Research Square

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Eco-Friendly Aqueous Extraction of Momordica charantia Fruits using Gelucire® 44/14: Phytochemical Fingerprinting and Antioxidant Activity

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Abstract

Conventional extraction methods for isolating phytoactives from *Momordica charantia Linn.* fruit rely on use of hazardous organic solvents. In this study, phytoactives of Momordica were extracted using an eco-friendly extraction method utilizing Gelucire® 44/14. Additionally, prepared extract was standardized as per CDSCO, India guidelines using LC-MS/MS and HPTLC to claim it as phytopharmaceutical drug. Various ratios of powder with gelucire® 44/14 and gelucire® 50/13 were prepared. Optimization of ratio based on highest percent of Momordicinin recovery. HPTLC method was developed to simultaneously quantify five phytoconstituents viz. β-sitosterol, gallic acid, quercetin, kaempferol, and rutin in the optimized extract (G44MC 1:2 ratio). Extract's antioxidant potential estimated using DPPH, Folin–Ciocalteu, and aluminum chloride colorimetric assays for total phenolic and flavonoid content. Results showed higher extraction of Momordicinin by gelucire® 44/14 (0.56 ± 0.02% w/w) compared to gelucire® 50/13 (0.42 ± 0.07% w/w) and ethanolic extract (0.40 ± 0.06% w/w). G44MC with ratio of 1:2 (fruit powder:gelucire) considered optimized and showed presence of β-sitosterol, gallic acid, quercetin, kaempferol, and rutin analysed using HPTLC method developed for simultaneous estimation. The prepared extract showed concentration dependent antioxidant potential revealed by DPPH assay. Present work reports eco-friendly, efficient, and solvent-free method for extracting phytoactives of momordica.

1. Introduction

Momordica charantia Linn. (MC), commonly known as bitter gourd or karela, is an important medicinal plant widely used in traditional medicine, particularly Ayurveda, to treat diabetes, inflammation, infections, and gastrointestinal disorders and to support wound healing. [1, 2] It contains bioactive compounds such as phenolics, flavonoids, glycosides, saponins, triterpenoids, and sterols, which are known to contribute to its antidiabetic, antioxidant, hypoglycemic, and anti-inflammatory activities. [3]

Herbal medicines are often preferred for chronic diseases because they are natural, economical, and typically produce fewer side effects than synthetic drugs. However, many phytoconstituents of MC have poor water solubility and stability, which impacts its therapeutic effectiveness. In addition, factors such as plant part selection, extraction method, solvent system, and processing conditions can affect the extract's phytochemical composition and biological activity.

Conventional extraction methods commonly use alcoholic solvents, acetone, and chloroform to extract and isolate phytoconstituents from plants. Although these methods provide good extraction efficiency, they have several limitations, including toxicity, solvent residue contamination, environmental pollution, and degradation of heat-sensitive compounds during processing.

[4]. Although water is a safer alternative with a Generally Recognized as Safe (GRAS) status, its higher polarity limits the extraction of semi-polar and lipophilic bioactives, known to demonstrate therapeutic effects by the extract. It for this reason there is need to develop safer and more effective extraction techniques for herbal preparations.

Lipid-based pharmaceutical excipients such as gelucires of specific grades have attracted significant interest in herbal drug delivery systems owing to their lipophilicity alongwith water solubility due to HLB range between 13–15. Gelucire® 50/13 (G50) and Gelucire® 44/14 (G44) are non-ionic lipid-based carriers composed of glycerides and polyethylene glycol (PEG) esters of fatty acids. These semi-solid, hydrophilic excipients are commonly used in pharmaceutical formulations to improve drug solubility and release characteristics. G50 has a melting point of about 50°C and an HLB value of 13, whereas G44 shows a melting at 44°C and an HLB value 14. Gelucire has been extensively explored to enhance the solubility, dissolution, permeability, and oral bioavailability of poorly water-soluble phytoconstituents. Gelucire-mediated extraction can be considered as a safer and more efficient alternative to traditional solvent extraction methods, as it improves extraction efficiency and water dispersibility of phytoconstituents while minimizing the use of toxic organic solvents. [5, 6]

Effective analytical standardization is essential for verifying the identity, quality, and batch-to-batch consistency of herbal materials. This can be done using methods like, high-performance liquid chromatography (HPLC), High-performance thin layer chromatography (HPTLC) and liquid chromatography–mass spectrometry (LC-MS/MS). Amongst these, HPTLC offers rapid, cost-effective multicomponent profiling and supports both qualitative and quantitative measurements of marker compounds. Similarly, LC-MS provides structural and fragmentation information necessary for accurate compound identification. Additionally, evaluation parameters such as total phenolic and flavonoid content, as well as *in vitro* antioxidant activity, help establish comprehensive quality standards for extracts.

The current study aimed to develop a Gelucire-assisted aqueous extraction method for MC to prepare a phytoconstituent-rich extract using a GRAS-compliant, safe approach. The study investigated ways to improve the recovery of bioactive compounds and to systematically standardize analytical methods. Preparative HPLC was used to isolate Momordicinin (MMR) which was further characterized using UV spectroscopy, HPTLC, and LC–MS/MS. HPTLC method was developed for quantitative analysis of selective phytoconstituents viz. quercetin (QR), rutin (RU), gallic acid (GA), kaempferol (KA), and β-sitosterol (β-ST) from the Gelucire extract (GMC) while LC-MS/MS was utilized for qualitative analysis of the extract. Thus, this work provides a reliable method for standardization of gelucire assisted extract of M. charantia Linn. Combining amphiphilic extraction with advanced analytical techniques.

2. Material and method

2.1 Plant Material

Momordica charantia Linn. (MC) fruit was procured from a local vendor in Pune, Maharashtra, India. Fruits were identified and authenticated at the Agarkar Research Institute, Pune, Maharashtra, India, with specimen number (AUTH 23–126) of the plant.

2.2 Chemicals and Reagents

Momordicinin (MMR, 98% purity) was isolated at using preparative HPLC. Reference standards of gallic acid, rutin, quercetin, kaempferol, and β-sitosterol were procured from Yucca Enterprise, Mumbai, India. Gelucire® 44/14 and 50/13 were generously provided by Gattefossé, France, and used in their original form. Analytical-grade ethanol, n-hexane, toluene, methanol, formic acid, and ethyl acetate were obtained from Merck Pvt. Ltd., Mumbai, India. Post-chromatographic derivatization was performed with an anisaldehyde–sulfuric acid reagent to visualize non-UV-active compounds.

2.3. Preparation of *Momordica charantia Linn.* Extracts

2.3.1 Extraction and isolation of Momordicinin from *Momordica charantia Linn.* -

Fresh fruits of MC were collected from the local market in Pune. The fruits were rinsed, divided into small sections, sun-dried, and ground into a fine powder. The dried powder (300 g) was soaked in n-hexane for 48 hours to remove fats and sugars. After defatting, the marc was collected and dried to remove residual n-hexane. The dried powder was then added to 95% alkalized ethanol (600 mL) and extracted by ultrasound-assisted extraction (LABBMAN LMUC3D Digital Ultrasonic cleaner, Chennai, India) at 40 kHz, 40°C for 45 minutes, followed by maceration for 10 days to maximize the extraction of bioactive compounds. The solvent from the mixture was evaporated after filtration using a rotary evaporator (IKA WERKE

RV06ML, Stanfer, Germany) under reduced pressure for 15 min at 45°C, obtaining a sticky mass weighing 8.49 g, referred to as ETC MC (ethanolic extract of M. charantia). For further purification, 60 mL of distilled water was added to the sticky EMC mass to remove water-soluble impurities. The water-insoluble fraction was separated and dissolved in ethanol: water (50:50 v/v). Further liquid-liquid partitioning was performed using 60 mL ethyl acetate. Ethyl acetate fraction was collected after repeated washing and concentrated to dryness, yielding 5.3 g. The sample was resuspended in ethanol and injected into the preparative HPLC with a mobile phase of ACN: water (60:40) to isolate the MMR compound [7]. Multiple injections of the same sample were performed to obtain a single, sharp MMR peak. The isolated fraction was dried and stored at -20°C for further identification using UV, HPTLC, and LC-MS/MS.

2.3.2 Optimization of Gelucire-Assisted Extraction Component

MC extract was prepared using food-grade lipids, viz., Gelucire® 50/13 and 44/14. The lipids, in molten form, were mixed with 1 g of MC powder at various ratios (1:0.5, 1:1, 1:1.5, 1:2, 1:2.5, 1:3). To each of these mixtures, 10 mL of deionized water was added, and the mixture was stirred for 1 hour to obtain a dispersion. The dispersion was subsequently centrifuged at 10,000 rpm for 30 minutes at 4°C (Cryocentrifuge 2810R, Eppendorf, USA). The supernatant was removed and filtered through Whatman No. 41 filter paper (Whatman, Middlesex, UK) to obtain an eco-friendly extract. The extracts (termed as

G44MC/G50MC extract or GMC extract) with varied plant-to-lipid ratios were then analyzed by HPTLC to optimize the ratio having maximum yield of marker compound MMR.

