JOURNAL OF TROPICAL LIFE SCIENCE
2020, Vol. 10, No. 3, 197 – 205
http://dx.doi.org/10.11594/jtls.10.03.03

Research Article

Bactericidal Potentiality of Purified Terpenoid Extracts from the Selected Sea Weeds and its Mode of Action

Sabira Siraj Sumayya [1], Abdulhadeef Shereefa Lubaina [2], Kumaraswamy Murugan [3]*

[1] Department of Botany, Sree Neelakanta Govt Sanskrit College, Pattambi, Kerala 695034, India
[2] Department of Botany, Christian College, Kattakada, Kerala 695572, India
[3] Center for Innovation in Science and Social Action (CISSA), Thiruvananthapuram, Kerala 695010, India

Article history:
Submission April 2020
Revised July 2020
Accepted July 2020

**Corresponding author:*
E-mail: harimurukan@gmail.com

ABSTRACT

Terpenoids are hydrocarbons involved in a variety of basic functions in plants such as growth, development and other physiological events. Terpenes and their associated molecules safe guard the organisms from pest, pathogen and herbivores. Similarly, therapeutically terpenoids function as antimicrobial agents against bacteria, fungi and viruses. The mechanism of bactericidal activities may be via inhibiting the synthesis of essential molecules like proteins, nucleic acids, cell-wall components, cell membrane derailment, bacterial DNA replication or inhibition of metabolic pathways. The crude methanolic extracts of the seaweeds were subjected to silica gel column chromatographic purification and eluted with different combinations of ethyl acetate: petroleum ether solvent systems. The eluted fractions were further subjected to thin layer chromatography and fractionated by GC-MS. The fractions obtained from *Hypnea musciformis* revealed the terpenoids such as eicosane, heneicosane, 2-pentadecnone, hexadecanoic acid methyl ester, n- hexadecanoic acid, hexadecanoic acid ethyl ester, heptadecanoic acid methyl ester, 11-octadecanoic acid metyl ester, whereas *Kappapycus alvarezii* displayed hexadecane, eicosane, heptadecane, octadecane, heneicosane, tricosane, hexadecanoic acid, methyl ester and beta amyrin. Similarly, *Gracillaria dura* revealed hexadecanoic acid methyl ester, n- hexadecanoic acid, 11-octadecanoic acid and phytol. Subsequently, the bactericidal activities of the purified terpenoid extracts from the sea weeds were carried. Initially, the extracts were tested for their *in vitro* antibacterial activity against six bacterial strains such as three Gram-positive (*Staphylococcus aureus*, *Streptococcus mutans*, *Enterococcus faecalis*) and three Gram-negative bacteria (*Klebsiella pneumoniae*, *Escherichia coli*, *Pseudomonas aeruginosa*) by disc diffusion method. The results revealed that the purified terpenoid extracts of *G. dura* exhibited significant bactericidal potentiality against *S. mutans* as compared to other strains. The zone of inhibition, MIC and MBC values narrate the efficacy of the purified terpenoid extract of the species. Remarkable leaching of metabolites like protein and DNA further substantiates the MIC and MBC results. Scanning electron microscopic observations such as clumping, irregularity of cells and ballooned walls reflect the possible membrane damage accounted in the cells by the terpenoid extracts. Further studies are planned to validate the above data by using molecular tools.

Keywords: Bactericidal activity, Disc diffusion method, Sea weeds, Terpenoids, Membrane damage

Introduction

Terpenes are hydrocarbons consist of 5-carbon isoprenoid units as their building blocks. They are produced via non-mevalonate (precursor methylerythritol phosphate) or mevalonate pathway (from acetyl CoA precursor). Their cyclic structure forms the backbone which may be multiples of isoprene units to form monoterpenes, sesquiterpenes; longer chains of diterpenes and

How to cite:
Sumayya SS, Lubaina AS, Murugan K (2020) Bactericidal Potentiality of Purified Terpenoid Extracts from the Selected Sea Weeds and its Mode of Action. Journal of Tropical Life Science 10 (3): 197 – 205. doi: 10.11594/jtls.10.03.03.

