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Research Article

ANTIOXIDANT AND FREE RADICAL SCAVENGING POTENTIAL OF DIFFERENT SOLVENT EXTRACTS OF *INDIGOFERA TINCTORIA* L. LEAVES

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ABSTRACT

Indigofera tinctoria L. (Fabaceae) is traditionally used in Indian and Chinese medicinal systems for various ailments including cancer, liver disorder, inflammation, ulcers and nervous disorders. Since the said curative effects are often related to antioxidant properties, different solvent (petroleum ether, benzene, chloroform, ethyl acetate, methanol and water) extracts of *I. tinctoria* leaves were evaluated for the antioxidant and free radical scavenging potentialby employing different *in vitro* assays such as reducing power assay, DPPH•, ABTS•+, NO• and•OH radical scavenging capacities, peroxidation inhibiting activity through linoleic acid emulsion system, antihemolytic assay through hydrogen peroxide induced hemolytic cells and metal ion chelating ability.Ethyl acetate, water and methanol extracts exhibited higher phenolic and tannin contents whereas benzene and ethyl acetate extracts showed higher flavonoid contents. Though all the extracts exhibited reducing power, the ethyl acetate extract was found to have more hydrogen donating ability. Ethyl acetate extract further exhibited higher DPPH• and NO• scavenging activities. All the studied extracts exhibited 22-56% •OH scavenging activities at 250µg concentration in the reaction mixture and strong peroxidation inhibition against linoleic acid emulsion system (87-96%). Methanol extract registered strong ABTS•+ scavenging activity (1434.5 ± 236.8 µmolg^{-1}DM). The potential of multiple antioxidant activity of this plant is evident as the extracts possessed antihemolytic and metal ion chelating activities.

Keywords: Antioxidant; Free radicals; Total phenolics; *Indigofera tinctoria*; DPPH•; ABTS•+; Antihemolytic activity

INTRODUCTION

Reactive Oxygen Species (ROS) effect oxidative damage to various biomolecules including proteins, lipids, lipoproteins and DNA [1]. This oxidative damage is a critical etiological factor implicated in several chronic human diseases including cardiovascular dysfunctions, atherosclerosis, inflammation, carcinogenesis, drug toxicity, reperfusion injury and neurodegenerative diseases. The putative protective effects of antioxidantagents of natural origin against oxidative stress defense and different human have received increasing attention in recent times, especially within biological, medical, nutritional, and agrochemical areas. Thus antioxidant compounds can be used to counteract oxidative damage by reacting with free radicals, chelating free catalytic metals and also by acting as oxygen scavengers.

Indigofera tinctoria L. (Fabaceae), an erect, pubescent shrub has been a part of Indian and Chinese medicinal systems since time immemorial. The plant is known for the natural blue dye (indigo) obtained from its leaflets and branches. It is widely used for the treatment of several nervous disorders, liver ailments, epilepsy, cancer, inflammation, bronchitis and as ointment for sores, old ulcers and hemorrhoids [2,3]. It has a broad range of pharmacological activities including anticancer [4], anti-hyperglycemic [5], antidiabetic [6], hepatoprotective [7,8], antinociceptive [9], anti-inflammatory [10], antibacterial [11] and antiepileptive [12] activities. Further, antioxidant activity of aqueous acetone extract of aerial parts [13] and floral extract [14] was also reported. Indirubin, the active constituent from the leaves of this plant is a promising anticancer drug [15]. The furano-flavonoids (pseudosemiglabrin, semiglabrin and glabritephrin) and a rare flavonoid glycoside: kaempferol-4'7-dirhamnoside isolated from *I. tinctoria* were proved as good antidyslipidemic agents [16]. Rotenoids (deguelin, dehydrodeguelin, rotenol, rotenone, tephrosin and sumatrol) isolated from *I. tinctoria* were found to be toxic to larvae of *Anopheles stephensi* and adults of *Callosobruchus chinensis* [17]. In order to understand the highly acclaimed properties of *I. tinctoria* and its usage in the traditional systems of medicine, we have attempted to evaluate its antioxidant potential through the use of several *in vitro* assay systems and this will provide insight information with reference to their medicinal value.

