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Evaluation of antioxidant potential of *Trema orientalis* by *in vitro* assay methods

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Abstract
By using the FRAP (Ferric reducing antioxidant power assay) and DPPH (2, 2-diphenyl-1-picrylhydrazyl) assays, a study was conducted to evaluate the potential *in vitro* antioxidant activity of the methanolic extract of *T. orientalis*. The experiments were carried out using aliquots of methanolic extract of *T. orientalis* (METO) in various doses. In an UV spectrophotometer, absorbance was measured at 517 nm using ascorbic acid as the reference. The parameters for the antioxidant activity are found using the spectrophotometric measurement of the color change. When compared to ascorbic acid, which served as the reference, the *in vitro* antioxidant analysis using the ferric reducing antioxidant test and the DPPH assay at 200 µg/ml and 250 µg/ml doses of METO demonstrated minimal antioxidant activity. Since most tannins and flavonoids are phenolic compounds, they may be responsible for METO's antioxidant properties. The solvent and plant materials used in the antioxidant analysis study contributed a portion to METO's lower antioxidant potency compared to standards. *T. orientalis* has been found to have reduced antioxidant potential, which could be further investigated to assess the therapeutic antioxidant efficacy in domestic animals in clinical circumstances.

Keywords: *Trema orientalis*, Antioxidant, METO, FRAP and DPPH

1. Introduction
Historically, a considerable portion of the population in developing nations have relied upon the products obtained from forests to treat ailments in humans and animals. Various medicinal and aromatic plants are often used for medicinal therapeutic utility. Approximately 12.5% of the 4,22,000 species of plants that are known to exist worldwide are thought to have therapeutic qualities. But only few plants among them are under cultivation [1]. More than 1.5 million people are believed to use the traditional medicinal plants for curative, preventative, and promotional purposes. It is estimated that rural populations use almost 25,000 potent plant-based medicines. Approximately 7,800 pharmaceutical production facilities are located in India, which might use approximately two thousand tonnes of herbal raw materials in a year [2]. The separation and identification of biologically active substances and molecules from the medicinal plants have contributed in discovery of new medicinal products, which in turn resulted in improving the pharmaceutical and health care sectors [3]. Because of safer pharmacological potentiality and significant therapeutic benefits, herbal medicines are in greater demand than ever before. However, in order to put the herbs-based medications on the market as main line therapies, efforts have to be made to investigate, standardize and validate them for their potency, safety and efficacy [4]. However, ethnopharmacological investigations are highly challenging since herbal medicines typically comprise a variety of pharmacologically active chemicals derived from different plants and sometimes it is not clear which of these components has the therapeutic effect [5]. *Trema orientalis* (L) Blume is a modest to mid-sized tree belonging to Cannabaceae family. The current study was carried out to evaluate the *in vitro* antioxidant properties of *T. orientalis* plant extract by using FRAP assay (Ferric reducing antioxidant power assay) and DPPH assay (2, 2-diphenyl-1-picrylhydrazyl).

2. Materials and Methods
The aerial portions of recently harvested *T. orientalis* plant have been collected from multiple regions of Soraba, Shivamogga district, Karnataka during April and May 2022. Dr. Rajeshwari, N. Professor, Department of Botany and Seed Technology, Sahyadri Science College, Shivamogga, verified the plant's taxonomic identification.

The plant material was further processed by soaking in methanol solvent and then filtration and concentration. Finally obtained 12.38% of final yield of greenish black coloured methanolic extract of *Trema orientalis* (METO) with powdery consistency. This METO is used for FRAP and DPPH *in vitro* assay methods.

2.1 *In vitro* antioxidant activity of the extract

2.1.1 Ferric reducing antioxidant power assay (FRAP assay)

The overall antioxidant capacity of a plant extract might be ascertained using the ferric reducing antioxidant power test (FRAP). The flavonoids and phenolic acids found in medicinal plant extracts have substantial antioxidant properties because of their capacity to build interactions with atoms of metals, especially iron and copper. The strategy is predicated on the concept that antioxidant activity increases along with reaction mixture absorption. The samples' antioxidant component reacts with potassium ferricyanide, trichloroacetic acid, and ferric chloride to produce a colorful complex that is detected by UV light Spectrophotometer at a wavelength of 700 nm [6].

