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Synthesis and Evaluation of Analgesic and Anti-inflammatory Activities of Most Active Free Radical Scavenging Derivatives of Mangiferin

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Authors' contributions

This work was carried out in collaboration between all authors. Author SM designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript and managed literature searches. Authors SB, SR, BST and VPV managed the analyses of the study and literature searches. All authors read and approved the final manuscript.

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Original Research Article

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ABSTRACT

In the present study, structural modification of mangiferin was carried out for structure activity relationship studies. One new compound and three known derivatives were

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prepared from mangiferin. All these compounds were synthesized and purified by standard procedures, identified by using physical and spectral (IR, ^{1}H NMR, ^{13}C NMR and MS) properties. The synthesized mangiferin derivatives were tested for *In vitro* antioxidant properties. Benzyl and methyl substituted mangiferin showed poor antioxidant activity than mangiferin. However, mangiferin derivatives substituted with acetyl and benzoyl groups were showed potent antioxidant activity than mangiferin in lipid peroxidation, p-NDA, deoxyribose and alkaline DMSO methods. But both the compounds failed to show potent analgesic and anti-inflammatory activities. In all these methods, standard drugs showed better activity than mangiferin and its derivatives.

Keywords: Mangiferin derivatives; antioxidant; analgesic; anti-inflammatory.

1. INTRODUCTION

Mangiferin (1,3,6,7-tetrahydroxyxanthone-C2-β-D-glucoside) is a pharmacologically active phytochemical compounds present in large amounts in the bark, fruits, roots and leaves of *Mangifera indica* Linn.(family: *Anacardiaceae*). The plant is indicated in traditional medicine for the treatment of various diseases. The root, bark, leaves and flowers are astringent, refrigerant, styptic, vulnerary, laxative, cardiotonic, haemoptysis, haemorrhages, constipating and diabetes [1,2]. Mangiferin showed antidiabetic [3], anticancer [4,5], antioxidant [6], anti-inflammatory [7], antiviral [8] and antimicrobial [9] activities.

Free radicals play important roles in many physiological and pathological conditions. In general, excess of free radicals caused by the imbalance between free radicals generation and scavenging may contribute to disease development. Painful stimulation increases the production of free radicals with increased lipoperoxidation. The application of antioxidants increases the antioxidative capacity and thus enhances the protection against the consequences of pain. Antioxidants are known to protect CNS against free radicals and also decrease the sensation of pain. The role of reactive oxygen species in the pathophysiology of inflammation is well-established. Free radicals can damage membranes, proteins, enzymes and DNA, increasing the risk of diseases such as cancer, Alzheimer's, Parkinson's, angiocardiopathy, arthritis, asthma, diabetes, and degenerative eye diseases. Natural products, natural products derivatives, synthetic compounds with natural products-derived pharmacophore and synthetic compounds designed from natural products are also important to manage pathological conditions of those diseases caused by free radicals.

Bhatia et al. [10] reported acetylation and methylation of mangiferin. Further, Hu et al. [11] synthesized a series of substitutional benzyl mangiferin derivatives. However, literature showed that detailed pharmacological investigation of mangiferin derivatives was not reported. Hence, the structural modification of mangiferin was taken up for the development of new pharmacologically active compounds.

2. MATERIALS AND METHODS

2.1 General

IR spectrum was recorded using FT-IR, Perkin Elmer 8400 series instrument. NMR spectrum was obtained on a DDR X - 400 MHz and 100 MHz Bruker Daltonics, Germany. Absorbance was recorded by using Elisa Reader, Bio-Rad Laboratories Inc, California, USA,

model 550. Mass spectrum was recorded by using Shimadzu MS-2010 A, Koyoto, Japan. Melting points (uncorrected) were obtained on a melting point apparatus, Lab India, Mumbai. 2,2-Diphenyl-2-picryl hydrazyl (DPPH) and 2,2'-azinobis (3-ethylbenzo-thiazoline-6-sulphonic acid) diammonium salt (ABTS) were obtained from Sigma–Aldrich Co., St. Louis, USA. Rutin and *p*-nitroso dimethyl aniline (*p*-NDA) were obtained from Acros Organics, NJ, USA. Naphthyl ethylene diamine dihydrochloride (NEDD) was from Roch-Light Ltd., Suffolk, UK, ascorbic acid, nitro blue tetrazolium (NBT) and butylated hydroxy anisole (BHA) were from SD Fine Chemicals Ltd., Mumbai, India. H_2O_2 and 2-deoxy-d-ribose was from Hi-Media Laboratories Pvt. Ltd., Mumbai, India. Sodium nitroprusside were from Ranbaxy Laboratories Ltd., Mohali, India. Sulphanilic acid used was from E-Merck (India) Ltd., Mumbai, India and carrageenan were obtained from Sigma Aldrich Co, St Louis, USA. Pentazocine was obtained from Ranbaxy, New Delhi, India. Diclofenac sodium was obtained from Wochardt Ltd, Mumbai, India. Rutin was obtained from Acros Organics, New Jersy, USA. Ascorbic acid was obtained from S.D. Fine Chem, Ltd., Biosar, India. All other chemicals used were of analytical grade.

