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Antimicrobial activities of *Chaetomorpha antennina* dichloromethane extract against isolated urinary tract infection pathogens

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ABSTRACT

During the treatment of infectious diseases, upon repeated administration of an antibiotic, most microbial pathogens generate resistance to that antibiotic. Multiple drug resistance is also a widespread problem. Thus, it is the need of the hour to identify alternative antibacterial drugs. Seaweeds are one of the major repositories of numerous pharmacologically active secondary metabolites. The goal of this investigation is to evaluate the antimicrobial activity of *Chaetomorpha antennina* dichloromethane (DCM) extract against isolated urinary tract infection pathogens from urinary tract infection (UTI) infected patients. Antimicrobial activity, minimum inhibitory concentrations (MIC), minimum bactericidal or candicidal concentration (MBC/MCC) and biofilm inhibition activity of *C. antennina* extract was observed against the 7 isolated microbial strains: *Enterococcus avium, Enterobacter cloacae, Staphylococcus hominis, Staphylococcus epidermidis, Pseudomonas aeruginosa, Proteus mirabilis, and Escherichia coli* and *Candida albicans* (yeast). All isolated strains from UTI infected patients were later sequenced, identified and submitted in PubMed. The crude DCM extract of C. *antennina* showed remarkable inhibitory effects ranging between 2mm and 9mm, and its activity was recorded in the following order of *E. coli >E. avium> E. cloacae* and *S. hominis* >S. epidermis > P. aeruginosa > *P. mirabilis > C. albicans.* The MIC ranged between 500 to 750µg/ml, and the MBC was recorded in the range of 750 to 1000µg/ml. The crude DCM extract of *C. antennina* was also observed to have biofilm inhibitory activity at different concentrations against isolated UTI pathogens. This investigation elucidates that *C. antennina* DCM extract possesses antimicrobial activity against isolated UTI pathogens.

Keywords: Antimicrobial activities, *Chaetomorpha antennina,* Minimum inhibitory concentrations and Urinary tract infection.

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1. INTRODUCTION

Urinary tract infection (UTI) is the condition where pathogenic bacteria invade the urethra and bladder and obstruct urine flow by causing inflammation, intolerable pain, and cloudy urine, along with nocturia and haematuria [1]. UTIs are one of the common bacterial infections that affect more than 150 million people per year worldwide, causing morbidity in infant boys, old men, and females of all ages [2]. The causative agents for UTIs are Gram-negative and/or Gram-positive bacteria as well as certain fungi. The most commonly prevailing infectious agent for UTIs is uropathogenic *Escherichia coli* (UPEC), followed by *Klebsiella pneumonia*, *Staphylococcus saprophyticus, Enterococcus faecalis*, group B *Streptococcus, Proteus mirabilis, Pseudomonas aeruginosa, Staphylococcus aureus*, and *Candida* spp [1, 2]. Antibiotics such as trimethoprim, sulphamethoxazole, and ciprofloxacin are among the most commonly recommended therapeutics for UTIs [3]. However, the rise of antibiotic resistance and high recurrence rates of such common infections leave a great impact on our society [2]. Thus, to combat antibiotic resistance, it is very important to identify safer and effective alternatives to treat UTIs. For centuries plants have been used around the world as herbal drugs and remedies for various diseases and infections [4].

Seaweeds are a great source of pharmaceutically important bioactive secondary metabolites that exhibit broad spectrum of various biological activities [5, 6, and 7]. Several seaweeds around the world, growing in varying habitats, have been explored extensively to isolate drugs or biologically active secondary metabolites. *Chaetomorpha antennina* (Bory) Kutzing is a genus of green algae in the family Cladophoraceae. The algae belonging to this genus have cylindrical cells with macroscopic filaments, characterized by unbranched filaments, which make them very distinctive from its closest relatives that are branched species under the

genus Cladophora. *C. antennina* have been reported to have various biological activities like antibacterial activity [8], antiplasmodial activity [9], antioxidant activity [10] etc.. Hence, the present investigation aims to determine the antimicrobial activity of the *C. antennina* extract against isolated UTI bacterial pathogens.

2. MATERIALS AND METHODS

Collection, identification and extraction of secondary metabolites from Indian marine green seaweeds

Young and healthy green seaweeds were collected from Kanyakumari coastal area that lies between 77°05' and 77°36' east longitude and 8°05' and 8°19' north latitude. The collected seaweed species were identified upto species level as *C. antennina* with the help of key identification reference described by Meshwarai [11]. Further, the seaweed was taxonomically identified as *C. antennina* by Dr. G.V.S. Murthy, Joint Director, Botanical Survey of India (BSI), Tamil Nadu Agricultural University Campus, Coimbatore, and the voucher specimen was deposited with registration number BSI/SRC/9/28/2015-16/91. Later, the voucher specimen of the seaweed *C. antennina* was stored in the Department of Microbiology, Sri Paramakalyani College, Alwarkurichi 627 412, Tamil Nadu, India, for future references.

