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GC/MS identification and applications of bioactive seaweed extracts from Mediterranean coast of Egypt

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ABSTRACT

The antibacterial activity of different seaweed species against five fish pathogens (*A. hydrophila, V. parahaemolyticus, V. alginolyticus, V. damsela* and *Vibrio* sp.) has been evaluated. The ethanolic extract of *C. sinuosa* (CSE1) and dichloromethane extract of *C. officinalis* (COM) exhibited the highest antibacterial activity with activity index (AI) = 1.4 ±0.74 and 1.2 ±0.71, respectively. When the extracts were encapsulated into calcium alginate beads their antibacterial activity against the most susceptible bacterial pathogens showed that the significant growth inhibition was against *V. parahaemolyticus* after 5 min post addition of the beads recording 1.4 and 2 fold decrease in the growth, respectively. Recycling of the (CA/COM) beads was carried out for 7 successive cycles with success in elimination of *V. parahaemolyticus*. The potential applications of encapsulated extracts for elimination of microbial load in fish rearing water and antifouling activity were proved in the current investigation. The chemical composition of the bioactive extracts (CSE1) and (COM) was determined using GC/MS analysis which revealed the presence of several constituents that have been reported to exhibit antimicrobial activity. Additionally, the dichloromethane extract (COM) produced six major components including *n*-nonadecane, 1,2,3-propanetricarboxylic acid, 2-(acetyloxy)-, tributyl ester, 2-methylhexadecan-1-ol, 1-docosene, 1-eicosanol and chloroacetic acid, octadecyl ester. However, the ethanolic extract (CSE1) was characterized by the presence of fatty acids, fatty acids ethyl esters and aromatic hydrocarbon where the most abundant compound was *n*-tridecanoic acid ethyl ester.

INTRODUCTION

Adverse effects of infectious diseases on global aquaculture are alarming since they cause total or partial loss of production (FAO, 2016; Bondad-Reantaso *et al*., 2005; Ward *et al*., 2016). It is well known that fishes are harmful carriers of some infectious diseases leading to opportunistic microorganisms such as *Salmonella* and *Vibrio* species. In order to get over these problems, synthetic chemicals and non-biodegradable polymers have been used to control the infections. Accumulated chemicals can cause environmental pollution, which may be toxic to the non-target organisms (Randhawa & Kullar, 2011). Moreover, commercial use of antibiotics and veterinary drugs are limited for use in aquaculture to control the bacterial and other opportunistic infections. Currently, degradable natural compounds from biochemical origin are used as an eco-friendly approach in aquaculture for the control of infection and thus promote growth (Stalin *et al*., 2008).

Seaweeds are rich in bioactive compounds that are safe and nontoxic compared with chemical synthetic drugs (Fernando *et al*., 2018; Wang *et al.,* 2018). Recently, majority of potential antibacterial natural products are produced from marine resources (Goyal *et al*., 2017; Nalini *et al*., 2018). Seaweed extracts or their isolated components have been utilized for treatment and/or prevention of shrimp and fish diseases (Chakraborty *et al*., 2014; Tomazelli Jr. *et al*., 2017) because of their nutritional, immunomodulatory, antiviral, antibacterial, and growth promoting activities (Cruz-Suárez *et al.,* 2008; Fleurence *et al*., 2012; Milledge *et al*., 2016; Pádua *et al.,* 2015; Sanjeewa *et al*., 2016). Encapsulation has been used for the protection of natural molecules against environmental conditions (Azzi *et al*., 2017; Gharib *et al.,* 2017). The encapsulation of bioactive agents may enhance their solubility, stability, efficiency, and bioavailability as well as control their release (Parris *et al*., 2005; Ephrem *et al*., 2018). Alginate microbeads have been used for the encapsulation of both low molecular and macromolecular weight agents (Ephrem *et al*., 2018; Kavoosi *et al*., 2018).

Biofouling process takes place when any natural or artificial surfaces submerged into the marine environment and colonized by micro- and macro-organisms (Clare, 1996), which in turn causes major ecological and economic impacts, especially when it occurs on ship hulls or aquaculture facilities (Yebra *et al*., 2004; Piola *et al*., 2009). Since several commercial biocides such as TBT based antifouling paints have been recently banned, the screening for alternative eco-friendly antifoulants appears to be urgent (Dobretsov *et al.,* 2015). Seaweeds could be an important source, since they produce metabolites such as amides, fatty acids, terpenoids, pyrroles, lactones and steroids with antifouling potential.

The present investigation is focused on the study of the antibacterial activity of hexane, dichloromethane and ethanolic extracts of five Mediterranean seaweeds belong to Chlorophyta (*Ulva fasciata, Ulva linza*), Rhodophyta (*Corallina officinalis*, *Jania rubens*) and Phaeophyta (*Colpomenia sinuosa*)) collected from the coast of Alexandria (Egypt) against five fish pathogens (*A. hydrophila, V. parahaemolyticus, V. alginolyticus, V. damsela* and *Vibrio* sp.). The bioactive components of dichloromethane and ethanolic extracts from *C. officinalis* and *C. sinuosa*, respectively were investigated using gas chromatography/mass spectroscopy (GC/MS) analysis. The effect of the encapsulation process and other potential applications of both extracts have been investigated.

MATERIALS AND METHODS

Seaweed collection

The five seaweed species used in this study; *Ulva fasciata, Ulva linza, Corallina officinalis*, *Jania rubens* and *Colpomenia sinuosa* were collected from the coast of Alexandria, Egypt at Abu Qir Bay and the Eastern Harbour (El-Manshia). Seaweeds were identified according to Aleem (1993). After collecting the samples, they were washed with fresh water followed by distilled water to remove any associated epiphytes and debris. Then they were dried under shade for one week and in oven at 45 °C for 24 h. The dried seaweeds were ground to powder with electric grinder mixer.

Preparation of seaweed extracts

The crude seaweed extracts have been obtained using *n*-hexane, dichloromethane and 70% ethanol. The powdered sample of each species (25g) was macerated in *n*-hexane (200 ml) followed by dichloromethane (200 ml) then 70% ethanol (125 ml) at room temperature for one week with regular shaking. After filtration, solvents were evaporated under reduced pressure at 45°C to produce dry *n*-hexane (UFH1, UFH2, ULH and CSH), dichloromethane (UFM1, UFM2, ULM, COM, JRM and CSM) and ethanolic (UFE1, COE1, JRE1 and CSE1) extracts. The extraction procedure with each solvent was repeated once or twice again. The ethanolic extracts (UFE2, UFE3, ULE2, COE2 and JRE2) have been directly produced by maceration in 70% ethanol for 15 days. The obtained crude extracts were then kept at -20°C until tested (Wefky *et al*., 2009; Shobier *et al*., 2010; Shobier *et al*., 2016).

