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Assessment of antioxidant activity, total phenolic content and *in-vitro* toxicity of Malaysian red seaweed, *Acanthophora spicifera*

Nurul Aili Zakaria[1*], Darah Ibrahim[1], Shaida Fariza Sulaiman[2] and Nor Afifah Supardy[1]

[1]*Industrial Biotechnology Research Laboratory, School of Biological Sciences, Universiti Sains Malaysia, Pulau Pinang, Malaysia*
[2]*Plant Phytochemistry Laboratory, School of Biological Sciences, Universiti Sains Malaysia, Pulau Pinang, Malaysia*

ABSTRACT

Various extracts of local marine red algae, Acanthophora spicifera were evaluated for antioxidant activity, total phenolic content and in-vitro toxicity. The antioxidant activity was measured using DPPH (1, 1-diphenyl-2-picrylhidrazyl) method, while Folin-Ciocalteu method was used for the total phenolic content. The cytotoxicity of the extracts were evaluated using in-vitro brine shrimp lethality assay. Chloroform extract showed the highest antioxidant activity of 50.098 ± 2.104% and EC_{50} value of 0.789 mg ml^{-1}. A positive correlation between total phenolic content and antioxidant activity of the extracts was observed for both extractions. Ethyl acetate extract from Soxhlet, exhibited the highest total phenolic content of 40.583 ±1.161 GAE; µg mg^{-1} dry extract and consequently had strong antioxidant activity (45.596 ±1.198%). The extract showed cytotoxic effect with $LC_{50}$of 635.47µg ml^{-1} for acute and 275.72 µg ml^{-1} for chronic, respectively.

Keywords: *Acanthophora spicifera*; Antioxidant activity; DPPH; Total phenolic content; *In-vitro* toxicity.

INTRODUCTION

Antioxidant compounds play an important role in various fields such as medical field (to treat cancers, cardiovascular disorders, and chronic inflammations), cosmetics (anti- ageing process),

food industries (food preservative) and others [1]. Over the years, the search for new antioxidant compounds from natural products has mounted. This is due to health concerns regarding the potential toxic and side effects generated from synthetic antioxidants, as well as changes in consumer preferences for natural products [2]. Many commercialized synthetic antioxidants, such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) are used under strict regulation in certain countries because of their potential health hazards. Thus, the search for alternative antioxidants from natural products has increased and among them, aquatic plants have gained the focus. Seaweeds or marine macroalgae have been known as rich sources of various natural antioxidants. Compounds such as polyphenols, catechin, flavonols, flavonol glycosides, and phlorotannins have been discovered from methanol extract of red and brown algae. The uniqueness of their molecular skeleton and structures has contributed to the strong antioxidant activity. Polyphenols for instant uses its phenol rings as electron traps for free radicals.

For the past 30 years, brine shrimp (*Artemia salina*) has been utilized in various bioassay systems. The *in-vitro* toxicity test using brine shrimps lethality assay is a simple, common, inexpensive, and rapid method to predict the antitumor and pesticidal activities [3]. It has also been used to evaluate the cytotoxic potential of compounds isolated from macroalgae [4].

Acanthophora spicifera (Vahl) Borgesen (spiny seaweeds) is an erect edible marine plant which belongs to the largest family of rhodophyta, Rhodomelaceae. It is widely distributed in tropical and subtropical areas [5] and locally known as "Bulung Tombong Bideng" in Malay. Despite the broad diversity of seaweeds along the long and wide coastal lines of Malaysia, limited studies have been reported on the antioxidant and cytotoxicity of *A. spicifera* from Malaysian waters. To the best of our knowledge, only ecological studies have been documented on the *A. spicifera* from Malaysian waters [6, 7]. Hence, this present study is aimed to investigate the antioxidant activity and cytotoxicity of this local algal. In addition, correlation between total phenolic content and antioxidant activity were also studied. Thus, finding of this study would provide an additional useful data on biological activities of this species. Besides, it also helps to promote the usage and consumption of algae as natural food products that contains edible polyphenols as antioxidants.

