Antibacterial activity of methanol extract of Gracilaria edulis (Rhodophyseae) in Sri Lanka

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Abstract

Seaweeds are important living organisms in the marine environment because they are recognized as a potential source of bioactive natural products. The methanol extract of Gracilaria edulis seaweed was prepared to screen its antibacterial activity against four common bacterial pathogens using the disc diffusion method. The tested pathogenic strains were Escherichia coli, an extended spectrum beta lactamases (ESBL) producing coliform, Pseudomonas aeruginosa and Staphylococcus aureus. The Minimum Inhibitory Concentrations (MIC) of the extract against the 4 organisms was determined using the broth macro dilution method. Methanol saturated sterile discs were used as the negative control and did not show any inhibitory zone against the tested 4 organisms. Four antibiotics, such as ceftazidime, cefotaxime, augmentin and ampicillin were used for comparison. The zone of inhibition (ZOI) produced by methanol extract of G.edulis was maximum against S.aureus (16±0.5 mm), medium against E.coli (12±0.8 mm) and ESBL producing coliforms (12±0.5 mm) but with minimum activity against P.aeruginosa (10±0.8 mm). In comparison, the ZOI produced by ceftazidime, cefotaxime and augmentin against E.coli were 26±0.8 mm, 32±0.8 mm and 18±0.8 mm respectively, against the ESBL producing coliform 25±0.5 mm, 32±1.4 mm, and 16±0.5 mm respectively and against S.aureus 9±0.5 mm, 21±1.9 mm and 30±0.5 mm respectively. A ZOI of 16±1.3 mm and 20±1.9 mm was exhibited only by ceftazidime and cefotaxime against P.aeruginosa. Ampicillin was relatively inactive against all test strains, giving a ZOI of 11.7±0.5 mm and 11.5±0.5 mm against E.coli and the ESBL producing coliform respectively. The MIC of the methanol extract was lowest for S.aureus at 1.25 µg/ml, 2.5 µg/ml for E.coli and ESBL producing coliform and 5 µg/ml against P.aeruginosa. The activity of the methanol extract of G.edulis against S.aureus, an ESBL producing coliform and E.coli is promising antibacterial and needs to be studied further.

Key words: Gracilaria edulis, methanol extracts, antimicrobial activity, zone of inhibition, minimum inhibitory concentration.

1. INTRODUCTION

Marine macroalgae are commonly called as seaweeds. Seaweeds are the renewable living resources which are also used as potential food applications and feed fertilizer in many parts of the world. It is used as sea vegetable for human diets in several Asian countries (Nisizawa, 2002). Nutritional interests of edible seaweeds are low calorie food, but rich in vitamins, minerals and dietary fibers (Ito and Hori, 1989). In addition which are having carotenoids, essential fatty acids, proteins, polysaccharides and fibers (Darcy-Vrillon, 1993; Lahaye, M., 1991). The unsaturated lipids are present in very small amounts, which protect from cardiovascular pathogens. Seaweeds are able to produce a great variety of secondary metabolites characterized by a broad spectrum of biological activities due to that they are considered as source of bioactive compounds. Bioactive Compounds with cytostatic, antihelmintic, antifungal, antibacterial and antiviral activities have been detected in green, brown and red algae (Newman, *et al*., 2003; Lindequist and Schweder, 2001). The Seaweeds extracts were preventive and curative agent for various diseases such as antibiotics, cough remedies, antihelminthics, antihypertensive, antidiarrhoea and antitumour. The chemical investigation of marine algae with a special accent shows on their bioactive properties (Siddhanata, *et al*., 1991). The secondary metabolites functions are defense mechanisms against herbivore, fouling organisms and pathogens, chemical defence mechanisms against herbivore; for example grazer induced mechanical damage

triggers the production of chemicals that act as feeding detergents or toxins in seaweeds (Kolanjinathan, *et al*., 2014).

