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SYNERGISTIC ANTIBACTERIAL EFFECT OF *MENTHA SPICATA* AND *CLADOPHORA GLOMERATA* AGAINST SOME PATHOGENIC BACTERIA

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SUMMARY

This study sought to investigate the importance of the inhibitory effect of medicinal plant extracts synergistically with algae extracts against pathogenic microbes. Herein, *in vitro* antibacterial effects of the *Mentha spicata* and *Cladophora glomerata* extracts underwent evaluation against three bacterial strains, i.e., *Escherichia coli*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus*. The antibacterial activity of each extract, tested alone and in combination, helped evaluate their synergistic antibacterial potential. The results revealed the *M. spicata* extract alone has higher antibacterial potential against all the tested bacterial strains than the *C. glomerata* extract. Both extracts in combination showed a higher synergistic antibacterial activity against all bacterial strains than those of the standard drugs gentamicin and ciprofloxacin. Gas chromatography-mass spectrometry (GC-MS) analysis authenticated the presence of different bioactive compounds, including 14 species of saturated, monounsaturated, and polyunsaturated fatty acids in the *M. spicata* extract. However, eight fatty acids were in the *C. glomerata* extract, as well as some essential oils, hydrocarbons, and sesquiterpenoids. The potent synergistic antibacterial effects of both extracts could most likely refer to the combined activity of bioactive constituents, especially palmitic, behenic, linolenic, oleic, arachidonic, 7-hexadecenoic, hexanoic, doconexent, and tetradecanoic fatty acids.

Keywords: *Mentha spicata*, *Cladophora glomerata*, fatty acids, antibacterial potential, synergistic effects

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Key findings: In the presented study, the remarkable combined antibacterial activity of the *M. spicata* and *C. glomerata* extracts revealed the synergistic effects of their bioactive compounds against the tested bacterial strains.

INTRODUCTION

The presence of secondary bioactive metabolites, such as phenolics, flavonoids, fatty acids, alkaloids, terpenes, and tannins, mostly characterizes medicinal plants. These natural constituents are well known for their high economic and pharmacological values (Znini *et al.,* 2011). Several medicinal plants, such as the *Mentha* sp*.,* thyme, flax, and cloves, contain these bioactive substances and have been beneficial for inhibiting the growth of microbes, including fungi and pathogenic bacteria (El-Kamali and El-Amir, 2010; Mahalingam *et al.,* 2011). Similarly, micro- and macro-algae also possess various value-added secondary metabolites. The marine and freshwater algae have had reports of positive health implications for humans (El-Sheekh *et al.,* 2023).

Furthermore, microalgae have become sources of peptides, fatty acids, vitamins, minerals, amino acids, and various pigments like chlorophyll and carotenoids with different applications and bioactive effects (Al-Hasso *et al.,* 2022). The marine and freshwater algae exploration for human nutrition and pharmaceutical purposes depends on their biochemical compounds (Mohamed and Saber, 2019). Reports have indicated a wide spectrum of algae-based antimicrobial effects against various microorganisms (bacteria and fungi) in past studies (Al-Hayali and Al-Katib, 2020; El-Sheekh *et al.,* 2023; Al-Hashimi *et al.,* 2024), and these effects are mainly algal species-specific and also dependent on the solvent used.

Mentha spicata is a perennial plant and widespread in the tropics and subtropics. Mint belongs to the family Lamiaceae, and it contains several bioactive compounds, which have proven antiseptic and therapeutic traits. Industrially, the *Mentha* plant has a wide use, such as in the preparation of toothpaste and soaps and as preservatives for canned foods. Given its effective therapeutic values, the active substances derived from mint have been applicable in the treatment of various diseases, including stomach and intestinal diseases, while also having effective antiseptic properties (Singh *et al*., 2015). Various studies have proven that the *Mentha* plant can protect against cancerous diseases due to its potent antioxidant compounds, as well as encompassing effective substances used as a rinse to treat mouth and throat ulcers (Motamed and Naghibi, 2010). The filamentous green macroalga *Cladophora* (family Cladophoraceae, phylum Chlorophyta) is a cosmopolitan species and usually occurs in nutrient-rich freshwater-to-brackish waters (Usha *et al.,* 2019).

