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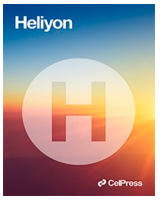

Research article

Antifouling activity of Malaysian green seaweed *Ulva lactuca* and its isolated non-polar compound

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ABSTRACT

Marine natural products especially seaweeds have gained much attention to combat biofouling. *Ulva lactuca* was determined for its antifouling activity and characterized the isolated non-polar metabolite involved. The methanolic crude extract (MCE) of *U. lactuca* was screened using crystal violet assay against biofilm-forming bacteria *Pseudomonas aeruginosa* and was further tested on laboratory and field tests. Then, it was fractionated and isolated using Liquid-Liquid Fractionation (LLE) and Column Chromatography (CC). The isolated compound was characterized using Liquid Chromatography-Mass Spectrometry (LC-MS), Nuclear Magnetic Resonance (NMR), and Fourier Transform-Infrared Spectroscopy (FTIR). The current study showed that the growth of biofilm produced by *P. aeruginosa* was inhibited by MCE at concentrations of 0.0156 mg/mL. The laboratory test indicated UL5% demonstrated a higher bacterial reduction of bacterial colonies with 1.903×10^6 CFU/mL better than blank paint. According to the field test, crude panels of UL5% were successful in reducing the settlement of fouling organisms due to less macrofouler growth compared to blank paint. The isolated compound A4 was identified as hexadecanoic acid ($C_{16}H_{32}O_2$) through NMR with a molecular mass of 256 g/mol detected using LC-MS. The characterization through FTIR obtained functional groups consisting of CH_3, CH_2, C=O, and OH. Therefore, *U. lactuca* produced hexadecanoic acid as one of the promising compounds from the seaweed group as an eco-friendly antifouling agent.

1. Introduction

The adhesion phenomena of fouling organisms towards wet unprotected living or non-living surfaces also man-made structures are problems that are normally encountered in marine environments globally. It includes bacteria, protozoa, algae (microfoulers), barnacles, tubeworms, and bryozoans (macrofoulers) [1]. Biofouling leads to various negative impacts such as decreased efficiency of

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ships and other marine devices, increased fuel and maintenance costs, and increased corrosion rate [2]. A study reported that as much as US $15 billion has been spent on biofouling cleanup [3]. For example, other than increasing the weight of offshore oil platforms, fouling organisms also reduce their capability to withstand tsunami and storm risks [4]. According to a previous study, marine biofouling is influenced by several elements including seawater temperature, depth, and light availability, currents and distance to shore and material, topography, wettability, and the color of the substrate [5]. The biofouling mechanisms involve four levels (Fig. 1) including (1) the process of biofilm conditioning, in which organic matter such as polysaccharides, lipids, glycoproteins, and proteoglycans accumulate within minutes; (2) within hours, primary colonizers (unicellular organisms) secrete extracellular polymeric substances (EPS) to substrates and develop the biofilm; (3) accumulation of more complex communities such as microalgae, and multicellular organisms; (4) maturation and dispersal process in which larger marine invertebrates such as barnacles, mussels and macroalgae begin to grow [6,7].

Many years ago, tributyltin (TBT) and triphenyltin (TPT) were developed as antifouling coatings but turned out to be toxic and non-environmentally friendly to the marine environment [8]. In the year 2008, the International Maritime Organization (IMO) banned the use of TBT for antifouling coating [9]. Then, copper is used as the main biocide ingredient in antifouling paint, and it also results on giving harmful effects on the fouling community and non-target species [10]. This matter has led to the finding of a new antifouling paint formulation which more environmentally based. There is a study indicating that marine organisms are suitable for the formulation due to a broad range of biological activities especially in antifouling activities and one of them is seaweed [11].

Seaweeds are eukaryotes that are classified into three groups chlorophyta (green seaweed), rhodophyta (red seaweed), and ochorophyta (brown seaweed) [12]. It has been used widely for cosmetics, food, medicines, agriculture, and sources of energy [13]. The unique characteristics have increased the possibility of being applied in various kinds of applications. For example, the adaptation to extreme conditions and their growth ability [14]. Most importantly, past studies have confirmed that seaweeds provide rich sources of secondary metabolites with a wide spectrum of biological activities such as alkaloids, glycosides, saponins, tannins, flavonoids, and steroids [13,15]. The biological activities that have been reported active in seaweeds include antibacterial, antiviral, antifungal, antineoplastic, antifouling, anti-inflammatory, antitumor, antimycotic, and cytotoxic activities [16]. Up to now, antifouling compounds from seaweeds have been reported from groups of alkaloids, terpenoids, sterols, fatty acids, amides, lactones, pyrroles, and lipopeptides [17].

Ulva lactuca is categorized in the Chlorophyta group and distributed from marine to freshwater globally [18]. Azizi et al. [19] also reported *Ulva* as the most extensive algae in coastal benthic communities around the world and advantaged the coastal ecosystem management in the environment eutrophication process. *Ulva* species also popularly known as 'sea lettuce' or 'green laver' indicates a green to dark green color depending on its existence whether at the beach or underwater [20]. Some of the compounds that are contained in *Ulva* are carotenoid, chlorophyll, phenolic, fatty acid, and polysaccharides [21]. Despite that, they are popular for their uniqueness which includes high growth rates because of geographic location/season and high content of polysaccharides [22]. However, various species are known as signs of ecological disturbances like green tides or algal blooms which happened due to intensive aquaculture, agriculture, industry, and wastewater disposal [23,24]. Therefore, the current study aims to investigate the methanolic crude extract (MCE) of Malaysian green seaweed *U. lactuca* and characterize its isolated non-polar compound that contributes to their antibacterial and antifouling activity in producing new antifouling paint, which is greener and safest for the marine environment.

2. Materials and methods

2.1. Sample collections and preparation

Samples of *U. lactuca* (Fig. 2) was collected during low tides at Tanjung Pengelih, Kota Tinggi, Johor, Malaysia (1°22′07.3″N 104°05′35.7″E) in September 2021. The collected samples were washed using distilled water to remove debris and dried under shade conditions. Then, it was ground with an electrical grinder to obtain a fine powder (400 mesh sieve) known as biota [25].

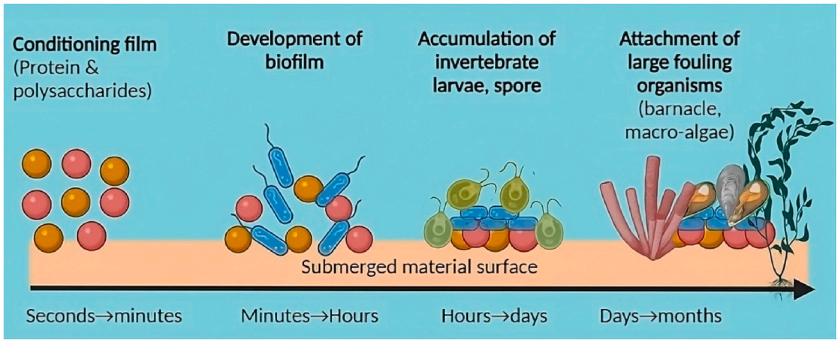

Fig. 1. Schematic diagram of four stages of marine biofouling process.

