Preformulation, stress stability studies and HPLC-UV method development and validation for 95 % ethanol extract of Moringa oleifera Lam. Leaves

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Original Article

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ABSTRACT

Introduction: There is a continuous expansion in number of botanical medicinal products and increase in consumers who often use it. One of such natural source products with versatile traditional uses as treatment for variety of diseases is Moringa oleifera Lam.

Objectives: After the pharmacological activity of a new drug candidate is approved, the subsequent development of this product requires substantial information about its physical and chemical properties before designing its dosage form.

Methods: Organoleptic properties, physicochemical characteristics, solubility profile, swelling index, partition coefficient, analyses of thermal behaviour, stress stability, heavy metals and microbial limit tests were performed in preformulation studies of 95 % ethanol extract of M. oleifera leaves.

Results: The results of preformulation studies illustrated several characteristic properties that should be consider during formulation of Moringa extract. In addition to that, a HPLC-UV method for simultaneous detection and quantification of three reference markers was developed and validated.

Conclusion: The 95 % ethanol extract of Moringa leaf is relevant to the development of phytomedicines, dietary supplements or cosmetics. To the best of our knowledge, the present study is the first preformulation study which aimed to determine the physicochemical properties of 95 % ethanol extract of Moringa oleifera leaf.

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Key Words: HPLC-UV, moringa oleifera, preformulation study, Stress stability.

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INTRODUCTION

After the pharmacological activity of a new drug candidate is approved, the subsequent development of this pharmaceutical product requires substantial information about its physical and chemical properties before designing its dosage form. Prior to preclinical and clinical studies, introductory information, named 'preformulation' data, on the physical and chemical properties of the new drug candidate is required. Preformulation studies have evolved in the early 1960s as a consequence of advances in pharmaceutical product development to ensure the quality of the final product[1]. The United States Food and Drug Agency (FDA) stated that 'quality cannot be tested into the product, but rather it should be built into the product'[2]. From the FDA's viewpoint, conducting preformulation studies before selecting and designing the dosage form of a new drug is essential. Preformulation studies provide vital information to support the selection of pharmaceutical dosage forms and suitable excipients.

Traditionally, formulation of new drug entities was based on the 'trial-and-error' strategy, which is highly reliant on formulator experience. This formulation approach is time consuming, tedious and costly[3]. A 'quality-by-design (QbD)' approach is best to be considered for rapid product development while ensuring quality and avoiding hindrances[4]. A fundamental element of the QbD approach is preformulation studies. A thorough understanding of the physical and chemical properties of a pharmaceutical

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product will eventually rationalise its formulation design[5]. Preformulation studies of botanical medicinal materials pose more challenges relative to those of pure synthetic drugs because of the complex nature and uncertainty of active phytocompounds. Unlike the preformulation studies in synthetic drugs, in which a large guidance information and numerous analytical techniques are available, the preformulation studies in a phytopharmaceutical is very bereft and required an adaptation, modification and, some time, development of different measurement techniques[6].

Moringa oleifera Lam is one of such plants with a wide uses in almost all traditional medicine references with writings dating back as far as 150 AD[7]. All parts of Moringa tree possess medicinal properties, but the leaves are the most useful part due to its richness of biologically active phytoconstituents. Several phytochemicals were isolated and identified in M. oleifera leaves (Table 1). Ethanol (95 %) extract of M. oleifera leaves showed a potential bioactivity as anti-nociceptive and anti-inflammatory than do 50 % ethanol or water extracts[8]. Moreover, promising results in ameliorating rheumatoid arthritis disease progression using complete Freund's adjuvant (CFA)-induced arthritis in rats were obtained[9,10]. These finding encourages for formulation of 95 % ethanol extract of Moringa leaves as a standardised oral dosage form. To formulate a high-quality pharmaceutical dosage form while minimising development time and cost, a preformulation studies should be conducted.

Table 1: Phytochemicals isolated, identified and quantified in Moringa oleifera leaves

Phytochemicals	Leaf material	Concentration	References
	Polyphenols		
Total phenols	Dry leaf powder	20.90 mg (GAE)/g to 288.00 mg (GAE)/g	[11,12]
Chlorogenic acid	Dry leaf powder	0.018 mg/g to 0.489 mg/g	[13]
Caffeic acids	Dry leaf powder	ND to 0.409 mg/g	[14]
Ellagic acid	Dry leaf powder	ND to 0.189 mg/g	[14]
Ferulic acid	Dry leaf powder	0.078 mg/g to 0.128 mg/g	[14]
o-coumaric acid, p-coumaric acid, synaptic acid, syringic acid, and gentisic acid	Freeze-dried leaves		[15]
	Flavonoids		
Total flavonoids	Dry leaves Freeze-dried leaves	5.059 mg/g 61.62 mg/g	[16] [17]
Quercetin	Dry leaf powder	0.207 mg/g to 7.57 mg/g	[18]
Myricetin	Dry leaf powder	5.804 mg/g	[19]
Rutin	Dry leaf powder	1.231 mg/g	[20]
Kaempferol	Dry leaf powder	ND to 4.59 mg/g	[21]
Isorhamnetin	Dry leaves Freeze-dried leaves	0.118 mg/g 0.72 mg/g	[13]
	Glucosinolates		
Total glucosinolates	Young dried leaves Older dried leaves	116 mg/g of dry weight 63 mg/g of dry weight	[22]
Sinalbin	Dry leaves	ND to 2.36 mg/g of dry weight)	[23]

In this work, preformulation studies involve determination of organoleptic properties, physicochemical characteristics, solubility profile, swelling index and partition coefficient; analyses of thermal behaviour and physical state; and tests on stress stability, heavy metals and microbial limit. In addition to that, a HPLC-UV method for simultaneous detection and quantification of three reference markers was developed and validated as per ICH-Q2R1 (2005) recommendation.

