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Research Article

FREE RADICAL SCAVENGING ACTIVITY, TOTAL PHENOLIC CONTENT AND TOXICITY LEVEL OF *HALIMEDA DISCOIDEA* (DECAISNE) EXTRACTS (MALAYSIA'S GREEN MACROALGAE)

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ABSTRACT

The antioxidant activity of *Halimeda discoidea* crude extracts (methanol, diethyl ether, ethyl acetate, butanol, chloroform and hexane) were determined through the α,α-diphenyl-1-pirylhydrazyl (DPPH) radical scavenging assay. Total phenolic content (TPC) of the extracts were determined according to the Folin-Ciocalteu method and results were expressed as gallic acid equivalent. The results showed that chloroform extract exhibited the strongest antioxidant activity and highest total phenolic content. The efficient concentration (EC_{50}) of chloroform extract was tested at 6 concentrations (4 µg/ml to 250 µg/ml). The EC_{50} for chloroform extract was 136.28 µg/ml. Chloroform extract was further tested for its toxicity level. Acute and chronic toxicity activities of the extract were determined through the brine shrimp lethality bioassay test. Lethality concentration (LC_{50}) values for both acute and chronic tests were 134.87 µg/ml and 96.2 µg/ml, respectively. In conclusion, chloroform extract of *Halimeda discoidea* possesses good antioxidant property and could be a potential anticancer agent.

Keywords: *Halimeda discoidea*; Antioxidant activity; Free radicals; Toxicity test; Phenolic compounds; Gallic acid.

INTRODUCTION

Reactive oxygen species (ROS) can be defined as derivatives of oxygen molecules with one unpaired electron [1]. It is formed during the redox reaction of a molecule whereby one electron is removed from the paired electron and caused the molecule to become unstable and very reactive. A complete redox reaction of oxygen will produce water molecule and also some by-products, which are the ROS. Normal metabolic pathways of living organisms such as aerobic respiration and photosynthesis process will generate free radicals and other ROS like hydrogen peroxide and hypochlorite ions that tend to attack other molecules [2]. Examples of macromolecules that became the victims of the free radicals' attack are such as the lipids, carbohydrates, deoxyribonucleic acid (DNA) and other protein molecules.

Damages to the functioning cells later on will cause chronic diseases such as cancers, coronary heart disease, Alzheimer and Parkinson [3,4]. Antioxidant is needed to counter act the oxidation process by slowing down or completely terminate the process. Antioxidants are the reductive agents that act on the oxidants thus inhibiting the oxidation of other substances [5].

Cells in human are protected by two defensive systems against these ROS, which are the endogenous and exogenous antioxidants. Endogenous antioxidants that naturally present in the body are divided into two; enzymatic antioxidants, including glutathione peroxidases, superoxide dismutases and catalase, [2] and also non-enzymatic antioxidant, including α-tocopherol (vitamin E), β-carotene, ascorbate (vitamin C), and glutathione [6]. Incomplete protection of endogenous antioxidants is covered by the antioxidants from the outside source, which is known as exogenous antioxidants; including vitamin A, C, E, polyphenols and carotenoids that are obtained through daily food consumption [7].

Knowing that antioxidant compounds are crucially needed to maintain a healthy body, the demand and production of synthetic antioxidants has gone apace across time. Buthylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), propyl gallate (PG), octyl gallate (OG) and dodecyl gallate (DG) and quercetin are the examples of synthetic antioxidants and they are known to have better activity than natural antioxidants. However, consumers are more preferred to consume natural antioxidants than synthetic one, concerning to the safety issues of the synthetic products [8]. Epidemiological and clinical studies on plant-based foods such as vegetables, fruits and medicinal plants have proved that terrestrial plants are rich with antioxidants compounds

Macroalgae is also another source of natural antioxidants. Like other terrestrial photosynthesizing plants, they are exposed to very high ultra violet (UV) radiation, high salinity and intensive photosynthetic activities throughout the year that will lead to the formation of oxidative stress. The presence of carotenoids, photosynthesis pigments and other antioxidative agents is the adaptation mechanism to protect them from the oxidation damages [9,10]. The antioxidant activities of naturally occurring phenolic compounds in some marine macrolagae have previously been studied and the results showed they possessed promising antioxidative activities [11,12].

