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Research Article

PHYTOCHEMICAL INVESTIGATION AND *IN VITRO* ANTIOXIDANT ACTIVITIES *INDIGOFERA CORDIFOLIA* SEED EXTRACTS

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ABSTRACT

In present study, we investigated the *in vitro* antioxidant and free radical scavenging activities of crude methanolic and aqueous extract of *Indigofera cordifolia* (*Fabaceae*) seeds. The antioxidant and free radical-scavenging activities of *Indigofera cordifolia* seed extracts were evaluated using different tests involving inhibition of DPPH, ABTS, TBARS, NO, superoxide anion and hydroxyl radical. The reducing power, total phenolic and flavanoid content of the extracts were also dtermined using standard phytochemical reaction methods. The aqueous and methanolic extracts exhibited wide range of total phenolic, 2.29± 0.003 mg and 3.97±0.006 mg gallic acid equivalent and flavanoid contents, 5.72±0.083 mg and 10.10±0.10 mg rutin equvalent/ g of extract powder respectively. The present findings suggest that the crude aqueous and methanolic extract of *Indigofera cordifolia* is a potential source of natural antioxidants. The methanolic extract showed both, the highest antioxidant activity and phenolic contents than aqueous extract.

Keywords: DPPH, Antioxidant, *Indigofera cordifolia.* Free radical scavenging activity

INTRODUCTION

It has been found that oxygen free-radicals, or generally known as reactive oxygen species (ROS) viz. hydroxyl, nitric oxide (NO), superoxide are generated in living organisms through numerous metabolic pathways and work in an intricate way in the biological systems, their overproduction harms the membrane lipids, cellular proteins and enzymes, i.e. oxidative stress[1]. The oxidative damage caused by ROS is involved in various diseases such as cancer, diabetes, cardiovascular diseases, atherosclerosis, hypertension, ischemia/reperfusion injury, neurodegenerative diseases, inflammation and ageing [2]. The cell can reduce the impact of ROS either by an endogenous system implicating enzymes such as catalase and superoxide dismutase or by an exogenous system using antioxidants, vitamin C and α-tocopherol [3].

Antioxidants are helpful in protecting cells from such oxidative damage [4,5], and play an important role in protecting the body tissues against free radicals damage. Antioxidants refer to a group of compounds that are able to delay or inhibit the oxidation of lipids or other biomolecules (DNA, proteins) and thus, prevent or repair the damage of the body cells that is caused by free radicals [6,7]. The lucrative Phenolic contents and related antioxidant potential in plants has paved way maximum research in this area. for The recent researches show great interest in medicinal plants due to their phenolic concentrations and related total antioxidant potential [8, 9, 10].

I .cordifolia is wildly growing plant in Rajasthan (India) and is traditionally used as a as cattle fodder. [11]. *Indigofera cordifolia* is employed for treatment of hepatitis and diabetes in folk medicine in Western Rajasthan for the times unknown. *I. cordifolia* is a source of rotenoids [12]. In the present study, we investigated the methanolic and aqueous extract of *Indigofera cordifolia* seeds for its antioxidant and free radical scavenging activity. This evaluation is related to the total phenolic content and antioxidant activity to find out new potential sources of natural antioxidants.

MATERIALS AND METHODS

Chemicals

Folin-Ciocalteu reagent, Na_2CO_3, glacial acetic acid, nicotinamide adenine dinucleotide (NADH), thiobarbituric acid (TBA), nitroblue tetrazolium (NBT), Phenazine methosulphate (PMS), DPPH (2,2-diphenyl-1-picryl-hydrazyl), 2-deoxy-2-ribose, FeSO4, EDTA, ascorbic acid, Trichloro acetic acid (TCA), sodium dodecyl sulphate (SDS), sodium nitroprusside, naphthyl ethylenediamine dihydrochloride, sulphanilamide, phosphoric acid, trichloro acetic acid (TCA), Potassium ferricyanide, ferric chloride, hydrogen peroxide were obtained from Merck, SD fine chemicals, Himedia or Sigma. All other reagents used were of analytical grade.

Instruments

The instrument used during study were UV spectrophotometer (Shimadzu- UV-1650), cold centrifuge machine (HERMLE, Z36HK).

Collection of plant materials

Seeds of *Indigofera cordifolia* were collected during the month of November 2011 from Farm at Barmer District, Rajasthaan state of India. The plant material was authenticated by Dr. Ganesh Iyer, Botanist at Ruia College, Matunga, Mumbai. Freshly collected plant materials were cleaned to remove adhering dust and then dried under shade. The dried samples were powdered in mixer grinder and used for solvent extraction.