There are numerous research reports are illustrated inhibiting activities from macroalgae against yeasts, fungi and human pathogens. Selective utilization of seaweeds as potential source of pharmaceutical agents has been increasing in recent years which many of the seaweeds possess bio-active components that inhibited the growth of some of the Gram positive and Gram negative bacterial pathogens. For many years antibiotic treatment has been applied to cure microbial diseases. The treatment of these infectious diseases by applying products from macroalgae appears as a possible alternative. So the interest in seaweeds as a promising potential source of pharmaceutical agents has increased during the last years (Mayer and Hamann, 2002; Newman, *et al*., 2003; Lindequist and Schweder, 2001). Among the marine organisms, seaweeds play a vital role as a source of biomedical compounds for pharmacological and biological activities (Manilal, *et al*, 2010). Macroalgae are used to isolate about 2400 natural products belonging to the classes Rhodophyceae, Phaeophyceae, and chlorophyceae. Bioactive secondary metabolites are indicators to detect the antimicrobial activities. Compounds derived from the seaweeds are having broad range of biological activities such as antibacterial activity (Chakraborthy, *et al*., 2010). Acrylin acid halogenated aliphatic compounds, chlorellin derivatives phenolic inhibitors, more recently guaiane sesquiterpenes and labdane diterpernoids were also antimicrobial agents detected from seaweeds (Chakraborthy, *et al*., 2010).The highest antibacterial activity was found in the class Rhodophyceae (80%) followed by the Chlorophyceae (62.5%) and the Phaeophyceae (61.9%) (Padmakumar and Ayyakannu, 1997).

To date many chemical unique compounds of seaweeds such are primary and secondary metabolites with various biological activities have been found and some of them are under investigation and are being used to develop new pharmaceuticals. Methanol is a suitable solvent to extract bioactive components and show the greatest inhibition diameters against Gram Positive and Gram Negative bacterial isolates (Kolanjinathan and Saranraj, 2014). The genus *Gracilaria* is an important component of the macroalgae especially found in Kalpitiya Sea in the Sri Lanka. Many of these species, especially *G.edulis* however, are poorly defined, minimally described and inadequately illustrated its antimicrobial activities. Particularly the *G.edulis* is one of the Rhodophyta and having good source of bioactive components. Hence this study was undertaken to screen the antibacterial activities of methanol extract of *G.edulis* for common disease causing selective bacterial pathogens.

2. MATERIALS AND METHODS

Collection of seaweeds:

The seaweeds sample *G.edulis* was collected from Kalpitiya Puttalam in North West coast of Sri Lanka.

Preparation of seaweed extracts:

The collected seaweed sample *G.edulis* was cleaned with tap water to remove associated debris and necrotic parts. The seaweed was dried at room temperature for 1week until brittle easily by hand. The 1.0 kg of seaweed material was ground to a fine powder using electrical blender after completely drying. 40 g of powdered sample were taken and it was extracted successively with 200 ml of methanol solvent in whattman filter paper No.1. Until the extract was clear. The extracts were evaporated to dryness and finally the resulting pasty form extracts were stored in a refrigerator at 4°C for future use.

Collection of Test Microbial Cultures:

Four different common bacterial cultures were used in this present study. Those are obtained from Pathophysiological lab, Faculty of Health Care Sciences, Eastern University, Sri Lanka. *Pseudomonas aeruginosa,* Bacteria that produce enzymes called extended-spectrum beta-lactamases (*ESBLs*), *Escherichia-coli*, *Staphylococcus aureus*.

Minimum Inhibitory Concentration (MIC):

The *G.edulis* extracts minimum inhibitory concentration (MIC) against common pathogenic bacterial isolates was tested in Mueller Hinton broth by Broth macro dilution method. The *G. edulis* extracts were dissolved in 5% DMSO to get 128 mg/ml stock solutions. 0.5 ml of stock solution was incorporated into 0.5 ml of Mueller Hinton broth for bacteria to obtain a concentration of 1.25, 2.50, and 5mg/ml (for *G.edulis* extracts). Devoid of seaweeds

extracts/FAME active principle and 50µl of standardized suspension of the test organism were added to culture tubes. The culture tubes were incubated in bacteriological thermostat incubator (India, B1021+) at 37°C for 24 hours. The lowest concentrations was found to each mentioned in above microbes which did not show any growth after macroscopic valuation was determined as Minimum inhibitory concentration.

