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Molecular diagnosis and phytochemical analysis of alcoholic extracts of *Moringa oleifera* leaves by GC-MS

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Abstract
A phytochemical study of *Moringa oleifera* leaves was carried out using gas chromatography mass spectrometry to determine the phytochemical components by GCMS analysis present in the alcoholic extract of *Moringa* plant. It was 2.035 ppm for Ethanol, 2-(2-hydroxyethoxy)-, 1-nitrate and the lowest peak area was 3.012 ppm for Sulfurous acid, bis(1-methylethyl) ester. Phytochemicals produced by plants can act as alternatives to antibiotics and anti-ulcers and antivirals for many infectious agents or act as nutritional agents for non-communicable diseases. They can also act as antioxidants and flavoring agents. A series of experiments were conducted, including diagnosing the plant under study molecularly, and registering the plant species in the Global GenBank of the International Center for Biotechnology Information (NCBI). I carried the serial number MT495787.

Keywords: *Moringa oleifera*, GC-MS analysis, chemical compound

Introduction

Medicinal plants have played a major part in conventional medicine and herbal therapy in many nations across the world, and 80% of the world's population continues to rely on them due to their affordability, accessibility, low cost, promising effectiveness, and lack of the negative side effects associated with the use of chemical drugs. Most therapeutic plants are non-toxic, however a few are quite poisonous. For both people and animals. (Okoye *et al*., 2014) [14] many plants have been used in the treatment of some diseases due to their medicinal properties, treatment of certain diseases or disease prevention, and other ways to increase the body's immunity and vitality since ancient times (Ullah *et al*., 2020) [18]. The Moringa plant belongs to the Moringaceae family. It is popularly called the horse radish tree, the Drumstick tree, or the coffee oil tree in English. It is also known as the "tree of life" or "the miracle tree." It is an evergreen, perennial tree that grows to a height of 10-12 meters with The stem may reach 45 cm. The plant is slender with drooping fragile branches. The leaves are feathery, pale green, and triple compound, 30-60 cm long. The flowers are white or creamy with a pungent odor (Paul & Didia, 2012) [16]. It has enormous medical and non-medicinal benefits. Traditionally, the plant is used For the treatment of wounds, pain, ulcers, liver disease, heart disease, cancer, and infections (Pareek *et al*., 2023) [15]. (Mahato *et al*., 2022) [12] (Paul & Didia, 2012) [16].

The plant is used in botanical medicine as an antioxidant, antimicrobial, anti-inflammatory, antipyretic, anti-ulcer, antidiabetic, and antipyretic (Ijioma *et al*., 2014) [7] The active substances used in traditional medical treatment are obtained from whole plants or from their parts such as seeds, roots, stem, leaves, flowers or bark (de Sousa Araújo *et al*., 2016) [3] and the fruits of the plant have their own phytochemical as they contain active compounds Despite their potential medicinal properties, different Moringa species are known worldwide for their diversity of uses. These species include Moringa longituba, Moringa drouhardii, Moringa ovalifolia, etc. (Leone *et al*., 2015) [11]. The three methods that are regarded as classic-soxolite, maceration, and hydro-distillation-that are used in the extraction or mixing process determine the temperature at which bioactive compounds are extracted. (Azmir *et al*., 2013) [1].

Therefore, the study aimed to investigate some active compounds in the alcoholic extract of the Moringa plant and to diagnose them molecularly.

Materials and Methods

Plant collection: Moringa leaves were obtained from one of the home gardens in Karbala Governorate in April 2022. The utilized plant pieces had been washed with normal water, then distilled water, dried by air., then processed in an electric mill to produce vegetable powder. Making of alcohol-based extracts For each plant sample, 50 g of dry vegetable powder was weighed. 500 ml of 70% ethyl alcohol was then combined with the 50 g of dry vegetable powder in a 1000 ml glass flask, sealed with cotton and aluminum foil, and allowed to stand at room temperature for 24 hours. The mixture was then filtered through multiple layers of medical gauze for disposal. After centrifuging the particulate matter for ten minutes at a speed of 3000 r/min, the extract was filtered using 0.1 Whatman filters. Not at all. to produce a transparent solution that may be used in gas chromatography-mass spectrometry (GC-MS) (Hernández-Pérez *et al*., 1994) [6].

Chemical and molecular properties of the studied plants Chemical properties

GC-MS technique for the qualitative and quantitative study of chemical components in plant samples use of gas chromatography-mass spectrometry to determine the active ingredient level in leaves. Identification of Active Ingredients The components were discovered by comparing the resulting spectrum of the unknown component to the known stored components in the NIST library using the National Institute of Standards and Technology (NIST) database.

Ministry of Science and Technology

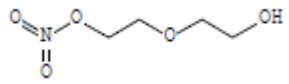
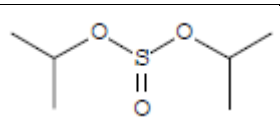
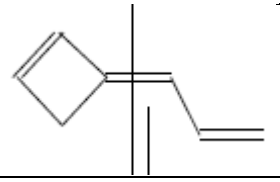
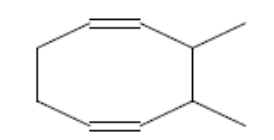
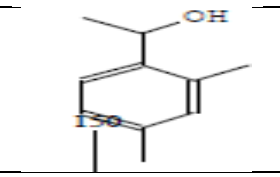
Molecular identification of Moringa plants Nitrogenous base sequence analysis of duplicated DNA.

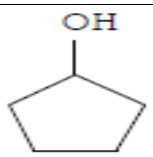
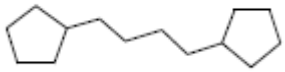
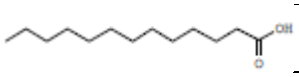
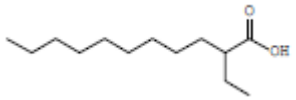
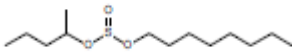
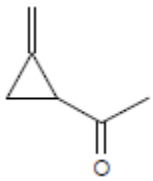
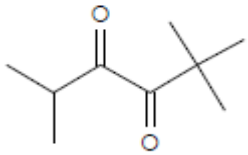
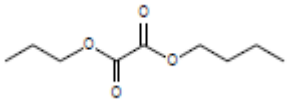
1. In order to establish the nitrogenous base sequence, the plant samples that were subjected to polymerase chain reaction multiplexing using the forward and reverse primers (ITS1 and ITS4) were sent to Macrogene, a South Korean company. This allowed for the ribosomal internal transcribed spacer (ITS) region duplicated completely, which is important for plant diagnosis.
2. The plants that had been identified were added to the Global Gene Bank.
3. The tree was identified for the plants under research using the Chromas tool to determine the similarities among the plants under study and those recorded globally. For Biotechnology Information (NCBI) National Center for Biotechnology Information in the United States and belonging to the same plants identified globally.

