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PHENOTYPING, VIRULENCE CHARACTERISTICS OF *AEROMONAS* SPECIES AND THE EFFECTS OF ESSENTIAL PLANT OILS AS ANTIMICROBIAL AGENTS AGAINST PATHOGENIC ISOLATES FROM DIFFERENT SOURCES

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ABSTRACT

Aeromonas species are increasingly recognized as enteric pathogens. Faecal samples from 20 cow, 45 sheep; 60 goat and 60 camels were examined for the presence of *Aeromonas* species, which was also sought in the available drinking water (55 well water and 52 drinking chlorinated tap water were also examined). *Aeromonas* species was isolated more frequently from goats (21.7%) than from other animal groups sampled and isolated more frequently from well water (38.2%) than chlorinated supplies (23.0%). *A. hydrophilia* was the most dominant species isolated from different kinds of samples (13.4%). Whereas *A. sobria* and *A. caviae* were isolated in much lower rates 4.7 and 2.1% respectively. There was significant association between the isolation of *Aeromonas* species from all animal faeces and its presence in drinking water. All isolated strains were examined for the characteristics that are reputed to have roles in pathogenicity. The data reported in this study indicates that the distributions of virulence factors, that regulate the pathogenicity of Aeromonads, are different in clinical and enviromental samples. *Aeromonas* isolates exhibited multi-drug resistanc amoxicillin, carbenicillin and ampicillin. The most potent antibiotics against *Aeromonas* species isolated in this study were ceftriaxone, ceftazidime, cefotaxime, cefepime. Essential oils have been tested for in vitro and in vivo antimicrobial activity. Clove, Olive and Peppermint oil exhibited a wide spectrum of antimicrobial activity against all strains used in this study, showed a zone of inhibition ranging from 10.00±0.8 to 14.82±0.41 mm in diameter. Minimum Inhibitory Concentration (MIC) for selected oils ranged from 12.8 to 25.6 mg mL^{-1}. Treatment of mice with essential oil for 15 days led to enhance antibody levels in all treated groups and significant clearance of *A. hydrophilia* from animals. The treated animals had minimal histopathological changes and lower bacterial loads in the organs examined. In conclusion, these findings indicate that aeromonads have the potential to cause human illness and confirm the role of water as vehicles for *Aeromonas* diseases. This study also demonstrated that the multi-factorial nature of the diseases and the influence of environmental conditions in the expression of the putative virulence properties. These results suggested the potential value of essential oils as an additional or supporting treatment in gastrointestinal inflammations.

Keywords: Aeromoans Species, Plant Oils, Antibiotics, Clove, Olive and Peppermint Oil

1. INTRODUCTION

Aeromonas species are facultative anaerobic Gram-negative bacteria that belong to the family Aeromonadaceae which are found in sea, river, fresh and ground water (Hassan *et al*., 2012). There are accumulating data indicating that *Aeromonas hydrophila* (*A. hydrophila*) is the causative agent for several diseases

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in cold blooded animals including fish and reptiles and in warm-blooded animals such as mammals and birds (Kuhn *et al*., 1997). In humans, *Aeromonas* causes diarrhea, gastroenteritis and extraenteritic conditions such as septicemia, wound infection, endocarditis, meningitis and pneumonia (Ali and Hossein, 2010). There is little information on the incidence of *Aeromonas* species in mammals, other than man. Wohlgemuth *et al*. (1972) isolated it from a case of bovine abortion and Sanyal *et al*. (1975) isolated it from the faeces of a calf. Annapurna and Sanyal (1977) isolated *A. hydrophila* from faeces of domestic animals-cow, buffalo, goat and chickens-although no large-scale study has been reported to date. Moreover, there are reports indicating that *A. hydrophila* in particular is one of the food borne pathogens in seafood and shellfish and in foods from other sources such as raw red meat, poultry, dairy products and vegetables (Daskalov, 2006). The pathogenesis of *Aeromonas* infection is complex and multifactorial. *Aeromonas* species secretes many extracellular proteins, including amylase, chitinase, elastase, aerolysin, nuclease gelatinase, lecithinase, lipase and protease. These proteins are known as virulence factors that cause disease in fish and humans. Aerolysin is a representative virulence factor of *Aeromonas* and was reported to function as hemolysins and cytolytic enterotoxins (Bhowmik *et al*., 2009).

Antibiotic resistance and reduced efficacy of the contemporary drugs due to intense usage of antimicrobial agents has been identified in animal pathogens (Grim *et al*., 2013). Additionally, antibiotic resistance can be transmitted horizontally from one bacterium to another and this way can be passed through human pathogens (Okmen *et al*., 2012). Finally, antibiotics can accumulate in animals which create a potential risk for the consumers (FAO\OIE\WHO, 2006). All these outcomes reported from agricultural industries increased awareness towered the negative impacts of indirect exposures to antimicrobial agents. This increasing public awareness led to search for green solutions such as organic food products that are free of organic or synthetic chemicals. Medicinal plants have been used for treatment of common infections since ancient times (Turker *et al*., 2009). Hence, it is important to isolate and determine the frequency of occurrence of *Aeromonas* species in the natural surface waters and clinical samples from different animals and to characterize them in detail. We report here its ability to produce virulence-associated factors as well as to determine the antibiotic profile of isolated *Aeromonas* species. The aim of this study also was to investigate the antimicrobial potential of three different plant essential oils (Clove, Olive and Peppermint oils) against the selected *Aeromonas* species isolates.

2. MATERIALS AND METHODS

2.1. Study Area and Sample Collection

In kingdom of Saudi Arabia, different sites located in Taif governorate and its surroundings, were selected.

2.2. Faecal Samples

A Total of 185 Faecal samples (20 from cow; 45 from sheep; 60 from goat and 60 from camels) were collected from diseased animals.

Faecal samples (rectal contents) were taken from rectum under a separate polyethylene bag.

Most establishments at which animals were sampled had well (untreated) water as the available drinking water.

2.3. Water Samples

A total of 107 water samples (55 well water and 52 drinking chlorinated tap water) were collected bi-weekly from January 2013 to Septemper 2014.

Wells water samples were collected in sterile 250 mL glass bottles (5 cm below the water surface).

These water sources are used for drinking animals and for domestic purposes such as bathing, washing of clothes and utensils and cooking, by the community.

From each available chlorinated drinking tap water, about 300 mL a sample was also collected in a sterile water sampling bottle. Inoculations into selective media were conducted within 24 h after collection of the water samples.

2.4. Isolation and Identification of Bacteria

Ten milliliters of each water sample or 5 g of each fecal sample was inoculated into 10 mL double-strength alkaline Peptone water (PH 8.6) and incubated at 37°C for 24 h. A sample from this enrichment culture was streaked with a loop on Thiosulfate-Citratebile Salts-Sucrose (TCBS) agar (Eiken) and incubated for 24 h at 37°C (Kuhn *et al*., 1997).

