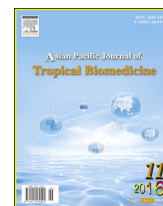




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journal homepage: www.elsevier.com/locate/apjtbOriginal article <http://dx.doi.org/10.1016/j.apjtb.2016.08.011>Secondary metabolites and their biological activities in Indonesian soft coral of the genus *Lobophytum*

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ABSTRACT

Objective: To investigate the antioxidant, antibacterial, antimalarial activities and cytotoxicity of the *n*-hexane, ethyl acetate, *n*-butanol, and aqueous fractions from a crude extract of *Lobophytum* sp.

Methods: This organism was collected from the Selayar Islands (South Sulawesi). The antioxidant activity was performed by the 1,1-diphenyl-2-picryl hydrazyl radical scavenging method. All fractions from the crude extract of *Lobophytum* sp. were examined for their cytotoxicity using the brine shrimp lethality bioassay and heme polymerization inhibitory activity assay for antimalarial activity.

Results: It was found that the ethyl acetate, *n*-butanol and aqueous fractions exhibited heme polymerization inhibitory activity with IC₅₀ values of 11.7, 14.3 and 12.0 µg/mL, respectively, while the *n*-butanol fraction showed moderate antioxidant activity and cytotoxicity with IC₅₀ values of 150.00 and 92.74 µg/mL, respectively.

Conclusions: This study provides information on antioxidant, antibacterial and antimalarial activities as well as the cytotoxicity of all fractions from the crude extract of *Lobophytum* sp. This is a new report of antimalarial substances derived from *Lobophytum* sp.

1. Introduction

Marine organisms are an important source of new bioactive molecules; thus the scientific community worldwide is focusing its efforts on the isolation and characterization of biologically active natural products [1]. Since the early days of marine natural products research in the 1960s, sponges and soft corals have famously yielded the largest number of new metabolites reported per year compared to any other plant or animal phylum known from the marine environment [2]. Octocorallia comprises approximately 3200 species of soft corals (Alcyonacea) found

in all marine environments and 94% of new compounds from cnidarians were discovered from soft corals or Alcyonacea [3]. Soft corals belonging to the genus *Lobophytum* (Alcyoniidae) have been shown to be a rich source of macrocyclic cembranoids and their cyclized derivatives are commonly described as defensive substances against predators such as other corals and fishes. Some of these metabolites are of considerable interest and merit continuous attention due to their unique structures and significant biological activities, including anti-tumor, anti-viral, and anti-inflammatory properties [4–10].

As part of our continuing research program aimed at the discovery of bioactive metabolites from marine organisms, we have recently been studying the chemical composition of marine invertebrates from the Indonesian coast which is considered to be one of the richest biodiversity hot spots in the ocean. In this context, we have started the analysis of a specimen of *Lobophytum* sp. collected from the Selayar Islands (South Sulawesi) and examined the antioxidant, antibacterial and antimalarial activity as well as cytotoxicity of fractions (*n*-hexane, ethyl acetate, *n*-butanol, and water) from the crude extract of *Lobophytum* sp.

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2. Materials and methods

2.1. Chemical and reagents

n-Hexane, ethyl acetate, *n*-butanol, methanol, dichloromethane, and 2,2-diphenyl-1-picrylhydrazyl (DPPH) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Butylated hydroxytoluene (BHT) and dimethyl sulfoxide were purchased from Merck (Kenilworth, NJ, USA). All chemicals used throughout experiment were analytical grade.

2.2. Animal material

Specimens of *Lobophytum* sp. (500 g wet weight) were collected in April 2015 from the Selayar Islands (South Sulawesi) at a depth of 3 m. Each sample of *Lobophytum* sp. was rinsed with sea water and immediately kept in ice. After being sent to the laboratory, immediate storage in the freezer was necessary to reduce any possible degradation. A voucher sample of *Lobophytum* sp. (SLYR SC-001) was deposited at the Research Center for Oceanography, Indonesian Institute of Sciences.

