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In vitro anti-biofilm and anti-bacterial activity of *Junceella juncea* for its biomedical application

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PEER REVIEW

Peer reviewer

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Comments

The authors have evaluated the *in vitro* anti-biofilm and anti-bacterial activity of *J. juncea* for its biomedical application. Based on the results the authors have proposed that methanolic extract of *J. juncea* was found to be impressive in hampering biofilm forming pathogens and showed good antibacterial activity. In general the article is well organized; material and methods appear to be reproducible.
(Details on Page 934)

ABSTRACT

Objective: To investigate the anti-biofilm and anti-bacterial activity of *Junceella juncea* (*J. juncea*) against biofilm forming pathogenic strains. **Methods:** Gorgonians were extracted with methanol and analysed with fourier transform infrared spectroscopy. Biofilm forming pathogens were identified by Congo red agar supplemented with sucrose. A quantitative spectrophotometric method was used to monitor *in vitro* biofilm reduction by microtitre plate assay. Anti-bacterial activity of methanolic gorgonian extract (MGE) was carried out by disc diffusion method followed by calculating the percentage of increase with crude methanol (CM). **Results:** The presence of active functional group was exemplified by FT-IR spectroscopy. Dry, black, crystalline colonies confirm the production of extracellular polymeric substances responsible for biofilm formation in Congo red agar. MGE exhibited potential anti-biofilm activity against all tested bacterial strains. The anti-bacterial activity of methanolic extract was comparably higher in *Salmonella typhii* followed by *Escherichia coli, Vibrio cholerae* and *Shigella flexneri*. The overall percentage of increase was higher by 50.2% to CM. **Conclusions:** To conclude, anti-biofilm and anti-bacterial efficacy of *J. juncea* is impressive over biofilm producing pathogens and are good source for novel anti-bacterial compounds.

KEYWORDS

Junceella juncea, Anti-biofilm, Anti-bacterial, FT-IR, Congo red agar, Microtitre plate assay

1. Introduction

As far as biodiversity is concerned, marine environment is one among the most richest and complex ecosystem to be studied for numerous reasons. Prevailing heterogeneity conditions induce marine organisms to develop a variety of complex macromolecules with unique structural and functional features provided, potential applications in pharmaceutical field[1]. Exploring the sea in search of new novel drugs have been extracted from marine animals like sponges, coral reefs, bryozoans, gorgonians, tunicates and others[2]. Till date, more than 20 000

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compounds have been discovered but only a few are being commercialized[3]. As a consequence of overall exploitation, marine ecosystem is being disturbed by physical damage such as trawling nets, anchoring of boats, recreational diving and washed out ashore by tidal currents. These washed out deposits are always rich an inexpensive source of bioactive compounds which are on the other side considered as waste products polluting the environment with an unpleasant odour. Keeping this in concern and as an initiative, some alternative measure is to be created to convert these disposed biomaterials into effective bio-products.

The discovery of anti-microbials like penicillin from *Penicillium notatum*, various other antibiotics have been initiated the search for naturally available bioactive molecules from living organisms[4]. Many of the bioactive molecules are secondary metabolites, generated in response to external pressures such as competition for space and potential predators. Marine bioactive compounds are frequently strong and often are highly specific in their defence activities due to a diversified exposure[5]. Though marine organisms are very sessile, they hold a brilliant stock of such anti-microbial metabolites factory to avoid formation of host hazardous biofilm on their own exposed surfaces; provided the overwhelming majority of planets' microbial biomass prefers to be in a biofilm state[6]. Hence, these organisms can be adopted for development of various bioactive molecules for inhibition of biofilms and later for human use.

Gorgonians are sessile colonial animals look similar to plants with about 500 described species. Like other coelenterates, octocoral polyps have a single aperture for both food intake and excretion. Thus, polyps must produce an enormous anti-microbial compounds to devoid microbial infection[7]. Taking this as an initiative, we report on anti-biofilm and anti-bacterial activity of methanolic gorgonian extract (MGE) from *Junceella juncea (J. juncea)* against pathogenic strains. Crude methanol (CM) is used as comparative agent, a positive control to depict the activity of gorgonian extract. Apart from this, novel bioactive compounds from waste disposed along the shore have been utilized for its unique importance and biomedical application.

