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Sonali Yadav
Research Scholar Department of Bioscience and Biotechnology, Banasthali Vidyapith, Rajasthan, India

Jyoti Mathur
Associate Professor, Department of Bioscience and Biotechnology, Banasthali Vidyapith, Rajasthan, India

Correspondence:
Jyoti Mathur
Associate Professor, Department of Bioscience and Biotechnology
Banasthali Vidyapith Rajasthan, India
Email: contact.srivastava@gmail.com

Varietal level comparison of *Moringa oleifera* Lam. for phytochemical constituents and antifungal properties against *Fusarium oxysporum* and *Albugo candida*

Sonali Yadav, Jyoti Mathur

ABSTRACT

Background: The main objective of this work was to examine the chemical components, antioxidant and antifungal activities of an ethanolic extract of three *Moringa oleifera* varieties (PKM-1, PKM-2, and ODC-3) against selected fungal strains. The ethanolic extract was prepared using the Soxhlet extraction method. The extracts of three different varieties were tested for antioxidant activity as well as antifungal activity against *Fusarium oxysporum* and *Albugo candida* using the disc diffusion method, and minimum inhibitory concentration (MIC) values were determined using the micro dilution method. Using the FTIR technique, spectrophotometer equipment was used to identify the distinctive peak values and functional groups. HPLC and GCMS were used to determine the phytochemical profiles of the three variants. **Results:** The preliminary phytochemical investigation revealed the presence of numerous secondary metabolites in all three *M. oleifera* types. The PKM-1 cultivar had a considerably higher total phenolic content (99.93 ± 0.239 mg GAE /g) than the PKM-2 and ODC-3 varieties of *M. oleifera* (81.58 ± 0.369 and 91.73 ± 0.254 mg GAE/g). The flavonoid content of the PKM-1 variety (135.3 ± 0.494 mg QE/g) was maximum than that of the PKM-2 and ODC-3 varieties (106.9 ± 0.553 and 117.1 ± 0.413 mg QE/g). In the DPPH and H_2O_2 assays, the PKM-1 variant was the most potent inhibitor (IC_{50}) compared to the other two varieties. Antifungal activity testing demonstrated that all the three varieties suppress the growth of both the fungal strains such as *F. oxysporum* and *A. candida* with a mean zone of inhibition. FTIR examination of ethanol extract of *Moringa* types (PKM-1, PKM-2, and ODC-3) revealed the presence of distinct functional chemical classes at a variety of characteristic bands. GCMS analysis confirmed the presence of 25 metabolites in *M. oleifera* PKM-1 and 23 compounds in PKM-2 varieties. **Conclusion:** The results showed that *M. oleifera* has a diverse set of phytoconstituents with considerable antioxidant and antifungal effects, confirming its applicability in a wide range of applications.

Keywords: *Albugo candida*, Antioxidants, Antifungal, Bioactive compounds, *Fusarium oxysporum*, *Moringa oleifera*, Phytoconstituents.

INTRODUCTION

Plants and biologically active compounds constitute the foundation of both contemporary and conventional medicine, and they are now frequently utilized in the manufacture of commercially produced pharmaceuticals [1]. According to scientific and reputable reports, herbs account for around 25% of all prescribed medicines globally[2]. Plant extracts and essential oils, and many types of bioactive compounds are known as antibacterial and antioxidant compounds with little or no harmful effects [3], which are critical in the treatment of many illnesses [4] Secondary metabolites with enormous bioactive principles, known as phytochemicals, are abundant in plants and are currently exploited as the foundation of medication development [5].Plant-based antimicrobials possess a huge number of therapeutic benefits, are less harmful to the environment, and are not related to the side effects that are frequently associated with synthetic drugs [6]. Phytoconstituents have been shown in investigations to be useful in the treatment of gastroenteritis, febrile disorders, reproductive and hepatic problems, trypanosomiasis, injuries, cardiopulmonary and respiratory difficulties, and parasitic infections [7]. Plant extracts continue to be significant sources of many medical substances, such as the antibacterial drugs used to treat infectious diseases [8, 9]. Similarly, antioxidants may be able to trap reactive oxygen species produced during regular metabolic actions and defend beside oxidative stress. Antioxidants also slow the progression of many chronic illnesses [10]. As a result of their therapeutic and physiological benefits, medicinal plants with antibacterial and antioxidant potential are gaining popularity. *M.oleifera*, along with *Anoma* and *Hyperanthera*, is one of the *Moringaceae* family genera [11]. This species is mostly well-known as the "drumstick" or "horseradish" and is found in many continents [12].

The species is useful in many ways such as its seed are used to filter water, the leaves are supplement to nutrition, the oil is used to make biofuel, the trunks is used in manufacturing of gum and rest of the plant tissue are being used for medicinal purpose [13].

M. oleifera species comprises a diversity of phytocompounds, including phenols, tannins, alkaloids, saponins, flavonoids, steroids, glycosides, anthraquinones, glycosylates, fatty acids, carotenoids, highly accessible minerals, and foliate etc. [14]. Zeatin, a substance that has been employed as a plant growth stimulant and aids in increasing agricultural yields, is abundant in *M. oleifera* [15]. The plant is also rich in retinol, potassium, calcium ascorbic acid and essential amino acids [16]. In comparison to African native samples, the Indian variants (PKM-1 and PKM-2) contained the highest levels of flavonoids [17].

F. oxysporum and *A. candida* cause wilt and white rust disease in crops and reduces the crop production. *Fusarium* wilt is caused by the fungus *F. oxysporum*, in plants viz. *Solanum lycopersicum*, *Solanumme longena*, *Tritium aestivum and Piper nigrum*. *A.candida* cause white rust disease in members belongs to the family *Brassicaceae* and infects plants *Capsella, Arabis, Lepidium, Brassica* and *Arabidopsis thaliana*. As a result, the current research was undertaken to analyses the phytochemical constituent in three different varieties of M. oleifera, then to evaluate its antifungal properties against *F. oxysporum* and *A. candida*, and finally to assess the phytochemical profiles using Fourier Transform Infrared (FTIR), High Performance Liquid Chromatography (HPLC), and Gas Chromatography-Mass Spectrometry (GCMS).

MATERIAL AND METHODS

Plant sample

Three different types of *M. oleifera* plants were procured from the Jatropha research Centre for biofuel in Jaipur, Rajasthan. The plant was checked for health and infection-free. The leaves were cleaned, dried at 100°C in a hot air oven, and then ground into a fine powder. Until further use, this powder was kept in an airtight container.

Preparation of leaves extract

All three types' dried leaves were ground up and then consecutively extracted with ethanol (25 g/250 mL) using a Soxhlet apparatus at 50–80°C for 15 hours. The component from the ground-up box was dried out and used in solvent extraction. The various extracts' solvents were evaporated in a Rota evaporator and stored at 4 °C for further use. By resuspending dried plant extracts in dimethyl sulfoxide (DMSO) to create a solution of 10 mg/mL for each extract, antioxidant and antifungal activity was examined.

Qualitative phytochemical investigation

Phytochemical profiling was done in ethanolic extracts of three different varieties confirm the presence of their typical bioactive compounds using analytical methodologies. The profiling was performed for alkaloids, proteins, flavonoids, glycosides, steroids, saponins, tannins, anthraquinones, reducing sugar, carbohydrates, phytosterols and fats and fixed oils. As analytical results from these tests, precipitate formation or color intensity were evaluated.

Test for alkaloids

For estimation of alkaloids Mayer's test was performed. Few drops of Mayer's reagent were added to 2-3 mL of filtrate. The occurrence of cream-colored precipitates confirmed the alkaloids presence [18].

Test for amino acids

For estimation of amino acids Xanthoproteic test was performed by adding 1mL of determination of Flavonoids by adding 2 mL of 2.0 % sodium hydroxide to crude extract. The appearance of a bright yellow colour, which turned transparent after two drops of diluted acid were added, indicated the presence of flavonoids [19].

Test for glycosides

Test for glycosides were done by Legal's Test. Few drops of 10% NaOH were added to make the concentrated extracts alkaline. After then, the solution was treated with newly synthesized sodium nitroprusside. The emergence of blue hue in the extract indicated the glycoside presence [20].

Test for steroids

By adding 5 mL of aqueous plant crude extract to 2 mL of chloroform and concentrated sulfuric acid, the presence of steroids was determined. The presence of steroids is indicated by the emergence of a red colour in the lower chloroform layer[21].

