Assessment of free-radical scavenging activities of mangiferin from *Curcuma amada* obtained by non-conventional extraction methods: A comparative study

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Mangiferin (MgFn) is a naturally occurring bioactive glucosylxanthone having numerous pharmacological activities. A feasible microwave assisted extraction (MAE) technique for extraction of MgFn from *Curcuma amada* (mango ginger) was developed in our laboratory. To prove the efficacy of MAE technique, it was compared with other methods like maceration extraction (ME) and ultrasonic sound assisted extraction (UAE). A high yield of MgFn was obtained by MAE (1.472 mg/g) compared to 1.017 mg/g yield by UAE and 0.837 mg/g by ME technique in acetone-water (50:50 v/v). Scanning electron microscopy (SEM) photographs of *C. amada* cells were taken, which were subjected to different extraction forces mentioned above. The efficacy of MgFn as a radical scavenger was studied using 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), nitrate scavenging and ferric reducing power assays. The IC\textsubscript{50} value of MgFn extracted with MAE was recorded to be 26 µg/mL; showing it to be an effective radical scavenger more efficient than ascorbic acid.

**Keywords:** IC\textsubscript{50} value, mangiferin, microwave assisted extraction, nutraceuticals aspect, radical scavenging

**Introduction**

Mangiferin (MgFn) is a bioactive molecule (1,3,6,7-tetrahydroxyxanthone-2-glucopyranoside), chemically belonging to the “xanthonoid” group and to be more specific a “glucosylxanthone” having significant antioxidant property\textsuperscript{1}. The presence of hydroxyl groups in flavonoid molecules conjugated with catechol moiety plays a significant role in radical scavenging property\textsuperscript{2}. MgFn with similar kind of structure has strong antioxidant property in lipid peroxidation and immunomodulation\textsuperscript{3}; antibacterial, antifungal, anti-inflammatory, cytotoxicity, antiallergic, biopesticide, hypotriglyceremic, CNS depressant and analgesic activity; and platelet aggregation inhibitory activity\textsuperscript{4}. Recent studies have shown that reactive oxygen species (ROS) is the causative agent for more than 100 health problems\textsuperscript{5}. ROS has many harmful effects in living cells as they impart damage to basic molecule like DNA, fatty acids, amino acids and enzymes\textsuperscript{6}. Pharmaceutical industries are using different non-nutrient chemical as dietary supplements, called nutraceuticals, to promote human health extensively\textsuperscript{7}. From past few decades, different parts of the plants have been investigated by researchers for their use as phytochemicals. Regular synthetic anti-oxidative molecules like butylated hydroxyanisole (BHA) or butylated hydroxytoluene (BHT) are suspected for being responsible for liver damage and other health hazards\textsuperscript{5}. As a result new findings and efficacy evaluation of anti-oxidative molecules from natural origins is in demand.

MgFn can be found widely in genus *Mangifera* and certain species of genus *Iris, Anemarrhena, Salicia, Cyclopia* etc. *Curcuma amada* Roxb. (Family: Zingiberaceae) is a perennial herb having a good source of MgFn, which has been extracted by our research group using microwave assisted extraction (MAE) technique\textsuperscript{8,9}. A variety of extraction methods have been introduced in recent years to obtain bioactive molecules from various natural origins\textsuperscript{10}. Besides conventional methods like solvent extraction, heat reflux extraction and Soxhlet extractions, emerging trends like MAE, ultrasound-assisted extraction (UAE), high hydrostatic pressure (HHP) etc. are grabbing attention due to lower solvent and time requirement\textsuperscript{1}. The basic mechanisms lying in these methods are generally relevant to selection of

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solvents, solubility of the product, disruption of cells, mass transfer rate etc.\textsuperscript{1,10}.

Several reports are available on MgFn extraction methods or its antioxidant property but so far no comparative study has been done on MgFn extraction or its radical scavenging efficacy. Our previous reports showed good yield of MgFn from \textit{C. amada} by optimized MAE method\textsuperscript{8} and a preliminary study on DPPH scavenging activity\textsuperscript{9}. In the present study, authors have compared the efficacy of MgFn extracted by different methods in terms of sensitivity towards different radicals and ability to remove DPPH, ABTS, nitrite and iron radicals.

Materials and Methods

Chemical, Reagents and Biological Materials

Pure standard mangiferin, DPPH (1,1-diphenyl-2-picrylhydrazyl), ABTS (2,2'-azinobis-(3-ethylbenzo thiazoline-6-sulfonic acid), Griess reagent (modified), methanol, acetone, acetonitrile, ethanol, acetic acid were provided by Sigma-Aldrich, India. Potassium persulphate (K$_2$S$_2$O$_8$), ascorbic acid (Vit C), sodium nitrite (NaNO$_2$), ferric chloride (FeCl$_3$), citric acid were purchased from Merck, India. Potassium ferricyanide [K$_3$Fe(CN)$_6$], trichloroacetic acid (TCA), potassium acetate, aluminium chloride (AlCl$_3$) were provided by Hi-Media, India.

