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Original Article

FORMULATION, EVALUATION, AND *IN VIVO* ANTI-INFLAMMATORY AND ANTI-ARTHRITIC ACTIVITIES OF *MORINGA* GRANULES

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ABSTRACT

Objective: Consumption of crude natural products like plants and herbs for mitigation or treatment of illnesses usually accompanied with inconsistent therapeutic effects because of poor solubility and low bioavailability of active phytochemical(s) in addition to product instability. To overcome all of above mentioned drawback ethanol extract of *Moringa oleifera* leaf was formulated as standardised solid dosage form.

Methods: Different types of materials as an adsorbent, surfactant and other necessary excipients were tested to be use in formulation of *Moringa* granules utilising wet granulation method. The formulated *Moringa* granules was then evaluated for organoleptic properties and physical characteristics, *in vitro* dissolution test, compatibility, drug content, heavy metal tests and microbial limit tests. Additionally, the *in vivo* anti-inflammatory against Carrageenan-induced paw oedema and anti-arthritic activity against CFA-induced arthritis were also assessed.

Results: 95% ethanol extract of *M. oleifera* leaves was successfully formulated as standardised granules for oral administration utilising simple and low-cost techniques. Dissolution rate for the marker compounds was increased by an average of 1.076 fold. Animal groups given the prepared *Moringa* granules showed an improvement in the anti-inflammatory activity and the anti-arthritic activity compared to animal groups given crude extract at the same dose level. Additionally, all the treatment groups showed a significant difference at $P<0.05$ and $P<0.01$ compared to control group.

Conclusion: To the best of our knowledge, this work was the first to use gum Arabic in the formulation of a standardised botanical pharmaceutical dosage form of *M. oleifera* crude extract. Additionally, formulation of *Moringa* granules apparently improves the drug release profile and bioactivity compare to Crude *Moringa* extract.

Keywords: *Moringa oleifera, Moringa* granules, Organoleptic properties, Anti-inflammatory, Anti-arthritic

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INTRODUCTION

The interest in botanical products has been regenerated because of the discovery of many novel natural bioactive phytochemicals [1]. However, the therapeutic potential of these compounds is often limited by their poor solubility, low bioavailability and product instability [2]. In this regard, pharmaceutical scientists should explore delivery systems that can enhance drug solubility and bioavailability and improve product stability. The complexity of phytoconstituents and the indecision about the phytochemical(s) responsible for bioactivity are the most challenging problems in formulation and evaluation of herbal products.

Moringa oleifera Lam is well-known and most widely cultivated species of *Moringa*ceae, which is a mono-generic flowering plant family that comprises 13 species. *M. oleifera* has been founded in many tropic and sub-tropic regions worldwide. The plant is referred to by a number of names such as horseradish tree, drumstick tree, ben oil tree, miracle tree, and "Mother's Best Friend". The leaf of *M. oleifera* is the most valuable part of the plant used as food and traditional medicine source. Large number of nutrients as such proteins, minerals and fibres that affords an important role in the human diet as well as it is a valuable nutritive, healing and socioeconomic potentials [3]. An online survey indicates a large number of *M. oleifera* medicinal and dietary supplement products, alone or in herbal combinations, available in the global markets. The products are mostly in oral dosage form as tablets, capsules, tea bags, elixir, syrup and leaf powder for decoction. Our survey and that conducted by [4] revealed that almost all of the reviewed products shared one or more features of the following variable in product composition, not standardised against a bioactive phytochemical marker(s) and inconsistence in medicinal indications and uses despite originating from the same plant part and using the same dose strength and same manufacturing processes.

In this study, an ethanol extract of *M. oleifera* leaves had been formulated as a standardised granules, evaluated for their organoleptic properties, physical and chemical characteristics, and subjected to *in vitro* dissolution test. Additionally, *in vivo* bioactivity of the formulated granules as anti-arthritis and anti-nociceptive were also evaluated. To the best of our knowledge, this the first time that ethanol extract of *Moringa oleifera* leaves was formulated as a solid dosage form with standardised content of marker compounds, standardised drug release rate and improved bioactivity.

MATERIALS AND METHODS

Chemicals and reagents

Astragalin (Kaempferol-3-*O*-glucoside) 98% purity, Extrasynthese (Genay, France); Cryptochlorogenic acid (4-*O*-Caffeoylquinic acid) 98% purity, Chemfaces (Wuhan, China); Ethanol 95%, Fisher Scientific (Selangor, Malaysia). λ-Carrageenan, Indomethacin, Complete Freund's Adjuvant (CFA) (1 mg/ml) of heat killed dried *M. tuberculosis* (strain H37Ra, ATCC 25177) suspended in 1.5 ml mannide monooleate and 8.5 ml, and Tween (Polysorbate) 20, 40, and 80all purchased from Sigma-Aldrich, St., MO, USA. Gum Arabic and HPMC, Methocel K100M CR were purchased from Colorcon Ltd, New Hampshire, USA. Magnesium stearate, RandM Marketing, Essex, UK; Microcrystalline cellulose (Avicel PH200) 200, FMC BioPolymer, Newark DE, USA.

Equipment and instruments

BUCHI R-114 rotary vacuum evaporator, BUCHI Labortechnik AG (Flawil, Switzerland); Drying oven, Memmert GmbH and Co. (Schwabach, Germany); Shimadzu HPLC system, Shimadzu Corporation (Kyoto, Japan); Digital micrometer, Mitutoyo, ID-C1012EXBS, Kawasaki, Japan; Electrolab ETD-1020 Tap Density

Tester (Globe-Pharma) Ireland; Fourier-transform infrared spectrometer Thermo Nicolet Nexus 470, USA.

