Phycochemistry of some *Sargassum* spp. and their cytotoxic and antimicrobial activities

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Purpose
A comparative study on the chemical composition as well as cytotoxic and antimicrobial activities of the brown algae *Sargassum asperifolium*, *Sargassum dentifolium*, and *Sargassum linifolium* (family: Sargassaceae) from the Red Sea, Hurghada, Egypt, is carried out.

Methods
The volatile constituents obtained by hydrodistillation as well as the isolated unsaponifiable matter and the fatty acids were analyzed using the gas chromatography/mass spectrometry technique. Antitumorigenic activities of the crude extracts of the three algae have been evaluated *in vitro* on different human cell lines. Furthermore, the antimicrobial activities of the volatile constituents, successive extractives, unsaponifiable matter, and fatty acids have been tested on 11 different microorganisms.

Results
The analysis of the volatile fraction led to the identification of sexual pheromones, terpenes, phenolic compounds, free fatty acids, and esters. The most abundant sterols of unsaponifiable matter were fucosterol and cholesterol in all algae. Palmitic acid was found in all investigated algae as a major fatty acid. Biological screening proved that the tested algae have various cytotoxic and antimicrobial activities.

Conclusion
S. asperifolium, *S. dentifolium*, and *S. linifolium* are rich in cytotoxic and antimicrobial bioactive metabolites.

Keywords:
antimicrobial activity, cytotoxic activity, *Sargassum asperifolium*, *Sargassum dentifolium*, *Sargassum linifolium*

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Introduction

Marine natural resources are a treasury of a large group of structurally unique secondary metabolites useful to medicine, which have yielded a large number of drug candidates [1].

The anticarcinogenic properties of brown seaweeds are well known in some cultures such as traditional Chinese medicine [2] and in ancient Ayurvedic texts [3]. In addition, they are mentioned in the Ebers Papyrus of the ancient Egyptians, who used seaweed to treat breast cancer [4].

There are numerous reports on compounds that have been derived from *Sargassum* spp. with a broad range of biological activities. Patra and colleagues, 2007, reported that the methanol extract of *Sargassum* spp. showed strong antioxidant activity and had antimicrobial activity against Gram-positive and Gram-negative bacteria [5]. Further, the methanol extract of *Sargassum swartzii* had chronic and acute anti-inflammatory effects [6], whereas the methanol extract of *Sargassum henslowianum* and *Sargassum siliquastrum* and the ethanol extract of *Sargassum dentifolium* acted as antidotes against the hepatotoxicity induced by carbon tetrachloride [7,8]. Other studies have reported that the hot water extract of *Sargassum horneri* is the most potent anticoagulant and has a high activated partial thromboplastin time [9], and that polysaccharides isolated from *Sargassum trichophyllum* show antiviral activity against herpes simplex virus type 2 [10].

Tang *et al.* [11] isolated several sterols from *Sargassum carpophyllum* that showed various cytotoxic activities against several cancer cell lines. Moreover, farnesylacetones isolated from *S. siliquastrum* showed a moderate vasodilatation effect on the basilar arteries [12]. Chandraraj *et al.* [13] reported that the ethyl acetate fraction of *Sargassum ilicifolium* stimulated *in-vitro* chemotatic, phagocytic, and intracellular killing of human neutrophil immunostimulants and showed prominent immunostimulator activity *in vivo*. *S. siliquastrum* acts as a food preservative that reduces the microbial count of bread and increases the time of storage [14].

The hydroalcoholic extracts of *Sargassum asperifolium*, *Sargassum dentifolium*, and *Sargassum linifolium* have shown

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various insecticidal and antiviral activities *in vitro* on isolated cell lines of *Spodoptera littoralis* (Sl52 cells) and *Spodoptera frugiperda* (Sf9 cells) with or without inoculation of nucleopolyhedrovirus and *in vivo* on *S. littoralis* nucleopolyhedrovirus replication [15]. Furthermore, Aboutabl *et al.* [16] have reported that the different extracts of *S. dentifolium*, collected from the Mediterranean coast of Egypt, showed potential insecticidal activity against *S. littoralis* at different stages of the life cycle.

Numerous substances such as 24-vinylcholest-4-ene-24-ol-3-one, saringosterone, saringosterol, and a hydroazulene diterpene dictyone were isolated from *S. asperifolium* [17]. Abdel-Fattah *et al.* [18] isolated sargassan (a sulfated heteropolysaccharide) from *S. linifolium* and Aboutabl *et al.* [16] isolated diisooctyl phthalate from *S. dentifolium*.

Other constituents such as pheromones [19], phlorotannins [20], polyphenols, benzoquinone, hydroquinones with a diterpenoid side chain, cyclopentenones, bisnorditerpene derivatives [21], and phthalic acid derivatives [22,23] have been isolated from different *Sargassum* spp.

The current literature and the lack of the data and information on the composition of the volatile matter and other active constituents of *S. asperifolium*, *S. dentifolium*, and *S. linifolium* led us to isolate and identify their volatile constituents and lipoidal matter. During our search for active cancer chemoprotective agents in these marine algae, we also evaluated the volatile constituents, successive extracts, and unsaponifiable and saponifiable matter as antimicrobial agents.

Materials and methods

Thallus material

The three brown algae *S. asperifolium* (Hering and G. Martens ex J. Agardh), *S. dentifolium* (Agardh), and *S. linifolium* (C. Agardh) (family: Sargassaceae) were collected at about 2–4 ft under the water surface on the Red Sea coasts in Hurghada, Egypt, during May 2007 and authenticated by Prof. S.A. Shaalan, Faculty of Science, Alexandria University.

Preparation of crude extract

In total, 100 g of the air-dried powdered thallus from each collected sample was extracted successfully with 70% methanol. Each extract was filtered and evaporated under vacuum.

Preparation of successive extracts

In total, 100 g of the air-dried powdered thallus from each collected sample was extracted exhaustively with petroleum ether (40–60°C), ether, chloroform, ethyl acetate, and methanol in a Soxhlet apparatus, followed by maceration in water. Each extract was filtered, evaporated under vacuum, and weighed.

Isolation of the volatile constituents

Pure and fresh homogenized algae (1 kg) were hydrodistilled in a modified Likens–Nickerson apparatus [24] using *n*-pentane (AR grade). The *n*-pentane layer was evaporated under pressure to yield a pale-yellow oil.

Isolation of lipoidal matter

Each petroleum ether residue was saponified using 0.5 N alcoholic KOH. The unsaponifiable matter was extracted with ether, washed with water, dried over anhydrous sodium sulfate, evaporated to dryness, weighed, and analyzed by gas chromatography/mass spectrometry (GC/MS). The fatty acids were liberated by acidification of the saponifiable matter and then extracted with ether and dried *in vacuo*. The fatty acids obtained were methylated (MeOH, 4–5% dry H_2SO_4) to yield the methyl ester derivatives and then analyzed by GC/MS.

