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Scientific Research

Study of the Synergistic effect of *Thymus vulgaris* fatty acids and Spirogyra, against some pathogenic bacteria

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ABSTRACT

Multidrug-resistant bacteria are a growing global concern, necessitating urgent action to discover alternatives to antibiotics. The current research focused on the discovery of the identified terpene extracted from *Thymus vulgaris* (T) and *Spirogyra* spp, respectively, using the saponification process and a GC-MS device. The extracts were tested for their antibacterial activity, either alone or in combination, and compared with the activity of each extract alone. The results indicated an apparent synergetic effect when the combination of two extracts was used against *P. aeruginosa* and *E coli*. The inhibitory and synergistic activities of the separated active compounds showed inhibitory effects against the tested bacteria, promising to develop a new treatment to cope with increasingly rised multidrug-resistant bacteria by recruiting natural plant products.

1- INTRODUCTION

Multi-drug-resistant bacteria are increasingly posing a significant threat to human health [1]. Finding alternatives has recently become a research target to investigate the proposed effects extracted from natural plant products [2,3]. The active substances in medicinal plants are typically present in very low concentrations and result from the plant's secondary metabolism [4]. Examples of effective compounds include glycosides, alkaloids, fatty acids, tannins, and other groups that have been demonstrated in numerous studies to have a therapeutic impact for various diseases [5,6] . These compounds can help to eliminate free radicals, antibiotic-resistant pathogenic microbes, and fight cancer cells [4]. Many plants, scientifically and traditionally recognized for their medicinal properties, are widely used in traditional medicine, including mint, cloves, garlic, cinnamon, sage, and thyme [7,38]. Research has focused on the inhibitory activity of various effective compounds, such as alkaloids, fatty acids, phenolic compounds, and tannins, often using them in raw extracts. However, the purification of these compounds into single, pure forms remains a challenge for scientists, as the process requires advanced equipment and modern techniques [8]. One of these medically known plants is *Thymus*, commonly referred to as thyme, which is considered one of the most aromatic herbs and a perennial shrub. Highly regarded for its uses, more than many other species. It includes approximately 300 species and is found in the Mediterranean region, Asia, southern Europe, and North Africa [9]. Because of its pharmacological and biological properties, *Thyme* has been widely used in traditional medicine to treat a variety of illnesses, including coughing, bronchitis, and chest congestion, as well as it's known to have a strengthening impact on heart muscles, expand arteries, prevent atherosclerosis, ease joint pain, and address gastrointestinal disorders, high cholesterol, and renal colic [10–12].

Several recent scientific studies have proven that thyme contains phenols, including thymol at 40% and carvacrol at 15%, along with other compounds in the essential oil, such as methyl ester (2%), linalool, sinol, and simene. Pinene, by 15%, along with the compounds in the essential oil, such as methyl ester (2%), linalool, sinol, and simene. well as tannins and soaps(Al-araji et al., 2011). Additionally, certain effective compounds, such as fatty acids and phenols, showed to exhibit inhibitory activity against several bacteria and fungi [13].

Spirogyra is considered as a type of macroalgae that have high nutritional value, as it composed of proteins, carbohydrates, and fats [14]. It is also characterized by having an active compound that used as food and treatment for some common illnesses [15]. The active compounds in *Spirogyra* spp**.** include phenols and polysaccharides. Flavonoids, terpene compounds, fatty acids, and other compounds have also been identified. It has been exposed to have antioxidant activity and antimicrobial properties. [16]. In addition, *Spirogyra* spp**.** has antibacterial and antifungal properties and is indicated to be effective in treating cancers and diabetes [16–19].

2-MATERIALS AND METHODS

Plants collection and classification:

The leaves of *Thymus* plant were collected from Mosul city gardens and its verified based on the sources of plant taxonomy (**Figure 1a**) [20].

Collection of Spirogyra:

Samples of *Algal* were collected in spring season from the area of banks of Tigris River in Mosul Forest area, Iraq (**Figure 1b**). Large samples were kept and stored in plastic bags and then transported to the laboratory. The biomass was then removed with tap water and dried to facilitate storage. Morphological identification of this species was based on taxonomic references [21].

