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Research Article

Phytochemical Screening and *In Vitro* Antioxidant Activity of Ethanolic Extract of *Cassia occidentalis*

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ABSTRACT

The ethanolic extract of the leaves of *Cassia occidentalis (Co)* were subjected to phytochemical analysis by standard qualitative analysis and the *invitro* antioxidant activity was evaluated by determination of total antioxidant capacity, 1.1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity, hydrogen peroxide (H_2O_2) radical scavenging activity, superoxide scavenging activity and Ferric reducing anti oxidant potential (FRAP). The analyses revealed that the ethanolic extract of *Co* was able to efficiently scavenge the free radicals in a dose dependant manner. The results were compared with the standard antioxidant ascorbic acid. The results have shown that crude ethanolic extract of the leaves of *Co* showed excellent antioxidant activity due to the presence of bioactive compounds namely alkaloids, betacyanin, cardiac glycosides, coumarins, flavonoids, phenols, steroids, saponins, tannins, terpenoids, anthraquinones and emodins.

Keywords: *Cassia occidentalis,* phytochemical analysis, antioxidant activity.

INTRODUCTION

Natural sources like plants play a vital role in drug development. This is due to the promising biological activity of plant materials in treating infectious diseases successfully without any side effects. This attracted the scientist worldwide and started exploring the plant kingdom. One among such herb is *Cassia occidentalis* Linn. which belong to the family Leguminaceae. The potential activity of these herbs in prevention and treatment of diseases depend on their bioactive compounds or phytochemicals. Plant-derived bioactive compounds have been found to stimulate osteoblast differentiation and bone formation. These bioactive compounds include flavonoids, coumarins, lignins, polyphenols, terpenoids, carotenoids, alkaloids and anthraquinone (emodin)[1]. Plant extracts containing the phytochemical anthraquinone (emodin) have gained momentum in being used in cosmetic, dye, food, and pharmaceutical industries due to their wide therapeutic and pharmacological properties[2]. The leaf is recognized as anti-neuralgic, purgative (in treatment of diarrhea and dysentery) and vermifuge.

Oxidative stress is caused due to the release of free radicals like superoxide anions, hydrogen peroxide, and hydroxyl, nitric oxide and peroxy nitrite. These free radicals plays an important role in the pathogenesis of neurodegenerative disorders, atherosclerosis, diabetes, inflammation,[3] aging,[4] cancer, coronary heart disease and Alzheimer's disease[5]. Antioxidants are compounds which act as radical scavengers when added to the food products prevent the radical chain reaction of oxidation, delay or inhibit the oxidation process and increase the shelf life by retarding the processes of lipid per oxidation[6]. This article takes interest in proving the antioxidant activity of *Cassia occidentalis* along with the detailed analysis of the phytochemicals present in this herb.

MATERIALS AND METHODS

Chemical reagents

The solvents used were of HPLC grade. The standards (BHT, ±-tocopherol, L-Ascorbic acid and gallic acid) and chemicals used were obtained from Hi-Media lab. Ltd, Mumbai, India. 1,1 diphenyl-2-picryl hydrazyl (DPPH) radicals were purchased from Sigma Chemical Co, St. Louis, MO, USA.

Preparation of plant extract

Healthy leaves of *Co* were collected and washed with double distilled water, dried under shade and powdered. The dried powder was extracted using 95% ethanol using Soxhlet apparatus. Using rotary evaporator, the final trace of the solvent was removed. The dried crude ethanolic extract was stored at 4ºC until use.

Phytochemical Screening

Test for Alkaloids

About 0.5 g of *Co* extract was dissolved in 5% Hydrochloric acid, filtered and treated with Dragendorff's

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reagent and Mayer's reagent separately. Appearance of turbidity or precipitate indicates the presence of alkaloids[7].

Test for Anthocyanin and Betacyanin

About 2ml of *Co* extract was mixed with 1 ml of 2N sodium hydroxide and heated for 5 min at 100°C. Presence of anthocyanin was confirmed by the bluish green colour formation and betacyanin was confirmed by yellow coloration[8].

Test for Cardiac Glycosides (Keller-Killiani Test)

0.5 mL of the *Co* extract was mixed with 2 mL of glacial acetic acid and few drops of 5% ferric chloride. To the sides of the tube, 1 ml of concentrated sulphuric acid was added. A brown ring appears indicating the presence of cardiac glycosides[8].

