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Research Article

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Development of wound dressing film using methanolic extracts of freshwater microalgae and determining its wound healing ability

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ABSTRACT

Wound healing is a complex process in which bacterial infection is a major cause of delayed wound healing. The occurrence of drug resistance among bacterial pathogens has led to the discovery of new antimicrobial agents from new sources. The present study aimed to identify microalgal metabolites with antibacterial activity and to develop a wound dressing film with their potential healing activity. Microalgal samples were collected from three different freshwater habitats, isolated, making them pure cultures, and the physico-chemical properties of water samples from the respective sampling sites were analyzed. Among the three microalgal isolates, *Chlorella* sp. NRMC-F-0350 showed antibacterial activity against clinical isolates of *E. coli*, *Klebsiella pneumoniae* and *Staphylococcus aureus*. Additionally, functional groups present in *Chlorella* sp. NRMC-F-0350 was identified using FTIR analysis. GC-MS analysis identified several antibacterial compounds, *viz.* benzoxazole, 1,2 Benzene dicarboxylic acid, sistosterol, 9-Octadecanoic acid, eicosane and hexadecane. Wound dressing films were developed and showed evident antibacterial as well as significant wound healing activity (84.5%). Therefore, the developed films can be used as a potential wound dressing material.

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INTRODUCTION

A wound can be described as the anatomic destruction of a tissue that may be caused by physical, chemical, thermal, microbial, or immunological damage. Wound healing is the re-establishment of the anatomy and function of an injured tissue for the purpose of restoring approximate prewound characteristics. Based on the repair process, wounds can be of two types: acute or chronic wounds. Acute wounds are tissue injuries that mostly tend to heal entirely, usually within a time period of 8–12 weeks and with the least scarring. Chronic wounds generally tend to reoccur and have a healing time extending beyond 12 weeks (Gonzalez *et al.*, 2016). One of the major factors for wound healing is bacterial colonization and infection. Bacterial infection in wounds can be caused by a broad range of pathogens such as *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella* sp, *Streptomyces* sp, *Propionibacterium* sp. and *Coryneybacterium* sp. (Edwards and Harding, 2004).

Infection in a wound causes a large barrier to healing and can have a bad effect on the patient's quality of life and also the healing rate of the wound. Infected wounds are very painful and hypersensitive. That also results in increased inconvenience for the patient. The most common microorganisms which have known to be associated with wound infection include *S. aureus*, which was considered in several studies, has been found to account for 20-40% and *P. aeruginosa*, which accounts for 5-15% of the nosocomial infections, with this infection can cause burns and surgery. Many other pathogens such as Enterococci and members of Enterobacteriaceae have been involved, especially in immune compromised patients that lead to abdominal surgery (Stojadinovic *et al.*, 2008).

Wound repair needs a sterile or healthy environment for the body to function normally. That will result in a good healing process with very little scar formation. The important procedure required to keep the process of relieving in progress is to sterilize

the wound from any microbial infection (Mubarak *et al*., 2013). Repeated usage of different kinds of antimicrobial agents has caused pressure that results in the origination of antibiotic resistant microbial strains, which, in turn, has made research complicated, which causes continued search for new antimicrobial agents. Unfortunately, the decreased rate of new drug discoveries and the very high cost of finding new effective antimicrobial agents make the condition increasingly worrisome. Due to the multiple antibiotic resistance activities of wound pathogens, there is a special need to discover new antibacterial drugs from different natural sources to manage such wound infections (Simes *et al*., 2018).

Researchers have found that microalgae have a significant attraction as a natural source of bioactive molecules, because they have a high potential to synthesize bioactive compounds in culture, which are very hard to generate through artificial means of chemical synthesis (Thajuddin & Subramanian., 1994; Parveez *et al*., 2017). Many of those compounds are accumulated inside the microalgal cells; others are excreted during growth into the environment. The rapid growth and simple nutrient requirements attracted researchers to explore the potential of microalgae species all over the world. To survive against microbial pathogens, they need to develop tolerance/defence mechanisms. These strategies resulted in the synthesis of a high diversity of antimicrobial compounds with different mechanisms of action. Microalgae contribute to the countless chemical or bioactive compounds that they are able to synthesize because of their varied span in an ecosystem. Hence, microalgae are the biggest hope for discovering new types of metabolites. It has been discovered that microalgae have many bioactive compounds which also have guaranteed anticipatory applications, such as antifungal, anticancer, and antibacterial activity (Thangaraj *et al*., 2020; Dhanasekaran *et al*., 2020). Therefore, the present work focuses on the development of novel wound dressing films from freshwater microalgae extracts against bacterial pathogens. The developed films were tested for their wound healing ability.