EXPERIMENTAL SECTION

Materials & Chemicals

DPPH (1, 1-diphenyl-2-picrylhydrazyl) free radical, gallic acid, Folin- Ciocalteu reagent, L-ascorbic acid (L-asc), quercetin, butylatedhydroxytoulene (BHT) and sodium bicarbonate were obtained from Sigma-Aldrich, USA. While, *Artemia salina* eggs was purchased locally from an aquarium shops in Sg. Dua, Penang. All the chemicals and solvents used were of analytical grade.

Preparation of algal material

Acanthophora spicifera (Vahl) Borgesen was collected from a floating buoy used for aquaculture cage, at Pulau Gedung, Penang, by hand picking method. The *A. spicifera* was identified by referring to book authored by Ahmad Ismail entitle "Rumpai Laut Malaysia" [8] and authenticated by Associate Professor Shaida Fariza Sulaiman from the School of Biological Science, Universiti Sains Malaysia, Penang, Malaysia. The voucher specimen was deposited at

the Herbarium School of Biological Sciences, Universiti Sains Malaysia. The algal was cleaned according to [9], with slight modification. The fresh collected samples were soaked and thoroughly cleaned under running tap water to remove the sand and salt contents. The sample was also gently brushed with soft brush to remove attached epiphytes, other marine organisms, and debris. The cleaned sample was then dried in a dryer at 50-60 °C for 72 hr. The dried sample was then kept in sealed plastic bag at dry and cool place to prevent from deterioration. The dried sample was grinded into coarse powdered form using an electrical blender prior to extraction.

Sample extraction

Soxhlet extraction

Briefly, 20 g of powdered algal sample contained in a Whatman No.1 filter paper thimble was placed into an extraction chamber. The extraction chamber was then connected to a flask containing 200 ml organic solvent with increasing polarity; hexane, ethyl acetate, chloroform and methanol, subsequently (1:10, w/v). Constant heat source was supplied for this procedure (40-50°C). All the extracts were concentrated under reduced pressure using a rotary evaporator and left air dried in a fume cupboard to obtain paste extract. The dried paste extracts were then stored at 4 °C for further bioassay.

Solvent partitioning extraction

Thirty gram of dried powdered sample was soaked in 300 ml of methanol at a ratio of (1:10) (w/v) for 72 hr. Frequent stirring using a glass rod was applied to ensure uniform mixing. The mixtures were then filtered with double-layered cheese cloth and followed by Whatman No.1 filter paper. The extract was then evaporated to dryness under reduced pressure using a rotary evaporator at 50 °C. The concentrated methanol extract was then left air dried in a fume cupboard. The methanol paste crude extract was then further partitioned with ethyl acetate, followed by diethyl ether and butanol using separating funnel [10]. All procedures were conducted in a fume cupboard. Extracts collected were evaporated to dryness using a rotary evaporator before left air dried in a fume cupboard. Dried paste extracts were stored at 4 °C for further analysis.

Preparation of stock extracts

Extract was prepared at 100 mg ml^{-1} by dissolving 0.1 mg of paste crude extracts in 1 ml DMSO (Dimethyl sulfoxide) for brine shrimp lethality assay. Meanwhile, 2 mg ml^{-1} extracts were prepared in methanol for DPPH free radical scavenging assay and total phenolic content (TPC).

Antioxidant activity

DPPH free radical scavenging assay

The method of [11] with a slight modification was used for evaluating the DPPH (1, 1-diphenyl-2-picrylhydrazyl) radical scavenging ability of the extracts. Briefly, 100 µl of freshly prepared methanolic DPPH (0.16 mM) was added to 100 µl of 2000 µg ml^{-1} samples in 96 well plates, yielding a final concentration of 1000 µg ml^{-1}. The 96 well plates was wrapped with aluminum foil and incubated at 37 °C for 30 min. Methanol and butylatedhydroxytoluene (BHT), L-ascorbic as well as quercetin (100 µl) with the addition of 100 µl of DPPH solution (without sample) acts as negative control and reference standards, respectively. Changes in the absorbance of the samples were measured at 515 nm using a microplate reader Multiskan EX (Thermo

Electron Corporation, Finland). The ability of samples to scavenge the DPPH free radical was evaluated using formula given by [10].

