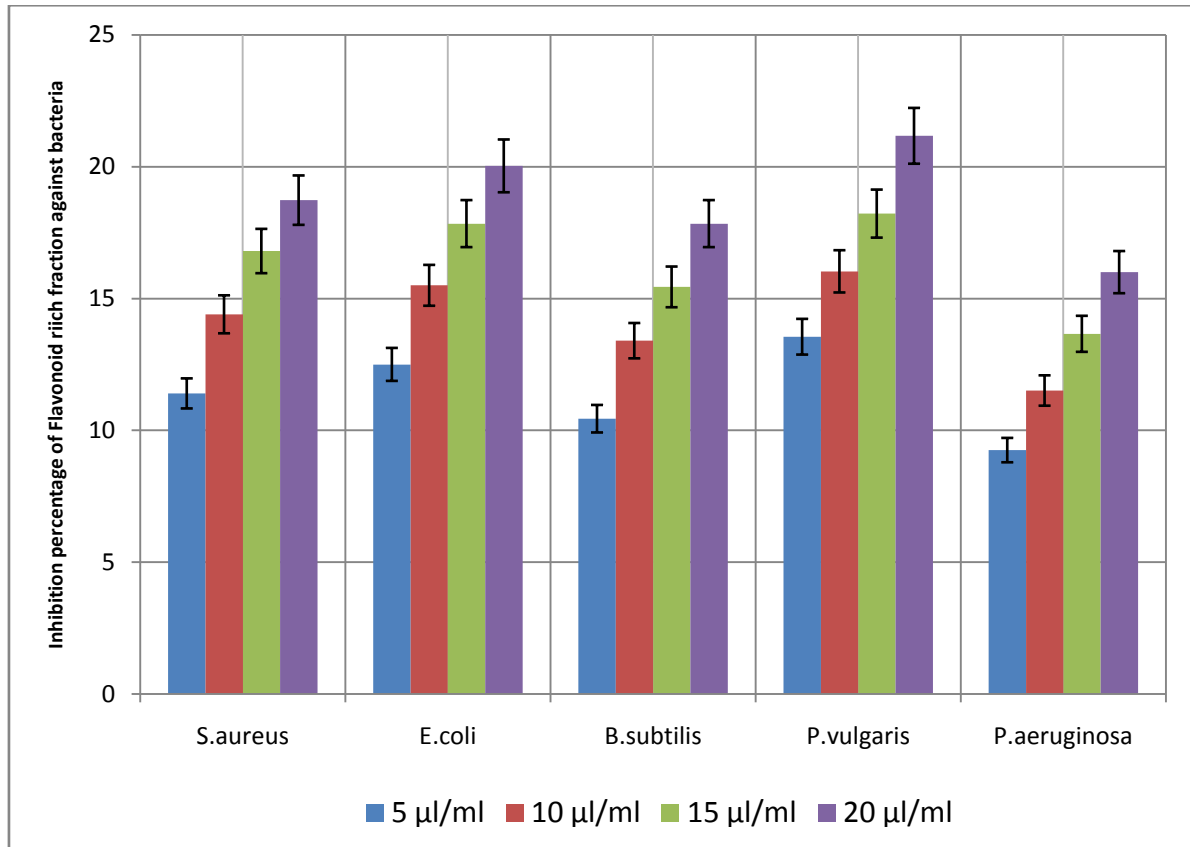
Antibacterial activity of flavanoid rich fraction of *Cassia auriculata* petals

