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RESEARCH ARTICLE

Validated HPTLC Method for Detection of Kaempferol in *Moringa oleifera* Leaf Extract and Investigation of its Antidiabetic Potential by *In Vitro* and *In Silico* Studies

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Abstract

Background: Exploring traditional treatments offers valuable foundations for the innovation of new pharmaceuticals for the management of diabetes mellitus and its complications. Validation of plant extract by suitable chromatographic techniques is a part of standardization procedures. *Moringa oleifera* is one such traditionally used plant to be explored. **Aim:** The study explores the antidiabetic potential of *M. oleifera* through both *in vitro* and *in silico* explorations along with HPTLC method development and validation for estimation of kaempferol in *M. oleifera* extract. **Method:** Plant extract was prepared by maceration method using ethanol: water (70:25) and followed by Soxhlet(95% ethanol). Pharmacognostical analysis was conducted.*In silico* and *in vitro* antidiabetic and antioxidant studies were conducted, as well as quality control analysis was done using HPTLC method the mobile phase used was toluene: ethyl acetate: formic acid: methanol: (6:3:0.3:1v/v/v/v). **Results:** HPTLC analysis showed the presence of kaempferol, and the method was validated as per ICH guidelines. According to reports on molecular docking studies, several phytocompounds inhibited when porcine pancreatic α-amylase (PPA) complexed with a carbohydrate inhibitor (PDB ID: 4W93).The hydroalcoholic extract was discovered to have the ability to inhibit α-amylase, with IC_{50} values of 57.38588±1.92 µg/mL and 32.51564±1.59 µg/mL for standard acarbose, respectively. **Conclusion:** Overall, this research provides comprehensive data for the estimation of kaempferol in *Moringa oleifera* by HPTLC and presents valuable evidence on its antidiabetic potential both *in vitro* and *in silico*.

Keywords: Antidiabetic, Antioxidant, HPTLC, Kaempferol, *Moringa oleifera*

Abbrevation: HPTLC: High-performance thin-layer chromatography; ICH: International Council for Harmonisation; LOD: Limit of detection; LOQ: Limit of quantification; µg: microgram; ml: millilitre; µl: micro litter, nm: nanometre; RSD: Relative standard deviation

1. Introduction

Every civilization has relied on a variety of medicinal plants as a source of medicines since antiquity to cure a variety of diseases. Due to their affordability and lack of side effects, there has been an increased interest in recent years in utilizing the several *Ayurvedic* medicinal plants[1]. Ancient medicinal traditions like *Ayurveda*, which document the use of therapeutic plants, have significantly contributed to the development of numerous contemporary medications[2].

Moringa oleifera is a medicinally significant plant, often known as horseradish, belongs to family Moringaceae and resides primarily in the sub-Himalayan part of north-western India. It is used in *Ayurvedic* texts to prevent or to treat several illnesses.

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Traditionally, the seeds, fruits, leaves, and roots of this plant are employed to address conditions such as helminthiasis, abdominal tumors, ulcers, prostate issues, scurvy, as well as for their antioxidant, antifungal, and antibacterial actions[3]. Due to its antioxidant and other beneficial properties, the WHO has designated *M. oleifera* as a recommended source of food for the treatment of malnutrition[4].

Numerous phytochemicals, including phenols, carotenoids, minerals, fatty acids, amino acids, vitamins like B1, B6, C, and E, folic acid, riboflavin, and nicotinic acid are found in the plant. Parts of plants consist of a variety of secondary metabolites, including phytates, oxalates, alkaloids, cardiac glycosides, glucosinolates, saponins, tannins, flavonoids, phenolic, carotenoids, isothiocyanates, and polyphenol[5].

Diabetes mellitus encompasses a range of conditions marked by inadequate insulin secretion, primarily stemming from persistent insulin insufficiency, leading to elevated blood sugar level. As a prevalent non-communicable disorder, it poses a significant global health concern and contributes to fatalities in developed nations. Exploring traditional treatments offers valuable foundations for the innovation of new pharmaceuticals for the management of diabetes mellitus and its complications[6].

The active components in plant extracts are measured using several kinds of chromatographic methods. One of the credible techniques for the accurate identification and evaluation of biomarkers in plant extracts as well as commercial formulations is High Performance Thin Layer Chromatography (HPTLC). It is also more dependable for the quantification of phytoconstituents at microgram and even at nanogram levels. Like HPLC and GC, HPTLC is accurate for determining and identifying complicated herbal extracts as well as developing chromatographic fingerprints. In contrast to HPLC and GC, HPTLC can simultaneously identify and quantify many chemicals in a variety of samples on a single plate. Complex herbal extracts can be determined and identified with great accuracy, and chromatographic fingerprints can be created which would help in quality assurance of desired products[7,8].

Molecular docking plays a crucial and expanding role in rational drug development, serving as an effective in *in silico* screening method. This computational technique efficiently identifies ligands that align energetically and geometrically with a protein's binding site[9]. Recent advancements in computational, proteomics, and genomics research have led to the development of docking techniques that consider protein-ligand interaction flexibility and diverse binding conformations. Enhancedreceptor flexibility contributes to more accurate predictions of binding poses and a realistic representation of protein-ligand interactions. *In silico* docking is instrumental in assessing potential ligands for diabetes-related targets, including Aldose reductase, Glycogen synthase kinase-3, peroxisome proliferator-activated receptor-γ, pyruvate dehydrogenase kinase isoforms 2, 11-hydroxysteroid dehydrogenase and Glucokinase[10,11].

In this study we assessed the antidiabetic potentials of *M. oleifera* by *in vitro* and *in silico* studies and developed the standard chromatographic fingerprints for the hydroethanolic extract using kaempferol as biological marker. Thus, the rapid growing herbal drug industries and other researchers can utilize the developed standards for proper standardization of commercial products containing *M. oleifera.*

2. Materialsand Methods

2.1 Chemicals and Reagents

All the chemicals and reagents used were of analytical grade. Standard kaempferol (98% purity) was obtained from sigma aldrich (Steinheim, Germany). Pre-coated silica gel F 60 HPTLC plates were procured from Merck (Darmstadt, Germany).

2.2 Plant Collection and Authentication

Moringa oleifera was collected from the natural surroundings near Belagavi, Karnataka, India. The plant was subjected for authentication at the Indian council of medical research in Belagavi, Karnataka, India, and was assigned specimen number RMRC-1753.