MATERIALS AND METHODS

Materials and equipment

All chemicals and solvents used were analytical grade reagents (AR). Astragalin (Kaempferol-3-O-glucoside) 98% purity reference standard (RS) was purchased from Extrasynthese, Genay, France; Cryptochlorogenic acid (4-O-Caffeoylquinic acid) 98% purity RS was purchased from Chemfaces, Wuhan, China and Isoquercetin (Quercetin-3-O-glycoside) 90% purity RS was purchased from Sigma-Aldrich, St., MO, USA. Chloroform, dichloromethane, ethyl acetate, 95 % ethanol, n-hexane, acetonitrile, acetone and methanol were purchased from Fisher Scientific, Selangor, Malaysia. Rotary evaporator, EYEL4, China; Water bath and drying oven, Memmert, Germany; Pyris6 DSC Differential scanning calorimeter, Perkin Elmer, Netherlands; Miniflex II powder X-ray diffractometry (PXRD), Rangaku, Japan; Thermo Nicolet Nexus 470 Fourier-transform infrared spectrometer, USA; Lambda 25 spectrophotometer, Perkin Elmer Inc., MA, USA; Shimadzu HPLC equipped with a double LC-20AD pump, a SPD-20A UV-Visible detector and SIL-20AHT auto-sampler, Shimadzu Corporation, Kyoto, Japan and Temperature Humidity chamber (floor stand-B), Tech-Lab Scientific Sdn. Bhd., Balakong, Malaysia.

Plant collection and preparation

Fresh leaves of M. oleifera Lam. were collected from the Butterworth area, Penang, Malaysia in November 2015 and authenticated by Dr. Rahmad Zakaria of Plant Sciences. A voucher specimen (No. 11626) was reserved in the herbarium of the School of Biological Sciences, Universiti Sains Malaysia. The fresh leaves were cleaned and dried in an oven at 40 °C for 3 to 5 days to obtain constant-weight dry mass. The dry leaves were pulverised using a grinding mill to a particle size of about 0.5 mm, packed into a polyethylene bag and stored in a sealed container in a dark, cool room until use.

Preparation of extract

Extract was prepared by macerating 1000 g of the dried Moringa leaves in 5000 mL of 95% ethanol at 45±2 °C for 48 h with occasional shaking. At the end of the maceration period, the extract was filtered initially with muslin cloth followed by Whatman No.1 filter paper and then concentrated by a rotary evaporator to about 10% of the original volume. The concentrated extract was dried in a drying oven at 45±2 °C until constant-weight dry mass was obtained. The collected dried extracts were stored at −20 °C until use.

Physical properties

The 95% ethanol extract of Moringa leaf was subjected to analysis of organoleptic properties including colour, odour, taste and texture. Loss on drying at 65 °C, pH of 5% (w/v) aqueous suspension at 25 °C and hygroscopicity were determined using the method described in USP37-NF32[24].

Solubility profile

The solubility profile of the 95% ethanol extract of Moringa leaf was determined using the method described in the completeness of solution <641> USP 37-NF32 through saturation shake-flask method. In brief, 100 mg of the extract was placed in a volumetric flask and added with 0.2 mL of solvent under vigorous shaking for observation of solubility. A stepwise volume increase of 0.2 mL was applied with shaking until the complete dissolution of the extract. Various solvents with different polarities were tested.

Partition coefficient

Partition coefficient was estimated using shake flask method described by the OECD[25] guideline for testing chemicals (test No. 107). An accurately weighed sample of 5 g of Moringa extract was suspended in 50 mL of distilled water pre-saturated with n-hexane, and the pH was adjusted to 2 by adding 0.1 N HCl. The solution was then carefully transferred into a 250 mL separatory funnel, added with an equal volume of n-hexane (50 mL), shaken for 15 min and allowed to stand for 1 h for phase separation. The n-hexane layer (upper layer) was removed and transferred to a clean pre-weighed 250 mL glass beaker. The procedure was repeated twice. All n-hexane fractions were collected, combined and completely dried in a drying oven at 40 °C. The beaker was weighed prior to the analysis. The increase in the beaker weight indicates the weight of phytoconstituents dissolved in n-hexane (M2). The weight of phytoconstituents remaining in the aqueous layer is the difference between the original weights of Moringa extract (M1) and (M2). Partition coefficient (P) was calculated using the following equation and expressed as logarithm to base 10:

$$P = \frac{M2}{M1-M2} \text{ ..... Eq. (1)}$$

Swelling Index

Swelling index was determined using the method described by Panda and Ansari[26]. In brief, 1 g of the Moringa leaf extract was moistened with 95% ethanol and transferred into a 25 mL graduated cylinder. The volume occupied by the extract was measured. The extract was added with up to 25 mL of distilled water, shaken every 10 min for 1 h and allowed to stand for 4 h. The volume occupied by the swollen Moringa leaf extract was measured. Swelling index was expressed as percent increase in the volume (in mL) occupied by 1 g of the sample after swelling and calculated as follows:

$$Si = \frac{V2-V1}{V1} \times 100 \text{..... Eq.(2)}$$

Where V1 is the volume of 1 g of Moringa extract before immersion in water, and V2 is the volume of 1 g of the Moringa extract after immersion in water.

Differential scanning calorimetry (DSC)

Thermal analysis of the 95% ethanol extract of Moringa leaf was conducted to evaluate thermal behaviour and melting point. DSC was applied to examine solid-state characteristics. In brief, 8–10 mg of the Moringa extract was placed in a standard thermal aluminium pan with a comparable lid (Perkin Elmer, USA) and heated from 0 °C to 200 °C at a 10 °C/min heating rate. An empty aluminium pan with lid was used as reference.

