

Antioxidant activity of methanolic leaf extract of *Moringa peregrina* (Forssk.) Fiori.

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Abstract

Natural antioxidants have an important role in the prevention of many age-related diseases and promotion of health. Among natural antioxidants from plants, flavonoids and other phenolic compounds are potent antioxidants and chelating agents. *Moringa peregrina* (Forssk.) Fiori (Moringaceae) is a small desert tree distributed from tropical Africa to east India. *Moringa* tree is also growing in south-east of Iran. The antioxidant activity of *M. peregrina* methanolic leaf extract on 2,2-diphenyl-1-picrylhydrazyl and superoxide anion radicals was determined in *in vitro* experiments. It exhibited the scavenging activity on DPPH and superoxide anion radicals with IC₅₀ of 8.06 ± 0.29 µg/ml and 47.93 ± 1.33 µg/mL, respectively. Moreover, total phenolic content of the leaf extract was determined and using ¹HNMR, mass and spectroscopic methods, the structure of the isolated flavonoid glycoside, rutin, as one of the compounds responsible for reported antioxidant activities was identified.

Keywords: Antioxidant activity; *Moringa peregrine*; Rutin; DPPH assay; Superoxide anion radical

INTRODUCTION

Antioxidants provide protection against degenerative diseases including cancer, coronary heart, and Alzheimer's diseases (1). Reactive Oxygen Species (ROS), contribute to cellular aging, mutagenesis, carcinogenesis, and coronary heart disease, likely through destabilization of membranes, DNA and protein damage, and oxidation of low-density lipoprotein (LDL) (2). Mechanism of action of antioxidants includes the suppression of ROS formation, the inhibition of enzymes or chelating of elements involved in free-radical production. Furthermore, antioxidants scavenge reactive species, and upregulate antioxidant defences (3).

Plants are rich sources of natural antioxidants, the best known are tocopherols, carotenoids, vitamin C, flavonoids, and different other phenolic compounds (1). Recently, among natural antioxidants,

flavonoids have received increasing attention. As compared with vitamin C and E, dietary flavonoids are considered to be more powerful antioxidants (4).

Flavonoids are known to be highly effective antioxidants by scavenging oxygen radicals, by having interesting anti-cancer, hypolipidemic, anti-ageing, and anti-inflammatory activities (5). Moreover, the protective effects of flavonoids in biological systems are attributed to their capacity to scavenge free radicals, chelate metal catalysts, activate antioxidant enzymes, reduce alpha-tocopherol radicals, and inhibit oxidases (2). Furthermore, phenolic compounds have phenolic hydroxyl groups which can dissociate to negatively charged phenolates. Dissociated phenolics can form hydrogen and ionic bonds with various proteins, which lead to a disturbance of their 3D-structures and in consequence to a change in their bioactivity (6).

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Moringa peregrina (Forssk.) Fiori. (Moringaceae) occurs in the Middle East to India. In Iran it is a desert tree growing in Sistan and Baluchestan province and is locally called Gas-e-rowghan or Gaz Rokh (7,8).

Several reports on antioxidant activity of leaves and seeds of another *Moringa* species exist (9,11). Leaves of *M. oleifera* contain flavonoid pigments such as kaempferol, rhamnetin, isoquercitrin, and kaempferitrin (1).

To the best of our knowledge no effort has been made to evaluate *in vitro* antioxidant activity of leaves of *M. peregrina*. Therefore, in the present study, the antioxidant activity of the leaf extract of *M. peregrina* was evaluated. In addition, total phenolics determination as well as isolation and identification of the flavonoid rutin as the main active antioxidant were carried out.

MATERIALS AND METHODS

General methods

UV spectra were measured on Biowave II UV spectrophotometer. ¹HNMR (400 MHz) was recorded on Bruker Avance spectrometer (spectra was taken in CD₃OD, using TMS as internal standard). MALDI-TOF and ESI-MS spectra were obtained on Bruker Biflex and Shimadzu LC-MS respectively. The MPLC was carried out using Butchi pump on microcrystalline cellulose (Merck, 20-100µm). HPTLC analysis carried out on silicagel G F₂₅₄, (20 × 20 cm, layer thickness 0.25 mm; Merck) detection by spraying with natural product reagent. The HPLC equipment used included Waters 515 HPLC pump and Waters 2996 PDA detector.

