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In vitro Antioxidant, Antimicrobial, Haemolytic and Cytotoxic Activity of Brown Alga *Padina gymnospora* from South East Coast of India

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The objective of this study was to investigate the antioxidant potential, antimicrobial, haemolytic and cytotoxic activities of petroleum ether extract of *Padina gymnospora*. The total phenolic and flavonoid content was estimated by Folin's ciocalteau and Aluminium chloride methods. Antioxidant potential of the extract was estimated by Phosphomolybdneum method, Diphenyl picryl hydrazine free radical scavenging method and the Superoxide free radical methods. Antimicrobial activity was performed by Disc diffusion method against seven bacterial and five fungal strains. *P.gymnospora* exhibited 61.52 mg GAE/g equivalents of phenolic content, 54.2 ± 0.15 mg Quercetin/grams equivalents of flavonoid content. The total antioxidant activity was found to be 74.23% and scavenging activity of DPPH 62.9% and superoxide ion 59.4% at 1000μg/ml. *P. gymnospora* extract has shown strong activity against *E. coli, S. aureus, A. niger, C. albicans* and moderate activity against *B. cereus*, *B. subtilis*, *K. pneumoniae, P. aeroginosa, A. flavus, A fumigatus, T. viridae* and *T. rubrum.* The crude extract was screened for the haemolytic activity towards human erythrocytes. The haemolytic activity was performed by spectroscopic method for different concentrations (250, 500, 750, and 1000) μg/ml) and it has shown the dose dependent hemolysis activity. The cytotoxic activity of crude extract against Hel 92.1.7 leukaemia cell line was evaluated by MTT assay which shown remarkable decrease in the number of cells by dose dependent manner with IC_{50} of 102.3 μg /ml. The cytotoxic activity was found might be due to the induction of apoptosis in Hel 92.1.7 leukaemia cell line which was demonstrated by DNA fragmentation analysis. The present findings showed that the petroleum ether extracts of *Padina gymnospora* have strong antioxidant activity, antimicrobial, haemolytic as well as cytotoxic activity which might be due to the presence of phenolic compounds and other secondary metabolites.

Key words: *Padina gymnospora,* antioxidant, antimicrobial, cytotoxicity, DNA fragmentation.

Marine macroalgae are commonly called as seaweeds, are the renewable living resources which are also used as food, feed and fertilizer in many parts of the world. Seaweeds are of nutritional interest as they contain low calorie food, but rich in vitamins, minerals and dietary fibres (Ito and Hori, 1989). Seaweeds contain bioactive compounds which can benefit for human health (Kuda *et al.*, 2002). They are considered as a rich source of bioactive compounds as they are able to produce a various secondary metabolites characterized by a broad spectrum of biological activities (Chew *et al.*, 2008). It has been used as a food and medicine in Asian countries. The high levels of non-digestible polysaccharide in their cell walls make seaweeds a rich-source of dietary fibre (Ruperez and Saura-Calixto 2002). Similar to photosynthesizing plants, seaweeds are exposed to a combination of light and oxygen to the

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formation of free radicals and strong oxidizing agents (Matsukawa *et al.,* 1989). Seaweeds are also known to contain various antioxidant molecules such as ascorbate, glutathione (GSH)as well as secondary metabolites- carotenoids, mycosporine-like amino acids (mycosporine-glycine); catechins (catechin, epigallocatechin, epigallocatechin); gallate, phlorotannins (phloroglucinol), and tocopherols (α-, χ-, δ-tocopherols). Antioxidant compound plays a vital role against various ailments (Kohen and Nyska, 2002). Antioxidants inhibit or prevent oxidation of a substrate and evolve to protect biological systems against damage induced by ROS (reactive oxygen species). Antioxidant activity of seaweeds is essential since these species are strongly exposed to UV radiation in the tropical environments. However, natural sources are considerably received more attention by the consumers and the researchers because of the concern about the potential toxic effects of synthetic antioxidants (Safer and al-Nughamish, 1987). Brown marine algae have been used in traditional medicine for treating some health ailments (Yubin and Guangmei, 1989). The brown alga, *Padina gymnospora* belongs to the family Phaeophyceae, is of common occurrence in the Gulf of Mannar. The aim of the study was to evaluate the bioactive potential which includes phytochemical analysis, antioxidant potential and antimicrobial activity of this common marine algae *Padina gymnospora*.