MATERIALS AND METHODS

Chemicals

Potassium ferricyanide, ferric chloride, 2,2- diphenyl-1-picryl-hydrazyl (DPPH), potassium persulfate, 2,2'-azinobis(3-ethylbenzothiozoline-6-sulfonic acid) diammonium salt (ABTS), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 3-(2-pyridyl)-5,6-bis(4-phenyl-sulfonic acid)-1,2,4-triazine (Ferrozine), linoleic acid, ferrous chloride, ammonium thiocyanate, hydrogen peroxide, ferrous ammonium sulfate, ethylenediaminetetracetic acid (EDTA) disodium salt, trichloroacetic acid (TCA), naphthylethylenediaminedihydrochloride (NEED), sodium nitroprusside and sulphanilamide were obtained from Himedia, Merck or Sigma. All other reagents used were of analytical grade.

Plant material

The fresh leaves of *I. tinctoria* were collected during the month of June, 2012 from the arid regions around Karur, Tamil Nadu, India. The plant species was authenticated by Botanical Survey of India, southern circle, Coimbatore, and voucher specimen (BUBH2021) was deposited in the Department of Botany Herbarium, Bharathiar University, Coimbatore. Freshly collected plant materials were cleaned to remove adhering dust and then dried under shade. The dried samples were powdered in a Willy Mill to 60- mesh size and used for solvent extraction.

Preparation of extracts

Coarse powder from the shadow dried leaves was extracted in soxhlet extractor successively with different solvents in the increasing order of polarity (petroleum ether, benzene, chloroform, ethyl acetate and methanol). Each time before extracting with the next solvent, the material was dried in hot air oven at 40°C. Finally, the material was macerated using hot water with occasional stirring for 16 h and the water extract filtered. The extracts were concentrated by rotary vacuum evaporator (Yamato BO410, Japan) 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 polyphenols and also for the *in vitro* assessment of antioxidant potential through various chemical assays.

Determination of total phenolics and tannin content

The total phenolic content of different solvent extracts was determined by Folin-ciocalteu method. Using the same extract, the tannins were estimated after treatment with polyvinyl polypyrrolidone (PVPP). The amount of total phenolics and tannins were calculated as the tannic acid equivalents (TAE) via, Siddhuraju and Becker [18] and Siddhuraju and Manian [19].

Determination of total flavonoid content

The total flavonoid content was determined according to the method described by Zhishen *et al* [20]. 0.5 ml aliquot of the extract (2 mg/2 mL) was mixed with 2 mL of distilled water and subsequently with 0.15 mL of a 5% $NaNO_2$ solution. After 6 min, 0.15 mL of 10% $AlCl_3$ solution was added and allowed to stand for 6 min, and then 2 mL of 4% NaOH solution was added to the mixture. Immediately, water was added to bring the final volume to 5 mL, and then the mixture was thoroughly mixed and allowed to stand for another 15 min. Absorbance of the mixture was determined at 510 nm versus water blank. The analysis was performed in triplicate and the results were expressed as rutin equivalent.

Total antioxidant activities

Reducing power

The reducing power of different solvent extracts of *I. tinctoria* leaves was determined by the method reported by Siddhuraju *et al* [21]. 20-100µg of extracts were taken in 1 mL of phosphate buffer and 5 mL of 0.2M phosphate buffer (pH 6.6) was added. To this, 5 mL of 1% potassium ferricyanide solution was added and the mixture was incubated at 50°C for 20 min. After the incubation, 5 mL of 10% TCA was added. The content was then centrifuged at 1000 rpm for 10 min. The upper layer of the supernatant (5 mL) was mixed with 5 mL of distilled water and 0.5 mL of 0.1% ferric chloride. Then the absorbance of the reaction mixture was read spectroscopically at 700 nm.

Free radical scavenging activity on DPPH$^{\bullet}$

The DPPH radical scavenging activity of different solvent extracts of *I. tinctoria* was measured according to the method of Blois [22]. IC_{50} values of the extract i.e., concentration of extract necessary to decrease the initial concentration of DPPH by 50% was calculated.

