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Research Article

Studies on Antibacterial Activity and Phytochemical Analysis of *Gracilaria corticata* (J. Agardh), *Gracilaria dentata* (J. Agardh) and *Gracilaria pygmaea* (Børgesen) against Diarrheal Causing Pathogen *E. coli* and *Salmonella* typhi

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Abstract

Enteric pathogens are the most frequent cause of diarrheal illnesses that account for an annual mortality rate of five million people worldwide, the second most common cause of death after cardiovascular illnesses. Prominent pathogenic enteric include strains of *Escherichia coli* and *Salmonella typhi* are responsible for diarrhea. The antibacterial characteristics of methanol, acetone, diethyl ether and ethanol extracts of three *Gracilaria* species (*Gracilaria corticata* J. Agardh, *Gracilaria dentata* J. Agardh and *Gracilaria pygmaea* Børgesen) collected from different shores (Buleji, Paradise Point, Manora channel and Mubarak village) of Pakistan coast. All these species were tested in vitro for their antidiarrheal activities against diarrhea causing *E. coli* and *Salmonella typhi* in five children of different ages (one, two, five, nine and ten years) stool culture that were affected by diarrhea. Phytochemical screening of the plant revealed the presence of Saponins, Tannins, Phenolic compounds, Alkaloids, Carbohydrates, Protein and amino acids and Glycosides. Acetone and Ethanol were the best extraction against the *E. coli* and *Salmonella typhi* used in this experiment. The aim of the study is to create social awareness among public, patient, *physician and government* about the economic and medicinal value of seaweeds.

Keywords: Seaweeds; Diarrhea; Karachi Coast; Stool; Therapeutic Agent; Antibacterial Activity

Introduction

The biological activity has a role to play in the beneficial or adverse effects of any drug or chemical for the living cells or tissues. Today, microbiologist and pharmacologist have increased their attention towards marine plants, which constitute the potential bioactive substances [1]. Many biological active components have been isolated from marine algae, and some of them are under investigation and are being used to develop new pharmaceuticals [2].

Enteric pathogens are the most frequent cause of diarrheal illness that account for an annual mortality rate of five million people worldwide and the second most common cause of death after cardiovascular disorder. In developing world diarrhea is responsible for high morbidity and mortality particularly among children under the age of five years. The two main reasons for the high morbidity and mortality could be attributed to poor diet and dirty water which contribute to the prevalence of the disease. Prominent pathogenic enteric *Escherichia coli* and *Salmonella typhi* strains are responsible for diarrhea.

The antimicrobial activity is considered as a characteristic of seaweeds to synthesize bioactive secondary metabolites or organic compounds that at are not directly involved in the normal growth, development and reproduction of an organism. The bioactivity of different types of algae have been tested in different areas of the world by using different organic solvents like ethanol, chloroform, methanol, acetone, petroleum ether and diethyl ether against wide range of microorganisms e.g., both gram positive and gram negative bacteria, fungus and viruses. There are many studies on

antibiotic activities of seaweeds and antibacterial compounds which are derived from seaweeds have been reported [2-7]. Seaweed of high potential consist of various compound like terpenoids, phlorotannins, steroids, phenolic compounds, halogenated ketones and alkanes, cyclic sulfides, fatty acids that showed the antibacterial activity most especially against humans pathogens, gram positive and gram negative bacteria [2,8].

Evaluation of biochemical and chemical component of some seaweeds occurring at Karachi coast had been reported by Abbas., *et al*. [9], Abbas and Qari, [10] Khan., *et al*. [11] Khan and Qari [12,13], Qari., *et al*. [14], Qari and Siddiqui [15-17], Qari and Qasim [18] and Qari [19]. The present study was carried out with seaweeds. The present study was undertaken with objective to assess the three red species of seaweeds (*G. corticata*, *G. foliifera* and G. pygmaea) potential as a source of antibacterial substances and to screen the phytochemical constituents present in the selected seaweeds against diarrhea caused by enteropathogenic organisms like *E. coli* and *S. typhi*.

Materials and Methods

Pakistan has about 1000 km long coastline bordering Arabian Sea in the south at 23°50´N latitude that covered the Sind (250 km) and Balochistan (800 km) coast. The most significant ports of Arabian Sea are Karachi, Pakistan, and Mumbai, India [13]. The coast of Karachi is sandy, rocky or muddy in nature and relatively shallow and flat-bottomed stretching over 200 miles of continental slope [20]. The shore is totally submerged under water at the time of high tide and at low tide it is exposed. The seawater is rich with nutrients and salinity ranged from 35 to 38 ‰, temperature 21 to 30 °C and pH 6.5 to 7.2 [21,22].