Disc Preparation:

No.1 Whattman filter paper 5 mm diameter disc were pretreated. These were sterilized in the mermet (schcvubate) hot air oven at 160°C for 1 hour. The solvent extracts of *G.edulis* were mixed with 1ml of Dimethyl sulfoxide (DMSO) and methanol. The discs were impregnated with 20µl of methanol solvent extracts of *G.edulis* sea weeds at 5mg/ml concentration to check their antibacterial activity.

Determination of antibacterial activity of *G.edulis*

Autoclaving

The thread test tubes with lids, needle with cotton swap, petridishes and conical flasks were autoclaved with use of equis autoclaver Taiwan excellence at 121°C for 15 minutes in 1.5lbs.

Preparation of Nutrient agar:

4.2g of Nutrient agar (m001Hi media) powder was added in conical flask with150ml of distilled water. This mixture was heated while stirring to fully dissolve all components. The dissolved mixture was autoclaved at 121°C for 15 minutes. Autoclaved nutrient agar was allowed to cool but not to solidify. Nutrient agar was poured into each plate and leave plates on the sterile surface until the agar is solidified. The lid of each Petri dish was replaced and stored in a refrigerator.

Bacterial Inoculum Preparation:

Bacterial inoculum was prepared by inoculating a loopful of test organisms in 5 ml of Nutrient broth and incubated at 37°C for 3-5 hours till a moderate turbidity was developed. The turbidity was matched with 0.5 Mc Farland standards and then used for the determination of antibacterial activity.

Preparation of Mueller-Hinton agar:

5.7g of medium was added into conical flask with 150 milliliter of purified water. This mixture was heated while stirring to fully dissolve all components. The dissolved mixture was autoclaved at 121°C for 15 minutes. It was cooled to 45°C. Cooled Mueller Hinton agar was poured into sterile petri dishes to a level and plates were kept horizontally to give uniform depth. It was allowed to solidify at room temperature. Prepared media was stored in refrigerator.

Disc Diffusion Method:

Disc diffusion method (Bauer *et al*., 1966) was used to determine the antibacterial activity of *G.edulis* extract. A bacterial suspension was prepared from the nutrient agar contained pure culture (number 0.5 in McFarland scale about 1.5 x 108 bacteria ml). Bacterial suspension was spread 1 on Mueller-Hinton agar using a cotton swab. The Mueller Hinton agar plates were inoculated with test bacterial organisms (*P.aeruginos, ESBL* bacterias, *E.coli, S.aureus)* by spreading the bacterial inoculum on the surface of the media. One of the air dried discs contained *G.edulis* methanol extracts at 5mg/ml concentration were placed on the surface of the Mueller Hinton agar plates. Rest of the discs containing standard concentration of Ceftazidime (30 PB05-CAZ), Cefotaxime (30 OE25-CTX), and Augmentin (30 OJ10-AUG) were used as antibiotics. Ampicillin was used as positive control. Methanol saturated sterile discs were used as negative control. The discs were placed on Mueller Hinton agar plates inoculated with each of the previously mentioned microorganisms. The plates were incubated at 37°C for 24 hours. The *G.edulis* antibacterial activity was evaluated by measuring the diameter of the inhibition zone (in mm). Six replicates were maintained to check the consistency of the experiments or results.