METHODOLOGY

Physico-chemical, pure culturing and microscopic analysis

Samples were collected in and around Tiruchirappalli, Tamilnadu in the year 2020 from three sites. Water and microalgae samples were collected from ponds, lakes, and rivers in both plastic vials and polyethylene bags with code numbers concerning the place and area of collection.

Pure Culture and Microscopic Analysis

The properties of the collected water samples were identified by its evaluation of physico-chemical and biological parameters. The analysis such as pH, alkalinity, total alkalinity, calcium, magnesium, chloride, nitrate, nitrite, total phosphorous, and inorganic phosphorousby using standard methods. The collected microalgal samples were isolated and transferred to tubes and then to conical flasks with BG 11 medium (Rippka *et al*., 1979). They were maintained under fluorescent lamps (1400lux): 14+10L/D at room temperature and observed under a microscope for topological characterization.

Extraction of Bioactive Metabolites

The isolated cyanobacterial cultures were cultivated in the medium and the dried biomass was sonicated with liquid nitrogen and then the extraction procedure was carried out with 95% methanol (Mubarak *et al*., 2008). The extracts from microalgae were centrifuged at 4000 rpm for 10 min, and then they were further concentrated in a vacuum with reduced pressure. The stock solutions of extract were prepared in DMSO at 50 mg ml^{-1} for testing the antimicrobial activity.

Antibacterial Analysis

The antibacterial sensitivity rate of the crude extracts was tested against the bacterial strains *viz Escherichia coli*, *Staphylococcus aureus* and *Klebsiella pneumoniae* by the well diffusion method. agar (Beef extract: 0.2g, Acid digest of casein: 1.75g, Starch: 1.5g, Agar 1.5g; Total pH: 7.3 0.1for 100ml) was prepared and sterilized and poured into plates. Test pathogens of the overnight cultures were grown and 0.1% of the liquid culture of each test organism was streaked with the help of a cotton swab throughout the Petri plate by rotating the plate at different angles. Wells were cut using a 6mm borer on the agar surface of each plate. Crude extracts collected from cyanobacteria were loaded into the wells and the plates were incubated in an incubator at 37oC for 48h. The antibacterial activity was determined by measuring the inhibitory zones around the wells on all the plates with test pathogens. The formation of clear zones was observed and measured in millimetres (Dhanasekaran *et al*., 2005).

FTIR Analysis

FTIR analysis is used to determine the functional groups present in the microalgae extracts. The scanning was done at a frequency wavelength of 400-4000 cm-1. The scanning result was displayed in transmission analysis.

GC/MS Analysis of Crude Extract

Methanol extracts were examined by GC/MS at TÜV SÜD South Asia Pvt. Ltd. The GC/MS analysis was performed using a Thermo Scientific, Trace GC Ultra/ISQ Single Quadrupole MS, TG5MS fused silica capillary column (15 m, 0.251mm, 0.1 mm film thickness). An electron ionization system with an ionization energy of 70 eV was used for GC/MS detection. Helium gas was used as the carrier gas at a constant flow rate of 1mL/min. The injector and MS transfer line temperature were set at 280°C. The identified components were quantified and investigated using a percent relative peak area. A tentative identification was made of the compounds based on the comparison of their relative retention time and mass spectra with those of the NIST, WILLY library data of the GC/MS system.

Preparation of Wound Dressing Film

The wound dressing film was developed by the modified method described by Natarajan *et al*., 2018. Polyvinyl alcohol (2%) is mixed with distilled water and stirred at room temperature. Under stirring conditions, 2% of microalgae extracts were added drop wise. 2% citric acid is added to the solution and stirred for 30mins. The films were poured into a plastic petri dish and kept at 60°C for overnight.