2.3 Pharmacognostical Study

Raw plant material was evaluated for Pharmacognostical parameters like physicochemical examinations including total ash, acid insoluble ash, water-soluble ash, extractive value, and moisture content also, microscopical and chemical evaluation was performed as per standard procedures to assess the quality[12,13].

2.3.1 Preparation of Plant Extract

Extraction was conducted using mixture of ethanol and water (70:25). 50 gm of dried plant material was macerated frequently for 7 days using the above solvent. Later it was filtered, and marc was further subjected for Soxhlet extraction using ethanol (95%). Crude extract was obtained by mixing both the extracts and concentrated using a rotary evaporator[14].

2.3.2 Preliminary Phytochemical Analysis

Implementing an assortment of preliminary phytochemical screening methodologies as per standard procedures, it was achievable to identify phytoconstituents like alkaloids, flavonoids, glycosides, saponins, triterpenoids, steroids, tannins, and phenolics[15].

2.3.3 Determination of Total Phenolic Content

TPC was determined by employing the conventional Folin-Ciocalteu (FC) reagent assay method. Gallic Acid (GAE) was used as a standard. Sodium carbonate and 1mL of plant extract were combined with 1mL of FC reagent (previously diluted 1:10 v/v with distilled water) the mixture was kept for 15 minutes at room temperature. Using a UV/visible spectrophotometer, the color generated was read at 765 nm. The results were represented as GAE/gm of dried extract using standard graph[16].

2.3.4 Determination of Total Flavonoid Content

According to aluminum chloride colorimetric method with slight modification, TFC was determined. Ethanol 95% was used to dissolve 10 mg of quercetin, which was then diluted to 2, 4, 6, 8 and 10 µg/ml. Each 1 mL concentration of standard solution was combined with 3 mL of 95% ethanol, 0.2 ml of 10% aluminum chloride, 1 M potassium acetate0.2 mL, and 5.6 mL of distilled water. The mixture was incubated for 10 minutes at room temperature. Absorbance was recorded using a UV-Vis spectrophotometer at 376 nm. The amount of total flavonoid was calculated as the QE/g of samples[17].

2.4 *In Vitro* Study

2.4.1 Determination of Antioxidant Activity

Antioxidant and free radical scavenging activities were performed through *in vitro* methods which involved the examination of extract efficacy using various assays, including DPPH free radical assay, ferrous ion chelating activity, nitric oxide scavenging activity, superoxide radical scavenging activity, and ABTS radical scavenging activity, according to established protocols[18,19].

$$\%\text{Inhibition} = \frac{\text{Control} - \text{Sample}}{\text{Control}} \times 100$$

2.4.2 Determination of Alpha-amylase Inhibitory Activity

According to a specified technique, the hydroethanolic plant extract was examined for its inhibitory effects on porcine pancreatic amylase. 20–100 µg /mL plant mixture was prepared from this 0.5mL of the extract solution was mixed with the (0.5mL) enzyme solution the mixture was then incubated at 25°C for 30 minutes. To the resultant mixture 1mL of starch solution (0.5% in phosphate buffer) was added then incubated at 25°C for 5 min. DNS (3,5-dinitrosalicylic acid) solution 1mL was added, then this mixture was subjected for heating for 15 minutes at 85°C. At 540 nm the absorbance was measured after the mixture was diluted with distilled water. The following equation was used to calculate the percentage inhibitory activity[20].

$$\%\text{Inhibition} = \frac{\text{Control} - \text{Sample}}{\text{Control}} \times 100$$

2.5 Determination of Kaempferol by LCMS

For the identification of bioactive compounds plant extract was subjected for LC MS/MS (LTQ XL, thermo electron corporation, USA) with an electrospray ion interface and a peak scientific-nitrogen generator working in positive ion polarity. The optimized condition was used to inject a sample, and chromatograms were recorded[21,22].

2.6 Network Enrichment Analysis

2.6.1 Identification of Phytocompounds and their Targets Involved in Diabetes Mellitus

The selection of phytocompounds in *M. oleifera* was based on a thorough review of the existing literature and the exploration of public repositories, including Dr. Duke's DB and IMPATT. The SMILES notation for each selected compound was gathered via the PubChem database[23] and subjected to protein-based prediction in DIGEP-Pred, assessing the probable activity (Pa) to probable inactivity (Pi) ratio[24]. Upon inputting the search term "diabetes mellitus" into the gene cards database[25]. We identified targets associated with diabetes mellitus. Genes associated with diabetes mellitus were identified and cross-referenced with the potential target genes of active components found in *M. oleifera*. To pinpoint the disease targets relevant to *M. oleifera's* effective constituents, we utilized venny 2.1.0. This analysis highlights the key disease targets for the active ingredients in *M. oleifera*, emphasizing their therapeutic potential.

2.6.2 Construction of Network

We utilized cytoscape 3.7.1 to construct a network model that delineates the intricate relationships among "component-disease targets-pathway" for *M. oleifera*. Active components linked with "diabetes mellitus" were integrated into the software, facilitating a comprehensive analysis that culminated in a network visualization depicting varying intensities of modulation, from high to low. This model was designed to pinpoint the specific disease targets impacted by *M. oleifera's* phytoconstituents. Within this network representation, components, disease targets, and pathways are represented by nodes while edges illustrate the connections between these elements. The network was methodically constructed and analyzed to visually assess and interpret the modulation effects from high to low levels.

2.6.3 Analysis of GO and KEGG Enrichment

Gene ontology analysis was conducted to categorize the identified targets into biological processes, cellular components, and molecular functions. From each category, the top ten entries were selected based on gene count for further analysis. Additionally, we performed Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis to annotate the pathways associated with the identified targets, thereby enhancing our understanding of their functional implications[26].