2.2 Plant Material

The leaves of *Mangifera indica* were collected from Namakkal, Tamilnadu, India and authenticated by Medicinal Plants Survey and Collection Unit, Ootacamund, Tamil Nadu, India, where a voucher has been deposited for further reference(Voucher No: J322/2014).

2.3 Extraction and Isolation of Mangiferin

Shade-dried and powdered leaves of *Mangifera indica* (1 kg) were soxhleted for 18-20 h with petroleum ether (60–80°C). The defatted plant material was extracted with ethanol under reflux for 18-20 h. On keeping overnight a solid matter separated out, it was filtered and washed with petroleum ether repeatedly, affording yellow powder of mangiferin [3]. After repeated crystallization of the powder in aqueous ethyl acetate and methanol pale-yellow needle-shaped crystals of mangiferin were obtained (1, 3, 6, 7-tetrahydroxyxanthone-C2-β-D-glucoside, mp 270-272°C, yield 4.5 g, 0.45%), and exhibited violet color with ferric chloride. It was found to be homogenous by HPTLC when separated using the solvent system ethyl acetate: methanol: water: formic acid (6:2:1:1, R_f = 0.76). It was characterized by comparing its melting point, IR, NMR and MS data with literature values [12].

2.4 Structural Modification of Mangiferin

The structural modifications of mangiferin was carried out by using the following standard procedure.

2.4.1 Benzoylation of mangiferin

Mangiferin (0.844 g, 2 mmol), benzoyl chloride (0.560 g, 4 mmol), pyridine (0.474 g, 0.6 mmol) and basic alumina (2 g) were added in a 50 ml beaker [13]. The mixture was stirred to obtain a free flowing powder, at room temperature. Then the temperature was increased upto 70°C for 30 min. After cooling to room temperature, the product was extracted with methylene chloride (3 x 15 ml). The combined extracts were washed with water and dried over sodium sulfate. The product obtained after removal of solvent under reduced pressure was crystallized from a suitable solvent (Ethyl acetate-petroleum ether; ethanol), yielded tribenzoyl mangiferin and designated as compound MGN 1 (Fig. 1).

