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Biological Active Metabolite Compounds for Antibacterial Agent From Extremophile Microalgae

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Abstract

The present investigation aimed to investigate and identify antimicrobial activity of four extremophile microalgae including *Tetraselmis suecica*, *Halochlorella rubescens*, *Cocomyxa dispar* and *Scendesmus parvus*. The comparative study of bioactive compounds in extraction yield, phytochemical screening and antimicrobial activities using disk diffusion method against pathogenic bacteria *Escherichia coli*, *Pseudomonas aeruginosa*, *Streptococcus* sp., and *Staphylococcus aureus* from two extraction solvent were evaluated. The study indicated that methanol extract gave higher extraction yield of all tested microalgae compared to those chloroform as solvent. Phytochemical screening analysis reveals the presence of flavonoids, alkaloids, tannins and saponin in methanol extract of *T. suecica*, *C.dispar* and *H. rubescens*. The study also showed that the microalgae crude extract exhibited effective inhibitory activity against all tested bacteria. The highest zone inhibitory of 11.33±1.23 mm and 11.67±0.37 mm was observed for *H. rubescens* chloroform extract and *S. parvus* methanol extract against *S. aureus*. The microalgal crude extracts were further chemically characterized by gas chromatography mass spectroscopy (GC−MS). GCMS profiling of both methanol and chloroform extract of all tested microalgal reveals the presence of different bioactive compound such as palmitic acid, octadecanoic acid, neophytadiene, phytol, loliolide and stigmasterol, with important antimicrobial properties. This finding indicates a promising antimicrobial activity of microalgal extract and can be possible to further develop for biologically active compound in various application such as aquaculture, nutraceutical, and food supplements.

Keywords: bioactive compound, phytochemical, antimicrobial, microalgae

1. Introduction

Microalgae is a unicellular photosynthetic microorganism and can be found in wide range of habitats including marine and fresh water environments (Mandal and Mallick 2014). Divers microalgae strains contain various metabolites such as lipid, carbohydrate, protein and minerals make its possibly potential source for various applications (Hachicha *et al.* 2022). Nutrient-rich microalgae biomass has been reported to have potential application as supplement for animal feed and health products (Agrawal and Verma 2022, Wang *et al.* 2021). Microalgae possess more advantages over other plant materials as such microorganisms are easy to cultivate, exhibit high growth rate, and are environmentally friendly and renewable. In addition, the microalgae-extracts from different microalgae strains consist of bioactive constituents that offer unlimited source of new bioactive compounds with different biological activities (Falaisae 2016., Hussein *et al.* 2020, Silva *et al*. 2021, Ghasemi *et al*. 2006).

Studies on screening and identification of metabolites from various microalgae strains found that bioactive compounds extracted such as fatty acids, acrylic acid, halogenated aliphatic compounds, terpenes, sulphur containing hetero cyclic compounds, carbohydrates and phenols demonstrated potential antimicrobial properties (Najdesnki *et al*. 2013, Toshkova-yotova *et al.* 2022, Kokou *et al*. 2012, Jun *et al*. 2018). These substances can inhibit the growth of microorganisms or eradicate them. In addition, the bioactive metabolites, such as phenolic compounds (e.g. simple phenols), phenolic acids (e.g. derivatives of benzoic acid and cinnamic acid), coumarins and flavonoids, from microalgae have also received much attention. These metabolites are known to have a wide range of pharmacological activities including, antimicrobial, antiviral, antifungi, anti-inflammatory, anti-oxidant and anticancer activities.

Isolation and characterization of bioactive secondary metabolites from various microalgae have been reported by many studies (Acurio *et al*. 2018, Makridis *et al*. 2006). The usage of extremophile microalgae in biotechnological applications like production of bioactive compounds has

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much greater potential. In general, production or accumulation of secondary bioactive metabolite is to aid cells in their interaction with the environment and in protection from predators as well as abiotic stress. Such microorganisms maintain their vitality by producing more stable materials in adapting to the changing environmental conditions. It is known that bioactive compound yield and its activity extracted from microalgae are significantly influenced by microalgae strain, cultivation condition, type of solvent and extraction method (Lomakool *et al*. 2021, Monteiro *et al*. 2020, Stirk *et al*. 2020). The accumulation of secondary bioactive metabolite can be influenced by several factors, and stress condition has been identified to be among the major factors that could affect the quality of the metabolites (Lafarga *et al*. 2021, Little *et al*. 2022). Microalgae that are able to grow in stress and extreme condition require the microalgae to maintain their metabolisms by adapting the changes which could require high energy consumption. This condition will eventually reduce photosynthesis activity, resulting in accumulation of intermediate compound such as secondary metabolite within the cell. A Study by Killic *et al.* (2018) on the biological and antimicrobial activity of *Dunaliella* sp. indicated that higher activity was observed from the microalgae extract cultivated under elevated NaCl concentration. Another study by Ameri *et al.* (2021) also found that changes of light exposure during cultivation condition could significantly affect the antimicrobial activity of *Chlorella* sp. extract. However, to the best of our knowledge, there are limited information of microalgae grown supplemented with elevated CO_2 conditions on their biological and antimicrobial activity. In addition, despite the many studies on the antimicrobial and antioxidant activity from different strains, no such investigation has been reported on the extremophile *Tetraselmis suecica*, *Halochlorella rubenses*, *Scenedesmus parvus* and *Cocomyxa dispar* strains. Thus, this study was designed to screen and evaluate ability of these locally isolated microalgae to produce antimicrobial and antioxidant compounds. The scientific novelty of this study lies in the identification of the extremophile microalgae strain that poses potential biological and antimicrobial activity which can be further developed for a new antimicrobial drug from renewable resources.

