Evaluation of *in vitro* Antioxidant Activity of hydro-alcoholic seed extratcs of *Cassia fistula* linn.

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ABSTRACT

Antioxidant activity has been assessed by *In vitro* method for phytochemical fraction of plant, viz hydro alcohol extract of *Cassia fistula* seeds. The present study was aimed to investigate the antioxidant activity of extracts of dried seed powder of *Cassia fistula* Linn. (Family: *Leguminosae). Cassia fistula* Linn. has been reported to possess hypoglycaemic, anticancer, abortifacient, anticolic, antifertility, estrogenic, laxative, antimicrobial, antipyretic, anti-inflammatory, smooth muscle stimulant, antiarthritic, antitussive, purgative, analgesic, antiviral, hepatoprotective, and anti implantation activity. The primary phytochemical study and *in vitro* antioxidant study was performed on hydro alcoholic extract of shade dried seeds. Modern phytochemical screening of the plant has shown the presence of phenolic compounds, fatty acids, flavonoids, tannins and glycosides. The extracts were evaluated for their phenolic content & antioxidant activity. Phenolic content was measured by using Folin-Ciocalteu reagent & was calculated as Gallic acid equivalents. Antiradical activity of hydro alcoholic extract was measured by DPPH (2,2-diphenyl-1-picryl hydrazyl) assay & was compared to ascorbic acid (vitamin C), and Ferric reducing power of the extract was also evaluated by Oyaizu method. In the present study three methods were used for evolution of antioxidant activity. The first two methods were for direct measurement of radical scavenging activity & remaining one method evaluated the reducing power. The present study revealed that the hydro alcoholic extracts of seeds have significant radical scavenging activity. In this study, *Cassia fistula* was identified as potentially novel source of free radical scavenging compound. The results clearly indicate that *Cassia fistula* is effective against free radical mediated diseases and also helpful to draw special attention for further studies.

Keywords: *In vitro* Antio xidant activity, *Cassia fistuala*, free radical scavenging activity (DPPH assay), Reducing power, total phenol content.

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INTRODUCTION

Since ancient times, the medicinal properties of plants have been investigated in the recent scientific developments throughout the world, due to their potent antioxidant activities. As antioxidants have been reported to prevent oxidative damage caused by free radical, Natural antioxidants present in the plants scavenge harmful free radicals from our body. Free radical is any species capable of independent existence that contains one or more unpaired electrons which reacts with other molecule by taking or giving electrons, and involved in many pathological conditions.[1] Free radicals can be described as chemical species that have an unpaired electron and play very important role in human health and beneficial in combating against several diseases like cardiovascular disorders, lung damage, inflammation etc. These free radicals are highly unstable and when the amount of these free radicals exceed in the body, it can damage the cells and tissues and may involved in several diseases. Thus there is the need of antioxidant of natural origin because they can protect the human body from the diseases caused by free radicals.[2,3]

An antioxidant is a molecule capable of slowing or preventing the oxidation of other molecules. The most common reactive oxygen species (ROS) include superoxide (02^{-}) anion, hydrogen peroxide (H202), peroxyl (ROO^{-}) radicals, and reactive hydroxyl (OH^{-}) radicals. The nitrogen derived free radicals are nitric oxide (NO) and peroxynitrite anion (ONOO).[4] Under normal state of affairs, the ROS generated are detoxified by the antioxidants nearby in the body and there is symmetry between the ROS generated

and the antioxidant present. However due to ROS over production and/or derisory antioxidant argument, this equilibrium is hindered favoring the ROS gain that culminates in oxidative hassle. The ROS readily attack and induce oxidative damage to various biomolecules including proteins, lipids, lipoproteins and DNA.[5] This oxidative damage is decisive etiological factor concerned in quite a lot of chronic human diseases such as diabetes mellitus, cancer, atherosclerosis, arthritis, and neurodegenerative diseases and also in the ageing course.[6] Based on growing interest in free radical biology and the lack of effective therapies for most chronic diseases, the expediency of antioxidants in protection against these diseases is defensible. Epidemiological studies have brought into being that the intake of antioxidants such as vitamin C reduces the risk of coronary heart diseases and cancer.[7] It is possible to reduce the risk of chronic diseases and prevent disease progression by either enhancing the body's natural antioxidant defenses or by supplementing with proven dietary antioxidants.[8]

