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Original Article

Microbiology Section

Antibacterial Activity of *Ulva lactuca* against Multidrug Resistant and Betalactamase Producing Isolates from Food Samples- An In-vitro Study

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ABSTRACT

Introduction: Antibiotic resistance is a problem of deep scientific concern, both in hospital and community settings. Due the presence of highly resistant microorganisms in food, it may lead to serious health issues to the human beings and other animals also. This can be controlled by novel control methods using natural products. Algae are one such wonderful source that helps in treating such dreadful microorganisms. *Ulva lactuca* incorporate discrete active compounds, hence, exhibit antibacterial property to control the Multidrug Resistant (MDR) bacteria. The present research aims in exploring the potential antibacterial property of such dreadful bacteria.

Aim: To evaluate the potential bioactive compound of *Ulva lactuca* and its antibacterial activity against MDR and betalactamase producing food isolates.

Materials and Methods: The present pilot in-vitro study was carried out at Arignar Anna Government Arts College, Namakkal, Tamil Nadu, India, in the period of December 2017 to December 2020. Total 18 bacterial isolates were isolated from five retail chicken meat samples. These were then, tested for its antibiotic resistance property using standard antibiotic discs. The algae *Ulva lactuca* was isolated and extract was prepared using ethanol and chloroform solvents, followed by which the phytochemical studies were performed. These extracts were then tested, against the selected organisms for its potential activity.

Results: Among the 12 antibiotics tested, all isolates were resistance to variety of antibiotic classes, mainly aminoglycosides, cephalosporins and fluoroquinolones and also 55% of the bacteria were able to produce betalactamase enzyme. The ethanol extract of *Ulva lactuca* was highly active against all isolates and exhibited a range of 10±1.24 mm to 22±1.24 mm inhibition zone. The chloroform extract, exhibited less potency, which exhibited 10±0.816 mm to 13.16±1.027 mm inhibition zone. Each organic solvent showed positive result for following metabolites- alkaloids, carbohydrates, flavonoids, sterols, tannins and terpenoids.

Conclusion: The ethanolic extract of *Ulva lactuca* was very effective against the selected MDR and betalactamase producing food isolates. Therefore, it could be suggested as an antibacterial agent in the future. Further studies are needed to reveal the behavioural mechanisms of this plant and its pharmacological effects.

Keywords: Antibiotics, Biofilm, Foodborne infections

INTRODUCTION

Recently, there have been significant concerns for human health regarding the misuse and indiscriminate use of antibiotics in veterinary medicine. One of the major impacts of antibiotic residues in animal-derived foods is the spread of antibiotic resistant microorganisms. Antibiotic resistant pathogenic bacteria can cause foodborne illnesses in humans that are challenging to treat. They can distribute resistance genes to other microbes through the food chain [1].

Approximately, 1.8 million people die from foodborne illnesses in underdeveloped countries each year due to food borne pathogens. Among the food samples, meats are main reservoirs of foodborne pathogens such as *Listeria monocytogenes*, *Vibrio parahaemolyticus*, *Escherichia coli*, *Salmonella* spp., *Vibrio cholerae*, and *Staphylococcus aureus*. This is caused by cross-contamination through poor personal hygiene and sharing dirty utensils [2].

A number of mechanisms are there through which bacteria can evolve antibiotic resistance. Among them Extended-Spectrum Beta-Lactamase (ESBL) is important enzyme, which deactivate the beta-lactam antibiotics through hydrolysis of beta-lactam ring. Moreover bacterial biofilms are a significant global health issue that contribute to persistent chronic infections because of their resistance to antibiotics, host defence mechanisms and other external stressors [3].

In this situation, there is an urgent need to develop a new and natural antibiotic, as there is a growing concern about pathogens in the food that are resistant to many drugs. Micro and macroalgae, phytoplankton, cnidarians, molluscs, corals, sponges, bryozoans and tunicates are the most common marine organisms that are focused for screening to identify their antimicrobial potential, especially to identify the compounds, that target pathogenic bacteria [4].