triterpenes [1]. Many terpenes display poor microbicidal activities. Terpenoids are terpenes derivatives i.e., modified terpenes through addition or removal of functional groups [2]. Thus, the antimicrobial potentialities of terpenoids are regulated by their functional groups. For example, the shifting or removal of a CH_3 group and addition of O_2 by enzymes result into derived terpenes. The OH group of the phenolic terpenoids and delocalized electrons are the microbicidal factors. Peppermint derived terpenes like linalool, menthol, carvacrol, thymol, citronella demonstrated synergistic activity against antibiotic resistant *E. coli O157:H7, Salmonella typhimurium, Staphylococcus aureus* and *Listeria monocytogenes* via disrupting *Klebsiella pneumoniae* carbapenemase via oxidative stress [3]. There are challenges involved in experimenting with terpenoids. Acquisition of terpenoids will demand higher cost when compared to synthetic chemicals. Natural phytochemicals have a molecular mass ≤ 500 g/mol may have the calibre to act as adjuvants for microbicides and exhibit synergistic roles [4]. Investigation of novel microbicidal agents via biotransformation is a recent trend practiced by molecular pharmacologists. Combination therapy with a low molecular weighed biomolecules like terpene derivatives has showed optimal outputs. Terpenes derivatives have proved microbicidal activities against susceptible and resistant pathogens [5]. Wagner and Merzenich [6] documented that synergic research approach was more effective in designing new generation phytopharmaceuticals for effective antimicrobial activities. In this juncture, the present study was carried to analyse the bactericidal potentialities of purified terpenoid extracts from the selected seaweeds and their plausible mode of action.

Material and Methods

The marine seaweeds such as *H. musciformis, K. alvarezii* and *G dura* were collected from the Mandapam coast (latitude 9˚17'N, longitude79˚ 22'E), Gulf of Mannar. The algal thalli were washed thoroughly to remove all the debris. Then, sliced into pieces, shade dried and powdered. Initially, each algal powder was subjected to Soxhlet hot continuous extraction with 250 ml of methanol as per the protocol narrated by Oussalah *et al.* [3]. The supernatants were gently decanted into a pre-weighed glass vials through Whatman No. 1 filter paper and concentrated to dryness using a rotary evaporator. The dried extracts were made up to a dosage of 10 mg/ml (stock solution) to be used in subsequent assays and stored at 4°C in tightly stoppered glass tubes [3, 4].

The purification of methanolic extracts was carried out using silica gel Column Chromatography (CC) with different solvent combinations of petroleum ether: ethyl acetate. The fractions so obtained was collected and further analysed by thin layer chromatography and fractionated by GC-MS. The TLC was carried out by using the solvent combinations of toluene and ethyl acetate in 6:1 ratio [3,4].

GC-MS analysis

Adeyemi [7] protocol was adapted for GC-MS analysis. The column purified terpenoid extract was injected into a HP-5 column (30 m X 0.25 mm i.d with 0.25 ìM film thickness), Agilent technologies 6890 N JEOL GC Mate II GC-MS model. Experimental conditions of GC-MS system were as follows. The flow rate of mobile phase (Helium: carrier gas) was set at 1 ml/min. In the gas chromatography part, temperature programme (oven temperature) was 40°C raised to 250°C at 5°C/min and injection volume was 1 µl. Samples dissolved in chloroform were run fully at a range of 50-650 m/z and the results were compared by using NIST Spectral library.

Strains of bacteria

For the *in vitro* bactericidal tests, three Gram-positive (*Staphylococcus aureus*, *S. mutans Enterococcus faecalis*) and three Gram-negative bacteria (*Klebsiella pneumoniae, Escherichia coli, Pseudomonas aeruginosa*) were purchased from Microbial Type Culture Collection and Gene Bank (MTCC), Chandigarh. None of the strains were multidrug-resistant categories.

Bactericidal assay-Agar disc diffusion assay

The bactericidal activity of the purified terpenoid extracts from the selected sea weeds was carried using the agar disc diffusion method of Bajpai et al. with marginal modifications [8]. Fresh sterile nutrient agar medium was prepared and cooled at 40–45°C before being poured into the sterilized petri plates (90 mm diameter). Ster-

ile Whatman No. 1 paper disc was used (6 mm in diameter). Varied concentrations of the purified terpenoid extracts were made with DMSO (100 mg/ml). Fresh bacterial cell suspensions were adjusted to 0.5 McFarland turbidity standards to prepare 1×10^8 bacterial/ml inoculum.

Bacterial suspension of each strain was inoculated on Mueller-Hinton agar plates, and the plates were then permitted to dry for 5 min. The sterile filter paper discs were soaked in 10 µL of each algal terpenoid extracts (concentrations of 0, 0.5, 1.0, 2.0, 3.0, and 5.0 mg/mL). The extract-soaked filter paper discs were then placed on the inoculated Mueller-Hinton agar plates. Gentamicin (10 µg/disc) disc was used as the positive control, and 10% DMSO-soaked filter paper disc was used as the negative control. Plates were incubated for 24 h at 32 ± 2°C. After incubation, the zones of inhibition were recorded as the diameter of the growth-free zones measured in mm using a Vernier caliper [9, 10]. The agar disc diffusion protocols were performed in triplicates.

