A comparative study on the antioxidant activity of methanolic extracts of *Terminalia paniculata* and *Madhuca longifolia*

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ABSTRACT

Introduction: *Terminalia paniculata* and *Madhuca longifolia* are widely used plants in the folk medicine for different ailments. Many of their traditional uses are based on the antioxidant property. But, till now, the antioxidant property of these plants has not been scientifically proved. Hence, in the present work, a comparative study of the antioxidant property of these plants has been taken up. **Materials and Methods:** The methanolic extracts of *Terminalia paniculata* and *Madhuca longifolia* were evaluated by *in vitro* methods for the free radical scavenging and antioxidant properties. The models used were ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid), DPPH (2,2-diphenyl-1-picrylhydrazyl), Nitric oxide, hydroxyl radical and hydrogen peroxide scavenging. Butylated hydroxyanisole, butylated hydroxytoluene, and Ascorbic acid were used as the standard antioxidants for comparison. **Results:** Both the extracts possessed significant antioxidant property. *M. longifolia* exhibited higher activity in case of DPPH, ABTS, hydrogen peroxide radical scavenging, whereas, *T. Paniculata* exhibited higher scavenging efficacy in case of nitric oxide and hydroxyl radicals. The antioxidant property of these plants might be due to the phenolic content. Conclusion: The antioxidant potential of these plants suggest their possible use to reduce the oxidative stress and hence, possible use as supplements in diabetes, liver problems, inflammatory conditions or cancer like diseases which are due to increased oxidative stress. Further studies are needed to prove their pharmacological applications.

Keywords: Antioxidant, Free radical, DPPH, ABTS, Nitric oxide radical, Hydroxyl radical, Hydrogen peroxide radical

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INTRODUCTION

Free radicals are atoms or groups of atoms with an odd (unpaired) number of electrons and can be formed when oxygen interacts with certain molecules. Free radicals due to environmental pollutants, radiation, chemicals, toxins, deep fried and spicy foods as well as physical stress, cause depletion of immune system antioxidants, change in gene expression and induce abnormal proteins.[1,2] Increased free radical formation may produce a continuous level of oxidative damage[3,4], which leads to many diseases such as atherosclerosis, cancer, stroke, asthma, arthritis and other age related diseases.[3,5]

However, the generated free radicals are removed from the body through the antioxidant defense mechanisms. Antioxidants are considered as possible protection agents reducing oxidative damage of human body from reactive oxygen species (ROS) and retard the progress of many chronic diseases as well as lipid peroxidation.[6] Therefore, there is a lot of ongoing research on such substances for their potential usefulness as dietary supplements and as adjuvants for use in therapeutic management of free radicals related disorders.

Synthetic antioxidants like butylated hydroxy anisole (BHA), butylated hydroxy toluene (BHT), tertiary butylated hydroquinone and gallic acid esters, have been suspected for liver damage and carcinogenesis.[7] Hence, strong restrictions have been placed on their application and there is a trend to substitute them with naturally occurring antioxidants.[8,9] Therefore the importance of searching for and exploiting natural antioxidants, especially of plant origin, has increased greatly in recent years. There is a growing interest in natural additives as potential antioxidants.

Terminalia paniculata (Family: Combretaceae) is most frequently found in valleys and on lower slopes preferring fairly moist situations. From the heartwood, Ellagic acid, 3, 3'-O-dimethylellagic acid-4- glucoside, O-pentamethyl flavellagic acid, 3, 4, 3'-O-trimethyl flavellagic acid and β sitosterol have been isolated. The bark contains 14% tannins which have a pyrogallol nucleus along with gallic acid.[10] Saponins in *Terminalia paniculata* have spermicidal activity.[11]