Test for saponins

In order to conduct the saponin test, 2 mL of distilled water and 2 mL of the aqueous extract were added, and the mixture was stirred for 15 minutes in a graduated cylinder. The formation of a 1 cm foaming layer was a positive reaction to the presence of saponin [22].

Test for tannin

Gelatin Test: Add a small amount of 10% sodium chloride to a 1% gelatin solution. When a 1% solution of tannin is administered to the gelatin solution, tannin precipitates the gelatin from the solution [22].

Test for reducing sugar

Fehling's solution test was performed for qualitative determination of reducing sugar. 1 mL of Fehling's A and B solutions were dissolved and heated for a min. Sample was added in the dissolved mixture followed by heating in water bath for 5-10 minutes. The existence of carbohydrates was yellow and, later, red precipitates [23].

Test for Carbohydrates

For the purpose of qualitatively estimating carbs, the Molisch's Test was used. A test tube containing 5 mg of extract in a 5 mL aqueous solution received two drops of Molisch's reagent. In the same test tube, 1 mL of concentrated H_2SO_4 was allowed to drip down the side without being mixed, establishing a layer below the aqueous solution. The development of a red ring at the point where the two liquids merged demonstrated the presence of carbohydrates. We stood or shook until a solution was found. After that, 5 mL of water was added to the solution and it was combined. A faint violet precipitate started to form right away [18].

Test for Anthraquinones

0.5 g of the extract was heated with 10 mL of H_2SO_4 for the test, which was then filtered. The filtrate received 5 mL of $CHCl_3$. One millilitre of 10% NH_3 was then added after the $CHCl_3$ layer had been pipetted into a different test tube. The resulting solution was tested to see if it turned violet, indicating the presence of anthraquinones [19].

Test for phytosterols

Liebermann-Burchard Test was performed for qualitative determination of phytosterols by adding 2 mL $CHCl_3$ to the extract followed by addition of 1-2 mL of acetic anhydride and 2 drops of concentrated H_2SO_4 in a test tube. The presence of sterols was shown by the presence of red, blue, and eventually green color [18].

Test for triterpenoids

Salkowski Test was performed for qualitative determination of phytosterols by adding few drops of $CHCl_3$ to 5 mL (1 mg/mL) of fraction followed by addition of 3 mL of concentrated H_2SO_4. Change in color from reddish brown to black indicates terpenoids presence [21].

Test for fats

Paper spot Test for saturated and unsaturated oils was performed by placing single drop of oil on filter paper. Saturated Oils leave a lasting stain on the paper, whereas unsaturated oils did not show any stain [20].

Quantitative estimation of phytochemicals

Assessment of Total phenolic content (TPC)

With a few minor deviations from the standard procedure, the Folin-Ciocalteau colorimetric technique was used to determine the TPC of ethanolic extracts of var-1, var-2, and var-3 leaves. [23, 24]. A total of 1 mL of water was added after each extraction of about 15 mg of leaf extracts, bringing the total to that size. Then, 2.5 mL of 20% Na_2CO_3 and 500 L of Folins-phenol reagent (1:1 with water) were added to the solution. The mixture was thoroughly stirred before being allowed to develop colour in full darkness for 30 minutes. After incubation, the absorbance at 725 nm was measured. After creating a calibration curve for gallic acid, linearity could be achieved. Using the standard curve, the TPC of the extracts was determined as mg of gallic acid equivalent (mg GAE/g extract).

Assessment of Total flavonoid content (TFC)

The TFC of the ethanolic extracts of Var-1, Var-2, and Var-3 leaves was calculated using the aluminum chloride colorimetric technique 150 L of $NaNO_2$ (5%) solution was added to 1 ml of plant extracts, and the mixture was then diluted with distilled water to reach a final volume of 200 L. 150 L of a 10% $AlCl_3$ solution was added after 5 minutes of incubation and let to stand for 6 minutes. Then, 2 mL of NaOH (4%) solution and 5 mL of distilled water were added. After carefully stirring, the liquid was allowed to sit at room temperature for 15 minutes. The absorbance was calculated at 510 nm. Using the standard curve, the TFC was expressed as mg QE/g dry weight of quercetin equivalent in the extract.

Antioxidant activity of *M. oleifera*

To generate the range of concentrations for antioxidant testing, each sample was diluted in 95 percent methanol (CH_3OH) to a concentration of 1 mg/mL.

Determination of DPPH free radical scavenging activity

Different leaf extract concentrations (20, 40, 60, 80, and 100 mg mL-1) were made using analytical methanol. Ascorbic acid was utilized as a common antioxidant. By dissolving 24 mg of DPPH in 100 mL of methanol and keeping it at 20°C until needed, a stock solution was created. The working solution was created by dilution of the DPPH solution with methanol using a spectrophotometer to obtain an absorbance of around 1.018 0.02 at 517 nm. 1 mL of extract and 0.5 mL of 1.0 mM DPPH were combined. After giving the reaction mixture a good shake, it was left to incubate in the dark. After 0 minutes, 5 minutes, 10 minutes, 20 minutes, and 30 minutes, the absorbance at 517 nm dropped. The identical process as before was used to make the control, but it included no sample. Three times the experiment was run, each time using vitamin C as a positive control. Based on the proportion of DPPH radicals scavenged, the following equation was used to compute the scavenging activity:

$$\% \text{ inhibition} = \frac{\text{Absorbance (control) at 517 nm} - \text{Absorbance (sample) at 517nm}}{\text{Absorbance (control) at 517 nm}} * 100$$

Hydrogen peroxide radical scavenging activity

Using the standard method, a 2mM H_2O_2 solution (pH 7.4) was prepared in a 50 mM phosphate buffer [25]. Aliquots of various fractions (0.1 mL) were placed in test tubes and their proportions were increased to 0.4 mL with 50M phosphate buffer (pH 7.4). The tubes were vortexed after administering 0.6 mL of H_2O_2 solution, and the absorbance of the H_2O_2 at 230 nm was measured after 10 minutes against a blank. The following equation was used to calculate the ability to scavenge H_2O_2:

$$\% \text{ of } H_2O_2 \text{scavenged} = \frac{(\text{Absorbance of control} - \text{Absorbance of sample})}{\text{Absorbance of control}} \text{ x } 100$$

Antifungal activity of *M. oleifera*

The selected fungal pathogens were *F. oxysporum* (MTCC-1008) and *A. candida* (MTCC-3102). The fungus was first grown on Potato dextrose agar (PDA) slants for 7 days at 35°C to prepare the inoculums for each isolate. The slant was carefully rubbed with a sterile cotton swab before being placed in a sterile tube with fresh Potato dextrose broth (50 mL).

Disc diffusion method

Each fungal culture was inoculated in 5 mL of potato dextrose broth and incubated for 2 days at 37°C. The disc diffusion assay was performed to determine antifungal properties [26]. The inoculum was dispersed on potato dextrose agar medium with a sterilized glass spreader. Small circular paper discs (6mm in diameter) impregnated with a defined amount of each extract was individually placed on the surface of the inoculation plates. The plates were kept at room temperature for extract absorption in the medium before being incubated at 37°C in the incubator for 24 to 48 hr. The diameter of the inhibitory zone was used to assess antifungal activity. For all experiments, triplicates were used.

Minimum inhibitory concentration (MIC)

With some slight adjustments, the MIC was calculated using the micro dilution technique [27]. The experiment with potato dextrose broth was carried out in 96-well micro titter plates. For fungal strains, each well of the 96-well plates was filled with 50 µL of PDB broth. 50 µL of the stock solution of the examined extract was added to the first row of the plate. A micropipette was then used to perform the 2-fold serial dilutions. Excluding the positive control, 10 µL of inoculant was added to each well after reaching concentrations ranging from 100 to 0.1953 mg/mL. Leaf extracts in media were used as a negative control. The test plates were then incubated at 28°C for 24 hr. The minimal dose at which no activity could be detected in the wells of the microtiter plates was identified as the MIC value during incubation.

Fourier transforms infrared spectrophotometer analysis

One of the greatest techniques for figuring out what sorts of chemical linkages (functional groups) are present in a substance is FTIR. Utilizing dried powders of varied solvent extracts of each plant material, the FTIR analysis was carried out. By encasing 10 mg of the dried extract inside a 100 mg potassium bromide pellet, a translucent sample disc was created. The powdered samples from each plant species were analyzed using an FTIR Spectrometer (Shimadzu, IR Affinity1, Japan) with a 400–4000 cm-1 scan range and a 4 cm–1 resolution [28].

