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Bacteria Associated with Diseased Cage Cultured *Oreochromis niliticus* and Control Trial with Goldenseal (*Hydrastis canadensis L.*)

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ABSTRACT

Goldenseal (*Hydrastis canadensis*) has been collected as a medicinal plant for hundreds of years. In the present study it used as natural product in treating bacterial fish pathogen in floating cage cultured Nile tilapia (*Oreochromis niliticus) in* El- Bostan, Damietta. Samples from diseased cages were collected and subjected to full clinical, post mortem and bacteriological investigation. The bacterial isolates were *Aeromonas hydrophila*, *A. sobria*, *Pseudomonas fluorescens* and *Citobacter freundii*. The identified isolated strains were examined against many antibiotic discs using disk diffusion methods comparing with discs from *Hydrastis canadensis* extract and interpretations the zones of inhibition. The diseased farm was treated for one week with Hydrastis *canadensis* at 15 ppm compared with other antibiotics. Survival rate were 87% in the cages treated with *Hydrastis canadensis* and was 84% in the cages treated with antibiotics.

Keywords: Goldenseal, floating cages, antibiotic and isolates

INTRODUCTION

Aquaculture has an important role in development of many national economic and play a key role in rural development, also has a main role in meeting demand for aquatic animal production (Hoylor and Bland, 2001). Aquaculture industry is gradually developed in the world as well as in Egypt. The healthy keeping fish depend on the relationship between environment and pathogens. The high stock density of intensive fish cultures leads to increase the feeding uptake and so, the higher wastes excreta that's form upper stress on fish heath resulting in bacterial diseases problems. Beside many stresses facing fish during culturing process as trauma from handling, transportation and partial harvesting act as predisposing factors for invasion of diseases factor specially bacteria (Mitchell, 1977).

The risk associated with using of chemical substances and antibiotics in control of bacterial infection may lead to antibiotic resistance bacteria, increased human infection and increasing of antibiotic residue in fish.

Goldenseal (*Hydrastis canadensis L.*), a member of the buttercup family (*Ranunculaceae*), has approximately the same native range and environmental requirements as ginseng (moist woodlands of the eastern U.S.). A perennial, goldenseal has an erect hairy stem

that grows to about a foot in height, with three or four yellowish scales at the base of the plant (Foster, 1993). The dried roots and rhizomes of Goldenseal is the part used for medicinal purposes contains not less than 2 percent of hydrastine and not less than 2.5 percent of berberine calculated on the dried basis (Avula et al., 2012).

With the introduction of antibiotics, US domestic use of goldenseal declined but it remained a valuable commodity in Europe. Over the past 20 years, with resumed interest in herbal medicines, golden seal has regained popularity in the US. It is now one of the most popular medicinal plants used in North America and remains a staple of most herbal practitioners (Upton, 2001). The herb is generally known for its superb antibiotic treatment and viral suppression properties. Native Americans used it hundreds of years ago for inflammation of the mouth and eyes. Today, it is an effective antibiotic for treating many infections (Das et al., 2013).

Golden Seal is not only used for its antibiotic properties but is works well in treating many types of problems such as , viral infections can be quite effectively responding to the treatment with it. Also, other health issues such as ringworm, candida, and skin problems can be treated with Golden Seal (Orr, 2010).

As an anti-bacterial, goldenseal extract does not appear to work directly on the bacteria but it is unclear exactly how it does work. It is the compound berberine that appears to act as an 'antibiotic' to the mucous membranes not by killing germs directly, but by increasing the flow of healthy mucous, which contains its own innate antibiotic factors and promotes easier removal of the bacteria by inhibiting their ability to adhere to tissue surfaces (Upton, 2001).The studies in the laboratory have shown that direct contact with berberine helps stop bacteria and some types of fungus from growing ((Madis, 1997).

Berberine has been shown to kill a wide range of other types of germs such as those that cause candida (yeast) infections and various parasites such as tapeworms and Giardia. It contains calcium, iron, manganese, vitamin A, vitamin C, vitamin E, B-complex, and other nutrients and minerals, as well as traces of essential oil, fatty oil and resin activate white blood cells, making them more effective at fighting infection and strengthening the immune system. As a complete herb goldenseal works on the immune system preventing antibiotic resistance (Hermann and von Richter, 2012).

