Arabian Journal of Chemistry (2015) 8, 32–37

King Saud University

Arabian Journal of Chemistry

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ORIGINAL ARTICLE

Antibacterial β-amyrin isolated from *Laurencia microcladia*

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Received 26 March 2013; accepted 21 September 2013
Available online 2 October 2013

KEYWORDS

Marine algae;
Laurencia microcladia;
Triterpens;
β-Amyrin;
Antibacterial activity

Abstract The present study aimed to isolate β-amyrin for the first time from *Laurencia microcladia* Kützing distributed at Dahab Coast, Aqaba Gulf, Egypt. The successive extraction of the ethanolic extract of the alga showed that, the petroleum ether extract was the best extractive solvent which contains the isolated active compound. Based on IR, MS, and ^{1}H NMR analyses, the active principle is proposed to be triterpens having the empirical formula $C_{30}H_{50}O$ with a melting point range 191–194 °C. The importance of this study was to draw attention and study the economic importance of different types of marine algae that grow on the Egyptian shores as they contain varying antimicrobial effectiveness. β-Amyrin obtained in this study from *L. microcladia* was known for its potent antibacterial activity and commonly used medically in many areas. These results provide evidence, to intensify the study on this *L. microcladia* to be used as a source of β-amyrin.

Abbreviations: MIC, minimum inhibitory concentration; LD_{50}, medium lethal dose; TLC, thin layer chromatography; PC, paper chromatography; UV, ultra violet; IR, infrared spectrum; ^{1}H NMR, nuclear magnetic resonance; MS, mass spectrometry; IR (KBr), infrared spectrum in the presence of potassium bromide

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Peer review under responsibility of King Saud University.

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1. Introduction

Marine algae produce a cocktail of metabolites with potential commercial value. Their medical and pharmaceutical applications have been investigated for a few decades (Bazes et al., 2009). Many compounds were discovered in the last years. The need for new drugs keeps this field open as many algal species are poorly screened. The ecological role of marine algal metabolites has somehow been overlooked. This new research field will provide valuable and novel insight into the marine ecosystem dynamics as well as a new approach to comprehend-

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http://dx.doi.org/10.1016/j.arabjc.2013.09.033

ing biodiversity. Furthermore, understanding interactions between compound production by algae and the environment, including anthropogenic or global climate changes, is a challenging target for the coming years.

Research on the active ingredients of metabolites has been more focused on macroalgae than on phytoplankton. However, phytoplankton could be a very promising material since it is the base of the marine food chain with quick adaptation to environmental changes, which undoubtedly has consequences on secondary metabolism (Cabrita et al., 2010).

Among all marine macroalgae, red algae are the main producers of bioactive compounds. *Laurencia perforate* is considered one of the most prolific genera (Faulkner, 2001 and Wright et al., 2003). A bioactive compound from *Laurencia* species is relatively high (Hill, 2007). Diterpenes, triterpenes, and C_{15}-acetogenins are the main secondary compounds of this genus (Faulkner, 1995) with antimicrobial, antifeedant, antihelmintic, and cytotoxic properties are generally associated (Davyt et al., 2001; Topcu et al., 2003 and Sun et al., 2005). Also, recent studies have shown promising antimalarial activity in *Laurencia microcladia* (Leon-Deniz et al., 2009).

One of the most important compounds of triterpens is β-amyrin. This compound is more potent than aspirin inhibiting collagen-induced platelet aggregation. In addition, β-amyrin includes skin care applications, anti-irritant, anti-inflammatory action as well as enhancement of the sun protection factor of organic sunscreens and emollient effect (Kweifio-Okai et al., 1995 and Ching et al., 2010). The present study aimed to evaluate the antibacterial activities as well as the chemical composition of β-amyrin isolated for the first time from *L. microcladia*.

2. Materials and methods

2.1. Collection of algal species

The benthic algal samples were collected at a depth of 1–2 m from the coast of Dahab Coast, Aqaba Gulf, Egypt during the summer of 2012. The samples were washed in seawater at the sampling station and cleaned of epiphytes and necrotic parts were removed and transferred to the laboratory under refrigerated conditions. After their arrival at the laboratory, the material was rinsed in sterile seawater and 5% ethanol in order to remove any associated microflora or other contaminating materials. Algal samples were air-dried in shade, reduced to fine powder, packed in tightly closed containers and stored for antimicrobial and physicochemical studies (Bazes et al., 2009).

