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PHYTOCHEMICAL PROFILE AND *IN-VITRO* ANTIOXIDANT POTENCIES OF *OROXYLUM INDICUM STEM* BARK EXTRACT

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Keywords:

Phytochemical, Antioxidant, *Oroxylum indicum*

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ABSTRACT: Background: Phytochemical profile and antioxidant studies are based on exploring plants for their use in the development of novel therapeutic drugs. *Oroxylum indicum* (OI), a valuable medicinal plant is native to India, found mainly in the Himalayan foothills and the Western Ghats. It has been used by traditional medicinal practitioners for a variety of ailments and is categorized as vulnerable medicinal plant by the government of India. **Objectives:** The present study was carried out to evaluate the phytochemical profile and antioxidant potential OI stem bark ethanol extracts. **Methods:** Phytochemical profiling and *in-vitro* antioxidant potential of stem bark were determined using standard protocols. The crude extract was subjected to LC-MS analysis for the identification of bioactive compounds. **Results:** The phytochemical analysis revealed the presence of compounds, namely glycosides, alkaloids, flavonoids, and tannins. At various concentrations tested, the increase in the free radical scavenging activity was observed with an increase in the concentration of extracts. The reducing power of the extract increased in a dose-dependent manner with an increase in concentration. The extract exhibited the ability to chelate metal ions as they reduced the concentration of catalyzing transition metal in lipid peroxidation. OI ethanolic extract showed remarkable ability to inhibit peroxidation in linoleic acid. The total antioxidant capacity of the extract increased linearly in a dose-dependent manner expressed as quercetin equivalents. **Conclusion:** The obtained results in our study may contribute to further standardization, validation, and research in *Oroxylum indicum* stem bark-based drugs, which are used in traditional and modern phytomedicine.

INTRODUCTION: Oxidative stress induced by reactive oxygen species (ROS) is involved in the pathogenesis of a variety of diseases such as cancer, atherosclerosis, hypertension, and coronary artery diseases.

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ROS causes tissue injury to biomolecules such as lipids, protein, and DNA damages and oxidation of important enzymes [1]. The intake of antioxidants has been effective in ameliorating the detrimental effects caused by oxidative stress.

The antioxidants prevent the initiation or propagation of oxidative chain reaction by free radical scavenging mechanism, quenching of singlet oxygen, and by their reducing properties, thereby prevention of these diseases [2]. Several studies have reported that these antioxidant compounds possess antitumor, antimutagenic,

antiviral, and anticarcinogenic activities [3]. There has been a global trend towards the utilization of the natural phytoconstituents present in herbs, oilseeds, berry crops, fruits, and vegetables, and so forth in recent times [4]. *Oroxylum indicum* (L.) Kurz is distributed throughout Southeast and South Asian countries. It is native to India, found mainly in the Himalayan foothills, Western Ghats, and is cultivated throughout the Indian forest regions. It has been categorized as a vulnerable medicinal plant by the government of India. *Oroxylum indicum* is widely used by the Indian population for the treatment of various ailments [5]. The therapeutic efficacy of many indigenous plants used for various diseases has been described by traditional herbal medicinal practitioners. In some parts of the world, natural products, which are the source of synthetic and traditional herbal medicine, are still the primary healthcare system. Since ancient times, plants have the basis of many traditional medicines and still continue to provide new remedies to mankind [6, 7].

Plants have served as a valuable starting material for drug development in all facets of life. The most important of these bioactive constituents of plants are steroids, terpenoids, carotenoids, flavonoids, alkaloids, tannins, and glycosides. The vital action of these chemicals with respect to human beings is that they function as antioxidants that react with the free oxygen molecules or free radicals in our bodies [8, 9]. Scientific validation of various medicinal properties of medicinal plants would facilitate the detection of the bioactive principles and subsequently may lead to drug discovery and development. Further, it may also contribute to the separation of pharmacologically active Phyto compounds, which could benefit the scientific world [9]. With this background, the present research study was carried out to evaluate the phytochemical constituents and antioxidant potential of the stem bark extracts of *Oroxylum indicum* (L.).

METHODS: The entire experiments were carried out at the Central Research Laboratory, K.S Hegde Medical Academy, Deralakatte, Mangaluru.

