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Research Article

PURIFICATION AND PHARMACEUTICAL APPLICATION OF BIOACTIVE NATURAL PRODUCT OF SEAWEED TREATED AGAINST MICROBIAL PATHOGENS

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ABSTRACT

Seaweeds are considered a source of bioactive compounds as they are able to produce a variety of metabolites with the broad spectrum of biological activities. The phytochemical analysis was performed to confirm the presence of phytochemical constituents and, carbohydrate, saponin, flavonoids, alkaloid, terpenoid, quinone and phenols were found positive. Antimicrobial activity was green seaweed was tested against clinical pathogens. A maximum inhibitory activity was recorded for *Escherichia coli* with the zone of inhibition 21mm in diameter. Besides the seaweed extracts was found to inhibit *Shigella flexneri*. The antifungal activity was tested against the selected pathogens; *Pencillium sp, Aspergillus niger, Aspergillus flavus, Aspergillus sp* and *Odium caricae*. The seaweed extract showed maximum activity against *Aspergillus sp* with zone on 41mm. The haemolytic activity of the extracted seaweed revealed a complete absence of haemolytic effect in blood agar plates. Functional groups modification was carried out with FTIR. The presence of aromatic compounds appeared in the seaweed extract such as, aromatic α, β, unsaturated aldehyde, amines, nitro compounds and amino groups were prominently seen. Wound healing studies in mice was carried out for 8 days between control and experimental treated mice. The seaweed extracts was found effective in wound closure which appeared to be complete during 8th day. A drastic difference between control and experimental treated mice was noted. Molecular analysis patterning to protein profile by SDS-PAGE in burn wound healed mice was observed for 8 days.

Keywords: Seaweeds, Phytochemical, Antimicrobial, Antifungal, Wound healing, FTIR, SDS-PAGE.

1. INTRODUCTION

Recently, seaweeds have received significant attention for their potential as natural antioxidants, antibacterial and cytotoxic properties. Seaweeds are also known to possess valuable medicinal components such as anticoagulants, antiangiogenic and antiadhesive activities [1]. Natural product is an organic compound that is synthesized from living organisms. Many natural products have very complex structures. The metabolic active compounds have already isolated from seaweeds. Marine algae are known as a potential source of bioactive substances. Natural products are often divided into two major classes; the primary and secondary metabolites. Primary metabolites are components of basic metabolic pathways that are required for life. They are associated with essential cellular functions such as nutrient assimilation, energy reduction and growth development. Primary metabolites involved with energy production include respiratory and photosynthetic enzymes [2].

Secondary metabolites were commonly used within the field of medicinal chemistry and pharmacology. Most of the secondary metabolites produced by seaweeds have bactericidal or the antimicrobial compounds derived from seaweeds consist of diverse groups of bacteriostatic properties, brominates phenols, oxygen heterocyclic, terpenols, sterols, polysaccharides, dibutenolides, peptides and proteins [3]. The function of these secondary metabolites was found to possess defense mechanism against herbivores, fouling organisms and pathogens chemicals defense mechanisms against herbivores. For example, grazer-induced mechanical damage triggers the production of chemicals that acts as feeding detergents or toxins in seaweeds [4].

Many algae highly produced primary and secondary metabolites may be these potential bioactive compounds are used in many pharmaceutical industry. The interest in marine organisms as a potential and promising source in marine organism has increased during the last decades

[5]. Marine algae were exploited mainly for the industrial production of phycocolloids such as agar-agar, alginate and carrageenan, not for health aspects. Bio stimulant properties of seaweeds are exploited for use in agriculture and the antimicrobial activities for the development of novel antibiotics. Seaweeds have some of the valuable medicinal components such as antibiotics, laxatives, anticoagulants, anti-ulcer properties, neurotoxins and suspending agents in radiological preparations. Algae and their extracts can be treasure of biologically active compound. Their beneficial properties for humans, animals and plants were recognized in the past and are appreciated nowadays, in the development of new biotechnological products. Seaweeds contain many different polysaccharides, whose chemical structure relates to the corresponding taxonomic classification of algae and their cell structure [6].