3. RESULTS AND DISCUSSION

Fig1:Sample microbes (*E.coli,ESBL,P.aeroginos,S.aureous*)

Fig2:Autoclaving

Fig3:Pureculture preparation in the nutrient agar plates

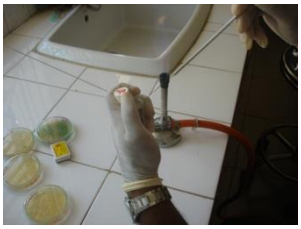


Fig4:Bacterial suspension

Fig5:Bacterial pure culture in Muller hinton agar plates

Fig6:Antibacterial disc on Muller hinton agar plates

Fig7:Incubate the microbes with antibacterial disc in Muller hinton agar plates

Fig8:Measure the minimum inhibitory zone

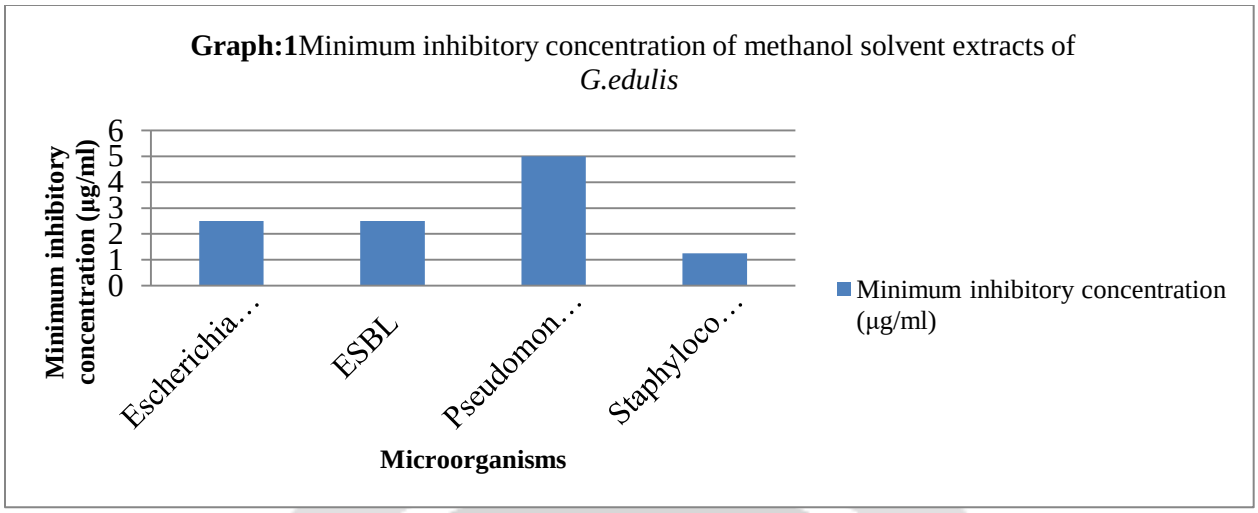
Table 1: Minimum inhibitory concentration of methanol solvent extracts of *G.edulis*

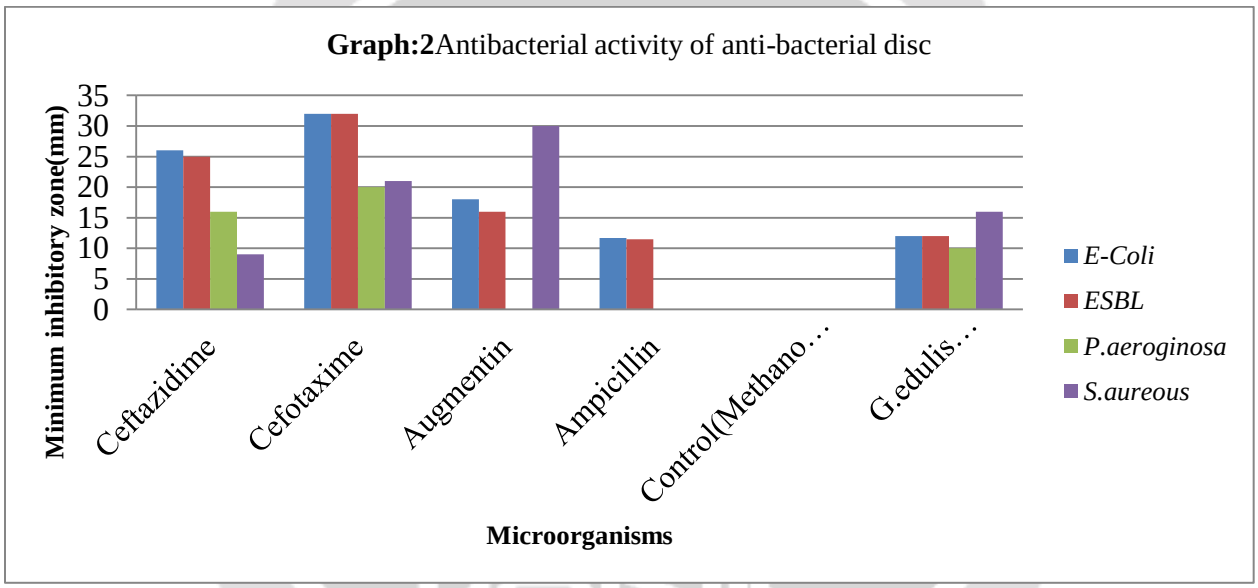
Microorganisms	**Minimum inhibitory concentration (µg/ml)**
Escherichia coli	2.50
ESBL bacterias	2.50
Pseudomonas aeruginosa	5.00
Staphylococcus aureus	1.25