Chemicals

All solvents used for the preparation of crude samples and separation process, diphenylboric acid 2-amino ethyl ester (natural product), rutine, and nitroblue tetrazolium (NBT) were purchased from Merck (Darmstadt, Germany). 2,2-diphenyl-1-1-ptycrylhydrazyl (DPPH), (-)-Epigallocatechin gallate (EGCG) 95%, Folin-Ciocalteu reagent, nicotinamide adenine dinucleotide

(NADH), phenazine methosulfate (PMS) were obtained from Sigma-Aldrich GmbH.

Plant material

Leaves of *M. peregrina* were supplied in 2008 by the Department of Natural Resources of Chabahar in Sistan and Baluchestan province of Iran. Voucher specimens of the seeds, leaves and stems (No. 2025) were deposited in herbarium of the Pharmacognosy Department of the school of Pharmacy and Pharmaceutical Sciences of Isfahan University of Medical Sciences (Iran).

Sample preparation

About 100 g of air-dried leaves of *M. peregrina* were powdered and defatted with n-hexane and then extracted with methanol: water (80:20) at room temperature by maceration with occasional stirring. After filtration the solvent was evaporated under reduced pressure and the dry extract stored under refrigerator until used for further analysis (9). In both antioxidant activity assays, different concentrations of leaf extract were used to find out the concentrations which could include 50% inhibition to calculate IC₅₀.

2,2-Diphenyl-1-ptycrylhydrazyl free radical scavenging activity

The DPPH radical scavenging assay is a standard procedure to determine the antioxidant activity of plant extracts and natural compounds. The free radical scavenging activity of methanolic leaf extract was measured using the method described by Blois (12) with some modifications. 500 µl of methanolic DPPH solution (0.2 mM) was added to 500 µl of leaf extract at different concentrations (5-40 µg/ml) or to 500 µl of methanol as the control. After 30 min, the absorbance was measured at 517 nm (13). EGCG with different concentrations (1, 2, 3 and 4 µg/ml) was used as positive control. The radical scavenging activity of DPPH was calculated using the following equation:

$$\text{DPPH scavenging effect (\%)} = [(A_0 - A_1) / A_0] \times 100$$

where, A₀ is the absorbance of the control reaction and A₁ is the absorbance in the presence of methanolic leaf extract or EGCG.

Superoxid anion radical scavenging activity

Superoxide anion scavenging ability of the leaf extract was determined by the modified method of Robak and Gryglewski (14). Different concentrations of methanolic extract (25-200 µg/ml) or 150 µl of water (as the control) were added to a solution mixture that contained 150 µl of NADH (156 µM) and 150 µl of NBT (630 µM) in 400 µl of 0.1 M phosphate buffer pH 7.4. The reference solution contained 150 µl of NBT (630 µM) in 700 µl of 0.1 M phosphate buffer pH 7.4. The reaction was started after addition of 150 µl PMS (30 µM) to all mixtures. Five min later, the absorption was measured at 560 nm. EGCG with different concentrations (1, 5, 10, 15 and 20 µg/ml) was used as positive control.

In this system, superoxide anions, derived from dissolved oxygen by PMS/NADH coupling reaction, reduce NBT. Antioxidants have the ability of inhibiting the blue NBT formation which results in the decrease of the absorbance at 560 nm.

The inhibition of superoxide anion generation was calculated as percentage using the following formula:

$$\text{Inhibition (\%)} = [(A_0 - A_1) / A_0] \times 100$$

where, A_0 is the absorbance of the control reaction and A_1 is the absorbance in the presence of methanolic leaf extract or EGCG.

Determination of total phenolics

Antioxidant compounds generally contain phenolic groups and hence, the amounts of phenolic compounds in the methanolic extract of *M. peregrina* leaves was determined by Folin-Ciocalteu reagent assay (15,16). To 1 ml of the extract in methanol, 5.0 ml of Folin-Ciocalteu reagent and 4.0 ml of sodium carbonate were added. After shaking, it was kept for 2 h in darkness and the absorbance was measured at 750 nm. Using gallic acid monohydrate, a standard curve was prepared. The total phenolic content was calculated and expressed as gallic acid equivalent in mg/g of the extract.