2.6.4 Molecular Docking Analysis

To determine the favorable conformation and affinity for binding with the appropriate targets, the potential compounds were taken into consideration, and molecular docking experiments were conducted. The protein data bank (PDB ID: 4W93) makes the crystal structures of Porcine Pancreatic Amylase (PPA) complexed with the carbohydrate inhibitor acarbose accessible. Protein architectures were minimized and optimized prior to docking utilising the previously reported technique. It uses a grid-based ligand docking approach to look for favorable interactions between a ligand and a receptor molecule, such as a protein. The interactions of each complex were evaluated with the aid of the Schrödinger suite, and 3D poses representative of the molecular recognition interactions were generated. We have used physico-chemical and ADME properties, which help in predicting both physico-chemical significant descriptors and pharmacokinetically important properties of the molecules, which were calculated using the qikprop module of Schrödinger. The ligprep module of the schrödinger software was used to optimize the 11 molecules that were constructed using maestro molecule builder and the OPLS_2005 force field. Ionizer was used to identify all potential protomers and ionization states for ligands at a pH of 7.4[27].

2.7 HPTLC Fingerprinting and Validation

Qualitative and quantitative screening of plant material was conducted by high performance thin layer chromatographic technique to confirm the presence of kaempferol as a bioactive marker and according to the ICH guidelines method was validated[28,29].

2.7.1 Chromatographic Conditions and HPTLC Fingerprinting for Kaempferol

On 20 × 10 cm aluminum plates covered with a 0.25 mm layer of silica gel $^{60}F_{254}$ (Merck, Germany), chromatography was conducted. For the activation of plates methanol was used and heated to 110°C for 5 minutes prior to use. Using a CAMAG (Muttenz,

Switzerland) linomat IV sample applicator with a 100µl syringe, samples were applied in 4 mm bands spaced 6 mm apart. The application rate was kept constant at 5 µl/S. After development, a CAMAG HPTLC scanner 3 with automated visioncat software was used to perform densitometric scanning at 254nm on the dried plate. The method development of kaempferol was verified by superimposing the UV spectra of extract and standard kaempferol with the same R_f values, scanned at 269 nm under a D2 light.

2.7.2 Preparation of Solvent System

Many solvent systems were tried, and the better resolution was found in toluene: ethyl acetate: formic acid: methanol (6:3:0.3:1 v/v/v/v).

2.7.3 HPTLC Method Validation

The established analytical technique was verified for following standards such as linearity, Limit of Detection (LOD), Limit of Quantification (LOQ), precision, specificity, and robustness in accordance with the recommendations of ICH Q2 (R1) guidelines[30].

2.7.4 Linearity and Range

By using various sample concentrations, the range of the standard is determined, and the limit is selected based on the reference's linearity curve. Furthermore, the wavelength at which the parameters will be applied has been selected.

2.7.5 Limit of Detection

The limit of detection was determined using the formula below, which is based on the slope obtained from the linearity and the standard deviation.

2.7.6 Limit of Quantification

Based on the slope and standard deviation, the limit of the quantification parameter is derived similarly to the limit of detection.

2.7.7 Precision

The intraday and intern day precision parameters are determined independently. Inter day precision is assessed by repeating the same protocol over three non-consecutive days. Intraday precision is evaluated by repeating the same procedure three times within the same day. The calibration curve with the highest concentration at the center is selected for the precision study. According to the ICH criteria, the precision study calculations must have a relative standard deviation (RSD) of less than 2%.

2.7.8 Specificity

Sample and the standard were applied on the plate for the specificity parameter. The resulting chromatograms from the developed plates are scanned and then analyzed for the R_f value.

2.7.9 Robustness

The parameters of the procedure, such as slight variations in the solvent system ratio, chamber saturation time, and solvent volume, are intentionally modified for the robustness parameter. The resulting changes must meet the criteria of %RSD (Relative Standard Deviation) being less than 2%.Top of Form

2.8.0 Quantification

To quantify the kaempferol in extract final concentration of 5.0mg/mL was prepared. 10µl of sample was applied on HPTLC plates and developed. The quantity of kaempferol in the sample solution was determined from the calibration curve using peak areas of the compounds recorded[31].

3. Results and Discussion

3.1 Pharmacognostical Evaluation of the Plant Material

To identify and standardize this plant, pharmacognostical analysis is essential. *M. oleifera* morphological and pharmacognostical aspects were examined in this study. The results revealed distinctive characters for identification of the plant (Tables 1 and 2; Figure 1).

Table 1. Organoleptic characters

Parameters	*Moringa oleifera* (leaf)
Color	Yellowish green
Oduor	Pungent
Taste	Acrid
Size	2.9cm long and 1.5 cm in width
Shape	Oval

Table 2. Physicochemical evaluation

Parameters	*Moringa oleifera* (%W/W)
Total Ash value	10.5±1.33
Acid insoluble ash	3.05±1.01
Water soluble ash	1.81±0.18
Moisture content	2.46±0.464
Alcohol-soluble extractive	12.06±0.784
Water Soluble extractive	9.86±0.555
Ether Soluble Extractive	4.53±0.478

*Data is expressed as Mean ±SD (n=3)

Figure 1. *Moringa oleifera* leaf.

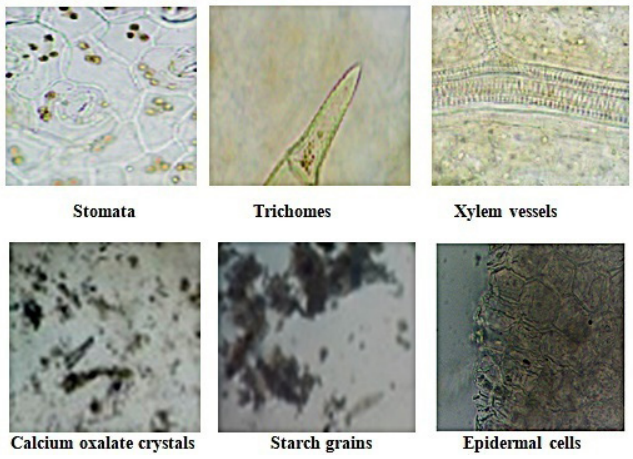

Figure 2. Microscopical characters.

3.2 Microscopical Description

The different microscopical features like stomata, trichomes, xylem and phloem vessels, starch grains and epidermal cells are shown in Figure 2.

3.3 Extract Preparation and Phytochemical Screening

Extraction was carried out by maceration and soxhlet method, menstrum of both the methods mixed and evaporated by rotary evapurator. Extract was collected, dried and kept in well closed container for further analysis. Phytochemical screening identified different sceondary metabolities like alkaloids, flavonoids, tannins, glycosides, terpenoids and steroids Table 3.

