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Research Article

Chemical composition and antibacterial activity of *Azadirachta indica* seed oil from Chad

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Chad.

Abstract: This study focuses on extracting oil from the seeds of the *Azadirachta indica* species (neem oil), known in Chad as mim or neem, utilizing a cold-press extraction method. It aims to characterize the oil's chemical composition through Gas Chromatography-Mass Spectrometry (GC-MS) analysis and evaluate its antibacterial efficacy using the well diffusion and microdilution techniques. The antibacterial potential was assessed against four food-borne pathogens: *Staphylococcus aureus*, Methicillin-resistant *Staphylococcus aureus* (MRSA), *Escherichia coli*, *Salmonella* sp., and a soil bacterium, *Bacillus subtilis*. The results from the GC-MS analysis indicated a predominant presence of fatty alcohols, notably Stigmasta-3,5-diene (49.00%), a steroid alcohol, and Tetradecen-11-yn-1-ol (35.37%), a long-chain fatty alcohol. Additionally, lesser quantities of compounds such as (E, E)-7,11,15-Trimethyl-3-methylene-hexadeca-1,6,10,14-tetraene (a diterpene) at 9.68%, Squalene (a triterpene) at 1.77%, and 2,4,4-Trimethyl-3-hydroxymethyl-5a-(3-methyl-but-2-enyl)-cyclohexene (a sesquiterpene) at 4.19% were identified. The analysis showed that neem oil is rich in fatty alcohols and phytosterols, with lower terpenes and phenolic compounds. It showed no significant antibacterial activity against the tested bacteria. This suggests that from cold-press extraction, neem oil may not effectively combat food-borne pathogens and soil bacteria due to its fatty alcohol and phytosterol content, along with the bacteria's resistance. Increasing the concentration of crude oil in the antibacterial test could lead to positive results. The findings indicate a need for further research to isolate stronger antibacterial molecules in neem oil by separating its components, focusing on extraction methods and solvent polarity.

1. INTRODUCTION

Azadirachta indica A. Juss (called in the local Chadian language mim or neem or mimay) is a fast-growing tropical evergreen tree that belongs to the family of Meliaceae and is easily available in the Chadian areas and tropical and semitropical regions of Africa, Asia like India,

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Pakistan, and America, with various medicinal values (Gupta *et al.*, 2019; Pankaj *et al.*, 2011). The tree is often covered with delicate flowers in the early summer. The neem tree produces green drupes that turn golden yellow in June-August. Its flowers droop in panicles up to 25 cm long (Gupta *et al.*, 2019). Neem tree parts, including leaves, bark, fruit, flowers, oil, and gum, are believed to treat conditions like cancer, hypertension, heart disease, and diabetes (Islas *et al.*, 2020). The neem tree is a potential solution to prevent the Sahara Desert from spreading southward in Africa (Kumar & Navaratnam, 2013). For centuries, neem has been used to treat human diseases (Arulkumar *et al.*, 2019).

The neem tree's fruits and seeds are used to extract oil for medicinal applications (Abbas *et al.*, 2020), and are widely used in pharmaceuticals, agriculture, and other fields (Das *et al.*, 2021). Currently, researchers and communities are showing interest in various parts of the neem plant to extract benefits. However, among all the parts, the oil seems to be the most popularly used (Patel *et al.*, 2016). The seeds from neem fruits contained oil varying in the range of 25–45% oil load (Das *et al.*, 2020). Neem oil is also used as an insecticide, lubricant, and for treating diseases like diabetes and tuberculosis (Puri, 1999; Ragasa *et al.*, 1997). Neem oil is a medicinally and chemically complex lipid that has a wide range of pharmacological activities (Momchilova *et al.*, 2007; Someya *et al.*, 2018).

Gas chromatography-mass spectrometry (GC-MS) is an important technique that is now being used to identify bioactive chemicals both qualitatively and quantitatively in crude plant extracts in leaves and seeds (Muzahid *et al.*, 2023).

Few studies in Chad reported the chemical composition and the medicinal properties of neem seed oil even if neem is a well-known plant for the primary cure treating many diseases in Chad. This research study focused on the chemical composition of neem seed oil by GC-MS analysis, and the evaluation of its antibacterial activity against fourth food-borne pathogenic bacteria strains namely *Staphylococcus aureus* (*S. aureus*), Methicillin-resistant *Staphylococcus aureus* (MRSA), *Escherichia coli* (*E. coli*), *Salmonella* sp. and against the soil bacterium *Bacillus subtilis* (*B. subtilis*).

2. MATERIAL and METHODS

2.1. Plant Material

Neem seeds were collected from N'Djamena (Chad) in February 2023. Almond (kernels) contained in the seeds of neem was obtained after hulling the grains (Figure 1). Once obtained after shelling, the almonds are stored in a hermetically sealed box for the future cold-pressure extraction of their oil.

Figure 1. a: Neem plant and fresh seeds. b: Neem seeds dried. c: almond of seeds.

2.2. Oil Extraction Method

Neem oil from seed almonds is obtained by chemical or mechanical extraction. Chemical extraction offers an oil yield of around 100%, however, the characteristics (iron, copper, and phosphorus contents) show, among other things, that neem oil from cold pressing has a satisfactory quality (Svitlana *et al.*, 2012). The best oil yields by pressing have been obtained by several authors such as (Soetaredjo *et al.*, 2008; Svitlana *et al.*, 2012) at the temperature of 30°C and 25°C respectively.

In this study, the physicochemical properties of neem almond seed and the oil extracted from it are the Mass of pressed almonds: 0.400g, Pressure temperature: 25°C. Liquid at room temperature, Progressive solidification at 16°C, Volatility (very odorous and pungent character), PH: 2, Solubility: Insoluble in water and methanol. Soluble in hexane, Density: 0.92-0.97, Saponification index: 175-205 (Figure 2).

There are many methods to extract oil from neem seed, including the mechanical method such as hydraulic press, screw press, or cold press, and the chemical methods such as solvent extraction, steam distillation, and enzyme extraction (Çakaloğlu *et al.*, 2018). In this study, we have mainly chosen the mechanical pressing extraction method due to its high oil yield even with small quantities of well-crushed almonds and also given that this type of extraction does not affect the quality of the oil obtained according to the literature (Çakaloğlu *et al.*, 2018; Nahak & Sahu, 2011; Svitlana *et al.*, 2012; Tsimidou *et al.*, 2020). According to Liauw *et al.* (2008), in their study on the quality of neem seed oïl, they found that an increase in temperature led to a decrease in iodine value but an increase in saponification, acid, and peroxide value. This suggests that higher temperatures resulted in higher neem oil yield but lower neem oil quality.

The extraction was carried out at room temperature without thermal pretreatment of the almonds. The oil was obtained by cold extraction under vacuum pressure from the oil press machine (1500W, 110V, Oil Press Professional 2019, Spain). Simply put, the pre-treated neem seeds are loaded into the feeding hopper. With the pushing action of the rotating screw shaft in the pressing chamber of the screw oil press machine, the material is pushed forward continuously. At the same time, due to the reduction of the screw pitch of the oil press, the increase of the width of the screw thread, the diameter of the root circle gradually increased (and the space of the oil press chamber decreased with the advance of the material), the material volume was compressed and a strong extrusion pressure was generated. In this way, the oil is squeezed out from the gap of the pressing cage, and the dry materials are pressed into oil cake blocks and discharged from the end of the pressing shaft automatically. The optimal extraction pressure for whole, uncrushed almonds is approximately 30.4MPa ± 4.1 (300bar). The extraction rate is 40.1% ± 1.1. The optimal height of the pressing cage is 40 mm, with a speed of around 1MPa/s and a delay time of 353s (approximately 6min). The oil obtained is pure, however a decantation was done to make at least the base even purer. This oil should be stored dry, away from heat and light. To avoid degrading the active ingredients, it is essential not to heat it above 60°C (Figure 2).