2.5. Identification of *Aeromonas* Species

The biochemical reactions of *Aeromonas* species were given in **Table 1** (Using Cowan and Steel"s Manual for the Identification of Medical Bacteria (3rd Edition):

- Detection of extracellular enzymes: (Protease, gelatinase and haemolysis production)

- Protease activity was assayed by method described by Sechi *et al*. (2002)
- Gelatinase production was determined using Luria Broth agar containing gelatine (30 g L^{-1}), as described by Sechi *et al*. (2002)
- Hemolysin production was assayed as described by Santos *et al*. (1999)
- Cytotoxin production
- Preparation of cell-free culture supernatants. Trypticase Soy Broth (TSB; Hi Media) was used for assessing production of various toxins and the cell-free filtrate was used for the tissue culture (Bhowmik *et al*., 2009)
- Tissue culture assay. The tissue culture assay was performed using HEp-2 cells as described previously (Bag *et al*., 2008). Morphological changes and cytotoxic effects were recorded after 24 h incubation using an inverted microscope (Olympus)
- Adhesion to epithelial cells HEp-2 cells were used for the adhesion assay as reported by Sechi *et al*. (2002)
- Slime test The test was performed according to Freeman *et al*. (1989)
- Crystal violet binding. The ability of *Aeromonas* species to bind crystal violet was determined as described by Paniagua *et al*. (1990)
- Hemagglutination test. The method described by Atkinson and Trust (1980) was followed for the evaluation of the hemagglutination ability of strains
- Serum resistance test. The susceptibility of bacteria to human serum was determined as described previously by Bag *et al*. (2008)

2.6. Antibiotic Susceptibility Testing

Aeromonas spp. strains isolated in the present study were subjected to susceptibility testing against 28 antimicrobials commonly used. Susceptibility was determined by the disk-diffusion technique of Kirby-Bauer on Mueller-Hinton agar plates (Oxoid Basingstoke, UK) with inocula adjusted to an optical density of 0.5 McFarland standard units (CLSI, 2010). Disks containing Ampicillin (AMP10 μg), Carbenicillin (CAR100 μg), Amoxicillin (AML10 μg), Amoxicillin/ Clavulanic acid (AMC30 μg), piperacillin (PRL100 μg), piperacillin/tazobactam (TZP110 μg), Ticarcillin (TIC75 μg), Ticarcillin/clavulanic acid (TIM85 μg), cephalothin (KF30 μg), Cefoxitin (FOX30 μg), Cefotaxime (CTX30 μg), Cefoperazone (CFP30 μg), cef-Tazidime (CAZ30 μg), Ceftriaxone (CRO30 μg), Cefepime (FEP30 μg), Aztreonam (ATM30 μg), Imipenem (IMP10 μg), gentamicin (CN10 μg), Kanamycin (K30 μg), Tobramycin (TOB10 μg), Amikacin (AK30 μg), Netilmicin (NET30 μg), tetracycline (TE30 μg), ciprofloxacin (CIP5 μg), Norfloxacin (NOR10 μg), Erythromycin (E15 μg), Trimethoprim/Sulfamethoxazole (SXT25 μg) and Ch-Loramphenicol (C30 μg) were used. All disks were obtained from Oxoid. After 24 h incubation at 30°C, organisms were classified as Sensitive (S), Intermediately resistant (I) or Resistant (R) on the basis of the size of the zone of bacteria growth inhibition according to the guidelines of the CLSI (2010).

2.7. Essential Oils

Clove, Olive and Peppermint oils used in this study were obtained from Aggarwal's Pharmaceuticals (Delhi, India) and SK Products (Meerut, India). Stock solutions were prepared by emulsifying oils (5%) in distilled water. The oils were sterilized at 10 lb for 30 min before use.

Determination of antimicrobial activity of essential oils and their principal constituents: The antimicrobial activity was investigated using a modified agar well diffusion technique (Wan *et al*., 1998).

Determination of Minimum Inhibitory Concentrations (MIC): The agar dilution method recommended by the National Committee for Clinical Laboratory Standards (Prudent *et al*., 1995) was used with modification described by (Delaquis *et al*., 2002).

2.8. Animals and Treatments

About 50 mice male BALB/c mice, aged 6 to 8 weeks and weighing 25±5g (Biological Supply Center, King Fahd Centre for Medical Researches, College of Medicine, King Abdul-Aziz University, Jeddah, KSA) were used. The animals were disease-free. Animals were divided into five groups, each of which contained randomly selected 10 mice were housed in propylene cages and had free access to an antibiotic-free diet and water ad libitum. The photoperiods were adjusted daily to a cycle of 12 h of light and 12 h of darkness. The environmental temperature and relative humidity was constantly maintained at 21±2°C and 50-70%, respectively.

Groups of 10 mice were fed on a standard laboratory chow diet with with oral supplementation of 1% (w/v) clove oil 0.5 mL (group1), 5% (w/v) olive oil 0.5 mL (group2) or 5% (w/v) Peppermint oil 0.5 mL (group3) for 15 days (Farzaneh *et al*., 2011). Control positive mice (group4) were fed on a standard laboratory chow diet and received daily oral normal saline 0.5 mL for 15 days. A. hydrophlia strain was isolated from clinical source and positive to all virulence tests used for challenge of all animal groups. All animal groups

were injected intramuscularly in the left hindquarter of each test mouse with of *A. hydrophila* bacterial suspension (0.1 mL; 3×lo7 cfu). Control negative mice (group5) were fed on a standard laboratory chow diet and not infected.

2.9. Mortality Rates

Mortality rates calculated as numbers of dead mice all over the experimental period in relation to all inoculated mice in each group.

2.10. Samples Collected From Experimental Animals

Blood samples were collected from all mice each group 1, 2, 3 and 4 weeks post-challenge of *A. hydrophilia*. The serum was separated by centrifugation and stored at -20°C until used for ELISA testing for the final titers of IgG to assess the potential therapeutic and eradicative effect of essential oil against *A. hydrophila*.

The post mortem examination was performed either on the dead mice all over the experiment within 15 min of death or to the sacrificed mice after the end of the experiment. Tissues specimens were collected from all animals (intestine, lung, liver and spleen) and divided into 2 portions, first part subjected to bacteriological examination for isolation and identification of *A. hydrophila* while the second one were fixed in 10% buffered neutral formalin for histopathological studies (Chang and Miller, 2006).

Exraction of cell envelopes. Cell envelopes were extracted from *A. hydrophila* isolated strain as described by Lugtenberg *et al*. (1975).

Indirect ELISA. The indirect ELISA was performed to measure antibody responses in all mouse sera against cell envelopes of *A. hydrophilia* according to the method described by Tissen (1985).

