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Chemical Analysis and Antibacterial-Antibiofilm Properties of Waste Biomass of the Invasive Alga Species *Rugulopteryx okamurae* in Morocco

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ABSTRACT

Since 2017, the invasive brown alga *Rugulopteryx okamurae* has posed significant biological, ecological, and socio-economic challenges in Morocco due to its rapid proliferation and biomass accumulation. This study investigates its antimicrobial and antibiofilm properties, along with its chemical composition. Methanol, chloroform, and ethyl acetate (EtOAc) extracts were evaluated for antibacterial activity against six human pathogens, including clinical and reference strains of *Escherichia coli* and *Staphylococcus aureus*. Fourier-transform infrared spectroscopy (FT-IR) analysis identified functional groups such as hydroxyl (O–H), carbonyl (C=O), and polysaccharides—indicative of the presence of bioactive compounds. Gas chromatography-mass spectrometry (GC-MS) revealed several key components: oleic acid butyl ester in the EtOAc extract, methyl tetradecanoate in the chloroform extract, and pentadecanoic acid methyl ester in the methanolic extract. Antibacterial potential was assessed using microdilution and growth curve assays, with minimal inhibitory concentrations (MICs) and minimal bactericidal concentrations (MBCs) ranging from 3.12 to 50mg/ mL. Growth curve analysis showed a bacteriostatic effect, demonstrated by extension of the lag phase and suppression of bacterial growth rates. Among the extracts, the EtOAc extract exhibited the highest antibiofilm activity, reducing biofilm formation by 73% in *E. coli* (clinical strain TT-23) and 70% in *S. aureus* (clinical strain 70). These findings suggest that *R. okamurae* harbors bioactive compounds with significant antibacterial and antibiofilm potential, offering not only a means to mitigate the ecological impacts of this invasive species but also promising applications in antimicrobial agent development.

INTRODUCTION

In recent years, the Moroccan coasts, including both the Mediterranean Sea and the Atlantic Ocean, have become hotspots for the introduction of non-indigenous marine species **(Menioui, 2021)**. Marine algae constitute a significant portion of invasive aquatic species. Among the most rapidly proliferating is the brown alga *Rugulopteryx okamurae*, a species native to the Pacific Ocean **(El Madany *et al.*, 2024)**. This alga is classified among the worst invasive species in the region due to its rapid spread and substantial impacts on ecosystems, biodiversity, fisheries, coastal tourism, and mariculture, particularly in the Strait of Gibraltar and surrounding areas **(Hwang *et al.*, 2009; García-Gómez *et al.*, 2019; Tsirintanis *et al.*, 2022)**. These effects are particularly evident along the Moroccan coastline, where the invasive *R. okamurae* has become a dominant seaweed (Fig. 1). In addition to the well-documented massive invasions along the Mediterranean coast, *R. okamurae* has been increasingly reported on the Atlantic coast as well **(Bernal-Ibáñez *et al.*, 2022; Faria *et al.*, 2022a; Fernández-Herrera *et al.*, 2023; Pereira, 2024)**.

Fig. 1. *Rugulopteryx okamurae* occurrence between Azla-Amsa coast, Morocco

Marine algae represent a significant proportion of invasive aquatic species. Among the most rapidly spreading is the brown macroalga *Rugulopteryx okamurae*, native to the Pacific Ocean **(El Madany *et al.*, 2024)**. This invasive species possesses several life history traits that enable its successful establishment in a wide range of environments across the Atlantic and Mediterranean coasts **(Laamraoui *et al.*, 2024)**. Key traits include rapid growth and reproduction, high dispersal capacity, and broad environmental tolerance **(Laamraoui *et al.*, 2024; Pereira, 2024)**.

R. okamurae poses a significant threat to local biodiversity by disrupting marine ecosystems **(Faria *et al.*, 2021b; Sempere-Valverde *et al.*, 2021; Pereira, 2024)**. It displaces numerous native species and alters both the productivity and biodiversity of the

invaded ecosystems **(Sempere-Valverde *et al.*, 2021)**. In addition to ecological impacts, the species also imposes considerable socio-economic burdens. Municipal cleaning operations in Morocco and Spain have removed thousands of tons of detached *R. okamurae* biomass from tourist beaches, leading to substantial coastal management costs **(Ocaña *et al.*, 2016; El Madany *et al.*, 2024)**. In southern Spain, the economic impact of beach cleaning alone has been estimated at over than one million euros annually **(Altamirano *et al.*, 2016)**. The fishing industry has also been affected, with significant financial losses due to the entanglement of fishing nets with *R. okamurae* biomass, disrupting operations and reducing productivity **(Báez *et al.*, 2013)**.

To mitigate these negative effects, several valorization strategies have been proposed for the biomass collected from beaches. These include its use in anaerobic co-digestion, composting, biofertilizer production, and the development of bio-based plastics, offering sustainable alternatives for biomass management **(Barcellos *et al.*, 2023)**. Due to its exceptionally high primary productivity in invaded regions, including Morocco, Spain, and Portugal, large-scale accumulation of *R. okamurae* biomass has prompted extensive research not only on limiting its spread but also on identifying opportunities for its valorization in biotechnological applications **(García-Gómez *et al.*, 2019; Barcellos *et al.*, 2023; Pereira, 2024)**.

Recent studies have highlighted *R. okamurae*'s potential in the biomedical and pharmaceutical sectors, citing its anti-inflammatory, antimicrobial, and α-glucosidase inhibitory activities. These properties suggest additional applications in the food industry, making the species a promising source of innovative natural products **(Barcellos *et al.*, 2023)**. Harnessing the bioactive potential of this invasive macroalga offers a dual benefit—contributing to environmental sustainability and providing economically viable pathways for biomass utilization.

In Morocco, *R. okamurae* has been reported along both the Atlantic and Mediterranean coasts, where it has caused significant ecological and economic impacts **(El Madany *et al.*, 2024)**. However, research on its potential applications remains limited in the Moroccan context **(Cebrián-Lloret *et al.*, 2024)**. This study aims to raise awareness within the scientific community about the untapped potential of this abundant and year-round available biomass.

Specifically, we evaluated the antibacterial and antibiofilm activities of three solvent extracts (methanol, chloroform, and ethyl acetate) derived from *R. okamurae* collected from the Amsa–Tetouan coastline, targeting two human pathogens: *Staphylococcus aureus* and *Escherichia coli*. Additionally, we assessed the impact of these extracts on bacterial growth kinetics to further explore their antimicrobial potential. The chemical composition of the bioactive compounds was analyzed using gas chromatography–mass spectrometry (GC-MS), while Fourier-transform infrared

spectroscopy (FT-IR) was employed to identify and characterize the functional groups present in the algal extracts.

MATERIALS AND METHODS

1. *R. okamurae* harvesting, identification, and preparation

The invasive brown alga *Rugulopteryx okamurae* (Heterokontophyta, Phaeophyceae) (Fig. 2) was collected in January 2023 from a depth of 0.5– 1m along the shoreline of Amsa (Tetouan, Morocco) at 35°32'50.69"N / 5°14'9.70"W. Identification of the alga was conducted in the Laboratory of Applied Phycology-Mycology, Faculty of Science, Tetouan, using anatomical and morphological characteristics observed under light microscopes (Olympus, Tokyo, Japan) and a stereo-microscope (OZL 451, KERN, China).

After collection, the algae were rinsed with clean water to remove salt and debris, then dried in an oven at 60°C for 24h. The dried material was ground into a fine powder using a blender and stored in plastic bottles at room temperature (RT) until further use. A specimen of the collected algae was deposited in the Herbarium of the Phycology and Mycology Laboratory, Faculty of Sciences, Abdelmalek Essaâdi University, Tetouan, Morocco (HTET), under the registration number HTET 1011.