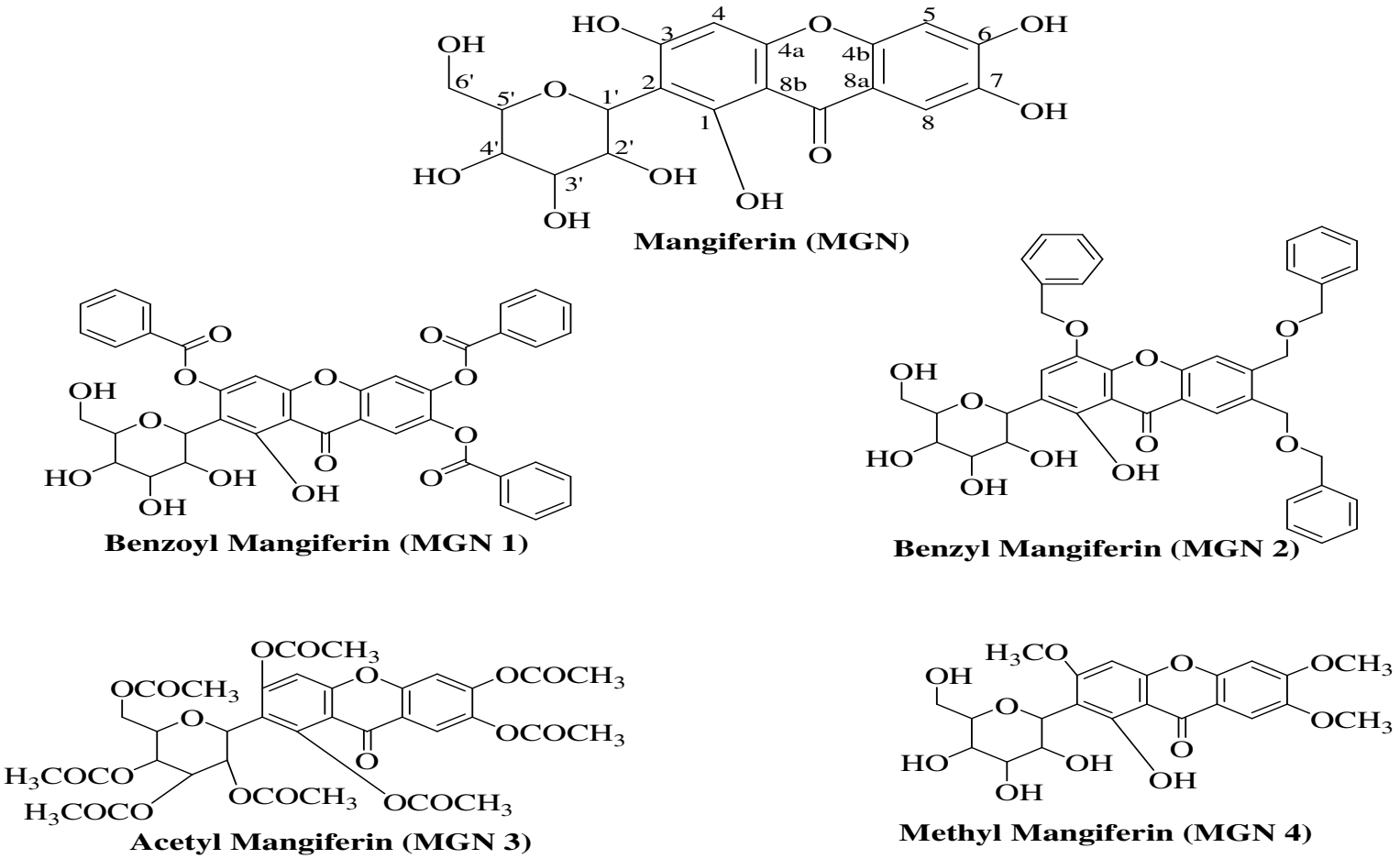

Fig. 1. Structure of mangiferin and its derivatives

2.4.2 Benzylation of mangiferin

A solution of mangiferin (0.422 g, 1 mmol) in dry DMF (20 ml) was treated with benzyl chloride (0.504 g, 4 mmol) and K_2CO_3 (0.1 g) with stirring at 60°C for 10 h [11]. The reaction mixture on evaporation in vacuo gave a residue and was submitted to column chromatography using dichloro methane and methanol as eluent to give tribenzyl mangiferin, designated as MGN 2.

2.4.3 Acetylation of mangiferin

A solution of mangiferin (0.422 g, 1 mmol) in pyridine (0.395 g, 5 mmol) was treated with acetic anhydride (0.306 g, 3 mmol) and then stirred at 40°C for 36 h at room temperature [10]. The reaction mixture was poured into ice-cold water and extracted with chloroform soluble fractions. On evaporation in vacuo gave a residue and was submitted to column chromatography using chloroform and methanol as eluent to give octaacetyl mangiferin, designated as MGN 3.

2.4.4 Methylation of mangiferin

A solution of mangiferin (0.422 g, 1 mmol) in acetone (150 ml) was treated with dimethyl sulphate (0.882 g, 7 mmol) and K_2CO_3 (0.1 g) with stirring at room temperature for seven days [10]. The reaction mixture on evaporation under hood gave a residue and was submitted to column chromatography using chloroform and methanol as eluent to give trimethyl mangiferin, designated as MGN 4.

2.5 *In vitro* Antioxidant Activity

The *in vitro* antioxidant activity of mangiferin and its derivatives were carried out by using the following standard procedure.

2.5.1 Preparation of test and standard solutions

All the synthesized compounds including mangiferin and the standard antioxidants (ascorbic acid, rutin, butylated hydroxy anisole and alpha tocopherol) were dissolved in distilled dimethyl sulphoxide (DMSO) separately and used for the *in vitro* antioxidant assays using seven different methods except the hydrogen peroxide method. For the hydrogen peroxide method (where DMSO interferes with the method), the compounds and the standards were dissolved in distilled methanol and used. The stock solutions were serially diluted with the respective solvents to obtain lower dilutions.

2.5.2 *In vitro* antioxidant activity

The synthesized compounds and mangiferin were tested for their *in vitro* antioxidant activity using the standard methods. In all these methods, a particular concentration of the compounds or standard solution was used which gave a final concentration of 1000–0.45 µg/ml after all the reagents were added. Absorbance was measured against a blank solution containing the compounds or standard, but without the reagents. A control test was performed without the compounds or standards. Percentage scavenging and IC_{50} values ± S.E.M. (IC_{50} value is the concentration of the sample required to inhibit 50% of radical) were calculated.

2.5.2.1 Scavenging of ABTS radical cation

Accurately 54.8 mg of ABTS was weighed and dissolved in 50 ml of distilled water (2 mM). Potassium per sulphate (17 mM, 0.3 ml) was then added. The reaction mixture was left to stand at room temperature overnight in dark before usage. To 0.2 ml of various concentrations of the compounds MGN, MGN1-4 or standards, 1.0 ml of distilled DMSO and 0.16 ml of ABTS solution were added to make the final volume to 1.36 ml. Absorbance was measured after 20 min by spectrophotometrically at 734 nm.

2.5.2.2 DPPH radical scavenging method

A 10 µl aliquot of the different concentrations of the compounds MGN, MGN1-4 and standards were added to 200 µl of DPPH in methanol solution (100 µM) in a 96-well microtitre plate (Tarson Products (P) Ltd., Kolkota, India). After incubation at 37℃ for 20 min, the absorbance of each solution was determined at 490 nm using ELISA reader (Bio-Rad Laboratories Inc., CA, USA, Model 550) [14].

2.5.2.3 Scavenging of hydrogen peroxide

A solution of hydrogen peroxide (20 mM) was prepared in phosphate-buffered saline (PBS at pH 7.4). Various concentrations of the compounds MGN, MGN1-4and standard in methanol (1 ml) were added to 2 ml of hydrogen peroxide solution in PBS. After 10 min the absorbance was measured at 230 nm [15].

2.5.2.4 Lipid peroxidation inhibitory activity

Lipid peroxidation inhibitory activity of all the compounds MGN, MGN1-4 and standard was carried out according to the method of Duh et al. [16]. Egg lectin (3 mg/ml, phosphate buffer, pH 7.4) was sonicated. The test sample of different concentrations was added to 1ml of liposome mixture, control was without test sample. Lipid peroxidation was initiated by adding 10 µl of ferric chloride (400 mM) and 10 µl L-ascorbic acid (200 mM). After incubation for 1 h at 37℃, the reaction was stopped by adding 2 ml of 0.25 N HCl containing 15% trichloro acetic acid and 0.375% thiobarbituric acid and the reaction mixture was boiled for 15 min then cooled, centrifuged and the absorbance of the supernatant was measured at 532 nm.

