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DETERMINATION OF FREE RADICAL SCAVENGING ACTIVITY OF *OROXYLUM INDICUM* AND *QUERCUS INFECTORIA* BY REVERSED-PHASE HPLC.

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ABSTRACT

In the present study, free radical scavenging activities of the two plants, *Oroxylum indicum* (L) Kurz and *Quercus infectoria* G. Olivier were investigated. Free radical scavenging activities of stem bark of *O. indicum* and insect galls of *Q. infectoria* were characterized by the HPLC method developed by D. Chandrashekhar, et al, 2005 using Waters 100 RP-18e column (250 mm × 4 mm, 5 µM) in which stable 1,1-diphenyl-2-picrylhydrazyl (DPPH•) free radical was used. The mobile phase used was a mixture of methanol and water (80: 20, v/v) pumped at a flow rate of 1 ml/min. the DPPH peaks were monitored at 517 nm. In the present investigation it was found that both the plant extracts had remarkable free radical scavenging activities. Ascorbic acid and trolox were used as the standard antioxidants. The IC_{50} values (inhibition concentration 50) of the ethanolic extracts of stem bark of *O. indicum* and insect galls of *Q. infectoria* were found to be 72 ± 2.32 and 58 ± 3.17 µg/ml, respectively as determined by the HPLC method, and correlated well with that determined by colorimetric method, i.e., 71.5 ± 3.64 µg/ml for *O. indicum* and 61.5 ± 2.42 µg/ml for *Q. infectoria*. The present study suggested that both the plants have potent antioxidant activity.

KEYWORDS: **ANTIOXIDANTS, DPPH, FREE RADICAL SCAVENGING ACTIVITY, REVERSED-PHASE HPLC**

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INTRODUCTION

The value of traditional medicine has increased all over the world in recent years and an extensive activity of research is done on different plant species for the their therapeutic purposes, experimental evidence suggests that free radicals (FR) and reactive oxygen species (ROS) are involved in a large number of diseases [1, 2]. Antioxidants are the substances that prevent or control the damage or destruction by oxidation [3, 4]. A large number of plants have been found to contain a lot of antioxidants that prevent oxidative damages. Recently, much attention has been directed towards the development of plant based medicines with strong antioxidant properties. *Oroxylum indicum (L.)* Kurz. (Family Bignoniaceae) (Also known as Syonaka, Sonpatha India) is a medicinally important, medium sized, deciduous tree distributed in India, Sri Lanka, China, Thailand, Philippines and Indonesia. In India it is distributed in Eastern and Western Ghats and North-East regions. Various parts of this plant are utilized to cure many diseases. In India roots and stem barks are used in Ayurvedic preparation called "Dasamoola" i.e. used as an astringent, anti-inflammatory, anti-helminthic, diuretic, anti-fungal, anti-bronchitic, antileucodermatic, anti-rheumatic, anti-anorexic and for the treatment of leprosy and tuberculosis [5]. Decoction of root bark is also effective to cure nasopharyngeal cancer [6]. Seed extract exhibits anti-microbial, analgesic and anti-inflammatory properties [7]. Leaf extracts exhibit antioxidant activity [8, 9]. The stem bark exhibits free radical scavenging activity [10]. *Quercus infectoria* G. Olivier. (Family Fagaceae) known as Majuphal in India, is a small tree found in Greece, Asia Minor, Iran and India. The galls arise on young branches of this tree as a result of attack by the gall-wasp Adleria gallae-tinctoria. In Asian countries, the galls of *Quercus infectoria* have been used for centuries in oriental traditional medicines for treating inflammatory diseases [11]. It is a well known plant in Indian traditional medicine and has been used as dental powder and in the treatment of toothache and gingivitis [12, 13]. The galls of *Q. infectoria* have also been pharmacologically documented to possess antiviral [14], antibacterial [15], antifungal [16], larvicidal [17], anti-inflammatory and antioxidant activities [18]. A number of methods have been developed to determine the free radical scavenging activities of various antioxidants using stable free radical species like 1,1-diphenyl-2-picrylhydrazyl (DPPH•) and 2,2-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS•±)[19]. The colorimetric estimation of DPPH is a simple method but it has certain drawbacks as it is not applicable to colored foods and certain commercial drugs such as nimesulide, etc. [20, 21]. D. Chandrashekhar developed and standardized a DPPH-HPLC method to compare the antioxidant activities of known standard antioxidants and some plant extracts and drugs [22]. In the present study, we investigated the free radical scavenging activity of ethanolic extracts of stem bark of *O. indicum* and insect galls of *Q. infectoria* by both HPLC-DPPH method and colorimetric method.

