



Inhibitory Potentials of *Kappaphycus Alvarezii* on Bacteria Isolated from Clinical Samples

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Article History	Abstract
Received: 06 June 2023 Revised: 05 Sept 2023 Accepted: 10 Nov 2023	<i>Kappaphycus alvarezii</i> is one of the high stress tolerant sea weed with good biopotentials. This was collected, processed, extracted and subjected to antibacterial activity against MDR UTI isolates. All the isolates were identified using standard biochemicals and other textual procedures along with 16S rRNA sequencing. Antibacterial activity was assessed by disc diffusion method and followed standard textual procedures to assess MIC, MBC, Percentage inhibition, IC ₅₀ and LC ₅₀ . Six different test organisms were used for studying antibacterial activity. Antibacterial activity of all extracts on different extracts were varied from 7.2±0.6mm zone of inhibition. Concentration required to inhibits the growth of the UTI causing test organisms found to varied from 383.3±57.7µg/ml to 1033.0±76.3 µg/ml. extracts inhibited the growth of bacteria especially <i>Staphylococcus aureus</i> var <i>haemolyticus</i> was up to 98.2%. antibacterial substances like flavonoids and phenolic compounds could be responsible for this kind of activity.
CC License CC-BY-NC-SA 4.0	Keywords: <i>Kappaphycus alvarezii</i> , sea weed, rhodophyta, antibacterial activity, UTI

1. Introduction

Now a day bacterium becomes resistant to a greater number of antibacterial agents, which could lead to the development of new versions of infection. To overcome this problem there is a new interest among scientists for the discovery of bioactive chemicals and biomolecules from the nature especially marine sources. One among the natural source of biochemical is seaweed [1]. Seaweeds could be from chlorophyta, rhodophyta and ochrophyta and are grows in extreme environments [2, 3]. *Kappaphycus alvarezii* is one among the most important red algae (rhodophyta) which possess multipotent biological activities. These algae grow effectively in marine ecosystem especially Indo-Pacific region as it is native to this region best growth is noted in euhaline and polyhaline region. It is also growing best at subtidal region and belongs to Areschougiaceae. This also grow best at alongside of coral reefs. Red algae like *Kappaphycus alvarezii* possesses primary and secondary metabolites. These metabolites include protein phenolic compounds, phenols, flavonoids, long chain fatty acids, pigments and sterols [3, 4, 5]. Among the secondary metabolites water soluble phenolic compounds, which are found in the vacuoles of red algae available as monomeric and polymeric forms and linked with aromatic hydrocarbon with hydroxyl groups [4, 6, 7]. To understand the efficiency of whole *Kappaphycus alvarezii* this present work is undertaken to screen antibacterial efficiency of virulent and MDR strains of bacteria isolated from clinical samples.

2. Materials And Methods

Selection and Collection of red algae *Kappaphycus alvarezii*

Kappaphycus alvarezii is a red tropical seaweed that is important for the economy. It is a major source of carrageenan, which is used in many food, medicine, industrial and biological uses. *Kappaphycus alvarezii* was collected from the artificially cultivated *Kappaphycus alvarezii* in a seashore (Lat 9.971071° and long 79.199178°) in Kottaiappattinam, Pudukottai district, Tamil Nadu 614 619, India. The seaweed was collected during north east monsoon season of the year 2021 (01.11.2021). The

seaweed was first washed in salt water and then in fresh water. Seaweeds are dried in the sun and then ground into a powder with a motorized mixer.

Preparation of extract

The active parts of *Kappaphycus alvarezii* powder were extracted with water, chloroform, and ethanol by keeping the algae in respective solvents for 24 hours at room temperature while shaking every now and then. After 24 hours, the solution was filtered through Whatman filter No. 1 paper and concentrated with a rotating evaporator until it was dry. The leftovers were used to study the phytochemistry.