Plant Material: Oroxylum Indicum (OI) stem bark was collected from the Mangaluru region. The stem bark was chopped into small pieces and washed using distilled water to remove all unwanted plant materials and sand, air-dried under light exposure (27 °C-30 °C for 5 days). The dried stem bark was powdered using a mixer grinder and then subjected to soxhlet extraction using 99% ethanol for 72 h.

The stem bark extract was evaporated to dryness using a rotary flash evaporator (Rotavapsuperfit PBU – 6) for 15 min at 60 °C and then stored in an airtight container in a refrigerator until further use. The crude bark extract obtained was subjected to preliminary phytochemical analysis following standard procedures.

Phytochemical Screening of the Plant Extract: A small portion of the dry bark extract was used for the phytochemical tests for compounds which included steroids, triterpenoids, glycosides, saponins, alkaloids, flavonoids, tannins, proteins, free amino acids, carbohydrates, and vitamin C following the method of Raman and Harborne with slight modifications [10, 11].

Few drops of acetic anhydride were added to the crude ethanolic extract. It was boiled and cooled. Concentrated sulphuric acid was then added from the sides of the test tube and observed for the formation of a brown ring at the junction of two layers. Green coloration of the upper layer and the formation of deep red color in the lower layer indicate a positive test for steroids and triterpenoids, respectively.

The test solution was treated with few drops of glacial acetic acid and Ferric chloride solution and mixed. Concentrated sulphuric acid was added from the sides of the test tube and observed for the formation of two layers. The lower reddish-brown layer and upper acetic acid layer, which turns bluish-green, indicate a positive test for glycosides.

The test solution was dissolved in bromine water, which was observed for the formation of a yellow precipitate, indicating a positive result for glycosides. When mixed with water and shaken, the test solution would result in the formation of froth, which is stable for 15 minutes for a positive result. To 5 ml of the test solution, a drop of sodium bicarbonate solution was added. The mixture was shaken vigorously and left for 3 min. It was observed for the formation of honeycomb-like froth for the positive result.

Hager's Test: Test solution, when treated with few drops of Hager's reagent (saturated picric acid solution) would result in the formation of a yellow precipitate, indicating a positive result for the presence of alkaloids. Wagner's test (Iodine in Potassium iodide): The test solution was treated with few drops of Wagner's reagent. It was then observed for the formation of a reddish-brown precipitate which would indicate the presence of alkaloids. Ferric chloride test - The test solution was treated with few drops of Ferric chloride solution is which would result in the formation of blackish-red color indicating the presence of flavonoids. Alkaline reagent Test - Test solution, when treated with sodium hydroxide solution, shows an increase in the intensity of yellow color, which would become colourless on the addition of few drops of dilute Hydrochloric acid, indicating the presence of flavonoids.

Lead Acetate Solution Test: Test solution when treated with few drops of lead acetate (10%) solution would result in the formation of a yellow precipitate, indicating the presence of flavonoids. Shinoda test - A few fragments of magnesium ribbon and concentrated hydrochloric acid were added to the test solution and observed for the appearance of red to pink color after few minutes, indicating the presence of Flavonoids.

Test solution, when treated with gelatin solution, would give white precipitate indicating the presence of tannins. Test solution when treated with 10% sodium hydroxide solution and two drops of 0.1% copper sulphate solution would result in the formation of violet/pink color indicating the presence of proteins. Test solution, when boiled with 0.2% solution of Ninhydrin, would result in the formation of purple color, suggesting the presence of free amino acids. Benedict's test - Few drops of Benedict's reagent (alkaline solution containing cupric citrate complex) was added to the test solution and boiled in water bath, observed for the formation of reddish-brown precipitate to show a positive result for the presence of carbohydrate.

DNPH Test - Dinitrophenyl hydrazine dissolved in concentrated sulphuric acid was added to the test solution. It was observed for the formation of a yellow precipitate, which would suggest the presence of vitamin C.