Gas chromatography/mass spectrometry analysis

GC/MS analysis was carried out using a Finnigan SSQ 7000 (ThermoFinnigan, San Jose, California, USA) GC/MS spectrophotometer equipped with library software Wiley 138 and NBS 75 under the following conditions: DB-5-fused silica capillary column, 30 m in length, 0.32 mm ID, and with a film thickness of 0.25 μm; carrier gas, helium at a flow rate of 10 ml/min; temperature programmed to 60–260°C at a rate of 4°C/min (volatile constituents), 70–290°C at a rate of 5°C/min (unsaponifiable matter), 60–220°C at a rate of 4°C/min (fatty acid methyl ester derivatives), chart speed: 0.5 cm/min, ionization voltage 70 eV, and detector: flame ionization detector.

The identification of the constituents was carried out depending on the fragmentation of the spectra obtained and by comparing them with those of available authentic standards such as an alkane standard mixture, hexadecanol, palmitic acid, geranylgeraniol, α-copanene, longifolene, aromadendrene, D-limonene, caryophyllene, germacrene D, phytol, β-ionone, cholesterol, campesterol, stigmasterol, β-sitosterol, fucosterol, and ergosterol (Sigma-Aldrich Chemie GmbH, Germany). In addition, brassicasterol, 22-dehydrocholesterol, fucostenone, clerosterol, and avensterol have been isolated previously and identified by our research group at the Pharmacognosy Department, NRC, Egypt, or by published data [25–28], and a library database [Wiley (Wiley Institute, USA) and NIST (National Institute of Technology, USA)]. Quantitative determination was carried out on the basis of peak area measurements of the GC chromatograms.

Antitumor activity

Cells

Authentic culture, H460 (lung carcinoma human cell line), Hela (cervix carcinoma human cell line), HepG2 (liver carcinoma human cell line), Mcf7 (breast carcinoma human cell line), Molt4 (leukemia carcinoma human cell line), and U251 (brain carcinoma human cell line) were obtained from the American Type Culture Collection, USA.

Culture media

The cells were suspended in RPMI 1640 medium supplemented with 10% fetal calf serum, 1% antibiotic–antimycotic mixture (10 000 U/ml K-penicillin, 10 000 µg/ml streptomycin sulfate, and 25 µg/ml amphotericin B), and 1% L-glutamine (all purchased from Lonza, Braine-l'Alleud, Belgium).

Assay method for cytotoxic activity

The cytotoxicity against H460, Hela, HepG2, Mcf7, Molt4, and U251 was determined at the National Cancer Institute, according to the method used by Skehan *et al.* [29]. Adriamycin (Doxorubicin; Pharmacia, Stockholm, Sweden) 10 mg vials were used as the reference drug.

The cell lines were plated in 96-multiwell plates (10^4 cells/well) for 24 h before treatment with the tested samples to allow the attachment of cells to the wall of the plate. Then, a 50 µl aliquot of serial dilutions of the crude extract (1.0, 2.5, 5, and 10 µg/ml) was added and the plates were incubated for 48 h at 37°C in a humidified incubator containing 5% CO_2 in air. Triplicate wells were prepared for each individual dose. Cells were fixed, washed, and stained with sulforhodamine B stain (Sigma, USA). Excess stain was washed with acetic acid and the attached stain was recovered with Tris EDTA buffer (Sigma, USA). The color intensity was measured in an ELISA reader spectrophotometer (Tecan Group Ltd.-Sunrise, Mannedorf, Switzerland).

Microbiological activity

The antimicrobial activity of the volatile constituents, successive extracts, saponifiable matter, and fatty acids of algae examined was determined against that of several microbes using the antibiotic assay method [30]. Pure strains of bacteria, yeasts, and fungi were kindly provided by the Microbial Genetics Department, National Research Center, Egypt. The bacterial strains used were *Bacillus cereus* (Gram positive, G^+), *Bacillus subtillis* (G^+), *Staphylococcus aureus* (G^+), *Escherichia coli* (Gram negative, G^-), *Pseudomonas aeruginosa* (G^-), and *Pseudomonas fluorescens* (G^-). The yeast strains were *Saccharomyces carles* and *Saccharomyces cerevisiae*, whereas the fungi were *Aspergillus flavus, Aspergillus niger*, and *Diplodia oryzea*.

The bacteria were cultured on Lauria–Bertani Medium [31], whereas the yeast strains were cultivated on Yeast Extract Peptone Medium [32]. The fungi were cultured on Potato-Dextrose Agar growth medium [33]. The oils, successive extracts, unsaponifiable matter, and fatty acids were sterilized by filtration through a bacterial membrane filter (0.45 µm, 2.5 mm diameter; Millipore, Billerica, Massachusetts, USA). A concentration of 100 µg/disc was used. The discs (6 mm diameter), after being air dried, were firmly applied to the surface of inoculated agar plates. The diameters of inhibition zones were measured per applied disc after incubation at 37°C for 24 h with the bacteria strains, whereas those containing yeast and fungi were incubated at 30°C for 48–72 h. Amoxycillin (Medical Union Pharmaceuticals Co., Ismailia, Egypt) as an antibacterial agent (100 µg/disc) and canesten (Alexandria Co., Alexandria, Egypt) as an antifungal agent (100 µg/disc) were used as reference drugs.

Statistical analysis of data

All values were expressed as means, with three replicates for each treatment. Data were subjected to a paired sample *t*-test using SPSS (version 17.0; SPSS Inc., Chicago, Illinois, USA). *P* less than 0.05 was considered as significant.

Results and discussion

The yields of volatile oils of fresh algae *S. asperifolium*, *S. dentifolium*, and *S. linifolium* were 0.038, 0.041, and 0.043% (w/w), respectively.

Fifty-seven, 53, and 54 compounds were identified, which represent 93.93, 92.36, and 89.43% of the total volatile compounds released from *S. asperifolium*, *S. dentifolium*, and *S. linifolium*; respectively. Table 1 shows that the volatile constituents of the algae are composed of alcohol (15.74, 16.44, and 15.76 %), aldehyde (–, 0.25 and 0.09%), esters (27.98, 29.05, and 10.08%), free acids (6.17, 9.43, and 1.85%), halogenates (0.33, 0.22, and 0.25%), C_{11} hydrocarbon pheromones (2.34, 5.89, and 24.38%), sesquiterpenes (1.31, 3.44, and 0.72%), hydrocarbons (28.28, 22.95, and 26.36%), ketones (11.43, 2.75, and 9.72%), and miscellaneous compounds (0.35, 1.94, and 0.22%), respectively.