Test pathogenic bacteria

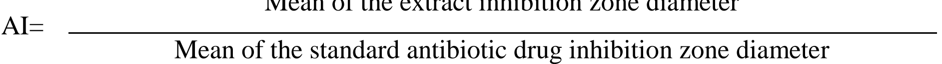
The test microorganisms included *Vibrio alginolyticus, V. parahaemolyticus, V. damsela*, *Vibrio* sp. and *A. hydrophila*, were kindly obtained from Fish Diseases Department, Faculty of Veterinary Medicine, Alexandria University, Egypt.

Antibacterial assay

Antibacterial activity of the seaweed extracts against different pathogens was performed using well-cut diffusion technique (Hassan, 2016). Cephalexin (30 µg), Rifampicin (5 µg) and Piperacillin (100 µg) were used as positive controls and DMSO was used as a negative control. After incubation at 37°C for 24 h, the positive results were detected as clear zones around wells and measured in mm.

Activity index

The activity index (AI) was used for comparing the antibacterial activity of each tested seaweed extract against all bacterial pathogens with that obtained from standard antibiotic.



$$AI = \frac{\text{Mean of the extract inhibition zone diameter}}{\text{Mean of the standard antibiotic drug inhibition zone diameter}}$$

Encapsulation and antibacterial activity of the most promising extracts

The most promising seaweed extracts were separately mixed with sodium alginate solution (2% w/v) then dropped from a hydrodermic syringe to 100 ml of (2%) calcium chloride solution with constant stirring at 65 °C and left to harden for 1 h. Finally the beads were washed with sterile distilled water and kept for use (Shobier *et al*., 2010). The antibacterial effect of the encapsulated extracts was estimated using shake-flask method (Ye *et al.,* 2005). Briefly, predetermined amount of each formed encapsulated extract was inoculated into 50 ml cell suspension of the tested pathogen with continuous shaking at 150 rpm at rotary shaker. 1 ml of sample solution was drawn and bacterial growth (optical density at 550 nm) was detected and compared with that inoculated with free extract and free bacteria (Kim *et al*., 2007).

Recycling of the encapsulated extract

The reuse of the encapsulated extract was carried out by removing the medium and washing the beads after each cycle and then a new sterilized medium (50 ml) was added, and new cycle was run. This process was repeated several times. At

the end of each cycle the bacterial growth was estimated and expressed as optical density at 550 nm (Kim *et al*., 2007).

Applications of the encapsulated extracts

Elimination of bacterial load in fish rearing water

Certain volume of the encapsulated extracts of *C. officinalis* (CA/COM) and *C. sinuosa* (CA/CSE1) were separately amended in 50 ml of fish rearing water, collected from El-Mex farm and incubated at 30 ^{o}C for 24h. One flask was kept as control (without addition of the encapsulated extracts). After that, estimation of total viable bacterial count and *Vibrio* spp. Counts was carried out and compared with control.

Antifouling activity

Antifouling activity was evaluated according to Kumaran *et al.* (2011). Briefly, seawater (200 ml) was mixed separately with the encapsulated extracts of *C. officinalis* and *C. sinuosa* in a conical flask containing cover glass and incubated overnight at 30°C followed by dying with crystal violet solution (0.4%) for 10 minutes, and washing with water, then drying at room temperature and checked under the microscope. One flask without extract was kept as control.

Gas chromatography / Mass spectrometry (GC/MS) analysis

GC/MS analysis was accomplished using GC instrument (Agilent 7890A) equipped with an HP-5MS column (30 m × 250 µm × 0.25 µm film thickness) and coupled with MS detector (Agilent 5975C). The initial oven temperature was programmed to be held at 90°C for 1 min then risen to 300 °C for 30 min at a rate of 8°C /min. Helium was used as a carrier gas at a flow rate of 1.5 ml/ min. The injection volume of each sample was 1 µl in the splitless mode where the injector temperature was 290°C. Mass spectrum was operated at 70ev and mass range from 60-600 amu.

Beads characterization

The formed beads were coated with a layer of gold to examine their morphological and surface structure by scanning electron microscope (SEM) JEOL JSM-5300 at the required magnification and an acceleration voltage of 25 kV. Fourier transform infrared spectra (FTIR) were recorded on Bruker VERTEX 70 spectrometer in the range between 4000 and 400 cm^{-1} connected with platinum ART unit.

Statistical analysis

The experiments were performed in triplicate, and the results were recorded by the mean values and the standard deviations (SD). The experimental data were subjected to analysis of variance (one-way ANOVA) to determine significant differences where the term significant difference was referred to $p \leq 0.05$.

RESULTS AND DISCUSSTION

Antibacterial activity of the seaweed extracts

The present study concerned with screening for the antibacterial potential of different seaweed extracts against selected fish pathogens. Results revealed that the ethanolic extract of *C. sinuosa* (CSE1) exhibited the highest antibacterial activity

showing average zone of inhibition 17.2 mm and activity index 1.4 ±0.74 followed by the dichloromethane extract of *C. officinalis* (COM) with average zone of inhibition 14.4 mm and activity index 1.2 ±0.71 (Table1 and Fig. 1). In accordance with the current study, Bansemir *et al.* (2006) studied the antibacterial potential of the components extracted from 26 algae species by methanol, water and dichloromethane against five fish pathogens and stated that the highest activities were recorded for the components extracted by dichloromethane due to the hydrophobic nature of some constituents, such as fatty products (Vatsos & Rebours, 2015). On the other side, Redjem *et al*. (2013) reported that the extracts obtained by using polar solvents exhibited the highest antibacterial activity against the tested fish pathogens. Further, the yield of extractable materials and antimicrobials from different seaweed species depend on the solvent type. Antibacterial potential of algae against fish pathogens was reported in other studies (Rizzo *et al.,* 2017; Saleh & Al-Mariri, 2017). Natural factors, such as environmental conditions (climate, location, salinity, and temperature), pollution, the life stage, reproductive state, growth conditions, age of the seaweed as well as collection time and epiphytic organisms affect the chemical composition and antimicrobial activity of seaweeds revealing that this activity is not attributed to a single compound, but it could be related to some of them and to a combination of metabolites (Trigui *et al.,* 2013; Stabili *et al*., 2014; Pérez *et al*., 2016).