Assessment of *In-vitro* Antioxidant Potential of OI Stem Bark Ethanolic Extract:

DPPH Radical Scavenging Assay: This assay is based on the ability of 2,2 – diphenyl-1-picrylhydrazyl (DPPH•), a stable free radical, to decolorize in the presence of antioxidants, is a direct and reliable method for determining radical scavenging action. The free radical scavenging capacity of OI ethanolic extract was determined using DPPH assay according to the method of Blois *et al.,* (1958), which was slightly modified.

Freshly prepared DPPH solution (0.004% w/v) in 99% ethanol was added to test solutions (0-500 µg/ml). The mixture was incubated at room temperature in the dark for 20 min. Following incubation, the mixture was vortexed, and the optical density was measured at 517 nm using a spectrophotometer. 99% ethanol was used as blank. A control sample was prepared to contain the same volume without any extract. Quercetin was used as a reference standard. All tests were performed in triplicates. Percentage scavenging of the DPPH free radical was calculated using the following equation,

DPPH radical scavenging activity (%) = (Acontrol-ATest) / Acontrol × 100.

Where A control is the absorbance of the control reaction and A test is the absorbance in the presence of the extracts or standard [12].

Ferric Reducing Antioxidant Power Assay (FRAP): Different concentrations of the sample solutions (0-500 µg/ml) of OI ethanolic extract were taken, and the volume of each was made upto 1 ml with distilled water. 0.5 ml of potassium hexacyanoferrate (potassium ferricyanide) 1% was added and incubated at 50 °C in a water bath for 20 min. The control tube was maintained without adding the extract.

After incubation, 0.5 ml of TCA (10%) was added to terminate the reaction. The upper portion of the solution was mixed with 1 ml distilled water, and 0.1 ml $FeCl_3$ solution (0.1%) was added. The reaction mixture was left for 10 minutes at room temperature and the Optical density was measured at 700 nm against an appropriate blank solution. Quercetin was used as reference standard. All tests were performed in triplicates [13].

Ferrous Ion Chelating Activity: Measured by the formation of ferrous ion ferrozine complex. Ferrozine combines with ferrous ions forming a red coloured complex which absorbs at 562 nm.

The ferrous ion-chelating (FIC) assay reported by Singh and Rajini (2004) was adopted 14.2 mM $FeSO_4$ (100µl) was mixed with different concentrations of extracts (0-125 µg/ml), followed by 5 mM ferrozine (500 µl). Absorbance was measured at 562 nm after 10 min. The ability of extracts to chelate ferrous ions was calculated as follows,

Percentage of inhibition = $(A_{control}-A_{Test})$ / Acontrol × 100

Linoleic Acid Peroxidation Inhibition Assay: The basic principle of this assay is to estimate the total amount of peroxides generated during lipid peroxidation. Peroxides react with ferrous chloride and form ferric ions. These ferric ions combine with ammonium thiocyanate and a ferric thiocyanate complex is formed whose colour intensity is measured at 500 nm.

The reaction mixture contained 500 µL of linoleic acid solution (20 mM), 500 µL Tris HCl (100 mM, pH 7.5), 100 µL $FeSO_4.7H_2O$ (4 mM) and 100 µL of OI ethanolic extract. Linoleic acid peroxidation was initiated by the addition of 100 µL of ascorbic acid (2 mM). The reaction was incubated for 30 min at 37°C and terminated by the addition of trichloroacetic acid (5.5%). One milliliter of the mixture was added to 250 µL of thiobarbituric acid (TBA) in 50 mM NaOH, followed by heating for 10 min. The mixtures were centrifuged at 3500 rpm for 10 min and the absorbance of the supernatant was read at 532 nm [15].

Total Antioxidant Capacity: The total antioxidant capacity (TAC) based on the reduction of Mo(VI) to Mo(V) by the extract and subsequent formation of green phosphate/Mo(V) complex at acid pH. Exactly 100 µL of the OI ethanolic extract of different concentrations (0-500 µg/ml) was pipetted out into a clean test tube and 100 µL of 5% TCA was added to it to precipitate out the proteins in the sample, the mixture was then allowed to stand for about five minutes and centrifuged. 100 µL of the clear supernatant was transferred into a clean test tube and 1 mL of TAC reagent was added to it and the mixture was then incubated in water bath at 90 ºC for 90 min.