2. Materials And Methods

2.1. Microalgae strains

Four different extremophile microalgae, namely *Halochlorella rubescens*, *Tetraselmis suecica*, *Coccomyxa dispar* and *Scenedesmus parvus*, were used throughout the experiment. Microalgae *T. suecica* and *H. rubescens* were obtained from CSIRO Australia, while *C. dispar* and *S. parvus* were isolated from abandoned mining area at Tasik Katak (2°7'60" N · 103°51'0" E), Pulau Pinang Malaysia. The microalgae were grown in Bold Basal's Medium (BBM) medium that consisted of the following chemicals: 25 g/L sodium nitrate ($NaNO_3$), 7.5 g/L magnesium sulphate heptahydrate ($MgSO_4.7H_2O$), 2.5g/L sodium chloride (NaCl), 7.5 g/L dipotassium phosphate (K_2HPO_4), 17.5 g/L potassium dihydrogen phosphate (KH_2PO_4), 2.5 g/L calcium chloride dehydrate ($CaCl_2.2H_2O$), 8.82 g/L zinc sulphate heptahydrate ($ZnSO_4.7H_2O$), 1.44 g/L manganese chloride tetrahydrate ($MnCl_2.4H_2O$), 0.71 g/L molybdenum trioxide (MoO_3), 1.57 g/L copper sulphate pentahydrate ($CuSO_4.5H_2O$), 0.49 g/L cobalt nitrate hexahydrate ($Co(NO_3)_2.6H_2O$), 11.42 g/L boric acid (H_3BO_3), 50 g/L ethylenediaminetetraacetic acid (EDTA), 31 g/L potassium hydroxide (KOH), 4.98 g/L iron sulfate heptahydrate ($FeSO_4.7H_2O$) and 1 mL sulfuric acid (H_2SO_4). The cultivation process was conducted in two phases. Initially, the seed microalgae culture was grown in BBM medium at 32±3 °C, pH 7 and exposed to light intensity of 1500 lux for 14 days. The active microalgae culture was then centrifuged and standardized its optical density (OD_{680}) to 1.0. A total of 10% (v/v) of the standardized active microalgae seed culture was then added in the 1L Schott bottle, and the mixture was kept at 32 °C, pH 7 under light intensity of 1500 lux supplemented with 5% CO_2 intermittently for 14 days.

The growth of the microalgae was monitored every 24 hours. For this analysis, a total of 1 mL of the sample was withdrawn and was measured using spectrophotometer (Hach, DR-5000). The relationship with the microalgal cell concentration was determined by correlating the absorbance at 680 nm and dry cell weight (DCW). The microalgal DCW was calculated using the following equation (1-4):

$$\text{Biomass concentration}_{(H.rubescens)} = 0.549\,(OD_{680}) - 0.0046 \quad (1)$$

$$\text{Biomass concentration}_{(T.suecica)} = 0.524\,(OD_{680}) - 0.0129 \quad (2)$$

$$\text{Biomass concentration}_{(C.dispar)} = 0.2845\,(OD_{680}) + 0.0016 \quad (3)$$

$$\text{Biomass concentration}_{(S.parvus)} = 0.574\,(OD_{680}) - 0.00469 \quad (4)$$

After completed the cultivation period, the cell was harvested by centrifugation and kept for oven dried at 24 to 48 hours at 60°C. The dried microalgae biomass was then kept in tight container for further analysis.