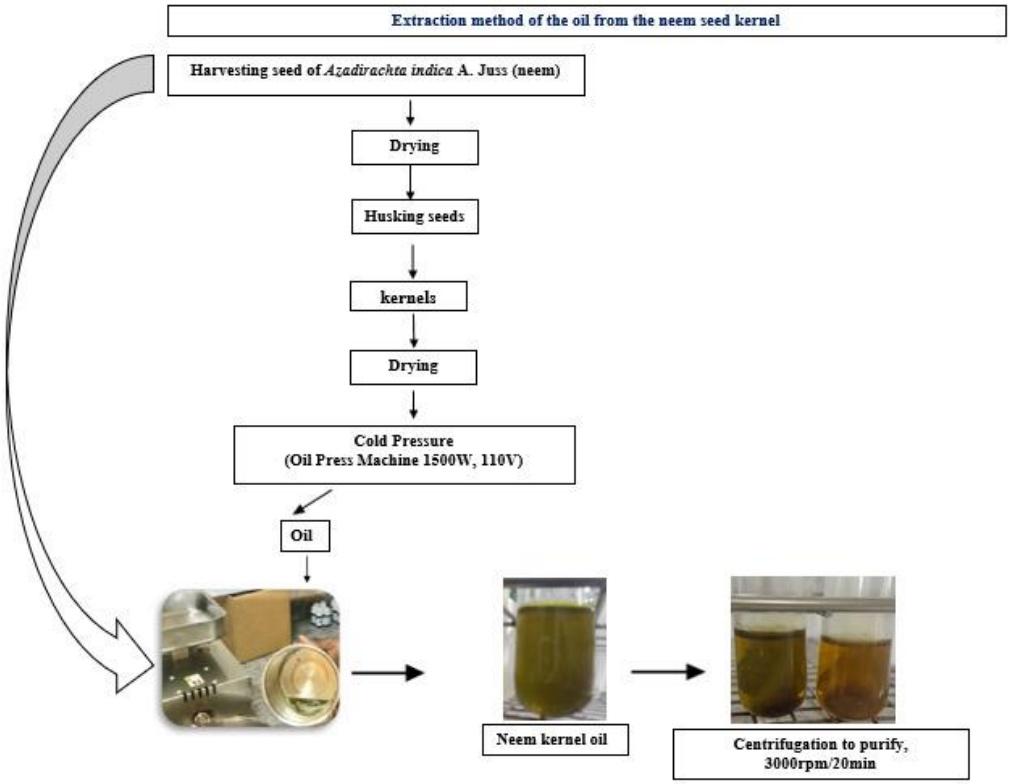


Figure 2. Process followed during the automatic cold pressure extraction of neem seed.

2.3. GC-MS Analysis

GC-MS separates chemical mixtures and identifies the components at a molecular level by heating them and carrying the gases through a column with an inert gas. The separated substances then flow into the MS (Rood, 2000; Medeiros, 2018). The analysis of the volatile fraction of the oil was carried out by a gas chromatography system coupled with mass spectroscopy type I Perkin Elmer Mass Spectrometer Clarus R SQ 8S with an auto-injector of the "AOC- 20i". The analyses are then carried out on an SLB-5ms column (30m length × 0.25mm diameter × 0.25µm film thickness). The initial temperature will be set at 50°C, then increased to 250°C as injector temperature (rate of increase: 3°C/min; holding time: 5min) (Muzahid *et al.*, 2023). The oil was solubilized in 0.5ml of n-hexane and injected after mixing with 1ml of n-hexane (split ratio: 0.5:1). The injection volume for the neem oil sample was 1µL Methylation was unnecessary as the sample was already dissolved in hexane. Hexane was used as the injection solvent to improve fraction separation, as it is a nonpolar solvent that speeds up the column process (Haleyur *et al.*, 2016). The process of pyrogenation was utilized in the GC-MS analysis. Pyrogenation, an alternative to the process of pyrolysis in GC-MS analysis, involved heating the sample to decompose and produce smaller molecules. These smaller molecules were then separated by gas chromatography and detected using mass spectrometry.

The ionization mass spectroscopic analysis was done with 70eV. Mass spectra were recorded across the range from 45*m/z* to 350*m/z* for 50.0min. The solvent cut time was 3min and the total run time was 50.0min. The bioactive compounds were identified based on retention time, MS similarity matching fragment ions generated, and an LRI filter, and the percentage of these bioactive compounds was evaluated from the total peak area (Muzahid *et al.*, 2023). Other parameters are: Linear speed: 30.0cm/s; Inlet pressure: 26.7KPa. Interface temperature: 250°C.

2.4. Antibacterial Activity

2.4.1. *Bacterial strain and growth conditions*

Five bacteria were tested: *Staphylococcus aureus* ATCC 25,923, *Methicillin-resistant Staphylococcus aureus* (MRSA), *Salmonella* sp., *Escherichia coli* K12, and *Bacillus subtilis* 6633. The bacteria are stored on an inclined agar medium (0.15) at 4°C and revived them twice

in Lysogeny Broth (LB) at 37°C for 18 to 24hours before testing. We used final inoculum concentrations of 10^6CFU/mL of bacteria as per the guidelines of the National Committee for Clinical Laboratory Standards, USA (CLSI, 2012).

2.4.2. Disc Diffusion Method

Glass cylinders were placed on a layer of Muller-Hinton agar. Lauria Bertoni medium containing 0.15% agar with bacteria. 50µL of neem almond oil was added to the cylinders. No solvent is used in order to only promote the activity of the sample. Petri dishes were incubated (37°C for 24h) and the inhibitory zone was observed (Belmehdi *et al.*, 2021; Bouhdid *et al.*, 2010; Laghmouchi *et al.*, 2018). To determine the minimum inhibitory concentration (MIC), 96-well sterile microplates were used. Initial sample 100µL of oil was diluted in wells from highest to lowest concentration, and each well received 50µL of bacterial inoculum (CLSI, 2012). Control wells had no oil sample. The microplates were then incubated for 18hours at 37°C. The MIC was determined by verifying growth with resazurin as a redox indicator. After 2hours of waiting, we found that the lowest amount of neem oil that didn't change the colour of the resazurin dye was the minimum inhibitory concentration (MIC) (Belmehdi *et al.*, 2021). The standard control antibiotic is chloramphenicol, and all tests were evaluated three times.

3. FINDINGS

3.1. GC-MS Analysis

The seed oil of neem dissolved in *n*-hexane was analyzed by GC-MS, and the results showed majority of compounds included a high quantity of long-chain fatty alcohol and phytosterol (steroid alcohol), and less quantity of terpenes (Figure 3, Table 1). Among the fatty alcohols, Stigmasta-3,5-diene (a phytosterol) was the most abundant (49.00%), followed by 13-Tetradecen-11-yn-1-ol (35.37%) (a long chain fatty alcohol).