A blank was also maintained simultaneously by substituting 100 µL of water instead of sample in the reaction mixture. Following the incubation, the reaction mixture was cooled and the optical density of the greenish to bluish colour formed was read at 695 nm against blank [16].

Liquid Chromatography: Mass Spectrometry Analysis (LC-MS): The OI stem bark ethanolic extract was subjected to LC-MS in order to identify the presence of various polyphenolic compounds. For LC-MS analysis, the dried crude extract was dissolved in ethanol at the concentration of 1 mg/mL and filtered through 0.22 μm syringe filter. The plant sample was analyzed on LC-MS system with Diode array detector.

Statistical Analysis: The obtained results of antioxidant study were compiled using Prism 7.0 software and the results were expressed in percentage.

RESULTS: The ethanol stem bark extract of *Oroxylum indicum* revealed the presence of glycosides, alkaloids, flavonoids, and tannins. The results of the preliminary phytochemical screening are shown in **Table 1**.

TABLE 1: PHYTOCHEMICAL IDENTIFICATION TEST

Phytochemical test	*Oroxylum indicum* stem bark ethanol extract
I. Test for triterpenoids & steroids Liebermann Burchard test	—
II. Test for glycosides	
Keller Killiani test	+
Bromine water test	+
III. Test for saponins Foam test	—
IV. Test for alkaloids	
Hagers's test	+
Wagner`s test	+
V. Test for flavonoids	
Ferric chloride test	+
Alkaline reagent test	+
Lead acetate solution test	+
VI. Test for tannins	
Gelatin test	+
VII.Test for Proteins	
Biuret test	—
VIII.Test for free aminoacids	
Ninhydrin test	—
IX. Test for carbohydrate Benedict`s test	—
X. Test for Vitamin C DNPH test	—

'+' and '-'indicates the presence and absence of phytochemicals respectively.