Halimeda species, including *Halimeda discoidea* is a member of Chlorophyta (green algae) that grows widely in the tropical ecology [13].This algae has the ability to produce a large amount of calcium carbonate precipitates due to the photosynthesis and respiration process [14]. Calcification and chemical constituents within this alga makes it less preferred by the sea grazers [15]. The aim of this present study was to investigate and evaluate the antioxidant activities of the polar, semi-polar and non-polar extracts of *Halimeda discoidea* by measuring the 2,2-diphenyl-1-picrylhydrasyl (DPPH) radical scavenging activity. Evaluation on the total phenolic content in the extracts was done through Folin-Ciocalteau method and the relationship between the antioxidant activities and total phenolic content of the extracts was investigated. The most active extract that exhibited antioxidant activity was further studied for its toxicity property through the brine shrimps lethality test.

MATERIALS AND METHODS

Collecting and drying

Halimeda discoidea was collected from Kera Island of Malaysia by hand picking in August and October 2008, during low tide. The identification of this species was based on the morphological examination from the book of 'Rumpai Laut Malaysia' [16], and was authenticated by Professor Dr Shaida Fariza Sulaiman from the School of Biological Sciences, Universiti Sains Malaysia. The fresh sample was cleaned under running tap water to remove the epiphytes and sea debris that attached on the surface of the plant. The sample later was dried in the oven at 45 °C for 4-7 days. The dried alga was ground into powder form for extraction.

Extraction

Two extraction methods were applied to give eight different extracts with different polarities (methanol, ethyl acetate, diethyl ether, butanol, chloroform, and hexane). The first method was solvent-

solvent partition method. A 40 g of powdered alga was first soaked in 400 ml of 100 % methanol for 3 days at room temperature (30 ± 2 °C) and further partitioned by diethyl ether, ethyl acetate and butanol, respectively. The second method of extraction was carried out using the Soxhlet apparatus. A 20g of powdered alga was extracted with 400 ml of hexane in Soxhlet extractor for 2-3 days. The dried residue of the alga was subsequently extracted with chloroform, ethyl acetate and methanol under the same condition. The resulting eight extracts were concentrated with a rotary evaporator at 40°C, 150 rpm and let to dryness. The paste of each extract was kept at 4 °C until further use.

Assay of DPPH-free radical scavenging activity

All samples including quercetin (positive control) were weighed accordingly and dissolved in 100% methanol to obtain stock solution samples with the initial concentration of 500 µg/ ml. Assays were performed in flat bottom 96-well microtiter plates (TPP Switzerland) according to a reference[17] with minor modifications. Briefly, 100 µl of test samples were added to 100 µl of 0.16 mM methanolic DPPH solution in the 96-well microtiter plate. A blank of 200 µl of each extract (sample without DPPH solution) was prepared due to the colour intensity of the extracts. Each sample was tested in triplicate and each sample will have the final concentration of 250 µg/ml in the 96-well microtiter plate. The plate was wrapped with aluminium foil and incubated in the dark at room temperature for 30 minutes, and its absorbance was read at 515 nm using microplate reader (Thermo, Multiskn EX). The ability to scavenge the DPPH radical was calculated using the following equation.

$$Scavenging\ effect\ (\%) = [1 - (A\ sample - A\ sample\ blank\ ) / A\ control\ ] * 100$$

A_{sample} is the absorbance of the test sample plus DPPH solution

$A_{sample\ blank}$ is the absorbance of the test sample only (sample without DPPH solution)

$A_{control}$ is the absorbance of the control (DPPH solution without sample).

To determine the EC_{50} (efficient concentration to cause 50 % reduction of DPPH's initial value), serial dilution of extract that exceeded 50% of scavenging activity was carried out, yielding six different concentrations (125.00 µg/ml, 62.50 µg/ml, 31.25 µg/ml, 15.63 µg/ml, 7.80 µg/ml and 4.00 µg/ml). The method used was the same as the screening assay of DPPH free radical scavenging activity. EC_{50} for the positive control was also measured. The EC_{50} of the samples were determined using GraphPad Prism software (GraphPad, USA).