Past studies authenticated the use of plants bioactive compounds in the treatment of various diseases affecting humans and to be safe from the side effects of chemical drugs, as well as their high costs. Presently, effective bioactive compounds of plant and algal origin are beneficial to treat bacterial diseases, especially resistant strains (Sultan, 2020). This study aimed to evaluate the in vitro antibacterial activities of petroleum ether extracts of the medicinal plant *Mentha spicata* and the filamentous green macroalga *Cladophora glomerata* against three human pathogenic bacterial strains: *Escherichia coli*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus*. The antibacterial activity of each medicinal plant extract proceeded in testing alone and in combination to evaluate their synergistic antibacterial potential. Analysis of the crude plant and algal extracts succeeded in characterizing their bioactive compounds.

MATERIALS AND METHODS

Plant materials

The collected *M. spicata* leaves were obtained from the gardens in Mosul City, Iraq, gathered in March. Their verification used the relevant past

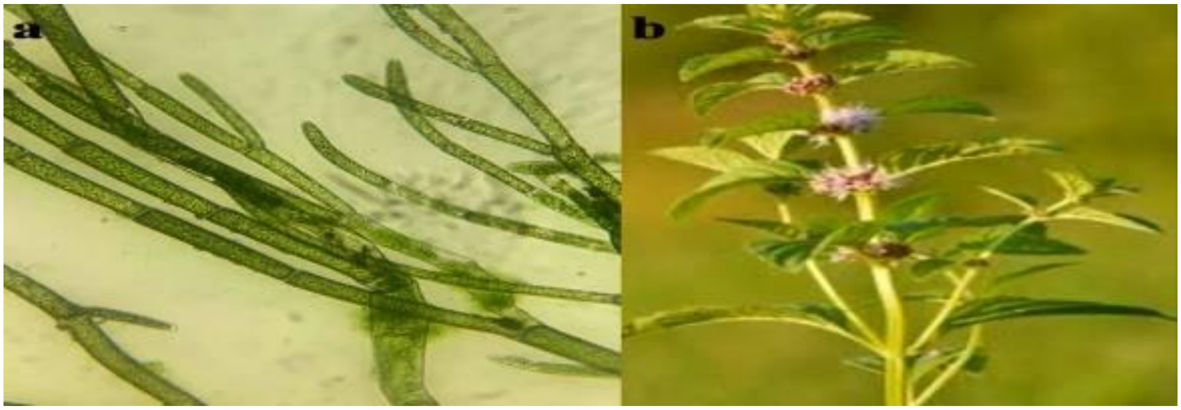


Figure 1. Morphotaxonomic details of *Cladophora glomerata* (A) and *Mentha spicata* (B).

literature on plant taxonomy (Figure 1a) (Singh *et al*., 2015).

Algal sampling and identification

The collection of *C. glomerata* specimens continued by hand and forceps in 100 ml sterile clean polyethylene terephthalate (PET) bottles from the Digla River at Mosul City, Iraq (Figure 1b). The specimens' transport as chilled in the icebox proceeded to the laboratory for further investigation. The algal specimens, cleaned well in the laboratory, used distilled water to remove debris and epiphytes. The above-mentioned step received further verification by microscopic examination. *C. glomerata* specimens obtained scrutiny for morpho-taxonomic identification, taking the light micrographs by BEL® photonics biological microscope (BEL® Engineering Co., Monza, Italy). The specimens' morphological determination utilized the relevant past literature (Škaloud *et al*., 2018).

***M. spicata* and *C. glomerata* extracts' preparation**

The preparation of petroleum ether extract used a Soxhlet following a protocol (Sultan, 2020). This chosen non-polar solvent served to separate fatty acids. The extraction process commenced at a boiling point of 40 °C–60 °C, while placing 50 g of dried *Mentha* leaves in the bag with a Soxhlet device used 250 cm^3 of the solvent. The extraction process proceeded at the rate of 20 h. The sample concentration got rid of the excess solvent using a rotary evaporator. The crude extract, once placed in sealed, opaque bottles, remained in the refrigerator until further use (Sultan *et al.,* 2020a). For the *C. glomerata* extract, 50 g of dried biomass extraction by Soxhlet apparatus also applied the aforementioned conditions and followed the protocol (Varga *et al.,* 2015).

