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Antioxidant Assessment For Various Solvent Fractions Of *Cassia fistula* Linn. Flowers

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Abstract: *Cassia fistula* Linn., an Indian Labernum, has been used in the treatment of various diseases in different parts of the world since time immemorial. The antioxidant activity of the 1 mg/ ml of the crude solvent (methanol, ethyl acetate, chloroform and water) extracts of their flowers were evaluated *invitro* by DPPH radical scavenging activity, reducing power and inhibition of lipid peroxidation against the standard (α-tocopherol), accompanied by phenolic and flavonoid content. Richest antioxidant content was observed in the methanol extract and the potency was revealed in its SC_{50}, EC_{50}, IC_{50} values in contrast to the other extracts, which appraises its antioxidant and free radical propensities. The present study shows a particular specificity not only in the contents and antioxidant assays, but also in their effect as a nutritional supplement. Thus *Cassia fistula* flowers can be used as a phytotherapeutic agent and as a protective shield against numerous free-radical mediated diseases.
Key words: *Cassia fistula* Linn., antioxidant activity, butylated hydroxyanisole, DPPH, free radicals, lipid peroxidation, reducing activity.

INTRODUCTION

In the developed world, reliance on surgery and synthetic drugs is more usual, but in recent years most people are complementing their treatment with natural supplements[1]. Plants, the natural reservoir of medicinal agents is almost free from the toxic side effects which are complimented by synthetic chemicals. Large body of evidence has accumulated to demonstrate the promising potential in treatment of human diseases. Attempts are now being made to find plant products, which may economically be useful for commercial exploitation and more relevant to the need of the society. Currently it is estimated that almost 50% of the synthetic medicines are derived from, or patterned after their phytochemicals. Many medicinal plants contain large amounts of antioxidants other than vitamins C, E and carotenoids[2]. The antioxidative effect is mainly due to phenolic components such as flavonoids, phenolic acids, phenolic diterpenes which is mainly due to their redox properties that can play an important role in absorbing and neutralizing free radicals or decomposing peroxides[3,4]. Free radicals are highly reactive species, generated as a result of erroneous metabolism. Based on numerous evidences on the strong biological activity of phenolic compounds and due to the scarcity of data for their content in foods, current study is focused on the determination of total phenolic and flavonoid content in *Cassia fistula* flowers.

The genus Cassia belongs to medically and economically important family Leguminosae (Syn. Fabaceae), sub-family, Caesalpinioidae[5]. In the last 10 years, there has been an increased interest in biological and medicinal potential of many members of Fabaceae. Native to India, Srilanka and Amazon, *Cassia fistula* Linn. a semi wild Indian Labernum commonly known as 'Golden shower tree', an ornamental tree got diffused in various countries like South Africa, Mauritius, China, West Indies, East Africa and Brazil due to its beautiful bunches of yellow flowers. In Indian literature, this plant has been reported useful against skin diseases, liver troubles,

tuberculous glands and in the treatment of haematemesis, pruritis, leucoderma and diabetes[6]. In Srilanka, *Cassia fistula* is used in treating bone fracture[7]. Its antifungal, antibacterial, laxative and antitussive properties have also been established[8].

Known for years as 'Purging Cassia', *Cassia fistula* is currently advertised/ marketed as an effective astringent, purgative/ laxative, and is used for colic and flatulence. The active part used in medicine is the fruit or pods, which are pounded to release the sweetish pulp. The leaves are known for their laxative, antiperiodic, ulcer healing and anti-rheumatic properties. A recent literature search resulted in articles testing *Cassia fistula* as a potential marker for evaluating air pollution levels, as a possible agent to remove heavy metals from waste sites, and as a source of potassium in adult diets. The aqueous extract of *Cassia fistula* root had *invitro* DPPH- radical scavenging and deoxyribose damage protection properties[9]. Trolox equivalent antioxidant capacity (TEAC) and Ferric reducing antioxidant power (FRAP) assays showed that the reproductive organs of *Cassia fistula* have highest antioxidant properties compared to vegetative part[10]. Several methods have been developed to measure the free radical scavenging capacity (RSC), regardless of the individual compounds which contribute towards the total antioxidant capacity of a plant product in scavenging free radicals from natural sources, an important strategy to improve human health condition and life quality.