Determination of total phenolic content (TPC)

Total phenols of extracts were determined according to the Folin-Ciocalteu method done by [18] with slight modifications. All samples were weighed accordingly and dissolved in 1 ml DMSO solution so that the stock concentration was 1.0 mg/ml. A 0.5 ml aliquot of extract sample from the stock solution was added with 1 ml of diluted Folin-Ciocalteu reagent in the universal bottle and the contents were mixed thoroughly. After 3 minutes, 3 ml of natrium carbonate solution was added and the mixture was again mixed thoroughly. For sample with the initial concentration of 1.0 mg/ml, the final concentration will be 0.1 mg/ml. The control contained all the reaction reagents except the sample. All the universal bottles were wrapped with aluminium foil because the reaction is light sensitive. After 2 hours of incubation at room temperature (30 ± 2 °C), the absorbance was measured at 760 nm with DMSO as blank using a spectrophotometer (Genesys 10uv). Readings were taken in triplicates and was compared to a gallic acid calibration curve. Total phenolics content was expressed as gallic acid equivalent (GAE) in micrograms per milligram of sample.

Toxicity assay

Hatching of brine shrimp

Artificial sea water was prepared by dissolving 40 g of sea salt in 1 litre of distilled water. The artificial sea water solution was filtered twice using Whatman No. 1 filter paper. The brine shrimp eggs (*Artemia salina*) were hatched in a shallow container filled with three quarter of the prepared artificial sea water. A light source was placed in order to attract the nauplii (larvae). The shrimp was allowed to hatch and mature as nauplii for two days (48 hours). Constant temperature (30 ± 2 ºC), sufficient light and oxygen supply were maintained in order to give good aeration.

Preparation of test sample

A hundred milligram of the chloroform extract was accurately weighed and dissolved in 1 ml of dimethyl sulfoxide (DMSO). From this stock solution (100 mg/ml), a series of extract solutions with different concentrations; 10, 50, 100, 500, and 1000 µg/ml were prepared. For control group, 3 universal bottles containing 10 brine shrimp nauplii in 5ml sea water with 50 µL DMSO to each universal bottle was used.

Bioassay

Ten brine shrimp nauplii were added to each universal bottle with the aid of Pasteur pipette. The counting of 10 ± 2 was considered, as counting exactly 10 nauplii was not possible. The test was done in triplicates. After 6 and 24 hours, the number of dead nauplii was counted. From the data, the percentage mortality of brine shrimp nauplii was calculated for all samples including the control as proposed by [19]. The LC_{50} (lethality concentration that cause 50 % of mortality) values were calculated by the extrapolation of the graph.

Statistical analysis

Values expressed as means of three replicates determinations ± standard deviation. Data were analyzed using one-way analysis of variance (ANOVA) followed by Tukey HSD test by using the SPSS 15.0 for Windows. A significant difference was considered at the level of $p < 0.05$.

RESULTS AND DISCUSSION

The test of DPPH reduction potential by antioxidant compounds in both terrestrial and aqueous plants have been widely carried out [20,21,22]. This DPPH-scavenging assay is a simple, rapid and accurate method that can be applied to both water-soluble and lipid-soluble samples. DPPH solution appears dark purple in colour and within the solution there is a large number of stable DPPH• free radicals being trapped. During the incubation time, these free radicals are paired off with the hydrogen acquired from the antioxidants and cause the purple colour to decolorize into yellow colour that is stoichiometric with respect to the number of electrons captured [23].

Table 1 shows the percentage and the standard deviation of DPPH-scavenging activity for the crude extracts and the quercetin. All eight extracts as well as the positive control were found to scavenge the free radicals at various degrees. The results revealed that non-polar chloroform extract was the strongest extract exhibited the scavenging activity and other extracts seemed to have moderate to weak scavenging activities. At the concentration of 250 µg/ml, chloroform extract of *Halimeda discoidea* has exceeded 50 % of scavenging activity, which was (76.32 %), followed by diethyl ether (37.00 %), ethyl $acetate^{Soxhlet}$ (18.47 %), $methanol^{partitoning}$ (17.44 %), hexane (13.24 %), butanol (11.48 %), $methanol^{Soxhlet}$ (11.36 %) and ethyl $acetate^{partitioning}$ (10.27 %) consecutively. The positive control, quercetin showed significantly higher scavenging activity than all the crude extracts, with 99.13 % of inhibition activity. A dose-dependent study was performed on both chloroform extract and quercetin. EC_{50}, the effective concentration of samples required to scavenge 50 % of free radicals was measured to evaluate the parameter for antioxidant effectiveness [24]. Table 2 shows the antioxidant activity of the chloroform extract and quercetin at different concentrations, ranging from 4 µg/ml to 250 µg/ml. The result has showed that the chloroform extract exhibited the concentration-dependent pattern, in which the higher the sample' concentration, the higher the percentage of scavenging activity and vice versa. The EC_{50} value for both chloroform and quercetin were 136.28 µg/ml and 1.72 µg/ml, respectively.