Madhuca longifolia is member of Sapotaceae family. It is a large, evergreen tree with spreading crown and dark grey or brownish scaly bark. Leaves are thin broadly ovate-lanceolate, acute, glabrous and are clustered near the ends of branches. Flowers are pale yellow and fleshy. Fruits are ovoid and yellow when ripe. Flowers are the rich source of sugars, vitamin A, ascorbic acid, thiamine, riboflavine, Ca, P, Fe, Mg, Cu, anthocyanins, betains, salts of malic and succinic acid.[12] The bark of this plant contains 17% tannins and is used for treatment of rheumatism, ulcers, itches, bleeding and spongy gums.[13] It is also considered as good remedy for inflammations, sprains and pruritus.[13]

Even though many of the traditional medicinal uses of these plants can be attributed to the antioxidant potential of these plants, there is no scientific evidence of the antioxidant property of both the selected plants. Hence, in the present study, comparison of antioxidant property of *Terminalia paniculata* and *Madhuca longifolia* has been taken up to provide a scientific basis for the ethnopharmacological uses of these plants.

MATERIALS AND METHODS

Chemicals

The chemicals used in the present study were ascorbic acid (ASC), 1, 1-diphenyl, 2-picryl hydrazyl (DPPH), N-(1-naphthyl) ethylene diamine dihydrochloride (NEDD), sodium nitoprusside, sulphanilic acid, sodium chloride, ferric chloride, disodium hydrogen orthophosphate, potassium dihydrogen phosphate, 2,2´-Azino-bis (3-ethylbenzothiazoline -6-sulfonic acid) (ABTS), butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), 1,10-phenanthroline and Folin-Ciocalteu reagent. All chemicals and solvents used were of AR grade and were obtained from HiMedia, Mumbai, Ranbaxy, New Delhi and CDH, New Delhi.

Plant material

Stem-bark of *Terminalia paniculata* (TP) and *Madhuca longifolia* (ML) were collected in the month of September 2007 from the areas in and around Udaipur, Rajasthan, India. The plants were authenticated by the Department of Biotechnology, National Institute of Medical Science University, Jaipur, India. The voucher specimens of these pants are preserved in the University with numbers. (CSU/128/2007) and (CSU/129/2007).

Extraction

The plants parts were shade dried, powdered and extracted individually with methanol by hot continuous percolation using Soxhlet apparatus. The extracts were filtered, concentrated and kept in vacuum desiccators for complete removal of the solvent.

Phytochemical analysis of extracts

The methanol extracts of TP and ML were subjected to qualitative determination of different phytochemical constituents such as alkaloids, glycosides, phenols, tannins, proteins, carbohydrates, fats and oils, coumarins, by standard methods reported earlier.[14]

Antioxidant assays

DPPH radical scavenging

The DPPH scavenging activity of TP and ML was measured by a method reported earlier.[22] Solution of DPPH in methanol (0.1 mM) was prepared and 1 mL of this solution was added to 3 mL of various concentrations of TP, ML and reference compound (10-50 μg). After 30 min, absorbance was measured at 517 nm. BHA, BHT and ASC were used as reference materials. All the tests were performed in triplicate and the graph was plotted with the mean value. The percentage of inhibition was calculated by comparing the absorbance values of control and samples.

ABTS radical scavenging

The ABTS scavenging activity of ML and TP was determined using a method reported by Re, et al.[16] The ABTS free radical was produced by the reaction between 7 mM aqueous ABTS solution and 2.45 mM potassium persulfate, stored in the dark at room temperature for 12 h. Before usage, the ABTS solution was diluted with phosphate buffer (0.1 M, pH 7.4), to get an absorbance of 0.700 ± 0.025 at 734 nm. The stock solutions of TP and ML were prepared by dissolving 10 mg of the extract separately in 10 mL distilled water. Then, 1 mL of ABTS solution was added 3 mL of TP and ML solution in methanol at different concentrations (25-150 μg/mL). After 30 min, the absorbance was measured at 734 nm

for each concentration using methanol as blank. All determinations were carried out in triplicate. The capability of the methanol extracts to scavenge the ABTS radical was calculated using the following equation:

$$\text{Inhibition (\%)} = \frac{A_0 - A_S}{A_0} \times 100$$

Where, A_0 was the absorbance of the blank (without extract) and A_S was the absorbance in the presence of the extract.