Dictyopterene D′, which is an odoriferous C_{11} hydrocarbon, was a major constituent in *S. linifolium* (20.26%) and was also identified in the oil of *S. asperifolium* and *S. dentifolium*. Another C_{11} hydrocarbon pheromone dictyopterene C was detected in *S. asperifolium*; dictyopterene A was detected in *S. dentifolium* and *S. linifolium.* These hydrocarbons have been detected here for the first time in *S. asperifolium*, *S. dentifolium*, and *S. linifolium*. However, ectocarpene and dictyotene have been detected previously in *S. asperifolium* [34].

Characteristic aroma dictyopterenes have been identified as constituents of brown algae with male gamete-attracting activity [19].

Bis-2-ethylhexyl phthalate was identified as the principal constituent in *S. asperifolium* and *S. dentifolium* (24.25 and 25.28%, respectively), and this was also found in the *Sargassum wightii* [22], *S. dentifolium* [16], and *Sargassum* spp. [23]. The biosynthesis of di-(2-ethylhexyl) phthalate by red alga *Bangia atropurpurea* has been described by Chen [35]. Furthermore, di-(2-ethylhexyl) phthalate showed antimicrobial activity against various microorganisms [36], antileukemic and antimutagenic [14]. In addition, dibutyl phthalate has been detected in some edible brown algae such as *Undaria pinnatifida* and *Laminaria japonica* as a natural product [37].

Furthermore, sesquiterpenoid compounds α-copaene, β-bourbonene, longifolene, γ-elemene, aromdenderene, and muurola-4(14),5-diene have been detected for the first time in *Sargassum* spp. under study. These compounds were detected in *Dictyopteris* spp. [38]. β-Ionone was detected

Table 1 Chemical composition of the volatile constituents of brown algae *Sargassum asperifolium*, *Sargassum dentifolium*, and *Sargassum linifolium*

Compounds	Base peak	Molecular weight	Main fragments	Relative %		
				Sargassum asperifolium	*Sargassum dentifolium*	*Sargassum linifolium*
Acid				6.17	9.43	1.85
Propionic acid	59	74	45, 41, 43, 58, 75	–	5.66	–
Tetradecanoic acid	73	228	60, 43, 58, 85, 129	0.91	1.31	–
Palmitic acid	43	256	41, 57, 55, 73	5.26	2.46	1.85
Alcohol				15.74	16.44	15.76
Cis-9-octadecen-1-ol	41	268	43, 55, 57, 83, 69, 97	1.93	1.43	2.26
4-Nonylphenol	135	220	57, 121, 43, 71	–	–	0.35
1-Hexadecanol	43	242	55, 41, 83, 97, 69, 57	5.77	11.90	10.69
9-*cis*-Octadecanol	41	268	43, 55, 57, 69	4.65	–	0.10
Phytol	71	296	43, 41, 57, 55, 123	0.71	1.17	1.43
Geranylgeraniol	41	288	159, 105, 69, 91	2.68	1.94	0.93
Aldehyde				–	0.25	0.09
2,6-Di-*t*-butyl-4-hydroxybenzaldehyde	219	234	57, 41, 220, 91	–	0.25	0.09
Esters				27.98	29.05	10.08
Isobutyl phthalate	149	278	41, 57, 223, 150	0.95	0.32	0.57
Dibutyl phthalate	149	278	41, 55, 150, 223	1.30	1.13	0.72
Methyl eicosa-5,8,11,14,17-pentaenoate	79	316	91, 67, 105, 41, 119	1.23	2.02	1.07
Dioctyl adiptate	129	370	57, 43, 55, 41, 71	0.25	0.30	–
Bis(ethyl hexyl) phthalate	149	390	167, 279, 57, 43, 71	24.25	25.28	7.72
Halogenates				0.33	0.22	0.25
2,2-Dicholoro-3-methylbutane	43	140	41, 42, 69, 105	–	–	0.11
Iodo-2-methylundecane	57	262	43, 71, 41, 55, 185	–	0.22	0.14
1-Chlorooctadecane	57	288	41, 43, 55, 69, 71, 85	0.33	–	–
Hydrocarbons				31.93	32.28	51.46
Nonane	43	128	57, 41, 42, 85, 71	1.37	–	0.69
2,6-Dimethylheptane	43	128	57, 41, 42, 71, 57	0.31	–	0.17
Decane	43	142	57, 41, 71, 85, 55	1.14	0.23	0.92
1-Undecene	41	154	43, 55, 56, 69	–	0.05	0.65
Undecane	57	156	43, 41, 71, 85	1.14	0.56	0.65
Dictyopterene D	91	148	79, 91, 105, 41, 77, 119, 66	2.02	4.63	20.26
Dictyopterene C	79	150	91, 77, 41, 93, 65, 51, 66	0.32	–	–
Dictyopterene A	79	150	93, 91, 77, 41, 67, 66, 55	–	1.02	3.18
6-[(1*E*)Butenyl]-cyclohepato-1,4-diene	79	148	91, 41, 66, 119, 55	–	0.24	0.94
Dodecane	57	170	43, 71, 41, 85	0.85	0.62	0.52
Tridecane	57	184	43, 71, 85, 55	0.26	–	0.19
Decahydro-1,4-ethanonaphthalene	67	164	82, 41, 80, 54, 121, 55, 93	–	0.24	1.91
α-Copaene	105	204	119, 161, 91, 41, 93	0.19	0.25	0.35
β-Bourbonene	81	204	79, 80, 123, 41	0.36	0.25	0.10
Tetradecane	43	198	57, 71, 41, 85, 55	0.76	0.60	0.58
Longifolene	91	204	41, 161, 105, 79, 93	–	0.63	–
γ-Elemene	41	204	121, 93, 91, 67, 105	0.44	–	–
Aromodendrene	41	204	91, 79, 105, 67, 93	0.40	1.18	–
Muurola-4(14)5-diene (*cis*)	161	204	41, 105, 91, 119, 133	0.92	0.44	0.27
Pentadecane	57	212	43, 41, 85, 71, 55	1.56	2.38	2.75
Cuparene	133	202	132, 145, 41, 91	–	0.69	–
Hexadecene	43	224	41, 55, 69, 83, 97	0.35	–	0.12
Hexadecane	57	226	43, 71, 41, 55, 85	1.65	1.37	0.99
8-Heptadecene	55	238	41, 43, 69, 82	–	0.34	0.56
1-Heptadecene	43	238	41, 55, 57, 97, 83	–	0.57	1.59
Heptadecane	57	240	43, 71, 41, 85	3.50	4.41	6.44
4,9-Di-nor-propyldodecane	57	254	43, 41, 71, 55, 85	–	0.30	–
1-Octadecene	43	252	55, 57, 41, 69, 97	0.33	0.10	0.42
Octadecane	43	254	41, 57, 55, 71, 85	3.00	3.08	2.50
Eicosane	43	282	57, 41, 71, 55, 85	0.21	0.59	1.11
Totarene	41	272	43, 55, 81, 175	0.45	0.29	0.07
Heneicosane	43	296	57, 41, 71, 55	0.70	0.51	1.06
1-Docosene	41	308	55, 43, 83, 97, 69	–	0.11	0.25
Docosane	43	310	57, 43, 71, 55, 85	0.65	0.35	0.57
Dictyone	159	304	43, 71, 286, 107, 145	8.13	5.14	–
Tricosane	57	324	43, 41, 71, 55, 85	–	–	0.61
Tetracosane	57	338	43, 71, 85, 55, 41	0.20	0.25	0.29
Pentacosane	57	352	71, 43, 85, 55, 113	0.50	0.35	0.23
Hexacosane	57	366	43, 71, 85, 55, 69	–	0.20	0.22
Heptacosane	57	380	43, 71, 85, 41, 55	0.22	0.31	0.18
Octacosane	57	394	43, 71, 85, 41, 55	–	–	0.12
Ketones				11.43	2.75	9.72
3-Methyl, 2-hexanone	43	114	42, 41, 55, 57	8.38	–	5.37
3-Methyl-2-heptanone	43	128	42, 41, 57, 58	0.19	–	0.17
2,6,6-Trimethyl-2,4-cycloheptadien-1-one	107	150	91, 108, 41, 77, 53, 55	0.51	0.37	0.32
β-Ionone	177	192	43, 121, 105, 135, 77	1.16	0.85	1.39
6,10,14-Trimethyl,2-pentadecanone	43	268	58, 71, 57, 109, 124	1.19	1.53	2.47

