In-Vitro Antioxidant Activity of *Cassia occidentalis* Seeds

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Summary

In vitro antioxidant activity of methanol extract of *Cassia occidentalis* seeds was determined by DPPH free radical scavenging, FRPA, Lipid peroxidation by thiobarbituric acid assay methods. The analysis had shown the maximum percentage inhibition in case of DPPH method as 66.53% at 160µg/ml and 61.07% in lipid peroxidation at 1000µg/ml. Further, reducing power of methanol extract of seeds increased in concentration-dependent manner. Total phenolic content estimation was done by using Folin-Ciocalteu reagent and was found to be 0.75% w/w. The present study revealed that the methanol extract of seeds have antioxidant potential and represent a potential source of medicine.

Keywords: Antioxidant, *Cassia occidentalis,* DPPH, Legumenocae

Introduction

Many plants contain natural antioxidants that act in metabolic response to the endogenous production of free radicals and other oxidant species. These responses are either due to ecological stress or promoted by toxins produced by pathogenic fungi and bacteria (1). Antioxidant activity plays a vital role in various pharmacological activities such as anti-aging, anti-inflammatory, anti-atherosclerosis, anti-cancer activities (2-3). Antioxidant nutrients such as Vit-E, β carotene, lycopene are regularly found to reduce the risk of lung, prostate, stomach cancers as well as oral precancers in epidemiologic studies (4).Currently available synthetic antioxidants like butylated hydroxy anisole (BHA), butylated hydroxy toluene (BHT), tertiary butylated hydroquinone and gallic acid esters, 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. Moreover, these synthetic antioxidants also show low solubility and moderate antioxidant activity (5, 6). Recently there has been an upsurge of interest in the therapeutic potential of medicinal plants as antioxidants in reducing such free radical-induced tissue injury (7). Besides, well known and traditionally used natural antioxidants from teas, wines, fruits, vegetables and spices, some natural antioxidants (e.g. rosemary and sage) are already exploited commercially either as antioxidant additives or as nutritional supplements (8). It has been mentioned that antioxidant activity of plants might be due to their phenolic compounds (9). Flavonoids are a group of polyphenolic compounds with known properties which include free radical scavenging, inhibition of hydrolytic oxidative enzymes and anti-inflammatory action (10).

Cassia occidentalis L. (Kasaundi) of family leguminosae is extensively used in the indigenous and folk-lore medicine systems for a variety of purposes (11-12). In Unani medicine, it is used as an antidote, blood purifier, expectorant, anti-inflammatory agent and a remedy for the treatment of liver diseases (13). The ethanolic extract of leaves of this plant was found to possess the anti-hepatotoxic activity against carbon tetrachloride- and thioacetamide-induced liver damage (14). The anti-inflammatory activity of its constituents isolated from root and stem have also been reported (15). The seeds are brewed into a coffee-like beverage to treat asthma and the flower infusion is used for bronchitis in the Peruvian Amazon (16). The present study was done to evaluate the antioxidant property of *Cassia occidentalis.*

Material and Methods

Plant material

Seeds of *Cassia occidentalis* were collected in the month of October- November, 2008 from the local area of Moga and were authenticated by Dr. H.B. Singh Director, Department of Raw Material Herbarium & Museum, National Institute of Sciences Communication and Information Resources (NISCAIR), New Delhi, vide their letter no. NISCAIR/RHMD/Consult/-2008-09/1126/157.

Preparation of extracts

Cassia occidentalis seeds were defatted with petroleum ether and then extracted in Soxhlet apparatus with methanol and filtered to yield the extract. The dried extract was used for the evaluation of antioxidant activity by using 1, 1-diphenyl, 2- picryl hydrazyl (DPPH) radical scavenging activity, lipid peroxidation with thiobarbituric acid and reducing capacity.

Phytochemical screening

Presences of various phytoconstituents were determined by performing different qualitative tests on methanol extract (17).