Currently available synthetic antioxidants like BHT, butylated hydroxyl anisole (BHA) and tertiary butylated hydroquinones have been suspected to cause or prompt negative health effects. Hence, strong restrictions have been placed on their application and there is a trend to substitute them with naturally occurring antioxidants.[9] Several studies revealed that phenols, mainly the type of flavonoids, from some medicinal plants are safe and bioactive, and have antioxidant properties and exert anticarcinogenic, antimutagenic, antitumoral, antibacterial, antiviral and anti-inflammatory effects.[10] Therefore in current years, substantial attention has been directed towards credentials of plants with antioxidant ability that may be used for human expenditure.

Due to their redox properties, acting as reducing agents, hydrogen donors, singlet oxygen quenchers and chelating metals.[11-13] The medicinal properties of plants have been investigated in the recent scientific developments throughout the world, due to their potent antioxidant activities, no side effects and economic viability,[14] in recent years one of the areas, which attracted a great treaty of attention, is antioxidant in the control of degenerative disease in which oxidative dent has been implicated. Several plant extracts have been shown to antioxidant activity.[15-17] Preliminary phytochemical analysis of *Cassia fistula seeds* indicated the presence of relatively high levels of flavonoids, glycosides, anthraquinones steroids, terpenoids and such phenolic compounds. Hence the present investigation was undertaken to determine the antioxidant potential of *Cassia fistula seeds*.

OBJECTIVE

Recently interest has increased considerably in finding natural occurring antioxidants for use in foods or medicinal materials to replace synthetic antioxidants that are being restricted due to their side effects such as carcinogenicity. One among such natural plants is *Cassia fistula* Linn. Commonly known as Indian Laburnum, is distributed in various countries including Asia, Mauritius, South Africa, Mexico, China, West Indies, East Africa and Brazil as an ornamental tree for its beautiful bunches of yellow flowers. Recognize by the British pharmacopoeia.[18] The seeds are reported to have demulcent and lubricating effect, bitter, acrid, cooling, emollient and useful in skin diseases, pruritus, burning sensation, dry cough and bronchitis.[19]

Thus, present study was undertaken to evaluate the *in vitro* antioxidant effect of hydro alcoholic extract of seeds. The main constituents present in seeds are tannins, fatty acids isoflavonoids, flavonoids, glycosides, anthraquinones, and phenolic compounds. The objective of the present study was to evaluate the antioxidant potential and free radical scavenging activity of a hydro alcoholic extract of *Cassia fistula* Linn. The extract was examined for different reactive oxygen species (SOS) scavenging activities such as DPPH assay, ferrus Reducing capacity, and total phenol content.

MATERIALS AND METHODS

Collection and Identification of Plant material

The fresh *Cassia fistula* seeds were collected from the local garden area of Jamnagar, Gujarat, India in the month of June-Aug 2009. The plant was authenticated by the department of pharmacognosy, I.P.G.T. & R.A. Jamnagar, Gujarat, India. Plant parts were collected on the basis of the information provided in the ethanobotanical survey of India. Each specimen/plant materials were labeled numbered, and annoted with the date of collection, locality and their medicinal uses were recorded.

Preparation of plant extract

The extraction of the *Cassia fistuala* seeds were carried out using known standard procedures.[20] The plant materials were dried in shade and powdered in a mechanical grinder. The powder(25.0gm) of the plant materials were initially de-fatted with petroleum ehrer (60-80°C), followed by 900 ml of hydro alcohol by using a Soxhlet extractor for

72 hrs at a temp. not exceeding the boiling point of the solvent. The extracts were filtered using Whattman filter paper (No.1), while hot and concentrated in vacuum under reduced pressure using rotary flask evaporator and dried in a desiccator. The hydroalcoholic extract yield a dark brownish solid residue weighing 1.875 gm (7.5% w/w) respectively. More yields of extracts were collected by this method of extractions. The extracts were then kept in sterile bottles, under refrigerated conditions, until further use. The dry weight of the plant extracts were obtained by the solvent evaporation and used to determine concentration in mg/ml. the extract was used directly for DPPH assay, total phenol and ferrus reducing power content and also for assessment of antioxidant capacity through various chemical assays.