Primary antioxidant test on TLC

Methanolic extract of leaves was primarily developed on TLC plates with solvent system ethylacetate: formic acid: acetic acid: H₂O

(100:11:11:26). After developing, the plates were observed under UV at 366 nm with and without exposure to ammonia and then sprayed with DPPH reagent (0.2% in MeOH). The DPPH test is performed as a rapid screening method anti radical activity determination on TLC plates. Further isolation and purification methods for identification of the bold spot found on TLC were designed.

Isolation and identification of antioxidant

The dried methanolic extract residue (10 g) was chromatographed on microcrystalline cellulose MPLC on 46 × 3.6 cm column, Eluted with chloroform/isobutanol (100:0 → 0:100) followed by isobutanol/ water (5% acetic acid) mixture with increasing polarity (flow rate 25 ml/ min) to give 32 fractions (Fr. 1-32). Fr. 25a-27a (0.45 g) was subjected to Sephadex LH-20, eluted with methanol to obtain yellow needle crystals (9.31 mg) as pure compound. The structure of the isolated compound was determined by comparison of its UV, NMR and MS data with the literature and co-TLC with reference compound (17,18). More confirmation was obtained using the analytical HPLC system. The samples were eluted isocratically using a mobile phase composed of methanol: water (1:1) (pH=2.8 by phosphoric acid) on a Waters µ-Bondapak C₁₈ column (3.9 × 150 mm, particle size 10 µm).

Sugar analysis of isolated compound

To achieve acidic hydrolysis of the isolated compound, 1 ml of 6% HCl was added to 0.5 mg of the sample, and maintained on steam bath for 45 min. Then cooled for TLC analysis which performed on cellulose with n-Butanol: acetic acid: water (4:1:5) and ethylacetate: pyridine: water (12:5:4) solvent systems (17,19) beside standard sugars. The developed chromatograms were sprayed by aniline hydrogen phthalate reagent to detect hydrolyzed sugars.

Statistical analysis

Statistical comparisons were made with two-tailed unpaired student's *t test* assuming equal variance. The IC₅₀s were reported as means ± standard deviation of mean.

RESULTS

DPPH assay

M. peregrina leaf extract reduced DPPH radicals significantly as compared to the control ($P<0.05$). The leaf extract showed IC_{50} value of $8.06 \pm 0.29 \mu\text{g/ml}$ (Fig. 1) compared with polyphenol, EGCG from green tea, which was used as the positive control with IC_{50} value of $1.54 \pm 0.19 \mu\text{g/ml}$ (Fig 2). The extract was significantly ($P<0.05$) less active than

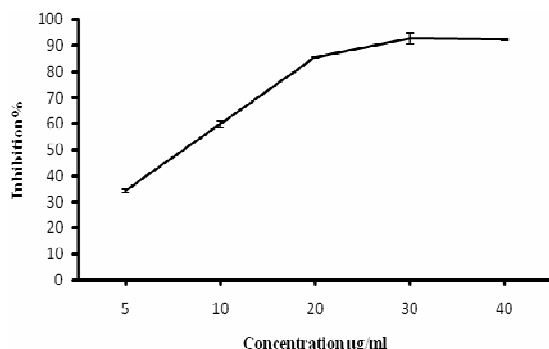


Fig. 1. Anti radical activity of *M. peregrina* leaf extract determined by DPPH assay. Values are mean \pm standard error of triplicate determinations.

EGCG. There were significant differences ($P<0.05$) between DPPH scavenging activity of different concentrations of leaf extract except 30 and 40 $\mu\text{g/ml}$ (Table 1).

Superoxide anion assay

The superoxide anion radical scavenging activity of the leaf extract was assayed using PMS-NADH system. Significant differences were observed between antioxidant activity of various concentrations of leaf extract except

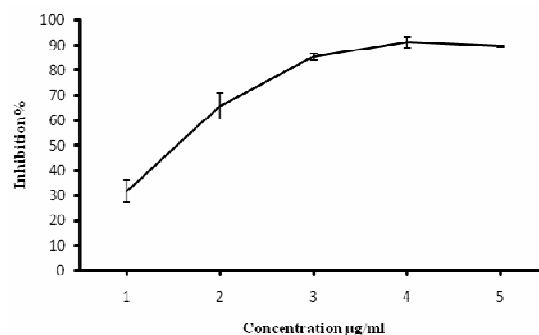


Fig. 2. Anti radical activity of epigallocatechin gallate (EGCG) determined by DPPH assay as positive control. Values are mean \pm standard error of triplicate determinations.

