RESEARCH ARTICLE

Identification of Bioactive Niaziridin in Horseredish Tree Extract by Analytical Tool

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ABSTRACT

Moringa oleifera Lam. is mostly known as the „potent medicinal flora" in terms of enormous therapeutic efficacy due to the availability of certain biologically active molecules. This has dual characteristics: it can be used as food and an active ingredient in many pharmaceutical formulations. Flavonoids, phenols, and alkaloids are some of *Moringa's* most abundant bioactive compounds, and they are responsible for its significant therapeutic properties. The current study is based on extracting and identifying major bioactive molecules from *M. oleifera* leaf and seed parts using a high-performance liquid chromatography technique to formalize its therapeutic potency of bioactive compounds. *M. oleifera* leaves and seed extracts in methanol were successfully analyzed in a single run using the HPLC method designed for this purpose. A Sunfire C18 column measuring 4.6 x 250 mm was used for the 220 nm HPLC analysis, and the solvent system consisted of 0.1% acetate buffered by acetonitrile (65:35 v/v) at a flow rate of 1.2 mL/min. The peak observed at 3.269 minutes in leaf extract and 3.289 minutes in seed extract shows the presence of niaziridin. The recovery values of niaziridin in *M. oleifera* leaves and seed extracts were 45.90 and 66.85%, respectively. Chromatograms show that seed extract has a larger amount of nitrile glycoside than leaf extract. The present investigation used an HPLC process to investigate the characteristics of niaziridin. This research is useful for standardizing *M. oleifera* leaf and seed extracts and producing pharmaceutical drugs from *M. oleifera*.

Keywords: *Moringa oleifera*, Drug resistance, Niaziridin, High-performance liquid chromatography, Biochemical

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INTRODUCTION

Hippocrates, the father of medicine, classified 300 medicinal plants on the basis of biological pharmacological activities like antipyretic, antarthritic, styptic, antimalarial, demulcent, anthelmintic, diuretic, sedative, narcotic, antiepileptic, anticancer, and astringent.[1] *Moringa oleifera,* also known as "the drumstick," is viewed as one of the world's most helpful trees. Leaves and seeds extracted from *M. oleifera* are found to show cell reinforcement movement. A few studies, notably in the cardiovascular field, are also evaluating the cancer-prevention agents of chemical compounds confined to *M. oleifera*. *M. oleifera Lam*. is an intriguing medicinal flora belonging to the Moringaceae family[2-3] with edible pods and leaves. The high-valued bioactive compounds available within different plant parts of *M. oleifera*[4] are numerous and include many secondary metabolites such as phenols, flavonoids, isothiocyanates, niazirin, glucomoringin, rutin, astragalin, etc. [5-8] The leaves and seeds of *M. oleifera* are being promoted as a potential primary treatment for coagulants in effluent from paper mills. Primary treatment of paper mill effluent could benefit from *M. oleifera* leaves and seeds as a coagulant treatment process.[9] *M. oleifera* leaf which has been acid activated, is another option for recovering dyes and heavy metals from water.[10]

Gold nanoparticles were synthesized in a sustainable way using *M. oleifera* flower petals.[11] Leaves and other parts of the tree were used as a pharmaceutical and antibacterial chemical source. [12-14] *Moringa* pods have three sides, are linear in shape, and drop down from the tree; they can range in length from 250–450 mm and contain 20 round seeds. [15] Using niaziridin as a marker, this chapter aims to examine the qualification and standardisation of *M. oleifera* leaf and seed content. The HPLC method we suggest is simple and precise, and it may be used to locate, observe, and measure niaziridin in *M. oleifera.*

Bioactive Compound Niaziridin

Niaziridin is a nitrile glycoside that is a medicinally valuable phytochemical of *M. oleifera*. Nitrile is a chemical compound made up of an organic hydrocyanic acid derivative and a glucoside, in which one or more sugars are linked to non-sugar

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molecules. Niaziridin, a bioavailability facilitator, promotes the efficient transfer of chemicals, medicines, and minerals across the membrane. The aziridine-rich fraction of *M. oleifera* pods was found to increase the bioavailability of drugs, vitamins, and nutrients by facilitating their absorption through the gastrointestinal membrane and increasing the bioactivity of commonly used antibiotics like tetracycline, rifampicin, and ampicillin against gram-positive and gram-negative bacteria.[16] Thus, niaziridin can be utilized in combination treatment with medications and supplements, bringing about decreased drug-related harmfulness, diminished expense, and a shorter course of chemotherapy. The original nitrile glycoside niaziridin may have bio-enhancing effects, therefore better extraction and chromatographic conditions were developed to rapidly and precisely screen niaziridin from *M. oleifera*. The part with the most extreme bio-enhancing action was named "pharmacologically active standardized extract."