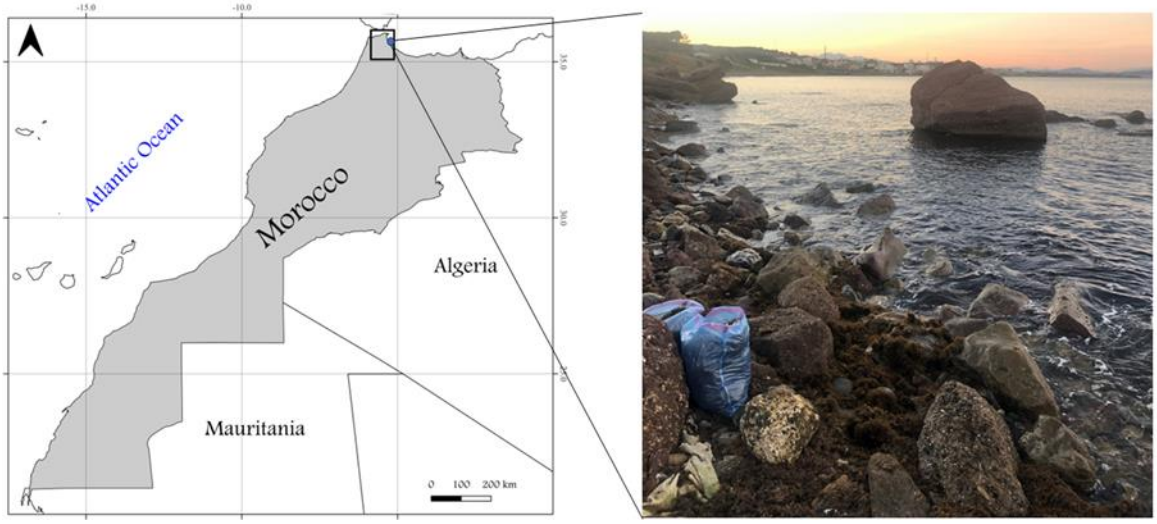

Fig. 2. Geographical location of the sampling site.

Three organic extracts of *R. okamurae* were prepared using solvents of different polarities: methanol (99.9 % purity), ethyl acetate (EtOAc) (≥99.5 % purity), and chloroform (99-99.4 % purity). A 1:10 ratio of dry algae powder to solvent was used, following the protocol described by **O'Sullivan *et al.* (2014)**. The mixture was agitated continually in the dark for 72h at RT. It was then filtered through Whatman nº. 1 paper

and concentrated under reduced pressure at 45°C using a rotary evaporator. The resulting crude extracts were stored in the dark at 4°C for subsequent biological activity assays.

2. Chemical analysis

2.1. Fourier-transform infrared (FT-IR) spectroscopy

The functional groups present in the dried algal powder of *Rugulopteryx okamurae* were analyzed using a Shimadzu FTIR-8400S spectrometer (Japan). The device was equipped with a Smart iTR accessory featuring a diamond attenuated total reflection (ATR) crystal, allowing spectral analysis in the range of 400–4000 cm^{-1}. This technique provides valuable information about the major functional groups in the dried algal matrix, contributing to the identification of bioactive chemical constituents **(Moubayed *et al.*, 2017; Bajad *et al.*, 2024)**.

2.2 Gas chromatography–mass spectrometry (GC-MS) analysis

Chemical profiling of the crude extracts was conducted using gas chromatography (GC) on a TRACE 1300 TSQ 8000 Evo system, coupled with mass spectrometry (MS). The GC was fitted with an Elite-5MS capillary column (5% diphenyl / 95% dimethylpolysiloxane; dimensions: 30 m × 0.25 mm × 0.25 μm). For each analysis, 1μL of extract was injected in split mode. Helium served as the carrier gas at a constant flow rate of 1mL/ min.

The inlet oven temperature was initially held at 110°C, while the injector temperature was set at 250°C. Detection was performed via electron ionization (EI) at an energy level of 70 eV, and the mass spectrometer operated across a scan range of 60–600 amu. Identification of chemical compounds was carried out by comparing the acquired spectra with entries in the NIST 2017 Mass Spectral Library.

3. Biological activities

3.1 Antibacterial activities

To evaluate the antimicrobial activity of *R. okamurae* extracts, two complementary methods were employed:

1. Broth Microdilution Assay, used to determine the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC), both expressed in mg/mL.
2. Growth Kinetic Curve Assay, used to assess the effect of the extracts on bacterial growth dynamics.

A total of six bacterial strains were tested, including four multidrug-resistant (MDR) clinical isolates and two reference strains:

- *Staphylococcus aureus* (clinical strain, Code 70)
- *S. aureus* (clinical strain, Code 34)
- *Escherichia coli* (clinical strain, Code TT-12)
- *E. coli* (clinical strain, Code TT-23)
- *E. coli* ATCC 25922 (reference strain)

- *S. aureus* ATCC 25923 (reference strain)

The antibiogram profiles of the clinical strains are presented in Table (1). All strains were provided by the Laboratory of Food Science and Health, Department of Biology, Abdelmalek Essaadi University, PO Box 2121, 93002 Tetouan, Morocco. These strains were also used in the evaluation of antibiofilm activities, detailed in the following section.

Table 1. Antibiotic resistance profiles of clinical strains

Clinical strains	Antibiotic[a] resistance
S. aureus (70)	OX, CRO, TZP, VAN, AMX, RIF
S. aureus (34)	OX, VAN, GEN, TET
E. coli (TT-12)	AMX, CPR, CAZ, CLT, CXT, AMK, NAL, TOB, CS
E. coli (TT-23)	AMX, CAZ, CIP, CXT, TOB, NAL, CS

[a]**AMX**: Amoxicillin, **CXT**: Cefotaxime, **CAZ**: Ceftazidime, **TOB**: Tobramycin, **AMK:** Amikacin, **NAL**: Nalidixic Acid, **CPR**: Ciprofloxacin, **CS:** Colistin, **OX**: Oxacillin; **TZP**: Tazobactam-piperacillin, **CIP**: Ciprofloxacin, **CRO**; Ceftriaxone. **VAN**; Vancomycin. **RIF**; Rifampin. **TET**; Tetracycline; **GEN**; Gentamycin, **CLT**: Cephalothin

The Minimum Inhibitory Concentration (MIC) was determined using the microdilution assay described by **Sarker *et al.* (2007)**, with slight modifications. The experiment was conducted in sterile 96-well microplates. Serial dilutions of 50µL of *R. okamurae* extracts were prepared to yield final concentrations ranging from 50 to 0.39mg/ mL. Then, 50µL of Mueller-Hinton Broth (MHB) inoculated with bacterial suspension at a final concentration of 5×10^5 CFU/mL was added to each well.

All tests were performed in triplicate and incubated at 37°C for 24 hours. After incubation, 10µL of 0.015% (w/v) resazurin solution was added to each well, followed by 30 minutes of incubation. A color change from purple to pink indicated bacterial growth.

To determine the Minimum Bactericidal Concentration (MBC), 10µL samples from wells with no visible growth were inoculated on Plate Count Agar (PCA) and incubated under the same conditions. MBC was defined as the lowest concentration at which 99.99% of bacteria were killed, i.e., no growth observed on PCA plates.

Growth-kinetic curve assay

The effect of *R. okamurae* extracts on bacterial growth kinetics was evaluated using a growth curve assay. Overnight bacterial cultures were diluted in sterile phosphate-buffered saline (PBS; 0.01 M, pH 7.4) to approximately 1×10^5 CFU/mL ($OD_{600} \approx 0.01$). A 250 µL aliquot of bacterial inoculum was mixed with 250µL of each seaweed extract at four concentrations: 0.12 MIC, 0.25 MIC, 0.5 MIC, and 1.0 MIC.

Bacterial cultures without extract served as the positive control. The mixtures were incubated at 37°C with agitation, and optical density at 600nm (OD_{600}) was measured hourly throughout the growth period. All experiments were conducted in triplicate.

3.2 Biofilm inhibitory potential

Biofilm inhibition was assessed following the method described by **O'Toole (2011)**, with modifications. All six bacterial strains were cultured overnight at 37°C in Luria-Bertani (LB) broth and adjusted to 5×10^5 CFU/mL. A total of 100µL of bacterial suspension was dispensed into 96-well microplates, followed by 100µL of each seaweed extract. Plates were incubated for 24 hours at 37°C to allow biofilm formation. Untreated wells containing only bacteria served as negative controls.