Powder X-ray diffractometry (PXRD)

PXRD pattern of the 95 % ethanol extract of Moringa leaf was determined using a powder X-ray diffractometer at 15 mA, 30 kV and 4°/min angle speed over the 2θ range of 5° to 45° by using Cu Kα radiation wavelength of 1.5406 Å.

Fourier-transform infrared spectroscopy (FTIR)

The 95% ethanol extract of Moringa leaf was prepared into a disc of 1% (w/w) in dry potassium bromide and scanned for transmittance within 3000–400 cm−1 at a resolution of 0.44 cm−1. IR spectrum was printed with individual peaks labelled with their corresponding wavelengths.

Ultraviolet -visible (UV-Vis) spectrophotometer of

Solutions of the three standard reference markers, namely 4-O-caffeoylquinic acid (cryptochlorogenic acid), quercetin-3-O-glucoside (isoquercetin) and kaempferol-3-O-glucoside (astragalin), were prepared separately in methanol at concentration of 100 µg/mL each. Equal volumes of each standard reference marker solution were mixed together to prepare a markers mixture solution. Pure marker solutions and markers mixture solution were then scanned at wavelength range of 200 to 500 nm against methanol as blank. A solution of 95% ethanol extract of Moringa leaf in methanol at concentration of 100 µg/mL was prepared and scanned at wavelength range of 200 to 700 nm against methanol as blank.

Development and validation of HPLC-UV quantitation method

Method validation involves plotting of calibration curve, selectivity, limit of detection (LOD), limit of quantification (LOQ), accuracy, precision and recovery from matrix effect analysis determined as described by the ICH-Q2(R1) guideline (2005). The developed HPLC-UV method was used for quantitative analysis of 95% ethanol extract of Moringa leaf.

Chromatography conditions

The analysis was performed using Agilent's Zorbax Eclipse plus C18 column (250 mm x 4.6 mm i.d., 5 µm particle size) pre-connected to Agilent's guard column (50 mm x 4.6 mm i.d.). The HPLC analysis was carried out at 45 ℃ oven temperature, total run time of 25 min, λ max 337 nm, a binary gradient elution mode with 1.2 mL/min flow rate and 20 µL injection volume. The mobile phase consisted of 0.1% (v/v) formic acid in water at pH 2.7±0.1 (solvent A) and acetonitrile (solvent B). The gradient elution was programmed as following: 0-0.5 min, 5% B; 0.5-11min, 20% B; 11-13min, 20 %B; 13-16min, 43% B; 16-18.5min, 43%B; 18.5-25min, 5 %B followed by a 10-min equilibration period prior to the next sample injection.

Construction of calibration curve and method validation

A stock solution of the three mixed reference markers, namely cryptochlorogenic acid, isoquercetin and astragalin, was prepared at concentration of 200 µg/mL each in 50 % methanol. From the stock solution, a series of dilution with mobile phase was carried out to produce working standard solutions with concentration range of 1.5625 - 200 µg/mL and used for plotting of calibration curves. The calibration curves of each standard were constructed to establish a proportional relationship by plotting the mean of peak areas of triplicate analysis against its corresponding concentrations. The linearity of the constructed curves was evaluated by linear regression analysis and expressed as the squared correlation coefficient (r2). For 95 % ethanol extract of Moringa leaf, a solution of 10 mg/mL in 50 % methanol was prepared. LOD was calculated as signal to noise ratio (S/N) of 3:1 and LOQ was calculated as signal to noise ratio (S/N) of 10:1.

Quantification of reference markers in 95 % ethanol extract of M. oleifera leaf

To quantify the concentration of reference markers in 95% ethanol extract of Moringa leaf, the linear regression equations of the calibration curves of each reference marker were used as following:

$$Concentration\ (\mu g/mL) = \frac{\text{peak area} + \delta}{m} \quad \text{..... Eq. (3)}$$

Where (δ) is y-intercept and (m) is the slope of the calibration curve.

Stress stability

Stability studies of the 95% ethanol extract of Moringa leaf in acidic, alkaline and oxidative stress conditions were conducted using the method described by Hamrapurkar, Patil[27] with minor modifications in volumes used. Photostability of the extract was also investigated as recommended by the ICH Harmonised Tripartite Guideline-Q1B[28]. A solution of three mixed standard reference markers, namely, cryptochlorogenic acid, isoquercetin and astragalin, was prepared at the concentration of 100 µg/mL in 50 % (v/v) methanol. A solution of 10 mg/mL Moringa extract was also prepared in 50% (v/v) methanol and used as sample solution.

Acid hydrolysis

Acid hydrolysis was performed by adding 2.5 mL of 50 % (v/v) methanol and 2.5 mL of 0.1 M HCl to 1 mL aliquot of the mixed standard solution or Moringa extract solution. The samples were stored at 45 °C for 6 h, cooled to room temperature, neutralised to pH 7 by adding 0.1 M NaOH and adjusted to a volume of 10 mL by adding 50% (v/v) methanol. Any physical changes, such as changes in colour, odour and clarity of solutions, were observed. Percent degradation of the reference markers in the mixed standard solution and Moringa extract solution was determined using HPLC–UV method before and after adding acid and calculated using the following formula:

$$Acid\ hydrolysis\ \% = \frac{C1 - C2}{C1} \times 100 \quad \text{..... Eq. (4)}$$

where C1 is the concentration of the reference marker before adding the acid, and C2 is the concentration of the reference marker after adding the acid.