Seeds are utilized for the production of cooking oil,[17] biodiesel,[18] source of water purification agent [19] and mosquito repellent[20] This HPLC analysis aims to determine how much niaziridin is present in *M. oleifera* leaf and seed samples. To establish which extracts of *M. oleifera* had a significant amount of bioactive chemicals, their total niaziridin concentrations were measured using HPLC techniques.

MATERIALS AND METHODS

Plant Sample Collection and Pre-treatment

Fresh leaves and seeds of *M. oleifera* were collected from the local area of Jhansi (U.P.) India, in their natural habitat. Collected parts were identified by Mr. Jagdesh Arya, research officer in botany at the Central Council for Research in Ayurvedic Sciences, Jhansi, U.P., where a voucher number (28717) was deposited for the specimen. The leaves and seeds were sorted, destalked individually, and washed under running tap water for 30 minutes at room temperature for estimation of niaziridin. The leaves were dried in a 60℃ hot air oven for 24 hours. The samples were dried, then powdered, strained, and kept at room temperature (28–30℃) in low light until use. The seeds were stored in a dry, cool place until they were ready to be air-dried and then crushed into a powder.[19]

Chemicals and Reagents

For the biomolecular quantification of various plant parts through HPLC analysis, reagents like acetonitrile, methanol, phosphoric acid and acetate buffer of HPLC grade (Rankem Pharmaceuticals. Rankem Pharmaceuticals, Gurgaon, Haryana, India) were used. Both the LR and HPLC grade solvents were bought from Rankem Pharmaceutical Company, Gurgaon, Haryana, India. The niaziridin standard was purchased from Sigma-Aldrich (St. Louis, MO, USA) (purity > 99% by HPLC).

Sample Extraction

A 5 gm of powder was placed in the thimble and extracted for 3 hours in 200 mL of methanol using a soxhlet extractor. The powdered sample is kept in a porous material like muslin cloth or filter paper in a thimble. A rotary shaker evaporator concentrated the extract for 48 hours and was stored in a 4°C water/air-proof bottle until use. The solvent was removed to yield a solid mass, which was then analyzed in the same manner. The extract was used to determine its analytical parameters.[20]

Table 1: Preparation procedure of Acetate Buffer (0.1 molar, pH 5.0)

Components	Amount	Concentration
Sodium Acetate (mw: 82.03 g/mol)	5.772 g	0.07 M
Acetic Acid (mw: 60.05 g/mol)	1.778 g	0.03 M

Preparation of Standard Solution

A standard stock solution of niaziridin was prepared in a 5 mL volumetric flask by mixing 1.7 mg of accurately weighted niaziridin in approximately 3 mL of solvent (0.1 % Acetate Buffer: Acetonitrile in a ratio of 65:35, respectively) (Table 1).

The solution was sonicated for 5 minutes after being adjusted to pH 5.0 with 10 N HCl. Finally, make the volume 1-L with the help of distilled water (solvent). The stock solutions of the samples and standard were prepared in methanol. HPLC determined the purity. Niaziridin resolution was investigated using different ratios of solvents. All successive HPLC analyses were carried out with the optimised mobile phase. The sample concentration was 20 mg/mL, while the standard concentration was 20 µg/mL.

Preparation of Sample Solution

HPLC-grade methanol was used to dissolve the measured crude dry extracts. 2.8 mg of accurately weighed methanol extract was dissolved in 1-mL of solvent to prepare the stock solutions of samples. For later use, all stock solutions were frozen at 20°C. Leaves and powdered seeds from the *M. oleifera* plant were sonicated at 30°C for 15 minutes. Sonicated flask contents (extracts and standard) were filtered using a 0.45 m syringe membrane filter (Type Millipore) before being evaluated by high-performance liquid chromatography (HPLC).[20] The filtrate was extracted and stored for further use.

