

Screening of Potential Bioactive Compounds from *Padina Gymnospora* Found In the Coast Of St. Martin Island Of Bangladesh

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Abstract

Seaweed is the most popular marine resource which plays an important role biologically in different research area in the world. In spite of this significance of seaweed very few algae based studies have been conducted in Bangladesh. For the first time this study aimed to find out the potential bioactivity of *Padina gymnospora* collected from the St. Martin Island of Bangladesh. The present study investigated the potential bioactivities of *Padina gymnospora*. Initially Study subject was extracted with five different solvents. Crude extracts were screened for crucial phytochemicals. DPPH radical scavenging activity was implemented for antioxidant test. The antibacterial activities were examined by agar well diffusion method. Cytotoxic test were conducted following Brine Shrimp Lethality Assay. The result showed that Methanol and ethanol crude extracts exhibited the presence of the highest number of bioactive compounds. The highest % inhibition ($48.28 \pm .47$) along with a significant IC_{50} value of $369.99 \mu\text{g/ml}$ ($p < 0.05$) was found in ethanol crude. Isopropanol followed by methanol crude extract has shown strong activity against *B. cereus*, *S. aureus*. Chloroform crude extract was highly effective against *Salmonella* species while ethanol crude extract showed the highest activity against *S. aureus*, *Salmonella* species and *S. hominis*. It is also recorded that the isopropanol and chloroform crude extracts showed highly cytotoxicity ($IC_{50} < 100 \mu\text{g/ml}$) ($p < 0.05$). The present findings suggested that *Padina gymnospora* has substantial bioactivity due to the presence of phenolic compounds, flavonoids, steroids and other secondary metabolites.

Keywords: Bioactive compounds; *Padina gymnospora*; Antimicrobial activity; Antioxidant; Cytotoxic; St. Martin Island

Introduction

Seaweed is one of the major marine sources considering its various potential aspects in different areas of research in the world^[1]. Researchers from different countries are looking forward to fight back with various complications like aging problems, microbial attack, cancer as well as malnutrition using natural products isolated from different marine sources^[2]. Now-a-days seaweeds found in Bangladesh, 193 species where approximately 140 species found in different region of St. Martin Island, appear to be a substantial marine living existence^[3].

Different bioactive properties like antioxidant^[4,5] antibacterial^[6,7], hemolytic activity^[8], cytotoxic^[9] found in seaweed have received major attention from different parts of the diversely blue world^[4-11]. Especially their least side effect along with negligible health risk leads themselves to the pinnacle of biological research and drugs industry^[12].

Nevertheless, the bioactivity along with potential bioactive compound based study on *Padina gymnospora*, a brown seaweed, found in the coastal area at St. Martin Island of Bangladesh, has not been studied yet^[3]. Therefore, our present

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study was undertaken to investigate its pharmacological properties by focusing on the antioxidant potential, antibacterial activity, cytotoxic effect of methanol, ethanol, aqueous, chloroform and isopropanol crude extract of *P. gymnospora* along with the screening of its major bioactive compounds.

Materials and Methods

Sample Collection and Processing

Study samples were collected at the end of November, 2019 from the Chera Island (Chera Dwip), an extension of St. Martin Island of Bangladesh. Sample was cleaned properly later drowned in 50% ethanol for the preservation. The collected sample was identified as *Padina gymnospora* based on its morphology [13]. After four weeks sample was washed thoroughly with sterile distilled water in the laboratory subsequently air-dried in the dark condition and oven dried at 37°C, cut into small pieces later ground with mortar and pestle until reach fine powder shape.

