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Original Article

COMPARATIVE ASSESSMENT OF ANTIOXIDANT POTENTIAL OF *CASSIA AURICULATA* (LINN.) FLOWER, LEAF AND SEED METHANOLIC EXTRACTS

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ABSTRACT

Objective: The present study aimed to investigate the antioxidant potential of the methanolic extract of flowers, leaves and seeds of *Cassia auriculata* (Linn.) along with the quantification of total phenolics and flavonoids content.

Methods: The antioxidant activity of flowers, leaves and seeds of *Cassia auriculata* (Linn.) was assessed with the help of various *in vitro* antioxidant assay systems as 2,2-Diphenyl-1-picrylhydrazyl (DPPH) scavenging assay, reducing power assay and Nitric oxide scavenging assay. For the determination of phenolic content the Folin Ciocalteu method and for flavonoids Aluminium Chloride spectrophotometric method was adopted.

Results: The flowers, leaves and seeds methanolic extracts of *Cassia auriculata* (Linn.) were screened for different phyto chemicals present and the major antioxidants polyphenols and flavonoids were quantified. All the extracts showed significant antioxidant activity in all assays with the same trend as in total phenolics and flavonoids content. Flowers show most antioxidant activity in all assay systems due to higher phenolics and flavonoids content. That indicated the direct correlation among antioxidant potential, total phenolics and flavonoids content.

Conclusion: The result implies that the *Cassia auriculata* (Linn.) plant parts especially flowers can be serving as natural sources of antioxidants and could be used in the treatment of diseases that have free-radical origin and as a substitute for synthetic drugs.

Keywords: *Cassia auriculata* (Linn.), Antioxidant potential flower, Leaf, Seed extracts.

INTRODUCTION

Free radicals are known to play an important role in the origin of life and biological evolution implicating their beneficial effects on the organism. Free radicals are highly reactive chemical species regularly produced in the human system by normal biological reactions and by various exogenous factors. Many environmental pollutants are oxidants or catalyze the production of reactive oxygen species (ROS) directly or indirectly [1]. The equilibrium between the production and the scavenging of ROS may be perturbed by various biotic and abiotic stress factors such as salinity, UV radiation, drought, heavy metals, temperature extremes, nutrient deficiency, air pollution, herbicides and pathogen attacks. These disturbances in equilibrium lead to the sudden increase in intracellular levels of ROS that can cause significant damage to cell structures [2].

Antioxidants are radical scavengers which protect the human body against free radicals which may cause pathological conditions such as cancer, cataract, coronary heart disease, stroke, rheumatoid arthritis, diabetes, Alzheimer's disease and ageing process [3]. Recently, there has been a considerable interest in finding the natural antioxidants from plant materials to replace synthetic ones. Plant phyto chemicals not only counteract free radical induced oxidative stress but also overcome the side effects of synthetic antioxidants [4]. The antioxidant property of plants is due to the presence of active constituents like phenolics, flavonoids, terpenoids, carotenoids, phytoestrogens, minerals, anthrocyanins, coumarins, lignans, catechins and isocatechins. In addition to these compounds, vitamins C and E, beta carotene and tocopherol are also known to possess antioxidant potential [5, 6]. With this background and abundant source of unique active components harbored in plants, the present study was taken up on medicinal plant namely of *Cassia auriculata* Linn

Cassia auriculata (Linn.) is a common Indian medicinal plant belonging to family Caesalpiniaceae and well known for its phytochemical compositions [7, 8], pharmacological applications and therapeutic potential [9]. Each part of this plant has medicinal value. The leaves are anthelmintic and good for ulcers, skin diseases and leprosy [10]. The seeds are believed to be refrigerant and alexipharmic. They are used in chronic purulent ophthalmic and conjunctivitis, cough, asthma, gout, gonorrhoea, dysentery and diabetes. The flowers are used in throat troubles, urinary disorders and as astringent [11]. An aqueous extract of the leaves and flower possesses hypoglycemic activity. Flowers also showed the hyperlipidimic activity in Triton induced hyperlipidimic rats [12]. Recently it has been reported as a potent anticancer herb [13]. *Cassia* species are rich sources of polyphenols, anthraquinone derivatives, flavonoids and polysaccharides [14]. Many works already explained the antioxidant properties of the various extracts of *Cassia auriculata* [15-19].