Table 3. Phytochemical evaluation of raw materials

Sl. No.	Phytoconstituents	Extract
1	Carbohydrates	+
2	Saponins	+
3	Tannins and Phenols	-
4	Flavonoid	+
5	Alkaloids	+
6	Terpenoids	+
7	Glycosides	+
8	Steroids	+
9	Amino acids	-

+ Present- Absent

3.4 Quantitative Estimation of Secondary Metabolites Total Flavonoid and Total Phenolic Content

TFC of the plant extract was determined as per standard procedure with reference to standard quercetin. The equation Y= 0.0368x +0.0629 demonstrates linear regression with a correlation of 0.9996. Using the above linear regression equation, flavonoid concentration in the extract was expressed in mg QE/gm of extract and was found to be 31 ± 0.30. TPC of the plant extract was determined as per standard procedure with reference to standard gallic acid. The existence of distinct components with phenolic moiety in their structures is indicated by the more considerable amount. Y = 0.034x+0.0481 represents linear regression equation while the correlation coefficient was found to be 0.9985. The amount of phenol content present in the extract in terms mg GAE/gm of extract was found to be 37.97±0.38 by using the above linear regression equation.

3.5 *In Vitro* Results

3.5.1 *In Vitro Antioxidant Activity*

Evaluation of antioxidant activity of hydroalcoholic extract of *M. oleifera* encompasses a range of tests. The effectiveness of extract in neutralizing free radicals depends on their phytoconstituent composition and the testing conditions, making it essential to employ multiple assays for a comprehensive assessment of diverse antioxidant mechanisms. The hydroalcoholic extract displayed significant activity in scavenging free radicals. Notably, both the standard substances and hydroalcoholic extracts exhibited percentage inhibition values in correlation with concentration. IC_{50} values of *M. oleifera* across various antioxidant tests displayed in (Table 4).

3.5.2 *Alpha-amylase Inhibitory Activity*

Alpha-amylase inhibitory activities of hydroalcoholic extracts of *M. oleifera* was assessed against porcine pancreatic α-amylase. The extracts produced excellent α-amylase inhibitory activity with IC_{50} value of 57.38588±1.92 µg/LmL where as IC_{50} value of acarbose was 32.51564±1.59 µg/mL. The obtained results are mentioned in Table 5 and Figure 3.

Table 4. *In vitro* antioxidant assay

Samples	*M. oleifera* Extract	Standard
DPPH (µg/mL)	57.983±1.83	12.86±4.86(AA)
Ferrous ion (µg/mL)	94.232±1.53	45.97±3.21(EDTA)
NO (µg/mL)	106.54± 0.42	67.827±1.62(AA)
ABTS (µg/mL)	89.535±1.56	29.583±2.94(BHA)
NBT	174.74±3.86	112.48±3.64(BHT)

Values are mean ± S.D(n=3)

Table 5. α-amylase inhibitory activity

Conc. (µg/mL)	Acarbose	Extract
	%Inhibition	%Inhibition
20	44.44±0.83	24.89548±0.28
40	53.58±1.75	36.374±0.53
60	64.81±1.49	48.23261±0.74
80	76.582±0.54	61.30749±0.42
100	83.29±0.85	70.27746±1.37
IC_{50} value	32.51564±1.59	57.38588±1.92

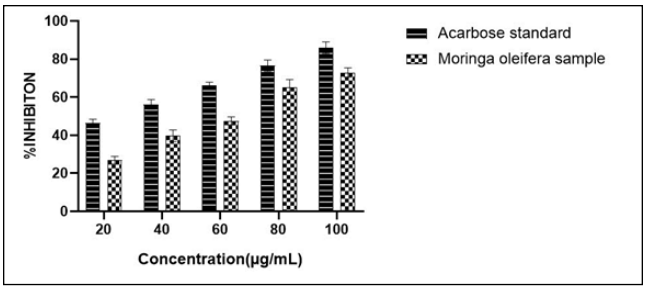

Figure 3. α-Amylase inhibitory activity.

3.6 Determination of Kaempferol by LCMS

Peaks with RT of 4.27 min eluted with kaempferol-like UV spectra and with [MH] ions at m/z = 447 and 489. Product ion scans of these two kaempferol derivatives demonstrated fragments at m/z 285.34 which are characteristic of kaempferol aglycone. Peak was identified as kaempferol depending upon molecular ion peaks and product ion scans. Figures 4A and 4B indicate the LC-MS spectra of kaempferol.

3.7 Network Studies

3.7.1 *Identification of Phytocompounds and their Targets Involved in Diabetes Mellitus*

A comprehensive examination of literature and database references provided information on 48 chemical components present in *M. oleifera*. PubChem was utilized for the validation of the plant's chemical composition, confirming the structures of 11 identified chemical components listed in Table 6. This data served as a foundation for target prediction, where a comparison with genes linked to diabetes mellitus in gene cards databases revealed 11 potential targets for the treatment of diabetes mellitus with *M. oleifera*. The molecular pathway enrichment analysis of 11 protein targets of phytocompounds was found to involve 96 molecular pathways. Among 96 pathways, 08was significantly associated with diabetes mellitus and its associated complications in Table 7.

3.7.2 *Network Construction*

The network was constructed between protein, pathways and phytoconstituents. The network had 104 edges and 30 nodes luteolin and genistein were projected to influence the greatest number of proteins among the 11 bioactive compounds. However,

