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 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.

KEYWORDS: Freshwater microalgae, wound infection, wound dressing film, wound scratch assay
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 these 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 microalgal 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 microalgal 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. The extracts were loaded into the wells and the plates were incubated in an incubator at 37°C 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 (Dhanasckaran et al., 2005).

**FTIR Analysis**

FTIR analysis is used to determine the functional groups present in the microalgal 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 2cm×2cm sizes and placed on the plates. The plates were incubated in an incubator at 37°C 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 μl 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 *Hypericum perforatum* oil (100λ) 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 652 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).

| S. No | Properties              | Area 1 | Area 2 | Area 3 |
|-------|-------------------------|--------|--------|--------|
| 1     | pH                      | 6.7    | 6.5    | 7.3    |
| 2     | Alkalinity (mg l^-1)    | 1.16   | 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).

**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, Hexadecenoic acid, 1-Triacontanol, Gamma sististerol, 1,2 Benzene dicarboxylic acid, Stigmstane and Benzoxazole.

| S. No | Sample              | Inhibitory Zone (mm) |
|-------|---------------------|----------------------|
| 1     | Phormidium sp.      | 0                    |
| 2     | Oscillatoria sp.    | 0                    |
| 3     | Chlorella sp. NRMC-F-0350 | 29 24 27     |
| 4     | Methanol            | 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           |
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.

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
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. pneumonia, 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, Pleurostrum terrestre, Dictyochaerium 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 benzoazole (Mishra et al., 2019), 1,2 Benzene dicarboxylic acid (Vijayakumar et al., 2018), siostester (Subramanian et al., 2014), 9-Octadecanoic acid (Rahman et al., 2014), eicosane (Alsan 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
Biodegradable wound dressing films were prepared using microalgal 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. Microalgal 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.

ACKNOWLEDGEMENT

Authors gratefully acknowledge University Science Instrumentation Center (RUSA) DST-PURSE Phase II and NRMC-F Sponsored by DBT, Govt. of India.

REFERENCES:

Ahsan, T., Chen, J., Zhao, X., Irfan, M. & Wu, Y. (2017). Extraction and identification of bioactive compounds (eicosane and dibutyl phthalate) produced by Streptomyces strain KK852460 for the biological control of Rhizoctonia solani AG-3 strain KK852461 to control target spot disease in tobacco leaf. AMB Express, 7(54), 1-9. https://doi.org/10.1186/s13668-017-0351-z

Al-Harbi, N. A., Shyam Kumar, R., Muthukumar, C. & Thajuddin, N. (2012). Antibacterial activity of Mentha piperita against selected food spoiling bacteria. African Journal of Biotechnology, 11(90), 15690-15694. https://doi.org/10.5897/AJB12.083

Ananthi, N., Bala krishnan, U., Sudha, S., Babu, M., Velmathi, S., Mubarak Ali, D. & Thajuddin, N. (2010). A Study on synthesis, characterization, and electrochemical and antibacterial properties of cobalt (ii) complexes of salen-derived ligand. The IUP Journal of Chemistry, 3(3), 28-41.

Balaperiasamy, B., Parveez Ahammed, A., Thajuddin, N. & Panneerselvam, A. (2014). Evaluation of Hypericum wightanum Wall.ex Wight and Arnextracts for antibacterial activity against human pathogens. International Journal of Current Research, 6(9), 8669-8672.

Bhadyur, P. & Wright, P. C. (2004). Exploitation of marine algae: Biogenic compounds for potential antifouling applications, Planta, 219, 561-578. https://doi.org/10.1007/s11259-004-4130-7

Cardozo, K. H. M., Guaratini, T., Barros, M. P., Falcão, V. R., Tonon, A. P., Lopes, N. P., Campos, S., Torres, M. A., Souza, A. O., Colecipolo, P., & Pinto, E. (2007). Metabolites from algae with economical impact, Comparative Biochemistry and Physiology - C Toxicology and Pharmacology, 146(1-2), 60-78. http://doi.org/10.1016/j.cbpc.2006.05.007