Hence, the aim of the present study is to assess the antioxidant activity and free radical scavenging properties in the flower extracts.

MATERIALS AND METHODS

Chemicals

1,1-diphenyl-2-picrylhydrazyl (DPPH), α-tocopherol, gallic acid, (+)-catechin and solvents like hexane, chloroform, ethyl acetate, methanol were purchased from Sigma Chemicals Pvt. Ltd.

Plant material

The fresh and healthy flowers of *Cassia fistula* were collected from Mt.Shevaroys, Salem, Tamil Nadu, India. This plant sample was authenticated by a botanist, (Specimen Voucher no. 780) BSI- TNAU, Coimbatore. Samples were washed well with tap water prior to distilled water deprived of dusts and insects, dried, powdered in a Willy Mill to 60-mesh size.

Phytochemical screening

Flowers were subjected to qualitative preliminary phytochemical screening to identify the presence of alkaloids, flavonoids, saponins, phenolics, tannins, carbohydrates, proteins/ aminoacids, glycosides, terpenoids, resins[11].

Soxhlet extraction

The powdered flowers were subjected to soxhlet extraction at 40°C using solvents like hexane (to remove fats and lipids), chloroform, ethyl acetate, methanol and then with water. Flavonoids being highly polar, it was extracted stepwise in the order of increasing polarity. All the solvent extracts were dried in a rotary evaporator, powdered and assayed for the following parameters.

Determination of total phenolic content

Contents of total phenolics in the extracts were estimated by a colorimetric assay based on procedures[12]. Basically, 1ml of extract sample was mixed with 1ml 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 dark for 90 min, after which the absorbance was read at 725 nm (Analytik Jena 200-2004 spectrophotometer). Gallic acid [0.01-0.4 mM; y=2.94848 x -0.99914) was used for constructing the standard curve and the results were expressed as mg of gallic acid equivalents per gram of extract (GAE_S).

Determination of total flavonoid content

Flavonoid contents in the extract were determined by the colorimetric method[12]. The flower extract (250 µL) was mixed with 1.25 ml of distilled water and 75 µl of 5% sodium nitrate solution. After 5 min, 150 µl of 10% $AlCl_3.H_2O$ solution was added. After 6 min, 500 µl of 1M sodium hydroxide and 275 µl of distilled water were added to prepare the mixture. The solution was mixed well and the absorbance was read at 510 nm. (±) Catechin (0.25-2.500 mM; Y= 0.2903; R^2= 1) was used to calculate the standard curve and the results were expressed as mg of % RSA (±) Catechin equivalents per gram of extract (CE_S).

DPPH- radical scavenging activity

Various concentrations of *Cassia fistula* flower extracts (0.3 ml) were mixed with 2.7 ml of methanol solution containing DPPH radicals (6×10^{-5} mol/ l). The mixture was shaken vigorously and allowed to stand for 60 min in the dark. The mixture was shaken vigorously. The reduction of the DDPH 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_S is the absorbance of the extract sample and A_{DPPH} is the absorbance of the DPPH solution[12]. α-tocopherol was used as standard.

Table 1: Phytochemical screening in various extracts of *Cassia fistula* Linn.