Nitric oxide radical scavenging activity

The nitric oxide scavenging activity of different solvent extracts along with the reference standard tannic acid and quercetin was measured according to the method of Sreejayan and Rao [23]. The analysis was performed in triplicate. The sample concentration providing 50% inhibition (IC_{50}) under the assay condition was calculated from the graph of inhibition percentage against sample concentration.

Hydroxyl radical scavenging activity

The scavenging activity of different solvent extracts on hydroxyl radical was measured according to the method of Klein *et al* [24]. 250 µg of different solvent extracts were added with 1 mL of iron-EDTA solution (0.13% ferrous ammonium sulfate and 0.26% EDTA), 0.5 mL of EDTA solution (0.018%), and 1 mL of DMSO (0.85% v/v in 0.1 M phosphate buffer, pH 7.4). The reaction was initiated by adding 0.5 mL of ascorbic acid (0.22%) and incubated at 80-90° C for 15 min in a water bath. After incubation the reaction was terminated by the addition of 1 mL of ice-cold TCA (17.5% w/v). Three milliliters of Nash reagent (75.0g of ammonium acetate, 3.0 mL of glacial acetic acid, and 2 mL of acetyl acetone were mixed and raised to 1 L with distilled water) was added and left at room temperature for 15 min. The reaction mixture without sample was used as control. The intensity of the color formed was measured spectroscopically at 412 nm against reagent blank. The % hydroxyl radical scavenging activity was calculated as follows:

% HRSA=1-(difference in absorbance of sample/difference in absorbance of blank) × 100

Antioxidant activity by radical cation ($ABTS^{\bullet+}$)

The total antioxidant activity of the samples was measured by ABTS radical cation decolorization assay according to the method of Re *et al*. [25] described by Siddhuraju and Manian [19]. ABTS•+ was produced by reacting 7 mM ABTS aqueous solution with 2.4 mM potassium persulfate in the dark for 12-16 h at room temperature. Prior to assay, this solution was diluted in ethanol (about 1: 89 v/v) and equilibrated at 30°C to give an absorbance at 734 nm of 0.700 ± 0.02. The stock solution of the sample extracts were diluted such that after introduction of 10µL aliquots into the assay, they produced between 20-80% inhibition of the blank absorbance. After the addition of 1 mL of diluted ABTS•+ solution to 10µL of sample or Trolox standards (final concentration 0-15µM) in ethanol, absorbance was measured at 30°C exactly 30 min after the initial mixing. Appropriate solvent blanks were also run in each assay. Triplicate determinations were made at each dilution of the standard, and the percentage inhibition was calculated of the blank absorbance at 734 nm and then was plotted as a function of Trolox concentration. The unit of total antioxidant activity (TAA) is defined as the concentration of Trolox having equivalent antioxidant activity expressed as µmol/g sample extracts on dry matter.

Chelating capacity

Chelating property of different solvent extracts was assessed by bipyridyl assay [26]. The reaction mixture contained 0.25 mL of extracts having the concentration of 1 mg, 0.25 mL of 1mM $FeSO_4$ solution, 1 mL of 0.2 M Tris-HCl buffer (pH 7.4), 1 mL of 2,2' bipyridyl solution (0.1% in 0.2 M Tris-HCl), 0.4 mL of 10% hydroxylamine-HCl and 2.5 mL of ethanol. The final volume was made up to 5 mL with deionized water and the absorbance was determined at 522 nm. The chelating activity of the extracts was evaluated using EDTA as standard. The results were expressed as mg EDTA equivalent / g sample extracts.

Antihemolytic activity

Antihemolytic activity of the extracts was assessed as described by Naim *et al* [27].The erythrocytes from cow blood were separated by centrifugation and washed with 0.2 M phosphate buffer (pH 7.4). The erythrocytes were then diluted with phosphate buffered saline to give 4% suspension. 500µg of extract/ mL of saline buffer were added to 2 mL of the erythrocyte suspension and the volume was made up to 5 mL with saline buffer. The mixture was incubated for 5 min at room temperature and then 0.5 mL of H_2O_2 solution in saline buffer was added to induce the oxidative degradation of the membrane lipids. The concentration of H_2O_2 in the reaction mixture was adjusted to bring about 90% hemolysis of blood cells after 120 min. After incubation the reaction mixture was centrifuged at 1500 rpm for 10 min and the extent of hemolysis was determined by measuring the absorbance at 540 nm corresponding to hemoglobin liberation.