Table 1: Diameter of the inhibition zones (mm) of crude extracts against different fish pathogens

Species	Extract	Sample Code	Inhibition zone diameter (mm)					
			A. hydrophila	*Vibrio* Sp.	*V. parahaemolyticus*	*V. alginolyticus*	*V. damsela*	AVG
U. fasciata (El-Manshia; Jan. 2018)	Hexane	UFH1	0	10	12	16	0	7.6
	Dichloromethane	UFM1	0	11	0	0	0	2.2
	Ethanolic	UFE1	0	0	0	0	0	0
		UFE2	0	15	12	0	12	7.8
U. fasciata (Abu Qir Bay; Feb. 2018)	Hexane	UFH2	0	0	0	0	0	0
	Dichloromethane	UFM2	0	0	0	0	0	0
	Ethanolic	UFE3	12	0	0	0	0	2.4
U. linza (Abu Qir Bay; Feb. 2018)	Hexane	ULH	0	0	0	0	0	0
	Dichloromethane	ULM	0	0	0	0	0	0
	Ethanolic	ULE2	0	0	0	0	0	0
C. officinalis (El-Manshia; Jan. 2018)	Dichloromethane	COM	17	15	20	20	0	14.4
	Ethanolic	COE1	11	12	13	0	12	9.6
		COE2	0	10	0	0	0	2
J. rubens (Abu Qir Bay ; Feb. 2018)	Dichloromethane	JRM	0	17	16	0	0	6.6
	Ethanolic	JRE1	0	0	0	0	0	0
		JRE2	0	12	13	18	0	8.6
C. sinuosa (Abu Qir Bay; Feb. 2018)	Hexane	CSH	11	12	15	11	0	9.8
	Dichloromethane	CSM	0	0	0	0	0	0
	Ethanolic	CSE1	15	18	19	19	15	17.2
Piperacillin	(100 µg)		20	14	18	0	8	12
Rifampicin	(5 µg)		12	0	15	0	10	7.4
Cephalexin	(30 µg)		0	0	33	0	0	6.6

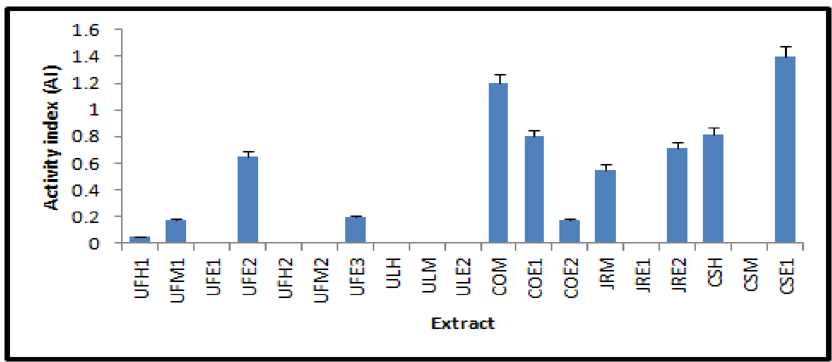


Fig. 1: Activity index of the crude seaweed extracts against the tested bacterial pathogens. Bars are expressed as mean ± SE.

Antibacterial activity of the encapsulated extracts (CA/COM) and (CA/CSE1)

The most effective extracts in term of antibacterial activity were selected and encapsulated into calcium alginate beads, to be further investigated against the most sensitive bacterial pathogens. Antibacterial activity of the encapsulated *C. officinalis* dichloromethane extract (CA/COM) was tested against *V. parahaemolyticus, V. alginolyticus* and *A. hydrophila,* while the encapsulated ethanolic extract of *C. sinuosa* (CA/CSE1) was tested against *V. parahaemolyticus, V. alginolyticus* and *Vibrio* sp. using shake flask method. Results (Fig. 2 A) indicated that the highest significant (*P*=0.031) bacterial inhibition caused by (CA/COM) extract against *V. parahaemolyticus* was after 5 min, where the bacterial growth (OD_{550}) reached $95x10^{-3}$ recording 2 fold decrease in the bacterial growth compared with free bacteria ($190x10^{-3}$) at the same time and the effect was prolonged up to 35 min, followed by an increase in the bacterial growth, however still more active than the free extract. Similar behavior was observed against *V. alginolyticus* and *A. hydrophila* (Fig. 2 B, C), where the bacterial reduction started 5 min post addition of the encapsulated extract (CA/COM) with 1.8 and 1.6 fold decrease in the bacterial growth, respectively compared with free bacteria at the same time.

Regarding the effect of *C. sinuosa* ethanolic extract (CA/CSE1) on the growth of the tested pathogens, elimination of *V. parahaemolyticus* and *Vibrio* sp. was significant (*P*= 0.013 and 0.042) and observed after 5 min with 1.4 and 1.3 fold decrease in growth, respectively compared with the growth of free bacteria after 5 min (Fig. 2 D, F). Delayed effect against *V. alginolyticus* was observed to be after 10 min post beads addition, recording 1.6 fold decrease in the bacterial growth compared with growth of free bacteria (Fig. 2E). The encapsulated beads (CA/CSE1) exhibited conservative effect against *Vibrio* sp. during 10-30 min, followed by slight decrease at 35 min. Overall results concluded that the encapsulation of *C. sinuosa* and *C. officinalis* extracts revealed better significant antibacterial effect (significant at $P<0.05$) compared with the free extract during the period of study. This effect may be attributed to the hydrophobicity of the calcium alginate beads entrapping the extract, which provides protection of the bioactive metabolites, controlled release of the bioactive components and allowing more surface contact (Millette *et al*., 2007). In a previous study by Shobier et al. (2010), the encapsulated methanolic extract of *Pterocladia capillacea* showed good antibacterial activity against *Aeromonas hydrophila*, *Pseudomonas fluorescens* and *Vibrio anguillarum*.