2.2. Preparation of the microalgae crude extract

Approximately, 1000 mg of the dried microalgae samples were weighed, and then soaked in two different solvents namely methanol and chloroform. Each of the microalgae biomass was pre-treated using ultrasonication at 40 °C, 100 W and 40 kHz for 20 min and then kept for mixing for 72 h at room temperature. After the extraction process, the extracts were filtered, and the supernatant were collected, while the pellet fraction was re-extracted for three times to ensure the maximum metabolite recovery. The supernatant was then concentrated by evaporation to dryness at 40 °C using rotary evaporator prior to be used for the phytochemical screening. The yields of extracts were calculated by using the following formula (Norul Azilah *et al*. 2020):

$$Percentageyield = \frac{\text{weight of dry crude extract}}{\text{weight of dried microalgae}} \text{X}100$$

2.3. Phytochemical screening

The presence of secondary metabolites in the extract was investigated using qualitative phytochemical analysis. Its standard protocols were used to test for terpenoids, flavonoids, tannins, saponins, and sterols. Each test's aqueous extract was made by dissolving 40 mg of crude extract in 5 mL of solvent and filtering the mixture.

2.3.1. Tests for terpenoids

Determination of terpenoid in the microalgal crude extract was performed using Salkowski test (Das *et al*. 2014). In this analysis, a total volume of 2 mL of chloroform was added into the tube containing 2 mL of aqueous extract, and the sample was mildly shaken for 5 minutes. Then, 2 mL of concentrated sulfuric acid was added into the mixture to form a layer. The presence of reddish-brown color at the interface of the mixture indicates the terpenoids detected in the sample.

2.3.2. Test for flavonoids

Determination of flavonoids in the microalgal crude extract samples was performed using Alkaline reagent test (Munir *et al.* 2020). In this analysis, three to four drops of dilute sodium hydroxide (NaOH) solution were dropped to the tubes containing 2 mL of aqueous extract. Then, 2 mL of dilute acid was added to the tubes. The changes in the color and formation of a strong yellow color in the mixture indicates the presence of flavonoids in the extract.

2.3.3. Test for tannins

Determination of tannins in the microalgal extract was carried out via Lead acetate test as per described by Abdel-Karim *et al.* (2020). A total 3 mL of an aqueous extract was placed in a test tube, and then a few drops of $FeCl_3$ solution were added. The presence of greenish-black precipitate indicates the presence of tannins in the sample.

2.3.4. Test for saponins

Determination of saponins in the microalgal crude extract was performed via Foam test with slight modifications (Kancherla *et al*. 2019). Firstly, a total 50 mg of microalgal extract was mixed with 20 mg of sodium bicarbonate ($NaHCO_3$) diluted with distilled water and the sample was shaken vigorously until the formation of honeycomb-like froth. The formation of one centimetre layer of foam indicates the presence of saponins.

2.3.5. Test for sterols

Sterol in the extracted sample was determined by Salkowski reaction test as per described by Mojab *et al.* (2003). A total of 40 mg of extracted sample was dissolved in 2 mL of chloroform and filtered. Then, the filtrated sample was treated with 1mL of concentrated sulfuric acid (H_2SO_4). The presence of sterols in the microalgal extracted sampled was confirmed by the 2 phase formation with red color in the chloroform phase.

2.4. Antibacterial test

The antibacterial activity test of microalgal crude extract was performed by disc diffusion method (Mohamad-Hanafiah et al. 2015). The bacterial tests such as *Bacillus cereus, Staphylococcus aureus, Pseudomonas aeruginosa* and *Escherichia coli* were used in this study. Those bacteria were obtained from School of Industrial Technology, Universiti Sains Malaysia. The bacteria inoculum containing $1x10^8$ cell/mL was uniformly spread over the nutrient agar/Mueller-Hinton agar (MHA)/molten agar using sterilized cotton buds. For this experiment, the crude extract with 10 µg/ml was applied on the sterile disc and solvent was kept to evaporate prior to be placed on the inoculated plate. Similarly, commercial antibiotic such as Gentamycin (50 µg/disc) and Ampicillin (50 µg/disc) was used as positive control while solvent as negative control. All the plates were then incubated at 37 ± 2 °C for 24 hours, and the diameter of the minimum zone of inhibition was measured in mm. For each test, three replicates were performed.

2.5. Minimal inhibitory concentration (MIC)

In order to identify the optimum antimicrobial concentrations of microalgae extract against different bacteria, the minimal inhibitory concentration (MIC) was performed via two-fold serial dilution method in 96 well plates. In this analysis, the microalgal extracts were diluted in 1ml sterile deionised distilled water and a total of 100 µl was added in 96 well plates. Then, a total of 100 µl the standardized bacteria with concentration of 10^8 CFU/mL was added into the well to final volume 200 µL/well. Wells containing only sterile MHB were used as a negative control and gentamicin serves as a positive control. The plate was incubated at 37° C for 24 hours. MIC values were taken as the lowest concentration of extracts that produced no visible bacterial growth when compared with the control tubes after incubation period.

