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Assessment of the antibacterial activity of macroalgae *Cladophora crispata* extract against extended spectrum beta-lactamase producing *Escherichia coli* isolated from diarrheic children

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Abstract:

Background: Diarrhea is a major cause of morbidity and mortality in children under 5 years old in developing countries.

Objective: This study designed to investigate the occurrence of extended spectrum beta-lactamase producing *Escherichia coli* (*E. coli*) as etiological infectious agents of diarrhea in children, assessment of their susceptibility/resistance to antibiotics, and to evaluate the antibacterial activity of the macroalgae *Cladophora crispata* extract towards these bacteria .

Materials and Methods: 150 diarrheic stool samples were collected from children less than 5 years and cultured. The isolated bacteria were subjected to various identification and biochemical tests. Then all the isolated bacteria (*E. coli*) were subjected to ESBL-producing screening by double-disc synergy test, the antibacterial activity of algal extract and antibiotic susceptibility test against tested bacteria were determined by using agar well diffusion and disc diffusion methods respectively .

Results: Out of total 150 samples, only 18 isolates revealed the presence of extended spectrum β-lactamase producing *E. coli*, which showed highly susceptibility to gentamicin and amikacin, *Cladophora crispata* extract revealed a strong antibacterial activity against ESBL-producing *E. coli*.

Conclusion: *Cladophora crispata* extract showed a strong antibacterial activity against ESBL-producing *E. coli* which can be exploited as source of antibacterial drugs to control and treatment diarrheal infection caused by these bacteria.

Keywords: Antibiotic susceptibility ;antibacterial activity; *Cladophora crispata*; algae; children diarrhea; *Escherichia coli*, extended-spectrum beta-lactamase.

Introduction:

Diarrhea is one of public health problem and major causes of morbidity and mortality in children under 5 years old in developing countries, especially in areas of inadequate water supplies, sanitation and little or no health education (Amaya *et al.*, 2011). Diarrhea is defined as three or more episodes of watery loose stools in the last 24 hours. Every episode of diarrhea in children could cause malnutrition, reduced resistance to infections with potential consequences of impaired physical and cognitive growth development (Mandomando *et al*., 2007; Riddle *et al*., 2016).

Escherichia coli is a gram negative bacteria responsible for a wide variety of hospital and community-onset infections, especially of diarrhea , urinary tract infections, bloodstream infections, surgical site infections, pneumonia , and sepsis (Pitout, 2012), *E. coli* is most frequently associated with diarrhea that is often accompanied by fever, vomiting and dehydration which can be fatal in young children, especially those already in poor health and malnourished (Shweash *et al*., 2014).

E. coli is highly resistant to many antibiotics which can result from the widespread

use of commercially available antimicrobial agents and thus pose a serious threat to global health (Hijazi *et al*., 2016). This resistance developed due to their ability for production of beta-lactamases enzymes, which are capable for hydrolyzing penicillins, broad-spectrum cephalosporins to such as cefotaxime, ceftazidime and monobactams such as aztreonam (Bush and Fisher, 2011). Therefore, the infections caused by ESBLs- producing *E. coli* are serious and more difficult to treat because of antibiotic resistance. So, determination of antimicrobial susceptibility and extended spectrum β-lactamase (ESBLs) production patterns are essential for treatment of related infections. In addition the information about the etiology of diarrhea is useful in planning and implementing control strategies to reduce diarrhea caused childhood morbidity and mortality in a country (Amaya *et al.*, 2011).

Cladophora is a branching, filamentous, green macroalgae belonging to the type of chlorophyta algae found in both marine and fresh water. *Cladophora* grows primarily on rocky substrates, it often becomes detached and accumulates along the shoreline, forming large algal mats (Dodds and Gudder, 1992; Leliaert and Boedeker, 2007).

Cladophora species have been used as a food source and also have many important health benefits such as rejuvenation, induction of appetite and expediting of recovery from many common maladies (Fahprathanchai *et al*., 2006). In addition, *Cladophora* extracts were used for the treatment of diabetes and diabetic complication as well as helpful in the retardation of cardiovascular diseases and preservation of healthy cardiovascular function (Daniels, 2004).