Many of them are soluble dietary fibres which have positive effect on digestive tract of animals. Also, seaweed derived polysaccharides are effective and non-toxic antioxidants. Among many different algal polysaccharides, the most important are galactans, fucoidan, laminarin and alginate [7]. Very important bioactive proteins can be extracted from macroalgae are lectins, which bind with carbohydrates and participate in many biological processes like intercellular communication. They have antibacterial, antiviral or anti-inflammatory activities. The protein content of brown seaweeds is generally less, whereas higher protein contents are recorded in green and red seaweeds [8].

Seaweed pigments can be divided into three major groups: chlorophylls, carotenoids and phycobiliproteins. Carotenoids are organic pigments present in chloroplasts and chromoplasts. Different species of algae contain different kinds of carotenoids, which are very strong antioxidants. The most important carotenoids are β-carotene, fucoxanthin and tocopherol. They constitute a few percent of algal dry weight phycobiliproteins have not only antioxidant, but also anti-inflammatory, antiviral and neuroprotective properties [9]. Wound is the disruption of cellular and functional continuity of living tissue, produced by physical, chemical, electrical or microbial insults to the tissue. Wound healing is the dynamic process take place by regeneration or repair of broken tissue. The healing cascade is activated when platelets come into contact with exposed collagen leading to platelet aggregation and the release of clotting factors resulting in the deposition of a fibrin clot serves as a matrix [10].

Seaweeds are considered as a source of bioactive compounds and they possess antiviral, antifungal, antibacterial, antihelminthic and wound healing properties. In the present study, the marine green seaweeds were collected from Gulf of Mannar and its antibacterial, antifungal and haemolytic activities were investigated. In addition, the wound healing activity of chloroform extract of seaweeds was evaluated in mice model.

2. MATERIAL AND METHODS

2.1. Collection of samples

The seaweeds were collected in bulk quantity from Mandapam coastal area, Gulf of Mannar. The collected samples were cleaned well with seawater to remove all the extraneous matter such as epiphytes, sand particles, pebbles, shells and brought to the laboratory in sterile bags. The samples were then washed thoroughly with tap and distilled water and spread in dark at room temperature for drying. After drying, the dried samples were powdered and subsequently stored at 4°C.

2.2. Extraction of seaweed extract by Soxhlet Apparatus

The chemical extraction was done by following the method [11]. Two (2)g of seaweeds powder was extracted in Soxhlet apparatus using chloroform (100ml) as solvent for 8 hours at 65°C. Soxhalation is a process of continuous extraction in which the same solvent can be circulated through the extracted several times. The process involves extraction followed by evaporation of the solvent. The vapours of the solvent were taken in a condenser and the condensed liquid will be returned to the same for continuous extraction.

2.3. Phytochemical Analysis

The phytochemical analysis for constiutents such as carbohydrates, tannins, saponins, flavonoids, alkaloids, quinones, glycosides, terpenoids, phenols, potein was studied [17].

2.4. Determination of antimicrobial susceptibility test

The antibacterial activity of seaweed extract obtained with chloroform was evaluated by the agar disc diffusion method [18]. The 24 hours old culture was inoculated for the assay. Four bacterial pathogens *i.e. Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa and Shigella flexneri* were used for antimicrobial study. A sterile cotton swab was dipped into the bacterial suspension and

then evenly swabbed over the entire surface of a sterile Muller Hinton agar plate to obtain uniform inoculums. The discs were punched on the seeded plates using a sterile cork borer and plates were allowed to dry for 5 minutes. The solvent extraction of chloroform extract was dispensed into each well using a sterile micro pipette. The Nalidixic acid disc was used as positive control. The plates were incubated for 24 hours at 37°C. The antibacterial activity was determined by measuring the diameter of zone of inhibition (mm).

2.4.1. Determination of antifungal activity for extracted seaweeds

The fungal pathogens were maintained on potato dextrose agar. Antifungal activity was determined against five fungal pathogens *i.e. Penicillium sp, Aspergillus niger, Aspergillus flavus, Odium caricae and Aspergillus sp,* using the agar disc diffusion method described by Mayer [12]. Young fungal cultures were incubated for 2-3 days at room temperature. The antifungal activity of seaweed extract obtained with chloroform was evaluated by the agar disc diffusion method. A sterile cotton swab was dipped into the fungal suspension and then evenly swabbed over the entire surface of a sterile, Muller Hinton Agar plate to obtain uniform inoculums. The discs were punched on the seeded plates using a sterile cork borer and the plates were allowed to dry for 5 minutes. The solvent extraction of chloroform extract was dispensed into each disc using a sterile micro-pipette. The penicillium disc was used as positive control. After 72 hours at 30°C the plates were observed for the presence of inhibition zones.