2.4 Characterization of the extract

2.4.1 HPLC instrumentation for MMR isolation

MMR was purified via preparative HPLC using a Jasco system (PU-2087 Plus, UV-2075 Plus) with ChromNav software. The process employed a Jasco Hypersil ODS RP-C18 preparative column (10 µm, 10 × 250 mm) along with a guard column (4.6 × 10 mm, 5 µm, Supelco). The residue obtained (5.3 g) was dissolved in ethanol and reinjected 2 ml into the same setup to generate a single, sharp peak. Separation was achieved using an acetonitrile: water (60:40, v/v) mobile phase at a flow rate of 3 mL/min under isocratic conditions. The mobile phase was filtered through a 0.45 mm Millipore membrane and degassed prior to use. The detection wavelength of UV detector was set at 230 nm. Multiple injections were made to isolate the peak corresponding to MMR for further purification. MMR thus obtained was used as a marker in further studies after appropriate analytical studies.

2.4.2. Identification of MMR from the ethyl acetate fraction of the ethanolic extract.

UV spectroscopy (100 ppm) and HPTLC (20 ppm) were used to preliminarily characterize the isolated MMR fraction. The dried fraction was dissolved in ethanol and analyzed within the wavelength range of 200 to 800 nm utilizing a UV-visible spectrophotometer (Lab India, UV3092, India). For HPTLC analysis, the fraction was chromatographed under optimized conditions [7].

2.5 Qualitative Profiling of Ethyl Acetate Fraction, Isolated Fractions, and G44 MC Extract Using LC–MS

LC–MS analysis was conducted utilizing an LC–MS/MS system (Agilent 6460 Triple Quadrupole LC/MS, Agilent Technologies, Santa Clara, CA, USA) equipped with electrospray ionization (ESI). Chromatographic separation was performed on a C18 column (150 × 4.6 mm, 5 µm) at 25°C using mobile phase 0.1% formic acid in water (A) and acetonitrile (B) with a flow rate of 0.8 mL/min. The total run duration was 15 minutes with gradient elution of 0–0.5 min, 90:10 (A: B); 0.5–5 min, 90:10 to 50:50; 5–12 min, 50:50 to 10:90 and 12–15 min, 10:90 to 90:10.

The sample injection volume was 10 µL. Mass spectrometric detection was conducted in full-scan mode across an m/z range of 100–1000 using ESI in both positive and negative ionization modes. The compounds were identified using their exact mass-to-charge ratio (m/z), fragmentation patterns, and retention time. The resulting spectra provided an initial profile of the phytoconstituents present in the extract, which was then compared with mass spectral databases and relevant literature (Table 1).

Table 1
Phytoconstituents identified by LC-MS-MS in extract of *M. Charantia Linn*.

Compound	Mol.Weight	RT (min)	Observed m/z	Ion Type	Major Fragment Ions (m/z)
ETC MC extract :					
1.Momordicinin (EA fraction)	438.7	0.009–0.242	437.3	$[M-H]^-$	391.2, 360.2, 338.2, 309.1
2. Momordicinin (Isolated fraction)	438.7	0.009–0.362	437.1	$[M-H]^-$	393.2, 365.1, 333.2, 304.1
G44 MC extract :					
1. Gallic acid	170.12	2.04	169.0	$[M-H]^-$	125
2. Quercetin	302.24	7.11	301.1	$[M-H]^-$	197.1, 147.0, 117.9
3. Kaempferol	286.24	11.46	285.1	$[M-H]^-$	293.1
4. Rutin	610.52	13.48	609.3	$[M-H]^-$	301
5. β-Sitosterol	414.72	11.87	413.1	$[M-H]^-$	395.1, 397.1, 405.2
6. Momordicinin	438.7	7.31	437.1	$[M-H]^-$	345.2, 303.1, 243.0, 187.1

Table 2
Momordicinin extracted using Gelucire® 50/13 and Gelucire® 44/14 at various ratios. G50MC; ratio code for *Momordica charantia Linn.* powder: Gelucire® 50/13: G44MC; ratio code for *Momordica charantia Linn.* powder: Gelucire® 44/14: a Mean ± SD (n = 3): b Optimized ratio chosen Gelucire grade. ETC MC extract; ethanolic extract of *Momordica charantia Linn.*

Batches	Crude drug: Gelucire	MMR content (% w/w in powder)
G50MC1	1:0.5	0.07 ± 0.02
G50MC2	1:1	0.13 ± 0.04
G50MC3	1:1.5	0.21 ± 0.01
G50MC4	1:2	0.42 ± 0.07
G50MC5	1:2.5	0.44 ± 0.01
G50MC6	1:3	0.47 ± 0.03
G44MC1	1:0.5	0.26 ± 0.01
G44MC2	1:1	0.30 ± 0.05
G44MC3	1:1.5	0.41 ± 0.03
G44MC4[b]	1:2	0.56 ± 0.02
G44MC5	1:2.5	0.56 ± 0.04
G44MC6	1:3	0.57 ± 0.04
ETC MC extract	-	0.40 ± 0.06

2.6 MMR-Based Standardization of Ethanolic and Gelucire Extracts

2.6.1 HPTLC instrumentation

Samples of MMR (10 µL), EMC extract (5 µL), and GMC extracts (7 µL) were applied as 6-mm-wide bands onto precoated silica gel 60 F_{254} aluminum plates (20 × 10 cm, 250 µm thick; E. Merck, Germany) using a CAMAG syringe and a CAMAG Linomat IV automated applicator (Switzerland). The distance between bands was set at 6 mm, with an application rate of 0.1 µL/s. CATS 4.0 software was used for data collection, and densitometric analysis was conducted using a CAMAG TLC Scanner III. The scanning was carried out at a speed of 10 mm/s, with slit dimensions set to 6.0 × 0.45 mm. Chromatographic separation was achieved using methanol:toluene:n-hexane (1.5:2:1, v/v) for MMR. Linear ascending development was carried out in a twin-trough glass chamber saturated with the mobile phase for 20 minutes at room temperature.

2.6.2 Calibration curve of MMR using HPTLC

A 1000 ppm stock solution of MMR was prepared in ethanol. The calibration curve was constructed using volumes of 1.5–1.9 µL of stock solution, corresponding to 1.5–1.8 µg/spot. The MMR content was measured by assessing the absorbance of the sample at 230 nm with a UV-Visible spectrophotometer (V-530, JASCO, Japan) upon appropriate dilution.

2.6.3 Estimation of MMR content in ethanolic and Gelucire Extracts

A liquid-liquid partitioning method using ethyl acetate was used to estimate MMR content of EMC and GMC extracts. The ETC MC (10 mg in 10 mL) and GMC (MC powder: Gelucire at a 100:200 mg ratio in 10 mL water) were partitioned with 5 mL of ethyl acetate. In each case, the ethyl acetate layer was isolated and evaporated to dryness under reduced pressure. The resulting residue was reconstituted in 0.5 mL of ethanol and then filtered through a 0.45 µm membrane filter. Quantification of the resulting MMR solutions from the GMC and ETC MC extracts was performed using HPTLC. The GMC extract was optimized based on the highest content of MMR, which is known to be the primary therapeutic component of MC.

2.7 Simultaneous Estimation of Phytoconstituents in Optimized Extract

2.7.1 HPTLC instrumentation

According to Central Drugs Standard Control Organization (CDSCO), India, an extract when claimed as a phytopharmaceutical drug needs qualitative and quantitative estimation of a minimum of four bioactives. [8] Thus, in the present study, it was decided to standardize the optimized extract in terms of five phyoconstituents (GA, KA, RU, QR, and β-ST) along with the primary compound MMR. In order to qualitatively and quantitatively estimate the stated phytoconstituents, a simultaneous estimation method was developed using HPTLC. The mobile phase used for the same consisted of formic acid, water, methanol, ethyl acetate, and toluene in the ratio 2:1:1.7:12:6 (v/v) for separation.

2.7.2 Calibration curve of selected phytoconstituents using HPTLC

To achieve a concentration of 1000 ppm, 10 mg of each reference standard was dissolved in methanol, and the volume was adjusted to 10 mL to obtain individual stock solutions of GA, KA, RU, QR, and β-ST. Each stock solution was then diluted to 100 ppm, and 6 µL of the solution was applied to the HPTLC plate separately. To facilitate simultaneous estimation, a mixture of standard solutions was prepared by combining 1 mL of each 1000 ppm stock solution of GA, KA, RU, QR, and β-ST. Further, the resulting solution was diluted to a final volume of 10 mL with methanol, yielding a final concentration of 100 ppm for each compound. In order to prepare a calibration curve for each compound, the mixed standard solution was filtered through a 0.45 µm membrane filter and applied in volumes of 3, 4.5, 6, 7.5, and 9 µL per band, which corresponded to 0.3–0.9 µg/spot.