Three species of Gracilaria (*G. corticata, G. dentata* and *G. pygmaea*) were collected from four different coastal areas of Karachi: Buleji, Paradise Point, Manora Channel and Mubarak Village. These coastal areas were easily approachable and known for the good and healthy algal flora and fauna. The species selected for the present study were found on the exposed coast of Buleji, Paradise Point, Manora Channel and Mubarak Village at low tide abundantly.

In present investigation four different solvents namely methanol, ethanol, acetone and diethyl ether were used in the preparation of crude extracts of shade dried seaweeds. The temperature was maintained at 30-50°C depending upon the solvent used. The extracts were then concentrated under reduced pressure in rotary evaporator and kept in cold environment (deep freezer) until tested.

The stool culture of two strain *Escherichia coli* and *Salmonella typhi* belonging to five children of different ages were obtained from Pathology laboratory, Civil Hospital, Karachi. The cultured sample was maintained on Brain Heart Infusion (BHI) agar medium at 4°C until testing [23].

Antibacterial activity of extraction of seaweeds was evaluated using the agar diffusion technique in petri dishes [23]. 25 µl of each extract was loaded on sterile filter paper discs 6 mm in diameter, and air-dried. Indicator microorganisms were spread on Mackankey agar plates with sterile effusion and the discs were placed on plates. After 20-24 hours incubation at 35-37°C a clear zone of inhibition of antibacterial activity around a disc was produced. Diameters of the zones of inhibition were measured in millimeters. Each test was prepared in duplicate. Discs loaded with the extracting agents were tested as controls that did not show any inhibition zone.

Qualitative phytochemicals were analyzed by using the standard method of Harborne [24]. The carbohydrate, glycosides, tannin, phenol, saponins, protein, steroids, alkaloids, and flavonoids were qualitatively analyzed in all three *Gracilaria* species of seaweeds.

Results

The results of primary screening tests of three species of seaweeds *G. corticata*, *G. foliifera* and *G. pygmaea* of Gracilariaceae showed that the acetone and ethanol extracts were active against both tested bacterial strains and showed a greater zone of inhibition than the extracts obtained with other solvents (methanol and diethyl ether). Amongst the bacterial strains tested, the gram negative bacterium *E. coli* was most susceptible to both solvent ethanol and acetone extracts as compared to the *S. typhi*. There was significant variation found in antibacterial activity between species, solvents, bacterial strains and ages of the children.

In *G. corticata* collected from Buleji coast ethanol and acetone extractions results showed positive results against *E. coli* and *S. typhi* also as compared to other solvents. The ethanol (10 mm) as well as acetone extracts of *G. corticata* collected from Buleji coast exhibited greater zone of inhibition (10.5 mm) against *E. coli* in stool culture of one and five years children samples of diarrhea respectively as compared to other culture samples of diarrhea whereas extraction of ethanol and acetone of same species collected from Paradise Point showed more effective results (8.2 mm and 9.2 mm respectively) against *E. coli* in five years old child stool culture samples of diarrhea (Figure 1). The ethanol and acetone extracts

of seaweeds collected from Manora showed high antimicrobial activity (10.2 mm and 8.5 mm respectively) against *E. coli* in one year old child stool culture sample like the other sites (Figure 1 and Plate 1A).

