World Applied Sciences Journal 6 (7): 868-879, 2009
ISSN 1818-4952

Investigations on *Vibrio* Sp Isolated from Diseased Crayfish *(Procambarus clarkii)* with Emphasis *on* Biochemical Characteristic and *in Vitro* Antibacterial Effects of Some Plants Extracts

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Abstract: The present study was designed to isolate and characterize pathogenic bacteria from the haemolymph and hepatopancres of diseased crayfish *Procambarus clarkii*. The pathological effects of the bacterial pathogen on hepatopancreas and muscles were also investigated. In addition, the effect of aqueous, ethanolic and methanolic extracts of *Aster squamatus*, *Inula erithmoides*, *Pluchea dioscoridis* and *Cyperus articulatus* on growth of isolated bacteria from investigated animal was studied. Diseased crayfish were collected from El- kased drain at Mehalt Menof, in Gharbia governorate, Egypt. Gram negative pathogenic bacteria namely *Vibrio* were isolated from haemolymph and hepatopancres of the diseased crayfish using TCBS agar as culture media. According to morphological, physiological and biochemical characteristic, the isolated bacteria were identified as *Vibrio alginolyticus* and *Vibrio parahaemolyticus*.Histopathological, hepatopanreas of diseased animal showed irregular tubules and the basal lamina appeared to become detached from the epithelial cells. B- cells were increased in number and associated debris in their vacuoles. Also, abdominal muscles of diseased crayfish showed shrinkage of myofibers led to diffuse area of myofiber disorganization. The preliminary phytochemical screening of the studied plants extracts revealed the presence of glycosides, tannins, steroids, terpens, mucilage and resins while, saponins was absent in extract of *Inula erithmoides* and flavonoids were detected in *Aster squamatus* and *Cyperus articulatus*. *Cyperus articulatus* have broad spectrum activities against the tested organisms followed by *Pluchea dioscoridis*. The aqueous extract appeared to be the most effective followed by ethanol while, methanol extract of all tested plants gave negative results except the methanol extract of *Cyperus articulatus* which gave positive results at higher concentrations (750-1000 mg ml^{-1}). It was concluded that, *Cyperus articulatus*, *Pluchea dioscoridis* and *Aster squamatus* can control bacterial diseases in crayfish ponds.

Key words: Histopathology, Phytochemistry, Antibacterial effects, Plant extracts, *Vibrio* sp., Freshwater Crayfish

INTRODUCTION

Crayfish *Procambarus clarkii* dwell in freshwater areas whereas there is risk to be infected by a number of pathogens. These pathogenes associated with the crayfish diseases are classified into six main taxonomic groups such as viruses, bacteria, rickets- like organisms, fungi, protests and metazoan [1, 2]. Bacterial diseases have been implicated as major causes of mortality both in wild populations and aquaculture crustaceans [3]. Bacteria belonging to the genus *Vibrio* are widespread in aquatic habitats of various salinities. They are common in marine and estuarine environments and on the surface of marine plants and animals [4]. Some species are found in freshwater [5,6]. Vibriosis is a bacterial disease responsible for mortality of cultured shrimp worldwide [7- 9]. Outbreaks may occur when environmental factors trigger the rapid multiplication of bacteria already tolerated at low levels within shrimp blood [10] or by bacterial penetration of host barriers. The diseased agents cause a distinct pathological effect of an infected population, reduced growth rate and increased mortality.

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There is very little information on the overall types and occurrence of bacteria in freshwater crayfish in Europe and elsewhere.

Histopathology may be utilized as diagnostic techniques when investigating the cause of mortality in fresh water crayfish populations. It revealed host defense reactions in different organs of some aquatic crustacean [11, 12]. Also, other more specific diagnostic techniques such as bacterial culture are utilized [13-16].

Various chemotherapeutics have been applied for treatment of bacterial diseases, however, these may leave drug residue in the treated animal. At the same time, the use of antibiotics in aquaculture may introduce potential hazard to public health and to environment by emergence of drug resistant microorganisms and antibiotics residues [17]. At the same time the development of drug resistance pathogens against commonly used antibiotics has necessitated a search for new antimicrobial substances from other sources including plants [18] for funding potential new compounds for therapeutic use [19]. It may be possible that herbs can be used in treatment of bacterial diseases in aquatic animals. They natural products which are safe for consumers and many kinds of herbs may be used [20]. The medicinal value of plants lies in some chemical substances that produce a define physiological action on living organism. The most important of these bioactive compounds of plants are alkaloids, flavonoids, tannis and phenolic compounds [21].