Densitometric scanning of the HPTLC plates was performed at wavelengths corresponding to the absorption maxima of respective compound. The usual isosbestic wavelength of 371 nm was used to scan QR, RU, and KA, while GA was scanned at 279 nm. The developed plate was initially derivatized with anisaldehyde–sulfuric acid reagent (anisaldehyde: concentrated sulfuric acid: glacial acetic acid: methanol, 1:10:20:170, v/v/v/v) and subsequently heated to 105°C for 5–10 min to generate a distinctive coloured band for the detection of β-ST. The derivatized plate was subsequently scanned densitometrically at 521 nm to detect and quantify β-ST.

2.7.3 Sample Preparation for Simultaneous Estimation of Actives in G44MC Extract

The optimized extract of MC powder and G44 at a 1:2 ratio in 10 mL of water was prepared and filtered through a 0.45 µm membrane filter. Ethyl acetate (5 mL) was then used to perform liquid-liquid partitioning. To obtain Fraction A, the ethyl acetate layer was separated, dried, and reconstituted with 1 mL of methanol. The residual aqueous layer was evaporated to dryness and reconstituted with 1 mL of a 70:30 (v/v) methanol-water mixture to obtain Fraction B. Ultimately, a total extract volume of 2 mL was obtained by combining Fractions A and B, which was used for additional analysis.

2.8 Phytochemical analysis

Phytochemical screening for the G44 MC extract was performed qualitatively, following established protocols with minor modifications [9, 10]. Standard colorimetric tests were employed to detect major phytochemical classes. All tests utilized small extract volumes (0.5–5 mL), with outcomes interpreted via characteristic colour changes or precipitates as detailed below.

2.8.1 Alkaloid Test (Wagner’s Test)

To 0.5 mL of G44MC extract, 2–3 drops of Wagner’s reagent was added. The formation of a reddish-brown precipitate indicates the presence of alkaloids.

2.8.2 Flavonoid Test (Concentrated H_2SO_4 Test)

The G44MC extract (1 mL) was treated with 2–3 drops of concentrated sulfuric acid. Formation of an orange to reddish coloration indicated the presence of flavonoids.

2.8.3 Tannin Test (Braymer’s Test)

G44MC extract (1 mL) was mixed with distilled water (3 mL). In the solution thus obtained, 3–4 drops of 10% ferric chloride was added. The presence of tannin was indicated by development of blue-green colour.

2.8.4 Saponins Test (Froth Test)

G44MC Extract (1 mL) was diluted with water (5 mL) and shaken vigorously for one minute within a graduated cylinder. The formation of a stable froth confirmed the presence of saponins.

2.8.5 Carbohydrate Test (Benedict’s Test)

To 2 mL of the G44MC extract, few drops of Benedict's reagent was added and then gently heated. The presence of carbohydrates in the extract was confirmed if an orange-red precipitate is observed.

2.8.6 Amino Acid Test (Ninhydrin Test)

Few drops of 5% ninhydrin solution was added to G44MC extract (2 mL) and heated. The presence of amino acids in the extract was confirmed if a violet color is developed in the tube.

2.8.7 Phytosterols Test (Salkowski’s Test)

G44MC extract (5 mL) was mixed with chloroform (2 mL) followed by the application of a few drops of concentrated H_2SO_4 to the wall of the tube. The appearance of a reddish-brown ring confirmed the presence of phytosterols.

2.8.8 Phenol Test (Lead Acetate Test)

G44MC extract (5 mL) was mixed with 10% lead acetate solution (3 mL). The appearance of a white precipitate confirmed the presence of phenols.

2.8.9 Fixed Oils and Fats Test (Saponification Test)

G44MC extract (1 mL) was heated with 0.5 N alcoholic KOH (2 mL) and a few drops phenolphthalein indicator for a duration of 2 hours. The presence of fixed oils and fats was confirmed upon the formation of soap/emulsification.

2.8.10 Anthocyanins Test (HCl Test)

G44MC extract (2 mL) was mixed with 2–3 drops of conc. HCl. Mix gently, and the pink-red solution signified the presence of anthocyanins.

2.8.11 Terpenoids Test

G44MC extract (5 mL) and chloroform (2 mL) was combined and evaporated. Subsequently, concentrated sulfuric acid (H_2SO_4, 3 mL) was added, and the mixture was heated to boiling. The emergence of a grey coloration signified the presence of terpenoids.

2.9 Determination of Total Phenolic Content

The Folin–Ciocalteu (FC) colorimetric method was used to record the total phenolic content of the fruit extract, with only slight modification. [11] For analysis, G44MC extract (0.2 mL) was combined with distilled water (0.6 mL). To this solution, 10% (v/v) aqueous Folin–Ciocalteu reagent (0.2 mL) was added. After five minutes, 8% (w/v) sodium bicarbonate ($NaHCO_3$, 1 mL) solution was introduced in the solution, followed by 3 mL of distilled water. A blank solution was similarly prepared taking 0.2 mL of ethanol instead of G44MC extract.

All reaction mixtures were incubated at 50°C for 30 minutes in order to enhance color development in the mixture. The absorbance of the samples was measured at 765 nm using a UV-visible spectrophotometer. Standard GA solutions ranging from 100 to 500 µg/mL were prepared to construct a calibration curve. The total phenolic content was then calculated from this curve and expressed as GA equivalents (GAE) using Eq. (1).

$C = c \times V / m$

where…

C = Total phenolic content (mg GAE/g of powder),

c = conc. of GA determined from the calibration curve (mg/mL),

V = vol. of extract used in the reaction (mL), and

m = weight of the powder (g).

2.10 Determination of Total Flavonoid Content

The total flavonoid content in the G44MC extract was estimated using an aluminum chloride colorimetric assay with slight modifications. Quercetin was used as the standard reference. Briefly, the aqueous extract or a quercetin standard solution of 10–50 µg/mL (0.6 mL) was mixed with ethanol (1.2 mL) and 2% w/v aluminum chloride solution in ethanol (1.2 mL). After 60 minutes of incubation, the absorbance of the mixture was measured at 517 nm using a UV-visible spectrophotometer. Quercetin was used to plot the standard curve, and the result was indicated as mg of quercetin equivalent per gram (mg QE/g) of dry fruit powder. [12]

2.11 DPPH free radical-scavenging activity

The DPPH radical-scavenging assay was used to record the antioxidant potential of the prepared extract [13]. Serial dilutions of G44MC extract at 100, 200, 300, 400, and 500 µg/mL were prepared. Each dilution (1 mL) was mixed with DPPH solution (0.15 mM in ethanol, 1 mL). All samples were incubated in the dark at room temperature for 30 minutes after gentle vortexing. A negative control was prepared by mixing ethanol (1 mL) with DPPH solution (1 mL). Ascorbic acid, prepared at the same concentrations as the extract dilutions (100–500 µg/mL), was used as the positive control. The spectrophotometer was calibrated using a 1:1 mixture of distilled water and ethanol. After incubation, the absorbance of each sample was recorded at 517 nm using a UV-visible spectrophotometer (Lab India, UV3092, India). The DPPH radical-scavenging activity percentage was calculated using the following formula:

% Scavenging activity = (Abs Control - Abs Sample) / Abs Control × 100

Where,

Abs Control—The absorbance measured for the control sample containing DPPH and ethanol.

Abs Sample—The absorbance measured for the mixture of DPPH radicals and the sample.

Each measurement was conducted thrice and presented as mean % inhibition. This method provides a simple, effective, and straightforward way to calculate inhibition by plotting percentage scavenging activity against sample concentration.

3. Results and discussion

3.1 Characterization of the Extract

3.1.1. Isolation of MMR

Preparative HPLC was used to isolate MMR. The ethyl acetate fraction (5.3 g) collected after treatment of MC powder, as described in section 2.3.1, was dissolved in ethanol at 1 mg/ml. The fraction (30 ppm, 2 mL) was injected into the preparative HPLC at 230 nm. The chromatogram showed multiple phytoconstituent peaks, with the major, well-resolved peak at rt 12.05 (Fig. 1A) corresponding to MMR as per the reported literature [7] The peak fraction observed at rt 12.05 was collected, and multiple injections of the same fraction were performed to obtain measurable amount of MMR. The collected fraction, when injected in HPLC, showed a characteristic peak at 11.8 min (Fig. 1B), confirming successful isolation of MMR.

3.1.2. Characterization of Isolated Marker Compound (MMR)

The isolated MMR fraction showed a characteristic, distinctive $\lambda_{\max}$ at 230 nm when analysed by UV spectroscopy (Fig. 2A). Moreover, the HPTLC densitogram recorded at 230 nm also showed a single peak. The results of UV-visible spectroscopy and HPTLC were in agreement with previously reported literature. The spectra demonstrated reliable spectral consistency for the compound. Moreover, the densitometric scanning performed during HPTLC analysis (Fig. 2B) was also in accordance with the previously reported literature. HPTLC chromatography showed a sharp, well-resolved peak at Rf 0.71 (Fig. 4), matching closely with the reported Rf value of 0.76, authorizing the successful isolation of MMR [7]. The single prominent peak in the densitogram suggested high purity of isolated MMR. LC-MS/MS analysis also showed a parent ion at 437.1 m/z (Fig. 3A) and diagnostic fragment ions at 393.2, 365.1, 333.2, 304.1 m/z consistent with the triterpenoid structure further strengthening our observations.