Table:2. Antibacterial activity of Anti-bacterial disc (inhibition of growth expressed as mm diameter of inhibition zone).

Antibiotic components	***E-Coli***	***ESBL* bacterias**	***P.aeroginosa***	***S.aureus***
Ceftazidime	26(±)0.8	25(±)0.5	16(±)1.3	9(±)0.5
Cefotaxime	32(±)0.8	32(±)1.4	20(±)1.7	21(±)1.9
Augmentin	18(±)0.8	16(±)0.5	00	30(±)0.5
Ampicillin	11.7(±)0.5	11.5(±)0.5	00	00
Control(Methanol saturated sterile disc)	00	00	00	0
G.edulis methanol extract	12(±)0.8	12(±)0.5	10(±)0.8	16(±)0.5

+/- Standard deviation





G.edulis was collected from the sea water of Kalpitiya along the North West coastal region of Sri Lanka. *G.edulis* was evaluated for their antimicrobial activity. Table 2 revealed that *G.edulis* algal extracts prepared with methanol which was inhibited growth of the pathogenic bacterias such as *E.coli, ESBL, P.aeroginosa* and *S.aureus*. The antibacterial activity of methanol extract of *G.edulis* against four common pathogenic bacterial strains was presented in Table2. The methanolic extract of *G.edulis* showed considerable antibacterial activity against the common pathogenic bacterial test organisms. The *G.edulis* methanolic crude was shown strong inhibitory effects against all the selected test microorganisms at different concentrations from 1.25 to 5.00 (5µg/disc). The Minimum inhibitory concentration (MIC) values of *G.edulis* methanol extract against four common Pathogenic bacteria was shown from 1.25 to 5µg/ml (Table: 1). Minimum concentration of *G.edulis* methanol was identified to inhibit the growth of those microbes (Graph: 1).

The zone of inhibition ranged between 10-16 mm. The maximum zone of inhibition activity 16(±)0.5mm was recorded from the extract of *G. edulis* against *S. aureus* and minimum 10(±)0.8mm against *P. aeruginosa*. The methanolic extract of red seaweed *G.edulis* was showed strong activity against *S.aureus* at 5µg/disc concentration.

From this study it was found that the Gram positive bacteria are more susceptible than Gram negative bacteria on *G.edulis* methanolic extracts due to the differences in their cell wall structure and their composition. The methanol extract of *G.edulis* (5.0 mg/ml) shows highest mean zone of inhibition (16±0.5mm) against the Gram positive

bacterial cocci *S.aureus*. For Gram negative bacterium, the maximum zone of inhibition was recorded with methanol extract of *G.edulis* against *Escherichia coli* (12±0.8mm) followed by *ESBL* (12±0.5mm) and *P.aeruginosa* (10±0.8mm).

The negative control as methanol impregnated discs showed no zone of inhibition against all common tested bacterial pathogens and the 5mg/ml concentration of ampicillin impregnated positive control discs were screened 11.7±0.5mm, 11.5±0.5mm, 00mm and 00mm for *Escherichia coli, ESBL* bacterias, *P.aeruginosa and S. aureus* respectively.