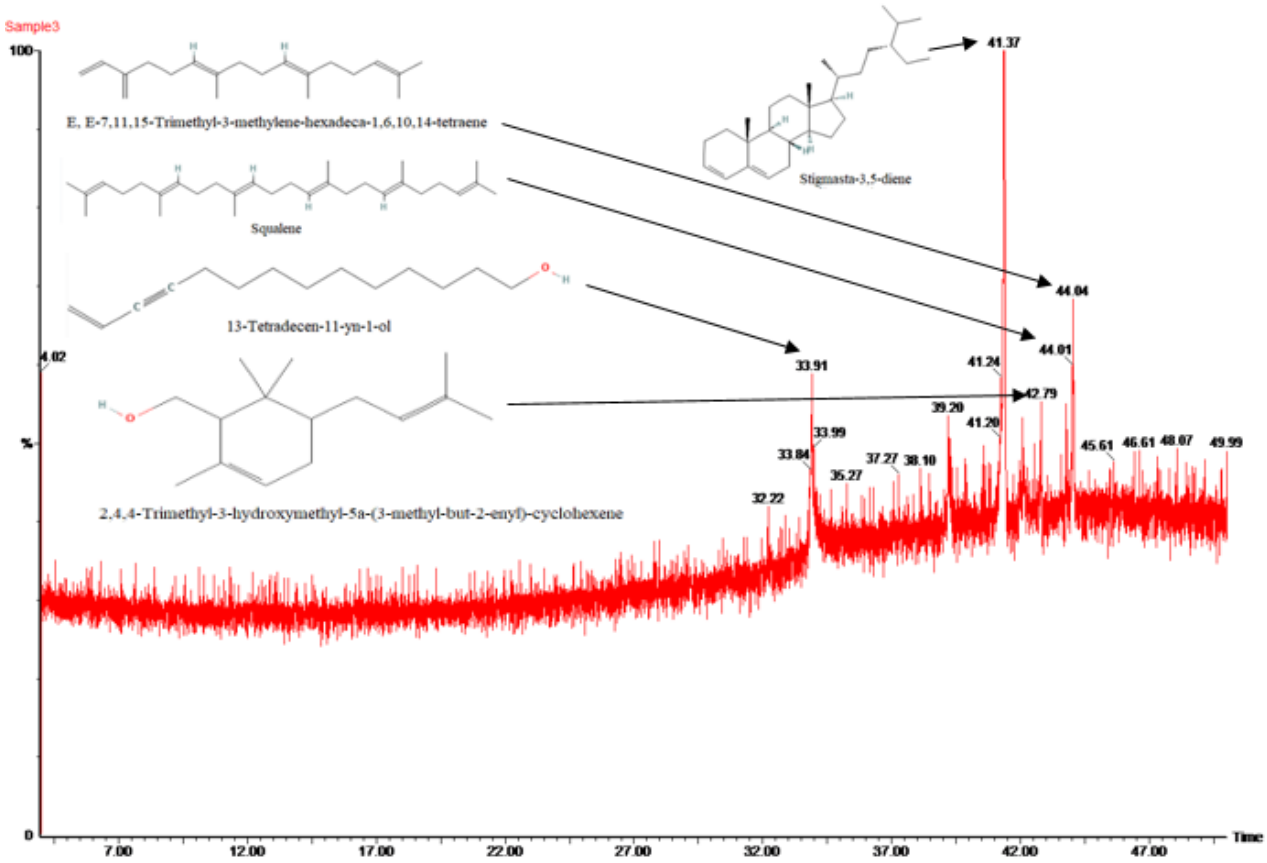


Figure 3. GC-MS chromatogram of neem seed oil.

Table 1. Showing the peak assignment of major and minor compounds detected in neem seed oil by GC-MS analysis.

Compound	RI	RT	Peak air	Area (%)	Compound	Chemical nature	Formula and Molecular weight (g/mol)	Biological Property	References of the biological property in the literature
1	-	33.91	9528973	35.37	13-Tetradecen-11-yn-1-ol	Fatty alcohol	$C_{14}H_{24}O$ (208.34)	Toxic to brine shrimp larvae	(Adedoyin *et al.*, 2013)
2	2719	41.37	13199198	49.00	Stigmasta-3,5-diene (derivate from B-Sitosterol)	Phytosterol or fatty steroid alcohol	$C_{29}H_{48}$ (396.73)	Anti-microbial	(Murniasih *et al.*, 2023)
3	-	42.79	1127420	4.19	2,4,4-Trimethyl-3-hydroxymethyl-5a-(3-methyl-but-2-enyl)-cyclohexene	Sesquiterpene	$C_{15}H_{26}O$ (222.37)	Anti-bacterial	(Karthik *et al.*, 2015) (Sharma *et al.*, 2018)
4	2832	44.01	475554,3	1.77	Squalene (Spinacene)	Triterpene	$C_{30}H_{50}$ (410.73)	Treats cardiovascular disease. As pesticide, as alternative for antivenom, as anticancer agent. As emollient, antioxidant, and for hydration and its antitumor activities	('Izzah Ibrahim *et al.*, 2020) (Tulashie *et al.*, 2021) (Farjaminezhad & Garoosi, 2020) (Fox *et al.*, 2023) (Huang *et al.*, 2009)
5	-	44.04	2606637	9.68	(E, E)-7,11,15-Trimethyl-3-methylene-hexadeca-1,6,10,14-tetraene (β-Springene)	Diterpene	$C_{20}H_{32}$ (272.5)	Antimicrobial	(Kamazeri *et al.*, 2012)
				Total: 100%					

The other compounds such as the diterpene (E, E)-7,11,15-Trimethyl-3-methylene-hexadeca-1,6,10,14-tetraene (9.68%), the sesquiterpene 2,4,4-Trimethyl-3-hydroxymethyl-5a-(3-methyl-but-2-enyl) cyclohexene (4.19%) and triterpene squalene (1.77%) were showed as the minority compounds present. The percentage of similarity ranged from 40.8 to 0.15%. The data from GC-MS chromatogram of neem oil n-hexane fraction displays the raised retention time peaks at 33.91, 41.19, 43.77, 44.04, and 44.35 representing respectively 13-Tetradecen-11-yn-1-ol, Stigmasta-3,5-diene, 2,4,4-Trimethyl-3-hydroxymethyl-5a-(3-methyl-but-2-enyl)-cyclohexene, squalene, and (E, E)-7,11,15-Trimethyl-3-methylene-hexadeca-1,6,10,14-tetraene (Figure 3).

3.2. Antibacterial Activity

The results of the disc diffusion method of neem seed oil showed no inhibition zone against the bacterial strains tested *S. aureus*, *MRSA*, *Salmonella* sp., *E. Coli*, and *B. subtilis* (Figure 4). In the MIC test, resazurin was completely reduced by bacterial metabolism (Figure 5).

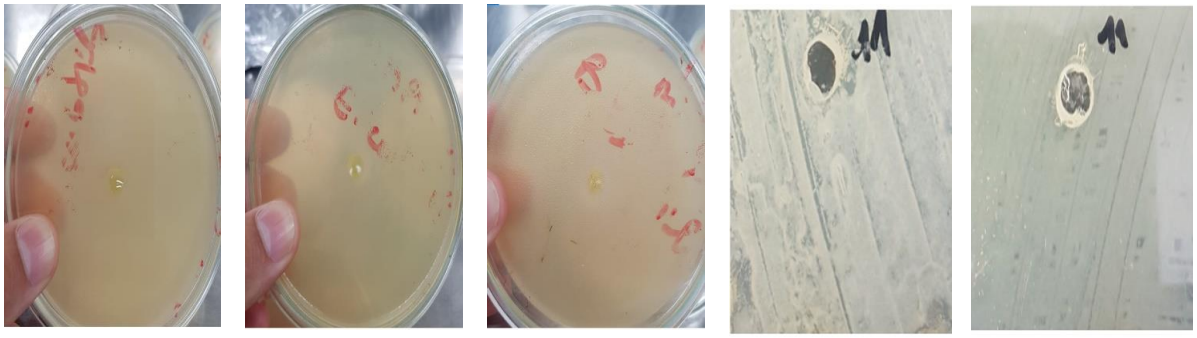


Figure 4. No antibacterial activity of neem seed oil on bacteria tested: From left to right respectively *S. aureus*, *E. coli*, *B. subtilis*, MRSA, and *Salmonella* sp.