High performance liquid chromatography analysis (HPLC)

To estimate the flavonoids quercetin and rutin in the *M. oleifera* leaf extract, HPLC analysis was performed using a photodiode array detector on a reverse phase C-18 column (4.5250 mm). For isocratic elution, a mobile phase of acetonitrile and water (60:40 v/v ratios) was utilised with a flow rate of 1.0 mL/min. With a run time of 10 minutes, the injection volume was set at 20 L for each sample [29]. To filter the mobile phase, 0.45 m membrane filters were utilised. Standard curves for the commercial standards of quercetin (QU) and rutin (RT) were plotted in order to estimate the flavonoid content. In order to verify the analyses' chromatographic peaks, the retention times and UV spectra were compared to those of the reference standards. Each and every chromatographic procedure was carried out at ambient temperature, Regular flavonoids [30].

Gas Chromatography-Mass Spectrometry (GCMS) analysis

The GC-MS analysis of M. oleifera leaf extract was carried out on an Agilent 7890A system outfitted with an MS detector and split less injection mechanism. In the GC, an HP-5MS capillary column (30 m 0.25 mm; film thickness 0.25 m) was installed [31]. The temperature schedule was as follows: injector temperature 260 °C, initial oven temperature 70 °C, then 5 minutes at 10 °C/min to 150 °C, 15 minutes at 12 °C to 200 °C, and 15 minutes at 12 °C/min to 220 °C. At a pressure of 17.69 psi and a flow rate of 0.6 mL/min, helium was used as the carrier gas. After dissolving the samples in methanol, a 1 L aliquot was automatically injected. MS was set up to scan. The mass range was chosen to be between 50-550 m/z. NIST libraries were used to identify the MS spectra of sample components.

Statistical analysis

All tests were performed in triplicate, and the results were expressed as mean standard deviation. The statistical software SPSS was used to analyses the data (17.0).

RESULTS AND DISCUSSION

Qualitative analysis of phytochemicals

The present study revealed that the ethanolic extract of var-1, var-2 and var-3 cultivars of *M. oleifera* leaves contained alkaloids, flavonoids, steroids, saponins, reducing sugar, phytosterols, fats, and fixed oils. However, proteins and amino acids were found only in ethanolic extract of var-1 variety of *M. oleifera* as compared to var-2 and var-3 varieties. Glycosides, anthraquinones, tannin, triterpenoids, and carbohydrates were absent in the ethanolic extract of all the three varieties viz. var-1, var-2 and var-3 of *M. oleifera* leaves. Saponins were found in higher amount in var-1 and phytosterols were found in higher amount in var-3 variety of *M. oleifera*. The tabulated data of the qualitative analysis of ethanolic extract of all the three varieties of *M. oleifera* is shown in Table 1 and Fig. 1.

Table 1: The screening of phytochemicals of the ethanolic leaf extract of *Moringa oleifera* PKM-1 (var-1), PKM2-2 (var-2) and ODC-3 (var-3)

S.no	Phytochemical	Test	Observation	PKM 1	PKM 2	ODC-3
1	Alkaloids	Mayer's test	Development of Yellow Precipitation	++	++	++
2	Protein and amino acids	Xanthoproteic test Ninhydrin test	Development of yellow colour Development of blue Colour	++	Ab	Ab
3	Flavonoids	Alkaline reagent test	Development of yellow to colorless	++	+	++
4	Glycosides	Legal's test	No formation of pink to red colour	Ab	Ab	Ab
5	Steroids	Sulfuric acid test	Development of Yellow green fluorescence	++	+	+
6	Saponins	Foam test	Development of Foam	+++	++	++
7	Tannin	Gelatin test	Development of precipitation	Ab	Ab	Ab
8	Reducing sugars	Fehling's test	Development of precipitation	+	++	++
9	Carbohydrates	Molish's test	No formation of	Ab	Ab	Ab

			violet ring			
10	Anthraquinones	Sulfuric acid test	No change in color	Ab	Ab	Ab
11	Phytosterols	Liebermann-Burchard's test	Development of brown ring	+	++	+++
12	Triterpenoids	Salkowski's test	No change in color	Ab	Ab	Ab
13	Fats and fixed oil	Filter paper press test	Observe the oil Content	+	++	++

Note- +++: highly present, ++: moderately present, +: low, Ab: absent.

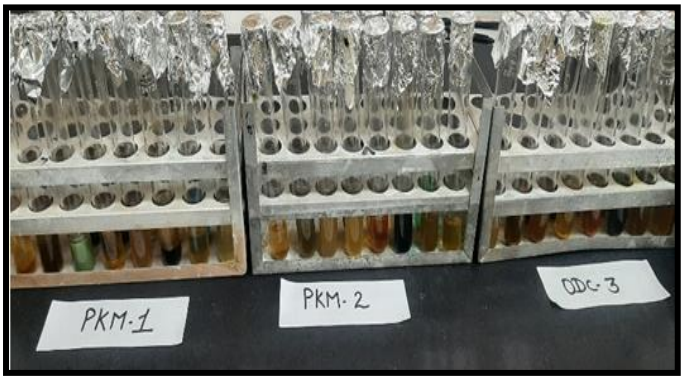


Figure 1: Phytochemical analysis of var-1, var-2 and var-3 these variety of *M. oleifera* leaves

Quantitative estimation of secondary metabolites

According to the makeup of the secondary metabolites in the leaf extract, *Moringa oleifera* leaves have potent antibacterial activity agents.

Estimation of Total phenolic content (TPC)

The TPC of the ethanolic extract of leaves of *M. oleifera* varieties (var-1, var-2 and var-3) is shown in table 2. The var-1 (99.93 ± 0.239 mg GAE/g) variety of *M. oleifera* showed the higher phenolic content followed by the var-3 (91.73 ± 0.254 mg GAE/g) and var-2 (81.58 ± 0.369 mg GAE/g) varieties of *M. oleifera*. The linear correlation between concentration and absorbance, [32] which gave an R^2 value of 0.991, served as evidence that the data were accurate (Fig. 2a).

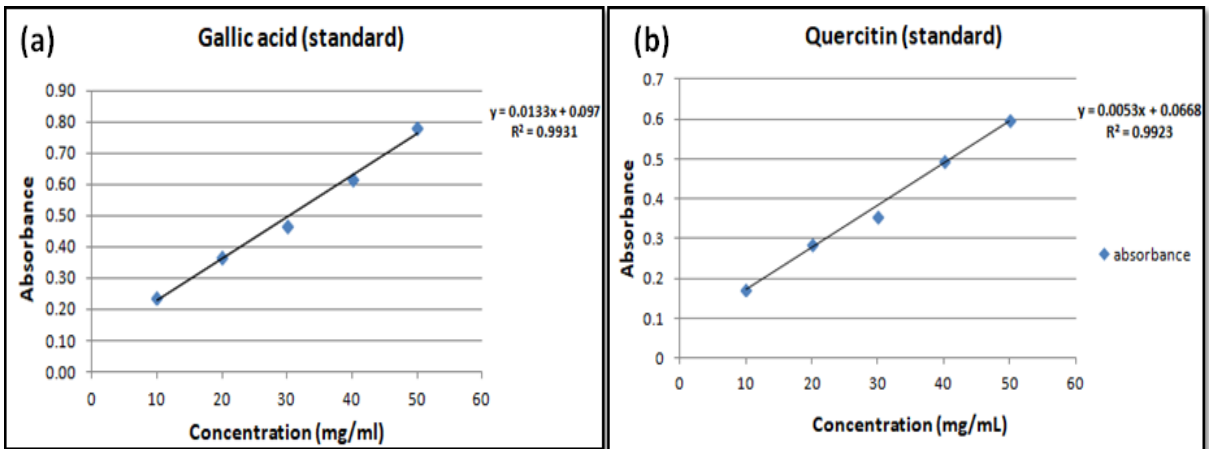
Estimation of Total flavonoid content

The TFC of the ethanol extract of leaves of *M. oleifera* types (var-1, var-2 and var-3) is shown in table 2. The maximum flavonoid content was found in the PKM-1 variety of *M. oleifera* (135.3 ± 0.494 mg QE/g), followed by the ODC-3 (117.1 ± 0.413 mg QE/g) and PKM-2 varieties (106.9 ± 0.553 mg QE/g). The linear association between absorbance and concentration validated the results, and an R^2 value of 0.992 was obtained (Fig. 2b).