After incubation, non-adherent cells were removed, and wells were washed twice with PBS (0.01 M, pH 7.4). Plates were air-dried at 37°C for 30 minutes. Then, 125µL of 0.1% (w/v) aqueous crystal violet (CV) was added and left at room temperature for 15 minutes. Excess stain was removed by three washes with distilled water, and plates were left to dry at 37°C.

To quantify biofilm biomass, the bound CV was solubilized with 150µL of 30% (v/v) acetic acid (15-minute incubation), and the solution was transferred to a fresh microplate. Absorbance was measured at 596nm, and biofilm inhibition was calculated using the following formula:

$$\text{Biofilm eradication (\%)} = [(\text{OD control} - \text{OD test}) / \text{OD control}] \times 100$$

4. Statistical analysis

All data were analyzed using Microsoft Excel 2016 (Microsoft Corp., Redmond, WA, USA). Results are expressed as mean ± standard deviation (SD) from triplicate experiments (n = 3).

RESULTS

1. FT-IR analysis

The analysis of the algae *Rugulopteryx okamurae* using Fourier-transform infrared (FTIR) spectroscopy enabled the identification of several characteristic transmission bands, each associated with specific functional groups. The results reveal a remarkable chemical diversity in the composition of this alga, encompassing both organic and inorganic structures. This chemical diversity could have significant implications for biotechnological and environmental applications. The results are presented in Fig. (3).

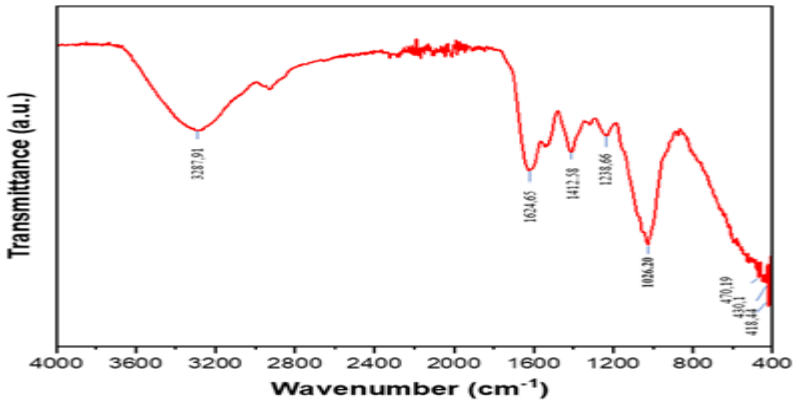

Fig. 3. Fourier infrared spectroscopy (FTIR) characterization of *Rugulopteryx okamurae* (Powder).

The band at 3287.91 cm^{-1} is attributed to the stretching vibrations of N–H and O–H bonds, indicating the presence of water-associated structures, hydroxyl functional groups, and amide linkages. These features are typically observed in proteins, polysaccharides, and other biological macromolecules **(Pereira *et al.*, 2003; Zamanileha *et al.*, 2024)**.

The band at 1624.65 cm^{-1} corresponds to C=O bending vibrations, suggesting the presence of carbonyl groups—especially amides, which are characteristic of proteinaceous or nitrogenous organic compounds **(Fabian & Schultz, 2000; Nakamoto, 2008)**.

At 1412.58 cm^{-1}, the deformation vibration of C–H bonds is observed, typical of methylene (–CH_2–) and methyl (–CH_3) groups commonly found in organic molecules **(Kemp, 1991)**.

The band at 1238.66 cm^{-1} is associated with C–O stretching, indicative of esters, ethers, or alcohols, commonly present in polysaccharides or glycosidic structures **(Guo *et al.*, 2018; Kassem *et al.*, 2023)**.

A strong band at 1026.20 cm^{-1}, also attributed to C–O vibrations, supports the presence of complex sugars or polysaccharides in the alga **(Hong *et al.*, 2021; Kassem *et al.*, 2023)**.

Lower-frequency bands at 470.19 and 430.1 cm^{-1} correspond to the vibrations of metallic atoms or inorganic structures, suggesting the alga may contain mineral elements or metal complexes **(Marcilla *et al.*, 2009; Vandanjon *et al.*, 2023)**.

Finally, the band at 418.44 cm^{-1} is associated with C–H bending or polysaccharide skeleton vibrations, potentially linked to sugar moieties or specific inorganic elements within the alga's composition **(Marcilla *et al.*, 2009; Vandanjon *et al.*, 2023)**.

In summary, FT-IR analysis of *Rugulopteryx okamurae* reveals the presence of several key functional groups, including hydroxyl, carbonyl, amide, ester, and C–O linkages, indicating a rich composition of proteins, amino acids, polysaccharides, and other bioactive compounds. These molecular signatures support the potential of *R.*

okamurae as a source of pharmaceutically and industrially valuable bioactive constituents.

2. Antibacterial activity of *R. okamurae* extracts

The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values of *R. okamurae* extracts against six bacterial strains, including four multidrug-resistant (MDR) clinical isolates and two reference strains, are summarized in Table 2.

For Gram-positive strains, particularly *Staphylococcus aureus*:

- The clinical isolate S. aureus Code 70 showed the greatest susceptibility to the methanolic extract, with both MIC and MBC values of 6.25mg/ mL.
- The clinical strain S. aureus 34 exhibited the highest sensitivity to the ethyl acetate extract, with MIC and MBC values of 3.12mg/ mL, outperforming methanol (12.5mg/ mL) and chloroform (50mg/ mL).
- The reference strain S. aureus ATCC 25923 displayed consistent susceptibility across all solvents, with MIC values ranging from 3.12 to 6.25mg/ mL. Methanol again showed the strongest effect, with both MIC and MBC at 3.12mg/ mL.

These results highlight the potent antimicrobial activity of *R. okamurae* extracts, particularly those prepared with ethyl acetate and methanol, and underscore their potential application against resistant strains of *S. aureus*.

Table 2. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of *R. okamurae* extracts tested against four clinical bacterial isolates (*S. aureus* (70), *S. aureus* (34), *E. coli* (TT-12) and *E. coli* (TT-23)) and both references bacterial (*E. coli* ATCC 25922, *S. aureus* ATCC25923).

		MIC value (mg/mL)			MBC value (mg/mL)		
		EtOAc	**Methanol**	**Chloroform**	**EtOAc**	**Methanol**	**Chloroform**
Gram^{+}	*S. aureus* (70)	12.5	6.25	25	12.5	6.25	25
	S. aureus (34)	3.12	12.5	50	3.12	12.5	50
	S. aureus ATCC 25923	3.12	3.12	6.25	6.25	3.12	6.25
Gram^{-}	*E. coli* (TT-23)	25	50	12.5	25	50	25
	E. coli (TT-12)	50	6.25	12.5	50	6.25	12.5
	E. coli ATCC 25922	6.25	12.5	6.25	6.25	12.5	6.25

Regarding *Escherichia coli* strains, *E. coli* (TT-23) showed high MIC and MBC values with ethyl acetate (25 and 50mg/ mL, respectively) and methanol (50mg/ mL for both). Chloroform was more effective with MIC and MBC values of 12.5mg/ mL. *E. coli* (TT-12) showed enhanced sensitivity to methanol with MIC and MBC values of 6.25mg/ mL, while ethyl acetate presented higher MIC and MBC values (50mg/ mL). The reference strain *E. coli* ATCC 25922 had low MIC and MBC values for all three solvents tested, ranging from 6.25 to 12.5mg/ mL, showing general sensitivity to the extracts. Ethyl acetate and chloroform both had MIC and MBC values of 6.25mg/ mL, while

methanol had slightly higher values at 12.5mg/ mL. These findings indicate that the choice of extraction solvent significantly impacts the antimicrobial effectiveness of the extracts against *E. coli*, with chloroform being the most effective, followed by methanol and ethyl acetate. This highlights the importance of optimizing extraction solvents to maximize the antimicrobial activity of the extracts.