Fig. 2. *U. lactuca* that collected at Kota Tinggi, Tanjung Pengelih (Johor), Malaysia.

2.2. Methanolic crude extract of U. lactuca

The methanolic crude extract (MCE) of *U. lactuca* was prepared using 1000 g of biota through the maceration process [26] using methanol (1:10 w/v ratio) and under room temperature. This process was repeated thrice to obtain the maximum yield of crude extract [27]. The extract was filtered using Whatman No. 1 filter paper and was condensed using a rotary evaporator (R300, BUCHI, Switzerland) at 40 °C under reduced pressure [25]. Up to 176.6 g of dried crude extract was obtained throughout the extraction process and it was kept in the cold room (4 °C) for further use.

2.3. Phytochemical test of MCE of U. lactuca

Phytochemical screening was performed to determine the presence of secondary metabolites in the MCE of *U. lactuca*. This involved the detection of alkaloids, saponins [28], tannins, saponins, flavonoids, terpenoids, phlobatanins [29], and glycosides [30]. Table 1 simplified the tests involved.

2.4. Fractionation and isolation of non-polar compounds from MCE of U. lactuca

2.4.1. Fractionation using liquid-liquid extraction (LLE)

The MCE of *U. lactuca* was first partitioned using LLE as proposed by Abu et al. [31] with some modifications. First, 30 g of MCE was dissolved in 150 mL of distilled water (1:5 w/v). Then, it was added into a separatory funnel with a ratio of 1:3 (v/v) of water and 100 % hexane for separation of non-polar compounds. After that, it was shaken for 2 min before being allowed to settle down at room temperature. The bottom layer (aqueous layer) was re-extracted with the same solvent for five times while the upper part (organic

Table 1
The phytochemical test methods.

Tests	Methods
Alkaloids	One mL of crude extract was added to two mL of Mayer's Reagent. The white color or pale precipitate indicates the presence of alkaloids [28].
Tannins	One mL of crude extract was dissolved in two mL of distilled water. Then, a few drops of 5 % ferric chloride were added. The presence of tannins was observed through the formation of the dark green color [29].
Saponins	Five mL of crude extract was diluted in five mL of distilled water and was shaken for 15 min. Then, it was warmed and the formation of stable layers of foam indicates the presence of saponins [28].
Flavonoids	One mL of crude extract was dissolved in diluted NaOH and mixed with HCL. Flavonoids were present when the yellow color of the solution was turned colorless [29].
Terpenoids	0.5 mL of crude extract was added to two mL of chloroform. Then, three mL of concentrated H2SO4 was added. The changes in the reddish-brown color of the interface were observed [30].
Coumarins	Two mL of aqueous crude extract was added with three mL of 10 % NaOH. The presence of coumarins was detected through the formation of a yellow color solution [29].
Phlobatanins	One mL of crude extract was dissolved in distilled water and filtered. The filtrate was then boiled with 2 % HCl and a red precipitate was formed [29].
Glycosides	A small amount of crude extract was dissolved in one mL of water and a few drops of aqueous NaOH were added. The yellow color shows the existence of glycosides [30].

layer) was collected and combined. The organic layer was condensed with a rotary evaporator (R300, BUCHI, Switzerland) to obtain a hexane layer crude extract. This method was continued using different polarities of the solvent including ethyl acetate, chloroform, and methanol.

2.4.2. Isolation and purification of active fraction using column chromatography (CC)

A cylindrical glass column of 30 cm height and 4.5 cm diameter was used for the isolation of active fraction. Slurry packing of the column was done by mixing 150 g of silica gel (230–400 mesh) with *n*-hexane before adding it to the column [32,33]. Hexane fractions were loaded onto a wet column by wet packing and were eluted with 100 % hexane followed by hexane: acetone with increasing polarity. The fractions were collected in 100 mL flasks and were concentrated using a rotary evaporator. There were 60 fractions collected and subjected to Thin Layer Chromatography (TLC). The ones with identical spots were pooled together and weighed to obtain 21 fractions. Then, fraction 8 (116.2 mg) was subjected to the next isolation using a column of 2 cm diameter with gradient elution of hexane: ethyl acetate. Each fraction was collected in 50 mL volume. From this, 20 fractions were successfully eluted and 7 fractions were obtained after pooling together. Based on TLC, fraction 6 (14.3 mg) with the best profiles proceeded for third isolation with chloroform and ethyl acetate as the mobile phase and 11 fractions were collected before being combined to get 4 fractions. The white solid compound labeled as A4 (3 mg) was obtained through the purification of fraction 4C (10 mg) using an isocratic system of hexane: ethyl acetate (9:1). The flow of isolation and purification were simplified in Fig. 3.

2.5. Crystal violet assay

Crystal violet assay was completed as mentioned by O'Toole [34] and Leroy et al. [35] with some modifications applied to monitor the capability of samples to inhibit the growth of biofilm produced by bacteria. In this study, *P. aeruginosa* was used as it is one of the biofilm-forming bacteria [36]. The bacteria were obtained from culture stock at the Institute of Climate Adaptation and Marine Biotechnology (ICAMB), Universiti Malaysia Terengganu (UMT), Malaysia, and were incubated in rich medium Luria-Bertani (LB) broth overnight before proceeding with biofilm development. 100 μL of M63 media and 100 μL samples which were already dissolved in a certain concentration of dimethyl sulfoxide (DMSO) were loaded into a 96-well microtiter plate before serial dilution. Then, it was left to be incubated for 1 h. After 1 h, 130 μL of M63 and 20 μL of bacteria were inoculated into the well of the sample and incubated in a shaker (120 rpm) for 24 h at 37 °C. As for negative control, the well was only filled with media and without the addition of bacteria. The non-adherent cells were stained, washed with sterile distilled water, and set to dry before being stained with 250 μL of 1 % crystal violet. Then, the well was stained again with sterile distilled water thrice. After drying in the oven, 250 μL of 30 % acetic acid was added to each well as the solubilizer and incubated for 15 min at room temperature. The plate was agitated and a Multiscan Spectrophotometer (ThermoFisher Scientific, Waltham, MA, USA) was used to measure optical density at OD_{595}.