Nitric oxide radical scavenging

Nitric oxide radicals were generated from sodium nitroprusside solution at physiological pH.[17] Four milliliters of sodium nitroprusside (10 mM) was mixed with 1 mL of the test extract / ascorbic acid in phosphate buffer saline (pH 7.4). The test extracts were prepared in different concentrations (10-60 μg/mL). The mixture was incubated at 25°C for 150 min. To 0.5 mL of the incubated solution, 1 mL of sulphanilic acid reagent was added, mixed well and allowed to stand for 5 min to complete diazotization. Then, 1 mL of NEDD was added, mixed and allowed to stand for 30 min in diffused light for the formation of a pink colored chromophore. The absorbance of these solutions was measured at 540 nm against the corresponding blank solutions.

Hydrogen peroxide radical scavenging

Ability of TP and ML extracts to scavenge H_2O_2 radical was determined according to the method of Ruch, et al.[18] A solution of H_2O_2 (40 mM) was prepared in phosphate buffer (pH 7.4). TP and ML in different concentrations (25-75 μg/mL) in 3.4 mL phosphate buffer were added to a H_2O_2 solution (40 mM, 0.6 mL). The absorbance of the reaction mixture was recorded at 230 nm against phosphate buffer without H_2O_2 as blank. The percentage of H_2O_2 scavenging of TP, ML and standard compounds was calculated according to the equation given under ABTS scavenging method.

Hydroxyl radical scavenging

The scavenging activity for hydroxyl radicals was measured with Fenton reaction.[19] The reaction mixture contained 60 μL of 1.0 mM $FeCl_3$, 90 μL of 1 mM 1,10-phenanthroline, 2.4 mL of 0.2 M phosphate buffer (pH 7.8), 150 μL of 0.17 M H_2O_2, and 1.5 mL of extract at various concentrations. Addition of H_2O_2 started the reaction. After incubation at room temperature for 5 min, the absorbance of the mixture was measured at 560 nm in a spectrophotometer. The hydroxyl radicals scavenging activity was calculated according to the equation given under ABTS scavenging method.

Determination of total phenolic content

Total phenol content of the extracts was determined by using the Folin-Ciocalteu method.[20] This test is based on the oxidation of phenolic groups with phosphomolybdic and phosphotungstic acids. After oxidation, the absorbance of the green-blue complex formed was measured at 750 nm. In a test tube, 200 μL of the extract (10 μg, 20 μg….50 μg) was mixed with 1 mL of Folin-Ciocalteu reagent and 800 μL of sodium carbonate (0.7 M). After shaking, it was kept for 2 h reaction time. The absorbance was measured at 750 nm. A standard curve prepared using gallic acid monohydrate, was used to calculate the total phenol content, which was expressed as gallic acid equivalent in mg/g of the extract.

Statistical Analysis

All experiments were conducted in triplicate. Students' T-test and ANOVA were used for determination of statistical significance and $p < 0.05$ were regarded as significant.

RESULTS

TP and ML were extracted with methanol to get 7.58 and 3.85% w/w of extracts respectively. The phytochemical analysis of the ML extract showed the presence of alkaloids, coumarins, tannins and phenolics and TP extract showed the presence of carbohydrates, glycosides, coumarins, tannins and phenolics.