3.2 LC–MS/MS Identification of Phytoconstituents

The LC–ESI–MS/MS analysis conducted in negative-ion mode showed numerous bioactive phytoconstituents in the ethyl acetate fraction of the 95% alkalinized ethanol extract of MC. Similar peaks were also observed in the preparative HPLC for ethyl acetate isolated fraction and the G44 extract.

(A)

(A)

(A)

Identification of phytomolecules was based on deprotonated molecular ions $[M-H]^-$, fragmentation patterns, and comparisons of derived m/z values with theoretical molecular weights and previously reported values for GA, QR, KA, RU, β-ST, MMR [14–20]. The isolated fraction from ethyl acetate collected from ETC MC showed a characteristic peak at 437.1 m/z, confirming its enrichment with MMR and revealing that it is soluble in organic solvent (Fig. 3A and Table 1). Similarly, the G44MC extract showed a characteristic peak at 437.1 m/z, thereby confirming the presence of MMR. Thus G44/14 extraction lead to micellar solubilisation of lipophilic MMR into aqueous phase yielding an eco-friendly extract (Fig. 3B and Table 1). Moreover, the G44MC extract exhibited a more diverse phytochemical profile, with molecular ions corresponding to β-SIT (413.1 m/z), GA (169.0 m/z) (Fig. 3C and Table 1), QR (301.1 m/z), KA (285.1 m/z), RU (609.3 m/z), and MMR (437.1 m/z).

Thus, the G44MC extract contained phenolic acids, flavonoids, and triterpenoids. Overall, LC–MS/MS profiling of the G44MC extract confirmed the presence of various secondary metabolites. Amongst the quantified phytoconstituents GA and RU are water soluble while QR, KA, β-ST, and MMR are entirely water-insoluble suggesting ability of G44/14 to extract compounds with diverse physicochemical properties. The quantification of selected phytomolecules was done using HPTLC.

3.3 Optimization of Gelucire Extract based on MMR using HPTLC

HPTLC was used to quantify the marker compound MMR in the prepared extracts. The method demonstrated excellent linearity across the concentration range of 1.5–1.8 µg/spot, with a regression equation of $y = 111x + 41.9$ and a correlation coefficient of $R^2 = 0.9991$. The calibration curve derived from the isolated MMR was used to quantify MMR in the prepared extracts. Under the optimized chromatographic conditions, the isolated MMR showed a distinct peak with an Rf value of 0.719 (Fig. 4A). All ratios of G44MC and G50MC extracts and the ethyl acetate fraction from ETC extract showed the presence of the marker compound (MMR), with a peak at the same Rf value as isolated MMR (0.71) (Fig. 4B, C, D).

Extraction efficiency of ETC MC extract, G44MC and G50MC was evaluated based on MMR content. Both G44MC and G50MC series demonstrated a progressive increase in MMR content as the proportion of Gelucire increased from 1:0.5 to 1:3. A statistical comparison using paired t-test of the two series (G50MC vs. G44MC) across identical crude drug-to-Gelucire ratios (1:0.5 to 1:3) revealed remarkable differences in MMR content (% w/w in powder). G44MC consistently showed higher MMR content than G50MC at every corresponding ratio ($t = 9.429$, $p = 0.0002$). G44MC showed higher baseline and peak MMR percentages compared to G50MC. For instance, at the lowest ratio (1:0.5), G44MC showed nearly

four times higher MMR content than G50MC. Further both series appear to plateau at higher Gelucire ratios (around 1:2 to 1:3), where the incremental gains in MMR content became marginal. Thus G44MC demonstrated superior compatibility, solubilisation with the crude drug extract. The higher MMR recovery with G44 may be attributed to its physicochemical properties. G44 contains lauroyl groups from medium-chain C12 fatty acids, whereas G50 contains stearoyl groups from long-chain fatty acids. The shorter fatty acid chain in G44 might have interacted more effectively with the plant matrix, improving MMR solubilization. In addition, G44 has a lower melting point (44°C) than G50 (50°C). Therefore, it can become fluid at a lower temperature, which may have improved wetting, micelle formation, and mass transfer during extraction. These findings are consistent with previous reports by [21] and [22]. Thus G44MC4 batch was considered as optimized since further increase in gelucire did not show remarkable extraction output and higher ratios would increase unnecessary lipid/excipient load in the formulation system.

3.4 Simultaneous Estimation of Phytoconstituents by HPTLC

HPTLC analysis showed successful simultaneous separation of GA, RU, QR, KA, and β-ST in the standard solution and the G44MC extract. The method demonstrated satisfactory selectivity, with selected active bands occurring at the characteristic Rf value without interference from other constituents. The chromatographic bands were distinct, well-defined, and effectively resolved, with no significant tailing or overlap.

3.4.1 Chromatographic profile

Images of HPTLC plates under various detection modes is depicted in Fig. 5. UV-quenching zones of phenolic compounds were clearly visible at 254 nm (Fig. 5A), whereas the flavonoid fraction showed strong fluorescence at 366 nm (Fig. 5B). The derivatization process showed a distinctive coloured band under visible light representing β-ST (Fig. 5C). Densitometric imaging provided precise peak characterization. The presence of GA in G44MC extract was confirmed by a distinct, symmetric peak at 279 nm (Fig. 6A–B) with Rf 0.408 ± 0.006 closely matching the Rf value of the standard (0.417 ± 0.003), indicating no co-elution. The flavonoid analysis at 371 nm (Fig. 6C–D) achieved excellent separation of RU (Rf 0.060 ± 0.002 (std) and Rf 0.061 ± 0.001 (extract), QR (Rf 0.583 ± 0.004 (std) and Rf 0.585 ± 0.002 (extract)), and KA (Rf 0.635 ± 0.005 (std) and Rf 0.636 ± 0.003 (extract) while maintaining baseline resolution ($\Delta Rf \geq 0.05$). The baseline resolution is crucial for precise quantification of compounds in complex matrices. [23, 24]. A prominent β-ST peak was observed at Rf 0.774 ± 0.003 (std) and Rf 0.786 ± 0.012 (extract) after post-derivatization scanning at 521 nm (Fig. 6E–F). [25]. The method specificity for all five markers was confirmed by the minimal Rf shift when compared to the standard, along with comparison of the spectra at the peaks, which together indicated uniform peak purity and no matrix interference.

3.4.2 Rf values of the standard and the sample

Rf values for marker compounds and corresponding resolved peak bands in the extract sample were determined and scanned at specific detection wavelengths as mentioned in Table 3.

Table 3
The corresponding peaks in the *Momordica charantia Linn.* extract at various detection wavelengths, along with the densitometric characteristics and Rf values of standard markers.

Compound	Rf (Standard)	Rf (Sample)	Wavelength
GA	0.417 ± 0.003	0.408 ± 0.006	279
RU	0.060 ± 0.002	0.061 ± 0.001	371
QR	0.583 ± 0.004	0.585 ± 0.002	371
KA	0.635 ± 0.005	0.636 ± 0.003	371
β-ST	0.774 ± 0.003	0.786 ± 0.012	521

3.4.3 Calibration Curves of Selected Phytoconstituents

In order to standardize the extract GA, RU, QR, KA and β-ST phytoconstituents were quantified using HPTLC. Calibration curves of all the selected phytoconstituents were constructed using marker compounds. The curves were linear over 0.3–0.9 µg/spot with correlation coefficients (R^2) > 0.99 for all the constituents, suggesting their suitability in the standardization of the extract (Table 4).

Table 4
Calibration curve parameters for gallic acid, rutin, quercetin, kaempferol, and β-sitosterol determined by the HPTLC method.

Compound	Linearity Range (µg/spot)	Regression Equation (y = mx + c)	Correlation Coefficient (R^2)
Gallic acid	0.3–0.9	y = 0.0157x + 0.0034	0.9966
Rutin	0.3–0.9	y = 0.0054x + 0.0002	0.9963
Quercetin	0.3–0.9	y = 0.0109x + 0.0015	0.9904
Kaempferol	0.3–0.9	y = 0.0155x − 0.001	0.9957
β-sitosterol	0.3–0.9	y = 0.0018x + 0.0011	0.9925

3.4.4 Quantitative Estimation

The optimized extract showed highest amount of β-ST (0.436% w/w) while GA was present in least amount (Table 5). The method demonstrated excellent specificity, with consistent Rf values and no matrix interference. Simultaneous estimation of chemically diverse compounds was enabled by multi-wavelength detection and derivatization. The reasonably acceptable amounts of phytoconstituents as quantified in the optimized extract could be attributed to the superior solubilizing power of gelucire,

extracting compounds with diverse physicochemical properties. Moreover, the extract would have numerous phytoconstituents, which could be beneficial in demonstrating its pharmacological activities however quantification of all of them is not possible. Additionally, the newly developed HPTLC method was found to be highly effective at quantifying multiple bioactives simultaneously, yielding consistent and reliable results. The method is straightforward, cost-effective, and suitable for routine quality control of MC.