Identification of the algae was verified according to Agardh (1820, 1848), Børgesen (1931), Børgesen and Fremy (1936), Nasr (1947), Taylor (1957, 1966), Mohsen (1972), Abbott and Hollenberg, 1976, Aleem (1978)and Lipkin and Silva (2002).

2.2. Antimicrobial screening of algae

Antibacterial activities of algae extracts were tested against pathogenic bacteria by agar-well diffusion method as described by Attaie et al. (1987). Representatives of Gram-positive bacteria; namely, *Bacillus subtilis* NCTC 1040, and *Staphylococcus aureus* NCTC 7447 and Gram negative bacteria; namely, *Salmonella typhi* ATCC 19430 , *Escherichia coli*, NCTC 10416 and *Pseudomonas aeruginosa* ATCC 10145a (kindly supplied from Biotechnological Research Center, AL-Azhar University for boys) were used as test organisms.

Briefly, 25 μL of each extract was loaded on sterile filter paper discs (12.7 mm in diameter) and air-dried. Indicator microorganisms were seeded in nutrient agar plates with sterile effusion and the discs were placed on plates. After incubation for 24 h at 37 °C, a clear zone formed around a disc was the evidence of antimicrobial activity. Diameters of the zones of inhibition were measured in millimeters. Each test was prepared in triplicate. Discs loaded with the extracting agents were tested as controls.

2.3. Preliminary phycochemical screening of L. microcladia

Phycochemical investigation was conducted as described by Gibbs (1974) for volatile, saponins, alkaloids, steroids, and/or triterpens substances, Rizk (1982) for carbohydrates and/or glycosides, Geissman (1962) for flavonoids, and Stahi and Schild (1981) for anthraquinone.

2.4. Isolation of the active compound

Defatted powder (200 g) of the alga was extracted in a Soxhlet apparatus with 95% ethanol. The ethanol extract was concentrated under reduced pressure (25 g), and diluted with water (300 ml), filtered over a piece of cotton then successively extracted with petroleum chloroform and methanol. Each extract was dried over anhydrous sodium sulfate for 48 h, and concentrated to yield 1and 2 g dry extracts, respectively.

2.5. Adsorbents and solvent systems

Aluminum sheets 20×20 Cm Silica gel G 60 F254 Merck KGaA 64271 Darmstadt, Germany, were used for thin layer chromatography (TLC). Silica gel of particle size 60 (70–230 mesh) was used for column chromatography. Whattman paper No. 3 for paper chromatography (PC). Solvent systems: (a) chloroform–methanol (80–20), (b) ethyl acetate:benzene (86:14), and (c) *n*-butanol: acetic acid: water (4:1:5) were used for developing the chromatoplates. Visualization of chromatograms was achieved under UV before and after exposure to ammonia vapor or by spraying with aluminum chloride, all solvents used were of analytical grade.

TLC examination of all extracts using systems (a) revealed the presence of clear spots. Accordingly the extract (1 g) was applied on column chromatography packed with silica gel G (410 g) and eluted gradually with systems (a). One hundred fractions of 20 ml each were collected and reduced to four sub-fractions and each fraction was concentrated under reduced pressure to yield 0.2, 0.1, 0.1, and 0.2 g, respectively. All fractions were subjected to re-isolation on silica gel columns from which only one compound was isolated.

2.6. Apparatus

As cited in Asthana et al. (2009), ^{1}H NMR spectra, using external electronic referencing through the deuterium resonance frequency of the solvent, were recorded on a JEOL

ECA 600 NMR spectrophotometer fitted with an auto 5 mm X/H probe. IR spectrum was recorded on JASCO FT/IR-5300 (Easton, MD) as film on a KBr-disc. Electronspray ionization mass spectra were recorded on Micromass Quattro 11 triple quadrupole mass spectrometer. The melting point of the active compound was determined using a Kofler hot-stage apparatus.