In the DPPH scavenging study, TP and ML exhibited marked DPPH free radical scavenging activity in a concentration-dependent manner. The IC_{50} value of ML (20.01 μg/mL) was found to be better than that of BHT (64.28 μg/mL). The scavenging effect of TP, ML and standards on the DPPH radical decreased in the order: BHA > ML >TP> BHT. The IC_{50} values for these compounds were found to be 92.7, 51.0, 42.2 and 12.6%, respectively, at 10 μg/mL concentration (Fig. 1). The results were found to be statistically significant ($P<0.01$) at 10 μg/mL concentration. The results show that both the plants extract exhibited concentration-dependent free radical scavenging activity, the most effective being ML which exhibited significantly higher DPPH scavenging activity (51.0% inhibition) when compared with the highest concentration of standard BHT (12.6% inhibition).

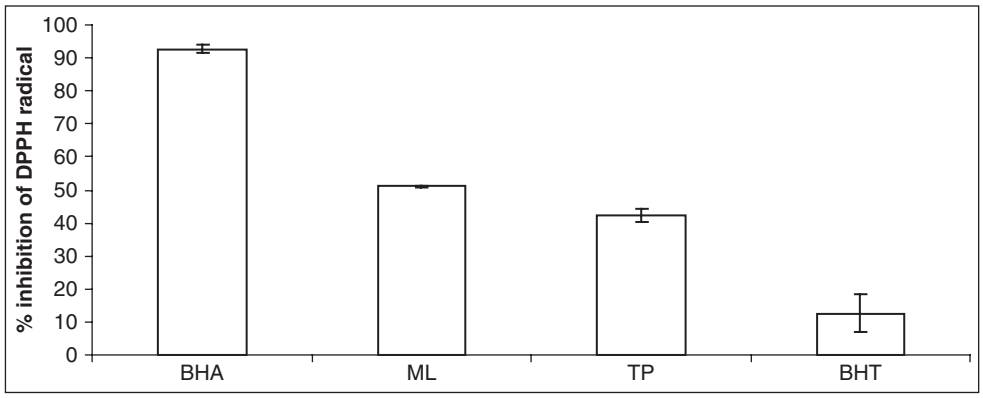

Figure 1. DPPH radical scavenging activity of methanol extracts of TP and ML.

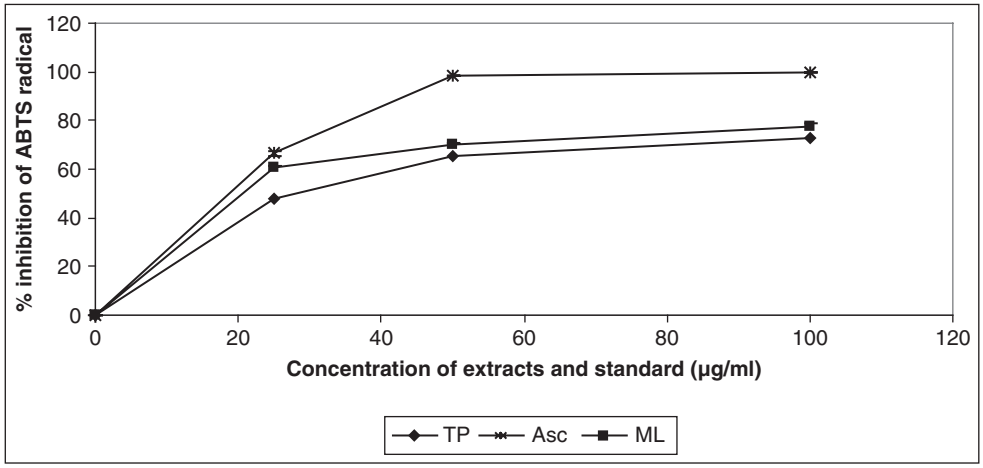

Figure 2. ABTS radical scavenging activity of methanol extracts of TP and ML.

In the ABTS test, TP and ML had effective scavenging activity. The effects of various concentrations of ML, TP and standard (ASC) on the ABTS were found to be 98.41, 60.94 and 47.71%, respectively, at 25 µg/mL concentration (Fig 2). There was a significant decrease ($p<0.01$) in the concentration of ABTS due to the scavenging capacity of TP and ML with ASC standard at 25 µg/mL concentration. The IC_{50} value of TP and ML were found to be 64.32 and 53.82 µg/mL. In this test also, ML exhibited better antioxidant property than TP.