The result of total phenolic content of the crude extracts is also summarized in Table 1. Total phenolic content varied from 6.99 ± 0.25 µg GAE/mg dry weight in methanol (Soxhlet extract) to 45.25 ± 0.33 µg GAE/mg dry weight in chloroform extract. As expected, chloroform extract that exhibited the highest antioxidant activity as compared to other extracts contains the highest total phenolic content. Figure 1 shows the relationship between the total phenolic content and DPPH-scavenging activity of the extracts. The statistical analysis showed a positive correlation with $p < 0.05$, $r^2 = 0.929$, indicating a strong correlation between the antioxidant activity and the total phenolic content.

Table 1: Antioxidant activity of various extracts of *H. discoidea* at 0.25 mg/ml

Samples	DPPH Scavenging Activity (%) ± SD	Total phenolic content (µg Gallic acid equivalent/ mg)
a) Solvent-solvent partition		
Methanol	17.44 ± 1.42^{b,c}	7.21 ± 0.84^{a}
Diethyl ether	37.00 ± 1.63^{d}	21.74 ± 0.19^{d}
Ethyl acetate	10.27 ± 1.00^{a}	14.71 ± 0.44^{e}
Butanol	11.48 ± 1.28^{a}	9.28 ± 0.27^{b}
b) Soxhlet apparatus		
Hexane	13.24 ± 1.38^{a,b}	12.68 ± 0.51^{c}
Chloroform	76.32 ± 2.88^{e}	45.25 ± 0.33^{f}
Ethyl acetate	18.47 ± 1.05^{c}	13.77 ± 0.06^{c,d}
Methanol	11.36 ± 0.92^{a}	6.99 ± 0.25^{a}
Quercetin	99.13 ± 0.11 ^{f}	-

Statistical analysis was determined using One Way ANOVA and Tukey HSD tests. Each value is the mean ± SD of 3 replicates. Means with the same letter are not significant at p<0.05.

Table 2: Antioxidant activities of chloroform extract and quercetin at different concentrations

Concentrations (µg/ml)	Log C	DPPH Scavenging Activity (%) ± SD	
		Chloroform extract	Quacetin
4.00	0.60	7.57 ± 0.57 ^{a}	89.73 ± 0.51^{a}
7.80	0.89	9.06 ± 0.60 ^{a}	94.10 ± 0.54^{a}
15.63	1.19	10.49 ± 0.57^{a}	95.80 ± 0.61^{a}
31.25	1.49	17.44 ± 0.22^{b}	95.56 ± 0.56^{a}
62.50	1.80	24.21 ± 4.40^{c}	96.44 ± 1.17^{a}
125.00	2.10	45.91 ± 0.62^{d}	93.16 ± 9.07^{a}
250.00	2.40	76.32 ± 2.88^{e}	99.13 ± 0.11^{a}

Statistical analysis was determined using One Way ANOVA and Tukey HSD tests. Each value is the mean ± SD of 3 replicates. Means with the same letter are not significant at p<0.05.

