

Full Length Research Paper

***In vitro* antibacterial activity of alkaloid extracts from green, red and brown macroalgae from western coast of Libya**

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Marine organisms and microorganisms are known to be a rich source of alkaloids with unique chemical feature and interesting biological activities. The current study presents the antibacterial effect of the alkaloid extracts of some green, red and brown algae were collected from western coast of Libya, against, *Escherichia coli*, *Salmonella typhi*, *Klebsiella* spp., and *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Bacillus subtilis*, *Bacillus* spp. and *Staphylococcus epidermidis* were investigated. Although alkaloid extracts of green algae inhibited all tested bacteria, maximum effect was exhibited by brown and red algae species. Thus, *Cystoseira barbata* alkaloid extract showed remarkable inhibition of human pathogen *Klebsiella* spp. *Dictyopteris membranacea* alkaloid extract also demonstrated similar considerable effect against *S. typhi* with MIC value 1.56 mg/ml. The pronounced antibacterial activity of *C. barbata* and *D. membranacea* can be attributed to their high alkaloid contents. These results suggest that red and brown algae secondary metabolites are important sources that could produce potential chemotherapeutic agents.

Key words: Macroalgae, alkaloids, antibacterial activity.

INTRODUCTION

Algae are a large and diverse group of organisms from which a wide range of secondary metabolites have been isolated. A number of these compounds possess biological activities such as toxicity, antibacterial, antifungal, antiviral, antitumour and other specific activity (Cannell, 1993). These bioactive compounds include alkaloids (Guven et al., 2010), polyphenols) Pereira et al., 2002), terpenoids (Cen-Pacheco et al., 2010), flavonoids (Stafford, 1991), tannins (Serrano et al., 2009) and acetogenins (Narkowicz and Blackman, 2006) which are applicable for antioxidant (Rocha et al., 2007), antimicrobial

(Li, 2009; Saidani et al., 2011), antiviral (Romanos et al., 2002; Mayer et al., 2009), anti-inflammatory and anticancer activities (Jaswir and Monsur, 2011; Bhakuni and Rawat, 2005; Vasanthi et al., 2004; Natarajan and Kathiresan, 2010). Nevertheless, in Libya this kind of study has not been well explored, despite the wealth of Libyan marine flora. Therefore, the present study is to investigate the alkaloid compounds extracted from macro-algae from the Libyan coast.

Alkaloids are heterocyclic nitrogen compounds, naturally occurring in plants, microbes, animals and marine

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organisms. The first medically useful example of an alkaloid was morphine, isolated in 1805 from opium *papaver somniferum* (Fessenden and Fessenden, 1982). Although, alkaloids have been extensively studied in plants and few studies in marine algae are reported due to the fact that alkaloids of marine algae are relatively rare compared with terrestrial plant alkaloids (Guyen et al., 2010). The alkaloids found in marine algae can be classified into three groups: Phenylethylamine alkaloids, indole and halogenated indole alkaloids, and other alkaloids. Structurally, phenylethylamine and indole groups are the most alkaloids isolated from marine algae. Biological activities of halogenated and non-halogenated forms have been reported as bioactive compounds and as biological probes for physiological studies (Kasim et al., 2010). In addition, *Caulerpin* isolated from macroalgae, was the only indole alkaloid from marine sources which has been reported to have anti-inflammatory potentials (Carolina et al., 2011; Everton et al., 2009). In addition, there are two derivatives: lophocladine A and lophocladine B which have been isolated from a red alga *Lophocladia* spp., collected from Fijian Island, New Zealand (Gross et al., 2006) and their anticancer activity has been proved successfully in various cancer cell lines (Patricia et al., 2010).