Antioxidant activity in linoleic acid emulsion system

The antioxidant activity of different solvent extracts was determined using the thiocyanide method [28]. Each sample (500µg) in the respective solvent was taken in screw cap tube and the solvent was evaporated to dryness. The tubes were then added with 0.5 mL of absolute ethanol, 0.5 mL of 2.51% linoleic acid in absolute ethanol, 1 mL of 0.05 M phosphate buffer (pH 7), and 0.5 mL of distilled water. The reaction mixture was incubated in dark at 40°C in an oven. A control without sample extract was used. Aliquots of 0.1 mL were taken at every 6 h during incubation and the degree of oxidation was measured by sequentially adding ethanol (9.7 mL, 75%), ammonium thiocyanate (0.1 mL, 30%) and ferrous chloride (0.1 mL, 0.02 M in 3.5% HCl). After the mixture was rested for 3 min, the peroxide value was determined by monitoring absorbance at 500 nm until the absorbance of the control reached the maximum. The antioxidant activity was calculated as percentage of inhibition relative to the control.

AA = 100 - (sample absorbance at 54h - sample absorbance at 0h /control absorbance at 54h-control absorbance at 0h) ×100

Statistical analysis

The data were subjected to one-way analysis of variance (ANOVA) and the significance of the difference between means was determined by Duncan's multiple range test (P<0.05) using Statistica (Statsoft Inc., Tulsa, USA). Values expressed are means of three replicate determinations ± standard deviation.

RESULTS AND DISCUSSION

Percentage yield, total phenolics, tannin and total flavonoid content

The yield of different solvent extracts during successive solvent extraction was calculated and presented in Table 1. The per cent yield was maximum in methanol extract (18.7%) followed by water extract (14.8%). The ethyl acetate extract gave the minimum yield (1.5%) when compared with other solvent extracts. In the current study, the total phenolics and tannin content of different solvent extracts of *I. tinctoria* leaves were estimated and expressed as tannic acid equivalent. As shown in Table 1, the total phenolic content was maximum in water extract (263.4 ± 10.5 mg/g extract) followed by ethyl acetate extract (257.9 ± 0.63 mg/g extract). Of all the solvents used, the minimum total phenolic contents were recorded in lesser polar solvent extracts. Further, methanol was found to be the most efficient solvent to extract antioxidant tannins as it registered higher levels of tannin content (115.75 ± 1.65 mg/g extract). This is in agreement with the reports of Yen *et al.* [29]who found that methanol is an effective solvent for extraction of antioxidants. Nonetheless, the flavonoid content was more in benzene extract (7.53 ± 0.22 mg/g extract) followed by ethyl acetate extract (5.22 ± 0.37mg/g extract). The higher contents of total phenolics in the highly polar solvent (ethylacetate, methanol and water) extracts of *I. tinctoria* might be contributed by the presence of furano-flavones, flavonol glycosides and high molecular tannins [16].

Table 1: Yield percent and total phenolics, tannin and total flavonoid contents of different solvent extracts of *I. tinctoria* leaves

Sample	Yield (%)	Total phenolics (mg/g extract)	Tannin (mg/g extract)	Flavonoids (mg/g extract)
Petroleum ether extract	3.4	97.07± 5.4^{c}	2.20 ± 1.1^{c}	-
Benzene extract	3.8	138.46 ± 7.6^{b,c}	21.24 ± 12.8^{b,c}	7.53 ± 0.22^{a}
Chloroform extract	2.3	176.92 ± 3.5^{a,b,c}	43.22 ± 8.8^{b,c}	0.64 ± 0.07^{d}
Ethyl acetate extract	1.5	257.87 ± 6.3^{a}	55.31 ± 14.6^{b}	5.22 ± 0.37^{b}
Methanol extract	18.7	191.94 ± 3.0^{a,b}	115.75 ± 16.5^{a}	1.27 ± 0.03^{c}
Water extract	14.8	263.37 ± 10.5^{a}	65.20 ± 56.4^{b}	0.70 ± 0.03^{d}

Values are means of three independent analyses ± standard deviation (n = 3). Mean values followed by different superscript letters indicatesignificant statistical difference (P<0.05).