The present work aimed to obtain natural product used in treating bacterial fish pathogen without harmful residue on public health in alternative to the synthetic products.

MATERIALS AND METHODS

Fish

One hundred diseased male monosex Nile tilapia (*Oreochromis niloticus*) weighted 75 – 100 gm were collected from floating cages (50.000fish/cage), (8m×12m×12m) in El-Bostan, Damietta and transported in ice box to the (CLAR) and subjected to full clinical, post mortem and bacteriological investigation.

Water Quality Examination

The examination of water quality were done in the places of fish culturing for measuring temperature, dissolved oxygen, water saturation, ammonia, total alkalinity, hardness, nitrate and salinity. The tests were done according to techniques described by American Public Health Associated standard methods **(APHA, 1985).**

Clinical Investigation

Clinical examination of affected fish was done out as described by **Scaperclous et al.,** (**1992**) to determine the clinical alteration on the skin, scales, eyes, abdomen, tail and fins. Also, fish behavior due to bacterial infection was examined before sampling.

Postmortem Examination

The post mortem was done on living and freshly dead fishes to examine all internal organs including gills, spleen, kidney, liver and intestine for detection the abnormalities. The examination was done according to **Scaperclous et al., (1992**).

Bacteriological Examination

Samples for bacteriological examination were taken from gills, liver, spleen, kidney, eyes and fins under aseptic conditions. The samples inoculated into tryptic soya broth (T.S) and brain heart infusion (BHI) then incubated at 25 – 29°C for 48 hr. After that time streaked over tryptic soya agar and incubated at same temperature for another 48 hr. The suspected purified colonies were picked up and streaked on selective aeromonas agar base supplemented with ampcillin (20 mg) and selective pseudomonas agar base. The identification of isolated bacteria was carried out according to **Bergery,** (**1994**) using routine study of the morphological characters and biochemical reaction.

Pathogenicity Test

The pathogenicity of isolate strains was done using 50 Nile tilapia 75 gm ± 5 gm divided into 5 groups. Each group contains 10 fishes. The fishes kept in glass aquaria (40×40×80 cm of each one) at 27 °C. The isolates were prepared and the dose was estimated with Mcfarland barium sulphate standard tube (Difico) at 0.05×10^6 cell/ml and injected intra peritoneum (I/P) to four groups and one group represented as control. The inoculated fishes were observed for 21 days for recorded the mortality and disease symptoms. According to Qunine et al., (1994).

Antibiogram Sensitivity Test

The identified bacterium isolates of sampled diseased fish were examined against many antibiotic discs using disk diffusion methods on Muller's and Hinton agar medium comparing with discs from *Hydrastis canadensis* extract and interpretations the zones of inhibition. The technique was done according to the limits given by Scaperclous *et al.,* (1992)

Estimation Sensitivity of Hydrastis canadensis

Different concentration 00 ppm, 10 ppm, 15 ppm and 20 ppm of *Hydrastis canadensis* extract was prepared and used to determine sensitivity concentration against defined bacterium isolates of diseased fish. The technique was done according to **Wolf and Snieszko, (1963).**

Determination Application Dose of Hydrastis canadensis

Forty Nile tilapia 75 gm ± 5 gm was collected from diseased cages and divided in 4 groups. Each group contains 10 fishes. The fishes kept in glass aquaria (4040×80 cm of each one) at 27 °C and treated with *Hydrastis canadensis* extracted in doses 00ppm, 10 ppm, 15 ppm and 20 ppm for one week **Mathias et al., (1976).**

Effect of Hydrastis canadensis on Bacterial Fish Pathogen at Field Application

The diseased cage farm which collected our samples was treated with the *Hydrastis canadensis* at results of recognized dose and other antibiotics obtained from sensitivity test for one week. The control group also was done. The technique was done according to Wolf and Snieszko, (1963).