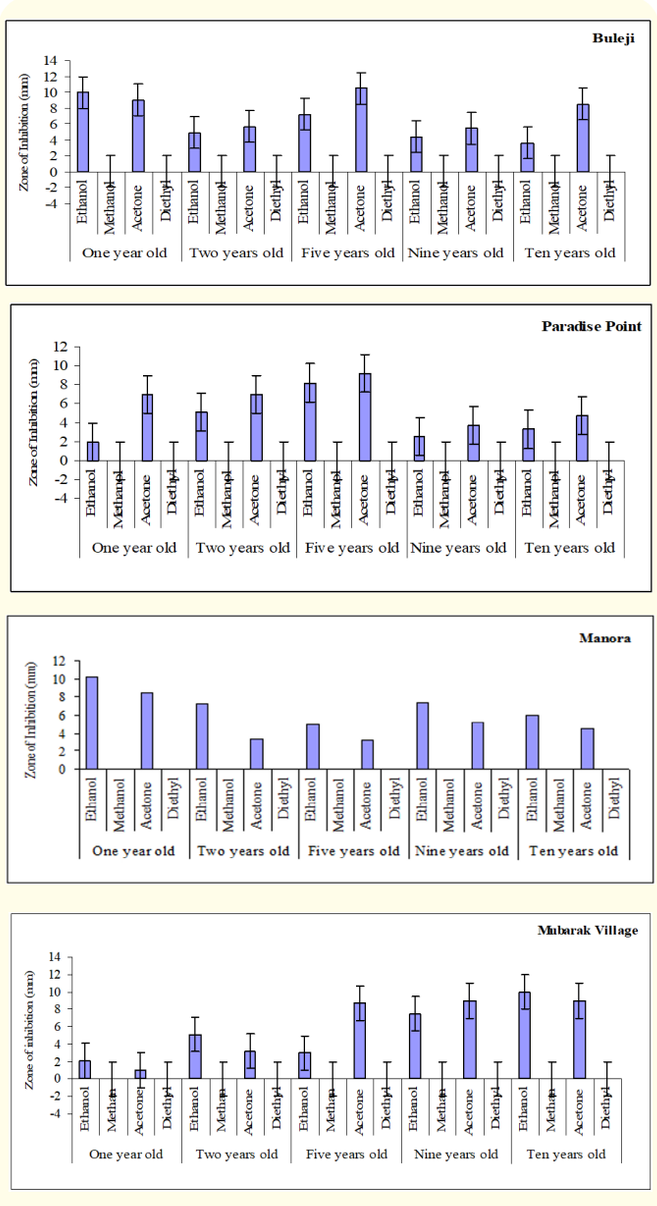

Figure 1: Diameter averages (mm) of the zone of inhibition in different solvent extracts of *Gracilaria corticata* collected from Buleji, Paradise Point, Manora and Mubarak village against *E. coli* in stool culture of children of different ages.

The pathogen *S. typhi* was also used in testing of antibacterial activities of crude extracts of red seaweed *G. corticata* collected at Buleji, Paradise Point and Manora. The ethanol extract of *G. corticata* collected from the Buleji showed great zone of inhibition (8.7 mm) in two years old child stool culture sample and acetone extract showed great activity (13.2mm) in five years old child stool culture sample. On the other hand ethanol extract of Paradise Point sample showed excellent activity (7.4 mm) in one year old child stool culture while acetone extract of *G. corticata* showed greater zone of inhibition (8.1 mm) against *S. typhi* in one year old child stool culture sample. The ethanol extract of *G. corticata* collected from Manora gave better results in five years old child culture sample against *S. typhi* (12.5 mm) while acetone extract gave better results in two years old child stool culture sample (10.9 mm) as compared to other culture samples (Figure 2 and Plate 1B).

