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Phytochemical screening and evaluation of antioxidant and cytotoxic activities of *Halimeda opuntia*

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Abstract

Bioactive properties of seaweeds isolated from the Bay of Bengal of Bangladesh have hardly been studied. In the present study, *Halimeda opuntia*, a calcareous seaweed, which was collected from the St. Martin's Island, was used to investigate its antioxidant and cytotoxicity profile along with the phytochemical constituents by using the ethanolic and methanolic extracts. Through phytochemical screening, we confirmed the presence of phenolic compounds and steroids in all extracts. Soaking samples in solvent with occasional shaking for 5-7 days may lead to extraction of higher quantities of compounds and thus higher bioactivities compared with 2-2.5 hours of shaking. Using the well-known 2, 2-diphenyl-1-picrylhydrazyl (DPPH) assay, we confirmed concentration-dependent DPPH scavenging activity. For cytotoxicity study, we used brine shrimp lethality assay and found considerable cytotoxicity highlighted by two ethanolic extracts that were highly toxic (LC_{50}< 100 µg/mL) to brine shrimp Nauplii. So, *H. opuntia* can be a good source of novel and potent cytotoxic compounds possibly with anti-tumor properties, which should be further clarified. Thus, our study clearly suggested that due to the presence of considerable amounts of bioactive compounds, *H. opuntia* would be a valuable source of antioxidants with cytotoxic properties.

Keywords: Antioxidant; Cytotoxic; Steroids; *Halimeda opuntia*

Introduction

Free radicals are one of the tycoons in the pathology of numerous diseases that are akin to various types of aging associated disorders due to lipid peroxidation, protein peroxidation, DNA damage and cellular degeneration in the cells[1]. Antioxidants scavenge these free radicals leading to the primary protection from chronic diseases. Though synthetic antioxidants are being used but the key concern is their health risks and acute toxicity. In this regard, natural antioxidant sources with least side effects are paramount and marine resources have received a great attention[2].

Halimeda is a genus of warm temperate to tropical calcareous macroalgae[3]. Various pharmacological properties like antibacterial[4-6], antifungal[5,7], hepatoprotective[8], cytotoxic[5]of this genus were previously reported, which were isolated from different parts of the ocean with biodiversity[4–9]. There is no report on the bioactive properties of *Halimeda opuntia* species from the Bay of Bengal of Bangladesh origin, a highly diversified continental shelf. There are at least 193 seaweed species in Bangladesh and a minimum of 140 of them are found in the St. Martin's Island[10].

The aim of this study was to evaluate the antioxidant and cytotoxic assays to decipher the pharmacological effects and inspect phytochemicals of *H. opuntia* from the Bay of Bengal of Bangladesh. Though numerous studies have shown the pharmacological importance of this species, which have been done in different continental shelf of the world, there still remains ample scope for further research due to biodiversity. So far, for the first time an attempt was taken to abet the antioxidant and cytotoxic effect of its ethanolic and

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methanolic extracts in South Asian territory. Accordingly, we disclose herein the potent antioxidant and cytotoxic properties of *H. opuntia* to further establish the scientific basis of this species from the Bay of Bengal.

Results

Phytochemical Screening

All extracts contained phenolic compounds and steroids. EtOH (Preserve) and EtOH (Crude) had the highest quantity of both these compounds. Soaking the powdered samples in solvent with occasional shaking for 5-7 days extracted higher quantities of phenolic compounds and steroids than shaking them for 2-2.5 hours. Saponins were only detected in EtOH (Preserve) Table 1.

Table 1: Qualitative analysis of the phytochemicals of *H. opuntia* extracts.