Bacterial isolates

The strains of the bacteria *P. aeruginosa*, *E. coli,* and *S. aureus* incurred testing in this study. The obtained isolates came from the germ bank at the College of Science, University of Mosul, and underwent isolation from infected patients. The nutrient agar medium, purchased from the Oxoid Company, began preparation following the company's instructions. These comprised dissolving 23 g of the medium in 1 liter of distilled water, adjusting the pH to 7.2, and sterilizing the medium with an autoclave. The bacterial isolates used remained at the Department of Biology, College of Education for Pure Sciences, University of Mosul, Iraq.

Antibacterial screening of the *Mentha* and *Cladophora* extracts

The study followed the standard protocol described by Perez *et al.* (1990). Briefly, creating 6-mm holes in the middle of the nutrient agar media had the bacterial suspensions added for incubation for 14–16 h. The *Mentha* plant and *C. glomerata* extracts' injection inside the wells had a concentration of 400 mg/cm^3 (Asri and Sultan, 2024). The synergistic antibacterial effect of the combined extracts also succeeded in testing. The inoculated Petri dishes received incubation at 37 °C for 24 h. The standard antibacterial medications gentamicin and ciprofloxacin were the treatments because of their wide-range properties, as well as being used as positive controls. The assay ensued in triplicate, with the plates monitored to observe the presence or absence of any bacterial growth (Nascimento *et al.,* 2000) and to measure the diameters of the inhibition zones (CLSI, 2011). The obtained results' expression was mean values ± standard deviation (SD).

Extraction of fatty acids and transmethylation

The process of saponification transpired to obtain the free forms of fatty acids from both the crude algal and plant extracts. The experiment progressed according to the protocol described by Arthur (1972). In extracting fatty acids, adding the KOH (7.5) molar solution to each extract occurred before heating them up for 90 min at a temperature of 100 °C. The cooling of the mixture happened at room temperature, and then adding 100 ml of distilled water followed to become an emulsion. Afterward, placing the mixture in a separating funnel had the ether added 25 × 2 ml to remove the unsolved fat. From there, taking the aqueous solution and acid used 20% concentrated sulfuric acid to reach pH 2. The obtained fatty acids in a separating funnel used 25 × 2 ml of ether. The gathered samples attained preservation in opaque glass bottles before being kept in the refrigerator until used in the biological experiment. The separated fatty acids underwent esterification before characterization by the GC-MS to make them less polar and increase their volatility (Sultan *et al.*, 2020b). The preparation of fatty acid methyl esters attained placement in tubes tightly capped with a Teflon-lined cap and subsequently incubated at 60 °C for 30 min. The acquired upper petroleum ether layer, which contains the esterified fat, proceeded for injection into the device (AOAC, 1995).

GC-MS analysis

Fatty acid methyl esters and other phytochemical compounds gained analysis by a Shimadzu QP2010 quadrupole GC-MS equipped with a carbowax (30 m × 0.25 mm ID, 0.25 µm film thickness) in a gas chromatograph system (Agilent Technologies Co., Santa Clara, USA) at the GC-Mass Lab, College of Agriculture, University of Basrah, Iraq. The carrier gas was helium with a constant flow rate of 1 ml min^{-1}. The oven temperature, initially kept at 40 °C for 3 min, reached ramp-up at 25 °C for 1 min to 280 °C for 3 min, with 1.0 µl of each sample solution being injected. The mass spectra by electron ionization (EI) was at 70 eV. The compounds' characterization was by comparison of their mass spectra with those of the NIST mass spectral databases (Rasheed *et al*., 2024).

Data analysis

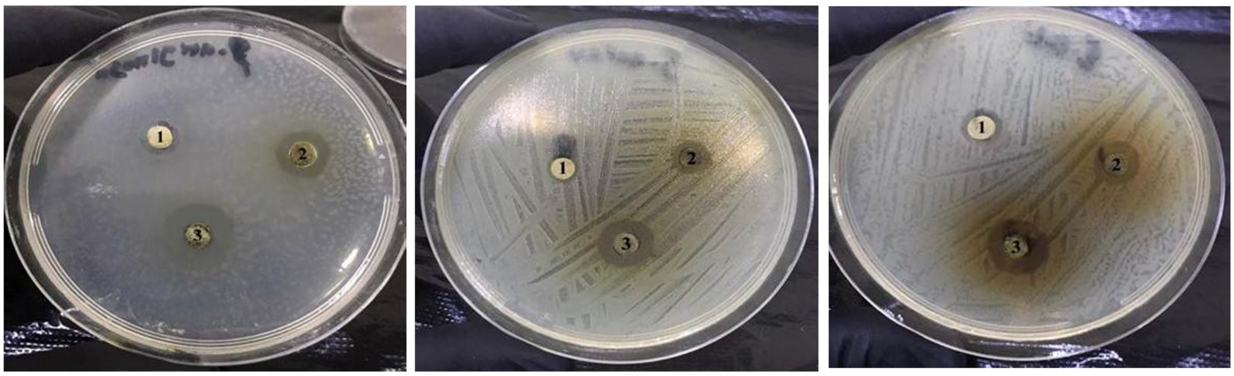
For data analysis, the use of the SAS program analyzed all the data by testing the significant differences between the mean values and using the complete random design (CRD). The means comparison and separation proceeded according to Duncan's multiple-range test (DMRT) at a significant level of $p \leq 0.01$.

