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Evaluation of the Biological Activities of Two Macro-Algae Collected from the Red Sea of Jeddah

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Abstract

Marine algae were used in many biological applications. Two marine algal samples, *Halimeda tuna* and *Dictyota dichotoma*, were collected from Obhur region, Jeddah, Saudi Arabia, washed with water, dried and extracted with methanol. The antimicrobial activities were conducted against some pathogenic bacteria. The results showed that the extracts of both *Halimeda tuna* and *Dictyota dichotoma* were active against at least one of the tested organisms. The highest antimicrobial activities of the extracts *Halimeda tuna* and *Dictyota dichotoma* were against *Staphylococcus aureus* and *Streptococcus pneumonia*. On contrast, both *Halimeda tuna* and *Dictyota dichotoma* showed weak inhibition against *Pseudomonas aeruginosa* and *Acinetobacter baumannii.* Moreover, the mixture of the two algal extracts showed excellent inhibition for all the tested bacteria. In addition, a toxicological experiment was conducted for the two algal extracts using *Artemia salina* as test organism. No toxicity was found for the two tested methanolic extracts. Furthermore, moderate antitumor activity was recorded for the two tested algal extracts against two cell lines, MCF-7 (breast cancer) and HepG2 (hepatocellular carcinoma) *using in vitro* MTT and Neutral Red assays. Also, the chemical analysis of each algal extract was carried out. In conclusion, these algal extracts inhibited some pathogenic microbes and can be used as antimicrobial agents. In conclusion, the two collected macroalgae showed antibacterial activities specially against *Salmonella* which contaminate food, thus the powder or the extracts of these two algae can be used safely as food additive.

Keywords: Marine Algae, Algal extracts, pathogenic organisms, Antimicrobial Activity.

Intoduction

The renewable living marine algae are resources of carbohydrates and proteins. They are the richest source of secondary metabolites for microbial inhibition (Asha *et al*., 2012). In the last years, pathogenic bacteria dramatically infected human and animals, therefore new antibiotics to treat these pathogens are needed**.** Moreover, the resistant to antibiotics among these various pathogens are increased (Jyoti *et al*., 2014, Umemura et al., 2022). New types of natural and safe antibiotics to inhibit the growth and development of these pathogens are necessary and urgently need (Alaribe *et al*., 2011). Many bactericidal or bacteriostatic active materials are obtained from different algal species (Govindasamy *et al*., 2011).

The Gram positive (*Staphyloscoccus aureus, Streptococcus pneumonia)* and the Gram negative *Escherichia coli, Salmonella, Pseudomonas aeruginosa* and *Acinetobacter baumannii* were the most dangerous pathogens. They cause many severe infections in immune compromised patients.

These previous pathogens cause serious infections which life-threatening the human live. *Staphylococcus aureus* caused shock syndrome, wound infection, food poisoning, septicemia and blood toxicity (Mylotte *et al.,* 1987) while *Escherichia coli* which are found in intestine of human caused septicemia, urinary tract inflammation and coleocystis. In hospitals**,** increasing resistance of the two pathogens, *S. aureus* and *E. coli* to antibiotics, were increased and resistance to drugs are mainly obtained after infection of people who were previously exposed to certain antibiotics ((Arredondo-García *et al*.2008, Mubita, *et al*.2018, Saleh *et al.*, 2018, Le Vavasseur and Zeller**,** 2022). Therefore, treatment failure make scientists try to found new drugs to cure them.

As a consequence of an increasing demand in screening for new therapeutic drugs from natural products, there is a greater interest towards marine organisms. Several marine organisms produce bioactive metabolites in response to ecological pressures such as competition for space, maintenance of unfouled surfaces, deterrence of predation and the ability to successfully reproduce (Konig *et al.*, 1994). Terrestrial and aquatic plants, macroalgae (Seaweeds) and marine animal's extracts may be used as natural products that may be used as an alternative materials. They provide a rich source of structurally diverse secondary metabolites. Microalgae species contained bioactive or secondary metabolites (phenolic agents, terpenoids and tannins) which can be used as antibacterial and, antifungal. Indeed, their abundance worldwide, easily to get them with low cost make them a potent candidates to be used as a medications. These secondary metabolites offers defense against herbivores, fouling organisms and pathogens; they also play a role in reproduction, protection from UV radiation and as allelopathic agents (Watson and Cruz-Rivera, 2003). Many algal species and seaweeds may have antimicrobial potentials due to the presence of many bactericidal or bacteriostatic substances (Glombitza, 1979; Michanek, 1979; Fenical and Paul, 1984; Paul and Puglisi, 2004). The bactericidal agents found in algae include amino acids, terpenoids, phlorotannins, acrylic acid, phenolic compounds, steroids, halogenated ketones and alkanes, cyclic polysulphides and fatty acids (Watson and Cruz-Rivera, 2003).