2. Materials and methods

2.1. Sample collection and Solvent extraction

Gorgonians were collected from trawl fishing nets along the shore of Tuticorin, southeast coast of India. Animals were identified as *J. juncea*, surface sterilized and eventually stored at −20 °C in laboratory. The specimens were extracted with methanol (3×) condensed using rotary evaporator and stored at 4 °C for further analysis.

2.2. Fourier-infrared spectroscopy analysis

The extracted samples were lyophilized and finely powdered with potassium bromide (KBr) prior to Fourier-Infrared spectroscopy (FT-IR) analysis. The vibrational frequencies of chemical groups present in the samples were recorded with Perkin-Elmer FT-IR spectrum spectrophotometer operated at a resolution of 2 cm^{-1} ranging from 4 000-400 cm^{-1}.

2.3. Bacterial strains

Test bacterial strains include *Escherichia coli* (MTCC 1687), *Salmonella typhii* (MTCC 531), *Shigella flexneri* (MTCC 1457) and *Vibrio cholerae* (MTCC 3906) were obtained from microbial type culture collection (MTCC), Institute of Microbial Technology, Chandigarh, India.

2.4. Biofilm formation in Congo red agar

The ability of biofilm formation was studied by the method previously adopted by Freeman *et al*[8]. Briefly, brain heart infusion (BHI, 37 g/L), sucrose (50 g/L) and agar No. 1 (10 g/L) was prepared and autoclaved at 121 °C for 15 min. Congo red dye (0.8 g/L) was also prepared simultaneously and added to warm (55 °C) BHI agar. The media was poured on to Petri dishes, inoculated and incubated for 24-36 h at 37 °C.

2.5. Microtitre plate assay (MTP)

To evaluate the efficacy of MGE in interrupting biofilm formation, MTP assay was carried out accordingly by Christensen *et al.*[9] using 96 well-flat bottom polystyrene titre plates. Individual wells were filled with 180 μL BHI broth followed by inoculation with 10 μL of overnight pathogenic bacterial culture. To this 10 μL MGE was added from the prepared stock solution of 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 μg/mL respectively along with control (without test sample) and incubated at 37 °C for 24 h. After incubation, content in the wells were removed, washed with 0.2 mL of phosphate buffer saline (PBS) pH 7.2 to remove free floating bacteria. The adherence of sessile bacteria were fixed with sodium acetate (2%) and stained with crystal violet (0.1%, w/v). Excessive stain was removed by deionized water wash and kept for drying. Further, dried plates were washed with 95% ethanol and optical density was determined using a microtitre plate reader (BIORAD) at 595 nm.

2.6. Anti-bacterial assay

Disc diffusion method was adopted to screen the anti-bacterial potentials of MGE and CM against test pathogens on Luria Bertani (LB) agar plates. This is a valuable and inexpensive test to demonstrate the susceptibility of a particular compound against pathogens by measuring the relative zone of inhibition. To achieve this, sterile antibiotic disks (HIMEDIA Laboratories, India) of 6 mm diameter were loaded with 30 μL of test sample and air-dried aseptically. Exponential bacterial cultures were swabbed on to the LB agar plates and impregnated with sample loaded disks. The plates were incubated at 37 °C for 24 h, meanwhile the experiments were performed in triplicates. Percentage fold increase between MGE and CM was calculated using the formula (A-B)/B×100.

3. Results

3.1. Solvent extraction and FT-IR prediction

MGE of *J. juncea* is yellow in colour and was insoluble in water but partially soluble in other examined solvents such as acetone and ethyl acetate. FT-IR prediction elucidates intense bands at 3 934, 3 818, 3 404, 2 954, 2 065, 1 641, 1 375, 1 035 and 666 cm^{-1} (Figure 1).

3.2. Biofilm production on Congo red agar

Biofilm formation of all tested strains on BHI agar supplemented with Congo red dye appeared as dry crystalline black with uniform colonies. The biofilm produced by bacteria is made up of exopolysaccharide matrix that protects microbes from host immune system and anti-microbial therapy[10]. Based on our results, *E. coli* displayed highest biofilm forming ability compared to *S. typhii* followed by *V. cholerae* and *S. flexneri*.

