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Phenolic content, anti-oxidant, anti-plasmodium and cytotoxic properties of the sponge *Acanthella cavernosa*Masteria Yunovilsa Putra^{1*}, Tutik Murniasih¹, Joko Tri Wibowo¹, Tri Aryono Hadi¹, Febriana Untari¹, Amalia Choirun Nisa², Respati Tri Swasono²¹Research Center for Oceanography, Indonesian Institute of Sciences, Jl. Pasir Putih I, Ancol Timur, Jakarta 14430, Indonesia²Department of Chemistry, Faculty of Mathematics and Natural Sciences, Universitas Gadjah Mada, Sekip Utara, Yogyakarta 55281, Indonesia

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ABSTRACT

Objective: To investigate the total phenolic content, anti-oxidant capacity and cytotoxic activity present in the *n*-hexane, ethyl acetate, *n*-butanol and aqueous fractions of an extract collected at Selayar Island, Indonesia.**Methods:** The antioxidant activity was performed by the 1,1-diphenyl-2-picrylhydrazyl radical scavenging method and β -carotene bleaching assay. All fractions from the crude extract of *Acanthella cavernosa* (*A. cavernosa*) were examined for their cytotoxicity using brine shrimp lethality bioassay and heme polymerization inhibitory activity assay for antimalarial activity.**Results:** The highest phenolic content was found in the *n*-butanol fraction, followed by the ethyl acetate, aqueous and *n*-hexane fractions. The highest antioxidant activity, as determined by the β -carotene bleaching assay, was observed in the *n*-hexane fraction. On the other hand, the *n*-hexane fraction was most effective in suppressing 1,1-diphenyl-2-picrylhydrazyl radicals and neutralizing 50% of free radicals at the concentration of 171.86 μ g/mL. Various fractions of the *A. cavernosa* extract showed the ability to inhibit heme polymerization indicating an anti-Plasmodium function. In this regard, the ethyl acetate fraction achieved an IC₅₀ value of 3.3 μ g/mL. The aqueous fraction showed moderate cytotoxic activity against the brine shrimp *Artemia* sp.**Conclusions:** This study provided information on antioxidant, total phenolic content and antimalarial activities as well as the cytotoxicity of all fractions from the crude extract of *A. cavernosa*. The natural anti-Plasmodium compounds are of particular interest. Further studies are needed for a more extensive screening and characterization of the bioactive components in this sponge.

1. Introduction

Many investigations have shown that sponges of the genus *Acanthella* yield a large number of highly diversified natural products such as diterpenes and sesquiterpenes that contain various nitrogenous functional groups, including isonitrile-, isothiocyanate-, isocyanate- and formamide groups. Diterpenes and sesquiterpenes isolated from this genus have also been reported to exhibit cytotoxic, anthelmintic, anti-malarial, anti-microbial, anti-fungal and anti-fouling activities[1-3].

As a part of our research on bioactive secondary metabolites from Indonesian marine organisms, we studied the chemical composition and biological characteristics of the marine sponge *Acanthella cavernosa* (*A. cavernosa*) collected from Selayar Island. We examined various phytochemical properties, including the total phenolic content (TPC), anti-oxidant capacity, anti-plasmodium activity as well as cytotoxicity of different fractions (*n*-hexane, ethyl acetate, *n*-butanol and water) prepared from a crude extract of *A. cavernosa*.

2. Materials and methods

2.1. Chemicals and reagents

Ethyl acetate, *n*-hexane, *n*-butanol, methanol, chloroform, 2,2-diphenyl-1-picrylhydrazyl (DPPH) and β -carotene were purchased from Sigma Chemical Company (St. Louis, USA). Butylated hydroxytoluene (BHT), linoleic acid, gallic acid, ferrous chloride, sodium carbonate, Tween-40, dimethyl sulfoxide (DMSO) and

*Corresponding author: Masteria Yunovilsa Putra, Research Center for Oceanography, Indonesian Institute of Sciences, Jl. Pasir Putih I, Ancol Timur, Jakarta 14430, Indonesia.