Minimum inhibitory concentration (MIC) and Minimal bactericidal concentration (MBC)

The minimum inhibitory concentration (MIC) for terpenoid extract was evaluated according to method described by Bereksi *et al.* [9] and as per the Clinical and Laboratory Standards Institute (CLSI) protocol [11]. Sterile 96-well microplates were employed. Briefly, a 24-h culture of all bacterial strains was prepared after incubation at 37°C. The Mueller-Hinton broth containing 1.5×10^8 CFU/mL from each strain was prepared equivalent to 0.5 McFarland standards and 100 mL of it was added to each well of the 96 well sterile microplate. Different dilutions were made from the terpenoid extract in each row by serial dilution; in well numbered from 0.0625 to 10.0 mg/mL. Positive control of culture medium containing bacteria was prepared without the extract and negative control of pure extract. Microplates were incubated for 24 h at 37°C and were examined for turbidity Absorbance Microplate Readers at 570 nm. The lowest dilution of the extract with any turbidity was considered the MIC. All experiments were repeated thrice.

Minimum bactericidal concentration (MBC) was determined by the microplate dilution method on the 96-well sterile plate by adding MTT reagent (3-(4, 5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide). The dose of the extracts which could reduce the viable cell number was determined by MTT method. MTT colorimetric assay is based on the capacity of viable cell succinate dehydrogenase enzymes to reduce the water soluble yellow MTT into formazan, an insoluble colored product which is recorded by ELISA Microplate Reader. The reduction of MTT occurs only in metabolically active viable cells. Briefly, bacteria treated with extracts for 24 h at 36°C were incubated with 20 µl of MTT solution for 4 h at 36°C that was added to each well. (MTT reagent was prepared as 5 mg/mL in phosphate-buffered saline). Subsequently, the supernatant was slowly removed and 100 µL DMSO (dimethyl sulfoxide) was added to each well. DMSO is a solvent of formazan crystals and caused variable degrees of intensity of color spectrum from purple to white that is a measure of viable cells. The absorption of wells was measured at the wavelength of 570 nm by ELISA reader. In this method, the wells without any absorbance due to formazan crystals were considered to contain only dead cells. The last wells without formazan crystals were considered as MBCs.

Estimation of 260-nm absorbing material and protein release

The leakage of 260-nm absorbing molecule from the bacterial cells was measured as per the protocol of Du *et al.* [11] with little modifications. Cells from the 100 ml bacterial suspension were extracted by centrifuging at 6,000 x *g* for 10 min, washed thrice with 0.1M PBS (pH 7.4), and resuspended in PBS (0.1M, pH 7.4).The 100 ml of bacterial suspensions was incubated at 37°C under agitation in an incubator shaker for 5 h in the presence of terpenoid extracts of three different doses (control, 1×MIC, and 2×MIC). Then, the suspensions were centrifuged at 6,000× *g* for 10 min. The supernatants were diluted with phosphate-buffered saline (PBS) (0.1 M, pH 7.4). Then, the OD at 260 nm of the supernatants was recorded using 96-Well Plate Reader M200 per 4 h. Results were expressed as OD of 260-nm absorbing compounds. For protein leakage, the above protocol was repeated and the OD was noted at 280 nm. The amount of protein released was quantified from the standard curve of bovine serum albumin (BSA) [12].

Scanning electron microscopic analysis (SEM)

Kaya et al. [13] method was employed for the scanning electron microscopic study. The susceptible species to the terpenoid extracts such as *E. coli* and *S. mutans* were subjected to SEM analysis. Small agar pieces were cut out from the inhibition zone of the agar diffusion assay and were fixed in 3 % (v/v) glutaraldehyde buffered with 0.1 M sodium phosphate buffer (pH 7.2) for 1 h at room temperature and then washed thrice in sodium phosphate buffer. Further, the pieces were post-fixed in 1 % (w/v) osmium tetroxide for 1 h and then washed thrice in the buffer. Subsequently, the blocks were dehydrated in a graded alcohol series. The last stages of dehydration were performed with propylene oxide. The samples were dried and were mounted onto stubs using double-sided carbon tape, and then were coated with a thin layer of gold by a Polaron SC 502 sputter coater. They were viewed in a Jeol JSM 6060 LV SEM.

Statistical analysis

The data were reported as mean ± SD values obtained from a minimum of three determinations. Data were analysed using one way Anova using GraphPad Prism5 software. A significant difference was considered at the level of $P < 0.05$ and 0.01.

Results and Discussions

The crude algal powder was extracted with 250 ml of methanol for 8h using hot continuous soxhlet method. The extract was filtered and concentrated to dryness using rotary evaporator. The crude methanolic extract was subjected to purification by column chromatography (silica gel G of mesh size 230-400). Different combinations of petroleum ether: ethyl acetate (PE: EA) solvents were employed for the elution of terpenoids. Elution was started with 100% PE and gradually increased the polarity. The eluted fractions were analysed for the presence of terpenoids and subjected to TLC using 6 ml toluene: 1ml EA solvent system as mobile phase. Further, the bands obtained from TLC were subjected to GC-MS analysis. In fraction eluted using 95:5 (PE: EA) of *H. musciformis* showed a single prominent band in TLC and in GC-MS revealed the components such as eicosane, heneicosane, 2-pentadecnone, hexadecanoic acid methyl ester, n-hexadecanoic acid, hexadecanoic acid ethyl ester, heptadecanoic acid methyl ester, 11-octadecanoic acid metyl ester. 50:50 solvent combination of *K. alvarezii* showed two bands of terpenoids and its