Constituents	Name of the test	*Cassia fistula*				
		HE	CH	EA	ME	WA
Alkaloids	Wagner's Meyer's Dragendorff's	- + +	- + -	- - -	- - -	- - -
Flavonoids	Ferric chloride Shinoda's Fluorescence	- - -	+ + -	+ + -	+ + -	+ + -
Flavones	Alkaline Mg-Hcl Con. H_2SO_4	- - -	- - +	+ + +	+ + +	- - +
Flavonols	Alkaline Mg-Hcl Con. H_2SO_4	- - -	+ + -	+ + +	+ + +	- - +
Isoflavonoids	Alkaline Mg-Hcl Con. H_2SO_4	- - -	- - -	- + +	+ + +	+ + +
Anthocyanins	Alkaline Mg-Hcl	- -	- -	- +	+ +	- -
Phenolics	Ferric Chloride Lead acetate Dichromate	- + +	+ - -	+ + +	+ + +	+ + +
Tannins	Gelatin KOH	- -	+ +	+ +	+ +	+ +
Saponins	Foam	-	-	-	-	-
Carbohydrates	Molisch's Fehling's Barfoed's Benedict's Borntrager's	- - + + +	+ + + + -	- - + + -	+ + + + -	- + + + -
Proteins/ aminoacids	Millon's Ninhydrin	- -	- +	+ +	+ +	+ +
Steroids	Libermann's & Burchard's Salkowski's	+ +	- +	- -	- -	- -
Terpenoids	Hager's Knollar's	+ +	- -	- -	- -	- -
Glycosides	Keller- killiani Brown ring	- -	+ +	+ +	+ +	- -
Fats/ Oils	Biuret's	+	+	-	-	-

HE: Hexane; CH: Chloroform; EA: Ethyl acetate; ME: Methanol; WA: Water;
+: Presence; -: Absence

Reducing power

Various concentrations of *Cassia fistula* flower 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 centrifugated at 1000 rpm for 8 min. (HERMLEZ 300 K centrifuge). The upper 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 spectrophoto - metrically at 700 nm[12]. α-tocopherol were used as standards.

Inhibition of lipid peroxidation using thiobarbituric acid reactive substances (TBARS)
The procedure[13] using a Fenton reaction-induced lipid peroxidation has been adapted for this assay. The extracts of all species in concentration of 100 µg/ ml have been mixed with 300 µl Tris- HCl buffer, pH=7.5, 500 µl of 20 mM linoleic acid and 100 µl of 4 mM $FeSO_4$. The peroxidation was started with the addition of 100 µl of 5 mM ascorbic acid. The reaction mixture was incubated for 60 min at 37°C. Thereafter, 2 ml of 10 % ice cold trichloroacetic acid was added and 1 ml aliquot of the samples was added with 1 ml of thiobarbituric acid. The TBA/ sample mixture was heated in the water bath at 95°C for another 60 min. the absorbance was read at 532 nm and the percentage of linoleic acid peroxidation inhibition was calculated using appropriate controls. α-tocopherol was used as positive control.

Statistical analysis
All the experiments were carried out in triplets and the results are expressed as mean values and standard error or standard deviation (SD).