Seaweed extracts proved their efficacy worldwide with board spectrum as antibacterial agents. Little is known about selected seaweeds efficacy against multidrug resistant bacteria. Thereby, the current investigation highlight the seaweeds activity against bacteria and the most potent extracts can be handled in the future in performance investigation (Shanmughapriya *et al*., 2008, Zammuto et al., 2022). In the present study, we tried to evaluate the antimicrobial characteristics of methanolic extracts of two marine algae collected from the coast of red sea (Jeddah) located at Obhur against some resistant bacteria and two cell lines.

Material and methods

Chemicals and used media

Methanol 99.9%, chloroform 99.9%, absolute diethyl ether 99.9% and Dimethyl sulfoxide (DMSO) were gotten from (Synthetic substances shop). The accompanying media were arranged and sterilized at 121 ◦C for 20 min via autoclaving at an autoclave. For solid medium, 2% agar was added.

Algae specimens

The pieces of two marine algae were gathered from Obhur , Jeddah in Saudi Arabia from depths of about 70 m during spring and summer 2018 By a diver (Dr ML Salmn). The two algal samples were recognized and identified at Biology Department, Faculty of Science, KAU. The samples were cleaned completely with water to remove any residue or sand particles. After that the algal samples were dried under shade at room temperature for various time until constant weight. Sub-

sequent to drying, each algal material was ground in crushing machine (moulinex) into powders and sieved.

Tested bacteria

All tested bacterial species were obtained from king Fahad General Hospital, Jeddah, Saudi Arabia. The tested bacterial were: *Staphylococcus aureus* ATCCBAA977 *Streptococcus pneumonia* ATCC49619, *Pseudomonas aeruginosa* ATCC27853, *Escherichia coli* ATCC35218, *E. coli* ATCC25922, *Acinetobacter baumannii* ATCC 17978 and *Salmonella typhi* ATCC *6539.* The test bacteria were grown on Nutrient agar at 37 C for 24 hrs.

Algal extraction

Preliminary experiment was carried out to select the best solvent. The two collected algal species were extracted with hot water methanol, n-Butanol, Ethyl acetate and Diethyl ether (2 g/10 ml w/v) for 12 hrs. The solvent was collected, dried and re-dissolved in 1 ml DMSO to detect the antibacterial activities of the extract using Agar well diffusion methods (Holder and Boyce, 1994, El Sayed and Aly, 2014). Furthermore, According to Hakkim *et al.* (2008) technique by few modification, dried powder portions of *Dictyota dichotoma* (20 g), *Halimeda tuna* (20 g) were extracted with 100 ml of methanol for 48 hrs at room temperature. The organic solvent was collected after filtration and dried at 40 C and the obtained material was dissolved in dimethyl sulfoxide (DMSO) and kept in little vials at low temperature 4 until used to detect the antibacterial, antitumor and toxic effects (Koba *et al.* 2009).

The antimicrobial activities of the algae extracts

Preparation of the inoculums:

Bacterial culture, 24 hrs old was prepared in broth medium and cells were collected and the turbidity was adjusted to 0.5 McFarland Nephelometer standard to get $1.5x10^8$ CFU/ml of bacteria (Mahesh and Satish, 2008). Then, 100 µl was used to inoculate each plate.

Preparation of stock solution of antibiotics:

Standard antibacterial agents were prepared by dissolving 20 mg of the powder in 30 ml DMSO and serial dilution were made, when necessary.