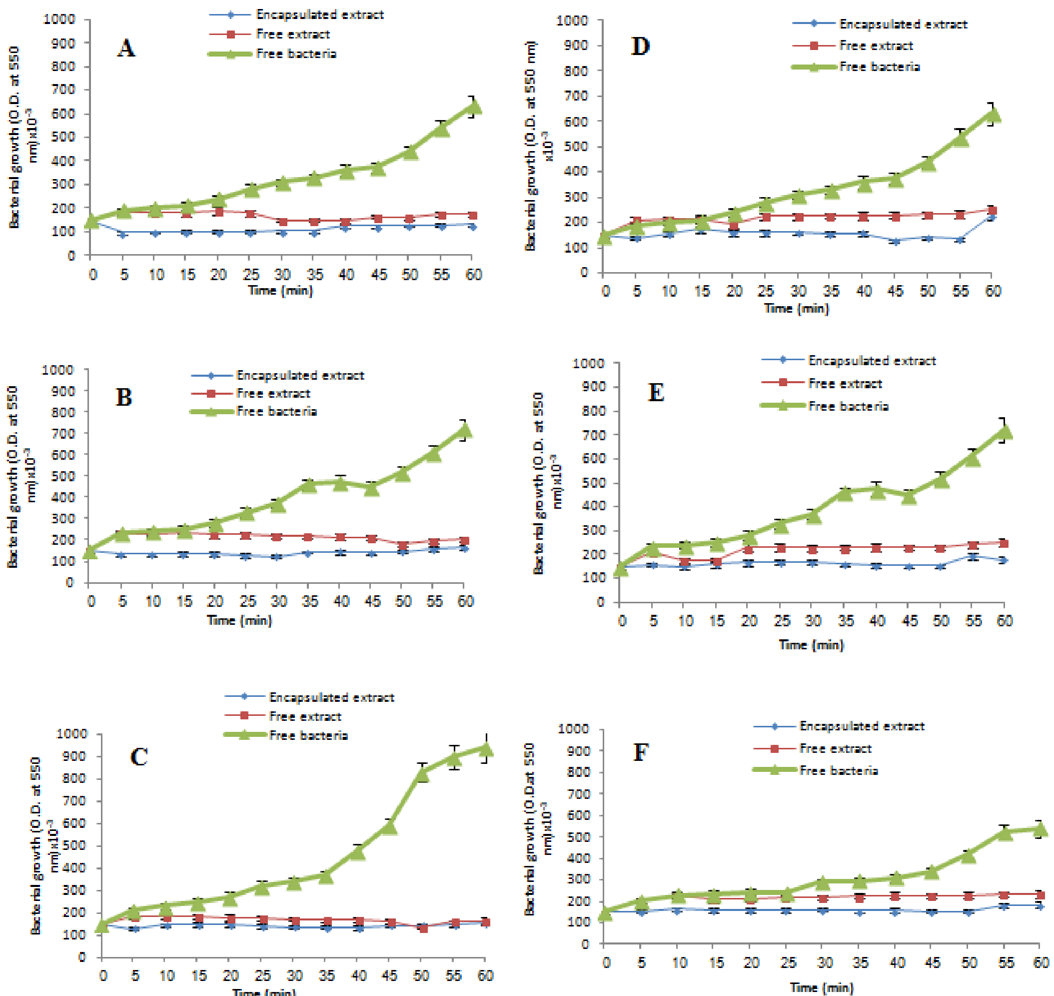


Fig. 2: Antibacterial activity of free and encapsulated extracts of *C. officinalis* against (A) *V. parahaemolyticus*, (B) *V. alginolyticus*, (C) *A. hydrophila* and of free and encapsulated extracts of *C. sinuosa* against (D) *V. parahaemolyticus*, (E) *V. alginolyticus* and (F) *Vibrio* sp.

Recycling of the most active extracts

The effective entrapped dichloromethane extract of *C. officinalis* (CA/COM) was recycled for 7 successive cycles. As shown in Fig. 3, the reduction of bacterial growth started at the first cycle and prolonged till the third cycle, followed by lower reduction in the bacterial growth, however still lower than the control (uninoculated with encapsulated beads). Our study was supported by study of Kim *et al*. (2007) who reported the potential inactivation of bacterial pathogens for successive cycles.

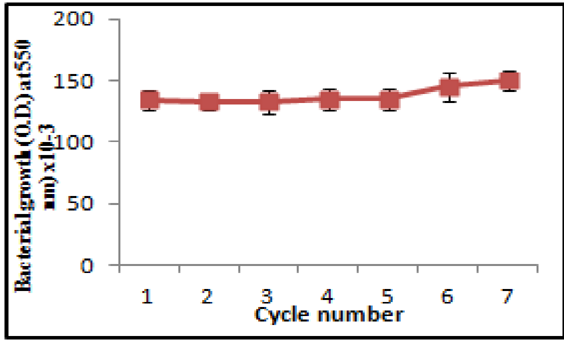


Fig. 3: Recycling of the encapsulated dichloromethane extract of *C. officinalis* (CA/COM).

Applications of the encapsulated extracts

Efficiency of the encapsulated extracts in elimination of bacterial load in fish rearing water

Infection with *Vibrio* is one of the most abundant and devastating diseases in aquaculture. Some *Vibrio* strains including *V. splendidus, V. harveyi* and *V. parahaemolyticus* are resistant to different antibiotics. Thus, there is a must to innovative eco-friendly alternatives. So the aim of the present experiment was a trial to eliminate the *Vibrio* load in fish rearing water collected from El-Mex fish farm. A determined volume of the encapsulated extracts was added to 50 ml of the fish rearing water and then estimation of *Vibrio* spp. and total bacterial counts (TVC) was carried out after 24 h. Results shown in Fig. 4 revealed the elimination of *Vibrio* spp. with 93.7% and 90% after addition of the encapsulated extract of *C. officinalis* (CA/COM) and *C. sinuosa* (CA/CSE1), respectively. On the other hand TVC was also reduced by 90% compared with the control (without addition of the encapsulated extract). Oliveira *et al*. (2014) stated that addition of the brown algae *Ascophyllum nodosum* as meals to the diet of Nile tilapia infected with *A. hydrophila* enhanced the sanitary status of the fish and occurrence of the infection was lower. Previous studies reported *in vivo* antibacterial activity of water, methanol and dichloromethane extracts of different algal species against *Vibrio anguillarum, Aeromonas salmonicida, Pseudomonas anguilliseptica*, *A. hydrophila*, *Yersinia ruckeri,* and shrimp *Vibrio* pathogens for aquaculture sanitary (Bansemir *et al*., 2006, Manilal *et al*., 2012; Rizzo *et al*., 2017; Wan *et al*., 2018).