MATERIALS AND METHODS

Sample collection

Seaweed *Padina gymnospora* (Kutzing.) Sonder was collected by handpicking from the low tide areas on the Mandapam coast of Rameswaram, Tamilnadu, India in the low tide areas. After collection, they were washed thoroughly with seawater to remove the epiphytes and dust particles, packed in a sterile polythene cover and kept in ice box transported to the laboratory for further process. Then, they were washed in tap water followed by distilled water, shade dried, grounded into the fine powder using mechanical grinder and finally stored in the refrigerator until use.

Sample preparation

Ten grams of *P. gymnospora* powder was soaked in 100 ml of petroleum ether solvent, kept in a shaker for 48 hours. It was centrifuged at 3000 rpm for 10 minutes. The supernatant was collected and filtered through Whatmann filter paper No.1. Then, it was condensed using a rotary evaporator and stored in the refrigerator at 4ºC for further use ((Demirel *et al.,* 2009).

Phytochemical screening

The phytoconstituents-carbohydrates, phenolics, alkaloids, flavonoids, cardiac glycosides, proteins, steroids, tannins, and terpenoids present in the sample qualitatively analysed by following the standard method described by Harbone1984.

Total Phenolic content

The total phenolic content present in the *P. gymnospora* extract was estimated by the Folin's ciocalteau method (Marinova *et al.,* 2005).1 ml of extract was added to 9 ml of distilled water. Different concentrations of Gallic acid were prepared to calibrate the standard curve. A reagent without the sample used as a blank. 1ml of Folin's ciocalteau reagent (1:1) dilution was added to it. After 5 minutes of incubation, 10 ml of 7% sodium carbonate solution was added to the mixture. Then, the solution was diluted and made upto 25ml in volumetric flask, incubated at dark for 90 minutes. Absorbance was measured against the reagent blank at 750nm in UV spectrophotometer. Total phenolic content present in the sample were expressed as mg/g Gallic acid equivalents. All the experiments were done in triplicates.

Total flavonoid content

The total flavonoid content present in the *P. gymnospora* extract was determined by Aluminium chloride method (Hoerudin 2004). 1ml of extract was diluted with 4 ml of distilled water. To this, 0.3 ml of 5% sodium nitrite was added and incubated for five min. 0.3 ml of 10% Aluminium chloride was added to it. 2ml of 1M sodium hydroxide was added and the total volume was made upto 10ml with distilled water. Quercetin was used as a standard. Different concentrations of Quercetin were prepared to calibrate the standard curve. A reagent without the sample used as a blank. Absorbance was measured against the reagent blank at 510nm in UV spectrophotometer. Total flavonoid content present in the sample were expressed as mg/g Quercetin equivalents. All the experiments were done in triplicates.

Total antioxidant activity

The total antioxidant activity of the extract was measured by the Phosphomolybdenum method according to the procedure described by Prieto *et al.*, 1999. Different concentrations of the sample (250, 500, 750, and 1000) µg / ml were prepared. 0.3 ml of the extract was added to 3ml of reagent which contains 0.6 M sulphuric acid, 28mM sodium phosphate and 4mM ammonium molybdate. A reagent without the sample used as a blank. Ascorbic acid used as a standard. Absorbance was measured against the reagent blank at 695nm in UV spectrophotometer. The percentage of antioxidant activity was calculated as by following the equation:

$$\frac{Ac - As \times 100}{Ac} \quad ...(1)$$

Ac = Absorbance of control; As = Absorbance of sample.