Fresh \textit{C. amada} (mango-ginger) was purchased from local market. It was manually peeled, chopped and washed with distilled water. Chopped pieces were freeze-dried, ground to powder and kept at 4°C for further use.

Preliminary Solvent Extraction of Mangiferin

The extraction of mangiferin from \textit{C. amada} was carried out in four different solvents, namely, acetone, acetonitrile, methanol and ethanol (1:1 dilution with water), following the method of Krivut \textit{et al}\textsuperscript{11} with modifications. Briefly, freeze dried sample was continuously stirred in different solvents (1:20 w/v %) for 30 min. Re-extraction was done by addition of $\frac{1}{4}$th of fresh solvent for another 20 min. Extracted solvent was concentrated by using rotary vacuum evaporator (Yamato, Japan), followed by filtration and consecutive treatment with chloroform and butanol (saturated with water) to remove the flavonoids. Final extract was evaporated in Lyophiliser (Digitech Instruments, India) and dissolved in DMSO for mangiferin estimation.

Maceration Extraction (ME)

Cold maceration extraction procedure was performed according to Ruiz-Montañez \textit{et al}\textsuperscript{1} with modifications. Briefly freeze-dried sample was taken with solvent (1:5 w/v %) in 250 mL Erlenmeyer flask and kept in incubator shaker (Innova®42 series-M1335-0010, Eppendorf-New Brunswick) for 30 h at 150 rpm agitation speed. Temperature was monitored at 20°C.

Microwave Assisted Extraction (MAE)

MAE of MgFn was carried out according to Kullu \textit{et al}\textsuperscript{9} with no further modification. Briefly 550 W power of microwave (Samsung Trio, Model CE117ADV; 230V$\approx$50 Hz) was used for an extraction time of 60 sec with a pre-leaching time of 30 min.

Ultrasonic Assisted Extraction (UAE)

UAE of MgFn was carried out by placing freeze-dried \textit{C. amada} with extraction solvent in a beaker with the help of ultrasound sonicator bath for 20 min (Rivotek by Riviera Glass Pvt. Ltd., Mumbai, India). Parameters like voltage, frequency and temperature were kept constant at 230 V, 50 Hz and 40°C, respectively.$^{1,12}$

Estimation and Analysis of Mangiferin

Estimation of MgFn was done by the method originally adopted by Joubert \textit{et al}\textsuperscript{13}. Briefly, 0.5 mL test samples were mixed with 1.5 mL ethanol, 0.1 mL 10% AlCl$_3$ (w/v) and 3 mL 0.03 M potassium acetate. The reaction mixture was vortexed, incubated at 30°C for 1 h and concentration of mangiferin was analysed using UV-Vis spectrophotometer (Techcomp, UV 2310) by measuring the absorbance at 410 nm. Qualitative estimation of MgFn was done by TLC (composition of mobile phase was ethyl acetate: formic acid: glacial acetic acid: water in the ratio of 100:11:11:26) and HPLC\textsuperscript{9,14}.

SEM Analysis

Morphological changes of \textit{C. amada} cells upon exposure to different extraction methods were investigated in terms of scanning electron microscopy (SEM) (Hitachi, S-530; Resolution: 5 nm; Magnification: 20-150000x).

DPPH Radical Scavenging Activity

Radical scavenging ability of the extracted MgFn was determined using DPPH by the method of Aquino \textit{et al}\textsuperscript{15} with minor modifications. Briefly, different concentrations of pigment was dissolved in methanol and mixed with equal volume of 0.1 mM
DPPH in methanol. Mixture was kept in dark for 15 min at 25°C. Change of absorbance was recorded at 517 nm. Methanol was taken as blank.

**ABTS Radical Scavenging Activity**

Antioxidant activity of the extracted MgFn was determined using ABTS (2,2’-azinobis-(3-ethyl benzothiazoline-6-sulfonic acid) adapting the method of Re et al with minor modifications. In brief, ABTS+mixture was generated though oxidation of 7 mM ABTS with 120 mM K₂S₂O₈ solution (Sigma-Aldrich, India) by incubating the mixture at 28°C for 14 h in the dark. Antioxidant activity was determined by adding 0.2 mL of plant extracts with 1.8 mL ABTS+radical cation mixture (diluted mixture having absorbance 0.75±0.2 at 734 nm). After 15 min of incubation, the absorbance was taken at 734 nm. ABTS+radical scavenging ability (%) of plant extracts was calculated based on the following equation:

\[
\text{ABTS+radical scavenging ability (\%) = } \left( \frac{\text{absorbance of control} - \text{absorbance of test sample}}{\text{absorbance of control}} \right) \times 100
\]

Where test sample and control was with and without extracted MgFn, respectively. Vitamin C was used as positive control.