Cladophora has been identified as a rich and renewable source of biologically active secondary metabolites including, alkaloids, polyketides, flavonoids cyclic peptide, polysaccharide, phlorotannins, diterpenoids, sterols, quinones, lipids and glycerols (Krish and Das, 2014 ; Al-Saif *et al*.,2014) which have broad spectrum of biological activities such as antibacterial, antioxidant, antifungal, antiparasitic, antiprotozoal, antimycobacterial, anticancer, antiproliferative and anti-inflammatory activities, that acts as potential bioactive compounds of interest for pharmaceutical applications (Spavieri *et al*., 2010; Soltani *et al*., 2011; Laungsuwon & Chulalaksananukul, 2013; Begum and Rao 2015; Douma *et al*.,2017).

This study designed to investigate the occurrence of extended spectrum beta-lactamase producing *E. coli* as etiological infectious agents of diarrhea in children, assessment of their susceptibility/resistance to antibiotics, and to evaluate the antimicrobial activity of the macroalgae *Cladophora crispata* extract towards these bacteria.

Materials and Methods:

Algal collection and extract preparation:

Cladophora crispata samples were collected as biomass manually from Al-Gharraf river, Thi-Qar Governorate, Southern Iraq. Algal samples were washed thoroughly with river water and rinsed many times with distilled water to remove the epiphytes and extraneous materials, then brought to the laboratory and identified based on morphological characteristics (Prescott, 1975; Bellinger and Sigee, 2010). The samples were then dried under shade and ground into the fine powder by electrical grinder.

10 grams of algae powder was extracted with 100 mL of (70 %) ethanol (1:10 w/v) solvents in a Soxhlet extractor for 10 hr. The extract was then filtered by Whatman number 1 sterile filter paper and concentrated in a rotary evaporator under reduced pressure until a crude solid extract was obtained, then crude extract was collected in airtight plastic vial and stored in refrigerator for further use (Sujatha *et al*., 2012).

Samples collection and bacterial identification:

A total of 150 fecal samples were collected from children less than 5 years suffering from diarrhea admitted in Bent AL-Huda Hospital at Thi-Qar province in Iraq, from May to August 2016, the samples were collected by sterilized screw capped bottles for further laboratory examinations.

All samples were cultured on MacConkey agar and incubated at 37°C for 24- 28 hours and observing for growth of the pink bacterial colonies. The presence of pink colonies on the plates indicated the presence of *E. coli*, then picked up and streaked each of these colonies on Eosin Methylene Blue agar for observing the metallic sheen. In addition to that, these bacterial also isolated and identificated according to their cultural, morphological, microscopically and biochemical characteristics (Kumar *et al*., 2014).

Detection of Extended-spectrum β-lactamase producing *E. coli* by double-disc synergy test (DDST):

A disc of ceftriaxone (30 µg) and ceftazidime (30 µg) were placed at a distance of 16-20 mm from the Augmentin disc (20µg amoxicillin plus10µg clavulanic acid) center to center on a Mueller-Hinton Agar (MHA) plate swabbed with the test isolate. After incubation, the plate at 37°C for 24 hours, the organisms were considered to be producing ESBL when the zone of inhibition around any of the expanded-spectrum cephalosporin discs showed a clear-cut increase towards the Augmentin disc (Kumar *et al*., 2014).

Antibiotic susceptibility test:

All the isolates were examined for their antibiotic susceptibility pattern using the Kirby-Bauer agar disc diffusion method (Bauer *et al*., 1966) by using ten commonly available antibiotic discs which are ticarcillin-clavulanic acid, amikacin, nitrofurantoin, imipenem, tetracycline, pipracillin, gentamicin, ampicillin, ticarcillin, and chloramphenicol. Bacteria from freshly grown culture was smeared onto the surface of Mueller Hinton agar by sterilized cotton swab, then left to dry for 30 min. Following this the antibiotic discs were placed on the surface of inoculated medium by sterile forceps with uniform distance between discs and pressed gently to ensure full contact and incubated at 37°C for 24 hours. After incubation, the diameter of inhibition zones produced by the antibiotic discs against tested bacteria were measured. Results were categorized as sensitive (S), intermediate (I) and resistant (R) and interpreted according to the Clinical and Laboratory Standards Institute (CLSI, 2011).