Reducing power

The reducing power of different solvent extracts of *I. tinctoria* leaves, which may serve as a significant reflection of the antioxidant activity was evaluated using potassium ferricyanide reduction method. All the extracts exhibited some degree of reducing power (Figure 1). The reducing power increases with increasing concentration. Among the different extracts, ethyl acetate extract exhibited the maximum reductive capability followed by methanol extract; however, their reducing power was inferior to tannic acid standard. Polyphenolic contents of all the sample extracts appear to function as electron and hydrogen atom donors and therefore should be able to terminate radical chain reaction by converting free radicals and reactive oxygen species to more stable products. Similar observation in terms of dose dependant activity between the polyphenolic constituents in terms of dose dependent and reducing power has been reported for several plant extracts [30,31].

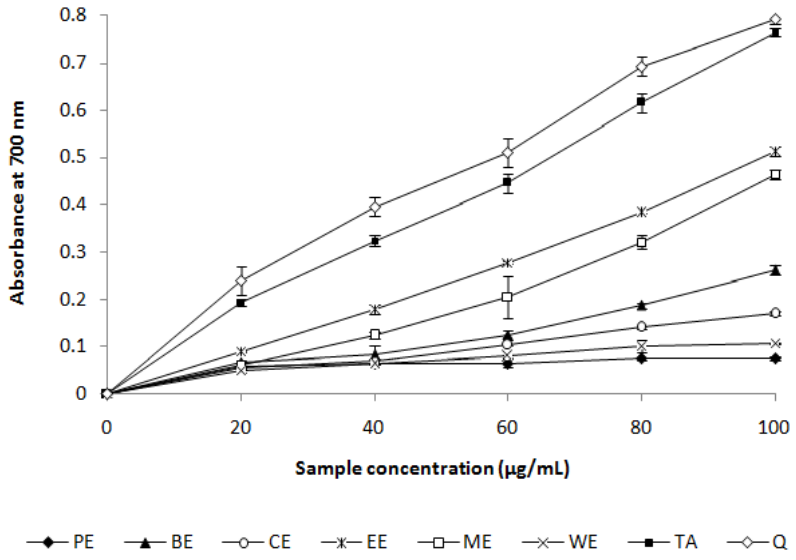

Fig. 1: Reducing power of different solvent extracts of *I. tinctoria* leaves

Values are means of three independent analyses ± standard deviation (n=3). PE- petroleum ether extract, BE- benzene extract, CE- chloroform extract, EE- ethyl acetate extract, ME- methanol extract, WE- water extract, TA- tannic acid, Q - Quercetin.

Free radical scavenging activity on DPPH·

The proton-radical scavenging action has been known as an important mechanism of antioxidation. DPPH radical is a stable organic free radical which has been extensively used for evaluating the free radical scavenging potential of natural antioxidants. In the present study, concentration of the sample extracts necessary to decrease initial concentration of DPPH· by 50% (IC_{50}) under experimental condition has been calculated and expressed in Table 2. A lower value of IC_{50} indicates higher antioxidant activity. Higher

DPPH• scavenging activity was shown by ethyl acetate extract (87.77±4.9µg/mL) followed by methanol extract (99.53± 0.9µg/mL). However, the DPPH• scavenging activity of all the sample extracts was inferior to the standard antioxidants quercetin(29.0 ± 4.50µg/mL) and tannic acid (37.81±2.52µg/mL). Similar to the reducing power, the extent of DPPH• scavenging activity also appears to depend on the phenolic and tannin concentrations of the extracts, the water extract being the only exception. The DPPH• scavenging activity can also be attributed to the nature of phenolics contributing to their electron transfer/ hydrogen donating ability [32]. The data obtained revealed that the extracts can act as free radical inhibitors and primary antioxidants that react with free radicals.