MATERIALS AND METHODS

Materials

DPPH, trolox, ascorbic acid were obtained from Sigma Aldrich, New Delhi, India. Methanol (HPLC grade, Merck specialities Pvt ltd., Mumbai India), ethanol (SD fine- chem. limited, Mumbai India) and deionized water purified by Millipore water purification system (Millipore Corporation, USA) was used. The stem bark of *Oroxylum indicum* (L) Kurz and insect galls of *Quercus infectoria* G. Olivier were procured from a local market of Old Delhi. The plant specimens were authenticated by Dr. H. B. Singh, Taxonomist, NISCAIR, Pusa, New Delhi, India.

Preparation of extracts

The plant materials were air-dried at room temperature (28 ± 2 ^{0}C) and then subjected to size reduction to get coarse powder and then passed through sieve no. 40 to get uniform powder. The dried powdered material (100 g) was packed in Soxhlet apparatus and then after defatted with petroleum ether extracted

with ethanol. The filtrates obtained were then concentrated under reduced pressure and evaporated at 40 ^{0}C by rotary evaporator (Rotavapor® R-210/R-215, BUCHI Labortechnik AG, Switzerland). The percentage yield of the ethanolic (OIEE) extract of stem bark of *O. indicum* was 2.15 %. The percentage yield of the ethanolic (QIEE) extract of insect galls of *Q. infectoria* was 18.6 %. The extracts were then dried in dessicator to remove moisture. The dried extracts were then stored in airtight containers at -20 ^{0}C until usage.

Sample preparation

Fresh DPPH (1,1-diphenyl-2-picrylhydrazyl free radical) stock solution (5 mL) at a concentration of 2.5mM/mL was prepared on each day of analysis. The stock solutions of trolox, ascorbic acid and plant extracts were prepared in methanol at a concentration of 1 mg/mL and stored at −20 ◦C. An aliquot of 100 µL of different concentrations of standard antioxidants and plant extracts in methanol were added to 100 µL of DPPH in solution (final concentration 250µM/200µL). The mixture was vortexed for few seconds and left to stand in the dark for 20 min at room temperature.

Colorimetric analysis

An aliquot of 100 µL of different concentrations of standard antioxidants and plant extracts in methanol were added to 100 µL of DPPH in solution (final concentration 250µM/200µL). The mixture was vortexed for few seconds and left to stand in the dark for 20 min at room temperature. Absorbance was measured at 517 nm by a UV/Visible spectrophotometer, SHEMADZU (Japan). The percent radical scavenging activity is determined from the difference in absorbance (*A*) of DPPH between the control and samples by using the following formula: Radical scavenging activity (%) = [Abs (control) – Abs (sample)]/ Abs (control) × 100. Where, Abs (control): Absorbance of DPPH radical ± methanol Abs (sample): Absorbance of DPPH radical ± extract/standard. IC_{50} value was determined from the graph plotted between radical scavenging activity (%) and concentration (µg/ml) by linear regression analysis. IC_{50} value is the concentration of the sample required to scavenge 50% DPPH free radical.

HPLC analysis

The sample was filtered through 0.2µmNylon membrane filter (Millipore, USA) and an aliquot (20 µL) of the sample was injected for HPLC analysis. The blank was prepared by adding 100µL of methanol to 100µL of DPPH stock solution (final concentration 250µM/200µL) and included before each run. The reversed-phase HPLC system consisted of a Waters HPLC system (6CE, USA), a system controller (600 controllers Waters), an auto-injector (Delta 600) and a photodiode array detector (PDA-996). Data analysis and processing were done by Empower Millennium 32 software (Version 2). Analyses were carried out using a Waters® 100 RP-18e column (250mm×4 mm, 5µM) (Waters, USA). Isocratic elution was carried out with methanol/water (80:20, v/v) at a flow rate of 1 mL/min. The DPPH peaks were monitored at 517 nm. The difference in the reduction of DPPH peak area (PA) between the blank and the sample was used for determining the percent radical scavenging activity of the sample by using the formula: Radical scavenging (%) = (PA BLANK – PA SAMPLE)/ PA BLANK × 100.