A. *Thymus* Plant

B. Spirogyra alga

Picture (1): *Alga* and Plant under microscope

Preparation of crude plant extracts using a Soxhlet:

The petroleum extract was prepared according to the method mentioned by the researcher using a Soxhlet device. A non-polar solvent, petroleum ether, was used to separate the fatty acids [22] . The separation was carried out at a temperature between 40 and 60°C, and 50 g of dried Thymus leaves were placed in the bag with the Soxhlet device, using 250 cm^3 of solvent. The separation process was repeated for 20 hours, and the extraction continued until the solvent became ineffective. The excess solvent from the sample was removing the using a rotary evaporator; then the prepared crude extracts were kept and stored in sealed, opaque bottles and in refrigerator until later use (Harborne, 1984; Najm and Sultan, 2023).

Preparing Spirogyra:

After collecting, identifying, and storing the algae in the laboratory, they are dried at 45°C in a thermal oven, then ground and mashed. Subsequently, 10 grams of the mass is dried before applying the Soxhlet method with the non-polar solvent petroleum ether at 60°C for 20 hrs., until the solvent becomes colorless (Verawaty et al., 2017).

Separation of fatty acids from the Thymus plant and the Spirogyra alga

The process of saponification was carried out to obtain the free form of fatty acids from the leaves of *Thymus* . The methods were conducted depending on (Arthur, 1972). Separation of fatty compounds from the crude extract achieved as follows: 5 g of the extract was taken then mixed with 100 ml of KOH solution (7.5 molar). The

mixture was heated for 90 minutes at 100°C. Subsequently, the mixture was afterword cooled at room temperature, after that, 100 ml of distilled water was added to form an emulsion.

The next step was placing the solution in a separating funnel. 25×2 ml of ether was added to remove the unsolved fat. Then, aqueous solution was combined with concentrated (20%) sulfuric acid to achieve a ph of (2). Afterward, the fatty acids were gained in a separating funnel using 25×2 ml of ether. The samples were kept and stored at 4°C until use. The samples were esterified prior analyzed via the GC-MS device to reduce the polarity and enhanced their volatility [22,23].

GC-MS fatty acids identification:

The separation of the fatty acids of *Mintha* and *Spirogyra* algae was conducted from the petroleum ether extract using a quadrupole gas chromatograph with mass spectrometry (GC-MS). This device was equipped from Shimadzu QP2010, with a Carbowax and a DB-MS 5 capillary column with a diameter of 30 m × 0.25 mm ID. The column temperature was set at 40°C, the injection temperature was approximately 280°C, and the pressure was 49.5 KPa. The GS-MS-QP2010 ultra programme was utilized, with the ion source temperature upheld at 200°C. The Machin was set at 0.97 kv, Start Time: 3 min, End Time: 26 min, ACQ Mode- Scan Event Time: 0.5 sec, Scan Speed: 1250 [26]. A 49.5 Kpa constant pressure was implicated to separate the fatty acid methyl ester, and the peaks were recognized via comparing the mass-spectra with the reference database. The NISI Library 2008 was used to designate the active substances present in the research samples [27].

Antimicrobial Efficacy: Antimicrobial aspect

Nutrient agar prepared from Oxoid and nutrient broth from Hi Media were made according to the companies' instructions for cultivating bacteria and adjusting the pH, and were sterilized under optimal conditions of the medium.

- **The antibiotics used are under study**

In this study, two antibiotics served as a control sample, prepared by the Turkish company Biqan Analyse Selted-TURKEY: Gentamicin (CN) 10 µg/tablet and Ciprofloxacin (CIP) 10 µg/tablet.

- **Sensitivity test (diffusion)**

The method described in [28] was adopted in the sensitivity test (diffusion by drilling), as a 6 mm diameter central holes were shaped in the nutrient agar, and the bacterial suspension was prepared in the nutrient broth after14-16 hours of incubation. The dishes were then incubated at 37°C for 24 hours. The physiological saline diluted bacterial suspension was compared with the standard control tube containing the MacFarland solution, which equals $10^{\wedge 8}$ cells/cm³, then 0.1 cm³ of the diluted bacterial suspension was transferred to the medium being studied. (CLSI, 2011).