Insufficient number of studies were carried out on bacterial disease of Egyptian freshwater crayfish [22] as well as the phytochemistry and the antibacterial effects of macrophytes extracts on these diseases. Thus, the present work was designed to isolate and characterize pathogenic bacteria from the haemolymph and hepatopancreas of diseased freshwater crayfish. Also, examination of pathological alterations in the hepatopancreas and muscles structure was another target. In addition the biochemical characteristics and the *in vitro* efficacy of some plant extracts such as *Aster squamatus, Inula erithmoides L., Pluchea dioscoridis L.* and *Cyperus articulatus* L against the isolated bacteria was performed as a preliminary step in testing the use of herbs for controlling diseases in aquaculture.

MATERIALS AND METHODS

Crayfish Samples: Adult specimens of the freshwater crayfish *Procambarus clarkii* measuring 9-12 cm in length were collected from El- kased drain at Mehalt Menof, in Gharbia governorate, Egypt. The animals were collected randomly during June 2008 by using trap nets. Collected samples were transferred alive to the laboratory.

Isolation of Bacterial Pathogen: 0.1 g from hepatopancreas of diseased animal was ground in 1 ml sterile saline (0.85%) and also 100 µl of haemolymph was suspended in 400 µl saline and used as a source of the pathogen. Both hepatopancreas and haemolymph suspensions were diluted ten folds and 100 µl of each were spread separately on the surface of thiosulphate citrate- bile salts (TCBS, Oxid) plates. The plates were incubated at 30°C for 2 days and then the appeared colonies were maintained on slants of the same medium at 4 °C.

Characterization of the Isolated Pathogen

Morphological Studies: Colony morphology was observed on thiosulphate citrate bile salts sucrose agar (TCBS) after 24 hrs of incubation at 30°C. The pure isolates were stained using Gram's technique [23].

Physiological and Biochemical Characteristic: Vibrio isolates were characterized by a number of tests, including: catalase activity [24], citrate utilization [25], production of indole acetic acid [26, 27], acid production from sugars[28] and growth in the presence of Nacl. The oxidation-fermentation test was performed on 18-24 h growth of the isolates from TSA with Oxidase Detection Strip (MB0266A, Oxide). Nitrate reduction test was performed by seeding a loopful of bacterial culture (TSA plate) in a tube containing 2 ml of nitrate broth (Beef extract, 3 g; Peptone, 5 g; NaCl, 10 g; dist. H_2O 1 L and pH, 7.0) supplemented with NO_3 (0.2% w/v) and 0.2% agar, after incubation at 30°C for 24 h, 0.1 ml of Griess reagent (Oxoid) was added. (Add 1 ml of dimethyl α-naphthylamine solution and Sulfanilic acid solution. The development of a red color indicated a positive result. Growth of the isolated bacterial pathogen in tryptic soy broth (TSB) at different temperature was tested at 4, 25, 35, 40 and 42°C [29]. Voges-Proskauer (VP) test was performed in MR-VP broth (Oxide). After 18-24 h incubation of the tested bacteria at 30°C, 2.5 ml of the culture was transferred to another tube with adding of 0.3 of alcoholic α-naphthol and 0.1 ml of 40% KOH solution, gently agitate the tube and allow to stand. Development of red color indicated a positive result.

Histopathology: Hepatopancreas and muscles were dissected from moribund and healthy crayfish then fixed in 10% formalin. Fixed samples were processed for paraffin sectioning. Sections were cut at 5µm, stained with

haematoxyline and eosin and examined with light microscope.

Plant Extracts Preparation: The plant used in this study *Aster squamatus* (Spreng.) Hierno (Asteraceae), *Inula erithmoides L., Pluchea dioscoridis L.* (Compositae) and *Cyperus articulatus* L (Cyperacea) were collected during May 2008 from El- Raswa station at the southern coast of lake Manzalh, Egypt. In retains to the laboratory the samples were identified [30, 31], washed with distilled water, air dried to constant weight, ground to fine powder and stored in clean bottles. The powdered samples were subjected to extraction using different organic solvents (methanol and ethanol) in addition to water [32, 33]. The extracts were filtered through Whatman No.1 filter paper and concentrated under vacuum at 4°C in Rota- vapor apparatus to dryness. The residues were dried to constant weights and kept under vacuum desiccators until used them.

The Elementary Analysis: The elementary analysis take place in all prepared plants extracts using Pertkin Elmer 2100 Flame Atomic Absorption Spectrophotometer with Auto- sampler.

Preliminary Phytochemical Screening: It was undertaken by a number of tests for glycosides [34], steroids and terpenes [35], flavonoids [36], tannins [37], saponins [38], resin [39] and mucilage [40].