Nitric oxide radical scavenging activity

The nitric oxide radical scavenging activity of different solvent extracts of *I. tinctoria* was determined, the IC_{50} under the experimental condition was also calculated and the results are presented in Table 2. The nitric oxide scavenging assay is based on the principle that sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide which interact with oxygen to produce nitrite ions that can be estimated using Griess reagent. In the present study, all the solvent extracts scavenged the nitric oxide radical and/or inhibited the nitrite formation but at much lower levels when compared with standard antioxidant tannic acid and quercetin. The extracts inhibit nitric oxide formation by competing with oxygen to react with nitric oxide directly and also to inhibit its synthesis. The ethyl acetate extract exhibited higher (11.09±0.19µg/mL) NO•scavenging activity followed by water (13.49±3.32µg/mL) and methanol (16.44±0.82µg/mL) extracts. The polyphenolic compounds present in *I. tinctoria* might be responsible for the observed scavenging activity. NO• scavenging activity of flavonoids and phenolic compounds are already well known [33].

Table 2: DPPH and nitric oxide radical scavenging activity of different solvent extracts of *I. tinctoria* leaves

Sample	IC_{50} (µg/mL)	
	DPPH.	No.
Petroleum ether extract	218.38 ± 17.3^{c}	89.64 ± 5.49^{a}
Benzene extract	233.31 ± 14.88^{b}	47.45 ± 6.45^{c}
Chloroform extract	104.35 ± 4.71^{d}	59.46 ± 2.3^{b}
Ethyl acetate extract	87.78 ± 4.86^{e}	11.09 ± 0.19^{d,e}
Methanol extract	99.53 ± 10.96^{d}	16.44 ± 0.82^{d}
Water extract	325.97 ± 3.40^{a}	13.50 ± 3.23^{d}
Quercetin	29.0 ± 4.50^{g}	3.07 ± 0.01^{e}
Tannic acid	37.81 ± 2.52^{f}	4.42 ± 0.73^{e}

Values are means of three independent analyses ± standard deviation (n = 3). Mean values followed by different superscript letters indicatesignificant statistical difference (P<0.05).

Hydroxyl radical scavenging activity

The hydroxyl radical is the most reactive free radical formed in biological systems and is considered to be one of the quick initiators of the lipid peroxidation process [34]. In the present study, ascorbic acid-iron-EDTA was used to generate hydroxyl radical. The •OH scavenging activity of different solvent extracts is presented in Figure2. All the extract samples exhibited •OH scavenging activity ranging between 22.41±5.5% and 55.14±7.52% at 250µg concentration in the reaction mixture. Generally, higher levels of •OH scavenging activity exhibited by the non-polar solvent extracts in the present study could be attributed to the active hydrogen donor ability of hydroxyl substitution provided by phenolic flavonoids extracted in the early stages of successive extraction. It may be concluded that the ability of different solvent extracts of *I. tinctoria* leaves to quench hydroxyl radicals seems to be directly related to the prevention of propagation of lipid peroxidation, thus reducing the rate of chain reaction.

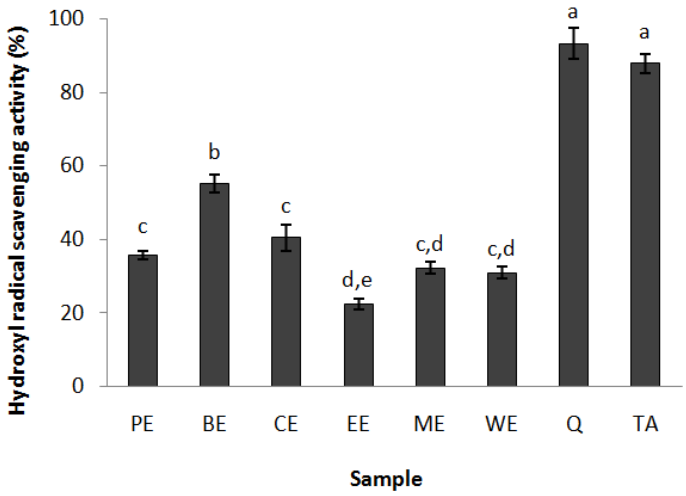

Fig. 2: Hydroxyl radical scavenging activity of different solvent extracts of *I. tinctoria* leaves

Values are means of three independent analyses ± standard deviation (n=3). Bars having different letters indicate significant statistical difference (P<0.05). PE- petroleum ether extract, BE - benzene extract, CE - chloroform extract, EE - ethyl acetate extract, ME - methanol extract, WE - water extract, Q - quercetin, TA - tannic acid.

