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In Vitro Antibacterial, Phytochemical and Molecular Characterization of *Moringa oleifera* Lam.

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Moringa oleifera Lam. popularly called as "miracle tree" is a fast-growing deciduous plant, originated in the Indian subcontinent and is commonly grown in tropical regions. Its extraordinary nutritional profile and a deluge of therapeutic benefits have attracted considerable attention worldwide. The leaves, in particular, are good source of calcium, potassium, proteins and vitamins A, C and E, making them an essential dietary supplement, especially in regions facing food insecurity. The leaves are also rich in antioxidants, including chlorogenic acid, quercetin and beta-carotene, which protect against oxidative stress and cellular damages. This study is an attempt to screen the plant extracts of M. oleifera for its antibacterial activity, qualitative phytochemical constituents and molecular characterization. The results indicate that crude and powdered fresh leaves extract showed no antibacterial activity whereas cold, hot and ethanol extracts, prepared from fresh and dried leaves of M. oleifera showed varying antibacterial properties. Phytochemical analysis revealed the presence of saponins, flavonoids, glycosides, alkaloids, carboxylic acids, coumarins, phenols, quinones, resins, phlobatannins, diterpenes and terpenoids. The study demonstrated efficient antibacterial action against human pathogens which can be attributed to the various phytochemicals present in this plant. To understand the genetic diversity exist in M. oleifera populations, matK and ITS regions were sequenced in five accessions collected from different parts of South Kerala. Five SNPs were detected in the ITS loci and no SNPs were detected in matK loci. Pairwise genetic distance were calculated based on ITS sequences and maximum genetic distance was found between Chengannur and Ochira accessions (0.006) whereas, minimum genetic distance was noted between Chengannur to Kallumala (0.001) and Thiruvananthapuram (0.001). Genetic distance data was subjected to cluster analysis using UPGMA dendrogram. Five accessions were entered into two distinct clusters. Accessions from Chennithala and Ochira were clustered together in one node with a bootstrap support of 98% whereas, the other three accessions, Chengannur, Kallumala and Thiruvananthapuram were clustered together with a boot strap support of 80%. Clustering pattern revealed the genetic structure exist in M. oleifera accessions studied.

Keywords: Antibacterial; Dendrogram; Moringa oleifera; Phytochemistry; UPGMA.

Moringa oleifera (Moringaceae) is luxuriously growing in tropical climatic zones and its leaves, fruits and flowers are consumed as vegetables. All the plant parts are utilised for their pharmacological, nutritional and water purifying properties[1]. Leaves are eaten as vegetables and are used in traditional pharmacology to treat many ailments[2]. The hepatoprotective[3]; antidiabetic[4];

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cardioprotective[5]; anticancer[6]; antioxidant[7]; neuroprotective and anti-neuroinflammatory[8]; anti-asthmatic[9]; anti-arthritic[10]; antimicrobial[11]; anti-ulcer[12]; CNS activity[13]; anti-allergic[14]; wound healing[15]; analgesic and antipyretic[16,17] activities were demonstrated earlier. The phytochemical profile of *M. oleifera* was reported earlier[18]. AFLP marker based genetic analysis of seven populations of *M. oleifera* from Kenya was also documented[19].

The population structure and genetic diversity of a global collection of 161 accessions of *M. oleifera* gathered from Asia, Africa, North and South America, and the Caribbean were also studied using 19 SSR markers and a partial sequence of the chloroplast gene *atp*B[20]. Eight Indian cultivars of *M. oleifera*, from different states in India, were examined for genetic variability using markers like cytochrome P450 gene, ISSR and RAPD[21]. Clustering pattern was independent of geographic origin of the accessions and concluded the spread of propagules and increased rates of gene flow in the studied area. This investigation aimed to screen the plant extracts of *M. oleifera* for its antibacterial activity, qualitative phytochemical analysis and molecular characterization of five accessions collected from various localities of South Kerala.

MATERIALS AND METHODS

Source plant

Tender leaves of *M. oleifera* were obtained from Kallumala region of Mavelikara municipality (Sl. No. 3; Table 1). The fresh and dried leaves are used for the antibacterial and phytochemical analysis. For molecular analysis, five samples were collected directly from home orchards (Table 1).