2.8. Minimum inhibitory concentration (MIC)

The minimum inhibitory concentration (MIC) was determined by the standard of agar dilution method described in the NCCLS guidelines (National Committee for Clinical Laboratory Standards, 1997). It was determined only for the chloroform method extract which recorded the highest antimicrobial activity. The lowest concentration of active principle that prevented microbial growth was considered to be the MIC. The test organisms were separately seeded in the agar medium. The wells (10 mm in diameter) were cut from the agar and 0.1 ml of extract solution (different concentrations) was transferred into them. After 24 h incubation period, the plates were examined and the inhibition zones were determined.

2.9. Toxicity test

2.9.1. Experimental animals

White male albino rats (*Rattus norvegicus*) weighing about 130–190 g were used as experimental animals in the present investigation. They were obtained from the animal house of Research Institute of Ophthalmology, El-Giza, Egypt. They were kept under observation for about 15 days before the onset of the experiment to exclude any intercurrent infection. The chosen animals were housed in metal (stainless steel) separated button cages at normal atmospheric temperature (25 ± 5 °C) as well as under good ventilation and received water and standard balanced diet.

2.9.2. Toxicological study (LD_{50})

LD_{50} of the isolated bioactive compound was determined as described by Finney (1964). For this purpose, 5 groups of 5 mature male albino rats (130–190 g body weight) each were used. The tested extract was administered orally in doses of 200–400 mg kg^{-1} body weight in addition to a group used as a control (given the solvent). Rats were kept under observation for 72 h during which the number of dead animals in each group was recorded.

2.9.3. Statistical analysis

Results from representative experiments are shown. They were expressed as mean ± standard deviation.

3. Results

In the present investigation, ethanolic extracts of 9 marine algal species were tested against 5 test organisms by agar diffusion method. The results of preliminary screening tests are summarized in Table 1, which revealed that, six algal species possess antibacterial activity. While the extracts of *Codium fragile*, *Cladophora vagabunda*, and *Padina boryana* did not show any activity during the experiment *L. microcladia* showed the largest clear zones against all tested Gram positive and Gram negative bacteria followed by *Ulva linza* and *Cystoseira rayssiae*. On the other hand, *Acanthophora nayadiformis* and *Osmundea hybrid*, were recorded moderate inhibitory activities, respectively. The extract of *Sargassum angustifoium* showed the lowest activities which appeared only against the two Gram positive bacteria. Regarding the susceptibility of the tested microorganisms, it was found that, the Gram positive bacteria were more sensitive to the algal extracts than the Gram negative (see Table 1).

As mentioned above, the red alga *L. microcladia* extract showed the highest antibacterial activity, and therefore the successive extractions of this extract using petroleum ether and chloroform in addition to the remained water extract were done. The experiment was conducted to detect the chemical analysis of the various components of the three extracts (Table 2), which proved that petroleum ether extract contained alkaloids, steroids, and/or triterpens while the chloroform extract contained only alkaloids. On the other hand, the aqueous extract did not contain any compound.

Table 1 Antimicrobial screening of ethanolic extracts from 9 marine macroalgae isolated from Dahab-Coast (Aqaba Gulf).

Algal extract	Diameter of inhibition zone (mm)				
	Staphylococcus aureus	*Bacillus subtilis*	*Salmonella typhi*	*Escherichia coli*	*Pseudomonas aeurginosa*
Discs loaded with ethanol as control	0.0	0.0	0.0	0.0	0.0
Chlorophyta					
Codium fragile	0.0	0.0	0.0	0.0	0.0
Cladophora vagabunda	0.0	0.0	0.0	0.0	0.0
Ulva linza	17 ± 0.1	19 ± 0.4	14 ± 1.0	7 ± 0.5	8 ± 1.0
Phaeophyta					
Padina boryana	0.0	0.0	0.0	0.0	0.0
Cystoseira rayssiae	12 ± 0.8	8 ± 1.0	10 ± 0.6	8 ± 0.3	5 ± 0.09
Sargassum angustifoium	8 ± 0.7	5 ± 0.8	0.0	0.0	0.0
Rhodophyta					
Acanthophora nayadiformis	10 ± 0.3	14 ± 0.2	12 ± 1.0	0.0	0.0
Laurencia microcladia	27 ± 2.0	32 ± 1.5	18 ± 2.3	19 ± 0.34	16 ± 0.55
Osmundea hybrida	10 ± 0.8	7 ± 0.5	10 ± 0.8	0.0	0.0

All values show mean of three replicates, ± standard deviation

Table 2 Preliminary phycochemical screening of the successive extracts prepared from *Laurencia microcladia*.