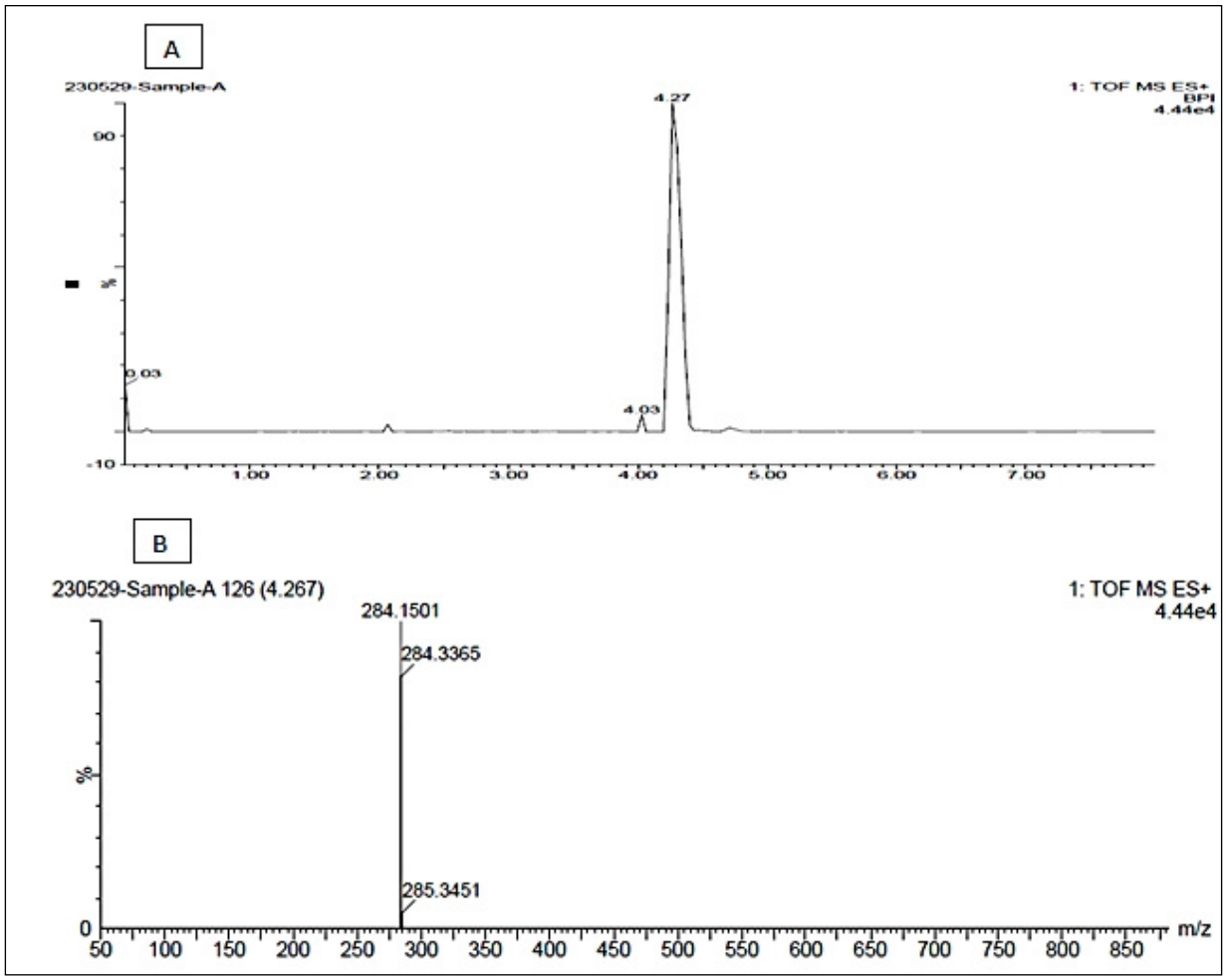

Figure 4. **(A).** LC of *M. oleifera*; **(B).** MS of *M. oleifera.*

Table 6. Summary of 11 phytoconstituents

Sr. No.	Compound Name	Mol weight (g/mol)	Mol. formula	Smiles
1	Quercetin	302.24	$C_{15}H_{10}O_7$	C1=CC(=C(C=C1C2=C(C(=O)C3=C(C=C(C=C3O2)O)O)O)O)O
2	Kaempferol	286.24	$C_{15}H_{10}O_6$	C1=CC(=CC=C1C2=C(C(=O)C3=C(C=C(C=C3O2)O)O)O)O
3	Apigenin	270.24	$C_{15}H_{10}O_5$	C1=CC(=CC=C1C2=CC(=O)C3=C(C=C(C=C3O2)O)O)O
4	Daidzein	254.24	$C_{15}H_{10}O_4$	C1=CC(=CC=C1C2=COC3=C(C2=O)C=CC(=C3)O)O
5	Epicatechin	290.27	$C_{15}H_{14}O_6$	C1C(C(OC2=CC(=CC(=C21)O)O)C3=CC(=C(C=C3)O)O)O
6	Genistein	270.24	$C_{15}H_{10}O_5$	C1=CC(=CC=C1C2=COC3=CC(=CC(=C3C2=O)O)O)O
7	Isorhamnetin	316.24	$C_{16}H_{12}O_7$	COC1=C(C=CC(=C1)C2=C(C(=O)C3=C(C=C(C=C3O2)O)O)O)O
8	Luteolin	286.24	$C_{15}H_{10}O_6$	C1=CC(=C(C=C1C2=CC(=O)C3=C(C=C(C=C3O2)O)O)O)O
9	Rutin	610.5	$C_{27}H_{30}O_{16}$	CC1C(C(C(C(O1)OCC2C(C(C(C(O2)OC3=C(OC4=CC(=CC(=C4C3=O)O)O)C5=CC(=C(C=C5)O)O)O)O)O)O)O)O
10	Astragalin	448.4	$C_{21}H_{20}O_{11}$	C1=CC(=CC=C1C2=C(C(=O)C3=C(C=C(C=C3O2)O)O)OC4C(C(C(C(O4)CO)O)O)O)O
11	Myricetin	318.23	$C_{15}H_{10}O_8$	C1=C(C=C(C(=C1O)O)O)C2=C(C(=O)C3=C(C=C(C=C3O2)O)O)O

Table 7. Molecular pathway enrichment analysis

Term ID	Term description	OGC	BGC	False discovery rate	Matching proteins in network(labels)
hsa04931	Insulin resistance	6	107	0.0000119	TNFRSF1A, PPARGC1A, CPT1A, PRKCD, MAPK8, PPARA
hsa04922	Glucagon signaling pathway	4	101	0.0011	EP300, PPARGC1A, CPT1A, PPARA
hsa04932	Non-alcoholic fatty liver disease	4	148	0.0025	TNFRSF1A, CASP8, MAPK8, PPARA
hsa05212	Pancreatic cancer	3	73	0.0038	KRAS, ERBB2, MAPK8
hsa04910	Insulin signaling pathway	3	133	0.0142	KRAS, PPARGC1A, MAPK8
hsa04930	Type II diabetes mellitus	2	46	0.0204	PRKCD, MAPK8
hsa03320	PPAR signaling pathway	2	75	0.0428	CPT1A, PPARA
hsa04068	FoxOsignaling pathway	4	127	0.0017	CAT, KRAS, EP300, MAPK8