2.5.2.5 Nitric oxide radical inhibition assay

The reaction mixture (6 ml) containing sodium nitroprusside (10 mM, 4 ml), phosphate buffer saline (1 ml), compounds MGN, MGN1-4 and standard solutions (1 ml) were incubated at 25ºC for 150 min. After incubation, 0.5 ml of the reaction mixture was removed and 1 ml of sulphanilic acid reagent (0.33% in 20% glacial acetic acid) was mixed and allowed to stand for 5 min for completion of diazotization reaction and then 1 ml of NEDD was added, mixed and allowed to stand for another 30 min in diffused light. The absorbance was measured at 540 nm against the corresponding blank solutions in a 96-well microtitre plate (Tarsons Product (P) Ltd., Kolkata, India) using ELISA reader (Bio-Rad Laboratories Inc., Model 550) [17,18].

2.5.2.6 Scavenging of hydroxyl radical by deoxyribose method

Various concentrations of the compounds MGN, MGN1-4 and standard in DMSO (0.2 ml) were added to the reaction mixture containing deoxyribose (3 mM, 0.2 ml), ferric chloride

(0.1 mM, 0.2 ml), EDTA (0.1 mM, 0.2 ml), ascorbic acid (0.1 mM, 0.2 ml) and hydrogen peroxide (2 mM, 0.2 ml) in phosphate buffer (pH, 7.4, 20 mM) to give a total volume of 1.2 ml. The solutions were then incubated for 30 min at 37℃. After incubation, ice-cold trichloro acetic acid (0.2 ml, 15%, w/v) and thiobarbituric acid (0.2 ml, 1%, w/v) in 0.25 N HCl were added. The reaction mixture was kept in a boiling water bath for 30 min, cooled and the absorbance was measured at 532 nm [19].

2.5.2.7 Scavenging of hydroxyl radical by p-NDA method

Various concentrations of the compounds MGN, MGN1-4 and standard in distilled DMSO (0.5 ml) were added to a solution mixture containing ferric chloride (0.1 mM, 0.5 ml), EDTA (0.1 mM, 0.5 ml), ascorbic acid (0.1 mM, 0.5 ml), hydrogen peroxide (2 mM, 0.5 ml) and *p*-NDA (0.01 mM, 0.5 ml) in phosphate buffer (pH 7.4, 20 mM), to produce a final volume of 3 ml. Absorbance was measured at 440 nm [20].

2.5.2.8 Scavenging of super oxide radical by alkaline DMSO method

To the reaction mixture containing 0.1 ml of NBT (1 mg/ml solution in DMSO) and 0.3 ml of the compounds MGN, MGN1-4 and standard in DMSO, 1ml of alkaline DMSO (1ml DMSO containing, 5 mMNaOH in 0.1ml water) was added to give a final volume of 1.4 ml and the absorbance was measured at 560 nm [20].

2.9 *In vivo* Analgesic and Anti-inflammatory Activities

The *in vivo* analgesic activity was carried out by using the following standard procedure.

2.9.1 Animals

The animals were obtained from the animal house of Sree Siddaganga College of Pharmacy, Tumkur, India, maintained under standard conditions (12 h light / dark cycle; 25 ± 3℃, 45-65% humidity) and had free access to standard rat feed and water *ad libitum*. All the animals were acclimatized to laboratory conditions for a week before commencement of the experiment. The experiments were performed during the light portion between 07:00-18:00 h to avoid circadian influences. Animal studies were performed according to the prescribed guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India, India.

2.9.2 Analgesic activity

Three different sets of mice were randomized into eight groups, each containing six animals and used in three different models for the evaluation of analgesic activity. Different doses of compounds MGN 1 and MGN 3 were prepared as suspensions in Tween-80 (1% v/v in saline). Two doses of mangiferin, MGN 1 and MGN 3 (10 and 20 mg/kg) were selected based on an earlier study [21].

Group I were treated with Tween-80 (1% v/v in saline) as normal vehicle control. Groups II-VII were treated with mangiferin, MGN 1 and MGN 3 at 10 and 20 mg/kg, respectively and Group VIII animals were treated with standard pentazocine at 20 mg/kg. All the treatments were administered intraperitoneally.

2.9.3 Eddy's hot-plate method

Mice were treated and placed on Eddy's hot plate kept at a temperature of 55 ± 0.5℃. A cut off period of 15 sec was observed to avoid damage to the paw. Reaction time and the type of response were noted using a stopwatch. The response is in the form of jumping, withdrawal of the paws or licking of the paws. The latency was recorded before and after 15, 30 and 45 min following the treatments. The percentage protection was calculated using the formula, protection (%) = (t-n/t) ×100, where, t = reaction time of treated group and n = reaction time of normal group [22].

2.9.4 Tail immersion method

In this method [23], 5 cm of the end of the mice tail was immersed in warm water maintained at 55±0.5℃. The tail withdrawal reflex was recorded before and after 60 min following the treatments. The percentage protection was calculated as per hot plate method.