Statistical analysis

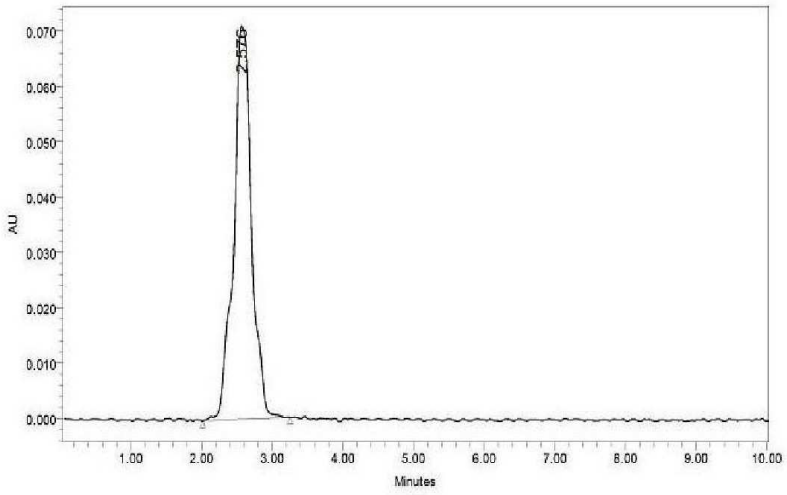
Values are expressed as the arithmetic means ± standard deviation. Statistical significance of the difference between various groups was evaluated by one-way analysis of variance (ANOVA), followed by the Tukey–Kramer tests. Analysis was performed using the statistical software Graph Pad InStat v3 (San Diego, CA). Differences were considered to be statistically significant when $p<0.05$ vs blank.

RESULTS

Optimization of mobile phase

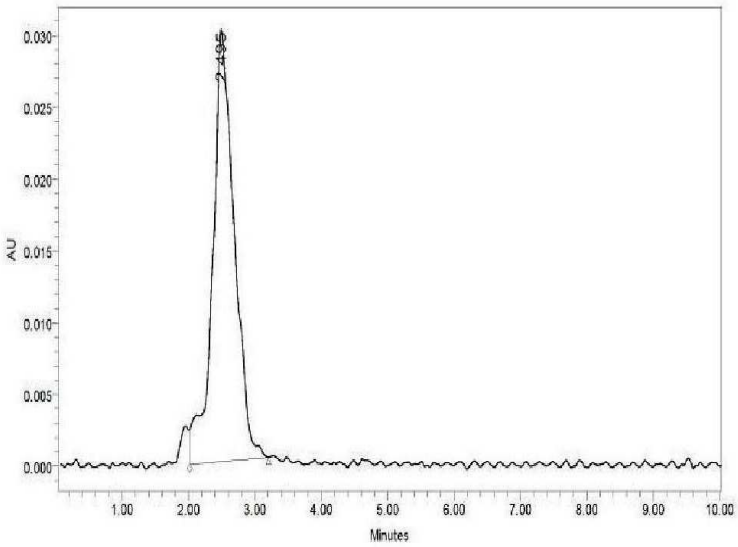
As DPPH was soluble in methanol, for HPLC mobile phase optimization different ratios of methanol: water including 50:50, 70:30 and 80:20 (v/v) was examined. When the methanol content was 80%, DPPH eluted as a sharp peak at 2.5 min. The peak was well separated

with no interferences, good resolution and acceptable tailing as shown in Fig. 1(a) (DPPH final concentration 250 µM/200 µL). The method was specific for DPPH with a run time of 10 min.