Priliminary phytochemical testing

The extracts were subjected to preliminary phytochemical testing to detect for the presence of different chemical groups of compounds. Air-dried and powdered plant materials were screened for the presence of saponins, tannins, alkaloids, flavonoids, triterpenoids, steroids, glycosides, anthraquinones, cumarin, saponins, gum, mucilage, carbohydrates, reducing sugars, starch, protein and amino acids as described in literatures.[21-23].

Table1. Phytochemical screening of *Cassia fistula* plant extracts.

Phytochemical composition of plant extracts	
Secondary Seeds	**Metabolite (Hydro alcoholic extract of *Cassia fistula*)**
Alkaloids	+
Tanins	+
Flavonoids	+
Saponins	+
Triterpenoids	+
Steroids	+
Glycosides	+
Coumarin	-
Anthraquinones	+
Reducing Sugars	+
Carbohydrates	+
Gum & Mucilage	-
Starch	-
Proteins	+
Amino acids	+

(+) Indicate Present and (-) Indicate Absent

Phytochemical evaluation

The hydroalcoholic extract of *Cassia fistula* seeds were subjected to the following chemical tests for the identification of various active constituents.

CHEMICALS & INSTRUMENT

Chemicals

2,2-Diphenyl-1-Picrylhydrazyl (DPPH, Lancaster-UK), Gallic acid (Loba-India), were purchased from Krishna scientific traders, Rajkot, Gujarat, India. *Folin Ciocalteu's* reagent, sodium carbonate, ascorbic acid, hydrogen peroxide, potassium ferricynide, trichloroacetica cid, ferric chloride, All other reagents were of analytical grade were obtained from the pharmaceutical chemistry laboratory of I.P.G.T & R.A., Jamnagar, Gujarat, India.

Instrument

UV spectrophotometer (Systronic double beam- UV-2201).

Centrifuge machine (Remi instruments-C24).

ANTIOXIDANT ASSAY

The antioxidant activity of Plant extracts were determined by different *in vitro* methods such as, the DPPH free radical scavenging assay, total phenol content by Folin-ciocalteu reagent and reducing power methods using OYAIZU method. All the assays were carried out in triplicates and average values were considered.

DETERMINATION OF TOTAL ANTIOXIDANT ACTIVITY

In-vitro antioxidant activity

Free radical scavenging activity (DPPH Assay)[24]

The anti-oxidant potential of any compound can be determined on the basis of its scavenging activity of the stable 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical as described by Sadhu *et al*[25]. DPPH is a stable free radical containing an odd electron in its structure and usually utilized for detection of the radical scavenging activity in chemical analysis.

The absorption maximum of a stable DPPH radical in methanol was at 517 nm. The decrease in absorbance of DPPH radical caused by antioxidants, because of the

reaction between antioxidant molecules and radical progresses, which results in the scavenging of the radical by hydrogen donation.

Preparation of standard solution

Required quantity of Ascorbic acid was dissolved in methanol to give the concentration of 5, 10, 15, 25, 50 and 60 μg/ml.

Preparation of test sample

Stock solutions of samples were prepared by dissolving 10 mg of dried hydro alcoholic extract in 10 ml of methanol to give concentration of 1mg/ml. then prepared sample concentrations of 5, 10, 15, 25, 50 and 60 μg/ml.

Preparation of DPPH solution

3.9 mg of DPPH was dissolved in 3.0 ml methanol; it was protected from light by covering the test tubes with aluminum foil.