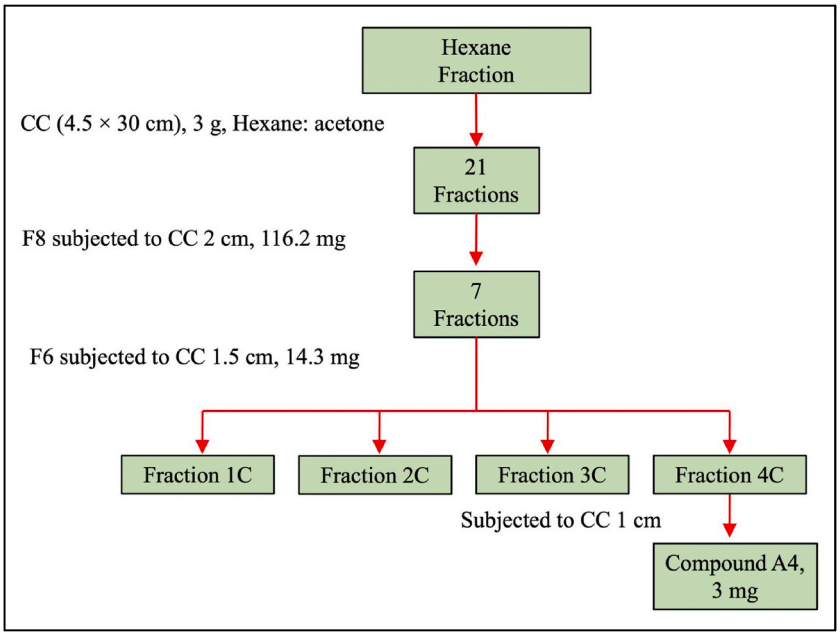

Fig. 3. Flows chart for fractionation, isolation, and purification of the compound from *U. lactuca*.

2.6. Application of MCE as paint additive for antifouling coating

2.6.1. Preparation of panels

The panels were square with 2.5 cm × 2.5 cm × 0.3 cm in size (Fig. 4). Before coating, the panels were run for sandblasting to remove the rust and were cleaned with methanol to remove oil, grease, and dust following the SSPC-SP1 standard as described by Idora et al. [37]. It was first coated with anticorrosive primer and was painted with blank paint (negative control), commercial antifouling paint known as reference paint 1 (RF1) and 2 (RF 2) (positive control), and paint incorporated with MCE of *U. lactuca*. Blank paint is referred to as paint with no antifouling agent. Blank and commercial paints were supplied by the industry (Local Oil and Gas Company, Bandar Baru Bangi, Selangor, Malaysia). Table 2 specifies the components contained in blank and commercial antifouling paint. Both paints comprise the same components except for dicopper oxide and zineb in commercial paints. Dicopper and zineb act as metal-based biocides for antifouling coating [38].

The incorporated paints were prepared by diluting crude extract in a small amount of solvent which is commercial thinner [37] (Xylene (CAS number: 1330-20-7), ethylbenzene (CAS number: 100-41-4) before being mixed with blank paint to form homogeneous paint at final concentrations of 5 % and 10 % weight per volume respectively [37,39]. An air spray compressor (Model ZL-550W × 2–50L, Uma) was used to paint the panels with an ultimate dry of film thickness were 150 μm for primer and 100 μm for the sample respectively. The dry film thickness was measured using a digital coating thickness gauge, PosiTector 6000 (Ogdensburg, NY, USA). Importantly, panels were left to fully dry before proceeding with testing [40].

2.6.2. Laboratory-based antibiofilm test using aquarium

Antibiofilm activity was further tested *in vitro* using an aquarium. The panels were suspended in an aquarium tank that was filled with 25 L of fresh seawater and 0.25 L of artificial seawater with waves mimicking conditions at room temperature [41]. The artificial seawater with composition NaCl (24.615 g/L), KCl (0.783 g/L), Na_2SO_4 (4.105 g/L), $MgCl_2(H_2O)_6$ (11.06 g/L), and $CaCl_2(H_2O)_2$ (1.558 g/L) [42] was added as an essential nutrient. On top of that, 0.4 % glycerol was also added to enhance the amount of nutrients and growth of biofilm [43]. The tested panels were retrieved at 24 h intervals and the generated biofilm was scraped off using a cotton swab and suspended in one mL of sterile artificial seawater. This suspension was serially diluted before being spread on Zobell marine agar plates. Then, agar plates were incubated at 37 °C, and the number of colonies was quantified using a microbial colony counter (LAPIZ, Medica) [44]. This experiment was done in triplicate.

2.6.3. Field test

Steel-coated panels were fitted to the stainless-steel frame and were submerged in the seawater at Tok Jembal Beach (Kuala Terengganu, Terengganu, Malaysia) on December 10, 2022, and then in strong brackish water at Kuala Kemaman (Kemaman, Terengganu, Malaysia) on December 12, 2022. In Fig. 5, the deployed sites are displayed. Specifically, panels that attached to a stainless-steel frame as shown in Fig. 6 were immersed for three months and the emergence of foulers was monitored through photographic observation. The fouler's growth was monitored monthly [37,40].

2.7. Characterization of isolated compound A4

2.7.1. Fourier Transform-Infrared Spectroscopy (FTIR) analysis

The isolated compound A4 was characterized using IR Tracer-100 FTIR (Shimadzu) to monitor the functional groups' presence. A 10 mg solid sample at room temperature was carefully put on the diamond attenuated total reflection (ATR) crystal. Then, the ATR anvil holder was pressed down in order to have full contact between the solid sample and the crystal. The spectra were measured against transmittance at wavelengths ranging from 4000 cm^{-1} to 500 cm^{-1} and generated by 42 scans with a resolution of 16 cm^{-1}. The baseline was adjusted after each scan using the background spectrum from a blank scan [45].

2.7.2. High-resolution Nuclear Magnetic Resonance (NMR) analysis

The ^{1}H NMR and ^{13}C NMR analyses were conducted for the identification of particular signals of isolated compounds using a high-

Fig. 4. Panels for testing method; Plain panels (A), Panels painted with anticorrosive primer (B), and panels painted with samples (C).

Table 2
The components contained in the blank and commercial paints.