The identified green seaweeds (*C. antennina*) were collected and rinsed with seawater, further rinsed with tap water to remove adhering particles and finally dried under shaded conditions (35°C to 37°C). The dried seaweeds were powered using mechanical blender. 100 g of dry seaweed powder were taken separately in a new screw cap bottle, and then soaked separately with 500 ml of dichloromethane (DCM) for 7days at 30ºC (cold extraction). After the 7 days period, the coloured mixture was filtered through cotton cheesecloth and finally filtered through Whatman No. 1 filter paper. The filtrate was then concentrated using vacuum rotary evaporator. The final crude extract was weighted and stored in a screw capped glass bottle at 4ºC.

2.1 Phytochemical screening:

The *C. antennina* DCM extract was analyzed for preliminary phytochemical screening such as alkaloids, tannins (ferric chloride test), saponins, glycosides, flavonoids, terpenoids, triterpenoids, phenol, steroids, protein and sugars following the standard protocols [12].

2.2 Selection of pathogenic bacterial strains

Urinary Tract Infective (UTI) clinical microbial pathogenic seven strains: *S.* hominis, *S. epidermidis, K. pneumonia, E. cloacae, E. coli, E. avium, P. aeruginosa* and *C.* albicans, were isolated and all the UTI pathogenic strains were identified upto species level through molecular taxonomy 16S rRNA sequencing method and collected sequence were submitted to NCBI database and the obtained Accession numbers for all seven strains were MG907042, MG911002, MH908781, MG913261, MG913260, MG913259, MG907041 and MG913256, respectively.

2.3. Antimicrobial properties of seaweed *C. antennina* crude extract

The agar well diffusion method was adopted for determining the antimicrobial activities of the *C. antennina* green seaweeds crude extract against the seven selected UTI pathogens. In brief, 12hrs cultures of the selected pathogenic bacterial strains were individually inoculated in Nutrient Broth (NB) and *Candida albicans* (yeast) was individually inoculated in Sabouraud's Dextrose Broth (SDB). They were further incubated for 12–48 h before being used for antibacterial and antifungal assay. Then 30 ml of sterilized Muller Hinton Agar (MHA) for antibacterial and Sabouraud's Dextrose Agar plates (SDA) were prepared and dispensed into Petri plates. After the solidification, wells with 6 mm diameter were punched in the MHA and SDA plates using sterile cork borer, and the test pathogenic bacteria (1×10^8 cells/ml) and *C. albicans* (1×10^7 cells/ml) strains were spread over the surface of respective agar plates using sterile swabs. Thereafter experimental wells were loaded with 100 ml DMSO containing 750 mg/ml of crude extracts, Rifampicin (50 mg/ml) and Flucona-zole (75 mg/ml) were used as positive control, while DMSO was used as negative control. Then all the plates were incubated for about 24 h at a temperature of 37±2 °C for bacterial strains and 30±2 °C for 72–96 h for *C, Candida* strains. The assay was carried out in triplicates. The inhibition level or zone was measured from the edge of the well to the clear zone in millimeter (mm).

2.4. Determination of MIC, MBC and minimum candicidal concentration (MCC) of crude *C. antennina* DCM seaweed extract

Minimum inhibitory concentration (MIC) of DCM crude extract of *C. antennina* was performed through micro broth dilution method in 96 well microtiter plate (Poly propylene plate) as described by Eloff [13] and Sharma and Kumar [14]. The different concentrations of crude extracts (250 to 1500 µg/ml) were prepared for bacterial strains and solvent was evaporated. Thereafter, 100 µl of bacterial (1×10^8 CFU/ml) and fungal spores (1×10^7 cell/ml) suspension was added to each well. Only bacterial or fungal spores suspensions were used as negative control, while broth containing standard drug was used as positive control. The 96 well microtiter plates were incubated at temperature of 37°C for 24 h for bacterial strains and 48 h for fungal spores. The assay was done in triplicates. The MIC values were the lowest concentration of the seaweed extract that showed no visible turbidity after the incubation period. The MBC or MCC was determined by streaking each well contain a loopful of inoculums in a nutrient agar plates. The growth and least concentration of extract that showed no visible growth on sub culturing was taken as MBC or MFC values [15].