Protocol for estimation of DPPH scavenging activity

Antiradical activity was measured by a decrease in absorbance at 517 nm of a solution of purple- coloured DPPH in methanol brought about by the sample [26-29]. A stock solution of DPPH (1.3 mg/ml in methanol) was prepared such that 75 μl of it in 3 ml methanol gave an initial absorbance of 0.9. Decrease in the absorbance in the presence of sample extract and standard at different concentrations was noted after 30 minutes. EC_{50} was calculated from % inhibition. A blank reading was taken using methanol instead of sample extract. Absorbance at 517 nm is determined after 30 min using UV-visible Spectrometer(Systronic double beam- UV-2201), Lower the absorbance of the reaction mixture indicates higher free radical scavenging activity. IC50 value denotes the concentration of sample required to scavenge 50% of the DPPH free radicals.

The capability to scavenge the DPPH radical was calculated using the following equation.

$$\text{DPPH Scavenged (\%)} = \frac{A_{control} - A_{test}}{A_{control}} \times 100$$

Where $A_{control}$ is the absorbance of the control reaction and A_{test} is the absorbance in the presence of the sample of the extracts. The antioxidant activity of the extract was expressed as IC50 and compared with standard. The IC50 value was defined as the concentration (in μg/ml) of extracts that scavenges the DPPH radicals by 50%.

Reducing power assay

For the measurement of the reductive ability, we investigated the Fe+3 Fe+2 transforma-tions in the presence of *Cassia fistula* hydro alcoholic extract using the method of Oyaizu. [30] The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity. Like the antioxidant activity, the reducing power of *Cassia fistula* extract and standard increase with increasing concentration.

Preparation of standard solution

3 mg of ascorbic acid dissolved in 3 ml of distilled water. Dilutions of this solution with distilled water were prepared to give the concentration of 10, 25, 50, 75 and 100 μg/ml.

Preparation of test sample

Stock solutions of samples were prepared by dissolving 10 mg of dried extract in 10 ml of methanol to give concentration of 1mg/ml. then prepares sample concentrations of 10, 25, 50, 75 and 100 μg/ml.

Protocol for reducing power

According to this method, the aliquot of various concentrations of the standard and test sample extracts (10 to 100μg/ml) in 1.0 ml of deionized 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 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. The upper layer of solution 2.5 ml 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 UV-visible spectrometer (Systronic double beam- UV-2201).[31] A blank was prepared without adding extract. Ascorbic acid at various concentrations (10 to 100μg/ml) was used as standard. As illustrated in figures Fe^{+3} was transformed to Fe^{+2} in the presence of *Cassia fistula* extracts. This results indicates that Increase in absorbance of the reaction mixture indicates increase in reducing power.

Total phenolic content

phenolic compounds are plant secondary metabolites produced either from phenylalanine or from its precursor shikimic acid.[32] The antioxidant potential of phenolic compounds has been shown in a number of *in-vitro* studies. They are capable of direct chain-breaking antioxidant action by radical scavenging. In addition to having potential for independent antioxidant action, polyphenols have been suggested to spare essential antioxidants.[33]

Preparation of standard solution

3 mg of Gallic acid dissolved in 3 ml of distilled water. Dilutions of this solution with distilled water were prepared to give the concentration of 25, 50, 75, 100, 200 and 250 **µg**/ml**.**

Preparation of test sample

Stock solutions of samples were prepared by dissolving 10 mg of dried methanolic extract in 10 ml of methanol to give concentration of 1mg/ml. then prepares sample concentrations of 25, 50, 75, 100, 200 and 250 µg/ml**.**