The prepared solution were Incubated at 37°C for 30 minutes, the solution was applied homogeneously on the surface of the medium using a sterile cotton swab. Then pits were filled with the addition of 100 microns of the active compounds under study at concentrations of (400, 200, 100) mg/cm³ in triplicate. The sample was then incubated at 37°C for 14-16 hours. The inhibition area was estimated using a graduated ruler [30]. The results were noted based on public health laboratories that follow the tests prescribed by the World Health Organization. And it was

compared with selected antibiotics under study as a bactericidal comparison sample. Bacterial sensitivity to fatty acids extracted from *Thymus* was examined in the presence of the algae extract used in this study. The sensitivity of bacteria to active compounds obtained from thyme was assessed alongside the involved algae in the research [31]. 0.1 cm^3 of the diluted bacterial suspension, as previously described, was transferred into the Nutrient Agar medium and it was spread uniformly across the surface using a sterile cotton swab. Then incubated for 30 minutes at 37 °C. During this retro, the fatty acids from *Thymus* extract were combined with the algae extracts in equal amounts at a concentration of 400 mg/cm^3.

After this step, three holes are created in a Petri dish which placed the fatty acid extract of the Thymus plant alone; the extract of one of the algae under study; the combine the two extracts at a concentration of 400 mg/cm^3. All samples were placed in one dish in triplicate and incubated at 37 °C for 16 to 14 hours. The inhibition zone was measured using a graduated ruler.

Statistical data analysis:

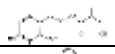
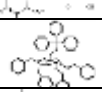
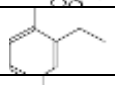
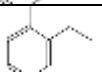
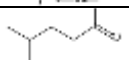
Statistical analysis program SAS was utilized to analyses the data and testing the significant differences between the means, employing the complete random design system (CRD). The comparison of the means was showed using Duncan's multiple-range test at a significance level of $p \leq 0.01$.

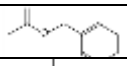
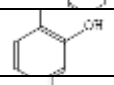
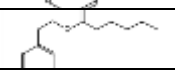
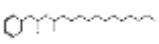
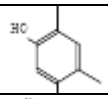
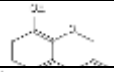
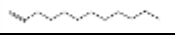
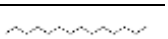
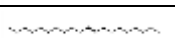
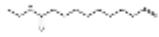
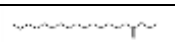
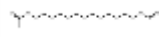
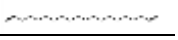
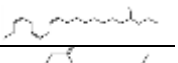
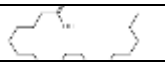
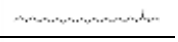
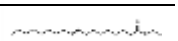
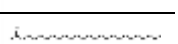
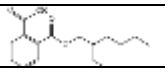
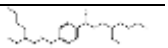
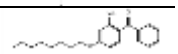
RESULTS AND DISCUSSION

Diagnosis of some fatty acids using Gas Chromatography Mass Spectrometry:

The GC-MS device was employed to analyses the separated fatty acids from the petroleum ether extract of *Thymus* leaves. Analysis curves and the concentrations of each fatty acid in the plant were obtained. Table 1 presents the fatty acids, their retention times, the percentages of each compound, and their presence within the plant. The diagnostic results revealed that Thyme is rich in fatty acids, both saturated and unsaturated, although most are unsaturated. The highest fatty acid identified was Acetic acid, phenylmethyl ester, with a concentration of 90% and a retention time of 7.882, alongside the other active compounds [32,33].

Table 1, Fatty acids of *Thyme* by GC-MS

Peak	R time	Area	Area%	Name	Type
1	1.19	1205777	3.028	Benserazide	
2	3.157	1865395	1.85	Benzyl 2,3,4-tribenzyl-trityl.beta.-d glucopyranoside	
3	5.349	1553994	1.54	3Benzene, 1-ethyl-2-methyl	
4	5.619	445369	0.44	Benzene, 1-ethyl-2-methyl	
5	5.808	4956195	4.90	Cyclotetrasiloxane, octamethyl	
6	6.075	4404455	4.36	Pentanoic acid, 4-methyl	