Previous studies revealed that seaweed extracts, especially polyphenols have antioxidant activity (Chandini et al., 2008; Ganesan et al., 2008); whereas alkaloids are commonly found to have antimicrobial properties against both Gram-positive and Gram-negative bacteria (Guyen et al., 2010) such as halogenated indole alkaloids which are isolated from red algae (Ayyad and Badria, 1994). These compounds have been approved for their antibacterial activity (Guella et al., 2006). The biological activity of marine indole alkaloids is clearly a product of the unique functionality and elements involved in the biosynthesis of marine natural products which increase the biological activity of seaweeds. For instance, bromination of many natural products has the potential to increase biological activity significantly (Gul and Hamann, 2005). The current study was undertaken to investigate the antibacterial effect of alkaloid extracts of 6 species of marine algae (two Chlorophyceae, three Phaeophyceae and one Rhodophyceae) collected from the Libyan western coast, against pathogenic Gram-negative bacteria: *Escherichia coli*, *Salmonella typhi*, *Klebsiella* spp., and *Pseudomonas aeruginosa* as well as Gram positive bacteria, *Staphylococcus aureus*, *Bacillus subtilis*, *Bacillus* spp., and *Staphylococcus epidermidis*.

MATERIALS AND METHODS

Sample collection

Ulva lactuca, *Codium tomentosum* (Chlorophyta), *Cystoseira barbata*, *Sargassum vulgare*, *Dictyopteris membranacea* (Phaeophyta) and *Gelidium latifolium* (Rhodophyta) were collected from western coast of Libya between February and spring, 2009.

The algal samples were taxonomically identified at Botany Department, Faculty of Science, Tripoli University. Algae were washed properly with distilled water, then they were shade dried at room temperature, after which they were crushed in an electric mill until a fine powder was obtained (Chiheb et al., 2009).

Bacterial strains

Eight bacterial strains (Gram positive and negative) were selected for the study. The Gram positive species were: *S. aureus* (*S. aur*) was obtained from the Clinical Microbiology Laboratory, Azzawiya Medical Center (Azzawiya, Libya). *B. subtilis* (*B. sub*) were kindly provided by Mohamed Elghazali, Department of Microbiology Biotechnology Research Center (Twaisha, Libya), while *Bacillus* spp. (*B. spp.*) and *S. epidermidis* (*S. epi*) were obtained from the Department of Microbiology, Faculty of Veterinary Medicine, Tripoli University. The Gram negative species *S. typhi* (*S. typhi*), *E. coli* (*E. coli*), *P. aeruginosa* (*P. aer*) and *klebsiella* spp. (*K. spp.*) were obtained from the Department of Microbiology, Faculty of Veterinary Medicine, Tripoli University, Libya.

Alkaloids extraction

Powdered algae materials (50 g) were extracted several times with methanol (300 ml). Methanol extraction was continued until the plant material gave a negative result for alkaloids (Mayer's test). The obtained methanolic extract was evaporated under reduced pressure at 40°C, to minimize any possible thermal degradation of the alkaloids and other thermo labile compounds. The crude alkaloid mixture was then separated from neutral and acidic materials, and water soluble ingredients by extraction with aqueous acetic acid, followed by dichloromethane extraction, then basification of the aqueous solution which was subjected to further dichloromethane extraction thereafter (Hadi and Bremner, 2001).

Thin layer chromatography (TLC)

Identification of alkaloids was further carried out by TLC using pre-coated silica gel 60 F264 plates (Wagner and Bladt, 2004). Different screening systems were used to obtain better resolution of the components. Dragendorff's reagent was used as a locating reagent (Harborne, 1992). R_f value of each spot was calculated as $R_f = \text{Distance travelled by the solute} / \text{Distance travelled by the solvent}$.

Determination of antibacterial activity

The antimicrobial activity test of algal crude extracts was performed *in vitro* using the "hole-plate diffusion method" (Sarvanakumar et al., 2009). Each test organism was maintained on nutrient agar slant and was recovered for testing by growth in nutrient broth (Biolab, Difco) for 14 h at 37°C before streaking. Cultures were routinely adjusted to a suspension of 1×10^6 to 2×10^6 CFU/ml using pre-made calibration curve representing viable cell count ($X \times 10^6$) against OD 660 nm (Y). The plates with bacteria were incubated at 37°C for 24 h. After incubation, the inhibition zones formed around the holes were measured. Methanol (100%) without seaweed extract was used as negative control and ciprofloxacin disc (30 µg) was used as the positive control.