Results

The alcoholic extract of *Moringa oleifera* leaves contains many chemical components. Table (1) and Figure (1) list the Chemical components found in the alcoholic extraction of *Moringa oleifera* leaves. These compounds were identified by mass spectrometry and gas chromatography, which revealed that the plant contained 14 different compounds. The compound ethanol had the highest peak area in the extract at 2.035 minutes. 2-(2-hydroxyethoxy)-, 1-nitrate, and The 3.012 per minute lowest peak area was observed for Sulfurous acid, bis(1-methylethyl) ester. The compounds showed different times of appearance, and several showed up in close succession.

Table 1: GCMASS analysis of alcohol extraction of *Moringa oleifera*

Peak	Area%	R. Time	Compound name	Chemical formula	structural formula
1	90.06	2.035	Ethanol, 2-(2-hydroxyethoxy)-, 1-nitrate	$C_4H_9NO_5$	
2	0.13	3.012	Sulfurous acid, bis(1-methylethyl) ester	$C_6H_{14}O_3S$	
3	2.29	3.803	Cyclobutene, 2-propenylidene	$C7H_8$	
4	0.92	8.693	1, 5-Cyclooctadiene, 3, 4-dimethyl-	$C_{10}H_{16}$	
5	0.24	13.338	1-(2, 4-Dimethylphenyl) ethanol	$C_{10}H_{14}O$	

6	1.75	15.509	Cyclopentanol	$C_5H_{10}O$	
7	0.45	19.950	Cyclopentane, 1,1'-(1, 4-butandiyl) bis-	$C_{14}H_{26}$	
8	0.86	21.334	Tridecanoic acid	$C_{13}H_{26}O_2$	
9	0.20	21.551	Undecanoic acid, 2-ethyl-	$C_{13}H_{26}O_2$	
10	0.67	22.808	Sulfurous acid, octyl 2-pentyl ester	$C_{13}H_{28}O_3S$	
11	1.90	23.158	9, 12, 15-Octadecatrienal	$C_{18}H_{30}O$	
12	0.24	23.302	Ethanone, 1-(methylenecyclopropyl)-	C_6H_8O	
13	0.16	26.167	3, 4-Hexanedione, 2, 2, 5-trimethyl	$C_9H_{16}O_2$	
14	0.14	28.072	Oxalic acid	$C_9H_{16}O_4$	
	100.00				

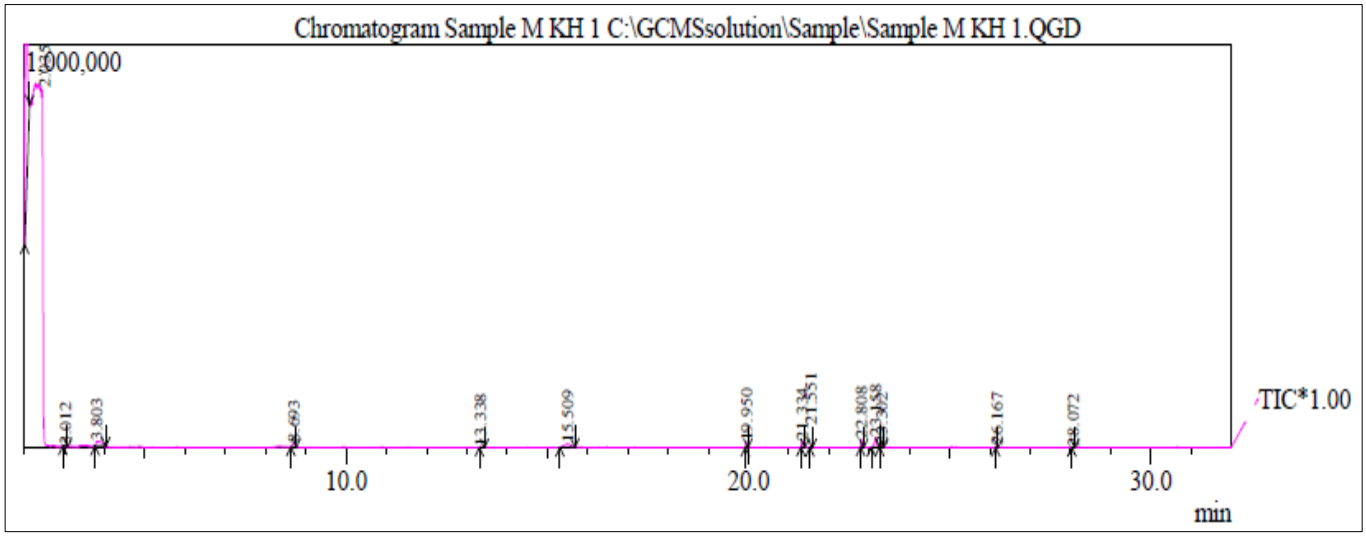
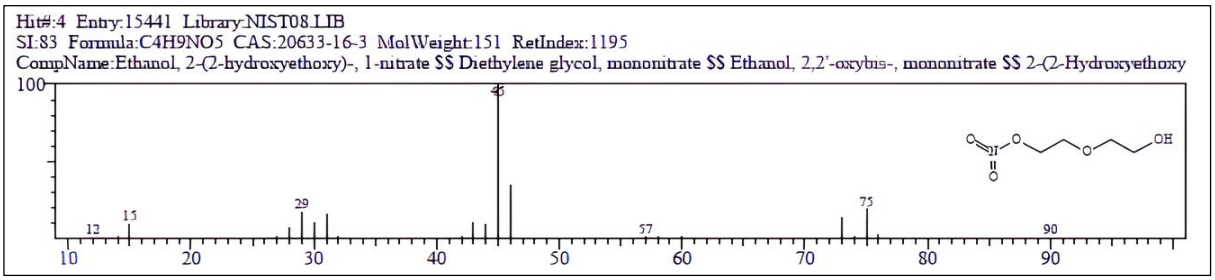
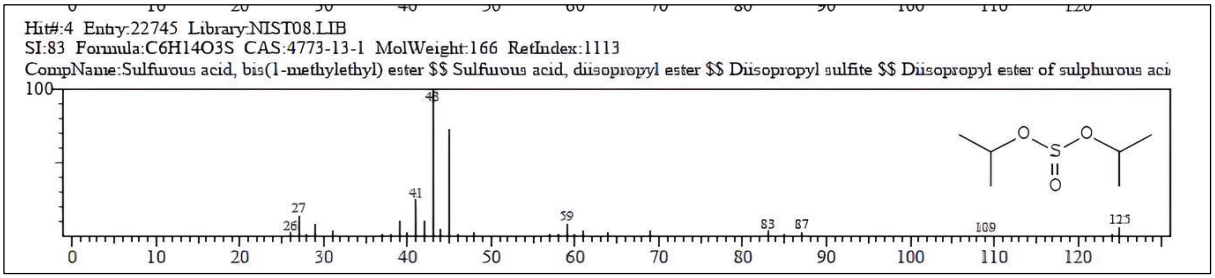
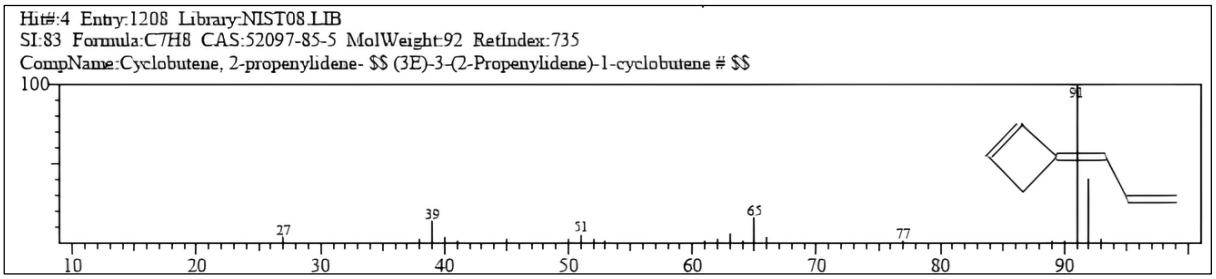