Reducing power

Reducing power of the extract was carried out by following the procedure described by Oyaizu (Oyaizu 1986). Various concentrations of the extracts 250, 500, 750, and 1000)µg/ml were prepared. Ascorbic acid used as a standard. A reagent without the sample used as a blank. 1 ml of the sample was added to the 2.5 ml of 0.2 M phosphate buffer (pH 6.6) and 2.5 ml of potassium ferric cyanate (1%) was added. The mixture was vortexed, incubated at 50º C for 20 minutes and 2.5 ml of trichloroacetic acid (10%) was added. It was centrifuged at 3000 rpm for 15 minutes. The top layer of the solution was diluted with the same volume of distilled water and 500µl of 0.1% ferric chloride was added. Then the absorbance was measured at 700nm against a blank. Increase in the absorbance indicates high reducing power

DPPH radical scavenging activity

Free radical scavenging activity of the extract was carried out by DPPH method (Mensor *et al.*, 2001) Different concentrations of the sample 250, 500, 750, and 1000) µg / ml were prepared. Ascorbic acid used as a standard. A reagent without the sample used as a blank. 1ml of DPPH (0.16mM in methanol) was added to the 2ml of extracts. Incubated at dark for 30 minutes and the absorbance were measured at 517nm in UV spectrophotometer. The percentage of scavenging activity was calculated by using the equation 1.

Superoxide anion radical scavenging activity:

Superoxide anion radical scavenging activity of *P. gymnospora* extract was measured based on the method described by Liu[16] with little modifications. Different concentrations of the extracts 250, 500, 750, and 1000) µg / ml were prepared. Quercetin used as a standard. To a 1 ml of the extract, 0.5ml of nitroblue tetrazolium (2.52mM), 0.5ml Nicotinamide dinucleotide (64µM) and 0.5ml Phenanzine methosulphate added. Incubated at room temperature for 15 min. Then, the absorbance was measured at 560nm and the scavenging activity on superoxide anion was calculated by using the equation 1.

Antimicrobial screening of the *Padina gymnospora*

Antimicrobial screening of the extracts were carried out by Disc diffusion method (Karthigadevi *et al.*, 2009). Muller Hinton agar was used for antibiotic susceptibility test. Bacterial strains namely *Escherichia coli, Proteus vulgaris Staphylococcus aureus, Klebsiella pneumnoniae, Pseudomonas aeruginosa, Bacillus cereus and Bacillus subtilis* were maintained in Luria bertani broth. Fungal strains such as *Asperillus niger, Aspergillus flavus, Aspergillus fumigatus, Candida albicans, Trichophyton viridae, Trichophyton rubrum* were maintained in a Potato dextrose broth**.** Fresh culture was swabbed on the sterile Muller Hinton agar plates using a sterile cotton swab. Each disc was impregnated with 30µl of extract by using micropipette. The plates were incubated at 37ºC for 24 hours. Ampicillin and Fluconazole was used as a positive control for bacteria and fungi respectively. Zone of inhibitions were measured in millimetre (mm). All the experiments were performed in triplicates.

Hemolysis activity

Hemolytic activity of the crude petroleum ether extract of *Padina gymnospora* was carried out using human blood (Bulmas *et al.*, 2003). The human blood was freshly collected and the blood was centrifuged, erythrocytes were collected and washed twice in sterile saline solution, centrifuged at 1500rpm for 5 min and the erythrocyte pellet was collected. Pellet was diluted with 1:9 (v/v) in saline solution and the crude *P. gymnospora* extract with the various concentrations from (250, 500, 750, and 1000) µg/ml was added. 10% triton X used as a positive control, Distilled water served as a negative control. Blank were prepared without

addition of blood. The mixture was vortexed and incubated at 37ºC for 1 hour, centrifuged at 8000 rpm for 10 min. The supernatant was collected and measured using UV spectrophotometer at 540 nm. Experiments were done in triplicates and the concentration of the extract inducing 50% hemolysis were calculated and represented graphically. The Percentage of hemolysis was calculated using the formula,

Percentage of hemolysis = (Abs of sample–Abs of blank) / (Abs of positive control) × 100