Tested compounds		EtOH			MeOH	
	Preserve	Crude	Soak	Shake	Soak	Shake
Phenolic compounds	+++	+++	++	+	++	+
Tannins	-	-	-	-	-	-
Steroids	+++	+++	++	+	++	+
Glycosides	-	-	-	-	-	-
Flavonoids	-	-	-	-	-	-
Alkaloids	-	-	-	-	-	-
Saponins	+	-	-	-	-	-

(-): not detectable, (+): low quantities, (++): moderate quantities, (+++): high quantities

DPPH Scavenging Activity

Highest DPPH scavenging activity (21.04 ± 1.04 % of inhibition) was observed for EtOH (Preserve) at 3.34 mg/mL Table 2. None of the extracts caused 50% of inhibition. So, IC_{10} or IC_{15} was determined. We used both linear regression and best-fitting models (4- and 5-parameter logistic regression for EtOH (Preserve) and EtOH (Crude), respectively) for determining IC_{15}. Values obtained from linear regression remained within the 95% confidence interval for IC_{15} estimated by 4- or 5-parameter logistic regression in both cases. Only linear regression was used to determine IC_{10} of other four extracts since fewer data points were available for them Table 3. Only 5-parameter logistic regression was used to determine IC_{10} and IC_{15} of L-ascorbic acid since its dose-response curve was hyperbolic (Figure 1A).

Table 2: DPPH scavenging activity of EtOH (Preserve), EtOH (Crude) and L-ascorbic acid

Sample	Concentration (mg/mL)	% inhibition	IC_{15} (mg/mL)a	IC_{15} (mg/mL)b	AEACc (mg ascorbic acid equivalent/100 g)
EtOH (Preserve)	0.1255 0.2505 0.501 0.8905 1.336 1.781 2.672 3.34	4.21 ± 0.8 5.31 ± 0.55 5.76 ± 0.63 6.29 ± 0.44 8.48 ± 0.41 13.01 ± 1.59 15.14 ± 1.68 21.04 ± 1.04	2.4 ± 0.21	2.43 (2.09-2.77)	32.72 ± 2.87
EtOH (Crude)	0.1255 0.2505 0.501 0.8905 1.336 1.781 2.672 3.34	4.22 ± 0.15 3.54 ± 0.53 4.99 ± 0.17 4.38 ± 0.69 7.58 ± 0.48 10.59 ± 0.59 14.17 ± 0.67 19.94 ± 0.43	2.65 ± 0.11	2.68 (2.46-2.89)	29.41 ± 1.27
L-Ascorbic Acid	0.15 x 10^{-3} 1.5 x 10-3 15 x 10^{-3} 15.625 x 10^{-3} 25 x 10^{-3} 31.25 x 10^{-3} 62.5 x 10^{-3}	3.69 28.35 92.09 95.17 95.52 96.56 96.11		7.8 x 10^{-4} (6.98 x 10^{-4} -8.64 x 10^{-4})	

[a] calculated with linear regression. Values are expressed as mean ± SD (n=3)
[b] estimated with 4-parameter and 5-parameter logistic regression for EtOH (Preserve) and EtOH (Crude), respectively. 95% CIs are in the parantheses
[c] calculated using values derived from linear regression. Results are expressed as mean ± SD (n = 3)

Table 3: DPPH scavenging activity of EtOH (Soak), EtOH (Shake), MeOH (Soak) and MeOH (Shake).

Extracts	Concentration (mg dry weight/mL)	% inhibition	IC_{10} (mg dry weight/mL)*	AEAC (mg ascorbic acid equivalent/100 g dry weight)*
EtOH (Soak)	12.5 25 50	1.38 ± 0.47 4.74 ± 0.68 15.83 ± 0.78	36.02 ± 1.7[a]	1.42 ± 0.07[a]
EtOH (Shake)	12.5 25 50	1.67 ± 0.28 6.73 ± 0.45 12.18 ± 0.35	40.75 ± 1.25[ab]	1.25 ± 0.04[b]
MeOH (Soak)	12.5 25 50	2.32 ± 0.41 7.1 ± 0.41 10.74 ± 0.49	44.58 ± 2.08[bc]	1.14 ± 0.05[c]
MeOH (Shake)	12.5 25 50	1.95 ± 0.65 6.59 ± 0.51 9.96 ± 0.64	48.12 ± 2.9[c]	1.06 ± 0.07[c]