Test Bacteria for antibacterial assay [8]

The test organisms used in this study were isolated from the urine samples from the UTI cases. Urine sample was directly inoculated on Hi-Chrome UTI agar, which differentiates causative agents of UTI. *Enterococcus faecalis* produce small blue coloured colonies, similarly *Escherichia coli* produce pink-purple colour colonies, *Klebsiella pneumoniae* produce mucoid blue to purple colonies, *Pseudomonas aeruginosa* produce colourless to greenish colonies and *Proteus mirabilis* produce light brown to golden yellow colonies. Similarly, *Shewanella* produces greenish colour colonies. *E. coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Proteus mirabilis*, *Enterococcus faecalis* and *Staphylococcus haemolyticus* were the test organisms used for antibacterial assay of *Kappaphycus alvarezii* extracts.

Differential Identification of clinical isolates

Selected colonies from selective and differential media were subjected to macroscopy, microscopy and biochemical tests for identification 9,10.

16s rRNA analysis of test organisms [11, 12,13,14]

The universal primers were used for the amplification of 16s rRNA gene were 5'AGA GTT TGA TCC TGG CTC AG'3 and R 5'ACG GCT ACC TTG TTA CGA CTT'3. Multiple sequence alignment was performed for homologous sequences and a phylogenetic tree was constructed using the neighbour joining method.

Assessment of Antibiotic Sensitivity Pattern of Uropathogens

Commonly used fifteen antibiotics were used to check antibiotic sensitivity pattern of the test isolates by making use of internationally accepted methods [15,16]

Determination of Antibacterial activity of extracts using disc diffusion method

Antibacterial sensitivity of the extracts was assessed by making use of well diffusion method [16]. The Mueller Hinton agar plate was prepared and inoculated with 0.1ml of test organisms (Suspension made with 0.5 Mc Farland's standard solution) by spread plate technique. Then, six well was made using cork borer with a diameter of 6 mm. By using a micropipette, five different concentrations of the plant extract solutions (0.5mg, 1mg,1.5mg, 2mg / well) was added to the well. The antibiotic ampicillin acts as a positive control and DMSO acts as a negative control.

Determination of the Minimum Inhibition Concentration (MIC)

The minimum inhibitory concentration (MIC) of extract was evaluated according to standard methods [17,18] with minor modification by employing 96-well micro plates (Table 1). Resazurin (7-Hydroxy-3H-phenoxazin-3-one 10-oxide) is a blue dye which can be irreversibly reduced to a pink and highly red fluorescent substance, resorufin by oxidoreductase within viable cells [19-22]. Changes of colour was observed and recorded. The lowest concentration prior to colour change was considered as the Minimum Inhibitory Concentration (MIC).

Determination of Minimum Bactericidal Concentration (MBC)

The MBC of the extracts were assessed by making use of the method Pfaller *et al.*, [23], which is also illustrated in table 1.

Determination of % inhibition

This involves calculating the extracts' inhibitory effect at a given concentration using the dilution and GC tubes' total viable count values. To compute it, the following formula was applied.

$$\frac{\text{Number of colonies in tube GC} - \text{Number of colonies in dilution tube}}{\text{Number of colonies in tube GC}} \times 100$$

Determination of IC₅₀

The FDA defines IC₅₀ as the amount of a medicine needed to inhibit organisms in in-vitro experiments 50% of the time. It is derived from the extract concentration and inhibition. The formula was utilised to determine the IC₅₀.

$$\frac{\text{Concentration of Extract}}{\% \text{ inhibition}} \times 50$$

**Table –1 ((100mg/10ml)
Assessment of MIC, MBC and IC₅₀**

Tube No.	1	2	3	4	5	6	7	8	9	10	GC
Volume of Mueller Hinton broth in µl	145	140	135	130	125	120	115	110	105	100	100
Volume of Extract / antibiotics in µl	05	10	15	20	25	30	35	40	45	50	0
Initial Total Extract concentration in µg	50	100	150	200	250	300	350	400	450	500	0
Bacterial Suspension in µl	50	50	50	50	50	50	50	50	50	50	50
Final Volume in µl	200	200	200	200	200	200	200	200	200	200	200
Final extract conc. µg/ml	250	500	750	1000	1250	1500	1750	2000	2250	2500	0
A.C = Antibiotic Control, G.C = Growth Control											
Incubate for 24 hours at 37°C											
Read OD at 620 nm, it will provide MIC value											
Inoculate each growth contents (0.1ml) on Mueller Hinton Agar by spread plate method (After Proper Dilution)											
Incubate for 24 hours at 37°C											
Count number of colonies											
Calculate % growth inhibition and MBC											
Finally calculate IC ₅₀											