Extract Preparation

3.0g of powdered sample was extracted in five different solvents (30 ml of 70% methanol, 50% ethanol, distilled water, chloroform, isopropanol) individually under shaking condition (25°C and 150 rpm) inside of a shaking incubator (Model : JSSI-070C, JSR, Korea) for 2 hours. The solution was filtered through Whatman No. 1 sterile filter paper (Qualitative, 102). The filtrates were dried using oven at 40°C and the dried precipitates were dissolved in the above five solvents. Concentrations for the extracts were 7.10 mg/mL, 7.05 mg/mL, 6.27 mg/mL, 6.82 mg/mL, 5.96 mg/mL for methanol, ethanol, distilled water, chloroform, isopropanol crude respectively. Later crude extracts were stored in airtight bottles at -20°C in a refrigerator before testing bioactivity.

Phytochemical Screening

Standard qualitative phytochemical procedures^[14-17] were followed to detect the presence of potential bioactive compounds in the sample. Phenolic compounds, tannins, alkaloids, steroids, glycosides, flavonoids, and saponins were subjected to identify in the collected sample. Test for Phenolic compounds using lead acetate, for alkaloids using Mayer's reagent, for tannins using ferric chloride, for flavonoids using alkaline reagent, for steroids as well as glycosides using Salkowski's test. Saponins test done by distilled water with vigorous shaking^[18].

Antioxidant Activity–DPPH Assay

The free radical inhibition activity of the four different extracts (methanol, ethanol, aqueous and chloroform) was determined using DPPH (2,2-diphenyl-1-picrylhydrazyl) following the well-established method with slight modification^[10,11,19,20]. 4mg DPPH (Cat. No.: sc-202591, Santa Cruz Biotechnology, USA) was mixed with 100 mL of 95% methanol. 3 mL of individual mixture (Crude extract: DPPH = 1:1) was prepared where four different crude extracts were subjected to dilution separately for making the concentration of 12.5 µg/mL, 25 µg/mL, 50 µg/mL, 100 µg/mL, 200 µg/mL and 400 µg/mL for each of the samples. Later the mixtures were incubated for about 1 hour in a dark condition and the decrease in absorbance was monitored at 517 nm in UV-VIS spectrophotometer (Model: UV-1900, Shimadzu).

Ascorbic acid is used as a standard. The percentage of scavenging (%SCV) was calculated as:

$$\% \text{ SCV} = 1 - (A_1 - A_0) / (A_2 - A_0) \times 100$$

Where,

A_1 = Absorbance of sample

A_2 = Absorbance of control

A_0 = Absorbance of blank

Cytotoxic Activity-Brine Shrimp Lethality Assay (BSLA)

Cytotoxic activity was investigated following with slight modification of previously published method^[10,11,21-23]. The 150mg of Brine shrimp (*Artemiasalina*) eggs were hatched in 1L sea water (pH=8.31) for 24 hours under continued aeration in a cylindrical container illuminated by a 60 watts bulb to produce nauplii. Six different concentrations of 45, 90, 135, 180, 225 and 270 µg/ml of sample extracts were prepared in each of the solvents of methanol, ethanol, distilled water, chloroform and isopropanol. Later 3 ml were transferred in Eppendorf and left open for 48 hours to evaporate the organic solvent before adding the nauplii. After 24 hours, the phototropic nauplii were collected. Ten brine shrimp nauplii were transferred in each of six test tubes. Four mL of seawater was added in each test tube of five different extract of the same samples before adding the nauplii. After 24 hours the observations were noted and survivors were counted and percent death at each dose level was calculated as:

$$\text{Mortality (\%)} = \frac{\text{Number of dead nauplii}}{\text{Number of dead nauplii} + \text{Number of alive nauplii}} \times 100$$

Potassium dichromate ($K_2Cr_2O_7$) was used as positive control and the above mentioned five different solvents were used as negative control. Concentration and the cytotoxic test for the positive control were similar as mentioned above and all the cytotoxic tests were performed in triplicate.