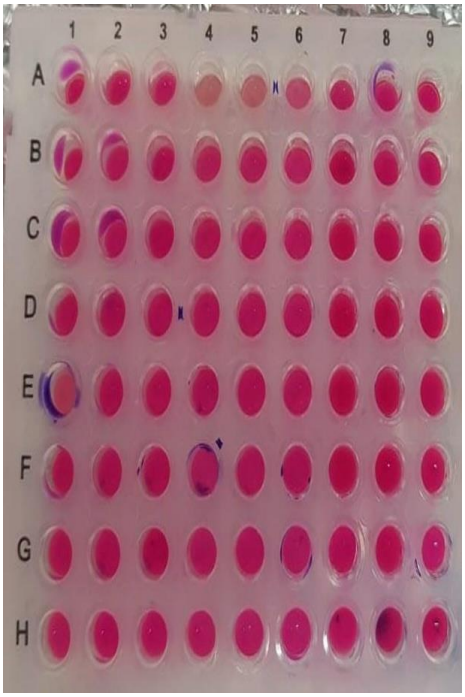


Figure 5. Resazurin dye test for determining minimum inhibitory concentration MIC (as % (v/v)) of neem seed oil. No MIC activity was revealed because resazurin was reduced here in a hot pink color.

4. DISCUSSION

This study demonstrated that neem oil obtained through pressing contains various compounds, which have been analysed through GC-MS. The mass spectra peak of the compound 13-Tetradecen-11-yn-1-ol in Figure 6 on the left is similar to peaks obtained from the National

Center for Biotechnology Information (NCBI) website data and PubChem Compound Summary for CID 543337 (National Center for Biotechnology Information (NCBI), 2024) (Figure 6, right). These peaks are shown at the m/z top peak at 55, m/z 2nd highest at 41, and m/z 3rd highest at 67 (Figure 6). The fatty acid alcohol 13-Tetradecen-11-yn-1-ol (7.83%) was identified as a major constituent of the stem essential oils from the Euphorbia heterophylla plant (Spurge Weed), extracted using the hydro distillation Clevenger method in the GC-MS analysis (Adedoyin *et al.*, 2013). The essential oil of the stem contains major compounds such as alcohols and epoxides, including 3,7,12,15-tetramethyl-2-hexadecen-1-ol, octadecanoic acid, oleic acid, linoleic acid, 1,2-epoxy-cyclododecane, cis-cis-7, 10, -hexadecadienal, 1,2-benzene dicarboxylic acid diisooctyl ester, phytol, and phthalic acid (Adedoyin *et al.*, 2013). The antimicrobial test showed moderate to low activity against *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Streptococcus pneumonia* and *Candida albicans* (Adedoyin *et al.*, 2013).

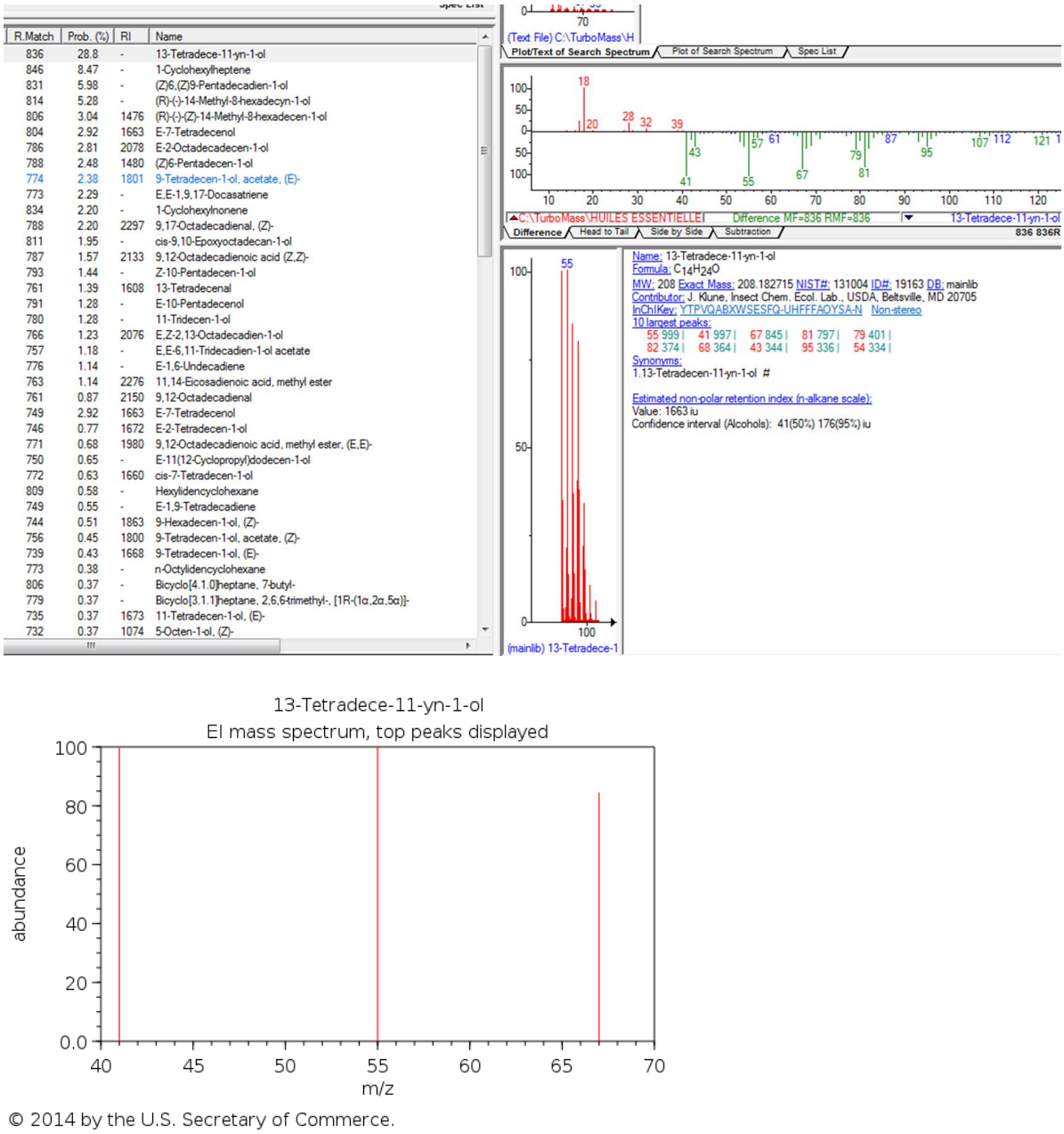


Figure 6. Mass Spectrometry GC-MS of 13-Tetradecen-11-yn-1-ol (above). The reference figure below is for the website of the National Center for Biotechnology Information website (National Center for Biotechnology Information (NCBI), 2024).

In our GC-MS analysis, the peaks of the compound Stigmasta-3,5-diene (fatty steroid alcohol) revealed in the Figure 7 in left are similar to the peaks obtained by the National Center for Biotechnology Information (National Center for Biotechnology Information (NCBI), 2024) (Figure 7, middle and the right).

The mass spectra displaying the peaks of the compound 2,4,4-Trimethyl-3-hydroxymethyl-5a-(3-methyl-but-2-enyl)-cyclohexene are depicted in Figure 8. The molecular structure indicates that the mass spectrum reveals a peak at 32.0575 Daltons. This peak can be attributed to the bombardment of the compound, which causes the fragmentation of two methyl (CH3) molecules.

