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EVALUATION OF ANTIOXIDANT PROPERTIES AND RADICAL SCAVENGING ACTIVITY ON ETHYL ACETATE EXTRACT OF *CASSIA OCCIDENTALIS*.L

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ABSTRACT

In the present study, methanol, ethanol and aqueous extracts of Cassia *occidentalis* were evaluated by employing various *in vitro* antioxidant assays. The aqueous extract of had shown highest total phenolic content (250mg/g), the further assays were carried on active fraction of aqueous that is fractioned ethyl acetate extract. The ethyl acetate extract of *C. occidentalis* had the most effective total antioxidant activity among the two separated extracts. The IC_{50} values of the extract and BHT were found to be 60.5 and 60.4 respectively. The ethyl acetate extract of *C. occidentalis* was found more effective in FRAP, Hydrogen and Nitric oxide scavenging activity than that of aqueous extract which shows that it has a strong antioxidant activity. This study revealed that ethyl acetate extract of the leaf of *C. occidentalis* is a potential source of natural antioxidant, which might be helpful in preventing the progress of various oxidative stresses.

INTRODUCTION

Free radicals are fundamental to any biochemical process and represent an essential part of aerobic life and our metabolism. Scientific evidence suggests that antioxidants reduce risk for chronic diseases including cancer and heart diseases. Antioxidants like vitamin C, vitamin E, carotenes, phenolic acid, phytate and phytoestrogens have been recognized to have potential to reduce the risk of diseases. Therefore, there has been an increasing demand to evaluate the antioxidant properties of direct plant extracts or isolated products from plant origin rather than looking for synthetic ones. ROS are continuously produced during normal physiological events and can easily initiate the peroxidation of membrane lipids, leading to the accumulation of lipid peroxides. ROS is capable of damaging crucial biomolecules such as nucleic acids, lipids, proteins and carbohydrates. ROS and RNS may cause DNA damage that may lead to mutation (Cakir *et al.,* 2006). If ROS are not effectively scavenged by cellular constituents, they lead to disease conditions (Halliwell *et al.,* 2003). ROS may interact with biological systems in a clearly cytotoxic manner. These molecules are exacerbating factors in cellular injury, aging process, colon cancer, coronary heart disease and atherosclerosis (Madhavi *et al.,* 1996). Recent research investigations have suggested that diets rich in polyphenolic compounds are associated with longer life expectancy (Hertog *et al.,* 1996). Moreover, these compounds have been found to be effective in many health-related properties, such as anticancer, antiviral, anti-inflammatory, effects on capillary fragility, and an ability to inhibit human platelet aggregation (Benavente *et al.,* 1997). *Cassia occidentalis* exerted many pharmacological effects included antimicrobial, anthelmintic, insecticidal, antioxidant, antianxiety, antidepressant, antimutogenic antidiabetic, wound healing, hepatoprotective, renoprotective, sun protective, smooth muscles relaxation, immune-modulating, antiinflammatory, analgesic, antipyretic and other effects (Ali Esmail *et al.,* 2015). *C.occidentalis* which is known to have a medicinal properties, reported that the leaf extract of the plant produced significant hepato-protection (Jafri *et al.,* 1999). Few observations have demonstrated that *C. occidentalis* seed extracts reduced the DNA degradation caused by iron (II)-driven Fenton reaction. They also noted that inhibition of DNA damage may be due to their strong ferrous ion chelation capability. In addition, they also proposed that it may be due to their very good scavenging