3. Results and Discussion

Kappaphycus alvarezii sea weed was collected from the Kottaipattinam sea shore and processed, dried, powdered and extracted with chloroform (KACE), ethanol (KAEE) and water (KAAE). Dried powder of the extracts was subjected for analyzing antibacterial activity by making use of UTI isolates. Six different UTI bacterial isolates were collected and identified by microscopic, growth on selective cum differential medium and Biochemical tests. These preliminarily identified organisms were submitted for 16SrRNA analysis. On the basis of these results the isolates were identified as *E. coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Proteus mirabilis*, *Enterococcus faecalis* and *Staphylococcus haemolyticus*. GenBank Accession Number of *Escherichia coli* isolate was **OK394043**. Similary GenBank Accession Number was obtained for other organisms they are as follows *Pseudomonas aeruginosa* (OK394057), *Klebsiella pneumoniae* (OK394046), *Proteus mirabilis* (OL451945), *Enterobacter faecalis* (OK360923) and *Staphylococcus aureus var haemolyticus* (MZ618708).

All the bacterial isolates were subjected for antibiotic sensitivity assay, which revealed that all the organisms were resistant to multiple number of antibiotics tested. Three different extracts were collected from the red seaweed *Kappaphycus alvarezii*. Among the extracts ethanolic extract (KAEE) of this alga produced higher zone of inhibition against all the test organisms followed by aqueous extract (KAAE) and chloroform extract (KACE). KAEE produced 15.4±1.8mm zone of inhibition against *Enterococcus faecalis* at 250µg/disc concentrations, which is followed by the same extract against *Staphylococcus aureus* (14.9±2.3mm zone inhibition) and KAAE against *Staphylococcus aureus* (14.8±1.0mm). Extracts of *Kappaphycus alvarezii* inhibited all organisms effectively than the commercial antibiotics (Table 2), which could be due to the flavonoid like chemicals. These chemicals along with phenolic compounds influence on active, passive and felicitated diffusion mechanism thereby reduce bacterial growth.