2.5. Haemolytic activity for extracted seaweeds

Haemolytic activity was performed in 5% sheep blood agar plates [13]. Fifty (50)µl of seaweed extract was spot inoculated on to blood agar plates and incubated for 24 hours at 37°C. After incubation, no zone around the seaweed extracts was noted.

2.6. Sample preparation of FTIR

FTIR analysis was performed using Perkin Elmer spectrometer system. The samples were prepared as follows: Dry (dehydrated) green seaweeds samples were analyzed, and the sample 25mg of green seaweed powder was mixed with few ml of ethyl alcohol and ground to obtain a fine paste were analysed in this technique. This technique was used to detect the characteristic peaks and their functional groups. The peak values of the FTIR were analyzed. Each and every analysis was repeated twice and confirmed.

2.7. Wound healing property of seaweed extract treated in mice

Mice were collected from the commercial market in Madurai and reared in laboratory. All animals had free access to pelleted food and water *ad libitium*. Temperature was maintained at 23±1°C.

2.7.1. Treatment

Animals were wounded under light chloroform anesthesia, aseptically. The animals were assigned into two groups. Group I animal received commercial ointment treatment. This was taken as control. Group II animal received seaweed extract ointment treatment. This ointment was prepared from green seaweed extract. No other topical or systemic therapy was given to animals during the course of this study.

2.7.2. Burn wound model

Hairs were removed from dorsal thoracic central region of anaesthetized mice. Full thickness from the marked area was burned to produce wound. Wound was cleaned with cotton swab soaked in alcohol. The two test formulations, commercial ointment and seaweed extract ointment were applied on wound once daily for 8 days starting from the first day of wounding; wound contraction was measured for 8 days.

2.8. Molecular weight determination in SDS-PAGE

The molecular weight of the granulation tissue of wound healing treated mice was determined by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) [14]. Polyacrylamide gel electrophoresis was done to separate proteins. The granulation tissue was separated using SDS-PAGE. The separation was done using 5% polyacrylamide stacking gel and 10% separating gel at a constant current for 3 hours. The gel was stained with Coomassie brilliant blue R250. The destaining was done using methanol and acetic acid in the ratio 5:1.

The gel electrophoresis apparatus was washed thoroughly with 10% SDS to remove the impurities. Stacking gel and separating gel was prepared using the above explained procedure. Spacer was washed thoroughly and it was then placed in the gel plate. Gel was prepared by pouring the separating gel at the bottom and stacking gel was poured on the top after polymerization of the separating

gel. Comb was placed immediately after pouring the stacking gel into the gel plate. The comb was removed carefully after the polymerization of stacking gel. The gel was kept in the gel tank and running carefully after the polymerization of stacking gel. The gel was kept in the gel tank and running buffer was added into the well. Then 20µl of sample was loaded into the well. Electrophoresis was carried out at 80v until the tracking dye reaches the end. The gel was separated from the gel plate carefully and it was stained in the staining solution for 3 hours. After 3 hrs, the gel was placed in the destaining solution so the excess stain get removed from the gel so that the bands can be visualized clearly.