The antibacterial activity of flavanoid rich fraction of *C. auriculata* petal at different concentration was screened by disc diffusion technique and the zone of inhibition was measured in mm diameter (Table 1). The flavanoid rich fraction was more effective against *P. vulgaris* and *E.coli* with a zone of inhibition percentage of 21.17 and 20.03 and was least effective against *P. aeruginosa*, *B. subtilis* and *S. aureus* with zone of inhibition percentage of 16, 17.84 and 18.73 respectively at the concentration of 20µl/ml (Graph-1). The flavonoid rich fraction of *C. auriculata* petal showed the maximum inhibitory activity at the highest concentration (20 µl/ml) than the lowest concentration (5 µl/ml) against gram negative bacteria such as *E. coli*, *P. vulgaris*, *P. aeruginosa* and gram positive *B. subtilis* and *S. aureus* (Table-2). The present findings corroborate with the report of minimum inhibitory concentrations of the flavonoid fractions from *Citrus bergamia* peel and the pure flavonoids, neohesperidin, hesperetin (aglycone), neoeriocitrin, eriodictyol (aglycone), naringin and naringenin (aglycone), were found to be in the range 200 to 800 mg/ml [21]. The petals of *C. auriculata* showed a broad spectrum of antimicrobial activity suggesting that the petals possess certain constituents with antibacterial properties that can be used as antimicrobial agents in designing drugs for infectious diseases caused by pathogens. The medicinal properties and pharmacological actions of *C. auriculata* is well used in traditional medicine. The plant *C. auriculata* contains many therapeutic uses and has certain biological activity against number of infectious diseases [22].

Table 1: Antibacterial activity of flavanoid rich fraction of *Cassia auriculata* petals

Flavanoid rich fraction Concentration ($\mu\text{l/ml}$)	Zone of inhibition [in mm diameter] ^a				
	<i>S. aureus</i>	<i>E. coli</i>	<i>B. subtilis</i>	<i>P. vulgaris</i>	<i>P. aeruginosa</i>
5	10.3 \pm 0.75	11.3 \pm 0.75	9.4 \pm 0.65	12.2 \pm 0.91	8.3 \pm 0.70
10	12.9 \pm 0.45	14 \pm 0.40	12.06 \pm 0.40	14.43 \pm 0.60	10.3 \pm 0.77
15	15.1 \pm 0.47	16.06 \pm 0.60	13.9 \pm 0.45	16.4 \pm 0.79	12.3 \pm 0.81
20	16.8 \pm 0.41	18.03 \pm 0.55	16.06 \pm 0.50	19.06 \pm 0.40	14.4 \pm 0.65

^a The antimicrobial activity was determined by measuring the diameter of zone of inhibition that is the mean of triplicates \pm SD of three replicates.

Fig. 1: Inhibition percentage of flavanoid rich fraction of *Cassia auriculata* petal against pathogenic bacteriaTable 2: Minimum inhibitory concentration of flavanoid rich fraction of *Cassia auriculata* petals against pathogenic Bacteria

Flavanoid rich fraction Concentration ($\mu\text{l/ml}$)	Optical density at 580 nm ^a				
	<i>S. aureus</i>	<i>E. coli</i>	<i>B. subtilis</i>	<i>P. vulgaris</i>	<i>P. aeruginosa</i>
Positive control	0.533 \pm 0.01	0.57 \pm 0.01	0.62 \pm 0.06	0.58 \pm 0.006	0.61 \pm 0.009
5	0.42 \pm 0.008	0.50 \pm 0.008	0.49 \pm 0.011	0.46 \pm 0.008	0.57 \pm 0.011
10	0.33 \pm 0.011	0.42 \pm 0.008	0.43 \pm 0.008	0.37 \pm 0.012	0.52 \pm 0.008
15	0.28 \pm 0.011	0.36 \pm 0.005	0.36 \pm 0.008	0.30 \pm 0.008	0.484 \pm 0.005
20	0.230 \pm 0.009	0.27 \pm 0.008	0.25 \pm 0.011	0.23 \pm 0.009	0.37 \pm 0.008

^aThe minimum inhibitory concentration was determined by optical density of inhibition that is the mean of triplicates \pm SD of three replicates.

Total phenolics and flavanoid content

In the present study, the phenolics content of petals of *C. auriculata* was found to be 72 % of catechol equivalents, while the flavanoid content was 68 % of quercetin equivalents. The obtained level of phenolics and flavanoid in *C. auriculata*, accordance with phenolics and flavanoid of *Cassia alata*. Phenolics and flavanoids are major group of secondary metabolites that are commonly found in plants and have multiple biological effects which include choleric and diuretic functions, decreasing blood pressure, reducing the viscosity of blood & stimulating intestinal peristalsis as well as antioxidant activity or free radical scavenging activities [23].

