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Research Article

ACTIVITY GUIDED ISOLATION OF ANTIOXIDANT COMPOUND FROM LEAVES OF *MANGIFERA INDICA*

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ABSTRACT

After reviewing the phyto-chemical composition of *Mangifera indica* leaf, activity guided fractionation was performed and the compound responsible for the antioxidant activity was isolated and characterized. An aqueous extract showed highest DPPH scavenging activity (91.48 ± 2.67 %). Active aqueous extract was fractionated using two different schemes. The methanolic fraction of aqueous extract showed highest DPPH scavenging activity (91.83 ± 2.02 %). Sub-fractionation of active methanolic fraction yielded two compounds designated as MG-1 and MG-2. MG-1 and MG-2 showed 5.31 ± 0.35 and 50.21 ± 3.11 % DPPH scavenging activity, respectively. The UV, IR, ^{1}H NMR, ESI-MS and HPLC analysis further confirmed MG-1 and MG-2 as isomangiferin and mangiferin, respectively.

Keywords: Antioxidant, *Mangifera indica*, Activity guided fractionation, DPPH, Mangiferin.

INTRODUCTION

There are increasing suggestions that reactive oxygen species (ROS) may play a role in the pathogenesis of cancer and other diseases including inflammation, bacterial infections and AIDS. ROS possess a strong oxidizing effect and induce damage to biological molecules, including proteins, lipids and DNA, with concomitant changes in their structure and function[1]. The epidemiological studies indicate that the major exogenous nutritional antioxidants, vitamin E, vitamin C and β-carotene, may be beneficial to prevent several chronic disorders[2]. Considerable interest has been raised in the possible reinforcement of antioxidant defenses, both for chemoprevention and treatment purposes.

Mangifera indica (L.) belonging to family Anacardiaceae is one of the most important tropical plants marketed in the world. *M. indica* is purported to posses numerous therapeutic uses viz. analgesic, anti-inflammatory, antioxidant and antidiabetic[3-5]. Phytochemical research of different parts of *M. indica* has demonstrated the presence of phenolic constituents, flavonoids and phytosterols[6-8]. There are several reports demonstrating the antioxidant activity of various parts of *M. indica viz.* bark and peels[7, 9].

After reviewing the phyto-chemical composition and prospective antioxidant potential, *M. indica* leaves were selected for the study. The objectives of the present study were to isolate and characterize the naturally occurring antioxidant compound(s) from *M. indica* leaves using a systematic approach of activity guided fractionation.

METHODS AND MATERIALS

General

UV spectra were recorded on a Jasco V-530 UV/VIS spectrophotometer (Jasco International co. ltd., Tokyo, Japan). ^{1}H NMR spectra was recorded on Varian Mercury YH300 spectrometer (Varian, CA, USA**)** using DMSO-d_6 as solvent. FT-IR data was collected using Bruker ALPHA C FT-IR (Bruker Optik GmbH, Baden-Württemberg, Germany). Electro-spray ionization mass spectra (ESI-MS) were obtained with a Micromass Quattro micro API LC-MS/MS instrument (Waters, MA, USA). HPLC analysis was performed on Jasco HPLC instrument (Jasco International co. ltd., Tokyo, Japan), using a HiQ Sil C_{18} column (Kya Tech., Tokyo, Japan) (250 x 4.6 mm i.d., 5 µm particle size). Silica gel 60 F_{254} TLC plates (Merck, Bundesland, Germany) were used for TLC analysis. 1,1-Diphenyl-2-picrylhydrazyl radical (DPPH) was purchased from Himedia (MH, India). L-Ascorbic acid was purchased from Finar chemicals ltd. (GJ, India). Mangiferin was purchased from Sigma-Aldrich (MO, USA). All solvents used for chromatography were of HPLC grade and obtained from Fisher Scientific (NJ, USA). All other reagents and chemicals used were of analytical grade.