Antibacterial Activity Assay

Antimicrobial assay was performed by agar well diffusion method in Luria Bertani (LB) media and the minimum inhibitory concentration of those extract was determined by dilution method. All the strains of bacteria was cultured in LB broth and incubated at 37°C for 18 hours in incubator (Model – JSGI - 050T, JSR, Korea). After incubation each stain were diluted with sterile distilled water. Prepared inoculums were incubated for 30 minutes at 37°C prior to use. Crude extracts (150 µl) were loaded into the respective wells. Solvents (Methanol, ethanol, distilled water, chloroform and isopropanol) were tested for their activity as negative control at the same time in the separate petridishes. The Ampicillin 20 µg/ml, Tetracycline 20 µg/ml, Kanamycin 20 µg/ml, Ciprofloxacin 20 µg/ml was used as a positive control. The Petridishes were then left for an hour with the lid closed so that extracts diffused to the media. The plates were incubated overnight at 37°C. After proper incubation (18 hours) the plates were observed for the zone of inhibition (ZOI) around well which is suggested by clean zone without growth. The ZOI were measured with the help of the ruler and mean was recorded for the calculation.

Determination of Minimum Inhibitory Concentration(MIC)

A stock solution of 0.5 mg/ml was prepared for five different crude extract (methanol, ethanol, aqueous, chloroform, isopropanol) of the study samples. This was serially diluted to obtain various ranges of concentrations between 0.5 mg/ml to 0.020 mg/ml.

Statistical Analysis

All data found in this study were expressed as mean values±SD of triplicate. Using one-way ANOVA the mean values were analyzed. The means of parameters were determined significantly ($p < 0.05$). Statistical Packages for Social Sciences ‘IBM SPSS statistics-20’ Software, Microsoft Excel programme 2007 were used for the data analysis. LC_{50} was determined using Probit analysis.

Results

Bioactive Compounds Screening

Phenolic compounds, flavonoids, steroid and tannins found maximum in ethanol crude extract of *P. gymnospora* followed by Chloroform and methanol crude extract while aqueous crude had all the tasted phytochemicals in a trace amount except the absence of alkaloid and saponins. The qualitative analysis of phytochemicals of four different crude extracts *P. gymnospora* is given in Table 1.

Table 1: Qualitative phytochemical analysis of crude extracts of *P. gymnospora*.

Attributes	Methanol	Ethanol	Aqueous	Chloroform
	Crude Extract	Crude Extract	Crude Extract	Crude Extract
Tannins	+	++	+	++
Phenolic compounds	++	+++	+	++
Alkaloids	+	++	-	-
Flavonoids	++	+++	+	++
Saponins	+	-	-	+
Glycoside	+	+	+	-
Steroid	+	+++	+	++

[N.B. n=3, (-): not detectable, (+): low quantities, (++): moderate quantities, (+++): high quantities]

Antioxidant Activity–DPPH Assay

Free radical scavenging is important to maintain the redox homeostasis^[24]. In this study, the antioxidant activity of the sample was determined using DPPH method. The reaction mixture color gradually changed into yellow from the initial purple color. The scavenging activity of methanol, ethanol, aqueous, chloroform extracts were compared with the standard ascorbic acid. Ethanol crude extract showed the highest antioxidant activity followed by methanol, chloroform and aqueous crude extract of *P. gymnospora*. The existent antioxidant of four different crude extracts level in the selected seaweed is mentioned in Table 2 and illustrated in figure 1(a, b). The IC_{50} value was also calculated where it also followed the similar tendency of efficacy with the highest IC_{50} value of ethanol extract.

Table 2: Antioxidant activities of *P. gymnospora* extracts.

Conc. (µg/mL)	% of Inhibition				
	Positive control	Different crude extracts of <i>P. gymnospora</i>			
	Ascorbic Acid	50% Ethanol	Chloroform	70% Methanol	Aqueous
12.5	10.01 ± 0.36	5.28 ± 0.37*	3.99 ± 0.29	3.19 ± 0.29	3.01 ± 0.14
25	17.84 ± 0.20	9.99 ± 0.19*	5.56 ± 0.16	5.01 ± 0.24	4.45 ± 0.42
50	25.46 ± 0.25	18.74 ± 0.14*	8.45 ± 0.42	7.99 ± 0.19	6.82 ± 0.54
100	50.99 ± 0.66	29.99 ± 0.24*	14.74 ± 0.26	12.01 ± 0.34	9.99 ± 0.34
200	77.01 ± 0.38	38.99 ± 0.53*	19.45 ± 0.35	16.74 ± 0.53	12.28 ± 0.62
400	77.70 ± 0.49	48.28 ± 0.47*	23.19 ± 0.24	19.56 ± 0.26	14.74 ± 0.43