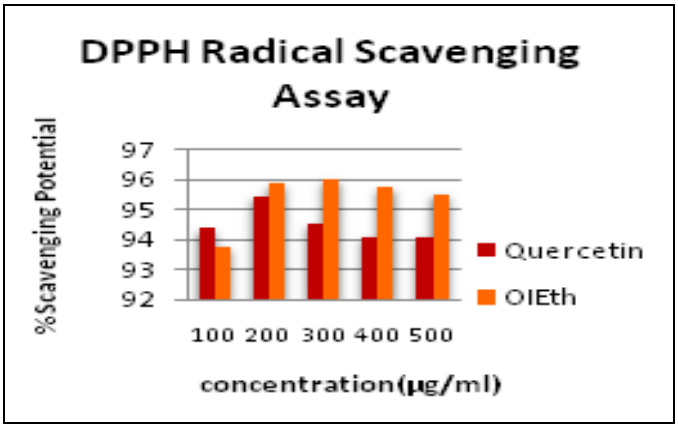

FIG. 1: DPPH RADICAL SCAVENGING PERCENTAGE OF OI EXTRACT

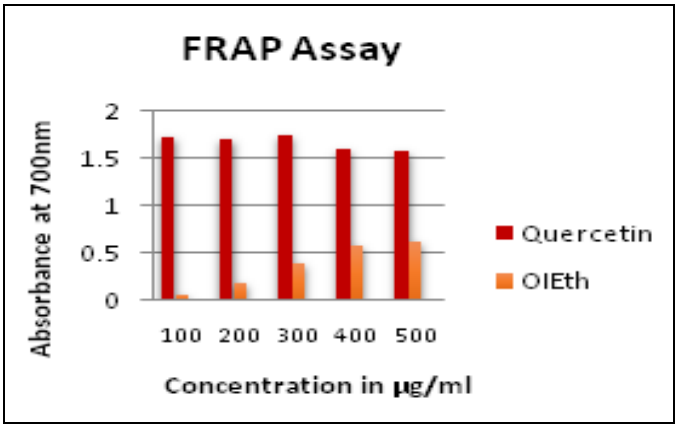

FIG. 2: FERRIC REDUCING ANTIOXIDANT POWER OF OI EXTRACT

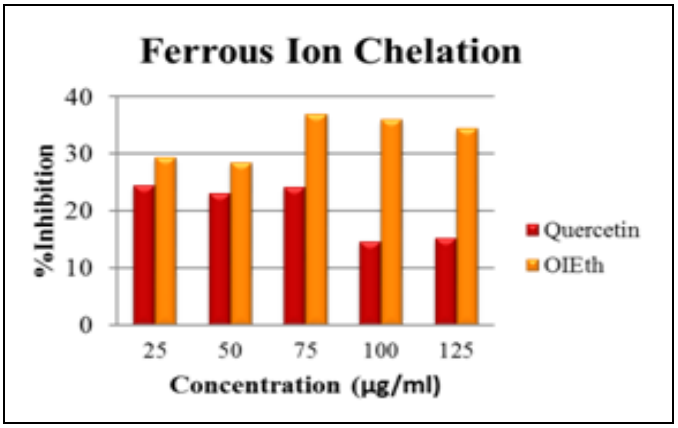

FIG. 3: FERROUS ION CHELATION ACTIVITY OF OI EXTRACT

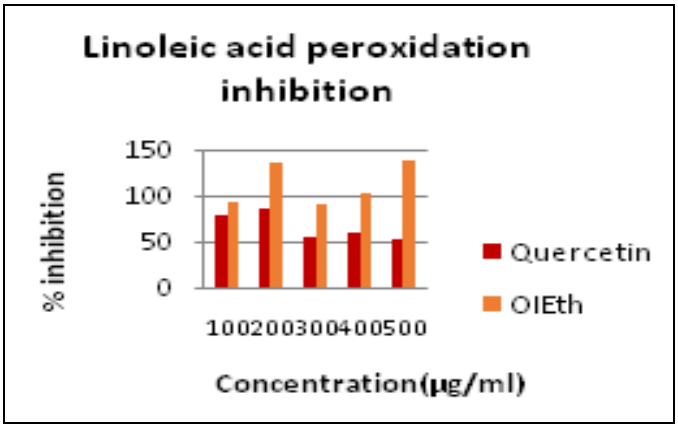

FIG. 4: LINOLEIC ACID PEROXIDATION INHIBITION PERCENTAGE OF OI EXTRACT

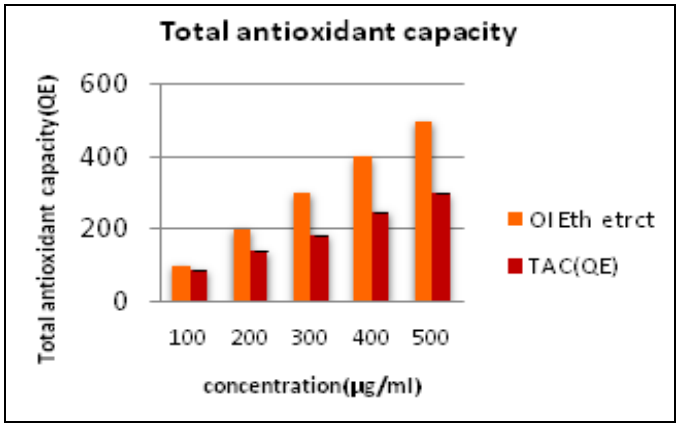

FIG. 5: TOTAL ANTIOXIDANT CAPACITY OF OIEXTRACT

TABLE 2: PHYTOCHEMICAL COMPOUNDS IDENTIFIED IN OI STEM BARK ETHANOL EXTRACT BY LC-MS ANALYSIS

Retention Time	Mass [M+H]	Mass [M-H]	Compound
6.9	549.3, 325.1, 163.0	623.4, 574.2	Unknown
8.12	549.2, 287.1	547.3, 285.1	Scutellarein
8.35	447.1, 287.1	891.4, 445.2	Baicalein
9.09	461.1, 301.1	919.4, 459.2	OroxylinA-7-O-beta-D-glucuronide
9.85	301.1	299.1	Hispidulin
10.48	594.2, 271.1	269.1	Oroxylin B
11.98	539.3, 255.1	537.2,253.1	Chrysin derivative
12.28	285.1	283.2	Oroxylin A