Antibacterial Activity of Wound Dressing Film

The antibacterial activity of the wound dressing film was evaluated against the three test organisms –*Escherichia coli*, *Staphylococcus aureus* and *Klebsiella pneumoniae*. The Muller-Hinton agar was prepared and sterilized and poured into plates. Test pathogens of the overnight cultures were grown and 0.1% of liquid culture of each test organism was streaked with the help of a cotton swab throughout the Petri plate by rotating the plate at different angles. Films were cut into 2cmX2cm sizes and placed on the plates. The plates were incubated in an incubator at 37oC for 48h. Films without extracts were used as a control.

Wound Healing Assay

The L929 cell lines were seeded into 24-well tissue culture dishes containing coverslips pre-coated with collagen type I (40 g/ml) for 2 h at 37 C, at a concentration of 3 10^5 cells/ml and cultured in a medium containing 10% FBS to nearly confluent cell monolayers. Then, a linear wound was generated in the monolayer with a sterile 100 l plastic pipette tip. Any cellular debris was removed by washing the coverslips with phosphate buffer saline (PBS). DMEM medium with dimethyl sulfoxide (0.25%) (control group), platelet derived growth factor (2 ng/ml) (as positive control), the commercial *Hypericumperforatum* oil (100l) and the crude extracts (5 to 100 g/l) were added to coverslips per dose and incubated for 12 h at 37°C with 5% CO_2. The cells were fixed with 4% paraformaldehyde for 15 min and stained with 4,6-diamino-2-phenylindole (DAPI) overnight. Three representative images from each coverslip of the scratched areas under each condition were photographed to estimate the relative migration of cells. The data was analysed using CellC software.

RESULTS

Physico-chemical analysis of water samples

Total of three water samples were collected and used for the analysis of physic-chemical properties. Physico-chemical analysis of water samples exposed the pH range from 6.5 to 7.3, alkalinity from 32 mg l^{-1} to 116 mg l^{-1}, total alkalinity from 208 mg l^{-1} to 632 mg l^{-1}, calcium from 528 mg l^{-1} to 2256 mg l^{-1}, magnesium from 320 mg $l^{-1}$to 720, chloride from 15.99mg l^{-1} to 103.99mg l^{-1}, nitrate from 8.2mg l^{-1} to 9.8mg l^{-1}, nitrite from 2.7mg l^{-1} to 5.3mg l-1, total phosphorous from 2.8mg l-1 to 5.4mg l-1, inorganic phosphorous from 4.2mg l-1 to 5.7mg l-1 (Table 1).

Isolation of Pure Culture and Microscopic Analysis

Water samples were plated directly on BG 11 media. The colonies showing predominant growth were selected and pure cultures were grown and maintained. About three microalgal species were isolated and observed under light microscopy to determine their shape and morphological characteristics. Figure 1, shows the microscopic images of the isolated *Phormidium* sp. is found to be dark green pigmented and filamentous in nature. In Figure 2, the pure culture of *Oscillatoria* sp. is green pigmented and filamentous. Figure 3 shows the pure culture of a single-celled, spherical, green *Chlorella* sp. NRMC-F-0350

Antibacterial Activity of Extracts

Antibacterial activity of the methanolic microalgae extracts was determined using well diffusion method. Three pathogens

Table 1: Physico-chemical analysis of water samples

S. No	Properties	Area 1	Area 2	Area 3
1	pH	6.7	6.5	7.3
2	Alkalinity (mg l^{-1})	116	76	32
3	Total alkalinity (mg l^{-1})	632	592	208
4	Calcium (mg l^{-1})	2256	696	528
5	Magnesium (mg l^{-1})	520	720	320
6	Chloride (mg l^{-1})	103.99	43.99	15.99
7	Nitrate (mg l^{-1})	9.8	8.2	9.1
8	Nitrite (mg l^{-1})	4.6	5.3	2.7
9	Total phosphorous (mg l^{-1})	3.3	2.8	5.4
10	Inorganic Phosphorous (mg l^{-1})	4.2	5.7	5.3