The microbial growth-kinetic assay was performed to assess the kinetics involved in the antibacterial potency of *R. okamurae* extracts (methanol, chloroform, and ethyl acetate). The evaluation of bacterial cell growth over 16h was compared to an untreated control culture. The three extracts exhibited concentration-dependent antibacterial effects. The treated cultures of all tested bacteria exhibited phenotypic growth that differed from the control culture in two key aspects: (1) the latent phase of bacterial growth was prolonged and/or (2) the growth rate of the planktonic culture reduced leading to a lower final absorbance value (Figs. 4, 5). As a result, sub-MIC concentrations of *R. okamurae* extracts caused a significant slowdown in bacterial growth.

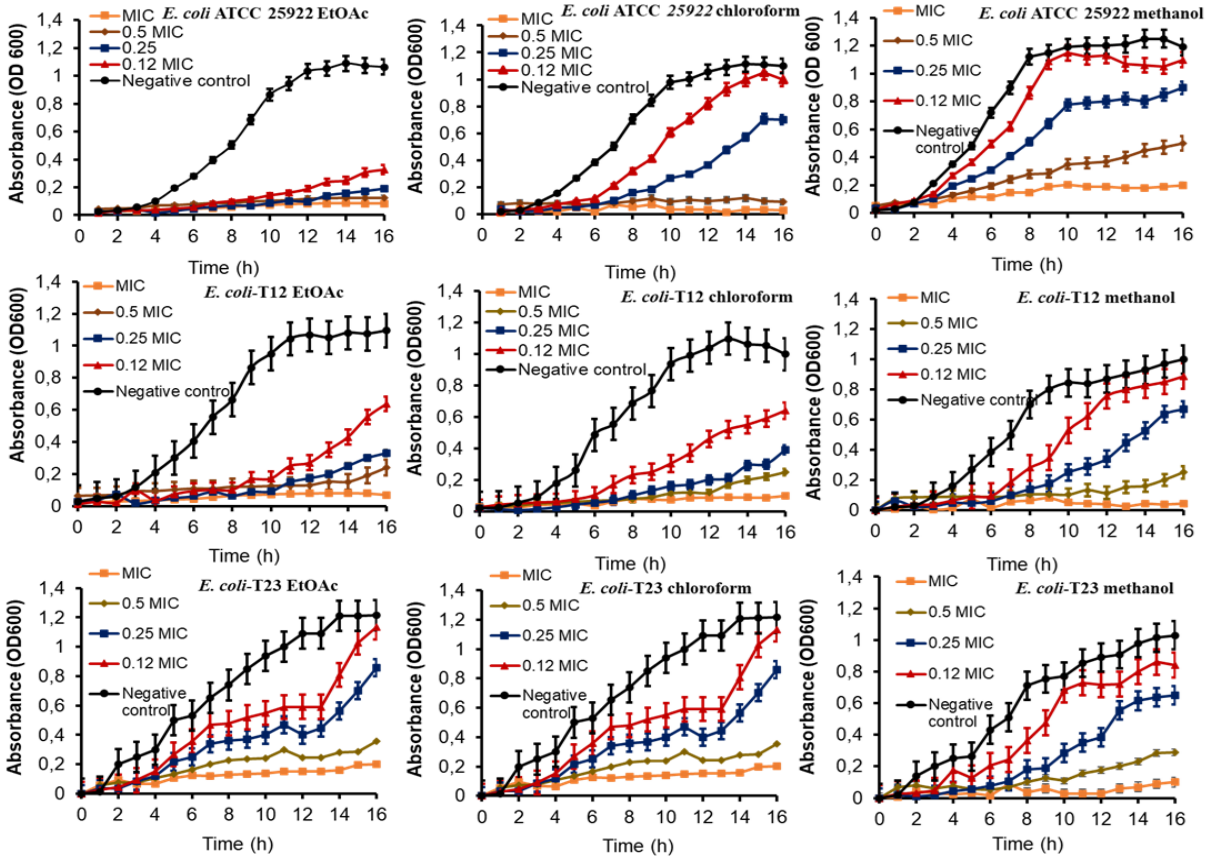

Fig. 4. Effect of *R. okamurae* extracts (Methanol, Ethyl acetate (EtOAc) and chloroform) on kinetics of bacterial growth (Gram negative; *E. coli (TT-12), E. coli (TT-23)* and *E. coli* ATCC 25922). MIC: minimum inhibitory concentration

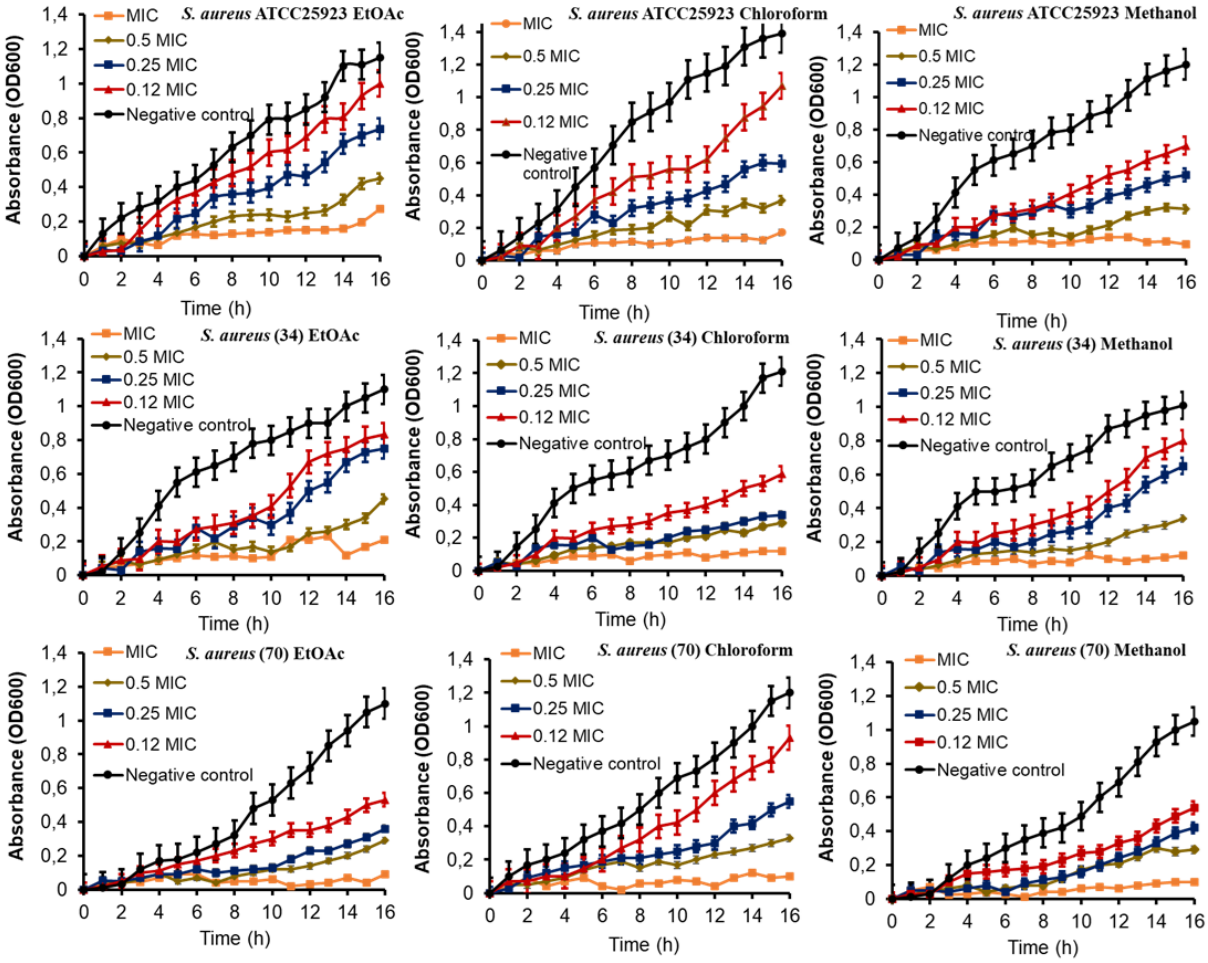

Fig. 5. Effect of *R. okamurae* extracts (Methanol, Ethyl acetate (EtOAc) and chloroform) on kinetics of bacterial growth (Gram positive; *S. aureus* (70), *S. aureus* clinical strain (34) and *S. aureus* ATCC25923) MIC: minimum inhibitory concentration.

