Full Length Research Article

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Research Journal of Agricultural Sciences **14**(6): 2003–2007 (Nov-Dec 2023)
DOI: http://rjas.org/Article/Article/5727

ISSN: 0976-1675 (P)
ISSN: 2249-4538 (E)

Exploring the Antibacterial Efficacy of *Padina pavonica* against Biofilm and ESBL-Producing Bacteria: A Comprehensive Study on Phytochemicals and Therapeutic Potential

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Received: 30 Oct 2023; Revised accepted: 07 Dec 2023; Published online: 28 Dec 2023

Abstract

The rise of antibiotic-resistant microorganisms within clinical environments, particularly those forming biofilms and those that are ESBL (Extended Spectrum Beta-Lactamases) producers, poses a significant threat to human health. Innovative strategies involving natural products may offer a solution to this challenge. Consequently, this research is designed to evaluate the potential antibacterial properties of the marine alga *Padina pavonica* against these resistant bacteria populations. A total of 7 genera of bacterial isolates were collected, and their biofilm and ESBL characterization revealed that 62.5% were biofilm producers, and 56.2% were ESBL producers. Simultaneously, *Padina pavonica* was collected from sea sources in Rameshwarm and subjected to solvent extraction to determine phytochemicals. Among the solvent extracts, ethanol showed predominance, and a majority of phenols, flavonoids, carbohydrates, and terpenoids were observed in all solvent extracts. The antimicrobial activity of ethanol extracts against the 7 genera revealed significant suppression of *E. faecalis* and Proteus sp. Additionally, various phytocompounds such as 9-Octadecenoic acid, Hexadecane, 1-iodo-, Tetradecanoic acid, Tridecan, 9-Eicosene, Phytol, and Gamma Elemene were observed. Overall, our study contributes to understanding the bioactive composition of marine algae, emphasizing the potential of these compounds for therapeutic applications, particularly in antimicrobial activities.

***Key words*:** *Padina pavonica,* Biofilm, ESBL, Tridecan, Phytol

The increasing threat of antibiotics, driven by bacterial infections, has prompted the development of comprehensive global health strategies. However, accurately quantifying the frequency, complications, and mortality associated with these infections remains a complex challenge. Developing countries are particularly affected because the lack of access to expensive antibiotics for most patients is compounded by uncontrolled antibiotic use, which contributes to the rise of antimicrobial resistance. Mitigating this issue requires a collective and concerted effort to ensure the sustainable and effective use of antimicrobial agents. The rapid spread of resistant organisms further strains healthcare systems amid a rise in infectious disease rates [1].

Growing apprehension is evident in numerous clinical scenarios where limited treatment choices compromise the ability to combat common infections. Bacterial resistance to initial drugs varies widely, ranging from none to nearly 100%. In some cases, resistance to second -and third-line drugs significantly impedes treatment efficacy. The intricacies of drug resistance underscore the urgency of international efforts to address AMR [2]. In this context, many studies have shown that algae and their molecules exert antioxidant activity through antimicrobial activity. Sapagh *et al.* [3] investigated the antimicrobial potential of *Arthrospira platensis* (cyanobacteria) and *Polysiphonia scopulorum* (Rhodophyta) against drug-resistant *Pseudomonas aeruginosa*. In a separate study, Morsi *et al.* [4] evaluated the antimicrobial activity of *Rhizoclonium hieroglyphicum* against a range of bacteria.

Biofilm formation is an important virulence factor contributing to the persistence of infections. Biofilms play an important role in facilitating the emergence of antimicrobial resistance, creating chronic infections and economic burdens such as high healthcare costs, prolonged hospital stays and difficulty in treatment [5]. Another problem for the medical world is the production of ESBL by bacteria. ESBL-producing isolates spread rapidly and cause poor outcomes with high mortality due to ineffective antibiotic therapy [6]. In this scenario, the significance of alternative drugs that are safe and devoid of drug resistance is paramount. Among the algae, brown algae of *Padina pavonica* provide unique benefits, like antimicrobial and antioxidant [7]. Recently Makhlof *et al.* [8] also observed the antibacterial, antioxidant, anticancer, and antibiofilm activities of the methanolic extract of *Padina pavonica* L. Nonetheless, none of the recent studies conducted by various authors have assessed the effectiveness of *Padina pavonica* against bacteria, and there is a notable absence of