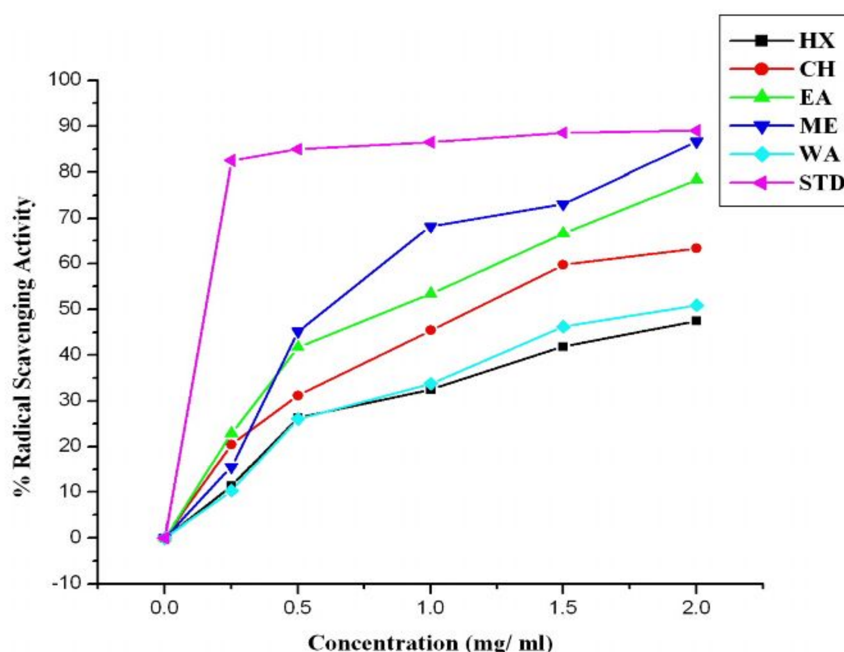
RESULTS

Phytochemical screening
The preliminary qualitative screening revealed the presence of useful constituents like alkaloids, phenolics, flavonoids and classes, tannins, carbohydrates, glycosides ,anthocyanins etc.,copiously present in almost all the extracts as listed in Table- 1.

Antioxidant content
The results of the antioxidant assay clearly outlined the richest source of phenolics in methanol extract (5.8%) and equal amount of flavonoids in both methanol and ethyl acetate extracts as 4.4% present in their whole respective extracts. Probably the low content of chloroform, ethyl acetate and water extract may be due to the fewer amounts of constituents extracted. Phenolic content obtained for chloroform, ethyl acetate, water were 2.5%, 4.1%, 4.0% and flavonoid content were 4.0%, 4.4%, 2.6% respectively.

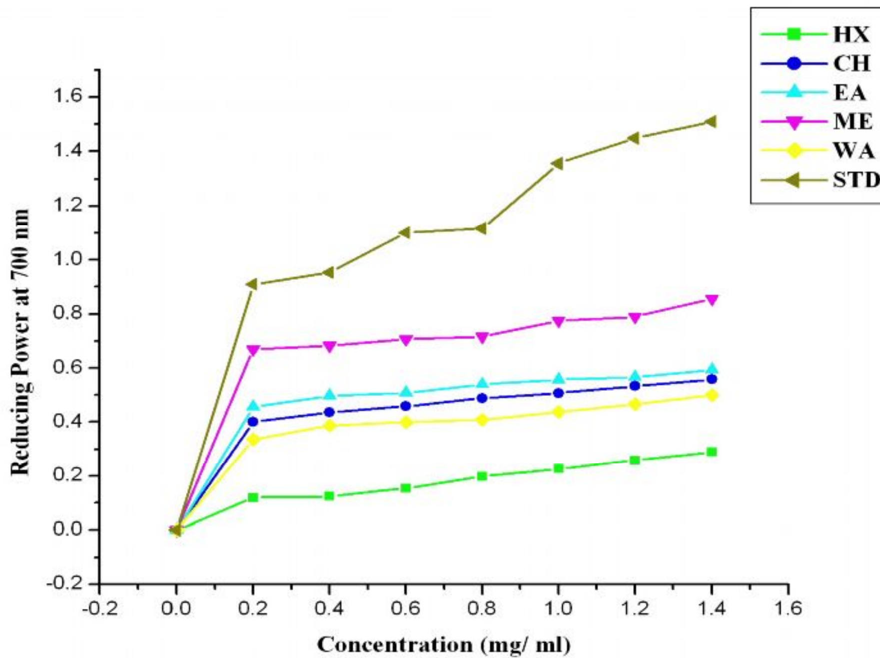
DPPH- radical scavenging activity
Methanol extract exhibited an excellent scavenging activity. A gradual increase in the % RSA with the increase in concentration can be made clear with fig 1, for all the tested extracts. SC_{50} value was found to be low for methanolic extract (0.602 µg/ ml) comparatively, proving its significant efficiency. Whereas the SC_{50} values for chloroform, ethyl acetate and water extracts were 1.1602, 0.845, 1.893 µg/ ml respectively, present in 1 mg/ ml of the crude. The standard α-tocopherol recorded 0.152 µg/ ml as SC_{50} value.