Protocol for Total phenol

Total Phenolic content was determined using Folin-Ciocalteu reagent, was established according to the method described by singleton and Rossi.[34] The powdered extracts of plant were dissolved in methanol to obtain a concentration of 10 mg/10ml. from this solution 1 ml was taken and give dilution up to 10 ml(100ug/ml of stock solution) with the same solvent.. considesr it as stock solution. From this stock solution make different concentrations 25 µg/ml, 50µg/ml, 75µg/ml, 100µg/ml, 200µg/ml, follow same procedures for standard. Gallic acid was used as a standard. 1.0 ml of Folin-Ciocalteu reagent was added in these concentrations samples and the content of the flask mixed throughly, The mixture was then kept for 5 min and to it 4 ml of 20% w/v sodium carbonate solution was added the volume was made with double distilled water. The mixture was kept for 30 minute until blue colour develops. The absorbance of blue colour developed which was recorded at 765 nm in UV spectrophotometer. The % of total phenolic was calculated from calibration curve of Gallic acid plotted by using similar procedure.[35, 36]

RESULTS AND DISCUSSION

Preliminary phytochemical screening

It was found that hydro alcoholic extract of *Cassia fistula* seeds contained tannins, fatty acids, glycosides, phenolic compounds and flavonoids in higher amounts.

Several concentrations of the *Cassia fistula* seed extaracts were tested for antioxidant activity in different *in vitro* models. It was observed that free radicals were scavenged by the *Cassia fistula* in a concentration dependent manner in all the assays.

DPPH Free radical scavenging activity

The decrease in absorbance of DPPH radical caused by antioxidants because of the reaction between antioxidant molecules and radical progress which results in the scavenging of the radical by hydrogen donation. It is visually noticeable as a change in colour from purple to yellow. *Cassia fistula* exhibited a comparable anti oxidant activity with that of standard ascorbic acid at varying concentration tested (5, 10, 15, 25, 50 µg/ml). There was a dose dependant increase in the percentage antioxidant activity for all concentrations tested (Table2,3). The extract at a concentration of 10µg/ml showed a percentage inhibition of 26.37±5.21 and for 60µg/ml it was 68.13±0.25. Ascorbic acid was used as the standard drug for the determination of the antioxidant activity by DPPH method. The concentration of ascorbic acid varied from 1 to 50 µg/ml. Ascorbic acid at a concentration of 5µg/ml exhibited a percentage inhibition of 44.50±0.59 and for 50 µg/ml 98.79±0.28 (Table 2,3). A graded increase in percentage of inhibition was observed for the increase in the concentration of ascorbic acid. The EC50 value of ascorbic acid was found to be 6.1 µg/ml. EC50 value of sample extracts was found to be 39.5µg/ml. All

Table 2. Shows percentage inhibition of standard at concentrations (µg/ml) in hydrogen peroxide scavenging model

Concentration (µg/ml)	% Inhibition	EC50
5	44.50±0.59	
10	52.74±0.22	
15	62.63±0.34	6.1 **µg**/ml
25	78.57±0.32	
50	98.79±0.28	
60		

Values are mean ± SD of three parallel measurements
STD-Ascorbic acid

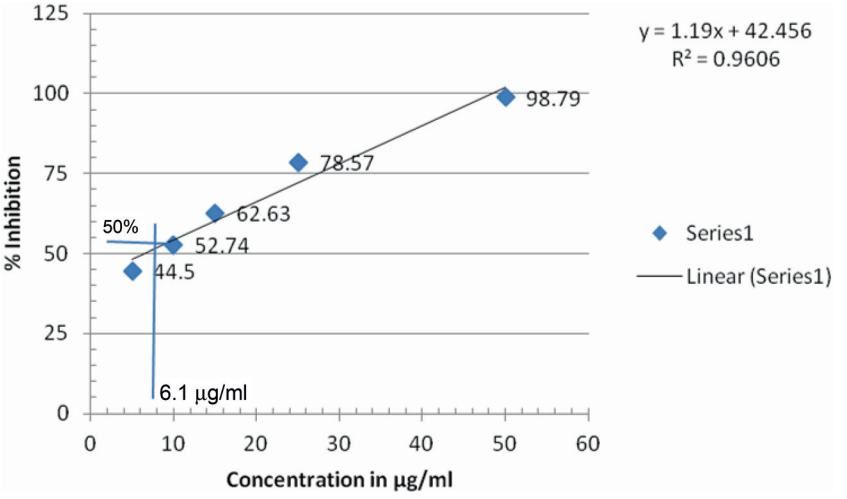

Figure 1. DPPH free radical scavenging activity of std ascorbic acid.