3. RESULTS

3.1. Isolation and Identification of *Aeromonas* Species

Analysis of the data presented in **Table 2** showed that, overall, *Aeromonas* was isolated more frequently from goats (21.7%) than from other animal groups sampled, whilst camels had the lowest rate of isolation (8.3%). Faces from cow and sheep revealed the isolation of *Aeromonas* species 10 and 13.3% respectively. All animals exhibited the diarrheic consistency of stool associated with the diseased animals. *Aeromonas* species was isolated more frequently from well water 38.2% (21 of 55 tested samples) than chlorinated supplies 23.0% (12 of 52 tested samples). *A. hydrophilia* was the most dominant species isolated from different kinds of samples 13.4% (39 out of 292 samples).Whereas *A. sobria* and *A. caviae* were isolated in much lower rates 4.7% (14 out of 292 samples) and 2.1% (6 out of 292 samples) respectively.

3.2. Putative Virulence Properties of the *Aeromonas* Strains

Relative frequencies of the different virulence properties of *Aeromonas* spp. (environmental and clinical strains) are given in **Table 3**.

Table 1. Identification tests applied for *Aeromonas* species

Biochemical tests	*Aeromonas hydrophila*	*Aeromonas caviae*	*Aeromonas sobria*
Indole production	+	+	+
Glucose fermentation	+	+	+
Lactose fermentation	+	-	-
Acid from sucrose	+	-	-
Acid from inositol	+	-	-
Gas production from glucose	+	-	+
Citrate as C source	+	+	+
Arginine hydrolysis	+	+	+

Table 2. of *Aeromonas* species from animal faeces and available water samples

	Cows -20		Sheep -45		Goats -60		Camels -60		Well water -55		Chlorinated tap water		Total (292)	
Aeromonas species	No.	(%)	No	(%)	No.	(%)	No.	(%)	No.	(%)	No.	(%)	No.	(%)
A. *hydrophelia*	2	10	4	8.9	9	15.0	3	5.0	13	23.6	8	15.4	39	13.4
A. sobria	0	0	1	2.2	4	6.7	1	1.7	5	9.1	3	5.8	14	4.8
A. caviae	0	0	1	2.2	0	0.0	1	1.7	3	5.5	1	1.9	6	2.1
Total	2	10	6	13.3	13	21.7	5	8.3	21	38.2	12	23.1	59	20.2

Percentage calculated according to number of samples collected from different sources

Table 3. Virulence phenotypes of environmental and clinical isolates of *Aeromonas* species

	A. *hydrophelia* isolates				*A. sobria* isolates				*A. caviae* isolates				Total	
	Environmental (21)		Clinical (18)		Environmental (8)		Clinical (6)		Environmental (4)		Clinical (2)		(59)	
Virulence test	No.	(%)	No	(%)	No.	(%)	No.	(%)	No.	(%)	No.	(%)	No.	(%)
Protease	14	66.7	16	88.9	3	37.5	4	66.7	1	25	1	50	39	66.1
Gelatinase	0	00.0	9	50.0	0	0.0	4	66.7	0	0	1	50	14	23.7
Haemolysis	3	14.3	7	38.9	2	25.0	2	33.3	1	25	1	50	16	27.1
Cytotoxin production	9	42.9	17	94.4	3	37.5	3	50.0	1	25	1	50	34	57.6
Adherence assay	6	28.6	16	88.9	4	50.0	3	50.0	0	0	1	50	30	50.8
Slim test	6	28.6	16	88.9	4	50.0	2	33.3	0	0	1	50	29	49.2
Serum resistance	9	42.9	17	94.4	3	37.5	4	66.7	0	0	1	50	34	57.6
Crystal violet binding.	6	28.6	12	66.7	2	25.0	4	66.7	0	0	2	100	26	44.1
Hemagglutination test	7	33.3	12	66.7	3	37.5	4	66.7	0	0	1	50	31	52.5

Protease Skimmed milk was hydrolyzed by 88.9% of *A. hydrophila* clinical strains and only by 66.7% of environmental strains. Whereas 66.7% clinical strains and 37.5% environmental strains of *A. sobria* isolates were able to hydrolyze Skimmed milk. Only one *A. caviae* isolate from each environmental and clinical sources was positive for Protease activity in percents of 25.0% and 50.0% respectively.

Gelatinase. Fifty percent of *A. hydrophila*; 66.7% of *A. sobria* and 50.0% *A. caviae* clinical isolates were gelatinase positive, whereas none of the environmental strains were able to degrade gelatine.

Haemolysin. About 38.9; 33.3 and 50.0% of clinical *A. hydrophila*; *A. sobria* and *A. caviae* strains were able to lyse rabbit erythrocytes and produced haemolysis on rabbit blood agar plates; slightly lower percentages of environmental *A. hydrophila*; *A. sobria* and *A. caviae* strains (14.3; 25.0 and 25.0% respectively) showed the same property.

Cytotoxin production Cytopathic effect was found in 42.9% of the environmental and in 94.4% of the clinical *A. hydrophila* isolates. About 37.5% of the environmental and 50% of the clinical *A. sobria* isolates produced a cytopathic effect (**Fig. 1b**). Finally one *A. caviae* isolate from each environmental and clinical source produced a cytotoxic response to HEp-2.

Adhesion test About 28.6% environmental and 88.9% clinical isolates of *A. hydrophila* strains were able to adhere to the epithelial cells, whereas 50.0% of each environmental and clinical *A. sobria* strains were able to adhere to the epithelial cells Only one *A. caviae* clinical isolate was able to adhere to the epithelial cells (**Fig. 1b, c**).

Slime test. The slime production test was positive for 28.6% of environmental and for 88.9% of clinical *A. hydrophila* strains tested, whereas 50.0 and 33.3% of *A. sobria* of environmental and clinical sources respectively were also positive. Only one *A. caviae* strain of clinical strains tested was positive in the slime test.