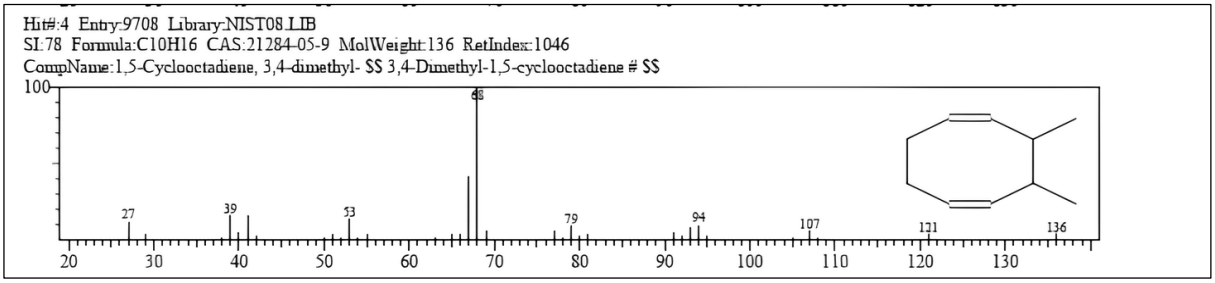
Fig 1: Shows GCMS chromatogram of ethanolic extract of *Moringa oleifera*

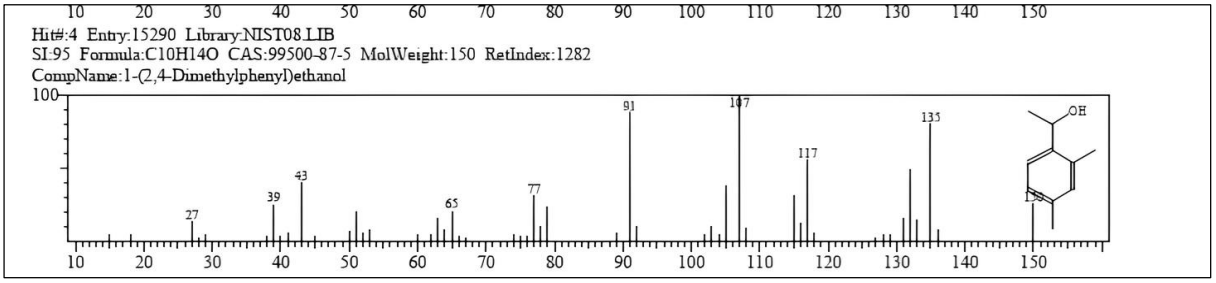
The mass spectrum of the plant components of *Moringa oleifera* was compared with those in the NIST database. Next, the 14 molecules were characterized and labeled in Figure 2.