RESULTS

Water Quality Examination

The results of water analysis resulted that presence low dissolved oxygen level, low temperature and slightly, increase in toxic ammonia in comparison with grad water quality as shown in Table (1).

Clinical Investigation

The mortality rate of tilapia in floating cages in El- Bostan, Damietta was more than 200 fish / day / cage. The clinical signs showed

Table (1): ***Water Quality Examination***

Item	Level
Temperature	24 ℃
Dissolved oxygen	3.1
Saturation	27.5
Total ammonia (mg/l)	1.82
Toxic ammonia (mg/l)	0.42
Total alkalinity (mg/l0	148.0
Total hardness (mg/l)	127.0
Nitrate	0.31
Salinity	0.050

in the moribund fish was off food, loss of balance, sluggish movement, swimming near to the water surface and gasping of air, roughness of skin, unilateral and bilateral exophthalmia, opaqueness of the eyes, hemorrhage at the base of the fine and around the mouth also presence of edema of the distend abdomen (Figure 1).

Postmortem Examination

The gills were congested in some cases and pale in other cases with presence of thick mucous covered it. Hepatomegaly with variable white foci on it's surface. The gall bladder was distended with bile. Spleen was enlarged and congested. Kidneys were dark red and oedematous. There is accumulation of bloody exudates in the abdominal cavity (Figure 2).

Bacteriological Examination

Two hundred and forty bacterial isolates with prevalence of 100% out of clinical examined fish according to morphological, culture and biochemical characters, they belonged to 4 bacterial genera. The bacterial isolates were *Aeromonas hydrophila*, *A. sobria*, *Pseudomonas fluorescens* and *Citobacter freundii* (Table 2 and 3).

Distribution of Isolated Strains

The distribution of isolated strains in different organs of infected *Oreochromis niliticus* were recorded in Table (4).

(A)

(B)

Figure (1): ***(A) mortality in the floating cages***
(B) hemorrhage at the base of the fine, around the mouth and abdomen distend

Figure (2): ***distended gall bladder, enlarged spleen, dark red kidneys and accumulation of bloody exudates in the abdominal cavity***

Table 2: ***Collective Data of Bacterial Isolates from Examined Fish***

A. hydrophila,	*A.sobria,*	*Ps.fluorescens*	*Citr. Freundii*	**Total**
98 (40.83%)	56 (23.33%)	68 (28.33%)	18 (7.5%)	240 (100%)

Chi^2 = 6.25**
Significant at (P< 0.01)

Table (3): ***Morphological and Biochemical Characters of Isolates Bacteria According to Bergy's Manual 1994.***

Test	*A. hydrophila,*	*A.sobria*	*Ps.fluorescens*	*C. freundii*
Gram Stain	-ve	-ve	-ve	-ve
Shape	Bacilli	Bacilli	Bacilli	Bacilli
Oxidase	+ve	+ve	+ve	-ve
O/F	F	F	O	F
Growth 0 % NaCl	+ve	+ve	+ve	+ve
Growth 0.5 % NaCl	+ve	+ve	-ve	-ve
Indole	+ve	+ve	-ve	+ve
Vogous Proskouer	+ve	+ve	-ve	-ve
Methyl Red	+ve	+ve	-ve	+ve
H_2S Production	-ve	-ve	-ve	+ve
Gelatin Liquefaction	+ve	+ve	+ve	-ve
Nitrate Reduction	+ve	+ve	+ve	+ve
Citrate Utilization	+ve	+ve	+ve	+ve
Arginin Hydrolysis	+ve	+ve	+ve	+ve
Sugar Fermentation				
Glucose	+ve	+ve	+ve	+ve
Sucrose	+ve	+ve	-ve	+ve
Lactose	-ve	-ve	-ve	+ve
Arabinose	+ve	-ve	-ve	+ve
Cephalothin 30µg (disc)	Resistance	Sensitive	Sensitive	Sensitive

Table (4): ***Distribution of Isolated Strains in Different Organs of Infected Oreochromis niliticus***