2.5 Antibiofilm properties of *C. antennina* DCM extract against UTI pathogens

The effect of biofilm inhibitory effects was evaluated in (96-well polystyrene flat-bottom) micro well plate method as described by CLSI [16]. Briefly, Different concentrations of test extract such as 250, 500, 750, 1000, 1250 and 1500µg/ml were prepared in fresh Trypticase Soya Yeast broth (TSY). A final concentration 1 x 10^6 CFU/mL of test bacterial inoculusm was aliquoted into each well of microplate and cultured in presence of different concentrations (250-1500µg/ml) of the seaweed extract. Wells that contained medium and without extracts or only with DCM were used as controls. All the plates were incubated at a temperature of 37 °C for around 24 h for bacteria and 48 h for fungi/yeast. After incubation, culture broth was removed and each well was washed thoroughly with sterile distilled water to remove free floating cells. Thereafter plates were air dried for 30 min and the biofilm formed was stained with 0.5% crystal violet for 15 min at room temperature. After that, the excess of crystal violet strain was removed through 50% ethanol. Finally, the dye bound to the cells in each well was solubilized by the addition of 200 μl of 95% ethanol. After 15 min of incubation, the absorbance was then measured using a

microplate reader at 570 nm wavelength. The percentage biofilm inhibition was determined using the formula:

Biofilm inhibition (%) = OD of the test - OD of the Negative Control / OD of the test samples

2.6 *In situ* visualization of biofilm Architecture on crude extract of *C. antennina*

In situ visualization of biofilm architecture was assessed in the crude DCM extract of *C. antennina* against predominate biofilm inhibitory effect rendering bacterial pathogens *S.* hominis, *P. mirabilis* and *C. albicans*. Briefly, cell suspension at a final density of 1×10^6 CFU/mL was treated with different concentrations of crude DCM extract of *C. antennina* in a 24-well micro plate containing sterile rectangular glass slides (1×1 mm). After incubating at 37 °C for 24 h for bacteria and 48 h for fungi/yeast, the slides were washed thrice with PBS (0.01 M, pH 7.2) and stained with 0.4% crystal violet solution for 5 min. Following staining, the slides were washed with sterilized water to remove the excess stain and air dried. The stained biofilm were observed *in situ* using a light microscope (Nikon Eclipse E200, Japan) at 400X magnification and images were captured using a phase contrast fluorescence microscopy using a digital camera [17].

2.7 Statistical analysis

The data acquired in the present investigation were analyzed by Mean± SD and One way and Two way-ANOVA by using computer software SPSS version 16 at $p<0.05$ and, Probit analysis by using EPA software.

3. RESULTS AND DISCUSSION

Antimicrobial drug resistance is the foremost problem faced all over the world against the present antibiotic therapy used in treating infectious diseases [18, 19]. Various research activities have been focused on seaweeds for isolating and developing newer antimicrobial agents. During the past four decades many novel bioactive compounds have been isolated from marine organisms [7, 19]. Since seaweeds have been known to show promising antimicrobial action, the present investigation was aimed to observe the antimicrobial activities from *C. antennina* against isolated UTI pathogens.

The preliminary phytochemical screening of the DCM crude extract of *C. antennina* showed the presence of alkaloid, flavonoids, tannins, glyceroids, terpenoids, phenols and sterols. Previously, Sivakumar and Safhi [19], Subathraa and Poonguzhali [20] and Abhishek et al. [21] reported that the presence of various phytochemical in the crude extracts of *C. antennina* such as carbohydrates, proteins, amino acids, alkaloid, flavonoids, tannins, terpenoids, glyceroids, phenols and sterols.

In disc diffusion method, the zones of inhibition were observed around the wells indicating antibacterial activities. The present results depicted antimicrobial properties of *C. antennina* DCM extract with a maximum inhibition zone of 9.17±1.041 mm that was recorded against *C. albicans* and the minimum zone of inhibition of 2.17±0.764 mm that was observed against to *E. avium*, whereas other UTI pathogens were observed to show only moderate inhibition zone against *C. antennina* DCM extract (Table 2). Thus, the results indicate that DCM extract of *C. antennina* have the potential to control the UTI pathogens. The results of this investigation was supported by Sivakumar and Safhi [19], who have reported antibacterial activity of petroleum ether extract of *Chaetomorpha antennina* against *Staphylococcus aureus*, *Bacillus cereus*, *Bacillus subtilis*, *Klebsiella pneumoniae*, *Escherichia coli* and *Pseudomonas aeruginosa*, with prominent zone of inhibition recorded as 7.3 ± 0.8 mm for *S. aureus*, 8 ± 0.6 mm for *E. coli* and 7.3 ± 1 mm for *P. aeruginosa*. This study outcome was also supported by Lima-Filho et al. [22], who have earlier reported that the DCM extracts of *Chaetomorpha* species have growth inhibitory effect against *Salmonella sp.*, *Salmonella pullorum, Salmonella enteric* subspecies *diarizonae*, *Salmonella enetrica* subspecies *houtenae* and *M. Morganii* with inhibition zone ranging from 10mm to 17mm. Additionally, Latha and Hemalatha [23] reported that the chloroform extract of *C. antennina* showed the moderate zone of inhibition ranging between 9 and 11 mm against the *S. aureus, E. coli* followed by *P. aeruginosa*. The above results may be due to the presence of pharmacologically active secondary metabolites in *C. antennina* DCM extract that possess antibacterial and anticandidal activities.