Fig. 3 depicts the scavenging activity of the TP, ML and ASC on the NO radical. The activity increased with increasing concentration of samples. As evident from Fig. 3, TP and ML demonstrated significant NO scavenging activities, when compared to the control. These differences were statistically significant ($p<0.05$) at 60 µg/mL concentration. Reducing power of TP, ML and standard compound exhibited the following order: ASC > TP >ML, which were 31.45, 20.63 and 15.21%, at 60 µg/mL, respectively. The IC_{50} valuees of TP and ML were found to be 140.26 and 197.30 µg/mL, respectively. The present results suggest that TP might be potent agent for scavenging of NO and the regulation of pathological conditions caused by excessive generation of NO and its oxidation product, peroxynitrite.

The ability of TP and ML to scavenge hydrogen peroxide radical is shown in Fig. 4. ML and TP exhibited 9.57 and 9.12% scavenging effect on H_2O_2 radical, at 25 µg/mL concentration. On the other hand, the standards, BHT, ASC and BHA exhibited 17.78, 16.22 and 7.46% hydrogen peroxide scavenging activity at the same concentration. These results suggest that both ML and TP have an effective hydrogen peroxide scavenging activity. At the above concentration, the hydrogen peroxide scavenging effect of ML, TP and standards decreased in the order of BHT > ASC > ML > TP > BHA. The IC_{50} values of TP and ML were found to be 208.74 and 214.09 µg/mL respectively. There was a significant decrease in the concentration of H_2O_2 ($p<0.05$) with TP and ML, when compared to the standards. The

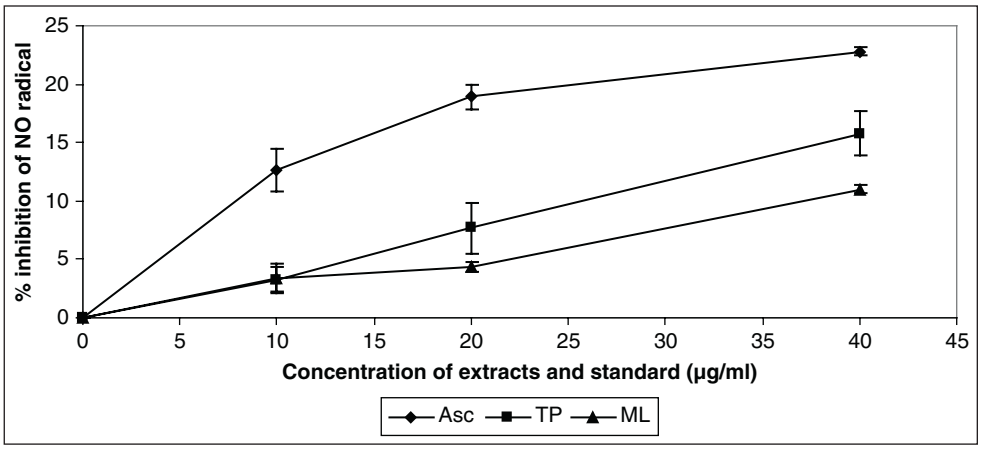

Figure 3. Nitric oxide scavenging activity of methanol extracts of TP and ML.