Plant material and extraction

In this study, the authentic young age leaves of *M. indica* were collected from botanical garden of Dr. Babasaheb Ambedkar Marathwada University, Aurangabad, Maharashtra, India during January 2010. A voucher specimen was deposited in the herbarium of Botany department, Dr. Babasaheb Ambedkar Marathwada University, Aurangabad under number 0791. Fresh leaves of *M. indica* (500 g) were ground to a coarse paste using twin blade mixer (Bajaj electrical ltd., MH, India). Initially an aqueous (aq.), aq.-methanolic (50 % v/v) and methanolic extracts were prepared and designated as MI-1, MI-2 and MI-3, respectively (Fig. 1).

Spectrophotometric DPPH scavenging capacity assay

The free radical scavenging capacity of *M. indica* extracts/fractions/compound(s) were determined using DPPH as described earlier with slight modifications[10,11].

The DPPH (5 mg) was weighed accurately using a pre-calibrated weighing balance (Model: CPA225D, Sartorius, Lower Saxony, Germany) and transferred to a 5 mL volumetric flask (Riviera glass pvt. Ltd., MH, India), dissolved (by sonication) and diluted with methanol to achieve 1 mg mL^{-1} strength (stock I). The stock I was diluted with methanol to obtain 0.3 mM DPPH solution. The control reaction mixture consisted of 100 µL of 0.3 mM DPPH solution and 3.9 ml of methanol whereas test reaction mixture consisted of additional 100 µL of 1 mg mL^{-1} extracts/fractions/compound(s) of *M. indica.* After an incubation period of 30 min, reduction of a DPPH free radical was measured by recording the absorbance (abs) of test and control reaction mixtures at 517 nm. Percent scavenging of the DPPH free radical was measured by using the following equation: % Scavenging activity = [(abs of control - abs of test)/ abs of control] x 100Eq. 1

The ascorbic acid was used as a reference standard in DPPH scavenging capacity assay. The antiradical activity IC_{50}, defined as the concentration of sample showing 50 % DPPH radical scavenging activity was determined for ascorbic acid and isolated compound (1-100 µM) from *M. indica*. The IC_{50} values were estimated from a graph of concentration vs. % scavenging activity.

Antioxidant activity guided fractionation

The MI-1, MI-2 and MI-3 extracts of *M. indica* were tested for their antioxidant potential using DPPH and the active antioxidant extract (MI-1) was further fractionated using two different schemes (Fig. 1). All the fractions of MI-1 *viz.* MI-1(1), MI-1(2), MI-1(3), MI-1(4) and MI-1(5) were dissolved in methanol, and tested for their antioxidant potential using DPPH scavenging capacity assay.