Table 1. Antioxidant activity of *M. peregrina* leaf extract with different concentrations against DPPH and superoxide anion radicals

DPPH assay concentration ($\mu\text{g/ml}$)	Inhibition (%)	Superoxide anion assay concentration ($\mu\text{g/ml}$)	Inhibition (%)
5	^a 34.22 \pm 0.81a	25	21.38 \pm 1.99a
10	59.77 \pm 1.24b	50	52.57 \pm 1.28b
20	85.27 \pm 0.45c	100	69.34 \pm 1.82c
30	92.73 \pm 1.95d	150	80.17 \pm 2.30d
40	92.43 \pm 0.33d	200	86.28 \pm 0.37d

^aValues are mean \pm standard error of triplicate analysis. Different letters within columns show significant differences ($P<0.05$).

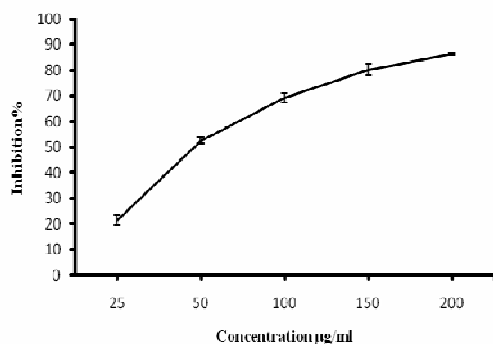


Fig. 3. Scavenging activity of *M. peregrina* Leaf extract on superoxide radicals. Values are mean \pm standard error of triplicate determinations.

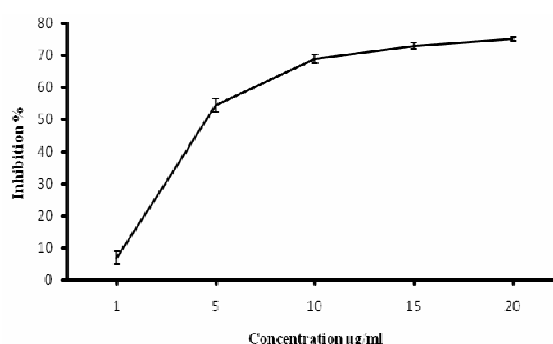


Fig. 4. Scavenging activity of EGCG as positive control on superoxide anion radicals. Values are mean \pm standard error of triplicate determinations.

between 150 and 200 $\mu\text{g/ml}$ (Table 1). The IC_{50} value of leaf extract was 47.93 ± 1.33 $\mu\text{g/ml}$ (Fig. 3) while EGCG as positive control was significantly more active ($P < 0.05$) with an IC_{50} of 4.64 ± 0.28 $\mu\text{g/ml}$ (Fig. 4).

Total phenolic content

The TPC was determined following a modified Follin-Ciocalteu method and result

was expressed as gallic acid equivalents. The total phenolic content (mg/g of extract) in sample determined based on calibration curve ($y = 0.0108x + 0.0056$, $R^2 = 0.9978$) was 88.05 ± 1.08 mg/g.

Identification of the isolated compound

Isolated compound was obtained as pale yellow solid with positive reaction to natural

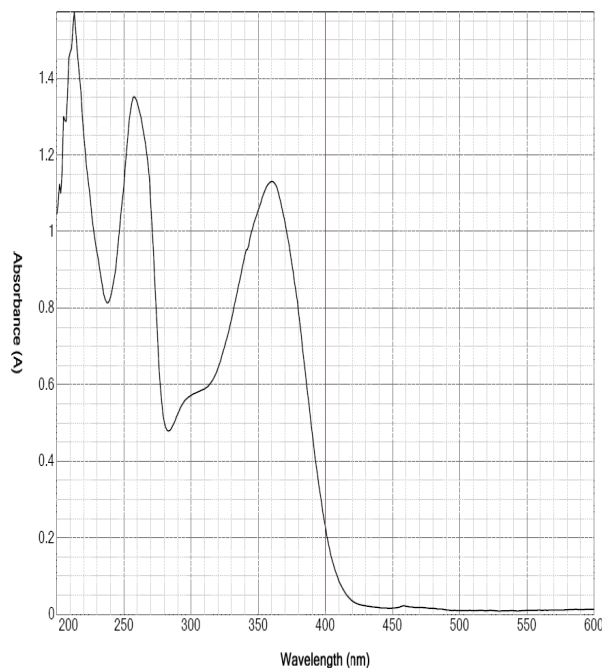


Fig. 5. UV spectrum (in CH_3OH) of isolated compound from *M. peregrina* leaf extract.