Values are expressed as mean ± SD (n=3).
*Values in the same column followed by different letter superscripts (a-c) are significantly different from each other ($p < 0.05$). Differences between values in the same row followed by the same letter superscript are not statistically significant. p values have been adjusted for multiple comparisons

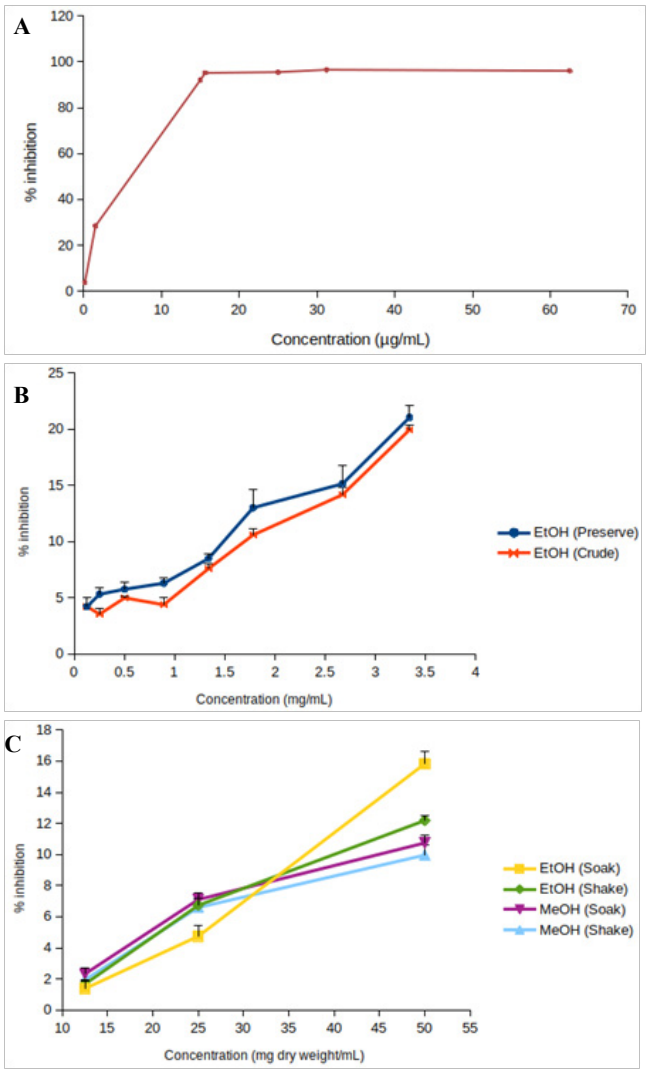


Figure 1: DPPH scavenging activity of (A) EtOH (Preserve) and EtOH (Crude), (B) EtOH (Soak), EtOH (Shake), MeOH (Soak) and MeOH (Shake), (C) L-ascorbic acid. Values are represented as mean (error bars indicate standard deviation) (n=3).

EtOH (Crude) seemed to have lower DPPH scavenging activity than EtOH (Preserve) (Figure 1B). But IC_{15} and AEAC of these two extracts were not significantly different from each other ($p > 0.05$). Among the other four extracts, EtOH (Soak) showed different pattern of inhibition (Figure 1C), significantly lower ($p < 0.01$) IC_{10} than both methanolic extracts (Figure 2A) and significantly higher ($p < 0.05$) AEAC (Figure 2B) than the other three extracts. Ethanolic extracts and extracts prepared by soaking method had significantly higher ($p < 0.05$) DPPH scavenging activity than methanolic extracts and extracts prepared by shaking method, respectively, but solvents caused more differences in this activity than methods applied (Supplementary figure 1). Lines in the interaction plots for IC_{10} (Supplementary figure 2A) and AEAC (Supplementary figure 2B) of EtOH (Soak), EtOH (Shake), MeOH (Soak) and MeOH (Shake) were not completely parallel to each other. But interaction between solvent and extraction method was not statistically significant ($p > 0.05$) (data not shown). So, extraction methods did not affect extraction by either solvent in a significantly different way.