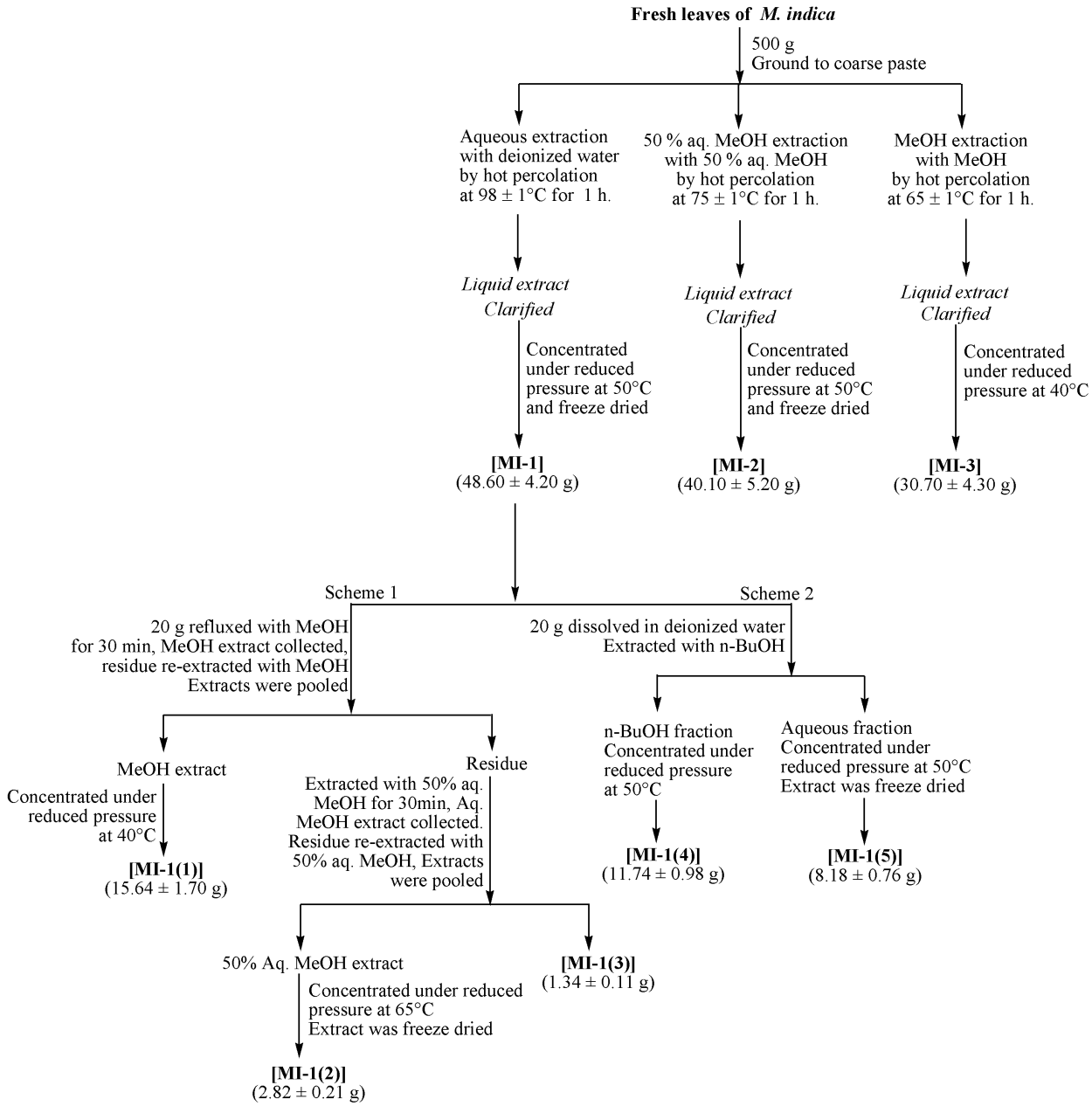

Fig. 1: Flow diagram showing preparation of various extracts of *M. indica* leaves *viz.* aq. extract (MI-1), 50 % aq.-methanolic extract (MI-2), methanolic extract (MI-3) and scheme 1 and 2 showing activity guided fractionation of aq. extract; MI-1.

Isolation of antioxidant compound from MI-1(1)

Activity guided fractionation of active fraction of *M. indica* was continued in search of compound(s) responsible for the said activity. MI-1(1) was dissolved in a minimum quantity of ethanol and adsorbed on silica gel (60–120 mesh). The adsorbed free flowing silica gel was charged onto a glass column (75 x 6 cm i.d.) pre-packed with silica gel (60–120 mesh). The column was initially eluted with dichloromethane and then with dichloromethane methanol mixture (99:1, 96:4, 94:6, 92:8, 88:12, 85:15 and 82:18 v/v). In all 85 sub-fractions (20 ml each) were collected. TLC was run in *n*-butanol: acetic acid: water (4:1:5 v/v) and patterns were visualized under UV light at 254 nm. Sub-fractions 31-40 and 41-50 showed identical TLC patterns with a single spot in solvent system *n*-butanol: acetic acid: water (4:1:5 v/v). Sub-fractions with identical Rf values were pooled and concentrated on a rotavapour under reduced pressure at 40 ± 1 °C. Crystallization was carried out in ethanol water mixture (4:1 v/v) and light yellow crystalline powders collected. These were designated as MG-1 [yield: 2.95 ± 0.19 g/20 g MI-1(1)] and MG-2 [yield: 9.69 ± 0.64 g/20 g MI-1(1)]. The MG-1 and MG-2 were tested for antioxidant activity using DPPH as described in **section Spectrophotometric DPPH scavenging capacity assay**. The MG-2 was characterized using UV, FT-IR, ^{1}H NMR, ESI-MS and HPLC techniques.