Paint	Components
Blank paint	Colophony (CAS number: 8050-09-7), Xylene (CAS number: 1330-20-7), Zinc Oxide (CAS number: 1314-13-2), Ethylbenzene (CAS number: 100-41-4), Hydrocarbons (C9 aromatic, CAS number: 64742-95-6), 1-methoxy-2-propanol (CAS number: 107-98-2), fatty acid (CAS number: 91001-64-8).
Reference Paint (Commercial paint)	Colophony (CAS number: 8050-09-7), Xylene (CAS number: 1330-20-7), Zinc Oxide (CAS number: 1314-13-2), Ethylbenzene (CAS number: 100-41-4), Hydrocarbons (C9 aromatic, CAS number: 64742-95-6), 1-methoxy-2-propanol (CAS number: 107-98-2), dicopper oxide (CAS number: 1317-39-1), zineb (CAS number: 12122-67-7)

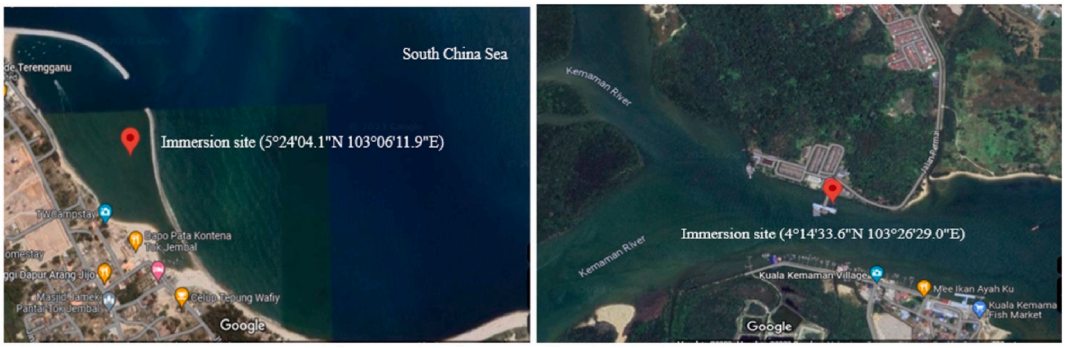

Fig. 5. Deployment site of panels in seawater at Tok Jembal beach(A) and Brackish water at Kuala Kemaman (B), Malaysia (https://www.google.com/maps/@5.4011273,103.1007347,773m).

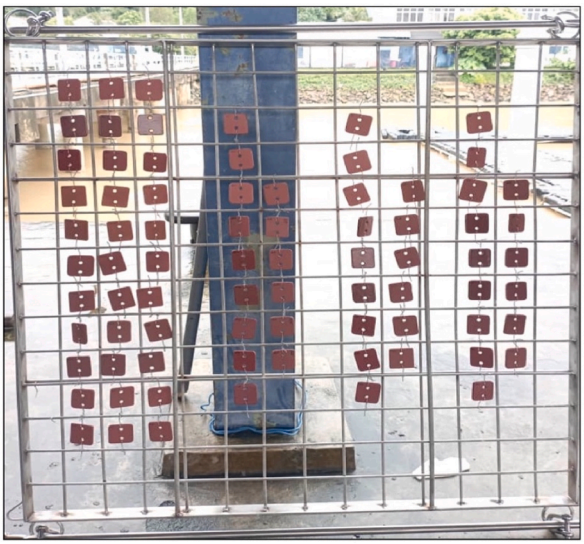

Fig. 6. Panels attached to stainless-steel frame for deployment.

resolution NMR system (Bruker, 600 MHz). Before being transported to the NMR tube, a 3 mg sample of the isolated compound A4 was dissolved in deuterated chloroform, $CDCl_3$. The internal standard used was tetramethylsilane (TMS) and all chemical shifts were recorded in part per million (ppm, δ) [46].

2.7.3. Liquid Chromatography-Mass Spectrometry (LC-MS) analysis

The LC-MS method was followed the procedure by Rofiee et al. [47]. The samples were dissolved in methanol (1 mg/mL) and the analysis was done using LC Agilent 1200. The Agilent ZORBAX Eclipse Plus C18 column Rapid Resolution HT (2.1 × 100 mm), 1.8 μm was used at temperature 40 °C and (A) 1 % formic acid in dH2O and (B) 1 % formic acid in acetonitrile were applied as the mobile phase with gradient elution. 2 μL of samples were injected and the flow rate of mobile phase was set at 0.25 mL/min. Besides, it was

operated in positive electrospray ionization (ESI) mode. The data (MS data) was analyzed using Agilent Mass Hunter Qualitative Analysis B.05.00 software (Agilent Technologies Santa Clara, CA, USA).

2.8. Statistical analysis

The data were expressed as mean ± standard deviation (SD). The data was analyzed using GraphPad Prism software version 8.0 (GraphPad Software, San Diego, CA, USA). A one-way analysis of variance (ANOVA) was used to compare the groups, followed by Tukey's post hoc test. When the *p*-value was less than 0.05, it was considered statistically significant ($p < 0.05$).

3. Results and discussion

3.1. Phytochemical test of MCE of U. lactuca

The preliminary phytochemical test was successfully performed towards the MCE of *U. lactuca* and the results were presented in Table 3. From Table 3, the active constituents in the MCE of *U. lactuca* were terpenoids and flavonoids. Previous studies have revealed that *U. lactuca* showed the presence of terpenoid, flavonoid, alkaloid, saponin, and glycosides with the absence of tannin and phlobatanin [48]. However, in the present study, the alkaloids, saponins, and glycosides were not active. The alkaloids were inactive, indicated by the absence of white color or pale precipitate when crude extract was added to Mayer's reagent. Besides, stable layers of foam were not formed indicating an absence of saponins. The glycosides were not active indicating that no yellow color materials appeared when crude extract was dissolved in water and aqueous NaOH. This might be due to the different locations of the sampling site. Samples from different locations are said to have differences in the production and accumulation of primary and secondary metabolites [49]. Moreover, the terpenoid and flavonoid compounds have been described as antifouling compounds in previous studies [17,50].

3.2. Fractionation and isolation of non-polar compound from MCE of U. lactuca

The hexane fraction gave the highest yield (10.68 g) followed by methanol (3.03 g), ethyl acetate (1.24 g), and chloroform (1.09 g). The isolation of non-polar compound from hexane fraction resulted in compound A4 which has the physical properties of a white solid and a mass of 3 mg.

In a recent study, the MCE of *U. lactuca* was said to have more non-polar compounds as they are extracted more during hexane partition through LLE. According to past findings, green seaweed normally produced a dominant pigment compound such as *a,b* chlorophylls, β-carotene, lutein, neoxanthin, violaxanthin, and zeaxanthin [51]. A study by Grosser et al. [52] reported that pigments (β-carotene) from *Ulva* have the potential as natural antifoulants when it normally extracted from a non-polar solvent (hexane). Following this, hexane fraction has proceeded for the isolation as many compounds here are said to produce a high possibility of bioactive constituents, especially for the antifouling. Thus, other than pigments, fatty acids, terpenoids, hydrocarbons, and alkaloids also may be isolated [53,54]. In addition, fatty acid derivatives were commonly isolated from hexane fraction [55].