2.9.5 Acetic acid induced writhing method

In the acetic acid induced writhing [22] in mice an intraperitoneal injection of acetic acid (1%, 10 ml/kg) was given 30 min after the treatments. The response is in the form of abdominal contractions, trunk twist and extension of hind limb. The number of writhing in each mouse was counted for 20 min from the injection of acetic acid. The percentage protection was calculated using the formula, protection (%) = (c-t/c) ×100, where, t = reaction time of treated group and c = reaction time of control group.

2.10 Anti-inflammatory Activity

The *in vivo* anti-inflammatory activity was carried out by using carragenan induced paw edema method.

2.10.1 Carrageenan induced paw edema in rats

Swiss albino rats (150-200 g) were divided into eight groups with six animals in each group. Group I was served as control and received Tween-80 (1% v/v in saline). Groups II-VII were received the treatments as described in analgesic activity. Group VIII was treated as positive control and received standard diclofenac (20 mg/kg). All the treatments were administered intraperitoneally. The initial hind paw volume of rats was determined volumetrically by using a plethysmometer [24]. A solution of carrageenan in saline (1%, 0.1 ml/rat) was injected subcutaneously into the right hind paw 30 min after the treatments. The animals in the control group received the vehicle only. Paw volumes were measured up to 6 h at intervals of 30, 60, 120, 180 and 360 min and percent increase in edema between the control and treated groups were compared. The percentage protection was calculated as acetic acid induced writhing method.

2.11 Statistical Analysis

The values were expressed as mean ± SEM. The statistical analysis was carried out by one way analysis of variance (ANOVA) followed by multiple comparison using the Dunnet's test. P values<0.05 were considered as significant.

3. RESULTS

3.1 Structural Modification of Mangiferin

The following results were obtained from various instrumental analysis of mangiferin and its derivatives

3.1.1 2-b-D-glucopyranosyl-1,3,6,7-tetrahydroxy-9*H*-xanthen-9-one, mangiferin (MGN)

Mangiferin was isolated from the leaves of *Mangifera indica,* found to be homogenous by HPTLC when separated using the solvent system ethyl acetate: methanol: water: formic acid (6:2:1:1, R_f= 0.76). Obtained as pale-yellow needle shaped crystals, mp 270-272°C; yield 4.5 g, 0.45%; IR u_{max}(KBr) cm^{-1}: 3367 (OH), 1651 (C=O), 1622 and 1585 (aromatic C=C), 1199 and 1095 (C–O), 1051, 829, 588; ^{1}H NMR (400 MHz, DMSO) δ: see (Table 1); negative ESI-MS: m/z calculated for 422.08, Found: 444 for [M +Na – H].

3.1.2 2-b-D-tetrahydroxyglucopyranosyl-3,6,7-tribenzoyl-1-hydroxy-9*H*-xanthen-9-one (MGN 1)

Obtained as yellow crystals, mp 181-183°C; yield 0.78 g, 56.07%; IR u_{max}(KBr) cm^{-1}: 3352 (OH), 1747, 1742 (carbonyl ester), 1618 (C=O), 1600 and 1556 (aromatic C=C), 1259 and 1091 (C–O), 1022, 821, 705, 567; ^{1}H NMR (400 MHz, DMSO) δ: see (Table 1); negative ESI-MS: m/z calculated for 734.16, Found: 755 for [M +Na – 2H].

3.1.3 2-b-D-tetrahydroxyglucopyranosyl-3,6,7-tribenzyl-1-hydroxy-9*H*-xanthen-9-one (MGN 2)

Obtained as yellow powder, mp 137-139°C; yield 0.53 g, 82.68%; IR u_{max}(KBr) cm^{-1}: 3392 (OH), 1647 (C=O), 1608 and 1577 (aromatic C=C), 1190 and 1080 (C–O), 1024, 808, 734, 696; ^{1}H NMR (400 MHz, DMSO) δ: see (Table 1); negative ESI-MS: m/z calculated for 692.23, Found: 689 for [M – 3H].

3.1.4 2-b-D-tetraacetoxyglucopyranosyl-1,3,6,7-tetraacetoxy-*H*xanthen-9-one (MGN 3)

Obtained as white amorphous powder, mp 141-143°C; yield 0.60 g, 79.58%; IR u_{max}(KBr) cm^{-1}: 1751 (Carbonyl ester), 1651 (C=O), 1620 (C=C), 1224 and 1055 (C–O), 1039, 920, 839, 601; ^{1}H NMR (400 MHz, DMSO) δ : see (Table 1); negative ESI-MS: m/z calculated for 758.17, Found: 755 for [M – 3H].

3.1.5 2-b-D-tetrahydroxyglucopyranosyl-3,6,7-trimethoxy-1-hydroxy-9*H*-xanthen-9-one (MGN 4)

Obtained as white amorphous powder, mp 193-195°C; yield 0.40 g, 86.58%; IR u_{max}(KBr) cm^{-1}: 3367 (OH), 1647 (C=O), 1606 (C=C), 1217 and 1064 (C–O), 1022, 819, 759, 619, 576; ^{1}H NMR (400 MHz, DMSO) δ: see (Table 1); negative ESI-MS: m/z calculated for 464.13, Found: 464.