2.6. Gas chromatography – mass spectrometry (GC-MS)

The phytocomponent in the microalgal crude extract was determined using gas chromatography-mass spectrometer (GC/MS) (QP2010 Ultra Gas Chromatograph Mass Spectrometer (Shimadzu, Japan)) equipped with SUPELCOWAX^{TM} 10 Capillary GC (L × I.D. 30 m × 0.25 mm, df 0.25 µm) with direct capillary column bpx5. Helium gas was used as a carrier gas at a constant flow rate of 1 ml/min. For each injection, the solvent delay was 2 min, and 1 µL of diluted samples was injected automatically using an auto sampler in the split mode. The injector temperature was 290 °C. All the analysis was conducted at the following column temperature program: initial temperature of 50 °C holding for 1 min, and the increased by 2 °C/min to 180 °C withhold 3 min then to 280 °C by 2°C/min withhold 5 min and at 300 °C increased by 2°C/min held for 7 min. The GC-MS was operated in the electron ionization (EI) mass spectra, and the data were collected at 70 eV ionization voltages over the range of m/z 40-650 in full scan mode.

2.7. Statistical analysis

All results were expressed in triplicate to calculate the mean ± standard error. In order to be considered statistically significant, a value of $P < 0.05$ was performed using Minitab 16 software.

3. Results

3.1. Microalgae growth profile

The growth profile of four microalgae strain was monitored for 14 days of cultivation and the growth profiles are presented in Fig. 1. The study indicated that all the three microalgae strain exhibited different growth pattern, and *H. rubescens* exhibited highest biomass production of 0.81±0.02 g/L followed by *C. dispar* after 15 days of cultivation. On the other hand, the lowest microalgae biomass production of *T. suecica* was observed for 0.54±0.02 g/L. The maximum biomass concentration of *S. parvus* of 0.67±0.03 g/L was obtained for 15 days of incubation.

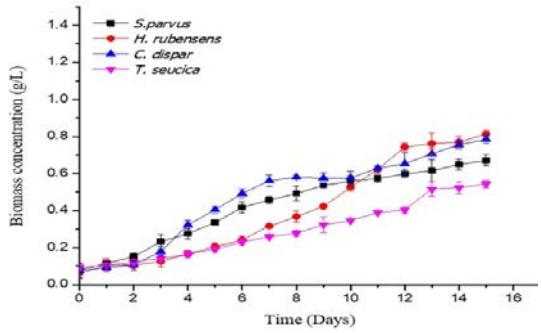

Figure 1. Growth profile of different microalgae cultivated at pH 7, light intensity 1500 lux, at 32±2.0℃ and supplemented with elevated CO_2 for 14 cultivation days.

3.2. Crude Extraction yield

Fig. 2 shows the crude extract yield obtained from different microalgae biomass using two different solvents. Overall, it was found that extraction using methanol gave better effect on extraction yield of all types of microalgae strain in comparison to extraction using chloroform. The highest crude extract yield was obtained from the extraction of *T. suecica* using methanol with 46.68% followed by extraction of *S. parvus* using similar solvent with yield of 31.90%. On the other hand, high extract yield for extraction using chloroform was observed for extraction using *C. dispar* biomass with yield of 15.12%. Only 3.54% of crude extract was successfully extracted from *S. parvus* using this solvent. It can be found that solvent and type of microalgae used for the extraction significantly affect the extraction yield.

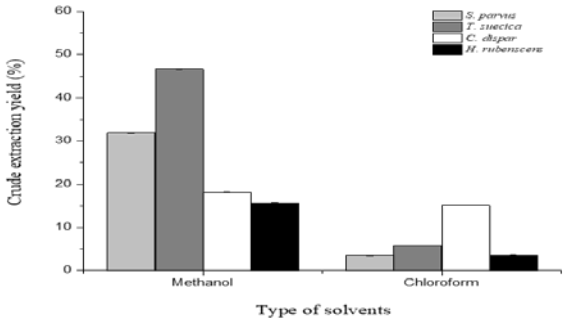

Figure 2. Crude extract yield (%) from different extremophile microalgae species extracted using methanol and chloroform

3.3. Phytochemicals screening

Primary screening of different phytochemicals bioactive compounds from *C. dispar*, *S. parvus* and *T. suecica* was conducted, and the results are presented in Table 1. The present study indicated that terpenoid is the only secondary metabolite that successfully recovered from all the microalgal biomass using different solvents. The study indicated that a higher number of bioactive compounds were successfully extracted from *C. dispar* and *T. suecica* using methanol as solvent. Flavanoids, terpenoids, saponin, tannins were present in methanol extract of *C. dispar*, whilst phytochemical compound, such as flavonoids, terpenoids, tannins, phenol and sterol were present in methanol extract of *T. suecica*. It was found that only terpenoids is present in the methanol extract of *Scenedesmus parvus*. Extraction using chloroform showed a fewer number of bioactive compound extracted from all the biomass tested. It was found that terpenoid, saponin and sterol were present in the chloroform extract of all microalgae biomass and flavonoids only present in the chloroform extract of *C. dispar*. In addition, flavonoids and terpenoids were present in both methanol and chloroform extract of *H. rubescens*. Compounds such as tannins and phenol were only detected in methanol extract; however, saponin and sterol were present in chloroform extract of *H. rubescens*. Phytochemicals extracted from these microalgal biomass are known to have medicinal importance.