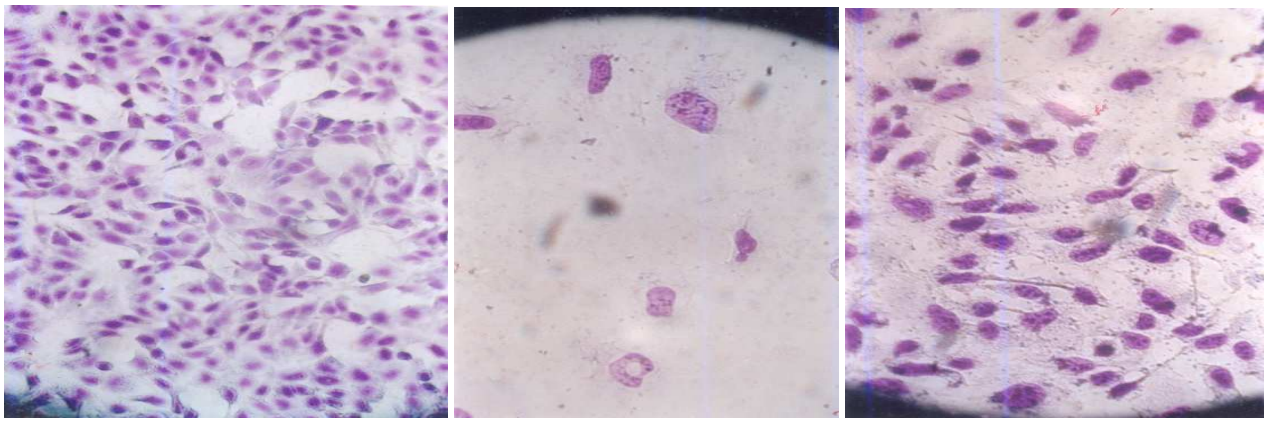
Serum resistance The ability of *A.hydropilia* isolates from environmental and clinical sources to survive in serum was 42.9 and 94.4% respectively while 37.5% environmental and 66.7% clinical *A. sobria* strains were able to survive in serum. Only one clinical *A. caviae* strain was positive.

Crystal violet binding About 28.6% environmental and 66.7% clinical *A.hydropilia* strains were able to bind crystal violet, whereas 25.0% environmental and and 66.7% clinical *A. sobria* strains were able to bind crystal violet. All clinical A.*caviae* strains were positive.

Hemagglutination test Hemagglutination ability of *A.hydropilia* tested on sheep blood cells was exhibited by 33.3% of environmental and 66.7 of the clinical strains. About 37.5% environmental and 66.7% clinical *A. sobria* strains were positive for hemagglutination property. Only one clinical *A. caviae* strain was positive.

3.3. Antibiotic Susceptibility Pattern Against test Organisms

Strains of *Aeromonas* spp. (n = 59) characterized biochemically (39 *A. hydrophila*, 14 *A. sobria* isolates and 6 *A. caviae*) were tested for susceptibility to a panel of 28 antibiotics. The results are presented in **Table 4** (in percen-tage). Our results show the existence of differences in some of the antibiotics tested according to the species and all *Aeromonas* isolates were resistance to amoxicillin, carbenicillin and ampicillin. Of the aminoglycosides antibiotics the most effective was amikacin (81.4%). Moreover, *Aeromonas hydrophila* showed sensitivity values to quinolones as ciprofloxacin and norfloxacin, about 39.0 and 37.3% respectively. High resistance to first and second-generation cephalosporins (cephalothin and cefoxitin, sensitivity 8.7 and 6.8% respectively) has been detected in motile aeromonad isolates. The most potent antibiotics showing 100% activity against *Aeromonas* species isolated in this study were ceftriaxone, ceftazidime, cefotaxime, cefepime, while cefoperazone showed 91.5%. Chloramphenicol showed the highest efficacy against the bacterial strains tested (93.2% sensitive). Tetracycline sensitivity was 22.0% for *Aeromonas* spp.



(a) (b) (c)

Fig. 1. Effects of culture filtrate of isolates of *Aeromonas* species on HEp-2 cells. (a) Confluent growth of HEp-2 cells; (b) cytotoxic effect associated with cell vacuolation (c) Adherence patterns of isolates of *A. hydrophila* on HEp-2 cells (Giemsa stain)

Table 4. Variations in susceptibilities (%) among environmental and clinical isolates of *Aeromonas* species strains to different antibiotics /disc-diffusion method

	A. *hydrophelia* isolates				*A. sobria* isolates				*A. caviae* isolates					
Antibiotics	Environmental (21)		Clinical (18)		Environmental (8)		Clinical (6)		Environmental (4)		Clinical (2)		Total 59	
used	No.	(%)	No.	(%)	No.	(%)	No.	(%)	No.	(%)	No	(%)	No.	(%)
AMP	0	0.0	0	0.0	0	0.0	0	0.0	0	0	0	0	0	0.0
CAR	0	0.0	0	0.0	0	0.0	0	0.0	0	0	0	0	0	0.0
AML	0	0.0	0	0.0	0	0.0	0	0.0	0	0	0	0	0	0.0
AMC	6	28.6	7	38.9	3	37.5	2	33.3	1	25	1	50	20	33.9
TIC	10	47.6	3	16.7	1	12.5	1	16.7	2	50	1	50	18	30.5
TIM	9	42.9	3	16.7	2	25.0	2	33.3	2	50	1	50	19	32.2
PRL	4	19.0	2	11.1	2	25.0	1	16.7	2	50	1	50	12	20.3
TZP	9	42.9	3	16.7	2	25.0	2	33.3	2	50	1	50	19	32.2
KF	2	9.5	1	5.6	0	0.0	1	16.7	1	25	0	0	5	8.7
FOX	1	4.7	2	11.1	0	0.0	1	16.7	0	0	0	0	4	6.8
CRO	21	100.0	18	100.0	8	100.0	6	100.0	4	100	2	100	59	100.0
CAZ	21	100.0	18	100.0	8	100.0	6	100.0	4	100	2	100	59	100.0
CFP	18	85.7	16	88.9	8	100.0	6	100.0	4	100	2	100	54	91.5
CTX	21	100.0	18	100.0	8	100.0	6	100.0	4	100	2	100	59	100.0
FEP	21	100.0	18	100.0	8	100.0	6	100.0	4	100	2	100	59	100.0
ATM	21	100.0	18	100.0	8	100.0	6	100.0	4	100	2	100	59	100.0
IMP	21	100.0	18	100.0	8	100.0	6	100.0	4	100	2	100	59	100.0
CIP	16	76.2	3	16.7	1	12.5	1	16.7	2	50	0	0	23	39.0
NOR	14	66.7	2	11.1	4	50.0	0	0.0	1	25	1	50	22	37.3
TOB	9	42.9	11	61.1	5	62.5	3	50.0	3	75	1	50	32	54.2
AK	17	81.0	14	77.8	6	75.0	6	100.0	3	75	2	100	48	81.4
K	11	52.4	12	60.0	4	50.0	4	66.7	2	50	1	50	24	40.7
CN	13	61.9	12	60.0	5	62.5	4	66.7	2	50	1	50	36	61.0
NET	12	57.1	17	85.0	6	75.0	5	83.3	3	75	1	50	44	74.6
TE	5	23.8	6	33.3	0	0.0	1	16.7	2	50	1	50	13	22.0
C	18	85.7	17	94.4	8	100.0	6	100.0	4	100	2	100	55	93.2
E	2	9.5	1	5.6	0	0.0	1	16.7	0	0	0	0	4	6.8
SXT	10	47.6	9	50.0	6	75.0	3	50.0	3	75	1	50	32	54.2

3.4. Effect of Essential Oils on Isolated *Aeromonas* Species Growth in Vitro

The anti-bacterial activity of three selected essential oils against isolated *Aeromonas* species is summarized in **Table 5**. The results revealed that the selected essential oils showed antibacterial activity with varying magnitudes.