Authentication of plant materials

The plant was authenticated by Dr. R. Zakaria, Plant Sciences; and sample was reserved in the herbarium of the School of Biological Sciences, Universiti Sains Malaysia (USM) under a voucher specimen No. 11626

Formulation of *Moringa* granules (*Moringa*-G)

The 95% ethanol extract of *M. oleifera* leaves prepared by maceration of dried, pulverised leaves in 95% ethanol at ratio of 1:5 and temperature of 45 °C for 72 h. The collected extract was first filtered using Whatman No.1 filter paper, concentrated using rotary evaporator then dried in drying oven at 45 °C for 48h. The obtained dry extract was formulated as common granules using wet granulation method. Selection of surfactant and adsorbent agent were based on the results obtained from preformulation studies of 95% ethanol extract of *M. oleifera* leaves [5]. Ethanol (95% v/v) was used as wetting agent enough to made *Moringa* extract as a paste. Tween 20 and gum Arabic were added and kneaded. The prepared mixture then speared on stainless steel tray and dried in drying oven at 45 °C for 48 h. The dried mass was screened through sieve No.22 (0.8 mm) and dried again in drying oven at 45 °C for 24 h and screened through sieve No. 35 (0.5 mm). The collected granules then evaluated.

Organoleptic properties and physical characteristics

The formulated *Moringa*-G were evaluated for their organoleptic properties, namely, appearance, colour, odour and taste. Particle size distribution was assessed using the sieve method. Loss on drying at 65 °C, the pH of 1% (w/v) solution at 25 °C and reconstitution time were determined as described by [6].

Angle of repose

A stainless-steel funnel with a 10 mm orifice diameter and 111 mm length from the top to the end of the orifice was fixed at 4 cm from the bench to the funnel orifice. In brief, 5 g of powder sample was charged into the funnel and allowed to fall. The height (h) and width (w) of the pile were measured. The results were considered valid only when a symmetric cone was obtained. The procedure was repeated in triplicate. Angle of repose (θ) was calculated as follows:

$$\boldsymbol{\theta = tan^{-1}\left(\frac{h}{0.5w}\right) \ldots\ldots Eq.\ (1)}$$

Bulk density and tapped density

In brief, 10 g sample was transferred into a 25 ml graduated cylinder with 0.5 ml mark. The cylinder was manually tapped gently twice on a table top surface, and the volume was measured. Bulk density was calculated as follows:

$$\rho bulk = \frac{W}{vb} \ldots\ldots\ldots \text{Eq. (2)}$$

Where w is the weight of the sample, and V_b is the volume occupied by the bulk powder.

The cylinder was fixed to the tap density tester and subjected to 500, 750 and 1250 taps at a rate of 250 taps/min. After each specified number of taps, the tapped volume was measured. Tapped density was calculated as follows:

$$\rho tapped = \frac{W}{vt} \ldots\ldots\ldots \text{Eq. (3)}$$

Where w is the weight of the sample, and V_t is the volume occupied by the powder after tapping.

Carr's compressibility index and Hausner's ratio

The results of bulk and tapped densities were used to calculate Carr's compressibility index (Eq. 4) and Hausner's ratio (Eq. 5). These parameters help in estimating the flow properties and compressibility of the powder.

$$CI = \frac{\rho tapped - \rho bulk}{\rho tapped} \times 100 \ldots\ldots\ldots \text{Eq. (4)}$$

$$\text{Hausner's ratio} = \frac{\rho tapped}{\rho bulk} \ldots \text{Eq. (5)}$$

***In vitro* drug dissolution and drug release profile**

In vitro drug dissolution was determined using USP-apparatus II (paddle apparatus). The samples of *Moringa*-G and *M. oleifera* crude extract were immersed in 500 ml of distilled water as dissolution medium at 37±0.5 °C and paddle speed of 100 revolutions per minute. Six dosage units were individually placed in each of the six vessels of the dissolution apparatus. Aliquots of the dissolution medium (3 ml) from each of the six vessels were withdrawn on 5, 15, 30, 45, 60, 90, 120 and 180 min, combined and filtered with a 0.45 mm syringe filter to achieve a pooled sample that will be used as test samples. The volume of the dissolution medium was kept constant by adding 3 ml of the solution pre-heated to 37 °C to fresh distilled water after each withdrawal. The samples were analysed for drug concentration using HPLC method described by [5]. Percent cumulative drug release for *Moringa*-G and *M. oleifera* crude extract were calculated and plotted against time. Graphical analysis method was used for comparison of the dissolution pattern, and the reference markers concentrations at each time point were used to compare the extent of dissolution. Percent dissolution efficiency (%DE) was used to characterise the release profile. DE can be calculated using the following equation:

$$DE\% = \left[\frac{\int_{t1}^{t2} y \times dt}{y100 \times (t2 - t1)}\right] \times 100 \ldots \text{Eq. (6)}$$

Where y is the percent dissolved reference marker at specified time and y_{100} is the maximum dissolution percent.

Compatibility of excipients

Fourier-transform infrared (FT-IR) spectroscopy analysis of 95% ethanol extract of *M. oleifera* leaves and formulated *Moringa*-G were conducted to evaluate drug-excipient compatibility. In brief, a 1% mixture of 95% ethanol extract of *M. oleifera* leaves/*Moringa*-G with IR-grade potassium chloride was prepared as disc and analysed by FT-IR spectroscopy with single-reflection ATR attachment within the IR range of 400 $cm^{-1}$to 3000 cm^{-1}.

Drug content

Drug content was calculated using HPLC method described by [4]. The test solution was prepared by accurately weighed a portion of prepared *Moringa*-G equivalent to 100 mg of *M. oleifera* extract, dissolved in 10 ml of 50% methanol, sonicated and filtered using 0.45 mm syringe filter to obtain 10 mg/ml solution. A standard calibration curve was plotted and mean drug content (%) was calculated as the average of three determinations (n=3).

Microbial limit tests

Microbial limit test was conducted to quantitatively estimate the total number of aerobic microorganisms, total yeasts and moulds, bile-tolerated Gram-negative bacteria, *Salmonella* sp., *Escherichia coli*, *Staphylococcus aureus* and *Pseudomonas aeruginosa* in accordance with [7], Appendix XVIB. The test microbes used in determination of microbial limit test were obtained from School of Biology, USM, Malaysia.

Heavy metals test

Heavy metal analysis was conducted as described by [7], Appendix IID. Inductively coupled plasma–atomic emission spectroscopy (ICP-AES) technique was used to determine the levels of cadmium, arsenic, lead and mercury.