Table 2: The quantification of ethanolic leaf extracts of *M. oleifera* variety.

***M. oleifera* varieties**	**Total phenolic content (mg GAE/ g)**	**Total flavonoid content (mg QE/ g)**
Variety-1	99.93 ± 0.239	135.3 ± 0.494
Variety-2	81.58 ± 0.369	106.9 ± 0.553
Variety-3	91.73 ± 0.254	117.1 ± 0.413

The data represent mean ± SEM of three replicates



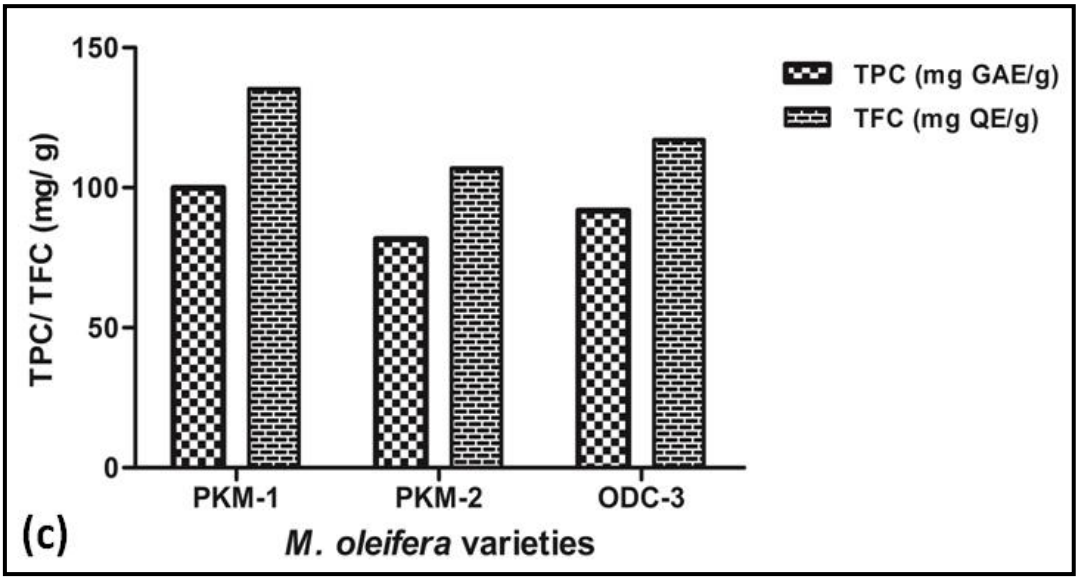


Figure 2: Standard graph of (a) Gallic acid and (b) Quercetin at different concentration (c) TPC and TFC of *M. oleifera* variety

Antioxidant activity of plant extract

Maximum numbers of phytoconstituents were present in ethanol extracts of *M. oleifera* leaves including flavonoids, tannins and phenolic compounds. Hence, these extracts were selected for in vitro antioxidant activity using DPPH, and H_2O_2 method to evaluate the free radicals scavenging capacity.

Evaluation of DPPH free radical scavenging activity

Table 3 and Fig. 3 have shown the potential of *M. oleifera* leaf extracts to scavenge DPPH radicals. *M. oleifera* solvent extracts of leaves demonstrated significant scavenging activity. Thevar-1, this plant had the highest inhibitory activity with the lowest IC_{50} value (14.22 ± 0.031 mg/ mL), followed by the var-2 (26.80 ± 0.231 mg/ mL). The var-3 of *M. oleifera* has the least scavenging activity (53.40 ± 1.126 mg/ mL). var-1, var-2, and var-3 of *M. oleifera* leaves ethanol extracts had IC_{50} values that were equivalent to Ascorbic acid (8.52 ± 0.033 mg/ mL).

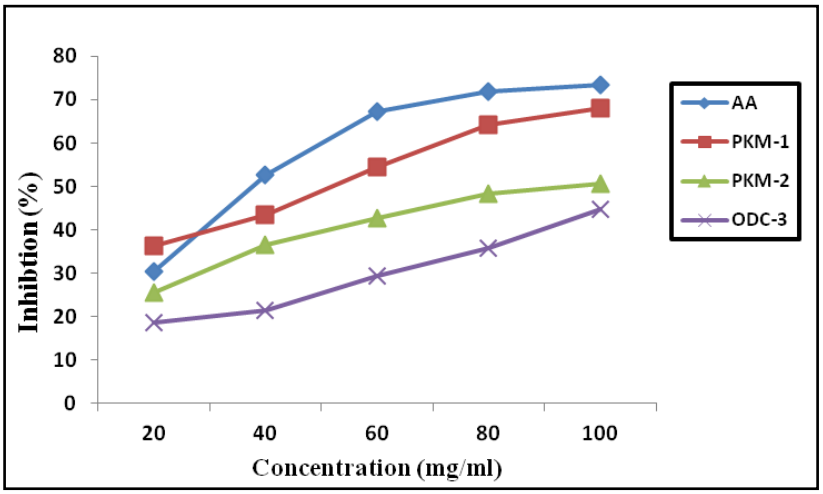


Figure 3: DPPH Inhibition (%) activity of ascorbic acid (AA) and variety-1, variety-2 and variety-3 of *M. oleifera* leaf extracts

Evaluation of Hydrogen peroxide radical scavenging activity

Table 3 and Fig. 4 show the effect of hydrogen peroxide on radical scavenging activity. The maximum H_2O_2 radical scavenging activity was seen in ethanol extract of thevar-1 (25.30 ± 0.101 mg/ mL) followed byvar-2 (48.1 ± 0.467 mg/ mL) of *M. oleifera*. The minimum inhibitory effect was seen in var-3 with (69.73 ± 0.314 mg/ mL). The value of var-1, var-2 and var-3 ethanol extract was equivalent to the value of ascorbic acid (12.37 ± 0.016 mg/ mL).

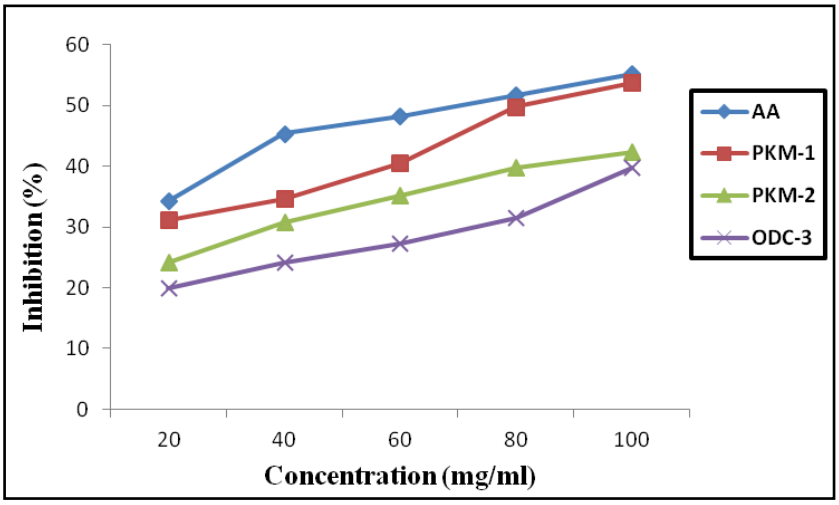


Figure 4: H_2O_2 inhibition (%) activity of ascorbic acid (AA) and var-1, var-2 and var-3 variety of *M. oleifera* leaf extracts

Table 3: Antioxidant potential of ethanolic leaf extracts of *M. oleifera* types

Extracts	DPPH activity (mg/ml)	H_2O_2 activity (mg/ml)
variety-1	14.22±0.031	25.30±0.101
variety-2	26.80±0.231	48.10 ±0.467
variety-3	53.40±1.126	69.73±0.314
AA	8.90± 0.033	12.19±0.016

The data is represented as mean ± Somewhere n = 3 and AA represents ascorbic acid

Antifungal activity of *M. oleifera* leaves

The antifungal efficacy of ethanolic extracts of *M. oleifera* types (var-1, var-2, and var-3) against two fungal strains (*F. oxysporum* and *A. candida*) was investigated at four different doses (100, 250, 500, and 1000 µg/ mL). The antifungal activity of extracts was measured in terms of fungal growth inhibition zones (ZOI) as shown in Fig. 5. Table 4 shows the results of antifungal activity. At a concentration of 250 - 1000 µg/ mL, the ZOI varied from 2.33 to 4.69 mm in the case of *F. oxysporum* and 2.033 to 2.5 mm in the case of *A. candida*, but no ZOI was seen at a concentration of 100 µg/ mL in either fungal strain. The ethanolic extract reduced the growth of *F. oxysporum* in the PKM-2 variety of *M. oleifera* at concentrations ranging from 250-1000 µg/ mL, ranging from 2.7 to 3.5 mm in diameter, while 100 µg/ mL showed no growth. However, in the case of *A. candida*, 100 and 250 µg/ mL concentrations revealed no zone of inhibition, while 500 and 1000 µg/ mL concentrations showed an inhibition zone ranging from 3.3 to 3.3 mm. In the ODC-3 variety, no ZOI was seen at concentrations of 100 and 250 µg/ mL in *F. oxysporum*, while in the case of *A. candida*, a concentration of 100 µg/ mL had no inhibitory effect. As a result, the concentrations of 500 and 1000 µg/ mL of all three varieties of *M. oleifera* leaves inhibited both fungal infections better than the concentrations of 100 and 250 g/ mL.