Plant extraction

The air dried powdered plant material were extracted in soxhlet extractor successively with petroleum ether followed by methanol and water. Each time before extracting with the next solvent, the material was dried in hot air oven at 40 °C. The extracts were concentrated by rotary vacuum evaporator and then dried. The dry extract obtained with each solvent was weighed. The percentage yield was expressed in terms of air dried weight of plant material. The extracts thus obtained were used directly for the estimation of total phenolic and also for the assessment of antioxidant potential through various chemical assays.

Determination of phenolic content

The total phenols were estimated according to the Folin-Ciocalteu method[13]. To 50 µl sample were added 250 µl of undiluted Folin-Ciocalteu-reagent. After 1 min, 750 µl of 20% (w/v) aqueous Na_2CO_3 were added, and the volume was made up to 5.0 ml with H_2O. The controls contained all the reaction reagents except the extract. After 2 h of incubation at 25°C, the absorbance was measured at 760 nm by using a spectrophotometer (Pharmaspec UV-1650, Shimadzu, Kyoto, Japan). and compared to a gallic acid calibration curve. Total phenols were determined as gallic acid equivalents (mg gallic acid/g extract), and the values are presented as means of triplicate analyses.

Determination of total flavonoid contents

Aluminum chloride colorimetric method was used for flavonoids determination[14]. Each plant extracts (0.5 ml of 1:10 g ml-1) in

methanol were separately mixed with 1.5 ml of methanol, 0.1 ml of 10% aluminum chloride, 0.1 ml of 1 M potassium acetate and 2.8 ml of distilled water. It remained at room temperature for 30 min; the absorbance of the reaction mixture was measured at 415 nm by using a spectrophotometer (Pharmaspec UV-1650, Shimadzu, Kyoto, Japan). Three replicates were made for each test sample. The total flavonoid content was expressed as rutin equivalents (mg RE/g extract).

Antioxidant activity

Free radical-scavenging activity on DPPH

The antioxidant activity of the extracts was determined in terms of hydrogen donating or radical scavenging ability, using the stable radical DPPH, according to[15]. A methanol solution of the *Indigofera Cordifolia* extracts at various concentrations (5-1000 µg/ml) was added to 0.5 ml of 0.1 mM methanolic solution of DPPH and allowed to stand for 30 min at 25°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

$$\% \text{ DPPH radical scavenging activity} = [(Abs_{control} - Abs_{sample})]/ (Abs_{control})] \times 100$$

where $Abs_{control}$ is the absorbance of DPPH + methanol; Abs_{sample} is the absorbance of DPPH + sample (i.e. extract or standard)

Superoxide radical-scavenging assay

Measurement of superoxide radical scavenging activity was done by using method[16]. The reaction mixture contained 1 ml of NBT(nitroblue tetrazolium) solution (150 µM prepared in phosphate buffer, pH 7.4), 1 ml of NADH(nicotinamide adenine dinucleotide) solution (468 µM prepared in phosphate buffer, pH 7.4) and methanol diluted sample extracts at various concentrations (5-1000 µg/ml) were added. Finally reaction were accelerated by adding 100 µL PMS(Phenazine methosulphate) solution (60 µM prepared in phosphate buffer, pH 7.4) to the mixture. The reaction mixture was incubated at 25°C for 5 min and absorbance was measured at 560 nm against methanol as control. Percentage inhibition of scavenging of superoxide radical was calculated as follows.

$$\% \text{ inhibition of Superoxide radical} = [(Abs_{control} - Abs_{sample})]/ (Abs_{control})] \times 100$$

Hydroxyl radical-scavenging assay

The hydroxyl radical-scavenging activity of aqueous and methanolic extract of *Indigofera cordifolia* was assessed by previously reported method [17]. The assay was based on quantification of the degradation product of 2-deoxy-2-ribose by condensation with thiobarbituric acid (TBA). Hydroxyl radical was generated by the Fe^{3+}– ascorbate – EDTA – H_2O_2 system (the Fenton reaction). The reaction mixture containing 0.1 ml of 2-deoxy-2-ribose (2.8 mM); 0.1 ml of phospahte buffer (20 mM, pH 7.4); 0.1 ml of $FeCl_3$ (100 µM); 0.1 ml of EDTA (100 µM); 0.1 ml of H2O2 (1.0 mM); 0.1 ml of ascorbic acid (100 µM) and various concentration of the extracts (50– 1000 µg/ml) in water were added and incubated for 1 h at 37 °C. After incubation, 0.5 ml of the reaction mixture was added with 1 ml of 2.8% TCA, 1 ml of 1% aqueous TBA and incubated at 90 °C for 15 min to develop the pink chromagen and cooled. After cooling, the absorbance was measured at 532 nm against an appropriate blank solution. The assay was performed in triplicates and the results were averaged. Mannitol was used as reference standard. The percentage inhibition of hydroxyl radical was calculated using the following equation:

$$\% \text{ Inhibition of Hydroxyl radical} = [(Abs_{control} - Abs_{sample})]/ (Abs_{control})] \times 100$$

Nitric oxide radical-scavenging assay

Nitric oxide scavenging activity was determined in terms of NO• generated from sodium nitroprusside in aqueous solution at physiological pH interacts with oxygen to produce nitrite ions, which were measured by the Griess reaction[18]. One milliliters of sodium nitroprusside (10 mM) in phosphate-buffered saline (pH 7.4) was mixed with 1 ml of test extracts at various concentrations (5-1000 µg/ml) dissolved in methanol and a control without a test compound, but with an equivalent amount of methanol. The mixture was incubated at 25°C for 30 min. After 30 min, 1 ml of the incubated solution was withdrawn and mixed with 1 ml of Griess reagent (1% sulphanilamide, 2% phosphoric acid and 0.1% naphthyl ethylenediamine dihydrochloride). The absorbance of the chromophore formed during the diazotization of the nitrite with sulphanilamide and the subsequent coupling with naphthyl ethylenediamine dihydrochloride was measured at 546 nm. The nitric oxide scavenging activity of the *Indigofera cordifolia* extracts is reported as a % inhibition and was calculated as follows

$$\% \text{ inhibition of Nitric oxide} = [(Abs_{control} - Abs_{sample})]/ (Abs_{control})] \times 100$$

Inhibition of microsomal lipid peroxidation

The lipid peroxidation level is measured as the thiobarbituric acid reactive substance (TBARS), by using rat liver homogenate for induction of lipid peroxidation, mediated by $FeSO_4$ as pro-oxidant and assessed [19]. Reaction mixture containing rat liver homogenate 0.1 ml (25% w/v in Tris–HCl buffer (20 mM, pH 7.0); 0.1 ml of $FeSO_4.6H_2O$ (0.16 mM); 0.1 ml ascorbic acid (0.06 mM) and various concentrations of aqueous and methanolic extracts of *I.cordifolia* (100–1000 µg/ ml) in water were incubated at 37°C for 1 h. After the incubation period, reaction mixture was treated with 0.2 ml SDS (8.1%); 1.5 ml TBA (0.8%); and 1.5 ml acetic acid (20%, pH 3.5). The total volume was made up to 4 ml with distilled water and then kept in a water bath at 95–100 °C for 60 min. After cooling, 1.0 ml of distilled water, 5.0 ml of n-butanol and pyridine mixture (15:1, v/v) were added to the reaction mixture, shaken vigorously and centrifuged at 4000 rpm for 10 min. The organic layer was removed and its absorbance was measured. The inhibition of *in vitro* lipid peroxidation by the measurement of thiobarbituric acid reactive substances (TBARS) in the extracts was measured spectrophometrically at 532 nm. The assay was performed in triplicates. Ascorbic acid was taken as reference standard. The percentage of inhibition of lipid peroxidation was calculated using the following equation:

$$\% \text{ Inhibition of lipid peroxidation} = [(Abs_{control} - Abs_{sample})]/ (Abs_{control})] \times 100$$

Reducing power

The reducing power of the methanolic and aqueous extracts of *Indigofera cordifolia* was determined [20]. Various concentrations of the extracts (100-1000µg/ml) in distilled water were added with 2.5 ml of 0.2 M phosphate buffer (pH 6.6), 2.5 ml of 1% potassium ferricyanide solution and incubated at 50 °C for 20 min. After incubation 2.5 ml of 10% TCA was added to the reaction mixture. The content was then centrifuged at 1000 rpm for 10 min. After centrifugation, the upper layer of the supernatant (2.5 ml) was mixed with 2.5 ml of distilled water and 0.5 ml of 0.1% ferric chloride. Then the absorbance of the reaction mixture was measure at 700 nm. Ascorbic acid (10-100 µg/ml) was used as positive control. The higher the absorbance of the reaction mixture the greater is the reducing power.