Table 5
Quantitative estimates of selected phytomarkers in *Momordica charantia Linn.* extract, reported as mg/g and % w/w.

Compound	mg/g of powder	Content (% w/w in powder)
GA	0.054 ± 0.07	0.0054 ± 0.0012
RU	0.47 ± 0.14	0.047 ± 0.008
QR	0.44 ± 0.21	0.044 ± 0.011
KA	0.97 ± 0.26	0.097 ± 0.0008
β-ST	4.36 ± 0.12	0.436 ± 0.095

3.5 Phytochemical analysis of G44 *Momordica charantia Linn.* extract

It is very well known that plant-derived sources contain a wide variety of phytochemicals, commonly seen as natural sources of bioactive compounds with various therapeutic applications. In medicinal plants, the fruit of MC contains a wide range of bioactive phytochemicals with various medicinal uses, including antidiabetic, antimicrobial, and antioxidant activities [3]. These plant-based remedies offer cost-effective, easily accessible treatment options with no side effects and proven efficacy. The qualitative analysis of optimized G44MC extract was done to confirm the presence of multiple phytochemicals (Table 6). Qualitative screening highlighted the impressive phytochemical richness of the G44MC extract with positive results across all categories tested viz. alkaloids, flavonoids, tannins, saponins, reducing sugars, phytosterols, phenols, lipids, and terpenoids. Thus, the optimized extract is believed to exhibit diverse therapeutic properties owing to extraction of various phytochemicals.

Table 6
Phytochemical Analysis of G44 *Momordica charantia Linn*. Linn extract

Phytochemical Test	Name of test	G44 MC extract
Alkaloids Test	Wagner's test	+
Flavonoids Test	Conc. H_2SO_4 test	+
Tannins Test	Braymer's test	+
Saponins Test	Froth test	+
Carbohydrates Test	Benedict's test	+
Amino acids Test	Ninhydrin test	-
Phytosterols Test	Salkowski's test	+
Phenols Test	Lead acetate test	+
Fixed oils and fats Test	Saponification test	+
Terpenoids Test	Chloroform + H_2SO_4 test	+
(+) signifies a positive test result		
(-) signifies a negative test result		

3.6 Total Phenolic Content—

Phenolic compounds play major role in demonstrating antioxidant activity and are the key bioactive components of plants with strong redox characteristics [26]. Folin-Ciocalteu assay was used to quantify the total phenolic content in the optimized G44MC extract. The calibration curve plotted for standard GA was found to be linear ($y = 0.0002x + 0.1844$, $R^2 = 0.9972$) spanning 100–500 µg/mL. The total phenolic content as expressed in GA equivalents (GAE) per gram of MC fruit powder was found to be 8.93 mg GAE per gram of powder which is in accordance with previous literature with TPC values of 19.005 ± 0.270 mg GAE/g dry extract [27], 2.24 mg GAE/g dry weight [28] and 8.48 mg GAE/g extract [29]. Variations across studies may arise from differences in cultivar, fruit maturity, drying methods, and extraction conditions.

3.7 Total flavonoid content

The aluminum chloride colorimetric method was used to estimate the total flavonoid content in the optimized G44MC extract and expressed as quercetin equivalents. The calibration curve plotted for quercetin demonstrated excellent linearity within the 10–50 µg/mL range, with the regression equation $y = 0.0133x - 0.0184$ and a correlation coefficient (R^2) of 0.998. The analysis of the extract revealed a concentration of 11.85 mg QE per gram of dry fruit powder.

Flavonoids are natural plant compounds with antioxidant activity that depends on the number and position of hydroxyl (OH) groups. Past literature reports include 1.24 mg RE/g dry powder [30], 17.7 mg

RE/g dry extract [31], and 842.27 mg QE/g extract [32]. Variations in reported TFC values may be due to differences in plant source, extraction method, and analytical conditions. The presence of flavonoids in the extract is believed to demonstrate potent antioxidant and free-radical scavenging activities as reported in the literature.[33]

3.8 DPPH free radical scavenging activity

The strong antioxidant potential of the extract, as demonstrated, is believed to exhibit strong antioxidant, anti-inflammatory, and antidiabetic activities, as reported in the literature. [34]. Additionally, the presence of flavonoids such as quercetin, rutin, and kaempferol in the extract may contribute to the biological activity of the extract [35]. DPPH free radical scavenging activity of the extract was estimated in order to confirm the results of the Folin-Ciocalteu assay.

Antioxidant ability of the extract was evaluated using the DPPH free-radical scavenging assay at concentrations ranging from 100 to 500 µg/mL.

DPPH radical-scavenging assay was used to estimate the antioxidant potential of the optimized G44MC extract. The assay involves the neutralization of DPPH radicals by phenolic and flavonoid compounds in the extract, resulting in a colour transition from purple to yellow in the sample, with absorbance at 517 nm. A noticeable, concentration-dependent reduction in DPPH absorbance at 517 nm with a corresponding increase in % scavenging activity demonstrated the free radical-scavenging activity of the extract. (Fig. 7).

At 500 µg/mL, G44MC extract achieved 68.34% DPPH radical scavenging activity, whereas the conventional aqueous extract of *Momordica charantia Linn fruit* typically shows 30.8–42.5% inhibition [36] and needs more than double this concentration (1000 µg/mL) to reach 50% scavenging. [33] The G44MC extract showed greater antioxidant power owing to its ability to extract diverse flavonoids, phenolics, and other vital bioactives from the fruit than standard water- or alcohol-based extracts [37]. Overall, the optimized extract demonstrating antioxidant potential is expected to show significant antidiabetic and anti-inflammatory activities. [38]

4. Conclusion

The present work was aimed at developing an eco-friendly extraction method using Gelucire® 44/14 for extraction of phytocomponents present in the fruit of MC. The optimized extract (in terms of fruit powder and gelucire) was standardized in terms of momordicinin, gallic acid, rutin, quercetin, kaempferol, and β-sitosterol complying with the CDSCO guidelines to be claimed as a phytopharmaceutical drug. The standardization was executed using HPTLC and LC-MS/MS methods. HPTLC method was developed for simultaneous estimation of gallic acid, rutin, quercetin, kaempferol, and β-sitosterol. The phytochemical screening of the extract confirmed the presence of several key bioactive compounds, highlighting its chemical richness. Further, the use of gelucire helped in the extraction of both polar and nonpolar phytoconstituents from the fruit of *Momordica charantia Linn.* The extract demonstrated concentration

dependent radical scavaning activity, confirming its capacity to neutralize free radicals and expected to exhibit broad therapeutic activities.

Declarations

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Author Contribution

Tejashri Deshmukh: Methodology, analysis and Writing original draft. Sharvil Patil: Conceptualization, Supervision and Review and editing the draft