N. B. Comparisons were made between ethanol extract groups with other three extract groups. Each value is presented as mean ± SD, (n=3), (* $p < 0.05$).

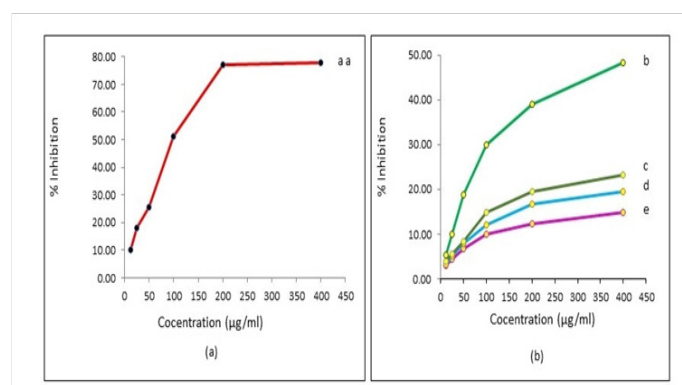


Figure 1: Antioxidant activity of *P. gymnospora* through DPPH assay. (a) Percentage inhibition of positive control (aa = Ascorbic Acid), (b) Percentage inhibition of four different crude extracts of *P. gymnospora* (b = EtOH, C = Chloroform, D = Methanol, E = Aqueous).

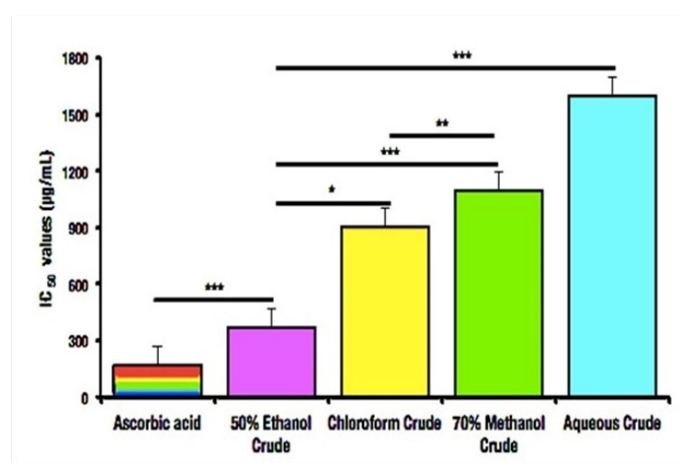


Figure 2: IC_{50} values of DPPH Assay determined from *P. gymnospora*.

Cytotoxic activity-Brine Shrimp Lethality Assay

According to the cytotoxicity test in this experiment, the mortality (%) of brine shrimp nauplii against the different crude

extracts of the study sample at various concentrations were different along with those of the positive control, Potassium Dichromate ($K_2Cr_2O_7$), (Table 3) indicates the future significance of the use of *P. gymnospora* in the treatment of cancer.

The 50 percent (%) Lethal Concentration (LC) of the study sample varies with the different crude extracts of the study sample depicted in figure 3.

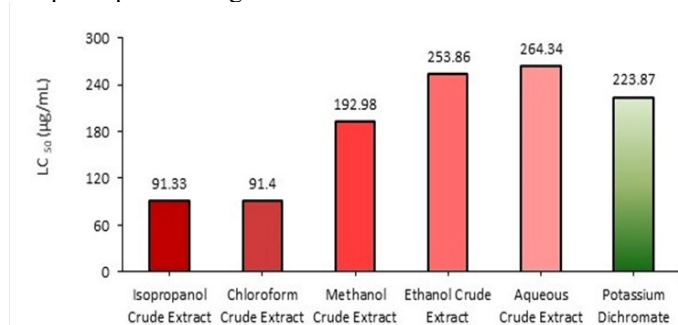


Figure 3: LC₅₀ Value of *P. gymnospora*.