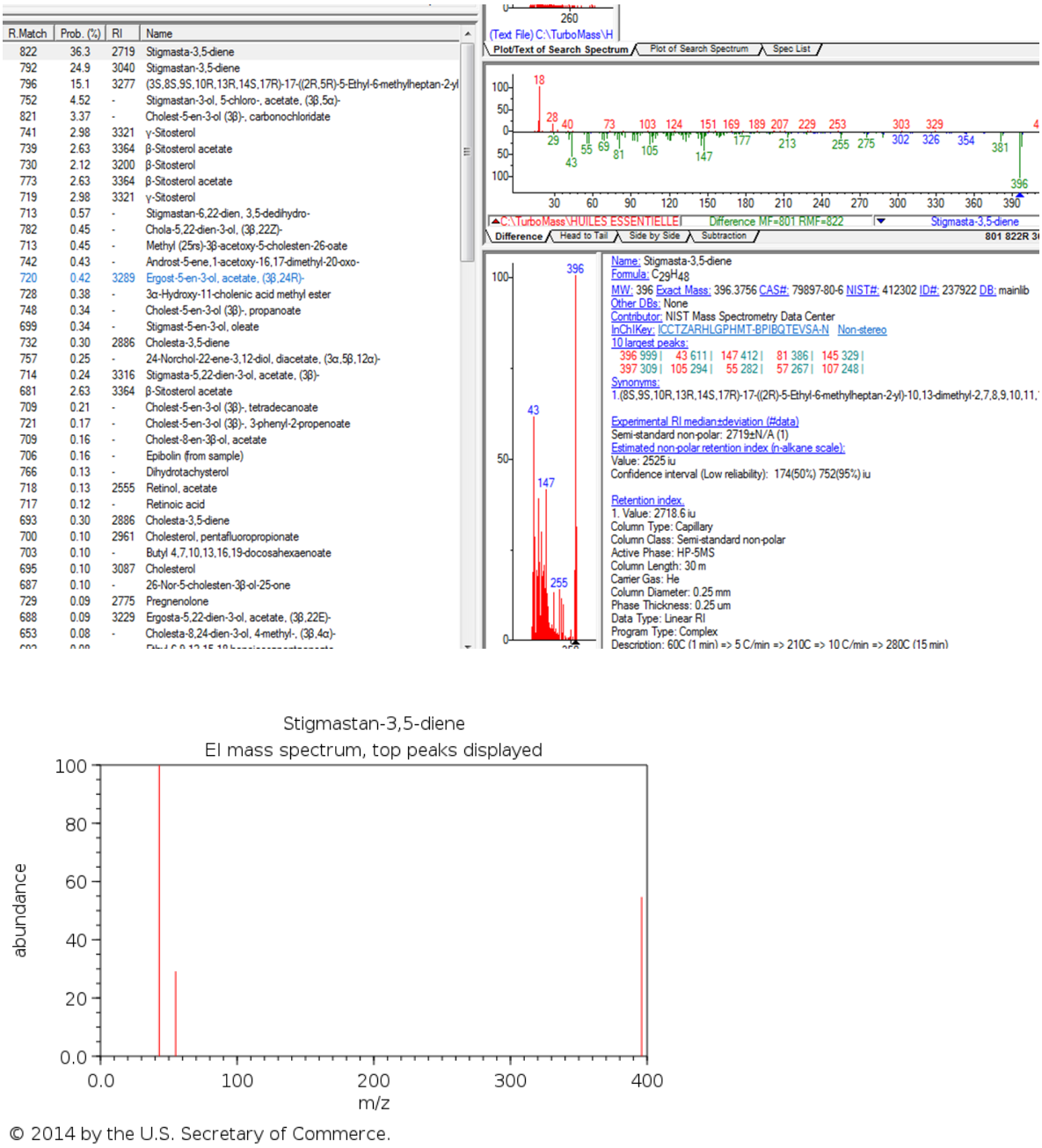


Figure 7. Mass Spectrometry GC-MS of Stigmasta-3,5-diene (above). The reference figure below is for the website of the National Center for Biotechnology Information website (National Center for Biotechnology Information (NCBI), 2024).

The peaks of the mass spectra for Squalene, as revealed by GC-MS on the left of Figure 9, are similar to the mass spectra peaks reported by the National Center for Biotechnology Information (National Center for Biotechnology Information (NCBI), 2024). The highest peak

is revealed at 69 Daltons, which is reported in our library on the left of Figure 9 and also on the right of Figure 9.

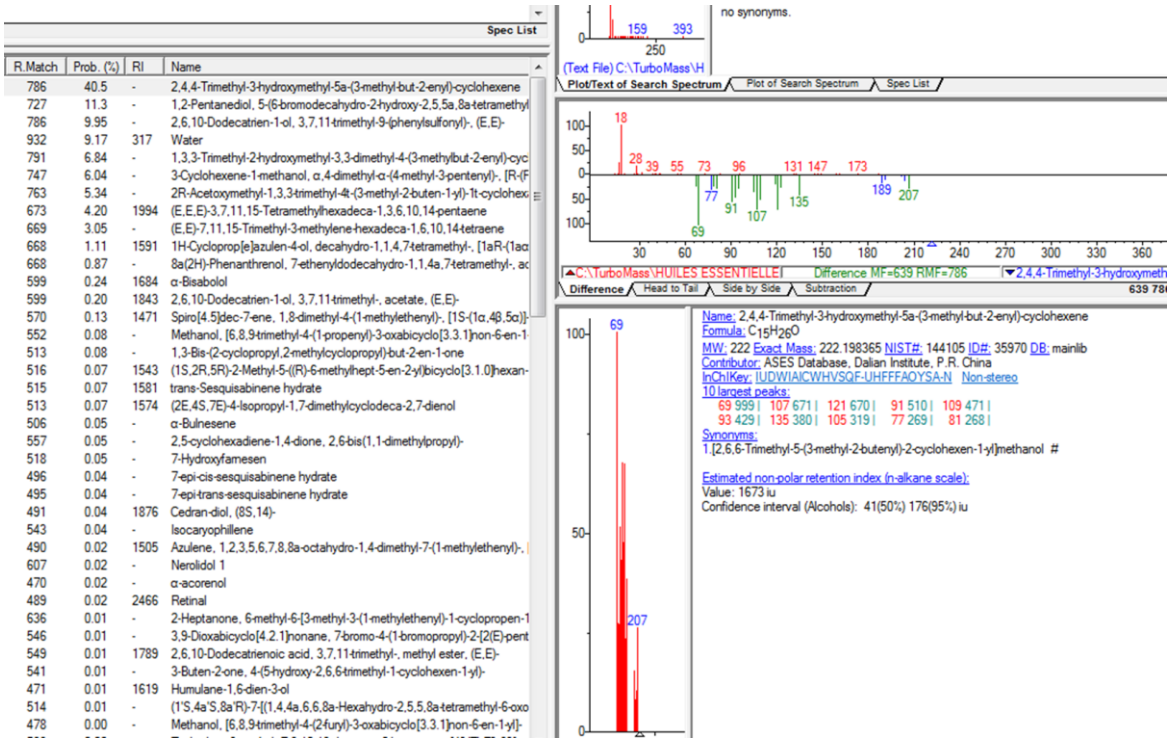


Figure 8. Mass spectrometry GC-MS of 2,4,4-Trimethyl-3-hydroxymethyl-5a-(3-methyl-but-2-enyl)-cyclohexene.