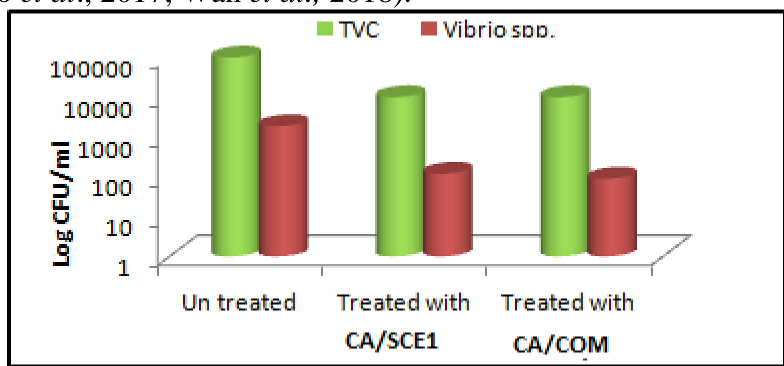


Fig. 4: Counts of total bacteria (TVC) and *Vibrio* spp. before and after treatment with the encapsulated extracts of *C. officinalis* and *C. sinuosa.*

Antifouling activity of the encapsulated extracts

Marine biofouling is a paramount phenomenon in the marine environment, which leads to serious problems to marine industries worldwide (Saha *et al*., 2017). The present experiment focused on the antagonistic effect of the encapsulated extracts of *C. officinalis* and *C. sinuosa* on biofilm formation. Fig. 5 A, B show the reduction of the bacterial load in the formed biofilm by the action of *C. sinuosa* and *C. officinalis* encapsulated extracts compared with the control (biofilm formed without addition of the encapsulated extract) (Fig. 5 C). This confirmed the potential antifouling activity of both encapsulated extracts. In accordance with the obtained results, Bazes *et al*. (2006) reported the antifouling activity of the marine alga *Ceramium botryocarpum* extracts against *Vibrio* sp. and *Pseudovibrio denitrificans* which are associated with immersed surfaces. The aqueous, ethanolic and dichloromethane extracts of 30 marine algae exhibited high levels of *in vitro* antifouling activity against 35 isolates of marine bacteria (Hellio *et al*., 2001; Pérez *et al*., 2016). Another report showed that the addition of algal extract to paint enhances the quality for 6 weeks (Chambers *et al.,* 2011).

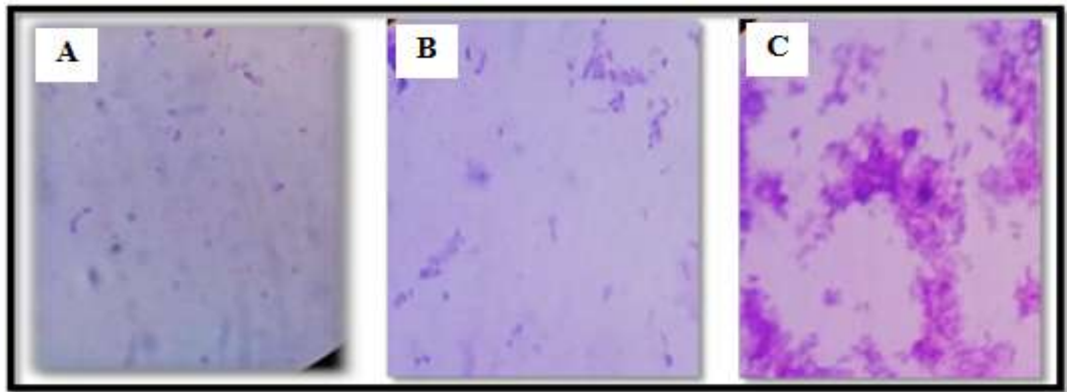


Fig. 5: Photographs illustrating the antifouling effect of the encapsulated extract of (A) *C. sinuosa*, (B) *C. officinalis* and (C) control (uninoculated).

GC/MS analysis of the most potential extracts (COM and CSE1)

The identification of the unknown phytochemicals of the *C. officinalis* and *C. sinuosa* crude extracts was confirmed by comparison of their mass spectra with those of standard compounds stored in the NIST library.

GC/MS analysis of the *C. officinalis* dichloromethane extract (COM)

The GC/MS chromatogram of the dichloromethane extract of *C. officinalis* harvested from El-Manshia exhibited 17 peaks (Fig. 6) of which 12 peaks were identified. The chemical compositions of the dichloromethane extract are shown in Table 2. The most abundant six constituents were *n*-nonadecane (RT = 16.95 min), 1,2,3-propanetricarboxylic acid, 2-(acetyloxy)-, tributyl ester (RT = 27.18 min), 2-methylhexadecan-1-ol (RT = 22.18 min), 1-docosene (RT = 25.89 min), 1-eicosanol (RT = 29.20 min) and chloroacetic acid, octadecyl ester (RT = 18.58 min). Ismail (2017) reported that *C. officinalis* contains high content of carotenoid (3.8 mg/g dry wt.), β-Carotene (3940.12 IU/100 g) and carbohydrates (27.98% of dry wt.). Moreover, *C. officinalis* exhibited good antioxidant activity (72.6%). Borik (2014) found that the volatile constituents of this alga consist of monoterpenes (1.17%), diterpenes (0.07%), aldehydes (1.42%), ketones (0.72%), alcohols (0.17%), esters (15.30%), aliphatic hydrocarbons (38.88%), cyclic hydrocarbons (0.35%), phenol (2.87%), heterocyclic (0.19%) and miscellaneous (0.87%). Djapic (2018) identified and quantified the chemical constituents of *C. officinalis* and indicated that the alga contains 8.42% monoterpenes, 2.02% sesquiterpenes, 4.32% diterpenes, 13.64% triterpenes, 14.92% acyclic alkanes, 2.06% branched alkanes, 5.44% alkenes, 0.6% aromatic compound, 2.88% organobromine and 0.6% organosulfur compounds. The triterpene squalene was the most predominant compound which was found in a percentage of 13.64%.