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activity towards free radicals. Himoliv is a polyherbal ayurvedic formulation in which *C. occidentalis* is an ingredient (20 mg/5 ml) (Bhattacharyya *et al.,* 2003) and its leaves have good anti-inflammatory activity as assayed by Sadique *et al.,* (1987). Methanolic fraction of the ethanolic extract of the leaves showed hepatoprotective activity. It enters into the formulation of LIV 52, an indigenous liver protective preparation. Liv 52 was found to reduce the carcinogenic ability of DMBA (7,12-dimethylbenz[a]anthracene)in mice (Saraf *et al.,* 1994). The propriety Herbal Preparation (PHP) comprising the plant as one of the ingredients provided significant protection against most of the biochemical alterations produced by CCl_4. The degree of protection is maximum at 12 days regimen at 250mg/Kg dose. Oral LD_{50} for the preparation is 2gm/Kg (Sharma *et al.,* 1999). The composite of Indian Herbal Preparation II (CIHP II) comprising the plant and other ingredients has been prepared. It has the properties of adaptogenic and improved maintenance of glucose levels. It also induced state of non-specific increased resistance during stress (Grover, 1995). The study by Odeja *et al.,* (2014) reported the antioxidant activities of the methanolic leaf extract of *C. occidentalis* by free radical scavenging capacity using hydrogen peroxide and the result showed that activity was increased by dose dependent mannaer.

The study done by Vedpriya *et al.,* (2011) reported the methanolic extract of leaves and stem were found to possess highest metal chelating and nitric oxide radical scavenging potential as compared to seed extract. The antioxidant potential of different fractions of whole plant of *Cassia occidentalis* was also evaluated using various in vitro assay including 1, 1- Diphenyl-2- Picrylhydrazyl (DPPH), nitric oxide scavenging activity, hydrogen peroxide scavenging activity, reducing power assay. The various antioxidant activities were compared with ascorbic acid and gallic acid as standard antioxidant. The results showed that ethyl acetate fraction of whole plant of *Cassia occidentalis* possess significant antioxidant activity than benzene fraction and methanol fraction (Vadnere *et al.,* 2011). The aim of this study was to investigate the total antioxidant activity of ethyl acetate leaf extract using Ferric ions Reducing Antioxidant Power assay (FRAP), DPPH radical scavenging, superoxide anion radical scavenging, hydrogen peroxide scavenging activities. Additionally, an important goal of this research was to compare *in vitro* antioxidative effects of *C. occidentalis* extracts with commercial and standard antioxidants such as BHT, ascorbic acid commonly used by the food and pharmaceutical industry.

MATERIALS AND METHODS

Chemicals: 1, 1-Diphenyl-2-picryl hydrazyl (DPPH) and quercetin were purchased from Sigma Chemical Co. (St., Louis, USA). Gallic acid, Folin-Ciocalteu reagent and methanol were purchased from Merck Co. (Germany).

Plant material

Leaves were shade dried for four days and then ground to coarse powder using high capacity grinding machine which was then stored in air-tight container and kept in cool, dark and dry place for the investigation. The powdered plant material was successively extracted in a Soxhlet extractor at elevated temperature using ethanol, methanol which was followed by aqueous and ethyl acetate. All extracts were filtered individually through filter paper and poured on Petri dishes to evaporate the liquid solvents from the extract to get dry extracts. After drying, crude extracts were stored in stock vials and kept in refrigerator for further use.

Bio assay guided extraction

The plant extraction was done using the method of Harpreet *et al*., (2010).To 1000g of leaf powder, 1500ml of water was added and allowed to soak overnight at room temperature and it was filtered through Whatman Filter Paper No.1. The filterate was collected and evaporated through rotary evaporator to have the dry crude aqueous extract (CA). CA was further partitioned by dissolving in Methanol and after some time the precipitates (PP) were formed. Both PP and Supernatant (SP) were separated and dried at room temperature separately. The dried SP was further fractioned in Water and Ethyl acetate (1:1) resulted in formation of two layers namely fractioned Ethyl Acetate (FEA) and aqueous Fraction (FA). These layers were separated and dried at room temperature.