According to the prominent Clarkson index of the cytotoxicity [25], the toxicity status of different crude extracts of *P. gymnospora* was determined.

Table 4: Toxicity Profile of *Padinagymnospora*.

Extracts	Status of toxicity
Methanol crude	Medium toxic
Ethanol crude	Medium toxic
Aqueous crude	Medium toxic
Chloroform crude	Highly toxic
Isopropanol crude	Highly toxic

Antibacterial activity assay

The antimicrobial activities of the tested crude extracts of *P. gymnospora* were evaluated by agar well diffusion method and the results were expressed as the diameter of inhibition zone (mm). Most of the extracts of *P. gymnospora* showed antimicrobial potency against the selected bacteria.

Determination of Minimum Inhibitory Concentration (MIC):

The smallest amount of compounds required to kill or inhibit the growth of microorganism *in vitro* is determined by the dilution method and considered as minimum inhibitory concentration (MIC). The MIC of five different crude extracts of the study sample against the tested organism is mentioned in the table 6.

Table 3: Mortality (%) of *P. gymnospora*, Potassium Dichromate (positive control) and the negative control.

Concentration (µg/mL)	Potassium Dichromate	Methanol Crude Extract	Ethanol Crude Extract	Aqueous Crude Extract	Chloroform Crude Extract	Isopropanol Crude Extract
45	10%	10%	10%	0%	30%	20%
90	20%	20%	20%	10%	50%	50%
135	30%	30%	30%	10%	60%	70%
180	40%	40%	40%	20%	60%	80%
225	50%	60%	50%	30%	80%	80%
270	60%	70%	50%	30%	90%	90%

Negative control showed no mortality

Table 5: Antibacterial activity of various crude extracts of *P. gymnospora*.

Test Organisms	Diameter of the Zone of Inhibition (mm)								
	Extracts of <i>Padinagymnospora</i>					Positive control			
	EtOH	MeOH	Aque.	Chlor.	Isop.	Amp.	Tet.	Kan.	Cipro.
<i>Bacillus cereus</i>	-	12.00 ± 0.41***a2	10.50 ± 0.41 ^{ta3}	15.00 ± 0.82**a1	27.50 ± 0.41 ^a	Nil	8.33 ± 0.58***a4	16.50 ± 0.50 ^{a5}	19.50 ± 0.50 ^{a6}
<i>Staphylococcus aureus</i>	18.17 ± 0.85***b1	11.17 ± 0.85 ^{tb2}	-	12.50 ± 0.41***b3	28.00 ± 0.82 ^b	3.67 ± 0.29	21.17 ± 0.76 ^{tb4}	16.33 ± 0.29 ^{tb5}	22.17 ± 0.76 ^{tb6}
<i>Klebsiella pneumonia</i>	13.00 ± 0.82**c2	10.45 ± 0.62	-	18.50 ± 0.41 ^c	13.50 ± 0.41***c1	Nil	7.50 ± 0.50	11.67 ± 0.29***c3	11.00 ± 0.58
<i>Salmonella species</i>	14.50 ± 0.41***d1	10.50 ± 0.41**d2	-	24.00 ± 0.82 ^d	10.50 ± 0.41 ^{td3}	Nil	1.83 ± 0.29 ^{td4}	8.00 ± 0.50 ^{td5}	4.67 ± 0.58
<i>Salmonella paratyphi</i>	09.50 ± 0.41 ^c	-	-	-	09.50 ± 0.82 ^e	15.33 ± 0.76 ^{te1}	19.67 ± 0.58**e2	17.83 ± 0.29**e3	14.67 ± 0.58 ^{te4}
<i>Staphylococcus hominis</i>	14.00 ± 0.82 ^f	-	-	-	10.50 ± 0.41 ^{fi1}	3.50 ± 0.5	20.83 ± 0.76 ^{fi2}	19.50 ± 0.87	22.17 ± 0.29 ^{fi3}