Animal ethics and laboratory animal

Animal's experimental protocol was approved by Animal Ethics Committee of Universiti Sains Malaysia No.: USM/Animal Ethics Approval/2016/(103) (764) and USM/Animal Ethics Approval/2016/(103)(807). Healthy male Sprague-Dawley rats 8-10 w old and weighing 150-180 g were used. They were fed with standard pellet diet and free access to water. They were housed in polypropylene cages, 3 animals per cage maintained under 12 hour

light, 12 hour dark cycle; 27±3˚C, 45 %±5% relative humidity and allowed to acclimatise for 7 d before starting the experiments.

***In vivo* anti-inflammatory activity**

The anti-inflammatory activity of *Moringa*-G was assessed using Carrageenan-induced paw oedema method and the selection of the administered dose was as described by [8]. The animals were fasted 12 h before experiment, but provided with water *ad libitum.* The rats were randomly assigned into four groups of six animals each (n = 6), weighed and numbered. Group I was the disease control group given only distilled water. Group II was orally treated with indomethacin (5 mg/kg body weight). Group III was orally treated with *M. oleifera* crude extract at a dose of 250 mg/kg body weight and group IV was orally treated with *Moringa*-G at dose equivalent to 250 mg/kg body weight. After 1 h of oral dose treatment, inflammatory paw oedema was induced by injecting 0.1 ml of freshly prepared 1% (w/v) carrageenan in normal saline into the sub-plantar region of the left hind paw of the rats. The thickness of the left hind paw was measured using a digital micrometre immediately before and at 1, 2, 3, 4 and 5 h after the carrageenan injection. The anti-inflammatory activity of *M. oleifera* crude extract and *Moringa*-G were calculated as percent increase in paw oedema by using the formula:

$$\% \text{ increase in paw oedema} = \frac{A-B}{B} \times 100 \ldots\ldots\ldots \text{Eq. (7)}$$

Where *A* is the paw thickness at the respective hours, and *B* is the paw thickness at hour 0 of the experiment. The results were expressed as mean % oedema±standard error of the mean (SEM) of six animals per group and statistically analysed compared with the disease control group.

***In vivo* activity against CFA-induced rheumatoid arthritis**

Animals and experimental design

The animals were randomly designed into five groups of five animals per group (n = 6). Group I: (Normal control) without CFA injection and treatment; group II: (CFA-control) CFA injection and vehicle 10 ml/kg/day; group III: CFA injection and oral treatment with indomethacin at 2.5 mg/kg/day; group IV: CFA injection and oral treatment with 250 mg/kg/day *M. oleifera* extract and group V: CFA injection and orally treated with *Moringa*-G at dose equivalent to 250 mg/kg/day. The daily oral dose for groups II to V were administered at 9 am and continued to day 21 post-injection. Measurements were conducted at 2 pm.

Induction of arthritis

Immunological arthritis was induced using the method described by [9]. The hind limbs of rats in groups II to V were shaved and sterilised with 70% (v/v) alcohol. Subsequently, 0.1 ml of CFA containing 1 mg/ml heat-killed *M. tuberculosis* were injected into the sub-plantar area of the left hind paw of each animal under mild anaesthesia with diethyl ether. The time of CFA injection was referred to as day 0.

Assessment of rheumatoid arthritis

CFA-induced arthritic paw oedema

The thickness of the left and right hind paws was recorded on day 0 before the induction of arthritis with CFA and considered as a baseline. The thickness of the paws was measured again on days 3, 6, 9, 12, 15, 18 and 21 post-CFA injection using a digital micrometre gauge. The percent increase in paw oedema was calculated using equation (7).

Change in animal's body weight

The body weight of the animals was monitored starting from day 0 and repeated on days 3, 6, 9, 12, 15, 18 and 21 post-CFA injection. Percent weight change was calculated using the following formula:

$$\% \text{ weight change} = \frac{\text{Wt} - \text{Wo}}{\text{Wt}} \times 100$$

Where W_t is the weight of the animal at time *t*, and W_o is the weight of the animal on day 0. The calculated weight change in the treatment groups was statistically compared with those in normal control and CFA-control groups.

Arthritic index

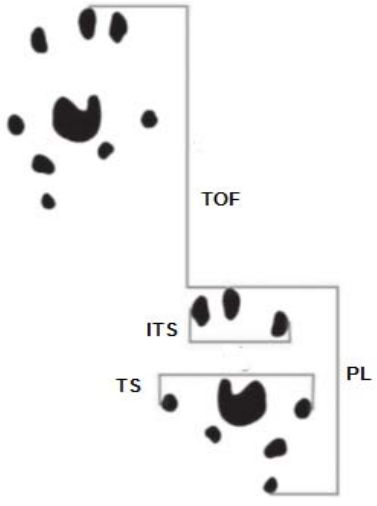
Arthritic index is a numeric system used to score the severity and disease progression of CFA-induced immunological arthritis. Development and severity of induced RA in rats were evaluated by a visual scoring system of the clinical signs and symptoms on a scale of 0–4 per limb by using the method described by [10] where 0: no change, 1: slight swelling and erythema of the finger, 2: swelling of the phalanx joint and digits, 3: severe swelling of the entire paws, and 4: deformity or ankylosis. The scores for the left and right hind limbs were combined, and the maximum score was 8. A score of 1 and above indicates arthritis. Measurement was started on day 3 post-CFA injection to avoid the pre-acute phase of CFA and then repeated on days 6, 9, 12, 15, 18 and 21 post-injection.

Walking track analysis

Walking track analysis was initially described by [11] as an approach to analyse changes in locomotion and functional behaviour. The footprints of each rat were recorded by immersing their hind paws in the ink and allowing them to walk over a paper sheet (8.7 cm wide by 43 cm long) fixed on a confined walkway corridor, with a dark shelter at the end. After two or three conditioning trials, when the rats were often stopped to explore the corridor, they began to walk steadily to the dark shelter end. The procedure was repeated on day 0 before CFA injection and on days 14 and 21 post-injection. The obtained data from the rat's footprint were used to calculate the functional index (FI) by using the following equation:

$$\text{FI} = [\left(\frac{\text{ETOF} - \text{NTOF}}{\text{NTOF}}\right) + \left(\frac{\text{NPL} - \text{EPL}}{\text{EPL}}\right) + \left(\frac{\text{ETS} - \text{NTS}}{\text{NTS}}\right) + \left(\frac{\text{EIT} - \text{NIT}}{\text{NIT}}\right)] \times \frac{220}{4}$$

where ETOF and NTOF are experimental (injected paw) and normal paw distance, respectively, to the opposite foot (mm); EPL and NPL are experimental and normal paw print length (mm), respectively; ETS and NTS are experimental and normal total paw spreading (mm), respectively; and EIT and NIT are experimental and normal intermediary toe spreading (mm), respectively. In the normal value range (±11%), 0 represents a totally normal paw and 100 represent a totally impaired paw.