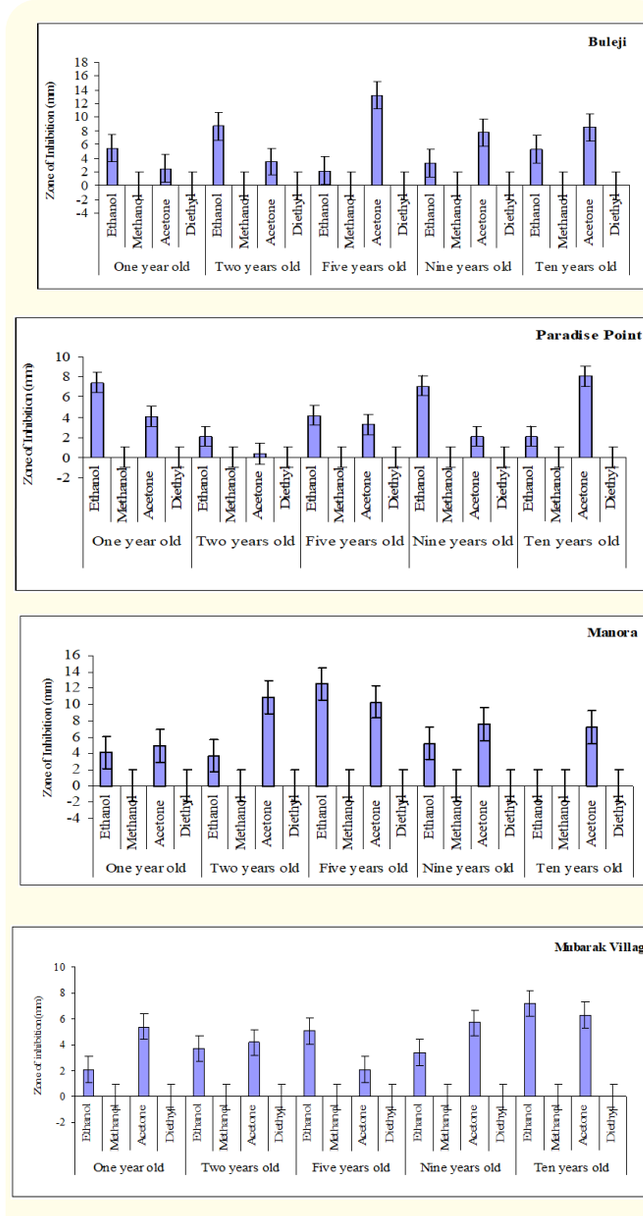

Figure 2: Diameter averages (mm) of the zone of inhibition in different solvent extracts of *Gracilaria corticata* collected from Buleji, Paradise Point, Manora and Mubarak village against *S. typhi* in stool culture of children of different ages.

G. foliifera was collected from Buleji and Paradise Point only. The ethanol as well as acetone extracts of *G. foliifera* Buleji coast samples were active against diarrhea causing bacteria *E. coli* strain tested and showed a greater zone of inhibition in ten (8.5mm) years old child stool culture sample as compared to the other culture samples. On the other hand acetone extracts of *G. foliifera* collected from Paradise Point showed large zone of inhibition (7.7 mm) in nine years old child stool culture sample of diarrhea against *E. coli* (Figure 3 and Plate 1C).

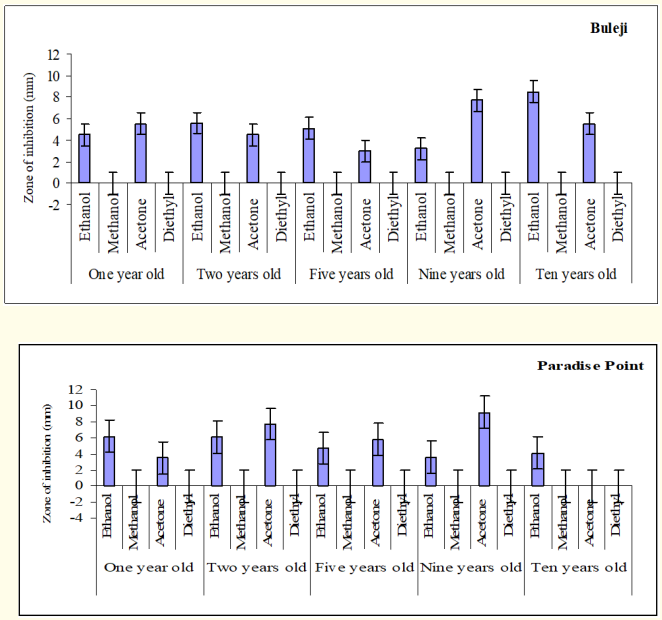

Figure 3: Diameter averages (mm) of the zone of inhibition in different solvent extracts of *G. foliifera* collected from Buleji and Paradise Point against *E. coli* in stool culture of children of different ages.

Acetone and ethanol also showed positive results against *S. typhi* as compared to methanol and diethyl ether. The extract of *G. foliiflora* Buleji sample in acetone possessed great activity against *S. typhi* in five year old child stool culture sample (9.1mm) while ethanol extract of sample of Paradise Point also given good results (8.2 mm) in two years child stool culture sample against pathogen *S. typhi* (Figure 4 and Plate 1D).

G. pygmaea was also collected from Buleji and Paradise Point. Figure 5 and 6 showed that the ethanol and acetone extracts of *G. pygmaea* of Buleji and Paradise Point were active against both organisms *E. coli* and *S. typhi* strains tested and had a greater zone of inhibitions. The ethanol extract of *G. pygmaea* collected from Buleji