Ulva lactuca (U. lactuca) also known as green algae (sea lettuce), is classified as macroalgae in the phylum Chlorophyta [5]. The *U. lactuca* contains number of secondary metabolites, which showed the antibacterial, anti-inflammatory, antioxidant and anticoagulant activities [6-9]. The presence of these secondary metabolites was determined to be the reason for the antimicrobial activity of *U. lactuca* against Multidrug Resistant (MDR) of bacterial isolates. However, information on the potential of *U. lactuca* as an antibacterial agent ESBL and biofilm producing isolates is still limited. The present study was undertaken to study the preliminary phytochemicals analysis and antibacterial activity of *U. lactuca* against multidrug resistance and biofilm producing food pathogens.

MATERIALS AND METHODS

This was a pilot in-vitro study, conducted at Arignar Anna Government Arts College, Namakkal, Tamil Nadu, india, in the period of December 2017 to December 2020. For the present study, all chemicals and culture media were purchased from Himedia, India. Total 18 bacterial isolates were isolated from five retail chicken meat samples. These

were then, tested for its antibiotic resistance property using standard antibiotic discs.

Study Procedure

Isolation and identification of bacterial isolates: A total of five samples of chicken meat were obtained from retail shops in and around Namakkal area. The samples were wrapped aseptically and shifted to the laboratory promptly and samples were reviewed and studied within 24 hours. All samples were crushed with phosphate buffer with the help of mortar and pestle. A loopful of crushed samples was inoculated into sterilised selective media agar plates. Perfectly label the inoculated plates and incubate the same at 37°C for 24 hours. Upon incubation, the colonies were identified based on the colony morphology in the selective media and subjected for further studies.

Antibacterial susceptibility testing: Antibacterial susceptibility test was accomplished by Kirby-bauer method using sterile Muller Hinton agar plates in conferment with Clinical and Laboratory Standards Institute (CLSI; formerly NCCLS) briefing [10-12]. A sequence of 12 antibiotics was used to test the competency of the bacterial isolates.

Biofilm assay: Biofilm assay was carried out by Freeman et al., 1989 procedure [12]. The isolates were inoculated by single streak method on sterile brain heart infusion agar media with supplemented with sucrose (5%) and congo red (0.08 g/L). All plates were incubated at 37°C for 24 hours. The formation of black colour indicates the positive result.

Collection of algal samples: The algal samples of *Ulva lactuca* were acquired from Rameshwaram seashore during winter season from the month of December to March (2018-2019) from a profound length of about 50 m of the sea surface water in rocky area. The samples were then, transferred to the laboratory in aseptic manner in sterilised plastic bags with sea water to hinder evaporation of the collected samples. The epiphytes and rock debris from the algal samples were cleaned with utmost care, followed by which it was rinsed gently with fresh water for the surface salt removal. A small quantity of seaweed sample collected was saved for its identification. Upon cleaning, it was dried and shade dried for about two to three days (48-72 hours) [13].

Identification of algae: The algae were identified based on their taxonomical characteristics which were published by Jha B et al., [14]. The identification procedures were carried out at CSMCRI Marine Algal Research Station, Maandapam, Rameshwaram, India.

Preparation of algal extracts: The algal biomass which was dried and powdered was extracted using different solvents (ethanol, chloroform and ethyl acetate). The biomass was soaked in respective solvents mentioned above (10 gm: 150 gm) and retained in a rotary shaker at 150 rpm at room temperature for 72 hours. After the specified time, the extracts were filtered separately using Whatman No.1 filter paper. The filtrates were dried by evaporation under reduced pressure in a rotary evaporator. The crude extracts were then dissolved in respective solvent to a final concentration of 100 mg/mL as stalk solution. It was stored at -20°C for further procedures [15].