Free radical-scavenging activity on ABTS_+

The method[21]. was adopted for the determination of ABTS activity of the plant extract. The working solution was prepared by mixing two stock solutions of 7 mM ABTS solution and 2.4 mM potassium persulphate solution in equal amount and allowed to react for 12 h at room temperature in the dark. The resulting solution was later diluted by mixing 1 ml of freshly prepared ABTS.+ solution followed by the measurement of absorbance at 734 nm after 7 min. The percentage of scavenging inhibition capacity of ABTS.+ of the extract was calculated and compared with Butylated hydroxyltoluene (BHT). The percent of scavenging inhibition capacity of ABTS.+ of the extract was calculated from the following equation:

$$\% \text{ inhibition} = [(Abscontrol - Abssample)]/(Abscontrol)]\times 100$$

RESULTS AND DISCUSSION

Determination of total phenolic content and total flavonoid content

Phenolic compounds are widely distributed in plants[22]. and have gained much attention, due to their antioxidant activities and free

radical-scavenging abilities,which potentially have beneficial implications for human health[23].The methanolic and water extracts obtained from the samples seeds were evaluated for the presence of phenolic compounds. The samples were evaluated using the Folin–Ciocalteu assay, which was suggested as a fast and reliable method to quantify phenolics in foods[24]. The total phenolic content of aqueous and methanolic extracts is 2.29 ± 0.003 and 3.97± 0.006 mg gallic acid equivalent/g of extract, respectively.The total phenolic content (TPC) was determined in comparison with standard gallic acid and the results expressed in terms of mg GAE/g. The highest TPC values were obtained for the methanolic extract than the aqueous extract.

It has been recognized that flavonoids show antioxidant activity and their effects on human nutrition and health are considerable. The mechanisms of action of flavonoids are through scavenging or chelating process[25]. The flavonoid contents of the aqueous and methanolic extracts in terms of rutin equivalent were between 5.72±0.083 and 10.10 ± 0.10 respectively.

Free radical-scavenging activity on DPPH

The free radical scavenging activity of aqueous and methanolic extract of *Indigofera cordifolia* was assessed by the DPPH assay. Table 1 shows the significant decrease in the concentration of DPPH radical due to scavenging ability of the *Indigofera cordifolia*. The results showed that *Indigofera cordifolia* (10-1000µg/ml) methanolic extract (IC_{50} 56.40) had high DPPH radical scavenging activity than the aqueous extract (IC_{50} 185.35). This indicates that methanolic extract as a good source of natural antioxidants. The DPPH method with the stable organic radical 1,1- diphenyl-2-picrylhydrazyl is used for determination of free radical scavenging activity, usually expressed as IC_{50}, the amount of antioxidant necessary to decrease the initial concentration of DPPH by 50%. This means that the lower is the IC_{50} value of the sample, the higher is its antioxidant activity[26].

Superoxide radical-scavenging assay

Superoxide anion is a reduced form of molecular oxygen created by receiving one electron. It plays an important role in the formation of other reactive oxygen species, such as hydrogen peroxide, hydroxyl radical, or singlet oxygen in living systems[27]. The effects of phenolic extracts of *Indigofera cordifolia* on superoxide radical were determined by the PMS-NADH superoxide generating system and the results shows significant reduction in superoxide radicals. Both the extracts had a scavenging activity on the superoxide radicals, in a dose dependant manner (10-1000µg/ml). Result showed that Methanolic extract(56.88)had highest superoxide radical scavenging activity than Aqueous extract (143.13) .

Hydroxyl radical-scavenging assay

Highly reactive free radical formed in biological systems are the hydroxyl radical and has been implicated as a highly damaging species in free radical pathology, able to damage almost every molecule found in living cells[28].

The hydroxyl radical scavenging effect was investigated using the hydroxyl radical-mediated deoxyribose degradation in a Fe^{3+}-EDTA-ascorbic acid and H_2O_2 (Fenton reaction) reaction mixture and the results are significant. The concentration of aqueous and methanolic extract of *Indigofera cordifolia* needed for 50% inhibition was found to be 119.29 and 32.40 µg/ml respectively. The results suggest that methanolic extract is a more potent scavenger of hydroxyl radicals than aqueous extract and mannitol.

Nitric oxide radical-scavenging assay

NO radicals induce inflammatory response and when they react with $O^{2\bullet-}$ radicals to form peroxynitrite , their toxicity multiplies, which damages biomolecules like proteins, lipids and nucleic acids[29].