Determination of minimum inhibitory concentration (MIC)

The MICs were determined by the agar dilution method (Daud and

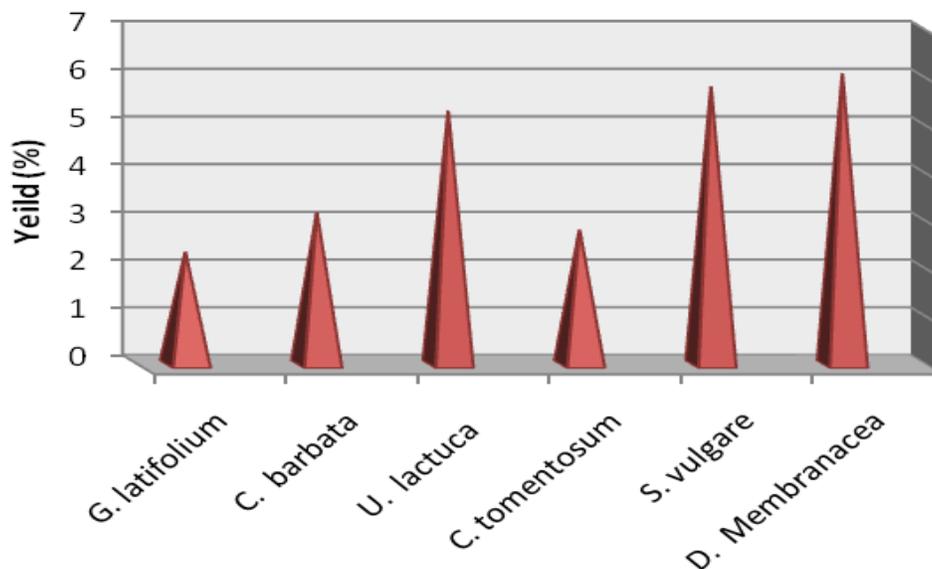


Figure 1. Percentage of alkaloid yield (mg alkaloid/g dry weigh) extracted from *U. lactuca*, *C. tomentosum*, *C. barbata*, *S. vulgare*, *D. membranacea* and *G. latifolium*.

Sanchaz, 2005). Two-fold serial dilutions of the original algae extract (100 mg/ml) were prepared in nutrient broth to obtain concentration from 100 to 1.56 mg/ml solvent. The plates incubated at 27°C for 18 h.

Bioautography method

Ten microliter (10 µl) of solutions corresponding to 1000 µg of alkaloids extract were applied to precoated Silica-gel TLC plates, developed with $\text{CHCl}_3/\text{MeOH}/\text{Na}_2\text{CO}_3$ (3:8:1, v/v) for each extract, and was evaporated to complete dryness. The dried plates were overlaid with nutrient agar medium seeded with *E. coli* (106 to 107 CFU/ml) and then incubated overnight at 37°C.

Statistical analyses

All assays were done in triplicate. All data are expressed as means \pm S.D. Data were analyzed by an analysis of variance ($P < 0.05$) and the means separated by one-way ANOVA with Turkey's b test using SPSS version 20.0.

RESULTS AND DISCUSSION

The qualitative phytochemical analysis for *U. lactuca*, *C. barbata*, *D. membranacea*, *C. tomentosum*, *S. vulgare* and *G. latifolium* showed the presence of alkaloids according to previous finding (Alghazeer et al., 2013). The present study was performed in order to extract alkaloids from the same species and then assess their antibacterial activity. Figure 1 shows alkaloid contents (% mg/g) extracted from green, red and brown algae species. The highest content was recorded for *D. Membranacea* (6.11%), *S. vulgare* (5.84%), *U. lactuca* (5.33%), (6.11%); whereas *C. barbata* and *C. tomentosum* showed moderate content of alkaloid (3.2 and 2.84%, respectively),