HPLC Instrumentation

The Waters 515 HPLC pump, the waters in line vacuum degasser AF, the Waters 2998 photo-diode array detector, and the Waters manual auto-injector were used for the HPLC analysis in this study. The system was a Waters HPLC system SPD-10A (Kyoto, Japan). Sun fire C18 (4.6 x 250 mm, 10 m) column with Waters temperature control module II and Waters pump control module II was used for the separation. The information was processed using a data system designed to empower chromatography.

Analytical Method

Using a gradient solvent system, elution was performed in a 0.1% acetate buffer: acetonitrile (65:35 v/v) mobile phase consisting of solvent A and solvent B, respectively. Isocratic elution was used for preliminary estimate of the standard, *M. oleifera* leaves, and seed extract. Using a flow rate of 1.2 mL/min and an elution period of 3.26 minutes at 30°C,

Table 2: Statistical validation of HPLC

Sl. No.	CONDITIONS	SELECTED MEASURES
1	Mobile phase	0.1 % Acetate buffer and Acetonitrile
2	Mobile phase ratio	65:35 v/v
3	Detection Wavelength	220 nm
4	Column Temp	30 °C
5	Injection Volume	20 µl
6	Flow rate	1.2 ml/min
7	Run Time	20 min
8	Mode of Operation	Isocratic

excellent resolution was achieved. In the HPLC equipment, 10 µL of standard was injected and 220 nm was used to monitor the photo-diode array detector. The samples of leaves and seeds were injected with a 20 mg/mL stock solution that was diluted to 20 µL. In order to determine each compound's qualitative HPLC analysis, we used the peak area and retention time. The chromatogram peaks were integrated manually using the standard method.

Peak area = peak height × half height breadth

Validation and Chromatographic Conditions of HPLC Analysis

The developed HPLC technique was formalized by defining the linearity, correlation coefficient, LoQ and LoD, relative standard deviation, accuracy, specificity, retention time precision, peak purity and selectivity of elutes (Table 2). Chromatography was performed on a Waters HPLC system, SPD-10A (Kyoto, Japan), equipped with a Waters 515 HPLC pump. The applied conditions for chromatography are given in Table 2. The linearity of the detector was tested using HPLC by using triplicate samples (10 µL) at a range of extract concentrations. Calibration curves were created by plotting the injected amount (g) against the corresponding peak areas (m Abs), and regression equations were derived.

Linearity

A calibration curve was created for each standard solution using least-squares regression with peak area on the Y axis and analyte concentration on the X axis. Each compound's relative standard deviation was determined after creating a calibration curve using synthetic standards of varied concentrations.[2-3]

Method Validation

Standard deviation (SD) and the slope of the calibration curve were used to derive the LoQ and LoD. The lowest analyte concentration detectable in a single sample injection. For the same reason, the LoQ refers to the smallest value of a target population that can be determined with enough precision and accuracy. In accordance with recommendations from the International Council for Harmonization (ICH), the signal-to-noise ratio was used to validate the LoD and LoQ. Both intra- and inter-day variation for each of the studies was validated to authenticate *M. oleifera* plant sample seeds and leaves, along with the calculation of individual relative standard deviation (RSD) values, maintaining them below 2% for accuracy and precision of the given compound analysis. Furthermore, each test sample was run three times, once on the same day and three times on different days, to increase precision. [21]

RESULT

Determination of Niaziridin

Herbal extracts must be standardized to assure their quality and biological activity. Insightful methods have been created for assessing the quality of *M. oleifera* leaves, which will be used to create uniform standards for the leaf concentrates. *Moringa* leaf extract methanol chromatogram showing four peaks that were identified. A peak retention duration of 3.269 minutes yielded a peak area of 45.90%, the highest possible. At about the same retention time (Table 3), 3.116 minutes, a second, larger peak was recorded, accounting for 28.45% of the total peak area. Niaziridin was found to have a retention duration of 6.228 minutes and a peak area of 9.69% in the third peak of the *Moringa* sample. We found that the fourth peak in the *Moringa* sample had a retention time of 3.636 minutes and a peak area of 8.36%.

The leaf sample peak and standard niaziridin peak area and retention time were compared. Sample compounds' peak regions matched their requirements. Phytochemicals caused leaf extract peaks to differ from seed extract. To characterise and quantify niaziridin, the detected chemical (Retention time, 6.206 minutes) must be evaluated using a more sensitive method.