With renewing interest, the present study comprises of the comparative assessment of an antioxidant potential of flowers, leaves and seeds of *Cassia auriculata* Linn. along with the quantification of total phenolics and flavonoids which are the important carriers of antioxidant activity that can help better on those Ethnomedicinal and Pharmacological applications, for their use and most effective new formulation aspects for future research.

MATERIALS AND METHODS

Chemicals and reagents

All the chemicals and solvents used were of analytical grade. Gallic acid, Sodium Carbonate, Potassium acetate, 2,2-Diphenyl-1-picrylhydrazyl (DPPH), Potassium ferricyanide, ferric chloride, Ascorbic acid, Sulphanilamide, Naphthyl-ethylene-diamine dichloride and Methanol were purchased from HiMedia, Mumbai, India. Aluminium chloride and Folin–Ciocalteau reagent purchased from SD Fine Chemicals limited, Mumbai, India. Trichloroacetic acid and Phosphoric acid were obtained from Qualigens fine chemicals, India. Spectrophotometric measurements were carried out using UV-Vis Spectrophotometer (UV mini-1240, SHIMADZU, India).

Collection and authentication of plant *Cassia auriculata*

Cassia auriculata was collected from the local medicinal flora of Amravati district, Maharashtra. The herbarium of the plant was

prepared, authenticated and the voucher specimen was submitted to Botanical Survey of India (BSI), Pune. (BSI/WRC/Tech./2013/203).

Preparation of sample

The collected plant was separated into flowers, leaves and seeds. Each part was cleaned and dried at room temperature. The dried plant parts were ground separately using an electric grinder to fine powder. The powder was stored in airtight containers until analysis.

Preparation of extract

The extract was prepared using ultrasound assisted extraction method as it is an inexpensive, simple, easier and efficient alternative to conventional extraction techniques. For ultrasound-assisted extraction, 500 mg of the powdered flower sample of *Cassia auriculata* was mixed with 15 ml of 80% methanol in a polypropylene centrifuge tube. An ultra sonicator probe horn was fitted into the sample tube with its tip immersed into the solvent. The ultrasonicator used was SONICS Vibracell that has a frequency of 20 kHz and a maximum power of 130 Watt that was operated at 50% pulse for 20 minutes. The procedure of ultrasonic extraction of plant material was repeated three times in the same way and extract was pooled together.

Phytochemical analysis of plant extract

The most effective components responsible for antioxidant potential seem to be flavonoids and phenolic compounds of many plants raw materials. Their metal chelating capabilities and radical scavenging properties have enabled phenolic compounds to be thought of as effective free radical scavengers and inhibitors of lipid peroxidation [20].

Estimation of total phenolic content

Antioxidant compounds generally contain phenolic group(s) and hence, the amounts of total phenolic compounds in the extracts of the flowers, leaves and Seeds were estimated by using Folin–Ciocalteau reagent with Gallic acid as standard. The total phenolic content was estimated as previously described by Singh *et al.* [21]. The aliquot of the extract was taken and made up to the volume of 1 ml with distilled water. Then 0.5 ml of Folin-Ciocalteu reagent (1:1 with water) and 2.5 ml of sodium carbonate solution (20%) was added sequentially to the test tube. Soon after overtaxing the reaction mixture, the tubes were placed in the dark for 40 min. and the absorbance was recorded at 725 nm using UV-Vis Spectrophotometer against the reagent blank. A standard curve was prepared using Gallic acid. The linearity obtained was in the range of 1-10 µg/ml. Using the standard curve, the total phenolic content was calculated and expressed as Gallic acid equivalent in mg/g of extract.