2.3. Extraction

Colonies of *Lobophytum* sp. were homogenized and repeatedly extracted with methanol: dichloromethane (1:1) at room temperature and the obtained combined material (10.88 g) was partitioned with *n*-hexane (non-polar) (3.4 g), ethyl acetate (semi-polar) (0.3 g), *n*-butanol (polar) (0.28 g) and water (6.9 g). Each fraction was subjected to preliminary phytochemical screening and tests for heme polymerization inhibitory activity, antioxidant, cytotoxicity, and antibacterial activity.

2.4. Phytochemical screening

All fractions were subjected to a preliminary phytochemical screening test for the presence of secondary metabolites utilizing the standard conventional protocol described by Senguntuvan *et al.* [11].

2.5. DPPH radical scavenging assay

DPPH radical-scavenging activity was determined as described by Li *et al.* [12] with some modifications. All fractions from the crude extract of *Lobophytum* sp. were prepared in different concentrations, ranging from 20 to 200 µg/mL for each sample and analyzed in triplicate. The methanol solution of the fraction of the tested sample (500 µL) was added to DPPH solution (1 mL) in 96-well plate and incubated in the dark for 30 min. Lower absorbance values were read at 517 nm using the microplate reader Infinite® 200 PRO (Tecan Austria GmbH, Grödig, Austria).

The reference standard compound being used was BHT and the experiment was done in triplicate. The IC₅₀ value of the sample, which is the concentration of a sample required to 50% inhibit of the DPPH free radical, was obtained by linear regression analysis of dose–response curve plotting between % inhibition and concentrations. The percentage of the DPPH scavenging effect was calculated using the following equation:

$$\text{DPPH scavenging effect (\%)} = (A_0 - A_1)/A_0 \times 100$$

where A₀ was the absorbance of the control reaction and A₁ was the absorbance in the presence of a test or standard sample.

2.6. Brine shrimp lethality bioassay

The cytotoxic activity of all fractions from the crude extract was evaluated using the brine shrimp lethality bioassay method with different concentrations (50, 100, 200 µg/mL) as described by Ullah *et al.* [13] with few modification. Each concentration was made in triplicate. The brine shrimp eggs were placed in 1 L of sea water, aerated for 48 h at 37 °C to hatch. After 48 h, 10 brine shrimp eggs were placed in a small container filled with sea water. The numbers of survivors were counted after 24 h. Larvae were considered dead if they did not exhibit any internal or external movement during several seconds of observation. The larvae did not receive food. To ensure that the mortality observed in the bioassay could be attributed to bioactive compounds, and not to starvation, we compared the dead larvae in each treatment to the dead larvae in the control.

2.7. Heme polymerization inhibitory activity assay

A heme polymerization inhibitory activity assay was conducted using a method developed by Basilico *et al.* [14] with some modification. A total of 100 mL solution of 1 mmol/L hematin in 0.2 mol/L NaOH was put into a 96-well microculture plate, and then a 50 mL assay solution with various concentrations, ranging from 0.3125 to 20 mg/mL, was added into each well. Glacial acetate acid (50 mL, pH 2.6) was added to the microculture to initiate a heme polymerization reaction. The microculture was then incubated at 37 °C for 24 h to obtain perfect polymerization. After the period of incubation, the microculture was centrifuged and the resulting deposits were washed three times using 200 mL of dimethyl sulfoxide. The solution of 0.1 mol/L NaOH (200 mL) was subsequently added to the deposits in each well of microculture. Absorbance values were read at 405 nm using a microplate reader, Infinite® 200 PRO (Tecan Austria GmbH, Grödig, Austria). The value of heme polymerization inhibitory activity was expressed in IC₅₀. Aquadest and chloroquine diphosphate were used for negative and positive control, respectively. The percentage inhibition of heme polymerization was calculated by the formula:

$$\text{Inhibition (\%)} = (\beta\text{-hematin}_0 - \beta\text{-hematin}_1)/\beta\text{-hematin}_0 \times 100$$

where β-hematin₀ was the concentration of negative control and β-hematin₁ was the concentration of fraction test.