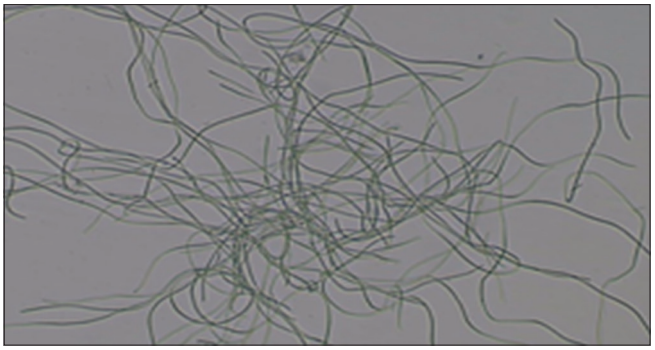

Figure 1: Microscopic analysis of *Phormidium sp.*

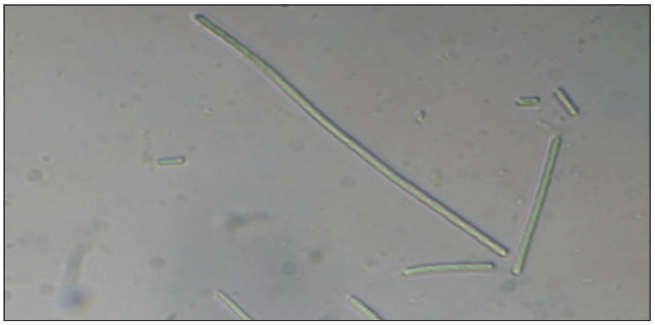

Figure 2: Microscopic analysis of *Oscillatoria sp.*

(*Escherichia coli*, *Staphylococcus aureus* and *Klebsiella pneumoniae*) were used to evaluate its antibacterial potential. From the analysis, two microalgal (*Phormidium* sp. and *Oscillatoria* sp.) extracts showed no zone of inhibition against all the three test pathogens. Whereas, *Chlorella* sp. NRMC-F-0350 showed antibacterial activity of 29mm against *Escherichia coli*, 24mm against *Staphylococcus aureus* and 27mm against *Klebsiella* pneumonia. Inhibitory zones were found to be higher for Gram negative pathogen than the Gram positive pathogen (Table-2). Therefore, *Chlorella* sp. NRMC-F-0350 extracts were used for further analysis and preparation of wound dressing films.

Fourier-transform Infrared Spectroscopy (FTIR) Analysis of *Chlorella* sp. NRMC-F-0350 Extract

FTIR analysis is used to determine the functional groups present in the extracts of selected microalgal species. Figure 4 shows the absorption bands observed in the analysis of *Chlorella* sp. NRMC-F-0350 extract. Absorption bands at 3759cm^{-1}, 3665cm^{-1}, and 3562cm^{-1} denote OH functional groups. Absorption bands at 2362cm^{-1}, 2313cm^{-1}, 1185cm^{-1}, 1056cm^{-1}, 891cm-1, and 846cm^{-1} correspond to ester (lipid) functional groups. A medium absorption band at 716cm^{-1} denotes a benzene derivative and a strong absorption band at 624cm^{-1} corresponds to C-S stretching (Table 3).

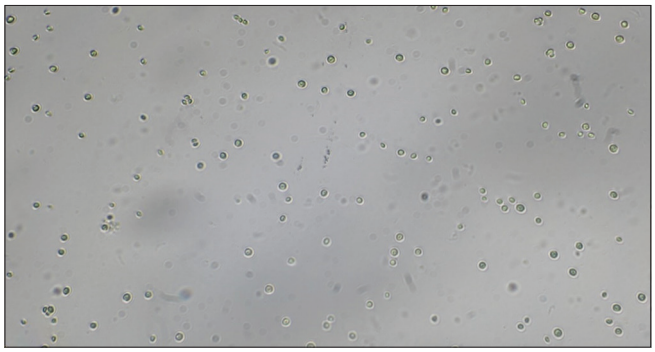

Figure 3: Microscopic analysis of *Chlorella sp. NRMC-F-0350*

GC-MS Analysis of *Chlorella* sp. NRMC-F-0350 Extracts

The bioactive compounds present in the *Chlorella* sp. NRMC-F-0350 extracts were identified by using GC-MS analysis. Figure 5 showed the chromatogram and Table 4 represents the bioactive compounds present in the *Chlorella* sp. NRMC-F-0350 extract. The major bioactive compounds present in the extracts were found to be Butanoic acid, 3-methyl-, 2-methylbutyl ester,Carbamic acid, methyl-phenyl ester, Benzene, 1,3-bis(1,1-dimethylethyl)-, Hexadecane, Pentanoic acid, 9-Hexadecenoic acid, Eicosane, 9-Octadecanoic acid, Hexanedioic acid, 1-Triacontanol, Gamma sististerol, 1,2 Benzene dicarboxylic acid, Stigmatane and Benzoxazole.