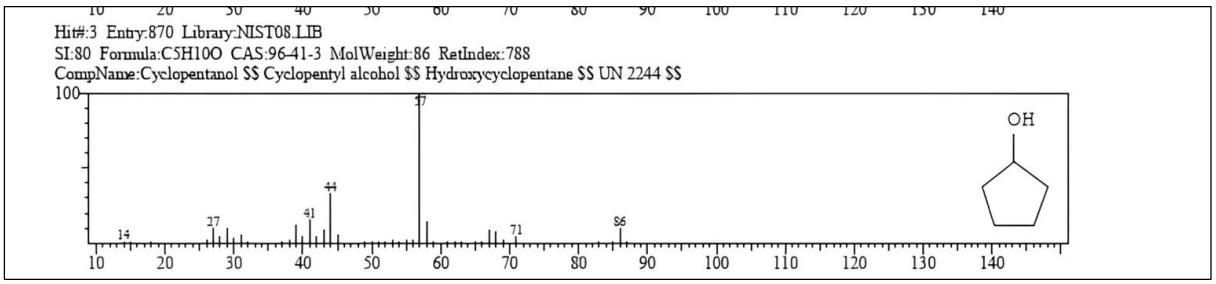

Hit#:4 Entry:15441 Library:NIST08.LIB
SI:83 Formula:C4H9NO5 CAS:20633-16-3 MolWeight:151 RetIndex:1195
CompName:Ethanol, 2-(2-hydroxyethoxy)-, 1-nitrate $$ Diethylene glycol, mononitrate $$ Ethanol, 2,2'-oxybis-, mononitrate $$ 2-(2-Hydroxyethoxy

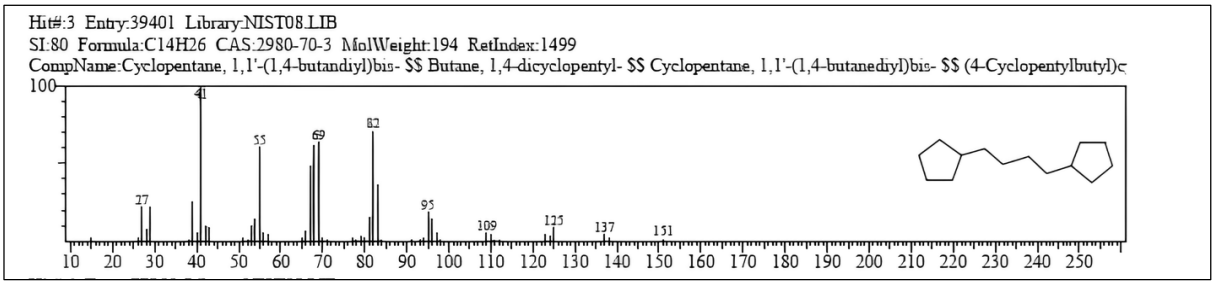

Hit#:4 Entry:22745 Library:NIST08.LIB
SI:83 Formula:C6H14O3S CAS:4773-13-1 MolWeight:166 RetIndex:1113
CompName:Sulfurous acid, bis(1-methylethyl) ester $$ Sulfurous acid, diisopropyl ester $$ Diisopropyl sulfite $$ Diisopropyl ester of sulphurous aci

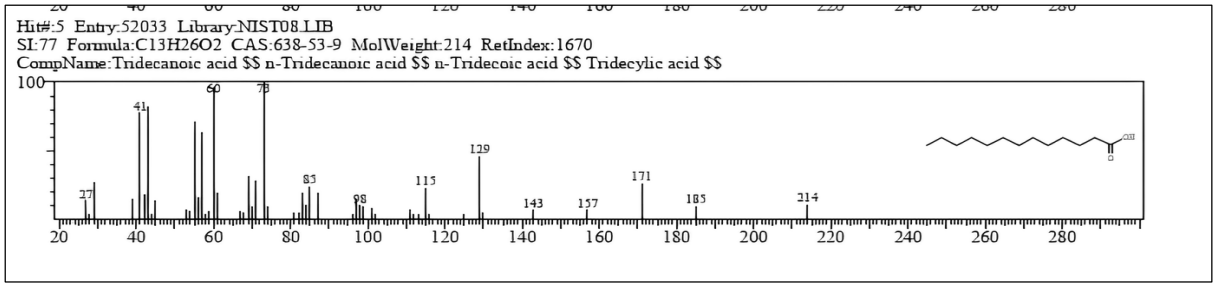

Hit#:4 Entry:1208 Library:NIST08.LIB
SI:83 Formula:C7H8 CAS:52097-85-5 MolWeight:92 RetIndex:735
CompName:Cyclobutene, 2-propenylidene- $$ (3E)-3-(2-Propenylidene)-1-cyclobutene # $$

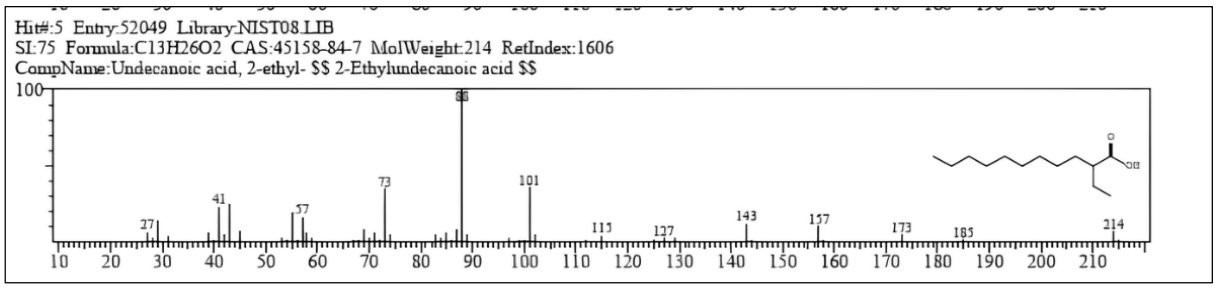

Hit#:4 Entry:9708 Library:NIST08.LIB
SI:78 Formula:C10H16 CAS:21284-05-9 MolWeight:136 RetIndex:1046
CompName:1,5-Cyclooctadiene, 3,4-dimethyl- $$ 3,4-Dimethyl-1,5-cyclooctadiene # $$

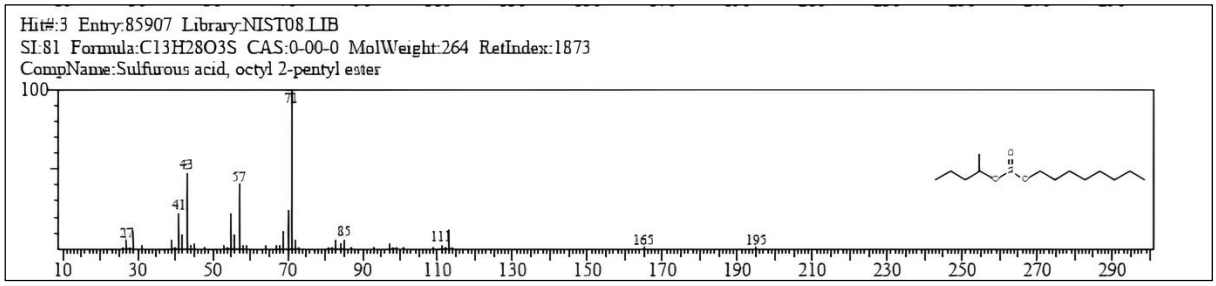

Hit#:4 Entry:15290 Library:NIST08.LIB
SI:95 Formula:C10H14O CAS:99500-87-5 MolWeight:150 RetIndex:1282
CompName:1-(2,4-Dimethylphenyl)ethanol