Table 1. Screening of phytochemicals of chloroform and methanol as solvent extracts of different extremophile microalgae species.

Phytochemical Constituents	*C. dispar*		*S. parvus*		*T. suecica*		*H. rubescens*	
	ME	CE	ME	CE	ME	CE	ME	CE
Flavonoids	+	+	-	-	+	-	+	+
Terpenoids	+	+	+	+	+	+	+	+
Tannins	+	-	-	-	+	-	+	-
Saponin	+	+	-	+	-	+	-	+
Phenol	-	-	-	-	+	-	+	-
Sterol	-	+	-	+	+	+	-	+

Note: ME= Methanol extract, CE= Chloroform extract (+) positive reaction (-) negative reaction.

3.4. Antimicrobial activity

Antibacterial activities of crude extract obtained from four species of microalgae *Coccomyxa dispar*, *Tetraselmis suecica*, *H. rubescens* and *Scenedesmus parvus* against *Staphylococcus aureus*, *Streptococcus* sp, *Pseudomonas aeruginosa* and *Escherichia coli* were tested out using the disk diffusion method, and the results are shown in Table 2. This study indicated that methanol extract of *S. parvus* was able to inhibit Gram - negative and +positive bacteria. This extract effectively inhibited *E. coli*, *P. aeruginosa*, *Streptococcus* sp. and *S. aureus* with inhibition zone of 7.00±0.89, 7.67±0.52, 11.67±0.37 and 10.00±0.78 mm, respectively. On the other hand, chloroform extract of S. parvus was only able to inhibit *E. coli* with inhibition zone of 10.00±1.78 mm. Interestingly, methanol extract of H. rubescence only effectively inhibited gram negative bacteria *E. coli* and *P. aeruginosa* with inhibition zone of 8.33±0.68 and 7.33±0.52mm, respectively. There is a slight inhibition zone observed for this extract tested on S. aureus. However, the chloroform extract of *H. rubenscens* exhibited inhibition activity on *S. aureus* with inhibition zone of 11.33±1.23 mm. This study also indicated that the methanol extract of acidophilic *C. dispar* only showed a good bacteria inhibition activity for *P. aeruginosa* with 8.33±1.86 mm. However, chloroform extract of *C. dispar* biomass showed inhibition zone for E. coli and *Streptococcus* sp. bacteria with similar inhibition of 7.67±0.93 mm. However, methanol extract of microalgae gave high antibacterial activity against gram negative bacteria; however, the highest inhibition zone was obtained when the microalgae extracted were tested on gram positive bacteria. The highest inhibition zone was observed for chloroform and methanol extract of S. parvus against gram positive *S. aureus* with inhibition zone of 11.67±0.37mm. According to the Table 3, the methanol

extract of *S. parvus* also shows high inhibition activity on *Streptococcus* sp. with inhibition zone of 10.67±0.78 mm.

Table 2. Zone of inhibition (mm) exhibited by methanol and chloroform crude extracts of different microalgae species

Inhibition Zone diameter (mm)	*Escherichia coli*		*Pseudomonas aeruginosa*		*Streptococcus sp*		*Staphylococcus aureus*	
	ME	CE	ME	CE	ME	CE	ME	CE
H. rubescens	8.33±0.68	10.00±0.57	7.33±0.52	8.17±0.21	6.17±0.26	6.83±0.68	6.00±0.00	11.33±1.23
T. Suecica	7.67±1.36	10.00±0.37	6.17±0.26	6.33±0.26	6.5±0.45	6.67±0.52	6.5±0.00	7.00±0.89
C. dispar	7.00±0.89	9.5±0.23	8.33±1.86	7.67±0.93	6.67±0.03	7.67±0.52	7.67±0.52	8.33±0.52
S. parvus	7.00±0.89	10.00±1.78	7.67±0.52	7.67±0.52	10.67±0.78	8.00±0.78	11.67±0.37	10.66±0.72

* Gentamycin (30 µg/mL) *E. coli* = 14±0.00 mm, *P. aeruginosa*= 11±0.2 mm, *Streptococcus* sp.= 18±0.00 mm and *S. aureus* = 20±0.00 mm

Minimal inhibitory concentration of microalgae extract using different solvent system against gram positive and gram-negative bacteria is shown in Table 3. According to the analysis, difference in the activity of microalgae extract to inhibit bacteria growth is significantly influenced by the type of solvent, and extract concentration. The MIC value for gram-negative bacteria ranged between 0.98 mg/mL to 17.45 mg/mL. Analysis on *P. aeruginosa* using methanol extract of *C. dispar* showed the lowest MIC of 0.98 mg/mL observed for gram negative bacteria, whilst the highest MIC for gram positive bacteria was obtained for *P. suecica* tested with chloroform extract *C. dispar*. In contrast, the MIC value for gram positive bacteria ranged between 1 mg/mL to 34 mg/mL. It can be found that *Streptococcus* sp. exhibited highest MIC value for both methanol and chloroform extract of *T. suecica*. The study indicated that MIC value for chloroform extract of *T. suecica* on *S. aureus* gave lowest MIC value. This study indicated that MIC value for *S. aureus* obtained from this study is lower than those reported in the earliest report.