Alkaline hydrolysis

Alkaline hydrolysis was performed by adding 2.5 mL of 50 % (v/v) methanol and 2.5 mL of 0.1 M NaOH to a 1

mL aliquot of the mixed standard solution or the Moringa extract solution. The samples were then stored at 45 °C for 6 h, cooled to room temperature, neutralised to pH 7 by adding 0.1 M HCl and adjusted to a final volume of 10 mL by adding 50% (v/v) methanol. Any physical changes, such as changes in colour, odour and clarity of the solutions, were observed. Percent degradation of the reference markers in the mixed standard solution and the Moringa extract solution was determined using HPLC–UV method before and after adding alkaline and calculated using the following formula:

$$Alkaline\ hydrolysis\ \% = \frac{C1-C2}{C1} \times 100 \ldots\ldots\ldots\ldots \text{Eq. (5)}$$

where C1 is the concentration of the reference marker before adding alkaline, and C2 is the concentration of the reference marker after adding alkaline.

Oxidative degradation

Oxidative degradation was performed by adding 2.5 mL of 50% (v/v) methanol and 2.5 mL of 30% H_2O_2 to 1 mL aliquot of the mixed standard solution or the Moringa extract solution. The samples were stored at 45 °C for 6 h, cooled to room temperature and adjusted to a final volume of 10 mL by adding 50% (v/v) methanol. Any physical changes, such as changes in colour, odour and clarity of the solutions, were observed. Percent degradation of the reference markers in the mixed standard solution and the Moringa extract solution were determined using HPLC–UV method before and after adding H_2O_2 and calculated using the following formula:

$$Oxidative\ degredation\ \% = \frac{C1-C2}{C1} \times 100 \ldots\ldots\ldots\ldots \text{Eq. (6)}$$

where C1 is the concentration of the reference marker before adding H_2O_2, and C2 is the concentration of the reference marker after adding H_2O_2.

Photo-stability

Two Moringa extract samples of 100 mg each were spread over two glass dishes and covered with a transparent glass cover. One of the Moringa extract sample was wrapped with aluminium foil. For reference markers, two samples of 1 mg each of cryptochlorogenic acid, isoquercetin and astragalin were weighed, mixed, spread over two glass dishes and covered with transparent glass cover. One sample of the reference marker mixed powder was wrapped with aluminium foil. All of the four samples were transferred to a temperature humidity chamber and exposed to a light source at 254 and 366 nm with an overall illumination of 1.2 million lux h for 200 h. The temperature was 25 °C, and the relative humidity was 75%. At the end of the exposure period, all of the samples were separately dissolved in 50% (v/v) methanol to prepare 10 mg/mL Moringa extract solution and 100 µg/mL mixed reference marker solution. All samples were evaluated using HPLC–UV method to determine photosensitivity. Percent degradation of the reference markers in the mixed standard solution and the Moringa extract solution was determined before and after exposure to light and calculated using the following formula:

$$Photo - degredation\ \% = \frac{C1-C2}{C1} \times 100 \ldots\ldots\ldots\ldots \text{Eq. (7)}$$

where C1 is the concentration of the reference marker before exposure to light, and C2 is the concentration of the reference marker after exposure to light.

Heavy metals

Heavy metal analysis was conducted using the method described in the British Pharmacopeia (BP) 2013 (Appendix IID). Inductively coupled plasma–atomic emission spectroscopy (ICP-AES) technique was used to determine the levels of cadmium, arsenic, lead and mercury.

Microbial limit tests

Microbial limit tests were performed to quantitatively estimate the total number of aerobic microorganisms, total yeasts and moulds, bile-tolerant Gram-negative bacteria, Salmonella sp., Escherichia coli, Staphylococcus aureus and Pseudomonas aeruginosa in accordance with the BP 2013 (Appendix XVIB).

Data analysis

All the data was expressed as mean ± SD and statistical difference between means were statistically analysed by IBM-SPSS version 20 software using one-way ANOVA followed by post hoc Dunnett-t test (2-tailed) at different variance level.

RESULTS AND DISCUSSION

Organoleptic properties

(Table 2) shows the organoleptic properties and physical attributes of the 95% ethanol extract of Moringa leaf. Organoleptic properties provide insights to the formulator with regard to problems concerning the Moringa extract that should be consider while designing the dosage form. Alkaline excipients should be avoided to prevent chemical incompatibility because the acidic nature of the Moringa extract. Given the sticky, greasy

Table 2: Organoleptic, some physical properties and solubility of 95 % ethanol extract of M. oleifera leaf.

Properties	Observation
Colour	Dark green to black colour
Odour	Strong tang characteristic odour
Taste	Acrid bitter and slight pungent taste
Texture	Sticky greasy mass
Loss on drying (at 65 ℃)	0.053% – 0.0783 %
pH of 5% aqueous suspension at 25 ℃	4.23
Hygroscopicity	Non
Partition coefficient (n-hexane/ water pH 2) at 25 ℃	kp = 6.87 log P =0.836
Swelling index	10.714%
Solubility	Practically insoluble in water and simulated gastric fluid pH 1.2, sparingly soluble in simulated intestinal fluid pH 6.8 and ethyl acetate, soluble in acetonitrile and acetone, and freely soluble in methanol and dichloro-methane.

nature of the Moringa extract, an adsorbent should be used to convert it into flowable powder. Flavouring and sweeteners are also required to mask the strong odour and bitter taste of the extract. A relatively high swelling index indicates that the Moringa extract itself acts as a disintegrant; as such, an external disintegrant may not be added.