Tel: +62 2164713850

E-mail: mast001@lipi.go.id

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ethylene diamine tetraacetic acid were purchased from Merck (Darmstadt, Germany). All chemicals used were of analytical grade.

2.2. Sponges

Sponges, identified as *A. cavernosa*, were collected by scuba diving in Selayar Island, South Sulawesi, Indonesia, at a depth of 10 m in June 2015. A voucher specimen was deposited in the Research Center for Oceanography, Indonesian Institute of Sciences, under the registration number SLYR SP-5.

2.3. Extraction in organic solvents

The sponge *A. cavernosa* (500 g wet weight) was homogenized and extracted with MeOH: CHCl₃ (3:1) at room temperature to obtain a crude extract. The extract was partitioned against various organic solvents to give fractions of *n*-hexane (non-polar), ethyl acetate (semi polar), *n*-butanol (polar) and water. The fractions were coded *n*-hexane (1.47 g), ethyl acetate (0.48 g), *n*-butanol (1.53 g) and water (1.46 g), respectively. Each fraction was subjected to a preliminary phytochemical screening to estimate its total phenol content, anti-oxidant capacity, anti-plasmodium activity, as well as cytotoxicity.

2.4. Phytochemical screening

All fractions were subjected to a preliminary phytochemical screening for the presence of selected secondary metabolites, following a standardized conventional protocol described by Abioye *et al.*[4].

2.5. TPC assay

TPC was measured using the Folin-Ciocalteu assay with slight modifications as described by Carciocchi *et al.*[5]. The various test samples, *viz.*, the calibration standards (10 µL of gallic acid), the fractions (1 mg/mL in DMSO) and quercetin (1 mg/mL in DMSO) as the positive control were added to Folin-Ciocalteu reagent (500 µL). After 5 min, 300 µL of Na₂CO₃ solution (115 g/L) was added to each reaction mixture and thoroughly mixed. The mixtures were incubated at room temperature for 2 h before measuring the absorbance at 765 nm using the Infinite® 200 PRO microplate reader (Tecan Austria GmbH, Grödig, Austria). TPCs in the various fractions of *A. cavernosa* extract were expressed in mg gallic acid equivalent (GAE)/g of extract.

2.6. DPPH radical scavenging activity assay

DPPH radical scavenging activity in fractions of the crude *A. cavernosa* extract was determined using the method described by Krishnanunni *et al.*[6] with slight modifications. The fractions of the sponge extract (500 µL) was mixed with 7 mL of methanol and then added to DPPH solution (1 mL) in 96-well plates. The mixture was kept at room and incubated in the dark for 30 min, and then the absorbance values were read at 517 nm using the Infinite® 200

PRO microplate reader (Tecan Austria GmbH). Methanol was used as the blank. DPPH solution without addition of the extract was served as the control, and BHT was used as the calibration standard. The percentage of DPPH scavenging effect was calculated using the following equation:

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

Where A_0 is the absorbance of the control, and A_1 is the absorbance of the extract/standard. The IC₅₀ value of the sample, the concentration of the sample required for 50% inhibition of the DPPH free radical, was obtained from the dose-response curve plotted as a linear regression between percent inhibition and concentrations.

2.7. Beta-carotene bleaching (BCB) assay

The BCB assay was performed using the method of Olugbami *et al.*[7] with some modifications. A stock solution of β-carotene/linoleic acid was initially prepared by dissolving 5 mg of β-carotene in 50 mL of chloroform. And 0.2 mL of the carotene-chloroform solution was pipetted into boiling linoleic acid (20 mg) and Tween-40 (200 mg). Chloroform was evaporated at reduced pressure for 5 min, and distilled water (50 mL) was added to the residue slowly with vigorous agitation to form an emulsion. An aliquot of 250 µL β-carotene/linoleic acid emulsion was pipetted into the 96-well plates containing 0.2 mL of the test solutions. An equal amount of methanol was used in the control. The plates were incubated at 50 °C, and the absorbance was then measured at 470 nm after 60 min using the Infinite® 200 PRO microplate reader (Tecan Austria GmbH). BHT was used as antioxidant reference compound. The anti-oxidative activity was expressed as percent inhibition with reference to the control after 60 min of incubation and calculated as follow:

$$\text{Degradation rate (DR) of } \beta\text{-carotene} = [\ln(a/b)/60]$$

$$\text{Antioxidant activity (\%)} = [(\text{DR control} - \text{DR sample})/\text{DR control}] \times 100\%$$

Where a is absorbance at time 0, and b is absorbance at 60 min.

2.8. Heme polymerization inhibitory activity assay

The heme polymerization inhibitory activity assay was conducted using the method developed by Basilico *et al.*[8]. A 100 mL solution of 1 mmol/L hematin in 0.2 mol/L NaOH was pipetted into a 96-well micro-culture plate, and 50 mL of the test sample of concentrations, ranging from 1.25 to 10.00 µg/mL were added to each well. A total of 50 mL of glacial acetate acid at pH 2.6 was added to the wells to initiate a heme polymerization reaction. The micro-culture plate was then incubated for 24 h at 37 °C to complete the polymerization process. Following this, the micro-culture plate was centrifuged, and the resulting deposits were washed 3 times with 200 mL of DMSO. NaOH (200 mL, 0.1 mol/L) was subsequently added to each well of the micro-culture plate. Absorbance values were read at 405 nm using the Infinite® 200 PRO micro-plate reader (Tecan Austria GmbH). Heme polymerization inhibitory activity was expressed as IC₅₀. Aquadest and chloroquine were used as negative and positive controls, respectively. The percent inhibition of heme polymerization was calculated by the following formula:

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

Where β-hematin₀ is the concentration of the negative control and

β -hematin₁ is the concentration of the test sample.

2.9. Brine shrimp lethality assay

The cytotoxic properties of all fractions of the crude extract were evaluated at concentrations of 10, 100 and 1000 $\mu\text{g/mL}$ using the brine shrimp lethality bioassay method expressed by Ahmed *et al.*[9] with slight modifications. Brine shrimp eggs were placed in 1 L of aerated sea water for 48 h at 37 °C to hatch. After 48 h, ten brine shrimp larvae were placed in a small container filled with sea water and different concentration of fractions. The number of survivors was counted after 24 h. Larvae were considered dead if they did not show any movement over several seconds of observation. The median LC₅₀, the concentrations at which 50% mortality of brine shrimp larvae occurred, were determined. We compared the dead larvae in each treatment with the dead larvae in the control to ensure that the mortality observed in the bioassay could be attributed to bioactive compounds and not to starvation.

2.10. Statistical analysis

All analyses were performed in triplicate and expressed as mean \pm SD. Results of the research were calculated using One-way ANOVA. Differences were considered statistically significant when $P < 0.05$. The software SPSS version 16.0 (SPSS Inc., Chicago, USA) was used for the analysis.

3. Results

The secondary metabolites presented in all the fractions of the *A. cavernosa* extract were presented in Table 1. Phytochemical screening of the fractions expressed the presence of phenols, terpenoids, alkaloids, flavonoids and saponins. Steroids were absent from all the fractions examined. The *n*-butanol and aqueous fractions showed the presence of alkaloids, while saponins were found in the ethyl acetate fraction. Terpenoids were present in all the three organic solvent fractions. Numerous ecological studies have shown that secondary metabolites produced by sponges often serve defensive purposes to protect them from predator attack, microbial infection, biofouling and overgrowth by other sessile organisms[10,11].

Table 1

Chemical constituents of *A. cavernosa* fractions.