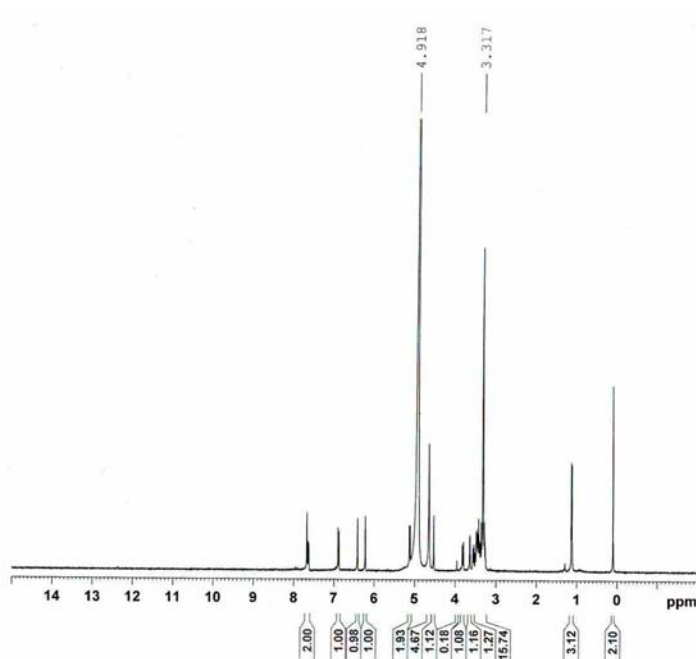


Fig. 6. ^1H NMR spectrum of isolated flavonoid (rutin) from leaves of *M. peregrina*

product reagent and to the yellow spot in purple background after spraying with DPPH reagent. The UV spectrum showed λ_{\max} (in MeOH) nm: 257, 267sh, 298sh, 360, characteristic of flavonol derivatives (Fig. 5). The ^1H NMR spectrum of the isolated compound in CD_3OD (Fig. 6) exhibited two sets of *ortho*- and *meta*- coupling aromatic protons at δ_{H} , 7.67 (1H, d, $J=2$ Hz, H-2'), 7.64 (1H, dd, $J=8.0, 2.0$ Hz, H-6') and 6.92 (1H, d, $J=8.0$ Hz, H-5') as well as 6.23 (1H, d, $J=2.0$ Hz, H-6) and 6.42 (1H, d, $J=2.0$ Hz, H-8). The ^1H NMR spectrum also supported the presence of two sugar moieties with the

anomeric proton signals at δ_{H} 5.12 (1H, d, $J=7.5$ Hz, Glc-H-1'') and δ_{H} 4.59 (1H, s, Ram-H-1''') related to glucose and rhamnose respectively which was confirmed more through acid hydrolysis and co-TLC with standard sugars. Therefore, based on UV λ_{\max} (in MeOH) nm: 257, 267sh, 298sh, 360, positive ESI-MS at m/z 633 $[\text{M}+\text{Na}]^+$ and negative ESI-MS at m/z 609.2 $[\text{M}-\text{H}]^-$ (Fig. 7), comparison with the reported data (16) and co-TLC with authenticated sample the isolated compound identified as rutin (quercetin 3-O- α -L-rhamnopyranosyl (1 \rightarrow 6)- β -D-glucopyranoside).