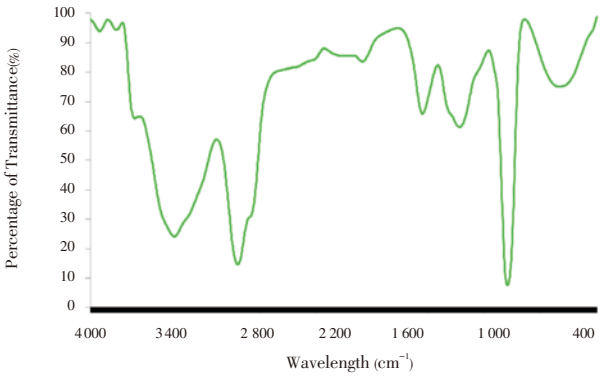

Figure 1. FT-IR prediction of MGE.

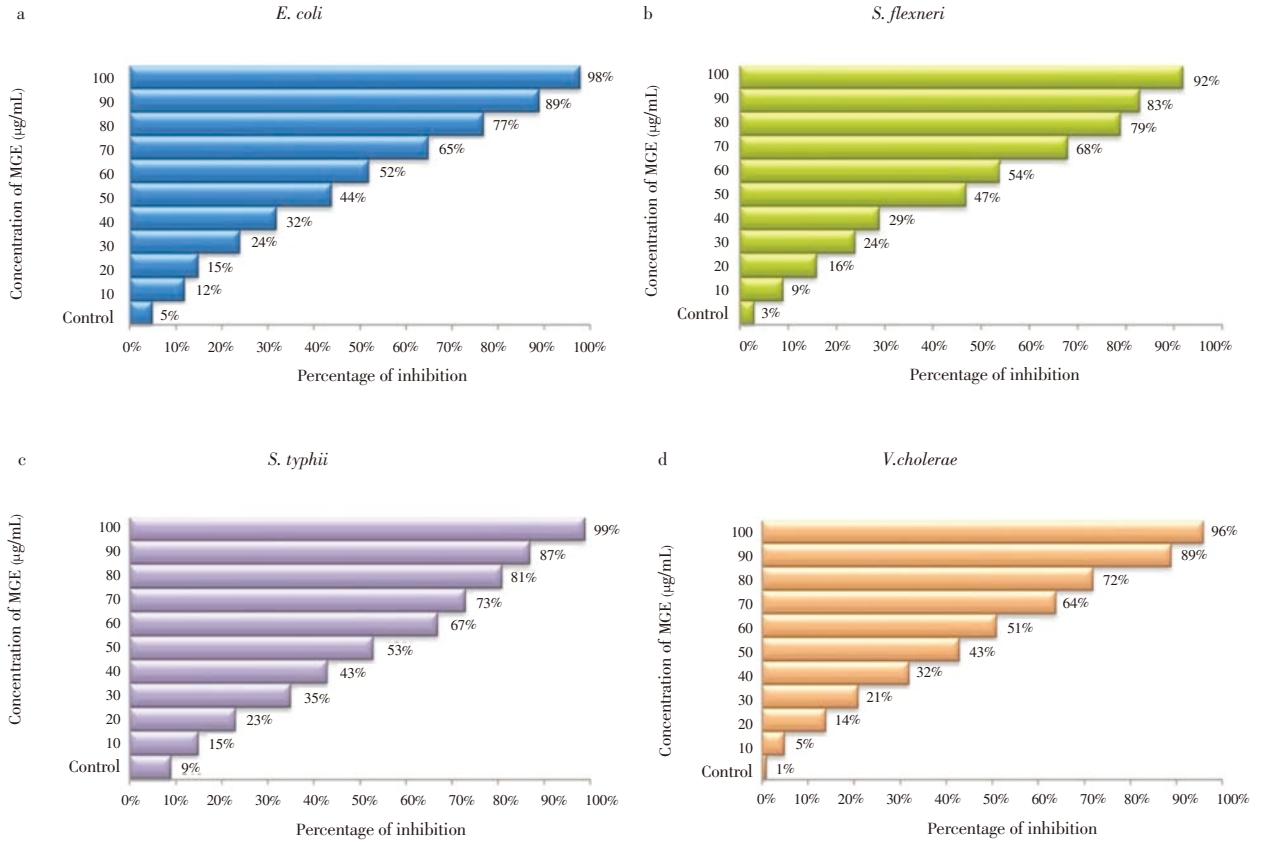

Figure 2. *In vitro* quantification of biofilm inhibition by MGE. a: *E. coli*, b: *S. flexneri*, c: *S. typhii*, d: *V. cholerae*.