X-ray radiographic assessment

On day 21 post-CFA injection, radiographs were taken with X-ray apparatus (PHILIPS Diagnose X-ray) at 55 kVp and 3 mA with focal film distance of 60 cm. The severity of joint deformation was blindly scored in accordance with the extent of oesteoporosis, joint spaces, soft tissue inflammation, subchondral erosion and joint ankylosis on

a scale of 0–4 as described by [10]; the scores are as follows: 0: no soft tissue inflammation and no degenerative joint changes; 1: slight soft tissue inflammation, joint space, subchondral erosion, oesteolysis and degenerative joint changes, 2: moderate soft tissue inflammation, joint space, subchondral erosion, oesteolysis and degenerative joint changes; 3: marked soft tissue inflammation, joint space, subchondral erosion, oesteolysis and degenerative joint changes; and 4: severe soft tissue inflammation, joint space, subchondral erosion, oesteolysis and degenerative joint changes. The X-ray images were analysed and scored independently by two certified radiologists who were blinded to the groups.

Haematology profile

On day 22 post-CFA injection, 4.5 ml of blood was collected from each animal through cardiac puncture under mild anaesthesia with diethyl ether and placed in suitable blood collection tubes (BD Vacutainer®). Haematological parameters including haemoglobin content (HGB), total red blood cell count (RBC), percent packed cell volume (% PCV) and total white blood cell count (WBC) were evaluated immediately after blood sample collection by using an automated haematology analyser (Beckman Coulter Blood Analyser). Erythrocyte sedimentation rate (ESR) was determined using the Westergren capillary tube method described by Guha *et al.* (2014).

Data analysis

The data obtained from *in vivo* experiments were expressed as mean±SEM; while other data were expressed as mean±SD. Statistical difference between means was analysed by IBM-SPSS version 20 software by using one-way ANOVA followed by post hoc Dunnett–t test (2-tailed) at different variance levels.

RESULTS AND DISCUSSION

Selection of adsorbent

The sticky, greasy texture of the *M. oleifera* extract requires the use of a suitable adsorbent to convert it into non-stick, freely flowable granules. Various adsorbents, natural or synthetic, were tested at the ratio of 1:1 to convert the *M. oleifera* extract into powder form (table 1). Three adsorbents showed a promising results, i.e. Avicel PH101, PVP-K12.5 and gum Arabic. For the three selected adsorbents, different ratios of adsorbents to the *M. oleifera* extract were tested (i.e. 0.5:1, 0.6:1 and 0.75:1) to determine the lowest useful ratio.

Table 1: Description of the granules when different adsorbents were mixed with 95% ethanol extract of *M. oleifera* leaf at 1:1 ratio

Adsorbent	Description
Avicel PH 200	Good shape coarse granules, dark green colour and need long drying time.
Avicel PH101	Good shape green colour granules.
Lactose monohydrate	Sticky soft very dark green colour mass and cannot be shaped as granules.
Starch (maize)	Soft light green colour mass and cannot be shaped as granules.
β-cyclodextrin	Sticky soft dark green mass and cannot be shaped as granules.
PVP-K12.5	Good shape green granules, hygroscopic during storage.
Gum Arabic	Good shape very dark green granules.

The prepared mixtures were evaluated for their flow properties and bulk and tapped densities (table 2). On the basis of the results of the angle of repose and density, gum Arabic was selected as the adsorbent to be applied at an adsorbent-to-*M. oleifera* extract ratio of 0.6:1.

Table 2: Evaluation of lowest ratio of adsorbents to *M. oleifera* extract for their flowability and density per adsorbent

Adsorbent	Lowest ratio	Angle of repose	Bulk density	Tapped density	Hausner's ratio
Avicel PH101	0.6:1	30.191±0.083	0.323±0.003	0.3488±0.0667	1.0788
Gum Arabic	0.6:1	33.690±0.112	0.4046±0.201	0.4563±0.412	1.1277
PVP-K12.5	0.75:1	37.364±0.0642	0.3448±0.0938	0.3726±0.113	1.0806

*Results were expressed as (mean±SD, n=3).

Selection of surfactant

Different types of surfactants, anionic and non-ionic, (Poloxamer 407, sodium lauryl sulphate, Tween 20, Tween 40, Tween 80, Span 20 and Span 40) were tested. Evaluation of the tested surfactants showed that Tween 20 was the best surfactant that improved the solubility of the *M. oleifera* extract. Tween 20 is a polyoxyethylene sorbitan monolaurate, that is, an amphipathic surfactants composed of fatty acid esters of polyoxyethylene sorbitan. Their popularity is largely due to their effectiveness at low concentrations and relatively low toxicities with an acceptable daily intake is up to 25 mg/kg body weight [12]. In addition, these surfactants are also widely used in the food industry because of their excellent emulsifying properties. Surfactant molecules form associates in the aqueous/non-aqueous solution beyond a certain concentration called micelles. The shape and size of the micelles are affected by many factors including temperature and concentration of surfactant. The consumption of missiles in the form of drug carriers is more valuable than other types of carriers owing to their tiny mass (~10–30 nm) and the enhanced stability of the drug in the course of missiles inclusion [13]. Accordingly, Tween 20 was selected to be used in the formulation of *Moringa*-G. Different concentrations of Tween 20 were then tested to determine the lowest useful concentration, which turned out to be 1% (w/w). Tween 20 was miscible with *Moringa* crude extract and other added excipients and can also improve bioavailability by acting as a P-glycoprotein inhibitor [14]. Identifying the solubility extent is important in determining the need for additional improvement steps.