3. RESULTS AND DISCUSSION

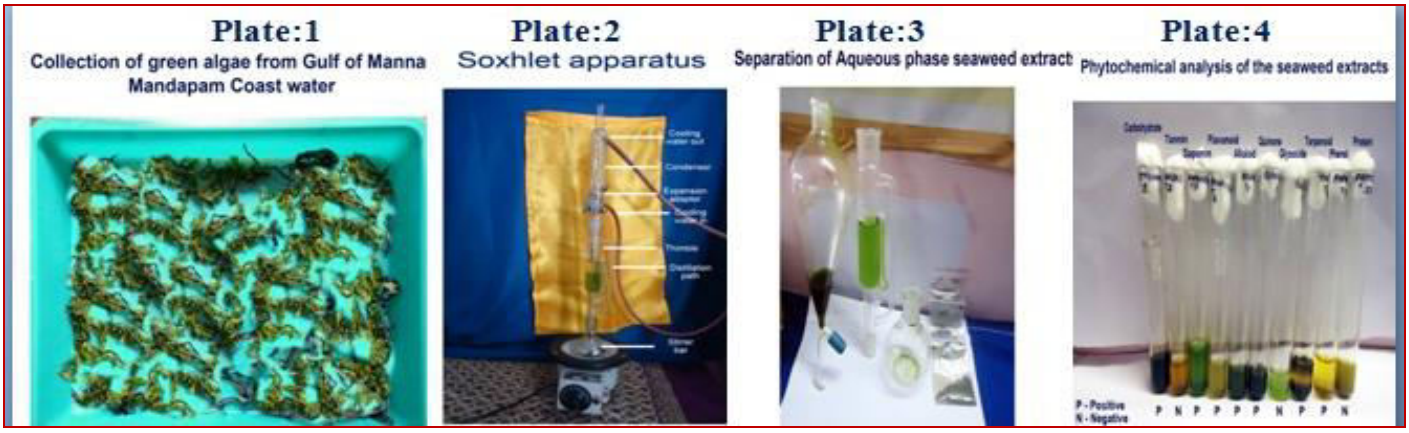
Seaweed was considered as a source of bioactive compounds as they are able to produce a great variety of secondary metabolites characteristics by a broad spectrum of biological activities.

3.1. Phytochemical analysis

In the present study, the marine green seaweed was collected from mandapam coast Gulf of Mannar (Plate: 1). The collected seaweeds were extracted by Soxhlet apparatus method (Plate: 2, 3). The seaweed extract was screened for the presence of phytochemical constituents. The phytochemical analysis of the seaweed extracts were depicted in Table 1. The extracts of green seaweed revealed the presence of carbohydrate, saponin, flavonoid, alkaloid, quinine, terpenoid and phenols (Plate: 4).

Table 1: Phytochemical analysis of seaweed (green seaweed-*Ulfa fasciata*)

S. No.	Characteristics	Observation
1	Test for carbohydrate	Positive
2	Test for Tannins	Negative
3	Test for Saponins	Positive
4	Test for Flavonoids	Positive
5	Test for Alkaloids	Positive
6	Test for Quinone	Positive
7	Test for Glycosides	Negative
8	Test for Terpenoids	Positive
9	Test for Phenols	Positive
10	Test for Protein	Negative



3.2. Antimicrobial activity of green seaweed extracts

The bacterial infection is one of the most serious global health issues in the 21st century. In the present study, the chloroform extract of green seaweed was investigated for antibacterial activity. According to the experimental result, the chloroform extract of green seaweed inhibited most of the tested bacterial pathogens. The results are presented in table 2. Seaweed extract revealed maximum inhibitory effect against *Escherichia coli* with 21mm in diameter, followed by *Staphylococcus aureus* with the inhibition zone of 20mm. The extract was also effective against the gram negative bacterial pathogen *Shigella flexneri*. The extract was not effective against the gram negative bacterial pathogen *Pseudomonas aeruginosa* (Plate: 5).

Table 2: Determination of antimicrobial susceptibility

S. No	Test organism	Zone of Inhibition	
		Standard	Extract
1	*Escherichia coli*	5mm	21mm
2	*Shigella flexneri*	9mm	17mm
3	*Pseudomonas aeruginosa*	No zone	No zone
4	*Staphylococcus aureus*	No zone	20mm

3.2.1. *Determination of antifungal activity of seaweed extract*

In the present study, the seaweed extract was evaluated for antifungal activity against fungal pathogens like, *Pencillium sp, Aspergillus niger, Aspergillus flavus, Odium caricae* and *Aspergillus sp*. The results were noted in table 3. The investigation made on chloroform extracts showed maximum activity against *Aspergillus sp* with 41mm. Similar observation made from chloroform extract showed an activity of 18mm against *Aspergillus niger* which is followed by an activity with zone of inhibition 15mm and 12mm against *Odium caricae* and *pencillium sp*, whereas seaweed extract against *Aspergillus flavus* produced a complete absence of zones (Plate: 6).