Zone of inhibition was not found in negative control

N.B. Each value is presented as mean ± SD, n=3. *p < 0.05, **p < 0.01, ***p < 0.001 were considered as statistically significant. Comparisons were made among a, b, c, d, e and f and their consecutive orders. EtOH – ethanol, MeOH – methanol, Aque – aqueous, Chlor – chloroform, Isop – isopropanol, Amp – ampicillin, Tet – tetracycline, Kan – kanamycin, Cipro – ciprofloxacin.

Table 6: Overview of MIC along with Zone of inhibition (ZOI) of *P. gymnospora*.

Tested organism	Attribute	MIC according to ZOI				
		70% MeOH	50% EtOH	Aqueous	Chloroform	Isopropanol
<i>Bacillus cereus</i>	MIC	50 µg/ml	Nil	70 µg/ml	40 µg/ml	30 µg/ml
<i>Staphylococcus aureus</i>	MIC	50 µg/ml	40 µg/ml	Nil	50 µg/ml	30 µg/ml
<i>Klebsiella pneumonia</i>	MIC	60 µg/ml	50 µg/ml	Nil	40 µg/ml	40 µg/ml
<i>Salmonella species</i>	MIC	60 µg/ml	40 µg/ml	Nil	30 µg/ml	40 µg/ml
<i>Salmonella paratyphi</i>	MIC	Nil	60 µg/ml	Nil	Nil	50 µg/ml
<i>Staphylococcus hominis</i>	MIC	Nil	40 µg/ml	Nil	Nil	60 µg/ml

N.B. MIC- Minimum Inhibitory Concentration (µg/ml), ZOI-Zone of Inhibition (mm).

Discussion

In the current study, we attempted to find out and characterize the major bioactive compounds found in *Padina gymnospora* using various different solvents. In addition, we also appraised its antioxidant, cytotoxic and antibacterial activity to establish the pharmacological significance. We confirmed the presence of tannins, phenolics, flavonoids, and steroids in all of the types of extracts from *P. gymnospora*, notably methanol and ethanol crude extracts contained higher amount of these bioactive compounds, which is similar with the previous finding though the species was different^[26,27]. Saponins, alkaloids and glycosides were also the additive findings of the methanol and ethanol extracts (Table 1). The presence of these bioactive compounds suggests the pharmacological properties of the *P. gymnospora*, which leads the subsequent antioxidant, cytotoxic and antimicrobial studies.

In the living system, free radicals are generating frequently as a response to metabolism^[28,29] and these free radicals are also scavenged by antioxidants that lead redox homeostasis in the body^[24]. Aberration of redox homeostasis compels various health hazards like cancer, diabetes, arthritis, neurological disorders etc^[30]. Seaweeds are the well-heralded sources of antioxidant^[31], and in our study, we also found significant amount of antioxidant response through DPPH assay. The 50% ethanol crude extract of *P. gymnospora* is substantially effective ($p < 0.05$) at the IC₅₀ level than other crude extracts in the present study. The 50% ethanol crude extract of this species had significantly more activity compared with all the concentrations used in the experiment ($p < 0.05$) than the chloroform crude extract ($p < 0.05$), 70% methanol crude extract ($p < 0.05$), aqueous crude extract of *P. gymnospora* ($p < 0.05$) (Fig. 1b, Fig. 2; Table 2), which was similar with the same genus but different species^[32]. It is well known that flavonoids, alkaloids and phenolic compounds, which were also common in our study, possess antioxidant activity due their capability to interact with protein phosphorylation, iron chelating, scavenging hydrogen peroxide by donating electron and thereby neutralizing it to water, absorbing and neutralizing

superoxide anion (O₂-•), hydroxyl radical or peroxy radicals, quenching singlet and triplet oxygen or decomposing peroxides as well as terminating the radical chain reaction involved in lipid peroxidation by converting free radicals and reactive oxygen species to more stable products^[33-38]. Thus, the antioxidant activity of *P. gymnospora* extracts might be accredited to these modes of action due to their flavonoid, alkaloid, and phenolic contents.