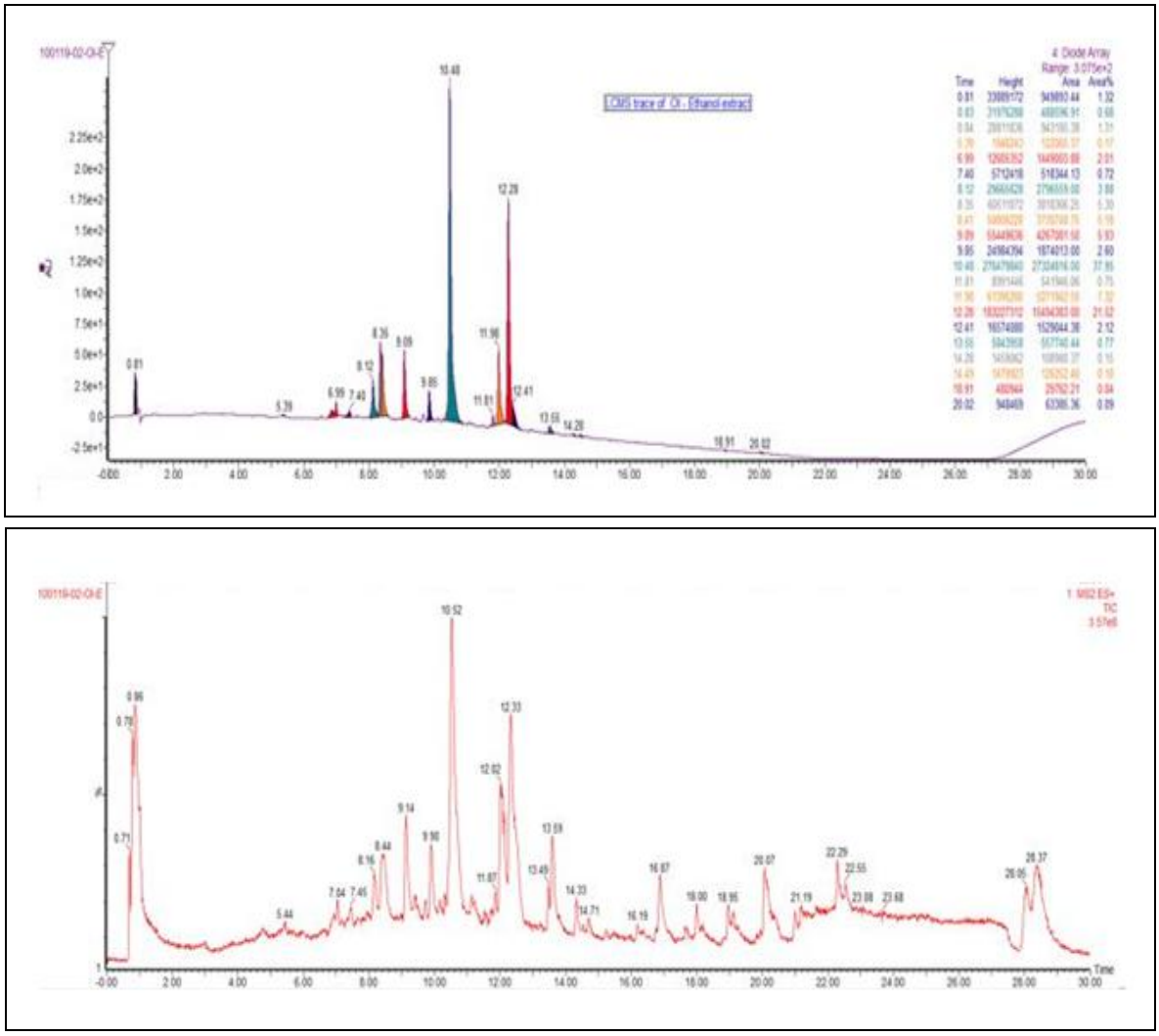

FIG. 6: LC-MS PROFILE OF OI STEM BARK ETHANOL EXTRACT

DISCUSSION: Phytochemical constituents occur naturally in plants and contributes to defense mechanism and protect from various pathological conditions. Plants polyphenols have been shown to exert multiple biological activities, which include free radical scavenging, antioxidant, anti-inflammatory, anti-carcinogenic *etc.* [17]