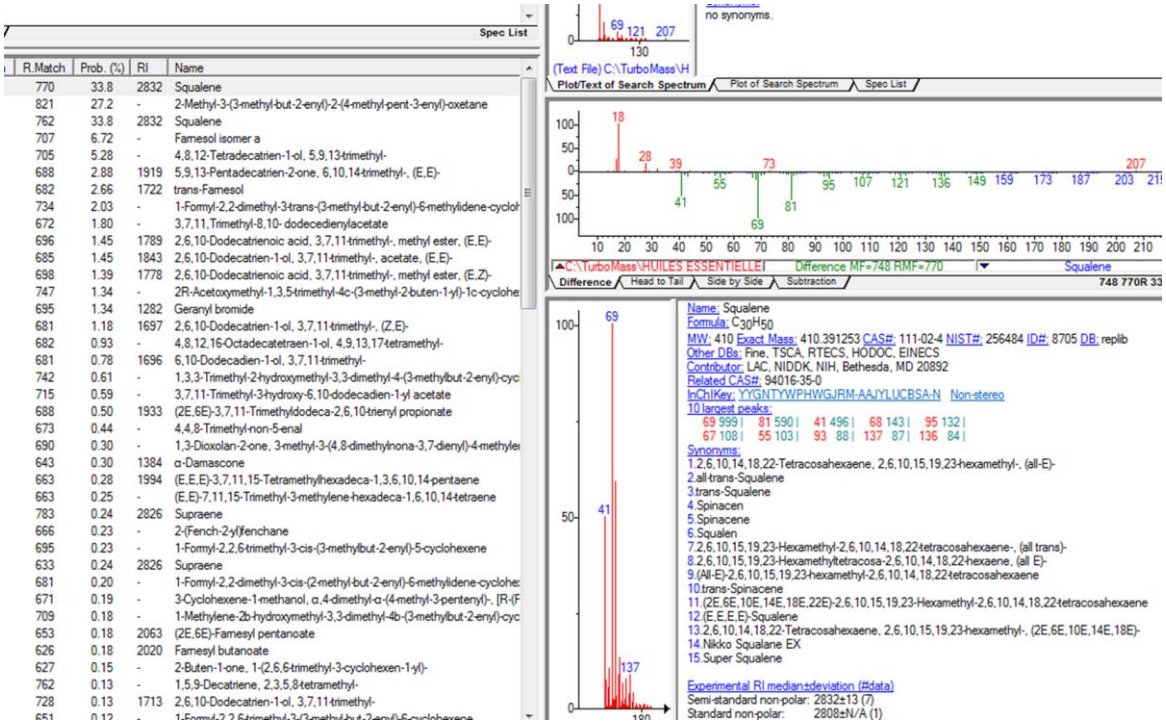


Figure 9. Mass spectrometry GC-MS of Squalene (above). The reference figure below is for the website of the National Center for Biotechnology Information website (National Center for Biotechnology Information (NCBI), 2024).

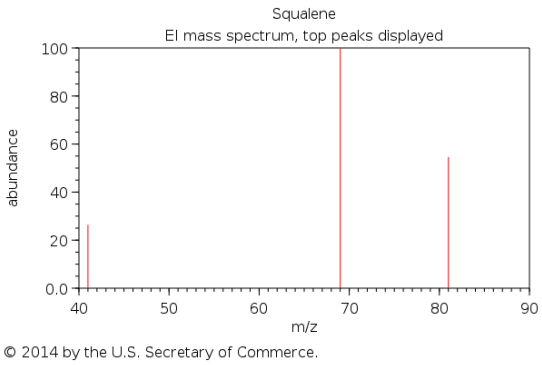
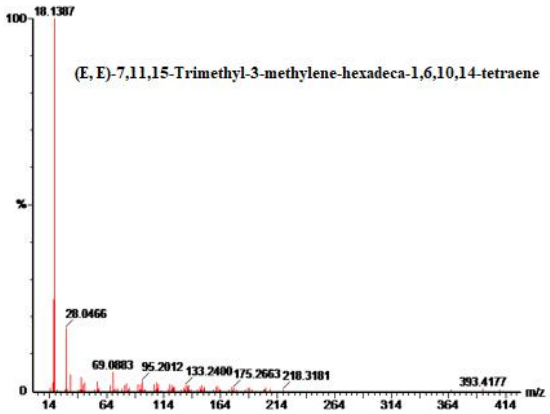


Figure 9. *Continue*.

The peaks of the (E, E)-7,11,15-Trimethyl-3-methylene-hexadeca-1,6,10,14-tetraene revealed by mass spectra GC-MS on the left of Figure 10 are similar to the peaks obtained by the National Center for Biotechnology Information shown in the right of Figure 10. The highest peak revealed at 69 Daltons is also reported by the library of the National Center for Biotechnology Information (National Center for Biotechnology Information (NCBI), 2024). The next peak at 95 Daltons is also similar to the peak reported by the National Center for Biotechnology Information (National Center for Biotechnology Information (NCBI), 2024) (Figure 10 right).

R.Match	Prob. (%)	RI	Name
744	13.9	-	(E,E)-7,11,15-Trimethyl-3-methylene-hexadeca-1,6,10,14-tetraene
702	11.2	-	5,6-Azulenedimethanol, 1,2,3,3a,8,8a-hexahydro-2,2,8-trimethyl-, (3aα,8β,8aα)-
742	8.15	1843	2,6,10-Dodecatrien-1-ol, 3,7,11-trimethyl-, acetate, (E,E)-
730	4.69	3031	1,6,10,14,18,22-Tetracosahexaen-3-ol, 2,6,10,15,19,23-hexamethyl-, (all-E)-(±)-
666	4.51	1876	Cedran-diol, (8S,14)-
728	4.34	1995	p-Camphorene
808	4.00	-	3-Cyclohexene-1-methanol, α,4-dimethyl-α-(4-methyl-3-pentenyl)-, [R-(R*,R*)]-
727	3.22	1960	m-Camphorene
637	2.85	-	1-Heptatriacotanol
674	2.74	1619	Humulane-1,6-dien-3-ol
664	2.63	-	α-Bulnesene
699	2.43	-	Isocaryophillene
631	2.33	-	2H-Pyran, 2-(7-heptadecynyloxy)tetrahydro-
647	2.15	-	α-acorenol
655	1.82	1505	Azulene, 1,2,3,5,6,7,8,8a-octahydro-1,4-dimethyl-7-(1-methylethenyl)-, [1S-(1α,7α,8aβ)]-
696	1.74	2302	2,6,10,14-Hexadecatetraen-1-ol, 3,7,11,15-tetramethyl-, acetate, (E,E,E)-
662	1.74	1492	Naphthalene, 1,2,3,5,6,7,8,8a-octahydro-1,8a-dimethyl-7-(1-methylethenyl)-, [1R-(1α,7β,8aα)]-
630	1.61	-	Butyl 6,9,12,15-octadecatetraenoate
624	1.61	-	Methyl 5,11,14,17-eicosatetraenoate
711	1.74	1492	Naphthalene, 1,2,3,5,6,7,8,8a-octahydro-1,8a-dimethyl-7-(1-methylethenyl)-, [1R-(1α,7β,8aα)]-
700	1.23	2234	(2E,6E,10E)-3,7,11,15-Tetramethylhexadeca-2,6,10,14-tetraen-1-yl formate
642	1.18	-	Pentacyclo[9.1.0.0(2,4).0(5,7).0(8,10)]dodecane, 3,3,6,6,9,9,12,12-octamethyl-, anti,anti,anti-
675	1.14	1493	1H-Cycloprop[e]azulene, 1a,2,3,5,6,7,7a,7b-octahydro-1,1,4,7-tetramethyl-, [1aR-(1aα,7α,7aβ,7bα)]-
622	1.09	-	2,3,3-Trimethyl-2-(3-methylbuta-1,3-dienyl)-6-methylenecyclohexanone
636	1.01	-	7-Oxabicyclo[4.1.0]heptane, 2,2,6-trimethyl-1-(3-methyl-1,3-butadienyl)-5-methylene-
632	0.89	-	2,6,11,15-Tetramethyl-hexadeca-2,6,8,10,14-pentaene
624	0.75	-	4,6,6-Trimethyl-2-(3-methylbuta-1,3-dienyl)-3-oxatricyclo[5.1.0.0(2,4)]octane
622	0.72	-	2H-Cyclopropa[g]benzofuran, 4,5,5a,6,6a,6b-hexahydro-4,4,6b-trimethyl-2-(1-methylethenyl)-
617	0.67	-	Ethanone, 1-(1,2,3,4,7,7a-hexahydro-1,4,4,5-tetramethyl-1,3a-ethano-3aH-inden-6-yl)-
700	0.64	1441	1H-3a,7-Methanoazulene, octahydro-1,9,9-trimethyl-4-methylene-, (1α,3aα,7α,8aβ)-
683	0.64	1635	Hinesol
618	0.59	-	Acetic acid, 3-hydroxy-6-isopropenyl-4,8a-dimethyl-1,2,3,5,6,7,8,8a-octahydronaphthalen-2-yl ester
614	0.54	-	Chola-5,22-dien-3-ol, (3β,22Z)-
627	0.50	-	Sandaracopimar-7,15-dien-6-one
628	0.48	1781	2(1H)Naphthalenone, 3,5,6,7,8,8a-hexahydro-4,8a-dimethyl-6-(1-methylethenyl)-
636	0.46	1486	Naphthalene, decahydro-4a-methyl-1-methylene-7-(1-methylethenyl)-, [4aR-(4aα,7α,8aβ)]-
614	0.46	-	Spiro[2.7]dec-4-ene, 1,1,5,6,6,9,9-heptamethyl-10-methylene-
687	0.44	1490	Bicyclo[7.2.0]undec-4-ene, 4,11,11-trimethyl-8-methylene-