Determination of partition coefficient is important for assessing the ability of a drug to penetrate biological barriers and the delivery of the drug to systemic circulation[29]. The 95% ethanol extract of Moringa leaf showed moderate lipophilicity, which suggests that, theoretically, the extract shows good permeability across the GIT membrane. The water solubility of the extract is predicted to be the limiting factor of absorption rate. When the aqueous solubility of a drug is less than 10 mg/mL (1% w/v), it is predicted to show problems in absorption, bioavailability and/or bioactivity. Solubility profiles, especially aqueous solubility, provide formulators with essential information regarding product development. Poor water solubility indicates the need for a surfactant/solubiliser or another method for enhancing solubility. In case of plant extract, common methods for determination of partition coefficient and solubility, like chromatography and spectrophotometry based on using a specified compound(s) as a reference marker, is not appropriate despite being more accurate. Since Moringa extract is a mixture of several phyto-materials, depending on such mentioned assessment method will determine the partition coefficient and solubility of the selected reference marker(s) but not entire Moringa extract. Alternatively, assessment of solubility by visual observation is an accepted method for approximating solubility[3]. The lipophilic–hydrophilic balance (HLB) is an important factor that affects the rate and extent of drug absorption of poorly water-soluble drugs. HLB affects the pharmacokinetic and pharmacodynamic behaviour of drugs[30]. Given that the Moringa extract is acidic with pH 4.23, the partition coefficient test was conducted at pH 2 to ensure that phytochemicals predominantly exist in their unionised forms[25] under conditions similar to that of gastric pH.

Differential scanning calorimetry (DSC)

The thermal analysis of the 95% ethanol extract of Moringa leaf (Figure 1) showed a broad peak at 115.44 °C, which reflects the complex nature and presence of multi-phytoconstituents in the extract. The heat transfer for a constant pressure process (Delta Cp) is 13.772 J/g °C, and the peak area is 218.72 mJ. The data obtained from DSC is essential to evaluate the possible change in physical state and thermal behaviour of M. oleifera leaves extract due to formulation, added excipients and manufacturing method.

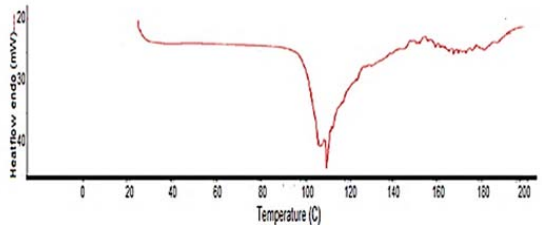

Fig. 1: Deferential scanning calorimetry (DSC) of 95% ethanol extract of Moringa leaf against empty aluminium pan as reference at at 10 °C/ minute heating rate.

Powder X-ray diffractometry (PXRD)

The PXRD pattern of the 95% ethanol extract of Moringa leaf is shown in (Figure 2) The PXRD spectrum reveals distinct peaks. This finding indicates that phytochemical compounds in the extract are present as crystalline materials with characteristic diffraction peaks at the 2θ diffraction angles of 10.66°, 11.54°, 12.31°, 14.18°, 18.78°, 22.08° and 24.46°.

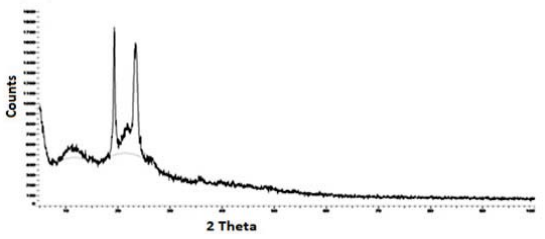

Fig. 2: Powder X-ray diffraction (PXRD) of 95% ethanol extract of Moringa leaf over the range of 2θ from 5 to 45°.

DSC and PXRD data show that the majority of phytochemicals in the Moringa extract are in the crystalline form. Hence, the solubility of the extract can be enhanced by converting the physical state from the less soluble crystalline state to the more soluble amorphous state.

Fourier transform infrared spectroscopy (FTIR)

The infrared spectra of the 95% ethanol extract of Moringa leaf (Figure 3) reveal major peaks as group frequencies, which were identified by Coates[31]. The strong bands at 2900–2800 cm−1 are due to the methyl/methylene C–H stretching from a hydrocarbon. The weak bands at 1736.11 cm−1 are due to the six-membered ring lactone, whereas the band at 1710.81 cm−1 is due to the aliphatic C=O bond for aliphatic esters or ketones. A broad band at 1655.87 cm−1 is due to amide or open-chain imino (−C=N−). A fingerprint region was found between 1500 and 800 cm−1. A band at 1579.32 cm−1 is due to aromatic ring stretching. Similar bands at 1554.12 and 1514.21 cm−1 are due to aromatic nitro compounds. A weak band at 1469.42 cm−1 is attributed to –OH bond. A band at 1388.77 cm−1 is due to dimethyl or 'iso'-bonds. The strong band at 1107.18 cm−1 is due to secondary alcohol or C–O stretching. The band at 964.37 cm−1 is due to aromatic C–H in-plane bending or trans-C–H out-of-plane bending. The weak band at 829.78 cm−1 is attributed to 1,4-disubstitution (para). Finally, the band at 722.16 cm−1 is due to monosubstitution (phenyl) or −OH out-of-plane bending.

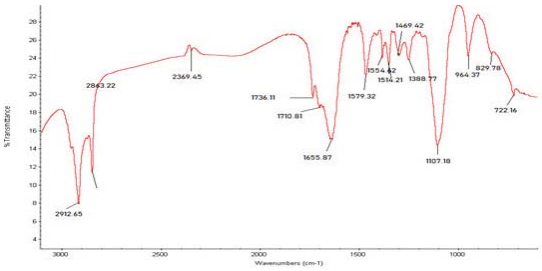

Fig. 3: Infrared spectrum 95% ethanol extract of Moringa leaf prepared as disc with potassium bromide with major group frequencies.

The sample is a mixture of several phytochemicals; as such, the FTIR spectrum of the 95% ethanol extract of Moringa leaf showed broad bands that may fall outside the quoted range. This result could be due to the effect of other functional groups within the molecule and the occurrence of hydrogen bonding. Thus, the strength and number of intermolecular interaction highly vary within the sample and lead to the broadening of the bands[31].