	RT	Area	% Area
1	2.576	1251141	100.00

A



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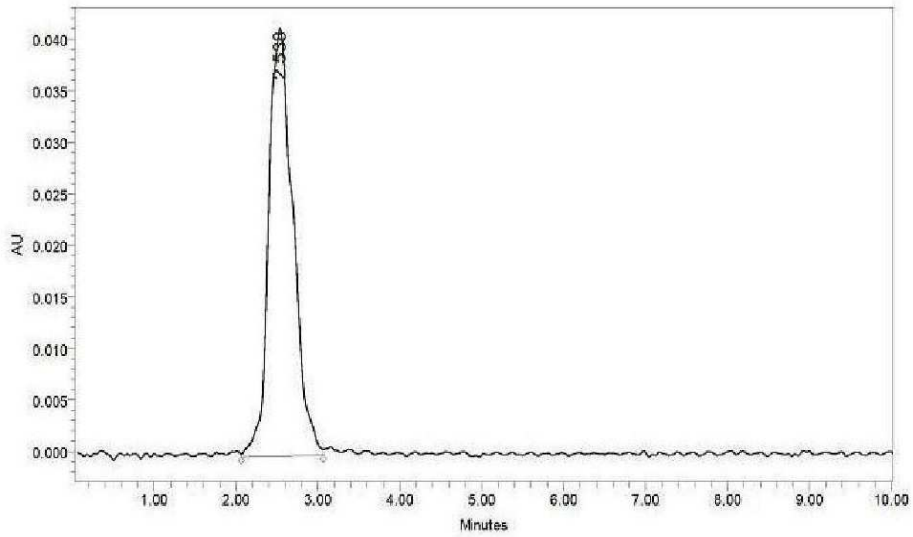

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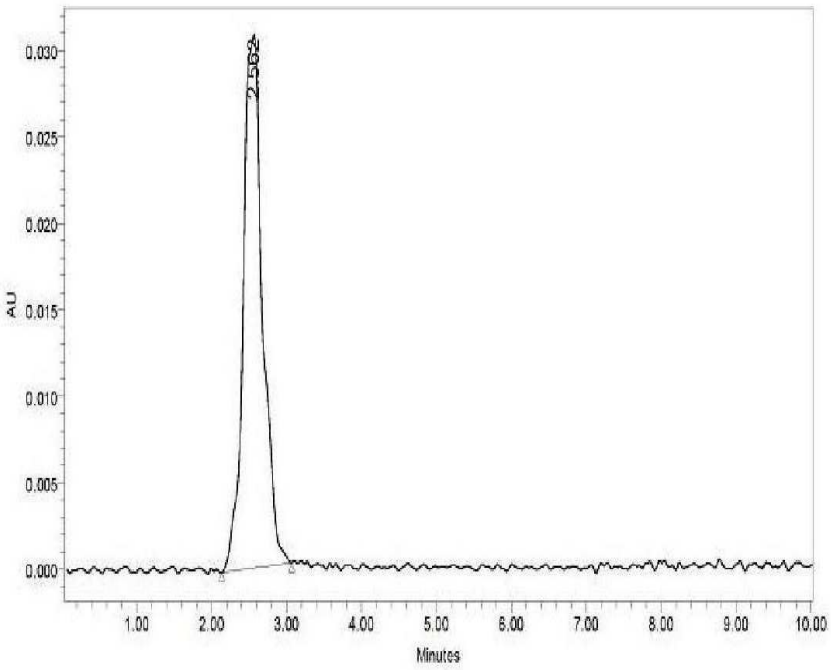
C



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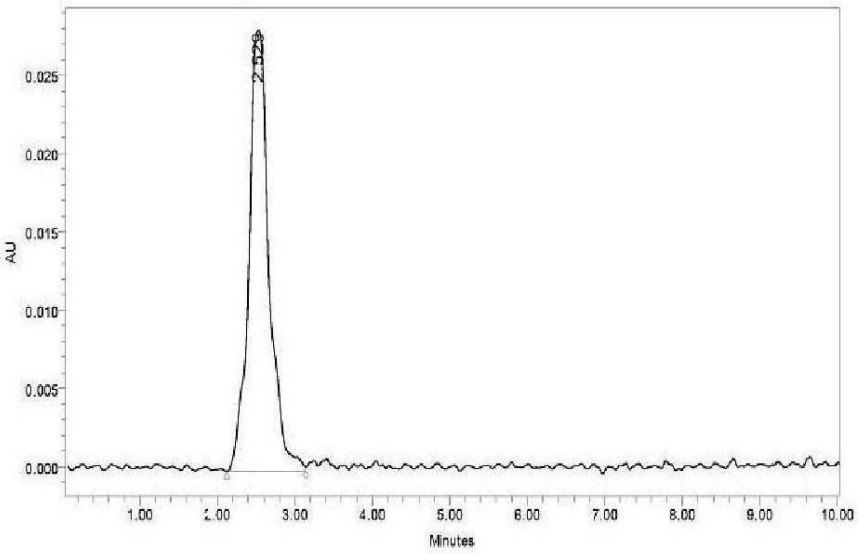


	RT	Area	% Area
1	2.529	631839	100.00

E

Figure.1.