while the lowest alkaloid content was obtained from *G. latifolium* (2.37%). Alkaloids present special interest because of their pharmacological activities. In fact, many reports revealed the presence of alkaloids in marine algae and some of them have been investigated for their biological activity (Güven et al., 2010; Kasım et al., 2010). Antimicrobial activities of alkaloid extracts from six seaweeds species represented by three Phaeophyta (*S. vulgare*, *D. membranacea* and *C. barbata*), two Chlorophyta (*U. lactuca* and *C. tomentosum*) and one Rhodophyta (*G. latifolium*) were examined against eight test bacteria (*Bacillus* spp., *B. subtilis*, *S. aureus*, *S. epidermidis*, *E. coli*, *kleb. spp.*, *P. aeruginosa* and *Salmonella typhi*). The inhibition zones of brown, green and red algae extracts against Gram positive and Gram negative bacteria ranged between 13 to 35, 12 to 29 and 15 to 34 mm, respectively, all values are shown in Table 1.

The alkaloid extract of *U. lactuca* showed a relatively high mean zone of inhibition (21 ± 0.11 mm) against the Gram positive *S. aureus*, *S. epidermis* then *Bacillus* spp. (17 ± 0.3 mm), *E. coli* (16 ± 0.12 mm) and *B. subtilis* (14 ± 0.10 mm). While the alkaloid extract of *C. tomentosum* showed a remarkable high inhibition zone against *S. epidermis* (29 ± 0.35 mm) then *Bacillus* spp. (20 ± 0.3 mm), *S. aureus* (16 ± 11) and *B. subtilis* (13 ± 0.09 mm). For Gram negative bacteria, maximum zone of inhibition was recorded with alkaloid extract of *U. lactuca* against *kleb* spp. (18 ± 0.15 mm) and *S. typhi* (17 ± 0.11 mm). Also, maximum inhibition zones were recorded by *C. tomentosum* alkaloid extract against *klebsiella* spp. (27 ± 0.35 mm) then *E. coli* (23 ± 0.11 mm), *S. typhi* (21 ± 0.23 mm) and *P. aeruginosa* (12 ± 0.09 mm) (Table 1). The alkaloid extract of *S. vulgare* and *C. barbata* showed highest mean zone of inhibition against the Gram positive

Table 1. *In vitro* antimicrobial activity of the algal alkaloids extracts (100 mg/ml) against gram positive and gram negative bacteria.

Algal species	<i>U. lactuca</i>	<i>C. tomentosum</i>	<i>G. latifolium</i>	<i>S. vulgare</i>	<i>D. membranacea</i>	<i>C. barbata</i>	^a Ciprofloxacin	^a Chloramphenicol	^a Neomycin
Test organism	DIZ (mm)	DIZ (mm)	DIZ (mm)	DIZ (mm)	DIZ (mm)	DIZ (mm)	DIZ (mm)	DIZ (mm)	DIZ (mm)
<i>E. coli</i>	16±0.12	23±0.11	29±0.54*	23±0.11	30±0.31*	22±0.22	23	19	-
<i>S. typhi</i>	17±0.11	21±0.23	18±0.11	25±0.6	35±0.74*	26±0.45	26	21	-
<i>Kleb. sp</i>	18±0.15	27±0.35*	15±0.11	24±0.09	28±0.6*	35±0.54*	24	18	-
<i>P. aer</i>	15±0.12	12±0.09	ND	ND	ND	ND	20	-	-
<i>B. sub</i>	14±0.10	13±0.09	ND	ND	23±0.6	15±0.12	29	-	21
<i>B. sp</i>	17±0.11	20±0.35	24±0.11	19±0.21	ND	31±0.15*	24	-	26
<i>S. aur</i>	21±0.15	16±0.11	15±0.12	13±0.09	16±0.11	22±0.15	25	-	20
<i>S. epi</i>	21±0.6	29±0.35*	34±0.6*	18±0.11	23±0.15	20±0.11	23	-	23

Data are expressed as the mean ± standard deviation (SD) of three replicates. * represent the statistical comparisons between alkaloid extracts and positive control by using ANOVA followed by post hoc Tukey's b test ($p < 0.05$). ND: not detectable.