Table 2: Antibacterial activity of microalgae extracts

S. No	Sample	Inhibitory Zone (mm)		
		E. coli	*S. aureus*	*K. pneumoniae*
1	*Phormidium sp.*	0	0	0
2	*Oscillatoria sp.*	0	0	0
3	*Chlorella sp* NRMC-F-0350	29	24	27
4	Methanol	0	0	0

Table 3: FTIR analysis of *Chlorella* sp. NRMC-F-0350 extract

S. No	FTIR peaks (cm^{-1})
1	3759.55
2	3665.05
3	3562.84
4	2362.37
5	2313.2
6	1185.04
7	1056.8
8	891.916
9	846.597
10	716.425
11	624.823

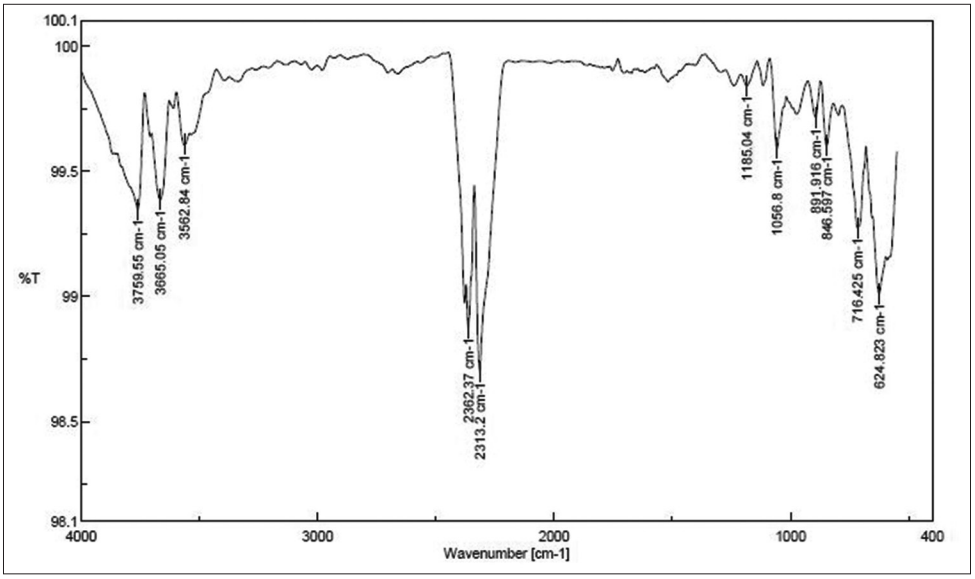

Figure 4: FTIR graph of the algal crude extract

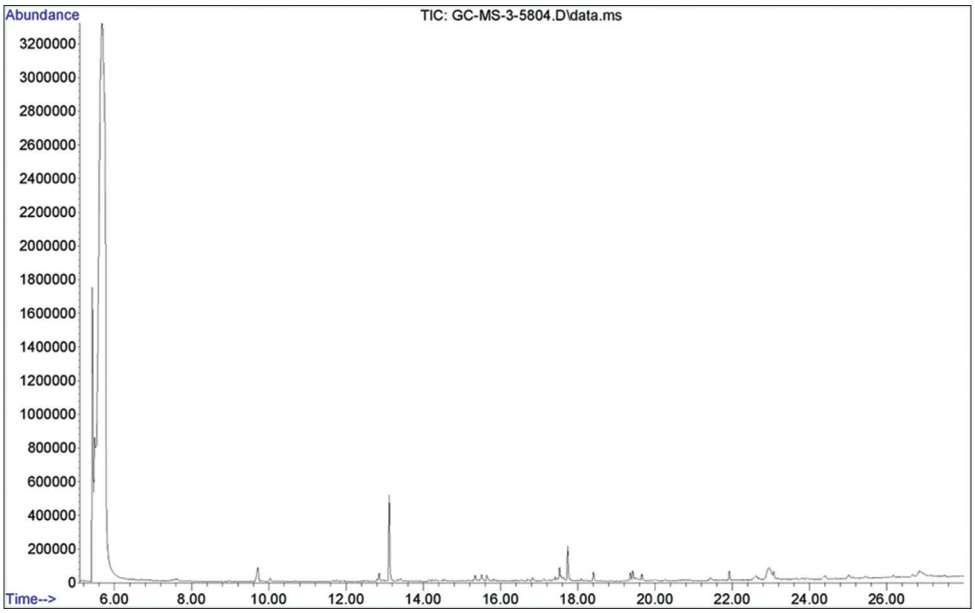

Figure 5: GC-MS Chromatogram of algal crude extract

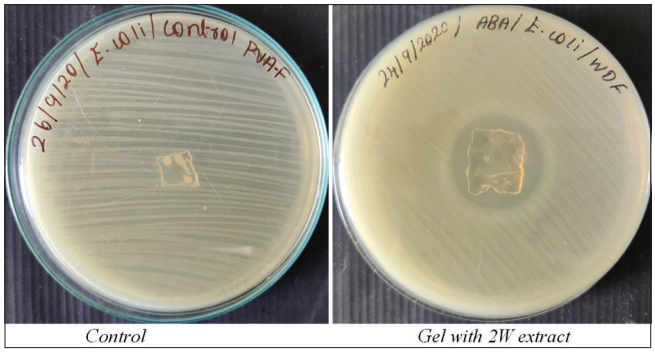

Figure 6: Antibacterial activity of the developed gels against *E. coli* Control Gel with 2W extract

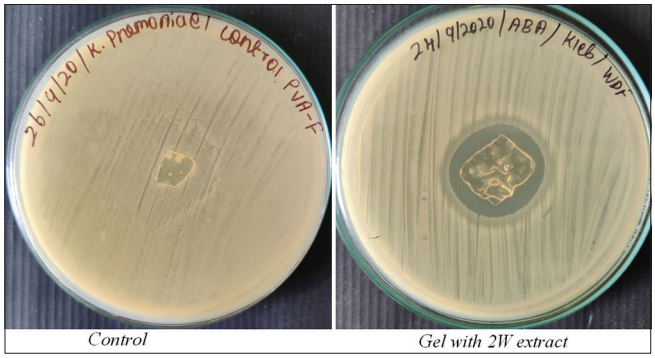

Figure 7: Antibacterial activity of the developed gels against *K. pneumonia* Control Gel with 2W extract

Antibacterial activity of wound dressing film