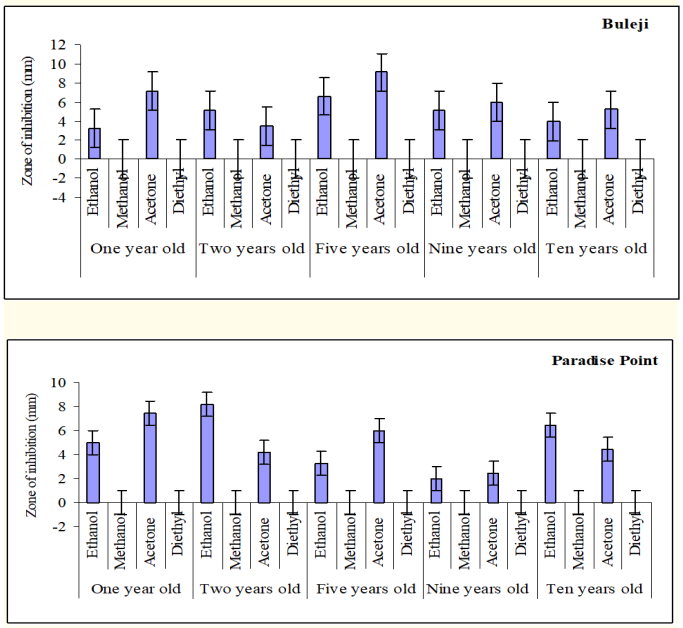

Figure 4: Diameter averages (mm) of the zone of inhibition in different solvent extracts of G. foliifera collected from Buleji and Paradise Point against S. typhi in stool culture of children of different ages .

showed greater zone of inhibition (5.5 mm) in one year old child stool culture sample against *E. coli* and acetone extracts of same site showed greater activity (7.0 mm) in ten year old child stool culture sample of diarrhea while both ethanol and acetone extracts of *G. pygmaea* collected from Paradise Point showed the good results (7.1 mm and 7.5 mm respectively) in ten years old child stool culture against *E. coli*. The diethyl ether and methanol extract of *G. pygmaea* of each site did not show any antibacterial activity except methanol extract of *G. pygmaea* of Paradise Point which showed greater zone of inhibition (4.1 mm) in five years old child stool culture sample against *E. coli* as compared to other solvents (figure 5 and plate 1E).

The ethanol extract of *G. pygmaea* collected from Buleji showed 7.2 mm diameter of the zone of inhibition and acetone extract showed 6.5 mm against *S. typhi* in nine and ten years old child stool culture sample respectively while ethanol and acetone extraction of sample from Paradise Point showed a greater zone of inhibition (8.1 mm and 8.6 mm respectively) in two and nine years children stool culture samples against *S. typhi* respectively (figure 6 and plate 1F).

In the present study, the phytochemical screening of three species *G. corticata, G. foliifera, G. pygmaea* with the four different

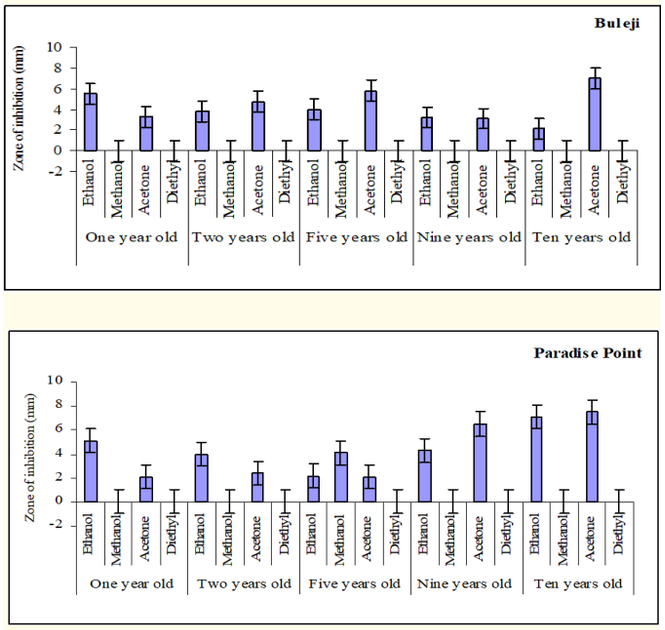

Figure 6: Diameter averages (mm) of the zone of inhibition in different solvent extracts of *G. pygmaea* collected from Buleji and Paradise Point against *S. typhi* in stool culture of children of different ages.