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Citation: Ravi P, Subramanian G, Jegadeeshkumar D. 2023. Exploring the antibacterial efficacy of *Padina pavonica* against biofilm and ESBL-producing bacteria: A comprehensive study on phytochemicals and therapeutic potential. *Res. Jr. Agril. Sci.* 14(6): 2003-2007.

research specifically targeting ESBL-producing bacteria. To fill this research gap, this study was conducted to investigate the role of phytocompounds against ESBL-producing bacteria.

MATERIALS AND METHODS

Collection of algal samples

Padina pavonica alga sample was harvested from the Rameswaram area, during December to March winter (2019-2020) in a rocky area at a depth of about 50 meters from the sea surface. Following collection, samples were transferred to the laboratory in sterilized plastic bottles containing seawater to prevent evaporation. Careful cleaning of epiphytes and removal of rock debris from algal samples was carried out, followed by gentle rinsing in fresh water to remove surface salt. A portion of the collected sponge samples was preserved for identification purposes. After the cleaning process, the samples were subjected to shade drying for two to three days [9].

Identification of algae

Algae identification was conducted utilizing the taxonomical characteristics outlined in the publication [10]. The identification procedures took place at the CSMCRI Marine Algal Research Station in Mandapam, Rameshwaram, India.

Preparation of algal extract

Dried algal biomass was extracted using different solvents (ethanol, chloroform, and acetone). The algal was soaked in their respective conditions (10 g: 100ml) of the above-mentioned solvents and retained in a rotary shaker at 150 rpm for 72 hours at room temperature. At specified times; extracts were filtered separately using the Whatman No.1 filter paper. The filters were dried by evaporation under the rotary evaporator. Raw juices were then available dissolved in the respective solvent to a final concentration of 100 mg/mL stalk solution. It was stored at 4 °C for further procedures [11].

Preliminary phytochemical analysis

The preliminary phytochemical studies for the algal extracts were accomplished by the previous study [12]. The phytochemicals like alkaloids, carbohydrates, flavanoids, phenols, saponins, tannins, terpinoids, quinines, glycosides, proteins, and steroids were analyzed.

Collection of clinical isolates

A total of 7 bacterial genera were collected from Microteck, a clinical laboratory, in Coimbatore. The collected isolates were confirmed with selective media and chromogenic media. The following isolates were subjected to antibacterial activity: including *E. coli, K. pneumoniae, P. aeruginosa, S. aureus, Proteus sp, E. faecalis,* and *Salmonella* sp.

Biofilm assay

The biofilm assay was carried out by Freeman *et al.* [13] procedure. The isolates were inoculated by the single streak method on sterile brain heart infusion agar media supplemented with sucrose (5%) and congo red (0.08 g/L). All plates were incubated at 37 °C for 24 hours. The formation of a black colour indicates a positive result.

Determination of ESBL producing isolates by phenotypic method

Confirmation of the ESBL- producing isolates was done by the phenotypic confirmatory test according to CLSI recommendations. In this test, the first generation of betalactam i.e. Amoxcillin disc (30µg) alone or in combination with clavulanic acid (10 µg) was used [14].

Antibacterial activity of Padina pavonica

Positive results indicating biofilm and ESBL-producing isolates were further subjected to antibacterial activity testing using extracts derived from *Padina pavonica*. To assess antimicrobial activity, the well diffusion method was used [15]. The fresh bacterial cultures, obtained from 24-hour-old broth, were evenly spread on sterile Muller Hinton agar plates. Wells were made using metal bores, and algal extracts at varying concentrations were carefully dispensed into the well, and appropriately labeled. The 10mcg of ampicillin was added in one well as a standard control and the respective solvent was added in another well as a negative control. The plates were then incubated at 37°C for 24 hours, and following incubation, they were observed for the formation of zones. The resulting zones were measured and recorded.