Table 4: Zone of inhibition (ZOI) obtained by the antifungal activity of sample extract against *F. oxysporum* and *A. candida*

Sample name	Average ZOI of *F. oxysporum*	Average ZOI of *A. candida*
variety-1	-	-
100µg/ml	-	-
250µg/ml	2.33±0.47	2.033±0.41
500µg/ml	4.66±0.51	2.46±0.59
1000 µg/ml	4.69±0.53	2.5±0.1
(variety-2) Control	-	-
100µg/ml	-	-
250µg/ml	2.7±0.49	-
500µg/ml	3.10±0.40	3.3±0.47
1000 µg/ml	3.5±0.1	3.3±0.49
(variety-3) Control	-	-
100µg/ml	-	-
250µg/ml	-	1.16±0.49
500µg/ml	2.33±0.47	2.93±0.59
1000 µg/ml	2.63±0.47	3.1±0.60

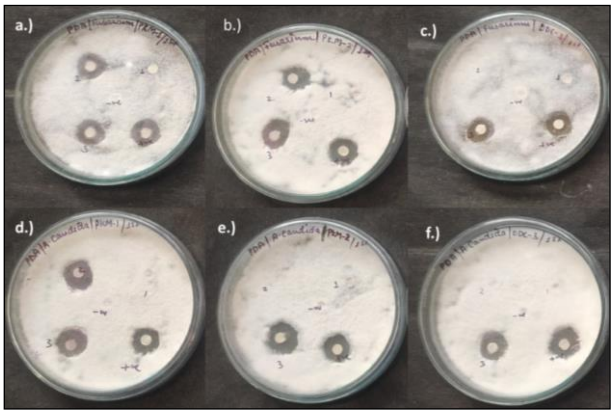


Figure 5: Antifungal activity of ethanolic extracts of three varieties with four concentrations (100, 250, 500 and 1000 µg/ mL). (a to c *Fusarium oxysporum* and d to *Albugo candida*)

Minimum inhibitory concentration

A micro dilution approach was used to determine the MIC, which was described as the lowest dosage that inhibited fungal growth. Table 5 shows that both fungal strains (*F. oxysporum* and *A. candida*) were susceptible to the MIC of *M. oleifera* leaves ethanolic extracts from the var-1, var-2 and var-3 types. The variety -1 had the lowest MIC of 25 and 12.5 µg/ mL against *F. oxysporum* and *A. candida* strains, but the variety-2 showed minimum inhibitory values of 6.25 and 3.13 µg/ mL against *F. oxysporum* and *A. candida*. At concentration of 1.56 and 25 µg/ mL, the variety-3 resulted into the least inhibitory impact against both fungal strains. Antifungal agents found in extracts were effective at inhibiting at low doses before becoming highly potent at maximum concentrations [33].

Table 5: Minimum inhibitory concentration of ethanolic extract of leaves of *M. oleifera* varieties against fungal pathogens

Microorganisms	Minimum inhibitory concentration (µg/ mL)		
	variety-1	variety-2	variety-3
F. oxysporum	25	6.25	1.56
A. candida	12.5	3.13	25

FTIR analysis of *M. oleifera* varieties

Based on the peak values in the IR radiation band, the FTIR spectrum was employed to determine the functional groups of the bioactive constituents present in the extract. When the extract was run through the FTIR, the peak ratios were used to segregate the functional groups of the components. The FTIR spectrums of ethanolic extracts of leaves of M. oleifera varieties (var-1, var-2 and var-3) are given in Fig. 6 a-c and Table 6. Variety-1 of *M. oleifera* exhibited characteristic bands ranging from 3290.27 to 1043.46 cm^{-1}indicating a presence of different compounds such as alcohol, alkane, thiocyanate, imine, alkane and anhydride. The characteristic absorption bands at the range of 3285.98 to 1043.49 cm^{-1}were exhibited by the variety-2 of *M. oleifera* leaves indicating the presence of alcohol, Aldehyde, alkyne, alkene, primary alcohol and anhydride compounds variety-3 of *M. oleifera* leaves exhibited the spectral bands ranging from 3244.60 to 969.71 $cm^{-1}$and indicated the presence of several compounds such as alcohol, thiocyanate, sulphate, sulfone, sulfoxide, tertiary alcohol and alkene.

Table 6: FTIR spectral peak values and functional groups obtained from ethanolic extract of *M. oleifera* varieties

M. oleifera varieties	Peak values	Functional groups	Compound class
variety-1	3290.27	O-H stretching	Alcohol
	2980.19	C-H stretching	Alkane
	2148.72	S – C ≡ N stretching	Thiocyanate
	1645.32	C = N stretching	Imine/ Oxime
	1418.05	C = C stretching	Alkane
	1085.11	C – O stretching	Primary alcohol
	1043.46	CO – O – CO stretching	Anhydride
variety-2	3285.98	O-H stretching	Alcohol
	2979.43	C-H stretching	Aldehyde
	2135.76	C ≡ C stretching	Alkyne
	1645.04	C = C stretching	Alkene
	1417.28	O – H bending	Alcohol
	1084.62	C – O stretching	Primary alcohol
	1043.49	CO – O – CO stretching	Anhydride
variety-3	3244.60	O – H stretching	Alcohol
	2162.68	S – C ≡ N stretching	Thiocyanate
	1643.69	C = C stretching	Alkene
	1385.94	S = O stretching	Sulfate
	1326.24	S = O stretching	Sulfone
	1192.00	C – O stretching	Tertiary alcohol
	1067.57	S = O stretching	Sulfoxide
	969.71	C = C bending	Alkene