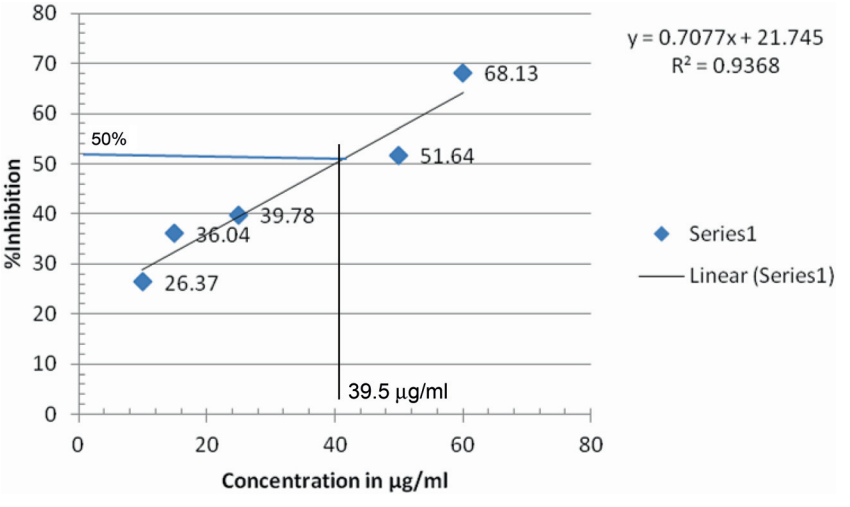

Figure 2. DPPH free radical scavenging activity of hydroalcoholic extracts of seeds.

Table 3. Shows percentage inhibition of hydroalcoholic extract of seeds at various concentrations (µg/ml) in hydrogen peroxide scavenging model.

Concentration (µg/ml)	% Inhibition	EC50
10	26.37±0.210	
15	36.04±0.280	
25	39.78±0.320	39.0 **µg**/ml
50	51.64±0.310	
60	68.13±0.250	

Values are mean ± SD of three parallel measurements
MCS-Hydroalcoholic extract of seeds.

determinations were done in triplicates and the mean values were determined. Hence DPPH is usually used as a substance to evaluate the antioxidant activity.

Reducing power assay

The reducing power of the hydroalcoholic extracts and standards increases with the increase in amount of sample and standard concentrations.(Table 4, 5). The Reducing power shows good linear relation in both standard (R^2=0.993) as well as sample extract (R^2=0.987) (Fig.3)

Total phenolic content

The total phenolic content of hydroalcoholic extract of *Cassia fistula* calculated as Gallic acid equivalent of phenols was detected.

The total phenol content shows good lienear relation in both standard as well as sample extracts(Fig.5,6). phenolic compounds are also very important plant constituents because of their hydroxyl groups confer scavenging ability.

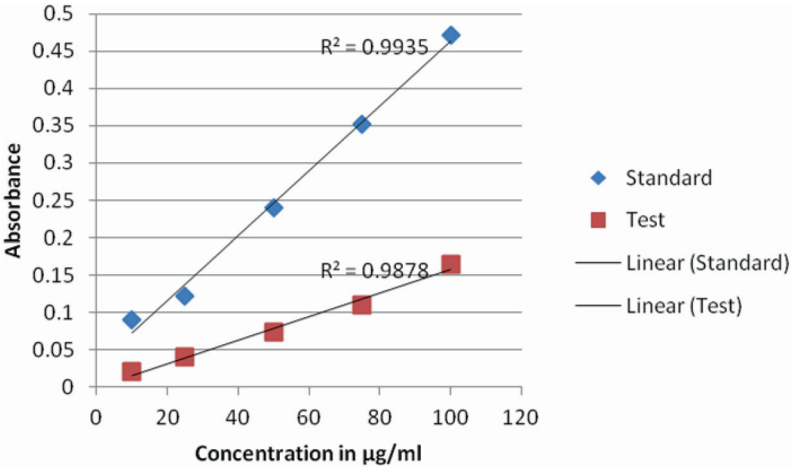

Figure 3. Ferric reducing power determination of standard and seed extracts.