G.edulis algal extracts prepared with methanol were recorded higher inhibitory activities against *S.aureus* compared to *E. coli*, *ESBL* bacterias and *P.aeruginosa* further observation from the results. *P. aeruginosa* was found to be highly inhibited by Cefotaxime compared to Ceftazidime but Augmentin was not inhibitted the growth of *P. aeruginosa*. Among the three antibiotics tested for inhibitory activities, Cefotaxime was showed relatively higher inhibitory activities against *E.coli, ESBL* bacterias, *P.aeruginosa*, *S.aureus.*

Whereas *G.edulis* methanol extracts, showed inhibition of growth of *E.coli and ESBL* bacterias were higher than Ampicillin and lesser than that compared with Ceftazidime, Cefotaxime and Augmentin. *P.aeruginosa* was not inhibited by ampicillin and Augmentin.(Table:2) but it was inhibited highly by Cefotaxime followed by Ceftazidime and *G.edulis* methanolic crude. *S.aureus* was inhibited highly by Augmentin followed by Cefotaxime, *G.edulis* methanol extract and Ceftazidime but which growth was not inhibited by Ampicillin among the antibiotics tested. Further the *G.edulis* methanol extract was found to be more effective against *S.aureus, E. coli,* and *ESBL* bacterias compared to that against *P.aeruginosa*.

Finally results concluded, methanolic extracts of selected seaweed of *G.edulis* were potentially a good source of antibacterial substances with a broad range of activities in retarding and preventing the growth of all selected common pathogenic bacteria.

Many research reports revealed antibacterial activity of red, brown and green algae against both Gram positive and Gram negative bacteria. The best antibacterial activity was screened with *G.edulis* (Selvi., 2000).The *G. edulis* of methanolic extract was inhibited most of the common pathogenic bacteria such as *E. coli, ESBL* bacterias, *P.aeruginosa*, and *S.aureus.* The *G.edulis* macroalgae was screened more or less equal zone of inhibition or slightly greater against some common bacterial pathogens when compared with positive control ampicillin impregnated positive control discs. In this study *E.coli, ESBL* bacterias and *S.aureus* were the most sensitive test microorganisms and *P.aeruginosa* was the most resistant to antibacterial activity.

According to earlier research study reports the extraction method and anti-bacterial activity efficiency depends on algal species research study revealed that, Methanol was the most effective solvent for the extraction of the bioactive compounds compare to the other solvents. The effectiveness of extraction methods were focused that methanol extraction yields the highest antimicrobial activity compare to the other solvents (Abirami and Kowsalya., 2011). The *G.edulis* methanol extract showed the greatest inhibition diameters against Gram positive and Gram negative bacterial isolates which was clearly noticed maximum inhibition to methanol (Kolanjinathan and Saranraj., 2014).

Muller-Hinton agar (MHA) was used as microbiological growth medium for antibiotic batch-to-batch reproducibility for susceptibility testing because it is a non-differential, non-selective medium which means that almost all organisms plated on here will grow. Additionally, starch of MHA ingredients is absorbed toxins released from bacteria. MHA allows for better diffusion of the antibiotics than most other plates due to its loose condition. Therefore MHA was selected to grow those bacterial strains. The agar medium were poured in plates to a depth of 4 mm because Plates that are too shallow will show false susceptible results as the antimicrobial compound will diffuse further than it should, creating larger zones of inhibition. Conversely, plates poured to a depth higher than 4 mm will result in false resistant results.

The Minimum inhibitory concentration (MIC) values of G.edulis methanol extract against four common Pathogenic bacteria was shown from 1.25 to 5µg/ml. (Table:1). This MIC was minimum concentration to restrict the growth of such germs. Therefor 5mg/ml G.edulis methanol extract minimum concentration was selected as higher than MIC and equal to antibacterial disc concentration.