Scavenging activity (%) = [1- (($A_{sample} - A_{sampleblank}$) / $A_{control}$))] x 100…… (i)

where, A_{sample} is the absorbance of test samples (DPPH solution plus samples), $A_{sampleblank}$ is the absorbance of samples only (sample without DPPH solution) and $A_{control}$ is the absorbance of control (DPPH solution without sample). The EC_{50} value (effective concentration with 50% radical scavenging activity) was obtained by linear regression analysis and expressed in µg ml^{-1}. All test samples were conducted in triplicate (n = 3).

Determination of total phenolic content

The total phenolic content (TPC) assay was performed in accordance to [12] with modifications. An aliquot of 0.5 ml of each sample was mixed with 1 ml of Folin-Ciocalteu reagent (10% in distilled water) in a universal bottle covered with aluminum foil. After 3 min, 3 ml of 1% sodium bicarbonate was added to each sample bottle, the universal bottles were cap-screwed and vortex. The samples were then incubated for 2 hr at room temperature in darkness. The absorbance was measured at 760 nm spectrophotometrically (Genesys UV 20, US). A standard curve of gallic acid solutions (ranging from 0 µg ml^{-1} to 250 µg ml^{-1}) was used for calibration. The experiment was done in triplicate. Results were expressed as microgram of gallic acid equivalents (GAE) per milligram of extract (GAE; µg mg^{-1} dry extract).

Brine shrimp lethality assay (BSLT)

The *in- vitro* toxicity of *A. spicifera* was performed on simple zoological animal, brine shrimp (*Artemia salina*) using a method by [3]. The eggs were hatched in artificial seawater with 3.8% salinity (38 g of sea salt in 1L distilled water) at 30 ± 2 °C for 48 hr to phototrophic mature shrimps called nauplii. The hatching process was done under light regime condition and oxygenated with an aquarium pump. Methanol extract from solvent partitioning extraction that exhibited potent antimicrobial activities against several bacteria was chosen. The extract was serially diluted by adding 0.5 µl, 2.5 µl, 5.0 µl,12.5 µl, 25.0 µl and 50.0 µl (not more than 50 µl in 5 ml solution) [3] from the stock plus 3.8% artificial sea water to attain final concentration of 10 µg ml^{-1}, 50 µg ml^{-1}, 100 µg ml^{-1}, 250 µg ml^{-1}, 500 µg ml^{-1} and 1000 µg ml^{-1}. The final volume of DMSO in the extract tested was fixed to not more than 1% (v/v). With the aid of a Pasteur pipette, 10-15 nauplii were loaded into each universal bottle containing the different concentrations of extract for treatment, including control. A universal bottle containing 50 µl DMSO diluted to 5 ml of artificial sea water was used as a positive control. While, 5 ml of only artificial sea water served as negative control. Each treatment and control were performed in triplicate (n = 3). The percentage of mortality (%) was evaluated after 12 hr (acute cytotoxicity) and 24 hr (chronic cytotoxicity) exposure time by counting the number of dead nauplii presence. Based on the percentage of mortality (%), the LC_{50} was determined using best-fit linear regression line equation. However, in case of control died the percentage of mortality was calculated as given below [13].

Mortality (%) = No. of survival in control (%) – No. of survival in treatment (%)…….. (ii)

Statistical Analysis

Linear regression analysis using best-fit line method was performed to determine the lethal concentration that was able to kill 50 % of the brine shrimps (LC_{50} value) treated with respective extract. A graph of log concentration tested was plotted versus percentage of mortality of the brine shrimps. For antioxidant activity (AOA) and TPC, one-way analysis of variance (ANOVA) followed by *post-hoc* test (Tukey) and Pearson's correlation analysis (bi-variate) were carried out to test the differences and correlation between the extracts using SPSS 15.0 software. The Pearson's correlation test was used to determine the correlation coefficient (r) and coefficient of determination (r^2) between TPC and AOA. DPPH activity was used as Y dependent variable and TPC as X independent variable. The significance of differences was defined at the 95% of confidence interval ($p < 0.05$). The EC_{50} values of AOA were determined using Graph Pad Prism 3.0 software (Graph Pad, USA).