Determination of total phenolic content

The total phenolic content in seeds of *Cassia occidentalis* was determined by using Folin Ciocalteu's method. Folin Ciocalteu reagent is a mixture of phosphomolybdate and phosphotungstate used for the colorimetric assay of phenolic and polyphenolic antioxidants. It works by measuring the amount of substance being tested needed to inhibit the oxidation of the reagent. The sample extract dilution was oxidized with Folin Ciocalteu reagent and the reaction was neutralized with sodium carbonate. The absorbance of the resulting blue color was measured at 765nm after 30 min (18).

Preparation of calibration curve using gallic acid as standard

10mg of standard gallic acid was accurately weighed and dissolved in 100ml distilled water in a volumetric flask (100µg/ml of stock solution). From the above stock solution 0.5 to 2.5 ml aliquots were pipette out into 25ml volumetric flasks. Added 10ml of distilled water and 1.5ml of Folin Ciocalteu reagent, diluted according to the label specification to each of the above volumetric flasks. After 5 min. 4ml of 20% sodium carbonate solution was added and volume was made up to 25ml with distilled water. Absorbance was recorded after 30 min. at 765nm and a calibration curve of absorbance *vs* concentration was plotted.

Preparation of test solution

1g of accurately weighed powdered drug was extracted with (3 × 15ml) 50% aqueous methanol by cold maceration for 2 h with intermittent shaking. Filtered and the final volume of the combined methanolic extract was made up to 50ml. From this test solution 1ml was pipette out into 25ml volumetric flask and same procedure was followed for colour development using Folin Ciocalteu reagent. The amount of total phenolics was calculated using the standard curve of gallic acid. Distilled water was taken as blank. Quantification was done on the basis of standard curve of gallic acid. Results were expressed as µg gallic acid equivalents (GAE) and percentage w/w. The percentage of total phenolics was calculated.

***In-vitro* assays for anti-oxidant activity**

Determination of DPPH radical scavenging activity

$DPPH^{\bullet}$ is a stable free radical at room temperature which when accepts an electron or hydrogen radical becomes a stable diamagnetic molecule (19). The reduction capability of the DPPH radical is determined by the decrease in its absorbance at 517nm, induced by antioxidants. The absorption maximum of a stable DPPH radical in methanol was at 517nm. On reaction with antioxidant or free radical there is decrease in absorbance of DPPH radical because of scavenging of the radical by hydrogen donation. There is change in color from purple to yellow which is visually noticeable. Hence, DPPH is usually used as a substrate to evaluate the antioxidative property (20). 0.1 mM solution of DPPH in methanol was prepared and 1.0mL of this solution was added to 3.0ml of extract solution in water at different concentrations (5-160µg/mL). It was incubated at room temperature for 45 min. and absorbance was measured at 517nm against the corresponding blank solution. The assay was performed in triplicates. Ascorbic acid was taken as reference standard. Percentage inhibition of DPPH free radical was calculated based on the control reading, which contain DPPH and distilled water without extract using the following equation:

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

Where; Acont is the absorbance of the control reaction and Atest is the absorbance in the presence of the sample of the extracts.

Reducing power assay

The different concentration of the extracts (25-400µg/mL) in 1 mL of deionized water were mixed with phosphate buffer (2.5mL, 0.2M, pH 6.6) and 1% potassium ferricyanide $[K_3Fe(CN)_6]$ (2.5mL). The mixture was incubated at 50°C for 20 min. The reaction was stopped by adding trichloroacetic acid (2.5mL, 10%) to the mixture, which was then centrifuged for at 1000 x g for 10 min. The upper layer of solution (2.5mL) was mixed with distilled water (2.5mL) and $FeCl_3$ (0.5mL, 0.1%), and the absorbance was measured at 700nm. Ascorbic acid was taken as a reference (21-22).