The MIC is the lowest concentration of antibiotics that inhibits the visible bacterial growth after overnight incubation using defined cut-off values for experimentally determined MICs [24]. The MBC or MCC/MFC is complementary to the MIC. The MIC test demonstrates the lowest level of antimicrobial agent that greatly inhibits growth, whereas the MBC demonstrates the lowest level of antimicrobial agent resulting in microbial death. In the present investigation, MIC, MBC and MCC of crude DCM extract of *C. antennina* were tested against isolated Urinary tract Infective (UTI) microbial pathogens and the minimum concentration for inhibition of bacterial and candida growth of UTI pathogens were recorded and the result are listed in Table 3. From the above results, it was observed that the tested UTI pathogens such as *S. hominis*, *S. epidermidis*, *P. mirabilis, E. avium*, *P. aeruginosa*, *C. albicans* showed MIC value of 500 µg/ ml and MBC/MCC value of 1000 µg / ml (Table 3). Similar findings were reported by Erturk and Tas, [25], who have reported that the extracts of all marine algae showed significant antimicrobial activity against pathogens like *S. aureus* and *S. typhimurium*. Additionally, there have been previous reports stating that brown, green and red algae have compounds with antiviral, cytostatic, antifungal, antihelminthic, and antibacterial activities [20]. Furthermore, Sivakumar and Safhi [19] reported antibacterial activity of petroleum ether extract of *Chaetomorpha antennina*. The above results (MIC, MBC and MCC) observed may be due to the presence of bioactive secondary metabolites in *C. antennina* DCM extract that have antibacterial and anticandidal activities.

The inhibition of biofilm formation is considered as one of the major drug targets for the treatment of numerous bacterial as well as fungal infections. Extensive studies on the development of drugs with antibioflim activities have been undertaken [26]. The antibiofilm properties was assessed for different *C. antennina* DCM extract concentrations (500, 750, 1000, 1250 and 1500 µg/ml) at $p>0.05$ significance and the results showed the presence of excellent biofilm inhibition effects against the isolated UTI pathogens and it ranged from 8 to 91% (Fig 1). The biofilm inhibition was found to be concentration dependent, and it increased as the concentration of *C. antennina* DCM extract increased. Moreover, the effective MIC, biofilm inhibitory concentration (BIC) and MBC, test concentrations of *C. antennina* extracts was documented for *S.* hominis, *P. mirabilis* and *C. albicans* by using light microscope and documenting any changes in the biofilm architecture, surface area colonization and thickness, and the following order of biofilm inhibition was observed: *C. albicans>S. hominis> P.*

mirabilis. From the results of light microscope photomicrographs, we could confirm the reduction in bacterial and *Candida* biofilms, architectural changes in the structural morphology of the three selected biofilms and we also observed very less adherence onto the glass pieces at higher concentrations (1250 to 1500 µg/ml) of *C. antennina* DCM extract (Fig. 2). Similar results were reported by Murugan et al. [27] who observed biofilm inhibitory activity of *C. antennina* diethyl ether, chloroform, acetone and methanol extracts. Moreover, seaweed extracts have been previously reported to have biofilm inhibition effect on *C. albicans* [28]. The presence of varios bioactive secondary metabolites in the *C. antennina* DCM extract may be the reason behind the antibioflim activity against *S.* hominis, *P. mirabilis* and *C. albicans*. Thus, all the results collectively indicate that the *C. antennina* crude DCM extract possess excellent antimicrobial activity against the isolated UTI pathogens.

4. CONCLUSION

The current study reveals the bioactive potential of *C. antennina* crude DCM extract and explores its bioactive potentials in the development of valuable antimicrobial and antibioflim therapeutics, which are both economical, naturally available and may be comparatively safer alternatives to commercially available drugs. Further studies are needed to investigate and study the individual secondary metabolites from *C. antennina* that are responsible for the antimicrobial and bioflim inhibition activities.

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