Preparation of *Moringa* granules

On the basis of the results of the test adsorbents and surfactant agents above, gum Arabic was selected as adsorbent and Tween 20 was selected as surfactant for preparation of *M*oringa granules. The granules were prepared through wet granulation at a dose size of 3 g/dose as described by [8] to be the minimum effective dose. No need for adding preservative as ethanol extract of *M. oleifera* leaves was known for its antimicrobial activity against wide range of microorganisms [15]. In addition to that, usually alcoholic extract in contrast to aqueous extract does not need for preservatives or a stabilizer [16, 17].

Organoleptic properties and physical characteristics of the prepared *Moringa* granules (*Moringa*-G)

The prepared *Moringa*-G was evaluated for organoleptic properties, including appearance, colour, odour and taste (table 3). Particle size distribution was slightly wide in range due to the use of manual granulation.

Loss on drying as within the acceptable limit of USP37-NF32 (2014). The powder flowability test results, including angle of repose, Hausner's ratio and Carr's index, indicate the good flowability of the granules.

FT-IR spectroscopy of *Moringa*-G

The FT-IR spectra of the 95% ethanol extract of *M. oleifera* leaves and the prepared *Moringa*-G showed the compatibility of the used excipients with the extract (fig. 1). All the major peaks of the *M. oleifera* extract and *Moringa*-G were present. The spectra did not show additional peaks, shifts or disappearance of characteristic peaks. This result confirms the absence of any chemical or physical interaction between the *M. oleifera* extract and excipients used for granule preparation.

Table 3: Organoleptic properties and physical characteristics for the prepared *Moringa* granules

Parameter	Result
Appearance	Coarse granules not stick to container wall or aggregate together with acceptable shape
Colour	Very dark green
Odour	Faint characteristic odour
Taste	Slight bitter pungent taste
Particle size distribution	3.6%:700 µm 79.4%:500 µm 11.6%:350 µm 4.5%:210 µm
Loss on drying (%) at 65 °C	0.428%
pH of 5% aqueous solution at 25 °C	4.48
Time of reconstitution	50–60 second
Angle of repose	33.690±0.112
Bulk density	0.4206±0.201g/cm^3
Tapped density	0.4563±0.412 g/cm^3
Hausner's ratio	1.1277
Carr's index	11.33%

*Results were expressed as (mean±SD, n=3).

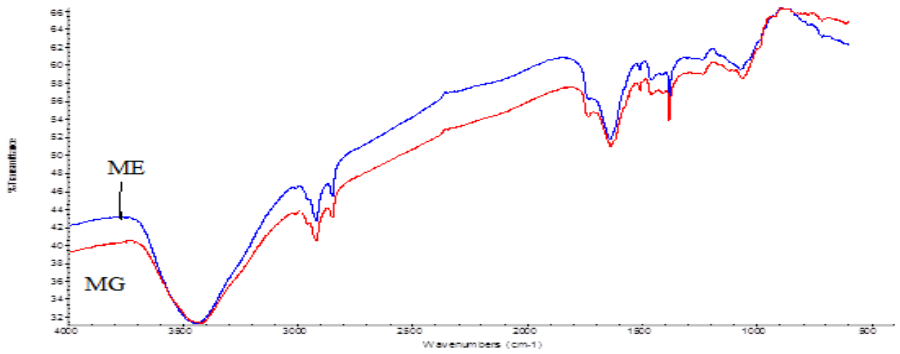


Fig. 1: FT-IR spectra of 95% ethanol extract of *M. oleifera* leaves (ME) and prepared *Moringa* granules (MG)

Drug content of the prepared *Moringa*-G

The drug content was determined using HPLC–UV method. The acceptance criterion of the initial concentration of the selected reference markers was 90%–110%. The percent drug contents for the three reference markers, namely, cryptochlorogenic acid, isoquercetin and astragalin, were 100.326±1.783, 99.631±1.322 and 97.406±1.392, respectively, which were equivalent to 3.555, 19.593 and 5.611 mg/g dry extract, respectively.

***In vitro* dissolution test for the prepared *Moringa*-G**

The dissolution test of the preparedness *Moringa*-G revealed an improved dissolution rate and extent compared with the crude *M. oleifera* extract (fig. 2). The prepared *Moringa granules* showed increase in dissolution rate of cryptochlorogenic acid, isoquercetin and astragalin by 0.66, 1.34 and 1.23 folds respectively.

This improvement in the dissolution profile could be due to the surfactant agent (Tween 20) adopted in the formula. Moreover, the gum Arabic fine powder used as an adsorbent and the formulation of *M. oleifera* extract as granules led to an increase in the surface area of the extract that exposed to the dissolution medium, thereby increasing the solubility. A low drug solubility is expected to yield a low dissolution rate. Based on the Noyes–Whitney law, the dissolution rate (dc/dt) is directly proportional to the concentration gradient ($C_0 - C_X$) and surface area.

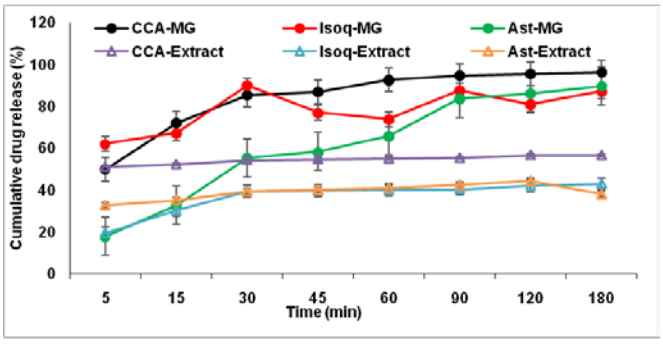


Fig. 2: *In vitro* drug release profile and dissolution rate of three reference markers present in prepared *Moringa* granules. Ast= astragalin; CCA= cryptochlorogenic acid and Isoq= isoquercetin. Data were presented as mean±SD (n=6)

Microbial limit tests of *Moringa*-G

Table (4) shows the microbial limit tests for the formulated *Moringa*-G which appeared to be free of any microbial contaminating.