2.8. Antibacterial activity

An antibacterial test was performed using minor modification of the agar diffusion method described by Touati *et al.* [15]. Briefly, the sample was prepared with a concentration of 100 µg/mL in MeOH. A 20 µL sample was dropped on a filter paper disc with 6 mm diameter. The paper disc was then placed on a Mueller–Hinton agar (Himedia, Mumbai, India) in a Petri dish that had been inoculated by test bacteria. The test bacteria used were *Escherichia coli* ATCC 25922 (*E. coli*), *Bacillus subtilis* ATCC 6633 (*B. subtilis*), wild-type *Vibrio eltor* (*V. eltor*), and *Staphylococcus aureus* ATCC 25923 (*S. aureus*). Inhibition of bacterial growth activity appeared as a

clear zone around the paper disc. The inhibition zone was observed after incubation at 30 °C for 20–24 h and then measured using a caliper. Each sample was tested in triplicate.

2.9. Statistical analysis

All experimental measurements were carried out in triplicate and are expressed as mean ± SD (n = 3). Results of the research were tested for statistical significance by One-way ANOVA. Differences were considered statistically significant at the P < 0.05 level. The statistical software package SPSS v.16 (SPSS Inc., Chicago, IL, USA) was used for the analysis.

3. Results

The phytochemical analysis of all fractions from the crude extract of *Lobophytum* sp. is presented in Table 1. Fractions extracted by using different solvents such as *n*-hexane, ethyl acetate, *n*-butanol and water of *Lobophytum* sp. were evaluated to detect secondary metabolites. The chemical analysis of all fractions indicated the presence of alkaloids, steroids, triterpenoids, flavonoids, saponins, terpenoids, and phenols. The identification of these chemical constituents showed the medicinal importance of *Lobophytum* sp. Terpenoids and steroids were found in the *n*-hexane, ethyl acetate and *n*-butanol fractions.

Table 1

Phytochemical analysis of all fractions from the crude extract of *Lobophytum* sp.

Chemical constituents	Fractions			
	<i>n</i> -Hexane	<i>n</i> -Butanol	Ethyl acetate	Aqueous
Alkaloids	+	–	+	+
Steroids	+	+	+	–
Flavonoids	+	+	+	–
Saponins	–	–	+	–
Terpenoids	+	+	+	–
Phenols	+	+	+	–
Tannins	–	–	–	–

+: Present; –: Absent.

3.1. DPPH radical scavenging assay

DPPH is a stable free radical and can accept an electron or hydrogen radical to become a stable diamagnetic molecule. In the present study, we provided information on the reactivity of different fractions with a stable free radical. The results of the free radical scavenging potential of all fractions tested by the DPPH method are presented in Figure 1. The *n*-butanol fraction

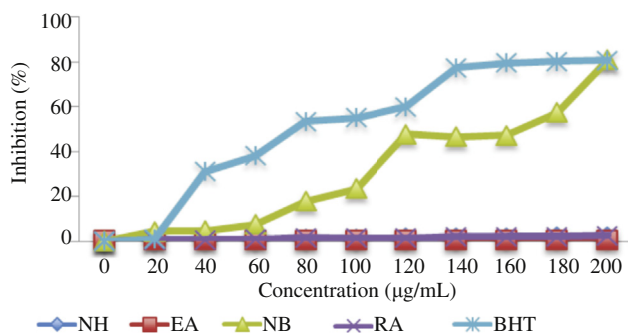


Figure 1. Radical scavenging potential of fractions by DPPH method at different concentrations.

NH: *n*-Hexane; EA: Ethyl acetate; NB: *n*-Butanol; RA: Aqueous.

showed the highest DPPH radical scavenging activity (IC₅₀ = 150.00 µg/mL) compared to other fractions. This assay provides information on the reactivity of different fractions with a stable free radical.