3.2 *In vitro* Antioxidant Screening of Mangiferin and Its Derivatives

Mangiferin showed potent antioxidant activity with IC_{50} values 1.72±0.07, 0.09±0.01, 8.39±1.09 and 32.58±0.34 µg/ml in DPPH, ABTS, H_2O_2 and nitric oxide methods, respectively. Mangiferin failed to show potent activity in rest of the methods. Benzoyl (MGN 1) and acetyl (MGN 2) substituted mangiferin were showed potent to moderate activity in all the methods. MGN 1 and 3 showed better activity thanmangiferin in deoxyribose, p-NDA and lipid peroxidation methods. In alkaline DMSO method, only MGN 1exhibited potent activity (Table 2). In p-NDA, lipid peroxidation and alkaline DMSO methods, MGN 1showed better activity than the standards. Mangiferin substituted with acetyl and benzoyl groups was beneficial for the activity. Based on these results, MGN1 and 3 along with mangiferin were chosen for comparing their *in vivo* analgesic and anti-inflammatory activities.

3.3 Analgesic Activity of Selected Mangiferin Derivatives

In the hot plate method, a significant increase in the response time was observed for mangiferin and MGN 1 at both the doses after 15, 30 and 45 min, MGN 3 at 20 mg/kg after 30 and 45 min, when compared to control. Similar results were observed for standard pentazocine at 20 mg/kg. MGN 1 showed better results than mangiferin. However, the strandardpentazocine produced better results compared to the other treated groups. The percentage protection after 45 min for all the compounds ranged between 51.59 to 72.45% (Table 3). A significant increase in the tail withdrawal reflex was observed for MGN 1 at both the doses and mangiferin at 20 mg/kg. MGN 1 showed better activity than mangiferin. The percent protection was found to be 9.11 to 55.34%.

Injection of acetic acid into control mice produced 43.83 ± 2.32 writhes. Pre treatment with mangiferin and its derivatives at both the doses significantly reduced the number of writhes (Table 3). Mangiferin at both the doses showed better activity than its derivatives. The percent protection was found to be 37.26 to 63.11%. However, the standard pentazocine showed better and significant results than all other treated groups.

3.4 Anti-inflammatory Activity of Selected Mangiferin Derivatives

In carrageenan induced paw edema in rats, mangiferin and MGN 1 produced significant activity at 20 mg/kg dose during 30 to 360 min measurements, compared to the control. Mangiferin, MGN 1 at 10 mg/ kg and MGN 3 at 20 mg/kg showed significantly reduced paw edema after 120, 180 and 360 min (Table 4). However, the standard diclofenac at 20 mg/kg also produced similar and better results than the tested samples. The percent protection was found to be 28.17 to 37.09 after 360 min measurements.

4. DISCUSSION

Chemical modification of mangiferin for obtaining compounds with better activity has also been tried. One new and three known mangiferin derivatives were prepared and characterized.

Table 1. ^{1}H NMR spectrum of mangiferin and its derivatives

Proton	Reported	MGN	MGN 1	MGN 2	MGN 3	MGN 4
1-OH	13.76 (1H)	13.50 (1H)	13.50 (1H)	13.50 (1H)	-	13.50 (1H)
6-OH	10.55 (1H)	10.80 (1H)	-	-	-	-
7-OH	10.55 (1H)	10.80 (1H)	-	-	-	-
3-OH	9.86 (1H)	9.80 (1H)	-	-	-	-
3′,4′-OH	4.86 (2H)	4.90 (2H)	4.95 (2H)	4.90 (2H)	-	4.90 (2H)
6′-OH	4.49 (1H)	4.49 (1H)	4.50 (1H)	4.50 (1H)	-	4.49 (1H)
2′-OH	3.87 (1H)	4.60 (1H)	4.65 (1H)	3.75 (1H)	-	3.75 (1H)
8-H	7.38 (1H)	7.40 (1H)	7.45 (1H)	7.45 (1H)	7.40 (1H)	7.45 (1H)
5-H	6.86 (1H)	6.90 (1H)	7.00 (1H)	7.30 (1H)	6.90 (1H)	6.65 (1H)
4-H	6.37 (1H)	6.40 (1H)	6.50 (1H)	6.75 (1H)	6.40 (1H)	6.65 (1H)
1′-H	4.60 (1H)	4.60 (1H)	4.85 (1H)	4.70 (1H)	4.90 (1H)	4.60 (1H)
2′-H	4.05 (1H)	4.05 (1H)	4.00 (1H)	4.00 (1H)	4.00 (1H)	3.95 (1H)
6′a-H	3.69 (1H)	3.69 (1H)	4.50 (1H)	3.40 (1H)	4.00 (1H)	3.65 (1H)
6′b-H	3.42 (1H)	3.41 (1H)	4.60 (1H)	3.20 (1H)	4.00 (1H)	3.30 (1H)
3′-H	3.18 (1H)	3.18 (1H)	4.60 (1H)	3.40 (1H)	5.80 (1H)	3.30 (1H)
4′-H	3.18 (1H)	3.18 (1H)	4.85 (1H)	3.20 (1H)	5.30 (1H)	3.30 (1H)
5′-H	3.18 (1H)	3.18 (1H)	4.90 (1H)	3.20 (1H)	5.20 (1H)	3.30 (1H)
CH_3CO	-	-	-	-	1.20-2.00(24 H)	-
Benzene ring	-	-	7.20-8.30 (15H)	7.30 -7.50 (15H)	-	-
$-CH_2$	-	-	-	5.25-5.40 (6H)	-	-
$-OCH_3$	-	-	-	-	-	4.60-4.90 (9H)
Total number of protons	18	18	30	36	34	24