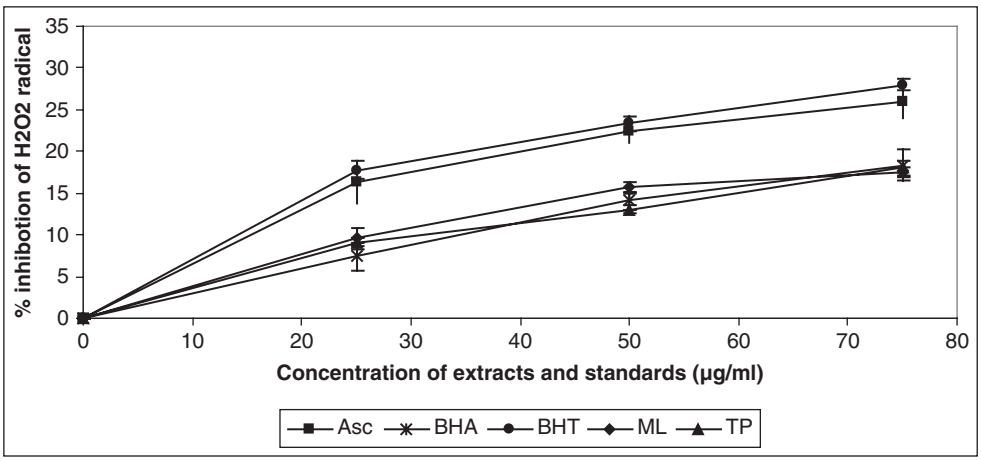

Figure 4. H_2O_2 scavenging effect of methanol extracts of TP and ML.

results suggest that ML can be a better antioxidant than TP for removing H_2O_2.

Fig. 5 shows the hydroxyl radical scavenging activities of TP and ML. Both TP and ML exhibited concentration dependent scavenging activities against hydroxyl radicals. The IC_{50} of TP and ML were 44.95 and 46.83 µg/mL, respectively. TP and ML showed significant decrease (p<0.001) in the concentration of OH, when compared with ASC at 20-40 µg/mL concentrations. In addition, the scavenging effect of TP, ML and standard on the OH decreased in the order: ASC > TP > ML, which were 74.07, 34.79 and 29.05%, at the 20 µg/mL concentration, respectively. TP exhibited higher OH• scavenging effect (34.79% inhibition) than ML.

The total phenolic content of TP and ML in terms of gallic acid equivalent was found to be 4.14 and 2.15 mg/g, respectively. The *in vitro* antioxidant activities of TP and ML might be due to the high phenolic content of both the plants. Apart from phenolic content, the methanol extract of TP bark also exhibited positive tests for carbohydrates and glycosides (positive test with Molisch's, Fehling's, Barfoed's and Benedict's reagent), coumarins and tannins, which can also be responsible for the antioxidant activity.[21]

DISCUSSION

Free radicals, oxidative stress and damage are implicated in many diseases such as ageing, stroke, asthma, cancer, atherosclerosis, diabetes and arthritis. Antioxidants can be given as food supplements to regulate such conditions.[1-6] Due to adverse effects associated with synthetic antioxidants, there is a quest for the search of a potent natural antioxidant.[7-9] Natural antioxidants can be classified as primary (chain-breaking) antioxidants, which can react directly with lipid radicals and convert them into stable products, or as secondary (preventive) antioxidants, which can lower the rate of oxidation by