Table 3: Determination of antifungal activity for extracted seaweeds

S. No	Test organism	Zone of Inhibition	
		Standard	Extract
1	*Pencillium sp*	No zone	12mm
2	*Aspergillus niger*	No zone	18mm
3	*Aspergillus flavus*	No zone	No zone
4	*Odium caricae*	2mm	15mm
5	*Aspergillus sp*	12mm	41mm

3.3. Haemolytic activity for extracted seaweeds

In order to access to ability of crude extract of green algae against normal erythrocytes, the haemolytic activity was performed in Blood agar plates. The results are shown in Table 4. The results revealed that the chloroform extract of green algae revealed no significant haemolytic effect towards the erythrocytes (Plate: 7).

Table 4: Haemolytic activity for extracted seaweeds

S. No	Test organism	Zone of inhibition (Extract)
1	Sheep Blood	No zone

3.4. FTIR Analysis

FTIR analysis was performed using Perkin Elmer Spectrophotometer. This technique was used to detect the characteristics peaks and their functional groups. The spectrum was used to identify functional group of active components based on peaks in the region of infrared. The prominent peak of 3886 $^{cm-1}$ indicated C-H stretching of methyl groups. Another peak at 2926.01 $^{cm-1}$ revealed the presence of alkenes. The results of FTIR with subsequent peaks where summarized. The peaks include 1836 $^{cm-1}$, 1649 $^{cm-1}$, 1423 $^{cm-1}$ and 1251 $^{cm-1}$ followed by 623^{cm-1}confirmed the presence of functional group such as aromatic α, β, unsaturated aldehyde, amines, nitro compounds and amino groups (fig.1).

Similarly, FTIR spectrum denoted prominant peaks when treated with organic solvent ethyl alcohol and the peaks include 3419 $^{cm-1}$, 2927 $^{cm-1}$.1653 $^{cm-1}$, 1427 $^{cm-1}$, 1055 $^{cm-1}$ and 646 $^{cm-1}$ presumed to be functional groups of aromatic aldehydes, carboxylic acid and nitro compounds (fig.1, 2).

3.5. Wound healing activity of seaweed extract on wound closure

Table 5 and fig. 3, depict wound healing activity in mice in burn of wound model treated with chloroform

extract of green seaweed. A significant reduction in the wound area was observed in control and experimental treated samples. Post wounding days were carried out with different the interval such as 1, 2, 4, 6 and 8th day. The control revealed 3.5cm of wound whereas a treatment extract showed 3.4cm during 1st dayand 2nd &4th day with control treated mice was found to exhibit 3.3cm and 3cm whereas extract treated samples showed 3cm and 2.6cm. On 6th day control treated mice was found to exhibit 2.7cm whereas extract treated samples showed 2.1cm. During 8th day wound closure in control treated mice revealed 1.0cm whereas extract treated revealed 0.7cm (Plate: 8, 9).