Assay of antimicrobial activity of algae extract:

Antimicrobial activity of algae extract was assessed by agar well diffusion technique, 0.1 mL of inoculum of the prepared bacterial suspensions was spread with a sterile cotton swab evenly onto Muller Hinton agar plate surfaces and left for 30 minutes to allow the absorption of the microbial inoculum. Wells of 8 mm in diameter on each agar plates were made by using a sterilized stainless steel borer and filled with 50 µl of algal extract. The prepared plates were left at room temperature for 30 minutes allowing the diffusion of the extract into the agar, then incubated at 37 °C for 24 hours. The antimicrobial activity was evaluated by measuring the inhibition zone around each well (Devi and Mehta, 2016).

Statistical analysis:

All the experimental results of were expressed as mean using Microsoft Excel 2010 software.

Results:

In this study, out of total 150 samples, only 60 isolates (40%) revealed the presence of *E. coli* depending on the basis of pink color production and metallic sheen in addition to their cultural, morphological, microscopically and biochemical characteristics. The double-disc synergy test showed 18 isolates (30%) only of *E. coli* produced extended-spectrum β-lactamase enzyme.

Algal extract showed a potent antibacterial activity against all (100%) isolates of ESBL-producing *E. coli* with (18 mm) zone of inhibition. In antibiotic susceptibility test, about (77.8%) and

(66.7%) of ESBLs- producing *E. coli* isolates showed highly sensitivity to gentamicin and amikacin with (19 mm) zone of inhibition, which could be inhibit the growth above 50%, while (44.4%) of ESBL- producing *E. coli* isolates showed sensitivity to chloramphenicol and tetracycline with (19 mm) and(16 mm) zone of inhibition, respectively. Whereas, all (100%) ESBL- producing *E. coli* isolates exhibited complete resistance against most of the commonly used antibiotics such as ticarcillin-clavulanic acid, ticarcillin, pipracillin, imipenem and ampicillin with (0 mm) zone of inhibition as shown in table 1.

Table 1:Antibacterial activity of algal extract and standard antibiotics against extended spectrum *β*-lactamase producing *E* coli (Number of isolates=18).

Antibiotics and algae extract	Sensitive		Intermediate		Resistant	
	No.(%)	IZ mm	No.(%)	IZ mm	No.(%)	IZ mm
Ticarcillin-clavulanic acid 75/10 µg	0 (0%)	0	0 (0%)	0	18 (100%)	0
Amikacin 30 µg	12(66.7%)	19	6 (33.3%)	15	0 (0%)	0
Nitrofurantoin300 µg	1 (5.6%)	17	10(55.6%)	15	7 (38.8%)	11
Imipenem 10 µg	0 (0%)	0	0 (0%)	0	18 (100%)	10
Tetracycline 30 µg	8 (44.4%)	16	2 (11.2%)	13	8 (44.4%)	3
Piperacillin 100 µg	0 (0%)	0	0 (0%)	0	18 (100%)	0
Gentamicin 10 µg	14(77.8%)	19	1 (5.6%)	14	3 (16.6%)	6
Ampicillin 10 µg	0 (0%)	0	0 (0%)	0	18 (100%)	0
Ticarcillin 75 µg	0 (0%)	0	0 (0%)	0	18 (100%)	0
Chloramphenicol 30 µg	8 (44.4%)	19	1 (5.6%)	15	9 (50%)	10
Algae ethanolic extract 100mg/mL	18 (100%)	18	0 (0%)	0	0 (0%)	0

No: number of isolate; %=percent of bacteria; IZ= mean of inhibition zone in mm.

Discussion:

Diarrheal diseases are a major cause of death in children less than 5 years of age. Information about household characteristics in a locality and their interrelation with diarrhea prevalence among the children aged five years is very crucial in the infection control measures, for effective reduction of childhood morbidity and mortality are required to make important policy decisions (Tambe *et al*., 2015; Riddle *et al*., 2016). Therefore, the antibiotic susceptibility testing plays a useful role in the outbreak setting and for surveillance of local trends in resistance patterns and mechanisms (Riddle *et al*., 2016).