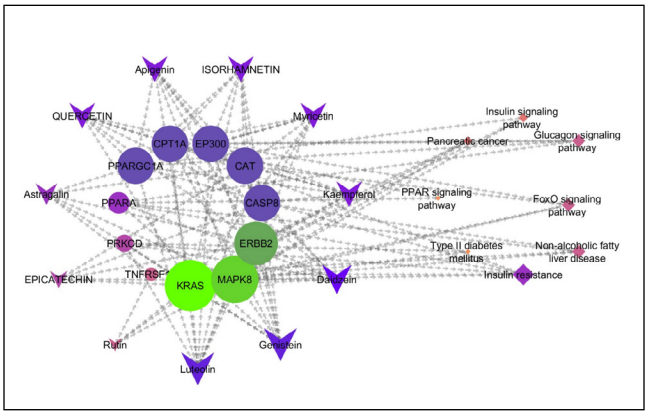

Figure 5. Network representation of phytoconstituents – targets and their pathways.

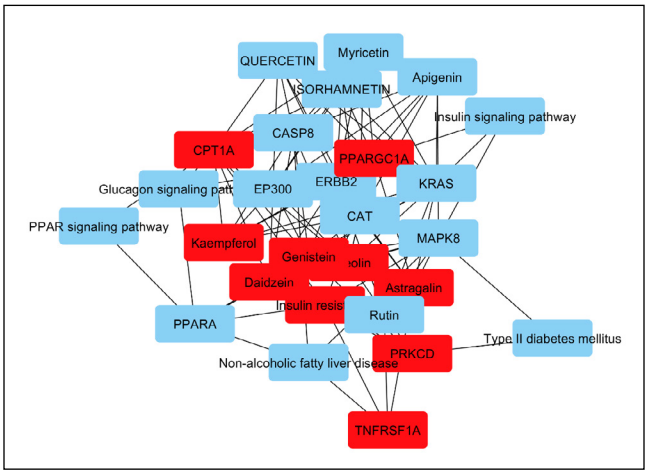

Figure 6. Cytohubba analysis of merged network.

enrichment analysis identified 11 molecules, namely luteolin, genistein, daidzein, kaempferol, myricetin, isorhamnetin acid, apigenin, quercetin, astragalin, epicatechin and rutin to regulate multiple pathways

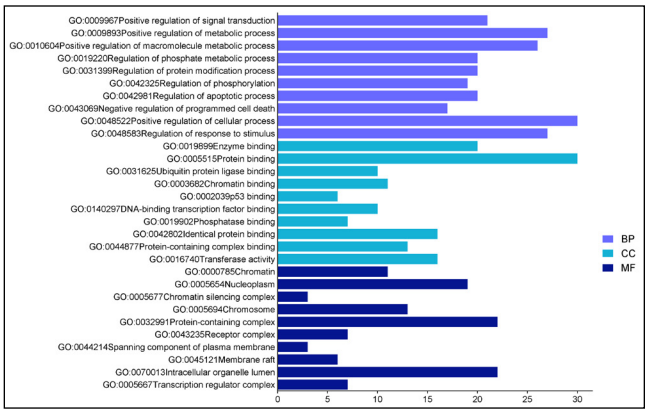

Figure 7. GO enrichment analysis of BP, CC, and MF.

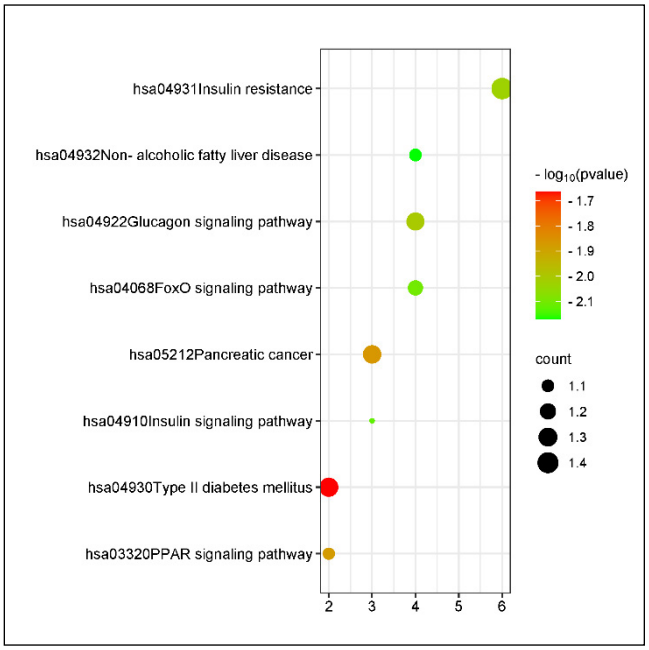

Figure 8. KEGG pathway bubble plot analysis.

Table 8. Binding affinity of phytocompounds

S. No.	Compounds (PDB ID:4W93)	Docking scores in Kcal/mol
1	Montbetrine	-14.659
2	Acarbose	-11.112
3	Rutin	-9.981
4	Astragalin	-8.737
5	Myricetin	-8.356
6	Quercetin	-7.679
7	Kaempferol	-7.665
8	Luteolin	-7.302
9	Isorhamnetin	-7.022
10	Epicatechin	-6.741
11	Genistein	-6.263
12	Apigenin	-5.86
13	Daidzein	-5.338

involved in diabetes mellitus and its complications. As seen in Figure 5 the combined action of amylase inhibitors indicated that the Insulin resistance pathway was regulated. The phytocompounds are represented in arrow shape and round shape indicates targets of DM. A diamond shape represents molecular pathways modulated by phytoconstituents. Merged network via cytohubba analysis is exhibited in Figure 6.