Table 1. Agar disc diffusion assay of purified terpenoid extracts from the selected seaweeds in terms of diameter of zone of inhibition (mm)

	Purified terpenoid extracts from *K. alvarezii*					
(concentration mg/mL)	0	0.5	1.0	2.0	4.0	5.0
Bacterial strains	Diameter of inhibition zone					
S. aureus	0	5.0	6.8	7.3	8	8.2
S. mutans	0	6.02	6.4	6.8	7.2	7.3
E. faecalis	0	0.98	1.3	1.8	2.0	2.2
K. pneumoniae	0	0.54	0.6	0.8	1.2	1.3
E. coli	0	1.54	1.8	2.4	2.9	3.0
P. aeruginosa	0	1.0	1.4	1.8	2.0	2.0
F ratio= 1591.48*						
$CD_{(0.05)}$ = 0.218						
SE= 0.0872						
	Purified terpenoid extracts from *H. musciformis*					
(Concentration mg/ml)	0	0.5	1.0	2.0	4.0	5.0
Bacterial strains	Diameter of inhibiton zone					
S. aureus	0	8.0	8.8	12.8	14.5	16.72
S. mutans	0	10	15.	18	19.2	21.6
E. faecalis	0	1.78	2.0	2.2	2.3	2.5
K. pneumoniae	0	1.3	1.6	1.8	2.2	2.3
E. coli	0	3.1	4.6	6.23	7.98	9.22
P. aeruginosa	0	1.6	3.6	5.0	6.2	7.4
F ratio = 2690**						
$CD_{(0.05)}$ = 0.183						
SE= 0.053						
	Purified terpenoid extracts from *H. musciformis*					
(Concentration mg/mL)	0	0.5	1.0	2.0	4.0	5.0
Bacterial strains	Diameter of inhibiton zone					
S. aureus	0	7.45	8.3	10	12.1	12.3
S. mutans	0	12.2	25	25.43	25.72	25.76
E. faecalis	0	3.4	3.8	4	4.1	4.1
K. pneumoniae	0	2.12	2.6	3.09	3.2	3.3
E. coli	0	6	8.3	10.1	13	13.54
P. aeruginosa	0	4.6	6.9	7.0	8.2	10.21
F ratio = 4598.981**						
$CD_{(0.05)}$ = 0.32245						
SE= 0.01981						

GC-MS fractionation showed the presence of hexadecane, eicosane, heptadecane, octadecane, heneicosane, tricosane, hexadecanoic acid, methyl ester; beta amyrin. The 90:10 (PE: EA) purified fraction of *G. dura* showed 2 bands on TLC with hexadecanoic acid methyl ester, n- hexadecanoic acid, 11-octadecanoic acid and phytol molecules in GC-MS.

Bactericidal assay – Agar disc diffusion assay

The bactericidal potentiality of different doses (0.0, 0.5, 1.0, 2.0, 4.0, 5.0 mg/mL) of the purified terpenoid extracts from *K. alvarezii, H. musciformis* and *G. dura* against Gram-positive (*Staphylococcus aureus, S. mutans Enterococcus faecalis*) and Gram-negative bacteria (*Klebsiella pneumoniae, Escherichia coli, Pseudomonas aeruginosa*) were qualitatively and quantitatively analysed in terms of diameter of inhibition zone (mm). As narrated in the Table1, the DIZ data for *S. mutans* increased significantly ($P < 0.01$) with the increasing dosage of *G. dura* terpenoid concentrations as compared to *K. alvarezii* and *H. musciformis*. The DIZ values for *S. mutans* treated with *G. dura* were ranged from 12.2 mm to 25.76 mm. DIZ values noticed for *E. coli* were 6 to 13.54 mm as compared to *S. mutans*. Meanwhile, minimal response was displayed by *E. faecalis, P. aeruginosa* and *K. pneumonia* (resistant strains). Antibacterial reports categorize the microbicidal potentiality in to three classes based on zone of inhibition i.e., strong (DIZ > 20 mm), moderate (12 mm < DIZ < 20 mm) and weak activity (DIZ < 12 mm) [14, 15]. Based on this, *G. dura* displayed strong activity against *S. mutans* and moderate to weak activity in other strains.