RESULTS AND DISCUSSION

Antioxidant activity

DPPH radical scavenging activity

The free radical scavenging activity of the different extracts of *A. spicifera* are shown in Table 1. Significant differences of antioxidant activity (AOA) were found for most of the extracts. On the contrary, no significant differences were found for nonpolar extracts of hexane, ethyl acetate, chloroform and diethyl ether with AOA ranging between 44.0 to 50.0%.

Table 1 Antioxidant activity and total phenolic content of various *A. spicifera* extracts

Extract	Total phenolic content[y] (GAE; µg mg^{-1} dry extract)	Antioxidant activity[z] (DPPH radical scavenging (%)
Hexane[s]	29.917 ± 0.382[b]	46.831 ± 0.685[ab]
Ethyl acetate[s]	40.583 ± 1.161[a]	45.596 ± 1.198[b]
Chloroform[s]	15.5 ± 1.192[d]	50.098 ± 2.104[a]
Methanol[s]	5.33 ± 0.144[f]	15.208 ± 4.186[e]
Diethylether[p]	30.417 ± 0.361[b]	44.218 ± 0.528[b]
Ethyl acetate[p]	25.125 ± 0.331[c]	25.468 ± 0.471[c]
Butanol[p]	9.375 ± 0.331[e]	6.819 ± 0.250[f]
Methanol[p]	7.837 ± 0.716[e]	20.751 ± 0.428[d]

[y]The experiments were measured according to the Folin-Ciocalteu method; [z]The experiment was performed using DPPH free radical scavenging assay. Antioxidant activity of standard used for DPPH assay were, Q: Quercetin (95.934 ±0.002%); BHT: butylatedhydroxytoluene (94.181 ±0.0015%); L-ASC: L- ascorbic acid (96.675± 0.002%).Each experiment was performed in triplicate (n = 3) and the results were mean ±SD. The differ ent letter indicates the significance differences at (p < 0.05); ([p]): solvent partitioning extracts, ([s]): soxhlet extracts.

In general, Soxhlet extracts showed more profound radical scavenging activity compared to extract from solvent partitioning. The AOA of Soxhlet extracts ranged from 15.208 ± 4.186 to 50.098 ± 2.104% while, the AOA for solvent partitioning extracts ranged from 6.819 ± 0.250 to 44.218 ± 0.528%. Chloroform extract exhibited the highest radical scavenging activity (50.098 ± 2.104%) followed closely by hexane (46.831 ± 0.685%) and both ethyl acetate extracts (45.596 ± 1.198%; 44.218 ± 0.528%). The other extracts showed moderate and low radical scavenging activity. The lowest radical scavenging activity (6.819 ± 0.250%) was found in butanol extract. Chloroform extract exhibited lower radical scavenging capability (EC_{50} = 0.789 mg ml^{-1}) than

the quercetin (EC_{50} = 3.4 x10^{-3} mg ml^{-1}) (data not shown). The AOA was found decreased as the solvents polarities increased (chloroform = hexane > ethyl acetate = diethyl ether > butanol > methanol). Thus, it proved that solvent polarity have great influences in the AOA and in efficiency to extract the antioxidant compounds [14]. It was also noticed that, the extracts that contained high AOA also exhibited high level of TPC. This finding suggests the antiradical property of algal-polyphenols in the extracts.

Based on the results, it can be concluded that Soxhlet extraction method was found to be a more convenient method compared with solvent partitioning in extracting the antioxidant compounds from this local *A. spicifera*. The combinations of heat, reflux, and direct contact between the algal material and the solvents in the Soxhlet extraction have increased the efficiency to extract the antioxidants [15, 16]. Percolation of the warm solvents through the sample will helps to break the cell wall of algal and hence extracted high amount of potential antioxidants compounds. Besides that, nonpolar solvents were found as the best extracting solvent to extract the antioxidant compounds. Hexane, ethyl acetate, diethyl ether, and chloroform showed higher free radical scavenging activity whereas, polar solvents such as butanol and methanol possess low scavenging activity. Similar pattern of finding was also discovered [17]. Their studies on the antioxidant activity of *A. spicifera* from Indian waters also reported that nonpolar solvent as the best solvent. The common major compounds that can be extracted using nonpolar solvents such as hexane, ether and ethyl acetate are terpenoids, flavonoids, and fatty acids [18]. Flavonoids are water soluble polyphenol that belongs to phenolic family. The capabilities of flavonoids from seaweed extracts to scavenge free radicals have been documented [19]. It was reported that, the position of hydroxyl groups in the structure of the flavonoids plays an important role in the scavenging activity.