Figure 1: DPPH radical scavenging activity of *Cassia fistula* Linn. flower extracts



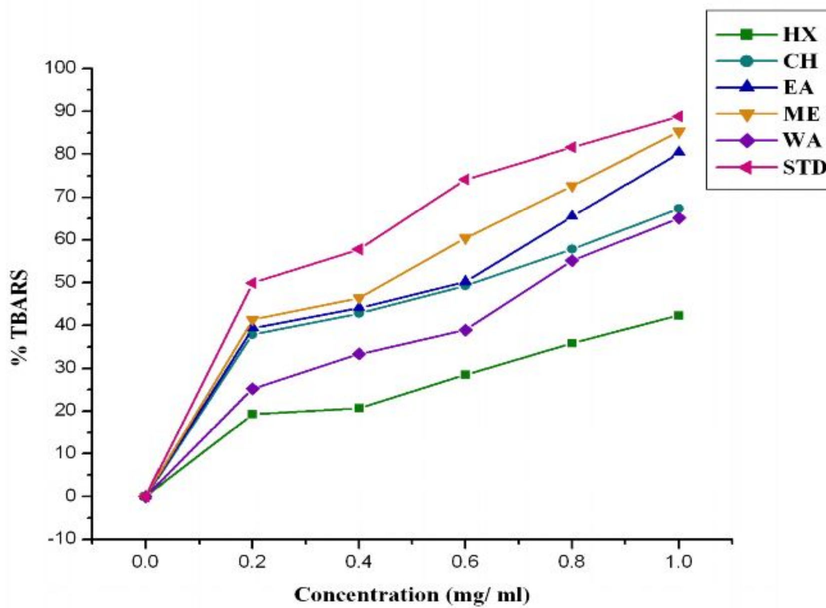
STD: α-tocopherol; CH: Chloroform; EA: Ethyl acetate; ME: Methanol; WA: Water

Figure 2: Reducing power of *Cassia fistula* Linn. flower extracts



STD: α-tocopherol; CH: Chloroform; EA: Ethyl acetate; ME: Methanol; WA: Water

Figure 3: Inhibition of lipid peroxidation of various concentrations of *Cassia fistula* flower extracts



STD: α-tocopherol; CH: Chloroform; EA: Ethyl acetate; ME: Methanol; WA: Water

Reducing power

An increase in absorption with the increase in concentration was exhibited by all the test extracts. Obviously, methanol extract recorded the highest reducing power in contrast with the other extracts. EC_{50} value of about 0.103 µg/ ml present in 1 mg/ ml of the crude has been calculated from **fig 2**. About 0.103, 0.106, 0.111 µg/ ml was calculated for chloroform, ethyl acetate and water extracts against the standard α-tocopherol whose EC_{50} was 0.035 µg/ ml.

Inhibition of lipid peroxidation (TBARS)

All the test extracts lowered the degree of lipid peroxidation induced by hydroxyl radical generated by an iron/ ascorbate system. For comparison, the efficient inhibition was given by methanolic flower extract with an IC_{50} value of about 0.447 µg/ ml. The chloroform and ethyl acetate extracts recorded 0.618 and 0.594 µg/ ml present in 1 mg/ ml of the crude. The highest IC_{50} value obtained for water extract (0.736 µg/ ml) showed its lower efficiency. The results are portrayed in fig 3.

DISCUSSION

In the present study, in order to characterize the extracts, different antioxidant assays such as inhibition of lipid peroxidation, DPPH scavenging activity, measurement of reducing potential combined with the estimation of total phenolic and flavonoid contents, showed a positive track of *Cassia fistula* flowers. The qualitative screening had revealed quite a number of useful chemical constituents, which may be responsible for pharmacological properties.