Heavy metals tests for *Moringa*-G

Heavy metals, including arsenic, cadmium, lead and mercury, in the formulated *Moringa*-G were determined through [7]. The results revealed a non-detectable level of testing heavy metals in the formulations (table 5). Hence, the suitability and safety of the formulated *Moringa*-G were confirmed.

***In vivo* anti-inflammatory assay**

Animals in the groups treated orally with either indomethacin, 95% ethanol extract of *M. oleifera* leaves, or *Moringa*-G significantly inhibited the increase in the paw thickness (P<0.05) compared with those in the control group (fig. 3). The oral administration of the 95% ethanol extract of *M. oleifera* leaves showed less significant inhibition effect on the increase in the paw thickness (P<0.05) than *Moringa*-G. The effect of *Moringa*-G was comparable with that of indomethacin in terms of the extent and onset of activity. The oral administration of crude *M. oleifera* extract or *Moringa*-G began 2 h post-injection and continued until hour 5 post-injection. Formulation of *Moringa* crude extract as a granule lead up to improvement of the anti-inflammatory activity.

The higher extent of activity and the faster onset of action of *Moringa*-G than those of the crude *M. oleifera* extract were attributed to the effects of the formulation of the extract in its pharmaceutical dosage form. Formulating *M. oleifera* extract as granules led to higher solubility and enhanced absorption and bioactivity compared with crude *M. oleifera* extract. Finding of [18], who formulated aqueous *M. oleifera* leaf extract as film dressing for wound healing, and [19], who formulated *M. oleifera* leaf powder as tablet, reported improvements in the *in vivo* bioactivity of the extract after formulation.

Table 4: Microbial limit tests for the formulated *Moringa* granules

Microbial type	Result	Limit	Method
Total aerobic microbial count (TAMC)	ND	<10 CFU/g	BP 2013, Appendix XVIB
Total combined yeasts/moulds count (TYMC)	ND	<10 CFU/g	BP 2013, Appendix XVIB
Bile-tolerant gram-negative bacteria	ND	<10 PN/g	BP 2013, Appendix XVIB
Salmonella sp.	Absent	in 10 g	
Escherichia coli	Absent	in 1 g	BP 2013, Appendix XVIB
Staphylococcus aureus	Absent	in 1 g	BP 2013, Appendix XVIB
Pseudomonas aeruginosa	Absent	in 1 g	BP 2013, Appendix XVIB BP 2013, Appendix XVIB

*ND: not detected and BP: British Pharmacopoeia

Table 5: Heavy metals tests for detection of arsenic, cadmium, lead and mercury levels in the formulated *Moringa* granules

Heavy metal	Result	Limit	Method
Arsenic	ND	<0.1 mg/kg	BP 2013, Appendix IID, ICP-AES
Cadmium	ND	<0.1 mg/kg	BP 2013, Appendix IID, ICP-AES
Lead	ND	<0.1 mg/kg	BP 2013, Appendix IID, ICP-AES
Mercury	ND	<0.1 mg/kg	BP 2013, Appendix IID, ICP-AES

*ND: not detected and BP: British Pharmacopoeia.

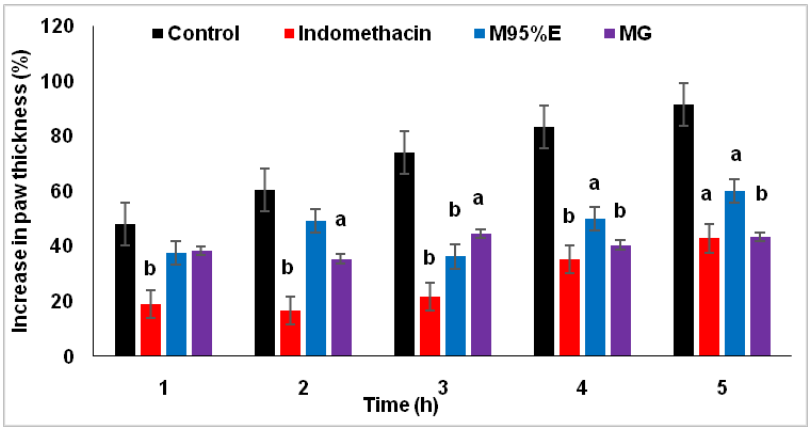


Fig. 3: Effect of oral administration of 95% ethanol *M. oleifera* leaves, indomethacin, and *Moringa* granules on carrageenan-induced hind paw oedema in rats. a: significant difference at P<0.05 and b: significant difference at P<0.01. Data were presented as mean±SEM for number of animals n=6

Effect of formulated *Moringa*-G on the paw thickness of rats

Oral administration of indomethacin, 95% ethanol extract of *M. oleifera* leaves, and *Moringa*-G significantly inhibited the increase in the paw thickness of rats with CFA-induced arthritis (P<0.05) compared with that in the CFA-control group (fig. 4). Based on statistical data analysis, the effect of *Moringa*-G was comparable with that of indomethacin. Furthermore, *Moringa*-G was generally more effective in inhibiting the increase in the paw thickness than *Moringa* crude extract.

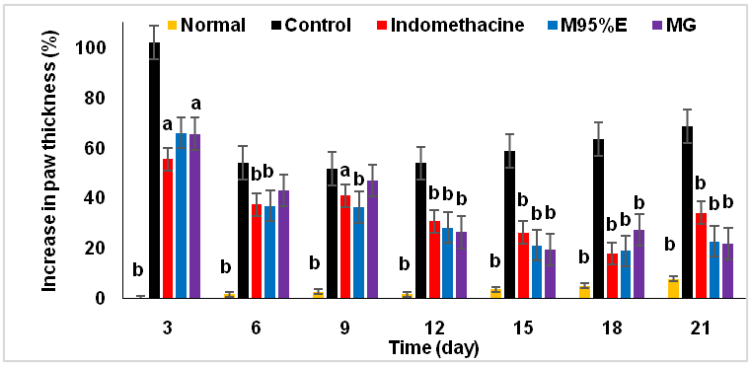


Fig. 4: Effect of oral administration of indomethacin, 95% ethanol extract of *M. oleifera* leaves, and *Moringa* granules on percent increase in paw thickness for CFA-induced arthritis rats. a: significant difference at P<0.05 and b: significant difference at P<0.01. Data were presented as mean±SEM for number of animals (n)=6

Effect of *Moringa*-G on the body weight of rats

Oral administration of 95% ethanol extract of *M. oleifera* leaves significantly increased the body weight of the animals (P<0.05) compared with that in the CFA-control group (fig. 5). Oral administration of indomethacin at 2.5 mg/kg/day and *Moringa*-G at dose equivalent to 250 mg/kg/day increased the body weight, but the effect was not significant. Rats in the normal group showed a steady increase in body weight. Rats in the group orally treated with crude *M. oleifera* extract showed higher percent weight gain than those in the normal group.