Test for Coumarins

0.5g of *Co* extract was moistened and shaken in a test tube which was further covered with filter paper moistened with 1N sodium hydroxide solution. The tube was allowed to stand in boiling water for few min. The filter paper was removed and exposed under UV light for yellow fluorescence which indicates the presence of coumarins[8].

Test for Flavonoids

0.5g of *Co* extract was mixed and gently shaken with petroleum ether in order to remove the fatty materials. The defatted residue was then treated with 20 ml of 80% ethanol and filtered. The filtrate was used for the following test:

To 3 mL of the filtrate, 4 mL of 1% aluminium chloride in methanol was added in a test tube. Yellow coloration occurs indicating the presence of flavonoids, flavones or chalcones.

To another 3 ml of filtrate, 4 ml of 1% potassium hydroxide was added leading to the formation of dark yellow colour indicating the presence of flavonoids[8].

Test for Phenol (Ferric Chloride Test)

1 ml of *Co* extract was mixed with 2 ml of distilled water followed by few drops of 10% ferric chloride. A blue or black colour was formed indicating the presence of phenols[7].

Test for Quinones

1 ml of *Co* extract was mixed with 1 ml of concentrated sulphuric acid leading to the formation of red colour which confirms the presence of quinones[8].

Test for Saponins (Foam Test)

To 2 ml of boiling water 0.5 g of *Co* extract was added in a test tube and allowed to cool. The mixture was shaken thoroughly until the foam appears indicating the presence of saponins[8].

Test for Steroids

0.5 ml of *Co* extract was added with 2 ml of chloroform and

1 ml of sulphuric acid. A reddish brown ring appears showing the presence of steroids[8].

Test for Tannins

0.5 g of *Co* extract was mixed with 20 ml of distilled water, boiled and filtered. The filtrate was further treated with 1 ml of 0.1% Ferric chloride. A brownish green or blue black colour forms indicating the presence of tannins[9].

Test for Terpenoids (Salkowski test)

To 0.5 ml of *Co* extract was mixed with 2 ml of chloroform and 0.5 ml of concentrated sulphuric acid was added leading to the formation of reddish brown coloration confirming the presence of terpenoids[8].

Test for anthraquinones

0.5 g of the extract was boiled with 10 ml of sulphuric acid and filtered while hot. The filtrate was shaken with 5 ml of chloroform. The chloroform layer was pipette into another test tube and 1 ml of dilute ammonia was added. The resulting solution was observed for colour changes.

Test for Emodins

2 ml of Ammonium hydroxide and 3 ml of benzene was added to the extract. Appearance of red colour indicates presence of emodins.

In Vitro Anti-Oxidant Activity

DPPH Radical Scavenging Activity

The scavenging activity for DPPH free radicals was measured according to the procedure described by Braca et al[10]. An aliquot containing 3 ml of 0.004% DPPH solution in ethanol and 0.1 ml of *Co* extract/ ascorbic acid at various concentrations (50 – 500 µg/ml) was prepared. The mixture was shaken vigorously and incubated at room temperature for 30min. The degree of decolorization of DPPH was determined by measuring the absorbance at 517 nm. Ascorbic acid was taken as a standard control for the assay. The percentage inhibition of DPPH radicals by the extract/compound was determined by comparing the absorbance values of the control and the experimental tubes where, 'A cont' is the absorbance of the control reaction and 'A test' is the absorbance for the presence of the sample in 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 extract that inhibits the formation of DPPH radicals by 50%. The inhibition curve was plotted for duplicate experiments and represented as % of mean inhibition ± standard deviation. IC_{50} values were obtained by probit analysis[11].

$$\text{DPPH Scavenged}\,(\%) = \frac{\text{A cont} - \text{A test}}{\text{A cont}} \times 100$$