Table 1 (*continued*)

Compounds	Base peak	Molecular weight	Main fragments	Relative % *Sargassum asperifolium*	Relative % *Sargassum dentifolium*	Relative % *Sargassum linifolium*
Miscellaneous				0.35	1.94	0.22
4,5-Dithiaoctane	43	150	41, 108, 57, 71, 113	0.35	0.12	0.22
Anethole	148	148	147, 79, 55, 117	–	1.61	–
Butylated hydroxytoluene	205	220	206, 57, 41, 177, 145	–	0.21	–
Total				93.93	92.36	89.43

Table 2 Chemical composition of the unsaponifiable fraction of the brown algae *Sargassum asperifolium*, *Sargassum dentifolium*, and *Sargassum linifolium*

Compounds	Base peak	Molecular weight	Main fragments	Relative % *Sargassum asperifolium*	Relative % *Sargassum dentifolium*	Relative % *Sargassum linifolium*
Alcohol				7.01	14.87	7.15
2-Butanol	45	74	43, 59, 44, 41	0.14	10.95	0.36
1-Hexadecanol	43	242	41, 55, 57, 97, 69	–	0.09	–
9-Heptadecanol	43	256	57, 71, 41, 58	–	0.04	–
Octadecanol	43	270	41, 57, 55, 111, 69, 97	0.24	–	–
Geranylgeraniol	41	288	159, 105, 69, 91	–	0.16	–
Phytol	71	296	43, 123, 41, 57, 81	5.48	3.08	5.67
1-Eicosanol	43	298	41, 55, 57, 69	0.11	0.12	0.22
1-Docosanol	43	326	55, 57, 41, 71, 97	–	–	0.24
1-Tricosanol	43	340	55, 57, 41, 69, 83	0.92	0.34	0.66
1-Tetracosanol	43	354	57, 55, 41, 83, 69	0.12	–	–
1-Hexacosanol	43	382	57, 55, 41, 83, 69	–	0.09	–
Aldehyde				0.27	0.11	–
2-Nor-heptylundec-2-enal	43	266	55, 41, 69, 95	0.03	0.11	–
Octadecanal	85	268	41, 57, 157, 139	0.24	–	–
Esters				3.58	14.01	10.46
Dibutyl phthalate	149	278	41, 150, 57, 55, 104	0.42	0.67	2.61
Undecyl laurate	43	354	57, 41, 55, 201, 69	–	0.05	–
Nor-butyl benzyl phthalate	149	312	91, 41, 56, 206, 104	0.70	–	–
Di-2-ethylhexyl phthalate	149	390	167, 279, 57, 43	0.80	11.12	6.07
Nor-docosyl acetate	43	368	83, 57, 97, 61, 69	–	1.09	–
Di-cyclohexyl phthalate	149	368	167, 55, 41, 150	1.66	1.08	1.78
Hydrocarbons				15.87	4.98	5.48
4,4-Dimethyl-1-pentene	57	98	41, 55, 43, 83	0.27	–	–
3-Ethyltridecane	43	212	57, 71, 85, 41, 55	0.03	0.08	–
Pentadecane	57	212	43, 41, 85, 71, 55	–	–	0.09
6,9-Dimethyltetradecane	43	226	41, 71, 85, 55	0.03	0.09	–
1-Heptadecene	43	238	41, 55, 57, 97, 83	0.02	–	–
Heptadecane	57	240	43, 71, 41, 85	–	0.01	1.02
Octadecane	43	254	41, 57, 55, 71, 85	0.05	0.22	0.22
5-Methyloctadecane	43	268	57, 85, 71, 84, 41	0.03	0.16	–
2,6,10,14-Tetramethylpentadecane	57	268	43, 71, 41, 85, 69	–	–	0.14
2-Phenyltridecane	105	260	91, 43, 41, 106, 104	0.02	–	–
8-Nor-hexylpentadecane	43	296	57, 71, 41, 85, 55	0.06	–	–
2-Nor-butyl-8-nor-hexylbicyclo(4,4,0) decane	41	278	95, 55, 81, 67, 43	0.13	0.53	–
Dictyone	159	304	43, 71, 286, 107, 145	–	1.43	–
Cyclodocosane	55	308	57, 43, 41, 69, 83	–	0.41	–
Nor-tricosane	57	324	43, 41, 71, 55, 85	0.20	–	0.38
2-Methyltricosane	43	338	57, 41, 55, 85, 97	–	–	0.28
Nor-pentacosane	43	352	57, 41, 69, 97, 83	0.17	0.24	0.17
Nor-heptacosane	57	380	43, 71, 55, 41, 85, 99	0.38	–	–
Nor-octacosane	57	394	43, 71, 85, 55, 41, 99	0.73	–	–
Nonacosane	57	408	43, 71, 55, 41, 85, 99	0.62	–	–
Squalene	69	410	81, 41, 95, 136, 55	13.13	1.81	3.18
Ketone				0.43	3.21	5.91
1,4-Benzoquinone	54	108	53, 52, 82, 50, 81	0.02	0.12	0.13
Hydroquinone	110	110	81, 53, 109, 55	0.04	2.27	3.44
2,6-Di-butyl-4-hydroxy-4-methyl-2,5-cyclohexadiene-1-one	165	236	180, 41, 57, 43	0.05	0.19	0.77
1,4-Naphthaquinone	158	158	104, 76, 102, 130	0.03	–	–
6,10,14-trimethylpentadecane-2-one	43	268	58, 41, 57, 71, 55	0.27	0.63	1.57
Benzophenone	105	182	77, 51, 106, 181	0.02	–	–
Phenol				2.03	19.64	18.45
2,6-Di-*t*-butyl-4-bromomethyl phenol	161	218	203, 175, 163, 176	–	0.07	0.17
2,6-Di-*t*-butyl-4-methyl phenol	205	220	206, 91, 57, 41	2.03	18.52	17.72
2,6-Di-*t*-butyl-4-formyl phenol	219	234	57, 220, 41, 191, 55	–	1.05	0.37