Antioxidant Assays

Reducing power assay of flavanoid rich fraction of *Cassia auriculata* petals

The reducing power assay, exhibited the presence of antioxidants in the extract, which resulted in the reduction of Fe^{3+} to Fe^{2+} by donating an electron. The maximum reducing property was found at 20 $\mu\text{l/ml}$ of flavanoid rich fraction of petals of *C. auriculata* (Fig-2). The reducing power of flavanoid rich fraction increased gradually in concentration dependent manner. The phenolic antioxidants usually scavenge free radicals by an electron-transfer mechanism [24]. The

reducing power capacity of extract may serve as a significant indicator of its potential antioxidant activity.

Metal chelating activity of flavanoid rich fraction of *Cassia auriculata* petals

In metal chelating assay, the absorbance of Fe²⁺ ferrozine complex decreased dose dependently. The maximum metal chelating activity

was found at 20µl/ml of flavanoid rich fraction of petals of *C. auriculata*. The result proved the flavanoid rich fraction possessed Fe²⁺ chelating activity & may play a protective role against oxidative damage induced by metal catalyzed decomposition reactions [25]. Ferrous iron can initiate lipid per oxidation by the fenton reaction as well as accelerating per oxidation by decomposing lipid hydroperoxides in to peroxy & alkoxy radicals [26].

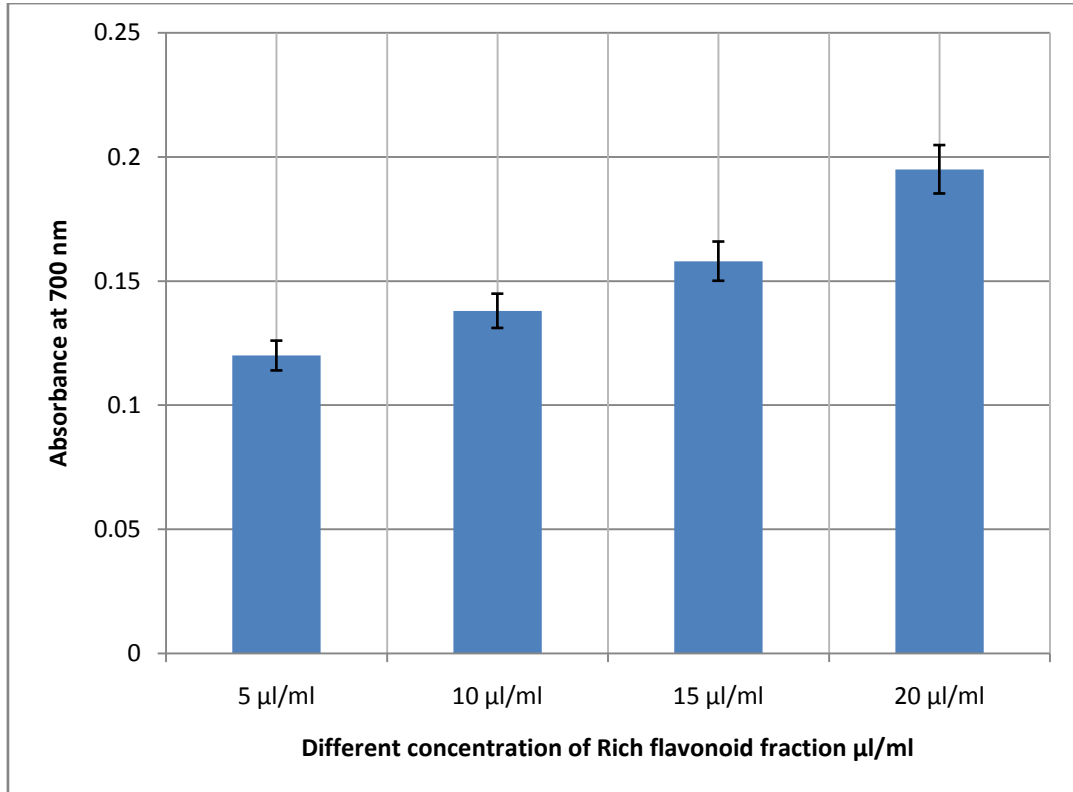


Fig. 2: Reducing power assay of flavanoid rich fraction of *Cassia auriculata* petals