Clove, Olive and Peppermint oil showed a zone of inhibition, ranging from 10.00±0.8 to 14.82±0.41 mm in diameter. They exhibited a wide spectrum of antimicrobial activity against all strains used in this study.

Minimum Inhibitory Concentration (MIC) for selected oils ranged from 12.8 to 25.6 mg mL^{-1} (**Table 6**). This study revealed that Clove oil showed maximum activity with MIC values 12.8 mg mL^{-1} followed by Olive and Peppermint oil with MIC values 25.6 mg mL^{-1} each against all the tested strains.

3.5. Effect of Essential Oils Against *Aeromonas* Hydrophilia in Vivo

Reactivity of cell envelop antigen with serum of tested animals was expressed in O.D. values (at 492 nm) and is shown in **Table 7**. It indicated that the sero-reactivity of infected-essential oil treated mice (groups 1,2 and 3) was higher than that recorded in infected-non treated group (4). These increases in antibody titer increase gradually till reach peak at 3 weeks post infection.

The effect resulting from orally administrated selected essential oils on mice experimentally infected with *A.hydrophilia* is shown in **Table 8**. In control positive group (group 4) not administrated with any essential oil and infected with *A.hydrophilia*, the cummmulative mortality of infected mice was 70%. No mortality was apparent in mice orally administrated with 1% (w/v) clove oil 0.5 mL (group1) or 5% (w/v) olive oil 0.5 mL (group2) for 15 days whereas 10% mortality in mice orally administrated with 5% (w/v) Peppermint oil 0.5 mL (group3).

Reisolation of *A.hydrophilia* from different organs of dead and sacrificed mice all over experimental period gave variable results. In infected-non treated group (group 4) *A.hydrophilia* isolated in a percent of 100%, 40, 70 and 80% from intestine, lungs, liver and spleen, respectively. While, in mice-treated with Clove (group1) or olive oil (group2) and infected, *A.hydrophilia* couldn't be isolated from any internal organ (**Table 6**). In group 3 treated with Peppermint oil and infected, *A.hydrophilia* isolated from intestine only in a percent of 10%.

Postmortem gross examination. The noninfected mice were used as controls. The noninfected control mice had normal organ architecture with no visible lesions in the intestine, lungs, liver and spleen.

In infected non essential oil treated group (group 4), among those mice which died, the most common gross pathological findings at necropsy were severe congestion in all internal organs with necrosis.

In essential oil treated mice (group 1, 2 and 3), significantly lowered the macroscopic damage scores in comparison to untreated animals (group 4).

Histological examination. The noninfected mice The noninfected control mice had normal organ architecture with no microscopic lesions in the intestine, lungs, liver and spleen (**Fig. 2a-d**).

In the infected non essential oil treated group (group 4), abnormal histology was present in internal organs of mice infected with the isolated *A. hydrophilia*. Necrosis of ileal villi accompanied by focally extensive infiltration of lamina propria by large numbers of polymorphonuclear neutrophils extending along the base of intestinal crypts and infiltration of villous lamina propria by large numbers of polymorphonuclear neutrophils with segmental sloughing of intestinal epithelial cells. Representative example of these lesions is shown in **Fig. 3a**.

Table 5. Inhibitory activity of tested essential oils against isolated *Aeromonas* species (Mean ± SD) (mm*)

	Isolates\zone of inhibition		
Oil name	*A. hydrophelia*	*A. sobria*	*A. caviae*
Clove oil	14.82±0.41	13.00±0.81	14.66±0.46
Olive oil	12.11±0.32	11.66±0.46	12.00±0.81
Peppermint oil	11.59±0.45	10.00±0.81	11.33±0.46

Table 6. Minimum Inhibitory Concentration (MIC) of selected essential oils (mg/mL)

	Isolates\ MIC		
Oil name	*A. hydrophelia*	*A. sobria*	*A. caviae*
Clove oil	12.8	12.8	12.8
Olive oil	25.6	25.6	25.6
Peppermint oil	25.6	25.6	25.6

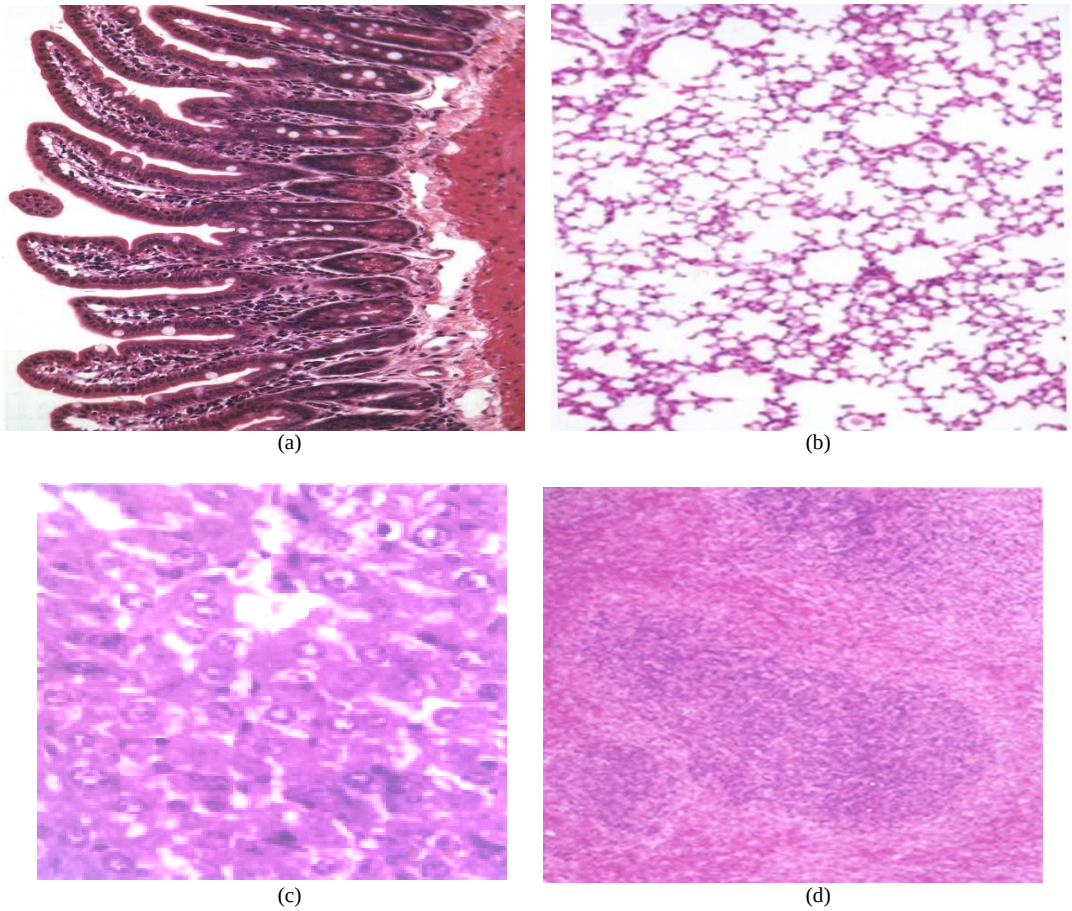
Table 7. Overall mean of ELISA optical density among experimental animal groups (Mean±SD)