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Figures

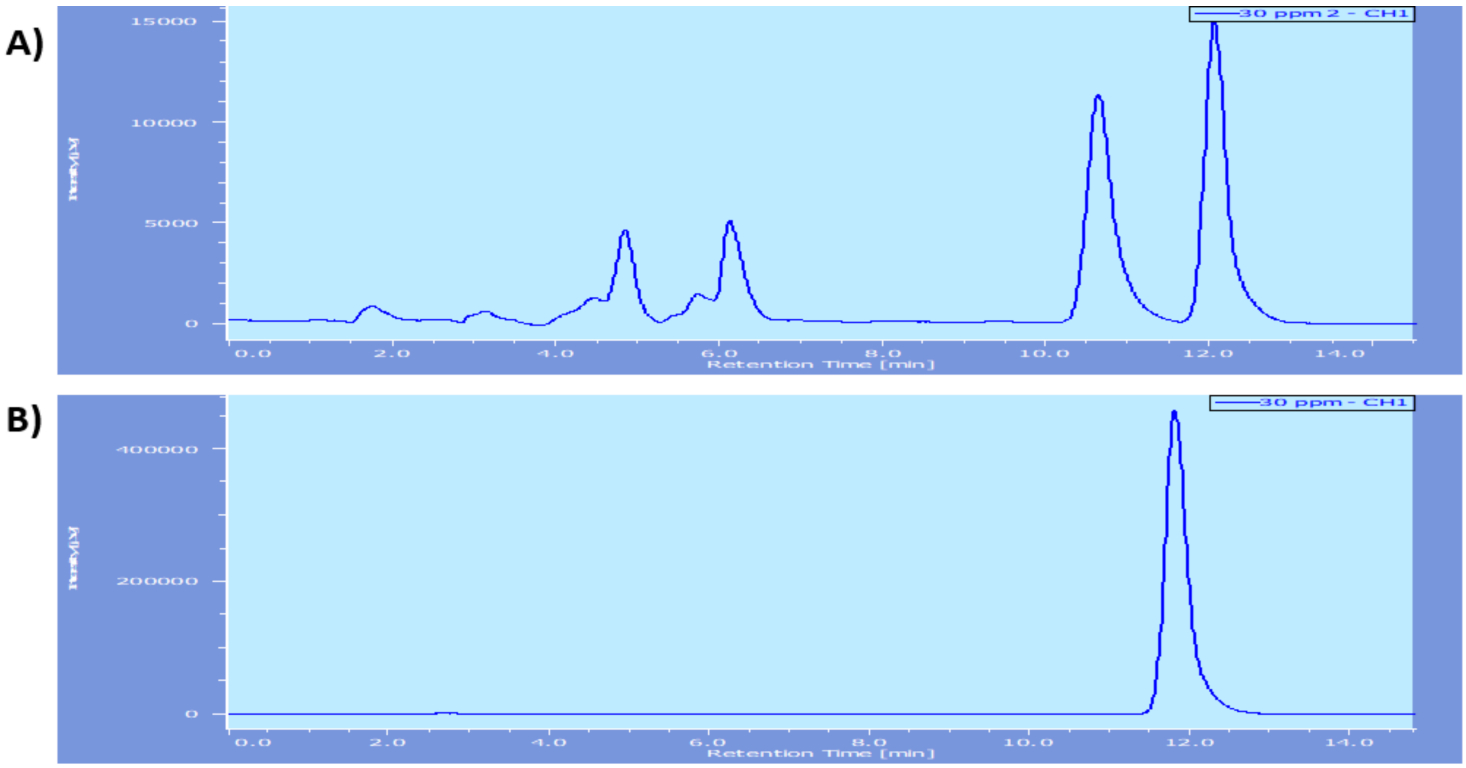


Figure 1

A) Chromatogram of ethyl acetate fraction B) Chromatogram of isolated compound MMR

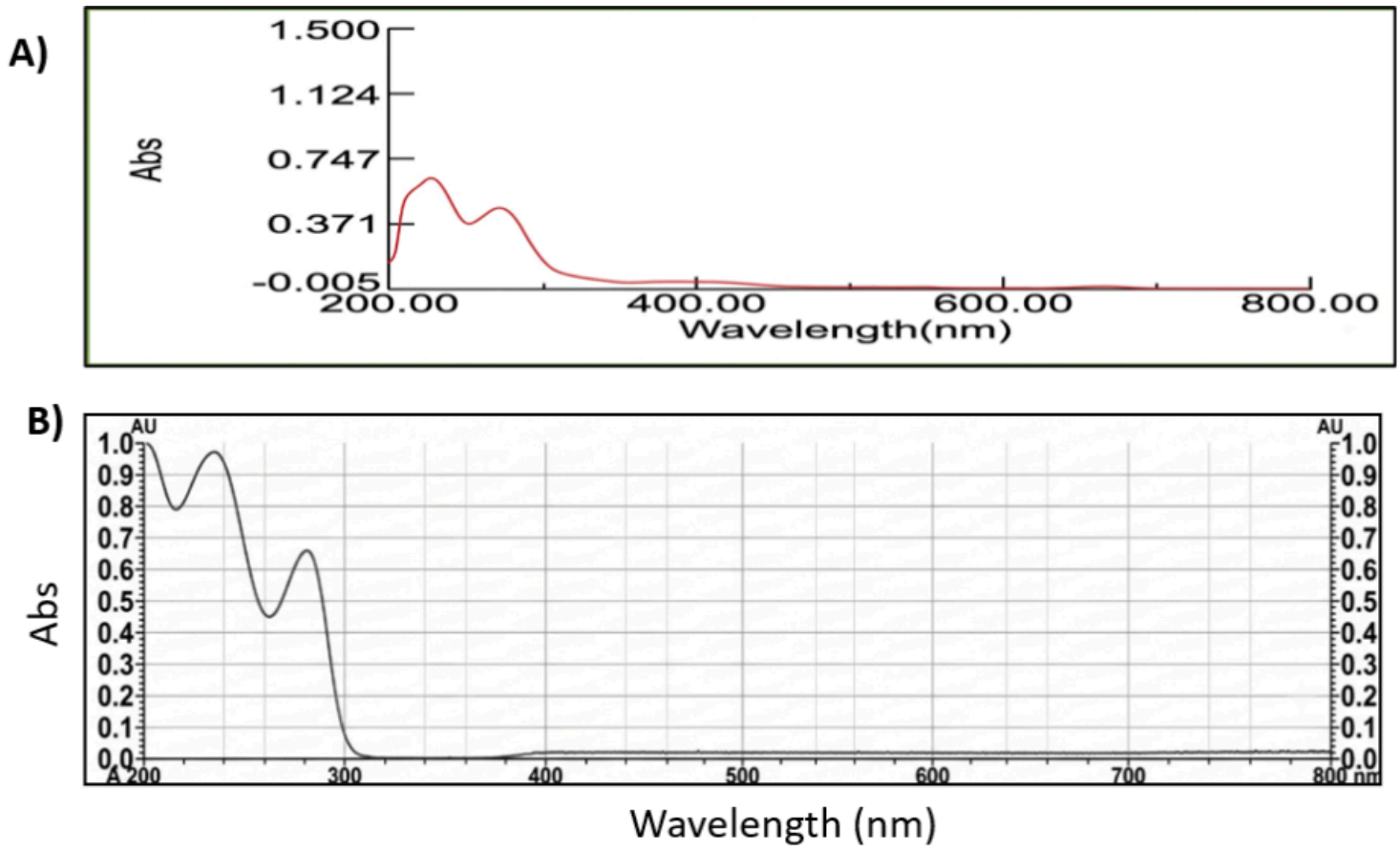


Figure 2

A) UV Spectrum of Isolated Compound MMR B) HPTLC Densitometric Spectrum of MMR

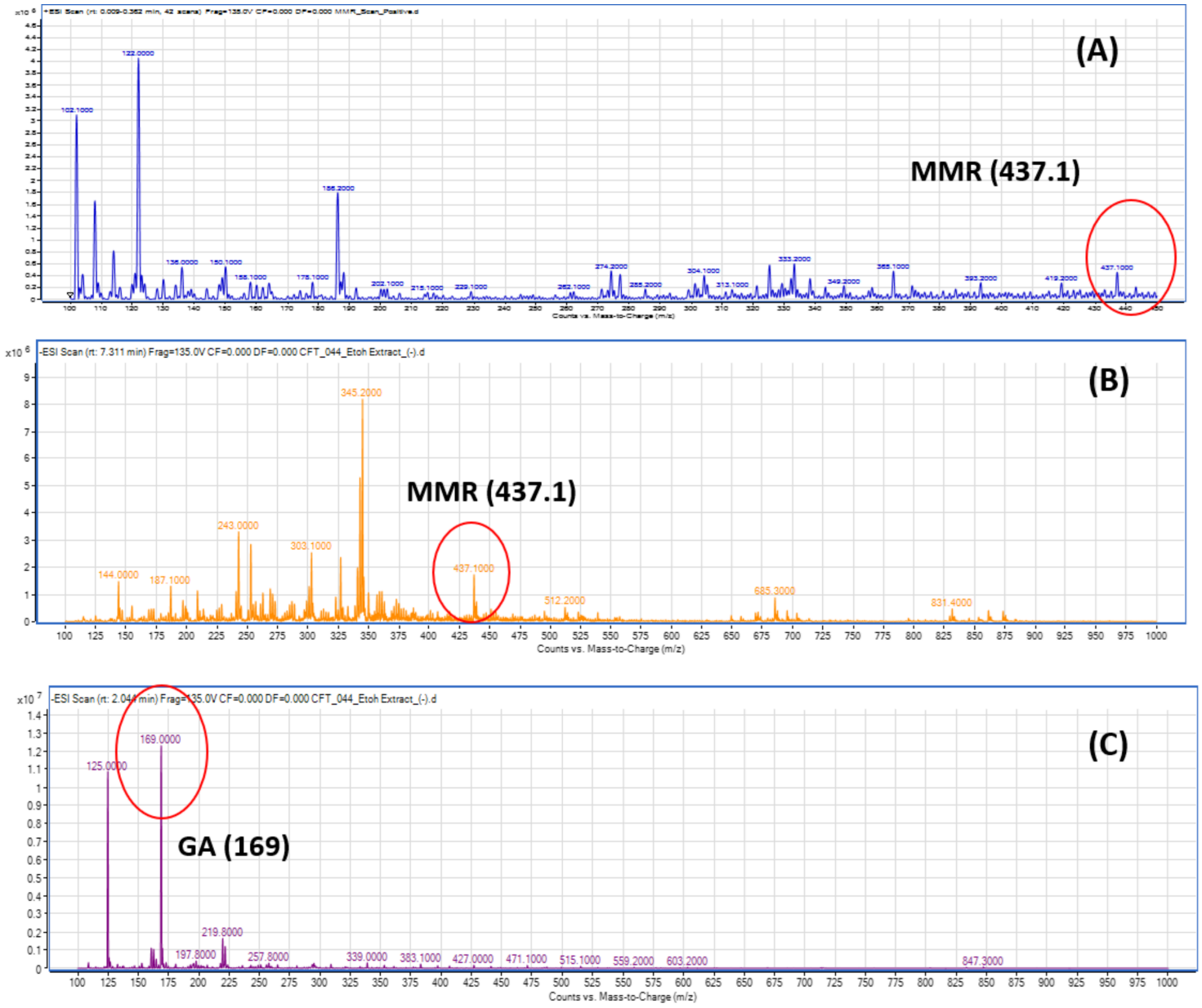


Figure 3

Representative LC–MS/MS chromatograms of A) Isolated fraction of Momordicinin (from ETC MC extract), B) Momordicinin (from G44MC), and C) Gallic acid (from G44MC), showing a distinct peak at its retention time and the corresponding mass spectral confirmation.