RESULTS AND DISCUSSION

The *M. spicata* extract exhibited higher antibacterial potential against the three tested bacterial strains (the inhibition zones were 10–15 mm *vs.* 8.3–8.7 mm) than the *C. glomerata* extract (Table 1, Figure 2). The present observations were highly coinciding with the previous studies of Singh *et al.* (2015) and Antolak *et al.* (2018). Interestingly, the combined extracts of *Mentha* and *C. glomerata* showed a higher synergistic antibacterial activity against all the bacterial strains than those of the standard drugs gentamicin and ciprofloxacin, and the inhibition zones were

Table 1. In vitro antibacterial activities of the crude petroleum ether extracts of *Mentha spicata* and *Cladophora glomerata* (400 mg/cm^3) using the well diffusion assay.

The extracts	Average diameters of inhibition zones (mm)		
	Staphylococcus aureus	*Escherichia coli*	*Pseudomonas aeruginosa*
Mentha spicata	15±1	10±1	12±1
Cladophora glomerata	8.3±0.6	8.3±0.6	8.7±1.2
M. spicata + *C. glomerata*	14±1	12±1	14±1
Gentamicin	11.7±0.6	10.7±0.6	-
Ciprofloxacin	10.3±0.6	10.3±0.6	11.7±0.6



Pseudomonas aeruginosa ***Staphylococcus aureus*** ***Escherichia coli***

Figure 2. Susceptibility of bacterial isolates investigated in the presented study to the crude extracts of *M. spicata* and *C. glomerata* (400 mg/cm^3). A: *Pseudomonas aeruginosa*, B: *Staphylococcus aureus*, C: *Escherichia coli*. 1: *M. spicata extract,* 2: *C. glomerata* extract, 3: *M. spicata* and *C. glomerata* extracts.

12–14 mm *vs.* 10.3–11.7 mm, respectively. Past findings based on different studies considerably support well the presented results (Al-Dulayymi, 2014; Mohammed and Al-Katib, 2023).

The *M. spicata* showed distinction by the presence of higher amounts of fatty acids and some other phytochemicals and essential oils, with different degrees of saturation indices (Table 2, Figure 3). In particular, palmitic, behenic, linolenic, oleic, cis-13-octadecenoic, pentanoic, 9-eicosene, 9-decenoate, propanedioic, hexanoic, 2-nonynoic, and propenoic were the most predominant fatty acids. These results distinctly coincide with the results reported by Varga *et al.* (2015) and Buleandra *et al.* (2016). The *C. glomerata* extract also enunciated remarkable amounts of fatty acids; however, less than those found in the *M. spicata* extract. Its predominant fatty acid species were cyclopropaneoctanoic acid, 2-2-[(2-ethylcyclopropyl) methyl cyclopropyl], palmitic, linoleic, arachidonic, linolenic, 7-hexadecenoic, tetradecanoic, and doconexent fatty acids (Table 3, Figure 4).

In general, the differentiation of algae comes from their antimicrobial potential because of the several bioactive compounds of pharmaceutical activities (Al-Hayali and Al-Katib, 2020; El-Sheekh *et al.,* 2023). The different compounds secreted from the extract can contain the specific substance of bacteria, which leads to the creation of concealment devices and the leakage of devices and ribonucleic acid (RNA) from the cells. Moreover, they increase the cell membrane permeability either by creating positive charges on anions in the mouth or by the molecule

Table 2. GC–MS analysis of the *M. spicata* extract.