Antibacterial Activity: The antibacterial activity of the plants extracts was performed against the two isolated strains by paper disc method [41]. Each extract was diluted to obtain different concentration of 1000, 750, 500 and 250 mg / ml. Sterilized filter paper discs having a diameter of 6 mm (Whatman No. 1) were impregnated with 40 µl of each dilution of each plant extract and placed on the surface of previously inoculated TSA plates with 100 µl of 5×10^5 cfu/ ml of the two bacterial isolates. The inoculated plates were kept at 4 °C for 2 h and incubated at 30 °C for 24 h. After the end of incubation period the diameter of zones of inhibition were measured in mm.

RESULTS

Gross Clinical Signs: The first recognizable sign of bacteraemia in crayfish was sudden change in the abdominal appearance from a streaked to uniformly white opaqueness. Crayfish with bacterial septicemia were typically lethargic and exhibit lack of muscle tone, loss of some appendages, reduce feeding, reduced response to stimulus and a tendency to lie on their sides. In some moribund animals, dorsal flexion of the abdomen was observed (Fig. 1b).

Table 1: Morphological, physiological and biochemical characteristics of two bacterial isolates from diseased crayfish *Procambarus clarkii*

Characteristics		Isolate No. 1	Isolate No. 2
Color in TCBS agar		Yellow	Green
Cell form		Curved rod	Straight rod
Oxidase		+	+
Catalase		+	-
Citrate		+	+
Indole		+	+
Acid form	Arabinose	-	+
	Inositol	-	-
	Lactose	-	-
	Mannitol	+	+
	Sorbitol	-	-
	Sucrose	+	-
Growth in (w/v)	0% Nacl	-	-
	3% Nacl	+	+
	6% Nacl	+	+
	8% Nacl	+	+
	10% Nacl	+	-
Nitrate reduction		+	+
Growth At	4 °C	-	-
	25 °C	+	+
	35 °C	+	+
	40 °C	+	+
	42 °C	+	+
Voges- Proskaur (VP)		+	-

(+): Positive (-): Negative

Isolation and Characterization: When the primary cultures from hepatopancreas and haemolymph samples of diseased crayfish grown on TCBS agar, yellow and green colonies were observed. These organisms exhibited negative reaction to Gram stain, suggesting that the bacteria belonged to the genus *Vibrio*. As shown in Table 1. the characterized pathogens were able to grew well at 35, 40 and 42°C but have no growth at 5°C in TCBS and grew well at 3, 6, 8% NaCl but the isolate No.2 showed no growth on 10% Nacl. The two isolates were positive for indole and nitrate reduction tests and could utilize citrate. The tested bacteria were able to produce acid from mannitol but not from inositol and lactose. On the other hand the isolate No. 1 was able produce acid from sucrose and isolate No. 2 produce acid from arabinose. The organism No. 1 showed positive Voges-Proskauer reaction. According to the above mentioned morphological and biochemical results, the two isolates No. 1 and No. 2 were identified as *Vibrio alginolyticus* and *Vibrio parahaemolyticus,* respectively.