	Weeks post infection			
Animals groups	1	2	3	4
I	0.480 ±0.006	0.696 ±0.019	0.821 ±0.009	0.806 ±0.010
2	0.495 ±0.009	0.743 ±0.009	0.811 ±0.018	0.793 ±0.021
3	0.413 ±0.31	0.762 ±0.031	0.791 ±0.021	0.757 ±0.015
4	0.309 ±0.111	0.397±0.087	0.409 ±0.018	0.411 ±0.017
5	0.121 ±0.003	0.099 ±0.007	0.103 ±0.009	0.112 ±0.009

Cut off value ≥2 was taken as positive

Table 8. Re-isolation of *A.hydrophilia* from different organs of dead as well as sacrificed mice during and at the end of the experiment

Animals groups	Intestine	Lungs	Liver	Spleen	Dead/total	Mortality rates (%)
1		0 (0%)	0 (0%)	0 (0%)	0 (0%)	0/100
2	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0/10	0
3	1 (0%)	0 (0%)	0 (0%)	0 (0%)	1/10	10
4	10 (100%)	4 (40%)	7 (70%)	8 (80%)	7/10	70
5	10 (0%)	0 (0%)	0 (0%)	0 (0%)	0/10	0



(a) (b) (c) (d)

Fig. 2. Histopathological analysis in the non infected mice which were used as controls. Tissues are as indicated (**Fig. 2a-d**), intestine; lungs; liver and spleen respectively with H&E staining had normal organ architecture with no microscopic lesions (Magnifications, ×100)

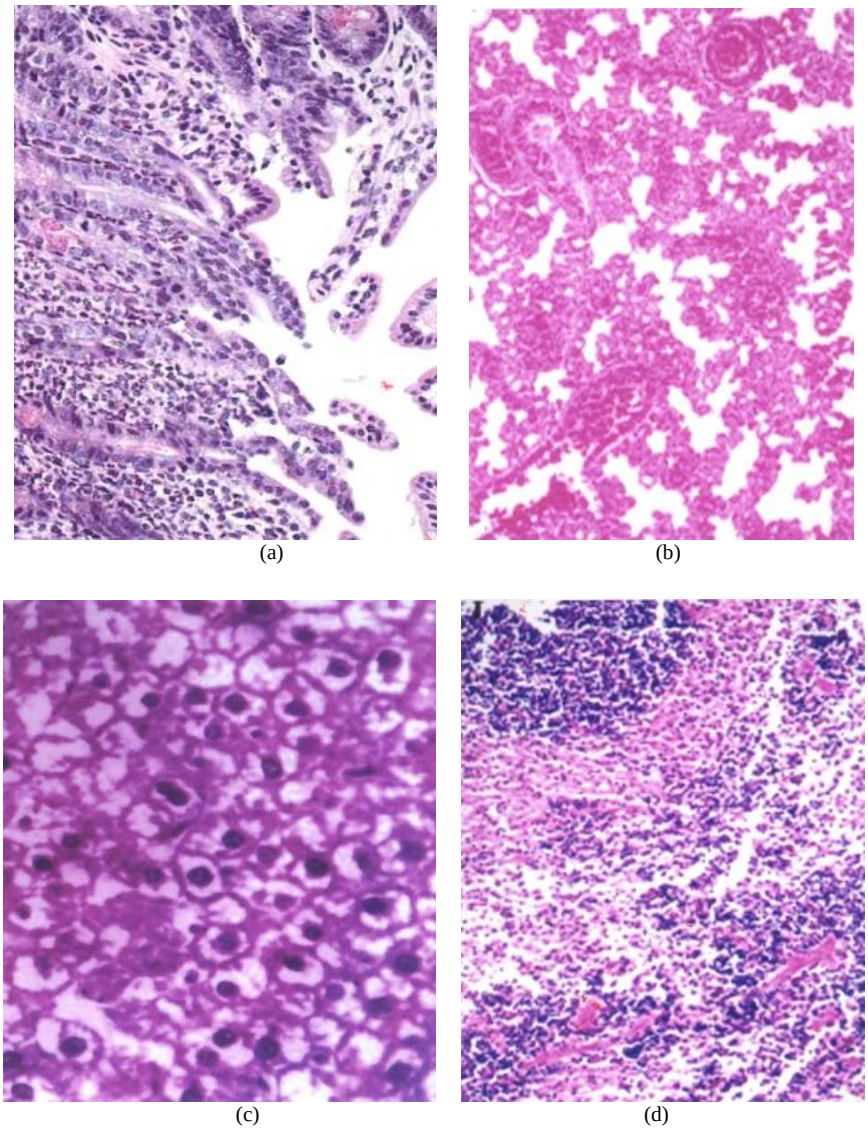


Fig. 3. Histopathological analysis with H and E staining in the infected nontreated mice (a) intestine showed necrosis of ileal villi accompanied by focally extensive infiltration of lamina propria by large numbers of polymorphonuclear neutrophils (b) Lung showed marked vascular congestion and alveolar hemorrhage (c) Liver showed coagulative necrosis of the hepatic parenchyma and vascular degeneration (d) Spleen showed marked necrosis in splenic follicle and apoptotic cells in the red pulp (Magnifications, ×100)

The lung section (**Fig. 3b)** had marked vascular congestion, alveolar hemorrhage and widening of the interstitium. The liver section (**Fig. 3c)** showed prominent coagulative necrosis of the hepatic parenchyma and vascular degeneration. Several inflammatory cells were seen in the sinusoids. In the spleen section of nontreated mice (**Fig. 3d**), the splenic follicle exhibited necrosis and apoptotic cells in the red pulp in proximity to the lymphoid follicle. Lymphocytic depletion was seen in the white pulp.