Preliminary phytochemicals analysis: The preliminary phytochemical studies for the algal extracts were accomplished by Solomon CU et al., procedure [16]. The phytochemicals like alkaloids, carbohydrates, flavanoids, phenols, saponins, tannins, terpinoids, quinines, glycosides, proteins and steroids were analysed.

Determination of antimicrobial activity: The antibacterial activity was determined by well diffusion method followed by Abdel-Khaliq A et al., [17]. Fresh bacterial cultures from 24 hour old broth were spread on sterile Muller Hinton agar plates. Metallic bores were used for making wells and the algal extracts were dispersed in the wells at different concentrations and labelled properly. The plates were incubated at 37°C for 24 hours and upon incubation, observed for the zone formation. The zones were measured and recorded.

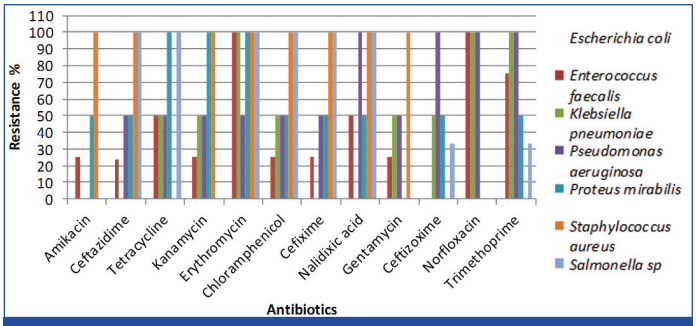
STATISTICAL ANALYSIS

Descriptive statistics were used and the data was presented in the form of percentages and mean and Standard Deviation (SD).

RESULTS

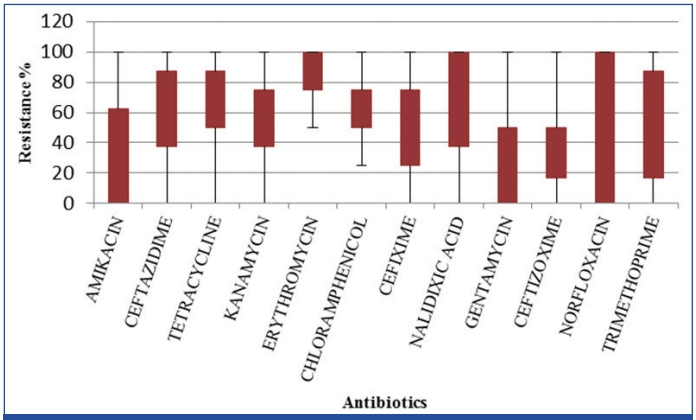
A total of seven genera of 18 bacterial isolates were isolated from five retail chicken meat samples. Among these populations isolated, 72.2% were identified as gram negative and 27.7% were found to be gram positive organisms. The predominant bacterial pathogen isolated was *E. faecalis* and *E. coli* (22.2%), followed by *Salmonella* spp. (16.6%) and lowest prevalence in *S. aureus* (5.5%).

In the present study, the authors monitor some antibiotics and their effect on three major bacterial isolates. Among the seven bacterial genera, single isolate of *S. aureus* (66.6%) was showed highest antibiotic resistance followed by *P. aeruginosa* (62.4%). In present study, 12 antibiotic tested, all isolates were resistance to atleast three drugs from a variety of antibiotic classes, mainly aminoglycosides, cephalosporins and fluoroquinolones [Table/Fig-1].



[Table/Fig-1]: Antibiotic resistance patterns of food isolates.