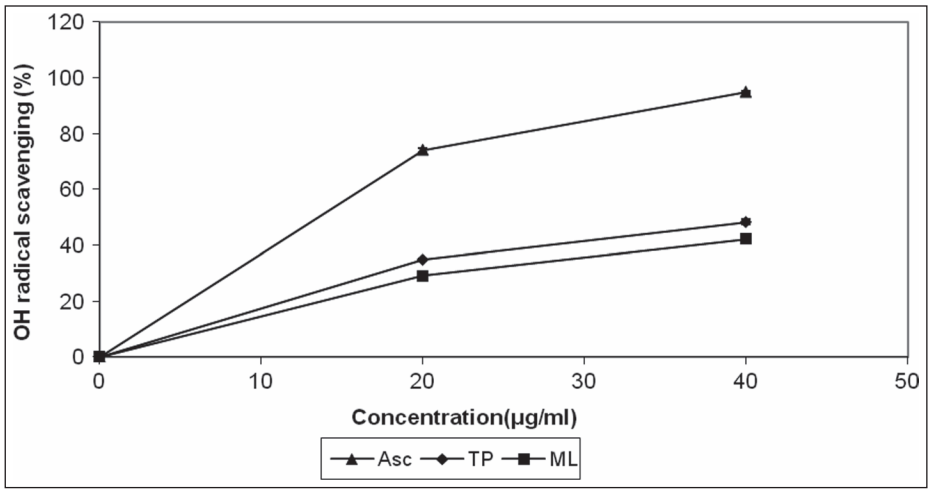

Figure 5. Hydroxyl radical scavenging effect of methanol extracts of TP and ML.

different mechanisms.[21] Primary antioxidants, most often, act by donating a hydrogen atom, while secondary antioxidants may act by binding metal ions which can catalyze the oxidation process, by scavenging oxygen, by absorbing UV radiation, by inhibiting enzymes or by decomposing hydroperoxides.[22] It is known that different natural phenolic compounds function as both primary and secondary antioxidants by different mechanisms.

In the present study, two traditionally used medicinal plants, *Terminalia paniculata* and *Madhuca longifolia*, were evaluated for their antioxidant potential to support the traditional claims of their medicinal uses. The plants were tested for antioxidant property using different *in vitro* methods.

DPPH is relatively stable nitrogen centered free radical that easily accepts an electron or hydrogen radical to become a stable diamagnetic molecule. DPPH radicals react with suitable reducing agent as a result of which, the electrons become paired off forming the corresponding hydrazine. ABTS radical scavenging radical activity is basically used to measure total antioxidant activity of the extracts. This activity shows that total antioxidant activity of ML was more than TP.

NO radicals play an important role in inducing inflammatory response and their toxicity multiplies only when they react with $O_2^{\bullet-}$ radicals to form peroxynitrite, which damages biomolecules like proteins, lipids and nucleic acids. Nitric oxide is generated when sodium nitroprusside reacts with oxygen to form nitrite plants inhibit nitrite formation by competing with oxygen to react with nitric oxide directly. The present study suggests that TP might be potent therapeutic agent for scavenging of NO and the regulation of pathological conditions caused by excessive generation of NO and its oxidation product, peroxynitrite.

Hydrogen peroxide is a weak oxidizing agent and can inactivate a few enzymes directly, usually by oxidation of essential thiol (-SH) groups. H_2O_2 can cross cell membranes rapidly and once inside the cell it can probably react with Fe^{2+} and possibly Cu^{2+} to form hydroxyl radicals and this may be the origin of many of its toxic effects. It is therefore advantageous for cells to control the amount of hydrogen peroxide that is allowed to accumulate. The results of present study showed that ML had strongest anti- H_2O_2 activity when compared with the standard BHA. The result suggests that ML can be a better antioxidant than TP for removing H_2O_2 and thus protecting food systems.

OH• has a short half-life and is the most reactive and damaging ROS. It causes oxidative damage to DNA, lipids and proteins. OH• is known to be capable of abstracting hydrogen atoms from membranes and they bring about peroxidic reactions of lipids. Since TP exhibited higher scavenging activity in this test, it can be anticipated that TP would show effect against lipid peroxidation on biomembranes.

The presence of phenolics, tannins and coumarins in both the selected plant extracts explains their potential free radical scavenging activity. These phytochemicals are active hydrogen donors, due to which they exhibit antioxidant potential.

CONCLUSION

From the above studies, it can be suggested that both the selected plants could be used as easily available sources

of natural antioxidants, which can be used as dietary supplements for reducing the oxidative burden in degenerative diseases such as cancer, inflammation, diabetes etc., which develop due to excess oxidative stress in the body. Further work is needed for establishing the potential of these plants in animal models and also to determine the possible adverse effects of these plants. Also, isolation and identification of antioxidant components and study on their individual antioxidant potential is needed, which could ultimately lead to the inclusion of these compounds in different antioxidant formulations.

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