Chemical test	Extract		
	Petroleum ether	Chloroform	Water layer
Volatile substances	−	−	−
CHO and/or glycosides	−	−	−
Tannins	−	−	−
Flavonoids	−	−	−
Saponins	−	−	−
Alkaloids	+	+	−
Steroids and/or triterpens	+	−	−
Anthraquinone	−	−	−

+, Present; −, absent.

The successive ethanolic extract of *L. microcladia* was assayed for antibacterial properties (Table 3). The antibacterial activity depends on both algal species and efficiency of extraction of their active(s) principle(s). For example, petroleum ether extract was effective against all tested bacteria, while the chloroform extract was recorded to have inhibitory activity against *Bacillus subtilis* only. On the other hand, the remaining water extract had no activity.

3.1. Chemical analysis

Chemical analysis of the petroleum ether extract of the alga *Laurencia* confirmed the extract to contain triterpens. The antibacterial test confirmed high effectiveness of this extract to discourage the growth of bacteria. These results lead to complete separation, purification, and identification of active substances contained in the petroleum ether extract.

The isolated compound was identified according to the melting point (191–194 °C m.p.), infrared spectrum (IR), nuclear magnetic resonance (^{1}H NMR) of the proton and mass spectrometry (MS). The infrared spectrum recorded in the presence of potassium bromide was as follows: IR (KBr): $\sqrt{}$max 3292 and 1035 cm^{-1} (OH group) and 2945, 2850, 1460 and 1385 cm^{-1} (aliphatic methylene and methyl group). ^{1}H NMR methyl single at 0.98, 0.79, 0.92, 0.96, 1.12, 0.82, and 0.86 and olefinic proton resonating at δ 5.17 (1H, *t*, *J* = 4 Hz, H-12). MS: *m*/*z* (rel int %) 218 (100), 203, 207, and 189 (pentacyclic triterpene amyrin) as described by Krishnaswamy (1999). The molecular formula indicates that it is a pentacyclic triterpene with molecular formula $C_{30}H_{50}O$, and this is supported by the characteristic play of colors given by the compound on treatment with the Liebermann–Burchard reagent (acetic anhydride–sulfuric acid). This compound can be identified as β-amyrin and the chemical structure is given in Fig. 1.

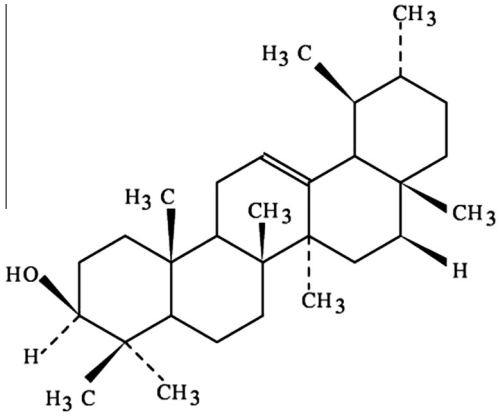

Figure 1 Molecular structure of β-amyrin.

The minimum inhibitory concentration (MIC) of the isolated compound was detected (Table 4). The data reported that the MIC against *Staphylococcus aureus* and *Salmonella typhi* was 2.5 mg mL^{-1}.

4. Discussion

The present data revealed a high antibacterial activity from the ethanolic extract of the red alga *L. microcladia* (Table 1). It is

Table 3 Antimicrobial activity of the successive extracts prepared from *Laurencia microcladia* against different bacterial targets.