3.2. Brine shrimp lethality bioassay

The brine shrimp lethality bioassay was used to predict the cytotoxic activity of the *n*-hexane, ethyl acetate, *n*-butanol and aqueous fractions from the crude extracts of *Lobophytum* sp. Plotting the log of concentration (log C) vs. the percent mortality (probits) for all test samples showed an approximate linear correlation (Figure 2). From the graphs, the median lethal concentration (LC₅₀), the concentrations at which 50% mortality of brine shrimp nauplii occurred, were determined. The LC₅₀ values of the *n*-hexane, ethyl acetate and *n*-butanol from the crude extract of *Lobophytum* sp. (Tables 2 and 3) were found to be 123.07, 109.41 and 92.74 µg/mL, respectively. The degree of lethality was directly proportional to the concentration of the extract. Maximum mortalities (93.33%) were observed at a concentration of 200 µg/mL in both *n*-hexane and *n*-butanol (Table 2).

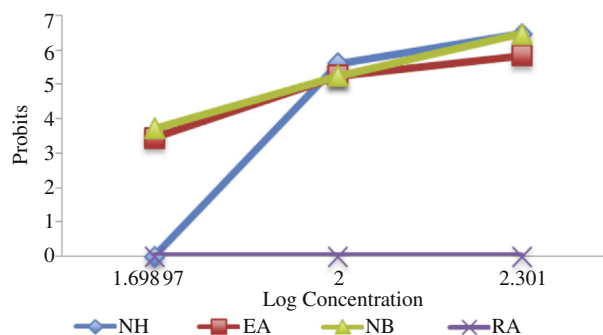


Figure 2. Plot of log concentration of four fractions vs. percent of shrimp mortality after 24 h of exposure (probits).

NH: *n*-Hexane; EA: Ethyl acetate; NB: *n*-Butanol; RA: Aqueous.

Table 2

Effect of *n*-hexane, ethyl acetate, *n*-butanol and aqueous fraction of the crude extract of *Lobophytum* sp. on brine shrimp.

Fraction	Concentration (µg/mL)	Log C	Mortality (%)	Probits	LC ₅₀ (µg/mL)
<i>n</i> -Hexane	50	1.699	0	0	123.07
	100	2.000	73.33	5.61	
	200	2.301	93.33	6.48	
Ethyl acetate	50	1.699	6.67	3.45	109.41
	100	2.000	60.00	5.25	
	200	2.301	80.00	5.84	
<i>n</i> -Butanol	50	1.699	10.00	3.72	92.74
	100	2.000	60.00	5.25	
	200	2.301	93.33	6.48	
Aqueous	50	1.699	0	0	0
	100	2.000	0	0	
	200	2.301	0	0	

3.3. Heme polymerization inhibitory activity assay

The ethyl acetate, *n*-butanol and aqueous fractions exhibited heme polymerization inhibitory activity with IC₅₀ values of 11.7, 14.3 and 12.0 µg/mL, respectively. When compared with

Table 3

The cytotoxic activity of *n*-hexane, ethyl acetate, *n*-butanol and aqueous fraction on brine shrimp.

Fraction	LC ₅₀ (µg/mL)	Regression equation	R ²
<i>n</i> -Hexane	123.07	$y = 10.76x - 17.49$	0.848
Ethyl acetate	109.41	$y = 3.969x - 3.093$	0.921
<i>n</i> -Butanol	92.74	$y = 4.584x - 4.018$	0.996
Aqueous	0	0	0

the IC₅₀ value of the positive control (chloroquine diphosphate), the ethyl acetate had a lower value which showed that the activity of ethyl acetate on heme polymerization inhibition was greater than that of chloroquine diphosphate (Figure 3).