Determination of total phenol content

Total phenols were determined by Folin-Ciocalteu reagent using the method of Makkar *et al.*, (1997). A diluted aliquot of each plant extract (0.5 mL of 0.1 g/mL) or gallic acid (standard phenol compound) was mixed with Folin-Ciocalteu reagent (5 mL, 1/10 diluted with distilled water) and aqueous Na_2CO_3 (4mL, 1 M). The mixtures were allowed to stand for 15 min and the total phenols were determined by spectrophotometrically at 765 nm. The total phenol content was expressed as gallic acid equivalents (GAE) in milligrams per gram of sample, using a standard curve generated with gallic acid.

Scavenging effect on DPPH radical

The antioxidant activity of the extracts was determined in terms of hydrogen donating or radical scavenging ability, using the stable radical DPPH, according to the method of Blois (1958). A methanol solution of the sample extracts at various concentrations was added to 5 ml of a 0.1 mM methanol solution of DPPH and allowed to stand for 20 min at 27°C. The absorbance of the sample was measured at 517 nm. Radical scavenging activity was expressed as the inhibition percentage of free radical by the sample and was calculated using the formula

% DPPH radical scavenging activity = (control OD - sample OD / control OD) x 100

Determination of Reducing Power

Total antioxidant activity is measured by ferric reducing antioxidant power (FRAP) assay of Benzie and Strain, (1999). FRAP assays uses antioxidants as reductant in a redox-linked colorimetric method, employing an easily reduced oxidant system present in stoichiometric excess.A sample of 100µl was mixed with 1.5ml of working FRAP reagent and incubated at 37°C for 4min. After incubation the absorbance at 593nm was measured. Ferrous sulphate standard was processed in the same way and the FRAP value was calculated from the standard graph.

Determination of the scavenging of Hydrogen peroxide

Scavenging activity of Hydrogen peroxide was determined by the method of Gow-Chin, Y. *et al.,*(1995).A solution of 4 mM H_2O_2 of 300µl (prepared in PBS) was added into 100µl solution of different extracts and incubated for 10min. The same procedure was performed for ascorbic acid. The absorbance of the solution was measured at 230nm against a blank solution containing the extract without H_2O_2.

Calculation : % of Scavenged H_2O_2 = $[(A_0-A_1)/A_0]*100$, where A_0=Absorbance of control and A_1=Absorbance in presence of sample/standard.

Nitric oxide scavenging activity

Scavenging activity of Nitric oxide was determined by the method of Garrat,(1964).The reaction mixture (3ml) containing sodium nitroprusside (10mM, 2ml), phosphate buffer saline (0.5ml) and the extract (1mg/ml, 100µl) / standard solution (ascorbic acid, 1mg/ml, and 100µl) was incubated at 25ºC for 150min. After incubation, 0.5ml of the reaction mixture was mixed with 1ml of sulphalinamide (1%) and allowed to stand for 5min for completing diazotization. Then, 1ml of naphthyl ethylene diamine dihydrochloride (0.1 % w/v) was added, mixed and allowed to stand for 30min at 25ºC. A pink colored chromophore was formed in diffused light. The absorbance of these solutions was measured at 540nm against the corresponding blank solution. Ascorbic acid was used as a standard.

Calculation: % of Scavenged NO = $[(A_0-A_1)/A_0]*100$, where A_0=Absorbance of control and A_1=Absorbance in presence of sample/standard.

Rancimat Analysis

The susceptibility of all oil samples to oxidation was studied by using the Rancimat apparatus. The test was performed on an automated Metrohm Rancimat model 679 at 120^o C and an air flow of 20 L/hr to determine the induction period (IP) of the individual oil.

Statistical analysis

The experimental results were performed in triplicate. The data were recorded as mean ± standard deviation and analyzed by SPSS (version 11.5 for Windows 2000, SPSS Inc.). One-way analysis of variance was performed by ANOVA procedures. Significant differences between means were determined by Duncans Multiple Range tests. $P<0.05$ was regarded as significant and $p<0.01$ was very significant.