Lipid peroxidation by thiobarbituric acid assay (TBA)

TBA reacts with malondialdehyde (MDA) to form a diadduct, a pink chromogen, which can be detected spectrophotometrically at 532nm (23). Normal male rats (250g) were used for the preparation of liver homogenate. The perfused liver was isolated, and 10% w/v homogenate was prepared with homogenizer at 0-4°C with 0.15M KCl. The homogenate was centrifuged at 8,000 x g for 15 min. and clear cell-free supernatant was

used for the study with *in vitro* lipid peroxidation assay. Different concentrations (25-1000µg/ml) of extract dissolved in methanol in test tubes. 1ml of 0.15 M KCl and 0.5mL of rat liver homogenates were added to the test tubes and peroxidation was initiated by adding 100µL of 0.2mM ferric chloride. After incubation at 37°C for 30 min. the reaction was stopped using 2mL of ice-cold HCl (0.25N) containing 15% trichloroacetic acid (TCA), 0.38% TBA, and 0.5% butylated hydroxytoluene. The reaction mixtures were heated at 80°C for 60 min. The samples were cooled and centrifuged; the absorbance of the supernatants was measured at 532nm. The percentage inhibition of lipid peroxidation is calculated by the formula:

Inhibition of lipid peroxidation (%) = 1 – (sample OD/blank OD) X 100

Results and Discussion

Phytochemical screening

The preliminary phytochemical screening of methanol extract revealed the presence of phenolics, flavonoids, anthraquinone, amino acids and steroids.

Total phenolic content

Phenolic constituents are very important in plants because of their scavenging ability due to their hydroxyl groups (24). These substances are known to possess the ability to reduce oxidative damage and act as antioxidants (25). They can trap the free radicals directly or scavenge them through a series of coupled reactions with antioxidant enzymes (26). In addition, it is reported that phenolic substances are associated to play important role in stabilizing lipid peroxidation (27). Total phenolics determined in extract were 0.75% w/w.

DPPH assay

DPPH stable free radical method is an easy, rapid and sensitive way to survey the antioxidant activity of a specific compound or plant extracts (28). Figure 1 illustrates decrease in the concentration of DPPH radicals due to the scavenging ability of methanol extract and standard. A 160µg/mL of methanol extract and ascorbic acid exhibited 66.53% and 85.79% inhibition, respectively. The results indicate that methanol extract of *Cassia occidentalis* reduces the radical to the corresponding hydrazine when it reacts with the hydrogen donors in the antioxidant principles (29).

Reducing power assay

Figure 2 shows the reductive capability of the methanol extract to ascorbic acid (standard). For the measurement of the reductive ability, we investigated the Fe3+ - Fe2+ transformation in the presence of methanol extract (21- 22). The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity (30). For the measurement of reducing ability, the Fe3+-Fe2+ transformation was investigated in the presence of *Cassia occidentalis*. The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity. The antioxidant activity has been attributed to various mechanisms, among which are the prevention of chain initiation, the binding of transition metal ion catalysts, decomposition of peroxides, the prevention of continued hydrogen abstraction, the reductive capacity and radical scavenging (31). Figure 2 shows the reductive capability of the methanol extract of *Cassia occidentalis* seeds.