3.3. Crystal violet assay

The present MCE of *U. lactuca* was shown to have antifouling properties, with different levels of effectiveness at different concentrations. This effect of MCE of *U. lactuca* was shown by the high percentage inhibition of the *P. aeruginosa* biofilm (Fig. 7). The lower concentrations of MCE of *U. lactuca* (0.0078 mg/mL and 0.0156 mg/mL) showed higher inhibition against *P. aeruginosa* (44.54 % and 74.26 % respectively) compared to the higher concentration (0.25 mg/mL; 12.78 %). These phenomena indicated that 0.0156 mg/ml is the most effective concentration compared to the others. Perhaps the higher concentration applied, the results show decreasing in terms of the biofilm inhibition. It has been shown that the MCE of *U. lactuca* was effective at the lowest concentration of *P. aeruginosa* biofilm. Besides that, Fig. 8 shows all fractions demonstrated good biofilm inhibition towards *P. aeruginosa* with different percentages at different series of concentrations. The highest inhibition was ethyl acetate fractions with 71 % achieved at an optimum

Table 3
Phytochemical screening of MCE of *U. lactuca.*

Test	Constituent
Alkaloids	–
Tannins	–
Saponins	–
Flavonoids	+
Terpenoids	+
Coumarins	–
Phlobatanins	–
Glycosides	–

*Note: The presence of constituents (+), the absence of constituents (−).

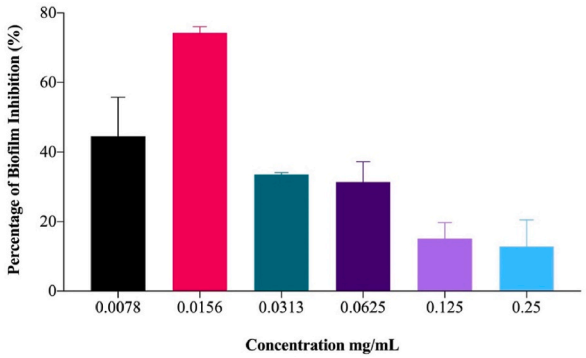

Fig. 7. Percentage of biofilm inhibition (%) against various concentrations of MCE of *U. lactuca* (0.0078 − 0.25 mg/mL). Positive control: wells with medium and bacteria. Negative control: wells only with medium and without bacteria. Data were expressed as mean ± SD (n = 3).

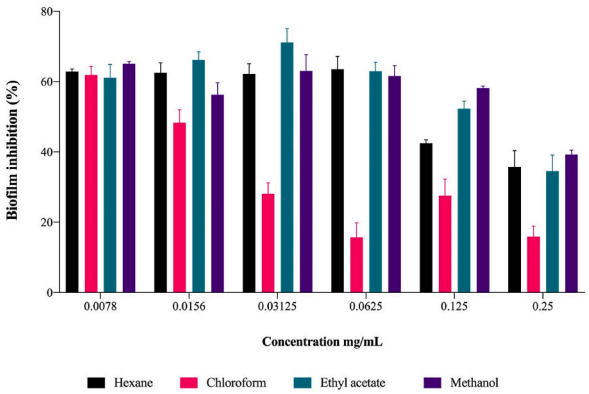

Fig. 8. Percentage biofilm inhibition of hexane, chloroform, ethyl acetate, and methanol fractions at series concentrations (0.0078 − 0.25 mg/mL). Data were expressed as mean ± SD (n = 3).

concentration of 0.031 mg/mL followed by hexane fractions by 64 % at a concentration of 0.0625 mg/mL. The chloroform fractions inhibited biofilm with 62 % at a concentration of 0.078 mg/mL before being decreased against increasing concentration while methanol with highest inhibition at 63 % at 0.031 mg/mL.

Primary testing on antifouling properties of MCE of *U. lactuca* was successfully done through preventive action of crystal violet assay. *P. aeruginosa* was chosen because it is one of the bacteria that can form a strong biofilm structure when attached to a conducive surface for biofilm formation [56]. In the current study, crystal violet served as a dye when it was attached to negatively charged molecules, staining bacteria, and the biofilm matrix as well [57]. Antifouling activity is considered active when the percentage of biofilm inhibition is ≥ 40 % [58]. The concentration of the sample that has been used was within the range of 0.25 g/mL to 0.00781 g/mL. Based on the current findings, MCE of *U. lactuca* inhibited the growth of biofilm that has been produced by *P. aeruginosa* with different levels of effectiveness at different concentrations. The percentage of biofilm inhibition was decreased against the increasing concentration. The optimum concentration to inhibit the biofilm was concentration 0.0156 mg/mL with 74 % inhibition. After reaching effective concentration, the efficiency of activity will decrease owing to the enzyme starting to achieve saturation level with the substrate [59]. Generally, increasing the concentration of substrate will increase the reaction rate but only up to a point. This is because when the substrate concentration further increases, the reaction rate approaches its maximum velocity and thus all active sites of the enzyme are occupied by the substrate molecules. The concept of enzymatic saturation is closely related to Michaelis-Menten kinetics [60]. For example, as the concentration of MCE increases, the reaction rate between molecules of MCE and the biofilm bacteria (*P. aeruginosa*) slowly decreases and leads to low inhibition. Another study revealed extract from marine macroalgae, *Cladostephus spongiosus* inhibits the growth of biofilm-forming bacteria in the same way [61]. Moreover, previous research also examined green seaweeds, *U. lactuca* to have antifouling properties regarding the crystal violet test [62]. The concentration of extract higher than 0.25 mg/mL will lead to the formation of biofilm. Moreover, the percentage of four fractions tested for biofilm inhibition was slightly decreased over the percentage of biofilm inhibition of MCE. This matter indicates the synergistic activity happened because the combination of many bioactive compounds in crude is said to have higher activity than the one with less bioactive compounds or act independently [63]. A study reported numerous compounds that act for antifouling including fatty acids, lipopeptides, amides, alkaloids, terpenoids, lactones, pyrroles, and sterols [23]. In this study, a fatty acid compound which is hexadecanoic acid has been successfully isolated and proven active for antifouling. This result is also supported by a previous study indicating the maximum

activity of antifouling produced by hexadecanoic acid [64]. Hence, the combination of other bioactive compounds together with hexadecanoic acid in crude may enhance the synergistic activity.

3.4. Application of MCE as paint additive for antifouling coating

3.4.1. Laboratory-based antibiofilm test

In the present study, the formation of biofilms on the coated panels was tested in an aquarium setup. The biofilm attachment was determined by measuring the bacterial counts from the biofilms attached. The current findings in the aquarium testing are illustrated in Fig. 9. From Fig. 9, the MCE of *U. lactuca* at UL5% (1.903×10^6 CFU/mL vs. 2.797×10^7 CFU/mL; $p < 0.0001$) and UL10 % (2.473×10^6 CFU/mL vs. 2.797×10^7 CFU/mL; $p < 0.0001$) significantly reduced the number of bacteria colonies compared to the negative control (BLANK). The effects of both concentrations of MCE of *U. lactuca* were comparable with the effects of the positive controls (RF1 and RF2).