GCMS analysis

Following the antimicrobial activity, secondary phytochemicals analysis was carried out with algal extract through GCMS analysis. The chosen potential algal extract underwent GC-MS analysis, which was conducted using a modified version of the analytical method described in the previous study [16]. Chromatograph interfaced to a mass spectrometer (GC-MS Perkin-Elmer) equipped with an Elite-1, fused silica capillary column (30 m´ 0.25 mm ID'1 m df, composed of 100% dimethyl poly siloxane).

RESULTS AND DISCUSSION

A total of seven genera of 16 bacterial isolates were clinical laboratory. Among these populations isolated, 71.4% were identified as gram-negative and 28.5% were found to be gram- positive organisms. The gram-negative isolates were identified as *Pseudomonas aeruginosa* and *Klebsiella pneumonia* each accounting for 25%, *E. coli*, *Salmonella* spp, and *P. mirabilis* each accounting for 16.6%. The gram-positive isolates identified as *Enterococcus faecalis* and *Staphylococcus aureus* accounting for 50%. All isolates were subjected to a biofilm assay; among them, 62.5% of isolates showed a positive reaction to biofilm. Among the genera, *S. aureus* and *K. pneumoniae* were the predominant isolates for biofilm formation. These biofilm-producing isolates were not easily eradicated, due to the antibiotic resistance developed by bacteria within the biofilm, the use of antibiotics alone for treating infections resulting from biofilm is ineffective [17].

The determination of ESBL (extended spectrum beta-lactamase) producing isolates was carried out using the double disk diffusion method. Our study revealed that among the 16 isolates analyzed, *E. coli and Salmonella sp* exhibited 100 percentage of ESBL activity. *K. pneumoniae* and *P. aeruginosa* showed ESBL activity in 66.6% of the isolates, while *P. mirabilis* and *E. faecalis* exhibited ESBL activity in 50% of the isolates.

These findings align with prior research conducted by previous studies [18-19], which reported elevated rates of ESBL production in *Salmonella sp* and *Escherichia coli* isolates. Similarly, studies by Wang *et al.* [20] and Patel *et al.* [9] documented substantial ESBL activity in *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* isolates, providing additional support for our results. Furthermore, the identification of ESBL activity in *Proteus mirabilis* and *Enterococcus faecalis* isolates is in accordance with the outcomes of studies conducted by previous studies [21-22].

In our study, aside from *Staphylococcus aureus*, 56.2% of the isolates demonstrated positive results for ESBL production. This aligns with the findings of Rodriguez-Bano *et al.* [23], which reported varying rates of ESBL production across different bacterial species. Notably, each of the seven bacterial species included in our study exhibited at least one isolate testing positive for ESBL production. Moreover, our results indicate that a majority of the ESBL-producing isolates displayed biofilm formation. This observation supports the conclusions drawn in previous studies [24-25], underscoring the association between ESBL production and biofilm in the clinical sector.

The emergence of ESBL and biofilm-forming bacterial isolates in clinical settings has become a significant public health concern. The ability of these demon bacteria is worrisome because it facilitates the spread of antibiotic resistance and can prolong infections in healthcare settings. Recently, antimicrobial resistance has become a public health problem threatening communities as it leads to high mortality [26].

Overall, our study contributes to the understanding of biofilm and ESBL production among clinical isolates and reinforces the need for effective strategies to address the spread of ESBL-producing bacteria and combat the associated antibiotic resistance. In this context, understanding ESBL formation and the antibacterial spectrum of bacterial isolates is important for providing reliable empirical antibiotic therapy to patients. Researchers are currently conducting extensive research on alternative therapy solutions, including most marine organisms. Among them, seaweed or macroalgae provide a great variety of metabolites and natural bioactive compounds, with antimicrobial activity. As a result, this study is conducted to obtain *Padina pavonica* to provide antimicrobial properties.