MIC and MBC of bacteria vs terpenoid extracts

The MIC values for purified terpenoid extracts from *G. dura, H. musciformis* and *K. alvarezii*, against *S. mutans* were 0.065, 0.5 and 1.5 mg/ml respectively. Similarly, the respective MBC values of the strain were 0.12, 1.25 and 3.0 mg/mL (Table 2). Generally, MIC values up to 0.5 mg/mL are considered strong, 0.6–1.5 mg/mL as moderate, and MIC above 1.5 mg/mL is with low antimicrobial power [16]. The results of the present study showed that the purified terpenoid extracts of the sea weeds had a strong bactericidal activity against *S. mutans*, while low with *E. faecalis, P. aeruginosa* and *K.pneumoniae*. Similarly, the sea weeds showed variations in their MBC and MKC activities between the strains. This may be attributed due to the variations of terpenoid molecules and their content in the purified extracts of the sea weeds. 11-octadecanoic acid and phytol were present only in the purified terpenoid extracts of *G. dura*. *K. alvarezii* revealed the unique molecules such as hexadecane, heptadecane, octadecane, tricosane and beta amyrin. *H. musciformis* showed the molecules such as 2-pentadecnone, hexadecanoic acid methyl ester, hexadecenoic acid ethyl ester and 11-octadecanoic acid methyl esters. Similar variations in the constituents such as sabinene, α-pinene, β-pinene, limonene, β-caryophyllene, and caryophyllene were reported among the four major cultivars of black pepper [17]. It was previously reported that the mode of action of essential oils was through altering the bacterial cell membrane permeably and also capable to disintegrate the outer membrane of Gram-negative bacteria [18]. Further part of the study was restricted to the strains such as *S. mutans* and *E. coli* against the terpenoid extracts of *G. dura* only.

Table 2. MIC and MBC of purified terpenoid extracts from the selected sea weeds (mg/mL)

Bacterial strains	*G. dura*		*H. musciformis*		*K. alvarezii*	
	MIC	MBC	MIC	MBC	MIC	MBC
S. aureus	1.5	3.0	2.5	4.0	3.5	7.0
S. mutans	0.065	0.12	0.5	1.25	1.5	3.0
E. faecalis	3.0	8.0	5.0	10.0	6.5	13.0
K. pneumoniae	6.0	12.0	7.5	15.0	8.5	18.0
P. aeruginosa	2.5	5.0	3.5	7.0	4.0	8.0
E. coli	1.25	2.5	2.0	4.0	3.0	5.0
F ratio	782.4**	578.5**	165.7*	96*	109*	102.1*
SE	0.043	0.017	0.0293	0.0310	0.044	0.009
CD (0.05)	0.713	0.356	0.287	0.176	0.231	0.177

Leakage of 260-nm absorbing material and protein

The leakage of nucleic acid and proteins from the *S. mutans* and *E. coli* cells against purified terpenoid extracts from *G. dura* was displayed in the Table 3. The absorbance values for nucleic acid leakage of *S. mutans* increased remarkably at 24 and 48 h at 1 x MIC concentration ($P < 0.01$), whereas effectively from 8 to 48 h with 2 × MIC dose ($P < 0.05$) treatments. The OD_{260} nm values of 2 × MIC were higher than that of 1 x MIC. However, the OD_{260} nm values for control were marginal throughout the experimental periods ($P < 0.01$). *E. coli* also showed a more or less similar trend but with less magnitude.

Similarly, the protein leakage values from *S. mutans* and *E. coli* cells increased at par with duration and concentrations of the terpenoid extract (1×MIC and 2×MIC ($P < 0.01$). The values of protein leakage at 2×MIC was maximal in *S. mutans* than that of 1×MIC i.e., 125.3 µg/mL at 48 h. Meanwhile, the similar value for *E. coli* was 100.3 µg/mL (Table 4.) The values of protein leak for control displayed the lowest values ($P < 0.05$). The 260 nm absorbing materials and proteins are used as biomarker for evaluating the irreversible disintegration to the cell membrane structure in the experimental groups when compared to control. Similar to the present data, the bactericidal potentiality of essential oil against membrane permeability of food-borne pathogens was recorded [19]. The vital biomolecules such as nucleic acids and proteins are compartmentalized in the cell membrane and also in the cytoplasm and are the key molecules for the active phase of life in the bacterial cells. The obtained data revealed that the exposure of *S. mutans* and *E. coli* to purified terpenoid extracts caused a drastic loss of 260 nm absorbing materials and proteins, suggesting an irrecoverable damage accounted in the cell membranes. Further, the leakage of nucleic acids and proteins may lead to functional disorders in the metabolic cycles of the cells and subsequently inhibits the bacterial growth.

Scanning electron microscopic observations

The morphological variation of *S. mutans* and *E. coli* cells treated with purified terpenoid extracts of *G. dura* and untreated control cells was evaluated by SEM. *S. mutans* control cells displayed the smooth surface of shape and its rigidity to form normal cocci chains (Figure 1A). Meanwhile, abnormalities were noticed on the cells after exposure with the terpenoid extract which was reflected by the formation of cavities initiated to lyse, shrunk, and collapse of cells (Figure 1B, C, and D). The disruption of the cells has continuously occurred to form debris i.e., most of the cells were lysed, shrunk drastically, and completely collapsed. The normal and smooth cocci chains shape was no longer observed but only the crumpled cell residues. Treatment and these destructions in bacterial cells may be due to severe morphological disorganization and invaginations resulted in to clumping (secrete sticky mucus) as a response to stress condition.