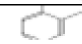
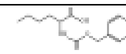
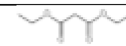
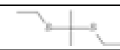
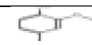
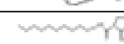
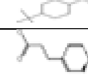
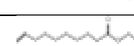
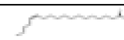
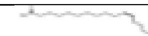
Peak	R time	Area	Area (%)	Name	Type
1	3.028	3302244	4.28	Butane-1,2,4-triol, 3-benzyloxy	
2	3.469	1045556	1.35	Bis(N)benzyldithiocarbamato) platinum (II)	
3	5.800	2230193	2.89	Benzene, 1-ethyl-2-methyl	
4	5.896	860535	1.11	Hexanoic acid, ethyl ester	
5	5.960	1063984	1.38	trans-3-Hexen-1-ol, trifluoroacetate	
6	6.075	5633703	7.29	2(3H)-Furanone, |dihydro-5-methyl-5-phenyl	
7	6.158	3227801	4.18	Pentanoic acid, 4-methyl	
8	6.378	914736	1.18	N-Benzyloxycarbonyl-dl-norleucine	
9	6.632	1776958	2.30	Propanedioic acid, diethyl ester	
10	7.879	591163	0.77	Acetic acid, phenylmethyl ester	
11	8.289	900240	1.17	Nonyl chloroformate	
12	8.431	925269	1.20	Dodecane	
13	8.742	1249915	1.62	2-Oxabicyclo [2.2.2] octan-6-ol,1,3,3-trimethyl	
14	9.267	929864	1.20	Benzofuran, 2,3-dihydro	
15	9.430	8325946	10.78	Propane, 2,2-bis(ethylthio)	
16	10.069	2937503	3.80	Benzeneacetic acid, 2-tetradecyl ester	
17	10.367	3924223	5.08	2-Methoxy-4-vinylphenol	
18	10.703	589714	0.76	2-Oxabicyclo [2.2.2] octan-6-ol,1,3,3-trimethyl	
19	10.839	692197	0.90	2-Imino-3-[n-decylthiocarbamyl] thiazolidine	
20	11.021	1736301	2.25	2-Oxabicyclo [2.2.2] octan-6-ol,1,3,3-trimethyl	
21	11.137	980516	1.27	p-tert-Butyl cyclohexyl-acetate trans	
22	11.315	1949728	2.52	Benzenepropanoic acid	
23	11.442	666053	0.86	1,4-Di-O-acetyl-2,5-di-O-methyl-3,6dideoxy-d-glucitol	
24	11.510	1232396	1.60	1-Tridecene	
25	11.629	637291	0.83	Dodecane	
26	12.006	414269	0.54	Propenoic acid, 3-(trans-3-phenyl-2-oxiranyl)-, ethyl ester (cinnamic acid, ethyl ester)	
27	13.060	2268684	2.94	Cis-1,4-dimethyladamantane	
28	13.160	841329	1.09	2(4H)-Benzofuranone, 5,6,7,7a-tetrahydro-3,6-dimethyl	
29	13.363	624440	0.81	2-Nonynoic acid, methyl ester	
30	14.063	778214	1.01	4-Methyl-2,5-dimethoxybenzaldehyde	
31	14.358	1299951	1.68	9-Eicosene, (E)	
32	14.618	1784037	2.31	1-tert-Butyl-3-(3methoxyphenyl) bicyclo[1.1.1]pentan	
33	15.594	781166	1.01	Ethyl 9-decenoate	
34	16.334	798048	1.03	Octanal, 2-(phenylmethylene)	
35	16.708	917396	1.19	2-Butanone, 4-(2,6,6-trimethyl-1-cyclohexen-1-yl)	

Table 2. (cont'd.)

Peak	R time	Area	Area (%)	Name	Type
36	16.870	1294967	1.68	9-Eicosene, (E)	
37	17.950	619582	0.80	Di-epi-. alpha. -cedrene-(I)	
38	19.023	5961482	7.72	n-hexadecanoic acid (palmitic acid)	
39	19.132	3436737	4.45	Docosanoic acid, ethyl ester (behenic acidethyl ester)	
40	20.282	1755583	2.27	1H-Indene,1-ethylideneoctahydro-7a-methyl	
41	20.783	735679	0.95	cis-13-Octadecenoic acid	
42	20.837	869589	1.13	cis-vaccenic acid (isomer of oleic acid)	
43	20.906	1400719	1.81	Ethyl 9,12,15-octadecatrienoate (linolenic acid ethyl ester)	
44	21.185	1817311	2.35	Docosanoic acid, ethyl ester (behenic acidethyl ester)	
45	24.276	508664	0.66	1,2-Benzenedicarboxylic acid, mono (2ethylhexyl) ester	