From the results, it can be noted that the methanolic extract were shown to possess significant antioxidant content, evidencing its ability of having extracted a considerable amount of polyphenols and flavonoids with specific distribution in the particular representatives. Rest three extracts cannot be considered to be inferior because satisfactory contents were obtained enough to show their antioxidant activity. Such strong content reflects their complexity of mechanisms for all the methodologies experimented. Thus these extracts shall be considered to contain conjugated ring structures and hydroxyl groups that have the potential to function as antioxidant *invitro* or cell function free systems by scavenging, singlet oxygen, lipid peroxy radicals and stabilizing free radicals involved in oxidative processes through hydrogenation or complexing with oxidizing species.

An excellent scavenging effect of the methanolic extract showed an array of its defense strategy against the stable free radical, DPPH. From the fig 1 it can be noted that a neat scavenging activity had taken place ie., a rapid mixing of the extract with DPPH solution leading to the quick disappearance of color of the free radical accompanied by a decrease in absorption with concentration. A decrease of the absorption with increase in concentration is the striking evidence for its scavenging activity. SC_{50} values of the chloroform, ethyl acetate and water extracts were higher than the methanol extract indicating their lower efficiency comparatively. This property of polyphenols scavenging the radicals, reasons the antioxidants for functioning against the disease causing free radicals which pulls the researchers to drive on the natural derivatives.

Furthermore, the absorption range plays a vital role in the observation of reducing power. It is noteworthy that a gradual increase in concentration is based on its electron donating activity that serves as an important action. This action was found to be intense in the methanolic extract with 50% efficient concentration (EC_{50}) of about 0.103 µg/ ml. Next stood the ethyl acetate extract ranging about 0.106 µg/ ml. Reports prove that the reducing properties are generally associated with the presence of reductones, which have shown to exert an antioxidant action by breaking the free radical chain by donating a hydrogen atom[14]. Another report[15,16] describes the relationship between the phenolic content and the reducing power, which shall be applied in this present work because an appreciable phenolic content has been recorded, for reasoning the reducing activity.

Fe^{2+} induced lipid peroxidation is an excellent system for assessing antioxidant action of the test extracts, due to chelation of Fe^{2+} or by scavenging free radicals. 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[12]. Moreover, all the solvent extracts tested were found to produce a dose-dependent inhibition of linoleic acid peroxidation. Among those, the most efficient was the methanol extract with an IC_{50} value of 0.447 µg/ ml which was found to compete effectively with the standard α-tocopherol whose IC_{50} was 0.202 µg/ ml. There was observed a rapid increase in the absorption when the concentration increased from 0.8 to 1 mg/ ml, indicating that the methanolic extract had terminated the peroxidation most rapidly than any other concentration. Fig 3 gives a clear idea about the concept of TBARS ie., the capacity of inhibition of lipid peroxidation is proportional to the extract concentration. The important mechanism of these extracts could be due to its direct radical scavenging ability revealed in its lipid peroxidation assay.

Hence to sum-up, a clear understanding about the relationship between the antioxidant content with the assays had proved the potency of the methanolic

extract. Methanol was found to be the most effective solvent in extraction of antioxidants from *Cassia fistula* flowers. This is in agreement with some reports[17,18] that the methanol is a widely used and effective solvent for extraction of antioxidants. Other three extracts have given a competence by recording good contents and SC_{50}/ EC_{50}/ IC_{50} data. It has provided an interest to isolate new compounds responsible for the antioxidant activity singly or in combination, that will ultimately lead to the application of these medicinal plants in pharmaceutical and cosmetic formulations.

CONCLUSION

Cassia fistula flower extract exhibited the presence of enormous amount of flavonoids and phenolic compounds which are responsible for the immense antioxidant property. The present 50% inhibition data for DPPH free radical scavenging activity, reducing power and lipid peroxidation are the basis for assessing their preventive role against free radical effect and will enrich the national food composition database. The antioxidant potential of *Cassia fistula* flowers can broaden its therapeutic applications towards the prevention of degenerative diseases.

Nature is still a perfect source for health promotion and for the supplementation of safe drugs. Development of natural antioxidants from flowers of *Cassia fistula* being safe, eveready and economic can replace synthetic antioxidants to act against the free radical mediated diseases.

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