Bacillus spp. (19 ± 0.21 mm and 31 ± 0.15 mm, respectively) then against *S. epidermis* (18 ± 0.11 ; 20 ± 0.11 mm, respectively), and *S. aureus* (13 ± 0.09 ; 22 ± 0.15). However, *D. membranacea* alkaloid extract had no effect on *Bacillus* spp., but showed high inhibition zones against *B. subtilis* and *S. epidermis* (23 ± 0.6 and 23 ± 0.15 mm). Maximum inhibition zone of Gram negative bacteria was recorded for alkaloid extract of *S. vulgare* and *D. membranacea* against *S. typhi* (25 ± 0.6 mm; 35 ± 0.74 mm) while the highest effect by the alkaloid extract of *C. barbata* was observed against *klebsiella* spp. (35 ± 0.54 mm). Whereas, *P. aeruginosa* was not susceptible to the alkaloid extracts of *S. vulgare*, *D. membranacea* and *C. barbata*.

The antibacterial activity of the alkaloid extract of *D. membranacea* and *C. barbata* were significantly high ($P < 0.05$) compared with the positive control (Ciprofloxacin and Chloramphenicol) against Gram negative bacteria (Table 1), the antibacterial activity of green, brown and red algae is well documented (Del Val et al., 2001) as well as their isolated alkaloids (Masuda et al., 1997; Kasim et

al., 2010). The alkaloid extract of *G. latifolium* showed significant high mean zone of inhibition against the Gram positive *S. epidermis* (34 ± 0.6 mm) compared with positive control (Ciprofloxacin and Neomycin) ($P < 0.05$) which is in consistent with earlier finding where alkaloid isolated from red algae exhibited different modes of bioactivity (Sato et al., 1998; Gross et al., 2006). The recorded inhibition zone against *Bacillus* spp. and *S. aureus* were 24 ± 0.11 and 15 ± 0.12 mm respectively, while no inhibition was observed against *B. subtilis*. For Gram negative bacteria, maximum zone of inhibition was recorded with alkaloid extract of *G. latifolium* against *E. coli* (29 ± 0.54 mm), *S. typhi* (18 ± 0.11 mm) and *Kleb. sp.* (15 ± 0.11 mm), while no inhibition was observed against *P. aeruginosa* (Table 1). The activity of red, green and brown algae against both Gram positive and Gram negative bacteria may be indicative of presence of broad spectrum antibiotic compounds or simply the content of pharmacological active constituents like alkaloids (Omulokoli et al., 1997; Phang et al., 1994).

Minimum inhibitory concentrations (MICs) of the

alkaloid extracted from algae (Figure 2) were found to be within the range of 100 to 1.56 mg/ml. The high levels of the MIC's of some alkaloid extracts can be attributed either to the presence of the active components in low concentrations, or to the presence of some antagonistic components that serve as growth promoters for the bacteria. The minimum inhibitory concentration (MIC) value of green algae (*U. lactuca*, *C. tomentosum*) against bacteria was ranged between 6.25 to 100 mg/ml. The lowest MIC value was recorded for *C. tomentosum* and *U. lactuca* extracts (6.25, 25 mg/ml respectively) against *S. epidermidis* while The minimum inhibitory concentration (MIC) value of brown algae (*S. vulgare*, *D. membranacea* and *C. barbata*) against bacteria was ranged between 6.25 to 100 mg/ml. The lowest MIC (1.56, 6.25 and 12.5 mg/ml) values were recorded for *C. barbata*, *D. membranacea* and *S. vulgare* extracts, respectively against *klebsiella* spp. (Figure 1). For alkaloid extracted from red algae (*G. latifolium*), the minimum inhibitory concentration (MIC) values of brown algae (*S. vulgare*, *D. membranacea* and *C. barbata*) against bacteria were