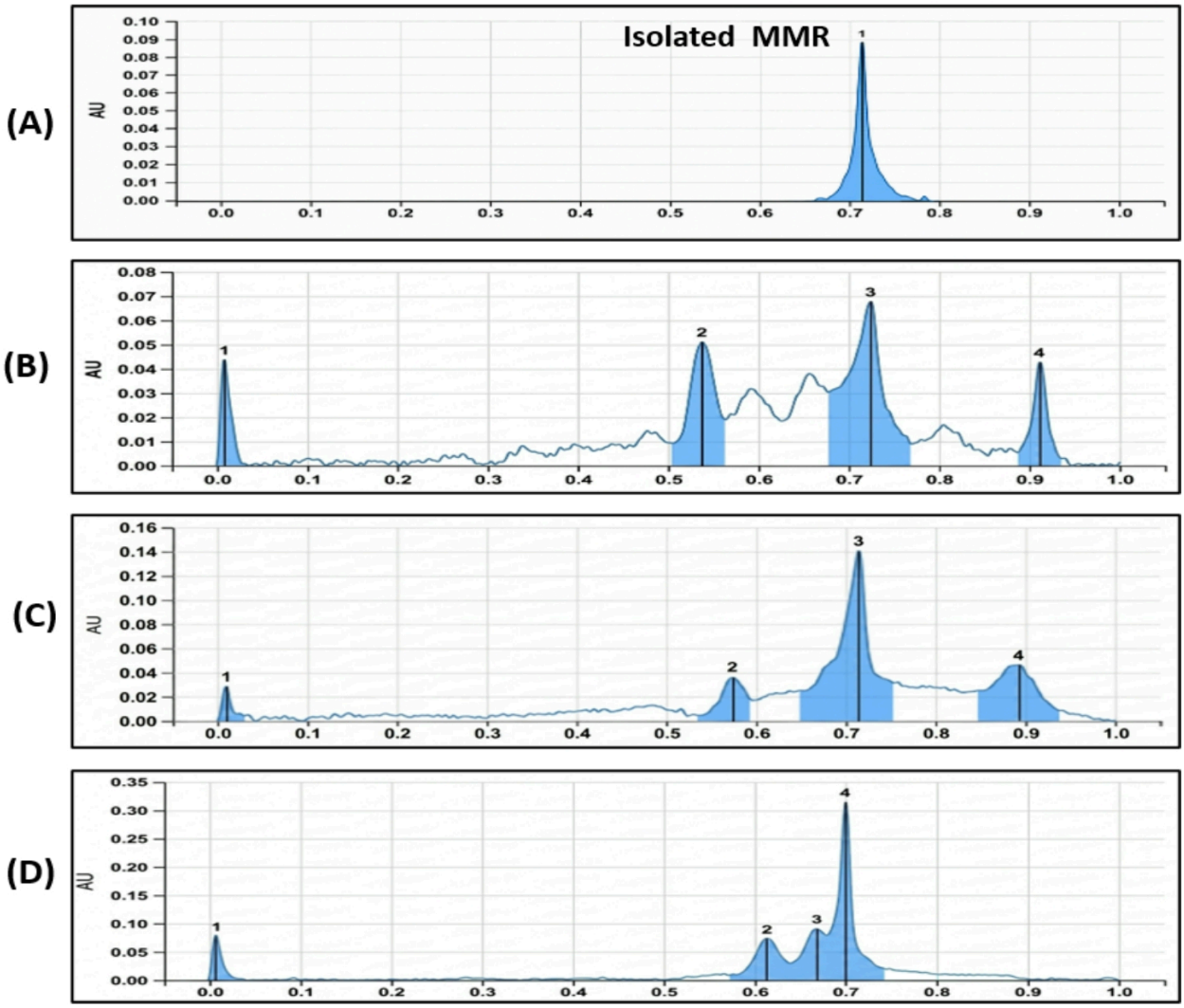


Figure 4

HPTLC chromatogram of A) Isolated Momordicinin; B) G50MC (Gelucire® 50/13 extract of *Momordica charantia Linn.*); C) G44MC (Gelucire® 44/14 extract of *Momordica charantia Linn.*); and D) ETC MC extract (ethanolic extract of *Momordica charantia Linn.*).

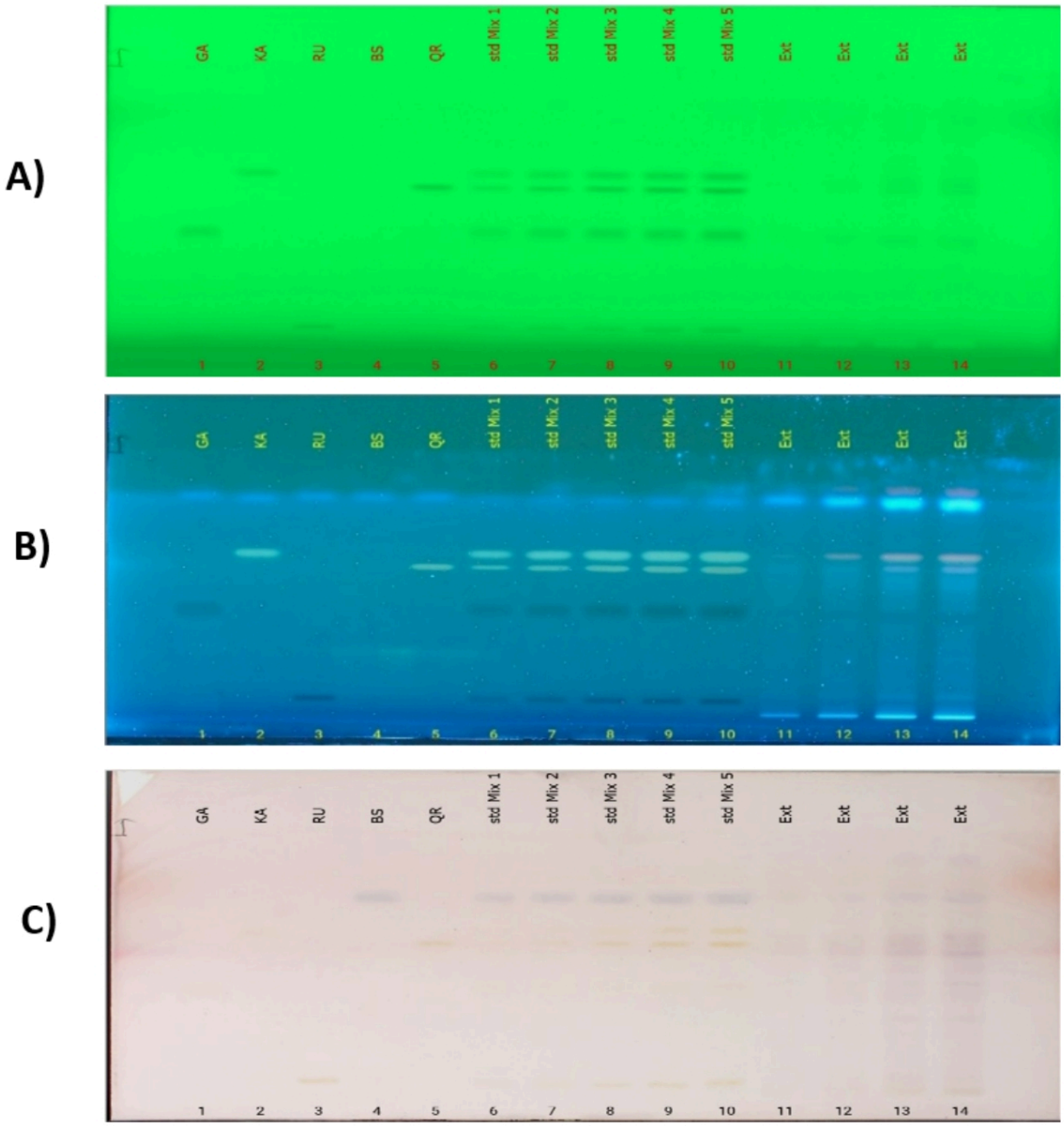


Figure 5

HPTLC chromatograms of standards (GA, KA, RU, BS, QR), std mixture (std mix), and extract sample (Ext) tracks under various conditions: (A) quenching bands of UV-absorbing chemicals at 254 nm; (B) fluorescent bands of flavonoids at 366 nm; and (C) visible light observation following derivatization.