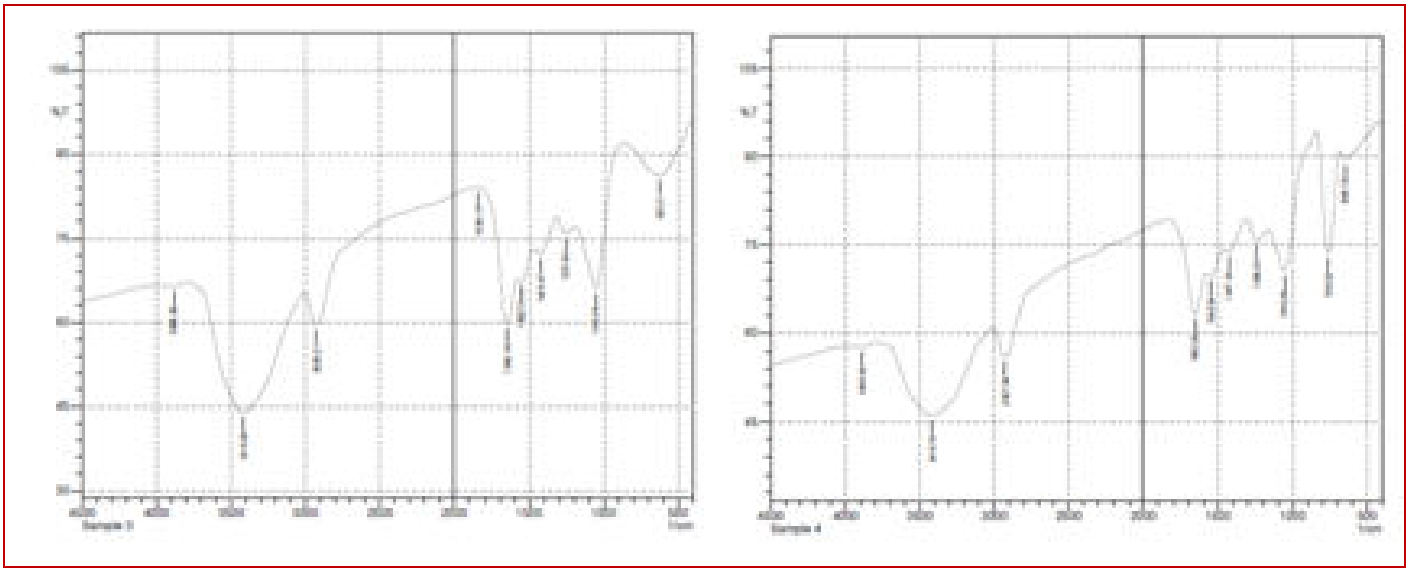


Fig: 1 and Fig: 2 FTIR analyses

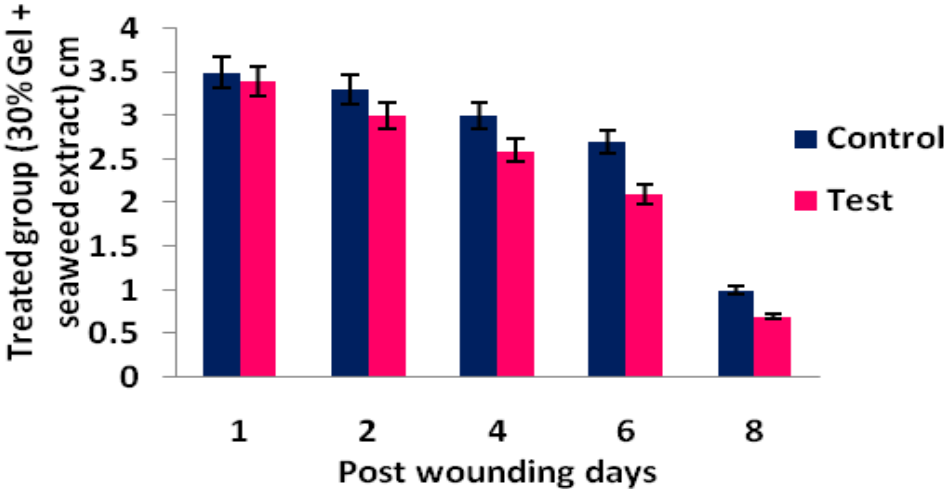
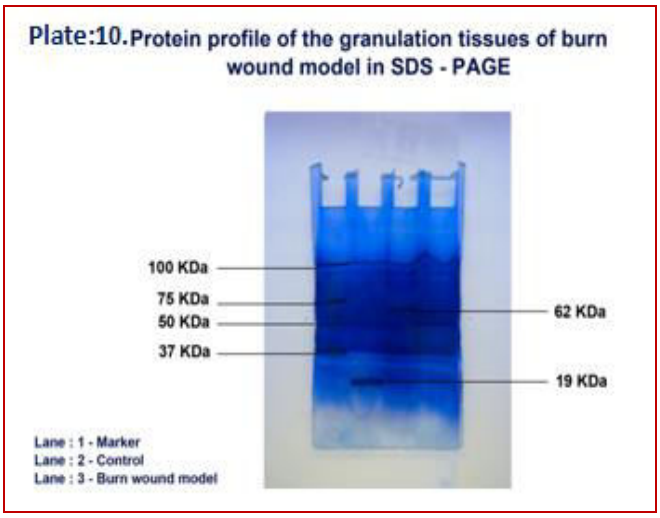


Fig. 3: Wound healing activity of seaweed extract on wound closure

3.6. Molecular weight determination in SDS-PAGE by burn wound healed mice

In burn wound model, granulations of tissues were excised after 8th day of wounding. In burn wound model, the molecular weight of protein was in variable in control mice whereas similar protein was observed in both the sea weed extract treated mice and standard treated wound mice. In sea weed treated groups as well as in standard drugs treated wounds protein profile was identical and particular protein of molecular weight of 62 KD were expressed in burn wound mice. A prominent appearance of protein with molecular weight of 19 KD was observed only in control mice which were absent in other groups (Plate: 10).