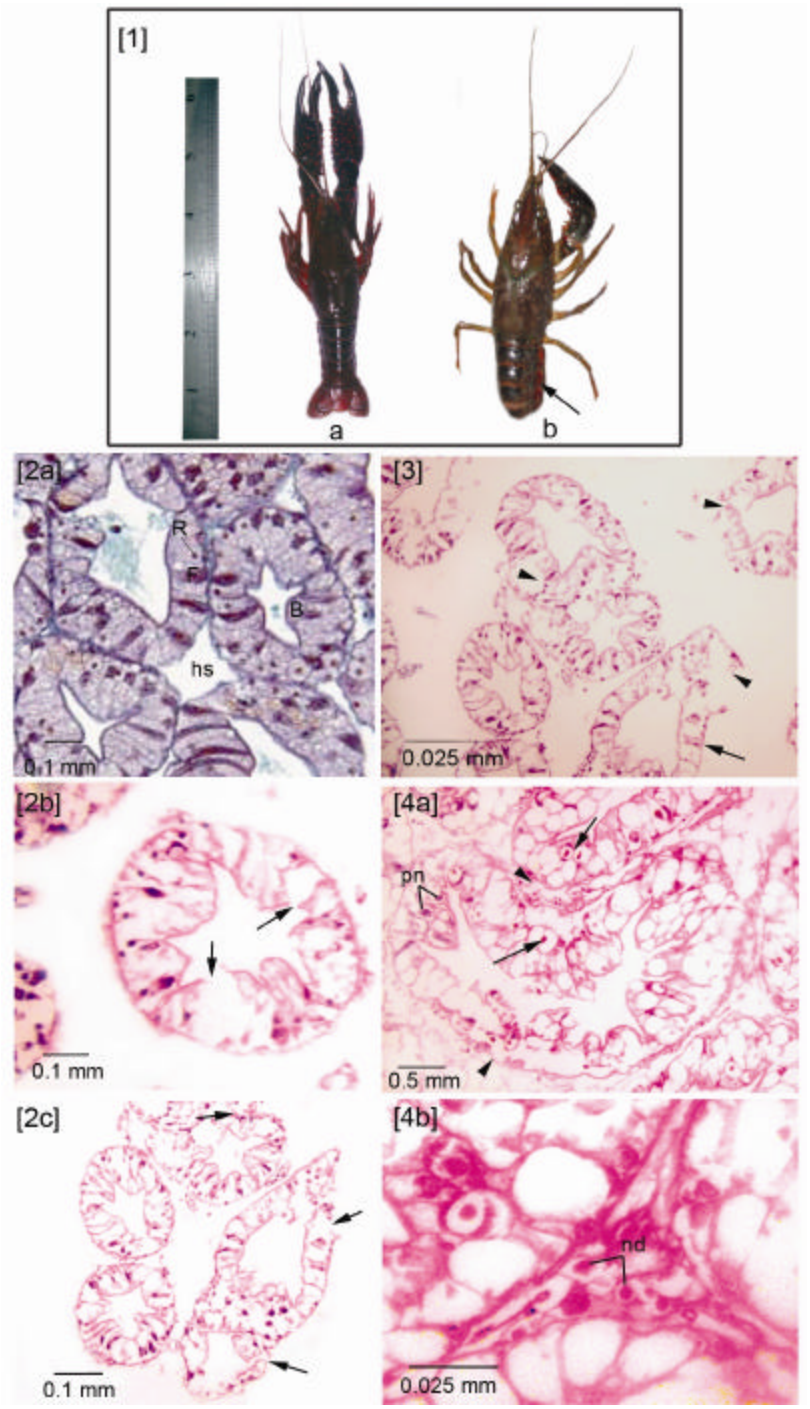

Fig. 1: (A) Healthy crayfish, (B) diseased crayfish. Arrow show dorsal flexion of its abdomen

Fig. 2: Photomicrographs of hepatopancreas of healthy (A) showing tubules containing (B) – cell, F-cell (F),R-cell (R) and haemal space (hs), (B & C) hepatopancreas from diseased crayfish showing varying degrees of necrosis of their tubules (arrow). Hand E stain. Bar= 0.1 mm.

Fig. 3: Photomicrograph of transverse sections showing irregular and elongation tubules of diseased hepatopancreas (arrow) and many cells rupture (head arrow). Stained with H and E. Bar = 0.025 mm.

Fig.4: Photomicrographs of hepatopancreas from diseased crayfish showing (A) increase in number of B- cells and associated with debris (arrow), indistinct cell boundaries (head arrow) and Pyknotic nuclei (pn), (B) increase in haemocytic nodule (nd) inhaemal space. Stained with H and E. Bar=0.5, 0.025 mm.