Bacteria	**Organ**						
	Skin	**Gill**	**Liver**	**Spleen**	**Kidney**	**Acetic Fluid**	**Total**
A.hydrophela	28(28.58%)	21(21.43%)	19(19.38%)	9(9.19%)	13(13.26%)	8(8.16%)	98
A. Sobria	15(26.88%)	12(21.52%)	10(17.86%)	6(10.53%)	9(16.07%)	4(7.14%)	56
Ps.fluorescence	14(20.59%)	15(22.06%)	13(19.12%)	8(11.76%)	12(17.65%)	6(8.82%)	68
Citr. freundii	5(27.78%)	10(55.56%)	-	-	-	3(16.66%)	18

Chi^2 = 25.80**
Significant at (P< 0.01)

Antibiogram Sensitivity Test

The antibiogram sensitivity test of the isolates was being done and the obtained results illustrated in Table (5).

Pathogenicity Test

The results of pathogenicity test as showed in Table (6) the *A. hydrophila* was highest viruralant followed by *A.sobria* and *P.fluorescens* while *Citobacter freundii* was mild pathogenic to the fishes. The inoculated strains re-isolated from the infected fishes and gave nearly same characters.

Antibiogram Sensitivity Test

The results of examined many antibiotic discs comparing with discs from *Hydrastis canadensis* extract against identified bacterium isolates of collected diseased cage fish were recorded in Table (4).

Estimation Sensitivity of Hydrastis canadensis

The isolated bacteria from the diseased fish were exposed to different concentration from *Hydrastis canadensis* 00 ppm, 10 ppm, 15 ppm and 20 ppm. The obtained results showed in Table (7).

Determination Field Application Dose of Hydrastis canadensis

The obtained results after treated samples from diseased fish by different concentration of *Hydrastis canadensis* for one week were summarized in Table (8).

Effect of Hydrastis canadensis on Bacterial Fish Pathogen at Field Application

The diseased farm from which collected our samples was treated with the

Hydrastis canadensis at 15 ppm and other antibiotics obtained from sensitivity test for one week. Total mortality and survival results were illustrated in Table (9) after one week..

DISCUSSION

The result obtained showed that mortality reached to 200 fish/ day/ cage for 20 days, in floating cages in El- Bostan, Damietta that suggested due to high stock density of cages which reach to 50,000 fish/ cage in dimension (8m × 12 m × 12m), using of supplemented artificial diet in the time fish can't reach to it from the overcrowded, beside organs of fish due to cannibalism resulted in accumulation of organic matter in the bottom of the cage at which the total ammonia was 1.82 mg/l and the toxic ammonia was 0.42 mg/l. The dissolved oxygen decreased to 3.1mg/l that leads to decreased the activity of nitrogenous bacteria which responsible about distractive ammonia to nitrate and nitrite. The increasing ammonia levels reflected on the general fish condition and become clear as general leucopenia state in the caged fish that referred to a decreasing in fish resistance against the causative diseased agents which normally inhabitance in water and fish. This obtained form is similar with results recorded by Plumb (1994).

The clinical investigation of infected fish showed sluggish movement as a result of rot of tail and pectoral fins, gasping and swimming near to water surface as a result of hyperplasia of epithelial lining of gills and accumulation of mucous on it this results agreed with that of Enany (1998).

Hemorrhage occurred on the base of fins and skin could be attributed to release of powerful bacterial protolytic enzymes which lead to electrolyte and loss of protein together with circulation disturbance (Morita, 1975).

The post mortem finding showed septicemic form that was congested liver, kidney, spleen and accumulation of bloody tinged exudates, this is similar recorded by Amlacher (1970). Distended gall bladder result from constriction of common bile duct due to the periductal hyperplasia of epithelial lining, uni or bilateral exophthalmia and opaqueness of