Wound dressing films were prepared using microalgal extracts. The developed wound dressing films were subjected to antibacterial analysis. The developed films were compared with plain films with no microalgal extracts. Films prepared using microalgal extracts showed significant antibacterial activity against all the test pathogens (*E. coli*, *S. aureus* and *K. pneumoniae*). Figure-6, 7, 8 showed the antibacterial activity of the developed wound dressing films against *E. coli*, *S. aureus* and *K. pneumonia* on comparison with no zones on plain films.

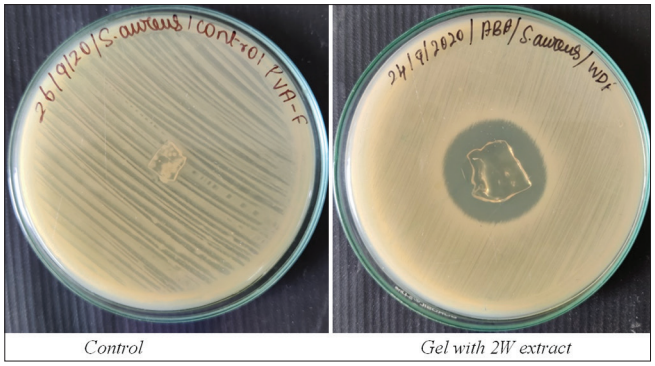

Figure 8: Antibacterial activity of the developed gels against *S. aureus*

Wound Healing Ability of the Developed Films

The wound healing ability of the developed films was determined using a wound scratch assay. Four concentrations (5g/l, 25g/l, 50g/l and 100g/l) of the films were used to determine the wound healing on L929 cell lines (Figure 9). 5g/l, 25g/l, 50g/l films showed significant wound healing at different intervals of time. 50g/l showed maximum healing of 84.5% compared with (97.2%) at 24h (Table-5).