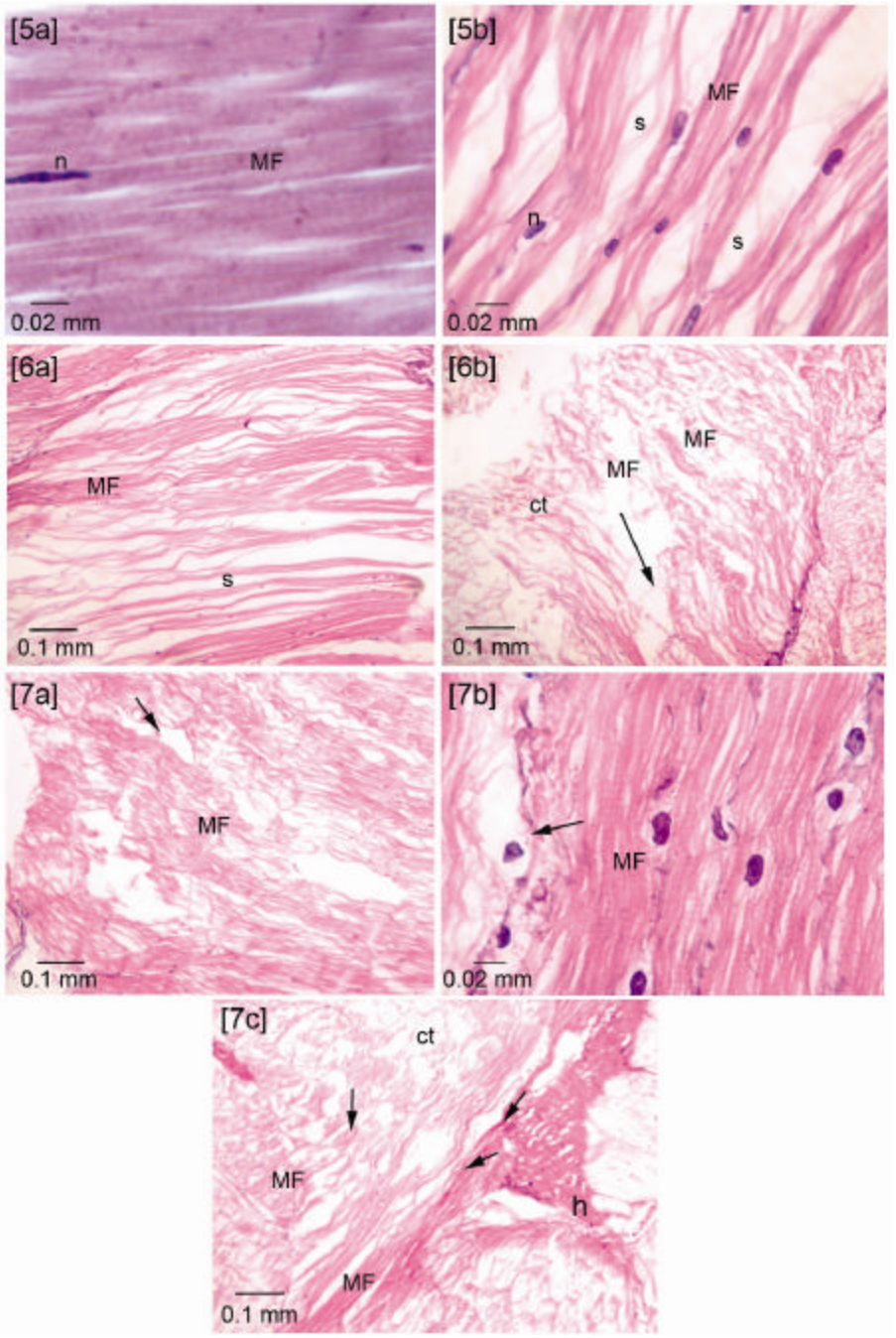

Fig. 5: Photomicrograph of transverse section in (a) abdominal muscles of healthy crayfish showing muscle fibers (Mf), nucleus (n), (B) Diseased abdominal muscles,. Stained with Hand E. Bar= 0.02 mm.

Fig. 6: (A) Photomicrographs of muscles from diseased crayfish showing early necrosis and separation (S) of muscle fibers,(B) Wave of the necrotic foci moving in the direction of the arrow, the area behind the arrow there is an increase in myofibril fragments (Mf) and connective tissue (ct). Stained with H and E. Bar= 0.1mm.

Fig. 7: Photomicrographs of diseased muscles showing muscle fibers lost their orientation (a, b), haemocytic infiltration(arrow),), myofibrillar fragments are condensing together to form densely stained eosinophilic materials. (arrow), connective tissue (ct), myofibril (Mf). Stained with Hand E. Bar= 0.1, 0.02, 0.1 mm.

Table 2: The weight of the extracts residues expressed as % of DW as well as its physical properties

	Aqueous. Extract		Ethanol extract		Methanol. Extract	
Plant Species	Weight of residues	Physical properties	Weight of residues	Physical properties	Weight of residues	Physical properties
A. squamatus	17.6	Dark brown mass	7.6	Brown mass containing some crystals	12	Brown resinous mass containing a lot of crystals
P. dioscorids	10.7	Dark brown mass	14.4	Dark green mass	13.4	Dark green resinous mass containing crystals
C. articulats	4.4	Faint brown mass	1.8	Faint yellow resins mass having little crystals	4.8	Faint brown resinous mass containing very few crystals
I. erithmoies	26	Dark brown mass	6.8	Dark green mass with some oils	12.2	Dark green resinous mass having a lot of crystals

Where: DW= Dry Weight

Table 3: Preliminary phytochemical screening of the studied plants

Tested substances	*A. squamatus*	*P. dioscoridis*	*C. articulatus*	*I. erithmoides*
Glycosides	+	+	+	+
Tannins	+++	+++	+++	-
Flavonoids	+	-	+	-
Saponins	+	++	+	-
Sterols&Terpenes	++	++	++	+++
Mucilage	++	++	+++	++
Resins	+++	+++	+	+