Leaf Extract preparation

100g of leaves were collected and ground using a mortar and pestle. The fresh extract obtained was stored in a clean airtight bottle for antibacterial analysis.10 ml of leaf juice were air dried and 0.1301 g fine powder was obtained and dissolved in DMS, stored in airtight bottle for antibacterial tests. In addition, 10 g of leaves were crushed directly and boiled in 40 ml of distilled water. The extract was stored in a refrigerator and centrifuged after 3 days for 10 minutes at 10,000 rpm. The supernatant obtained was gathered in a clean bottle and stored in a refrigerator. Likewise, 10 g of leaves were crushed in 40 ml cold distilled water and refrigerated. After 3 days, the stored material is centrifuged at 10000 rpm for 10 minutes. The supernatant obtained was stored in refrigerator for further analysis. 10g of dried leaves of *M. oleifera* were crushed and 40 ml ethanol was added, stored in refrigerator and after 3 days, the extract is centrifuged at 10000 rpm for 10 minutes and stored the supernatant in refrigerator. Similarly, hot water, cold water and ethanol were also carried out in same manner with dried powder too.

Microorganisms

Four bacterial strains were used in the study, namely, *Escherichia coli* and *Salmonella typhi* (gram negative) and *Bacillus cereus* and *Staphylococcus aureus* (gram positive).

Antibacterial Assay

Bactericidal activity of extracts was checked using agar disc diffusion method and was reported elsewhere[22]. Test microorganisms i.e., 10 µl from overnight broth cultures were seeded into nutrient agar medium by spread plate method. Agar discs were punched and soaked in plant extracts. Control disc were prepared by soaking in the solvents (negative control). The discs were inoculated and incubated the plates at 37^0C for 24 hours and the diameter of the zone of inhibition (mm) is noted. The antibacterial potential of various plant extracts prepared were determined by analysing the zone of inhibition.

Phytochemical analysis

Extracts from fresh leaf and dried powder using different solvents (hot water, cold water and ethanol) were checked for the presence of various secondary metabolites using the procedure described earlier[23,24,25]. Major pharmaceutically valuable phytochemical compounds like alkaloids, terpenoids, carboxylic acids, carotenoids, coumarins, lignins, flavonoids, diterpenes, phenols, free amino acids, quinones, resins, saponins, steroids, phytosteroids, tannins, xanthoproteins, glycosides, phlobatannins, proteins, and sugars were screened.

DNA Extraction

Extraction of DNA was carried out with Nucleospin® Plant II Kit (Macherey Nagel) as per instructions provided in the manual. The eluted DNA was stored at 4^0C and quality and quantity is checked by agarose gel electrophoresis.

PCR Analysis

PCR amplification reactions were done in a 20 µl reaction volume which contained 1X Phire PCR buffer, 0.2mM each dNTPs, 1 µl DNA, 0.2 µl PhireHotstart II DNA polymerase enzyme, 0.1 mg/ml BSA and 3% DMSO, 0.5M Betaine, 5pM of forward and reverse primers (Table 2).

DNA amplification was done in a PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems). Cycling settings were 98^0C for 5 minutes and then 40 cycles of 98^0C (5s), 50^0C for *mat* K/58^0C for ITS (10s), 72^0C (15s) and final extension at 72^0C (5 min). PCR products were checked in 1.2% agarose gels. After quality check, PCR products were subjected to ExoSAP-IT Treatment, by mixing 5 µl of PCR products and 2 µl of ExoSAP-IT (GE Healthcare). The mix is incubated for 15 min. at 37°C followed by enzyme inactivation at 80^oC for 15 minutes.

Sequencing using BigDye Terminator v3.1

The sequencing process was conducted in a PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems) using the BigDye Terminator v3.1 Cycle sequencing Kit (Applied Biosystems, USA), following the company's instructions. The PCR mix consisted of, 3.2 µM of primer (forward or reverse), 0.28 µl of the sequencing mix, 1.86 µl of 5x reaction buffer, 10 µl of sterile distilled water, and 10–20 ng of ExoSAP treated PCR product. The temperature profile for the sequencing PCR was as follows: 30 cycles for 30 seconds at 96 °C, 40 seconds at 50 °C, and 4 minutes at 60 °C were performed for each primer after an initial cycle that lasted two minutes at 96 °C.

Sequence Analysis

Sequence Scanner Software v1 (Applied Biosystems) and Geneious Pro v5.1 was used for aligning and editing of sequences[26].

Table 1. *M. oleifera* accessions used for molecular studies.