Table (5): ***Antibiogram Sensitivity Test***

			Tested Strains		
Antibiotic	**Code**	**Conc**	***A. hyderophila,***	***A. sobria***	***Ps. Fluresence***
Ciprofloxacin	CIP_5	5 µg	+++	+++	+++
Amikacine	AN_{30}	30mcg	+	+	-
Nalidixic acid	NA_{30}	30mcg	+	-	+
Amoxicillin	AX_{25}	25mcg	-	-	+
Colistin	CL_{10}	10mcg	-	-	-
Chloraumphencol	C_{30}	30 µg	-	-	-
Sulphatrimethoprim	SXT_{25}	25mcg	-	-	+
Cephaloridin	CD_{30}	30mcg	+	-	-
Lincomycin	L_2	2mcg	+	-	-
Rifampin	RD_{30}	5mcg	-	+	-
Erythromycin	E_{15}	15mcg	-	+	-
Penicillin	P_{10}	10 unit	-	-	+
Hydrastis canadensis	Hc	15ppm	++	++	++
(+) : sensitive	***(-) : resist***				

Table (6): ***Pathogenicity Test of the Isolate Strains***

Group	Inoculated Organism	Rout	Dose	Mortality	Survival
T_1	*A. hydrophila*	I/P	0.5 ml	9 (90.00)	1 (10.00)
T_2	*A.sobria,*	I/P	0.5 ml	8 (80.00)	2 (20.00)
T_3	*Ps.fluorescens*	I/P	0.5 ml	8 (80.00)	2 (20.00)
T_4	*Citobacter freundii*	I/P	0.5 ml	2 (20.00)	8 (80.00)
T_5	Sterile Broth	I/P	0.5 ml	0 (0.00)	10 (100.00)

Table (7): ***Estimation Sensitivity of Hydrastis canadensis***

Concentration	*A. hydrophila,*	*A.sobria,*	*Ps.fluorescens*	*Citr. freundii*
00 ppm	-	-	-	-
10 ppm	+	++	+	+
15 ppm	++	++	++	++
20 ppm	++	++	++	++

Table (8): ***Determination Field Application Dose of Hydrastis canadensis***

Group	Fish No.	*Hydrastis Canadensis*	Survival No. and Rate
1	10	00 ppm	1 (10.00)
2	10	10 ppm	8 (80.00)
3	10	15 ppm	9 (90.00)
4	10	20 ppm	9 (90.00)

Table (9): ***Mortality and Survival after Drugs Application***

Drug	Mortality %	Survival %
----------	74	26
Ciprofloxacin	16	84
Hydrastis canadensis	19	81

the eyes attributed to inflammatory local edema due to increases capillary permeability leading escape of plasma protein under the effect of exotoxins which produced by infected bacteria these results were recorded also by Sakr and Abou El Atta (2006).

The bacteriological examination showed that, isolation of two hundred and fourty belonged to *A. hyderophila, A. sobria, Ps. Florescences* and *Citrobacter ferundii* isolated from different organs (fins, skin, kidney, liver, spleen and ascetic fluid). The identification of isolated bacteria according to culture, morphological and biochemical characters according to Bergy's Manual (1994) as shown in Table (3). The isolation of *A. hyderophila* from diseased fish was 98 (28.33%), *A. sobria* was 56 (23.33%), *Ps. Florescences* was 68 (28.33%) and *Citrobacter ferundii* was 18 (7.5%). This results nearly similar with Enany, (1995). The high prevalence of aremonus could be attributed to it's presence as one of the normal intestinal flora and be pathogenic at stress factors Newman, (1982).

The results of virulence test showed *A. hyderophila* was highest virulent which caused 90% mortality by intra-peritoneal injection (i/p) followed by *A. sobria* caused 80%, , *Ps. Florescences* caused 80% and *Citrobacter ferundii* caused mild mortality that was 20% which indicated it isn't the main cause of infection. This result agrees with Sakr and Abou El Atta (2006).

The results of antibiogram revealed that, the isolated strains were sensitive to cibrofloxacin 5µg as showed in Table (4) these results agreed with Abou El Atta and Saleh (2010) and Enany et al., (2011).

The isolated bacteria from the diseased fish in the experiment has been indicated susceptibility to *Hydrastis canadensis* and that has been proved the presence of its affect on the bacteria. This is supported by Sandhu et al., (2003) who discovered that roots and rhizomes of goldenseal contain not less than 5 percent of hydrastine and not less than 10 percent of total alkaloids, including berberine and it is believed that the high content of these alkaloids gives its antibiotic activity and so, it is considered a natural antibiotic.