**Nitrite Scavenging Activity**

Nitrite scavenging activity of the obtained MgFn was estimated by the method of Choi et al with major modifications. In brief, 1 mL of each sample concentration was mixed with 0.5 mL of 1 mM NaNO₂ and 3 mL of citric acid (0.1 N, pH 3.0). Then each reaction mixture was diluted up to 10 mL volume by addition of deionised distilled water and incubated in 35°C for 1 h. After incubation, 2 mL of each sample was mixed thoroughly with 4 mL of 2% acetic acid and 1.0 mL of modified Griess reagent (Sigma Aldrich, USA) prepared according to manufacturer instruction. The mixture was kept at room temperature and absorbance was taken at 540 nm. Scavenging activity was calculated by the following formula:

\[
\text{Nitrite scavenging activity (\%) = } \left( 1 - \frac{A - C}{B} \right) \times 100
\]

Where A is the absorbance of the mixture sample during a reaction with 1 mM NaNO₂ after 1 h reaction, B is the absorbance of a mixture of distilled water and 1 mM NaNO₂ after 1 h reaction, and C is the absorbance of the sample.

**Ferric Reducing Power Assay**

Reducing power of the extracted MgFn was estimated with method by Gharibzahedi et al with minor modifications. Vitamin C was used as positive control.

**Statistical Analysis**

Data presented in this article show mean of three replicates with their standard deviation (mean±SD) using Microsoft Excel 2010.

**Results and Discussion**

**Quantification and Analysis of MgFn from C. amada**

Preliminary extraction of MgFn by different solvent showed that acetone is the best choice rather than acetonitrile, methanol and ethanol (data not shown). Hence further work was carried out with 50% aqueous acetone. Throughout the entire experiment, dilution of solvent (acetone) with water enhanced the extraction efficacy by swelling the plant material, thus ensuing easy mass transfer of MgFn from C. amada cells.

MgFn yield obtained from ME, MAE and UAE methods were found to be 0.837, 1.472 and 1.017 mg/g, respectively. This result supports the earlier report of Zou et al who suggested that MAE is a useful method to extract MgFn from plant parts rather than other conventional method. TLC of extracted MgFn revealed initial purity and Rf values were calculated to be 0.356, 0.358 and 0.355 for ME, MAE and UAE methods, respectively. These Rf values were close to the Rf value of the standard MgFn (0.356). The HPLC analysis of MgFn extracted by ME, MAE and UAE method were carried out and is shown in Fig. 1. A clear sharp peak with 100% peak area of MgFn was obtained by three extraction process. Retention time of MgFn obtained by ME, MAE and UAE method was calculated to be 9.124 (Fig. 1B), 9.131 (Fig. 1C) and 9.128 min (Fig. 1D), respectively, whereas standard MgFn had a retention time of 9.026 min (Fig. 1A).

**C. amada** cells were dried and further subjected to SEM analysis to visualise the effect on cells morphology by each extraction procedure. The control cells (C. amada cells without any extraction) showed smooth surface without any rupture (Fig. 2A). On the other hand, the extracted C. amada cells showed rough and shattered cells in comparison to the control. Further, the cells subjected to MAE were more disrupted (Fig. 2B) compared to the cells subjected to ME or UAE (Figs 2C & D). MAE may...
have led to loosening and disruption of cells leading to higher extractability of MgFn\textsuperscript{1, 12}, whereas ultrasonic sounds produces vibration and acceleration thus influencing mass transfer rates, interfacial area or the driving force\textsuperscript{1, 22}, which ultimately also leads to higher degree of extractability. Thus it could be said that nonconventional methods can be promoted over conventional extraction procedures for changing physical forces on the materials leading to higher bioactive yield\textsuperscript{1}.

**Radical Scavenging Activities of MgFn**

Natural plant extracts like *C. amada* show efficient ROS prevention\textsuperscript{6}. This may be due to the presence of compounds bearing phenolic hydroxyl group. When different oxidizing free-radicals react with MgFn, they convert into phenoxyl radicals, which gradually decay with time\textsuperscript{23}. *C. amada* rhizome retains its antioxidant activity even after cooking, making it widely acceptable in culinary preparations\textsuperscript{5}.