Suppression of nitric oxide release may be attributed to a direct nitric oxide scavenging effect as both the extracts decreased the amount of nitrite generated from the decomposition of sodium nitroprusside *in vitro*.The results show that concentration of aqueous and methanolic extract of *Indigofera cordifolia* needed for 50% inhibition was found to be 105.26 and 64.45µg/ml respectively. The results suggest that methanolic extract is a more potent scavenger of Nitric oxide than aqueous extract.

Inhibition of microsomal lipid peroxidation

Thiobarbituricacid reactive species (TBARS), the byproducts of lipid peroxidation that occur in non-polar region of the biological membranes , involve in the free radical induced cellular damage that lead to many human diseases[30].

Table 1 shows the antilipid peroxidation activity of *Indigofera cordifolia* aqueous and methanolic extracts by suppressing TBARS formation in rat liver homogenate. The IC_{50} value of aqueous, methanolic extract of *Indigofera cordifolia* and was found to be 146.24, 53.68µg/ml, respectively. The results suggest that methanolic extract is more potent scavenger of TBARS than aqueous extract.

Free radical-scavenging activity on ABTS_+:

The investigated extracts and fraction possessed the free radical-scavenging properties in different degrees. Their suppressive effect on ABTS cation radicals was assayed at various concentration regions: from 100 to 1000 µg/ml for the extracts and fraction, respectively. At this assay the MeOH fraction was the most active like DPPH method. The IC_{50} value of aqueous, methanolic extract of *Indigofera cordifolia* was found to be 2147.26±, 30.79± µg/ml, respectively. The scavenging activity of ABTS+ radical by the *Indigofera cordifolia* plant extract was found to be appreciable, this implies that the plant extract may be useful for treating radical related pathological damage especially at higher concentration[32].

Table 1: IC_{50} value of aqueous and metahnolic extract fo *Indigofera cordifolia* with their standard drug.

Name of Assay	IC_{50} value	
	Aqueous extract	Methanolic extract
DPPH	185.35	56.40
Super Oxide	143.13	56.88
OH Radical	119.29	32.40
NO Radical	105.26	64.45
ABTS	147.26	30.79
TBARS	146.24	53.68

Reducing power

The reducing power of aqueous and methanolic extracts of *Indigofera cordifolia* was estimated using potassium ferric cyanide method. The yellow color of the test solution changes to various shades of green and blue depending upon the reducing power of each extract. Fig.1 shows the reducing capacity of aqueous and methanolic extracts of *Indigofera cordifolia* compared to standard ascorbic acid (10-100 µg/ml) at 700 nm. The results suggests that both the extracts were found to possess good reducing power but not as efficient as standard ascorbic acid. The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity[31]. The reducing powers of methanolic and aqueous extract of *Indigofera cordifolia* increased with increasing concentration.

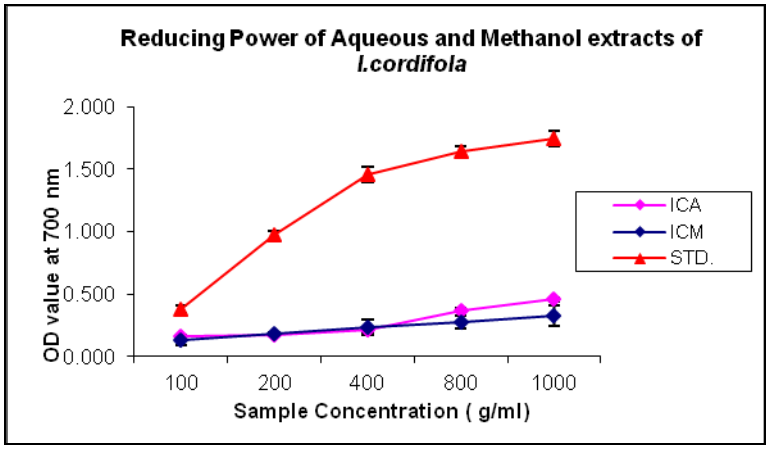

Fig. 1: Reducing capacity of aqueous and methanolic extracts of Indigofera cordifolia compared to standard ascorbic acid at 700 nm

CONCLUSION

The present investigation confirmed the *in vitro* antioxidant potential of the aqueous and methanolic extract of *Indigofera cordifolia*, with results comparable with those of standard compounds such as ascorbic acid and BHT. These data further support the view that the seeds of *Indigofera cordifolia* are promising sources of natural antioxidants, and could serve as inhibitors or scavengers of free radicals, acting possibly as primary antioxidants.

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