HPLC analysis

Reverse phase HPLC analysis of *M. indica* extracts/fractions/sub-fractions/compound(s) was performed as described by Qin et al using mangiferin as reference standard[12].

Statistical Analysis

All the procedures (extraction/fractionation/sub-fractionation) were performed in four replicates. Yield and DPPH scavenging activity of *M. indica* extracts/fractions/compound(s) were expressed in terms of Mean ± SE.

RESULTS AND DISCUSSION

Extraction and Antioxidant activity guided fractionation

The diverse physicochemical properties and wide spectrum of chemical classes of compounds present in plants makes separation and purification process of unknown, desired bioactive compounds a challenging task. Activity guided fractionation is the most frequently used method for separation of compounds possessing specific activity. It involves the techniques like column chromatography coupled with HPLC for the isolation of compounds and biological testing for the detection of desired activity within the separated extract/fraction/sub-fraction[13].

For quick access to detection and localization of the active compounds in a complicated plant extract, activity guided fractionation guides the separation, so it was used for isolation of antioxidant compound(s) from leaves of *M. indica*. Results of the antioxidant activity of *M. indica* extracts *viz.* MI-1, MI-2 and MI-3 are summarized in Fig. 2. The assay was validated using ascorbic acid as reference standard wherein it demonstrated 94.91 ± 2.78 % DPPH scavenging activity. The DPPH scavenging activity of MI-1 (91.48 ± 2.67 %) was found to be higher than that of MI-2 and MI-3. On the basis of DPPH scavenging activity, MI-1 extract was selected and further fractionated using two different schemes (Fig. 1). MI-1(1) extracts showed 91.83 ± 2.02 % DPPH scavenging activity (Fig. 2). The DPPH scavenging activity of MI-1(4) and MI-1(5) extracts was 79.29 ± 2.55 % and 75.78 ± 2.87 %, respectively (Fig. 2). MI-1(1) extracts were found to possess higher DPPH scavenging activity than MI-1(2), MI-1(3), MI-1(4) and MI-1(5).

MI-1(1) was further sub-fractionated by open column chromatography technique. MI-1(1) loaded silica column was washed initially with dichloromethane so as to remove the non-polar constituents present in MI-1(1). The remaining semi-polar to polar constituent(s) of MI-1(1) were separated using dichloromethane methanol mixture. Sub-fractions 31-40 (solvent; dichloromethane: methanol, 88:12 v/v) and sub-fractions 41-50 (solvent; dichloromethane: methanol, 85:15 v/v) yielded compounds, designated as MG-1 and MG-2, respectively. The MG-1 and MG-2 yield was 5.61 ± 0.38 and 18.41 ± 1.67 g/500 g *M. indica* leaves. The DPPH scavenging activity of MG-1 and MG-2 was found to be 5.31 ± 0.35 and 50.21 ± 3.11 %, respectively (Fig. 2).