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

3.1. TPCs

The TPC in various fractions of the *A. cavernosa* extract were estimated using the Folin-Ciocalteu reaction and the results were

expressed as GAE determined from the standard curve (Figure 1). TPCs varied widely among different fractions of the *A. cavernosa* extract. The highest amount was found in *n*-butanol fraction [(4.500 \pm 0.007) mg of GAE/g of fraction], followed by ethyl acetate [(3.800 \pm 0.041) mg of GAE/g of fraction], aqueous [(3.400 \pm 0.010) mg of GAE/g of fraction] and *n*-hexane [(2.800 \pm 0.080) mg of GAE/g of fraction] fractions in the decreasing order. Higher solubility of phenolics in polar solvents could have accounted for their higher presence in the *n*-butanol fraction, whereas the aqueous fraction contained the least phenolics.

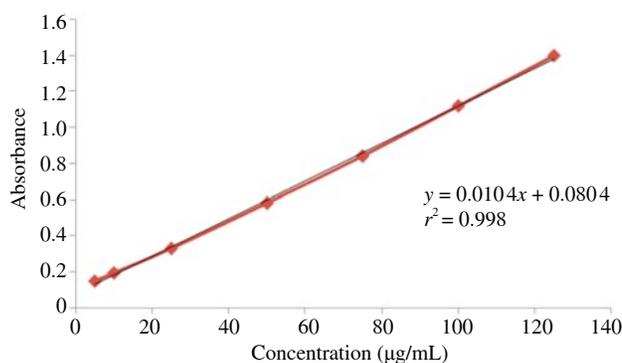


Figure 1. Gallic acid standard curve.

3.2. Anti-oxidant capacity

3.2.1. DPPH radical scavenging activity

The free radical scavenging activities in fractions of the *A. cavernosa* extract were tested using the DPPH method with the results expressed as percent inhibition and IC₅₀ values ($\mu\text{g/mL}$) (Figure 2). DPPH was a very stable free radical with a strong absorption maximum at 517 nm (purple color). In brief, antioxidants reacted with the stable free radical *i.e.* 2,2-diphenyl- β -picrylhydrazyl to convert it to 2,2-diphenyl- β -picrylhydrazine, with a loss of coloration that measured the antioxidant and free-radical scavenging potential of the test sample[12]. Antioxidant activities of the *A. cavernosa* fractions ranged from 16.40% to 40.57% inhibition of the free radical. By extrapolation of the results shown in Figure 2, the largest capacity for neutralizing DPPH radicals was found in the *n*-hexane fraction that neutralized 50% of free radicals at a concentration of 171.86 $\mu\text{g/mL}$.

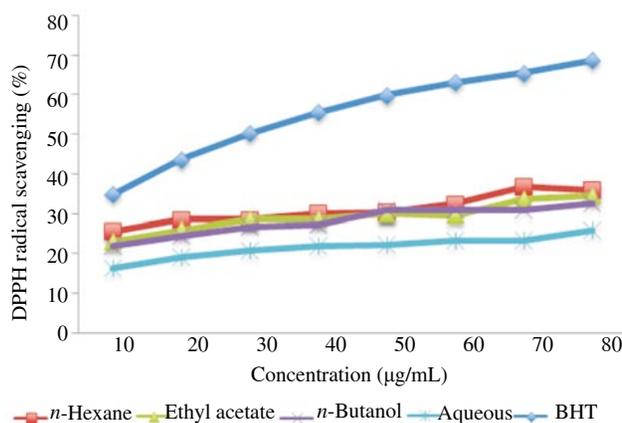


Figure 2. Scavenging effect of fractions of *A. cavernosa* extract on DPPH radicals. Values were expressed as mean \pm SD ($n = 3$). BHT was used as antioxidant reference compound.