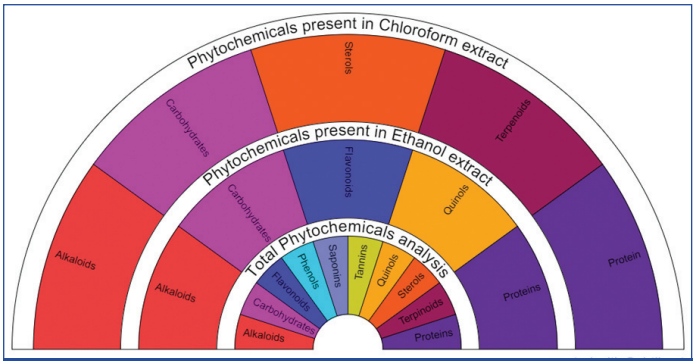
Moreover, significant MDR patterns was observed in *Salmonella* spp. Among the antibiotic tested, erythromycin, tetracycline, nalidixic acid and cephalosporins antibiotics were resistance to most of the *Salmonella* isolates [Table/Fig-2].



[Table/Fig-2]: Percentage of antibiotic resistance on food isolates.

In this investigation, next part of the study was determination preliminary phytochemicals on both solvent extract. Among the tested phytochemicals, alkaloids, carbohydrates, and protein were positive in both solvent extracts, flavonoids and quinones were showed in ethanol extract only. Sterols and terpenoids were present in chloroform extract only. The phenols and saponins were not observed in both extracts [Table/Fig-3].

The [Table/Fig-4] revealed that antibacterial activity of ethanol solvent extract of *Ulva lactuca*, among the seven genera, *E. faecalis* was highly suppressed, which exhibiting the zone of inhibition was ranged from 10±1.24 mm to 22±1.24 mm, and followed by *Proteus* spp. Presently, all isolates were suppressed while using 7.5 mg of extract and 2.5 mg of extract was suppressed only two isolates [Table/Fig-5]. Unlike the high potency of the ethanol extract, the chloroform extract exhibited less potency against these bacteria,

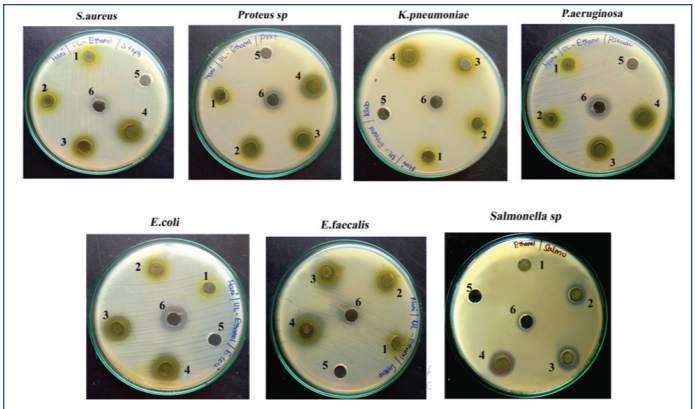


[Table/Fig-3]: Phytochemical analysis of ethanol and chloroform extracts of *Ulva lactuca*.

which exhibiting zone of inhibition was ranged from 10±0.816 mm to 13.16±1.027 mm [Table/Fig-6]. The negative controls (ethanol and chloroform) did not produce any zone of inhibition for all the bacterial strains tested, but positive control of ampicillin (10 µg/disc) produced mean zone of inhibition ranged from 11±0.81 to 15±0.81 mm [Table/Fig-7].

Isolates name	Con. of extract in mg zone of inhibition in mm				Ethanol	Ampicillin
	2.5	5	7.5	10		
E. coli	-	13±1.63	15±0.81	18±1.24	-	15±0.81
E. faecalis	10±1.24	14±1.63	18±1.24	22±1.24	-	14±1.63
S. aureus	-	10±0.81	14±1.63	17±1.24	-	12±0.81
K. pneumoniae	-	-	11±1.24	16±0.81	-	-
P. aeruginosa	-	-	12±0.81	14±1.63	-	14±1.24
P. mirabiis	10±1.24	12±1.24	14±1.63	17±1.24	-	12±1.24
Salmonella spp.	-	11±0.81	13±1.63	15±1.24	-	11±0.81