Table 2: Antibacterial activity of *Kappaphycus alvarezii* extracts

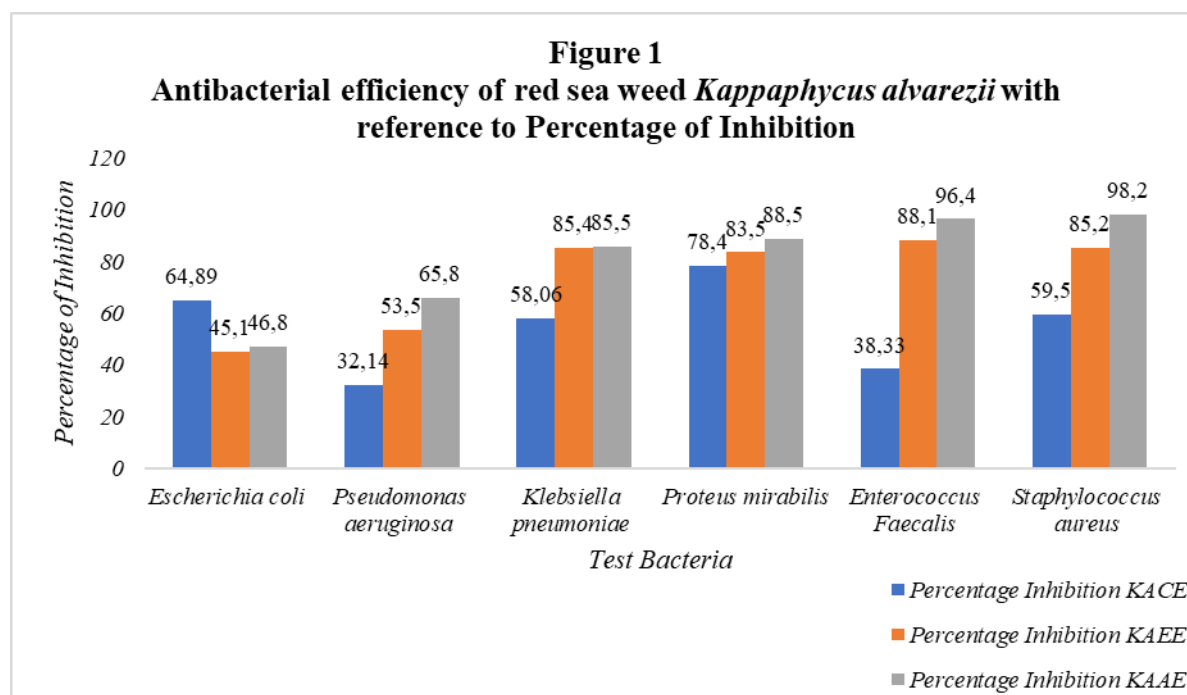
				KACE	KAEE	KAAE
1	<i>Escherichia coli</i>	11.6±0.60	-	07.2±0.6	11.6±1.5	10.7±1.7
2	<i>Pseudomonas aeruginosa</i>	11.5±1.57	-	07.8±1.2	12.8±1.0	12.2±1.4
3	<i>Proteus mirabilis</i>	14.8±1.57	-	07.3±0.4	13.6±0.8	13.3±0.5
4	<i>Staphylococcus aureus</i>	12.8±1.15	-	08.1±0.2	14.9±2.3	14.8±1.0
5	<i>Klebsiella pneumoniae</i>	12.5±0.30	-	08.6±1.6	13.6±1.2	10.3±1.1
6	<i>Enterococcus Faecalis</i>	11.3±2.67	-	09.3±2.1	15.4±1.8	14.2±2.5

Broth dilution along with pour plating were done to assess MIC, MBC and Percentage inhibition along with IC_{50} and LC_{50} of three extracts tested. Concentration required to inhibits the growth of the UTI causing test organisms found to varied from $383.3 \pm 57.7 \mu\text{g/ml}$ to $1033.0 \pm 76.3 \mu\text{g/ml}$. Though disc diffusion method illustrated good efficiency of KAAE than other extracts, here it was noted that better efficiency of KAAE (Table 3). Lower concentrations of extracts needed to inhibit the bacterial growth. KAAE was effectively inhibits *Staphylococcus aureus* var. *haemolyticus* at 383.3 ± 57.7 .

Table 3 Minimal inhibitory concentration of *Kappaphycus alvarezii* extracts

	Test Organism	KACE	KAEE	KAAE
1	<i>Escherichia coli</i>	533.3 ± 76.3	766.7 ± 28.8	750.0 ± 50.0
2	<i>Pseudomonas aeruginosa</i>	750.0 ± 50.0	716.3 ± 104.0	716.7 ± 28.8
3	<i>Klebsiella pneumoniae</i>	676.7 ± 929	466.7 ± 76.3	450.0 ± 50.0
4	<i>Proteus mirabilis</i>	525.0 ± 25.0	483.3 ± 28.8	466.7 ± 28.8
5	<i>Enterococcus Faecalis</i>	1033.0 ± 76.3	466.7 ± 28.8	350.0 ± 50.0
6	<i>Staphylococcus aureus</i>	625.0 ± 25.0	516.3 ± 76.3	383.3 ± 57.7

Percentage inhibition was calculated using number of colonies on growth control (Total TVC) versus number of colonies on MIC plates. Similar to MIC effect percentage inhibition also good in KAAE at respective MIC concentrations. Percentage inhibition ranges from 46.8 to 98.2% for KAAE, 45.1 to 85.4% for KAEE and 32.14 to 78.4% for KACE (Figure 1). Among the organisms *Staphylococcus aureus* var *haemolyticus* was best inhibited by KAAE (98.2%) followed by *Enterococcus faecalis* by KAAE (96.4%), *Proteus mirabilis* by KAAE (88.5%), *Enterococcus faecalis* by KAEE (88.1%), *Klebsiella pneumoniae* by KAAE (85.5%), *Klebsiella pneumoniae* by KAEE (85.4%) and *Proteus mirabilis* (83.5%). On the other hand, least, inhibition is by KACE against *Pseudomonas aeruginosa* (32.14%). All the extracts on different organism exhibited similar pattern of inhibition, highest by KAAE and lowest by KACE (KAAE>KAEE>KACE) but variability was noted on *Escherichia coli*, here this organism was best inhibited by KACE.