Determination of Total phenolic content

In this study, significant differences were found in the level of total phenolic content (TPC) for most of the extracts obtained from *A. spicifera*. The differences ranged from 5.33 ± 0.144 to 40.583 ± 1.161 GAE; μg mg^{-1} dry extract (Table 1). Similar level of TPC was reported between hexane extract from Soxhlet (29.917 ± 0.382 GAE; μg mg^{-1} dry extract) with diethyl ether (30.417 ± 0.361 GAE; μg mg^{-1} dry extract) and between butanol (9.375 ± 0.331 GAE; μg mg^{-1} dry extract) with methanol extract (7.837 ± 0.716 GAE; μg mg^{-1} dry extract) from solvent partitioning. It was also noticed that the TPC level declined as the solvent polarity increased. This was demonstrated by the TPC level of solvent partitioning extracts. The level of TPC decreased from 30.417 ± 0.361 to 7.837 ± 0.716 GAE; μg mg^{-1} dry extract, as the solvent polarity increases (diethyl ether > ethyl acetate > butanol > methanol). Nonpolar solvents were found to be the best extracting solvent to extract the crude phenolic compounds that are responsible for the high AOA from this local *A.spicifera*. Hexane, ethyl acetate, and diethyl ether yielded the crude phenolic compounds in a range of 25.125 ±0.331 to 40.583±1.161 GAE; μg mg^{-1} dry extract. This finding is not in agreement with [20], who compared the extraction efficiency of polyphenolic compounds from an algal of *Fucus vesiculosus* using solvents with increasing polarities. Their study reported that the extraction efficiency increases as the solvent polarities increases where 70% aqueous acetone (v/v) was found to be the best solvent.

The choice of solvents used for the extraction of phenolic compounds depend on its solubility property. Phenolic compounds are commonly more soluble in polar organic solvents than in

water. Thus, the preferable solvents that were commonly used were aqueous mixtures of methanol, ethanol, and acetone [21]. However, phenolic compounds extracted may differ for different species, extraction methods, and solvents used. Hence, polarity of solvents and extraction methods to be used must be taken into consideration in order to extract the phenolic compounds of interest. Due to differences in geographical, extraction methods, solvents, unit of measurements applied in the previous literature, fair comparison cannot be made with this study. However, similar observation was obtained by [17] who reported that non-polar solvents was also found as the best solvent used to extract the phenolics from *A. spicifera* collected from East and West coastal region of India.

Correlation between TPC and AOA

Pearson's correlation analysis was performed to investigate the relationship between TPC and AOA of the extracts. A significant and positive correlation was found between the TPC level and AOA for both extraction methods applied (Figure 1).