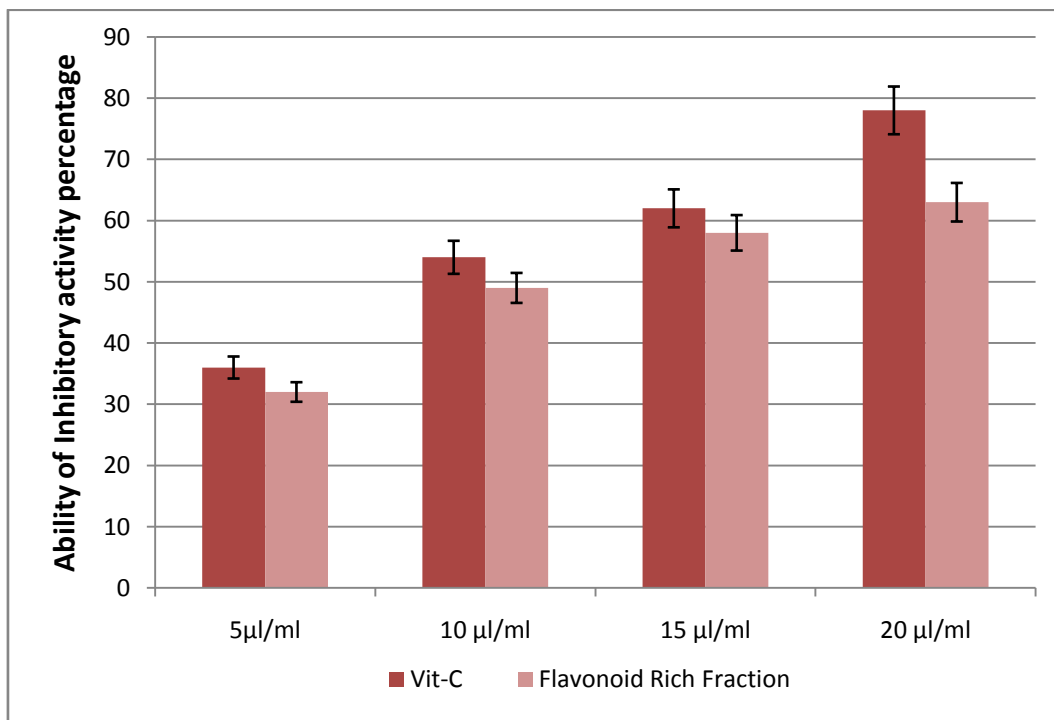


Fig. 3: Antioxidant activity in a hemoglobin-induced linoleic acid of flavanoid rich fraction of *C. auriculata* petals

Table 3: Metal chelating activity of flavanoid rich fraction of *Cassia auriculata* petals

Flavanoid rich fraction ($\mu\text{l/ml}$)	$^a\text{Fe}^{2+}$ chelating activity
5 $\mu\text{l/ml}$	64.55 \pm 6.83
10 $\mu\text{l/ml}$	80.16 \pm 6.58
15 $\mu\text{l/ml}$	101.28 \pm 7.31
20 $\mu\text{l/ml}$	129.28 \pm 9.75

^a Results are expressed as % inhibition of Fe^{2+} chelating with respect to control. Each value represents Mean \pm SD

Antioxidant activity in hemoglobin induced linoleic acid of flavanoid rich fraction of *Cassia auriculata* petals