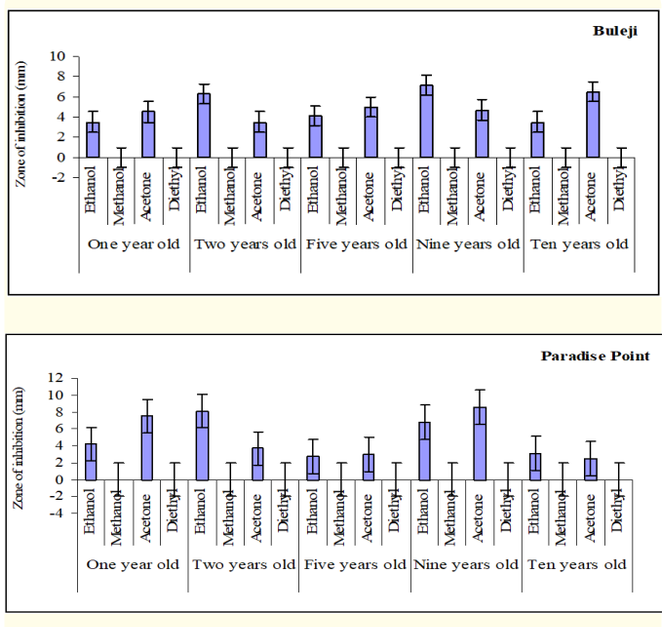

Figure 6: Diameter averages (mm) of the zone of inhibition in different solvent extracts of *G. pygmaea* collected from Buleji and Paradise Point against *S. typhi* in stool culture of children of different ages.

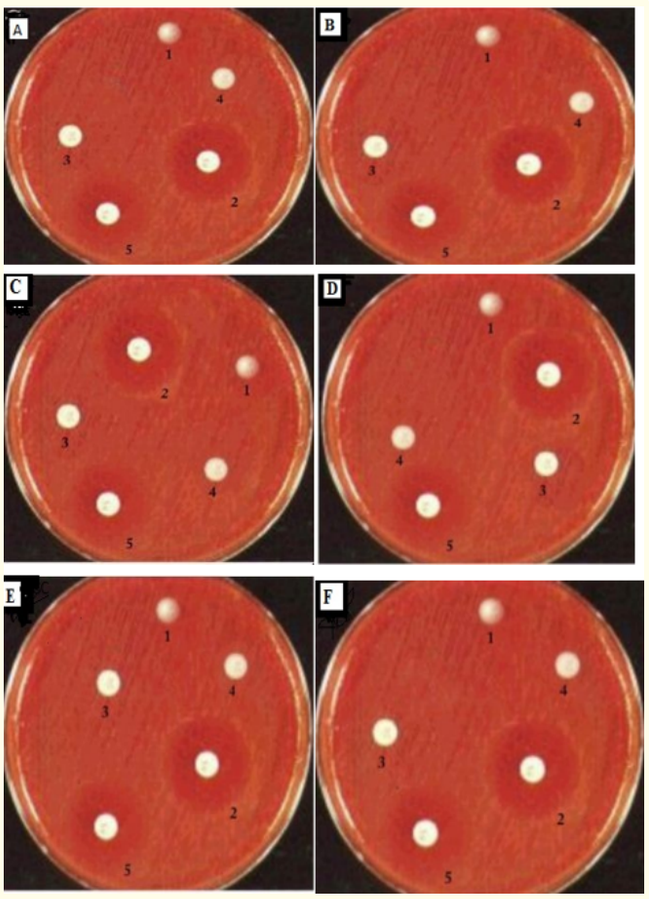

Plate 1: Inhibition zone obtained by the different solvent extract 1. Control 2. Ethanol 3. Methanol 4. Acetone 5. Diethyl ether of G. corticata (A, B), G. dentata (C, D) and G. pygmaea (E, F) against E. coli (A, C, E) and S. typhi (B, D ,F).

solvents (methanol, ethanol, acetone and diethyl ether) was performed for nine different chemical compounds. Total nine hundred tests (9 x 4 x 3 = 108) were conducted for the presence or absence of different chemicals compounds or secondary metabolites (carbohydrate, glycoside, tannin, phenol, saponins, protein, steroid, alkaloid and flavonoid) in extracts of all three studied species of seaweeds i.e., thirty six tests (9 x 4 x 1 = 36) were conducted in each species. Ethanol and acetone extracts of all samples showed better results as compared to other solvents (methanol and diethyl ether). Only forty three extracts showed the presence of chemical compounds whereas sixty five extracts showed negative results. Carbohydrate showed the maximum presence (11) followed by phenol (9), tannin (7), protein (6), flavonoid (5), glycosides (3) and saponins (2) in four different extracts (ethanol, acetone, methanol and diethyl ether). Among the four different extracts maximum

number tests showed by acetone (17) followed by ethanol (14), diethyl ether (7) and methanol (5). Carbohydrate, phenol and protein showed their presence in all four extracts (ethanol, acetone, methanol and diethyl ether), tanin and flavonoid in three (ethanol, acetone and diethyl ether) glycosides and saponins in two extracts (ethanol and acetone). It is also noted that in present study alkaloids and steroid were totally absent in all studied species extracts.