Estimation of total flavonoids content

The antioxidant activity of medicinal plants could be attributed to its flavonoid content. Flavonoids act as scavengers of various oxidizing species i.e. superoxide anion, hydroxyl radical or per-oxy-radicals, they also act as quenchers of singlet oxygen [22]. The Aluminum chloride colorimetric method was used to determine the total flavonoids content in *Cassia auriculata* plant extract using Quercetin as standard. The total flavonoids content was estimated according to the method of Chang *et al.* [23]. Briefly, 0.5 ml solution of extract in methanol was mixed with 1.5 ml of methanol, 0.1 ml of 10% aluminum chloride, 0.1 ml of 1 M potassium acetate, and 2.8 ml of distilled water, and left at room temperature for 30 min. The absorbance of the reaction mixture was measured at 415 nm with the help of UV-Visible spectrophotometer. The calibration curve was prepared by preparing Quercetin solutions at concentrations 10 to 100 µg/ml in methanol.

Antioxidant activity

The antioxidant potential of the plant extract was evaluated with the help of DPPH radical scavenging assay, Nitric oxide scavenging assay and reducing power assay.

DPPH radical scavenging assay

The anti-oxidant potential of a compound can be determined on the basis of its scavenging activity of the stable 2, 2-diphenyl-1-picrylhydrazyl (DPPH) free radical. DPPH radical scavenging activity of flower, leaves and seeds extracts of *Cassia auriculata* L. was determined according to the method reported by Blois [24]. An aliquot of 0.5 ml of sample solution in methanol was mixed with 2.5 ml of 0.3 mM methanolic solution of DPPH. The mixture was shaken vigorously and incubated for 37 min in the dark at room temperature. The absorbance was measured at 517 nm using UV-VIS spectrophotometer. Ascorbic acid and Quercetin were used as a positive control.

DPPH free radical scavenging ability (%) was calculated by using the formula.

$$\% \text{ Inhibition} = \frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}} \text{ X } 100$$

Reducing power assay

The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity. Substances, which have reduction potential, react with potassium ferricyanide (Fe3+) to form potassium ferrocyanide (Fe2+), which then reacts with ferric chloride to form ferric ferrous complex that has an absorption maximum at 700 nm. The reducing power of plants' extract of *Cassia auriculata* was determined according the method previously described by Oyaizu [25]. According to this method, the aliquot of various concentrations of the standard and test plant extracts (10 to 100µg/ml) in 1.0 ml of de ionized water were mixed with 2.5 ml of (pH 6.6) phosphate buffer and 2.5 ml of (1%) potassium ferricyanide. The mixture was incubated at 50 °C in the water bath for 20 min. After cooling, aliquots of 2.5 ml of (10%) trichloroacetic acid were added to the mixture, which was then centrifuged at 3000 rpm for 10 min. 2.5 ml supernatant of the solution was mixed with 2.5 ml distilled water and a freshly prepared 0.5 ml of (0.1%) ferric chloride solution. The absorbance was measured at 700 nm in a UV-visible spectrophotometer. A blank was prepared without adding extract. Ascorbic acid and Gallic acid at various concentrations (10 to100 µg/ml) were used as standards. An increase in absorbance of the reaction mixture indicates the increase in reducing power.

Nitric oxide scavenging assay

Nitric oxide (NO) was generated from sodium nitroprusside (SNP) and was measured by the Griess reagent. SNP in aqueous solution at physiological pH spontaneously generates NO which interacts with oxygen to produce nitrite ions that can be estimated by the use of Griess Reagent. Scavengers of NO compete with oxygen leading to reduced production of NO [26]. SNP (10 mM) in phosphate buffer saline (PBS) was mixed with different concentration of extracts (20-100µg/ml) and incubated at 25 °C for 180 minutes.

The samples from the above were reacted with Griess reagent (1% sulphanilamide, 0.1% naphthyl-ethylene-diamine dichloride and 3% phosphoric acid). The absorbance of the chromophores formed during the diazotization of nitrite with sulphanilamide and subsequent coupling with naphthyl-ethylene-diamine-dichloride was read at 546 nm. Inhibition of nitrite formation by the plant extracts and the standard antioxidant ascorbic acid were calculated relative to the control.

$$\text{Nitric Oxide scavenged (\%)} = \frac{\text{A control} - \text{A test}}{\text{A control}} \text{ X } 100$$

Where, A control = Absorbance of control reaction and

A test = Absorbance in the presence of the samples of extracts/Ascorbic acid.

