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The Inhibitory Effect of the Aqueous Extract of *Ceratophyllum demersum* on *Vibrio cholarae* that Isolated from *Cyprinus carpio*

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Abstract: The current study was conducted to determine the inhibitory effect of the aqueous extract of hornwort *Ceratophyllum demersum* on *Vibrio cholerae* bacteria that affects the common carp *Cyprinus carpio* L. in fish farms. The inhibitory effect of the aqueous extract was determined in petri dishes. The inhibition effects of the extract and six common antibiotics (Nalidixic acid, Nitrofurantoin, Gentamicin, Streptomycin, Tetracycline, and Amoxicillin) were compared; the results showed that the inhibition effect of the aqueous extract of hornwort was higher than the inhibitory effects of the six antibiotics. The stock solution 100% of the aqueous extract showed an inhibition effect reached 35.3 mm compared with 25 mm, 15 mm, 7 mm, 10 mm, 23 mm and 11 mm of Nalidixic acid, Nitrofurantoin, Gentamicin, Streptomycin, Tetracycline, and Amoxicillin antibiotics respectively. A positive correlation was recorded between concentration of aqueous extract and its inhibitory effect. The average of inhibition diameter was 23.3 mm, 27.6 mm, 28.6 mm, and 35.3 mm for the concentrations 25%, 50%, 75% and 100% of the stock solution, respectively.

Keywords: *Cyprinus carpio*, *Vibrio cholerae*, *Ceratophyllum demersum* extract, Antibiotics

Introduction

Species of the genus *Vibrio* are natural bacterial flora in fishes (Izvekova et al., 2007). They may become harmful to the fish when stress occurs and fish immunity decreases. These species are pathogenic to human, since it colonizes on fish (Al-Hussainy et al., 2017). They affect warm and cold water fishes, which show symptoms when temperature rises in late summer, especially in shallow water and near beaches. About 50 species of *Vibrio* were accommodated (Machen et al., 2008).

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Vibrio cholerae is a facultative anaerobic, Gram negative bacteria with one flagellum on the cell pole (Calderon, 2000). The infection with these bacteria cause intestinal inflammation, and their severity depends on the level of infection (Jameel et al., 2016). The infection spreads via water and contaminated food (Calderon, 2000).

Recent attention has been focused on the natural sources of the antibodies inherent in plants. Phenolic compounds are among the most prominent natural antibodies, including flavonids, tannins, carotenoids and phenolic acid. Phenols are aromatic compounds with one or more hydroxyl group (Pereira et al., 2009). They are found in almost all plant parts and considered as secondary metabolic products (Wang et al., 1998).

The hornwort (*Ceratophyllum demersum*) belongs to the Family Ceratophyllaceae. It's a worldwide submersed plant, and endemic plant in the Iraqi waters (Abdalhameed & Al-Hassany, 2019). In Basrah province, it is distributed in Abu El-Khaseeb irrigation systems, Shatt Al-Arab river, Basrah marshes and Garamat Ali river (Al-Mayah et al., 2016).

Al-Seedi et al. (2013) showed that the metabolic products of hornwort hampered the growth of many nitrogen-fixing bacteria, in addition to its role in curing wounds and ulcers, while Mjelde & Faafeng (1997) showed that the extracts of hornwort prevented the growth of phytoplankton. In addition, its leaves extract possesses high effectiveness in inhibiting the growth of yeasts, Gram negative and Gram positive bacteria (Dulger & Gonuz, 2004).

The present study aims to detect the inhibitory effect of the aqueous extract of hornwort on the cholera which infect common carp in Basrah province to avoid economic losses and preserve the environment through using organic substances as an antibiotics alternative.

Materials and Methods

Fish samples were collected in April, 2019 from Al-Hammar marsh. *Ceratophyllum demersum* was collected from the local water bodies in Basrah province. The plant was dried in a well-aerated shaded place at room temperature with continuous stirring to prevent their decomposition. After grinding, it was kept in a labeled paper bag at room temperature until use (Al-Kanany et al., 2011).

The plant was aqueously extracted according to Twaij et al. (1983). A volume of 250 ml of distilled water was added to 25 mg of dried powder of grinded plants in a 500 ml conical flask. The mixture was placed in blender at room temperature for three hours, then centrifuged for 15 minutes. The aqueous suspension was diluted with distilled water up to 250 ml of four concentrations of extract (25%, 50%, 75% and 100%).

Bacterial Isolation

Vibrio cholerae was isolated from the skin of 13 infected *Cyprinus carpio* after cleaning and washing the fishes with distilled water. The isolated bacteria were

cultured in selective medium: Thiosulfate citrate bile sucrose salt agar (TCBS) (Hi media- India). The plates were incubated at 37ºC for 48hr. Isolated bacteria was morphologically identified by microscope with Gram staining and cultured on nutrient agar and then identified by the Vitek II system (Biomerieux- USA) according to Al-Shammari et al. (2019).