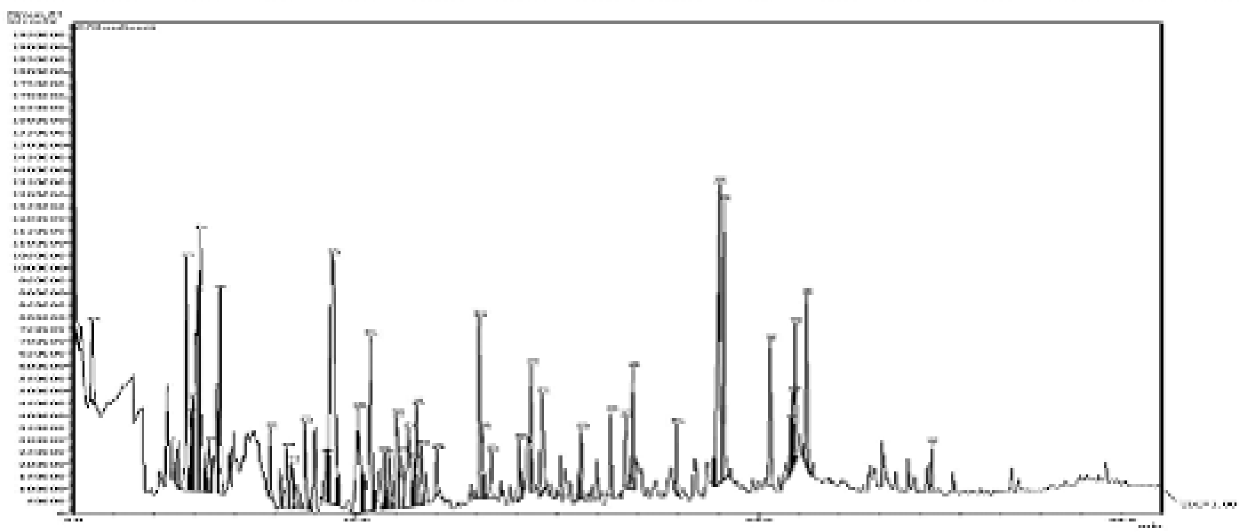


Figure 3. GC-MS chromatography of fatty acids and other phytochemicals of the petroleum ether extract of *M. spicata*.

composition or the distinctive activity that occurs inside the system, leading to the destabilization of partial sweating (Alghazeer *et al*., 2022). In the promising study, the antibacterial activity of the *C. glomerata* extract can attain authentication by the synergistic effect of its bioactive constituents, particularly the fatty acids. In line with the presented results, Peerapornpisal *et al.* (2006) highlighted that the ethanolic extract of *C. glomerata* had valuable therapeutic properties. Khalid *et al.* (2012) also reported the antibacterial activities of the methanolic extracts of *C. glomerata* and *C. okamurae* because of the 38 different fatty acids (15 saturated and 23 unsaturated species) found in both species. Supporting the present observations, Stabili *et al.* (2014) disclosed that *Cladophora rupestris* lipidic extract has remarkable antibacterial activities against *Enterococcus* sp., *Streptococcus agalactiae*, and *Vibrio cholera*, recognizing these antimicrobial effects due to fatty acids.

Table 3. GC-MS analysis (mainly fatty acids) of the *C. glomerata* extract.

No.	Peak	R time	Area	Area%	Name	Type
1	1	3.950	4693865	4.35	1-Hexanol, 2-ethyl-	
2	4	12.781	6456750	5.98	Tetradecanoic acid, 12-methyl-, methyl ester	
3	7	14.602	1777896	1.65	Doconexent(ω−3 fatty acid)	
4	9	14.777	3431917	3.18	9,12,15-Octadecatrienoic acid, methyl ester, (Z,Z,Z)- (linolenic acid methyl ester)	
5	10	14.859	5706074	5.29	7-Hexadecenoic acid, methyl ester, (Z)-	
6	12	15.112	17440529	16.16	Hexadecanoic acid, methyl ester (palmitic acid methyl ester)	
7	14	16.377	2214775	2.05	Arachidonic acid	
8	16	16.884	11186564	10.36	9,12-Octadecadienoic acid, methyl ester (linoleic acid methyl ester)	
9	17	16.961	22583093	20.92	Cyclopropaneoctanoic acid, 2-[[2-[(2-ethylcyclopropyl)methyl]cyclopropyl]	
10	18	17.061	14015979	12.99	Phytol	