Table 3. Minimal inhibitory concentration (MIC) of microalgae extract of different solvent system against Gram-positive and Gram-negative bacteria

Inhibition Zone diameter (mm)	*Escherichia coli*		*Pseudomonas aeruginosa*		*Streptococcus sp*		*Staphylococcus aureus*	
	ME	CE	ME	CE	ME	CE	ME	CE
H. rubescens	4.03	1.48	8.03	1.48	16.05	11.85	2.00	1.48
T. Suecica	17.45	8.50	4.36	17.00	34.00	24.9	1.00	0.74
C. dispar	1.93	15.73	0.98	23.80	15.75	7.70	1.92	1.96
S. parvus	9.40	23.80	4.70	23.80	23.80	18.8	9.40	11.90

3.5. Compound identification using Gas Chromatography Mass Spectrometry

Extremophile microalgae have been reported to have capability to produce wide range of secondary metabolite compound which can be beneficial in pharmaceutical and medical therapy. Thus, further identification of bioactive compound from microalgae was conducted using gas-chromatography mass spectrometry (GC-MS). Several phytochemical compounds have been detected to be available in the microalgal crude extract (Table 4). A total of 50 and 30 phytochemicals compound peaks were detected from extraction of all microalgae sample using chloroform and methanol, respectively. The present study revealed that various phytochemical compounds such as fatty acid, fatty alcohol, alkane, alkene, phytosterol and phenol were identified present in the extracted fraction.

The obtained GC-MS results indicated that different extraction solvents could significantly affect the phytochemical composition extracted from microalgal biomass. The present study showed that palmitic acid, oleic acid, linoleic acid and pentadecanoic acid are among the dominant fatty acids present in the crude extract. It was found that palmitic acid has been identified in all extracted sample of all microalgal strain. In addition, it was also found that extractions using different solvents have affected the presence of alkane group in the extract. Several compounds such as pentadecane, hexadecane, octadecane, eicosane, tetracosane and tetracosane are among the compounds from alkane group have been identified presence in the chloroform extracted sample. It was found that pentadecane was detected in both chloroform and methanol extract of all microalgal samples. On the other hand, alkane such as tetrapentacontane, eicosane, tetracosane, octadecane, and hexadecane were only detected in the chloroform extracted sample. This present study also indicated that other phytosterol compounds such as phytol, tocopherol, stigmasterol, campesterol, cholesterol, sitosterol, liolide and squalene were also detected. Phytosterol compound such as Campesterol and Sitosterol only identified in chloroform extract of *T. suecica*. In addition, Squalene was only detected in chloroform extracted of *C. dispar* and *S. parvus*. This study also found that phytone and phytol were detected in all the microalgal biomass strains except for methanol extract of *T. suecica* and *S. parvus*. The presence of phytosterol and phytol in these extremophile microalgae shows their potential to be used in nutraceutical and pharmaceutical industries because they are precursors of some bioactive molecules.

Table 4. Phytochemical compounds detected in different microalgal crude extract extracted using methanol and chloroform