- Not detected + Small amount

++Moderate amount +++High amount

Histopathology: Histologically, each tubule of hepatopancreas is surrounded by haemal spaces that contained haemocytes. The hepatopancreatic tubules were enclosed by basal lamina and contained a central lumen. The epithelial lining of the tubules consist of columnar cells: digestive cell (B), secretory cell (R) and absorptive cell (F) (Fig.2a). Diseased crayfish showed varying degrees of necrosis of their hepatopancreatic tubules (Figs. 2b,c, 3). The hepatopancreas of crayfish with a greater degree of external lesion was more damaged than that of less affected ones. High loads of *Vibrio* sp induced the rounding up and detachment of epithelial cells from the basal lamina of the hepatopancreas (Figs. 2c, 4a). In the R-cells the nuclei become pyknotic or lacking (Fig. 4a). In most severely affected animals, the spaces between tubules were eliminated and outer basal lamina confused with the next tubules (Figs. 2c, 3). Many cell contents were ruptured and unruptured ones showed few cytoplasm and karyolitic nuclei. As the apparent severity of necrosis increased, haemocytic nodules appeared in the haemal spaces (Figs.3, 4a, b).

Muscles of crayfish are normally composed of up to several hundred muscle fibers (Fig.5a). In diseased crayfish, light microscope revealed a loss of muscle fiber structure and expansion of the sarcomeric space (Fig. 5b). These muscle fibers became separated, with an amorphous fluid matrix filling intercellular spaces (Figs. 6a,b). Figure (7a,b) showed muscle fibers became loosed, the orientation of them were lost and was different than the healthy animals. Between the bundles of muscle fibers connective tissues were invested supplying them with more capillaries and many vacuoles were appeared between muscle bands. As shown in figure (7a,c), orientation of muscle fibers was confused in some places or disappeared in other places. Distances between muscle fibers disappeared and were fragmented causing vacuolar space.

Plant Extracts: The physical properties of both aqueous and alcoholic residues were compiled in (Table 2).

The residues in all cases were semi-solid, varied in their physical properties and their weights with plant material and the solvent used in extraction. The dried residues of *Inula erithmoides* aqueous extract recorded the highest weight (26%of dry wt.) followed by *Aster squamatus* (17.6 % of dry wt.). While on using ethanol and methanol for extraction, the highest residue weights were recorded for *Pluchea dioscoridis* (14.4 and 13.4%of dry wt with the two solvents, respectively), (Table 2). At the same time, all studied plants methanol extracts were

Table 4: Values of the measured elements in different plants extracts expressed as µg g-1 of DW

Plants	*A. squamatus*			*P. dioscoridis*			*C. articulatus*			*I. erithmoides*		
Elements	E	M	W	E	M	W	E	M	W	E	M	W
Fe^{++}	162.5	177.5	125	180	132.5	145	160	127.5	110	140	160	122.5
Mn^{++}	47.5	42.5	37.5	60	42.5	42.5	52.5	45	42.5	55	45	30
Cu^{++}	52.5	97.5	80	57.5	100	70	60	102.5	52.5	77.5	102.5	50
Zn^{++}	90	65	57.5	77.5	82.5	70	92.5	80	65	77.5	55	65
Pb^{++}	20	20	10	17.5	10	10	15	17.5	7.5	22.5	15	5

Where: E= Ethanol extract, M= Methanol extract and W= Water extract.

Table 5: Inhibition growth zone (expressed as cm.) of the isolated bacteria caused by different plant extracts. Where Org.1= *V. alginolyticus*, Org.2= *V. parahaemolyticus*

	Concentration (mg ml^{-1})							
	Organism 1				Organism 2			
Plants extracts	250	500	750	1000	250	500	750	1000
A. squamatus								
E	-ve	0.9±0.0	1.7±0.01	2.4±0.02	0.7±0.0	1.0±0.0	1.5±0.0	2.7±0.01
M	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
W	1.5±0.0	2.1±0.02	2.8±0.0	3.5±0.0	-ve	1.3±0.01	2.57±0.02	3.46±0.02
P. dioscoridis								
E	-ve	0.8±0.0	1.5±0.0	2.9±0.01	-ve	0.7±0.0	1.3±0.01	2.5±0.0
M	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
W	1.1±0.01	2.1±0.01	3.3±0.02	4.0±0.0	-ve	1.9±0.01	3.5±0.0	4.4±0.02
C. articulatus								
E	0.8±0.0	1.5±0.0	2.1±0.01	3.3±0.02	0.7±0.0	1.1±0.01	2.2±0.01	2.9±0.02
M	-ve	-ve	0.7±0.0	1.0±0.0	-ve	-ve	0.9±0.0	1.3±0.0
W	2.1±0.02	3.17±0.01	4.3±0.01	5.17±0.02	-ve	1.5±0.0	2.5±0.0	3.5±0.01
I. erithmoids								
E	-ve	-ve	-ve	0.8±0.0	-ve	-ve	-ve	-ve
M	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
W	-ve	-ve	1.5±0.0	2.1±0.01	-ve	1.3±0.0	1.7±0.01	2.2±0.0