Principle

The FRAP method's chemical process consists of a one-electron reaction using the single electron donors ArOH and Fe (TPTZ)2(III).

$$Fe\ (TPTZ)_2(III) + ArOH \Longrightarrow Fe\ (TPTZ)_2(II) + ArOH^{+}$$

The ferric reducing capacity of the extract (FRAP) is evaluated using this method of investigation. Low pH conditions result in the reduction of a ferric-trotripyridyltriazine (Fe III-TPTZ) complex to the ferrous (Fe II) form, which exhibits a vivid blue color with the highest absorption at 700 nm. The reduction of Fe III-TPTZ could be sped up by any half-reaction other than the Fe III/Fe II-TPTZ half-reaction that has a less-positive redox potential under the reaction circumstances. Test conditions promote the reduction of the complex provided in the presence of a reluctant (antioxidant), which results in the formation of colour [6, 7].

Equipments

Centrifuge, cuvettes, cyclo mixer, graduated centrifuge tubes, incubator, pipette, micro centrifuge tubes, micropipette, sonicator ultra sonic bath, UV-Spectrophotometer (Ultra violet), vortex shaker and water bath were the major laboratory equipments used in the study.

Reagents

Phosphate buffer (0.2M at pH 6.6)

In a 1-liter standard flask, 800 ml of distilled water was poured in along with 8 grams of sodium chloride, 0.2 grams of potassium chloride, 1.44 grams of disodium hydrogen phosphate and 0.24 grams of potassium dihydrogen phosphate. Hydrochloric acid was used to bring the pH down to 6.6 and deionized water is added to make up the volume.

a. Potassium ferricyanide (1%W/V)
b. Trichloroacetic acid (10%W/V)
c. Ferric chloride (0.1%W/V):
d. Ascorbic acid (0.1%W/V):

Procedure

By dissolving extract in methanol in different quantities and making serial dilutions, the stock solutions for the test samples were obtained. Similar to this, various measurements of ascorbic acid have been made by dissolving in distilled water and making successive dilutions. The 2.5 ml of various aliquots of *T. orientalis* methanolic extract were treated with 2.5 ml of 0.2 molar sodium phosphate buffer (pH 6.6) and 2.5 ml of 1% $K_3Fe\ (CN)_6$ solution. The concentrations of METO used were 25µg/ml, 50µg/ml, 100µg/ml, 150µg/ml, 200 µg/ml, and 250µg/ml. For 20 minutes, the mixture was incubated at 50 °C. A cyclomixer was used to vigorously vortex the reaction mixture after being incubated for 20 minutes at 50 degrees Celsius. After incubation, 2.5 ml of 10% trichloroacetic acid was incorporated to the mixture, and it was centrifuged for 10 minutes at a speed of 3,000 rpm. The next step was to blend 2.5 ml of supernatant with 0.5 ml of 0.1% ferric chloride and an equal quantity of deionized water. The colored mixture was measured at 700 nm with a bandpass of 1 nm using a UH 5300 UV Spectrophotometer in comparison to the blank and standard. In this case, ascorbic acid was used as a reference standard, and samples with ascorbic acid concentrations of 6.25 µg/ml, 12.5 µg/ml, 25 µg/ml, 50 µg/ml, 75 µg/ml and 100 µg/ml had reducing powers that were equivalent to those of the reference standard. The antioxidant ferric reducing potential was consequently determined [8, 9].

2.1.2 DPPH assay

The DPPH (2,2-diphenyl-1-picrylhydrazyl) assay, which is usually employed to measure the extent to which an assigned antioxidant compound scavenges free radicals, is a widely used colorimetric method for evaluating the antioxidant capabilities of pure substances. In a variety of solvent systems, such as ethanol, aqueous acetone, methanol, aqueous alcohol, and benzene, this test procedure is often employed to determine the antioxidant levels in vegetables, conjugated linoleic acids, herbs, edible seed oils, plant extracts and flours. Despite the fact that this radical shares only a few similarities with peroxyl radicals. The procedure is based on the antioxidant's capacity to scavenge DPPH, which would lower the initial concentration and changes the solution's colour from purple to yellow. The concentration of the DPPH solution, which varies from 22.5 to 250 mM for the solvents or solvent mixes needed for dissolving DPPH or to prepare the extracts. The duration of the reaction is all explained in the context of DPPH test methods [10, 11, 12, 13].

Principle

This analysis is based on the principle that the purple color transforms to yellow and the absorbance at 515 nm drops when DPPH takes a hydrogen (H) atom from the antioxidant molecule, reducing DPPH to DPPH-H. The criteria for antioxidant efficacy can be assessed by the spectrophotometric estimation of the colour change. The DPPH assay thus seems straightforward, however because of the persistent nitrogen radical it possesses, each antioxidant may react differently or not at all. Additionally, since the reaction involving DPPH and antioxidant process is reversible, the antioxidant would not be totally responsible for the decrease of the radical because DPPH-H will eventually change back to DPPH. Because of the reaction's reversibility, it was postulated that many antioxidants might be having limited antioxidant capacity [14, 15]. The principle of FRAP assay is presented in figure (1).