Cytotoxic activity against HEL 92.1.7 leukaemia cell line

Cytotoxic activity of the petroleum ether extracts of *P. gymnospora was* screened against the Hel 92.1.7 leukaemia cell line using the MTT assay (Denziot and Lang, 1986).1×10^6 Hel 92.1.7 in 100 µL RPMI medium with 10% FBS per well plated in a 96 well plates and incubated overnight at 37ºC in 5% CO_2 incubator. 0-100 µg / ml of the test samples were prepared in RPMI without FBS. 100 µL of RPMI was used as negative control and 5 µg / mL Doxorubicin was added as internal positive control for the assay. Wells without any cells are used as blank. Plate was gently shaken and incubated at 37ºC in 5% CO_2 for 48 hours. 20 µL of 5 mg / mL MTT in PBS was added to each well and incubated at 37ºC in 5% CO_2 for 4 hours. Then the medium was aspirated out and 200 µL of dimethylsulfoxide (DMSO) to each well. The optical density of each well was measured using microplate reader at 50 nm. Then, the percentage of inhibition of cell growth is calculated as follows:

Percentage of inhibition = 100 – (Sample / Control) x100

DNA fragmentation

$1x10^6$ Hel 92.1.7 cells were plated were plated per well of 6 well tissue culture plate and incubated at overnight at 37ºC at 5% Co_2 incubator. Then these cells were treated with 1.0 mg/ml of the test sample dissolved in RPMI medium containing 10% FBS. RPMI containing 10% FBS was used as a negative control. 0.2µM Camptothecin, 2µg/ml Doxorubicin hydrochloride used as a positive control. Then the plates were incubated for 48 hr. then the apoptotic DNA fragments were isolated by following the method (Stanojkovi*et al.,* 2013). Cells were harvested from the plate and washed with phosphate buffered saline and centrifuged to recover the pellets. Then, the pellets were treated 10 sec with 50µl of lysis buffer which contains 1% NP-40 in 20 mM EDTA, 50 mM Tris-HCl, (pH 7.5) and centrifuged at 2000 rpm for 5 min. Supernatant was collected and the process repeated again with the lysis buffer. After centrifugation for 5 min at 1600 x g the supernatant is collected and the extraction is repeated with the same amount of lysis buffer. Finally, the supematant was added to the 1% SDS and treated for 2 hr. with RNase A (final concentration 5µg/µl) at 56°C followed by digestion with proteinase K (final concentration 2.5 µg/µl) for 2 h at 37°C. After addition of 2 mL of 10 M ammonium acetate, the DNA was precipitated with equal volume of ethanol. Finally, the DNA pellet was dissolved in 50µl of TE buffer, and separated by agarose gel electrophoresis in 1.0% agarose gel.

Statistical analysis

Statistical analysis was performed by using Graphpad prism software. All the experiments were performed in triplicates. Values were expressed as mean ± SEM

RESULTS AND DISCUSSION

Marine organisms have a wide potential to produce a rich biologically active primary and secondary metabolites which have importance in pharmaceutical industry. Many novel compounds of marine origin have been isolated with broad spectrum of activities and some are still in research to develop new medicines against various ailments (Smith 2004). Phytochemical analysis of the petroleum ether extract of *Padina gymnospora* showed the presences of phytoconstituents are alkaloids, flavonoid, phenolics, carbohydrates, steroids, terpenoids, cardiac glycosides, tannins, proteins as depicted in the table 1. Total phenolic content present in it was found to be 61.51 ± 0.21 mg GAE/g equivalents. Phenolic compounds are commonly found in wide variety of plants and they have been found to have correlated with several biological activities including the antioxidant activity because they have high redox potential as they can acts as a reducing agents, hydrogen donors and oxygen quenchers (Kuda *et al.*, 2005, Li *et al.*, 2005). Sidhuraju *et al*., 2002 suggested the phenolic content could be related to the antioxidant activities. Patrica *et al.*, 2008 found that the presence of phenolic is responsible for antioxidant