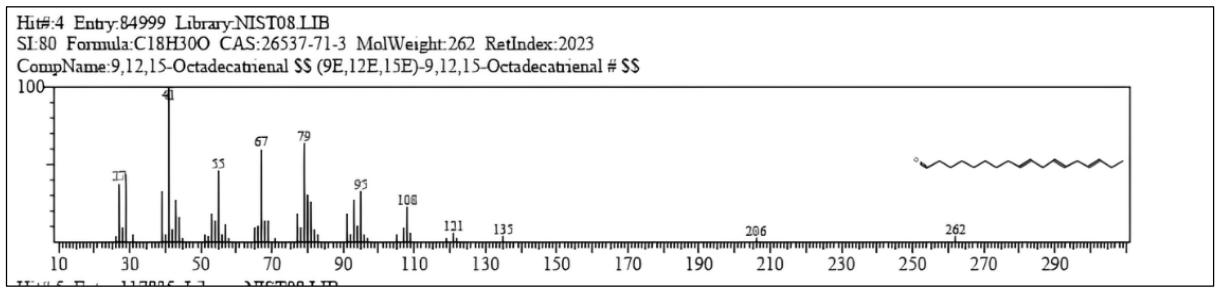

Hit#:3 Entry:870 Library:NIST08.LIB
SI:80 Formula:C5H10O CAS:96-41-3 MolWeight:86 RetIndex:788
CompName:Cyclopentanol $$ Cyclopentyl alcohol $$ Hydroxycyclopentane $$ UN 2244 $$

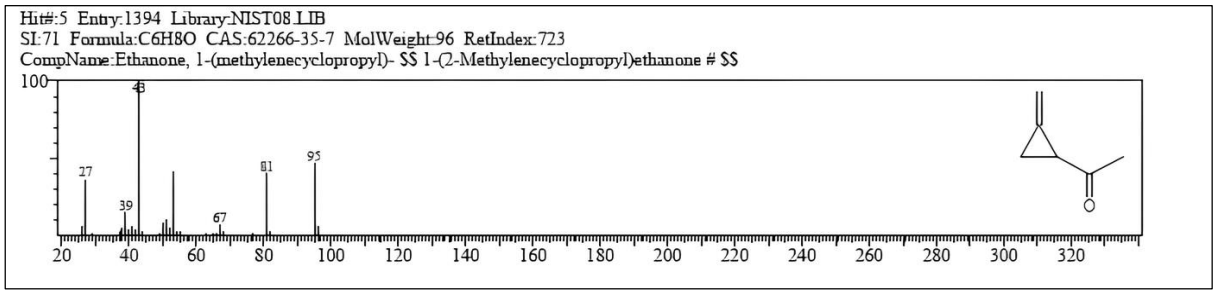

Hit#:3 Entry:39401 Library:NIST08.LIB
SI:80 Formula:C14H26 CAS:2980-70-3 MolWeight:194 RetIndex:1499
CompName:Cyclopentane, 1,1'-(1,4-butandiyl)bis- $$ Butane, 1,4-dicyclopentyl- $$ Cyclopentane, 1,1'-(1,4-butanediyl)bis- $$ (4-Cyclopentylbutyl)c


Hit#:5 Entry:52033 Library:NIST08.LIB
SI:77 Formula:C13H26O2 CAS:638-53-9 MolWeight:214 RetIndex:1670
CompName:Tridecanoic acid $$ n-Tridecanoic acid $$ n-Tridecoic acid $$ Tridecylic acid $$


Hit#:5 Entry:52049 Library:NIST08.LIB
SI:75 Formula:C13H26O2 CAS:45158-84-7 MolWeight:214 RetIndex:1606
CompName:Undecanoic acid, 2-ethyl- $$ 2-Ethylundecanoic acid $$


Hit#:3 Entry:85907 Library:NIST08.LIB
SI:81 Formula:C13H28O3S CAS:0-00-0 MolWeight:264 RetIndex:1873
CompName:Sulfurous acid, octyl 2-pentyl ester


Hit#:4 Entry:84999 Library:NIST08.LIB
SI:80 Formula:C18H30O CAS:26537-71-3 MolWeight:262 RetIndex:2023
CompName:9,12,15-Octadecatrienal $$ (9E,12E,15E)-9,12,15-Octadecatrienal # $$


Hit#:5 Entry:1394 Library:NIST08.LIB
SI:71 Formula:C6H8O CAS:62266-35-7 MolWeight:96 RetIndex:723
CompName:Ethanone, 1-(methylenecyclopropyl)- $$ 1-(2-Methylenecyclopropyl)ethanone # $$