Antimicrobial activities:

Antimicrobial activities of the algae extracts were tested against different test microorganisms using agar well diffusion method (Egorove, 1985). Petri plates were prepared by pouring 20 ml of Nutrient agar medium for bacteria. It was composed of g/l: Beef extract, 3; peptone, 5; agar 20 at pH 7.0. For solid medium, 20 agar was added. Optimally, within 15 min after adjusting the turbidity of the inoculum suspension, sterile cotton swab was dipped into the adjusted suspension. The swab should be rotated several times and presses firmly on the inside wall of the tube above the fluid level. This well remove excess inoculum from the swab and the dried surfaces of agar plates were inoculated by streaking the swab over the entire sterile agar surface. This procedure is repeated by streaking two more times, rotating the plate approximately 60 each time to ensure an even distribution of inoculum. Using sterile crokborer, three wells of 8 mm diameter in agar plate were made. Each well was filled with 100 µl of the tested algal extract. Plates were left for one hour at 4 and then incubated for 24 hrs at 37◦C. Inhibition zones (including the diameter of disc) were measured. The obtained results were compared with DMSO which was used as negative control.

The method of Chand et al. (1994) and modified by Aly and Gumgumjee (2011) was used to determine the minimal inhibitory concentrations (MICs) for the tested algal extracts or antibiotics against bacteria. This method is a simple colorimetric method used 96 well ELISA trays and fluorescein diacetate was used as indicator. Fractional inhibitory concentration (FIC) was calculated using this question (Petersen et al., 2006):

FIC index = MIC of extract in combination/MIC of extract alone + MIC of antibiotics in combination/MIC of antibiotics alone.

The combination defined synergy if $\sum FIC \leq 0.5$, additively if $0.5 < \sum FIC \leq 1$, indifference if $1 < \sum FIC \leq 4$ and antagonism as $\sum FIC > 4$

The cytotoxicity of each algal extract (LD_{50}) was determined after 24hrs using brine shrimp lethality test. Fixed number of live larvae was added to each algal extract and the average number of larvae that survived was calculated and LD_{50} was recorded (Meyer et al. 1982; Aly and Gumgumji 2011).

The antitumor activity of the algal extracts was determined against MCF-7 (breast cancer) and Hep G2 (hepatocellular carcinoma), tumor cell lines by two different methods, *In vitro* MTT and Neutral Red methods. The MTT method was applied in 96-well plates using the method described by Betancur-Galvis et al. (1999). Also, Neutral Red assay technique, described by Betancur-Galvis et al. (1999), was used to detect any activity of the two tested algal extracts on the two studied cell lines, Hep G2 and MCF-7. After 48 hrs, cells were collected, re-suspended in culture medium containing neutral red and after incubation for 3 hrs at 37◦C, the cells were washed, re-suspended acetic acid/ethanol/ water (1:49:50 v/v/v), the absorbance was detected at 540 nm and LD_{50} was recorded. The mean value of three replicates of each test was calculated and standard deviations was applied.

Detection of secondary metabolites*:*

The algal extracts were analyzed for the presence of any active constituent. Preliminary studies were carried out on the chemical analysis of those extracts. The prepared algal extracts were analyzed for the presence of anthocyanins, butacyanins, flavonoids, saponins, steroids and tannins (Aly et al., 2013, El Sayed and Aly, 2014).

Statistical analysis

Measurable investigations were performed utilizing the Factual Bundle Social Science (SPSS for windows, adaptation 16) (SPSS Inc., Chicago, IL, U.S.A). The inconstancy level of the outcome is communicated as mean ± standard deviation (Mean ± SD and analysis of variance using ANOVA were recorded to detect any significant difference at $P \leq 0.5$.).

Results

The two collected algal species were identified as *Halimeda tuna* and *Dictyota dichotoma* (Figure 1). *Halimeda tuna* is a green seaweed belong to order Bryopsidales and found in the Red sea Atlantic Ocean, the Indo-Pacific region and the Mediterranean Sea. Moreover, *Dictyota dichotoma* has a yellowish-brown or greenish color, belong to Brown algae and found at high temperate in the Red Sea. The membranous, flattened, dichotomously-branching body is semi erect with very small or no stalk attached to the seabed by rhizoids which can also absorb nutrients from water. The fronds up to 25 cm long and 1 cm wide, has reticulated shape with no midrib. The thallus branches seemed equal in length.