The adherence of biofilm was measured by counting the bacterial colonies that emerged after serial dilution. According to the data of the current study, the number of bacterial cells attached to blank paint panels (negative control) was 2.797×10^7 CFU/mL. It was found that panels coated with antifouling paint UL5 % and UL10 % reduced the number of bacterial cells of blank paint panels with 1.903×10^6 CFU/mL and 2.473×10^6 CFU/mL respectively. Panels with incorporated paint of MCE of *U. lactuca* successfully reduced the adherence of marine bacteria possibly as good as the commercial antifouling paint, RF1 and RF2. On top of that, the results corresponded to the crystal violet experiment, in which MCE of *U. lactuca* prevents biofilm growth with a higher percentage at lower concentrations, whereas panels reduced the number of bacterial cells more at a concentration of 5 %. There was another study validated the reduction of biofilm settlement using panels containing crude extracts of *Sonneratia lanceolata* and *Diadema setosum* through some bacterial cells performed by laboratory testing [40].

3.4.2. Field test

The tested panels were retrieved every month time interval for three months. Fig. 10 shows that the tested panels for Tok Jembal Beach (Fig. 10A) and Kuala Kemaman (Fig. 10B) were retrieved and photographed at different time intervals, respectively. From Fig. 10, it was observed that macroalgae grew heavily on the blank panels after 3 months in both immersion sites. However, significantly fewer macroalgae were found growing on the antifouling paint coated with the UL5% and UL10 % panels at Kuala Kemaman. According to Fig. 10B, the antifouling at a lower concentration of MCE of *U. lactuca* showed greater antifouling activity compared to the higher concentration. This matter relates to biofilm bacteria which it less susceptible to most chemicals and difficult to eliminate because of the protection by the compounds in the matrix itself [65]. Thus, too high a concentration of extract may not be necessary as it could lead to the promotion of biofilm as well as increase the amount of foulers on the panels. Therefore, low concentration shows that MCE has high efficiency as a natural antifoulant.

In terms of water, brackish water at Kuala Kemaman was considered to have low salinity compared to Tok Jembal Beach because it is a combination of seawater and saline-alkaline water [66]. During the first and second months at Tok Jembal Beach, more macroalgae started to grow on blank paint panels compared to crude and reference panels (RF). After three months of observation, panels of incorporated paints UL5% show the best concentration that can inhibit the growth of foulers approximately as RF1 and RF2 panels due to lesser macro foulers adhered at the panels. Thus, it shows that a low concentration of MCE *U. lactuca* is already enough to increase the effectiveness of antifouling activity where it is parallel with crystal violet and bacterial cell attachment assay. Chambers et al. [67] performed a field test study on the antifouling properties of red seaweed *Chondrus crispus* which caused in reduction of the fouling amount at low concentrations starting from 6 weeks which was better than the blank panel. In addition, the immersion of painted panels from paint incorporated with water-soluble polysaccharides, protein, and lipids from four algal extracts (*Ulva fasciata*, *Cymodocea nodosa*, *Padina pavonia*, and *Colpomenia sinusa*) at Eastern Harbor water produces favorable results, with a lower percentage of fouling organisms' growth compared to control [11]. Besides, bioactive compounds that are produced from the crude extract of natural products (seaweeds) occur naturally in the marine environment thus more environmentally friendly than using antifouling coating

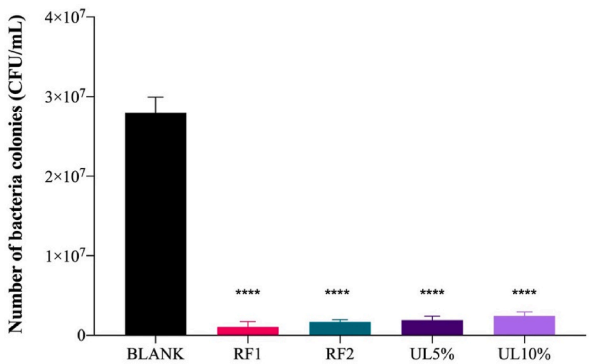

Fig. 9. Number of bacterial cells (CFU/mL) $\times 10^4$ attached against tested panels. Data were expressed as mean ± SD (n = 3). RF: Reference; CFU: colony-forming unit; Positive control: Commercial panel (RF1 & RF2); Negative control: Blank panel.

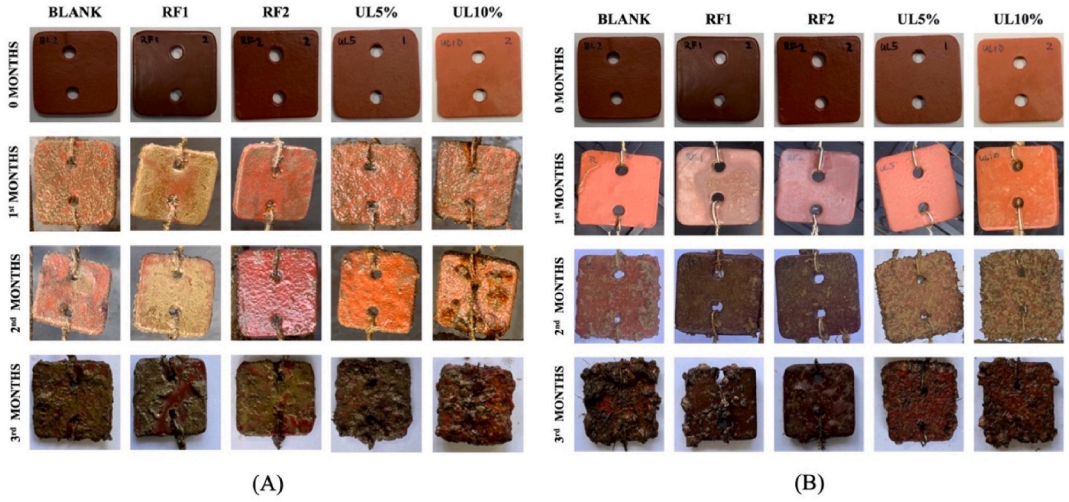

Fig. 10. Observations within 3 months of blank panels, reference panels, and antifouling paint panels of *U. lactuca* at Tok Jembal Beach (A) and Kuala Kemaman (B). RF: Reference. Positive control: Commercial panel (RF1 & RF2); Negative control: Blank panel.

which contains heavy metal substances [68]. From this, *U. lactuca* has good potential to be utilized and commercialized as an additive in antifouling paint since it can enhance the inhibition of biofilm and fouler more effectively at lower concentrations while still being compatible with the environment.

3.5. Characterization of isolated compound A4

As shown in Fig. 11 and Table 4, the peak that exists at 2916 cm^{-1} and 2847 cm^{-1} indicates the symmetrical stretching vibration of the -CH_3 and -CH_2 groups significantly [69]. The peak of the carboxylic acid functional group which is C=O stretching is verified by the peak formed at 1697 cm^{-1}. Besides, the band presence at 1466 cm^{-1} corresponds to the bend vibration of -CH_2 and -CH_3 groups of isolated compounds. The peak appears at 941 cm^{-1} and 1297 cm^{-1} representing the out-of-plane and in-plane vibration of the -OH group respectively while peaks 725 cm^{-1} and 687 cm^{-1} represent the swinging vibration of the -OH group [70].