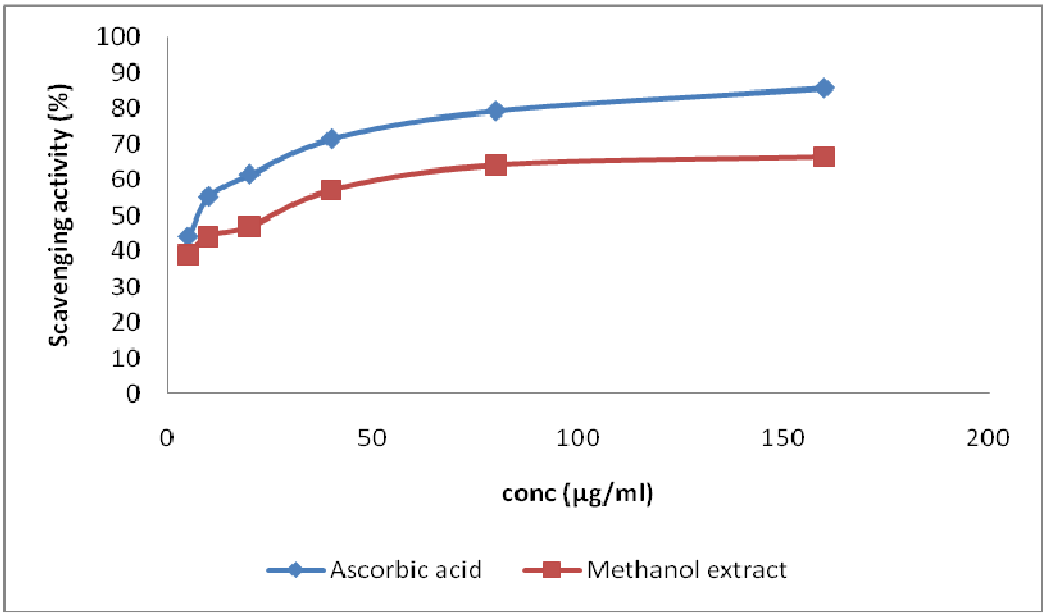

Fig. 1: Free radical scavenging activity of methanol extract of *Cassia occidentalis* seeds at different concentrations

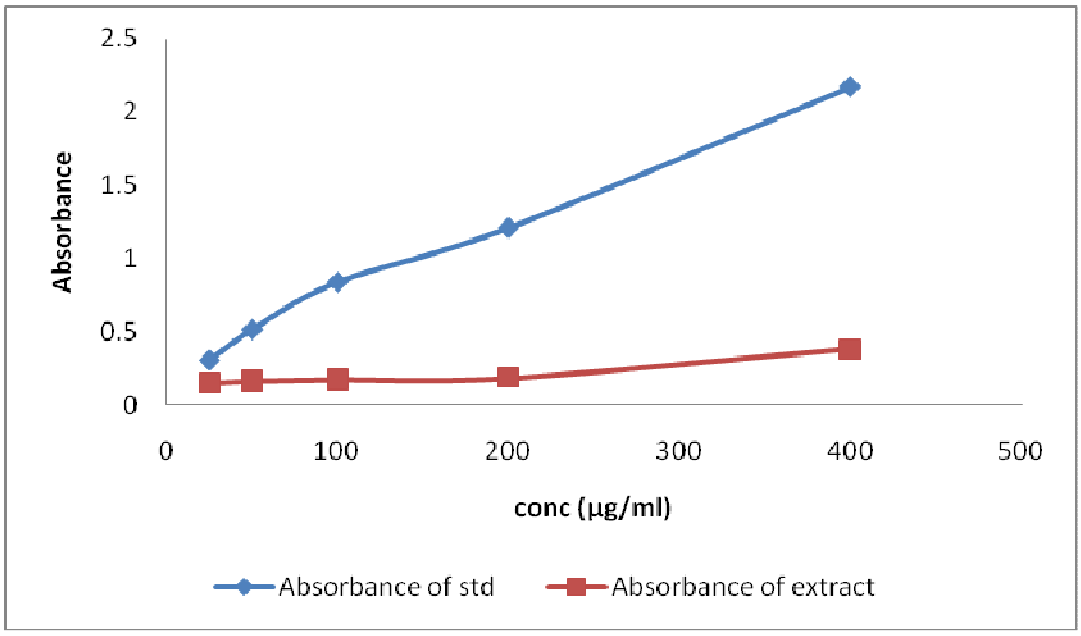

Fig.2: Reducing power assay of methanol extract of *Cassia occidentalis* seeds at different concentrations.