3.2.2. BCB assay

The BCB assay is one of the most frequently applied methods for the determination of antioxidant activity. In this assay, linoleic acid produced hydroperoxides as free radicals during incubation at 50 °C which attacked the β -carotene molecules, causing a reduction in absorbance at 470 nm. Among the different fractions compared, the highest antioxidant activity was observed in the *n*-hexane fraction. Comparing the results in DPPH and BCB assays, both showed that the *n*-hexane fraction had the highest free-radical scavenging activity. There was no disagreement between the two methods of determining anti-oxidant activity. In fact, the activity in the other fractions (ethyl acetate, *n*-hexane and aqueous fractions) were also ranked in the same order for both methods of analysis. In other words, the DPPH test agreed with the BCB assay. The result showed that there was considerable variation in antioxidant activities in different fractions ranging from 14.3%, 24.6%, 16.3% and 42.4% for aqueous, *n*-butanol, ethyl acetate and *n*-hexane fraction, respectively. The results demonstrated that antioxidant activities demonstrated by the BCB and DPPH assays were greater in a non-polar solvent (*n*-hexane) whereas the TPCs were better represented in a polar solvent (*n*-butanol). This result indicated the total antioxidant activity was not only due to the phenolic content, but a greater part of it was, in fact, attributed to other antioxidant components in the sponge extract.

3.3. Cytotoxic properties

The brine shrimp lethality assay was a rapid and inexpensive bioassay to test for cytotoxic properties of biological extracts which, in many cases, were correlated reasonably well with cytotoxic and anti-tumor activity[9]. Various species of marine invertebrates have been tested against the brine shrimp *Artemia* sp., and it was observed that some of these showed high toxicity. 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 sponge extract. The degree of lethality was directly proportional to the concentration of the fraction that inflicted maximum mortality (90%). By plotting the log concentration versus the percent mortality (probits) for the test samples, and an approximate linear correlation was obtained (Figure 3). From the graphs, the median LC₅₀, which were the concentrations at which 50% mortality of brine shrimp nauplii occurred, were determined. The LC₅₀ values of the *n*-hexane, ethyl acetate, *n*-butanol and aqueous fractions of the crude extract of *A. cavernosa* are shown in Table 2. In this regard, cytotoxic properties appeared to be presented in the aqueous fraction.

Table 2

Effect of the fractions of *A. cavernosa* extract on brine shrimp survival.

Fraction	LC ₅₀ (μg/mL)	Regression equation	R ²
<i>n</i> -Hexane	2831.4	$y = 1.96x - 1.5733$	0.895
Ethyl acetate	1055.6	$y = 0.85x + 2.43$	0.904
<i>n</i> -Butanol	4246.1	$y = 0.475x + 3.2767$	0.996
Aqueous	115.1	$y = 1.255x + 2.4133$	0.995

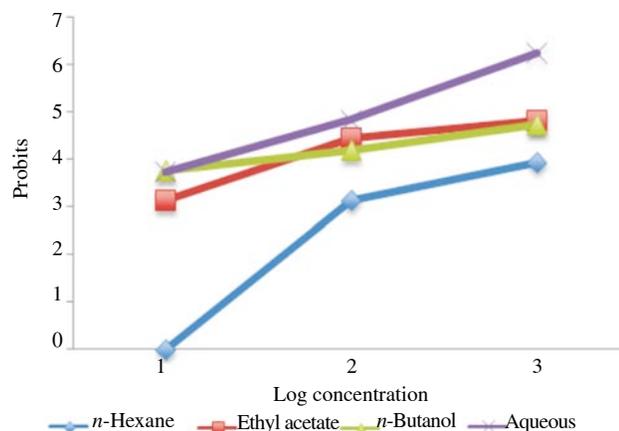


Figure 3. Plot of log concentration of the fractions of *A. cavernosa* extract fractions versus percent of shrimp mortality (probits) after 24 h of exposure.