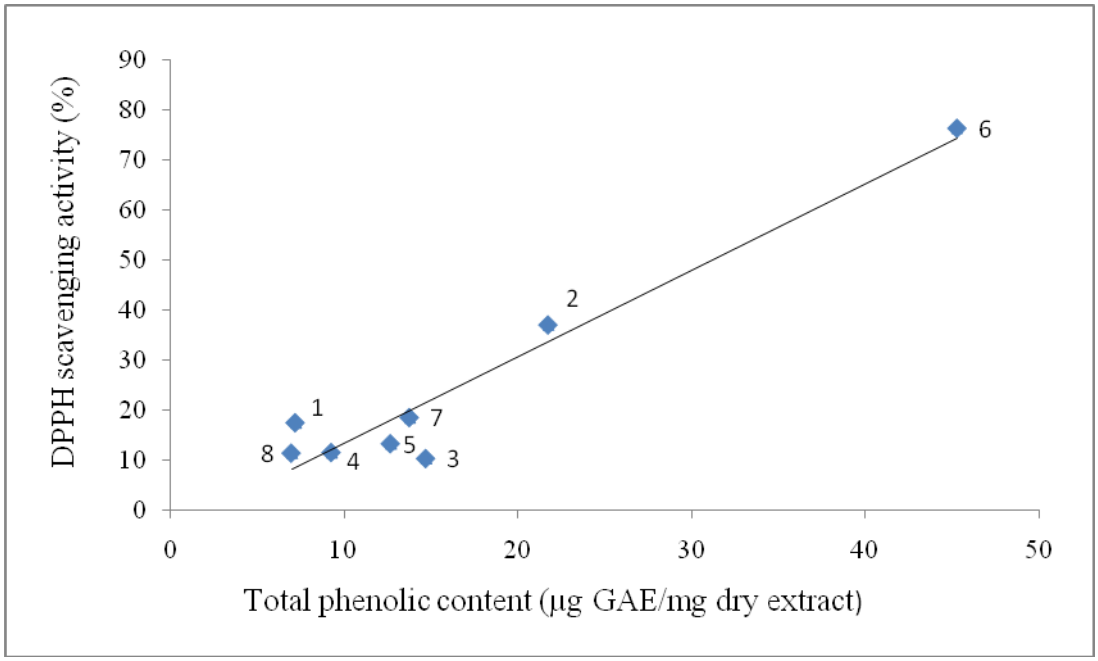

Fig. 1: Linear correlation between the percentages of DPPH-scavenging activity and total phenolic content of various extracts of *H. discoidea*

(1) Methanol (partitioning), (2) diethyl ether, (3) ethyl acetate (partitioning), (4) butanol, (5) hexane, (6) chloroform, (7) ethyl acetate (Soxhlet) and (8) methanol (Soxhlet)

Solvents with wide range of polarities (polar to non-polar) were used to maximize the extraction of antioxidant compounds available within the plant sample [17,20,25]. From the results, it shows that different solvents used did resulted in varying degrees of antioxidant activities. The varying degrees of antioxidative activity are suggested to be directly influenced by the types of solvent used as the solvent itself will determine the efficiency of the extraction to extract the antioxidant components. This is because different extracts are composed of different types and amount of antioxidant components, in which they will be successfully extracted out by the solvents or vice versa. Chloroform extract that appeared as the strongest free radical scavenger indicates that the non-polar solvent chloroform is the most suitable solvent to extract the highly potential antioxidants of *H. discoidea*. Therefore, it can be suggested that the antioxidant components within *Halimeda discoidea* are of medium polarity. The present result was in parallel with some antioxidant studies of other seaweeds that reported of more promising antioxidant activities of the less polar to non-polar extracts [12,17,20].

Lipid-soluble chlorophylls such as chlorophyll a, b and c that can be extracted by the non-polar chloroform organic solvent might be the major contributor to the antioxidant capability of *H. discoidea*. The presence of these photosynthetic pigments in marine primary producers is stimulated by the high solar radiation in hot climate such as tropical or during summer season. High UV-radiation will cause the algae to produce excessive reactive oxygen species (ROS) and other free radical species that will lead to the increasing level of cell's DNA and enzymes damages, lipid peroxidation and other macromolecules. Besides the photosynthetic pigments, the adaptive response towards the oxidative products produced during photosynthesis could also depend on the photosynthetic accessories like β-carotene and carotenoids in the algae [26] and other antioxidant compounds such as chlorophyll-related compounds, polysaccharides or mycosporines-like amino acids [27]. These pigments are responsible in absorbing the UV-substances and eliminating adverse effects of the photooxidative stress [10]. Several studies have also reported on high antioxidant activities of the *Halimeda* genus. Rivero et al. [28] who screened sixteen species of algae from Caribbean Sea including four species of *Halimeda* found that this genus showed the highest antioxidant activity among the other algal species. In addition, previous studies of DPPH-scavenging activity of dichloromethanol:methanol *Halimeda tuna* extract [29] and on hydrogen peroxide and methyl mercury-induced oxidative stress of *Halimeda incrassate* aqueous extract [30] also revealed that both species exhibited relatively high antioxidant activities.