The high total polyphenol content in *M. oleifera* leaves directly results from their high total antioxidant capacity. According to prior findings.[17, 22-23], we attributed the peak at about 3.116 min to Quercetin-3-O-glucoside (compound 1), a flavonoid found in all samples. Compound 2 (niaziridin) served as a reference compound because to its large percentage area (45.90%) and short retention time (3.269 minutes). Apigenin-C-glucoside (compound 3) was also found to be present,[24] with a peak obtained at 3.636 minutes. In addition to flavonoids,

Table 3: Peak areas and retention times of compounds present in M. oleifera leaves at 20 mg/mL

Sl. No.	Retention Time (min)	Area	% Area	Height	Compounds Name
1	3.116	744053	28.45	67392	Quercetin-3-O-glucoside
2	3.269	1200280	45.90	194717	Niaziridin
3	3.636	218607	8.36	20601	apigenin-C-glucoside
4	6.228	253316	9.69	24656	Hexadecylferulate

Table 4: Peak areas and retention times of compounds present in *M. oleifera* seeds at 20 mg/mL

Sl. No.	Retention Time (min)	Area	% Area	Height	Compounds Name
5	3.139	658819	16.77	91299	Quercetin-3-O-glucoside
6	3.289	2625810	66.85	350772	Niaziridin
7	3.657	229282	5.84	20980	Apigenin-C-glucoside
8	6.244	229731	5.85	22134	Hexadecylferulate

hexadecylferulate (peak at 6.228 minutes in HLC spectra) was found in all extracts (compound 4).

HPLC analysis of *M. oleifera* seeds extract showed presence of Quercetin-3-O-glucoside (compound 5), the peak attributed at 3.139 minutes (Table 4). Niaziridin (compound 6) was used as standard compounds that having large percentage area i.e. 66.85% and retention time was 3.289 minutes (Table 5). Apigenin-C-glucoside (compound 7), whose peak was found to occur at 3.657 minutes, was also confirmed to exist in this investigation. Hexadecylferulate (compound 8) peaked at 6.244 min in HPLC spectra, and its identity was established**.** Das *et al.*, 2020 also evaluated *M. oleifera* seeds extract, investigated phenolic content in the plant, and evaluated its microbial activity.[25] However, Kadam *et al.*, 2017 identified ethynylestradiol in the seed extract of *M. oleifera*.[26]

Maximum extraction of the target marker, i.e., niaziridin, from the pods was achieved at 30°C for 3.26 minutes. The HPLC profile of the isolated chemical niaziridin is shown in Figure 1, demonstrating a purity of 66.85%. *M. oleifera* seed concentrations have been standardised as a result of

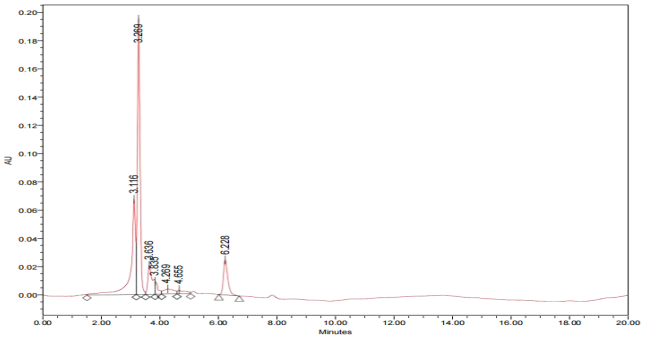

Figure 2: HPLC Chromatogram of the bioactive fraction of *M. oleifera* leaves

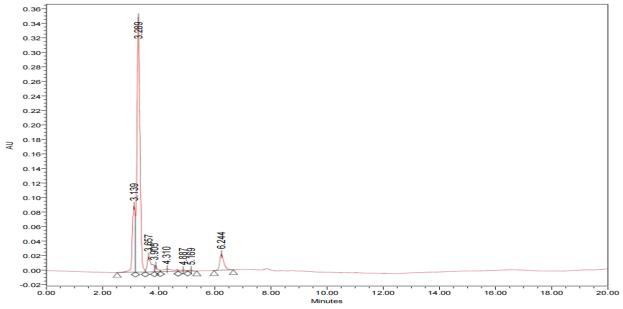

Figure 3: HPLC Chromatogram of the bioactive fraction of *M. oleifera* seeds

Table 5: Peak area and retention time of standard solution at 20 µg/mL

Sl. No.	Retention Time (min)	Area	% Area	Height	Compound
1	3.262	9385194	91.95	1322994	Niaziridin