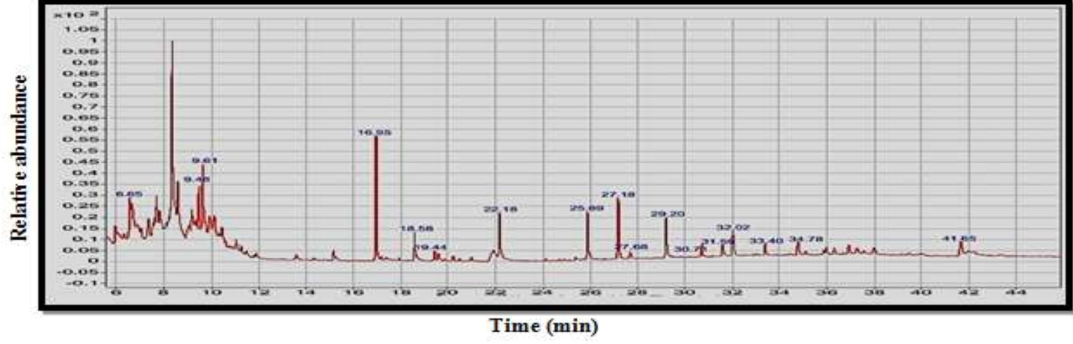


Fig. 6: GC/MS chromatogram of the dichloromethane extract of *C. officinalis* (COM).

Table 2: Chemical constituents and biological activity of *C. officinalis* dichloromethane extract (COM)

Peak no.	RT (min)	Component name	Molecular formula	MW (m/z)	Chemical structure	Biological activity	Reference
1	6.65	Unidentified	-	-	-	-	-
2	9.48	Unidentified	-	-	-	-	-
3	9.61	Unidentified	-	-	-	-	-
4	16.95	*n*-Nonadecane	$C_{19}H_{40}$	268		Cytotoxic, antimicrobial	Hsouna *et al.* (2011)
5	18.58	Chloroacetic acid, octadecyl ester	$C_{20}H_{39}ClO_2$	346	Cl O O	-------	-------
6	19.44	Cholestan-3-ol, 2-methylene-, (3β,5α)	$C_{28}H_{48}O$	400	HO	Anti-inflammatory	Al-Rubaye *et al.* (2017)
7	22.18	2-Methylhexadecan-1-ol	$C_{17}H_{36}O$	256	HO	-------	-------
8	25.89	1-Docosene	$C_{22}H_{44}$	308		Antibacterial	Beevi *et al.* (2014)
9	27.18	1,2,3-Propanetricarboxylic acid, 2-(acetyloxy)-, tributyl ester	$C_{20}H_{34}O_8$	402		Antibacterial	Al-Rubaye *et al.* (2017)
10	27.68	1,1-Dimethyltetradecyl hydrosulfide	$C_{16}H_{34}S$	258	SH	Antioxidant, antibacterial	Sivakumar (2014)

11	29.20	1-Eicosanol	$C_{20}H_{42}O$	298		Anticancer, antimicrobial	Wong & Kadir (2011); Karthi *et al.* (2015)
12	30.71	1,54-Dibromotetrapenta-contane	$C_{54}H_{108}Br_2$	914		-------	-------
13	31.59	3',8,8'-Trimethoxy-3-piperidin-1-yl-2,2'-binaphthyl-1,1',4,4'-tetrone	$C_{28}H_{25}NO_7$	487		Antioxidant	Nazareth & Vijayalakshmi (2014)
14	32.02	(17*E*)-17-Pentatriacontene	$C_{35}H_{70}$	490		Antibacterial, antiviral	Paramanantham & Murugesan (2014)
15	33.40	2-Octadecoxyethanol	$C_{20}H_{42}O_2$	314		Antimicrobial	Karthi *et al.* (2015)
16	34.78	Unidentified	-	-	-	-	-
17	41.65	Unidentified	-	-	-	-	-

GC/MS analysis of the *C. sinuosa* ethanolic extract (CSE1)

The chemical constituents identified by GC-MS analysis of the ethanolic extract of *C. sinuosa* collected from Abu Qir Bay presented in Fig. 7 and Table 3. *n*-Tridecanoic acid ethyl ester (RT = 22.38 min) was found as the major component followed by tetradecanoic acid, ethyl ester (RT = 18.66 min), *n*-hexadecanoic acid (RT = 22.03 min), ethyl (9*Z*,11*E*)-9,11-octadecadienoate (RT = 29.02 min), ethyl *n*-heptadecanoate (RT = 29.28 min), bis (2-ethylhexyl) 1,2-benzenedicarboxylate (RT = 31.61 min), 6,10,14-trimethylpentadecan-2-one (RT=19.64 min) and *n*-pentadecanoic acid ethyl ester (RT = 25.96 min) (Table 3). El-Shora et al. (2018) isolated the polysaccharide fucoidan from *C. sinuosa* which possessed appreciable antibacterial activity. Pasdaran et al. (2016) reported that the volatile oil of *C. sinuosa* contains 2-iodo-3-methyl-butane, 2-undecanone, 1-dodecanol, 2-tridecene, tridecane, neryl acetone, edulan I, pseudoionone, 1-tridecanol, 1-tetradecene, 1-tetradecanol, pentadecane, 7-pentadecanone, hexadecane, 1-hexadecanol and 8-heptadecene, 1-chloro.It was found that hexadecane and 7-pentadecanone are the main components of the volatile oil. However, a significant antibacterial activity was not observed.

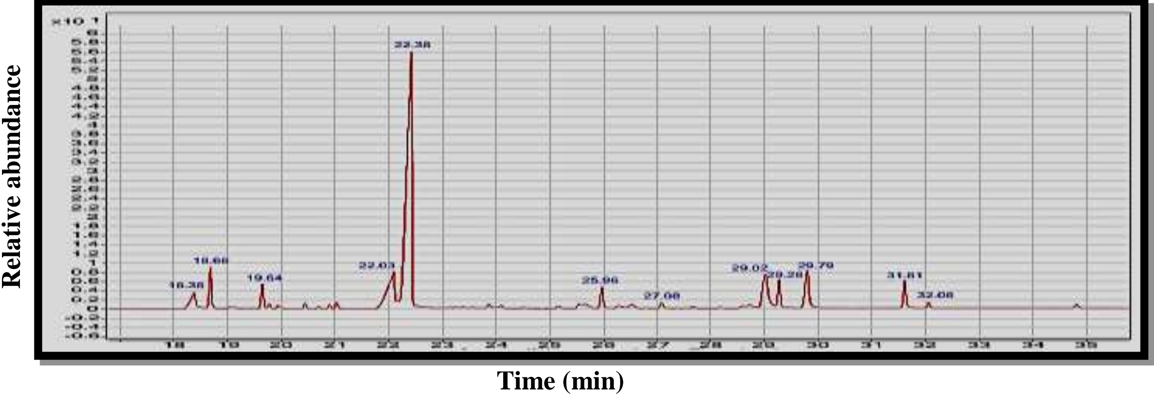


Fig. 7: GC/MS chromatogram of the ethanolic extract of *C. sinuosa* (CSE1).