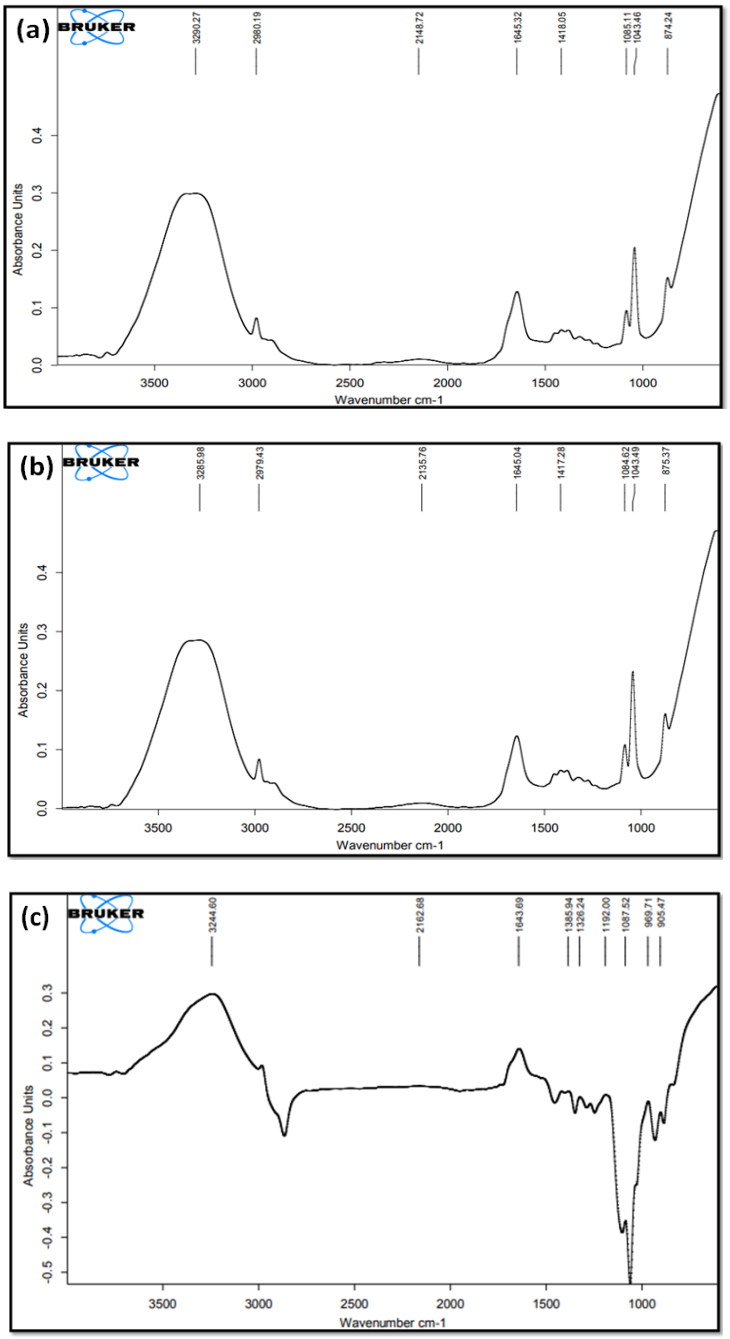


Figure 6: FTIR spectrums of ethanolic extract of *M. oleifera* types (a) variety- 1 (b) variety- 2 and (c) variety- 3

Flavonoids identification and quantification in leaves of *M. oleifera* leaves by HPLC

HPLC chromatogram of Flavonoid Quercetin (QU) and Rutin (RU) standard is recorded at 368 nm (fig 7). Symmetrical, sharp, and highly defined peaks were found for QU and RU. The RT for QU and RU were 5.187 and 5.289 minutes, respectively. The peak areas were 99.9% for QU and 98.7% for RU. Ethanol extracts of plant leaves were directly injected described above. The chromatograms of three variety of *M. oleifera* extract showed the existence of quercetin and Rutin as presented in Fig 8. The calculated contents of the QU were observed to be 7.13 % at 5.1 RT, 0.16% at 5.2 for RU and as shown in Table 7. The higher content of QU i.e. 7.13% was found in PKM-2 variety, 6.69% in var-1 and 1.97 in var-3. QU and RU were successfully determined in all three varieties, as shown in table 8.

Table 7: Validation data for standards of Quercetin and Rutin

Peak	Constituents	Retention time	Area %
1.	Quercetin (QU)	5.187	99.9%
2.	Rutin (RU)	5.289	98.9%

Table 8: Quantification of flavonoid content in the extract of *three different variety of M. oleifera*leaves.

Sample	Component	Content %	Retention time	Area %
variety-1	QU	6.69	5.187	17.95%
	RU	1.16	5.289	0.99%
variety-2	QU	7.13	5.187	1.93%
	RU	1.48	5.289	14.58%
variety-3	QU	1.97	5.187	2.41%
	RU	1.92	5.289	5.42%

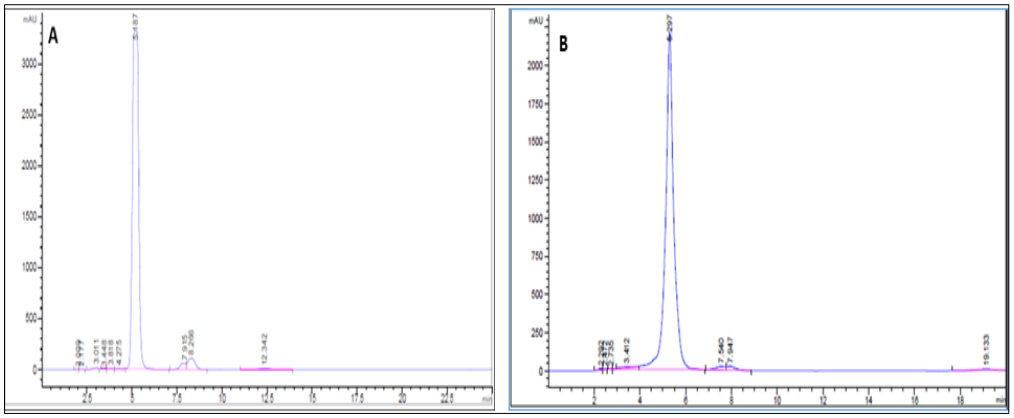


Figure 7: HPLC chromatograph of the standards (A) quercetin (B) rutin

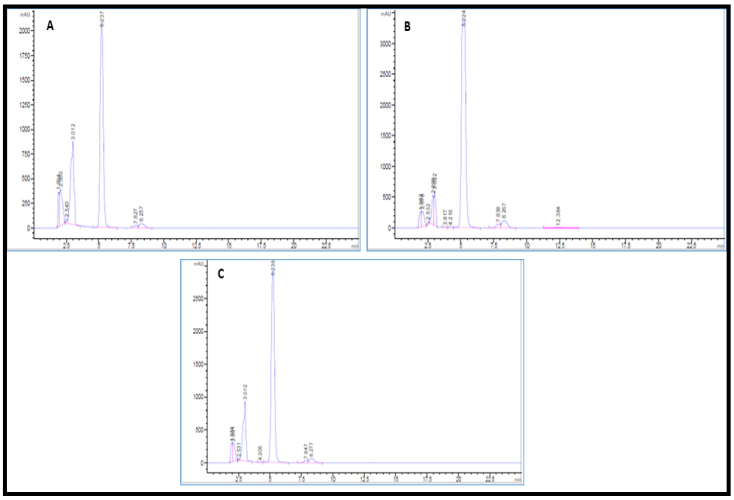


Figure 8: HPLC chromatograph of (A) variety-1 (B) variety-2 (C) variety-3 of *M. oleifera* leaves ethanolic extract

GC-MS analysis

GC-MS profiling of PKM-1 variety of *M. oleifera* leaves extract

Fig. 9 and 10 show the GC-MS analysis of an ethanolic extract of *M. oleifera* leaves from the PKM-1 variety, which contains 25 metabolites. Table 9 lists the phytocompounds, as well as their RT, peak area percentage, molecular formula, and important functions. Few significant compounds with high peak area percent are identified are Adenosine, 4'-de(hydroxymethyl)-4'-[N-ethylaminoformyl]- (8.27%), 4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl- (6.95%), Glycerin (6.77%), 1,2,3,5-Cyclohexanetetrol, (1.alpha.,2.beta.,3.alpha,5.beta) (5.90 %), n-Hexadecanoic acid (3.66%), Benzyl .beta.-d-glucoside (3.62%) and Benzoic acid (3.14%) with antioxidant, antimicrobial, anti-inflammatory properties.

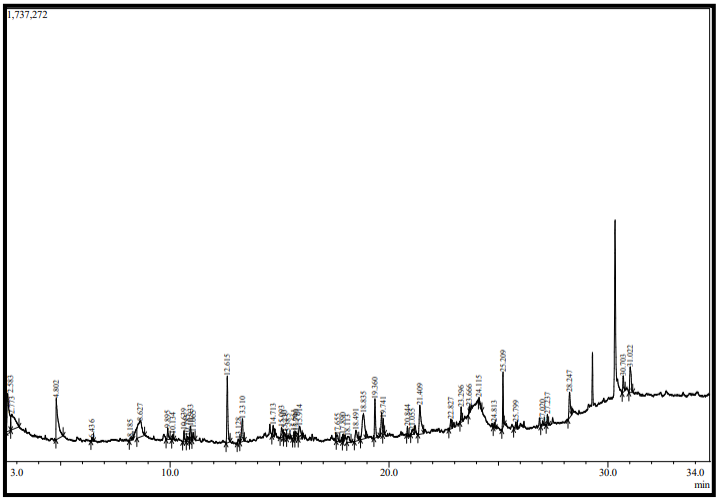


Figure 9: GC-MS chromatogram of variety-1 of *M. oleifera* leaves ethanolic extract

Table 9: List of compounds identified in the ethanolic extract of leaves of *M. oleifera* (Variety-1)