MgFn extracted by different methods were subjected to DPPH assay. Fig. 3 shows the DPPH free-radical scavenging activity of MgFn extracted from three different extraction methods. MgFn extracted by MAE had the highest radical quenching efficacy. In 50 µg/mL concentration, MgFn from MAE, UAE and ME showed 94.64, 82.88 and 71.36% DPPH inhibition, respectively, whereas positive control ascorbic acid (Vit C) showed only 35.34% inhibition. The IC\textsubscript{50} values for MAE and UAE extracted MgFn were found to be 35.66 and 29.6 µg/mL, respectively; while ME extracted MgFn showed a lesser IC\textsubscript{50} value of 26 µg/mL, but significantly superior compared to Vit C (IC\textsubscript{50}: 75.4 µg/mL). Thus, MgFn can act as a better
antioxidant as compared to regular standard (Vit C). Stoilova et al. and Dar et al. did similar kind of experiments and found that MgFn was more effective compared to “rutin” (a commercial antioxidant).

In case of ABTS assay, MgFn extracted by MAE method again showed best inhibition but in lower concentrations compared to DPPH assay. Antioxidant molecules donate electron into free ABTS radical to generate a non-radical form. At 20 µg/mL concentration, MgFn extracted by MAE showed the highest (96.48%) ABTS free-radical scavenging activity, followed by UAE (93.55%) and ME (86.74%); while positive control Vit C showed 91.24% inhibition but at 100 µg/mL concentration (Fig. 4). Further, IC$_{50}$ values of MgFn for MAE, UAE and ME methods were 2.49, 3.71 and 4.98 µg/mL, respectively. Thus MgFn extracted from various methods had better IC$_{50}$ values as compared to the IC$_{50}$ value (38.05 µg/mL) of Vit C, the regular standard. The present result supports the earlier studies of Malherbe et al. and Prema et al. where ABTS was found to be more sensitive to free-radical scavenging activity of MgFn from C. amada rhizome extract as compared to DPPH.

Nitrite is a common food processing and preservative agent but its reactive product nitrosamine is highly toxic to human health. The studies have shown that reductive power of a molecule is attributed to scavenge nitrosamines. The process of reducing nitrite can be explained by the bellow equation:

\[
\text{NO}_2^- + 16\text{H}^+ + 12\text{e}^- = 2\text{NH}_4^+ + 4\text{H}_2\text{O}
\]

Fig 3—Per cent DPPH inhibition by mangiferin extracted by different procedures. [ME, Maceration extraction; MAE, Microwave assisted extraction; UAE, Ultrasound assisted extraction; Vit C is the positive control. Each value represents the mean±SD of three replicates.]

Fig. 4—Per cent ABTS inhibition by mangiferin extracted by different procedures. [ME, Maceration extraction; MAE, Microwave assisted extraction; UAE, Ultrasound assisted extraction; Vit C is the positive control. Each value represents the mean±SD of three replicates.]

Fig. 5—Per cent NSA (nitrite scavenging activity) inhibition by mangiferin extracted by different procedures. [ME, Maceration extraction; MAE, Microwave assisted extraction; UAE, Ultrasound assisted extraction; Vit C is the positive control. Each value represents the mean±SD of three replicates.]
strong nitrite scavenging activity of the molecule by donating hydrogen ions. Thus MgFn extracted by MAE may be more prone to loose $H^+$ rather than MgFn extracted by other methods. This study also shows that MgFn from MAE was more efficient nitrite radical scavenger than ascorbic acid (Vit C).

Reducing power assay (RP) also followed similar dose dependant results as DPPH, ABTS and NSA in terms of MgFn extracted by MAE, UAE and ME. MgFn by MAE showed the lowest absorbance (OD$_{700}$: 0.0024 & 0.0273 at 0.625 & 10 µg/mL, respectively) compared to MgFn by UAE (OD$_{700}$: 0.0021 & 0.0218) and ME (OD$_{700}$: 0.0017 & 0.0198) at similar concentration (Fig. 6). On the other hand, Vit C showed higher absorbance (OD$_{700}$: 0.5021 & 0.5123 at 0.625 & 10 µg/mL, respectively) compared to MgFn. Higher absorbance value at 700 nm indicates stronger reducing power. It is clear from above results that Vit C is more sensitive and effective compared to MgFn in terms of reducing power ability. This may be due to higher hydrogen donating ability of the positive control sample.

Thus, the present study shows that MgFn can be used not only as food additives but also as an important pharmaceutical.

**Conclusion**

MgFn was extracted from *C. amada* rhizomes by different extraction procedures. It was found that MgFn extracted from non-conventional methods like MAE was more efficient than conventional procedures in terms of quantitative yield. It was also revealed that MgFn obtained by MAE had strong free-radical scavenging activity compared to standard anti-oxidative agents like ascorbic acid. Results also showed that MgFn from *C. amada* rhizomes is more sensitive towards ABTS compared to DPPH in terms of radical quenching ability. Obtained results established that MgFn from MAE has a strong antioxidant property and thus could be used as nutraceutical. Hence, MgFn extracted by MAE from *C. amada* rhizomes can be used in terms of both nutraceutical and pharmaceutical to solve wide range of human health problems by scavenging ROS.

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