In the essential oil treated groups (group1,2 and 3), intestinal segments from mice challenged with *A.hydrophilia* showed highly preserved histological architecture of the lamina propria and submucosa

throughout the small intestine (**Fig. 4a**). There were minimal damages to the epithelial layer and the heights of the villi were preserved with mild infiltration of inflammatory cells inside the villi. The lung (**Fig. 4b)** section showed resolution of stages of pneumonia had focal thickening of alveolar septa, lymphohistiocytic infiltrates and neutrophils with scanty edematous in some alveoli. There was no evidence of alveolar hemorrhage or vascular congestion. In the liver (**Fig. 4c**) section, occasional lobular and perivascular lymphohistiocytic infiltrates. Focal areas of necrosis; congested and dilated sinusoids were also observed.

The increased macrophages in the red pulp and marked lymphoid activation in the splenic follicles, with germinal center formation, were present in the spleen section (**Fig. 4d**). Tissues from *A. hydrophila* infected and essential oils treated mice were relatively normal. Overall; architectural analysis revealed to us that mice infected with *A. hydrophila* without treatment with essential oils exhibited more severe pathologies.

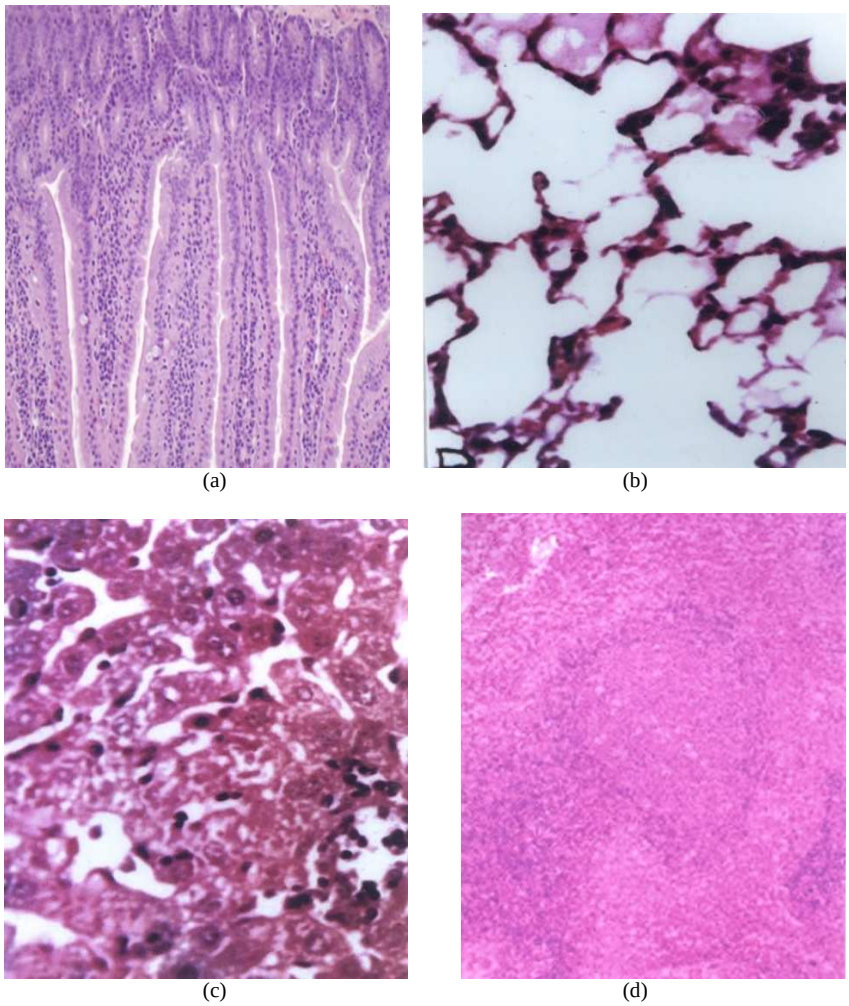


Fig. 4. Histopathological analysis with H&E staining in the essential oil treated mice (a) intestine showed minimal damages to the epithelial layer and the heights of the villi were preserved (b) Lung showed resolution of stages of pneumonia, lymphohistiocytic infiltrates and neutrophils with scanty edematous in some alveoli (c) Liver showed occasional lobular and perivascular lymphohistiocytic infiltrates (d) Spleen showed marked lymphoid activation in the splenic follicles, with germinal center formation, were present (Magnifications, ×100)

4. DISCUSSION

Aeromonas spp. are widely distributed in the environment, particularly in fresh and brackish water, sewage, marine water and drinking water. There are also various reports of the presence of these bacteria in food (Sechi *et al*., 2002). Most research on *Aeromonas* has focused on their potential to cause gastro-enteritis both in adults and infants, they are also recognized as causes of infections in immune-compromised hosts which may progress to septicemia (Kuhn *et al*., 1997). A high ability of *Aeromonas* species to live under a wide variety of environmental conditions in natural waters has been observed by Paniagua *et al*. (1990). The microorganism has been isolated over wide ranges of salinity, conductivity, temperature, pH and turbidity. In this study, *Aeromonas* strains were identified at the phenotypic level by integrating a conventional biochemical scheme with new biochemical key tests able to avoid a misidentification of each species with the nearest neighbours. The finding of 59 strains with phenotypes sharing characters belonging to different species, *A. hydrophila* and *A. caviae* and *A. sobria* isolates. The high incidence of *Aeromonas* species in farm animals may therefore, only reflect constant exposure to water containing the organism (Sechi *et al*., 2002). This would appear to be substantiated by the significant overall correlation found between its presence in animal faeces and the available drinking water. The cow, sheep, goats and camels groups studied were by nature 'free' roaming animals with ready access to untreated water with a high likelihood of contamination with *Aeromonas* species The highest incidence of isolation was from well water samples 38.2% (21 out of 55.samples) and chlorinated tap water samples 23.1% (12 out of 52 samples) which agreed with Araujo *et al*. (1991). The data reported in this study indicates that the distributions of virulence factors, that regulate the pathogenicity of Aeromonads, are different in clinical and enviromental samples. The haemolysin and protease production was found more frequently in the clinical samples whereas gelatinase, which may be important in colonization through the disruption of the intestinal barrier, was found exclusively in the clinical samples (Sechi *et al*., 2002). For many bacterial species the ability to adhere to eukaryotic cells is the first step in the colonization and development of disease. The cytopathic effect induced on the HEp-2 cells infected by *Aeromonas* spp. was more evident when the cells were infected with strains which produced toxins, such as haemolysin and protease. The study also found a greater prevalence of haemolysin, protease and gelatinase production, as well as higher adhesion capacity, among strains isolated from clinical samples. These findings are in agreement with those reported by Sechi *et al*. (2002). Overall, in this study, adhesive and cytotoxic properties were more present in clinical than in environmental strains. Similar results were reported by Carrello *et al*. (1988) who found that clinical strains of *A. hydrophila* were more adhesive on Hep-2 cells than environmental strains, while the opposite was found by Sechi *et al*. (2002) for isolates of A. veronii biovar *sobria*. In the present study, about (88.9%) of *A. Hydrophila* clinical isolates were positive for Congo red binding test (**Table 6**). Paniagua *et al*. (1990) reported that all motile *Aeromonas* take up Congo red dye. In our study, the majority of the clinical isolates of *A. hydrophila* (94.4%) showed serum resistance properties. Additionally, the bactericidal activity of antibodies and 'complement-like' bactericidal activity is operative in the intestinal mucosa, contributing to the colonization properties of a variety of bacterial pathogens. Hence, the serum resistance properties of *A. hydrophila* could also play an essential role in intestinal colonization. It has been suggested that the ability of some *A. hydrophila* strains to resist complement mediated killing could result in bacteraemia and other invasive diseases associated with *Aeromonas* infection (Merino *et al*., 1996).