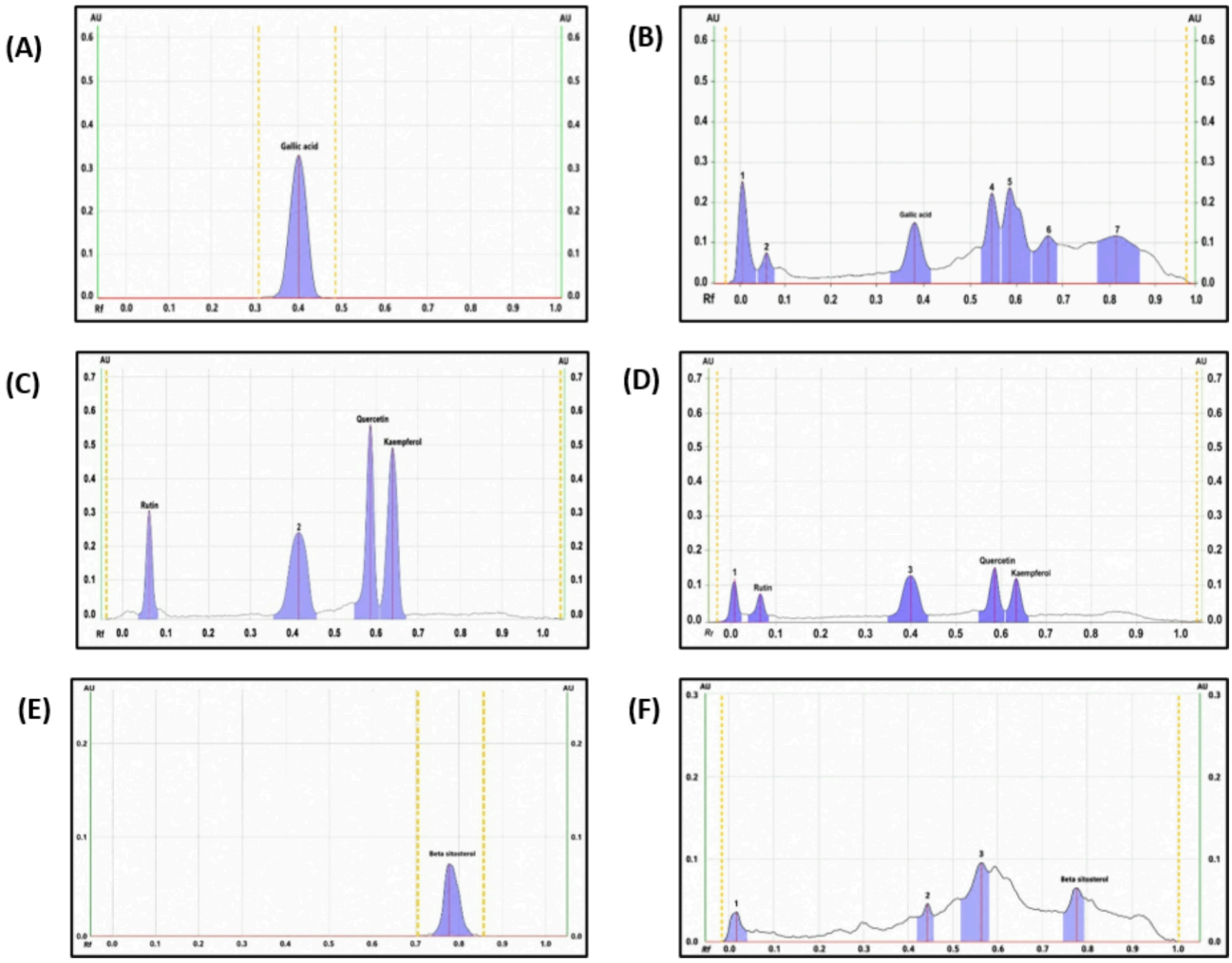


Figure 6

Densitometric HPTLC chromatograms at 279 nm: (A) Standard gallic acid; (B) G44 *Momordica charantia Linn.* extract showing the gallic acid peak. Densitometric HPTLC chromatograms at 371 nm: (C) Standard Rutin, Quercetin, and Kaempferol; (D) G44 *Momordica charantia Linn.* extract showing Rutin, Quercetin, and Kaempferol peaks. Densitometric HPTLC chromatograms at 521 nm after derivatization: (E) Standard β-sitosterol; (F) G44 *Momordica charantia Linn.*extract showing a β-sitosterol peak.

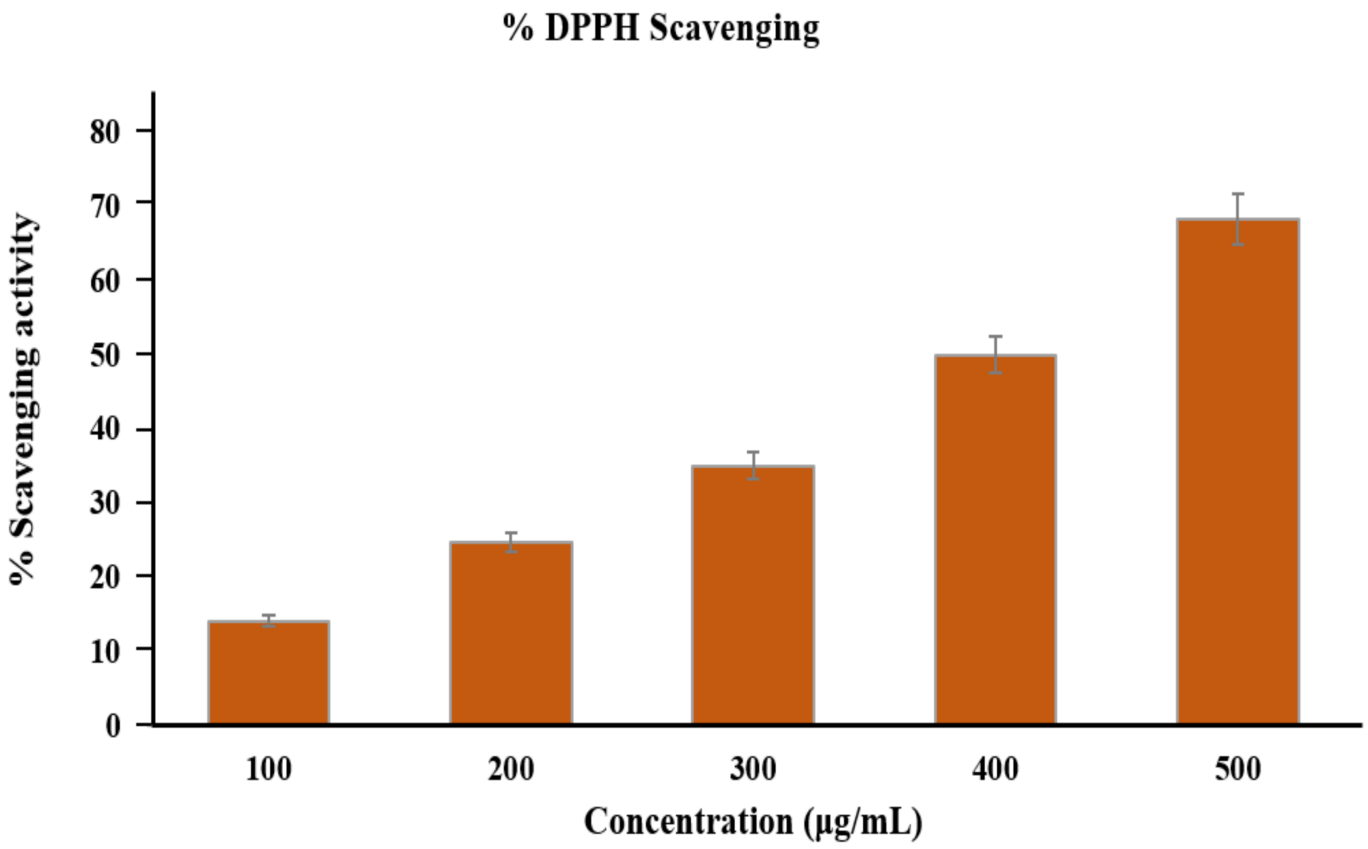


Figure 7

Concentration-dependent DPPH radical scavenging activity of optimized G44MC extract

Supplementary Files

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