3.3. Quantitative biofilm detachment

In vitro quantification of biofilm formation is monitored by MTP assay using crystal violet dye and measured spectrophotometerically. Crystal violet dye not only stains, but also screens a very small amount of adhered molecules that alter biofilm formation. A calibration curve was plot against *E. coli, S. flexneri, S. typhii* and *V. cholerae* at various concentration of MGE with control *i.e.*, without MGE to quantify biofilm density. After 12 h treatment, strains exhibit 98%, 92%, 99% and 96% reduction in biofilm formation when compared to control (Figure 2).

3.4. Anti-bacterial activity

Anti-bacterial effect of MGE (100 μg/mL) was screened against four biofilm forming pathogens. After 36 h of incubation, anti-bacterial property of MGE is significantly more than 13.0 mm against all pathogenic strains compared to CM by calculating zone of inhibition. Among all tested strains, *S. typhii* is more susceptible to MGE while *S. flexneri* is least susceptible (Figure 3a). Comparatively, overall percentage of increase between MGE and CM was found to be 50.2% (Table 1, Figure 3b). This clearly suggests MGE extract has well established bactericidal activity against biofilm forming pathogens.

Table 1
Zone of Inhibition against pathogenic bacterial strains. Disc diameter was 6 mm.

Microorganisms	A (mm)	B (mm)	Percentage of increase (%) (A-B)
E. coli	14.5	10.0	45.0
S. flexneri	13.0	9.0	44.4
S. typhii	15.0	11.0	36.4
V. cholerae	14.0	8.0	75.0

A: Methanolic gorgonian extract (MGE); B: Crude methanol (CM). The percentage of increase between MGE and CM was calculated using the formula (A-B)/B×100 indicates an overall increase of 50.2%.

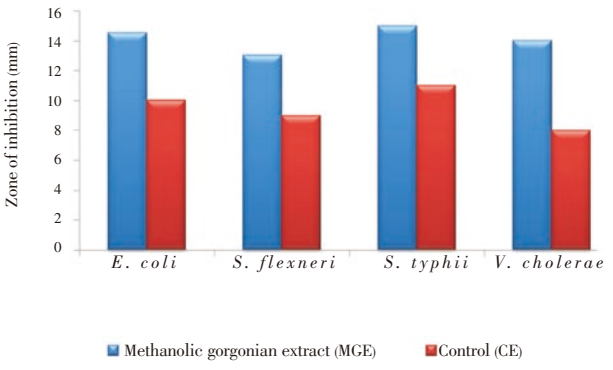

Figure 3. Anti-bacterial activity.
a: Zone of inhibition b: Percentage of increase.

4. Discussion

J. juncea posses a modern cembranoid carbocyclic system that recruits more attention of researchers due to its biologically importance[11]. Recent investigation on genus *Junceella* revealed 81 new metabolites and 74 diterpenoids compounds respectively[12]. Further, molecular frequencies of intense FT-IR bands indicate the presence of O-H & N-H stretching vibrations at 3404 cm^{-1} followed by C-H stretching group at 2954 cm^{-1}. A major peak at 1035 cm^{-1} indicates the presence of glycogen ν (CO), ν (CC), ν (CCO), (polysaccharides, cellulose). Similar infrared (IR) spectrum studies have been conducted in *Plumarella* sp. indicating the presence of α, β-unsaturated γ-lactone, double bonds and hydroxyls[13].