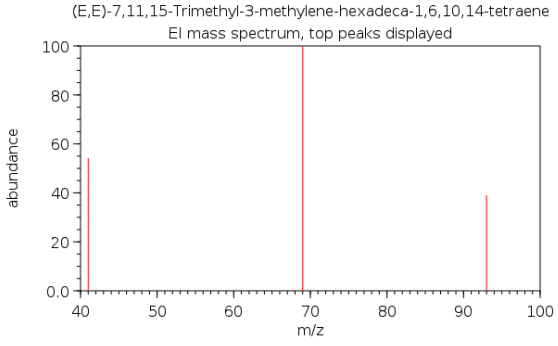


Figure 10. Mass spectrometry GC-MS of (E, E)-7,11,15-Trimethyl-3-methylene-hexadeca-1,6,10,14-tetraene (above). The reference figure below is from the website of the National Center for Biotechnology Information (National Center for Biotechnology Information (NCBI), 2024).

In our current study, GC-MS analysis detected several compounds in the neem plant that are similar to those reported in other GC-MS studies. One such compound is stigmasta-3,5-diene (see Figure 7), which is a derivative of beta-sitosterol (Babatunde *et al.*, 2019). Beta-sitosterol is a promising anticancer agent for both chemoprevention and chemotherapy (Wang *et al.*, 2023). The compound 2,4,4-trimethyl-3-hydroxymethyl-5a-(3-methyl-but-2-enyl)-cyclohexene (Figure 8) detected in our study is suggested to be the related compound of 1,3,3-trimethyl-2-hydroxymethyl-3,3-dimethyl-4-(3-methyl but-2-enyl)-cyclohexene found in oil extracts of neem obtained from steam and solvent extraction methods with two solvents, ethanol, and hexane (Babatunde *et al.*, 2019).

Squalene (Figure 9) was also detected in a study by Babatunde *et al.* (2019), which focused on the chemical composition of steam and solvent crude oil extracts of neem. As well, Tulashie *et al.* (2021) conducted a study on the characterization and assessment of the toxicity of neem extracts obtained using two different methods against fall armyworm (FAW) larvae. The study revealed that squalene is the major compound present in the neem seed oil extract, constituting 21.61048%. This compound may be responsible for the pesticide activity observed in the neem oil extract (Tulashie *et al.*, 2021).

This study is the first to report the presence of compounds 13-Tetradecen-11-yn-1-ol and (E, E)-7,11,15-Trimethyl-3-methylene-hexadeca-1,6,10,14-tetraene in neem oil obtained by cold pressing. The study conducted by Murniasih *et al.* (2023) found that the active compound Stigmasta-3,5-diene reported in antibacterial activity by Khan *et al.* (2016) was revealed in GC-MS analysis of ethyl acetate extracts of the marine invertebrate Lithistid sponge.

In a study conducted by Balasubramanian *et al.* (2014) the methanolic extract of neem leaves was obtained using a Soxhlet extractor and analysed through GC-MS. The analysis identified five major compounds in the extract: phytol, linolenic acid, homo-γ-linolenic acid, palmitic acid, and tridecylic acid. In this current study, *n*-hexane was selected as the fractional solvent for GC-MS analysis because it is inert and almost insoluble in water (~0.01 g/L). It provides excellent chromatography with split-less injections onto DB5 columns. With a boiling point of 69°C, it can take advantage of the 'solvent trapping effect' to produce good peak shapes with early/middle elutes when an initial column oven temperature of 45°C is used (Strutt, 2017). Also, hexane can produce good recoveries of low levels of semi-volatiles and often gives cleaner extracts. Peak shapes can be better than for dichloromethane with a split-less injector for some early/medium eluting compounds as you can use the 'solvent trapping effect' at a higher initial column oven temperature. Hexane is very inert and, it cannot react with analytes. However, dichloromethane for example can generate chlorine acid hydrogen (HCl) in a hot injector causing degradation and forming active sites in the injector and column (Strutt, 2017)

In this current study, regarding the diffusion disk method for antibacterial analysis, it has been observed that neem oil obtained through cold pressing is not effective in fighting bacteria due to the low concentration of active compounds, and also the GC-MS analysis showed that the oil has a high concentration of fatty alcohol and phytosterol, while antibacterial active elements such as terpenes, phenolics, saponins, tannins glycosides are present in lower quantities or are absent. Similar results were also found in the study of Cesa *et al.* (2019) when neem oil obtained by cold-pressure had weak or no activity against the bacterium *Helicobacter pylori*, and the fungi strains *Candida* spp. and *Malassezia furfur*.

The absence of antibacterial activity in our study could be also linked to the diffusion of the oil because it is hydrophobic. It also can be explained that the oil is mixed with other compounds which facilitate their diffusion, which shows a negative activity against such bacterial strains. It could also be due to the resistance of these strains. Moreover, if the concentration of crude oil used in the antibacterial test is increased, positive results could be obtained. However, it is possible to extract active ingredients with effective antibacterial properties through the fractionation of the oil based on the polarity of the solvent used to obtain an essential oil or organic extracts. The neem oil obtained from a company by cold pressing was tested for its

effectiveness against *Helicobacter pylori*. The oil was treated with diethyl ether and aqueous methanol, and then after fractioning, organic extracts were obtained. These organic extracts from the oil showed a significant ability to kill the bacteria (Blum *et al.*, 2019). The extract was tested against *H. pylori* strains, and its effectiveness was measured by its ability to inhibit (MIC) and kill bacteria (MBC). The extract's bactericidal activity was dependent on its concentration and incubation time. At 75-105 µg/mL, no bacteria were detected after 6 hours. (Blum *et al.*, 2019). Accordingly, many studies showed that neem essential oil has multiple biological properties including anti-inflammatory, antibacterial, antifungal, antipyretic, diuretic, hypoglycaemic, antiarthritic, anti-gastric ulcer, antimalarial, and spermicidal activities, and is more effective than vegetable oil (Ghosh *et al.*, 2015). In a study by Upadhyay *et al.* (2010), the antibacterial activity of neem essential oil was moderate against five Gram-positive bacteria *B. cereus*, *Lactobacillus acidophilus*, *Micrococcus luteus*, *S. aureus*, and *Streptococcus pneumoniae*; and two Gram-negative bacteria *E. coli* and *Klebsiella pneumoniae*. Neem essential oil effectively kills *L. acidophilus* and *S. pneumonia* bacteria, with a low MIC value of 0.125µL/mL against *L. acidophilus*. The minimum bactericidal concentration (MBC) value was also lowest against *L. acidophilus* and *S. pneumonia* at 0.25µL/mL. Neem oil's MIC value against *L. acidophilus* was lower than common antibiotics like tetracycline, ampicillin, and ciprofloxacin. Similarly, its MBC value against *L. acidophilus* was lower than these antibiotics. They conclude that neem essential oil could be an alternative to antibiotics for treating bacterial infections caused by *L. acidophilus* and *S. pneumonia* (Upadhyay *et al.*, 2010).