ESBL is known as Extended-Spectrum beta-Lactamase enzymes which are produced most antibiotic resistant bacteria and can make the infection harder to treat. The most common ESBL producing bacterial microbes are *Klebsiella and Escherichia coli (E.coli) which are* commonly live in the gut/bowel. *ESBL* is usually not a problem to healthy people. *ESBL*-producing germs in their bowel are usually harmless to Patients without infection. However in some patients infections do occur. Urinary tract infections (UTI's), wound infections and chest infections which are the most common *ESBL* infections. It is spread mainly by direct contact with the patient or their immediate environment. In this research reports one of the antibacterial disc with *G.edulis* methanolic extract prevented the growth of microbes from *ESBL*.

Furthermore earlier research results, genus *Gracilaria* was the most effective marine macroalgae against tested bacterial species followed by *D. ciliolata, U. reticulata, C. occidentalis* and *Cladophora socialis* (Meyer and Paul, 1992; Isnansetyo, *et al*., 2003, Burkholder, *et al*., 2009; Saeidnia, *et al*., 2009; Williamson and Carughi, 2010; Khalil and El-Tawil, 1982). The seaweeds have flavonoids, polyphenols and carotenoids referred to as antioxidants which protect the body's tissues against oxidative stress and associated pathologies such as inflammation and cancer (Tapiero, *et al.,* 2002). *G.edulis* was very important a red algae for antimicrobial activities such as which was used to prepare antibacterial components in the pharmaceutical industry (Kolanjinathan and Saranraj, 2014).

The most common pathogenic bacterial strains such as *E. coli, ESBL* bacterias, *P.aeruginosa*, and *S.aureus* growth were inhibited by methanolic extract of *G.edulis.* It is efficiently inhibited against *S.aureus* followed by *E. coli* and *ESBL* bacterias, minimally to *P.aeruginosa*. Methanolic extract of *G.edulis* was considerably high antibacterial activity than Ampicillin and lower than cefotaxime ceftazidime and augmentin to the *E. coli* and *ESBL* bacterias which was evident from inhibition zones produced. The inhibition zone diameter was high which revealed that antibacterial compound was retard or resistant the growth of that common pathogenic bacteria but low inhibition zone diameter was reveled which antibacterial disc/compound had more susceptible to those bacterial strains.

4. CONCLUSION

This research study revealed that methanol extracts of *G.edulis was* showed promising antimicrobial activity. This macroalgae could serve as important source of new antimicrobial agents. Antimicrobial activity of marine seaweed *G.edulis* was screened for uses in the traditional system of medicine to treat various infectionous diseases caused by the common bacterial pathogens. The antimicrobial activities were screened in the present study by using methanol extract of *G.edulis* seaweed. Methanolic extract of *G.edulis* seaweed was inhibited growth of common pathogenic bacteria which indicated by inhibition zone. Thus *G.edulis* was the richest source of novel bioactive compounds (primary and secondary metabolites). Inhibited zones were related to the presence of bioactive metabolites in the *G.edulis,* which were soluble in methanol. The data and results of this research were screen the antibacterial activity of *G.edulis* algae which was effective in inhibiting the growth of common pathogenic bacteria such as *E.coli, ESBL* bacterias, *P.aeroginosa* and *S.aureous*. *G.edulis* algal extracts prepared with methanol were recorded higher inhibitory activities against *S.aureus* followed by E. coli, *ESBL* bacterias, *and P.aeroginosa.* The obtained outcomes may provide a support to use of the *G.edulis* in Pharmaceutical industry. Further the Kalpitiya, Puttalam Sea has potential source to change pharmaceutically useful *G.edulis* seaweeds which can be harnessed for the development of drugs for use in management of common pathogens. This research dissemination also encourages cultivation of the highly valuable seaweed *G.edulis* in large scale to increase the economic status of the cultivators in the Sri Lanka. The present study suggests that methanolic extract of *G.edulis* possessed antibacterial activity against common bacterial pathogens thus supporting their usage, promising a future scope for use of this marine seaweed against another microbial population. This study suggests the possibility of using *G.edulis* seaweed extracts as natural antimicrobials in food industry. It was great findings for further research toward drug development and uncontaminated food for long period to preserve to serve.

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