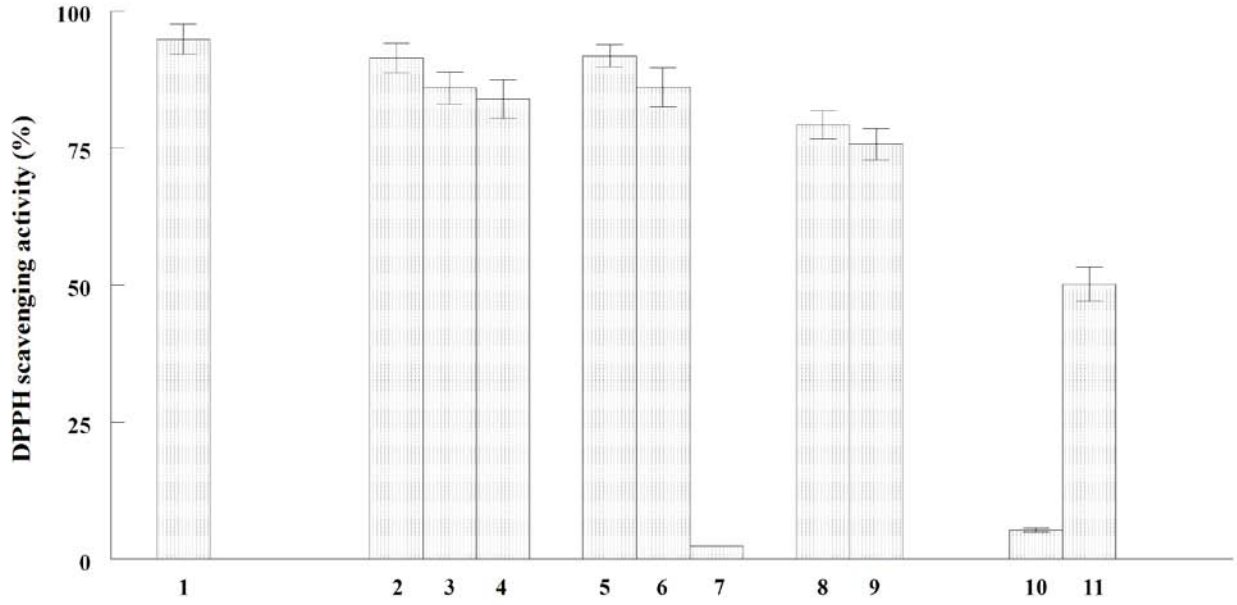

Fig. 2: DPPH scavenging activity (%) of various extracts/fractions/sub-fractions/compound(s) of *M. indica* leaves. 1, Ascorbic acid; 2, aq. extract (MI-1); 3, 50 % aq.-methanolic extract (MI-2); 4, methanolic extract (MI-3); 5, methanolic fraction of MI-1 [MI-1(1)]; 6, 50 % aq.-methanolic fraction of MI-1 [MI-1(2)]; 7, remaining residue after 50 % aq.-methanolic fraction of MI-1 [MI-1(3)]; 8, *n*-butanolic fraction of MI-1 [MI-1(4)]; 9, remaining aq. fraction after -butanolic fraction of MI-1 [MI-1(5)]; 10, MG-1 and 11, MG-2

Spectral Analysis

MG-1 and MG-2 was characterized using UV, IR, ^{1}H NMR, ESI-MS and HPLC techniques. MG-1 was obtained as pale yellow crystals, with melting point range of 241°C. UV spectrum of MG-1 (solvent: methanol) showed wavelength maxima at 256 and 365 nm. IR spectrum of MG-1 showed peaks at following wave numbers: 3339, 2964, 1640, 1620, 1470, 1410, 1298, 1182, 1024, 846, 771, and 631 cm^{-1}. ^{1}H NMR (400 MHz, DMSO-d$_6$): δ [ppm]: 13.72 (1H, s), 10.41 (2H, s), 9.91 (1H, s), 7.18 (1H, s), 6.63 (1H, s), 6.31 (1H, s), 4.92 (1H, d), 3.21-4.1 (sugar protons). ESI-MS spectrum of MG-1 represented $[M+H]^+$ ion at m/z 423. MG-2 was obtained as light yellow crystals, with melting point range of 259-260°C. UV spectrum of MG-2 (solvent: methanol) showed wavelength maxima at 258, 318 and 366 nm. IR spectrum of MG-2 showed peaks at following wave numbers: 3352, 2948, 1652, 1618, 1488, 1415, 1296, 1198, 1090, 1050, 1024, 830, 799, 753, 644 and 519 cm^{-1}. ^{1}H NMR data of MG-2 was obtained as follows: (400 MHz, DMSO-d$_6$, δ ppm): 13.42 (1H, s), 10.02 (3H, br), 7.01 (1H, s), 6.47 (1H, s), 6.10 (1H, s), 4.41 (2H, br), 4.29 (1H, d), 4.18 (1H, br), 4.04 (1H, br), 3.69 (1H, t), 3.34 (1H, d), 3.11 (1H, d), 2.88 (2H, m), 2.81 (1H, m). ESI-MS spectrum of MG-2 represented $[M+Na]^+$ ion at m/z 444.92.

Based on the above data, MG-1 and MG-2 were identified as isomangiferin and mangiferin, respectively[14, 15].