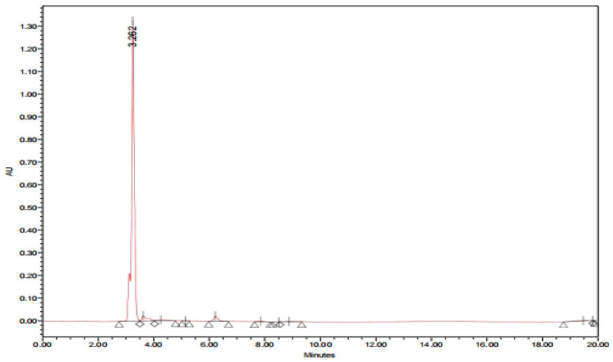

Figure 4: HPLC Chromatogram of standard niaziridin

sensitive methods for evaluating seed quality (Figure 2). Other compounds that were presented had the following retention times:

Four peaks were explained in the chromatogram created in the methanol seed concentrate of *Moringa*. When the peak had a retention time of 3.289 min and a peak region of 66.85%, the most elevated peak region was obtained. The second higher peak was acquired at about the same retention time of 3.139 minutes, which has covered 16.77% of the peak region. The third peak of the *Moringa* extract has shown a retention time of 3.657 minutes for niaziridin with a peak region of 5.84%. It was seen that the fourth pinnacle of the *Moringa* test had shown a maintenance time of 6.244 minutes with a peak region of 5.85%.

Niaziridin-related peaks in the extracts had a retention duration of 3.262 and a peak area of 91.95% (Figure 3). The conventional peak obtained a retention time of 3.262 minutes and a peak area of 91.95%. The spectra obtained from the *Moringa* methanol extract suggest that niaziridin and its derivatives may be present in higher concentrations than previously thought. Standard niaziridin was present in both plant extracts since the retention time of the standard and the measured spectra of the leaf and seed samples were identical on the chromatogram. The consistency of compound peaks with chemicals in the sample validated the method's specificity. There was niaziridin in the extracts because of peak at 3.269 min in the leaf extract sample and at 3.289 min in the seed extract.

DISCUSSION

The oxoanion quercetin 3-O-beta-D-glucopyranoside(1-) is formed when the hydroxy group at position 7 of the flavone moiety of quercetin is deprotonated. The dominant species at a pH of 7.3. It is the conjugate base of 3-O-beta-D-glucopyranoside, the sugar moiety of quercetin.

Apigenin 8-C-glucoside is a powerful glucosidase inhibitor and antioxidant. Retards the synthesis of inflammatory

cytokines such TNF-α, IL-1, IL-6, and IL-33. Displays *in-vivo* antinociceptive, anti-inflammatory, and antioxidant properties.

The coumaric acids and their derivatives, of which hexadecyl ferulate is a member, are a class of organic substances. Aromatic compounds hydroxylated on carbon atoms C2 (ortho-), C3 (meta-), or C4 (para-) of the benzene ring, including cinnamic acid and its derivatives. Hexadecyl ferulate has been found in potatos (Solanum tuberosum) and other root vegetables, although its concentration has not been determined. Because of this, hexadecyl ferulate may serve as a useful biomarker for determining whether or not certain foods have been consumed. A survey of published works reveals a dearth of information about hexadecyl ferulate. These three plants—Hendysarum polybotrys, Rubia tinctorum, and Euchresta horsfieldii—are the sources of this natural compound.

The isolation and validation of several naturally occurring plant product formulations necessitate rapid, robust and precise methodologies for the characterization of recognized molecular and structural categories. In this context, the high-pressure liquid chromatography (HPLC) method is a well-established technique that properly separates many types of compounds from a mixture depending upon their size, affinity to the solvent system, polarity, and several other factors.[2-3] The chromatograms produced by such a method have precise validation and result calculation. It is based on a differential migration protocol in which a mobile phase passes over a stationary phase and the mixture of compounds in a crude extract can be separated to get the desired components. Solutes with higher polarity in response to mobile phase solution will play a major role in separation through migration with analytes.[3]

For the separation of desired compounds, the various mobile phases used show different retention times depending on the movement of the compound in the selected mobile phase according to the solvent polarity. HPLC is the most frequently utilized separation technology in analytical science, with a global recovery rate of more than 65% of the compounds separated in plants and other systems. Examining the reference and the test samples, the technique was found sensitive and specific. Comparing the retention durations of the peaks for chemicals in the samples to those of standards confirmed that the compounds were present in the samples.