characterized by the presence of crystals in their dried residues. In addition, results showed that, the minimum amounts of dried residues were recorded for *Cyperus articulatus* extracts (4.4, 1.8 and 4.8 % of dry wt. for water, ethanol and methanol respectively).

Preliminary Phytochemical Screening: The present study revealed the presence of tannins, steroids, terpenes, mucilage and resins in all studied plants extracts while, saponins was detected in *Pluchea dioscoridis* and *Cyperus articulatus*. At the same time flavonoids were found only in *Aster squamatus* and *Cyperus articulatus* (Table 3).

Elementary Analysis: Different values were recorded for all the analyzed metals (Table 4) depending on plant species as well as the solvent used in extractions. The highest values of Cu^{++} were found in methanol extract ranged between 97.5 µg g^{-1} of DW in *Aster squamatus* and 102.5 µg g^{-1} of DW in *Pluchea dioscoridis* and *Inula erithmoides*. The minimum values of Fe^{++}, Mn^{++}, Zn^{++} and Pb^{++} were recorded in aqueous extract of all studied plants while, the maximum values of Zn^{++} were recorded in ethanol extract of *Aster squamatus* 90.0 µg g^{-1} of DW and *Pluchea dioscoridis* 92.5µg g^{-1} of DW. At the same time, the maximum concentrations of Pb^{++} were found in ethanol extract of *Inula erithmoides* 22.5µg g^{-1} of DW.

Antibacterial Activities: The antibacterial activities of *Aster squamatus*, *Inula erithmoides*, *Pluchea dioscoridis* and *Cyperus articulatus* extracts against the previously isolated *Vibrio* sp were investigated *In- vitro* and the results were summarized in Table 5.

The aqueous extract of *Aster squamatus, Pluchea dioscoridis* and *Cyperus articulatus* exhibited antibacterial effects against *V. alginolyticus* at all concentrations while, *V. parahaemolyticus* show resistance at low concentration 250 mg ml^{-1}. On using *Inula erithmoides* negative results were recorded with the two bacterial species at low concentrations 250 and 500 mg ml-1. Comparing the results of all tested plants extracts *Cyperus articulatus* being the most effective against *V. alginolyticus* causing inhibition zone 5.17±0.02cm while, *Pluchea dioscoridis* extract was the most effective against *V. parahaemolyticus* causing inhibition zone 4.4±0.02cm.

The results of antibacterial activity of the crude ethanol extract of all studied plants showed good antibacterial activity against the two isolates except, the extract of *Inula erithmoides* which gave negative results with *V. parahaemolyticus* at all concentrations while, with *V. alginolyticus* positive result(inhibition zone 0.8±0.0 cm) was recorded at higher concentration 1000 mg ml^{-1}. The methanol extract of all studied plants gives negative results with the two identified organisms except the extract of *Cyperus articulatus* which gave positive results at higher concentrations (750-1000 mg ml^{-1}).

DISCUSSION

A number of causative agents may be responsible for bacterial septicemia including Gram- negative and Gram positive species [42,43]. Bacterial septicemia is frequently described as opportunistic bacterial infection attributed to stressful condition [44]. Typically, mildly pathogenic strains of ubiquitous bacteria gain entry into the host, proliferate in the haemolymph and multiply in the body tissues [45]. In crustaceans there are often limited specific signs of a disease. Most dying crayfish exhibit non specific signs of disease such as anorexia, lethargy and possibly autotomy, all of which have been associated with infection by a wide range of pathogens of crustaceans. Therefore, clinical symptoms were less significance in crayfish pathology than in that of vertebrates [46]. Shell diseased male *Cancer pagurus* displayed a significantly higher percentage of limb loss than their disease- free counterparts[47]. The present study has shown that haemolymph infection frequently co- occurs with limb loss suggesting a potential route of microbial entry. However, bacteria may also gain entry to the haemocoel through breach of the cuticle either at the gill lamellae or through other external lesions.