No.	Sample Code	Geographic origin
1	SR912-CA	CHENNITHALA
2	SR912-CR	CHENGANNUR
3	SR912-KA	KALLUMALA
4	SR912-O	OCHIRA
5	SR912-TVPM	TRIVANDRUM

RESULTS AND DISCUSSION

***In vitro* antibacterial activity**

Dried leaf powder extracts have showed no bactericidal property. Cold water extract of fresh leaves showed no antibacterial effect without any zone of inhibition. Hot water extracts of mature fresh leaves displayed meagre activity on *E. coli* and *S. aureus* whereas no activity found against *S. typhi* and *B. cereus*. Ethanol extract of fresh leaves displayed a relatively better antibacterial assay on *B. cereus, E. coli*, *S. aureus* and *S. typhi* with their distinct diameter zones of inhibition recorded 15 mm, 20 mm, 15 mm, 10 mm respectively.

Phytochemical analysis

Hot, cold water and ethanol extracts from fresh and dried leaves were profiled to compare the difference between the treatments in phytochemical elusion. The study demonstrated the presence of common phytoconstituents like tannins, saponins, flavonoids, glycosides, alkaloids, carboxylic acids, coumarins, phenols, quinones, resins, xanthoproteins, phlobatannins, diterpenes, terpenoids, sugars, lignins, carotenoids, proteins and amino acids, sterols and phytosterols from the leaves of *M. oleifera*. Phytochemical profile of hot and cold water extracts of mature fresh and dry leaves were significantly different in the absence of certain constituents (Table 3). The findings in the study are *at par* with earlier reports[27]. Using Gas chromatography-mass spectrometry aided study, the presence of 16 components in

Table 2. Primers used for sequencing

Loci	Primer Name	Direction	Sequence (5' → 3')
mat K	390f	Forward	CGATCTATTCATTCAATATTTC
	1326r	Reverse	TCTAGCACACGAAAGTCGAAGT
ITS	ITS-5F	Forward	GGAAGTAAAAGTCGTAACAAGG
	ITS-4R	Reverse	TCCTCCGCTTATTGATATGC

the methanolic extract of leaves with highest percentage of 9-octadecenoic acid (20.89%) and L-(+)-ascorbic acid- 2,6-dihexadecanoate (19.66%) was recorded[28]. The synergistic effect of the phytochemicals may be the reason behind the use of the plant for various ailments, especially in indigenous system of medicine[29] and the present results also attested it.

Molecular diversity

Two gene regions, *mat* K and ITS, were selected to understand the genetic diversity of five accessions of *M. oleifera* obtained from various

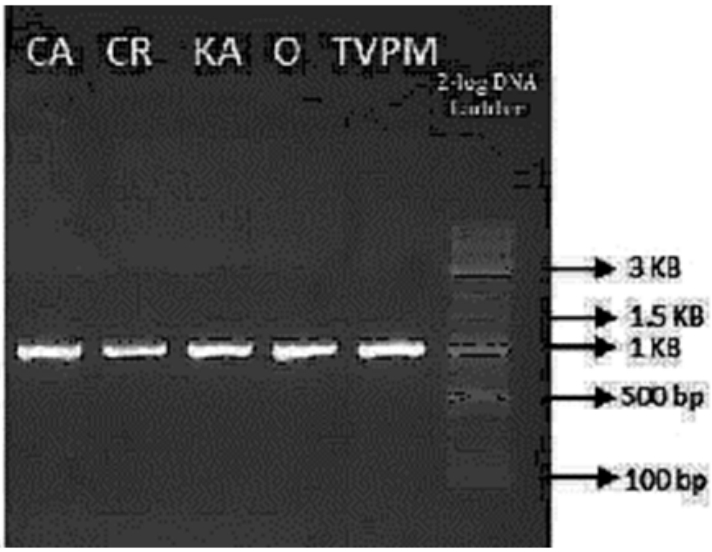

Fig. 1. Gel showing the amplification of *mat* K loci in the five accessions of *M. oleifera* and run on 1% Agarose gel. The line codes correspond to the code given in Table 1

Table 3. Phytochemical profile of *M. oleifera*

No.	Compounds	Fresh leaves			Dry leaves		
		Hot extract	Cold extract	Ethanol	Hot extract	Coldextract	Ethanol
1.	Alkaloids	+	+	+	+	+	+
2.	Carboxylic acid	-	-	-	-	-	-
3.	Coumarins	-	+	+	+	+	+
4.	Flavonoids	+	+	+	+	+	+
5.	Phenols	-	-	-	-	-	-
6.	Quinones	-	-	-	-	-	-
7.	Resins	-	-	-	-	-	-
8.	Saponins	-	-	-	-	-	-
9.	Sterols & Phytosterols	+	-	+	+	+	+
10.	Tannins	-	-	-	+	+	-
11.	Xanthoproteins	-	-	-	-	-	-
12.	Glycosides	-	-	-	-	+	+
13.	Phlobatannins	-	-	-	-	-	-
14.	Diterpenes	+	-	-	+	+	+
15.	Terpenes	-	-	-	-	-	-
16.	Carotenoids	-	-	-	-	-	-
17.	Lignins	-	-	-	-	-	-
18.	Proteins and amino acids	-	-	-	-	+	-
19.	Sugars	-	-	-	-	+	-