Table 1. Phytochemical analysis of *P. gymnospora*

Name of the test	*Padina gymnospora* Petroleum ether extract
Alkaloids	Present
Flavonoid	Present
Phenolics –	Present
Carbohydrates – Molisch's test	Present
Steroids andTerpenoids	Present
Cardiac Glycosides	Present
Tannins	Present
Proteins	Present

Table 2. Cytotoxic effect of *P. gymnospora* on Hel 92.1.7 leukaemia cell line

Conc.mg.ml	OD 590 nm	% Inhibition
1.56	0.31	1.35
3.125	0.30	4.61
6.25	0.28	10.09
12.5	0.26	17.00
25	0.24	23.35
50	0.19	37.29
100	0.13	58.55

activity. Phenolic compounds are one of the most effective antioxidants in brown algae reported by Nagai and Yukimotto 2003. Previous studies have been reported Phlorotannins and fucoxanthin are other bioactive compound present in the brown algae responsible for antioxidant activities (Chung *et al.*, 2002). Phlorotannins from brown algae has been reported to possess strong antioxidant activity than other polyphenols derived from terrestrial sources (Ahn *et al.*, 2007, Hemat 2007).

The total flavonoid present in the *P. gymnospora* was found to be 54.20 ± 0.16 mg Quercetin/grams equivalents. Few earlier studies demonstrated the presence of Flavonoid content in the seaweeds. Flavonoids act as potential antioxidants depend upon its molecular structure. The position of the hydroxyl groups and the other features in the structure plays a role in the antioxidant and free radical scavenging activities. Flavonoids are non-nutritive compound present

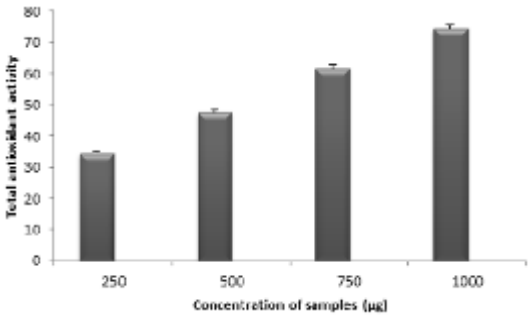


Fig. 1. Total antioxidant activity of *P. gymnospora*

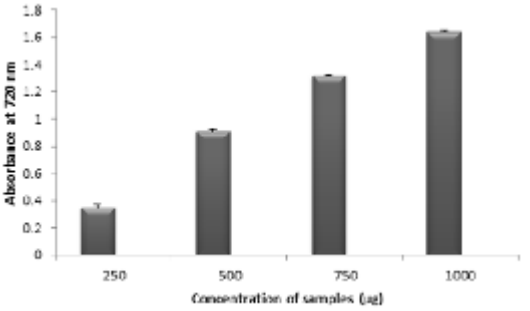


Fig. 2. Reducing potential of *P. gymnospora*

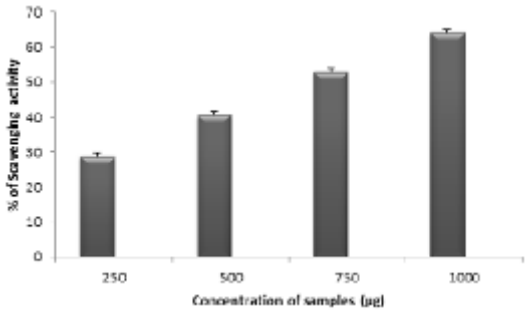


Fig. 3. DPPH scavenging activity of *P. gymnospora*

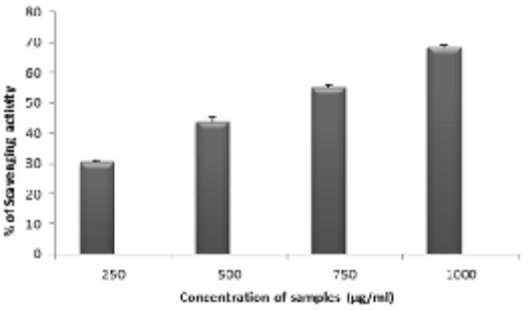


Fig. 4. Superoxide anion scavenging activity of *P. gymnospora*

in most of the seaweeds and they may help in the protection of various diseases alone or along with the antioxidant enzymes, vitamins to the total antioxidant defence of the body (Meenakshi *et al.*, 2009). The results of the present study also show that there is a strong correlation between the antioxidant property and the flavonoid, phenolic content.