Table 3. Release of 260 absorbing material in terms of OD_{260} from *S. mutans* and *E. coli* against purified terpenoid extracts from *G. dura*, Control, MIC 1×, and MIC 2×

	0h	4h	8h	12h	16h	20h	24h	48h
	Release of 260 absorbing material from *S. mutans*							
Control	0.047	0.047	0.047	0.047	0.047	0.047	0.047	0.047
MIC X	0.089	0.143	0.241	0.276	0.298	0.323	0.376	0.489
MIC 2X	0.095	0.187	0.294	0.365	0.504	0.599	0.631	0.754
F ratio= 1904.27**								
CD(0.05)= 0.316								
SE= 0.1076								
	Release of 260 absorbing material from *E. coli*							
Control	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.04
MIC X	0.067	0.131	0.195	0.23	0.280	0.30	0.34	0.40
MIC 2X	0.072	0.143	0.21	0.279	0.38	0.46	0.49	0.52
F ratio= 785.47**								
CD(.05)= 1.095								
SE=0.2317								

Table 4. Release of protein (µg/mL) from *S. mutans* and *E. coli* against purified terpenoid extracts from *G. dura* Control, MIC 1× and MIC 2×

Release of protein from *S. mutans*	0h	4 h	8 h	12 h	16 h	20 h	24 h	48 h
Control	1.46	1.48	1.487	1.49	1.493	1.497	1.49	1.53
MIC X	31.4	43.2	47.5	49.2	54.3	57.5	66.9	70.23
MIC 2X	35	67.8	76.6	90	98.4	108	116.5	125.3
F ratio	590**							
SE	0.1682							
$CD_{(0.05)}$	0.953							
Release of protein from *E. coli*								
Control	1.36	1.38	1.383	1.40	1.43	1.44	1.435	1.461
MIC X	26.5	30.2	36.4	39.2	43.3	46.5	54.2	60.3
MIC 2X	28.9	44.5	58.6	68.5	76.4	87.8	93	100.3
F ratio	1003**							
SE	0.1743							
$CD_{(0.05)}$	0.4872							

The control *E. coli* cells were about 3.5 µm long and showed smooth and intact surface (Fig. 2 A control). After exposure to terpenoid extracts, the cells were shortened to 1 µm with remarkable compactness (Fig. 2 B, C & D). Blisters were seen followed by protrusion of numerous small bubbles. Further, alterations of cell membrane were seen with abnormal cell shapes. Similarly, sticky membranes, deep craters, lysed and completely burst cells. SEM micrographs showed asymmetric cytoplasmic turbidity and also the irregularity of distribution.

Similar changes in the bacterial cells treated with oregano and thyme essential oils and there by confirmed their antimicrobial power against *E. coli* O157:H7 [19]. Burt [18] correlated the mode of action of essential oils with cell membrane damages in bacterial cells. Further, the mode of action may be multiple such as the damage of proteins on the membrane, the binding molecules of the envelop, leakage of cell contents, cytoplasmic coagulation and derailment of the proton motive force. The antibacterial activity of various leaf extracts of *Merremia emarginata* validates the present results displayed by the terpenoid extracts [19]. All the above research outputs indicate that purified terpenoid extracts of *G. dura* possess bactericidal activity as reflected by zone of inhibition, MIC and MBC values. The cell wall and cell membrane lysis further inhibit the bacterial growth. Leakage of nucleic acid and proteins further substantiates the SEM visuals. Further studies are warranted at clinical level to validate the obtained results. The current challenges and perspectives of the usage of aqueous

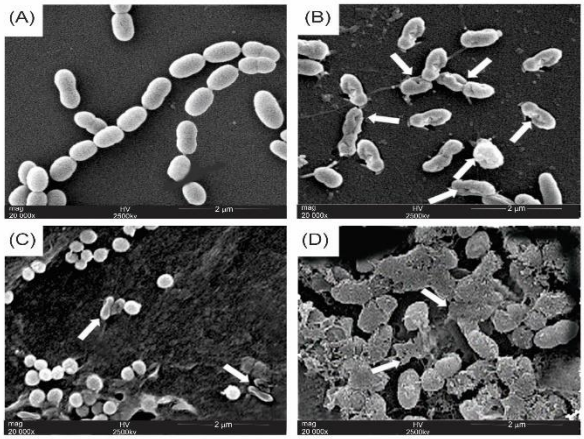

Figure 1. SEM images showing the morphological variations in *S. mutans* A – control, B, C, and D treated cells with abnormalities