HPLC chromatograms of DPPH: (A) Blank, and after incubation with—(B) Ascorbic acid 20 µg/ml, (C) Trolox 40 µg/ml, (D) Oroxylum indicum 80 µg/ml, and (E) Quercus infectoria 60 µg/ml. HPLC conditions: column, Waters® 100 RP-18e column (250mm×4 mm, 5µM); mobile phase, a mixture of methanol and water (80:20, v/v); flow rate, 1 mL/min; detection wavelength, 517 nm; injection volume, 20 µL.

Repeatability and reproducibility

The repeatability and reproducibility of the analytical method was confirmed from the peak area and retention times of the DPPH blank solution. The results are listed in Table 1.

Table 1

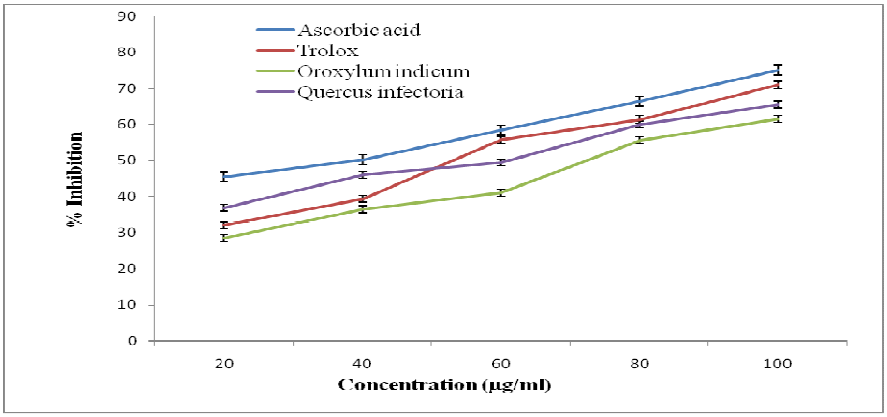
Repeatability and reproducibility of the retention time and peak area of DPPH blank (250µM/200µL) solution

Days	Retention time (min) Mean ± S.D	Peak Area Mean ± S.D
Day 1	2.512 ± 0.04	1253701 ± 37621.89
Day 2	2.476 ± 0.07	1228931 ± 56321.46
Day 3	2.576 ± 0.06	1251141 ± 46215.37

Each value is Mean ± S.D. of triplicate analysis.

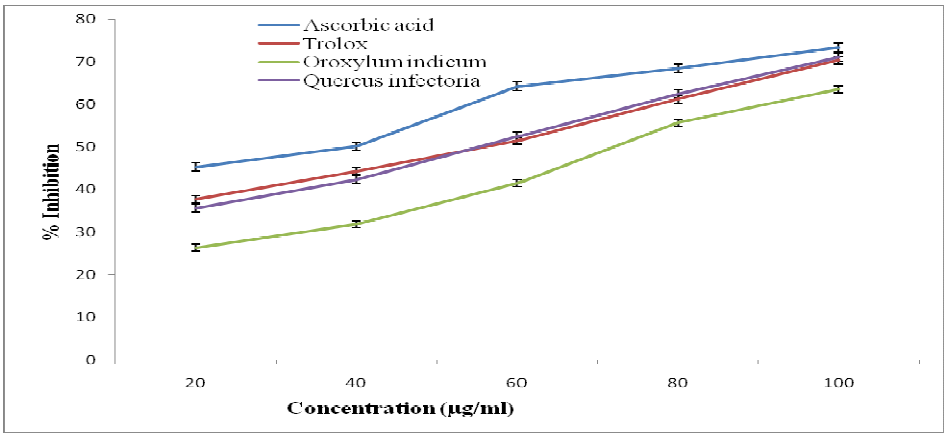
Radical scavenging activity

The radical scavenging activity of the standard antioxidants and plant extracts is given in Fig.2 and Fig.3. The extracts showed significant DPPH-scavenging activity between 20 and 100 µg/ ml. It was found that the radical-scavenging activities of all the extracts increased with increasing concentration.