In our study, ESBL-producing *E. coli* was the one of the most frequent causes of bacterial diarrhea in children aged less than five years. Similar findings reviewed that ESBL-producing *E. coli* is the one of the most common pathogens of diarrhea in children under 5 years old in India (Aggarwal *et al*., 2013; Kumar *et al*., 2014). In addition, several studies with stool samples from children under 5 years of age also reported that the ESBL-producing *E. coli* as the most frequent pathotype in children who had diarrhea which are in accordance with the results found in our study (Alizadi *et al*., 2015).

In the present study, the antibiotic susceptibility tests showed that extended spectrum beta –lactamase producing *E. coli* exhibited highly sensitivity to gentamicin and amikacin, while it showed a complet resistance against most of the commonly used antibiotics such as ticarcillin-clavulanic acid, ticarcillin, pipracillin, imipenem, and ampicillin. Similar findings were observed by Kumar *et al*. (2014) who reported that the extended spectrum beta –lactamase producing *E. coli* isolated from feces samples from neonatal diarrhea exhibited highly sensitivity to amikacin, aztreonam, and gentamicin, whereas exhibited highly resistant to ampicillin, cefdinir, co-trimoxazol, cloxacillin, erythromycin, lincomycin, norfloxacin,pefloxacin, pencillin, tetracycline, nitrofurantoin and vancomycin.

Other studies demonstrated that ESBL-producing *E. coli* susceptible to amikacin and gentamicin and resistant to azetreonam, cefepime, cefpodoxime, ticarcillin (Hijazi *et al*., 2016). In addition resistant to pipracillin and tetracyclie (Ochoa *et al*., 2009).

Wasito *et al*. (2016) revealed that extended spectrum beta-lactamase producing *E. coli* isolated from stools of pediatric diarrhea patients in Surabaya exhibited good susceptibilities to imipenem, amikacin and fosfomycin. whereas showed resistance to ampicillin, sulbactam/ampicillin, piperacillin, cefotaxime, ceftadizime, cefepime, cefpodoxime, aztreonum, tetracycline, chloramphenicol, nalidixic acid, ciprofloxacin and sulfamethoxazole-trimethoprim.

Contrary to our results, Wasito *et al*. (2016) reported that ESBL-producing *E. coli* isolated from children with diarrhea were susceptible to imipenem.

The antimicrobial activities of gentamicin and amikacin against extended spectrum beta-lactamase producing *E. coli* are mediated by inhibition of protein synthesis through their binding to 30S subunits of the intracellular ribosomes and results in the disruption of the normal cellular metabolism of the bacteria, and consequently leads to the death of the organism (Thenmozhi *et al*., 2014).

In this study, ESBL-producing *E. coli* showed resistance to β-lactam due to their ability for production of beta-lactamases enzymes, which are capable for hydrolyzing penicillins, broad-spectrum cephalosporins to such as cefotaxime, ceftazidime and monobactams such as aztreonam. The amount of beta-lactamase enzyme production depends on the concentration of the antibiotic and the time of exposure (Hijazi *et al*., 2016).

In addition, ESBL-producing *E. coli* exhibited resistant to a non-β-lactamase antimicrobials agents such as chloramphenicol and tetracyclines. The possible explanation for this observation may in fact be that ESBLs are encoded on plasmids and can be mobile and therefore, easily transmissible as resistance gene elements for other antimicrobials from one organism to another (Al-Yaqoubi and Elhag, 2008 ; Hijazi *et al*., 2016).

The rates of resistance obtained in this study were different from the rates obtained in the above mentioned studies. This could be attributed to the different antibiotic use policies of different centers or to more frequent use of antibiotics by the patients applied to private hospitals, owing to their higher socioeconomically status and easier attainment of antibiotics without prescription.

In the present study, ethanolic extract of *Cladophora crispata* showed a strong antibacterial activity against ESBL-producing *E. coli* . These results are in accordance with those obtained by several authors, they found that ethanolic extract of alkoidal compound of *Cladophora crispata* showed a potent antibacterial activity (13 mm) against *E. coli* (Athbi *et al*., 2011). Nearly similar results also obtained by other study which revealed that ethanolic extract of *Cladophora* showed higher inhibitory activities (20 mm) against *E. coli* (Al-Saif *et al*., 2014). Other studies confirmed the antibacterial activity of alcoholic extract of *Cladophora* sp. on several bacterial species which among them *E. coli* strains (Khalid *et al*., 2012).