3. Activity antibiofilm of *R. okamurae* extracts

The formation of biofilms by certain microorganisms is a key mechanism in the development of microbial resistance to antimicrobial compounds. Accordingly, *R. okamurae* extracts were tested for their capacity to suppress biofilm development by six bacterial strains. The findings are presented in Fig. (6).

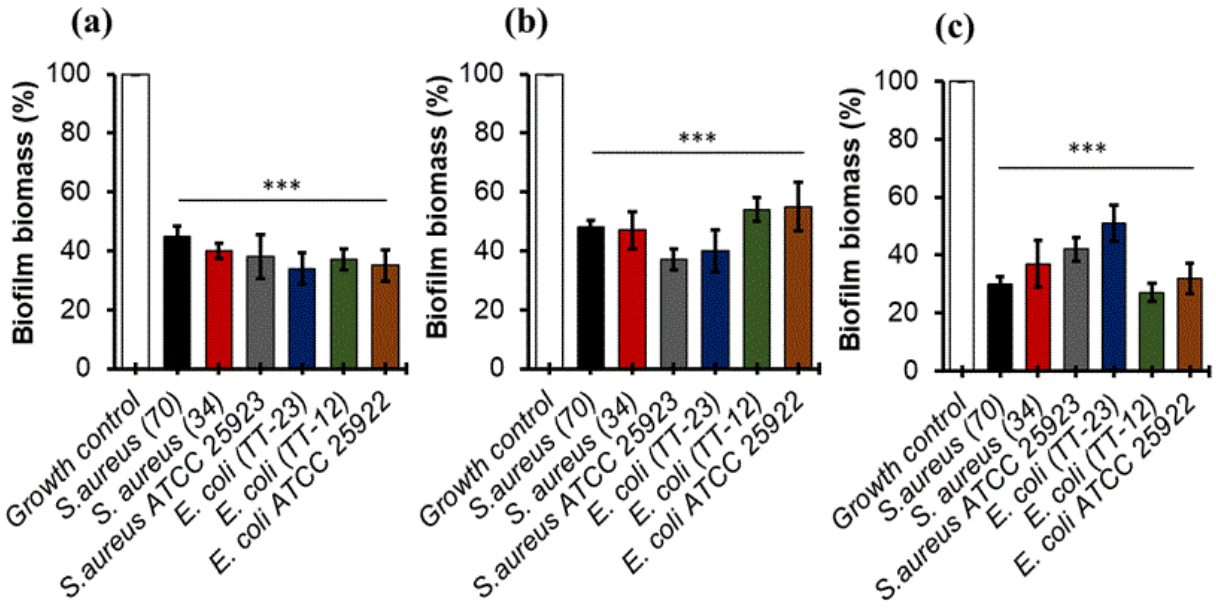

Fig. 6. *Rugulopteryx okamurae* extracts (a); methanol, (b); chloroform, (c) EtOAc; potential to inhibit; *E. coli* (clinical strains, TT-23), *E. coli* (TT-12), *E. coli* ATCC 25922, *S. aureus* (clinical strains,70), *S. aureus* (34) *S. aureus* ATCC25923 and biofilm formation

The ethyl acetate extract (Fig. 6c) of *Rugulopteryx okamurae* showed a high percentage of biofilm inhibition for almost all tested *E. coli* bacterial strains, ranging from 49% for *E. coli* (TT-23) to 73% for *E. coli* (TT-23). It also exhibited strong inhibition for *S. aureus* strains, with inhibition rates ranging from 58% for *S. aureus* ATCC 25923 to 70% for *S. aureus* (70).

The methanolic extracts (Fig. 6a) of *R. okamurae* showed similar results against all *E. coli* strains, with up to a 66% inhibition rate. They also demonstrated almost total inhibition of biofilm formation for *S. aureus* (70), *S. aureus* (34), and *S. aureus* ATCC 25923, with inhibition rates above 55%.

The antibiofilm efficacy of chloroform extracts against the six tested strains is illustrated in Fig. (6b). The biofilm produced by *S. aureus* strains was inhibited, with the most significant reduction of biofilm recorded for *S. aureus* ATCC 25923 (63%). The highest inhibition of biofilm production for *E. coli* clinical strain TT-23 was 60%.

The best inhibitory potential of biofilm formation was demonstrated for the ethyl acetate extract, which showed robust efficacy against *S. aureus* strains with an inhibition rate of up to 70%. It also recorded high inhibition percentages of biofilm formation against *E. coli* strains, with an inhibition rate of up to 73%.

4. Chemical components of different *R. okamurae* extracts

The bioactive compounds present in ethyl acetate, chloroform, and methanol extracts obtained from *R. okamurae* are illustrated in the GC-MS chromatogram (Figs. 7- 9).

Tabulated data include compound identities, retention times, molecular weights, and molecular formulae (Tables 3- 5). In the ethyl acetate extract (Table 3), the top 7 major compounds were Oleic acid, butyl ester (Fig. 7-b) (71.85%), 1-Heptadecyne (8.86%), 9-Octadecen-1-ol (Z) (8.86%), supraene (5.53%), Phosphorodithioic acid, S-[(tert-butylthio)methyl] O,O-diethyl ester (2.83%), Pentadecanoic acid, methyl ester (2.27%), and 9-Octadecenoic acid (Z)-, methyl ester (2.03%).

Table 3. Chemical composition of ethyl acetate extract of *Rugulopteryx okamurae* using GC/ MS analysis, RT = Retention time (min)

Peak number	RT (min)	Compound name	Molecular formula	Molecular weight (g/ mol)	Peak area %
1	25.67	Methyl tetradecanoate	$C_{15}H_{30}O_2$	242	0.86
2	32.04	10-Undecenoic acid, butyl ester	$C_{15}H_{28}O_2$	240	0.58
3	32.44	Pentadecanoic acid, methyl ester	$C_{16}H_{32}O_2$	256	2.27
4	37.66	2,6,10-Dodecatrien-1-ol, 3,7,11-trimethyl	$C_{15}H_{26}O$	222	0.42
5	37.85	9-Octadecenoic acid (Z)-, methyl ester	$C_{19}H_{36}O_2$	296	2.03
6	38.66	Methyl stearate	$C_{19}H_{38}O_2$	298	0.62
7	42.42	Dicamba	$C_8H_6Cl_2O_3$	220	0.30
8	50.79	Oryzalin	$C_{12}H_{18}N_4$	346	0.40
9	51.18	Endosulfan	$C_9H_6Cl_6O_3S$	404	1.22
10	54.95	9-Octadecen-1-ol, (Z)-	$C_{18}H_{36}O$	268	8.86
11	54.95	1-Heptadecyne	$C_{17}H_{32}$	236	8.86
12	55.35	Oleic acid, butyl ester	$C_{22}H_{42}O_2$	338	71.85
13	56.72	Phosphorodithioic acid, S-[(tert-butylthio)methyl] O,O-diethyl ester	$C_9H_{21}O_2P\ S_3$	288	2.83
14	56.95	Supraene	$C_{30}H_{50}$	410	5.53