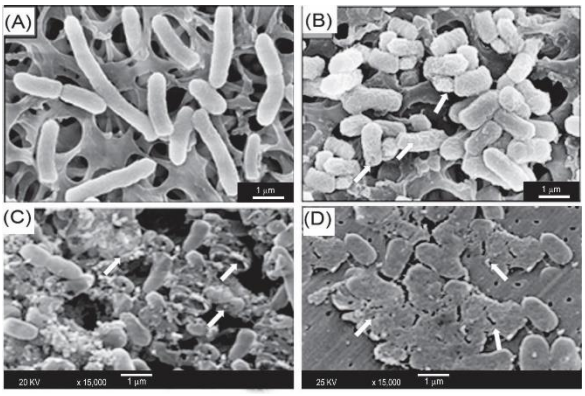

Figure 2. SEM images showing the morphological variations in *E. coli.* A – control, B, C, and D treated cells with damages, deformities & clumping

plant extracts were also validated against *Salmonella enterica* [21]. The antimicrobial values and

mode of action of some plant extracts against food pathogens and the spoilage microbes, antimicrobial activity of plant extracts against antibiotic susceptible and resistant bacterial strains causing wound infection, *in vitro* microbial power of medicinal herbals against human pathogenic bacteria, anti-bacterial activity of *Ricinus communis* against bacterial pathogens like *Escherichia coli* and *Klebsiella oxytoca* and antimicrobial activity of diverse extracts of *Ocimum basilicum* supports the present mode of action of terpenoids from *G. dura* in terms of its antibacterial efficacy [22, 23, 24, 25, 26, 27].

Conclusion

Among the three sea weeds evaluated, the *Gracillaria dura* presented potent bactericidal activities as compared to others. The terpenoid fractions of the species showed strong antibacterial activity against *S. mutans*. The obtained visuals of SEM indicate the mode of the bacterial cell death of the evaluated *S. mutans* was due to loss of cellular membrane integrity or function. Leaching of 260 molecules and protein substantiates the SEM data. The present study brings an insight about the antibacterial activity of the red algae. Similarly, an understanding for the future researches of synergism, mode of action and bioavailability of various sea weed terpenoid components.

References

1. Christianson DW (2017) Structural and Chemical Biology of Terpenoid. Chemical Reviews 117(17):11570–11648.doi: 10.1021 /acs.chemrev.7b00287.
2. Jiang Z, Kempinski C, Chappell J (2016) Extraction and Analysis of Terpenes/Terpenoids. Current Protocols Plant Biology 1(4):345–358.doi: 10.1002/cppb.20024.
3. Oussalah M, Caillet S, Saucier L, Lacroix M (2007) Inhibitory effects of selected plant essential oils on the growth of four pathogenic bacteria: *E. coli O157:H7, Salmonella Typhimurium, Staphylococcus aureus and Listeria monocytogenes*. Food Control 18(6): 414–420.doi: 10.1016/j.foodcont.2005.11.009.
4. Barbieri R, Coppo E, Marchese A et al. (2016) Phytochemicals for human disease: An update on plant-derived compounds antibacterial activity. Microbiological Research 3 (7): 196 - 214. doi: 10.1016/j.micres.2016.12.003.
5. Mahizan NA, Yang SK, Moo CL, et al. (2019) Terpene derivatives as a potential agent against antimicrobial resistance (AMR) Pathogens. Molecules 24(14): 2631 2641. doi :10.3390/molecules24142631.
6. Wagner H, Ulrich-Merzenich G (2009) Synergy research: Approaching a new generation of phytopharmaceuticals. Phytomedicine 16(4): 97–110. doi: 10.1016/j.fitote.2010.11.016.
7. Adeyemi M (2017) Phytochemical analysis and GC-MS determination of *Lagenaria breviflora* R. fruit. International Journal of Pharmacogonsy and Phytochemical Research 9 (7):1045-1051. doi: 10.25258/phyto.v9i07.11178.
8. Bajpai VK, Sharma A, Baek KH (2013) Antibacterial mode of action of *Cudrania tricuspidata* fruit essential oil, affecting membrane permeability and surface characteristics of food-borne pathogens. Food Control 32 :582–590 doi:10.3389/fmicb.2017.00552.
9. Bereksi MS, Hassaïne H, Bekhechi C, Abdelouahid DE (2018) Evaluation of antibacterial activity of some medicinal Plants extracts commonly used in Algerian traditional medicine against some pathogenic bacteria. Pharmacogn Journal 10 (3):507-512. doi: 10.5530/pj.2018.3.83.
10. Humphries RM, Ambler J, Mitchell SL et al. (2018) CLSI methods development and standardization working group best practices for evaluation of antimicrobial susceptibility tests. Journal of Clinical Microbiology 56 (4): e01934-17. doi:10.1128/JCM.01934-1.
11. Du W, Sun C, Liang Z et al. (2012) Antibacterial activity of hypocrellin A against *Staphylococcus aureus*. World Journal of Microbiology and Biotechnology 28(3): 3151–3157. doi: 10.1007/s11274-012-1125-z.
12. Xu JG, Hu QP, Wang XD et al. (2010) Changes in the main nutrients, phytochemicals, and antioxidant activity in yellow corngrain during maturation. J. Agriculture and Food Chemistry 58 (7): 5751–5756. doi: 10.1021/jf100364k.
13. Kaya I, Yigit N, Benli M (2008) Antimicrobial activity of various extracts of *Ocimum basilicum* L. and observation of the inhibition effect on bacterial cells by use of scanning electron microscopy. African Journal of Traditional, Complementary and Alternative Medicines 5 (4): 363-369 doi: 10.4314/ajtcam.v5i4.31291.
14. Rota MC, Herrera A, Martinez RM et al. (2008) Antimicrobial activity and chemical composition of *Thymus vulgaris, Thymus zygis* and *Thymus hyemalis* essential oils. Food Control 19 (4): 681–687. doi: 10.1016/j.foodcont.2007.07.00.
15. Weerakkody NS, Caffin N, Turner MS, Dykes GA (2010) *In vitro* antimicrobial activity of less-utilized spice and herb extracts against selected food-borne bac-