IC_{50} value of the extracts

The antioxidant activity of the extract was expressed as IC_{50}. The IC_{50} value was defined as the concentration (in) µg/ml of extracts that inhibits the formation of DPPH radicals by 50%. IC50 was calculated for all the extracts by plotting the percentage of DPPH radicals/NO scavenged versus the concentration of extract. Lower the IC_{50} value, higher the radical scavenging effect.

Statistical analysis

The experimental results were expressed as mean±standard deviation (SD) of three replicates. All statistical calculations and data

analysis were carried out using GraphPad Prism5 software program (GraphPad Software Inc., La Jolla, CA, USA). One way analysis of variance (ANOVA) followed by Tuckey's post-test analysis was used for comparison of the means. Differences among means were considered as to be significant at P<0.05.

RESULTS AND DISCUSSION

The significant correlation was observed in many studies between the phenolics contents and antioxidant activity [27, 28], the present study was conducted on the determination of antioxidant potential in flowers, leaves and seeds of *Cassia auriculata* (Linn.); they were screened for the quantification of phenolics and flavonoids. For the determination of antioxidant potential various *in vitro* assay systems was used like DPPH radical scavenging assay, Nitric oxide scavenging assay and reducing power assay since, evaluation of antioxidant properties of plants cannot be carried out accurately by single universal method.

Phytochemical analysis

Phenolics and flavonoids are the major constituents in plant extracts responsible for its antioxidant activity [29]. So, flowers, leaves and seeds of *Cassia auriculata* were screened for the quantification of phenolics and flavonoids. The Folin Ciocalteu method was used to determine the total phenolics content and Aluminium chloride colorimetric method was used to determine the total flavonoids contents in plant extracts. The total phenolics content is expressed in mg Gallic acid equivalent (GAE)/g dry weight of extract and total flavonoids contents is expressed in mg Quercetin equivalent (QE)/g dry weight of plant extracts (table 1).

Table 1: Total phenolics and flavonoids content in methanolic extract of plant parts of *Cassia auriculata* (Linn.)

Sample	TPC(mg GAE/g extract)	TFC(mg QE/g extract)
Flower	266.01±2.59[a]	66.060±3.030[a]
Leaves	176.91±1.30[b]	61.363±0.757[a]
Seeds	086.93±2.45[c]	53.787±0.757[b]

Each value represents mean±SD (n=3)

Different superscripts in the same column represent significant differences (p<0.05).

TPC = total phenolic content; TFC = total flavonoid content; GAE = Gallic acid equivalent; QE = Quercetin equivalent

The result represented in table 1 indicates the significant statistical difference in both phenolics and flavonoids content in the methanolic extract of flowers, leaves and seeds of *Cassia auriculata* (Linn.) with p<0.05. The total phenolics content was observed to be maximum in flowers followed by leaves and least in seeds. The flavonoids content was found to be more in flowers and leaves and significantly lower in seeds *(*p<0.05). Some previous studies [30-32] independently reported the phytochemical contents of flowers, leaves and seeds of *Cassia auriculata* (Linn.).

Antioxidant activity

DPPH radical scavenging assay

The stable DPPH radical model is a widely used, relatively quick and precise method for the evaluation of free radical scavenging activity. The DPPH assay is used to predict antioxidant activities by mechanisms in which antioxidants in a system act to inhibit lipid oxidation by scavenging of DPPH radical and this gives an idea of the free radical scavenging capacity of the system/substance being investigated [33]. Radical scavenging activity of *Cassia auriculata* plant extract against stable DPPH• (2,2-diphenyl-2-picrylhydrazyl) was determined spectrophotometrically. DPPH is a stable free radical containing an odd electron in its structure with the absorption maximum at 517 nm. When DPPH reacts with an antioxidant compound, which can donate hydrogen, it is reduced. The change in colour that is from deep violet to light yellow was measured at 517 nm on a UV/visible light spectrophotometer. The antioxidant activity of the extract was expressed as IC_{50}. All flower, leaves and seeds methanolic extract along with Ascorbic acid as positive controls significantly reduced the absorbance caused by DPPH free radical indicating their antioxidant potential with IC_{50} values as shown in table 2.