The two species after identification were extracted using different organic solvents (data not shown) and it was clear that methanol extracts of the two tested algal materials were the best solved. Thus, it was selected for the detail studies. The antimicrobial activities of each extract was determined using different bacterial pathogens. The results showed that the extracts of both *Halimeda tuna* and *Dictyota dichotoma* were active against at the tested bacterial pathogens and all the tested bacteria were inhibited by the algal extracts. The highest antimicrobial activities were against the two tested Gram positive bacteria, Staphylococcus aureus and Streptococcus pneumonia and *Dictyota dichotoma* extract showed more inhibition activates compared to *Halimeda* extract. *Salmonella*

typhi was affected by both algal extracts (Table 1, Figure 2). On contrast, both *Halimeda* and *Dictyota* extracts showed weak inhibition activities against *Psedomonas aeruginosa* and *Acinetobacter baumannii*. Moreover, the mixture of the two algal extract showed excellent activities against all the tested bacteria and there is a clear synergistic effect between both extracts. Minimal inhibitory concentrations (MICs, µg/ml) of the two tested algal methanolic extracts and their mixture using Fluorescein diacetate method were determined (Table 2). The MIC was ranged from 22.5 -37.5 µg/ml for *Halimeda* extract and lower concentration was concentration (22.5 µg/ml) was recorded for *Dictyota*. The MICs for the mixture of the two methanolic extracts were ranged from 7.5-15 µg/ml which is very good results to be used in many medical applications.

The antitumor activity (LD50, µg/ml) of the methanolic extracts of the two tested methanolic extracts against two different cell line were recorded and two different techniques were compared (Table 3). The recorded LD50 values were 250 -400 µg/ml for *Halimeda tuna* and from 300- 400 µg/ml for *Dictyota dichotoma*. Lower LD50 was recorded for the mixture of the two extracts which were ranged from 200- 300 µg/ml. The toxicity of the two extracts (LD50, µg/ml) were recorded using *Artemia salina* as test organism (Table 3). No clear toxicity was recorded for any algal extracts or their mixture up to 450 µg/ml. Table 4 showed the detected photochemical classes in the two examined algal extracts. It was clear that the two extracts contained Flavonoids, Anthocyanin, Betacyanin, Tannins and Steroids

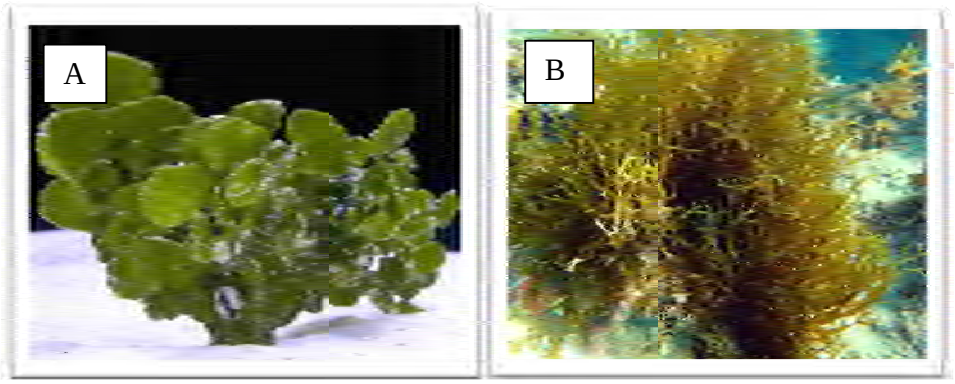

Figure 1. *Halimeda tuna* (A) and *Dictyota dichotoma* (B) .

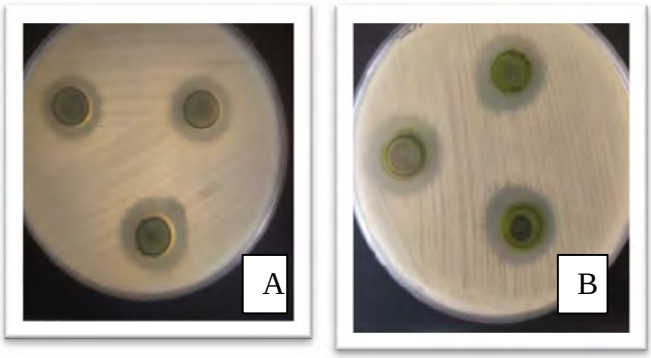

Figure 2. The effect of *Halimeda* extract on *Salmonella typhi* (A) and the effect of *Dictyota* extract on *S. aureus* (B)

Table 1. The inhibitory effect (diamter of the inhibition zon, mm) of two algal extracts, *Halimeda* and *Dictyota*) and their mixture on different bacterial pathogens, compared to the control antibiotic