Each value is Mean ± S.D of triplicate analysis.

Figure. 2
Radical scavenging activity of standard antioxidants and plant extracts as determined by HPLC method.



Each value is Mean ± S.D.of triplicate analysis.

Figure. 3
Radical scavenging activity of standard antioxidants and plant extracts as determined by spectrophotometric method.

Linearity graphs of standard antioxidants

The linearity graphs of all standard antioxidants were constructed by taking the mean value of the triplicate analysis. Linear regression equations for both the standard antioxidants indicated good correlation in the linearity range of 20–100 µg/ml (Table 2 and Table 3).

Table 2
Linearity graph of standard antioxidants and plant extracts by HPLC method

Compounds	Linearity range 20-100 µg/ml	
	Linear regression equation	Correlation coefficient (r)
Ascorbic acid	y = 0.3759x ± 36.586	0.9956
Trolox	y = 0.4979x ± 22.039	0.9891
Oroxylum indicum	y = 0.4257x ± 19.116	0.9869
Quercus infectoria	y = 0.3351x ± 32.273	0.9883

x denotes the concentration (µg/mL) of standard antioxidants and plant extracts and Y denotes the peak area. Each value is Mean ± S.D. of triplicate analysis.

Table 3
Linearity graph of standard antioxidants and plant extracts by Spectrophotometric method

Compounds	Linearity range 20-100 µg/ml	
	Linear regression equation	Correlation coefficient (r)
Ascorbic acid	y = 0.3725x ± 37.990	0.9767
Trolox	y = 0.4095x ± 28.450	0.9965
Oroxylum indicum	y = 0.4910x ± 14.280	0.9905
Quercus infectoria	y = 0.4551x ± 25.494	0.9980

x denotes the concentration (µg/mL) of standard antioxidants and plant extracts and y denotes the peak area. Each value is Mean ± S.D. of triplicate analysis.

Determination of IC_{50} values

The IC_{50} values of the standard antioxidants and plant extracts are given in Table 4. IC_{50} value was determined by linear regression analysis. All extracts had shown good reducing power ($p<0.001$).

Table 4
Free radical scavenging activity of antioxidants and plant extracts

Sample	IC_{50} value determined by	
	HPLC method (µg/ml)	Colorimetric method (µg/ml)
Ascorbic acid	35.68 ± 2.18[a]	32.24 ± 1.28[a]
Trolox	56.15 ± 1.56[b]	52.625 ± 1.74[b]
Oroxylum indicum	72.54 ± 2.32[c]	72.74 ± 2.64[c]
Quercus infectoria	52.90 ± 3.17[d]	53.84 ± 2.42[d]

Each value is Mean ± S.D. of triplicate analysis. Values with the different superscript letter across the same row and same column are significantly (P<0.05) different.

DISCUSSION

Table 4 shows that the ethanolic extracts of stem bark of *Oroxylum indicum* and insect galls of *Quercus infectoria* have antiradical activity by inhibiting DPPH radical as depicted by their IC_{50} value. IC_{50} value is the effective concentration at which antioxidant activity is 50%. DPPH is usually used as a substrate to evaluate anti-oxidative activity of the antioxidant. The method is based on the reduction of methanol DPPH solution in the presence of a hydrogen donating antioxidant due to formation of the non-radical form DPPH-H by the reaction. The extracts were able to reduce the stable radical DPPH to the yellow colored diphenylpicrylhydrazine. It has been found that antioxidant compounds reduce and decolorize DPPH by their hydrogen donating ability [23]. It appears that extracts of *O. indicum* and *Quercus infectoria* possess hydrogen donating abilities to act as an antioxidant. The IC_{50} values for ascorbic acid, trolox, *O. indicum* and *Quercus infectoria* found by HPLC method correlated well with that of colorimetric estimation as shown by Table 4.

CONCLUSION

On the basis of the results obtained in the present study, it is concluded that ethanolic extracts of stem bark of *Oroxylum indicum* (L) Kurz and insect galls of *Quercus infectoria* G. Olivier exhibit high free radical scavenging activities. These *in-vitro* assays indicate that the plant extracts are a significant source of natural antioxidants, which might be helpful in preventing the progress of various oxidative stresses. However, more detailed pre-clinical and clinical evidences are required to establish its potency.

CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

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