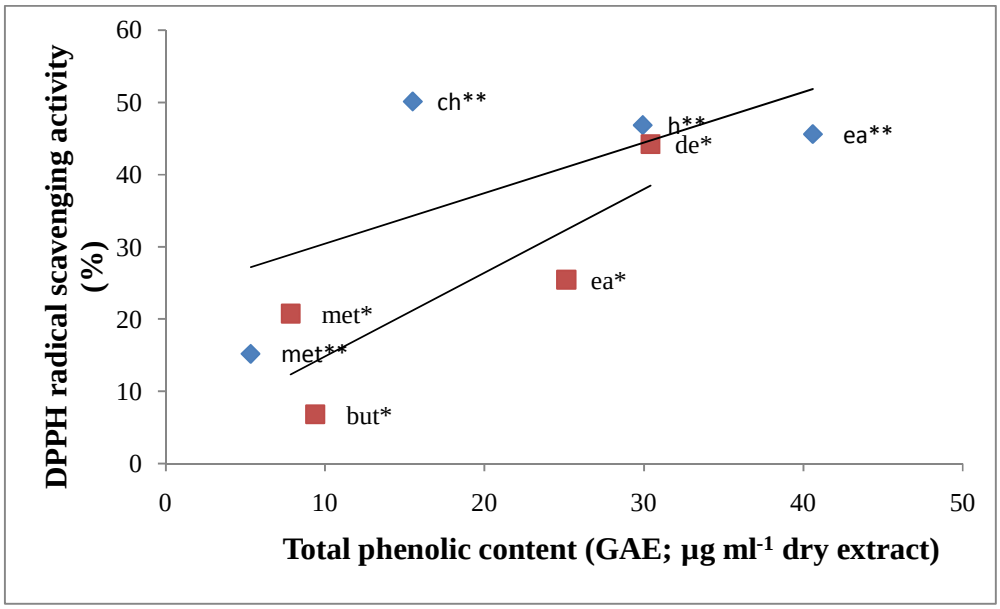

Figure 1 Linear correlations between total phenolic content and antioxidant activity of *A. spicifera* extracts based on extraction methods.

For both extractions, the variables were found significantly correlated at $p < 0.05$ (2-tailed case). The p- value calculated was $p < 0.019$ for soxhlet and $p < 0.001$ for solvent partitioning, respectively. (**) represent soxhlet extract; (*) solvent partitioning extract.

The correlations can be said as in dependent manner wherein, extract which have a high level of TPC would eventually possess high capacity of AOA as demonstrated in Table 1. A strong correlation was shown by solvent partitioning extracts (r^2 = 0.713; r = 0.844) wherein, a poor correlation was demonstrated by the Soxhlet extracts (r^2 = 0.444; r = 0.663). The result indicates that, the TPC and AOA of the extracts were positively correlated. The finding also was in line with several studies conducted [22]. Based on the result, it can be said that macroalgae polyphenols is a potent radical scavenger. The roles of phenolic compounds as the main contributors to the antioxidant activity of various seaweeds have been reported from previous studies [23].

Interestingly, not all the extracts showed a positive correlation between the TPC and AOA. Chloroform and methanol extracts gave contradictory results. Chloroform extract possessed the highest AOA (50.098 ± 0.940%) even though it contained low level of TPC (15.5 ± 1.192 GAE; µg mg^{-1} dry extract) than the other extracts such as ethyl acetate, diethyl ether, and hexane (Table 1). This finding might suggest the synergistic effect of the extracts. Other bioactive compounds present in the extracts could also contribute to the scavenging activity, which leads to this contrast. Non-phenolic compounds such as polysaccharides and proteins could also contribute to the antioxidant activity [24]. Furthermore, previous studies had stated that Folin- Ciocalteau reagent only gives basic estimation of total phenolic compounds present in the crude extracts which is not specifically for polyphenols [25]. Thus other compounds with aromatic rings such as sugar, polysaccharides, proteins and ascorbic acid might interfere in the reaction and resulted in inaccurate findings. Hence, further studies and more antioxidant assays are needed to characterize the active compounds that are responsible for the activity.

In-vitro toxicity test

In the brine shrimp lethality test, a dose-dependent relationship was observed wherein the percentage of mortality increased as the concentration of the extract increased (Figure 2).