The extract was obtained from *Padina pavonica* and subjected to preliminary phytocemicals analysis. Among the ten different phytochemical analyses, phenols, flavonoids, carbohydrates and Terpenoids were the majority observed in the extracts. The highest count of phytochemicals was observed in ethanol extract, followed by acetone extract. The saponins, tannins, and proteins were observed only in the ethanol extract. The sterols were not observed in any solvent extracts. A similar line of results was observed in the previous study [27], which found flavonoids, carbohydrates, tannins, and terpenoids. Phenolic compounds are plant secondary metabolites, and the isolation, characterization, and study of their biological activities have been the focus of much research. According to Mekinic *et al*. [28], phytochemicals with high phenolic content in seaweed have positive pharmacological and nutraceutical properties.

Building upon a previous investigation on the antibacterial activity of *Padina pavonica*, the current study was undertaken to assess its antibacterial efficacy against biofilm and ESBL-producing isolates. Among the four solvent extracts, the ethanol extract showcased predominant phytochemicals, prompting its selection for antibacterial testing. Notably, *Enterococcus faecalis*, *Proteus* sp and *Staphylococcus aureus*, exhibited significant suppression, with zone of inhibition ranging from 10mm to 20mm, while the least inhibitory activity was observed against *E. coli*. Out of the seven isolates tested, 2.5mg of the extract was effective in suppressing two isolates, while five isolates were inhibited by the extract (Fig 1).