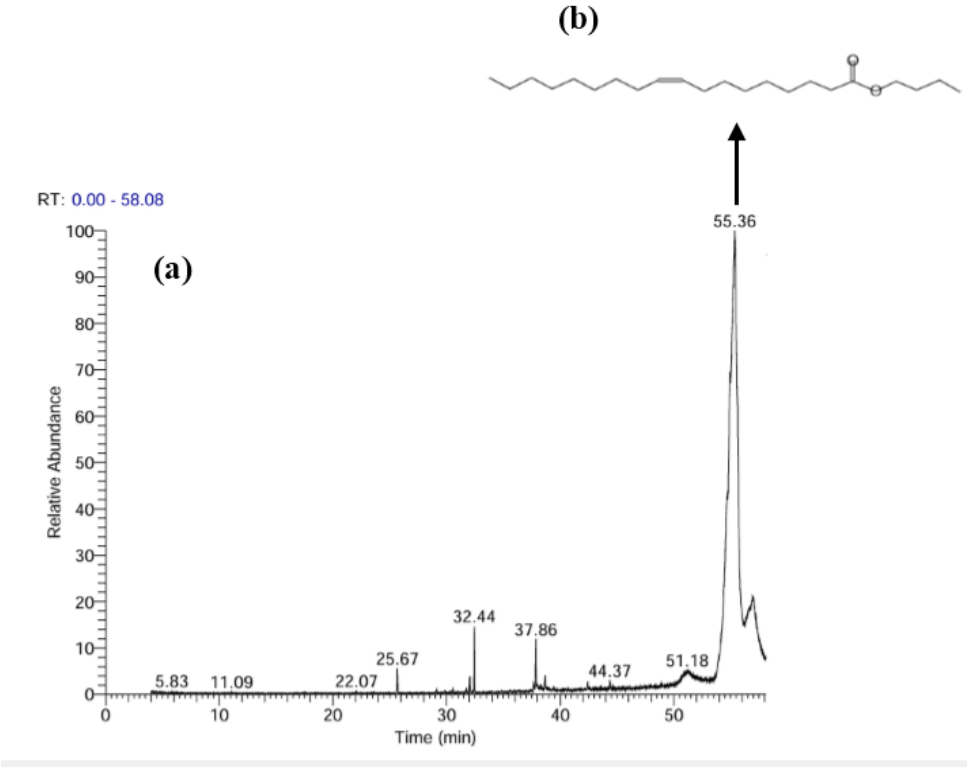

Fig. 7. GC/MS Chromatogram of ethyl acetate extract of *Rugulopteryx okamurae* **(a)**, Oleic acid, butyl ester **(b)**

In the chloroform extracts (Table 4), the main compounds were Methyl tetradecanoate (Fig. 8-d) (21.13%), 9-Octadecenoic acid (Z)-, methyl ester (20.61%), Pentadecanoic acid (8.55%), Methyl stearate (7.06%), Benzene, 1-fluoro-2-methoxy- (5.51%), Pentadecanoic acid, methyl ester (3.54%), 1,5,9-Cyclododecatriene (E,Z,Z) (2.68%), and Docosanoic acid (2.08%).

Table 4. Chemical composition of chloroform extract of *Rugulopteryx okamurae* using GC/MS analysis, RT = Retention time (min)

Peak number	RT (min)	Compound name	Molecular formula	Molecular weight (g/ mol)	Peak area %
1	25.67	Pentadecanoic acid	$C_{15}H_{30}O_2$	242	8.55
2	29.85	3,7,11,15-Tetramethyl-2-hexadecen -1-ol	$C_{20}H_{40}O$	296	0.86
3	30.58	3,7,11,15-Tetramethyl-2-hexadecen -1-ol	$C_{20}H_{40}O$	296	1.37
4	31.74	9-Octadecen-1-ol, (Z)-	$C_{18}H_{36}O$	268	1.62
5	32.44	Methyl tetradecanoate	$C_{15}H_{30}O_2$	242	21.13
6	37.66	1H-Fluorene, dodecahydro-	$C_{13}H_{22}$	178	4.50
7	37.85	9-Octadecenoic acid (Z)-, methyl ester	$C_{19}H_{36}O_2$	296	20.61

8	38.22	Oleic acid, butyl ester	$C_{22}H_{42}O_2$	338	1.68
9	38.66	Methyl stearate	$C_{19}H_{38}O_2$	298	7.06
10	38.99	Dieldrin	$C_{12}H_8Cl_6O$	378	0.92
11	39.45	Silanol, triphenyl-	$C_{18}H_{16}OSi$	276	0.85
12	42.41	1,5,9-Cyclododecatriene, (E,Z,Z)-	$C_{12}H_{18}$	162	2.68
13	42.60	Bicyclo[3.1.1]hept-2-ene-2-ethano l, 6,6-dimethyl	$C_{11}H_{18}O$	166	0.88
14	44.38	Pentadecanoic acid, methyl ester	$C_{16}H_{32}O_2$	256	3.54
15	44.64	2-Aminobiphenyl	$C_{12}H_{11}N$	169	1.08
16	45.52	Aldrin	$C_{12}H_8Cl_6$	362	0.80
17	48.93	Docosanoic acid	$C_{22}H_{44}O_2$	340	2.08
18	49.65	Endrin	$C_{12}H_8Cl_6O$	378	1.32
19	50.42	Benzoic acid, 3-methoxy-	$C_8H_8O_3$	152	1.05
20	53.43	Benzene, 1-fluoro-2-methoxy-	C_7H_7FO	126	5.51

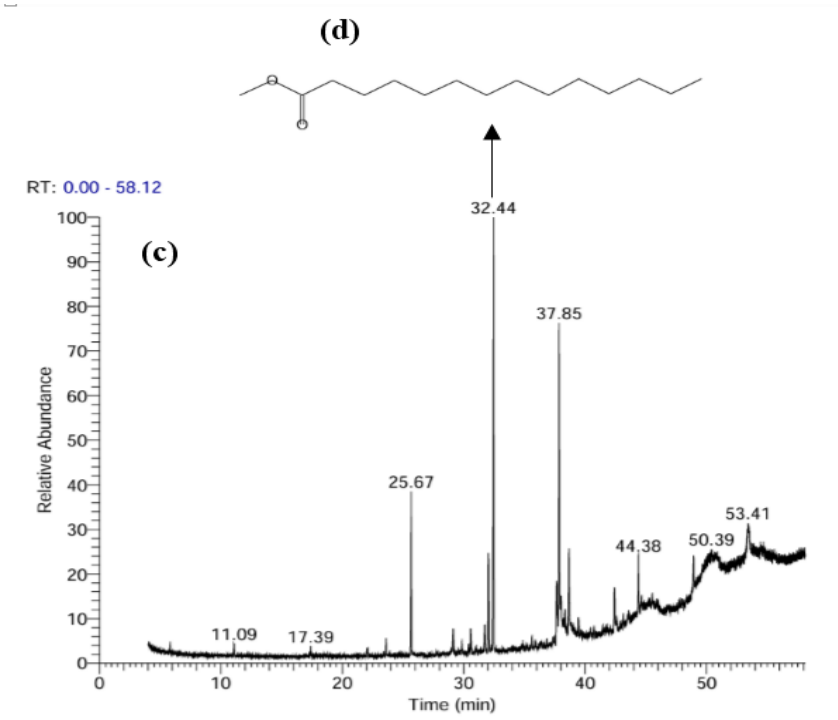

Fig. 8. GC/MS Chromatogram of chloroform of *Rugulopteryx okamurae* (c), Methyl tetradecanoate (d)

Finally, in the methanolic extract (Table 5), the top 10 predominant compounds were Pentadecanoic acid, methyl ester (18.50%), 1-Chloroeicosane (17.29%), 9-Octadecenoic

acid (Z)-, methyl ester (14.77%), Pentadecanoic acid (6.26%), Methyl stearate (4.69%), Oleic acid (4.24%), Dieldrin (2.93%), 9-Octadecen-1-ol (Z) (3.34%), Octadecanoic acid, butyl ester (3.03%), and 1,5,9-Cyclododecatriene (E,Z,Z) (2.35%). Pentadecanoic acid, methyl ester, 9-Octadecenoic acid (Z)-, methyl ester, and Methyl stearate were present in all three types of extracts.