S. No.	Compounds	Molecular weight	RT	Area %	Biological properties
1	Adenosine, 4'-de(hydroxymethyl)-4'-[N-ethylaminoformyl]-	$C_{20}H_{22}N_6O_6$	2.583	8.27	Not yet reported
2	2,4-Dihydroxy-2,5-dimethyl-3(2H)-furan-3-one	$C_6H_8O_4$	8.185	0.56	Dietary, flavor ingredient for food [34]
3	Glycerin	$C_3H_8O_3$	8.627	6.77	Used as ointment and moisturizers in cosmetics and pharmaceuticals [35]
4	Decane, 3,7-dimethyl-	$C_{12}H_{26}$	10.134	0.38	Not yet reported
5	Trans-Linalool oxide (furanoid)	$C_{10}H_{18}O_2$	10.639	1.07	Antimicrobial [36]
6	2-Hexanone, 3-methyl-4-methylene-	$C_8H_{14}O$	10.933	1.78	Not yet reported
7	4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-	$C_6H_8O_4$	12.615	6.95	Antioxidant [9]
8	Benzoic acid	$C_7H_6O_2$	13.310	3.14	Used as antimicrobial preservative in food and beverages [5]
9	5-Hydroxymethylfurfural	$C_6H_6O_3$	14.713	1.29	Antioxidant, Antimicrobial, Anti-inflammatory [30]
10	1,2,3-Propanetriol, 1-acetate	$C_5H_{10}O_4$	15.093	1.47	Not yet reported
11	Tetradecane, 5-methyl-	$C_{15}H_{32}$	15.385	0.44	Antimicrobial [3]
12	L-Proline, 5-oxo-, methyl ester	$C_6H_9NO_3$	17.655	0.97	Not yet reported
13	Niacinamide	$C_6H_6N_2O$	18.113	1.43	Antiaging
14	1,3-Dioxolane, 2,4,5-trimethyl-	$C_6H_{12}O_2$	18.491	2.21	Not yet reported
15	2,4-Di-tert-butylphenol	$C_{14}H_{22}O$	19.741	1.18	Anti-inflammatory, antioxidant and cytotoxic activity [9]
16	1,2,3,5-Cyclohexanetetrol, (1.alpha.,2.beta.,3.alpha,5.beta)	$C_6H_{12}O_4$	21.409	5.90	Not yet reported
17	4-Hydroxyphenylacetamide	$C_8H_9NO_2$	22.827	1.38	Not yet reported
18	Isopropyl myristate	$C_{17}H_{34}O_2$	23.666	0.42	Antioxidant, skin enhancer and pesticide [26]
19	L-Lyxose	$C_5H_{10}O_5$	24.115	0.68	Not yet reported
20	Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$	24.813	0.41	Anti-inflammatory [22]
21	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	25.209	3.66	Antioxidant, anti-inflammatory [37]
22	9H-Pyrido[3,4-b]indole	$C_{11}H_8N_2$	25.799	0.85	Not yet reported
23	Octadecanoic acid	$C_{18}H_{36}O_2$	27.237	0.79	Decrease the HDL level [38]
24	Benzyl .beta.-d-glucoside	$C_{13}H_{18}O_6$	28.247	3.62	Not yet reported
25	Beta.-l-Rhamnofuranoside, 5-O-acetyl-thio-octy	$C_{16}H_{30}O_5S$	30.703	1.64	Anti-inflammatory, Antimicrobial, antioxidant [39]

GC-MS profiling of variety-2 of *M. oleifera* leaves extract

GC chromatogram of the ethanolic extract of *M. oleifera* leaves extract of variety-2 showed 23 peaks (Fig. 11) which indicated the presence of 23 phytochemical compounds. The MS spectrum of ethanolic extract of variety-2 is shown in Fig. 12. The biological properties with their RT, peak area percentage and molecular formula of 23 phytochemicals are represented in Table 10. The most prevalent bioactive metabolites identified among the 23 identified were n-Hexadecanoic acid (4.79%), 2, 4-Di-tert-butylphenol (4.68%), 13-Docosenamide, (Z)- (4.50%), Methyl stearate (4.26%), 2,3-Anhydro-d-galactosan (3.60%), 4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl- (2.98%), Butylated Hydroxytoluene (2.73%), Hexadecanoic acid, 2-hydroxy-1-(hydroxyethyl)ethyl ester (2.53%) with antioxidant, antimicrobial, anti-inflammatory, anthelminthic, and antinociceptive properties.

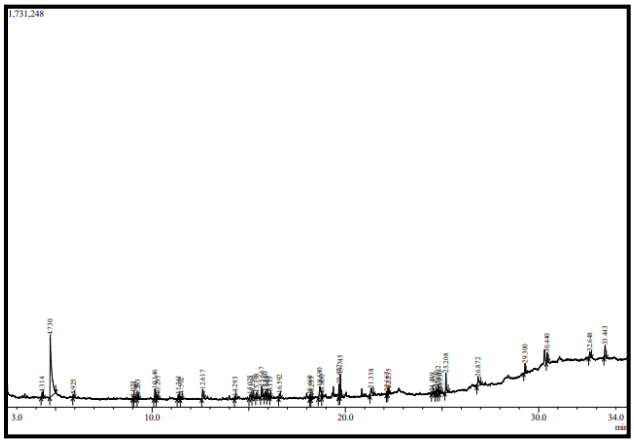


Figure 11: GC-MS chromatogram of variety-2 of *M. oleifera* leaves ethanolic extract

Table 10: List of compounds identified in the ethanolic extract of leaves of Variety-2 of *M. oleifera*

S. No.	Compounds	Molecular weight	RT	Peak value	Biological properties
1	Heptane, 2,4-dimethyl-	C_9H_{20}	4.314	1.36	No activity reported
2	1,3,5,7-Cyclooctatetraene	C_8H_8	5.925	0.60	No activity reported
3	Decane, 4-methyl-	$C_{11}H_{24}$	9.263	0.94	Antidermatitic [40]
4	Octane, 5-ethyl-2-methyl-	$C_{11}H_{24}$	10.146	2.32	No activity reported
5	Decane, 3,7-dimethyl-	$C_{12}H_{26}$	10.295	1.01	Antihelmintic, Antiparasitic, Antihelmintic, Membrane permeability inhibitor, Antimicrobial [41]
6	Octadecane, 1-(ethenyloxy)-	$C_{20}H_{40}O$	11.502	0.61	No activity reported
7	4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-	$C_6H_8O_4$	12.617	2.98	antioxidant [42]
8	1,2,3-Propanetriol, 1-acetate	$C_5H_{10}O_4$	15.075	1.60	No activity reported
9	Dodecane, 4,6-dimethyl-	$C_{14}H_{30}$	15.196	0.57	Antineoplastic, General pump inhibitor, Antifungal [33]
10	3-Acetoxydodecane	$C_{14}H_{28}O_2$	15.860	1.91	No activity reported
11	2-Methyltetracosane	$C_{25}H_{52}$	18.169	1.09	Free radical scavenger [42, 9]
12	2,6,10-Trimethyltridecane	$C_{16}H_{34}$	18.800	1.07	No activity reported
13	ButylatedHydroxytoluene	$C_{15}H_{24}O$	19.672	2.73	Antioxidant [43]
14	2,4-Di-tert-butylphenol	$C_{14}H_{22}O$	19.745	4.68	Antifungal, antioxidant [44]
15	2,3-Anhydro-d-galactosan	$C_6H_8O_4$	21.338	3.60	Preservative [45]
16	Eicosane	$C_{20}H_{42}$	22.167	0.70	Antifungal, Antitumor, [9] Antibacterial, Larvicidal, Cytotoxic, Antimicrobial
17	Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$	24.812	2.29	Anti-inflammatory [8]
18	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	25.208	4.79	Anti-inflammatory [10]
19	Methyl stearate	$C_{19}H_{38}O_2$	26.872	4.26	Anti- helminthic (Nematodes), Antinociceptive, Anti-inflammatory, intestinal Lipid metabolism regulator, Gastrin inhibitor [16]
20	Hexanedioic acid, bis(2-ethylhexyl) ester	$C_{22}H_{42}O_4$	29.300	2.06	No activity reported
21	Hexadecanoic acid, 2-hydroxy-1-(hydroxymethy)ethyl ester	$C_{19}H_{38}O_4$	30.440	2.53	Antioxidant [10]
22	Octadecanoic acid, 2,3-dihydroxypropyl ester	$C_{21}H_{42}O_4$	32.648	2.25	Anticancer, antimicrobial [6]
23	13-Docosenamide, (Z)-	$C_{22}H_{43}NO$	33.443	4.50	Antimicrobial, antioxidant [40]