teria. Food Control 21 (5): 1408–1414. doi: 10.1016/j.foodcont.2010.04.014.

16. Aligiannis N, Kalpoutzakis E, Mitaku S, Chinou IB (2001) Composition and antimicrobial activity of the essential oils of two *Origanum* species. Journal of Agriculture and Food Chemistry 49 (2): 4168–4170.doi:10.1021/jf001494m.
17. Menon AN, Padmakumari KP, Jayalekshmy A (2003) Essential oil composition of four major cultivars of black pepper (*Piper nigrum* L.). Journal of Essential Oil and Research 15 (1): 155–157. doi:10.1080/10412905.2003.9712099.
18. Burt S (2004) Essential oils: their antibacterial properties and potential applications in foods- a review. International Journal of Food Microbiology 94(2):223–253. doi:10.1016/j.ijfoodmicro.2004.03.022.
19. Burt SA, Reinders RD (2003) Antibacterial activity of selected plant essential oils against *Escherichia coli* O157:H7. Letters in Applied Microbiology 36: 162-167.doi: 10.1046/j.1472-765X.2003.01285.x.
20. Elumalai EK, Ramachandran M, Thirumalai T, Vinothkumar P (2011) Antibacterial activity of various leaf extracts of *Merremia emarginata*. Asian Pacific Journal of Tropical Biomedicine 1 (5): 406–408. doi:10.1016/S2221-1691(11)60089-0.
21. Santos SAO, Martins C, Pereira C et al. (2019) Current challenges and perspectives for the use of aqueous plant extracts in the management of bacterial infections: the case-study of *Salmonella enterica*. International Journal of Molecular Science 20 (4): 940 – 947. doi:10.3390/ijms20040940.
22. Gonelimali FD, Lin J, Miao W et al. (2018) Antimicrobial properties and mechanism of action of some plant extracts against food pathogens and spoilage microorganisms Frontiers in Microbiology 8 (2): 1639 -1643. doi: 10.3389/fmicb.2018.01639.
23. Atef NM, Shanab SM, Negm SI, Abbas YA (2019) Evaluation of antimicrobial activity of some plant extracts against antibiotic susceptible and resistant bacterial strains causing wound infection. Bulletin of the Natural Research Center 43 (5): 1-5.doi: 10.1186/s42269-019-0184-9.
24. Kalra SS Goldy Saxena (2011) Antimicrobial activity pattern of certain terpenoids. International Journal of Pharmaceutical and Biological Science 2 (1): 87-91. doi:10.1.1.294.1573.
25. Manandhar S, Luitel S, Dahal R (2019) In Vitro antimicrobial activity of some medicinal plants against human pathogenic bacteria. Journal of Tropical Medicine 54(2):1-7.doi:org/10.1155/2019/1895340.
26. Hajrah N, Abdul WM, Sabir J et al. (2018) Antibacterial activity of *Ricinus communis* L. against bacterial pathogens *Escherichia coli and Klebsiella oxytoca* as evaluated by Transmission electron microscopy, Biotechnology and Biotechnological Equipment 32(3): 686-691.doi: 10.1080/13102818.2018.1451778.
27. Kaya I, Yiğit N, Benli M (2008) Antimicrobial activity of various extracts of *Ocimum basilicum* L. and observation of the inhibition effect on bacterial cells by use of scanning electron microscopy African Journal of Traditional, Complementary and alternative medicine 5 (4): 363 – 369.doi: 10.4314/ajtcam.v5i4.31291.

This page is intentionally left blank