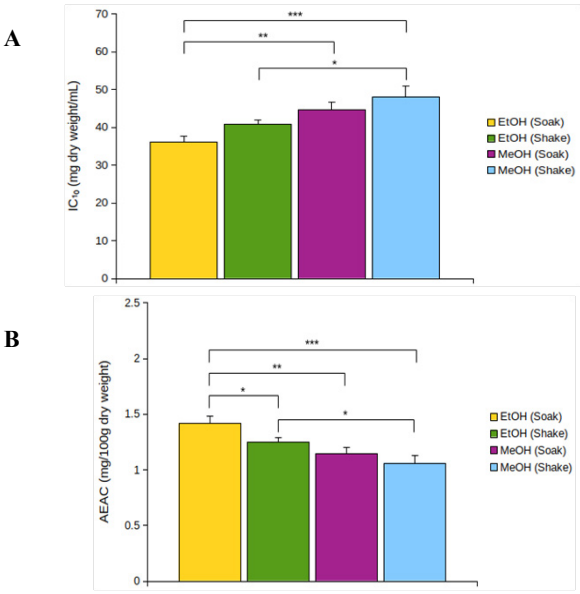
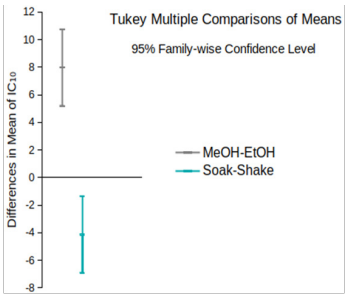
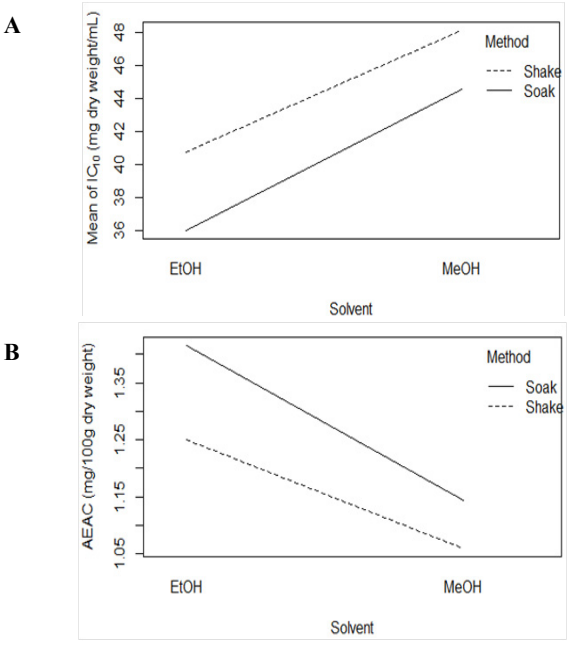


Figure 2: (A) IC_{10} and (B) AEAC of EtOH (Soak), EtOH (Shake), MeOH (Soak) and MeOH (Shake). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. p values were adjusted for multiple comparisons.



Supplementary figure 1: 95% confidence intervals of differences in the mean of IC_{10} of extracts prepared with ethanol and methanol and by soaking and shaking.



Supplementary figure 2: Interaction plot for (A) IC_{10} and (B) AEAC of EtOH (Soak), EtOH (Shake), MeOH (Soak) and MeOH (Shake).

Brine Shrimp Lethality Assay

100% mortality of nauplii was caused by EtOH (Preserve), EtOH (Crude) and potassium dichromate (Table 4), but not by the other four extracts (data not shown). LC_{50} was estimated at 95% CI using LW1949 package[11] and also calculated by Probit regression analysis. Values calculated using the latter method always remained within the 95% CI estimated using the former method (Table 5).