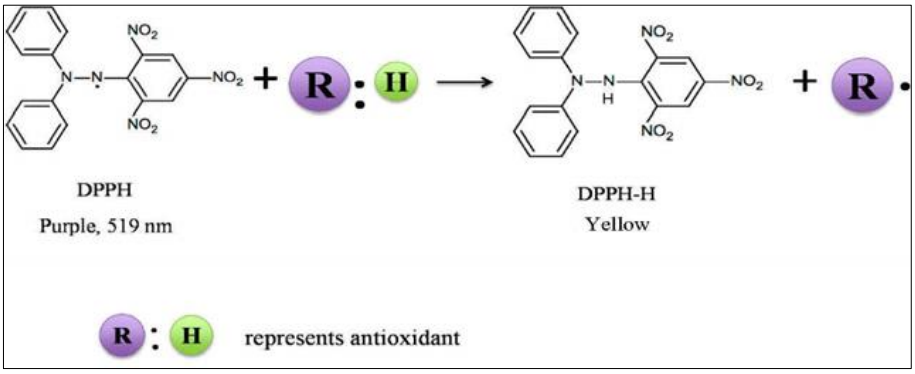

Fig 1: The mechanism of reaction of DPPH with antioxidant [16]

Where, R: H = antioxidant radical scavenger and R = antioxidant radical

Reagents and equipments

The reagents used in the present study included: Ascorbic acid, capped test tubes, cuvettes and DPPH reagent. Incubator, pipette, micro centrifuge tubes, micropipette, Sonicator, ultra sonic bath and UV-Spectrophotometer were the laboratory equipments used.

Procedure

The extract was dissolved in different quantities of methanol to make the stock solution for the sample and multiple dilutions were made at concentrations of 25 µg/ml, 50 µg/ml, 100 µg/ml, 150 µg/ml, 200 µg/ml, and 250 µg/ml. The ascorbic acid concentrations were produced by dissolving it in distilled water, which was then progressively diluted to yield concentrations of 6.25 µg/ml, 12.5 µg/ml, 25 µg/ml, 50 µg/ml, 75 µg/ml, and 100 µg/ml. Then 4 mg of the DPPH reagent was mixed in 100 ml of methanol to formulate the DPPH solution. 200 µl of the sample was transferred to 4 ml of the resulting DPPH solution in a test tube with a cover. Ahead of viewing the results, the test tube was stored at room temperature without exposure to light for 20 to 30 minutes. In an UV spectrophotometer, the absorbance was measured at 517 nm. The blank was precisely the same amount of the control sample produced from 95% methanol lacking any extract. The following equation was deployed to determine the percentage of the DPPH free radical that was scavenged:

Inhibition (%) = [(ABs - As)/ABs] x 100]

As- Absorbance of sample
Abs- Absorbance of blank.
[8, 17, 18, 19].

3. Results

3.1 *In vitro* antioxidant activity of the extract

3.1.1 Ferric reducing antioxidant power assay (FRAP assay)

The methanolic extract of *T. orientalis* at various concentrations i.e., 25, 50, 100, 150, 200 and 250 µg/ml produced absorbance of 0.114, 0.14, 0.214, 0.311, 0.374 and 0.441 µmol AAE/g (Ascorbic acid equivalent/gram) respectively. In the same assay the various concentrations of ascorbic acid Std. at 6.25, 12.5, 25, 50, 75 and 100 µg/ml produced absorbance of 0.11, 0.115, 0.229, 0.533, 0.663 and 0.787 µmol/ml, respectively. Absorbance exhibited by both test substance (METO) and the standard were compared and graph was plotted. It is depicted in table (1), figure (2) and figure (3).

Table 1: *In vitro* antioxidant activity of methanolic extract of aerial parts of *T. orientalis* by FRAP method

Concentration (µg/ml)	METO (µmol AAE/g)	Concentration (µg/ml)	Ascorbic acid (Std µmol/ml)
25	0.114	6.25	0.110
50	0.140	12.5	0.115
100	0.214	25	0.229
150	0.311	50	0.533
200	0.374	75	0.663
250	0.441	100	0.787