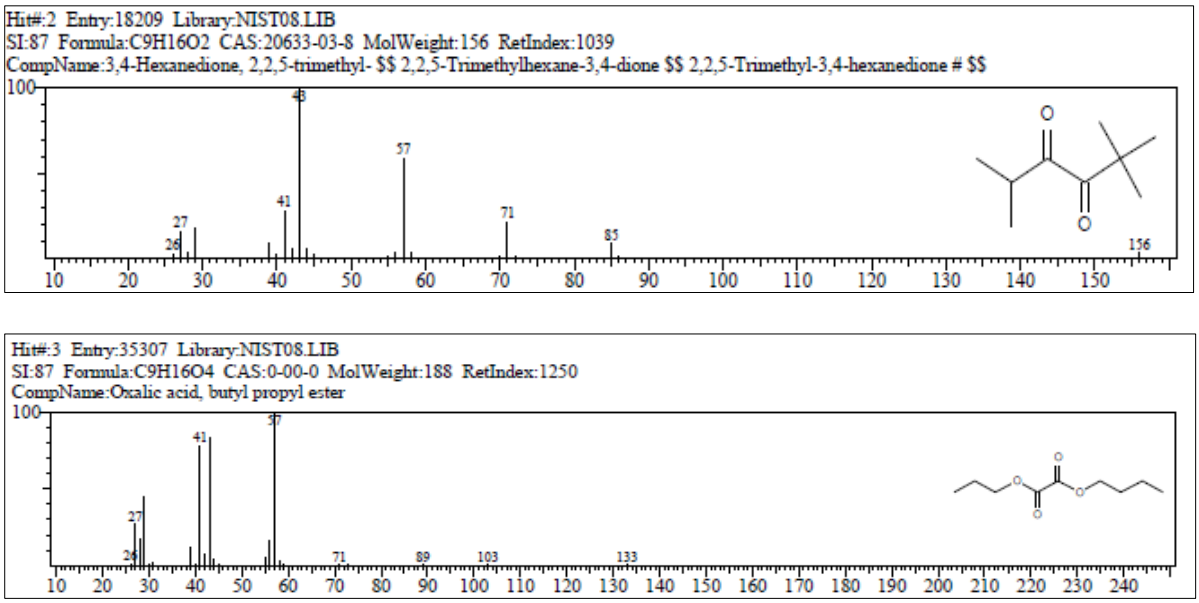


Fig 2: Shows the mass spectra of alcohol extract of *Moringa oleifera*

The results of the duplication of ITS regions between rDNA genes were shown by using forward and reverse primers of ITS1 and ITS4 primers for DNA samples extracted from the leaves of *Moringa* spp.; The replication results using the polymerase chain reaction (PCR) technique using specific primers and electrophoresis of the replication products showed the appearance of bands at approximately 700 bp compared to the DNA ladder (1000 plus), which indicates the association of the primers and the occurrence of replication as shown in Figure (3).

Figure (3) Electrophoresis of PCR products of *Moringa* spp. Using the primers ITS1 and ITS4 on a 1.5% agarose gel at 70 V for 1.5 hours, the appearance of the bands at approximately 700 bp compared with the DNA ladder (1000 plus).

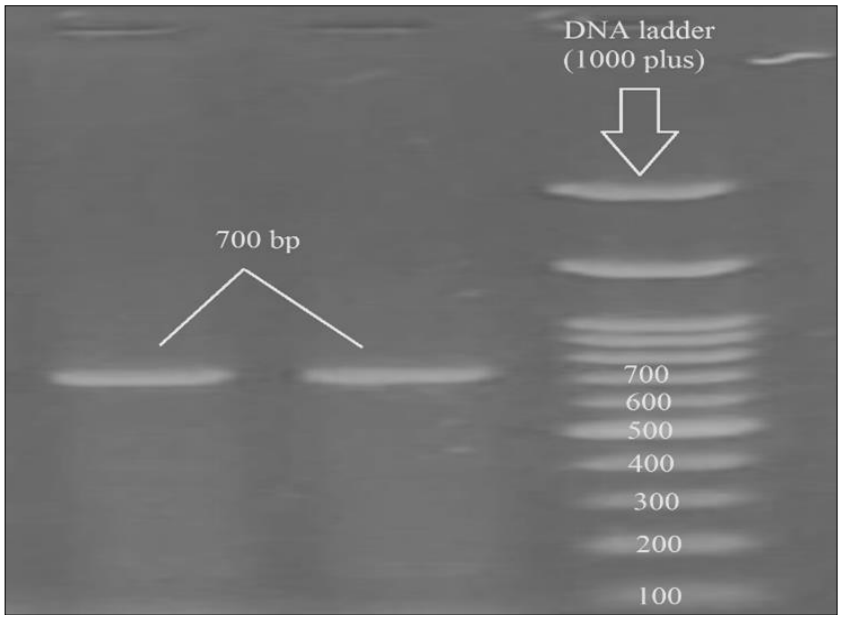


Fig 3: Electrophoresis of PCR products of *Moringa* spp. Using the primers ITS1 and ITS4 on a 1.5% agarose gel at 70 V for 1.5 hours, the appearance of the bands at approximately 700 bp compared with the DNA ladder (1000 plus).

Figure (4) also shows the results of alignment (match) between the plant sequences under study and the most closely related plant sequences *Moringa oleifera* ID: KT737750.1, and it is clear that the match rate is 99%.

Moringa oleifera isolate MO7 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence

Sequence ID: KT737750.1 Length: 687 Number of Matches: 1

Range 1: 260 to 644 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
689 bits(373)	0.0	381/385(99%)	0/385(0%)	Plus/Plus

```
Query  1    TGTGGTCTCTAAACGATGTCTAAAATGACTCTCGGCAACGGATATCTCGGCTCTCGCATC  60
Sbjct  260  ...C...C.....................................................  319

Query  61   GATGAAGAACGTAGCGAAATGCGATACTTGGTGTGAATTGCAGAATCCCGTGAACCATCG  120
Sbjct  320  .....................................................  379

Query  121  AGTTTTTGAACGCAAGTTGCGCCCGAAGCCATCAGGCCAAGGGCACGTCTGCCTGGGTGT  180
Sbjct  380  ...C.....................................................  439

Query  181  CAAGCACCGTCGccccgaccccccGCATCCCTTCGGGGAGGGGAGGGACCATCGCGGGG  240
Sbjct  440  .....................................................  499

Query  241  TGGATGTTGGCCTCCCGTGCGCCTCTGGCTCGCGGTTGGCTTAAAAAAGAGTCTCGAGCG  300
Sbjct  500  C.....................................................  559

Query  301  ACGCCGCGTCACGGCTTGGCGGTGGTAGAGAAAGCCTCGTGCTCCCTCGTGCGCGCGTCG  360
Sbjct  560  .....................................................  619

Query  361  CTCGCTCGCGGCTCACGGACCCAAC  385
Sbjct  620  .........................  644
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Fig 4: Aligns the sequence of nitrogenous bases in the BLAST program for *the Moringa oleifera* under study with the closest species registered in India under accession identification number ID: KT737750.1, with a percentage of 99%, which shows the sites of variation in the nitrogenous bases.

Figure (5) shows phylogenetic tree analysis of the *Moringa oleifera* sample, which corresponded to the Indian and Belgian samples by 99%, and the sample under study matched the samples from India by 98%, while the sample from America matched by 92% and matched the samples from Madagascar and Thailand by 83%.

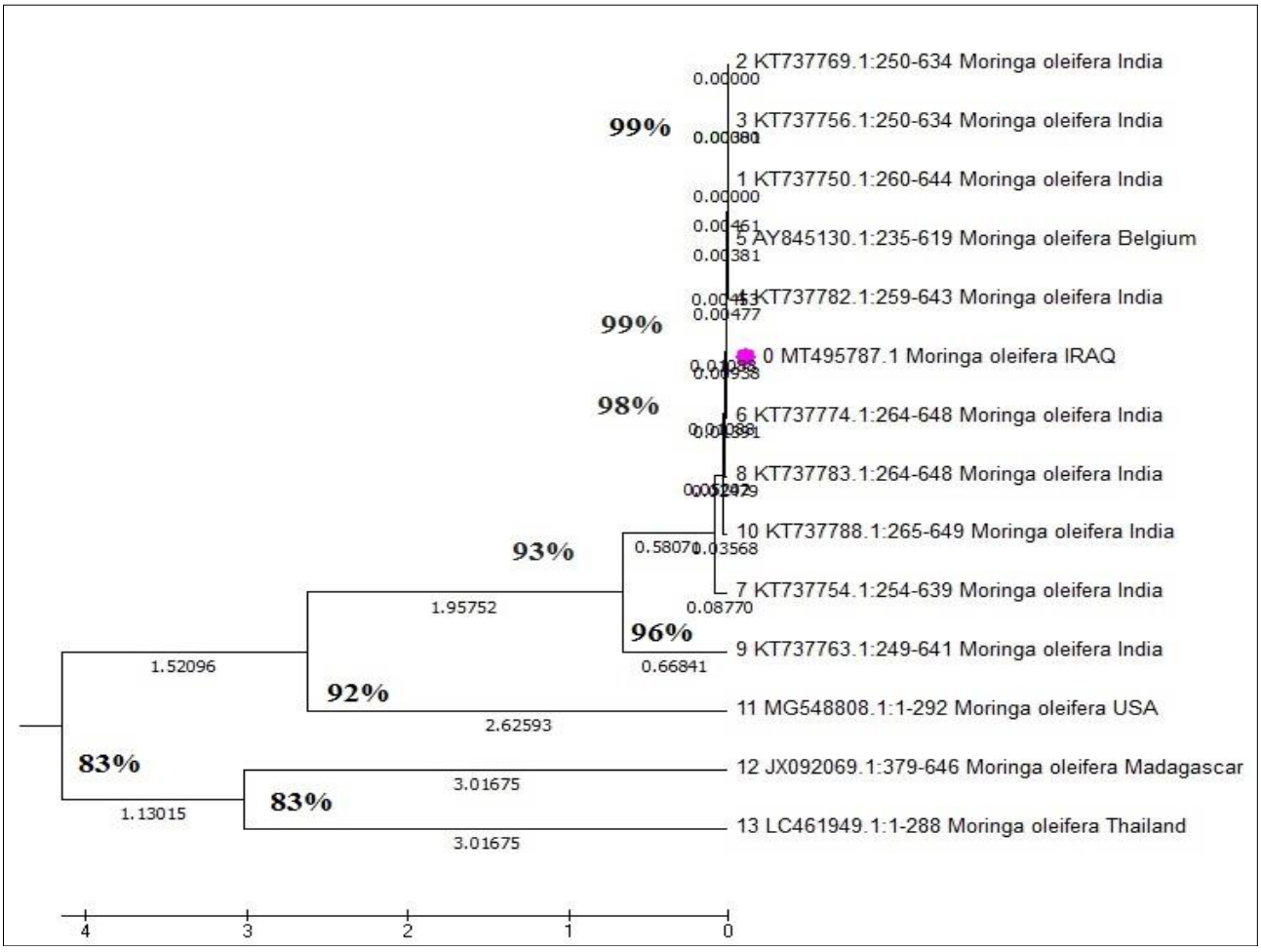

Fig 5: *Moringa oleifera* strains together in a phylogenetic tree. The bar represents the genetic distance resulting from the sequence variation.

After completing the evaluation of the sequences of the nitrogenous bases of the samples under study, these sequences were registered in the Global GenBank, and identification numbers were given to these data, as sample No. 1 of the *Moringa oleifera* plant carried the sequence MT495787.1, and the match rate was 99% with each of the

samples from India that carried the number. The sequences KT737750.1 and KT737769.1, respectively, are in GenBank. As shown in Table (2) and Figure (5), each isolate has an identification accession number.

```
Moringa oleifera clone SGY40 internal transcribed spacer 1, partial sequence;
5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2,
partial sequence
GenBank: MT495787.1