Total antioxidant activity of the *P.gymnospora* extracts were determined by the Phosphomolybdneum method based on the reduction of Mo (VI) to Mo (V) and the formation of green phosphate/Mo (V). These results shows petroleum ether extract exhibited increased antioxidant activity with increasing concentration as shown in the fig 1. Antioxidant potential of ascorbic acid has been used as reference standard with *P. gymnospora* extract antioxidant activity is compared (Aliyu *et al.*, 2012).

Antioxidant activity depends upon the concentration was evaluated as a function of reducing power as this gave general view of reductones present in the sample. Increase in absorbance indicates the increasing reducing power. Reducing power increases with increasing concentration of sample as shown in the fig 2. Similar findings have also been reported by researchers (Kuda *et al.*, 2007). Yuwada *et al.*,2010 reported Brown algae *Padina minor* from Andaman sea possessed strong antioxidant activity and anti-inflammatory activity. This property is associated with the presence of reductones that are reported to be terminators of free radical chain reaction (Duh *et al.*, 1999).

DPPH method is a quick, reliable and reproducible assay for investigating the *in vitro* antioxidant activity (Ara *et al.*, 2010). Rapid discolouration of the purple stable DPPH to light yellow diphenylpicryl hydrazine, suggesting that the radical scavenging activity due to its proton donating ability (Aliyu *et al.*, 2012) is seen. The activity of antioxidants on DPPH is due to their