7	6.856	552154	0.55	Benzene, 1-methyl-4(1methylethenyl)	
8	7.882	727666	0.72	Acetic acid, phenylmethyl ester	
9	9.258	1371679	1.36	1,2-Benzenediol	
10	9.385	563706	0.56	Hexanoic acid, 2-phenylethyl ester	
11	10.061	1605983	1.59	Benzeneacetic acid, 2-tetradecyl ester	
12	10.313	21924431	21.69	Phenol, 2-ethyl-4,5-dimethyl	
13	10.984	1082245	1.07	Phenol, 2-methoxy-3-(2-propenyl)	
14	11.283	1767373	1.75	4-Nitrosophenyl-.beta.-phenylpropionate	
15	11.509	1625560	1.61	1-Dodecene	
16	11.629	451671	0.45	Tetradecane	
17	14.230	2423077	2.40	Phenol, 4-methoxy-2,3,6-trimethyl	
18	14.360	3075575	3.04	9-Eicosene, (E)	
19	16.871	2262594	2.24	9-Eicosene, (E)	
20	17.851	683848	0.68	10-Undecenoic acid, ethyl ester	
21	18.737	879058	0.87	Phthalic acid, butyl tetradecyl ester	
22	19.048	9812395	9.71	n-Hexadecanoic acid	
23	19.139	7743442	7.66	Hexadecanoic acid, ethyl ester	
24	19.235	4511935	4.46	9-Octadecenoic acid, ethyl ester	
25	19.401	1324229	1.31	1,13-Tridecanediol, diacetate	
26	20.285	500140	0.49	1H-Indene, 1-ethylideneoctahydro-7a-methyl-, cis	
27	20.846	3677590	3.64	1,19-Eicosadiene	
28	20.908	3824848	3.78	Ethyl 9,12,15-octadecatrienoate	
29	21.044	1657150	1.64	Octadecanoic acid	
30	21.190	4365396	4.32	Docosanoic acid, ethyl ester	
31	21.278	1646785	1.63	(E)-9-Octadecenoic acid ethyl ester	
32	21.342	626246	0.62	Tricosyl acetate	
33	23.069	599212	0.59	1-Octacosanol	
34	23.159	1607127	1.59	(E)-9-Octadecenoic acid ethyl ester	
35	24.277	1148015	1.14	1,2-Benzenedicarboxylic acid, mono(2-ethylhexyl) ester	
36	25.279	1077908	1.07	Z-14-Octadecen-1-ol acetate	
37	26.023	532371	0.53	Terephthalic acid, di(2-ethylhexyl) ester	
38	26.266	1014539	1.00	Octabenzone	

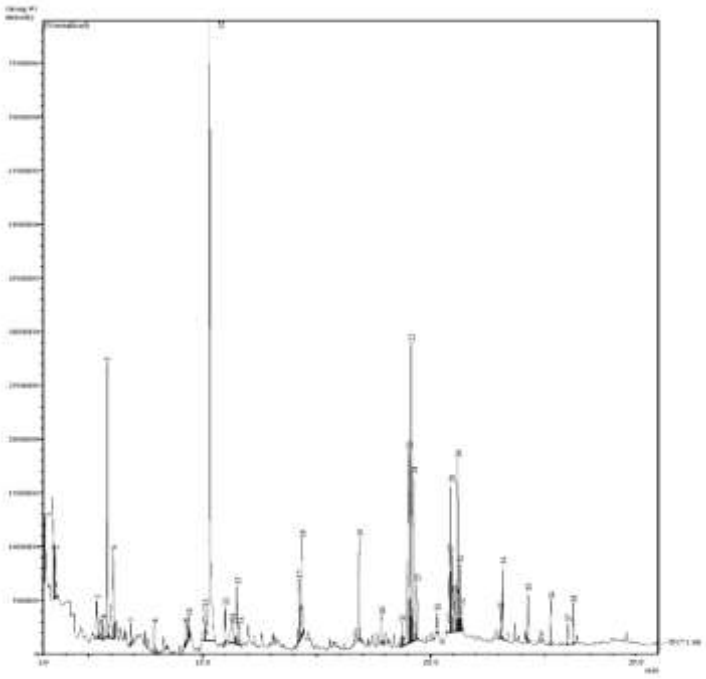


Figure (1). GC-MS testing of Thyme

Table 2. Data from the GC-MS analysis of fatty acids extracted from the Spirogyra alga.

Peak	R time	Area	Area%	Name
1	3.954	9552304	3.13	1-Hexanol, 2-ethyl
2	4.854	150825220	49.39	Glycerin
3	5.775	3534110	1.16	1,2-Benzenediol
4	5.853	5407327	1.77	(3-Acetylphenyl)carbamic acid, 4-butoxyphenyl ester
5	6.147	6435219	2.11	1,2-Benzenediol 5 6.147 6435219 2.11 1,2-Benzenediol
6	7.074	955126	0.31	Benzenepropanol, .alpha.-methyl-.beta.-nitro
7	7.574	4871524	1.60	Hydroquinone
8	8.203	2038345	0.67	Ethanol, 2-methoxy-, carbonate (2:1)
9	10.626	3445161	1.13	beta.-D-Glucopyranose, 1,6-anhydro
10	12.167	2163010	0.71	Cyclohexanol, 1R-4-trans-acetamido-2,3-trans-epoxy
11	12.575	2074960	0.68	Methyl(methyl 4-O-methyl-.alpha.-d-mannopyranoside)uronate
12	14.770	3877995	1.27	11,14,17-Eicosatrienoic acid, methyl ester
13	15.097	6772147	2.22	Hexadecanoic acid, methyl ester
14	15.368	526911	0.17	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)