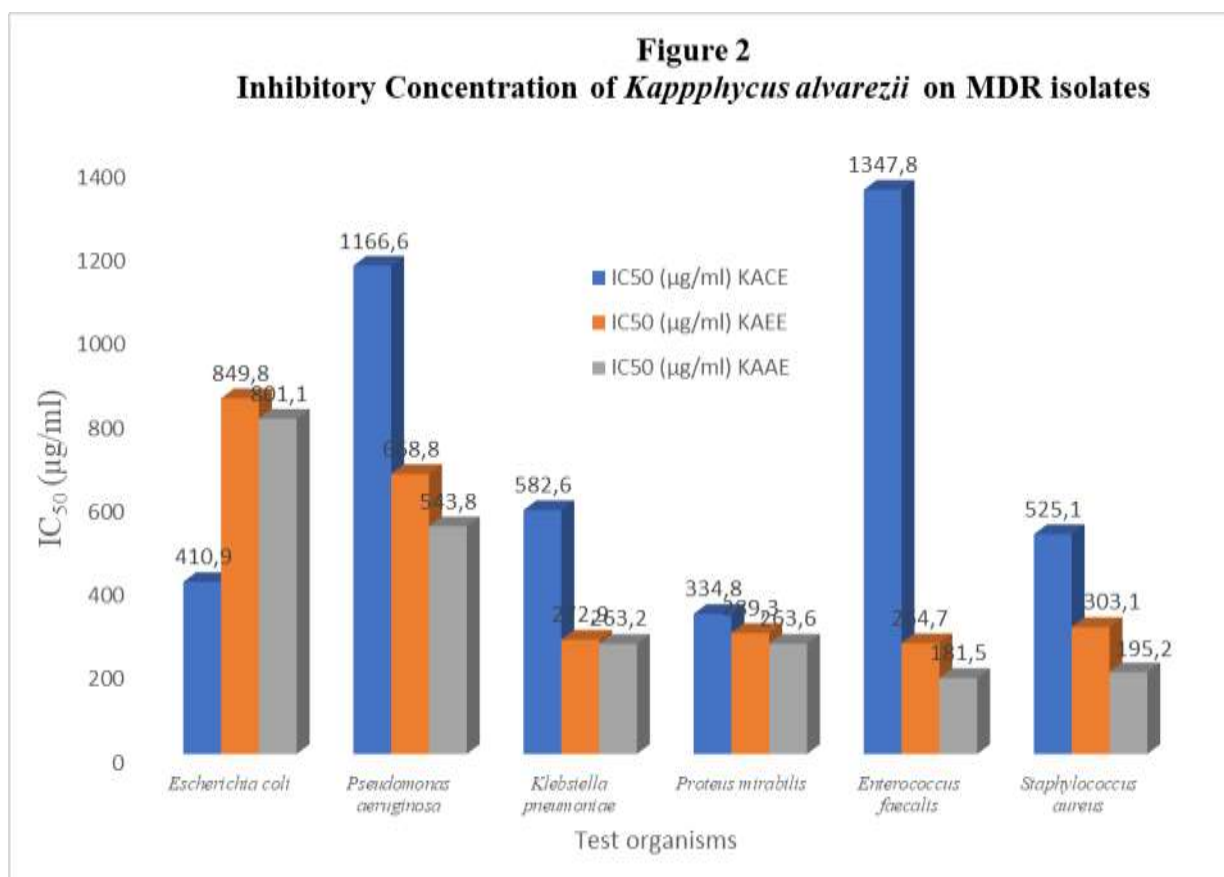
Greatly variable MBC effects was exhibited by the extracts of *Kappaphycus alvarezii*. Growth of *Enterococcus faecalis* is best controlled by KAAE with reference to MBC value ($516.6 \pm 50.0 \mu\text{g/ml}$). MBC value directly indicated the bactericidal efficiency of the test extracts, concentration of extracts required to kill 100% of the test organisms were varied from $516.6 \pm 50.0 \mu\text{g/ml}$ to $1283.3 \pm 28.8 \mu\text{g/ml}$ (Table 4). All the extracts provide efficient bactericidal activity on *Staphylococcus aureus* than other organisms. Overall gram-positive organisms were best controlled by KAAE and KAEE.