This result could be due to the high nutritional value and contents of essential amino acids and vitamins in the *M. oleifera* leaf extract. The insignificant increase in the body weight of rats in the group treated orally with indomethacin may be due to the adverse ulcerative and gastric disturbance effects associated with indomethacin [19]. For the rats treated with *Moringa*-G, the decrease in the body weight at the first 6 d, and the insignificant increase could be due to the effect of gum Arabic in reducing body weight and fat level [21].

Arthritic index

Oral administration of indomethacin at 2.5 mg/kg/day, 95% ethanol extract of *M. oleifera* leaves at dose 250 mg/kg/day, and *Moringa*-G at dose equivalent to 250 mg/kg/day significantly decreased the arthritic scores (P<0.05) compared with that in the CFA-control group (fig. 6). The animal group orally treated with *Moringa*-G achieved the lowest arthritic scores, i.e. less severe clinical signs of inflammation and arthritis, than the animal groups given with indomethacin and crude *M. oleifera* extract.

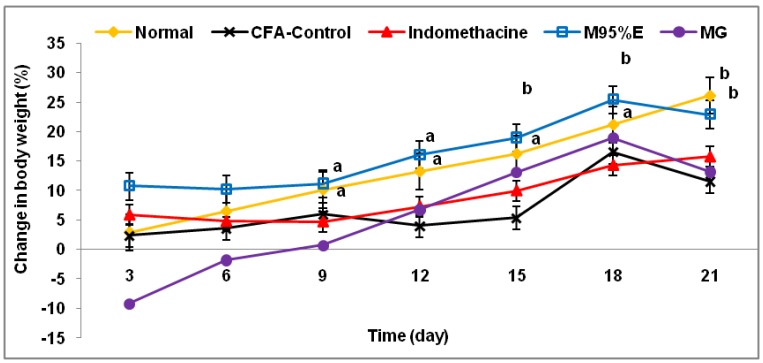


Fig. 5: Effect of oral administration of indomethacin, 95% ethanol extract of *M. oleifera* leaves, *Moringa* granules on percent increase in body weight for CFA-induced arthritis rats. a: significant difference at P<0.05 and b: significant difference at *P*<0.01. Data were presented as mean±SEM for number of animals (n)=6

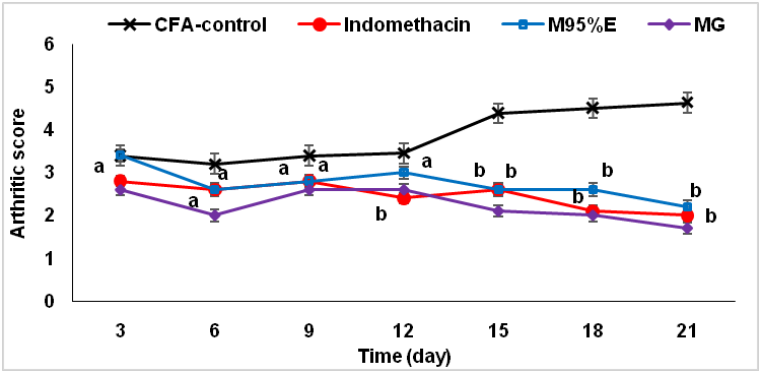


Fig. 6: Effect of oral administration of indomethacin, 95% ethanol extract of *M. oleifera* leaves, and *Moringa* granules on arthritic index of CFA-induced immunological arthritis rats. a: significant at P<0.05 and b: significant at P<0.01. Data were presented as mean±SEM for number of animals (n)=6

Walking track analysis using deMedinaceli method

Rats with CFA-induced arthritis treated orally with indomethacin at 2.5 mg/kg/day, 95% ethanol extract of *M. oleifera* leaves at 250 mg/kg/day, and *Moringa*-G at dose equivalent to 250 mg/kg/day exhibited functional index within the normal range (±11%) on day 21 (table 6). However, the CFA-control group showed an out of normal values after 3 w of CFA injection. Hence, *Moringa*-SD prevented the disruption of locomotion and gait behaviour.

X-ray radiography assessment

In the CFA-control group, soft tissue swelling (white arrows) and reduced joint spaces (black arrows) were observed, indicating the narrowing of the joint space under arthritic conditions (fig. 7). The average radiographic score for the CFA-control group (n=6) on day 22 post CFA injection was 9.66±1.16. Groups given indomethacin at 2.5 mg/kg/day, 95% ethanol extract of M. oleifera leaves at dose of 250 mg/kg/day, and Moringa-G at dose equivalent to 250 mg/kg/day for 21 d exhibited average radiographic scores of 3.17±1.08, 3.23±1.21, and 3.30±1.24, respectively. Statistical data analysis of the obtained scores (mean±SEM) revealed that 21 d of oral treatment with indomethacin, 95% ethanol extract of M. oleifera leaves, formulated Moringa-G significantly reduced (P<0.01) the radiographic score of rats with CFA-induced arthritis.

Haematology profile

All of the animals in the treatment groups and the normal control group were evaluated for haematological parameters including the HGB, total RBC, %PCV, total WBC and ESR (table 7). The erythrocyte profile of rats in the CFA-control group was below the normal value ranges. These abnormal results suggest the occurrence of iron deficiency anaemia, which is a clinical manifestation of RA. The erythrocyte profiles of rats in all of the orally treated groups were comparable with those in the normal group but significantly different (P<0.05) from those in the CFA-control group. These results could be due to enhanced solubility of phytonutrients in the *M. oleifera* extract and the consequent improvement in absorption.