places of South Kerala. The chloroplast region, *mat* K was selected as a maternally inherited, highly conserved loci, whereas, nuclear gene region, ITS as highly variable and depicts the biparental genetic diversity. Figs. 1 and 2 provide a representative gel image demonstrating the amplification of the two loci examined.

Sequences were aligned with the help of Geneious software. Five SNPs were detected in the ITS loci. *mat* K sequences were aligned and no SNPs were detected. Pair wise genetic distance between accessions were calculated based on ITS sequences (Table 4). Maximum genetic distance was noticed between Chengannur and Ochira

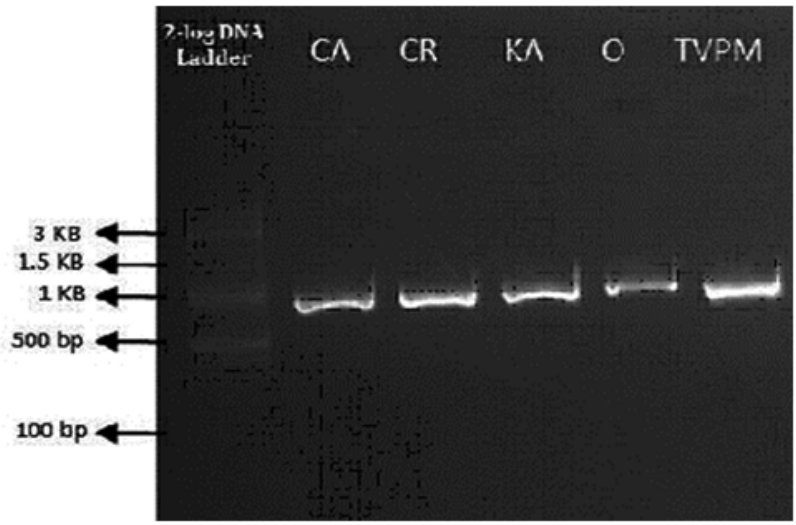

Fig. 2. Gel showing the amplification of ITS loci in the five accessions of *M. oleifera* and run on 1% Agarose gel. The line codes correspond to the code given in Table 1

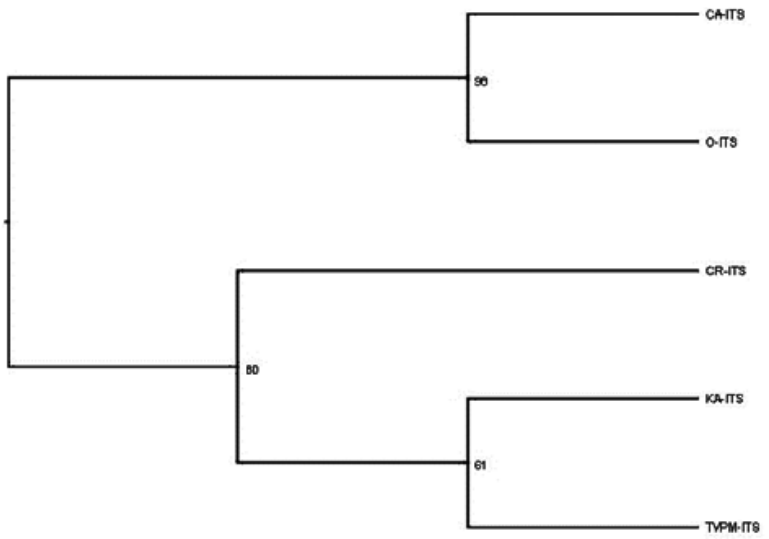

Fig. 3. UPGMA dendrogram of five *M. oleifera* accessions based on ITS sequences. Values at the node denotes bootstrap support