Table 5. Chemical composition of methanolic extract of *Rugulopteryx okamurae* using GC/MS analysis, RT = Retention time (min)

Peak number	RT (min)	Compound name	Molecular formula	Molecular weight (g/ mol)	Peak area %
1	22.07	Naphthalene, 1,2,3,4-tetrahydro-1,1,6-trimethyl	$C_{13}H_{18}$	174	0.90
2	23.59	Cyclotetrasiloxane, octamethyl-	$C_8H_{24}O_4Si_4$	296	0.91
3	25.66	Pentadecanoic acid	$C_{15}H_{30}O_2$	242	6.26
4	31.73	9-Octadecen-1-ol, (Z)-	$C_{18}H_{36}O$	268	1.01
5	32.04	Oleic Acid	$C_{18}H_{34}O_2$	282	4.24
6	32.44	Pentadecanoic acid, methyl ester	$C_{16}H_{32}O_2$	256	18.50
7	37.66	9-Octadecen-1-ol, (Z)	$C_{18}H_{36}O$	268	3.34
8	37.85	9-Octadecenoic acid (Z)-, methyl ester	$C_{19}H_{36}O_2$	296	14.77
9	38.02	Ethyl Oleate	$C_{20}H_{38}O_2$	310	1.23
10	38.66	Methyl stearate	$C_{19}H_{38}O_2$	298	4.69
11	39.45	Triphenylene, 1,2,3,4,5,6,7,8,9,10,11,12-dodecahydro	$C_{18}H_{24}$	240	0.93
12	42.41	1,5,9-Cyclododecatriene, (E,Z,Z)-	$C_{12}H_{18}$	162	2.35
13	42.59	Trifluralin	$C_{13}H_{16}F_3N_3O_4$	335	0.82
14	43.35	Octadecane, 1-bromo-	$C_{18}H_{37}Br$	332	2.15
15	43.48	Dodecanedioic acid, dimethyl ester	$C_{14}H_{26}O_4$	258	1.41
16	44.65	Oleic Acid	$C_{18}H_{34}O_2$	282	0.82
17	45.52	1-Naphthalenecarboxaldehyde, 2-methoxy	$C_{12}H_{10}O_2$	186	1.12
18	48.92	Octadecanoic acid, butyl ester	$C_{22}H_{44}O_2$	340	3.03
19	52.63	1-Chloroeicosane	$C_{20}H_{41}Cl$	316	17.29
20	53.05	Dieldrin	$C_{12}H_8Cl_6O$	378	2.93
21	53.34	Endrin	$C_{12}H_8Cl_6O$	378	1.01

22	58.00	1-Bromo-2,4,6-triisopropylbenzene	$C_{15}H_{23}Br$	282	1.59

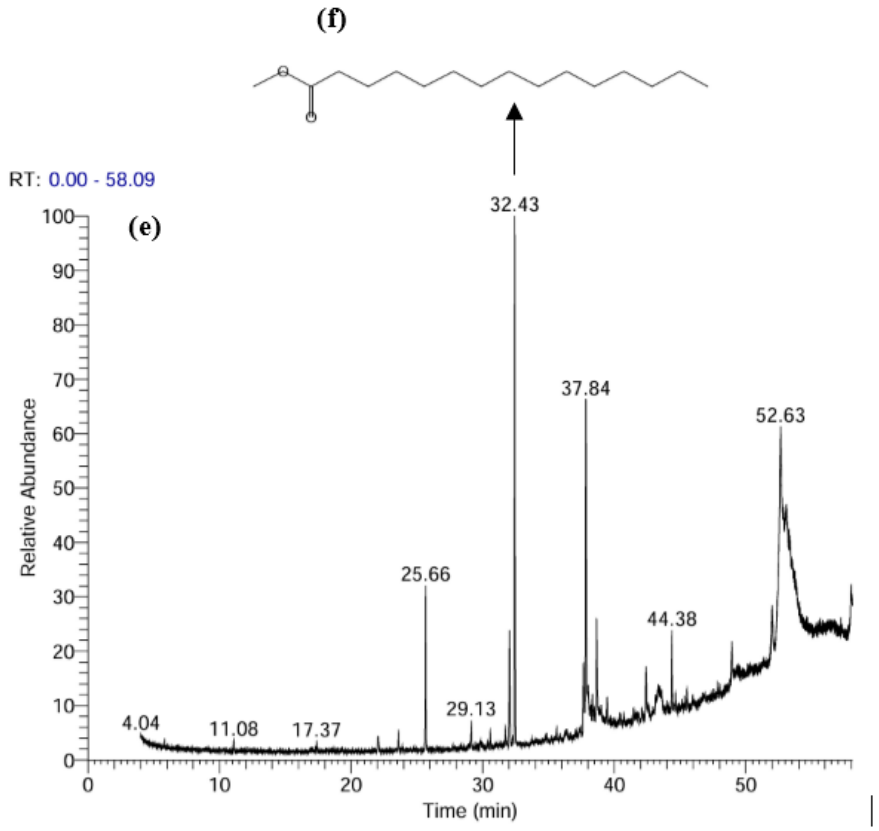

Fig. 9. GC/MS chromatogram of methanolic extract of *Rugulopteryx okamurae* (e) Pentadecanoic acid, methyl ester (f)

DISCUSSION

To the best of our knowledge, no previous research has reported the antibacterial and antibiofilm activities of organic extracts from *R. okamurae* against human pathogenic strains. This is the first study to identify bioactive compounds using GC-MS and to characterize functional groups using Fourier-transform infrared (FT-IR) spectroscopy. This study represents a significant step towards exploiting the massive biomass accumulated along the Moroccan coast and mitigating the ecological and socioeconomic impacts of this invasive alga. However, previous studies on bioactive compound production from *R. okamurae* have primarily focused on the extraction and characterization of antioxidant and carbohydrate compounds **(Barcellos *et al.*, 2023)**. While investigations into its antibacterial potential remain scarce.

According to a recent study by **Vega *et al.* (2023)**, the minimum inhibitory concentration (MIC) of a $_dH_2O$:EtOH (1:4) extract was reported at 25mg/ mL against

Staphylococcus aureus, with no observable effect against *E. coli*. In contrast, our results demonstrate strong antibacterial activity with MIC values of 3.12mg/ mL for both *Staphylococcus aureus* strains: *S. aureus* (clinical strain 34) and *S. aureus* ATCC 25923 and 6.12mg/ mL against *E. coli*.. In the same study, **Vega *et al.* (2023)** compared *R. okamurae* with *Asparagopsis armata*, another highly invasive alga, reporting that a dH_2O:EtOH (1:4) extract of *A. armata* inhibited *S. aureus* at 25mg/ mL and had no effect on *E. coli*.

Our study reports significantly lower MIC values (3.12mg/ mL for *S. aureus* and 6.12mg/ mL for *E. coli*), suggesting greater antibacterial potency of *R. okamurae* extracts. Further, VLC fractions of *A. armata* were found to be more effective, with IC_{50} values of 13.3µg/ mL (*S. aureus*) and 410.1µg/ mL (*E. coli*), highlighting the influence of extraction method and compound pu.

Differences in antibacterial activity across studies may be due to variations in methodology, solvent type, season of collection and environmental conditions. Compared to *Sargassum* sp., our extracts also showed superior antibacterial activity. **Alreshidi *et al.* (2023)** reported MIC and MBC values ranging from 6.25– 12.5mg/ mL and 50–>50mg/ mL, respectively, for methanol/water extracts of *Sargassum* sp. Meanwhile, a 70% ethanolic extract of *R. okamurae* collected from its native habitat in Jeju-do, Korea, exhibited MIC values ranging from 256 to >1,024µg/ mL against *Cutibacterium acnes* **(Lee *et al.*, 2021)**. These data underscore the high antibacterial potential of Moroccan *R. okamurae* extracts, particularly against *S. aureus* (3.12mg/ mL) and *E. coli* (6.25mg/ mL). Interestingly, there are no reports addressing the antimicrobial potential of organic extracts from *R. okamurae* against human pathogens in invaded area, making it difficult to compare our findings with other studies.

Biofilm production, characterized by a sticky exopolysaccharide matrix, is a key virulence factor in biofilm-related infections **(McCarty *et al.*, 2014)**. It has been documented that bacterial biofilms are more resistant to antimicrobial agents than planktonic cells, complicating their eradication. Therefore, biofilm removal is a major challenge that needs novel strategies/approaches to effectively combat biofilms. One such strategy involves the identification of natural bioactive molecules capable of efficiently suppressing biofilm formation, serving as a more environmentally friendly alternative to antibiotics or chemically synthesized toxic agents **(Flemming & Wuertz, 2019)**.