Table 4: Mortality (%) of brine shrimp nauplii at various concentrations of EtOH (Preserve), EtOH (Crude), potassium dichromate and negative controls.

Extracts	Concentration (µg/mL)	Mortality (%)
EtOH (Preserve)	668	100
	334	93.33
	167	80
	83.5	56.67
	41.75	16.67
	20.91	0
	668	100
	334	90
	167	73.33
EtOH (Crude)	83.5	53.33
	41.75	16.67
	20.91	0
	90	100
	81	100
	72	93.33
	63	86.67
Potassium Dichromate	54	70
	45	63.33
	36	46.67
	27	30
	18	23.33
	9	6.67
No mortality was observed in the Negative controls		

Table 5: LC_{50} and toxicity profiles of EtOH (Preserve), EtOH (Crude) and potassium dichromate

Extracts	LC_{50} (µg/mL)[a]	95% CI for LC_{50}(µg/mL)[a]	LC_{50} (µg/mL) (Probit Analysis)[b]	Toxicity Profile	
				Clarkson's Toxicity Index	Meyer's Toxicity Index
EtOH (Preserve)	86.41	68.21-109.46	84.81	Medium/ Highly Toxic	Toxic
EtOH (Crude)	94.24	72.6-122.34	92.63	Medium/ Highly Toxic	Toxic
Potassium Dichromate	32.19	27.73-37.36	31.95	Highly Toxic	Toxic

[a] Estimated using Litchfield-Wilcoxon method with LW1949 package

[b] Calculated using probit analysis. Regression equations were generated with Libre Office Calc.

According to Clarkson's toxicity index[12], extracts/ compounds with LC_{50} value of 100-500 µg/mL are medium toxic and those with LC_{50} value of 0-100 µg/mL are highly toxic. So, potassium dichromatewas highly toxic. EtOH (Preserve) and EtOH (Crude) were medium to highly toxic. According to Meyer's toxicity index[13], extracts with LC_{50}< 1000 µg/mL are toxic. So, both of those extracts were toxic based on this criterion. Both extracts were significantly less toxic than potassium dichromate (Figure 3A). Yields were not determined for the other four extracts. So, toxicity profile could not be ascertained. 95% CIs for their LC_{50} overlapped with one another (Figure 3B). So, it cannot be deduced with certainty that their toxicities were significantly different from each other. Among those four extracts, 95% CIs for LC_{50} of ethanolic extracts were, in general, wider than those of methanolic extracts. So, the tested concentrations provided more precise information about the cytotoxicity of methanolic extracts.

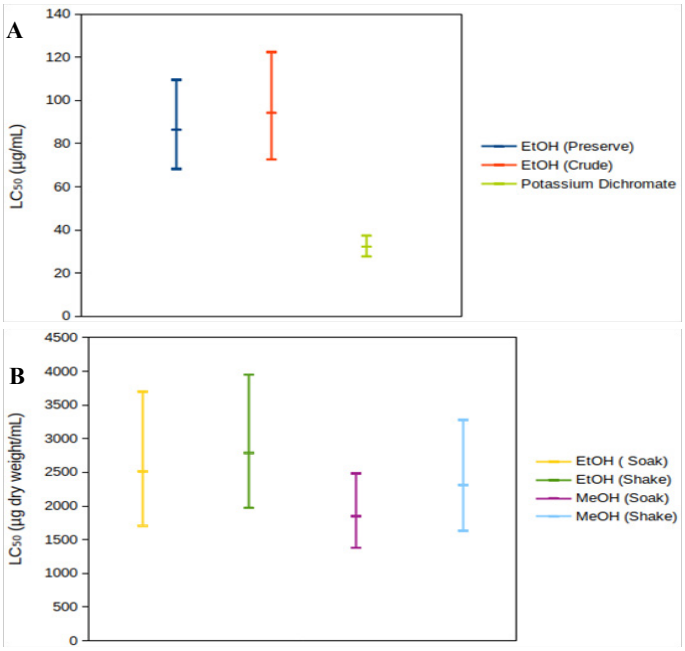


Figure 3: 95% confidence intervals for LC50of (A) EtOH (Preserve), EtOH (Crude) and potassium dichromate, (B) EtOH (Soak), EtOH (Shake), MeOH (Soak) and MeOH (Shake).