Extract	Bacteria				
	Diameter of inhibition zone (mm)				
	Staphylococcus aureus	*Bacillus subtilis*	*Salmonella typhi*	*Escherichia coli*	*Pseudomonas aeurginosa*
Control					
Petroleum ether	0.0	0.0	0.0	0.0	0.0
Chloroform	0.0	0.0	0.0	0.0	0.0
Water	0.0	0.0	0.0	0.0	0.0
Petroleum ether extract	35 ± 2.0	37 ± 1.0	28 ± 1.3	30 ± 2.1	23 ± 3.0
Chloroform extract	0.0	8 ± 0 .4	0.0	0.0	0.0
Water layer extract	0.0	0.0	0.0	0.0	0.0

Table 4 Minimum inhibition concentration of the isolated β-amyrin.

Compound concentration mg mL^{-1}	Diameter of inhibition zone (mm)	
	Staphylococcus aureus	*Salmonella typhi*
10	26	20
5	20	16
2.5	14	11
0.125	−ve	−ve

−ve, no inhibition zone.

noteworthy to mention that this alga exhibited a broad spectrum of antibacterial activity (Abdel-Raouf et al., 2008). It was confirmed by the successive extracts by petroleum ether, chloroform, and water (Table 3) which revealed the affectivity of petroleum ether in the extracts of the bioactive materials. The preliminary phycochemical screening of the successive extracts prepared from *L. microcladia* (Table 2) indicated the presence of alkaloids, steroids, and/or triterpens. Additionally, the chemical analysis of the petroleum ether extract indicated that the bioactive ingredient was β-amyrin. So the broad spectrum of antibacterial activity recorded by *L. microcladia* and their beneficial effects could be attributed to the presence of β-amyrin which has inhibitor properties retarding the growth of these bacteria and antagonizing the infection mechanisms of these microbes. Our finding agreed with Kurata et al. (1998) and Hill (2007), who reported that, the bioactive compounds from genus *L. microcladia* were relatively high with diterpens, triterpenes, and C_{15}-acetogenins. It was reported that these compounds exhibited antimicrobial, antifeedant, antihelmintic, and cytotoxic properties (Sun et al., 2005 and Cabrita et al., 2010).

In this study, *Codium fragile*, *Cladophora vagabunda*, and *Padina boryana* did not show any activity during the experiment. The variation in the antibacterial activity between the studied algal species may be due to the method of extraction, solvent used and season at which samples were collected (Febles et al., 1995; Lima-Filho et al., 2002; Tuney et al., 2006).

In the present study, it was observed that Gram positive bacteria were more sensitive to the algal extracts than the Gram negative. In this respect, Ozdemir et al. (2006), Salvador et al. (2007), Abdel-Raouf and Ibraheem (2008) and Ibraheem et al. (2012) reported that Gram-positive bacteria are more effectively controlled by the studied algal extracts compared to Gram negative bacteria. Similar observations also were recorded by Taskin et al. (2001, 2007), indicating that more sensitivity of Gram positive bacteria towards the algal extract was due to the differences in their cell wall structure and their composition. Gram negative cell walls are more complex than Gram positive cell walls, both structurally and chemically. Structurally, a Gram negative cell wall contains two layers external to the cytoplasmic membrane. Immediately external to the cytoplasmic membrane is a thin peptidoglycan layer, which accounts for only 5% to 10% of the Gram negative cell wall by weight. There are no teichoic or lipoteichoic acids in the Gram negative cell wall. External to the peptidoglycan layer is the outer membrane, which is unique to Gram negative bacteria. This outer membrane acts as a barrier to many environmental substances including antibiotics (Tortora et al., 2001).

At determination of LD_{50} of the isolated compound, it was found that, this bioactive compound failed to kill rats within 72 h in doses up to 395 mg kg^{-1}. This means that, the studied extract is considered to be safe for human use (LD_{50} is very low). This agrees with the findings of Galvin and Mikhail (1976) who mentioned that the substances possessing safety test up to 2000 mg kg^{-1} are considered non toxic.

5. Conclusions

Finally it can be concluded that, the extracts of *L. microcladia* used in the present investigation showed better antibacterial activity against pathogens used. The potential source of bioactive compound β-amyrin should be investigated with further studies.

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