RESULTS AND DISCUSSIONS

Natural antioxidants were closely related to their biofuntionalities, such as the reduction of chronic diseases like DNA damage, mutagenesis, carcinogenesis and inhibition of pathogenic bacterial growth which is often associated with the termination of free radical propagation in biological systems (Covacci *et al.,* 2001). Phenols are very important plant constituents. There is a highly positive relationship between total phenols and antioxidant activity of many plant species, because of the scavenging ability of their hydroxyl groups (Vinson *et al.,* 1998). It was also reported that Phenol compounds are effective hydrogen donors, making them very good antioxidants (Yen *et al.,* 1993). The amount of total phenol was calculated as gallic acid equivalents from the calibration curve. Higher the gallic acid equivalents will depict the stronger antioxidant activity. From the result, it is clear that FEA (361.67±0.6mg/g) has highest value when compared with standard ascorbic acid (153.33±1.2 mg/g) followed by CA (250.67±0.5mg/g) (Table 1).

Table 1. Total Phenol content of various extracts of *Cassia occidentalis*

S. No	Extract	Gallic acid equivalents(mg/g)
1	Methanol	23.33±1.5
2	Ethanol	26.67±0.6
3	CA	250.67±0.5
4	FA	29±1.0
5	FEA	361.67±0.6
6	Ascorbic acid	153.33±1.2

Data are presented as means ± standard deviation (n≥ 3).

DPPH radical scavenging assay

The radical scavenging activity, using a DPPH generated radical, was tested with different sample extracts, along with BHT. It was observed in the kinetics (Fig. 1) that the radical scavenging activity was very fast for the FEA extracts followed by BHT and CA but other extracts showed very slow kinetic behavior. In terms of percentage, the inhibiting activity (at 30 min) was calculated to be in the following order: FEA (86.52%), BHT (82.71%), FA (76.17%), CA (73.73%), Methanol (49.87%) and Ethanol (48.54%). These results reveal that leaf extracts contain powerful inhibitor compounds, which may act as primary antioxidants that react with free radicals.

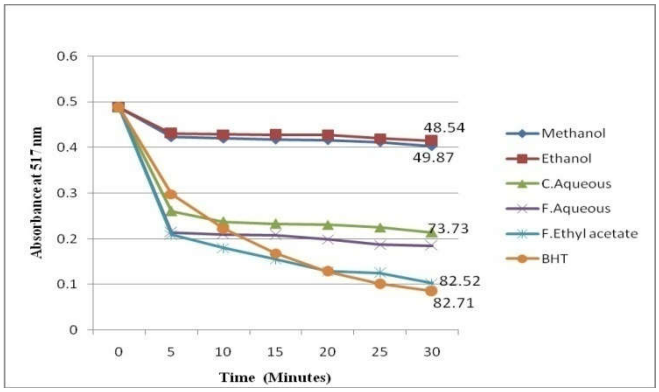

Figure 1. DPPH radical-scavenging capacity of extract using different solvents of *C. occidentalis* and butylated hydroxytoluene (BHT). Values at the end of kinetic curves indicate the DPPH radical - scavenging percent

Table 2. DPPH Radical Scavenging activity IC_{50} values

S. No	Extract	IC_{50} values
1	Methanol	100.26±.02
2	Ethanol	102.97±.06
3	CA	67.78±.20
4	FA	65.63±.02
5	FEA	60.59±.01
6	BHT	60.43±.17

Data are presented as means ± standard deviation (n≥ 3),**The IC_{50} was obtained by linear regression equations.

In order to quantify the antioxidant activity, the IC_{50}, which is the concentration of sample required to decrease the absorbance at 490 nm by 50%, was further calculated and is

shown in Table 2. The lower the IC_{50} value is, the greater the free radical-scavenging activity is. The free radical scavenging activity of ethyl acetate fraction (IC_{50} = 60.59±.01) was superior to all other fractions. The IC_{50} value of ethyl acetate fraction was slightly lower than synthetic antioxidant, butylated hydroxyanisole (BHA) (IC_{50} = 60.43±.17). The strongest activity of ethyl acetate fraction may be related to its higher phenol content (361.61 mg GAE/g) as measured by gallic acid test (Table 1).