Table 2: DPPH radical scavenging activity of methanolic extract of flower, leaves and seeds of *Cassia auriculata* (Linn.) with Ascorbic acid as positive control

Name of plant extract	IC 50 (µg/ml)
Flower extract	22.548±0.187[a]
Leaves extract	24.014±0.261[b]
Seeds extract	77.489±2.072[c]
Ascorbic acid	09.210±0.010[d]

Each value represents mean±SD (n=3)

Different superscripts in the same column represent significant differences (p<0.05).

The table 2 shows that all flower, leaves and seeds extract shows significant antioxidant activity in relation of positive controls with IC_{50} values in order of Seeds extract>Leaves extract>Flower extract>Ascorbic acid. Among flowers, leaves and seeds extract flower methanolic extract has a least IC_{50} which shows that methanolic flower extract is the potent DPPH radical scavenger (p<0.05).

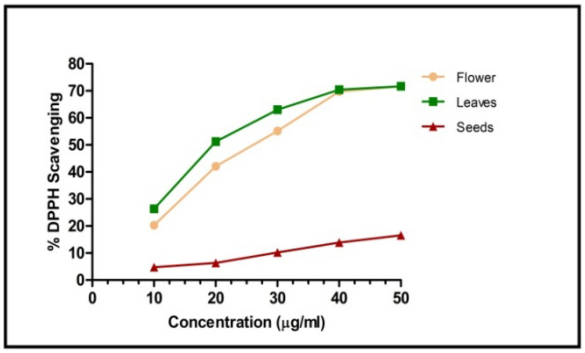

Fig. 1: % DPPH radical scavenging activity of methanolic extract of flower, leaves and seeds of *Cassia auriculata* (Linn.) at various concentrations which are presented as mean±SD of three observations

Fig. 1 illustrates a significant increase in the percentage inhibition of DPPH radical due to the scavenging ability of extracts and ascorbic acid. The scavenging of DPPH by the flowers, leaves and seeds extract was increased in dose-dependent way and was found to be highest in flower and leaves methanolic extract as compared to seeds (P<0.05). The potential of methanolic extracts of flower of *Cassia auriculata* (Linn.) as DPPH radical scavenger was also reported by [34, 35].

Reducing power assay

The reducing power of the plant extracts components may serve as a significant indicator of its potential antioxidant activity. In this assay, the yellow colour of the test solution changes to various shades of green and blue depends upon the reducing power of each compound. The presence of antioxidants in the extract causes the conversion of the ferricyanide (Fe^{3+}) complex to the ferrous ferrocyanide form (Fe^{2+}). Therefore by measuring the formation of pearls Prussian blue at 700 nm, the (Fe^{2+}) concentration can be measured. A higher absorbance at 700 nm indicates a higher reducing power. Observations in table 3 show the reducing power of flowers, leaves and seeds methanolic extract with reference to Ascorbic acid.

Table 3: Reducing ability of flowers, leaves and seeds methanolic extract of *Cassia auriculata* (Linn.) with Ascorbic acid as positive control

Sample Extract	Concentrations of samples (µg/ml)				
	10	30	50	70	100
Flower	0.421±0.006	0.509±0.013	0.619±0.03	0.681±0.005	0.896±0.016
Leaves	0.5±0.006	0.548±0.002	0.603±0.0058	0.635±0.01	0.676±0.02
Seeds	0.484±0.022	0.529±0.002	0.553±0.004	0.57±0.0025	0.595±0.014
Ascorbic acid	0.46±0.0025	0.483±0.0045	0.498±0.0035	0.537±0.003	0.606±0.015

Each value represents mean±SD. (n=3) with significant difference at P<0.05.

The reducing power of flower, leaves, seeds extract and Ascorbic acid is increased with increase in concentration in dose-dependent manner and was found to be highest at100 µg/ml. The reducing power was found to be highest in flower extract (P<0.05) as compared to leaves and seeds methanolic extract at the concentration of the 100 µg/ml indicated its potential antioxidant activity with respect to positive control Ascorbic acid. The dose dependant reducing power activity of flowers of *Cassia auriculata* (Linn.) was previously reported [30, 32, 36].