Classification	Compounds				*T. suecica*		*H. rubescens*		*C. dispar*		*S. parvus*	
		RT	Molecular weight	Formula	ME	CE	ME	CE	ME	CE	ME	CE
Fatty acid	Oleic acid (cis-9-0ctadecanoic acid)	78.861	296	$C_{18}H_{34}O_2$	1.49	-	0.13	9.34	0.96	2.92	-	0.44
	Linoleic acid (cis-9-12-octadecanoic acid)	78.221	294	$C_{18}H_{33}O_2$	1.68	-	3.68	-	0.38	21.08	-	-
	Palmitic acid (hexadecanoic acid)	71.413	256	$C_{17}H_{34}O_2$	1.55	5.33	16.76	9.34	11.98	0.46	3.71	18.04
	Eicosapentaenoic acid	66.297	316	$C_{21}H_{32}O_2$	-	-	0.28	0.07	3.01	-	-	-
	Tetradecynoic acid	12.869	238	$C_{15}H_{26}O_2$	-	-	0.44	-	0.48	-	-	-
	Octadecanoic acid	90.480	282	$C_{18}H_{34}O_2$	-	-	0.13	-	0.38	2.92	-	0.15
	Cis-9-hexadecenal	80.951	238	$C_{16}H_{30}O$	-	18.98	42.76	-	0.41	0.49	-	0.24
	Pentadecanoic acid	71.394	242	$C_{15}H_{30}O_2$	1.55	-		9.34	0.53	-	0.38	-
Fatty alcohol	Pentadecanol	55.071	228	$C_{15}H_{32}O$	-	-	-	0.15	-	-	-	0.17
	Hexadecanol	80.951	298	$C_{20}H_{42}O$	-	18.98	-	0.15	-	0.71	-	-
	Eicosanol	95.995	298	$C_{20}H_{42}O$	-	-	-	0.08	-	1.29	-	-
Alkane	Bromodocosane	136.195	446	$C_{22}H_{44}BrS_2$	-	-	-	0.17	-	-	-	-
	Tetrapentacontane	86.496	914	$C_{54}H_{11}O$	-	0.24	-	0.57	-	0.25	-	0.27
	Eicosane	55.054	282	$C_{20}H_{42}$	-	0.42	-	0.15	-	0.25	-	0.13
	Tetracosane	98.389	338	$C_{24}H_{50}$	-	0.28	-	0.19	-	0.16	-	0.39
	Octadecane	104.576	310	$C_{22}H_{46}$	-	0.91	-	0.16	-	0.49	-	0.28
	Pentadecane	66.263	268	$C_{16}H_{32}O_2$	-	0.57	16.67	0.16	-	11.08	3.71	18.04
	Hexadecane	133.364	420	$C_{20}H_{42}$	-	0.37	-	0.15	-	0.52	3.71	0.13
	Hexacosane	66.286	366	C26H54	-	0.57	-	0.15	-	-	-	-
	Neophytadiene	63.131	278	$C_{20}H_{38}$	-	2.85	0.22	8.69	1.87	11.88	-	1.64
	Tetracosane	87.913	338	$C_{24}H_{50}$		0.65		0.19		0.16		0.39
Phytolsterol	Phytol	96.456	296	$C_{20}H_{40}$	-	0.26	4.83	8.69	8.34	7.50	-	0.25
	Tocopherol	119.152	430	$C_{29}H_{50}O_2$	-	-	-	0.13	-	0.71	-	0.32
	Stigmasterol	127.488	412	$C_{31}H_{50}Br_2O_2$	-	-	-	0.76	-	-	-	0.32
	Cholesterol	125.402	400	$C_{28}H_{48}O$	-	-	-	0.51	-	0.46	-	1.49
	Sitosterol	123.003	400	$C_{29}H_{50}O$	-	5.36	-	-	-	-	-	-
	Loliolide	61.480	196	$C_{11}H_{16}O_3$	-	0.41	0.31	-	-	-	23.10	0.24
	Squalene			$C_{30}H_{50}$	-	-	-	-	-	0.68		0.32
Phenol	Phytone	63.730	268	$C_{18}H_{36}O$	-	3.48	-	0.91	0.58	0.66	-	0.20

4. Discussion

The bioactive compound extracted from different extremophile microalgae as antimicrobial agent was carried out against different pathogenic bacteria strain. This study indicated that all four tested microalgae strains were able to grow under high CO_2 concentration as carbon sources, which can be beneficial for CO_2 capture purpose. It is known that during cultivation process, microalgae when through photosynthesis process by utilizing CO_2 and sunlight to produce it biomass as well as main metabolite in its cell. In order to determine the effect of CO_2 on microalgae metabolite, further extraction of bioactive metabolites have been performed using methanol and chloroform as extraction solvent. It was found that different solvents used for extraction process significantly affect the percentage of crude extract from tested microalgae. Different solvents significantly affect the crude extract of different microalgae strains. This finding is in agreement with other studies on the bioactive compound extraction from different microalgae strains reported previously. A study by Fattah-Shaima *et al*. (2022) who compared methanol extraction yield of three microalgae biomass; *Chlorella sorokiniana* (UKM2), *Chlorella* sp. (UKM8) and *Scenedesmus* sp. (UKM9) indicated that different extraction yield was obtained from the extraction of type microalgae strains. The study found that the highest extraction yield of 29.5% was obtained from extraction of *Scenedesmus* sp. (UKM9). This study indicated that extraction efficiency is significantly influenced by the type of solvent used for the process. The differences of extraction yield obtained from different type of solvent could be explained by the solubility of microalgae metabolite into the solvent used. It is known that polar solvent could extract more metabolite and bioactive compound from the sample compared to non-polar solvent (Lezould *et al*. 2020, Jayakumar *et al*. 2020).