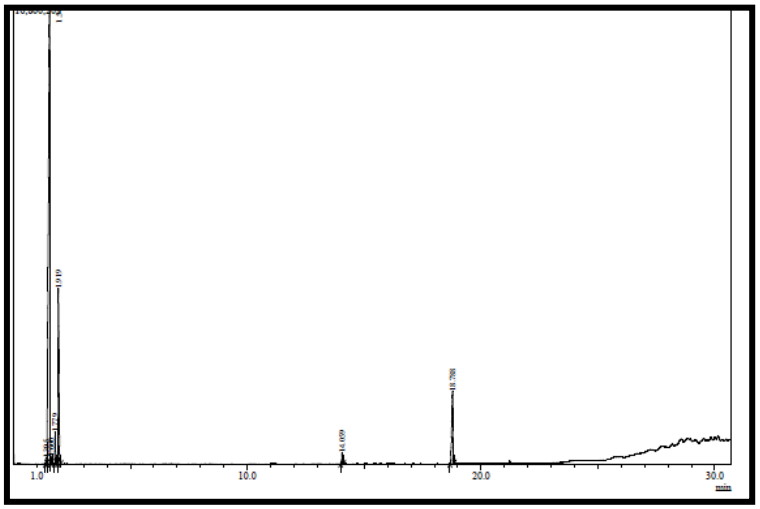


Figure 12: GC-MS chromatogram of variety-3 of *M. oleifera* leaves ethanolic extract

GC-MS profiling of variety-3 of *M. oleifera* leaves extract

GC chromatogram of the ethanolic extract of *M. oleifera* leaves extract of variety-3 showed 5 peaks (Fig. 12) which indicated the presence of 5 phytochemical compounds. The MS spectrum of ethanolic extract of variety-3 is shown in Fig. 12. The biological properties with their RT, peak area percentage and molecular formula of 5 phytochemicals are represented in Table 11. The most prevalent bioactive metabolites identified among the 5 identified weren- 3-Acetooxy dodecane (1.91%), 2-methyltetracosane (1.09%), Isopropyl alcohol (1.6%), Tri-chloral methanol (2.73%), 2-methnoxy-4 vinylphenol (4.68%), with antifoaming agent, anti-coagulant antimicrobial properties.

Table 11: List of compounds identified in the ethanolic extract of leaves of Variety-3 of *M. oleifera*

S. No.	Compounds	Molecular weight	RT	Peak value	Biological properties
1	3-Acetoxydodecane	$C_{14}H_{28}O_2$	14.860	1.91	No activity reported
2	2-Methyltetracosane	$C_{25}H_{52}$	18.169	1.09	Free radical scavenger [9]
3	Isopropyl alcohol	C_3H_7OH	1.6.8	1.07	Antiseptic activity [22]
4	Trichloromethane	$CHCl_3$	1.41	2.73	Antifoaming agent, anti-coagulant and anti-fungal activity [4]
5	2-methnoxy- 4 vinylphenol	C_9H_{1002}	14.74	4.68	Flavoring agent [46]

DISCUSSION

Primary components include simple carbohydrates, proteins and chlorophyll whereas secondary components include alkaloids, phenols, and Terpenoids [47]. Terpenoids possess a variety of important therapeutic potential, including anti-inflammatory, anti-mutagenic, anti-malarial, cholesterol synthesis inhibition, antiviral, and antibacterial properties [48]. Each phytochemical has shown the ability to perform some bioactivity, such as the antioxidant capacity of flavonoids, the antimicrobial, and antistress properties of alkaloids, and the anti-inflammatory potential of steroids [49].Tannins bind to and inhibit the synthesis of proline-rich proteins. Flavonoids have the potential to function as antioxidants due to their capacity to form a complex with extracellular, soluble, and bacterial cell wall proteins. Alkaloids can be found in medicinal plants and serve as anesthetics [50]. Several studies have found that glycosides lower blood pressure. According to the findings of this study, the identified phytochemical compounds may be bioactive constituents, and this plant is showing to be a wonderful reservoir of bioactive metabolites with great medicinal significance [51]. Wangcharoen and Gomolmanee conducted a similar investigation on thevar-1 type of *M. oleifera* and discovered var-1 to be a potent source of phenolic chemicals [45]. El Sohaimyand his colleagues found that methanol extract has the greatest quantity of TPC (48.35mg GAE/g) among the various extracts of *M. oleifera* leaves [52]. Gull and his colleagues found similar results, with ethanol extract having a lower TPC level (25.93 mg GAE/g) than methanol extract (48.35 mg GAE/g) [53].

Ghafar and his colleagues illustrated that the high TPC and TFC value in methanolic extract of M. oleifera seeds is the good source of natural antioxidant [54]. The DPPH approach is typically based on DPPH scavenging via the addition of an antioxidant that decolorizes the DPPH solution. DPPH radicals were considerably decreased by *M. oleifera* leaf extract. The degree of discoloration shows the antioxidant extract's scavenging power, which is linked to its radical scavenging activity [55]. H_2O_2 is extremely significant due to its capacity to penetrate biological membranes. H_2O_2 alone is not highly reactive; it is a mild oxidizing agent. However, it can be hazardous to cells because it can generate hydroxyl radicals in the cells [56, 57].

Furthermore, the growth inhibitory activity of extracts may be attributed to the interaction many bioactive components present in higher or lower amounts in the crude extract rather than the action of a solitary active molecule [58]. A similar pattern was reported in previous investigations into the antibacterial activity of different components of *M. oleifera* [59]. Both plant extracts functioned in a dose-dependent way; as the amount of the extract was reduced, so did the efficacy [60]. This is related to the pathogen's vulnerability to extract concentration, after which the extract destroys that microbe, which is unbearable for it [61]. Furthermore, the growth inhibitory effect of crude extracts may be attributed to the interaction of many active components present in varying concentrations in the bulk extract, rather than the action of a solitary active molecule [62]. Our findings were similarly consistent with previous findings from *M. oleifera* investigations [9,3,4]. The documented antifungal activity of *M. oleifera* morphological components could be related to the existence of phenols alkaloids, tannins, triterpenoids, terpenoids, flavonoids, and saponins. These phenolic components triggered enzyme denaturation, which could delay the amino acids required in spore germination [63]. Furthermore, the growth inhibitory effect of crude extracts may be attributed to the interaction of many active components present in varying concentrations in the bulk extract, rather than the action of a solitary active molecule (Rengasamy KR et al. 2019). Our findings were similarly consistent with previous findings from *M. oleifera* investigations. The documented antifungal activity of *M. oleifera* morphological components could be related to the existence of phenols alkaloids, tannins, triterpenoids, terpenoids, flavonoids, and saponins. These phenolic components triggered enzyme denaturation, which could delay the amino acids required in spore germination [63].

CONCLUSION

Study focused on comparative evaluation of three varieties (PKM-1, PKM-2, ODC-3) of highly medicinal plant Moringa. Efficacy of their potential as biocontrol agent has been test against two major pathogens *Fusarium oxysporum* and *Albugo candida* responsible for huge crop loss.

In this study, preliminary phytochemical analysis, quantitative estimation (TPC and TFC), antioxidant assays (DPPH and H_2O_2 assays), antifungal activity tests, and analysis of phytoconstituents with FTIR, HPLC, and GCMS were performed to validate the antioxidant and antifungal potential with chemical components of three varieties (var-1, var-2, and var-3) of *M. oleifera*. This is, as far as we are aware, the first investigation to examine the relationships between the phytoconstituents, antioxidants and antifungal properties of three distinct *M. oleifera* varieties. Therefore, we discovered that the leaves of *M. oleifera* are abundant in phenolic and flavonoid chemicals, which accounted for the potentially significant antioxidant and antifungal properties among the three different kinds. There salts proved that *M. oleifera* leaves could be a more viable source of antioxidants and antifungal, and have great promise for the production of health-promoting dietary supplements in the immediate future.

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Conflict of interest

The authors state that they do not have any conflict of interests.

Data availability

On reasonable request, the corresponding author will make the datasets used in this work public. This eliminates raw interview materials, which contain sensitive information about the participants.

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