3.4. Anti-plasmodium activity

Heme polymerization was a mechanism that released iron II ferriprotoporphyrin IX which was toxic to the malaria-carrying *Plasmodium falciparum* when it degraded hemoglobin as a source of nutrients. However, free ferriprotoporphyrin IX became detoxified when it was oxidized to iron III ferriprotoporphyrin IX, and then polymerized into inert crystals of hemozoin, a non-toxic pigment. β -Hematin used in the assay was a polymer identical to hemozoin at an acid pH reflecting the conditions of the lysosomal food vacuole. Hence, the heme polymerization inhibitory activity of a compound was directly related to its potential as an anti-malarial[13]. By extrapolation of the results shown in Figure 4, the IC₅₀ values of the ethyl acetate, *n*-hexane, *n*-butanol and aqueous of the sponge extract were estimated at 3.3, 4.6, 9.5 and 15.0 μg/mL, respectively. According to Baelmans *et al.*[14], a compound could be considered to have heme polymerization inhibitory activity if its inhibitory IC₅₀ value was smaller than the limit of chloroquine diphosphate, (37.5 mmol/L or 12.0 mg/mL). Thus, the *n*-hexane, ethyl acetate and *n*-butanol fractions all displayed heme polymerization inhibitory activity.

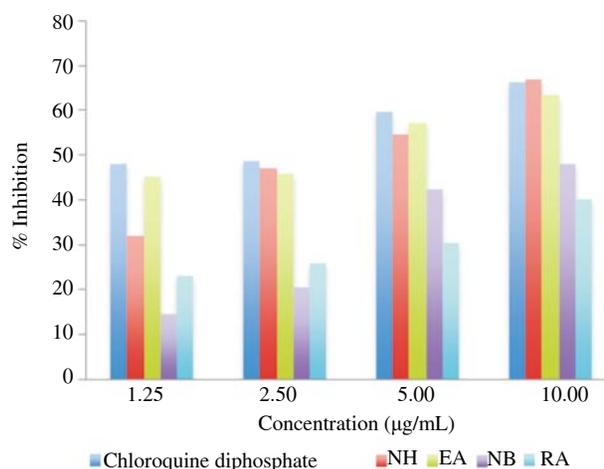


Figure 4. Heme polymerization inhibitory activity assay of fractions from *A. cavernosa* extract.

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

4. Discussion

The phytochemical screening of the fractions of the *A. cavernosa* extract displayed the presence of phenolics, terpenoids, alkaloids, flavonoids and saponins, but steroids were not detected from all fractions. Some of these secondary metabolites with their many biological properties could be of medicinal importance.

In the present study, all fractions of the *A. cavernosa* extract exhibited moderate radical scavenging activity by the DPPH scavenging assay. The *n*-hexane fraction was found to be most active in this respect, which neutralized 50% of free radicals at a concentration of 171.86 µg/mL. In the β-carotene-linoleic acid model system, the results showed no disagreement between the two methods of determining anti-oxidant. The results showed that the antioxidant activities demonstrated by the BCB and DPPH assays were greater in a non-polar solvent (*n*-hexane) whereas the TPCs were better represented in a polar solvent (*n*-butanol). This result indicated the total antioxidant activity was not only due to the phenolic content, but a greater part of it was, in fact, attributed to other antioxidant components in the sponge extract.

The brine shrimp (*Artemia*) lethality assay has been extensively used in the primary screening of crude biological extracts as well as isolated compounds to evaluate their toxicity towards brine shrimps. This response also provides an indication of possible cytotoxic properties^[15] that relate to anti-tumor, trypanocidal and pesticidal characteristics. The results in this study showed that the aqueous fraction, but not the organic fractions, of the *A. cavernosa* extract was cytotoxic against brine shrimp. According to Ullah *et al.*^[15] and Bhatti *et al.*^[16], the crude extract or fractions is toxic (active) if it has an LC₅₀ value of less than 1 000 µg/mL, while it is deemed non-toxic (inactive) if it is greater than 1 000 µg/mL.