Cytotoxic property of a compound is an important measure for the treatment of tumor due to its cell cycle inhibitory role. In this line, screening of some marine seaweeds with cytotoxic properties is additive in the field of chemotherapy. Generally, cytotoxic compounds present in seaweeds provide a protective defense to them from many herbivores^[39]. In our study, the cytotoxic effect of the bioactive compounds present in different solvents extracts of *Padina gymnospora* was studied using brine shrimp lethality assay (BSLA). From the examined crude extracts of this study subject, the isopropanol and chloroform crude extracts showed the highest toxic activity in brine shrimps. The LC₅₀ results obtained for isopropanol crude extract was 91.33 µg/mL and for chloroform crude extracts 91.48 µg/mL indicated higher toxicity whereas 70% methanol, 50% ethanol and aqueous crude extracts of the same sample showed the LC₅₀ values 192.98 µg/mL, 253.86 µg/mL, 264.34 µg/mL, respectively (Fig. 3; Table 3 & 4). It is well known that the phytochemical phenolic compounds and steroids are the tycoon concerning the cytotoxicity, which is also similar in line with our phytochemical analysis that suggests that the presence of these bioactive compounds would be the underlying factors for the cytotoxic properties of *P. gymnospora*.

The antimicrobial activity of the five different extracts of *Padina gymnospora* were determined by measuring the zone of inhibition in the Kirby Bauer well diffusion assay. Depending upon their solubility and polarity, different solvents shows the different antibacterial activity. The antibacterial activity of the 50% ethanol and isopropanol crude extracts of study sample showed a prospective activity against most of the pathogens. Isopropanol had higher antibacterial activity than that of extracts obtained with other organic solvents such as ethanol, methanol, chloroform, and distilled water (Table 5). The presence of phenolic contents and alkaloids may be responsible for the antibacterial activity and for more precise mechanism concerning to alkaloids, for example, sanguinarine, perturbs bacterial FtsZ-Z-ring formation and inhibits bacterial cytokinesis^[40,41]. 50% Ethanol extract of *P. gymnospora* are active against most of the gram positive and negative bacteria. In this present study, we concluded that Isopropanol extract followed by ethanol extract showed an adequate amount of phytochemical compounds hence it is taken for antibacterial activity testing.

The minimum inhibitory concentration (MIC) was observed in methanol, ethanol, aqueous, chloroform and isopropanol crude extract of the study sample against the six different test organisms. The lowest MIC (30 µg/ml) was found both in isopropanol crude extract against *Bacillus cereus* and *staphylococcus aureus* bacteria and in the chloroform crude extract against *Salmonella species*. The highest MIC (70 µg/ml) was recorded in aqueous crude extract against *Bacillus cereus*. On the other hand methanol and ethanol crude extracts also showed promising result in the determination of MIC. 50% Ethanol showed

the lowest MIC (40 µg/ml) against *Staphylococcus aureus*, *Salmonella* species, *Staphylococcus hominis*. 70% Methanol crude extracts provided the lowest similar MIC (50 µg/ml) against *Bacillus cereus* and *Staphylococcus aureus*(Table 6).

Conclusion

Padina gymnospora species used in the present study has a great potential for antimicrobial properties, in addition to cytotoxic activities as well as antioxidant activities under the oxidative stress condition. This study has generated a renewed interest in the possibility of the study sample to be used as a pharmaceutical resource especially their constituents may be applied as drug (antibacterial and anticancer agent) for human administration in different pathogenic condition.

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