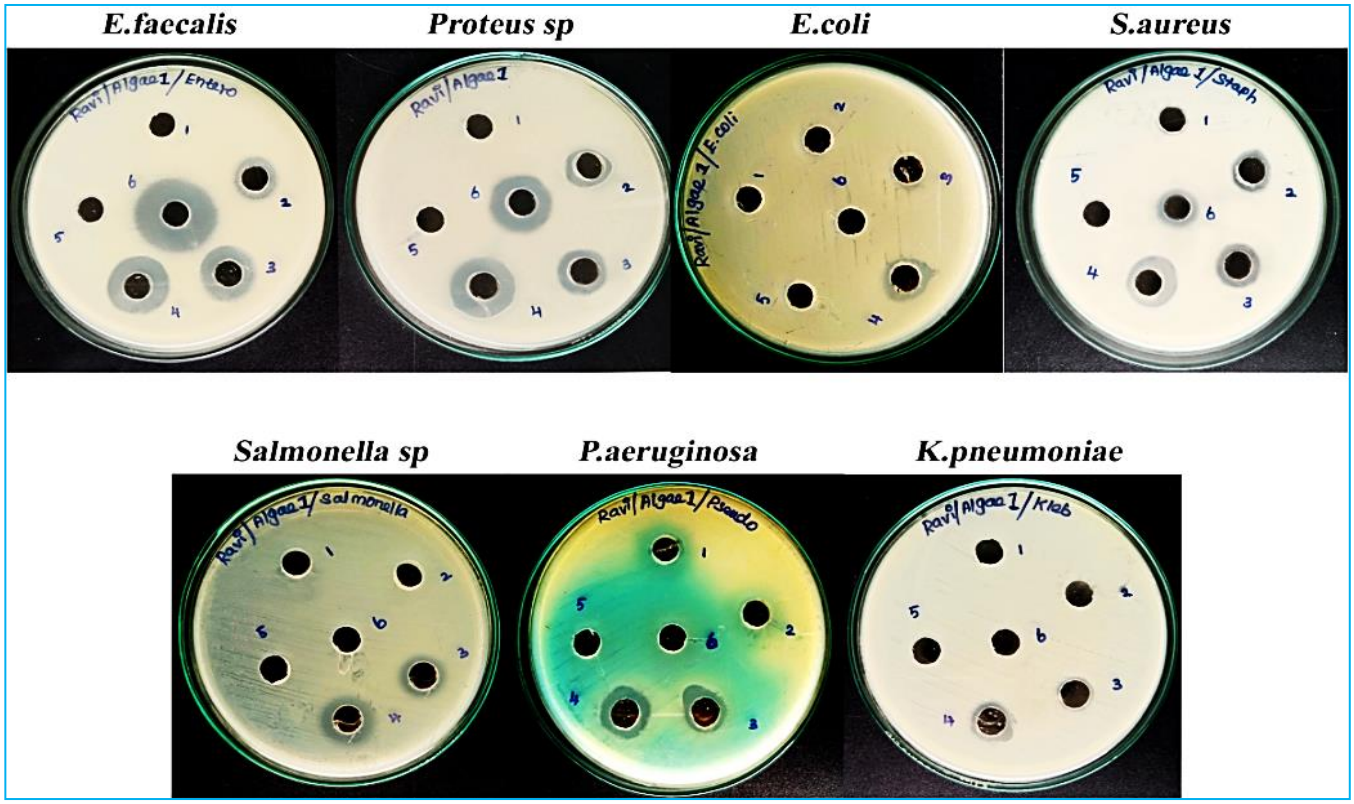

Fig 1 Antibacterial activity of *Padina pavonica* against Biofilm and ESBL producing bacteria

Seaweeds harbor a diverse array of distinctive secondary metabolites, demonstrating intriguing bioactivities. Marine algae represent a valuable reservoir of various bioactive compounds, encompassing dietary fibers, proteins, minerals, and vitamins. Additionally, they house noteworthy bioactive substances, including polysaccharides, polyphenols, phytochemicals, and polyunsaturated fatty acids, holding promise for therapeutic applications [29]. In the present study, various bioactive compounds were observed through GCMS analysis.

Table 1 Phytocomponents detected in the ethanol extract of *Padina pavonica* by GC-MS analysis

Retention time	Name of the compound	Molecular formula	M. Wt. $gmol^{-1}$	Area %
15.885	Heptacosane, 1-chloro-	$C_{27}H_{55}Cl$	415.179	0.36
17.463	9-Octadecenoic acid	$C_{18}H_{34}O_2$	282.5	3.17
20.074	Hexadecane, 1-iodo-	$C_{16}H_{33}I$	352.3377	4.97
20.651	Nonadecane, 9-methyl-	$C_{20}H_{42}$	282.5475	0.772
14.619	Tetradecanoic acid	$C_{14}H_{28}O_2$	229.36	1.13
21.274	Tridecane	$C_{13}H_{28}$	184.37	1.52
14.963	9-Eicosene	$C_{20}H_{40}$	280.53	0.99
17.585	Phytol	$C_{20}H_{40}O$	296.53	5.30
19.607	Gamma.-Elemene	$C_{15}H_{24}$	204.357	1.88

The following compounds were observed: hexacosane, 1-chloro-, 9-Octadecenoic acid, Hexadecane, 1-iodo-, Nonadecane 9-methyl, Tetradecanoic acid, Tridecane, 9-eicosene, Phytol, Gamma Elemene (Table 1). A previous study [30] observed the antimicrobial activity producing Tetradecanoic acid, Tridecane, and fatty acid of Tetradecanoic acid and 9-Octadecenoic acid from *Padina pavonia.* In 2019, Minetti *et al.* [31] also observed the various fatty acid phytocompounds of phytol and nonadecane from *Padina pavonica.* Recent studies have revealed that phytol has antimicrobial activity [32]. The cumulative evidence from these studies strengthens the understanding of the bioactive composition of marine algae, emphasizing the potential for these compounds to contribute to therapeutic applications such as antimicrobial activities. Bioactive composition of marine algae provides a robust foundation for recognizing their potential in therapeutic applications, particularly in the context of combating microbial infections.

CONCLUSION

In conclusion, the present investigation contributes to the growing body of knowledge on the bioactive compounds in seaweeds. The diverse array of identified compounds, in conjunction with previous research, highlights the multifaceted nature of marine algae and their potential significance in pharmaceutical and nutritional applications. Further research is warranted to elucidate the specific therapeutic benefits and mechanisms of action associated with these bioactive compounds.

Conflict of interest: *None declared*.

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