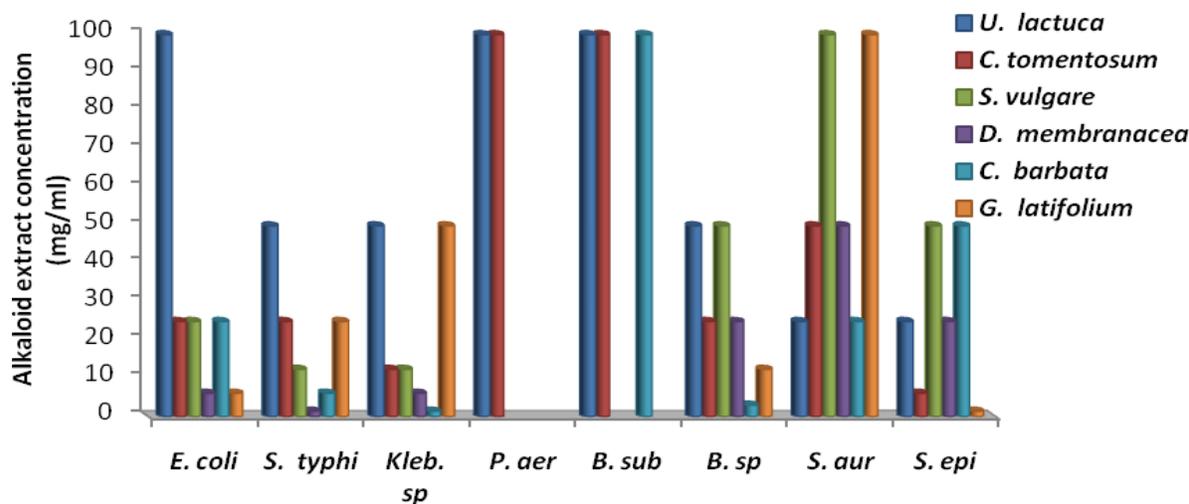


Figure 2. The *in vitro* antimicrobial activity of alkaloids extracts of tested algae expressed as minimum inhibitory concentration (MIC) (mg/ml) against some bacteria. *S. aur*: *Staphylococcus aureus*, *B. sub*: *Bacillus subtilis*, *E. coli*: *Escherichia coli*, *P. aer*: *Pseudomonas aeruginosa*, *B. sp*: *Bacillus* spp., *S. typhi*: *Salmonella typhi*, *S. epi*: *Staphylococcus epidermidis*, *Kleb. sp*: *klebsiella* spp.

Table 2. Location and prominence of zones of inhibition at different R_f values of alkaloid extracts against *E. coli*.

Algal species	Spot Number					
	S1		S2		S3	
	R_f	<i>E. coli</i>	R_f	<i>E. coli</i>	R_f	<i>E. coli</i>
<i>U. lactuca</i>	0.57	+	0.68	+	-	-
<i>C. tomentosum</i>	0.59	-	0.72	+	-	-
<i>G. latifolium</i>	0.57	++	0.61	-	0.68	+
<i>S. vulgare</i>	0.55	+	0.76	+	-	-
<i>D. membranacea</i>	0.54	++	0.64	-	0.75	++
<i>C. barbata</i>	0.52	+	0.74	++	-	-

Degree of inhibition: ++ = Prominent; + = moderate; - = Nil, *E. coli*: *Escherichia coli*; Mobile phase system is: chloroform: methanol: sodium carbonate (3:8:1, v/v).

ranged between 1.56 to 100 mg/ml. The lowest MIC (1.56 mg/ml) value was recorded against *kleb. sp* (Figure 1).

Preliminary TLC and the bioautographic assay tests were carried out on extracts to separate the compounds that were responsible for the inhibition tested the bacteria hence one spot may contain more than one compound. The results showed that the antibacterial assay can be attributed to the compounds observed at the various R_f values on the TLC separation. Although, sometimes the activity of compounds is not easily detected by this assay, if the compound does not diffuse through the agar, then the activity could be masked. The bioautography method was applied to the extracts using *E. coli* isolate to which all extracts exhibited antimicrobial activity (Table 1). The results showed good activity against *E. coli*, with prominent inhibition zones for the alkaloid of *D. membranacea*, *C. barbata*, *S. vulgare*, *G. latifolium* and *U. lactuca* extracts had two zones of inhibition, whereas,

the alkaloid of *C. tomentosum* extract exhibited one zone of inhibition (Table 2).

Conclusions

The results of this work indicate the presence of alkaloids in tested algae that play an indispensable role in antibacterial activity, however further studies to identify and characterize the specific active compounds, as well as the evaluation of the toxic aspects are recommended.

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