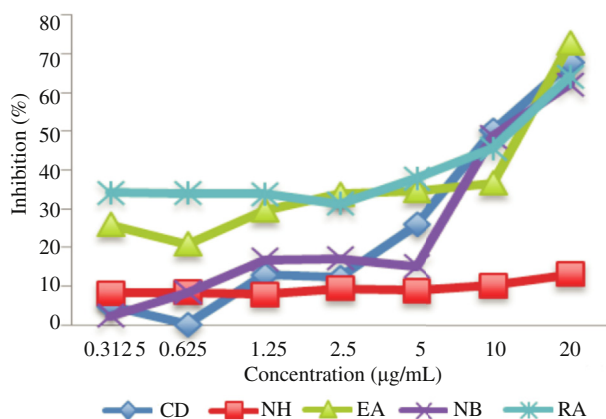


Figure 3. Heme polymerization inhibitory activity assay of fraction from the crude extract of *Lobophytum* sp.

CD: Chloroquine diphosphate; NH: *n*-Hexane; EA: Ethyl acetate; NB: *n*-Butanol; RA: Aqueous.

3.4. Antibacterial assay

All fractions of *Lobophytum* sp. had antibacterial activity against test bacteria from Gram-positive (*B. subtilis* and *S. aureus*) and Gram-negative (*E. coli* and *V. eltor*) (Figure 4). The averages of inhibition activity of all fractions were more than 10 mm but less than 15 mm. Based on category from Paudel *et al.* [16], all fractions of *Lobophytum* sp. had moderate antibacterial activity against all tested bacteria.

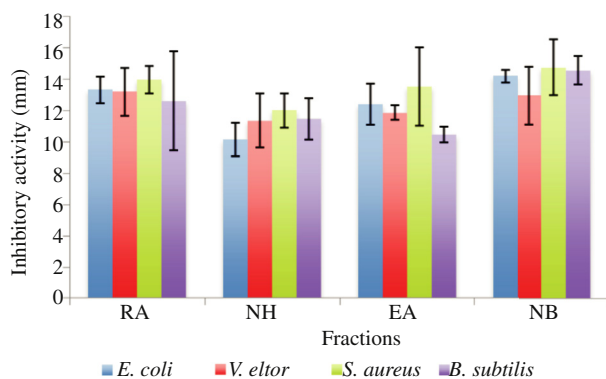


Figure 4. Antibacterial assay of fraction from the crude extract of *Lobophytum* sp.

RA: Aqueous; NH: *n*-Hexane; EA: Ethyl acetate; NB: *n*-Butanol.

4. Discussion

The chemical analysis of all fractions indicated the presence of alkaloids, steroids, triterpenoids, flavonoids, saponins, terpenoids and phenols. Several reports are available on terpenoids from the soft coral genus *Lobophytum*, which exhibited a high potential for biological activities such as anti-inflammatory, antimicrobial and antiviral activity. Most of the isolated terpenoids were diterpenoids or cembranoid compounds [17], which were found in high concentrations (up to 5% dry weight) in soft corals and possibly had a chemical defense role against predators such as fishes as well as microorganisms and other corals [18,19]. This study also provides information on antioxidant and antiplasmodium activities as well as the cytotoxicity of all fractions from the crude extract of *Lobophytum* sp.

The DPPH radical scavenging method is the most popular and widely used method for screening the free radical scavenging ability of compounds. This assay is sensitive and easy to perform and offers a rapid way to screen radical scavenging activity compared to other methods. DPPH is a stable radical, with a strong absorption maximum at 517 nm (purple color) in the UV spectrum [20]. The hydrogen atom or electron donation abilities of the corresponding extracts/fractions were measured from the bleaching of the purple-colored methanol solution of DPPH [21]. Among all fractions, the *n*-butanol fraction showed moderate antioxidant activity with IC₅₀ 150.00 µg/mL with radical scavenging activity. From the LC₅₀ values of brine shrimp lethality assay, it can be concluded that the *n*-butanol fraction has more potent cytotoxic compounds than the other fractions, with LC₅₀ value 92.74 µg/mL. Moreover, the crude extract or fractions resulting in LC₅₀ values less than 100 µg/mL were considered significantly active and indicated the presence of potent bioactive compounds for further investigation. Several studies have shown that brine shrimp assay has been an excellent method for preliminary investigations of toxicity, which could also have positive correlation with antitumor, trypanocidal and pesticidal activities.