Efficiency Test of *Ceratophyllum demersum* Extract against Isolated Bacteria

Whole diffusion method was applied (Bansode & Chavan, 2012) by using Mueller-Hinton agar (MHA) (Himedia- India) media. The media was left to stubbornness on the dishes after 15-30 minutes, then *Vibrio cholerae* was introduced evenly over the media by using cotton swap, followed by making a hole (8mm) on the MHA by a cork borer and adding 100 µL of different concentrations to each hole by fine and sterile pipette. The plates were incubated for 18 hrs at 37 ºC, separately, and the diameter of the clear zone around the bacteria was measured.

Antibiotic Sensitivity Test

Six antibiotic discs (Nalidixic acid 30 mcg, Nitrofurantoin 300 mcg, Gentamicin 10 mcg, Streptomycin 10 mcg, Tetracycline 30 mcg and Amoxicillin 25 mcg) were used for antibiotic sensitivity test. The agar diffusion method was used according to Jorgensen et al. (1999). Guidelines of using antibiotic discs on the media Mueller-Hinton agar, Antibiotic, Nalidixic acid 30 mcg, Nitrofurantoin 300 mcg, Gentamicin 10 mcg, Streptomycin 10, Tetracycline 30 and Amoxicillin 25 MCG were followed.

Statistical Analysis

The IPM SPSS statistics version 22 was used to analyze the results statistically. Significant differences were also compared with the revised least significant difference (RLSD) at a significance level of 0.01 (Al-Janae'e et al., 2018).

Results

The inhibitory effect of the aqueous extract of hornwort on *V. cholerae* that isolated from common carp was examined. Figure 1 shows the efficacy of the aqueous extract of hornwort on cholera bacteria, four concentrations were used (100%, 75%, 50% and 25%). The results show that there is an inhibitory effect of these concentrations on cholera bacteria, which has differences in the strength of inhibitory effect per concentration by measuring the diameter of the inhibition zone. The statistical analysis has shown significant differences ($P<0.01$) in the inhibitory effect of 100% of the full-strength solution compared with other concentrations. There were no significant differences ($P<0.01$) in this effect in case of both 50% and 75% of the full-strength solution.

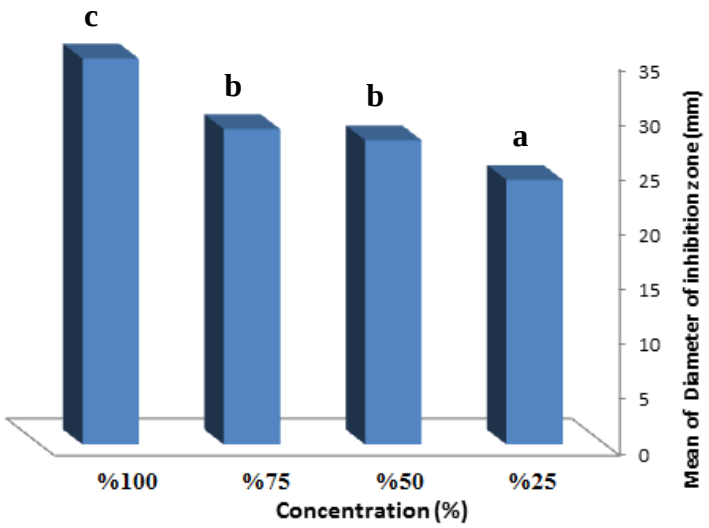

Figure 1: The mean of inhibition zones (mm) of different concentrations of *C. demersum*.

The results also show significant differences ($P<0.01$) in the inhibitory effect of the aqueous extract with a concentration of 100% of the full-strength solution compared with the inhibitory effect of all antibiotics. Table 1 shows the concentration of antibiotics and the diameter of the inhibitory zone for them. Figure 2 shows the inhibitory effect of the aqueous extract of hornwort compared with the antibiotics.

Table 1: Concentration of antibiotics and the mean diameter of the inhibitory zone.