Ultraviolet-visible spectrophotometry scanning (UV-Vis)

The UV-Vis spectra of each reference marker mixed three reference markers and Moringa extract solution were shown in (Figure 4). The ethanol extract showed absorption at 220 and 335 nm with almost similar spectra. The mixture of three reference markers showed a maximum absorption at 337 nm. The intra- and inter-molecular interactions and the presence of inorganic compounds leads to shift in λmax of Moringa extract solution and mixed reference markers solution[32].

HPLC-UV method development and validation

A HPLC-UV method for quantitative analysis of Moringa extract was successfully developed (Figure 5). The retention times (tR) of cryptochlorogenic acid, isoquercetin and astragalin were11.76, 17.47 and 18.99 min, respectively, showed no interference between the eluted analytes and clearly indicated for the specificity of the developed method. Specificity of the method, also known as selectivity, inspects any possible influences from sample matrices and/or co-existing phytocompounds which may lead to overlapping of separated peaks and interfere with the retention time of measured analytes. Linearity, intra-day and inter-day precision, accuracy and recovery of the method were calculated depending on constructed calibration curves of each reference markers (Figure 6)

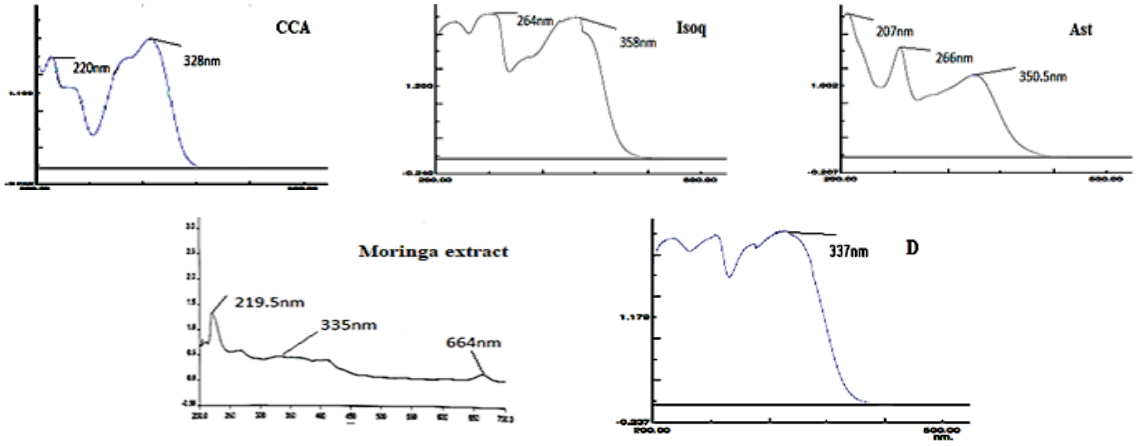

Fig. 4: The UV-vis spectrophotometer scan of 95% ethanol extract of M. oleifera leaf at concentration 100 µg/mL in methanol, three reference markers and a mixture of them at concentration 100 µg/mL in methanol. CCA: crypto chlorogenic acid, Isoq: isoquercetin, Ast: Astragalin and D: mixture of CCA, Isoq and Ast reference marker compounds.

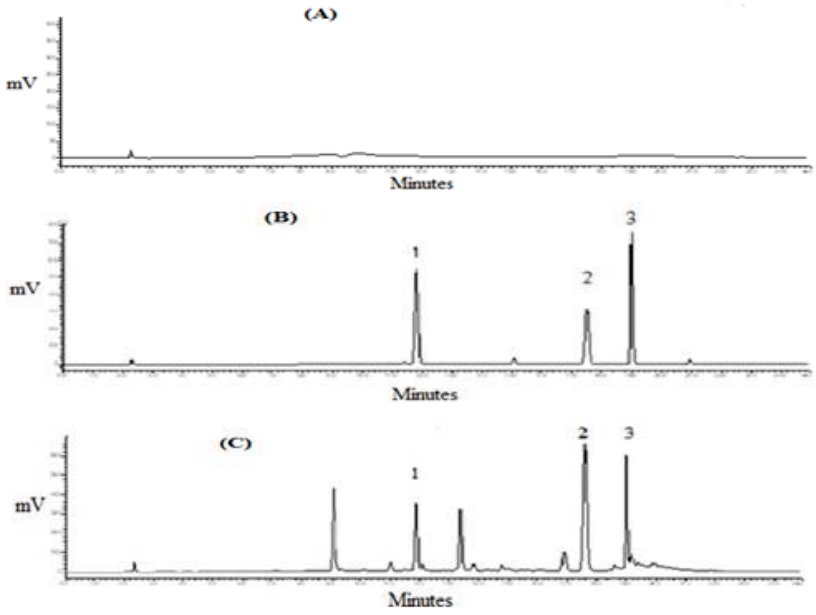

Fig. 5: HPLC chromatogram of (A) solvent only; (B0 mixed three reference markers at concentration 50 µg/mL each and (C) 95% ethanol extract Moringa oleifera leaf at concentration 10 mg/mL. 1: cryptochlorogenic acid (tR = 11.76 min); 2: isoquercetin (tR = 17.47 min) and 3: astragalin (tR = 18.99 min).