Table 2 (*continued*)

Compounds	Base peak	Molecular weight	Main fragments	Relative %		
				Sargassum asperifolium	*Sargassum dentifolium*	*Sargassum linifolium*
2,6-Di-*t*-butyl-4-methoxy phenol	221	236	57, 41, 91, 222	–	–	0.08
1,2-Bis-(3,5-di-*t*-butyl-4-hydroxyphenyl) ethane	219	438	43, 55, 57, 69, 220	–	–	0.11
Sterols				51.27	22.23	32.35
3-Hydroxy androst-2-en-17-one	91	288	105, 255, 41, 79, 55	–	1.93	5.33
Pregna-4,16-diene-3,20-dione	312	312	43, 136, 160, 159, 297	0.30	1.86	0.81
Dehydro-22-cholesterol	384	384	69, 300, 255, 133	1.99	1.16	1.17
Cholesterol	386	386	275, 301, 368, 107	12.21	5.92	10.05
Brassicasterol	398	398	380, 300, 271, 255	–	0.65	0.91
Ergosta-5,7,22-trien-3β-ol	396	396	381, 288, 271, 255	–	1.12	–
Δ^5 Ergosterol	400	400	385, 382, 367, 315	–	1.71	0.53
Campesterol	55	398	69, 81, 383, 253	0.28	–	–
Stigmasterol	43	412	55, 57, 41, 69, 314	–	0.20	2.92
Clerosterol	412	412	394, 314, 271, 371	2.63	–	1.05
β-Sitosterol	91	414	91, 55, 43, 95, 108	0.88	–	0.12
Fucosterol	314	412	55, 43, 69, 83, 281	29.35	7.13	7.34
24-Isoethylidene cholest-5-en-3β ol	314	412	55, 43, 69, 83, 95	–	–	0.34
24-Ethylcholesta-5,24(25) dienol	314	412	299, 281, 271, 296	–	0.55	0.58
Fucostenone	312	410	313, 297, 124, 231	3.63	–	1.20
Miscellaneous				1.71	0.69	0.64
2,2-Diethoxy ethanamine	47	103	75, 60, 89, 45	0.02	0.18	–
Dihydroactinidiolide	111	180	137, 109, 41, 67, 95	0.08	0.46	0.64
Phenanthrene	249	264	43, 55, 57, 121	0.04	–	–
4-Hydroxyoctadec-9-enolide	41	280	55, 67, 81, 54	1.05	0.05	–
2-Ethylhexyldiphenyl phosphate	251	362	43, 41, 55, 94, 250	0.52	–	–
Total				82.17	79.74	80.44

in all algae under investigation, and it has antibacterial and antifungal activity [39]. Two aliphatic chains diterpenes, phytol and geranylgeraniol, were identified in the tested algae, which had bactericidal activity against *S. aureus*. These diterpenes exerted both growth-inhibitory and growth-accelerating effects depending on their concentration [40].

The yields of unsaponifiable matter of *S. asperifolium*, *S. dentifolium*, and *S. linifolium* were 0.22, 0.22, and 0.61% (w/w), respectively. Fifty-seven, 44, and 40 compounds were identified, which represent 82.17, 79.74, and 80.44% of the total unsaponifiable matter of *S. asperifolium*, *S. dentifolium*, and *S. linifolium*, respectively. Table 2 shows that the unsaponifiable matter of *S. asperifolium*, *S. dentifolium*, and *S. linifolium* is composed of sterols (51.27, 22.23, and 32.35%), which represent the mean fraction of unsaponifiable matter, alcohol (7.01, 14.87, and 7.15%), aldehydes (0.27, 0.11%, and –), esters (3.58, 14.01, and 10.46%), hydrocarbons (15.87, 4.98, and 5.48%), ketones (0.43, 3.21, and 5.91%), phenols (2.03, 19.64, and 18.45%), and miscellaneous compounds (1.71, 0.69, and 0.64%).

Fucosterol was detected as a major sterol in the algae tested as other *Sargassum* spp. showed cytotoxic activity against various carcinoma human cell lines [11,41]. In addition, it showed antifungal activity against *Curvularia lunata*, *Stachybotrys atra*, and *Microsporum canis*. These results were obtained for the first time in this work for unsaponifiable matter from *Sargassum* spp.

The percentages of fatty acids of the brown algae *S. asperifolium*, *S. dentifolium*, and *S. linifolium* were 0.09, 0.041, and 0.043% (w/w), respectively. Table 3 shows that the saturated fatty acids represent the main fraction (56.12, 65.39, and 56.42%, respectively) of fatty acid, and palmitic acid was found in all *Sargassums* spp. under study as a major fatty acid. Furthermore, oleic acid represents the main unsaturated fatty acid of *S. dentifolium* and *S. linifolium*. However, 9,12-octadecadienoic acid represents the major unsaturated fatty acid of *S. asperifolium*.

Cytotoxic activity

The cytotoxic activity of crude extracts of *Sargassum* spp. under study against human cells H460, Hela, HepG2, MCF7, Molt4, and U251 cultured *in vitro* was examined. The percentages of inhibition and relative inhibition related to the reference drug doxorubicin are shown in Tables 4 and 5 and illustrated in Figs 1–3. The crude extract of *S. linifolium* has significantly promising *in-vitro* cytotoxic activity against HepG2 and Molt4, with an effective dose (ED_{50}) of 5.97 and 2.28 μg/ml, respectively, compared with the control, and at concentrations of 5 and 10 μg/ml, they showed good cytotoxic activity against HepG2, which was comparable to that of the reference drug doxorubicin.