Table 6: Effects of oral administration of indomethacin 2.5 mg/kg/d, 95% ethanol extract of *M. oleifera* leaves 250 mg/kg/d, and *Moringa* granules on functional index of CFA-induced arthritis rats

Group	Day 0	Day 14	Day 21
CFA-control	1.67±1.51	-1.41±1.99	-18.22±6.19
Indomethacin	-4.11±1.92	-1.55±6.12	-5.88±2.60
M95%E	-1.80±4.43	-3.06±4.27	-1.38±3.87
Moringa granules	2.23±2.45	-16.55±2.78	-7.89±2.16

*Results were expressed as (mean±SEM, n=6).

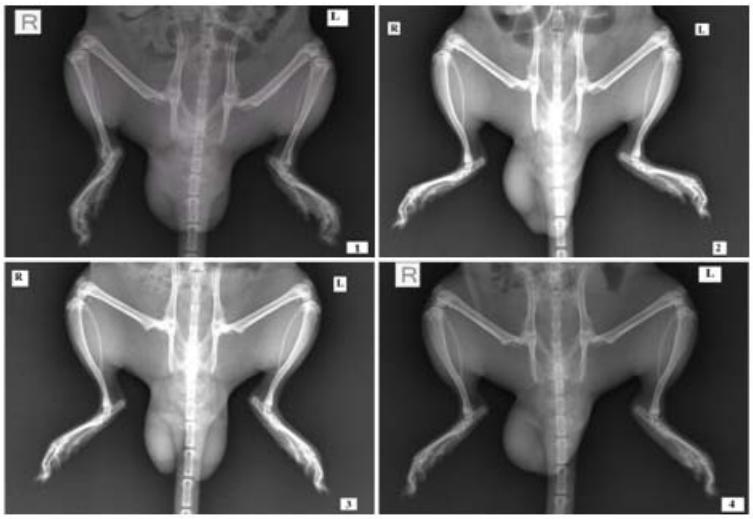


Fig. 7: X-ray radiographs for hind paws of CFA-induced arthritis rats at day 22 post CFA injection. 1: CFA-induced arthritis rat without treatment, 2: animal orally treated with indomethacin at dose 2.5 mg/kg/day, 4: animal orally treated with 95% ethanol extract of *M. oleifera* leaves at dose 250 mg/kg/day and 5: animal orally treated with *Moringa* granules at dose equivalent to 250 mg/kg/day

Table 7: Effects of oral administration of indomethacin, 95% ethanol extract of *M. oleifera* leaves, and formulated *Moringa* granules on haemoglobin content (HGB), total red blood cell count (RBC), % packed cell volume (%PCV), total white blood cells count (WBC) and erythrocyte sedimentation rate (ESR) in CFA-induced arthritis rats; results were expressed as mean±SEM and n=6

Haematology parameter	Normal range	Animal group				
		Healthy rats	CFA-Control	Indomethacin	M95%E	MG
HGB (g/dl)	13.5-18.4*	13.60±0.15[a]	12.25±0.79	13.30±0.45[a]	13.87±0.36[b]	13.65±0.74[a]
PCV (%)	38.9-54.9*	39.50±0.01[a]	35.75±0.02	39.50±0.02[a]	41.0±0.01[b]	39.75±0.17[a]
RBC (10[12]/l)	7.8-10.2*	7.86±0.09[b]	7.08±0.39	7.83±0.37[a]	7.95±0.23[a]	7.62±0.25[a]
WBC (10^9/l)	5.9-19.0*	9.70±2.19[a]	12.59±0.54	7.75±1.22[b]	9.76±0.74	10.48±0.24
ESR (mm/h)	0.5-1.45**	1.12±0.13[a]	2.04±0.04	0.45±0.05[b]	0.84±0.12[b]	1.01±0.09[b]

MG: *Moringa* granules; M95%E: 95% ethanol extract of *M. oleifera* leaf; *: normal range in rats according to Petterino and Argentino-Storino (2006); **: normal range in rats according to Ihedioha *et al.* (2017); a: significant at *P*<0.05 and b: significant at *P*<0.01 compared to CFA-control group, * Results were expressed as (mean±SEM, n=6).

The CFA-control group showed the highest WBC level, which is still within the normal range. The groups orally given indomethacin at 2.5 mg/kg/day showed a significant difference (P<0.05) in WBC compared with the CFA-control group. By contrast, the oral administration of crude extract of *M. oleifera* leaves and *Moringa*-G at dose equivalent to 250 mg/kg/day did not significantly affect the WBC compared with that in the CFA-control group.

Rats with CFA-induced arthritis treated orally with indomethacin at 2.5 mg/kg/day, 95% ethanol extract of *M. oleifera* leaves at 250 mg/kg/d, or *Moringa*-G at dose equivalent to 250 mg/kg showed significant decreases in the ESR (P<0.05) compared with rats in the CFA-control group.

CONCLUSION

In some cases processing of natural source products may effects on bioactivity of the final product, therefore, material processing was evaluated to ensure a consistent level of bioactivity. The main challenge in the formulation of herbal products is the complex nature of its constituents and the scarcity of fundamental information about the physical and chemical properties of plant extracts. The formulation of *M. oleifera* leaf extract as a standardised granule effectively improved their physical and chemical properties, particularly flowability, crystallinity degree, aqueous solubility, drug release profile and stability under stress conditions. In comparison with crude *Moringa* extract, the formulation of 95% ethanol extracts of *Moringa* leaves as a common granules improved their *in vivo* bioactivity. Haematological tests, body weight gain and arthritic index also indicated that the admissibility and health improvement induced symptoms of arthritis could be alleviated by the formulated *Moringa* extract were better than those promoted by to a greater extent than crude *Moringa* extract.

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AUTHORS CONTRIBUTIONS

Dr. Harith Jameel, the guarantor of this study, has designed, carried out the experiment, analyzed the results, and contributed in the preparation and revision of the manuscript. Assciate Prof Dr. Nurzalina Abdul Karim and Associate Prof Dr Roziahanim Mahmudhave designed, supervised the experimental process, and reviewed the manuscript.

CONFLICT OF INTERESTS

The authors declare no conflict of interest.

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