Determination of H_2O_2Radical Scavenging Activity

Scavenging activity of hydrogen peroxide (H_2O_2) by *Co* extract was determined by the method described by Ruch et al[12]. According to this method, about 4 ml of different concentration of (50 – 500 µg/ml) of *Co* extract was prepared with distilled water. To this solution, 0.6 ml of 4 mM H_2O_2 solution prepared in phosphate buffer (0.1 M pH 7.4) was mixed and incubated for 10 min. The absorbance of the solution was measured at 230 nm in UV spectrophotometer. Ascorbic acid was taken as a positive control compound and the percentage of inhibition was calculated by the given formula,

$$\text{H}_2\text{O}_2\ \text{Scavenged}\,(\%) = \frac{\text{A cont} - \text{A test}}{\text{A cont}} \times 100$$

where 'A cont' is the absorbance of the control reaction and 'A test' is the absorbance for the presence of the compound in the extracts. The inhibition curve was plotted for duplicate experiments and represented as % of mean

Table 1: Phytochemical analysis of *Cassia occidentalis.*

S.No	Phytochemicals	Analysis
1	Alkaloids	Present
2	Betacyanin	Present
3	Cardiac glycosides	Present
4	Coumarins	Present
5	Flavonoids	Present
6	Phenols	Present
7	Steroids	Present
8	Quinones	Present
9	Saponins	Present
10	Tannins	Present
11	Terpenoids	Present
12	Anthraquinones	Present
13	Emodins	Present

Table 2: DPPH Radical scavenging activity.

Concentration (µg/ml)	Ascorbic acid % Inhibition	*Co* % Inhibition
50	81.22±1.01	72.82±1.55
100	90.87±1.47	76.41±1.04
200	92.49±0.85	80.6±0.80
300	96.83±0.30	84.46±0.80
400	99.36±0.68	87.33±0.39
500	103.85±0.28	90.39±1.12
IC_{50} (µg/ml)	142	261

Table 3: Hydrogen peroxide scavenging activity.

Concentration (µg/ml)	Ascorbic acid % Inhibition	*Co* % Inhibition
50	40.65±0.99	30.98±1.27
100	44.17±0.55	32.76±0.81
200	45.31±0.41	35.37±0.57
300	45.8±0.18	38.24±0.43
400	47.18±0.36	39.81±0.28
500	47.54±0.68	41.48±0.57
IC_{50} (µg/ml)	104.12	172

Table 4: Total antioxidant capacity.

Concentration (µg/ml)	Ascorbic acid % Inhibition	*Co* % Inhibition
50	19.12±0.54	23.38±0.45
100	29.51±0.70	34.12±0.94
200	44.81±0.84	49.28±0.62
300	45.97±0.40	54.15±0.21
400	52.72±0.99	59.42±0.49
500	61.27±0.73	66.49±0.96
IC_{50} (µg/ml)	286	323

inhibition ± standard deviation. IC_{50} values were obtained by probit analysis[11].

Determination of Total Antioxidant Capacity

Prieto et al[13] have described phospho molybdenum method for assessing the total antioxidant capacity of plant extract. According to this method, plant extracts in an acidic medium reduces molybdenum and subsequently forms a green coloured complex. Based on this principle, 0.3 ml of *Co* extract was mixed with 3 ml of reagent solution containing 0.6 M sulfuric acid, 28 m M sodium phosphate and 4 mM ammonium molybdate. The reaction mixture was incubated at 95°C for 90 min and allowed to cool at room temperature. The absorbance of the mixture was measured at 695 nm using a UV spectrophotometer against blank. 0.3 ml of ethanol was used as blank. The antioxidant activity was expressed as the number of gram equivalents of ascorbic acid.

Table 5: Ferric reducing anti oxidant power (FRAP).

Concentration (µg/ml)	Ascorbic acid % Inhibition	*Co* % Inhibition
50	85.67±1.22	64.18±0.84
100	92.39±1.21	69.31±0.63
200	94.18±0.35	71.1±1.63
300	96.22±0.58	74.42±1.14
400	97.31±0.50	79.54±0.63
500	98.23±0.35	90.05+1.00
IC_{50} (µg/ml)	224	275

Table 6: Superoxide scavenging activity.