Whereas the crude extract of *S. dentifolium* showed significant cytotoxic activity against HepG2 with an ED_{50} of 11.03 μg/ml, H460 and MCF7 related to the control test and at concentrations of 5 and 10 μg/ml showed good cytotoxic activity against H460 in comparison with doxorubicin.

Furthermore, the crude extract of *S. asperifolium* at a concentration of 1 μg/ml showed high cytotoxic activity against H460, whereas it showed good cytotoxic activity at concentrations of 1 and 2.5 μg/ml against U251 and H460, respectively, when compared with doxorubicin as a reference drug.

Table 3 Methyl ester of the fatty acid composition of the brown algae *Sargassum asperifolium*, *Sargassum dentifolium*, and *Sargassum linifolium*

Compounds	Base peak	Molecular weight	Main fragments	% of total fatty acid derivatives		
				Sargassum asperifolium	*Sargassum dentifolium*	*Sargassum linifolium*
Saturated fatty acids				56.12	65.39	56.42
Methyl laurate	74	214	87, 43, 41, 55, 143, 171, 183	–	0.73	–
Methyl myristate	74	242	87, 43, 41, 55, 143, 199, 213	9.71	3.85	7.78
Methyl pentadecanoate	74	256	87, 43, 41, 55, 75, 143, 213	–	2.19	4.02
Dimethyl azelate	55	216	74, 83, 43, 59, 152, 41	–	9.80	–
Methyl palmitate	74	270	74, 43, 41, 55, 75, 143, 227	20.72	18.21	40.16
Methyl heptadecanoate	74	284	87, 43, 57, 75, 143, 241, 199	–	1.50	–
Methyl stearate	74	298	87, 43, 55, 75, 143, 255, 199	18.61	10.75	4.46
Methyl docosanoate	74	354	256, 43, 129, 87, 213, 185	4.38	11.12	–
Methyl tricosanoate	74	368	87, 43, 75, 57, 55, 143	–	3.44	–
Methyl tetracosanoate	74	382	87, 75, 55, 43, 41, 69, 143	2.70	3.80	–
Unsaturated Fatty Acids				43.40	34.61	43.58
Methyl palmitoleate	55	268	74, 69, 41, 87, 96, 236, 194	1.20	1.05	–
Methyl oleate	55	296	74, 41, 69, 43, 264, 87, 222	5.51	11.96	25.26
Methyl 9,12-octadecadienoate	41	294	67, 81, 95, 55, 79, 109	18.73	10.07	6.95
Methyl 12,15-octadecadienoate	67	294	81, 82, 95, 55, 109, 123	9.21	–	–
Methyl 9-*cis*,12-*cis*,15-*cis*-octadecatrienoate	79	292	67, 41, 93, 55, 107, 150, 194	7.15	6.70	–
Methyl eicosa-5,8,11,14,17-pentaenoate	79	316	91, 67, 105, 41, 119, 147, 201	–	1.92	–
Methyl heptadec-*trans*-10-en-8-ynoate	79	278	41, 67, 93, 91, 108, 121	1.60	2.91	2.49
Methyl eicosa-11-yn- *trans*-13-enoate	79	320	80, 150, 67, 93, 77, 55	–	–	5.97
Methyl 4,7,10,13,16,19-docosahexaenoate	79	342	91, 67, 41, 55, 77, 105	–	–	2.91
Hydroxylated fatty acids				0.48	–	–
Methyl 3-hydroxyoctadecanoate	43	314	103, 41, 57, 55, 82, 83, 79, 229	0.48	–	–

Table 4 Cytotoxic activity of the crude extract of the brown algae *Sargassum asperifolium* (S1), *Sargassum dentifolium* (S2), and *Sargassum linifolium* (S3) against different cultured human cell lines

Human cell line	Sample Concentration (μg/ml)	% of inhibition ± SEM 1	2.5	5	10	ED_{50}
H460	S1	28.00 ± 0.18*,**	24.00 ± 0.15*,**	0 ± 0.12**	−2.00 ± 0.03**	–
	S2	−50.00 ± 0.15*,**	12.00 ± 0.16*,**	30.00 ± 0.26*,**	34.00 ± 0.12*,**	>10
	S3	−130.0 ± 0.8*,**	−36.00 ± 0.05**	10.00 ± 0.24**	28.00 ± 0.22*,**	>10
	Dox	24.40 ± 0.05*	36.50 ± 0.10*	51.60 ± 0.09*	59.90 ± 0.12*	4.77
Hela	S1	−30.13 ± 0.04*,**	−45.60 ± 0.13*,**	−23.14 ± 0.09*,**	−12.72 ± 0.04**	–
	S2	−63.31 ± 0.03*,**	−34.62 ± 0.09*,**	−4.06 ± 0.04**	−40.61 ± 0.12*,**	–
	S3	−69.93 ± 0.11*,**	−39.74 ± 0.09*,**	−44.48 ± 0.07*,**	−32.50 ± 0.10*,**	–
	Dox	22.6 ± 0.08*	37.6 ± 0.09	79.7 ± 0.02*	80.90 ± 0.01*	3.64
HepG2	S1	−16.67 ± 0.11*,**	−0.55 ± 0.06**	15.75 ± 0.06**	37.59 ± 0.05*,**	>10
	S2	14.07 ± 0.02**	27.4 ± 0.05*,**	31.29 ± 0.03*,**	45.72 ± 0.06*,**	11.03
	S3	13.15 ± 0.06*,**	25 ± 0.07*,**	46.11 ± 0.06*,**	65.56 ± 0.06*,**	5.97
	Dox	64.90 ± 0.11*	90.7 ± 0.11*	86.90 ± 0.15*	95.00 ± 0.10*	0.80
MCF7	S1	−3.23 ± 0.03**	5.85 ± 0.07**	4.23 ± 0.07**	10.45 ± 0.07*,**	>10
	S2	13.19 ± 0.03*,**	22.77 ± 0.07*,**	11.32 ± 0.15*,**	35.33 ± 0.03*,**	>10
	S3	6.00 ± 0.09**	−56.59 ± 0.10*,**	−77.73 ± 0.10*,**	3.00 ± 0.04**	–
	Dox	31.80 ± 0.11	48.50 ± 0.013*	80.80 ± 0.03*	82.80 ± 0.02*	2.97
Molt	S1	−41.2 ± 0.18*	−75.12 ± 0.11*	−54.77 ± 0.20	−57.62 ± 0.29*	–
	S2	−9.54 ± 0.34	−43.77 ± 0.47*	−62.31 ± 0.02*	−65.32 ± 0.58*	–
	S3	67.40 ± 0.22*	53.78 ± 0.18*	25.13 ± 0.18*	44.73 ± 0.07*	2.28
	Dox	NT	NT	NT	NT	–
U251	S1	39.12 ± 0.03*,**	29.55 ± 0.07*,**	32.12 ± 0.01*,**	7.05 ± 0.05*,**	–
	S2	14.84 ± 0.08*,**	25.82 ± 0.07*,**	14.14 ± 0.05*,**	12.18 ± 0.04*,**	–
	S3	16.00 ± 0.12*,**	−0.75 ± 0.12**	−3.94 ± 0.20**	−17.76 ± 0.12*,**	–
	Dox	67.1 ± 0.20*	85.20 ± 0.05*	85.80 ± 0.05*	92.80 ± 0.05*	<0