Heme polymerization is a mechanism in releasing Iron II Ferriprotoporphyrin IX (FPIX) when *Plasmodium falciparum* degrades hemoglobin as a source essential of nutrients, which is free FPIX of a toxic substance. Free FPIX is oxidized to Iron III (FPIX), then polymerized into inert crystal of hemozoin, a non toxic malarial pigment. β-Hematin is a polymer identical to hemozoin, which is chemically indistinguishable from hemozoin, at an acid pH reflecting the conditions of the lysosomal food vacuole [22]. Therefore, the heme polymerization inhibitory activity of a compound is directly related to its potential as an antimalarial [14]. Among all fractions, the ethyl acetate and aqueous fractions were the most active fractions in inhibiting heme polymerization with IC₅₀ values of 11.7 and 12.0 µg/mL. According to Baelmans *et al.* [23], a compound could be considered to have heme polymerization inhibitory activity if it has heme polymerization inhibitory IC₅₀ values smaller than the limit of chloroquine diphosphate (37.5 mmol/L or 12.0 mg/mL). Thus, the ethyl acetate and aqueous fractions displayed heme polymerization inhibitory activity. This is a new report of antimalarial activity substances derived from *Lobophytum* sp. All fractions of *Lobophytum* sp. showed moderate antibacterial activity against *E. coli*, *V. eltor*, *B. subtilis*, and *S. aureus*.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgments

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References

- [1] Almeida MTR, Moritz MIG, Capel KCC, Pérez CD, Schenkel EP. Chemical and biological aspects of octocorals from the Brazilian coast. *Rev Bras Farmacogn* 2014; **24**: 446-67.
- [2] Ebada SS, Proksch P. The chemistry of marine sponges. In: Fattorusso E, Gerwick WH, Tagliatalata-Scafati O, editors. *Handbook of marine natural products*. Dordrecht: Springer Netherlands; 2012, p. 191-293.
- [3] Rocha J, Calado R, Leal M. Marine bioactive compounds from cnidarians. In: Kim SE, editor. *Springer handbook of marine biotechnology*. Heidelberg: Springer-Verlag Berlin Heidelberg; 2015, p. 823-49.
- [4] Wanzola M, Furuta T, Kohno Y, Fukumitsu S, Yasukochi S, Watari K, et al. Four new cembrane diterpenes isolated from an Okinawan soft coral *Lobophytum crassum* with inhibitory effects on nitric oxide production. *Chem Pharm Bull (Tokyo)* 2010; **58**: 1203-9.
- [5] Quang TH, Ha TT, Minh CV, Kiem PV, Huong HT, Ngan NTT, et al. Cytotoxic and anti-inflammatory cembranoids from the Vietnamese soft coral *Lobophytum laevigatum*. *Bioorg Med Chem* 2011; **19**: 2625-32.
- [6] Hong JY, Boo HJ, Kang JI, Kim MK, Yoo ES, Hyun JW, et al. (1S,2S,3E,7E,11E)-3,7,11,15-Cembratetraen-17,2-olide, a cembranoid diterpene from soft coral *Lobophytum* sp., inhibits growth and induces apoptosis in human colon cancer cells through reactive oxygen species generation. *Biol Pharm Bull* 2012; **35**: 1054-63.
- [7] Cheng SY, Chen PW, Chen HP, Wang SK, Duh CY. New cembranoides from the Dongsha Atoll soft coral *Lobophytum durum*. *Mar Drugs* 2011; **9**: 1307-18.
- [8] Hegazy MEF, Su JH, Sung PJ, Sheu JH. Cembranoids with 3,14-ether linkage and a secocembrane with bistetrahydrofuran from the Dongsha Atoll soft coral *Lobophytum* sp. *Mar Drugs* 2011; **9**: 1243-53.
- [9] Wang SK, Duh CY. New cytotoxic cembranoides from the soft coral *Lobophytum michaelae*. *Mar Drugs* 2012; **10**: 306-18.
- [10] Zhao M, Yin J, Jiang W, Ma M, Lei X, Xiang Z, et al. Cytotoxic and antibacterial cembranoids from a South China Sea soft coral, *Lobophytum* sp. *Mar Drugs* 2013; **11**: 1162-72.
- [11] Sengunttuvan J, Paulsamy S, Karthika K. Phytochemical analysis and evaluation of leaf and root parts of the medicinal herb, *Hypochoeris radicata* L. for *in vitro* antioxidant activities. *Asian Pac J Trop Biomed* 2014; **4**(Suppl 1): S359-67.
- [12] Li X, Wu X, Li Huang. Correlation between antioxidant activities and phenolic contents of radix *Angelica sinensis* (Danggui). *Molecules* 2009; **14**: 5349-61.
- [13] Ullah MO, Haque M, Urmi KF, Zulfiker AHM, Anita ES, Begum M, et al. Anti-bacterial activity and brine shrimp lethality bioassay of methanolic extracts of fourteen different edible vegetables from Bangladesh. *Asian Pac J Trop Biomed* 2013; **3**(1): 1-7.
- [14] Basílico N, Pagani E, Monti D, Olliaro P, Taramelli D. A microtitre-based method for measuring the haem polymerization inhibitory activity (HPIA) of antimalarial drugs. *J Antimicrob Chemother* 1998; **42**(1): 55-60.
- [15] Touati I, Chaieb K, Bakhrouf A, Gaddour K. Screening of antimicrobial activity of marine sponge extracts collected from Tunisian coast. *J Med Mycol* 2007; **17**: 183-7.
- [16] Paudel B, Bhattarai HD, Kim IC, Lee H, Sofronov R, Ivanova L, et al. Estimation of antioxidant, antimicrobial activity and brine shrimp toxicity of plants collected from Oymyakon region of the Republic of Sakha (Yakutia), Russia. *Biol Res* 2014; **47**: 10.
- [17] Blunt JW, Copp BR, Keyzers RA, Munro MH, Prinsep MR. Marine natural products. *J Nat Prod Rep* 2012; **29**: 144-222.
- [18] Gross H, Kehraus S, Nett M, König GM, Beil W, Wright AD. New cytotoxic cembrane based diterpenes from the soft corals *Sarcophyton cherbonnieri* and *Nephtea* sp. *Org Biomol Chem* 2003; **1**: 944-9.
- [19] Coll JC. The chemistry and chemical ecology of octocorals (Ctenophora, Anthozoa, Octocorallia). *Chem Rev* 1992; **92**: 613-31.
- [20] Phang CW, Malek SNA, Ibrahim H, Wahab NA. Antioxidant properties of crude and fractionated extracts of *Alpinia mutica* rhizomes and their total phenolic content. *Afr J Pharm Pharmacol* 2011; **5**(7): 842-52.
- [21] Rauf A, Uddin G, Khan H, Siddiqui BS, Arfan M, Yousuf M, et al. Analgesic and antioxidant activity of crude extracts and isolated fractions of aerial parts of *Hedera helix* L. *JSM Chem* 2014; **2**(2): 1012.
- [22] Hawley SR, Bray PG, Mungthin M, Atkinson JD, O'Neill PM, Ward SA. Relationship between antimalarial drug activity, accumulation, and inhibition of heme polymerization in *Plasmodium falciparum* *in vitro*. *Antimicrob Agents Chemother* 1998; **42**(3): 682-6.
- [23] Baelmans R, Deharo E, Muñoz V, Sauvain M, Ginsburg H. Experimental conditions for testing the inhibitory activity of chloroquine on the formation of beta-hematin. *Exp Parasitol* 2000; **96**(4): 243-8.