Lipid peroxidation assay

The liver of rat was used as a source of polyunsaturated fatty acids for determining the extent of lipid peroxidation. Malondialdehyde, a lipid peroxidation product, is an indicator of reactive oxygen species (ROS) generation in the tissue (32). During lipid peroxidation low-density lipoprotein (LDL) breaks down into TBARS and the amount of TBARS can be used as an index of lipid peroxidation (33). As phenolic antioxidants are suggested to act as inhibitors of LDL oxidation by means of free radical scavenging, so it is expected that the presence of these compounds in these extract might be responsible for inhibiting the LDL oxidation by donating the hydrogen atom (34). The main interest of the recent research suggests that LDL oxidation may play an important role in the pathogenesis of atherosclerotic complications, including coronary heart disease (CHD). The radical-mediated oxidative chain reaction is a possible mechanism involved in LDL oxidation. LDL oxidation is believed to be a complex and multistep process involving both lipid and protein fractions through different mechanisms (39). Antioxidants, including vitamins C and E, flavonoids, and other plant phenolics, have been shown to suppress LDL oxidation and delay the development of heart diseases (36). A 1000µg/mL of methanol extract and BHT exhibited 61.07% and 92.63% inhibition, respectively. Figure 3 shows the percentage inhibition of extract in comparison with the standard Butyl hydroxytoluene.

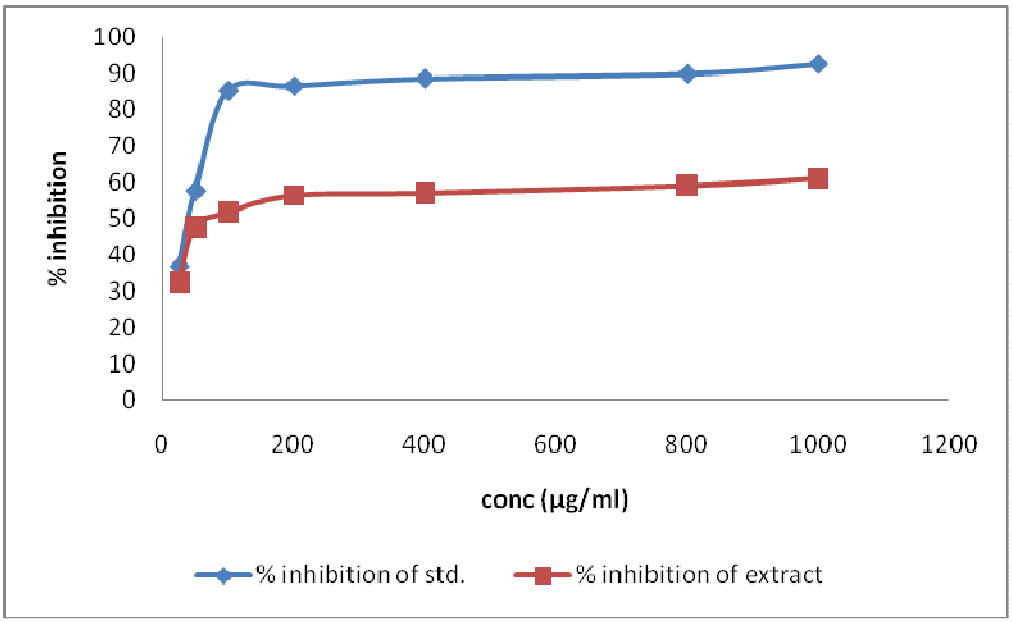

Fig.3: Lipid peroxidation inhibition activity of methanol extract of *Cassia occidentalis* seeds at different concentrations

Conclusion

The results of the present study has shown that the methanol extract of *Cassia occidentalis* Linn. Seeds possess antioxidant activity proved via DPPH radical scavenging activity, lipid peroxidation with thiobarbituric acid and reducing power assay. Preliminary phytochemical analysis indicates the presence of polyphenols (tannins, flavonoids) in methanol extract. Polyphenols like flavonoids and tannins are the well known natural antioxidants (37). So, the antioxidant potential of methanol extract of *Cassia occidentalis* may be due to the presence of polyphenolic content. However, the isolation and characterization of the responsible phytoconstituents is in progress.

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