Table 3: Chemical constituents of and biological activity of *C. sinuosa* ethanolic extract (CSE1)

Peak no.	RT (min)	Component name	Molecular formula	MW (m/z)	Chemical structure	Biological activity	Reference
1	18.36	*n*-Tetradecanoic acid	$C_{14}H_{28}O_2$	228		Antioxidant, anti-cancer, hypocholesterolemic, antibacterial	Rajeswari *et al.* (2013); Al-Saif *et al.* (2013)
2	18.66	Tetradecanoic acid, ethyl ester	$C_{16}H_{32}O_2$	256		Hypercholesterolemic	Kanimozhi & Ratha Bai (2012)
3	19.64	6,10,14-Trimethylpentadecan-2-one	$C_{18}H_{36}O$	268		Flavoring agent, antimicrobial	Uma Maheswari & Reena (2017)
4	22.03	*n*-Hexadecanoic acid	$C_{16}H_{32}O_2$	256		Antioxidant, antimicrobial, hypocholesterolemic, 5-alpha reductase inhibitor	Hema *et al.* (2011); Pietro *et al.* (2010); Hsouna *et al.* (2011)
5	22.38	*n*-Tridecanoic acid, ethyl ester	$C_{15}H_{30}O_2$	242		-------	-------
6	25.96	*n*-Pentadecanoic acid, ethyl ester	$C_{17}H_{34}O_2$	270		-------	-------
7	27.08	Ethyl (9*Z*,12*Z*)-9,12-octadecadienoate	$C_{20}H_{36}O_2$	308		Hypocholesterolemic, antiarthritic, hepatoprotective antihistaminic, anticoronary	Sudha *et al.* (2013)

8	29.02	Ethyl (9*Z*,11*E*)-9,11-octadecadienoate	$C_{20}H_{36}O_2$	308		Anti-cancer	Mohansrinivasan *et al.* (2015)
9	29.28	Ethyl *n*-heptadecanoate	$C_{19}H_{38}O_2$	298		Antimicrobial	Zheng *et al.* (2005)
10	29.79	Unidentified	-	-	-	-	-
11	31.61	Bis (2-ethylhexyl) 1,2-benzenedicarboxylate	$C_{24}H_{38}O_4$	390		Anti-cancer	Save *et al.* (2015)
12	32.06	Ethyl *n*-hexadecanoate	$C_{18}H_{36}O_2$	284		Antioxidant, nematicide,, hypocholesterolemic	Sethi *et al.* (2013)

Characterization of alginate beads

Scanning electron microscopic study

The beads were analyzed by scanning electron microscope to evaluate the morphology and the bead surface. SEM photographs of the outer surface of plain and encapsulated beads (CA, CA/COM and CA/CSE1, respectively) appear flattened, rough and very irregular with a porous structure which appears like that of an orange peel (Fig. 8 A, B, C). The drying of the beads had big effect on their morphology and texture which leads to the deformation of the surface. SEM images of the inner surface show that the beads have porous nature with a sponge like structure in which the extracts were entrapped (Fig. 9 A, B, C).

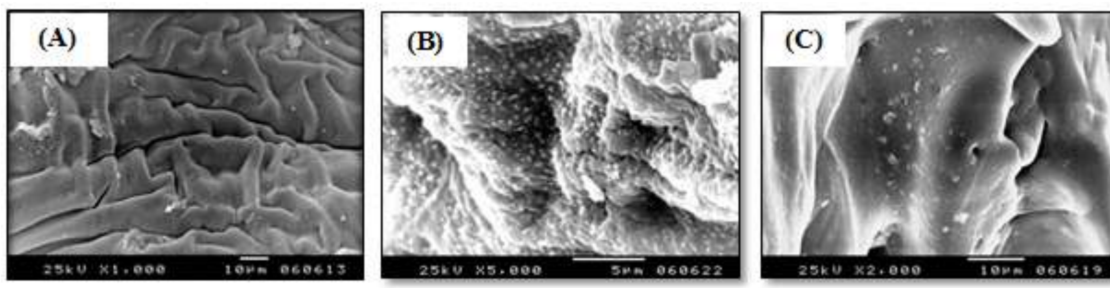


Fig. 8: SEM of the outer surface of (A) plain Ca-alginate beads (CA), (B) Ca-alginate beads encapsulated with *C. officinalis* extract (CA/COM) and (C) Ca-alginate beads encapsulated with *C. sinuosa* extract (CA/CSE1).

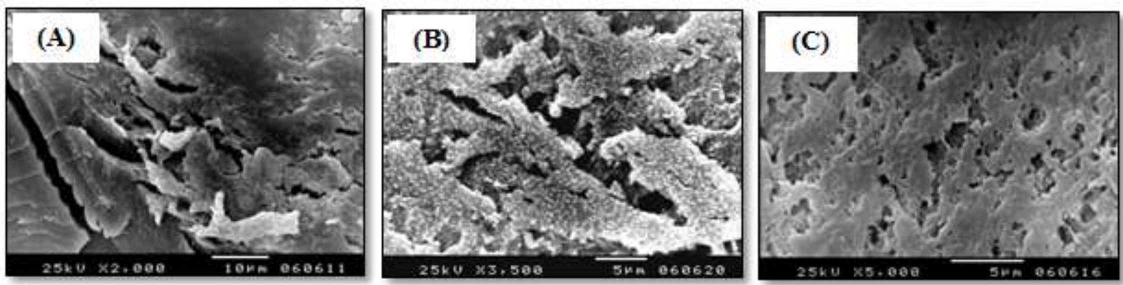


Fig. 9: SEM of the inner surface of (A) plain Ca-alginate beads (CA), (B) Ca-alginate beads encapsulated with *C. officinalis* extract (CA/COM) and (C) Ca-alginate beads encapsulated with *C. sinuosa* extract (CA/CSE1).