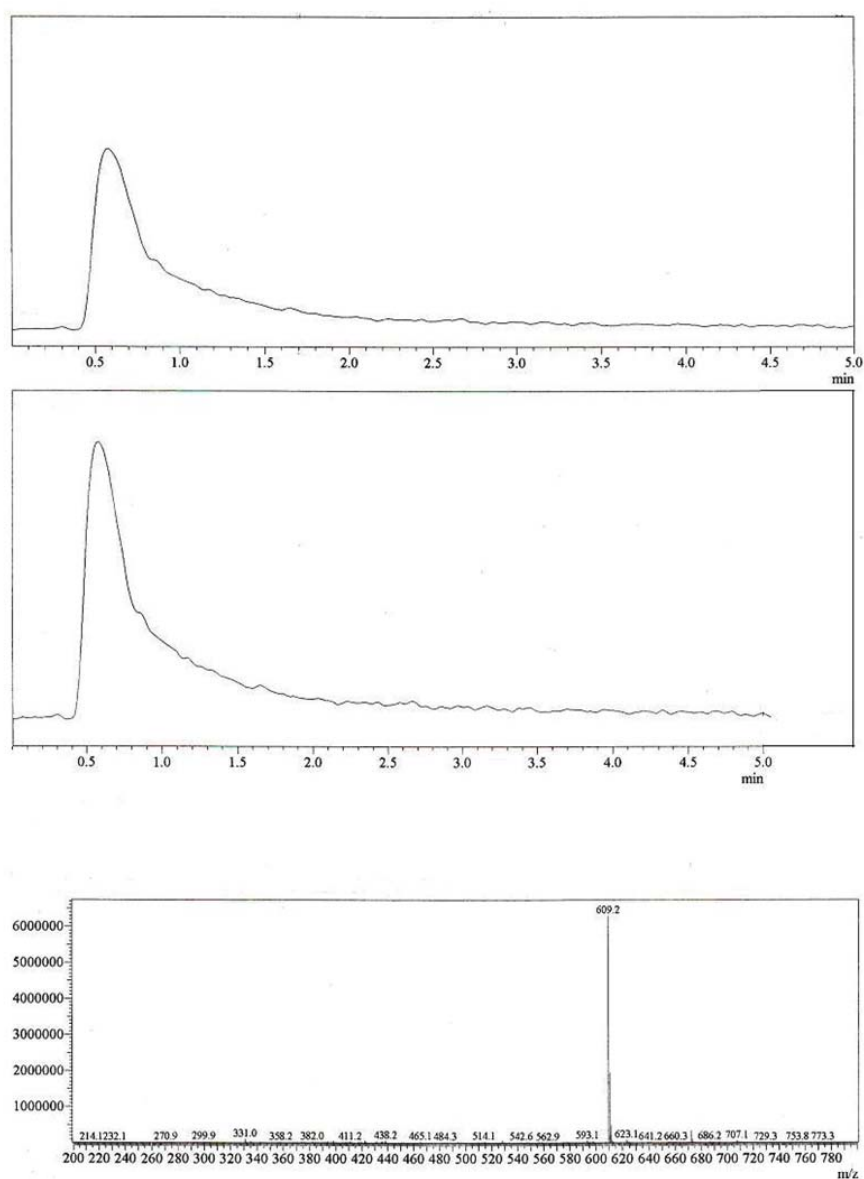


Fig. 7. LC/MS (ESI-) chromatogram of isolated compound from leaf extract of *M.peregrina*.

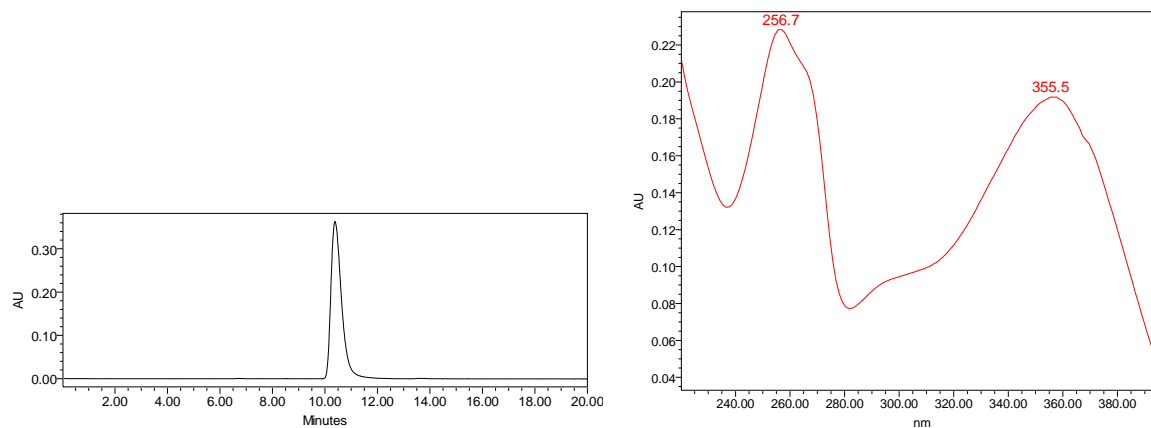


Fig. 8. HPLC chromatogram and UV spectrum of standard rutin.