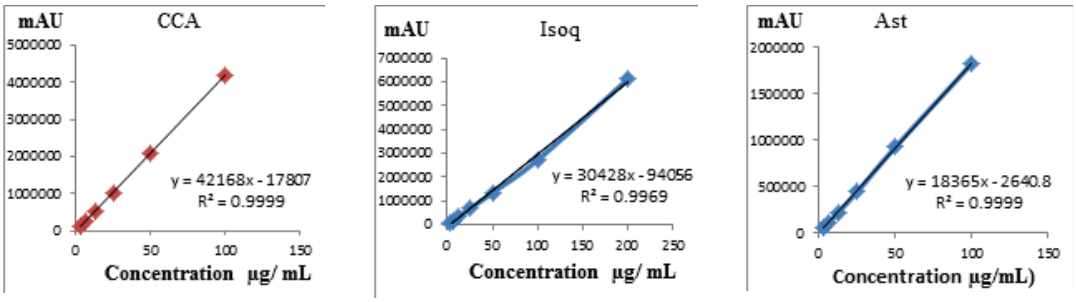

Fig. 6: Calibration curve of crypto chlorogenic acid, isoquercetin and astragalin at concentration range of 3.125 to 100 µg/mL

The linear range and correlation coefficients (r2) for each reference marker obtained from their calibration curves, LOD and LOQ were all shown in (Table 3).

Table 3: Calibration parameters, linear ranges, limit of detection and limit of quantification of reference standard compounds for HPLC-UV method validation calculated from calibration curves.

Standards	Regression equation	r^2	Linear range (µg/mL)	LOD (µg/mL)	LOQ (µg/mL)
Crypto chlorogenic acid	y=42168x – 17807	0.9999	3.125 - 100.00	1.093	3.643
Isoquercetin	y=30428x – 94056	0.9969	3.125 - 200.00	1.284	4.280
Astragalin	y=18365x – 2640.8	0.9994	3.125 - 100.00	0.883	2.945

The recovery values of the three reference markers were between 92.72 and 96.15% (Table 4) indicating that the plant matrix and other phytoconstituents present in the extract had a negligible effect on the assay method. (Table 5) shows the intra-day and inter-day accuracy and precision of the three reference markers within their linear range. The acceptance criteria included accuracy at range 85 – 115% of the investigated nominal values and precision ≤ 2% of percent relative standard deviation (%RSD)[33,34].

Table 4: The recovery quantity (mean ± SD, n=3), % recovery and % RSD of the three reference marker compounds of 95% ethanol extract of M. oleifera leaf.

Marker compound	Blank spiked sample (µg/mL)	Recovered quantity (µg/mL)	% recovery	% RSD
Crypto chlorogenic acid	50	46.94±1.79	93.87	2.798
	100	92.89±1.19	92.89	1.190
	150	139.08±1.81	92.72	2.800
Isoquercetin	50	47.59±2.17	95.18	1.400
	100	95.08±1.40	95.08	2.967
	150	143.58±1.68	95.72	1.678
Astragalin	50	47.84±1.11	95.68	3.169
	100	96.04±0.75	96.04	0.748
	150	144.23±0.93	96.15	3.218

Table 5: Intra-day and inter-day % accuracy and precision (%RSD) for the three reference marker compounds of 95% ethanol extract of M. oleifera leaf at their linear range.

Standards concentration (µg/mL)	Intra-day (n=6)		Inter-day (n=6)	
	Accuracy (% of true value)	Precision (% RSD)	Accuracy (%of true value)	Precision (% RSD)
Crypto chlorogenic acid				
3.125	102.95	0.485	102.43	0.930
6.25	99.99	0.917	101.30	1.074
12.5	99.39	0.983	98.82	0.861
25	100.24	0.544	100.28	0.633
50	100.08	0.990	100.30	1.127
100	101.74	0.654	101.11	0.854
Isoquercetin				
3.125	101.21	0.681	99.97	1.021
6.25	101.65	0.437	100.80	0.842
12.5	99.95	0.454	101.82	0.765
25	99.61	0.309	99.97	0.521
50	99.63	0.729	98.23	0.644
100	100.23	1.762	101.02	1.387
200	100.17	1.860	100.89	0.997
Astragalin				
3.125	101.66	0.425	99.06	0.502
6.25	101.50	1.065	101.09	1.284
12.5	99.29	0.352	102.86	0.741
25	102.44	1.910	99.70	1.713
50	100.59	0.744	101.20	0.909
100	100.48	0.968	102.22	1.112

In the present study, the intra-day accuracy and precision values were range from 99.29 to 102.95% and 0.309 to 1.910 respectively. For inter-day accuracy and precision analysis the values were from 98.23 to 102.86% and 0.502 to 1.713% respectively. The resolution of each reference marker's peak, tailing factor, retention coefficient (K`) and number of theoretical plates (NTP) all implied the robustness of used method as well as the efficiency of the column and the system (Table 6).

Table 6: Method robustness and column efficiency parameters for the developed HPLC-UV method for quantification of 95% ethanol extract of M. oleifera leaf.

Marker	Resolution	Tailing factor	Retention coefficient	NTP (USP)	Separation
Crypto chlorogenic acid	1.972	1.091	4.063	62664	1.042
Isoquercetin	4.436	1.038	6.479	94665	1.066
Astragalin	2.195	1.102	7.123	297634	1.020

The developed HPLC-UV method was applied to quantify the three selected markers in Moringa extract. Quantification (mean±SD) of crypto-chlorogenic acid, isoquercetin and astragalin in Moringa leaf extract were 3.544± 0.06, 19.667± 0.10 and 5.762± 0.12 mg/g dry extract respectively. The results indicated that the developed HPLC-UV method provided the best chromatographic conditions for separation and simultaneous quantification of cryptochlorogenic acid, isoquercetin and astragalin in M. oleifera extract. The method was selective, accurate, precise, robust and could simultaneously analyse three reference markers. The survey of published literatures about HPLC-UV quantitative analysis of M. oleifera leaf extract had found only two studies. The main criteria to consider the published study is for the availability of method validation data. The majority of published HPLC analysis methods for M. oleifera leaf were only used for identification of phytochemicals as a fingerprint profile not for quantification. Of that, Vongsak, Sithisarn[35], using hypersil BDS C18 column, gradient elution consisted of methanol and 1% acetic acid in water, UV detector at 334 nm and flow rate of 1 mL/min at room temperature, quantified three markers namely, crypto chlorogenic acid, isoquercetin and astragalin, in 70% ethanol extract of M. oleifera leaf and the results were 0.081, 0.120 and 0.153 % (w/w) respectively. Irfan, Asmawi[36] quantified cryptochlorogenic acid, isoquercetin and astragalin in butanol fraction of 95% ethanol extract of M. oleifera leaf using hypersil C4 column, gradient elution consists of acetonitrile and 0.1% formic acid in water, photo-diode array detector (DAD), flow rate of 1.2 mL/min and oven temperature at 45 ℃ and the results were 5.16, 23.90 and 56.58 µg/mL respectively.