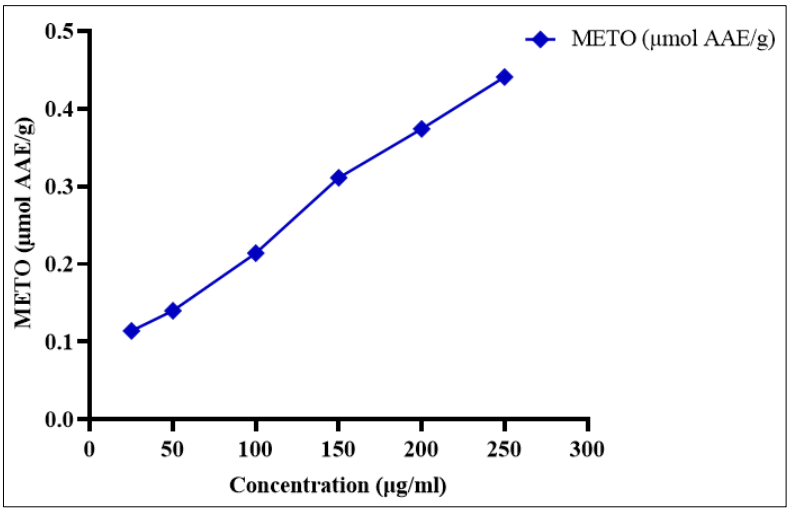

Fig 2: *In vitro* antioxidant activity of methanolic extract of aerial parts of *T. orientalis* by FRAP method

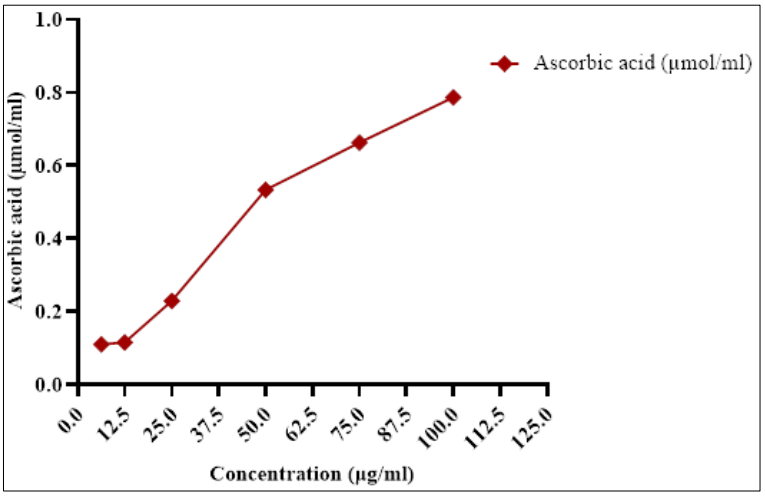

Fig 3: *In vitro* antioxidant activity of ascorbic acid by FRAP method

3.1.2 DPPH assay

The various concentrations of methanolic extract of *T. orientalis* at 25, 50, 100, 150, 200 and 250 µg/ml produced 4.676, 4.316, 6.619, 7.914, 10.288 and 10.503% inhibition respectively. Meanwhile, the different concentrations of ascorbic acid at 6.25, 12.5, 25, 50, 75 and 100 µg/ml produced 4.784, 9.496, 12.842, 23.849, 29.209 and 43.021% inhibition respectively. The percentage inhibition exhibited by both METO and the standard were compared and graph was plotted. The results are expressed in table (2) and figure (4).

Table 2: *In vitro* antioxidant activity of methanolic extract of aerial parts of *T. orientalis* by DPPH method

Concentration (µg/ml)	Ascorbic acid (% Inhibition)	Concentration (µg/ml)	METO (% Inhibition)
6.25	4.784	25	4.676
12.5	9.496	50	4.316
25	12.842	100	6.619
50	23.849	150	7.914
75	29.209	200	10.288
100	43.021	250	10.503

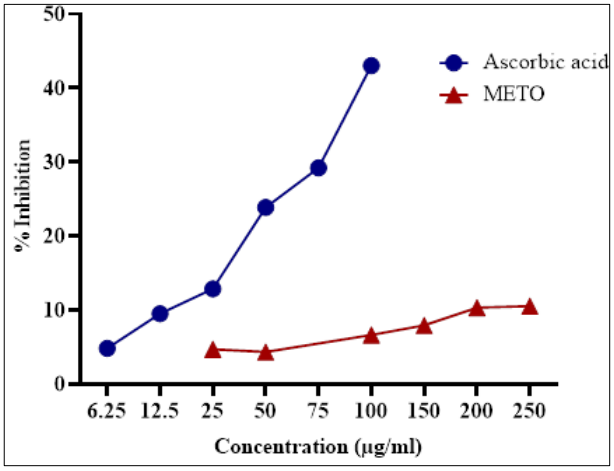

Fig 4: *In vitro* antioxidant activity of methanolic extract of aerial parts of *T. orientalis* by DPPH method