From the obtained results, the amount of total phenol and free radical scavenging activity is comparatively higher in crude aqueous extract than ethanol and methanol extract of the leaf of *C. occidentalis.* In order to separate active compound, the aqueous extract is fractioned as non-polar active ethyl acetate and polar active aqueous fraction by means of bioassay guided fractionation. Further analysis was carried out using different concentration of its fraction and compared with standard ascorbic acid.

Table 3. DPPH inhibition percentage of plant extract

S.No	Extract	Concentration (µg)		
		50	100	150
1	CA	59.9±1.01%	73.73±.02%	78.67±.01%
2	FA	73.87±.21%	76.17±.03%	80.11±.82%
3	FEA	78.38±.02%	82.52±.01%	85.31±.02%
4	BHT	80.22±.59%	82.71±..25%	87.21±.84%

Data are presented as means ± standard deviation (n≥ 3)

The Ethyl acetate extract of *C. occidentalis* exhibited a significant dose dependent inhibition of DPPH activity. The percentage inhibition was found to be 85.31% at 150µg/ml. By comparing its active fractions, the free radical-scavenging activities decreased in the order of FEA > CA > FA (Table 3).

Hydrogen peroxide Scavenging Activity

The ability of *C. occidentalis* to scavenge hydrogen peroxide was determined according to the method of Ruch *et al.,*(1989).The inhibitive effect of leaf extract was subjected to hydrogen peroxide scavenging assay. The FEA extract inhibition percentage was found to be higher (Table 4) when compared to ascorbic acid. The differences in H_2O_2 scavenging capacities between the extracts may be attributed to the structural features of their active components, which determine their electron donating abilities (Wettasinghe *et al.,* 2000). FEA and CA extracts exhibited 83.83% and 79.38% hydrogen peroxide scavenging activity, respectively, while, at the same concentration, ascorbic acid showed 86.07% activity (Ascorbic acid >FEA > CA > FA). The correlation between the *C. occidentalis* extract values and those of the controls was statistically significant (p< 0.05). The overall result showed that the plant *C. occidentalis* is a good hydrogen peroxide radical scavenger.

Table 4. Hydrogen Peroxide scavenging Activity of aqueous extract of *Cassia occidentalis* and its fractions

S.No	Extract	% H_20_2 Scavenging Activity Concentration (µg)		
		50	100	150
1	CA	78.58±.04	78.91±.08	79.38±.03
2	FA	68.76±.12	68.72±.06	68.96±.07
3	FEA	78.18±.07	79.21±.08	83.83±.11
4	Ascorbic acid	84.92±.19	85.17±.11	86.07±.40

Data are presented as means ± standard deviation (n≥ 3)

Reducing Power Assay (FRAP)

FEA (5300/150µg) and CA (4900/150µg) of leaf extract showed the highest reducing power and the values were compared to that of ascorbic acid (4840/150µg). FA extract exhibited lower reducing power activity at the concentration when compared with other extracts (Table 5). *C.occidentalis* had effective reducing power using potassium ferricyanide reduction method when compared to the standard (Ascorbic acid). All the extracts showed some degree of electron-donating capacity in a linear concentration-dependent manner. The reducing power followed the order: FEA > CA > Ascorbic acid> FA extract. The reducing power of the ethyl acetate extract was superior to that of the aqueous extract, which coincides with other reports. At different concentration, *C.occidentalis* demonstrated powerful reducing ability and these differences were statistically significant (p<0.05).The reducing power of *C.occidentalis* and ascorbic acid increased steadily with increasing the concentration of samples. Reducing power of *C. occidentalis* and standard compounds exhibited the electron donor properties of *C. occidentalis* and *C. occidentalis* thereby neutralizing free radical by forming stable products.