DISCUSSIONS

Wound healing is a complex process which involves several internal and external factors. One of the major reasons for delayed wound healing is bacterial infection. Bacterial infection

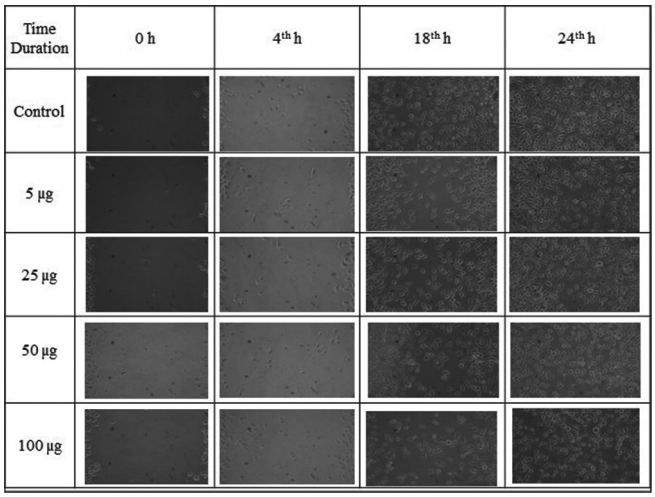

Figure 9: Wound healing ability of developed hydrogel using wound scratch assay

Table 4: GC-MS analysis of *Chlorella* sp. NRMC-F-0350 extract

S. No	Bioactive compounds	Retention time	Area (%)
1	Butanoic acid, 3-methyl-, 2-methylbutyl ester	7.609	0.27
2	Carbamic acid, methyl-phenyl ester	5.498	1.80
3	Benzene, 1,3-bis(1,1-dimethylethyl)-	9.720	0.62
4	Hexadecane	12.864	0.20
5	Pentanoic acid	13.119	1.98
6	Eicosane	15.352	0.20
7	9-Hexadecenoic acid	17.541	0.54
8	9-Octadecanoic acid	19.429	0.20
9	Hexanedioic acid	18.407	0.25
10	1-Triacontanol	22.618	0.43
11	Gamma sististerol	22.951	1.16
12	1,2 Benzene dicarboxylic acid	23.073	0.19
13	Stigmatane	25.040	0.26
14	Benzoxazole	26.851	0.55

Table 5: Wound healing ability of developed hydrogel using wound scratch assay

Sample	Conc. (µg/µl)	Wound area (µm)	Time intervals (h) and percentage of healing (%)			
			0	4	16	24
Chlorella sp.	5	4041	0	5	67	82
NRMC-F-0350	25	4025		3.1	70	86
	50	4059		2.4	73	84.5
	100	4049		4.9	73	82.1
Control	100	4097		11.4	83	97.2

worsens the condition and results in additional negative effects. Diabetic wounds are highly riskier due to the higher glucose levels. Improper treatment of diabetic foot ulcers may result in amputation (Falanga, 2005). In earlier days, topical applications of antibiotics were given to reduce bacterial colonization. The emergence of drug resistant bacteria has increased the alarming necessity for the development of new antimicrobial agents from new sources (Gopinath *et al*., 2015; Ganesh *et al*., 2009). Microalgae can be a potential resource as almost 18,500 new compounds have been identified till now. Several freshwater microalgae species at different geographical locations remain unexplored (Kumar *et al*., 2010; Balaperiasamy *et al*., 2014). In the present study, novel antibacterial agents from microalgae species were collected from ponds and lakes in Tiruchirappalli, Tamilnadu.