Discussion

In this study we confirmed the presence of phenolic compounds,which is similar with the previous report[14], along with steroids in *Halimeda opuntia*,.We further clarified the antioxidant and cytotoxic properties of this species.

We found significantly higher DPPH scavenging activity by soaking method compared to shaking method in EtOH (Soak) and MeOH (Soak) (Table 2; Figure 1B, 1C, 2A and 2B). *H. opuntia* was exposed to sunlight and so was expected to have efficient antioxidant mechanisms due to the of phenolic compounds[14,15]. But 50% DPPH scavenging activity may not be obtained by its crude solvent extracts even at high concentrations[8]. It doesn't necessarily indicate that *H. opuntia* doesn't produce effective antioxidants. In fact, fractions of the extracts enriched

in bioactive compounds may have better DPPH scavenging activity[16].

In brine shrimp lethality assay, all extracts showed cytotoxicity and two extracts- EtOH (Preserve) and EtOH (Crude)- were medium to highly toxic (Table 4 & 5), which is similar with a previous study[5]. Presence of phenolic compounds and steroids in all extracts may account for their toxicity to brine shrimps[17,18]. Generally, cytotoxic compounds in seaweeds, including *H. opuntia* , can act as chemical defense against herbivores[19,20]. Further studies are needed to develop a deeper understanding of cytotoxic compounds produced by *H. opuntia* and their anti-tumor properties.

In our phytochemical screening study, the highest quantity of phenolic compounds ensured the possible presence of antioxidant, anti-inflammatory, anti-allergenic, antithrombotic, anti-carcinogenic and hepatoprotective activities of *H. opuntia* . In addition, for the first time we disclosed the presence of steroids in this species, which will open a new avenue to explore the cellular signaling system by using *H. opuntia* as a source of steroids.

Materials and Methods

Sample Collection and Processing

Seaweed samples were collected in November, 2019 from the shallow water on the eastern side of Chera Island (CheraDwip), which is an indwelled extension of St. Martin's Island. They were then cleaned with clean seawater and completely immersed in 50% ethanol for preservation. The collected seaweed was subsequently identified to be *Halimeda opuntia* (Linnaeus) J.V. Lamouroux based on its morphology[21]. After about one month, the seaweeds were taken out of ethanol, washed with filtered water and segmented followed by air drying and oven drying at 37º C, then ground to powder with mortar and pestle. The powder was stored at -20ºC until further use.

Preparation of Extracts

5.0 g of powdered sample was soaked in 50 mL of 50% ethanol and 70% methanol, separately, with occasional shaking for 5-7 days. Extracts prepared in this way were referred to as EtOH(Soak) and MeOH (Soak), respectively. 5.0 g of powdered sample was also separately shaken in 50 mL of 50% ethanol and 70% methanol at 25ºC and 150 rpm with a table top shaking incubator (Model : JSSI-070C, JSR, Korea) for 2-2.5 hours. Extracts thus prepared were referred to as EtOH (Shake) and MeOH (Shake), respectively. In all cases, after the stipulated time the resulting extracts were filtered through Double Rings 11.0 cm filter paper (Qualitative, 102). The initial concentrations of these four extracts were expressed as 100 mg dry weight/mL[22]. 60 mL of the 50% ethanol used for preservation was taken and the solvent was evaporated in oven at 45ºC to give amorphous solid masses[23], which were weighed and dissolved again in 60 mL of 50% ethanol and this extract was referred to as EtOH (Crude). The 50% ethanol for preservation was indicated as EtOH (Preserve). Both these extracts had a concentration of 6.68 mg/mL. Comparison between the composition and the bioactivities of these two extracts may give useful information about the thermostability of bioactive compounds of *H. opuntia* . All extracts were stored at -20º C until further use.