3.7 Stress stability

(Table 7) shows the photostability and stability of the 95% ethanol extract of Moringa leaf and the mixed standard reference markers under acidic, alkaline and

oxidative conditions. For the mixed standard reference markers, cryptochlorogenic acid appeared to be unstable under acidic and alkaline conditions but was not affected by oxidative or light stress. Isoquercetin appeared highly unstable under alkaline condition but was not affected by acidic, oxidative and light stress conditions. Astragalin appeared to be stable under acidic, alkaline, oxidative and light stress conditions and showed no considerable degradation. For the 95% ethanol extract of Moringa leaf, cryptochlorogenic acid was unstable under acidic and alkaline conditions but not affected by oxidative or light stress. The Moringa extract matrix appears to provide a higher level of protection against such stress conditions than the standard reference markers. The extract appeared to be stable against light and oxidative stress because of the presence of high amounts of phytochemicals with strong antioxidant activity[8]. Accordingly, adding antioxidant or using a special light-protecting packaging container is unnecessary. Stress stability studies are less commonly used for assessing botanical mate¬rials and products than for synthetic drugs. The results of the stress stability studies should be considered during selection of dosage form and excipients and during manufacturing process and development of analytical methods to ensure maximum stability and bioactivity.

Table 7: Stress stability study of 95% ethanol extract of Moringa leaf and reference markers in acidic, alkaline, oxidative and light.

Acid stability (% degradation)				
Sample	Crypto chlorogenic acid	Isoquercetin	Astragalin	Physical changes
Standard	77.37±2.117	3.05±0.474	4.31±1.213	Clear with faint yellow discoloration
Crude extract	19.78±1.360	1.89±0.522	3.23±0.852	No observed change
Alkaline stability (% degradation)				
Standard	98.68±6.366	84.88±3.633	6.30±1.120	Hazy fiant yellow discoloration
Crude extract	67.36±3.778	69.00±2.629	9.42±0.883	Clear, decrease in green colour intensity,
Oxidative stability (% degradation)				
Standard	14.70±1.124	10.97±1.274	3.46±0.226	No observed change
Crude extract	2.75±0.351	-	1.96±0.056	No observed change
Photo-stability (% degradation)				
Standard	11.54±2.192	-	-	No observed change
Crude extract	3.76±1.016	1.59±0.478	-	No observed change

* (-) indicate no noticeable change.

3.8 Heavy metal tests

Determination of heavy metals including arsenic, cadmium, lead and mercury in the 95% ethanol extract of Moringa leaf was conducted using the method described in BP. The tested metals were undetected. Hence, the extract was not contaminated by dangerous toxic heavy metals (Table 8). The rapid industrialisation, urbanisation and extensive environmental pollution have led to high levels of dangerous toxic heavy metals in air, water and soil. Heavy metals (specific gravity greater than 5 g cm−3 and atomic weight of 63.5–200.6 g mol−1) are easily transmitted from the soil, irrigation water or air to plants and then accumulate and transfer to humans through the food chain[37]. Consumption of plant materials (as a whole or processed material) contaminated with heavy metal leads to potential health risk, serious ailment and even death[38]. Therefore, the levels of heavy metals in plant materials intended for human consumption must be determined. The result of the heavy metal analysis revealed the absence of such toxic elements, thereby confirming that the Moringa extract is safe and appropriate to be consumed by humans as medicine or dietary supplement.

Table 8: Heavy metal tests of the 95% ethanol extract of Moringa leaf.

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

3.9 Microbial limit tests

The 95% ethanol extract of Moringa leaf appeared to be free of microbial contaminants (Table 9). The results of the microbial limit test revealed the absence of contamination of virulent microorganisms possibly because of the antimicrobial activity of 95% ethanol used as extraction solvent. Moreover, acidic environment and low water content are unfavourable conditions for microbial growth[39]. Hence, the Moringa extract has been acknowledged for its antimicrobial activity[40].

Table 9: Limit of microbial test of 95% ethanol extract of Moringa leaf.

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	BP 2013, Appendix XVIB
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

*ND: not detected and BP: British Pharmacopoeia.

CONCLUSION

Preformulation studies of botanical medicinal materials pose more challenges relative to those of pure synthetic drugs because of the complex nature and uncertainty of active phytocompounds. For phytopharmaceutical products it is an essence step to conduct a preformulation studies for the raw materials. Preformulation studies provide important information to support the selection of suitable dosage form, optimal excipients to be used and appropriate manufacturing process, container and packaging type. In addition, preformulation studies help in development of analytical methods necessary for evaluating and standardising the raw material and final product. A thorough understanding of the physical and chemical properties will eventually rationalise the formulation design[5].

The results of preformulation studies of the 95% ethanol extract of Moringa leaf pointed out to some problems that should be considered and overcome during the development of the final product to ensure high product quality, such as in terms of texture, taste and solubility. The 95% ethanol extract of Moringa leaf is relevant to the development of phytomedicines, dietary supplements or cosmetics. This preliminary information is the first step for selection of suitable dosage form and formulation of Moringa extract as a standardised solid dosage form.

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CONFLICT OF INTEREST

There are no conflicts of interest.

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