15	17.049	6722101	2.20	Phytol
16	17.477	32682081	10.70	cis-Vaccenic acid
17	17.892	2552298	0.84	3-Dodecyne
18	18.274	1679879	0.55	9,12-Octadecadienoic acid (Z,Z)
19	19.553	1265584	0.41	Eicosanoic acid
20	20.660	1393237	0.46	1-Heneicosanol
21	20.750	370353	0.12	Tricyclo[4.2.1.0(3,7)]nonane-3,8-diol, (anti-8)
22	21.825	778671	0.25	Hydroxydehydrostevic acid
23	22.174	707799	0.23	Myristic acid
24	23.059	677142	0.22	9-Octadecenamide, (Z)
25	23.249	520232	0.17	2,6,10,14,18,22-Tetracosahexaene, 2,6,10,15,19,23-hexamethyl-, (all-E)
26	25.546	671997	0.22	Stigmast-5-en-3-ol, oleate

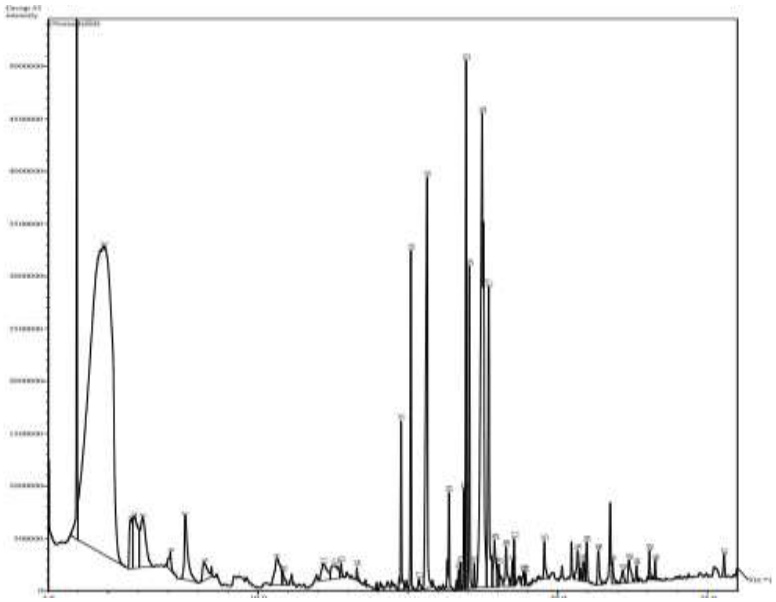


Figure (2). GC-MS analysis for Spirogyra alga Fatty acids

The effect of fatty acids extracted from the *Thymus* plant on the bacteria used under study, compared with standard antibiotics, was studied using the sensitivity test method (disk diffusion).

The extract from the Thymus plant showed an inhibitory effect against bacteria, with zones measuring 12, 10, 14, and 14 mm at a concentration of 400 mg/cm³ against Pseudomonas aeruginosa, Escherichia coli, and Staphylococcus aureus, respectively (Table 2). Additionally, the extract of Spirogyra sp. produced inhibition zones of 14 mm for *Staph aureus*, 14 mm for *E. coli*, and 8 mm for *P. aeruginosa* at the same concentration of 400 mg/cm³ (Bellinger and Sigee, 2010b; Rakaa and Obaid, n.d.; Varga et al., 2015) .