3.7.3 Gene Ontology Enrichment and KEGG Pathway Analysis

The GO analysis resulted in the identification of 541 GO terms, including 461 associated with biological processes, 26 related to cellular components, and 54 concerning molecular functions. The top 10 enriched terms from each of these three categories have been selected and are presented in Figure 7. The enriched BP terms highlighted key processes such as signal

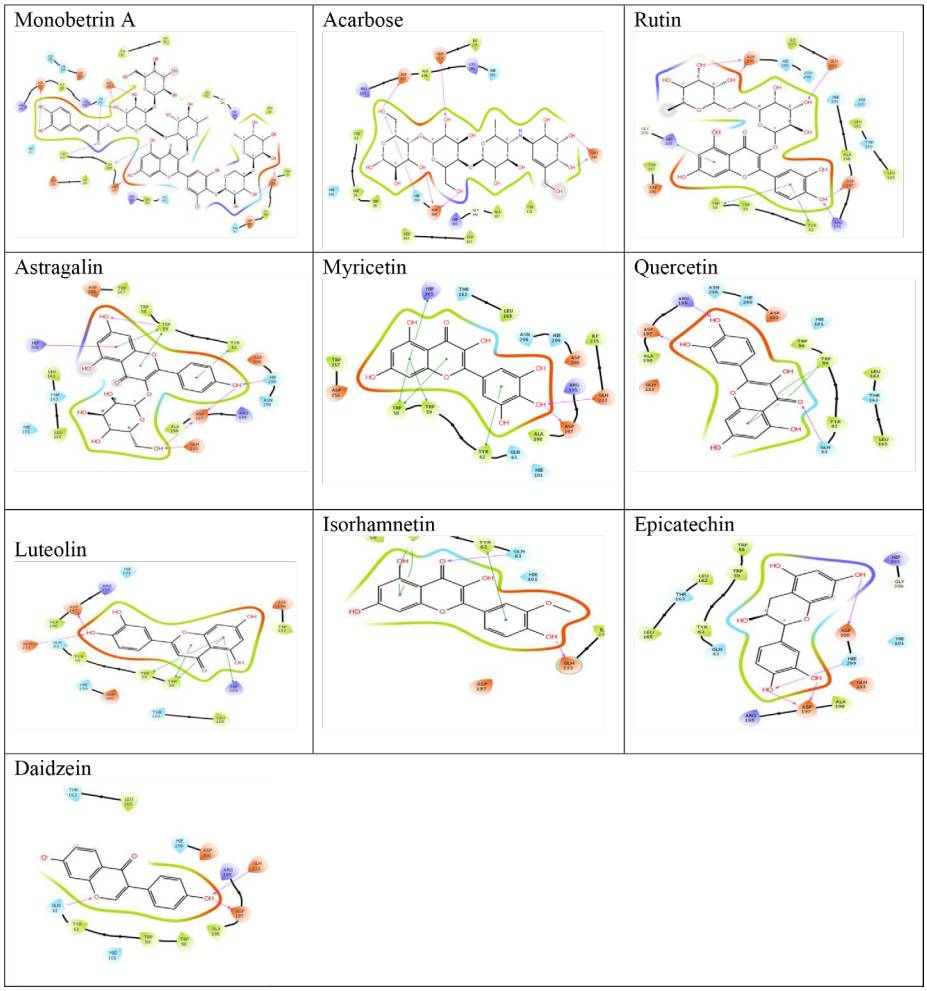

Figure 9. The interaction of each ligand with protein (4W93).

Table 9. The ADME profile of bioactive compounds

Title	CNS	QPlogPo/w	QPlogS	QPlogHERG	QPPCaco	QPlogBB	QPPMDCK	QPlogKhsa	HOA	PSA	ROF	ROT
Montbetrin A	-2	-6.717	0.33	-7.241	0.008	-9.709	0.002	-3.641	1	510.66	3	2
Acarbose	-2	-7.354	0.723	-5.727	0.04	-5.765	0.01	-2.582	1	317.25	3	2
Quercetin -2 0.383			-2.878	-5.067	19.286	-2.377	6.933	-0.343	2	142.67	0	1
Kaempferol	-2	1.059	-3.137	-5.173	53.753	-1.862	20.992	-0.189	3	121.15	0	0
Apigenin	-2	1.636	-3.335	-5.084	116.767	-1.429	48.555	-0.028	3	99.665	0	0
Daidzein	-1	1.814	-3.038	-5.158	382.728	-0.923	175.188	-0.125	3	80.486	0	0
Epicatechin	-2	0.483	-2.641	-4.8	54.786	-1.883	21.429	-0.415	2	115.52	0	1
Genistein	-2	1.716	-3.085	-5.063	163.955	-1.334	70.075	-0.086	3	99.973	0	0
Isorhamnetin	-2	1.194	-3.469	-5.164	53.471	-2.004	20.874	-0.152	3	128.55	0	0
Luteolin	-2	0.954	-3.06	-4.985	42.001	-1.93	16.079	-0.189	3	121.18	0	0
Myricetin	-2	-0.283	-2.637	-4.962	6.969	-2.897	2.307	-0.488	2	164.07	1	1
Rutin	-2	-2.359	-2.166	-5.163	1.66	-4.26	0.489	-1.272	1	265.45	3	2
Astragalin	-2	-0.626	-2.513	-5.129	16.774	-2.771	5.962	-0.788	2	190.50	2	2

Table 10. Results of HPTLC analysis

Sr. No.	Validation parameters	Kaempferol
1.	Linearity	
	Linearity range (ng/mL)	10-50
	Correlation-coefficient	0.9992
2.	LOD (ng/mL)	15.09
	LOQ(ng/mL)	45.72
3.	Precision	
	Intra-day (%RSD)	0.239±0.03
	Inter-day (%RSD)	0.743±0.092
4.	Robustness	
	Mobile phase volume (%RSD)	0.356±0.027
	Mobile phase composition (%RSD)	0.293±0.097
	Duration of saturation (%RSD)	1.252±0.034
5.	Accuracy	
	50% recovery	101.95±0.04
	100% recovery	100.35±0.27
	150% recovery	99.40±0.32
6.	R_f Value	0.66

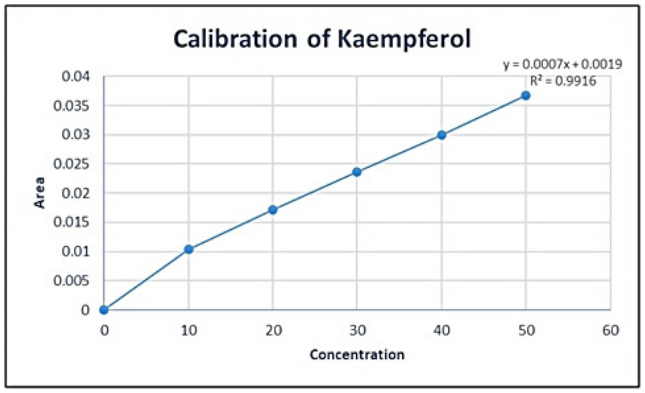

Figure 10. Calibration curve of kaempferol.