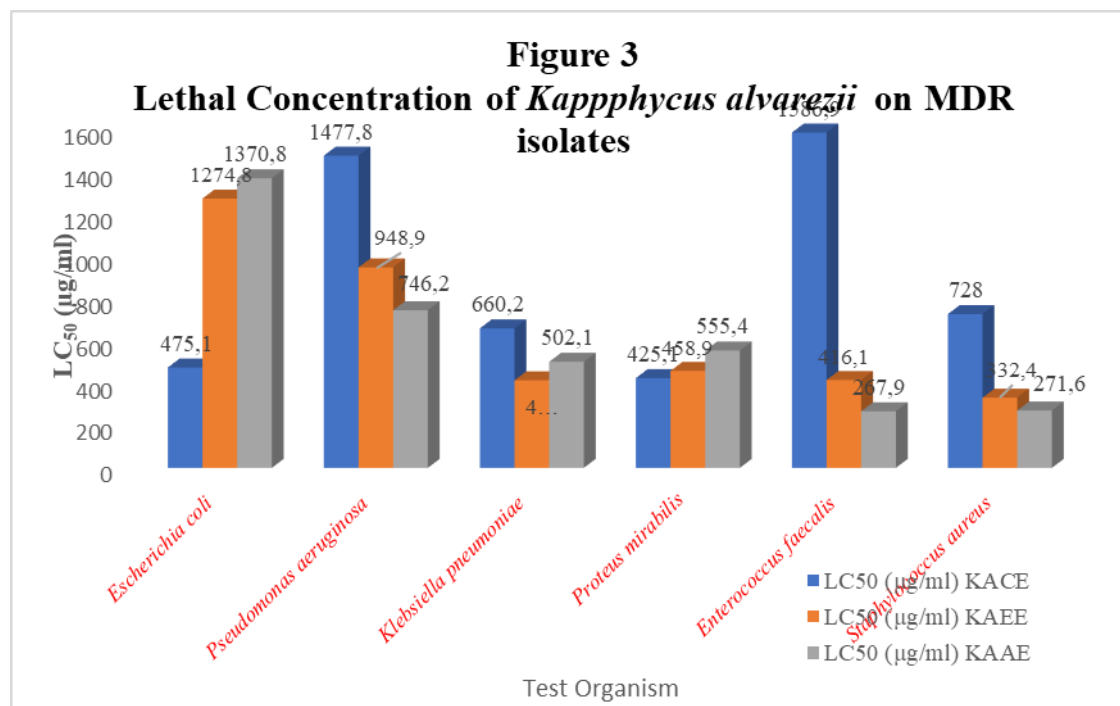
Table 4: Minimal Bactericidal concentration of *Kappaphycus alvarezii* extract

		KACE	KAEE	KAAE
1	<i>Escherichia coli</i>	616.6 ± 57.7	1150.0 ± 50.0	1283.3 ± 28.8

2	<i>Pseudomonas aeruginosa</i>	950.0±50.0	1016.6±115.4	983.3±104.1
3	<i>Klebsiella pneumoniae</i>	766.2±76.6	708.3±52.1	858.3±50.0
4	<i>Proteus mirabilis</i>	666.5±28.8	766.6±28.3	983.3±28.8
5	<i>Enterococcus faecalis</i>	1216.6±76.6	733.3±62.3	516.6±50.0
6	<i>Staphylococcus aureus</i>	866.5±28.8	566.6±76.1	533.3±57.7