Sargassum asperifolium (S1), *Sargassum dentifolium* (S2), and *Sargassum linifolium* (S3).
Each value represents the mean of percentage of inhibition cells of three replicates ± SEM.
Dox, doxorubicin; ED, effective dose; NT, not tested.
*Significantly different from the control value at $P<0.005$ according to a paired sample *t*-test.
**Significantly different from the reference drug doxorubicin value at $P<0.005$ according to a paired sample *t*-test.

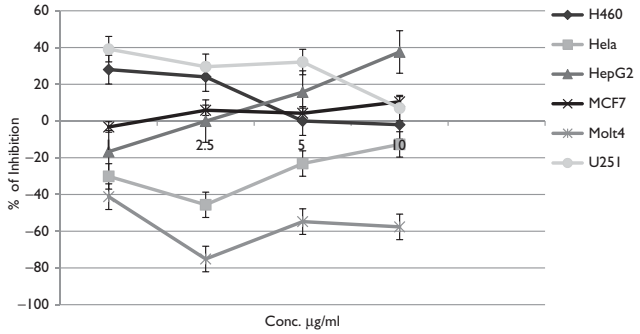
It is noteworthy that the authors have isolated many bioactive cytotoxic constituents such as diterpenes and polysaccharides from different marine algae [42–44]. Khanavi *et al.* [41] found that fucosterol, the most abundant phytosterol in the brown algae, is responsible for the cytotoxic effect against a breast carcinoma cell line [inhibitory concentration (IC_{50}) 27.94 μg/ml] and a colon carcinoma cell line (IC_{50} 70.41 μg/ml).

Table 5 Relative inhibition of growth of different human cell lines related to doxorubicin

	Relative inhibition to doxorubicin (%)																	
	H460			Hela			HepG2			MCF7			Molt4			U251		
Concentration (μg/ml)	S1	S2	S3	S1	S2	S3	S1	S2	S3	S1	S2	S3	S1	S2	S3	S1	S2	S3
1	114.75	–	–	–	–	–	–	21.67	20.26	–	52.76	24	NT	NT	NT	58.30	22.11	23.84
2.5	65.75	32.87	–	–	–	–	–	30.20	27.56	10.08	39.25	–	NT	NT	NT	34.68	30.30	–
5	–	58.14	19.37	–	–	–	18.12	36.00	53.06	5.25	14.05	–	NT	NT	NT	37.43	16.48	–
10	–	56.76	46.74	–	–	–	39.56	48.12	69.01	11.42	38.61	3.68	NT	NT	NT	7.59	13.13	–

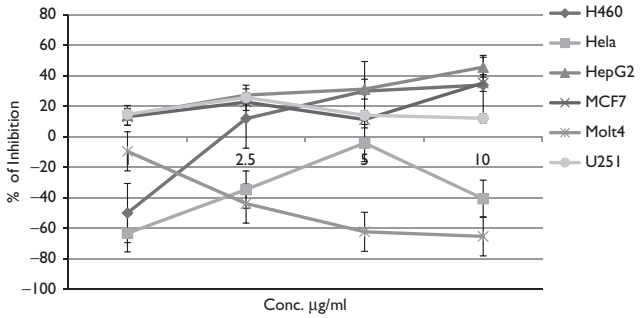
Sargassum asperifolium (S1), *Sargassum dentifolium* (S2), and *Sargassum linifolium* (S3).
The activity was evaluated according to the inhibition growth related to doxorubicin. Activity >75%, high; 75–50%, good; 50–25%, normal; and <25%, weak activity.
–, no cytotoxic activity; NT, not tested.

Figure 1



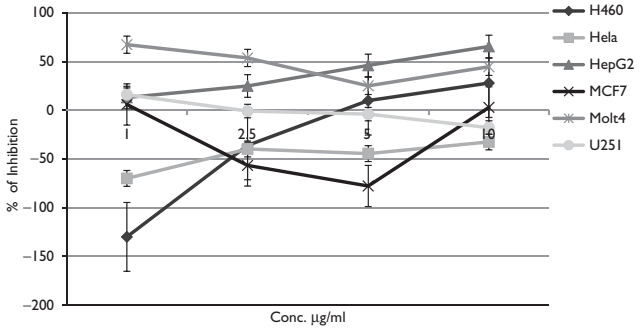
Cytotoxic activity of a crude extract of *Sargassum asperifolium* on different human cell lines.

Figure 2



Cytotoxic activity of a crude extract of *Sargassum dentifolium* on different human cell lines.

Figure 3



Cytotoxic activity of a crude extract of *Sargassum linifolium* on different human cell lines.

The antimicrobial activity

The antimicrobial activities of the volatile constituents, successive extracts, unsaponifiable fractions, and fatty acids fractions of *Sargassum* spp. under study are summarized in Table 6. The different fractions of *S. asperifolium* showed significant antimicrobial activity against *B. cereus* compared with amoxycillin as a reference drug. However, the various fractions of *S. dentifolium* showed pronounced antimicrobial activity against *S. carles* compared with canesten as a reference drug. Ether, ethyl acetate, and methanol fractions of *S. linifolium* were found to have potent antimicrobial activities against *D. oryzea* when compared with standard canesten.

It has been reported that the antimicrobial activity of some algal species is because of the presence of a mixture of fatty acids such as capric, lauric, linoleic, myristic, oleic, palmitic, and stearic acid [45]. It is clear from the present study that these fractions can be utilized as good natural antimicrobial agents in the pharmaceutical industry.

Conclusion

The volatile constituents as well as unsaponifiable matter and fatty acids isolated and identified from *S. asperifolium*, *S. dentifolium*, and *S. linifolium*, collected from the Red Sea coasts in Hurghada, for the first time comprise alcohol, aldehydes, esters, free acids, halogenates, C_{11} hydrocarbon pheromones, sesquiterpenes, hydrocarbons, and ketones. The different extracts of these three algae have various antimicrobial and cytotoxic activities and can act as promising natural sources of these bioactive products.