The effective concentration of applicable dose of *Hydrastis canadensis* was appeared at 15 ppm at which there is no significance different at 20 ppm (Table 7). So, that is preferable used first dose in application (15 ppm) from the economic and pharmakinetic view. This result is somewhat over than result recorded by Robbins, (1997) who suggested the applicable dose of *Hydrastis canadensis* preferable to be 10 ppm. That may be due to the different of work media and different of treated microorganisms.

The diseased farm from which collected our samples was treated with the

Hydrastis canadensis at results of recognized dose and compared with first antibiotic obtained from sensitivity test which was ciprofloxacin for one week. The cumulative mortality and survival percent during treated week were illustrated in Table (9). The obtained results showed better recovered percent of treated fish with the drug under investigation nearly to the applied ciprofloxacin that is due to the *Hydrastis canadensis* isn't only natural antibiotic but also, help the infection-fighting ability of the immune system. The results agree

with Blumenthal, (2003) who recorded the *Hydrastis canadensis* as natural antibiotic, anti-infective and immune stimulating qualities and its effectiveness against bacteria, protozoa, fungi, and Streptococci. And that is the cause effect of *Hydrastis canadensis* was nearly closed to the used antibiotic ciprofloxacin which has only effect on the infected bacteria and has no effective on the fish immune system or other organisms presence the environment share in the diseases.

The different between the survival rates in Table (8) and Table (9) is due to the variation in environmental condition between the laboratory at which there is constricted condition and the open field.

It can be calculated that the *Hydrastis canadensis* is natural plant can be used as antibiotic due to it has antibacterial effect and immuno-up-modulator. The plant didn't leave any residues in the flesh of fish and so the produced fish treated and reared with it be safe for human consumption.d

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البكتيريا التى تصيب أسماك البلطى النيلى فى أقفاص التربية ومحاولة السيطرة عليها بإستخدام مستخلص نبات زهرة الذهب

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*قسم صحة الأسماك ورعايتها، المعمل المركزى لبحوث الثروة السمكية بالعباسة
**معهد بحوث صحة الحيوان – معمل كفر الشيخ

يعتبر نبات زهرة الذهب من النباتات الطبية التى عرفت من مئات السنين لما له من تأثير مضاد للبكتيريا وأيضا منشط مناعى وفى الدراسة المقدمة تم استخدام نبات زهرة الذهب فى محاولة لعلاج حالة إصابة بكتيرية فى إحدى مزارع أقفاص تربية الأسماك فى منطقة البستان بمحافظة دمياط. حيث تم أخذ عينات من الأسماك المصابة وتم عمل إختبارات لهذه العينات والتى تمثلت فى الفحص الظاهرى، الصفة التشريحية، والعزل البكتيرى للمسبب المرضى وتصنيفه والتعرف عليه من خلال إختبارات الكمياء الحيوية للبكتيريا، ثم إختبار حساسية العزولات للمضادات الحيوية المختلفة وأيضا حساسية تلك المعزولات لمستخلص زهرة الذهب.

وقد وجد أن أفضل تركيز للعلاج بمستخلص زهرة الذهب هو ١٥ جزء فى المليون وعند المعالجة بهذا التركيز فى الأقفاص المصابة وجد أن نسبة الإعاشة كانت ٨١% وقد قابلت هذه النسبة المستوى العلاجى للأقفاص التى تم معالجتها بالمضاد الحيوي الأول فى إختبار الحساسية والذى كان السبروفلوكساسين والذى أعطى نسبة إعاشة ٨٤% ويعزى هذا إلى لما لزهرة الذهب من تأثير منشط للمناعة بالإضافة لكونها مضاد حيوى طبيعى كما أن لها تأثير بدرجات مخلفة على الفطريات والأوليات الأخرى.

وتوصى الدراسة بإستخدامه فى علاجات مزراع الأسماك لتأثيره الفعال حيث أنه مضاد حيوى طبيعى ليس له آثار جانبية على صحة الأسماك المعالجة به ولا يترك بقايا ضارة فى لحومها تضر الإنسان.