In the present study, *R. okamurae* extracts demonstrated a high level of biofilm inhibition: 73% against *E. coli* (clinical strain TT-23) and 70% against *S. aureus* (clinical strain 70). To our knowledge, no previous studies have documented antibiofilm activity from *R. okamurae*, making comparisons difficult. Similarly, **Pinteus *et al.* (2021)** reported that *Sargassum muticum* and *Asparagopsis armata* extracts inhibited *Vibrio parahaemolyticus* biofilm formation by 20–30% and 30–40%, respectively, and *Bacillus subtilis* biofilms by 49–59% and 30–50%, respectively. These results are notably lower than the antibiofilm activity observed in our work on *R. okamurae*. Accordingly, the

present study demonstrates that *R. okamurae* holds promising potential as a source of novel, strong chemicals that combat infectious diseases, particularly those caused by *S. aureus* and *E. coli*. On other hand, ethanol extracts of *Sargassum* sp. have been recorded to exhibit the strongest inhibitory ability, with 73.72 ± 0.03% reduction in biofilm formation of *S. aureus* ATCC 29213 strains at 12.5mg/ mL **(Alreshidi *et al.*, 2023)**.

Our results further indicate that the antibacterial activity against *Staphylococcus aureus* (Gram-positive) was higher than against *E. coli* (Gram-negative) strains, likely due to the increased resistance of Gram-negative bacteria to antibacterial agents. Exploiting invasive species to obtain natural bioactive metabolites offers a dual advantage: the abundant biomass available for extraction and the potential to alleviate the detrimental impacts of invasive species through specimen collection, thereby enhancing ecosystem integrity and sustainability.

Noting the high antibacterial activity against Gram-negative germs (*E. coli*) due to the chloroform extract, the GC-MS analysis revealed a predominance of Methyl tetradecanoate (21.13%), 9-Octadecenoic acid (Z)-, methyl ester (20.61%), Pentadecanoic acid (8.55%), Methyl stearate (7.06%), Benzene, 1-fluoro-2-methoxy- (5.51%), Pentadecanoic acid, methyl ester (3.54%), 1,5,9-Cyclododecatriene (E,Z,Z)- (2.68%), and Docosanoic acid (2.08%). These compounds are responsible for the antibacterial activity against this germ. They may also act synergistically with other compounds that are not as abundant among the major compounds. Numerous studies have documented the high antibacterial activity of these compounds **(Alreshidi *et al.*, 2023; Muzahid *et al.*, 2023; El Mouns *et al.*, 2024)**.

The biologically active compounds in *R. okamurae* were determined using polarity-based solvents (chloroform, ethyl acetate, and methanol) to maximize compound extraction. The different polarities of the solvents impacted the extraction of biologically active molecules from the algae. GC-MS analysis of the different extracts showed various potential bioactive compounds (Figs. 7–9 & Tables 3, 4, and 5). A total of 14, 19, and 20 compounds were determined in EtOAc, chloroform, and methanol extracts, respectively. Some compounds were found in all solvents, such as Pentadecanoic acid, methyl ester, 9-Octadecenoic acid (Z)-, methyl ester, and Methyl stearate.

In the ethyl acetate extract (Fig. 7 & Table 3), Oleic acid, butyl ester (71.85%) was the most abundant compound, followed by 1-Heptadecyne (8.86%) and 9-Octadecen-1-ol (Z) (8.86%). In the chloroform extract (Table 4), Methyl tetradecanoate (21.13%) and 9-Octadecenoic acid (Z)-, methyl ester (20.61%) were predominant. The methanolic extract (Table 5) contained mostly Pentadecanoic acid, methyl ester (18.50%), 1-Chloroeicosane (17.29%), and 9-Octadecenoic acid (Z)-, methyl ester (14.77%).

The obtained biologically active compounds have therapeutic significance for human health. Oleic acid, butyl ester (71.85%), was the major bioactive compound found in ethyl acetate extracts from *R. okamurae* (Table 3). Many studies have shown that this compound has high antibacterial, antifungal, and antioxidant properties **(Dwivedi *et al.*,**

2012; Pinteus *et al.*, 2020; Mabrouki *et al.*, 2022). A significantly greater percentage of Oleic acid, butyl ester was also determined in chloroform extracts. This compound's presence in all three different fractions suggests the potential therapeutic importance of *R. okamurae*. Overall, the results obtained indicate that the combined therapeutic or medicinal effects of these compounds may be attributed to Oleic acid, butyl ester.

It should be noted that the ethyl acetate extract contains supraene, a biologically active compound with anesthetic activity **(Hemmings & Hopkins, 2006)**. The methanolic extract of *R. okamurae* contains a high abundance of three compounds: Pentadecanoic acid, methyl ester (18.50%), 1-Chloroeicosane (17.29%), and 9-Octadecenoic acid (Z)-, methyl ester (14.77%), which possess antioxidant and antimicrobial properties **(Alreshidi *et al.*, 2023)**.

This work provides the first comprehensive approach concerning the use of *R. okamurae* as a source of bioactive compounds with antimicrobial potency. Several compounds were isolated, some of which presented strong antimicrobial activity. New applications for this biomass are needed to mitigate the economic cost related to the massive removal of *R. okamurae* from beaches. Considering the carbohydrate-rich composition of *R. okamurae*, it appears as a promising raw material to produce bio-actives, in line with the aims of the circular economy.

The present work indicates that the macroalga *R. okamurae* represents a promising raw material to produce active substances with antimicrobial activity, which can be converted into value-added products in the pharmaceutical field. Marine algae are known to contain several interesting compounds, including polyunsaturated fatty acids (PUFAs), polysaccharides, alkaloids, and bioactive peptides. In brown algae, the content of alginates, fucoidans, and phenolic compounds such as phlorotannins is significant **(El-Beltagi *et al.*, 2022)**. These biomolecules are currently being used in the pharmaceutical, food, and nutraceutical industries.

Based on the results of this work, it can be affirmed that the macroalga *R. okamurae* could be a sustainable source of nutritional, functional, or bioactive compounds with promising potential for application in diverse industrial fields, such as human food and pharmaceuticals. This transformation of threat into opportunity aligns with the framework of the circular economy.

The pharmacological potential of *R. okamurae* is represented by its antimicrobial and antioxidant properties, indicating its potential use in treating diseases and boosting immunity. However, further studies are needed to quantify and assess the active compounds of this invasive alga. Additionally, the availability and seasonal variations of *R. okamurae* biomass on the coast need to be studied in greater depth.

CONCLUSION

Based on the findings of this study, it can be concluded that the macroalga *Rugulopteryx okamurae* exhibits significant potential as a source of bioactive compounds

with notable antimicrobial and antibiofilm activities. Various organic extracts, such as methanol, chloroform, and ethyl acetate, demonstrated high inhibitory effects against multiple bacterial strains, including both Gram-positive and Gram-negative pathogens. The presence of compounds like Oleic acid, butyl ester, Pentadecanoic acid, methyl ester, and 9-Octadecenoic acid (Z)-, methyl ester in these extracts underscores their potential therapeutic importance.

Furthermore, the study highlighted the effectiveness of different solvents in extracting bioactive molecules, with chloroform and methanol showing particular promise. The substantial phenolic and flavonoid content, as well as the high concentration of fucoxanthin, likely contribute to the observed antimicrobial activities.

This research represents the first comprehensive approach to utilizing *R. okamurae* for its bioactive properties, proposing a sustainable method to mitigate the ecological and socioeconomic impacts of this invasive species. By transforming the biomass of *R. okamurae* into value-added products for pharmaceutical and nutraceutical applications, this study aligns with the principles of the circular economy. Moving forward, further studies are necessary to quantify and assess the seasonal variations and availability of *R. okamurae* biomass to maximize its potential in various industrial applications.

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