Analytical quality evaluation methods have been established to standardize *M. oleifera* leaf and seed extracts for the purpose of determining the quality of these leaves and seeds. It is essential to assess the observed compound using a more delicate strategy to characterize and qualify niaziridin. Leaves and pods are the source of the bioactive nitrile glycosides niazirin and niaziridin.[27] Antibiotics (including rifampicin, doxycycline, and ampicillin), vitamins, and minerals have all been shown to have greater bioavailability when taken with them because of their enhanced absorption through the gastrointestinal membrane.[28] An extract of *M. oleifera* pods was shown to considerably raise plasma concentrations of rifampicin when administered with the drug in a study examining the effect of the extract on the pharmacokinetic disposition of the antibiotic.[29]

The results showed that while individually glycoside could not inhibit bacterial growth, its combined effects reduced the MIC of tetracycline by a factor of up to 16 times. Efflux pump inhibition was found for both, but niaziridin was more successful. These glycosides decreased the concentration of tetracycline, which inhibits mutations.[16] This study is the first to show that glycosides, niazirin, and niaziridin can reverse drug resistance by inhibiting efflux pumps and modifying the expression patterns of drug-resistant genes. This research can potentially be useful in developing a synergistic antibacterial combination from the commonly cultivated *M. oleifera* to combat drug-resistant gram-negative bacteria.[30] The leaves have a variety of medicinal applications, including treating infections, diabetes, and heart problems, as well as relieving stomach pain, sprains, and high body temperatures.[31] Glucosinolate glycosides and complex flavonoid compounds have been found in the leaves of *M. oleifera*, and these compounds are responsible for the plant's anti-atherosclerotic, anti-oxidative, and anti-diabetic properties, as demonstrated by prior phytochemical studies.[32]

Niaziridin had a concentration of 0.015 to 0.005 in plant extracts derived from leaves and seeds. Compared to the plant samples used in the study, niaziridin concentrations in the commercial trial were lower. In another study, the HPLC analysis of *M. oleifera* leaf samples revealed detection at 334 nm wavelength with a minimal relative standard deviation below 2%.[21] Similar results from the above investigation indicate that for the separation of niaziridin, seeds of *M. oleifera* might be utilised as a possible source.[33]

A study confirmed the presence of nitrile glycoside (s) using a reverse phase high-performance liquid chromatography (HPLC) method using acetonitrile and water as the mobile phase (20:80 (% v/v)). The buffer is a phosphate solution with a pH of 3.8 and a flow rate of 0.7 mL/min at a wavelength of 220 nm.[34] Niaziridin and niazirin were being validated in some samples of *M. oleifera* but were not seen within the stem bark portions. Even isoquercitrin and quercetin 3-O-(6-O-malonyl)-β-D-glucopyranoside were found within the leaves of *M. oleifera*. In another study, isothiocyanates and astragalin were evaluated in the leaf and seed of *M. oleifera.* Phytomedicine has played an important role in pharmacotherapy.[3] As a result, *M. oleifera* has the potential to improve efficacy while also lowering drug treatment costs.

CONCLUSION

Results demonstrate the developed technique was extremely specific, accurate, and exact; it has the potential to be employed in the quality control of *M. oleifera* concentrates and formulations like tablets and capsules. Niaziridin was recovered at a rate between 45.90 and 66.85% across all *M. oleifera* extracts. The recent results supported the use of plant parts as traditional medicines, as the solvent extracts

include bioactive chemicals of therapeutic significance. Niaziridin's use in combination with other drugs and supplements can reduce side effects, lower expenses, and shorten chemotherapy regimens. This study could contribute to the evolution of gram-negative bacteria) that are resistant to antibiotics. It can also be used to examine the variance in bioenhancer content in standardized *M. oleifera* extracts from product to product. This method might be used to find out if this biomarker is present in other herbal extracts and to construct natural health products and finished products with these components in commercial nutraceutical products.

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CONFLICT OF INTEREST

In related to this research, the authors have no conflicts of interest.

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