The EC_{50} value for chloroform extract that was exceptionally higher than the one exhibited by the quercetin, indicates a weaker antioxidant property of the chloroform extract. Two factors that could root to the weak activity of the chloroform extract were assumed to be the kinetic behaviour of the antioxidant component and the antagonistic property of other components that co-exist with the antioxidants within the extract. Different antioxidants exert different scavenging actions, depending on their own reaction kinetic types and rates [31]. A slower rate of scavenging activity usually performs a very good and complete antioxidant activity, but the mechanism could be more complex. As for the antagonistic effect reason, the chloroform crude extract that was comprised of other components probably weaken the capability of the real antioxidant components. This fact was proved by two studies on antioxidant activity of two macroalgae, namely *Polysiphonia urceolata* [17] and *Rhodomela confervoides*[32]. Some fractions of both species exhibited higher antioxidant activity as compared to their crude extracts.

From the total phenolic content results, it can be summarized that the amounts of phenolics in the crude extracts have decreased simultaneously with the increasing polarity of the organic solvents used. The decreasing order was chloroform>diethyl ether>ethyl acetate>hexane>butanol>methanol. An inference was drawn, stating that this macroalgae *H. discoidea* is rich with the hydrophobic (less polar) phenolic compounds rather than the hydrophilic (polar) phenolic compounds. However, in between the ethyl acetate and butanol extract there was the hexane extract (which was the least polar extract). This exception could be explained by the assumption that the hexane extract of *H. discoidea* is composed of fairly equal amount of polar and non-polar phenolics. In contrast to the result obtained, less terrestrial and aquatic plant studies have reported the presence of phenolic compounds that are of the hydrophobic compounds, but more of the hydrophilic compounds[33,34]. Despite of which extract exhibited the highest or the lowest phenolic content, the overall result depicted the variedness of the phenolic compounds present in *H. discoidea*. In addition, a series of polyphenolic compounds and related phenolic compounds such as epigallocathecin, cathechol, caffeic acid, myricetin and hespedirin have been isolated from the same genus *Halimeda macroloba* [35]. The existence of phenolic compounds in green, brown and red macroalgae is understood to be associated with their protective mechanisms during certain adverse conditions. Higher amount of phenolic compounds is produced during the hot climate and during the early stage of the growth in order to prevent the photooxidative damage and sea grazers, respectively [36].

The positive correlation between the DPPH-scavenging activity and total phenolic content of the extracts suggests that the presence of phenolic contents within the algae might be the major contributors to the antioxidant activity of *H. discoidea*. Many studies have examined the role of phenolic contents in relation to the antioxidant activity [12,25,37] and the results revealed that phenolic compounds existed in the plants did significantly contributed to the antioxidant property. However, negative correlation between total phenolic contents and antioxidant capacity did exist in some studies [38,39]. In this kind of cases, it can be said that the antioxidant protection lies on the antioxidant compounds, not the phenolic contents of the samples. The mechanism of phenolic compound as an antioxidant relies on the structure of aromatic rings that attached to the hydroxyl groups [40]. The hydrogen atom of the hydroxyl group will be donated to the unstable free radicals and thus terminating the oxidative activity.

In this study, preliminary screening on toxicology of the chloroform extract was done to further explore its potential as antioxidant as well as anticancer agent. Figure 2 shows the results of acute and chronic toxicity of chloroform extract. The LC_{50} values of acute and chronic toxicity were found to be 134.87 µg/ml and 92.60 µg/ml, respectively. The results revealed that increase in time and concentration of samples did increase the mortality rate of the brine shrimps. Eventhough there was no large difference in both acute and chronic LC_{50} values, it still shows that a lower dose is needed to increase the number of death whenever prolong extract exposure towards the brine shrimps takes place.