FASTA Graphics
Go to:
LOCUS       MT495787                385 bp    DNA     linear   PLN 25-MAY-2020
DEFINITION  Moringa oleifera clone SGY40 internal transcribed spacer 1, partial
            sequence; 5.8S ribosomal RNA gene, complete sequence; and internal
            transcribed spacer 2, partial sequence.
ACCESSION   MT495787
VERSION     MT495787.1
KEYWORDS    .
SOURCE      Moringa oleifera (horseradish tree)
  ORGANISM  Moringa oleifera
            Eukaryota; Viridiplantae; Streptophyta; Embryophyta; Tracheophyta;
            Spermatophyta; Magnoliopsida; eudicotyledons; Gunneridae;
            Pentapetalae; rosids; malvids; Brassicales; Moringaceae; Moringa.
REFERENCE   1  (bases 1 to 385)
  AUTHORS   Shrook,G.Y.
  TITLE     Moringa oleifera
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 385)
  AUTHORS   Shrook,G.Y.
  TITLE     Direct Submission
  JOURNAL   Submitted (20-MAY-2020) Department of Biology, University of
            Kerbala / College of Education for Pure Science, iraq, Kerbala
            00964, Iraq
COMMENT     ##Assembly-Data-START##
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                     ribosomal RNA, and internal transcribed spacer 2"
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       61 gatgaagaac gtagcgaaat gcgatacttg gtgtgaattg cagaatcccg tgaaccatcg
      121 agtttttgaa cgcaagttgc gcccgaagcc atcaggccaa gggcacgtct gcctgggtgt
      181 caagcaccgt cgcccccgac cccccgcatc ccttcgggga ggggagggac catcgcgggg
      241 tggatgttgg cctcccgtgc gcctctggct cgcggttggc ttaaaaaaga gtctcgagcg
      301 acgccgcgtc acggcttggc ggtggtagag aaagcctcgt gctccctcgt gcgcgcgtcg
      361 ctcgctcgcg gctcacggac ccaac
//
```

Fig 6: Registration of *Moringa oleifera* in the World Gene Bank

Table 2: Percentage of genetic similarity between the isolate being studied and global isolates for plants with sequence numbers that have been found and registered in the Global Gene Bank

	Accession	Country	Source	Compatibility
1.	ID:MT495787.1	IRAQ	*Moringa oleifera*	99%
2.	ID: KT737750.1	India	*Moringa oleifera*	99%
3.	ID: KT737769.1	India	*Moringa oleifera*	99%
4.	ID: KT737756.1	India	*Moringa oleifera*	99%
5.	ID: KT737782.1	India	*Moringa oleifera*	98%
6.	ID: AY845130.1	Belgium	*Moringa oleifera*	98%
7.	ID: KT737774.1	India	*Moringa oleifera*	98%
8.	ID: KT737754.1	India	*Moringa oleifera*	96%
9.	ID: KT737783.1	India	*Moringa oleifera*	95%
10.	ID: KT737763.1	India	*Moringa oleifera*	93%
11.	ID: KT737788.1	India	*Moringa oleifera*	91%
12.	ID: MG548808.1	USA	*Moringa oleifera*	98%
13.	ID: JX092069.1	Madagascar	*Moringa oleifera*	99%
14.	ID: LC461949.1	Thailand	*Moringa oleifera*	83%

GC-MS analysis revealed the existence of several active chemicals with carbonyl and hydroxyl groups. Biological activities such as antioxidant, antibacterial, and anti-inflammatory properties are known to be possessed by carbonyls and double bonds, in addition to their significance in chemoprevention. Possibly because it was the solvent used to prepare the dry samples, the ethanol compound 2-(2-hydroxyethoxy)-,1-nitrate seemed to have the biggest peak area. These chemicals' bioavailability serves as a barometer for the plants under investigation's potential for therapeutic use. While some of them exhibit a variety of pharmacological effects, the various phytochemicals discovered in plants cannot be neglected (Shakour *et al*., 2023) [17]. In a study conducted by (Mishra *et al*., 2023) [13], the presence of different plant compounds in different extracts of Moringa leaves and seeds by gas chromatography, The presence of benzoic acid, 3, 4, 5, trihydroxy (gallic acid) was detected by GC-MS and HPLC analysis. Which can be used to develop the most promising therapeutic agent to treat various diseases. This compound can be used to prepare several mixtures of folk medicine such as safe and cost-effective medicine. A study conducted by (Bhalla *et al*., 2021) [2] on Moringa plants using GC-MS and HPLC analysis revealed the presence of an important biological compound, gallic acid (3, 4, 5-trihydroxybenzoic acid), in leaf and seed extracts. From m. Oliveira. The analysis showed the presence of 11, 15, 16 compounds in different extracts (MS1, MS2, MS3) of *M. oleifera* seeds. The main components observed in the aqueous extract of *M. oleifera* leaves (ML3) are alpha-linolenic acid, glycine, N-methyl-N-allyloxycarbonyl-, heptyl ester with the highest retention time (RT-39.79) followed by the bioactive compounds obtained in Ethyl acetate extract of *M. oleifera* seeds (MS1) is acetamide, N-(3-methylphenyl)-2-phenylthio- with a retention time (RT-39.29). Phytochemicals produced by plants can serve as antibiotic, anti-inflammatory, anticancer, and antiviral alternatives to many infectious agents or serve as nutritional agents for non-infectious diseases. They can also act as antioxidants and flavoring agents.

The study included diagnosing the moringa plant and registering it in the global gene bank. It was discovered that because of the varied phenotypic and polymorphism, using conventional approaches is usually insufficient. Additionally, the polymerase chain reaction (PCR) approach based on primers was used to identify it due to the differences in environmental circumstances. For molecular diagnosis. The results showed that this plant species is the first to be registered in the global gene bank, based on the data found in the recording and the analysis of the proximity and resemblance of the registered plants. The Genomic Tree was utilized to identify the link between specific species of each genus and to identify the species, as well as phenotypic features and properties, as a means of obtaining diagnostic accuracy in addition to traditional diagnosis. (Korf and Rehm, 2013) [8] (Hao and Xiao 2015) [5]. The ITS region has found widespread application in both taxonomy and molecular diagnostics due to its direct amplifying and high variability, even among closely related species (Kress *et al*., 2005) [9]. Amplification of the ITS region of rDNA has been used for the reason of distinguishing plant species (Gao *et al*., 2010) [4]. The DNA sequence is initially checked for its nucleotide composition and is then compared to other genetic strains around the world. To achieve this, the online NCBI-BLAST-Query tool for nucleotides was used, producing accurate results and generating comparisons with international strains showing a range of 98-99% similarity. For analysis, Molecular Evolutionary Genetic Analysis (MEG) software was used. Mega is a specialized application designed for comparative analysis of gene sequences that share homology, exploring Genetic associations, and observing patterns of DNA and Evolution of proteins. In addition, besides Tools for analyzing statistical data, Mega also provides several resources for extracting sequence data from online repositories, which can then be visualized through phylogenetic trees (Kumar *et al*., 2008) [10].

Conclusion

The combined findings underscore the rich chemical diversity and genetic uniqueness of the moringa plant. The identified compounds present potential therapeutic applications, while the molecular diagnostic approach enhances our understanding of its genetic characteristics, emphasizing the importance of integrating modern analytical techniques for comprehensive plant characterization.

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