The antibacterial activity of *Cladophora crispata* may be attributed to the presence bioactive compounds such as, alkaloids, tannins, fatty acids, polysaccharides, amino acids, proteins, terpenes, sterols, phenolics, flavonoids, aromatic organic acids, aldehydes and ketones(Athbi *et al*., 2011; Khalid *et al*., 2012; Krish and Das, 2014).

The antibacterial activity of algal alkaloids may be attributed to their ability for inhibition of RNA polymerase of *E. coli* result in inhibited both RNA and protein synthesis (Doan *et al*., 2001), alkaloid also inhibit DNA synthesis by inhibition of the topoisomerase enzyme that control the DNA replication of *E. coli* leading to morphological changes and cell lysis (Karou *et al*., 2005).

Algal tannins (phlorotannins) has been reported to produce antibacterial activity due to inhibition of oxidative phosphorylation, and their ability to bind with bacterial proteins such as enzymes and cell membranes, in which the phenolic aromatic rings and OH groups of the phlorotannins bind to the -NH groups of bacterial proteins by H-bond and hydrophobic interactions, causing cell lysis. (Wang *et al*., 2009 ; Wei *et al*., 2015).

Algal free fatty acids have been reported to act as inhibitors of the electron transport chain and normal oxidative phosphorylation in bacterial cell membranes. This interferes with adenosine triphosphate energy transfer, and inhibits enzymes such as bacterial enoyl-acyl carrier protein reductase, necessary for the synthesis of fatty acids within the bacterial cell result in formation of peroxidation and auto-oxidation degradation products and lysis of the cell (Zheng *et al*., 2005; Wang *et al*., 2009). Other study revealed that the bacterial cell wall were perforated when treated with algal extract due to free fatty acids, resulting in rupture of the cell wall, cytoplasmic leakage, shrinking of the protoplasm, cytoplasmic vacuolation, scattering of chromatin, distortion of the outer cell shape, and decreased cell size (El-Shafay *et al*., 2016).

Algal polysaccharides and sulphated polysaccharides have been used successfully for pharmaceutical and dietary applications. Their antibacterial mechanism is due to glycoprotein-receptors present on the cell-surface of polysaccharides which bind with compounds in the bacterial cell wall, cytoplasmic membrane, and DNA. This results in increased permeability of the cytoplasmic membrane, protein leakage, and binding of bacterial DNA (Amorim *et al*., 2012).

The antimicrobial activity of amino acids in the form of peptides, or proteins, has been demonstrated in a number of recent studies. The amphipathic conformation of peptides enables them to bind with polar and non-polar sites on bacterial cytoplasmic membranes, thereby interfering with cellular processes and propagation. Algal Lectins, are a diverse group of proteins had bactericidal properties due to their ability to selectively bind with lipopolysaccharides, β-glucans, and peptidoglycans on the cell surface of bacteria, for this lectin inhibit the growth of Gram-negative bacteria by binding to mannan which is a linear polymer of the saccharide monomer mannose found on the surface of bacteria (Nguyen *et al*., 2011; Cheung *et al*., 2015).

A number of terpene compounds from algae have been found to inhibit bacterial growth. the antibacterial activity of algae diterpenoid was due to its amphipathic structure of three polar alcohol groups, with non-polar aliphatic carbon and bromine atoms on the opposite side of the molecule, which enable it to bind with bacterial cell membranes lead to bacterial inhibition (Rodrigues *et al*., 2015). In addition, the antibacterial activity of terpenoid and phenolic compounds mediated by destabilization the lipopolysaccharide layer of gram negative bacterial membrane led to cell death (Thummajitsakul *et al*., 2012).

In conclusion, our findings showed that multidrug resistant ESBL-producing *E. coli* is one of major etiological infectious agents of diarrhea in children which showed highly susceptibility to gentamicin and amikacin. This study also revealed that ethanolic extract of *Cladophora crispata* possess a potent antibacterial activity which may constitute promising sources of bioactive metabolites that can be exploited as antibacterial drugs to control and treatment diarrheal infection caused by these bacteria.

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