Based on ^{1}H NMR (600 MHz, $CDCl_3$), the aliphatic carboxylic acid was present at the region between δ_H 0.85–2.34 (Fig. 12). The spectrum showed the existence of a set of long methylene protons which signifies 12 methylene groups overlapped as a multiplet peak at a chemical shift between 1.26 and 1.23 ppm. For ^{13}C NMR (Fig. 13), the spectrum showed the existence of seven signals including the signals of the carbonyl group at δ_C 178.83, a terminal methyl at carbon in position 16 (δ_C 14.12), and methylene carbon at C4-C15 which overlapped at the chemical shift in range 31.9–22.7 ppm. The chemical shifts (Table 5), formula, and structure were confirmed in a study by Ekeocha et al. [46] and Unnikrishnan et al. [71] on to same signals obtained. The chemical formula for this isolated compound A4 is considered $C_{16}H_{32}O_2$ which belongs to hexadecanoic acid and the chemical structure is shown in Fig. 14.

The hexadecanoic acid with structural formula, $C_{16}H_{32}O_2$ was identified using LC-MS with the ion of mass/charge (*m/z*) value in a positive mode where the addition of proton occurred to generate $[M+H]^+$ [72]. Following this, a saturated fatty acid compound, hexadecanoic acid was detected by [M+Na]+ion peak at *m/z* of 279.16 (Fig. 15). In this way, sodium ion (Na) has an atomic mass of 23 g/mol. The original mass of hexadecanoic acid which is 256 g/mol was added with 23 g/mol to obtain 279.16 g/mol. Therefore, the molecular mass obtained from LC-MS analysis was matched with the molecular formula, $C_{16}H_{32}O_2$ obtained from the NMR study.

The hexadecanoic acid or palmitic acid, belongs to saturated fatty acid which normally comprises in animals, plants, and microorganisms as well [73]. Hexadecanoic acid (C16:0) was informed as the most dominant saturated fatty acid contained in *Ulva* species [74] and was confirmed to inhibit the biofilm formation and settlement of fouling organisms indicating antifouling properties occurred in the MCE. This statement was supported by several studies together with Gao et al. [75]. This study indicates hexadecanoic acid produced from two bacterial strains; *Kytococcus sedentarius* and *B. cereus* to have high antifouling activity. Besides, a study also justified hexadecanoic acid to have wide-ranging antifouling activity due to the inhibition of biofilm towards *Vibrio alginolyticus*, *V. mimicus*, *V. parahaemolyticus*, *P. aeruginosa*, and *B. subtillis* [76].

4. Conclusions

The MCE of *U. lactuca* was recovered to possess antifouling properties upon testing against *P. aeruginosa*, through aquarium and field testing. Based on phytochemical analysis, MCE of *U. lactuca* extract exhibits the presence of flavonoid and terpenoid compounds which may help in preventing the growth of biofilm and fouler. Besides, the antifouling compound was also successfully isolated from MCE of *U. lactuca* which is hexadecanoic acid. Furthermore, the findings of this research revealed the new antifouling products that can act as biocides in paint which can replace toxic antifouling coatings as it is more environmentally friendly, efficiently work with minimum concentration, and suitable to be commercialized.

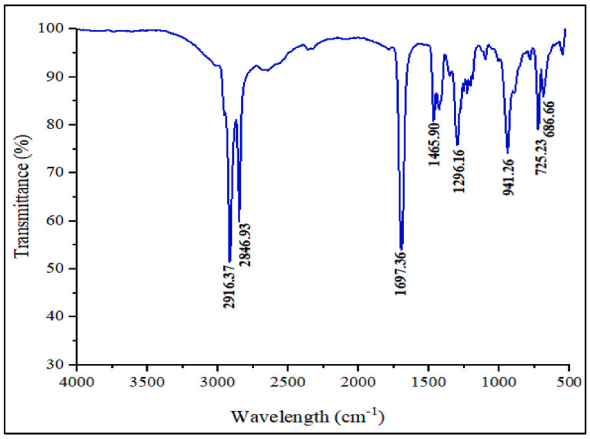

Fig. 11. The FTIR spectrum of isolated compound A4 from MCE of *U. lactuca*.

Table 4
FTIR peak assignment for isolated compound A4 from MCE of *U. lactuca*.

Wavenumber, cm^{-1} [70]	Band assignment	Approximated wavenumber, cm^{-1} (current study)
2913 and 2848	Symmetrical stretching vibration -CH_3 and -CH_2	2916 and 2847
1695	C=O stretching	1697
1464	Bend vibration -CH_2 and -CH_3	1466
939 and 1299	Out-of-plane and in-plane vibration -OH	941 and 1296
723 and 685	Swinging vibration -OH	725 and 687

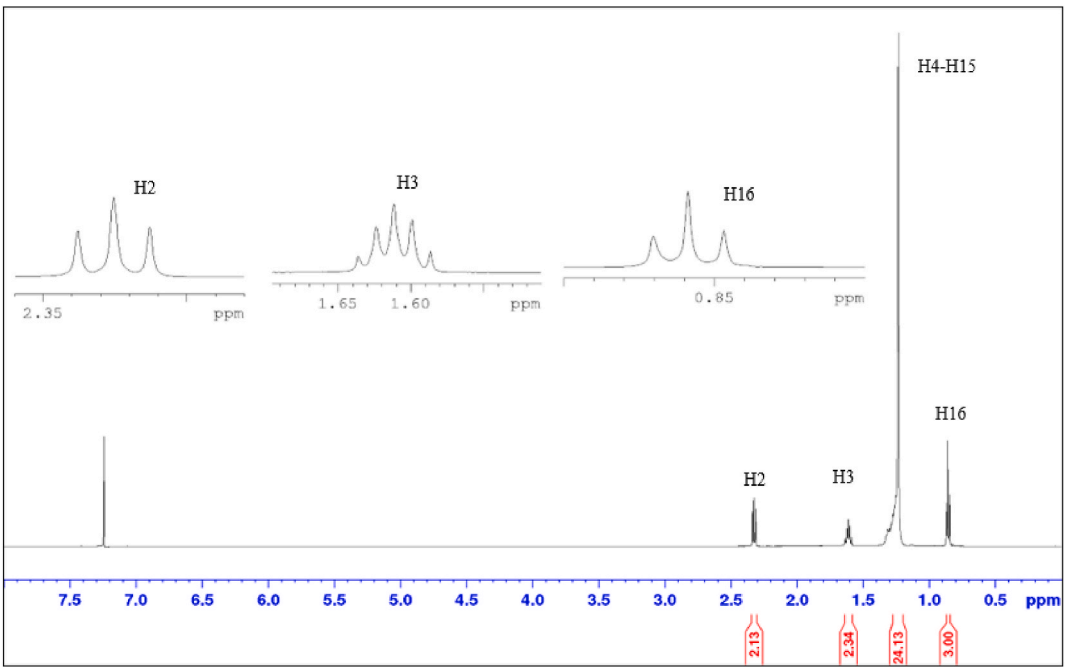

Fig. 12. Spectrum of ^{1}H NMR isolated compound A4 (600 MHz, $CDCl_3$).