Algae extract / Tested Bacteria	*Halimeda tuna*	*Dictyota dichotoma*	Mixture#	Control antibiotic**
Acinetobacter baumannii ATCC 17978	10.3 ± 0.17*	11.3 ± 0.47*	21.13 ± 0.23	23.17 ± 0.33
E. coli ATCC25922 (1)	13.1 ± 0.09*	17.6 ± 0.84*	23.01 ± 0.33	33.41 ± 0.31
E. coli ATCC35218 (2)	14.0 ± 0.82*	17.3 ± 0.53*	23.12 ± 0.32	33.00 ± 0.17
Klebsiella pneumonia ATCC 13883	14.3 ± 0.40*	17.3 ± 0.27*	25.11 ± 0.13	39.23 ± 0.49
Salmonella typhi ATCC 6539	14.5 ± 0.36*	17.4 ± 0.42*	26.13 ± 0.11	36.10 ± 0.56
P. earuginosa ATCC27853	10.3 ± 0.27*	11.9 ± 0.17*	21.30 ± 0.12	31.32 ± 0.90
Staphylococcus aureus ATCCBAA977	18.2 ± 0.29*	19.7 ± 0.29*	27.14 ± 0.90	43.12 ± 0.96
Streptococcus pneumonia ATCC49619	18.7 ± 0.52*	19.3 ± 0.47*	27.19 ± 0.22	47.13 ± 0.44

*significant results compared to control antibiotic , **Control antibiotic:Ampicillin trihydrate, #: mixture of the two algal species (W/W).

Table 2. Minimal inhibitory concentration (µg/ml) of the two tested algal methanolic extracts and their mixture using Fluorescein diacetate method

	Halimeda tuna	*Dictyota dichotoma*	Mixture#	FIC index	Effect **
E. coli ATCC25922 (1)	37.5	22.5	15.0	1.06	Indifference
E. coli ATCC35218 (2)	30.0	22.5	15.0	0.81	Additively
Klebsiella pneumonia ATCC 13883	30.0	22.5	15.0	0.81	Additively
Salmonella typhi ATCC 6539	30.0	22.5	15.0	0.81	Additively
Staphylococcus aureus ATCCBAA977	22.5	22.5	7.5	0.66	Additively
Streptococcus pneumonia ATCC49619	22.5	22.5	7.5	0.66	Additively

*FIC: Fractional inhibitory concentration, ** Effect: FIC ≥ 1 (Indifference), FIC ≤ 1 (Additively)

Table 3. Antitumor activity (LD50, µg/ml) of the methanolic extracts of the two tested methanolic extracts against two different cell line and their toxicity (LD50, µg/ml) using *Artemia salina* as test organism.

Tested extract / Tested cells	MTT Test (LD50, mg/l)		Neutral Red Assay (LD50, mg/l)		Toxicity (LD50, mg/l)
	Hep G2	MCF-7	HEp-2	MCF-7	Artemia salina
Halimeda tuna	400.0+50 *	250.0+12.5 *	450.0+12.5 *	400.0+12.5 *	≥450

Tested extract / Tested cells	MTT Test (LD50, mg/l)		Neutral Red Assay (LD50, mg/l)		Toxicity (LD50, mg/l)
	Hep G2	**MCF-7**	**HEp-2**	**MCF-7**	**Artemia salina**
Dictyota dichotoma	400.0+25 *	400.0+50 *	400.0+50 *	300.0+25 *	≥450
Mixture	200.0+50 *	200.0+50 *	300.0+25 *	300.0+50 *	≥450
#Bleomycin (control)	0.08+0.01	0.08+0.00	0.10+0.00	0.16+0.04	≥ 0.16

* Significant result at $p \leq 0.05$ compared to control (untreated cells), # : Antitumor agent (control).