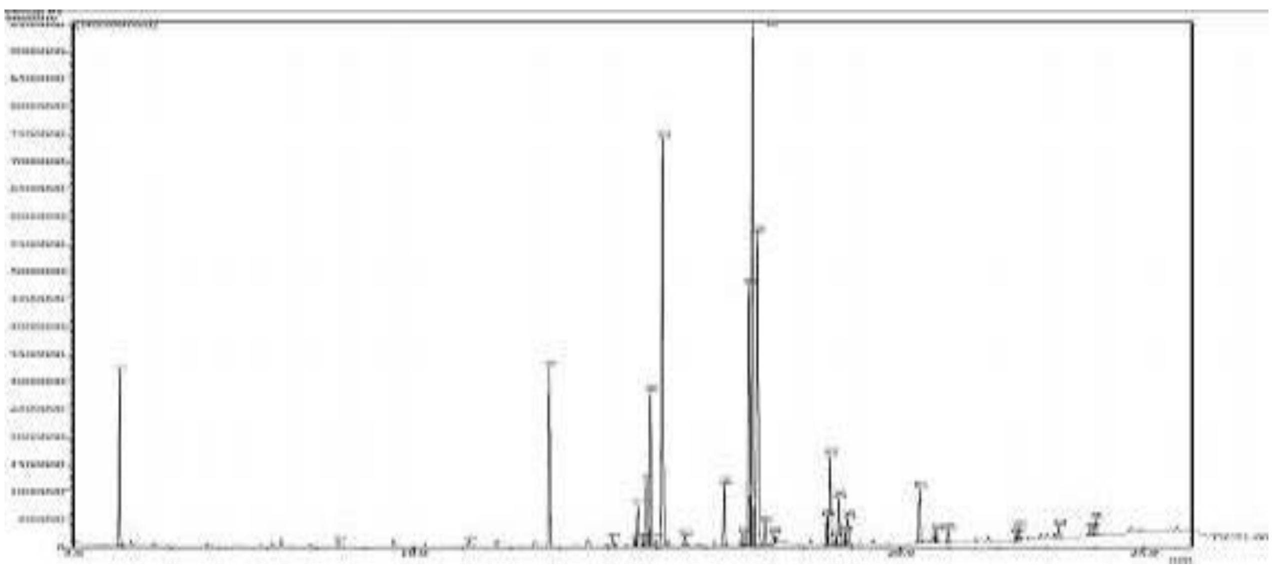


Figure 4. GC-MS chromatography of fatty acids and other phytochemicals detected in the petroleum ether extract of *C. glomerata*.

Additionally, the anti-pathogenic effects of the crude methanolic extract of *C. glomerata* showed antifungal activity against the plant pathogens *Pythium ultimum* and *Rhizoctonia solani*. This antimicrobial activity was ascribable to the combined effect of bioactive compounds, such as flavonoids, alkaloids, and phenolics (Al-Hayali and Al-Katib, 2020). Similarly, the explanation of the inhibitory antibacterial activity of *Mentha* was due to the presence of a wide range of fatty acid compounds (El-Gali and Hypa, 2018). Generally, the solvent used and the extraction process applied were largely affecting the types and amounts of the bioactive compounds by their effect on breaking down the cell wall (Messyasz *et al.,* 2018; Ali and Dwaish, 2018).

In the present study, the remarkable antibacterial activity of the combined *Mentha* and *C. glomerata* extracts exhibited the synergistic effect of their bioactive compounds against all the bacterial strains tested. These conclusions also coincide with the results obtained by Yazdani *et al.* (2011).

CONCLUSIONS

Mentha spicata fatty acids and *Cladophora* extract revealed the antibacterial effectiveness for some germs that infect humans individually. The inhibitory effect was higher when combining both extracts, except for staphylococcus, and this indicates the synergistic interaction effect of the active compounds found in both extracts.

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