Indeed, methanol is a polar solvent with capability to extract more polar compounds from the biomass. Low extraction yield observed for all the microalgae biomass tested could be linked to the rapid volatility and low polarity which will only extract fatty acid from the biomass.

Further phytochemical screening was performed to identify the presence of metabolite compounds in crude extract from *C. dispar*, *S. parvus*, *T. suecica* and *H. rubescens* using a different solvent. It was found that a different solvent used during extraction process could significantly affect the recovery of metabolite compound such as flavonoids, terpenoids, saponin and tannins from microalgae. The presence of these compounds in the crude extract derived from microalgae is important to ensure its biological activity performance. For instance, flavonoid extracted from various plant materials has been reported to show biological activities such as antimicrobial against wide range of human pathogen and anti-inflammatory (Cushnie and Lamb 2005, Nguyen *et al*. 2015). Similarly, tannins derived from medicinal plants is known to have antimicrobial activities, antiviral and antitumor activities (Maisetta *et al*. 2019., Kaczmarek *et al*. 2020). In addition, terpenoids which are known to be the major secondary metabolites from plants have also been reported to have antimicrobial and anti-inflammatory activities (Wang *et al.* 2019).

Antimicrobial assay conducted from this study indicates that crude extract derived from microalgae exhibited different performance against *E.coli*, *Pseudomonas aeruginosa*, *Streptococcus* sp. and *Staphylococcus aureus*. Various studies on the antibacterial activity of microalgae extract against bacteria vary considerably (Matharasi *et al*. 2018). Generally, gram positive bacteria are more easily to be killed than those gram negative. The effectiveness of antibacterial activity against different bacteria strain is significantly influenced by their cell wall structure (Hidhayati *et al.* 2022). Gram positive bacteria have a thick monolayer cell wall; nevertheless, gram negative bacteria consist of thin with three-layer cell wall structure which makes these bacteria more resistant toward any antibacterial agents. In addition, the effectiveness of antibacterial activity of microalgae extract could also be attributed to the presence of biologically active compounds available in the extracted fraction (Toma and Aziz 2022).

GCMS analysis obtained indicated that several bioactive metabolite compounds were detected present in the crude extract. The presence of wide range fatty acid such as palmitic acid and metabolite in microalgal extract has exhibited potential to have great benefit on human health. Similar observation has been reported on the GC-MS profiling of *T. chuii*, *Nannochloropsis* and *Chlorella* sp. which found that the dominant fatty acid compound identified from both microalgae is palmitic acid (Fattah-Shaima *et al*. 2022, Gnanakani *et al.* 2019, El-Sayed *et al.* 2014). Several studies have reported that fatty acid from microalgae can act as antimicrobial, anti-inflammatory, anti-cancer and give positive impact on the cardiovascular disease (Cepas *et al.* 2021, Ruffell *et al.* 2016, Vilakazi *et al.* 2021). A study by Maligan *et al.* (2013) on antimicrobial activity of *T. suecica* indicated that antibacterial compounds such as fatty acid and ester have a significant role to inhibit the growth of bacteria. The long chain fatty acid has been found to exhibit the microbial lyse activity and disrupt bacterial cell wall (Willet *et al.* 1966, Yoon *et al.* 2018). The presence of other metabolites such a fatty alcohol, alkane, and phenol has also been reported to contribute to the inhibition effect of pathogenic bacteria. For instance, phytosterol is a natural steroid-alcohol compound that possesses anti-oxidant, anti-inflammatory and anti-atherogenicity activity (Le-Goff *et al.* 2019). Some phytosterols have also been reported to have anti-fungal, anti-bacterial, anti-inflammatory, anti-tumor, anti-oxidant, and anti-ulcerative properties (Danesi *et al.*2016).

5. Conclusion

This study evaluated and characterized the antibacterial activity of extremophile microalgae extract against *E. coli*, *P. aeruginosa*, *Streptococcus* sp. and *Staphylococcus aureus*. It can be concluded that the types of solvent and microalgae strain significantly affect the extraction yield. Higher extract yield was observed for extraction using methanol compared to chloroform. The study also indicated that methanol extract of *S. parvus* was able to effectively inhibit the growth of *P. aeruginosa*, *Streptococcus* sp. and *S. aureus*. GC-MS analysis indicated that there are various bioactive compounds present in the microalgae extract such as palmitic acid, lolinoide, phytol, phytone and stigmasterol known to have antibacterial activity. Based on this present study, extremophile microalgae such as *Scenedesmus parvus*, *Coccomyxa dispar* and *Halochlorella rubescens* have a great potential source of bioactive compound with pharmaceutical value from renewable resource. It is also indicated that these microalgae strains have economic value that can be further improved for agriculture and biomedical purposes. However, further investigation on bioactive metabolite accumulation improvement, purification, *in vitro* and *in vivo* study is needed to elucidate the compounds responsible for antibacterial activity against these bacteria.

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