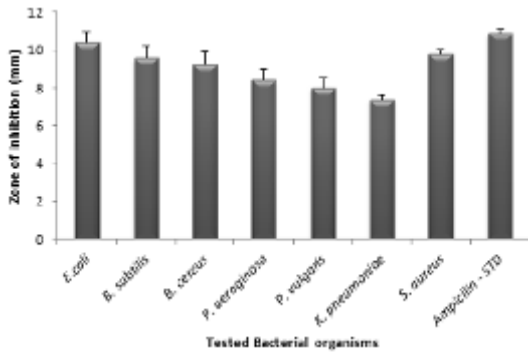


Fig. 5. Antibacterial activity of *P. gymnospora*

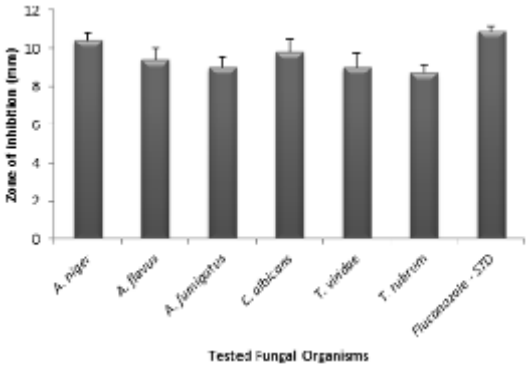


Fig. 6. Antifungal activity of *P. gymnospora*

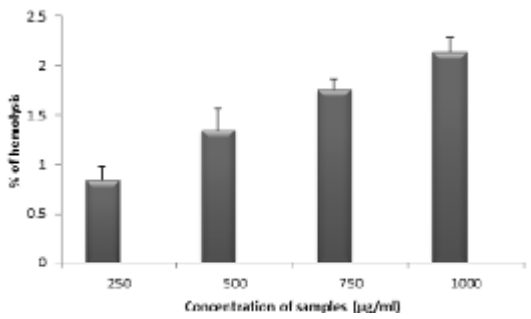


Fig. 7. Hemolysis activity of *P. gymnospora*

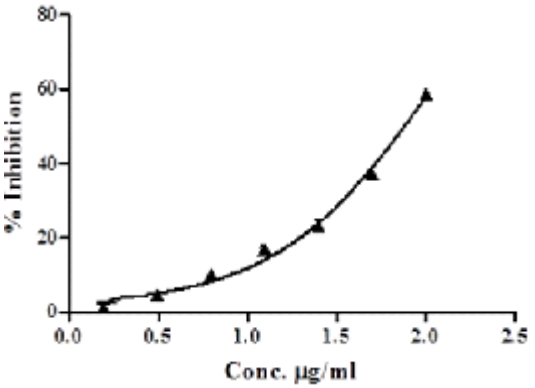


Fig. 8. Viability of Hel 92.1.7 leukaemia cell line after the treatment with *P. gymnospora*

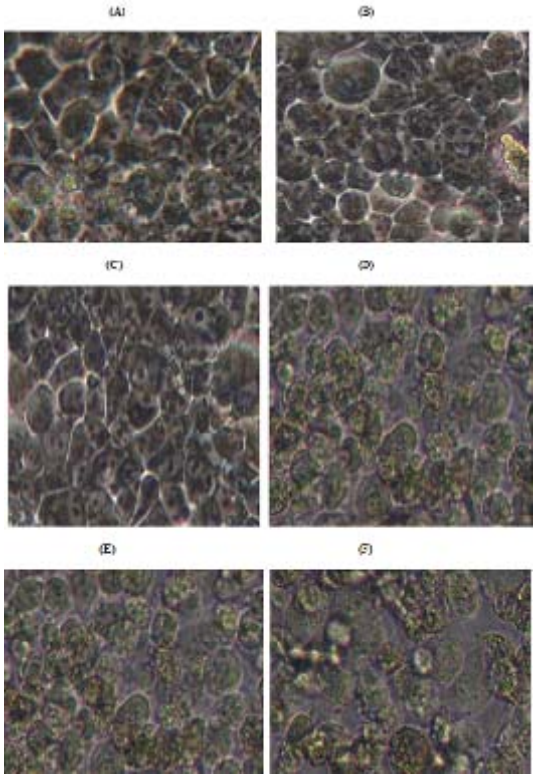
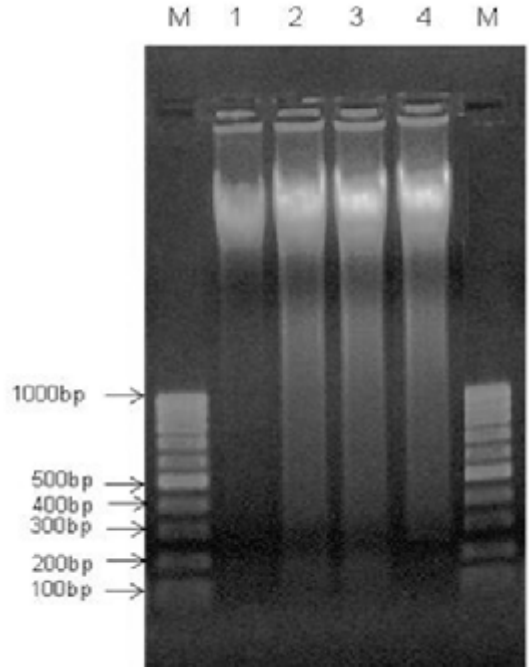


Fig. 9. Morphological changes induced by crude extract of *P. gymnospora* in Hel 92.1.7 leukaemia cell line. (A) Control (B) 6.25 (C) 12 (D) 25 (E) 50 (F) 75 G) 100 (µg/ml)



M- 100bp DNA ladder; 1-Control (medium); 2-Camptothecin treated; 3-Doxorubicin treated; 4-Sample treated

Fig. 10. 1% Agarose gel electrophoresis of DNA.

hydrogen donating capability (Gheraff *et al.*, 2011). DPPH radical scavenging activity of *P. gymnospora* extract as presented in the fig 3. The percentage of scavenging activity increases with increasing concentration of sample. These results clearly indicates concentration depend scavenging activity. The findings of the present study are similar to the findings of Wang *et al.*, 2009 who have also found that brown algae contained higher amount of polyphenols and DPPH radical scavenging activity.