Nitric oxide scavenging assay

Nitric oxide is responsible for the pathogenesis of various disorders. It also mediates smooth muscle relaxation, neuronal signaling, inhibition of platelet aggregation and regulation of cell-mediated cytotoxicity [37] In the present assay, the sodium nitroprusside spontaneously generates nitric oxide at physiological pH in aqueous solution. The nitric oxide generated is converted into nitric acid and nitrous acids on contact with dissolved oxygen and water. The liberated nitrous acid was estimated using the modified Griess reagent. The nitrous acid reacts with Griess reagent to form a purple azo dye. In the presence of antioxidants, the amount of nitrous acid would be decreased and the degree of decrease in the formation of purple azo dye would show the extent of scavenging [38].

In order to evaluate the antioxidant potency through NO scavenging by the plant samples, the change of optical density of NO was monitored and % NO scavenged are calculated from the decomposition of SNP *in vitro*. Fig. 2 illustrates a significant decrease in the NO radical which results in the increase in percentage inhibition due to the scavenging ability of extracts and ascorbic acid.

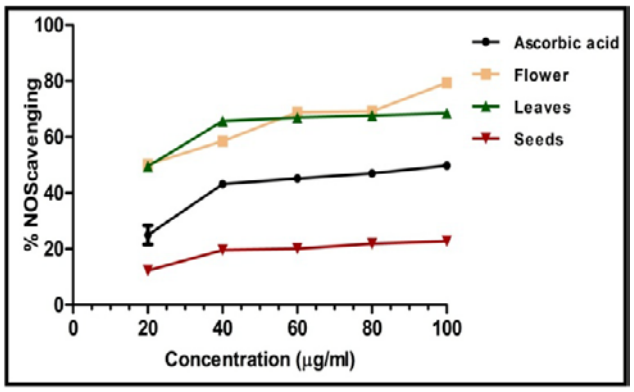

Fig. 2: % Nitric oxide scavenging activity of methanolic extract of flower, leaves and seeds of *Cassia auriculata* (Linn.) at various concentrations which are presented as mean±SD of three observations

The scavenging of Nitric Oxide by the flowers, leaves and seeds extract was increased in dose dependent manner and was found to be highest at 100 µg/ml for all extracts. The NO scavenging activity was observed to be 79.375±0.219 % in flower methanolic extract at 100 µg/ml which is significantly higher (P<0.05) as compared to leaves (68.535±0.275) and positive control Ascorbic acid (49.810±0.268) and significantly lower in seeds (22.755±1.619) (fig. 2). Greater Nitric oxide scavenging potential of flower methanolic extract can also be observed from its lower IC50 value as compared to leaves, Ascorbic acid and seeds (table 4). *In vitro* antioxidant activity of flower extracts and leaves extract of *Cassia auriculata* (Linn.) was observed previously using nitric oxide scavenging assay [34, 35, 39].

Table 4: Nitric oxide scavenging ability of flowers, leaves and seeds methanolic extract of *Cassia auriculata* (Linn.) with Ascorbic acid as positive control

Name of plant extract	IC 50 (µg/ml)
Flower extract	15.869±0.979[a]
Leaves extract	44.653±0.651[b]
Seeds extract	217.18±1.94[c]
Ascorbic acid	89.956±0.5[d]

Each value represents mean±SD (n=3)

Different superscripts in the same column represent significant differences (p<0.05).

CONCLUSION

In brief summarizing the above results, it is well cleared that flowers, leaves and seeds extract of *Cassia auriculata* (Linn.) exhibited significant antioxidant potential. Antioxidant potential of all the three plant parts was investigated with the help of various *in vitro* antioxidant assays like DPPH Scavenging assay, Reducing power assay and Nitric oxide scavenging assay. All the extracts show significant antioxidant activity in all assays; but results suggest the greater potential of methanolic flower extracts as compared to leaves and seeds. The greater antioxidant activity of methanolic flower extract may be related to the higher content of total phenolics and flavonoids. It indicates the direct correlation between antioxidant potential and total phenolics and flavonoids content. So, *Cassia auriculata* (Linn.) plant parts especially the flower methanolic extracts could serve as natural sources of antioxidants and could be used in the treatment of diseases which have the free radical origin. Further studies are still needed on the isolation and identification of bioactive components responsible and to clarify the *in vivo* potential of this plant parts in the management of human diseases resulting from oxidative stress.

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CONFLICTS OF INTERESTS

All authors have none to declare

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