Type of antibiotics	Concentration (Mcg/g)	Diameter (mm) of inhibition zone
Nalidixic acid	3	25
Nitrofurantoin 300	3	15
Gentamicin	1	7
Streptomycin	1	10
Tetracycline	3	23
Amoxicillin	2	11

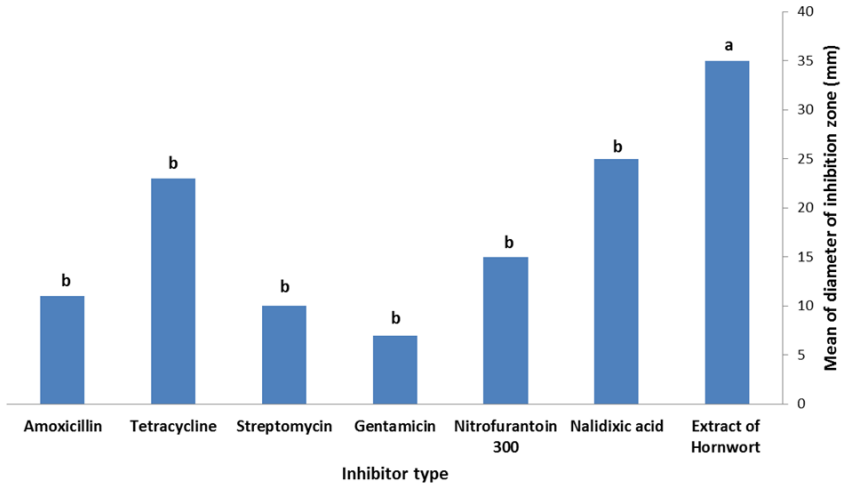

Figure 2: Effect of aqueous extract of *C. demersum* on *V. cholerae* compared with antibiotics.

Discussion

The results showed that there is a significant inhibition effect of the aqueous extract of hornwort on the bacteria, due to the presence of some effective substances. Eigemann (2013) indicated that hornwort produces chemical compounds with allelopathic potential which has a great role in the formation and change of the aquatic environments by influence the growth, survival, development, and reproduction of other organisms,. The allelopathy is a process that involves the production of secondary metabolic products from plants and microorganisms (Cheng & Cheng, 2015).

Al-Seedi et al. (2013) referred to the high allelochemical potential of hornwort by producing large quantities of polyphenols that impede the growth of some organisms. The chemical analysis of the aqueous extract of hornwort also showed that it contained resins, glycosides, tannins, flavones and alkaloids (Al-Kanany et al., 2011). The same study indicated that the 250 mg/ml of hornwort aqueous extract make 20 mm of inhibition area to *Staphylococcus aureus*.

Cowan (1999) indicated that proteins and enzymes are inhibited by the tannins in the bacterial cell wall as well as the adhesion of cells. The alkaloids have a lethal effect on microorganisms (Al-Maeeny et al., 2007). The effective substances in the aqueous extract dissolve and disrupte the adipose membranes, which affect the metabolic efficacy of cells and thus inhibits them (Hili et al., 1997). It also affects fat metabolism by affecting the efficacy of the 3-hydroxy-3methyl glutase enzyme, responsible for the construction of the mevalonic acid, which paves the construction of sterols to synthesize the enzyme Acetyl CoA (Singh et al., 2008), as well as inhibiting DNA by linking it (Ünver et al., 2009), or it may be due to the synergistic action of a combination of effective compounds such as phenols, flavones and alkaloids and the difference in the side strings (Hugo & Russell, 2004).

The results showed that the extracts concentrations of 25%, 50% and 75% were of less inhibition than 100%, as the concentration of the effective substances in the 100% concentration of the extract is higher than in the rest of the concentrations (Al-Janae'e et al., 2018). Ali et al. (2012) indicated that high concentrations lead to the shrinkage in the organism, while in a lower concentration, the dispersion of effective substances causes their separation from cells, resulting in a decrease in the inhibition efficacy of the substances (Hili et al., 1997), or the low concentration of effective substances makes it non-therapeutic (Al-Zubaidi et al., 2007). Also, the low concentrations of effective substances are unable to inhibit the growth of microorganisms (Thanaboripat et al., 2007).

The results also showed significant differences in the sensitivity of cholera bacteria to the extract of hornwort compared with antibiotics. This may be due to the difference in the type and concentration of their active substances (Al-Janae'e et al., 2017), or to their absorbability and movement within the cell (Pourmorad et al., 2006), The difference in bacterial sensitivity to these substances depends on the type, nature of composition, and thickness of the cellular membranes and their content of fat and proteins, in addition to the mechanism of transition operation of effective substances, wherefore some bacteria show a higher sensitivity against a specific compound (Lee et al., 1999). This difference may also be due to the presence of protective substances inside the cells of these bacteria that are either caused by their exposure to external efforts or that they have reached them through effective transport from the environment. These substances increase the protection of their cells depending on the type of microorganisms, type of external effect and other abnormal factors. The most important of protective substances are carbohydrates, mannitol, and glutamate (Salih & Hussein, 2014).

Through the study, it can be concluded that the aqueous extract of the hornwort plant with a concentration of 100% can be successfully used in inhibiting the cholera bacteria that infects common carp fish, and the study recommends testing the effect of this extract in treating infected fish in fish farms because it is less harmful to the environment and fish than antibiotics.

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