Table 6 Inhibitory response of the different fractions of the brown algae *Sargassum asperifolium* (S1), *Sargassum dentifolium* (S2), and *Sargassum linifolium* (S3) on the tested microbes in comparison with standard antibacterial and antifungal substances

		Mean of inhibition zones (mm ± SEM)										
Fractions	Algae species (100 μg/disc)	*Bacillus cereus*	*Bacillus subtillis*	*Staphylococcus aureus*	*Escherichia coli*	*Pseudomonas aeruginosa*	*Pseudomonas fluorescens*	*Saccharomyces carles*	*Saccharomyces cerevisiae*	*Aspergillus flavus*	*Aspergillus niger*	*Deplodia oryzea*
Volatile constituent	S1	18 ± 0.57*	8 ± 0.0[a]	–	11 ± 0.57 *	–	–	–	9 ± 0.57	–	–	–
	S2	–	–	–	13 ± 0.57*	8 ± 0.57	–	14 ± 0.0	9 ± 0.0[a]	–	–	9 ± 0.0*
	S3	–	9 ± 0.0[a]	9 ± 0.0[a]	–	–	9 ± 0.0[a]	–	10 ± 0.0[a]	–	–	8 ± 0.0*
Petroleum ether	S1	20 ± 0.57*	8 ± 0.0[a]	–	9 ± 0.0[a]	8 ± 0.0[a]	–	–	8 ± 0.57*	–	–	9 ± 0.57*
	S2	8 ± 0.0[a]	–	–	12 ± 0.0[a]	8 ± 0.0[a]	–	12 ± 0.0	–	–	–	10 ± 0.57*
	S3	14 ± 0.57*	8 ± 0.57*	–	–	8 ± 0.57	–	–	–	–	8 ± 0.0[a]	8 ± 0.57*
Ether	S1	14 ± 0.57*	12 ± 0.57*	10 ± 0.0[a]	12 ± 0.57*	8 ± 0.57	10 ± 0.57*	9 ± 0.0*	10 ± 0.57	–	8 ± 0.57	8 ± 1.15*
	S2	10 ± 0.57	11 ± 0.0[a]	10 ± 0.57*	10 ± 0.0[a]	–	–	14 ± 0.57[a]	12 ± 0.0[a]	–	–	–
	S3	12 ± 1.15	10 ± 0.0*	10 ± 0.0[a]	9 ± 0.0[a]	–	–	9 ± 0.57	11 ± 0.0[a]	–	8 ± 0.0[a]	14 ± 0.0[a]
Chloroform	S1	12 ± 0.0[a]	10 ± 0.0[a]	–	8 ± 0.0[a]	–	8 ± 0.57*	–	8 ± 0.57*	–	–	8 ± 0.0[a]
	S2	–	–	–	–	–	8 ± 0.0[a]	11 ± 0.57	–	–	–	–
	S3	–	10 ± 0.0[a]	–	–	–	10 ± 0.0[a]	–	9 ± 0.57	–	–	–
Ethyl acetate	S1	10 ± 0.57	–	9 ± 0.0[a]	10 ± 0.57*	8 ± 0.57	10 ± 0.0[a]	9 ± 0.0*	–	–	–	9 ± 0.0[a]
	S2	–	–	–	–	9 ± 0.57	9 ± 0.57*	9 ± 0.0*	–	–	–	9 ± 0.57*
	S3	–	9 ± 0.57*	–	–	8 ± 0.57	9 ± 0.57*	–	–	–	–	14 ± 0.57
Methanol	S1	11 ± 0.0[a]	9 ± 0.0[a]	8 ± 0.57*	8 ± 0.57*	–	–	–	8 ± 0.57*	–	–	9 ± 0.0[a]
	S2	–	–	8 ± 0.0[a]	–	9 ± 0.57	–	9 ± 0.57*	–	–	8 ± 0.57	8 ± 0.0[a]
	S3	–	12 ± 0.0[a]	12 ± 0.0[a]	–	9 ± 0.57	12 ± 0.0[a]	–	9 ± 0.0[a]	–	8 ± 0.0[a]	16 ± 0.0[a]
Water extract	S1	9 ± 0.57	8 ± 0.0[a]	–	11 ± 0.0[a]	8 ± 0.0[a]	–	–	9 ± 0.0[a]	9 ± 0.0*	–	–
	S2	–	–	9 ± 0.0[a]	–	8 ± 0.57	–	8 ± 1.15	–	–	–	–
	S3	–	–	–	9 ± 0.0[a]	9 ± 0.0[a]	10 ± 0.57*	–	9 ± 0.57	–	8 ± 0.0[a]	8 ± 0.0[a]
Unsaponifiable matter	S1	13 ± 0.57*	8 ± 0.0[a]	8 ± 0.0[a]	12 ± 0.0[a]	8 ± 0.57	–	9 ± 0.57*	12 ± 0.57	9 ± 1.15*	–	10 ± 0.57*
	S2	10 ± 0.0[a]	8 ± 0.0[a]	9 ± 0.57*	10 ± 0.57*	8 ± 0.0[a]	–	12 ± 0.57[a]	–	–	–	8 ± 0.0[a]
	S3	–	10 ± 0.57*	9 ± 0.57*	11 ± 0.57*	8 ± 0.0[a]	–	8 ± 0.57	10 ± 0.57	11 ± 0.57*	–	9 ± 0.57*
Fatty acid	S1	12 ± 0.57[a]	9 ± 0.57*	–	–	8 ± 0.57	9 ± 0.57*	9 ± 0.0*	8 ± 0.57*	9 ± 0.57*	–	14 ± 0.57
	S2	8 ± 0.0[a]	8 ± 0.0[a]	–	–	9 ± 0.57	9 ± 0.0[a]	12 ± 0.0	8 ± 0.0[a]	–	–	9 ± 0.57*
	S3	15 ± 0.57*	10 ± 0.0[a]	–	–	9 ± 0.0[a]	9 ± 0.0[a]	–	9 ± 0.0[a]	–	–	11 ± 0.0[a]
Reference drug	Amoxycillin	10 ± 0.0	24 ± 0.0	22 ± 0.0	16 ± 0.0	9 ± 0.57	26 ± 0.0					
	Canesten							12 ± 0.57	11 ± 0.0	20 ± 0.57	9 ± 0.0	14 ± 0.0

Each value represents the mean of inhibition zones (mm) of three replicates ± SEM.
[a]The correlation and *t* could not be computed because the standard error of the difference is zero.
*Significantly different from the reference drug at $P < 0.05$ according to a paired sample *t*-test.

Acknowledgements

Conflicts of interest

There are no conflicts of interest.

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