Table 2. *In vitro* antioxidant acivity of mangiferin and its derivatives using standard methods

Compound	IC_{50} values±SEM (µg/ml) by methods*							
	DPPH	ABTS	Deoxyribose	p-NDA	H_2O_2	Nitric oxide	Lipid peroxidation	Alkaline DMSO
Mangiferin	1.72±0.07	0.09±0.01	502.20±3.20	106.84±0.07	8.39±1.09	32.58±0.34	433.20±4.08	>1000
MGN 1	23.80±0.89	0.65±0.02	87.58±2.21	24.13±1.05	23.57±0.34	52.67±1.30	8.90±0.98	152.30±3.63
MGN 2	>1000	2.41±0.08	>1000	>1000	215.02±3.11	>1000	>1000	>1000
MGN 3	36.47±0.81	3.71±0.14	406.30±2.85	31.53±3.72	81.23±1.05	>1000	29.80±1.27	>1000
MGN 4	>1000	2.23±0.06	>1000	>1000	>1000	>1000	>1000	>1000
Ascorbic acid	4.92±0.28	11.25±0.49	-	>1000	193.45±2.30	-	-	>1000
Rutin	8.91±0.10	0.52±0.04	-	205.54±3.25	32.35±1.02	65.21±2.97	-	>1000
BHA	-	-	83.46±4.34	-	22.16±0.56	-	110.02±3.41	-

Average of three determinations

Table 3. Analgesic activity of mangiferin and its derivatives using standard methods

Treatment (dose, mg/kg, i.p.)	Hot plate test			Tail immersion	Acetic acid induced writhing
	Latency period , sec (% Protection)			Latency period, sec (% Protection)	No. of writhing(% Protection)
	15 min	30 min	45 min		
Control	2.32±0.25	2.27±0.26	2.43±0.27	4.39±0.60	43.83±2.32
Mangiferin (10)	3.21±0.25(27.73)	4.58±0.47^{b}(50.44)	6.46±0.71^{b}(62.38)	5.70±0.50(22.98)	23.33±2.99^{a}(46.77)
Mangiferin (20)	4.97±0.32^{a}(53.32)	5.46±0.54^{a}(58.42)	7.35±0.95^{a}(66.94)	8.69±0.49^{a}(49.48)	16.17±2.33^{a}(63.11)
MGN 1 (10)	3.90±0.47^{c}(40.51)	4.75±0.45^{b}(52.21)	7.14±0.50^{a}(65.97)	7.64±0.60^{c}(42.54)	24.17±2.93^{a}(44.85)
MGN 1 (20)	6.16±0.72^{a}(62.34)	6.66±0.27^{a}(65.92)	8.82±0.51^{a}(72.45)	9.83±0.89^{a}(55.34)	17.33±1.69^{a}(60.46)
MGN 3 (10)	3.08±0.19(24.68)	3.85±0.46(41.04)	5.02±0.42(51.59)	4.83±0.41(9.11)	27.50±2.32^{a}(37.26)
MGN 3 (20)	3.61±0.33(35.73)	4.58±0.50^{b}(50.44)	5.79±0.44^{b}(58.03)	7.04±0.81(37.64)	23.50±2.69^{a}(46.38)
Pentazocine (20)	5.11±0.32^{a}(54.60)	6.85±0.50^{a}(66.86)	10.61±1.14^{a}(77.10)	10.21±1.00^{a}(57.00)	7.67±1.45^{a}(82.50)

Values are given as mean±S.E.M. for groups of six animals each, Dunnet's test; values are statistically significant at $^{a}P<0.001$,$^{b}P<0.01$, $^{c}P<0.05$ between control and treated groups

Table 4. Anti-inflammatory activity of mangiferin and its derivatives against carrageenan induced paw edema in rats

Treatment (dose, mg/kg, i.p.)	Paw volume, ml after min (% Protection)					
	0	30	60	120	180	360
Control	0.84±0.02	1.63±0.04	1.77±0.05	1.90±0.07	1.96±0.07	2.13±0.06
Mangiferin (10)	0.91±0.04	1.51±0.07(7.36)	1.60±0.06(9.60)	1.56±0.03^{a}(17.89)	1.52±0.08^{a}(22.45)	1.48±0.08^{a}(30.52)
Mangiferin (20)	0.98±0.08	1.39±0.04^{b}(14.72)	1.46±0.04^{b}(17.51)	1.43±0.06^{a}(24.74)	1.38±0.05^{a}(29.59)	1.34±0.04^{a}(37.09)
MGN 1 (10)	0.98±0.05	1.55±0.04(4.90)	1.57±0.04(11.30)	1.59±0.04^{a}(16.32)	1.55±0.09^{a}(20.92)	1.52±0.05^{a}(28.64)
MGN 1 (20)	1.00±0.06	1.40±0.04^{b}(14.10)	1.40±0.06^{a}(20.90)	1.41±0.04^{a}(25.79)	1.36±0.03^{a}(30.61)	1.37±0.05^{a}(35.68)
MGN 3 (10)	0.95±0.05	1.58±0.03(3.07)	1.67±0.06(5.65)	1.60±0.07^{b}(15.79)	1.55±0.07^{a}(20.92)	1.53±0.06^{a}(28.17)
MGN 3 (20)	1.02±0.03	1.51±0.06(7.36)	1.55±0.04(12.43)	1.57±0.04^{a}(17.37)	1.45±0.08^{a}(26.02)	1.42±0.07^{a}(33.33)
Diclofenac (20)	0.89±0.04	1.22±0.05^{a}(25.15)	1.25±0.05^{a}(29.38)	1.30±0.03^{a}(31.58)	1.16±0.02^{a}(40.82)	1.13±0.03^{a}(46.94)