Table 4 . Preliminary phytocheical analysis of two algae extracts

Algae	The detected phytochemicals				
Saponins	**Flavonoids**	**Anthocyanin**	**Betacyanin**	**Tannins**	**Steroids**
Halimeda tuna	+	+	+	+	-
Dictyota dichotoma	+	+	+	+	+

+ Present, - absence

Discussion

The green seaweed *Halimeda tuna* found mainly in the Red sea Atlantic Ocean, the Indo-Pacific region and the Mediterranean Sea. *Halimeda tuna* is the type species of the genus *Halimeda* and the type locality is the Mediterranean Sea. It was reported that the previous algal species found also in warm shallow lagoons and sometimes in deep protected places with rocky habitats. *Halimeda tuna* has thallus (frond) with a single cell like a tube and had multiple nuclei grow attached to the seabed by a holdfast Each individual had green chloroplasts had carotenoids and chlorophyll and the contents of the cells are free to move inside the cell, and are rich with calcium carbonate. The algal species is often overgrown in summer by epiphytes and need increasing temperatures and high concentration of nutrients. Fragmentation was noticed as asexual stage of reproduction (Guiry**,** 2015, 2019). According to the brown algal isolate, *Dictyota dichotoma* was isolated from the Red Sea, Atlantic Ocean, Mediterranean Sea and Black Sea in addition to Indian Ocean. *It had two similar stages*, sporophytes and the gametophytes and this algal species was mainly noticed in summer. The cells contained chlorophyll in addition to other pigment and many secondary like diterpenes alkaloids, steroids, tannins, flavonoids and phenols (Deyab et al., 2016, Zouaoui and Ghalem, 2018, Buron et al., 2021). The extract of the previous algal material with methanol contained many secondary products like coumarins, quinones and glycosides which poses inhibition activities against some pathogenic bacteria and the pathogenic yeast, *Candida albicans*.

The main aims of this study was to determine and compare the ability of different selected algal species from Jeddah coast of Red Sea to produce bioactive compounds of potential therapeutic interests. The production of antimicrobial activities was considered to be an effective indicator of the capability of the seaweeds to synthesize bioactive secondary metabolites including some polysaccharides (Shanmughapriya *et al*., 2008). Screening of the seaweeds for inhibition activity are the area of many investigations. The aqueous extracts has weak activities against the bacterial pathogens while the organic extracts suppressed the growth of some bacterial and fungal pathogens. On con-

trast the antifungal activities of *D. dichotoma* extracts were weak and depend on the used solvents (Saleh *et al.*, 2018).The antibacterial activity of seaweeds may be influenced by some factors such as the habitat and the season of algal collection, the growth stage of the algae, the experimental methods etc. Susceptibility of the bacterial strains to the algal extracts was expressed as Zone of Inhibition (Saleh *et al.*, 2018) but variation in antibacterial activity may be due to the method of extraction and solvent used in extraction. Many solvents may be employed in screening seaweeds for antibacterial activity (Johnson and Raja, 2015).

The results found in this study reported that the methanolic extract was the best solvent and these results were supported and/or opposed in the data reported in literature (Alang *et al*., 2009). In this study the methanol extract inhibited the growth of some bacterial pathogens and these results were similar to that of Ghalem et *al*. (2018). Also, the tested methanolic extracts has moderate anticancer agent with no toxicity. On contrast of our results, a study found an excellent inhibitory effect, obtained using *Ulva lactuca* chloroform extract and this extract was more active on *S. aureus* and *E.coli* but the results of Pushparaj et al. (2014) showed no activity against *S. aureus* and *E. coli* of the chloroform extract *of U. lactuca*. Algotiml et al (2022) reported that the extracts of some marine algae like *Ulva rigida* (green alga), *Cystoseira myrica* (brown alga), and *Gracilaria foliifera* (red alga), had excellent reducing and capping activates which can be used to prepare AgNPs which had low cytotoxicity, anticancer and antimicrobial activities.

Conclusion

Microbial resistant is problems in a wide range of countries, from medicine and food industries to environmental aspects. The bioactive agents from marine algae had promising activities for several bio- prospecting agents such as nutraceutical, functional food, cosmetic and pharmaceutical applications. Several molecules, , isolated from green, brown and red algae have been reported to possess potent antimicrobial activities

The outcomes demonstrated the promising antimicrobial and antitumor activities of dissolvable concentrates of marine green and brown algae, collected from the Red sea coast, Saudi Arabia and propose that these algae can be consumed without toxicity. *Salmonella typhi* **w**as affected by both algal extracts which mean the possibility of adding the algal powder to minced meat or chicken to preserve it for long time. In this manner, investigation of such organic extract may be a likely asset of a variety of organically dynamic mixtures and the present outcomes will guarantee a beginning stage for using regular bioactive substances exhibits in the marine green or brown algae which may lead to improvement of new pharmaceutical agents.

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