The study of marine natural anti-microbial has included the assessment of crude extracts or isolated metabolites in anti-biofilm assays[14,15]. The first stage of biofilm development is by adsorption of macromolecules to the surface followed by the attachment of bacteria. These extracellular polymeric substances (EPS) are rich in water content (98%) and are primarily made up of polysaccharides. The slow growth rate of biofilm associated organisms minimizes the rate of anti-microbial susceptibility. Hence, an effective inhibition at early stage would prevent the formation of extracellular matrix and macrofoulers[16]. The present study showed similar frequency of biofilm production by *S. aureus* from clinical samples, environment and micro-biota of healthy individuals[17-19]. The interaction of Congo red with polysacchardies produced by bacterial strains elucidates black, crystalline colonies on BHI agar plates. Further investigations were preceded by the use of MTP assay along with BHI as sole source for growth at different test concentration (μg/mL) to inhibit biofilm formation. Crystal violet dye on MTP assay diffuses slime layer determining the biofilm recurrence spectrophotometerically. Here, MGE showed anti-biofilm activity on MTP assay inhibiting the growth and ability of bacterial strains to produce exopolysachharides as well. Almost 90% of biofilm formation is inhibited by increasing concentration of MGE extract at 100 μg/mL.

In spite of studies conducted on anti-fouling activity of some gorgonians[14], this is the first report considering anti-biofilm activity of crude extract using this gorgonians. *J. juncea* have a well established defence mechanism with biologically active compounds such as terpenes, acetogenins, mono- and poly-hydroxlated steroids[7,12]. These classes of chemicals have high anti-microbial, anti-viral, anti-fouling, anti-feedant, anti-deterrent, immunomodulatory and allelopathic properties[12,20] that are pharmacologically important.

In the present study, MGE extract showed good anti-bacterial activity against biofilm forming pathogens compared to CM. The maximum zone of inhibition was observed in *S. typhii* and minimum inhibition was recorded in *S. flexneri*. Previously, several of the report on anti-microbial activity of gorgonian extract have been studied and published[21-32]. Sastry *et al.*[22] have reported ethyl acetate extract of *J. juncea* showed enhanced anti-bacterial activity against *B. subtilis, B. pumilis* and *E. coli*. Similarly, Murthy[11] have purified a new 8-hydroxy briarane diterpenoids compound from *J. juncea* and tested it for anti-fungal activity. At this junction, the percentage fold increase of MGE was 50.2% when compared to CM as control indicated by zone of inhibition.

To conclude, the methanolic extract of *J. juncea* showed evidence of high anti-biofilm and anti-bacterial property against all tested pathogens. Consequently, further research is needed to elucidate the anti-microbial agents from *J. juncea* that are pharmacologically important using advance techniques in an ecofriendly approach.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgement

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Comments

Background

Exploring the sea in search of new novel drugs have been extracted from marine animals like sponges, coral reefs, bryozoans, gorgonians, tunicates and others. Till date, more than 20 000 compounds have been discovered but only a few are being commercialized. Gorgonians are sessile colonial animals look similar to plants with about 500 described species. Like other coelenterates, octocoral polyps have a single aperture for both food intake and excretion. In the present study, anti-biofilm and anti-bacterial activity of MGE from *J. juncea* against pathogenic strains were studied.

Research frontiers

Study are being performed to determine the bioactive compounds present in gorgonians that are biologically important from *Euplexaura anastomasans* (Korea), *Pseudopterogorgia elisabethae* (Columbia) and *Pterogoria anceps* (Caribbean) gorgonians have been done so far.

Related reports

There is not much literatures supporting the extraction of bioactive compounds from *J. juncea* for its anti-biofilm and antibacterial activity. YLN Murthy, 2012 have reported a new antifungal briarane diterpenoid compounds from the gorgonian *J. juncea*. Similarly, SH. Qi, 2009 have reported the antifeedant and antifouling briaranes from ethyl acetate/chloroform extract of *J. juncea*. The present differs from the above mentioned study with novelty and originality

Innovations and breakthroughs

The study have showed the anti-biofilm and anti-bacterial of crude methanolic gorgonian extract (*J. juncea*). The study is highly significant in the field of pharmacology in which the developing novel drugs which pose anti-microbial agents in near future.

Applications

It is very interesting to see the utilization of disposed waste in the marine environment (*J. juncea*) for the extraction of novel bioactive compounds that possess unique importance and biomedical application. Thus, from this study it have been shown that the methanolic extract of *J. juncea* is pharmacologically important.

Peer review

The authors have evaluated the Invitro anti-biofilm and anti-bacterial activity of *J. juncea* for its biomedical application. Based on the results the authors have proposed that methanolic extract of *J. juncea* was found to be impressive in hampering biofilm forming pathogens and showed good antibacterial activity. In general the article is well organized; material and methods appear to be reproducible.

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