Superoxide scavenging activity may be one of the potential health needs as it may be effective in reducing the level of 0^{2-} which is produced during the oxidative stress in the body. 0_2^-mediated stress is believed to be involved in the pathogenesis of cardiovascular and neurogenerative disorders (Singh 2006, Vijayabaskar and Shiyamala 2010). The *P. gymnospora* extracts were investigated for the superoxide scavenging assay and the results were clearly presented in the figure 4. Percentage inhibition of superoxide radical generation of different concentrations of extract were determined and compared with the standard α-tocopherol. The percentage inhibition of superoxide generation by 1000µg/ml was found to be 62.08%.

Antimicrobial activities of the crude petroleum ether extract of *P. gymnospora* against seven bacterial strains and five fungal strains were done and their zone of inhibition were measured and compared with standard antibiotic ampicillin and fluconazole. The *P. gymnospora* extract has shown the maximum activity against the bacterial strains and fungal strains as shown in the figure 5,6. Antimicrobial activity may be due to the presence of flavonoids and phenolic compounds (Murugan *et al.*, 2013). These results are supported by the findings of Shanumuga Priya *et al.*, 2008 where *Padina sps* and other seaweeds showed maximum activity against the multiresistant pathogens. Rosett and Srivastava have found

similar antibacterial activity with methanol and ethyl ether extract of brown algae. Similarly, Brown algae has been reported the higher antimicrobial activity than green and red algae. (Rao 1995, Selvi and Selvaraj 2002).

Haemolytic activity of the crude petroleum ether extracts of *P. gymnospora* were screened against normal chicken erythrocytes. Haemolytic activity of the plant is expressed in % of hemolysis and is expressed as mean ± SEM. Extracts exhibited haemolytic effect towards human erythrocytes. Haemolytic activity was found to be increasing with increase in concentration as shown in the fig 7.

Hel 92.1.7 leukaemia cell line was used to assess the cytotoxic activity of petroleum ether crude extract of *P. gymnospora.* Extracts inhibited the growth of Hel 92.1.7 cells in a dose dependent manner. It suppresses the growth of cells at the various concentrations after the 48 hr treatment as shown in the table 2. The IC50 value (Concentration causing 50% inhibition of the growth of the cell was used as parameter for cytotoxicity (Smith *et al.,* 1995). This test showed that the crude extract was toxic to the Hel 92.1.7 leukaemia cells and IC_{50} for Sample was found to be 102.3 µg /ml. The morphological changes after the treatment of extract as compared to control are shown in the figure 8. The main aim of an anticancer compound is to trigger the apoptosis signalling system in these cancer cells leads to the failure of proliferative activity (Lee *et al.*, 2004). These results revealed that the morphological changes were typical of apoptosis. Hence the crude petroleum ether extract has shown a remarkable effect on Hel 92.1.7 leukaemia cell line. In order to confirm that the effect of the extract was due to the induction of cell apoptosis, DNA fragmentation was done on Hel 92.1.7 leukaemia cells after treatment (Figure 10). There was a smear and faint band of 300 bp in Camptothecin and Doxorubicin treated cells. This also observed with the sample treated cells, and control cells indicating that there is induction of apoptosis by the sample.

P. gymnospora extract induced the similar pattern of DNA ladder in Hel 92.1.7 leukaemia cell line as revealed by DNA gel electrophoresis. Similar results have shown previously by the researchers on the bioactivity of some brown algae. Several apoptotic events such as DNA fragmentation and chromatin condensation were observed (Hyun *et al.,* 2009).

CONCLUSION

In recent decades, marine algae have been drawn attention by the researchers worldwide because of the valuable resource. Marine algae become an alternative source for the bioactive compounds with broad spectrum of activities. Based on the results, it could be concluded that the brown alga *Padina gymnospora* collected from Mandapam, South east coast of India, contains a bioactive compound with wide range of activities. Hence, this finding provides the path to find a potential compound for future researchers and pharmacists for further exploitation of natural compound for the development of novel drugs.

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