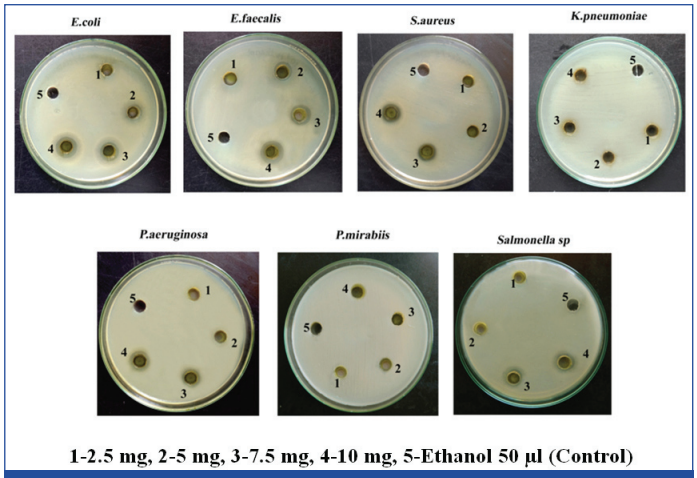
[Table/Fig-4]: Antimicrobial activity of ethanol extract of *Ulva lactuca* against food isolates.
The values presented as Mean±SD



[Table/Fig-5]: Antimicrobial activity of ethanol extract of *Ulva lactuca* against food isolates.

Isolates name	Con. of extract in mg zone of inhibition in mm				Chloroform
	2.5	5	7.5	10	
E. coli	-	10±0.816	11.16±1.027	12.33±1.247	-
E. faecalis	-	10±0.816	11±0.816	13.16±1.027	-
S. aureus	-	-	11.33±0.471	11.83±0.623	-
K. pneumoniae	-	-	-	-	-
P. aeruginosa	-	-	11.33±0.849	12±1.224	-
P. mirabiis	-	-	-	-	-
Salmonella spp	-	-	10.33±0.849	11±1.224	-

[Table/Fig-6]: Antimicrobial activity of chloroform extract of *Ulva lactuca* against food isolates.
The values presented as Mean±SD



[Table/Fig-7]: Antimicrobial activity of chloroform extract of *Ulva lactuca* against food isolates.

DISCUSSION

All isolates were confirmed with morphological and cultural characterisation with chromogenic media and selective media. This result revealed that such contamination occurred during the butchering of the animals and the processing of the meat. In fact, slaughtering chickens with *Salmonella* infections can contaminate the slaughter line, a significant source of cross-contamination [18]. The detection of *Salmonella* spp., in the present study is considered important, because *Salmonella* spp. the most important food borne pathogens worldwide. MRSA has been identified as a significant nosocomial pathogen and has been linked to food borne diseases [19]. Recently Likhitha P et al., observed the 7.6% of MRSA from food samples [20]. The present study is quite contrasting to that of the above study, where the percentage of *S. aureus* isolates was only 5.5%.

A major problem in poultry production worldwide today is infection by MDR bacteria. Poultry veterinarians are concerned because providing antibiotics to chickens at therapeutic and sub-therapeutic doses has always been an integral part of poultry production. Shrestha A et al., and Tawakol M et al., have observed the MDR of *P. aeruginosa* from poultry meat samples [21,22]. The similar line with the result was observed from Badr JM et al., study, they were observed the 100% of cephalosporins and 62.5% of aminoglycosides group of antibiotic resistance against to *P. aeruginosa* [23].

Moreover, significant MDR patterns was observed in *Salmonella* spp. This result was agreement with previous study of Hamed EA et al., they were also observed the similar line of the antibiotic resistance patterns [24]. Many of these antibiotics employed in poultry production, also serve as essential medicines for use in humans in many countries.