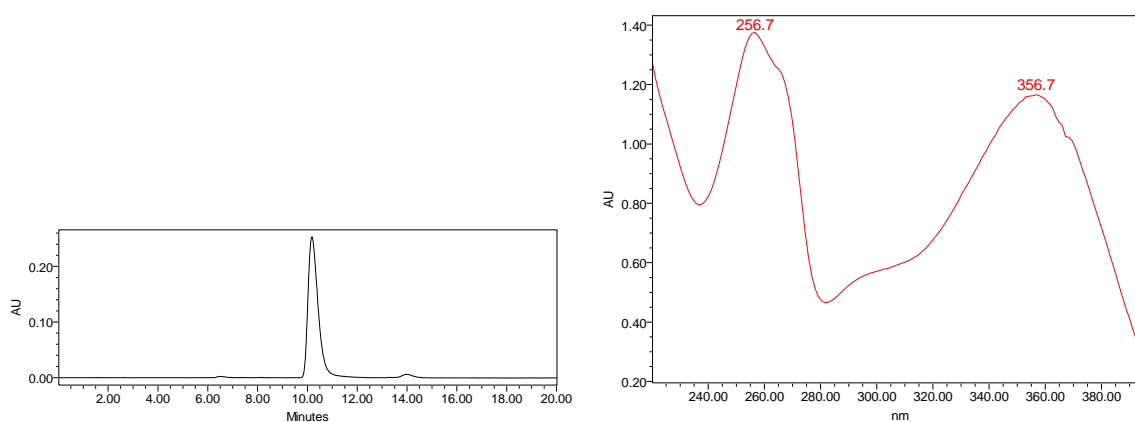


Fig. 9. HPLC chromatogram and UV spectrum of isolated compound from *M. peregrina* leaf extract.

Moreover, comparison of the retention times, UV spectrum (200–400 nm) and absorption maxima (λ_{\max}) of rutin with the isolated compound by HPLC suggested the presence of rutin (Fig. 8 and 9).

DISCUSSION

Antioxidant activity is evaluated by different methods but the most widely used methods are those that generate free radical species which are then neutralized by antioxidant compounds.

EGCG had stronger dose-dependent radical scavenging activity against DPPH and superoxide anion radicals which agrees with an earlier study (13). However, leaf extract as well as EGCG revealed dose-dependent anti radical activity.

The phenolic compounds may contribute directly to antioxidative effect. The presence

of 3-OH group as well as hydroxyl groups in ring B is related to the superoxide scavenging activity of flavonoids. Epigallocatechin with a hydroxyl group at C-3 and three hydroxyl groups in ring B shows high superoxide scavenging activity (20). It is also demonstrated that the principal site of antioxidant reactions in EGCG and epigallocatechin (EGC) was the trihydroxyphenyl B-ring, regardless of the presence of a 3-galloyl moiety (21). Therefore, the observed *in vitro* antioxidant activity of the leaf extract of *M. peregrina* may be attributed to its phenolic content (1) and also related to flavonoid rutin isolated from this plant which are in agreement with several reports on flavonoids in moringaceae family including quercetin 3-O-rhamnoglucoside and quercetin 3-O-glucoside in *M. stenopetala* and 3'-methoxylated quercetin, vitexin, quercetin 3-O-glucoside, quercetin 3-O-6''-malonyl-

glucoside and lower amounts of kaempferol 3-O-glucoside, and kaempferol 3-O-(6"-malonyl-glucoside) in *M. oleifera* leaves and myricetin in the seeds of *M. oleifera* which showed antioxidant activity (10,24,25).

Spraying the TLC with DPPH is a suitable method for detection of antioxidants in crude plant extracts or pure compounds isolated from plant material. This method was also used for screening the antioxidant activity of flavonoids from the leaves of *Licania licaniaeflora* (5).

CONCLUSION

Our study demonstrates that methanolic extract of *M. peregrina* leaves possesses substantial antioxidant activities. The antioxidant potential of *M. peregrina* leaves extract may be attributed to its phenolic content as well as the presence of the flavonoids, rutin, which identified in this work. Thus, the free radical scavenging ability of *M. peregrina* could provide health benefits to humans by protection against oxidative stress.

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