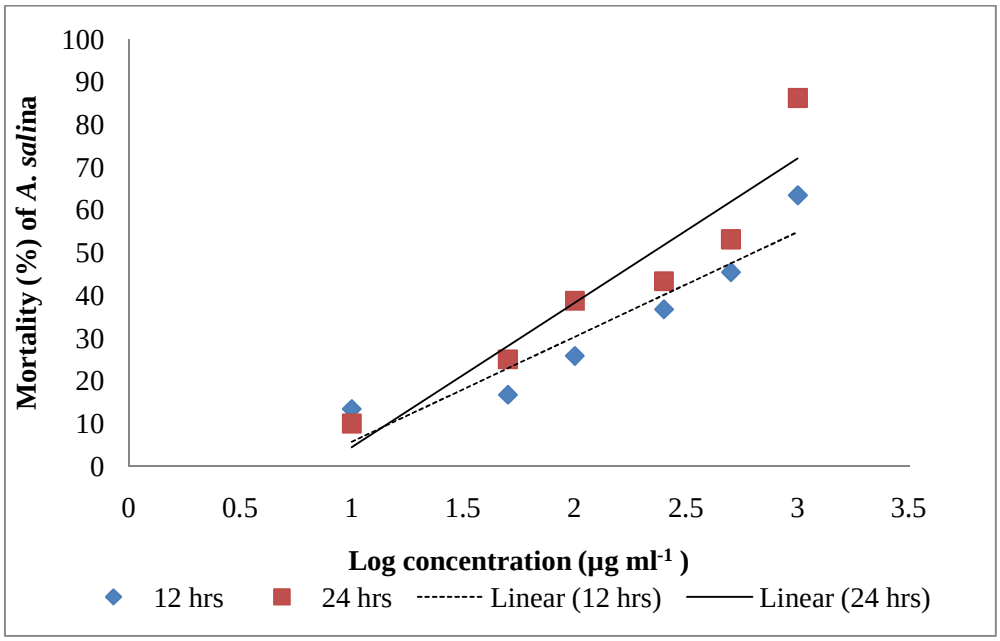

Figure 2 Cytotoxicity of *A. spicifera* extract against brine shrimps (*A. salina*) conducted at different exposure times
The LC_{50} values (concentration of extract that able to kill 50% of A. salina) were calculated and expressed in µg ml^{-1}.

This was confirmed by the observation made by Indabawa [26], who conducted the brine shrimps lethality test to detect the toxicity level of microcystin (cyanobacterial toxin) extracted from *Microcystis aeruginosa* isolated from burrow pits in Kano. The results of their works showed that the brine shrimps were killed at various dose levels tested ranging from 2 to 10 µg ml^{-1}. In the present study, methanolic extract of *A. spicifera* was found significantly toxic to brine shrimp at 12 and 24 hours. The finding of this study also revealed the effect of the time of exposure to the brine shrimp. Prolonged exposure to the extract resulted in decreasing LC_{50}

value. The LC_{50} value was found to be 635.47 µg ml^{-1} and 275.72 µg ml^{-1} for 12 hours (acute) and 24 hours (chronic) of exposure, respectively.

The results obtained were in line with Elmer-Rico [13] as they also recorded the decreasing LC_{50} values with the increase of exposure time. This is due to the tolerance of the nauplii tested towards toxic compounds. The nauplii of *A. salina* can survive for up to 48 hours without food as they feed on their yolks-sacs as the food source [27]. Thus, increased of exposure time more than 48 hours may lead to inaccurate results. In this study, BSLT assay served as a preliminary assessment to detect cytotoxic compounds present in the extract. Several compounds that were abundantly extracted from seaweeds such as terpenoids, fucoidans, and laminarins have been reported to exhibit anticancer, antiproliferative and antitumor properties [28]. Thus, this assay will hopefully provide an insight into the potential use of the cytotoxic compounds as a natural anticancer agent. The extract, fraction or isolated compounds were considered bioactive when the LC_{50} value was 1000 µg ml^{-1} or less [3,29]. Thus, the extract was said to be bioactive and exhibited a significant cytotoxic effects towards brine shrimps tested.

CONCLUSION

With these preliminary screening results, it was revealed that the different extracts of *A. spicifera* exhibited different levels of antioxidant and total phenolic contents. The TPC was found correlated with the antioxidant activity which indicates the roles of algal polyphenols as free radical scavengers for the extracts. It was also found that non polar solvents were more efficient in extracting the phenolic compounds with antioxidative property from this algal compared to polar solvents used. This finding proved the impacts of type of solvents used on both TPC and antioxidant activity. Besides, synergistic effects of the other active compounds presence in the crude extracts might also interfered with the activities. As for cytotoxicity test, the methanolic extract of *A. spicifera* demonstrated a significant cytotoxicity on the brine shrimps. In general, even though the antioxidant activity and the cytotoxicity possessed by the crude extracts did not appear to be superior to the standard used (Quercetin, BHT and BHA), the activity will more likely to be enhanced as further fractionation and purification process performed. Thus, future works are needed to purify and identify the bioactive compounds. The findings of the cytotoxicity and antioxidant properties of this *A. spicifera* are indeed highly valuable to promote the use of this seaweed as natural sources of potential antioxidants.

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