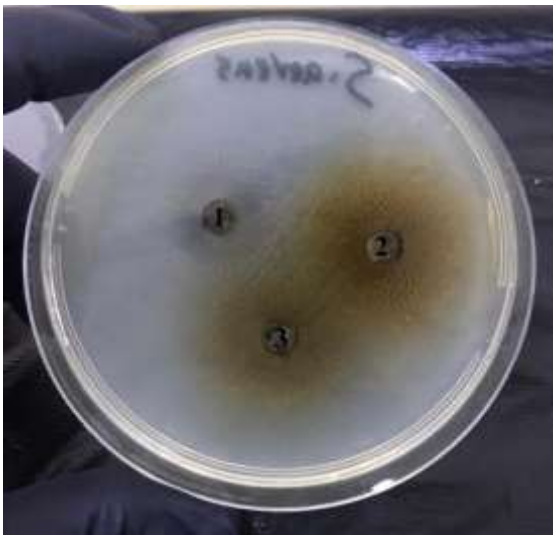
The results of the synergy experiment demonstrated the product of combining the fatty acids from the *Thymus* plant and the extract of Spirogyra algae.

The synergistic effect against *P. aeurogenosa* and *E. coli* measured 14 and 16 mm, which is greater than the effect observed with the two extracts alone. Conversely, the combination against *Staph. aureus* exhibited an antagonistic action with a diameter of 10 mm of inhibition, which is less than the effect of the two extracts on their own[31].

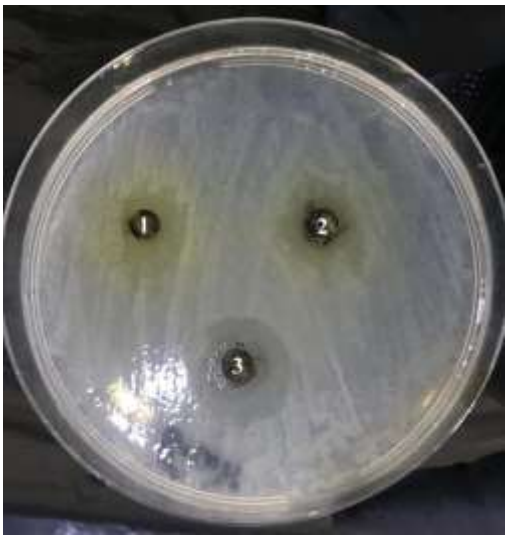
The inhibitory effect arises from active compounds extracted from the thyme plant, which research has shown to considerably hinder germ cell growth to different degrees, depending on the germ type. This effect may result from a reduction in energy compound production by preventing oxygen absorption or by disrupting various vital cellular processes, notably the inhibition of nucleic acid and protein synthesis. This has been supported by many studies. [35–37].

Table 2: Inhibitory activity of isolated fatty acids from the *Thymus* plant and their synergy with Spirogyra against the bacteria studied, compared to standard antibiotics at a concentration of 400 mg/cm³.

No.	400 mg/cm³		Bacteria species		
			Staph. aureus	***E. coli***	***P. aeurogenosa***
1	Fatty acids in Thymus (T)		14	10	12
2	*Spirogyra sp*. (S)		14	12	8
4	T + S		10	16	14
6	Comparison	Gentamicin (CN)	12	11	-
		Ciprofloxacin (CIP)	10	10	12



1= T , 2= S , 3= T &S in *Staph. aureus*



1= T , 2= S , 3= T &S in *E. coli*

1=T , 2= S , 3= T&S in *P. aeurogenosa*

Picture (1). Effect of separated fatty acids from the *Thymus* plant in synergy with Spirogyra at a concentration of 400 mg/cm³ on the bacteria under study.

4-Conclusion

The escalating threat of multidrug-resistant (MDR) bacteria necessitates novel therapeutic alternatives. This study investigated the antibacterial potential of terpene-rich extracts from *Thymus* vulgaris and Spirogyra spp., isolated via saponification and characterized using GC-MS. When tested individually against *Pseudomonas aeruginosa*, *Escherichia coli*, and *Staphylococcus aureus*, both extracts demonstrated moderate inhibitory activity. Notably, the combination of both extracts exhibited a clear synergistic effect, producing larger inhibition zones against *P. aeruginosa* (14 mm) and *E. coli* (16 mm) compared to either extract alone. In contrast, an antagonistic effect was observed against *S. aureus*. These findings highlight the promising synergistic potential of combining natural plant and algal products to combat specific MDR bacterial pathogens, warranting further purification and in-depth mechanistic studies.

Authors' Contributions

All authors contributed to the design of the study, data collection, data analysis, interpretation, and writing of the initial manuscript draft. Each author participated in subsequent manuscript revisions, editing, and review. Final approval of the manuscript was granted by all authors.

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Competing Interests

The authors disclose that they have no competing interests.

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