It is important to note that throughout period of wound treatment, the extract did not cause irritation or pain to the animals as the rats neither show any signs of restlessness nor scratching/biting of wound site when the extract were applied. In the present study, all the surgical interventions were carried out under sterile conditions and the mice were closely observed for any infection; those which showed signs of infection were separated and excluded from the study. The control of microbial infection of mice is necessary for better healing and its management [15].

Wound healing is a complex and dynamic process of restoring cellular structures and tissue layers in damaged tissue as closely as possible to its normal state. Wound contracture is a process that occurs throughout the healing process, commencing in the fibroblastic stage where by the area of the wound undergoes shrinkage. In the maturational phase, the final phase of wound healing the wound undergoes contraction resulting in a smaller amount of apparent scar tissue [16].

The preliminary phytochemical analysis of the chloroform extract of green sea weeds revealed the presence of carbohydrates, saponins, flavonoids, alkaloids, quinones, terpenoids, and phenols are known to promote the wound healing process mainly due to their astringent and antimicrobial property, which seems to be responsible for wound contraction and increased rate of epithelisation. Flavonoids are known to promote the wound healing process mainly due to their antimicrobial properties, which appear to be responsible for wound contraction and increased rate of epithelialization [17].

The algal crude extracts and active constituents of various algae have been shown to have antibacterial activity in vitro against Gram-positive and Gram-negative bacteria [18]. Seaweeds contains various compounds, such as polysaccharides, proteins, lipids, aminoacids, alkaloids, sterols and phenolic molecules, some of them show bioactivity against microorganisms [19] and viruses [20]. Presence of alkaloids and phenolic compounds may affect growth and metabolism of bacteria. They could have an activating or inhibiting effect on microbial growth according to their constitution and concentrations [21].

The seaweeds have an effective antibacterial activity against most of the human pathogenic bacteria. It was reported that 151 species of marine algal crude extracts showed inhibitory activity against pathogenic bacteria [22]. In the present study, *Ulfa fasciata* showed good antibacterial activity against gram positive bacteria such *staphylococcus aureus* in chloroform. Our results were in accordance with the findings of Goto [23].

In the present work, the crude extract of green seaweed was evaluated for antifungal activity against fungal pathogens. *Ulfa fasciata* showed maximum inhibition against *Aspergillus sp* with 41mm. Our results were similar to the findings of Kayalvizhi [24].

Limitation of extracts to be used in therapy is their potential to cause damage to mammalian cell. In the present study the crude extract of *Ulfa fasciata* produced pronounced haemolytic activity on sheep erythrocytes. Similar findings were reported by Pangestuti and Kim [25].

In the current study, topical application of the seaweed ointment and the extract given orally in varying concentration capable of producing signifying wound healing activity. Wound healing in mice lead to granulation tissue formation. Folded by re-epithelialisation, keratinocytes and migration from wound edge and wound bed and proliferate. A few days after injury, dermal, fibroblasts were stimulated by growth factor, as

TGB and PDGE to proliferate and migrate and to secrete extracellular matrix protein. This serves to physically close wound gap [26].

In burn wound model, a unique similarly was observed in protein profile of *Ulfa fasciata* and control treated group. It is assumed that the mechanism by which *Ulfa fasciata* enhanced the healing process. The protein with the molecular weight of 62 KDa was expressed in seaweed extract treated group indicated that this particular protein might be involved in the wound healing activity. Similar observations were reported in the *Acyranthes aspera* [27] Hippophae rhamnoides treated group [28] where granulation tissue also showed differential expression of some proteins as compared with the control and burn wound model.

4. CONCLUSION

Marine algae have several active components such as proteins, lipids, alkaloids, phenols etc. The results clearly demonstrated that seaweeds are an interesting source for biologically active compounds that may be applied for therapy of bacterial and fungal diseases instead of or in addition with the commercial antibiotics. It was also concluded that the chloroform extract of *Ulfa fasciata* had showed the significant wound healing activity. As infections being a major cause of morbidity and mortality in wound patients this seaweed extract may prevent infection that leads to high risk of sepsis and thereby prevents the prolongation of inflammatory phase. This seaweed extract may provide a better alternative of the infection management in the process of wound healing.

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