Table 5. Reducing ability of *Cassia occidentalis* on FRAP assay

S.No	Extract	FRAP values Concentration (µg)		
		50	100	150
1	CA	3100	3150	4900
2	FA	1666	2300	3800
3	FEA	3366	3800	5300
4	Ascorbic acid	3100	3600	4840

Data are presented as means ± standard deviation (n≥ 3)

Table 6. Nitric Oxide scavenging Activity of aqueous extract of *Cassia Occidentalis* and its fractions

S.No	Extract	% NO Scavenging Activity Concentration (µg)		
		50	100	150
1	CA	19.43	29.04	57.99
2	FA	8.59	17.04	55.03
3	FEA	17.04	36.05	68.88
4	Ascorbic acid	35.73	68.9	74.63

Data are presented as means ± standard deviation (n≥ 3)

Table 7. Induction Period of extracts using Rancimat at 120^{0}C

Particulars	Induction Time (Hours)
Sunflower oil without Antioxidant	3.27
Sunflower oil with 0.01% FEA	3.38
Sunflower oil with 0.02% FEA	3.37
Sunflower oil with 0.03% FEA	3.39
Sunflower oil with 0.01% FA	3.42
Sunflower oil with 0.02% FA	3.45
Sunflower oil with 0.03% FA	3.44

Nitric oxide Scavenging Activity

Nitric oxide (NO) is a potent pleiotropic inhibitor of physiological processes such as smooth muscle relaxation, neuronal signaling, inhibition of platelet aggregation and regulation of cell mediated toxicity. It is a diffusible free radical that plays many roles as an effector molecule in diverse biological systems including neuronal messenger, vasodilatation and antimicrobial and antitumor activities (Hagerman *et al.,* 1998). The scavenging of nitric oxide by plant extract was increased in a dose-dependent manner. At concentration of 150 µg/ml of extract maximum inhibition

percentage of nitric oxide generated by incubation was scavenged. The inhibitory effect of Nitric oxide scavenging activity was found to be lower when compared with other activities. FEA has given maximum inhibition percentage of 68.88% at 150µg which was equivalent to ascorbic acid at 100 µg (68.9%). The order of Nitric oxide scavenging activity of the plant extract is ascorbic acid>FEA>CA>FA (Table :6). The nitric oxide scavenging assay showed the half maximum inhibitory concentration of ethyl acetate extract, which was quite equivalent to standard.

The result indicated that the extracts might contain compounds that are able to inhibit nitric oxide and offers scientific evidence for the use of the plant in inflammatory condition. The overall result showed that the plant *C. occidentalis* is a good nitric oxide radical scavenger. Preliminary phytochemical analysis shows that the plant contains phenolic compounds (flavonoids and tannins). These compounds have been implicated in antioxidant metabolism (Chang *et al.,* 1999). The nitric oxide scavenging activity of flavonoids and phenolic compounds are known, so we can speculate that these constituents might be responsible for the observed scavenging activity. The FEA and FA extracts were tested for its heat stability at various concentrations (0.01, 0.02 & 0.03%) in sunflower oil using Rancimat analysis. Complete dissolution didn't happen when the FA and FEA extracts where added into sunflower oil. Also, the extracts didn't exhibit appreciable stabilizing property (Table 7).

Conclusion

According to data obtained from the present study, *Cassia occidentalis* was found to have an effective antioxidant and radical scavenging activity in different *in vitro* assays when compared to the standard antioxidant compounds such as BHT and ascorbic acid. Based on the discussion above, *Cassia occidentalis* can be used for minimizing or preventing free radical formation in pharmaceutical products, retarding the formation of toxic oxidation products, maintaining nutritional quality and prolonging the shell life of pharmaceuticals. However, further work is required to resolve the active chemical principles responsible for the observed properties and to ascertain their molecular mechanism.

Conflicts of interest: The authors declare no conflict of interest.

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