Three microalgae species were isolated and observed under microscope. The topology and structural characteristics were observed and a cyanobacteria species was identified. Antibacterial activity of the algal extracts was determined against common bacterial pathogens. *Chlorella* sp. NRMC-F-0350 showed potent antibacterial activity where the other two extracts had no activity. Antibacterial potential of *Chlorella* sp. NRMC-F-0350 was observed for Gram positive and Gram-negative pathogens. The order of antibacterial potential against the three test pathogens were *E. coli*, *K. pneumoniae*, *S. aureus*. Pratt *et al*., (1944) isolated the first antibacterial compound from a microalga, *Chlorella*; a mixture of fatty acids, *viz*. chlorellin, was found to be responsible for that inhibitory activity against Gram +ve and Gram -ve bacteria. Similarly other microalgae species like *Phaeodactylum tricornutum* (Desbois *et al*., 2009), *Haematococcus pluvialis* (Santoyo *et al*., 2009), *Skeletonema costatum* (Naviner *et al*.,1999), Euglena viridis (Das *et al*., 2005), *S. costatum*, *Staurastrum gracile*, *Pleurastrum terrestre*, *Dictyosphaerium pulchellum*, *Klebsormidium crenulatum*, *Chlorococcum* sp. (Bhadury and Wright, 2004) *Chlamydomonas reinhardtii*, *Chlorella vulgaris* exert antibacterial activity against various pathogens including drug resistant bacteria (Ghasemi *et al*., 2007).

Similarly, antibacterial activity of the various *Chlorella* strains isolated from the paddy field was evaluated by Ghasemi *et al*., 2007. Among the eight isolated strains, *Chlorella* vulgaris 025 showed significant antibacterial activity of 12mm against *S. aureus* and 8mm against S. epidermidis. Another study carried out by Hussein *et al*., 2018 investigated the antibacterial activity of the *Chlorella* vulgaris isolated from polluted water. Methanolic extracts showed 15mm against *E. coli* and 20mm against *S. aureus*. No inhibitory zones were observed for *K. pneumonia*. Whereas in the present study, methanolic extracts showed higher bacterial inhibition of 27mm against *E. coli*, 24mm against *S. aureus* and 29mm against *K. pneumonia*. Therefore, the isolated *Chlorella* sp can be used for the development of novel antibacterial drugs.

From FTIR analysis, the majority of the functional group were related to lipids and alcohol. GC-MS was performed to identify the bioactive compounds responsible for antibacterial activity. Some of the notable compounds with antibacterial mechanisms are found to be benzoxazole (Mishra *et al*., 2019), 1,2 Benzene dicarboxylic acid (Vijayakumar *et al*., 2018), sistosterol (Subramaniam *et al*. 2014), 9-Octadecanoic acid (Rahman et al., 2014), eicosane (Ahsan *et al*., 2017) and hexadecane (Naeim *et al*., 2020). The antimicrobial activity of microalgae has been attributed to compounds belonging to several chemical classes – including indoles, terpenes, acetogenins, phenols, fatty acids and volatile halogenated hydrocarbons (Cardozo *et al*., 2007); for instance, the antimicrobial activity of supercritical

extracts obtained from the microalga Chaetoceros muelleri were related to its lipid composition (León *et al*., 2007).

However, the antimicrobial activity detected in several pressurized extracts from *Dunaliella salina* may be explained not only by several fatty acids, but also by such compounds as a- and b-ionone, b-cyclocitral, neophytadiene and phytol (Herrero *et al*., 2006).

Biodegradable wound dressing films were prepared using microalgae extracts and these films showed evident antibacterial activity against all test pathogens. Further wound scratch assays are carried out to determine the wound healing ability of developed films. Compared to control of 100g/l showing 97.2% of healing, films at 50g/l showed 84.5%. Thus, the developed films with good wound healing activity can be used as wound dressing films against bacterial pathogens. This could also be used against drug-resistant pathogens. Detailed characterization of *Chlorella* sp. NRMC-F-0350 on intensive biochemical, pharmacological, and molecular studies is required for the confirmation and utilization of novel antibacterial compounds in health care aspects.

CONCLUSION

Three water samples were collected and its physic-chemical properties were determined. Microalgae species present in the water samples were isolated and pure cultures were observed under a microscope. Methanolic extracts of the microalgal extracts were used to determine antibacterial activity. The order of antibacterial activity of *Chlorella* sp. NRMC-F-0350 of against the three test pathogens such as *E. coli*, *K. pneumoniae*, *S. aureus*. Functional groups and bioactive compounds from *Chlorella* sp. NRMC-F-0350 extracts were evaluated using FTIR and GC-MS analysis. Wound dressing films were prepared and showed evident antibacterial activity. Developed films also showed significant wound healing activity. Therefore, the developed films can be used as a potential antibacterial dressing for enduring wound healing activity.

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