Concentration (µg/ml)	Ascorbic acid % Inhibition	*Co* % Inhibition
50	84.04±0.39	82.97±0.22
100	91.03±1.38	93.17+0.69
200	94.90±0.81	94.33+0.59
300	96.99±0.14	95.22±0.48
400	98.01±0.42	96.86±0.46
500	97.36±0.42	97.04±0.59
IC_{50} (µg/ml)	255	267

Ferric Reducing Anti-oxidant Power (FRAP ASSAY)

The reducing power of the extract was determined by the procedure given by Oyaizu et al[14]. According to this method, various concentrations of *Co* extracts (50 – 500 µg/ml) were prepared in 1ml of distilled water, mixed with 2.5 ml of 0.2M phosphate buffer pH 6.6 and 2.5 ml of 1% potassium ferricyanide. The mixture was incubated at 50°C for 20 min. To this mixture 2.5 ml of 10 % trichloro acetic acid was added and centrifuged at 3000 RPM for 10 min. 2.5 ml of supernatant was taken and mixed with 2.5ml of distilled water and 0.5ml of 0.1% ferric chloride. The absorbance was measured at 700 nm using spectrophotometer. Ascorbic acid was used as the positive standard solution and phosphate buffer pH 6.6 was used as blank solution. All the tests were performed in triplicate and the graph was plotted with the average of three observations.

Superoxide Scavenging Activity

In superoxide scavenging activity, alkaline DMSO (dimethyl sulphoxide in sodium hydroxide) was used as a super oxide generating system. In this method, 0.5 ml of *Co* extract with different concentrations (50-500 µg/ml) was taken and to this 1 ml of alkaline DMSO and 0.2 ml 20 mM of nitro blue tetrazolium (NBT) in phosphate buffer pH 7.4 was added. The experiment was performed in triplicate and the absorbance was measured at 560 nm[15]. The percentage scavenged was determined by the given formula,

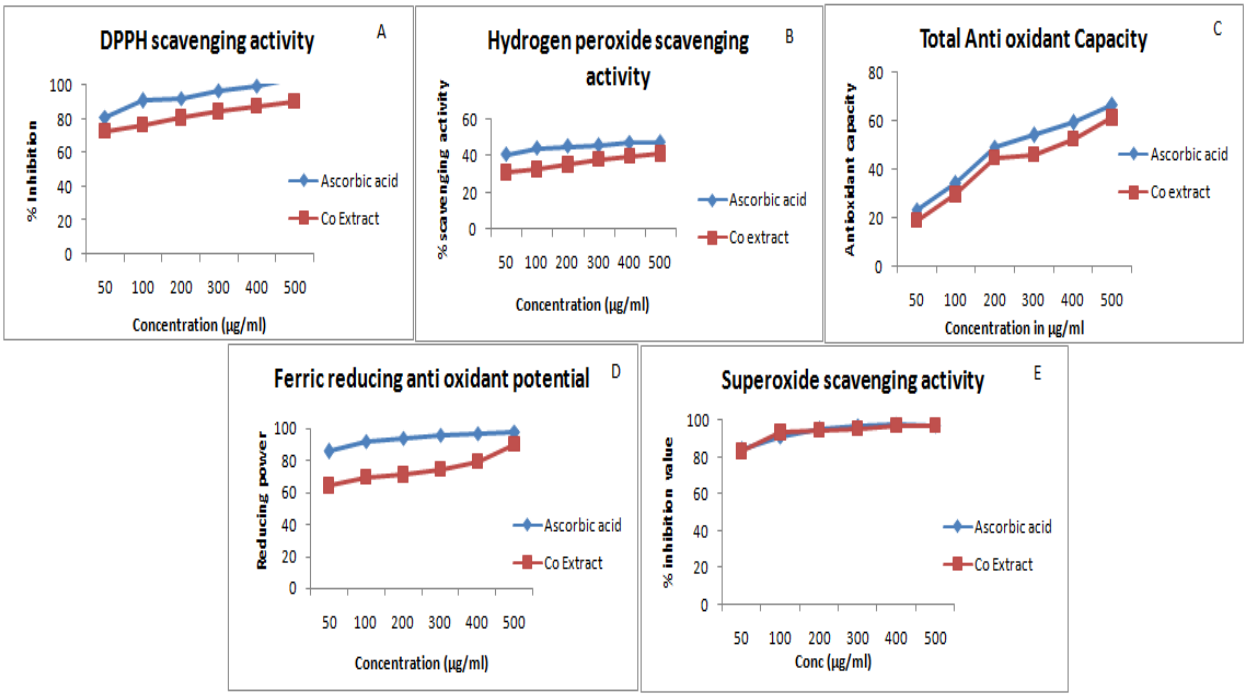

Figure 1A to 1E Antioxidant potential of *Cassia occidentalis*.

$$\text{Scavenged}\,(\%) = \frac{\text{A cont} - \text{A test}}{\text{A cont}} \times 100$$

where 'A cont' is the absorbance of the control reaction and 'A test' is the absorbance for the presence of the sample in the extracts. The amount of sample required to decrease the absorption of DPPH, FRAP, H_2O_2, total antioxidant capacity and superoxide by 50% were calculated graphically (% of inhibition was plotted against the concentration in µg/ml). DPPH, FRAP, H_2O_2, superoxide radical scavenging activity, and total antioxidant capacity was compared with a reference standard ascorbic acid.