FTIR study

The FTIR spectrum of plain beads (CA) shows a broad band at (3266 cm^{-1}) attributed to O-H stretching. The band around (2926 cm^{-1}) could be related to aliphatic C-H vibration. The two peaks around (1600 and 1418 cm^{-1}) are assigned to carboxylate $–COO^{-}$ asymmetric/symmetric stretching. The bands at (1078 and 1021 cm^{-1}) are assigned to the –C-O stretching of ether and alcoholic groups, respectively (Fig. 10 A). The spectrum of alginate beads encapsulated with *C. officinalis* dichloromethane extract (CA/COM) (Fig. 10 B) shows stretching frequencies of O-H groups (3279 cm^{-1}), $–COO^{-}$ asymmetric/symmetric stretching (1603 and 1415 cm^{-1}), –C-O stretching of the ether group (1080 cm^{-1}) of alginate and –C-O stretching band attributed to alcoholic group (1024 cm^{-1}). The FTIR spectrum of alginate beads encapsulated with *C. sinuosa* ethanolic extract (CA/CSE1) indicated that the position of the O-H stretching band is shifted to (3340 cm^{-1}). Other peaks appeared at 1600, 1423, 1080 and 1021 cm^{-1}, respectively (Fig. 10 C). These spectra revealed that all characteristic functional groups originally exist on the plain alginate beads are still

present after encapsulation except the two bands at (2322 and 2284 cm^{-1}) disappeared in the encapsulated beads.

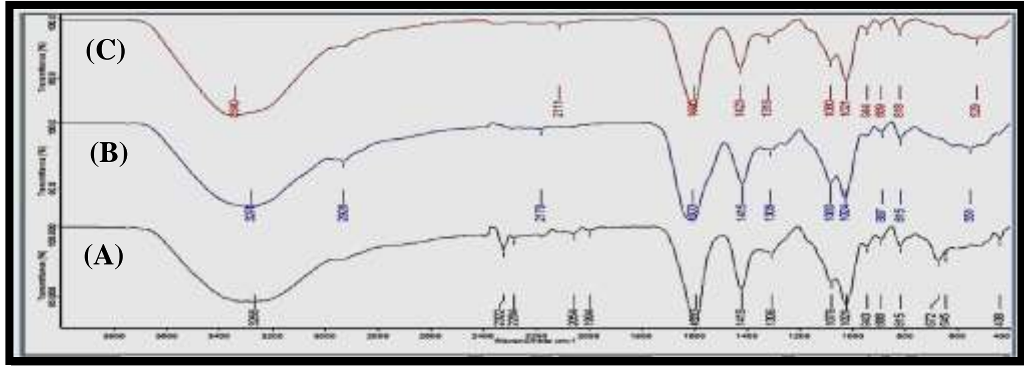


Fig. 10: FTIR spectra of (A) plain Ca-alginate beads (CA), (B) Ca-alginate beads encapsulated with *C. officinalis* extract (CA/COM) and (C) Ca-alginate beads encapsulated with *C. sinuosa* extract (CA/CSE1).

CONCLUSIONS

The investigated extracts of *C. officinalis* and *C. sinuosa* were found to contain some phytocomponents such as 1-docosene, 1,2,3-propanetricarboxylic acid, 2-(acetyloxy)- tributyl ester, 1,1-dimethyltetradecyl hydrosulfide, 1-eicosanol, (17*E*)-17-pentatriacontene, 2-octadecoxyethanol, *n*-tetradecanoic acid, *n*-hexadecanoic acid, ethyl *n*-heptadecanoate and 6,10,14-trimethylpentadecan-2-one possessing a wide-spectrum of antimicrobial activity. So these seaweeds may be used as a natural antibacterial agent for fish pathogenic bacteria. In order to control fish infection, these seaweed extracts could be further investigated as dietary supplements of farmed fish, in a modern and eco-sustainable aquaculture. The present study encourages the use of the encapsulated extract as biocontrol agent in the aquaculture.

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ARABIC SUMMARY

تعريف كروماتوجرافيا الغاز/ الكتلة وتطبيقات مستخلصات الطحالب البحرية النشطة بيولوجياً من ساحل البحر المتوسط لمصر

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تم في هذه الدراسة تعيين النشاط المضاد للبكتيريا لأنواع مختلفة من الطحالب البحرية ضد خمس ممرضات بكتيرية للأسماك (*A. hydrophila, V. parahaemolyticus, V. alginolyticus, V. damsela*, *Vibrio* sp.) حيث أظهر المستخلص الإيثانولي لطحلب *C. sinuosa* (CSE1) ومستخلص ثنائي كلورو ميثان لطحلب *C. officinalis* (COM) أعلى نشاط و كانت قيمة مؤشر النشاط (AI) = 1.4 ±0.74 و 1.2 ±0.71 لكل منهما على التوالي. وعند تغليفهما داخل كريات ألجينات الكالسيوم، فقد تبين من نشاطهما المضاد للبكتيريا ضد ممرضات البكتيرية الأكثر تأثراً أن أعلى تثبيط لنمو البكتيريا كان ضد *V. parahaemolyticus* وذلك بعد ٥ دقائق من إضافة كريات المستخلصين المغلفين مسجلاً انخفاضاً في النمو بمقدار ١.٤ و ٢ مرة على التوالي. و قد أعيد تدوير كريات مستخلص (CA/COM) لمدة ٧ دورات متتالية مع النجاح في القضاء على *V. parahaemolyticus*. كما تم إثبات فاعلية المستخلصين المغلفين في إزالة الحمولة الميكروبية الموجودة فى مياه تربية الأسماك و نشاطهما المضاد للحشف. وقد إستخدم تحليل كروماتوجرافيا الغاز/ الكتلة (GC/MS) للتعرف على مكونات المستخلصين الأكثر نشاطاً (CSE1) و (COM) و الذي أوضح عن وجود العديد من المكونات التي سبق التحقق من أن لديها نشاط مضاد للميكروبات. بالإضافة إلى ذلك، فان مستخرج ثنائي كلورو ميثان يحتوي على ٦ مكونات رئيسية تشمل:

n-nonadecane, 1,2,3-propanetricarboxylic acid, 2-(acetyloxy)-, tributyl ester, 2-methylhexadecan-1-ol, 1-docosene, 1-eicosanol and chloroacetic acid, octadecyl ester.

بينما تميز المستخلص الإيثانولي (CSE1) بوجود أحماض دهنية و إسترات إيثيل الأحماض الدهنية والهيدروكربونات الأروماتية حيث كان المركب *n*-tridecanoic acid ethyl ester هو الأكثر وفرة.