The antioxidant activity of flavanoid rich fraction of petals of *C. auriculata* was determined by the method [27] using hemoglobin induced linoleic acid system. The maximum inhibitory activity was 63% in 20 $\mu\text{l/ml}$ of flavanoid rich fraction of *C. auriculata* petals, which was lower percentage than vitamin-C (Fig-3). The extracts showed a rapid and concentration-dependent increase of antioxidant activity. The high reducing power indicated the compounds present in the extract were electron donors and could react with free radicals to convert them into more stable products & to terminate radical chain reaction. Antihemolytic activity and the relationship between iron ion chelating activity and protective activity against oxidative damage to erythrocyte membrane by quercetin have been reported previously [28]. It seems that high total phenol and flavonoid contents in the extract led to its potent antihemolytic activity.

Flavanoid rich fraction of *Cassia auriculata* petals expressed as capacity to bleach the stable ABTS radical.

The free-radical scavenging activity of *C. auriculata* flavanoid rich fraction was also tested by their ability to bleach the stable ABTS radical. This assay provided information on the reactivity of test compounds with a stable free radical. Because of its odd electron, ABTS gave a strong absorption band at 517 nm in visible spectroscopy. As this electron becomes paired of in the presence of a free-radical scavenger, the absorption vanishes, and the resulting decolorization was stoichiometric with respect to the number of electrons taken up. *C. auriculata* flavanoid rich fraction showed a significant and dose dependent ABTS quenching capacity. Higher concentration of *C. auriculata* flavanoid rich fraction 20 $\mu\text{l/ml}$ was more efficient concentration to bleach the stable ABTS radical (Table-4). The previous result has shown the antioxidant potential of the total ethanol and methanol extracts of *C. auriculata* flowers. The antioxidant activity was determined by an improved assay based on the decolorization of the radical monocation of 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging method, which supports our findings [29].

Table 4: Flavanoid rich fraction expressed as capacity to bleach the stable ABTS radical.

Flavanoid rich fraction $\mu\text{l/ml}$	Free radical-scavenging activity ^a
5 $\mu\text{l/ml}$	22.1 \pm 2.5
10 $\mu\text{l/ml}$	47.6 \pm 2.5
15 $\mu\text{l/ml}$	69.5 \pm 3.7
20 $\mu\text{l/ml}$	89.3 \pm 4.4

^aResults are expressed as percentage radical-scavenging activity with respect to control. Each value represents the mean \pm SD of five experiments.

Scavenging of hydrogen peroxide activity of flavanoid rich fraction of *Cassia auriculata* petals

The hydrogen peroxide scavenging assay evidently showed the flavanoid rich fraction of petals of *C. auriculata* was capable of scavenging H_2O_2 in a concentration dependent manner. The scavenging activity of H_2O_2 increased with increasing concentration. Hydrogen peroxide has strong oxidizing properties and can be formed *in vivo* by many oxidizing enzymes such as superoxide dismutase and can cross cellular membranes and may slowly oxidize

a number of intracellular compounds. The present result findings agree with previous data in which aqueous fraction of *Artemisia tschernieviana* containing higher phenol and flavonoid contents, scavenged hydrogen peroxide showed better activity than control group [30].

Table 4: Determination of scavenging of hydrogen peroxide activity of flavanoid rich fraction of *Cassia auriculata* petals

Flavanoid rich fraction ($\mu\text{l/ml}$)	^a Hydrogen peroxide scavenging activity %
5 $\mu\text{l/ml}$	25.15 \pm 5.70
10 $\mu\text{l/ml}$	36.62 \pm 5.28
15 $\mu\text{l/ml}$	45.60 \pm 4.64
20 $\mu\text{l/ml}$	53.06 \pm 3.88

^a Results are expressed as % of scavenging of hydrogen peroxide activity with respect to control. Each value represents Mean \pm SD

CONCLUSION

The natural antioxidant compounds of plant extracts will help to develop new drugs. The results indicated that flavanoid rich fraction of *Cassia auriculata* petals possessed abundant phenolic & flavanoid content and exhibited excellent antioxidant anti bacterial activities and might be useful in treating the bacterial disease associated with oxidative stress.

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