In the present study, we also evaluated all fractions of the *A. cavernosa* extract for their antimalarial potential by assessing the inhibition of heme polymer formation. The ethyl acetate fraction was found to be the most active fractions inhibiting heme polymerization with IC₅₀ values of 3.3 µg/mL.

In conclusion, the results in this study could be an effective introduction to the anti-oxidant capacities, anti-plasmodium properties and cytotoxic activities of the sponge *A. cavernosa*. The natural anti-plasmodium compounds are of particular interest. Further studies are needed for a more extensive screening and characterization of the bioactive components in this sponge.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgements

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References

- [1] Xu Y, Lang JH, Jiao WH, Wang RP, Peng Y, Song SJ, et al. Formamido-terpenes from the South China Sea sponge *Acanthella cavernosa*. *Mar Drugs* 2012; **10**: 1445-58.
- [2] Bugni TS, Singh MP, Chen L, Arias DA, Harper MK, Greenstein M, et al. Kalihinols from two *Acanthella cavernosa* sponges: inhibitors of bacterial folate biosynthesis. *Tetrahedron* 2004; **60**: 6981-8.
- [3] Grkovic T, Bleses JS, Bayer MM, Colburn NH, Thomas CL, Henrich CJ, et al. Tricyclic guanidine alkaloids from the marine sponge *Acanthella cavernosa* that stabilize the tumor suppressor PDCD4. *Mar Drugs* 2014; **12**: 4593-601.
- [4] Abioye EO, Akinpelu DA, Aiyegoro OA, Adegboye MF, Oni MO, Okoh AI. Preliminary phytochemical screening and antibacterial properties of crude stem bark extracts and fractions of *Parkia biglobosa* (Jacq.). *Molecules* 2013; **18**: 8485-99.
- [5] Carciochi RA, Manrique GD, Dimitrov K. Optimization of antioxidant phenolic compounds extraction from quinoa (*Chenopodium quinoa*) seeds. *J Food Sci Technol* 2015; **52**(7): 4396-404.
- [6] Krishnanunni K, Senthilvel P, Ramaiah S, Anbarasu A. Study of chemical composition and volatile compounds along with *in-vitro* assay of antioxidant activity of two medicinal rice varieties: Karungkuravai and Mappilai samba. *J Food Sci Technol* 2015; **52**(5): 2572-84.
- [7] Olugbami JO, Gbadegesin MA, Odunola OA. *In vitro* free radical scavenging and antioxidant properties of ethanol extract of *Terminalia glaucescens*. *Pharmacognosy Res* 2015; **7**(1): 49-56.
- [8] Basilico 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.
- [9] Ahmed MN, Yasin KA, Ayub K, Mahmood T, Tahir MN, Khan BA, et al. Click one pot synthesis, spectral analyses, crystal structures, DFT studies and brine shrimp cytotoxicity assay of two newly synthesized 1,4,5-trisubstituted 1,2,3-triazoles. *J Mol Struct* 2016; **1106**: 430-9.
- [10] Mehbub MF, Lei J, Franco C, Zhang W. Marine sponge derived natural products between 2001 and 2010: trends and opportunities for discovery of bioactives. *Mar Drugs* 2014; **12**: 4539-77.
- [11] Paul VJ, Puglisi MP, Ritson-Williams R. Marine chemical ecology. *Nat Prod Rep* 2006; **23**: 153-80.
- [12] Hossain MA, Shah MD. A study on the total phenols content and antioxidant activity of essential oil and different solvent extracts of endemic plant *Merremia borneensis*. *Arabian J Chem* 2015; **8**: 66-71.
- [13] 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.
- [14] 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.
- [15] Ullah MO, Haque M, Urmī KF, Zulfiker AH, 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.
- [16] Bhatti MZ, Ali A, Saeed A, Saeed A, Malik SA. Antimicrobial, antitumor and brine shrimp lethality assay of *Ranunculus arvensis* L. extracts. *Pak J Pharm Sci* 2015; **28**(3): 945-9.