Table 1 showed seventeen positive tests and remaining eighteen gave negative results in the phytochemical screening of *G. corticata*. The positive results showed the presence of carbohydrate, glycoside, tannin, phenol, saponin, protein, and flavonoid whereas steroid and alkaloid did not show any positive result for their presence. The carbohydrate and phenol showed the maximum presence in all four extracts followed by flavonoid (3), saponin and tannin (2), protein and glycoside only in one extract. Among the four extracts, ethanol extract showed the presence of maximum number (7) of compounds. Next to that, acetone (5), diethyl ether (3) and methanol (2).

Table 2 showed thirteen positive tests and remaining twenty three gave negative results in the phytochemical screening of *G. foliifera*. The positive results showed the presence of carbohydrate, glycoside, tannin, phenol, protein, and flavonoid. Carbohydrate, phenol and tannin showed the maximum presence in three different extracts followed by protein in two extracts, flavonoid and glycoside only in one extract. Among the four different extracts, acetone extract showed the presence of maximum number (6) of compounds and next to that, ethanol (4), diethyl ether (2) and methanol (1).

S. No.	Name of compound	Solvent				
		Ethanol	Methanol	Acetone	Diethyl Ether	Total presence
1	Carbohydrate	+++	+	+	++	4
2	Glycosides	+	-	-	-	1
3	Tannin	++++	-	+++	-	2
4	Phenols	+++	+	++++	+	4
5	Saponins	+	-	++	-	2
6	Protein	+++	-	-	-	1
7	Steroids	-	-	-	-	-
8	Alkaloids	-	-	-	-	-
9	Flavonoid	+	-	+++	+	3
		7	2	5	3	17

Table 1: Qualitative phytochemical studies of *Gracilaria corticate.*

+ = 20% measure, ++ = 40% measure, +++ = 60% measure, ++++ = 80% measure, - = absent

S. No.	Name of compound	Solvent				
		Ethanol	Methanol	Acetone	Diethyl Ether	
1	Carbohydrate	++	-	++	+	3
2	Glycosides	-	-	+++	-	1
3	Tannin	++++	-	++	+	3
4	Phenols	+++	+	+++	-	3
5	Saponins	-	-	-	-	-
6	Protein	++	++	+++	++	4
7	Steroids	-	-	-	-	-
8	Alkaloids	-	-	-	-	-
9	Flavonoid	-	-	++	-	1
		4	2	6	3	15

Table 2: Qualitative phytochemical studies of *G. foliifera.*

+ = 20% measure, ++ = 40% measure, +++ = 60% measure, ++++ = 80% measure, - = absent

A total of 11 tests exhibited positive and 25 gave negative results out of 36 tests for 9 different chemicals compounds in *G. pygmaea* extracts. The positive results showed the presence of carbohydrate, glycoside, tannin, phenol, protein, and flavonoid, saponin. Carbohydrate showed the maximum presence in all four extracts followed by tannin and phenol in two extracts, protein and flavonoid in one extract. Among the four different extracts, acetone extract showed the presence of maximum number (6) of compounds. Next to that, ethanol extract (3) and diethyl ether and methanol extracts showed (1) compound (Table 3).

S. No.	Name of compound	Solvent				
		Ethanol	Methanol	Acetone	Diethyl Ether	
1	Carbohydrate	+++	++	+++	+	4
2	Glycosides	-	-	+	-	1
3	Tannin	++++	-	+++	-	2
4	Phenols	+++	-	+++	-	2
5	Saponins	-	-	-	-	-
6	Protein	-	-	++	-	1
7	Steroids	-	-	-	-	-
8	Alkaloids	-	-	-	-	-
9	Flavonoid	-	-	++	-	1
		3	1	6	1	10

Table 3: Qualitative phytochemical studies of *G. pygmaea.*

+ = 20% measure, ++ = 40% measure, +++ = 60% measure, ++++ = 80% measure, - = absent

The completely randomized design with nested treatments analysis of variance (ANOVA) model were used to test the significant differences of antibacterial activity in between four different extracts (ethanol, methanol, acetone and diethyl ether) of species (*G. corticata*, *G. foliifera* and *G. pygmaea*), sites (Buleji, Paradise Point, Manora and Mubarak Village), bacterial strains (*E. coli* and *S. typhi*), age of children (one, two, five, nine and ten years). The results showed that there was no any significant variations found in between species, sites, bacterial strain and ages of children for ethanol, methanol, acetone and diethyl ether extract. The data for antibacterial activity of seaweed species (green, brown and red) at four sites (Buleji, Paradise Point, Manora and Mubarak Village) against bacteria (*E. coli* and *S. typhi*) in different ages of children, patient of diarrhea (one, two, five, nine and ten years) were analyzed by looking at the relationship between them. There was insignificant correlation found in between *Gracilaria* Extract and bacterial strain (*E. coli* and *S. typhi*).