Based on the LC_{50} results, the chloroform extract can be considered as moderately toxic considering to the 50 % of lethality it caused at the concentration of 100 µg/ml. This criterion is in agreement with the American National Cancer Institute that fixed the limitation of IC_{50} must be lower than 30 µg/ml, in order to consider a promising crude extract for further purification [41]. However in other toxicity study of seaweeds, based on the LC_{50} values obtained in this study the chloroform extract can be considered as highly toxic. This is because of the 50 % lethality it caused at the concentration less than 1000 µg/ml [42].

The toxicity property exhibited by this macroalgae is inferred to be due to its chemical constituents as the defence mechanism against sea herbivores. A work done by Paul and Hay, 1986 [15] that examined on macroalgae susceptibility to sea herbivores has revealed a positive correlation between chemical and morphological defences. Calcareous *H. discoidea* was reported to produce secondary metabolites called halimedatetraacetate that act as a powerful chemical deterrent to the grazers [43,44,45]. It is ecologically relevant for the sessile macroalgae to produce chemical constituents to repel the predatory pressure.

As for the conclusion, it can be concluded that the production of chemical defences and the higher antioxidant concentrations within this algal species is ecologically relevant. This species has to tolerate with the dangerous level of oxidative stress like sunlight and UV radiation, high salinity, desiccation during high temperature and also attacks from the sea grazers. These bio-properties have bring about the potential of chloroform extract of *H. discoidea* as one of the promising candidate with antioxidant property, total phenolic content as well as toxicity effect and these criterions are best suited for an anticancer agent. The data obtained will be beneficial for the future drug development purposes. The isolation of pure bioactive compounds of the active chloroform extract is in progress.

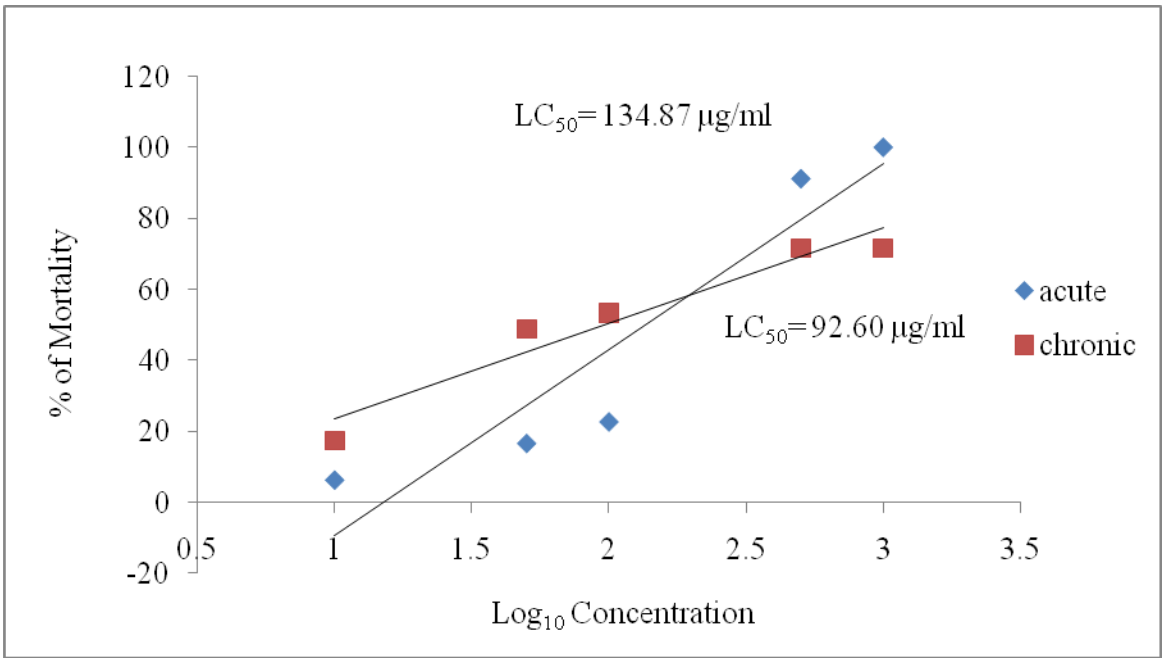

Fig. 2: Graph of percentage of mortality versus $\log_{10}$ concentration for acute (6 hours) and chronic (24 hours) toxicities of *H. discoidea* chloroform extract

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