There are several reports demonstrating mangiferin as main compound of *M. indica* peels and stem bark[7, 9]. Previously, Olabinri et al[16] has demonstrated the antioxidant potential of *M. indica* leaves wherein aq. extract of leaves showed 86.95 % DPPH scavenging activity. The investigation revealed the presence of flavonoids, polyphenols and tannins in *M. indica* leaves. The report demonstrated comparison of antioxidant potential of leaf extracts, without isolation of compound(s) responsible for the activity.

In the present experimental work, the natural antioxidant from leaves of *M. indica* is systematically isolated and identified using an activity guided fractionation technique. The results demonstrated that mangiferin is the key compound responsible for the antioxidant nature of *M. indica* leaves.

Spectrophotometric DPPH scavenging capacity assay [antiradical activity]

Fig. 3 shows the concentration-response curves for the DPPH scavenging activity of mangiferin isolated from *M. indica* leaves and ascorbic acid. IC_{50} values of mangiferin and ascorbic acid were 58.16 ± 0.15 and 21.57 ± 0.21 µM, respectively. The obtained IC_{50} values of mangiferin and ascorbic acid were comparable with earlier reported values *viz.* 55.90 and 13.74 µM, respectively[17, 18].

HPLC analysis

The typical chromatograms of standard mangiferin, active MI-1 extract, active MI-1(1) fraction and mangiferin from *M. indica* leaves are shown in Fig. 4A-D. As shown in chromatograms, mangiferin (retention time: 22.02 min) was found to be predominant compound in an active aq. extract and methanolic fraction of aq. extract of *M. indica* leaves.

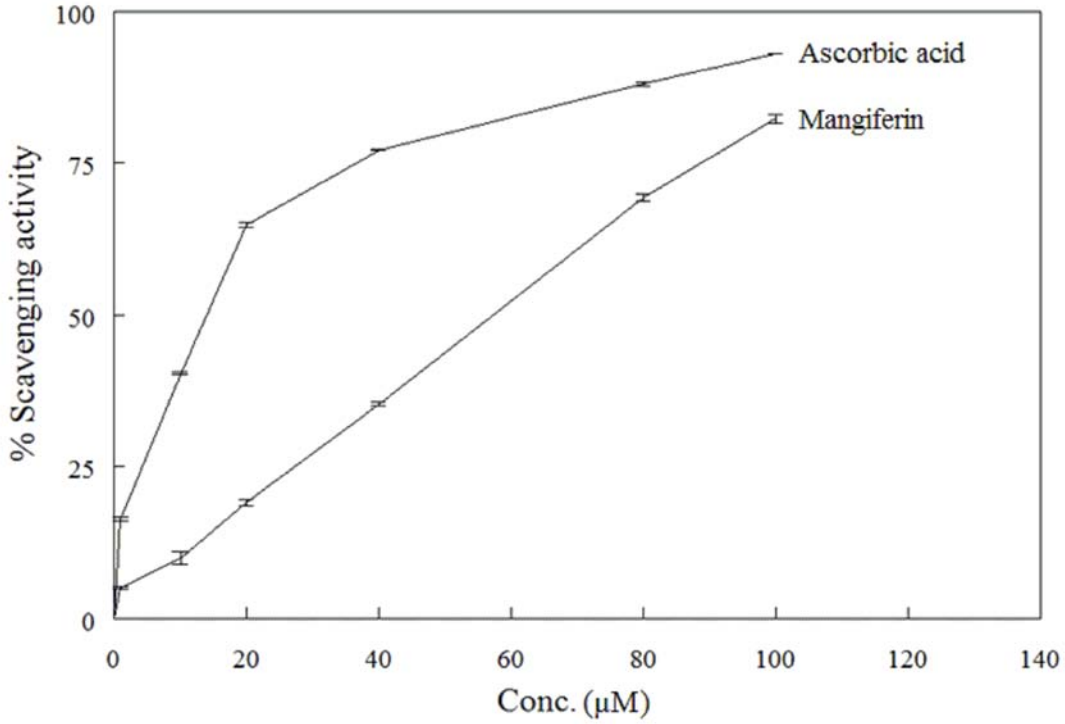

Fig. 3: Antiradical activity of MG-2 (mangiferin) and ascorbic acid [DPPH scavenging activity (%) of MG-2 and ascorbic acid at 0-100 µM]