Discussion

As it is evident from the data (Figures 1-6) the ethanol and acetone extracts of all three studied species (*G. corticata*, *G. foliifera* and *G. pygmaea*) showed good antibacterial activity against *E. coli* and *S. typhi*. Methanol and diethyl ether showed effective extraction solution in few species of seaweed. For example methanol extract of *G. pygmaea* collected from Paradise Point showed a greater zone of inhibition (4.1 mm) in five years old child stool culture sample against *E. coli* as compared to other three solvents: ethanol, acetone and diethyl ether.

The present results is in agreement with research findings generated by Freile-Pelegri and Morales [25] who found antimicrobial activity of the ethanol (89%) and lipid-soluble extracts (94%) of some algal specie against bacteria (*B. subtiles*). Present research finding strongly agree with the results of Karthikaidevi *et al.* [26] who found ethanol extract of marine algae with maximum zone of inhibition (13mm) as compared to other solvents (acetone, methanol, chloroform, diethyl ether, ethyl acetate and petroleum ether) against *Staphylococcus*. Pesando and Caram [27] and Naqvi., *et al*. [28] used ethanol solvent that showed the highest activity with some seaweeds while Tuney., *et al.* [23] studied on the effectiveness of extraction methods and asserted that methanol extraction yield higher antibacterial activity. Acetone extract of Indian marine algae also gave good antibacterial activity as recorded in the seaweeds of Karachi coast in the present study [29]. Pushparaj., *et al.* [30] reported ethanol and acetone extract exhibited the strongest activity as recorded in present investigation.

The maximum presence of carbohydrate, phenol and protein compounds in present study could be due to the fact that they are widely found in marine plants and involved in different biological and pharmacological activities including antioxidant and antimicrobial properties [31]. The present results showed high carbohydrate content (20-60%) that is agreed with the previous studies [32] who also reported maximum value of carbohydrate content present in Rhodophyceae members from the coast of Maharashtra coast of India. The high amount of carbohydrates in Rhodophyta is due to high polysaccharides content present in their cell walls [33]. It is also noted in present study that 60-80 % phenol and 40-80% tannins was present in all studied species (*G. corticata*, *G. foliifera*, *G. pygmaea*).

In present study the flavonoid showed positive results in three extracts (ethanol, acetone and diethyl ether) of all studied species. In a study conducted by Jeeva., *et al.* [34] with benzene, chloroform and other petroleum ether solvents were used for extraction but no positive results for alkaloids was detected like the present study. Saponins possesses numerous biological properties such as antimicrobial, antiflammatory and antifeedent [35] present study saponins showed only two positive results, one in acetone and second one in ethanol extract of *G. corticata* and *G. pygmaea* whereas Thinakaran and Siva Kumar [33] found positive results of saponins in methanol extract of species *G. corticata* but did not find any positive results in acetone extract.

Seaweeds are nutraceutical product that provides health and medical benefits, including the prevention and treatment of diseases [33]. The data generated in this present study showed that crude extracts of seaweeds with acetone and ethanol showed good activity against diarrhea causing organisms *E. coli* and *S. typhi* in stool samples of children, patient of diarrhea. Extracts of methanol and diethyl ether showed no activity against organism in the present investigation except in rare cases. The results of the present study also indicates and proved that seaweeds are rich source of secondary metabolites that are biologically active component with bactericidal and antibacterial properties. Hence the presence of carbohydrate, glycoside, tannin, phenol, saponin, protein, and flavonoid in the crude extracts of seaweeds suggested that seaweeds can be used as antibacterial agent in future. In view of this, it can be asserted that seaweeds found at the coast of Karachi abundantly have good activity against diarrhea causing organisms and potential sources of bioactive compounds that should be used for isolation of natural antibiotics. In conclusion, studied species of *Gracilaria* are beneficial for pharmaceutical use.

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Volume 2 Issue 3 March 2019