4. Discussion

The antioxidant potentiality of METO was assessed *in vitro using* the FRAP assay. The study revealed that, 200 µg/ml and 250 µg/ml concentrations of METO exhibited minor antioxidant activity *in vitro* in comparison to ascorbic acid, the standard. METO at 25 µg/ml produced 0.114 µmol AAE/g the lowest antioxidant activity and 250 µg/ml of METO produced 0.441 µmol AAE/g the highest antioxidant activity. The antioxidant activity exhibited by 250 µg/ml concentration of METO was equivalent to the antioxidant activity of 50 µg/ml concentration of ascorbic acid with the same potential, indicating that METO exhibited lesser antioxidant potential than that of ascorbic acid. The lesser antioxidant potentiality of METO might be attributed partly to the solvent and plant materials employed in the antioxidant assay. The outcomes from the current study were in agreement with [8, 17] As per [20] the iron chelating activity methanolic extract of *T. orientalis* leaves was 40.74% which indicated that the activity was just approximately 50% that of an EDTA ligand which was used as the control because of its 93.15% chelating activity. [8] had estimated the FRAP values of three plant herbs and expressed as gallic acid equivalents, as per their report, *T. orientalis* had greater FRAP values (8.866 mg gallic acid/g), proceeded by *L. interrupta* (3.115 mg/g) and C. *cinerea* had the lowest values (2.385 mg/g), which suggested that *T. orientalis* had a greater capacity of reducing ferric ions.

The DPPH test was performed to determine the antioxidant content of METO. The assay disclosed that, 200 µg/ml and 250 µg/ml concentrations of METO exhibited negligible *in vitro* antioxidant activity (% inhibition) in comparison to the standard ascorbic acid. METO at 25 µg/ml exhibited the lowest antioxidant activity with % inhibition of 4.676 and highest antioxidant activity at 250 µg/ml with 10.503% inhibition. The equivalent potential antioxidant activity of METO at 250 µg/ml was comparable to the antioxidant activity of ascorbic acid at 25 µg/ml concentration, suggesting that METO had less antioxidant potential than ascorbic acid. The results of the current investigation concurred with [17].

As per the study of [19], after being extracted in methanol, the leaves of *T. orientalis* exhibited a certain capability for scavenging free radicals, but not much in an aqueous extract. The capability of phytoconstituents to neutralize free radicals may be one of the mechanisms through which this plant works as a traditional medicinal product. The fact that most of the tannins and flavonoids are phenolic compounds might be the reason for their antioxidant properties.

The findings of [21] revealed that leaf extracts had the highest concentration of polyphenols and flavonoids, have an impact on antioxidant activity more efficient against free radicals and that bark extracts had the lowest concentrations, indicating the leaf extract had strong antioxidant ability. The higher

concentration of hydroxyl groups is likely to be the reason for the maximal antioxidant activity. It is assumed that the phenolic OH groups participate in redox reactions and have the potential to transmit hydrogen to free radicals, which neutralizes the free radicals that are the primary cause of oxidative stress [21] demonstrated that there is an association between the polyphenol and flavonoid content and the antioxidant activity because the antioxidant action is correlates directly to the level of polyphenols and flavonoids noticed within the test samples (Plant extract). Phenolic components have the capability to scavenge and to chelate metals due to their redox properties which enable them to serve as reducing agents, hydrogen donors and singlet oxygen quenchers [17].

5. Conclusion

Using the FRAP test technique, the *in vitro* antioxidant potential of METO was examined. In contrast to ascorbic acid, the gold standard for antioxidant capability, METO at 200 µg/ml and 250 µg/ml concentrations revealed relatively lower antioxidant activity. Moreover, ascorbic acid would require 50 µg/ml concentration when compared with METO which would require concentration of 250 µg/ml to produce similar absorbance and antioxidant activity. The antioxidant potency of METO was low compared to standard and this was partly attributable to the solvent and plant materials employed in the antioxidant analysis study.

The DPPH test was performed to analyze the antioxidant content of METO. At 200 µg/ml and 250 µg/ml concentrations METO exhibited lesser *in vitro* antioxidant activity (% inhibition) in comparison to the standard, ascorbic acid. METO at 250 µg/ml exhibited the highest antioxidant activity with 10.503% inhibition. The majority of tannins and flavonoids being phenolic substances, could be attributed for the cause of antioxidant characteristics of METO. The present study concluded that *T. orientalis* would exhibit antioxidant actions from therapeutic point of view and need to be explored further in the future.

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