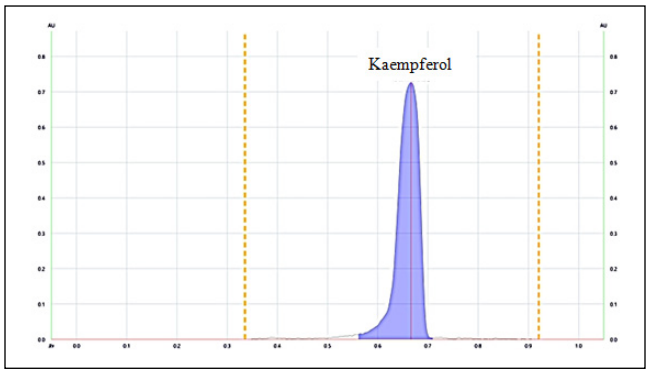

Figure 11. **A.** Standard spectra of kaempferol.

transduction, metabolic processes, phosphorylation, cellular processes, and responses to stimuli. The most notable CC terms involved various binding activities including enzyme, protein, chromatin, phosphatase, and transferase activities. MF terms primarily centered around structures such as chromatin, nucleoplasm, chromosomes, receptor complexes, membrane rafts, and transcription regulator complexes. Additionally, the analysis of the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways revealed 96 significantly enriched pathways. Figure 8 illustrates the top 8 KEGG pathways among the identified common

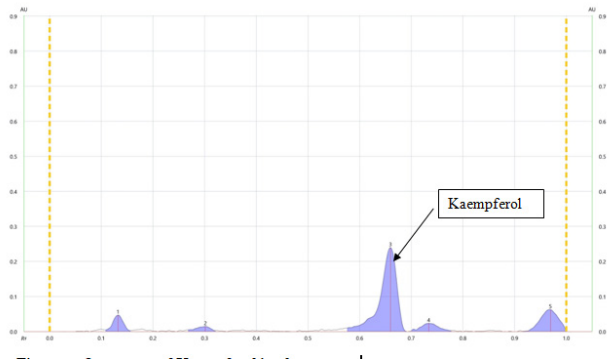

Figure 11. **B.** Spectra of kaempferol in extract.

targets, underscoring their crucial roles in modulating responses related to diabetes mellitus.

3.7.4 Molecular Docking

According to docking data, those compounds had good docking energy values Kcal/mol against the amylase co crystallize complex, compared to the conventional medication acarbose, which has -11.11 Kcal/mol. Among the selected compounds Rutin showed the highest binding affinity with 4W93 whereas Daidzein showed the least binding affinity shown in Table 8 and Figure 9.On looking hydrogen bond interaction, Rutin and Astragalin scored the highest number of hydrogen bond interaction towards 4W93 via four bonds. Amino acid residues of the protein that interact with Rutin are ARG: 195, ASP: 197, GLH233, ASP 300 and standard acarbose interacts with GLH 233, ASP 197, ASP 300, HIE 299, GLU 240 amino acids. The ADME profile of bioactive are described in Table 9.

3.8 HPTLC Fingerprinting and Validation

3.8.1 Method Development and Fingerprinting for Kaempferol

After analyzing the plates under UV light, it was determined that the optimal wavelength for detection was 269 nm. Following several experiments, the solvent system composition was finalized as toluene: ethyl acetate: formic acid: methanol: (6:3:0.3:1v/v/v/v). This choice resulted in an enhanced separation between the standard and the sample, and good spectra were obtained.

3.8.2 Method Validation

The method was developed and validated using a new solvent system composition at 269 nm. The chosen solvent composition was further validated. The information pertaining to linear regression is shown in Figure 10. The linearity was discovered to be in the 10–50 ng/band range, and the correlation coefficient was discovered as 0.9916.

The LOD and LOQ were determined using a signal-to-noise ratio of 3:1 and 10:1, respectively. The LOD was found to be 15.09 ng/band, and the LOQ was found to be 45.72 ng/band. Precision measurements were made on an intraday and interday basis, and the %RSD values were found to be within the required range of ≤2 for the Kaempferol. Table 10 shows the results for intraday and interday precision, respectively. Specificity parameters shows that the sample and standard shared the same R_f value 0.66.Figure 11A and 11B display the chromatograms for the standard and *M. oleifera* extract.

Variation in mobile phase composition resulted in a %RSD of 0.293%, changes in mobile phase volume showed a %RSD of 0.356%, and changes in the duration of chamber saturation exhibited a %RSD of 1.252%. These values fall within the acceptable range of ≤2%, confirming that the developed method was robust. The results of the developed method have been presented in Table 10.

3.8.3 Quantification of Kaempferol in the Extract

243.3ng/mL of kaempferol was found to be present in hydroalcoholic extract of *M. oleifera* leaf sample.

3. Conclusion

The examination of *M. oleifera* pharmacological aspects reveals compelling discoveries, highlighting its potent antioxidant activity and potential health benefits across diverse methods. Additionally, its antidiabetic potential is underscored through a comprehensive analysis involving *in vitro* experimentation, *in silico* molecular docking studies, and network investigations. Furthermore, in this groundbreaking research, a High-Performance Thin-Layer Chromatography (HPTLC) method has been innovatively developed and validated for the precise determination of kaempferol in the

hydroalcoholic extract of *M. oleifera*. Renowned for its demonstrated accuracy, simplicity, specificity, robustness, rapidity, and cost-effectiveness, this methodology extends its versatility beyond *M. oleifera*. It can be readily applied to analyze and quantify kaempferol in extracts from various plant species and herbal remedies available in the market. This study not only propels advancements in analytical chemistry through its innovative method but also contributes valuable insights to the interdisciplinary fields of pharmacology and herbal medicine, establishing *M. oleifera* as a pivotal player in natural product research and drug discovery.

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