Concentration required by the extracts to inhibit the growth was variable with the killing efficiency of the extracts. Higher concentration was required to kill the bacteria than the inhibition (Figure 2 and 3). IC₅₀ concentration required to inhibit the growth of *Enterococcus faecalis* was 1347.8 µg/ml whereas 1586.9 µg/ml of the same extract (KACE) was required to cause LC₅₀. Among the extracts aqueous extracts plays a vital role in inhibiting the growth of bacteria as well as killing of bacteria with one exception which was against *Escherichia coli*. KACE only produced good activity against *Escherichia coli*. Growth of *Proteus mirabilis* also best killed by chloroform extract with 425.1 µg/ml LC₅₀. Growth of *Klebsiella pneumoniae* also best arrested by KAEE with 414.3 µg/ml LC₅₀.





Antibacterial efficiency by ethanol and aqueous extract indicated the holistic efficiency of our traditional system of medicine practice. Both aqueous and ethanolic extract were equally good in creating antibacterial potential in response to antibacterial activity by zone of inhibition, MIC, MBC Percentage inhibition, IC₅₀ and LC₅₀ [24]. Few studies from India revealed the efficiency of *Kappaphycus alvarezii* extracts on plant and animal pathogens. This is the first study uses human isolates from UTI and assessed antibacterial activity by disc diffusion method. Most of the reports showed positive effect on bacterial isolates [25-30]. Bhuyer et al., [30] indicated the involvement of chemicals on microbial cells. The also indicated that Levoglucosenone is responsible for antibacterial effect.

Few studies indicated the effect of *Kappaphycus alvarezii* extracts. Chuah et al., [29] reported zero zone of inhibition of methanol extract on *Salmonella enterica*. This study noted the efficiency of test organism on gram positive organisms. KAAE & KAAE showed greater activity on Gram positive organisms than Gram negative organisms, which Could be due to the strength variability of bacterial cell wall. Many studies supported the efficiency of different seaweeds on food borne pathogens especially *Salmonella* species [31-36]. Galan *et al.*, [37] also reported the efficiency of rhodophyta members not *Kappaphycus alvarezii* extracts on fish pathogens.

Natural chemicals like tannins, phenolic compounds obtained from the seaweed could be responsible for these activities. One of the previous studies indicated the presence of steroids, terpenoids, flavonoids, phenolic compounds in both extracts of fruit [38]. Tannins are known to be useful in the treatment of microbial infection which could cause microbial damage through coagulation of cell wall proteins of pathogenic organisms. Flavonoids have been shown to exhibit their actions through effects on membrane permeability and by inhibition of membrane bound enzymes such as the ATPase and phospholipase [39]. They also serve as health promoting compounds as a result of their anion radicals [40]. Afonso et al., [41] also reported that hexadecanoid acid and sterol of seaweed are responsible for antibacterial activity.

Patni *et al.*, [42], Masika and Afolayan [43] and Karou *et al.*, [44] were stated the susceptibility pattern of gram-positive bacteria than gram negative bacteria. Antimicrobial property of a seaweed extracts were directly proposed to the active constituents. A wide range of bactericidal activity were assigned to tannins [45]. Scientists from all parts of the country Some authors have found that more highly oxidized phenols are inhibitor [46, 47] Flavonoids indicated the role of chemicals found in the rhodophyta members [49]. Terpenoids are active against bacteria [50], fungi [51], viruses [52] and protozoa [53]. Tannins also binds to proline rich protein and interfere with protein synthesis [54]. Reports from other countries also supports the antibacterial efficiency on different isolates like *Xanthomaons campestris* [55], *E. coli* [56], *Vibrio harveyi* [57], *Aeromonas salmonicida* [58], *Xanthomonas punicae* [59]. Few studies expressed the nature of seaweed extract as an agent of reducing dental caries [60], which also prevents periodontal diseases. All these antibacterial activities are due to compounds like phenolic compounds, sterols, flavonoids from seaweeds.

4. Conclusion

Kappaphycus alvarezii is one of the most important red algae which could be responsible for inhibiting gram-positive bacteria effectively than gram negative organisms. These activities were due to the available phytoconstituents like flavonoids, sterols and phenolic compounds.

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