Table 4. Pair wise genetic distance calculated for accessions of *M. oleifera*

	CA-ITS	CR-ITS	KA-ITS	O-ITS	TVPM-ITS
CA-ITS	0.000				
CR-ITS	0.006	0.000			
KA-ITS	0.004	0.001	0.000		
O-ITS	0.000	0.006	0.004	0.000	
TVPM-ITS	0.004	0.001	0.000	0.004	0.000

accessions (0.006) whereas, minimum was found between Chengannur to Kallumala (0.001) and Thiruvananthapuram (0.001). Genetic distance data was subjected to cluster analysis using UPGMA dendrogram (Fig 3). Five accessions were entered into two distinct clusters. Accessions from Chennithala and Ochira were clustered together in one node with a bootstrap support of 98%. Whereas, the other three accessions, Chengannur, Kallumala and Thiruvananthapuram were clustered together in one node with a boot strap support of 80%. A recent report discussed the genetic diversity of *Moringa* and its importance in the current nutritional security environment[30]. 164 genotypes of *Moringa* were identified by employing cluster analysis, principle coordinate analysis (PCoA), 3D plot and phylogenetic tree[31]. An indepth analysis of the *Moringa* gene pool for leaf micronutrient and phytochemical properties produced encouraging results[32].

The nutritional, therapeutic and industrial significance of *M. oleifera* was evaluated earlier[33]. Aqueous, extract of *M. oleifera* confirms the presence of secondary metabolites including Carbonic acid, Butanedioic acid, Citramalic acid, some esters etc. Also, 54 components were identified in methanolic *Moringa* leaves extracts, with 1,3-Propanediol, 2-ethyl-2- (hydroxymethyl) and Propionic acid as major components[34]. It should be noted that a number of factors, including the plant's cultivation location, soil type, water and fertilizer availability, industrialization process, and storage conditions, affect the phytochemical contents of *Moringa*. Taking these precedents into account, it can be inferred that the aforementioned reasons account for the variance in the nutritional and functional qualities of *M. oleifera* from different regions in Kerala.

CONCLUSION

Antibacterial efficiency of various leaf extracts (fresh and dried leaves) of *M. oleifera* was examined using Agar disc diffusion method. Dried leaf powder extracts have showed no antibacterial activity. Cold water extract of fresh leaves showed no antibacterial effect while hot water extract exhibited meager activity on *E.coli* and *S. aureus* whereas no specific action detected against *S. typhi* and *B. cereus*. Ethanol extract of fresh leaves displayed a relatively better bactericidal property on *B. cereus, E. coli*, *S. aureus* and *S. typhi* with distinct individual zones of inhibition measured in diameters documented 15 mm, 20 mm, 15 mm, 10 mm respectively. Different extracts of dried leaves showed no activity against the organisms tested. Qualitative phytochemical analysis of fresh and dry leaves of *M. oleifera* revealed the occurrence of secondary metabolites like tannins, saponins, alkaloids, carboxylic acids, coumarins, phenols, quinones, resins, xanthoproteins, phlobatannins, diterpenes, terpenoids, sugars, lignins, carotenoids, proteins and amino acids, sterols, phytosterols *etc*. Phytochemical profile of various extracts from mature fresh and dry leaves were significantly different. One chloroplast and one nuclear gene region, were chosen to observe the phylogenetic interrelationships between five accessions of *M. oleifera* obtained from different parts of South Kerala. Pair wise genetic distance between accessions were calculated based on ITS sequences. Maximum genetic distance was found between Chengannur and Ochira accessions (0.006) whereas, minimum was noticed between Chengannur to Kallumala (0.001) and Thiruvananthapuram (0.001). Genetic distance data was subjected to cluster analysis using UPGMA dendrogram. Five accessions were entered into

two distinct clusters. Accessions from Chennithala and Ochira were clustered together in one node with a bootstrap support of 98%. Whereas, the other three accessions Chengannur, Kallumala and Thiruvananthapuram were clustered together in one node with a boot strap support of 80%. Genetic characterization studies identified high genetic diversity in nuclear loci and no genetic variation at the chloroplast loci. These results will be helpful for future research endeavors that attempt to investigate the numerous biological potentials of *M. oleifera*.

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Conflict of Interest

The authors do not have any conflict of interest.

Data Availability Statement

This statement does not apply to this article.

Ethics Statement

This research did not involve human participants, animal subjects, or any material that requires ethical approval.

Informed Consent Statement

This study did not involve human participants, and therefore, informed consent was not required.

Author Contributions

Rari Vijayan, Rinu V : carried out the antibacterial and phytochemical assays; Dinesh Raj R : written the initial form of the manuscript; Asha Ramachandran and Dinesh Raj R :did the molecular works and finalized the manuscript.

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