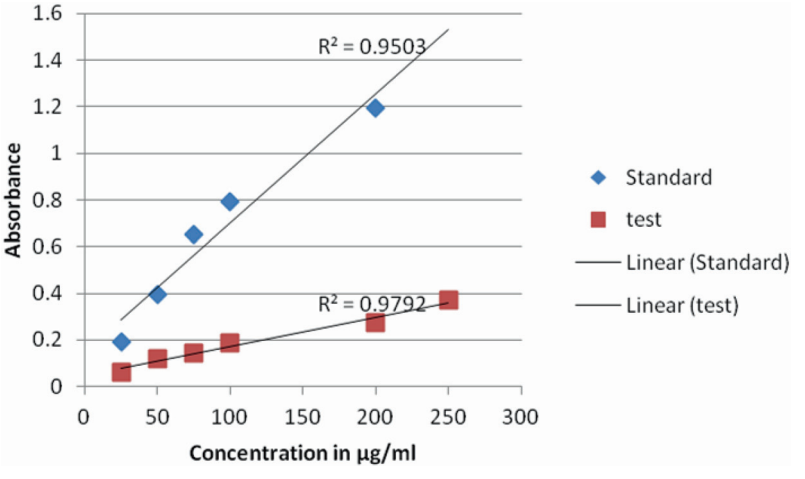

Figure 4. Total phenol content of standard and seed extracts.

Table 4. Shows the Absorbance of Standard at various concentrations (µg/ml) in ferric reducing power determination model.

Concentration (µg/ml)	Absorbance
10	0.090±0.002
25	0.122±0.003
50	0.240±0.002
75	0.352±0.004
100	0.471±0.001

Values are mean ± SD of three parallel measurements

Table 5. Shows the Absorbance of hydroalcoholic extract of seeds at various concentrations (µg/ml) in ferric reducing power determination model.

Concentration (µg/ml)	Absorbance
10	0.020±0.001
25	0.040±0.004
50	0.074±0.003
75	0.110±0.005
100	0.165±0.001

Values are mean ± SD of three parallel measurements

CONCLUSION

Cassia fistula seeds extracts showed antioxidant activity by inhibiting DPPH, and reducing power activities when compared with standard L-ascorbic acid. In addition, the hydroalcoholic extract of *Cassia fistula* found to contain a noticeable amount of total phenols, which plays a major role in controlling antioxidants. The results of this study show that the hydroalcoholic extract of *Cassia fistula* can be used as easily accessible source of natural antioxidants and as a possible food supplement or in pharmaceutical industry. However, the chemical constituents present in

Table 6. Shows the Absorbance of Standard Gallic acid at various concentrations (µg/ml) in total phenolic content determination model.

Concentration (µg/ml)	Absorbance
25	0.194±0.012
50	0.395±0.010
75	0.652±0.009
100	0.790±0.015
200	1.192±0.010

Values are mean ± SD of three parallel measurements

Table 7. Shows the Absorbance of hydroalcoholic extract of flowers at various concentrations (µg/ml) in total phenolic content determination model

Concentration (µg/ml)	Absorbance
25	0.062±0.001
50	0.121±0.003
75	0.143±0.005
100	0.185±0.002
200	0.272±0.002
250	0.370±0.005

Values are mean ± SD of three parallel measurements

the extract, which are responsible for this activity, like phenols, glycosides, flavonoids, alkaloides, steroids, terpenoides, tannins, reducing sugars and proteins present in the extract may be responsible for such activity. Some of these constituents have already been isolated from this plant. Hence, the observed antioxidant activity may be due to the presence of any of these constituents

However, the components responsible for the antioxidant activity of hydroalcoholic extract of *Cassia fistula* are currently unclear. Therefore, further works have been performed on the isolation and identification of the antioxidant components present in hydroalcoholic extract of *Cassia fistula.*

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