Values are given as mean ± S.E.M. for groups of six animals each, Dunnet's test; values are statistically significant at $^{a}P<0.001$,$^{b}P<0.01$, $^{c}P<0.05$ between control and treated groups

Mangiferin on benzoylation, showed an intense ionic peak at m/z 755 for [M+Na-2H) of tribenzoyl derivative in its positive mode ESI-MS spectrum. In its IR spectrum, it exhibited characteristic absorption bands at 3352 (OH), 1747, 1742 (ester carbonyl), 1618 (C=O), 1600 and 1556 (C=C of aromatic ring) and at 1091 cm^{-1} (C-O). The presence of signals at δ 7.20-8.30 (15H) indicated the presence of benzoyl groups in the molecule. Further disappearance of –OH group signals at 3, 6 and 7^{th} position in ^1H NMR suggesting the position of the benzoylation in the hydroxyl groups of the basic molecule.

Mangiferin on benzylation, showed intense ionic peaks at m/z 689, 599 and 510 for tribenzyl, dibenzyl and monobenzyl derivatives in their negative mode ESI-MS spectrum. In its IR spectrum it exhibited characteristic absorption bands at 3392 (OH), 1647 (C=O), 1608 and 1577 (C=C of aromatic ring) and at 1080 cm^{-1} (C-O). The presence of three signals at δ 5.40, 5.30 and 5.25 each for two protons accompanied with the aromatic protons at δ 7.30, 7.40 and 7.50 suggests the presence of a tribenzyl derivative of mangiferin.

Mangiferin on acetylation, showed an intense ionic peak at m/z 611, 653, 695 and 755 for [M–3H] ion of tetra acetyl, penta acetyl, hexa acetyl and octa acetyl derivatives, respectively in its positive mode ESI-MS spectrum. In its IR spectrum, it exhibited characteristic absorption bands at 1751 (ester carbonyl), 1651 (C=O), 1620 (C=C of aromatic ring) and at 1055 cm^{-1} (C-O). The ^1H NMR values showed signals at δ 1.20-2.00 (24H) indicating the formation of an octa acetyl derivative. The position of the acetylation in the octa acetyl derivative is at all the –OH groups, which is evident from the downfield shift of the corresponding protons in the ^1H NMR spectrum. The hydroxyl group signals disappeared in the acylated spectrum.

Mangiferin on methylation, showed an intense ionic peak at m/z 450, and a pseudo molecular ion peak at m/z 464 for $[M]^+$ ion and analyzed for $C_{22}H_{24}O_{11}$ in its positive mode ESI-MS spectrum. In its IR spectrum, it exhibited characteristic absorption bands at 3367 (OH), 1647(C=O), 1606 (C=C of aromatic ring) and at 1064 cm^{-1} (C-O). The presence of signals at δ 4.60-4.90 (9H) suggests a trimethyl derivative of mangiferin. The signals of the remaining protons are same as that of mangiferin. This suggests that methylation occurred in the xanthone nucleus (C-3, 6 and 7).

All the synthesised mangiferin derivatives were screened for their *in vitro* antioxidant activity using various standard methods. Mangiferin showed better activity in DPPH, ABTS, H_2O_2 and nitric oxide methods when compared to all the derivatives. Furthermore, mangiferin was poorly active in lipid peroxidation, p-NDA, deoxyribose and alkaline DMSO methods. A possible reason for these results is that mangiferin has ability to scavenge free radicals formed in the initial step of lipid peroxidation as has been noticed previously in rat liver microsomes [25]. MGN 1 and MGN 3 showed potent activity than mangiferin in lipid peroxidation, p-NDA, deoxyribose and alkaline DMSO methods revealed that acetyl and benzoyl group substitution is beneficial for the activity. However, methyl and benzyl mangiferin abolishes the antioxidant activity in most of the methods. Based on these results, compounds MGN 1 and MGN 3 along with mangiferin were chosen for comparing their *in vivo* analgesic and anti-inflammatory activities with mangiferin.

4. CONCLUSION

In analgesic and anti-inflammatory studies MGN 1 and MGN 3 did not produce significant activity when compared to mangiferin. However, MGN 1 showed slightly better results than

mangiferin in analgesic models, but it failed in acetic acid induced writhing and anti-inflammatory studies. Further studies are required to confirm its mechanism of action.

COMPETING INTERESTS

Authors declare that there are no competing interests.

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