Phytochemical Screening

Qualitative phytochemical tests for the identification of phenolic compounds, tannins, alkaloids, steroids, steroidal glycosides, flavonoids and saponins were performed using previously described methods[24-27]. Phytochemical screening of the extracts was performed with following tests: phenolic compounds with lead acetate test, tannins with ferric chloride test, and alkaloids with Mayer's test, steroids and glycosides with Salkowski's test, flavonoids with alkaline reagent test and saponins with frothing test.

DPPH Scavenging Activity

DPPH scavenging activity was assayed using a previously described method[28,29] with slight modifications. In brief, 4 mg of DPPH (Cat. No.: sc-202591, Santa Cruz Biotechnology, USA) was dissolved in 100 mL of 95% methanol. 2 mL of the each extract, after dilution with respective solvent, was mixed with 2 mL of DPPH solution making the final concentrations for EtOH (Preserve) and EtOH (Crude) 3.34, 2.672, 1.781, 1.336, 0.8905, 0.501, 0.2505 and 0.1255 mg/mL and those of the other four extracts 50, 25 and 12.5 mg dry weight/mL. These mixtures were kept in the dark at room temperature for 1 hour. Then absorbance was measured at 517 nm using a UV-VIS spectrophotometer (model: UV-1900, Shimadzu). All determinations were performed in triplicates. The free radical scavenging activity or % of inhibition was calculated using the following formula:

$$\% \text{ inhibition} = (1- (A_{sample}-A_{blank})/(A_{control}-A_{blank})) \times 100$$

Where A_{sample} = Absorbance of reaction in presence of the sample (sample dilution + DPPH solution)

$A_{control}$ = Absorbance of control reaction (sample solvent + DPPH solution)

A_{blank} = Absorbance of blank for each sample dilution (sample dilution + DPPH solvent)

L-ascorbic acid was used as a positive control and DPPH scavenging activity of the extracts was also expressed as ascorbic acid equivalent antioxidant capacity (AEAC) (mg ascorbic acid/100 g dry weight).

Brine Shrimp Lethality Assay (BSLA)

Brine shrimp lethality assay was performed using previously described methods[30-32]with modifications. In brief, artificial seawater was made by dissolving 37 g sodium chloride 1 L of sterile distilled water and adjusting its pH between 8.25 and 8.5 by adding 1.0 N sodium hydroxide (NaOH). 200 mg of brine shrimp (*Artemiasalina*) eggs were hatched to produce nauplii in 1 L of this water for 24 hours under strong aeration in a vessel illuminated by a 60 watts bulb. 500 µL, 250 µL, 125 µL, 62.5 µL, 31.25 µL and 15.65 µL of each extract were transferred in triplicates into separate test tubes and organic solvent was completely evaporated. 10 nauplii were added to each test tube and the final volume was adjusted to 5.0 mL by adding artificial seawater. After 24 hours, number of dead nauplii was counted with the aid of a magnifying glass. Mortality (%) was calculated using the following formula

Mortality (%) = (number of dead nauplii)/(number of dead nauplii+number of alive nauplii) x 100

Cytotoxicity of the positive control, potassium dichromate ($K_2Cr_2O_7$), was determined in the same way using final concentrations of 90, 81, 72, 63, 54, 45, 36, 27, 18 and 9 µg/mL in triplicates. 50% ethanol and 70% methanol were used as negative controls.

Statistical Analysis

Statistical analyses of the results were performed by using ANOVA (one & two-way), Tukey's Honest Significant Difference (HSD) test and two-sample t-test. LC_{50} values were estimated at 95% confidence interval (CI) using Litchfield-Wilcoxon method[33] with LW1949 package of R. LC_{50} was also calculated using Probit Analysis. All the triplicate data were expressed as mean±SD as appropriate. The limit of significance was set at $p<0.05$.

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Conflict of interest

We have no conflicts of interest to disclose in this study.

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