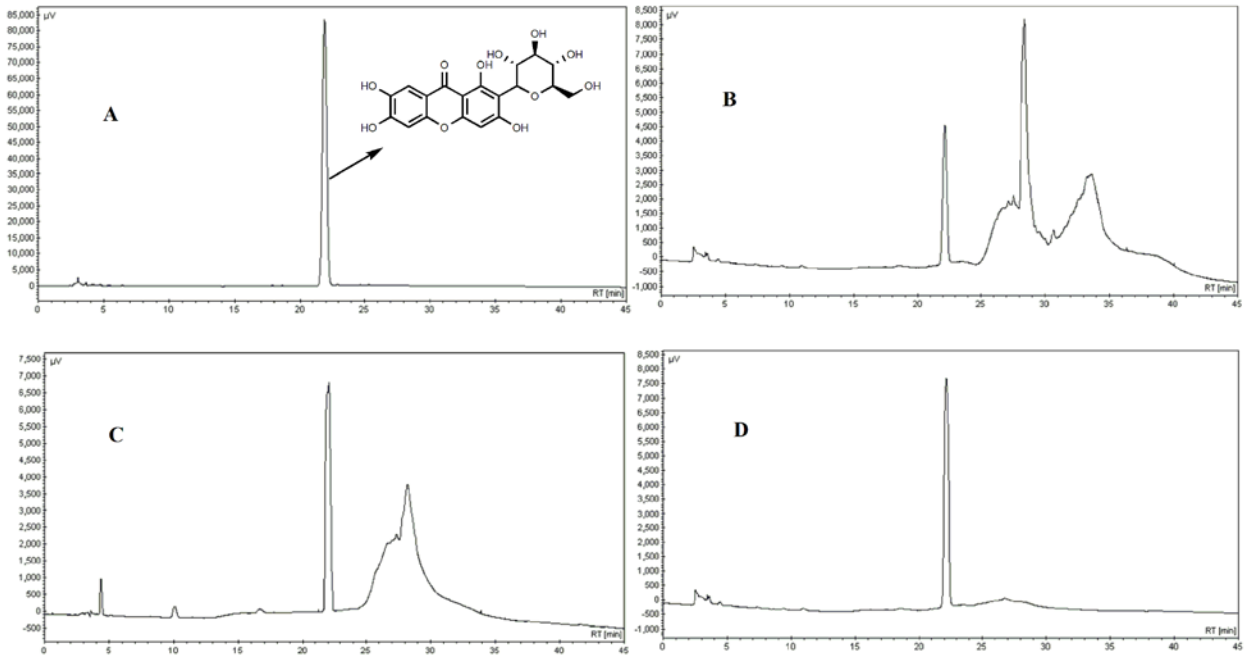

Fig. 4: HPLC chromatograms: A; mangiferin [standard], B; MI-1 [aq. extract of *M. indica* leaves], C; MI-1(1) [methanolic fraction of aq. extract of *M. indica* leaves] and D; mangiferin [isolated from *M. indica* leaves]

Abbreviations

MI-1: Aq. extract of *M. indica* leaves

MI-2: 50 % aq.-methanolic extract of *M. indica* leaves

MI-3: Methanolic extract of *M. indica* leaves

MI-1(1): Methanolic fraction of MI-1

MI-1(2): 50 % aq.-methanolic fraction of MI-1

MI-1(3): Remaining residue after 50 % aq.-methanolic fraction of MI-1

MI-1(4): *n*-butanolic fraction of MI-1

MI-1(5): Remaining aq. fraction after *n*-butanolic fraction of MI-1

MG-1: Compound isolated from MI-1(1)

MG-2: Compound, mangiferin isolated from MI-1(1)

CONCLUSION

The DPPH scavenging capacity assay based activity guided fractionation strategy was used to isolate the antioxidant compound from *M. indica* leaves. The two pure compounds were isolated from leaves of *M. indica*. One of the isolated compounds showed significant free radical scavenging activity which was further confirmed as mangiferin on the basis of UV, IR, ^{1}H NMR, ESI-MS and HPLC studies.

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