The widely distributed secondary metabolites in plants, namely, phenolic and flavonoids, are responsible for a wide range of biological activities such as anti-oxidant, anti-apoptosis, anti-aging, anti-carcinogen, anti-inflammation, anti-atherosclerosis, cardiovascular protection, and improvement of endothelial function, as well as inhibition of angiogenesis and cell proliferation activities [18, 19]. As per recent study reports, most dietary polyphenolic constituents derived from plants are more effective antioxidants *In-vitro* and thus might contribute significantly to the protective effects *in-vivo* [20]. The obtained results of phytochemical analysis indicate that *Oroxylum indicum* stem bark ethanolic extract hold promise as a source of pharmaceutically important phytoconstituents. Plant-derived antioxidant compounds have received considerable attention because of their physiological effects like antioxidant, anti-inflammatory, anti-tumour activities, and low toxicity compared with synthetic phenolics. *In-vitro* antioxidant studies are widely carried out to screen the antioxidant potential of phenolic and flavonoid compounds from plants [21].

The DPPH radical scavenging effect of OI ethanol extract at 0-500 µg/ml concentrations is summarized in **Fig. 1**. At various concentrations tested, an increase in the scavenging activity was observed with an increase in the concentration of extracts. According to our observations, the strong antioxidant activity of OI stem bark ethanolic extract might be due to the available hydroxyl group present in the substance [22]. The reducing

power of the extract increased in a dose-dependent manner with an increase in concentration which showed absorption maximum at 700 nm as shown in **Fig. 2**. The reducing power of the extracts is based on the hydrogen donating ability. The reducing property of a compound serves as a significant indicator of its potential antioxidant activity [23].

The increased, reducing power in OI ethanolic extract indicated that some components in the extract were strong electron donors that could react with the free radicals to convert them into more stable products to terminate radical chain reaction. The chelating effect on the ferrous ions by OI ethanolic stem bark extract is presented in **Fig. 3**.

The extract exhibited the ability to chelate metal ions as they reduced the concentration of catalyzing transition metal in lipid peroxidation. Antioxidants will slow down the peroxidation in lipids resulting in low production of peroxides. As a result, there will be less oxidation of ferrous into ferric, and therefore less amount of the complex will be formed [24].

Thus, the stronger the antioxidant, the less is the formation of ferric thiocyanate, and lower will be the absorbance. In our study, the OI ethanolic extract showed a remarkable ability to inhibit peroxidation in linoleic acid. The plant samples proved to be better antioxidants than Quercetin, as depicted in **Fig. 4**. The Total Antioxidant capacity of OI extract at different concentrations (0-500 µg/ml) is shown in **Fig. 5**. The Phospho-molybdenum method is based on the reduction of Mo (VI) to Mo (V) by the antioxidant compound and the formation of a green phosphate/Mo (V) complex, which has a maximal absorption at 695 nm. The total antioxidant capacity of the extract increased linearly in a dose-dependent manner which was expressed as quercetin equivalents.

The LC/MS analysis enabled us to identify 7 phenolic compounds in *Oroxylum indicum* stem bark ethanol extract as mentioned in **Fig. 6** and **Table 2,** namely, scutellarein, baicalein, oroxylin A-7-O-beta-D-glucuronide, Hispidulin, oroxylin B, chrysin derivative, oroxylin B. Overall, LC/MS analysis reflects the phenolic profile of OI stem bark extract [25].

CONCLUSION: Through our systematic phyto-chemical and antioxidant potential analysis study, the ethanolic *Oroxylum indicum* stem bark extract was found to be an excellent free-radical scavenger and a potent natural antioxidant. The present study revealed the presence of various important phyto-chemicals in the ethanolic *Oroxylum indicum* stem bark extract. These results may contribute to further standardization, validation, and research in *Oroxylum indicum* stem bark-based drugs which are used in traditional and modern phytomedicine. In order to discover and progress the alternative choice of using plant-derived bioactive compounds, intense profiling research needs to be done. The targeted compounds may be employed in biomedical and pharmaceutical research ranging from *in-vitro*, *in-vivo*, and clinical trial steps to evaluate the safety, efficacy and also toxic effects of the novel candidate compounds.

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CONFLICTS OF INTEREST: There are no conflicts of interest.

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