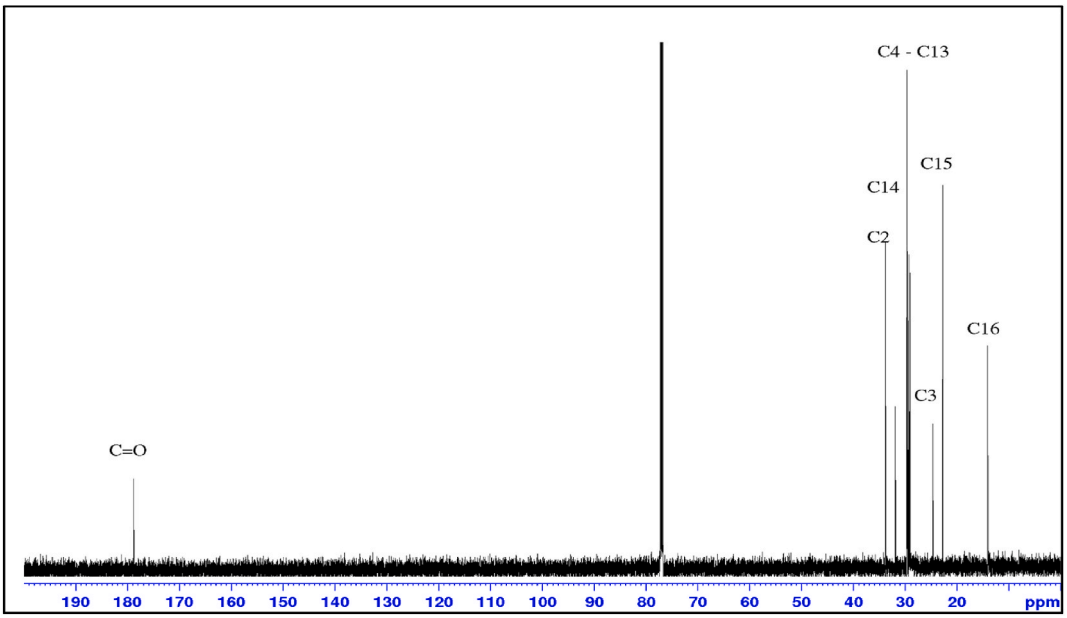

Fig. 13. Spectrum of ^{13}C NMR of isolated compound A4.

Table 5
^{1}H and ^{13}C NMR chemical shifts of isolated compound A4 and studied hexadecenoic acid (600 MHz, $CDCl_3$).

Position	Isolated compound A4		Hexadecanoic acid [46]	
	δ_H (ppm, *J* in Hz)	δ_C (ppm)	δ_H (ppm, J in Hz)	δ_C (ppm)
1	–	178.8	–	178.0
2	2.33 (*t*, *J*= 7.8 Hz)	33.8	2.32 (*t*, *J* = 7.3 Hz)	34.0
3	1.64 (*m*)	24.7	1.60 (*m*)	24.7
4–13	1.26 (*m*)	29.1–29.7	1.26 (*br, s*)	29.0–29.5
14	1.25 (*m*)	31.9	–	31.9
15	1.23 (*m*)	22.7	1.28 (*m*)	22.7
16	0.87 (*t*, *J* = 7.2 Hz)	14.1	0.89 (*t*, *J* = 7.3 Hz)	14.1

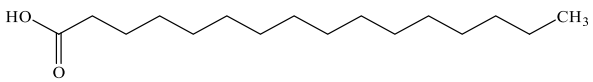

Fig. 14. Chemical structure of hexadecenoic acid.

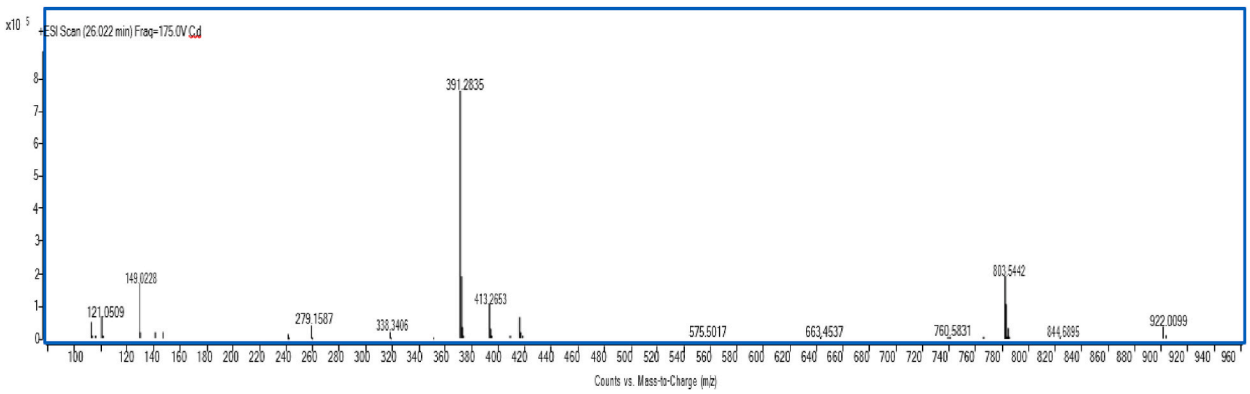

Fig. 15. Mass spectrum of hexadecanoic acid from MCE of *U. lactuca.*

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Data availability statement

Data are available upon request.

CRediT authorship contribution statement

Nik Nurhanis Amira Nik Mohd Sukrri: Writing – review & editing, Writing – original draft, Investigation, Formal analysis. **Ain Farina Farizan:** Writing – review & editing, Writing – original draft, Investigation, Formal analysis. **Mujahidah Mohd Ramzi:** Writing – review & editing, Writing – original draft, Investigation, Formal analysis. **Nurul Najihah Rawi:** Writing – review & editing, Writing – original draft, Investigation, Formal analysis. **Nor Izzati Abd Rahman:** Writing – review & editing, Writing – original draft, Investigation, Formal analysis. **Kamariah Bakar:** Visualization, Validation, Software, Investigation. **Julius Yong Fu Siong:** Investigation. **Ahmad Khusairi Azemi:** Writing – review & editing, Writing – original draft, Methodology, Formal analysis. **Noraznawati Ismail:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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