Antioxidant activity by radical cation ($ABTS^{\bullet+}$)

The ABTS radical cation scavenging activity of different solvent extracts of *I. tinctoria* leaves are presented in Table 3. In this assay, total antioxidant activity (TAA) reflects the ability of hydrogen donating antioxidants to scavenge $ABTS^{\bullet+}$, comparable with that of Trolox. Though all the samples exhibited considerable $ABTS^{\bullet+}$ scavenging activity, the methanol extract exhibited stronger TAA (14348.5±236.8µmol g^{-1} DM) followed by ethyl acetate and benzene extracts with TAA values of 12111.4±242.6 and 10346.7±60.2 µmol g^{-1} DM respectively. In the present study, the order of scavenging activity of sample extracts is quercetin >methanol extract > ethyl acetate extract > benzene extract >tannic acid >water extract > petroleum ether extract > chloroform extract. These results suggest that the investigated sample extracts may prevent or retard the *in vitro* formation of radical species related with oxidative stress. The extensive investigations on antiradical and antioxidant activities of small phenolics, including flavonoids and phenolic acids have been reported [35]. Apart from these, Hagerman *et al.* [36]have reported that the high molecular weight phenolics (tannins) have more ability to quench free radicals ($ABTS^{\bullet+}$) and that effectiveness depends on the molecular weight, the number of aromatic rings and nature of hydroxyl groups substitution than the specific functional groups. In similar lines, the present study also reveals higher $ABTS^{\bullet+}$ scavenging activity in the methanol extract of *I. tinctoria* that has registered the highest contents of tannins (Table 1). This is in

agreement with the reports of Bakasso *et al*. [13] that the higher $ABTS^{\bullet+}$ scavenging activity of acetone extract of *I. tinctoria* aerial parts is due to its high phenolic content.

Metal ion chelating property

The chelating effects of different solvent extracts of *I. tinctoria* are shown in Table 3. The final reaction mixture contained 1 mg of extract sample and the values are expressed as mg EDTA/g extract equivalent. All the extracts demonstrated marked ability to chelate ions. Among the samples tested, ethyl acetate (52.63±4.80 mg EDTA/g extract), water (45.16±1.33 mg EDTA/g extract) and methanol (41.02±1.16 mg EDTA/g extract) extracts were found to have higher chelating activity than other samples. The extracts may be able to play a protective role against oxidative damage by sequestering iron (II) ions that may otherwise catalyze Fenton-type reactions or participate in metal-catalyzed hydroperoxide decomposition reactions [37]. The iron (II) chelating properties of the additives may be attributed to their endogenous chelating agents, mainly phenolics. Certain phenolic compounds have properly oriented functional groups, which can chelate metal ions. Chelating agents may serve as secondary antioxidants because they reduce the redox potential thereby stabilizing the oxidized form of the metal ion [38]. Accordingly it is suggested that the low to moderate ferrous ion chelating effects of the different solvent fractions of *I. tinctoria* would be somewhat beneficial to protect against oxidative damages.

Antihemolytic activity

Antihemolytic activity of different solvent extracts of *I. tinctoria* leaves is shown in Table 3. In this study, we used a biological test based on free radical-induced erythrocyte lysis in cow blood. Lipid peroxidation of cow blood erythrocyte membrane mediated by H_2O_2 induces membrane damage and subsequently hemolysis. Addition of different solvent extracts of *I. tinctoria*at the concentration of 500 µg in the final reaction mixture strongly inhibited free radical-induced red blood cell hemolysis. The percentage inhibiting activity of all the extract samples ranged between 61% and 71%. A similar observation of hemolysis inhibition have also been reported from the extracts of *Camellia sinensis, Ficus bengalensis, Ficus racemosa* and *Melothria maderaspatana* [31,39]. In the present study, higher antihemolytic potential was exhibited by ethyl acetate extract which might be attributed to the presence of high concentration of phenolic substances (Table 1). The phenolic compounds in the extracts may probably have an important role to participate in the biological pathways in the protection of radical-induced hemolysis.