The products extracted from the seeds of neem are used in the control of pests and agricultural diseases, where they mainly act to inhibit the development of insects and mites. They contain insecticidal compounds, belonging to the group of limonoids known as meliacins or tetranortriterpenoids (azadirachtin), the principal representatives of the class of terpenoids that show insecticidal action (Schlesener *et al.*, 2013). Other findings about neem seed oil showed that it is the oil generally used with a mixture of some natural and synthetical chemical products to get good potential activity, such as insecticidal potency against pests inhibiting the development of insects and mites (Choupanian *et al.*, 2017), preserving fruit quality (da Silva *et al.*, 2020), prevention of cancer development and progression (Alzohairy, 2016), showing antipyretic and anti-inflammatory activities (Arora *et al.*, 2011; Kumar *et al.*, 2012; Naik *et al.*, 2014) and antibacterial activity against *Staphylococcus aureus* when the oil is mixed with various crosslinking agents (glyoxal/glycol, citric acid, 1,2,3,4-butane tetracarboxylic acid) (Joshi *et al.*, 2010). Hence, in a study by Choupanian *et al.* (2017), neem oil (3% azadirachtin), mixed with nanoemulsion formulations (non-ionic and ionic alkyl polyglucoside surfactant and, non-ionic and ionic polysorbate surfactant) and water significantly increased the mortality of two serious pest insect species *Sitophilus oryzae* and *Tribolium castaneum* when compared to non-formulated neem oil. They concluded that neem oil was found to be effective against *S. oryzae* and *T. castaneum* pests in stored products by using nanoemulsion formulations (Choupanian *et al.*, 2017). In another study, to maintain the quality of Guava fruit that is susceptible to attack by pests and diseases both pre-and post-harvest, the employment of techniques such as the use of neem oil-based products and chitosan (poly (1-4) 2 amino 2-deoxy p-D glucan) (biopolymers derived from polysaccharides) solution together with cold storage was used (da Silva *et al.*, 2020). Following the physical, chemical, and enzymatic analysis (such as the antioxidant analysis) of the Guava fruit during pre-harvest and storage, neem oil (0.5%) + chitosan (1%) proved to be effective in preserving and prolonging the quality of the guava fruit during storage for 8days at 24 ± 1 °C, and 16days at 10 ± 1 °C. They conclude that the application of neem oil (0.5%) + chitosan (1%) during pre-harvest in the guava fruit was efficient in preserving fruit quality at temperatures of 24 ± 1 °C and 10 ± 1 °C (da Silva *et al.*, 2020).

In the same way, Alzohairy, (2016) reported that neem and its compounds may prevent cancer by affecting various cell signalling pathways, though the exact mechanism is unclear. Polysaccharides G1A and G1B present in bark extracts have good antitumor activity (Gupta *et*

al., 2019). Neem has ingredients that help activate tumour suppressor genes and stop the activity of genes that cause cancer, like VEGF, NF-κB, and PI3K/Akt. Neem also helps activate apoptosis and slow down the NF-κB signal, and the cyclooxygenase pathway. Research shows that neem is a good way to help stop cancer and activate tumour suppressor genes (Alzohairy, 2016).

The neem seed oil contains limonoids, including nimbolide and azadirachtin (Sarkar *et al.*, 2021). Azadirachtin can trigger cell death through apoptosis and autophagy by activating the caspase cascade (Srivastava *et al.*, 2012). Especially, less p21 enhances the activation of the caspase pathway in the presence of azadirachtin (Srivastava *et al.*, 2012; Sarkar *et al.*, 2021). Neem extracts and seed oil can help protect cells by removing free radicals, reducing damage caused by ROS, normalizing lipid peroxidation, reducing cell death, and increasing the number of CD4+ and CD8+ T-cells (Sarkar *et al.*, 2021).

Earlier findings showed anti-inflammatory and antipyretic activities (Naik *et al.*, 2014) of neem seed oil, and chemo-preventive effects of leaf extract (Arora *et al.*, 2011). Neem seed oil was tested on albino rats to evaluate its pain-relieving effects. Results showed significant pain relief at 1 or 2 ml/kg doses, with a dose-dependent effect (Kumar *et al.*, 2012). The study carried out by (Naik *et al.*, 2014) showed neem seed oil reduces swelling in albino rats with hind paw oedema caused by carrageenan. Higher doses (up to 2mL/kg body weight) result in greater reduction with 53.14% inhibition in 4th hour after injection (Naik *et al.*, 2014). Results of the study of Ilango *et al.*, (2012) conclude that the treated animals with 100mg/kg dose of carbon tetrachloride extract (CTCE) of neem fruit skin and isolated ingredient azadiradione showed significant antinociceptive and anti-inflammatory activities respectively (Ilango *et al.*, 2012).

Natural products, due to their eco-friendliness and non-toxicity, hold great promise for niche applications such as medical and healthcare textiles. In the study of (Joshi *et al.*, 2010), neem seed extract was applied to the cotton fabric using different crosslinking agents, and the antibacterial activity of the finished fabrics was evaluated quantitatively against the Gram-positive bacteria *Staphylococcus aureus*. They used extracts of neem seeds (vegetable oil) when mixed with some chemicals such as cotton, glyoxal/glycol, citric acid, and 1,2,3,4-butane tetracarboxylic acid (BTCA) (crosslinking agents). The results showed significant antibacterial activity of seed extract-treated fabric with various crosslinking agents after one wash. In the experiment, the mixtures of cotton + glyoxal/glycol + seed extract (l0 %w/v), cotton + citric acid + seed extract (10% w/v), and cotton + BTCA+ seed extract (10% w/v) showed high antibacterial activity (more than 99%) against *Staphylococcus aureus* respectively with the values of 99.9, 99.0, 99.5 (%) compared to the control samples which showed less activity against the tested bacteria with values of 42.0, 13.0, 18.0 (%). They concluded that neem seed extract-treated cotton fabric exhibits effective and durable antibacterial activity even after multiple washes (Joshi *et al.*, 2010). Ethanolic extract of neem seed oil showed the highest free radical scavenging activity at 200µg/mL with 66.34 ± 0.06% (Nahak & Sahu, 2011).

5. CONCLUSION

GC-MS analysis of neem seed oil obtained by cold-pressure showed many effective compounds, among them two major and active substances first reported in the neem oil namely the phytosterol Stigmasta-3,5-diene (the derivate from B-Sitosterol) and the fatty alcohol Tetradecen-11-yn-1-ol. The antibacterial test results were negative against the food-borne pathogens *Staphylococcus aureus*, Methicillin-resistant *Staphylococcus aureus* (MRSA), *Escherichia coli*, *Salmonella* sp., and the soil bacterium, *Bacillus subtilis*. This suggests that cold-press extraction may not be effective against these strains due to the high levels of fatty alcohol and phytosterols. Resistance of these strains might also be a reason. Moreover, increasing the concentration of crude oil used in the antibacterial test could yield positive results. However, the individual compounds identified through GC-MS have potential biological activities and therapeutic uses. The study recommends further research into different extraction methods, such as steam distillation, to extract essential oil and explore its potential

medicinal applications, including its antibacterial, anti-insecticidal, anti-cancer, antimalarial, and antipathogen properties. When fractionating neem oil, it's important to consider both the extraction method, which aims to obtain essential oil, and the polarity of the extraction solvent in order to isolate components with effective biological activities. This study's results help us understand the impact of the extraction method of neem seed oil on biological activity. Additionally, the study reports that various bioactive components present in the neem plant could be used as herbal alternatives for treating various illnesses.

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Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the authors.

Authorship Contribution Statement

BMO: Investigation, Resources, Software, Formal Analysis, and Writing - original draft. **BMO** and **OB:** Materials, Method, Analysis, Interpretation. **OB** and **IB:** Visualisation, Critical Review. **SY** and **BBO**: Supervision, Visualization, Validation.

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