This resistance not only develops when given to growing poultry and also unhygienic slaughter practices are responsible for contamination of MDR isolates on meat samples. In the present study, 55.5% of the isolates were resistance to betalactam antibiotic of penicillin. As proof of this statement, 55% of the bacteria in the present study produced the betalactamase enzyme; among the seven genera *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* were highly produced. Moreover, the betalactamase producing isolates were resistance to other than betalactam antibiotics. This phenomenon was similar to previous study of Mukherjee M et al., reports [25].

Batabyal K et al., also observed the highest percentage of betalactam antibiotic resistance isolates from poultry meat samples in India [26]. The reason could be that those antibiotics are the most commonly prescribed treatment for bacterial infection and are the leading cause of antibiotic resistance in gram negative bacteria [27]. Furthermore, 72.2% of biofilm producing isolates were observed and unsurprising information is that, most of the bacteria produce the betalactamase enzyme and have high antibiotic resistance.

These isolates were limiting uptake of a drug, modification of a drug target and inactivation of a drug, therefore, not easily eradicate [3].

As an issue of deep scientific concern, the treatment of these drug resistance organisms is difficult to resolve, since, the prevalence of ESBL producing organisms is difficult to resolve for various reasons, including the difficulty of detecting ESBL production and inconsistency in reporting [28]. 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.

Number of studies has shown marine algae to possess antimicrobial, anti-allergic, and anticancer properties [29]. Green alga, *Ulva* spp., is documented to have antimicrobial activity against major pathogens such as *Staphylococcus aureus* and even MRSA [6,30]. Number of phytochemicals was responsible for the beneficial activity. *Ulva lactuca* is of the family Chlorophyta (green algae), which has plethora of secondary active metabolites [30]. In the present study, alkaloids, carbohydrates, and protein were positive in both solvent extract and flavonoids, tannins and quinones were showed in ethanol extract only. Anjali KP et al., also observed the various phytochemicals from solvent extract of *Ulva lactuca* [31].

The previous studies was performed outstanding antimicrobial activity of ethanol extract of *Ulva* spp., against several bacteria including *P. aeruginasa, E. coli, K. pneumonia* and *S. aureus* [32,33]. In 2017, El-shouny WA et al., determined the antibacterial activity of *Ulva* spp. against MDR isolates [34]. However, the antibacterial effect of *Ulva lactuca* against ESBL producing bacteria has not been studied so far. In the present study, it is clear that the antibacterial activity was without any difference between gram negative and gram positive isolates. It was also observed that, ethanol extract showed highest inhibitory activity because of its high polarity and it allows extracting all the phytochemicals.

Furthermore, phytochemicals analysis was observed from both extracts, those metabolites are responsible for beneficial activity. The flavonoids and tannins positive result in the ethanol extract and which were used therapeutically as antiviral, antibacterial, antiulcer and antioxidant agents. Many drugs containing tannin are well-known to possess general antimicrobial properties reported [35]. Based on the results of the present study, the phytochemicals extracted from green algae *Ulva lactuca* have shown a relatively strong antibacterial activity on bacteria and it could be considered as, a source of novel antibiotic.

Limitation(s)

The minimum inhibitory concentrations were not performed, the present study can only be considered preliminary and further studies are needed to explore the possibility of using it, as an antibacterial agent, against food pathogens and food preservative.

CONCLUSION(S)

The findings of the present investigation demonstrated that *Ulva lactuca* extracts in ethanol and chloroform had positive antibacterial effects on isolates of MDR bacteria, biofilm and ESBLs. The highest antibacterial activity was observed with the ethanolic extract, and further characterisation of the phytochemicals revealed the presence of beneficial compounds that, may account for the observed antibacterial activity. From this, it can be concluded that, the highly polar compounds present in the *Ulva lactuca* extract may be responsible for the significant antibacterial activity. Therefore, further studies are needed to evaluate the antibacterial activity of different extracts and to determine the chemical structure of the active ingredients.

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