Table 3: $ABTS^{\bullet+}$ scavenging activity, metal chelating activity and antihemolytic activity of different solvent extracts of *I. tinctoria* leaves

Sample	TAA* (µmol g^{-1} extract)	Metal chelating activity (mg EDTA/g extract)	Antihemolytic activity (%)
Petroleum ether extract	3249.62 ± 116.91^{e}	39.96 ± 1.43^{c}	50.35 ± 9.3^{b}
Benzene extract	10346.73 ± 60.22^{c}	23.30 ± 4.84^{d}	64.50 ± 8.0^{a}
Chloroform extract	2092.49 ± 142.70^{f}	39.54 ± 1.44^{c}	68.27 ± 5.09^{a}
Ethyl acetate extract	12111.36 ± 242.61^{b}	52.63 ± 4.80^{b}	71.61 ± 5.55^{a}
Methanol extract	14348.49 ± 236.79^{a}	41.01 ± 1.16^{c}	64.62 ± 6.57^{a}
Water extract	7646.74 ± 116.91^{d}	45.16 ± 1.33^{c}	61.46 ± 10.86^{a}
Quercetin	14984.2 ± 111.30^{a}	67.6 ± 2.1^{a}	69.31 ± 3.49^{a}
Tannic acid	10254.30 ± 56.2^{c}	61.5 ± 1.9^{a}	62.74 ± 4.46^{a}

* Total antioxidant activity (µmolTrolox equivalent $ABTS^{\bullet+}$ scavenging activity/g extract)

Values are means of three independent analyses ± standard deviation (n = 3). Mean values followed by different superscript letters indicatesignificant statistical difference (P<0.05).

Antioxidant activity in linoleic acid emulsion system

The antioxidant potential of different solvent extracts of *I. tinctoria* was evaluated employing linoleic acid peroxidation system (Figure3). During the linoleic acid peroxidation, linoleic acid hydroperoxides, generated from the peroxidation of linoleic acid and subsequently decomposed to many secondary oxidation products, or the intermediate products may be converted to stable end products and the substrate was exhausted (Chen *etal*., 1996). Addition of different solvent extracts of *I. tinctoria* into the emulsion was able to reduce the formation of hydroperoxide. All the different solvent extracts showed inhibition ability that was comparable with the standard quercetin at 250 µg in the final reaction mixture. At a concentration of 500µg in the final reaction mixture, all the extracts exhibited 87.81±1.65% to 96.79±0.53% peroxidation inhibition of linoleic acid after incubation for 54 h. Peroxidation inhibiting activity has also been reported for several other plant extracts [31,40].

The findings of the present study suggest that *I. tinctoria* leaves have potent antioxidative and protective effects against free radicals as assessed in different *in vitro* system. The antioxidant activity was directly related to the total amounts of phenolics and flavonoids found in the sample.

Further investigations on the isolation and characterization of individual phenolic compounds will be required to elucidate their different antioxidant mechanism and existence of possible synergism among the compounds.

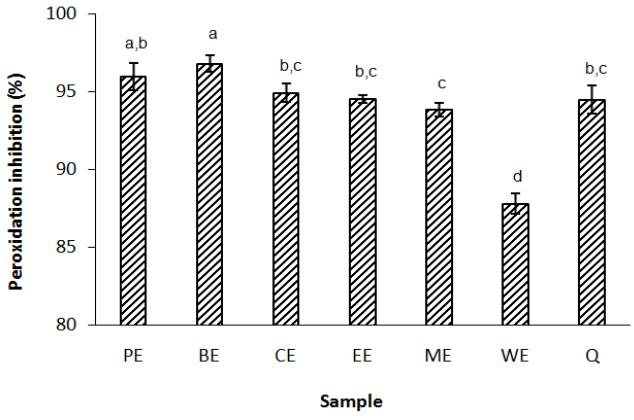

Fig. 3: Peroxidation inhibiting property of different solvent extracts of *I. tinctoria* leaves

Values are means of three independent analyses ± standard deviation (n=3). Bars having different letters indicate significant statistical difference (P<0.05). PE- petroleum ether extract, BE- benzene extract, CE- chloroform extract, EE- ethyl acetate extract, ME- methanol extract, WE- water extract, Q – quercetin.

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