RESULTS AND DISCUSSION

Phytochemical analysis

Table 1 shows the phytochemical analysis of *Co* leaf extract. The standard procedures showed that *Co* extract contains alkaloids, betacyanin, cardiac glycosides, coumarins, flavonoids, phenol, steroids, quinines, saponins, tannins, terpenoids, anthraquinones and emodins. These phytochemicals support the leaf extract to treat various ailments like rheumatism, diarrhea, dysentery and to treat cutaneous diseases etc. Plant-derived phytochemicals such as tannins, phenolic acids, flavonoids, anthocyanins and proanthocyanins, lignans, stilbenes, coumarins, quinones, xanthones, catechins, emodins etc., could delay or prevent the onset of degenerative diseases because of their redox properties, which allow them to act as hydrogen donors, reducing agents, hydroxyl radicals (-OH.) or superoxide radical (O_2) scavengers. Ethanolic extract of *Co* which was qualitatively assessed for phytochemical analysis revealed the presence of Alkaloids, Betacyanin, Cardiac glycosides, Coumarins, Flavanoids, Phenol, Steroids, Quinones, Saponins, tannins, terpenoids, Anthraquinones and emodins. These bioactive compounds may be the probable reason for the free radical scavenging activity of the extract.

In vitro Anti-oxidant activity

Table 2 to 6 and Figure 1A to 1E shows the DPPH, H_2O_2, total antioxidant capacity, FRAP, and superoxide radical scavenging activity of *Co* leaf extract. In these analyses standard ascorbic acid was used as a positive control. The antioxidant property was expressed as inhibition concentration, IC_{50}. The concentration of extract was used to calculate the inhibition concentration IC_{50} in µg/ml.

Co was able to effectively scavenge the free radicals in different concentrations in a dose dependant manner in all the assays. DPPH free radical scavenging activity is one of the commonly accepted models against lipid oxidation. The effect of antioxidants on DPPH radical scavenging was thought to be due to their hydrogen donating ability. Thus *Co* caused a very strong anti-oxidant activity against DPPH radicals showing hydrogen donating ability.

Superoxide (O_2^{-}•) is accepted to be a very harmful radical that can be converted into more reactive species, such as hydroxyl radical or peroxy nitrite, contributing to tissue damages and various diseases. Superoxides are produced from molecular oxygen due to oxidative enzymes of the body by auto-oxidation of catecholamines[16]. The scavenging activity of *Co* may be due to inhibitory effect of generation of superoxides in an invitro reaction mixture. Hydrogen peroxide (H_2O_2) is a weak oxidizing agent and crosses cell membranes rapidly to enter the cell. There, H_2O_2 can react with Fe+2 ions to generate the hydroxyl radical and this may be the origin of many of its toxic effects[17]. H_2O_2 is relatively stable in the absence of reducing compounds. Scavenging of H_2O_2 by *Co* may be attributed to their electron donating abilities[18].

FRAP assay is based on the ability of antioxidants to reduce Fe^{3+} to Fe^{2+} and is generally associated with the presence of reductones, that exert a very strong antioxidant action by breaking the free radical chain through donating a hydrogen atom[19]. The total anti-oxidant capacity of *Co* extract reveals that it exhibited a very strong anti oxidant potential. The FRAP activity and the total anti oxidant capacity is correlated to high phenolic and flavonoid compounds namely quercetin, and kempferol. Phytochemical analysis on the crude leaf extract of *Co* showed the presence of such phenolic and flavonoid compounds in the extract.

CONCLUSION

This study supports the traditional use of *Cassia occidentalis* and revealed the phytochemicals present in it. In addition, the study showed the excellent antioxidant activity of *Co* extract which could be a supporting factor for its pharmacological applications in treating various ailments and can be recommended to use as an anti-stressor.

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