Desbois, A. P., Mearns-Spragg, A. & Smith, V. J. (2009). A fatty acid from the diatom Phaeodactylum tricornutum is antibacterial against diverse bacteria including multi-resistant Staphylococcus aureus (MRSA). Marine Biotechnology, 11, 45–52. http://doi.org/10.1007/s10126-008-9118-5

Dhanasekaran, D., Thajuddin, N., Panneerselvam, A., & Chandraleka, S. (2014). Isolation, characterization of antibacterial methyl substituted β-Lactum compound from Streptomyces noursei DPTD21 in Salt pan Soil, India. Journal of Biologically Active Products from Nature, 4(2), 71-88. https://doi.org/10.20231/jbapn.2013.0013338

Dhanasekaran, D., Path, S. A., Thajuddin, N. & Panneerselvam, A. (2013). Extracellular biosynthesis, characterisation and in-vitro antibacterial potential of silver nanoparticles using Agaricus bisporus. Journal of Experimental Nanoscience, 8(4), 579 – 588. https://doi.org/10.1080/17458080.2011.577099

Dhanasekaran, D., Path, S. A., Suganya, P., Panneerselvam, A., Kumar, T. S., Alharbi, N. S., Arunachalam, C., Alharbi, S. A., & Thajuddin, N. (2020). Taxonomic identification and bioactive compounds characterization of Psiloyce cubensis DPT1 to probe its antibacterial and mosquito larvicidal competency. Microbial Pathogenesis, 143, 104138. https://doi.org/10.1016/j.micpath.2020.104138

Dhanasekaran, D., Panneerselvam, A., & Thajuddin, N. (2005). Diversity of antibacterial marine streptomycetes from coastal region of Cuddalore District, Tamilnadu, India. In: Proceedings of the International Conference on Recent Advances in Marine Antifouling Technology, 420 – 428.

Dhanasekaran, D., Rajakumar, G., Sivamani, P., Selvamani, S., Panneerselvam, A., & Thajuddin, N. (2005). Screening of salt pans Actinomycetes for antibiotic agents. The Internet Journal of Microbiology, 11(2), 1 – 6.

Edwards, R. & Harding, K. G. (2004). Bacteria and wound healing. Current Opinion in Infectious Diseases, 17(2), 91–96. http://doi.org/10.1097/00013422-200404000-00004

Falanga, V. (2005). Wound healing and its impairment in the diabetic foot. Lancet, 366(9498), 1736-1743. http://doi.org/10.1016/S0140-6736(05)67700-8

Ganesh Manikandan, S., Lakshmi narasimhan, C., Thajuddin, N., & Saravanan, S. (2009). Antibacterial activity of epicarp extract of Punica granatum L. against Methicillin resistant Staphylococcus aureus (MRSA). Medicinal and Aromatic Plant Science and Biotechnology, 3(1), 52 – 54.

Ghasemi, Y., Moradian, A., Mogheheghzadeh, A., Shokravi, S., & Morovvat, M. H. (2007). Antifungal and antibacterial activity of the microalgae collected from paddy fields of Iran: Characterization of antimicrobial activity of Chroococcus dispersus. Journal of Biological Sciences, 7(6), 904-910. http://doi.org/10.22295/jbbs.2007.009.910

Ghasemi, Y., Moradian, A., Mogheheghzadeh, A., Shokravi, S., Morovvat, M. H. (2007). Antifungal and Antibacterial Activity of the Microalgae Collected from Paddy Fields of Iran: Characterization of Antimicrobial Activity of Chroococcus dispersus. Journal of Biological Sciences, 7, 904-910. https://doi.org/10.3923/jbbs.2007.904.910

Gonzalez, A. C. D. O., Costa, T. F., Andrade, Z. A., & Medrado, A. R. A. P. (2017). Isolation, characterization of antibacterial methyl substituted β-Lactum compound from Punica granatum L. against Methicillin resistant Staphylococcus aureus (MRSA). Medicinal and Aromatic Plant Science and Biotechnology, 3(1), 52 – 54.

Gopinath, P. M., Narchonai, G., Dhanasekaran, D., Ranjani, A., & Thajuddin, N. (2015). Mycosynthesis, characterization and antibacterial properties of AgNPs against multidrug resistant (MDR) bacterial pathogens of female infertility cases. Asian Journal of Pharmaceutical Sciences,