This study found distinct changes from the normal histology of the hepatopancreas and muscles in diseased animals. In this study the pathogenicity of *Vibrio* sp. on *P. clarkii*, showed severe necrosis of the hepatopancreas which appear to be located within B- cell. Similarly [48-50] also showed damage of the hepatopancreas in combination with bacterial infections. Release of an exotoxin from Gram- negative bacteria in the tubules of *Palaemon eleganus* was believed to be the cause destruction of the hepatopancreas[48]. Bacterial toxins may cause initial lysis of cells, but once the R- cells have disintegrated, autolysis of the tubules may exacerbated by liberation of digestive enzymes. Also, the occurrence of melanised nodules in hepatopancreas suggest systemic bacterial infections in these animals [51].

The histological alterations in the abdominal muscles observed in the present study were similar to previously described as viral disease in muscles of *Macrobrachium rosenbergii* [52, 53]. Muscle disease were characterized by a loss of sarcomeric structure with necrotic lesions containing pyknotic nuclei, fragmented of myofibrils and connective tissue elements, eventual condensation of these elements into separate islands of heavily stained eosinophilic materials [54,55].These features were accompanied by progressive weeking of their feeding and swimming ability.

The phytochemical screening data of the studied plants have revealed among other constituents of their extracts the presence of tannins, steroids, terpenes, mucilage and resins. While saponins was not detected in extract of *Inula erithmoides* and flavonoids were detected only in *Aster squamatus* and *Cyperus articulatus*. These results are concomitant with those previously recorded changes [56, 57]. Besides, the results also indicated the activity of both ethanol and aqueous extracts of *Aster squamatus, Pluchea dioscoridis* and *Cyperus articulatus* against *V. parahaemolyticus* and *V. alginolyticus* at all concentrations but and as an unexpected the water extract of these plants exerted greater antibacterial activity than corresponding ethanol extract at the same concentration. These observations may be attributed to the nature of biologically active substances (tannins, steroids, terpenes, mucilage, resins, flavonoides and saponins) which, have been documented as a well known components for antibacterial activity [58]. The extracts of

Inula erithmoides were weakly effective on the studied bacterial strains except it is water extract which gives positive results at higher concentrations. This may be related to the absence of most active substances (tannins, flavonoides and saponins) from its extracts. However, the methanol extract of all studied plants did not show antibacterial activity *Cyperus articulatus* extract gave positive results at higher concentrations (750-1000mg ml^{-1}). These negative results and the very weekly inhibition effect of methanol extract may be related to the presence of active materials in the form of crystals which could be observed in all methanol extracts.

The metals analysis of investigated plants extracts showed the variations in elements concentrations with plant species and the solvent used in extraction. The lowest concentrations of all measured elements were found in water extracts reflecting, the difference in solubility of these elements in different solvents which may be related to the difference in chemical composition of the studied plants. At the same time comparing the antibacterial activity of the prepared plants extracts, the aqueous extracts appeared to be the most effective suggesting the dependency of antibacterial activity of the studied plants extracts on the presence or absence of the active substances while, the presence of some elements especially Fe^{++}, Cu^{++} and Pb^{++} at higher concentrations in both ethanol and methanol extracts may inhibit the effect of these active materials. Similar findings were previously found [59] while, [60] cited that, the stronger extraction capacity of ethanol could have produced greater active constituents responsible for antimicrobial activity.

Faker Eldin, *et al.* [61] studied the composition and antimicrobial activity of essential oil of *Pluchea arabica* from Oman and he recorded that, the essential oils of *P. arabica* was active against *Staphyllococcus aureua (ATTC 29213), Candida albicans (ATTCC 10231)* and *Bacillus* subtillis when tested against seven organisms.

In this work *Cyperus articulatus* extract was recorded to had the same active substances like *Aster squamatus* and *Pluchea dioscoridis* but it's the highly effective and this may be related to the presence of special substance called cyperones [62]. Two of these chemicals, called cyperotundone and alphacyperone, have been reported with antimalarial actions [63], as well as the ability to inhibit nitric oxide. Synthesis (a pro-oxidant) and prostaglandin synthetase (aspirin and ibuprofen are prostaglandin synthetase inhibitors) [64] The plant was also reported with antioxidant actions [65], antibacterial actions against Staphylococcus and Pseudomonas [66, 67] and anti-yeast actions against Candida [68].

It could be concluded that, *Cyperus articulatus*, *Pluchea dioscoridis* and *Aster squamatus* can control bacterial diseases in crayfish ponds. Therefore, *in vivo* studies on these plants are necessary and should seek to determined toxicity of active constituents of these plants.

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