Binding of crystal violet to virulent strains allows the rapid and simple differentiation of virulent and avirulent strains of *Aeromonas* species (Mahmoud and Tanios, 2008). Paniagua *et al*. (1990) found that all the CV+ strains examined harboured a plasmid and demonstrated other virulence-associated attributes that have been reported to be plasmid determined. In the present study, 66.7% *A. hydrophila* clinical isolates were positive for crystal violet binding activity (**Table 6**). The obtained results agree with Paniagua *et al*. (1990).

Many bacteria produce surface antigens which enable adherence to epithelial cells *in vivo* and may promote agglutination of different species of erythrocytes (RBCs) in vitro. Such adherence factors are in many cases encoded by plasmids (Burke *et al*., 1986). Haemagglutination of different animal erythrocyte systems have been used to type strains of mesophilic aeromonads. There is evidence that haemagglutination patterns vary under different test conditions Burke *et al*. (1986). In the present study non agglutinating isolates were more likely to be *A. caviae* than *A. hydrophila* or *A. sobria* and this confirms previous findings Burke *et al*. (1986).

In conclusion, these findings indicate that aeromonads have the potential to cause human illness and confirm the role of water as vehicles for *Aeromonas* diseases. This study also demonstrated that the multi-factorial nature of the diseases and the influence of environmental conditions in the expression of the putative virulence properties

Factors of virulence represented by extracellular proteases, hemolysins and cytotoxins were produced by nearly all virulent and avirulent strains of *A. hydrophila* to the same extent. This fact and the absence of correlation between the corresponding activities and the degree of virulence may indicate that qualitative factors rather than quantitative ones are involved in the pathologic process.

Antibiotics are generally administered to animals and human prophylactically and therapeutically against microbial diseases and subtherapeutically as growth promoters .Potential consequences of antibiotic use in culture and animal feeds are development of drug resistant bacteria, transfer of resistant characteristics to bacteria and reduced efficacy of antibiotic treatment for human and animal diseases.

An increasing incidence of multidrug resistance among *Aeromonas* spp. isolates, which are emerging opportunistic human pathogens, has been observed worldwide. This can be attributed to the horizontal transfer of mobile genetic elements like plasmids and class 1 integrons (Jacobs and Hafizah, 2006). *Aeromonas* spp. are known to be intrinsically susceptible to all antibiotics active against non-fastidious Gram-negative bacilli, except for many β-lactams, due to the production of multiple inducible, chromosomally encoded β-lactamases (Jones and Wilcox, 1995). In this study all strains were resistant to amoxicillin, carbenicillin and ampicillin.

Of the aminoglycosides antibiotics the most effective was amikacin (81.4%) which is similar to the findings of Jones and Wilcox (1995) who reported that *Aeromonas* spp. usually retain their aminoglycoside susceptibility.

The most potent antibiotics showing 100% activity against *Aeromonas* species isolated in this study were ceftriaxone, ceftazidime, cefotaxime, cefepime, while cefoperazone showed 91.5%. Commonly, these antibiotics used as first therapeutic options for *Aeromonas* infections in humans (Alcaide *et al*., 2010).

Chloramphenicol showed the highest efficacy against the bacterial strains tested (93.2% sensitive). Similarly, all of the investigated strains were susceptible to chloramphenicol, Jones and Wilcox (1995) reported that Chromamphenicol resistance is an extremely rare trait in *Aeromonas* spp. Tetracycline sensitivity was 22.0% for *Aeromonas* spp. Ko *et al*. (1996) found as many as 49% tetracycline-resistant *Aeromonas* spp., The increasing resistance to antibiotic represents the main factor justifying the need to find and/or develop new antimicrobial agents. Plant essential oils and extracts have been used for many thousands of years, in food preservation, pharmaceuticals, alternative medicine and natural therapies (Maddox *et al*., 2010). It is necessary to investigate those plants scientifically which have been used in traditional medicine to improve the quality of healthcare.

A method frequently used to screen plant extracts for antimicrobial activity is the agar well diffusion technique. The zones of inhibition formed by the essential oils (varied from10.00±0.81 to 14.82±0.41) are reported In **Table 5**. Almost all selected essential oils were found to be effective against the tested Aeromoas species (Wan *et al*., 1998). The results of this study revealed that, the essential oils Clove; Olive and Peppermint oil had inhibitory effect on isolated Aeromoas species. Clove had MIC 12.8 mg mL^{-1}, whereas Olive and Peppermint oil had MIC 25.6 mg mL^{-1} each (**Table 6**). Many authors ruled out the use of antibiotics and favored the use of essential oils as Clove; Olive and Peppermint oil that exhibited the antimicrobial activity against wide range of gram positive and gram negative bacteria in very low concentrations without mutageicity (Wan *et al*., 1998; Maddox *et al*., 2010). Essential oils have been tested for in vitro and *in vivo* antimicrobial activity and some have demonstrated to be possessing potential antimicrobial potential. Their mechanism of action appears to be predominantly on the cell membrane by disrupting its structure thereby causing cell leakage and cell death, secondary actions may be by blocking cell membrane synthesis and inhibition of cellular respiration. They readily penetrate into cell membrane and exert their biological effect because of high volatility and lipophilicity of essential oils (Inouye, 2003). A growing interest in using herbs and other material therapies in animal production has been made just a complementary medicine. These results showed that daily administration of essential oil enhance antibody levels in all treated groups (1 and 2 and 3). Similar results were reported by Subeena and Navaraj (2012) who Showed that these essential oils had some immmuostimlatory effects on these immunological factors such as antibody titters, total white blood cells and serum bactericidal activity in some test groups. Treatment with essential oil Clove or Olive for 15 days

led to significant clearance of *A. hydrophilia* from the all of animals exhibiting negative *A. hydrophilia* culture compared with Group 4 (the infected untreated); however, *A. hydrophilia* was detected in intestine of one remaining infected mice treated with Peppermint oil (Group 3) by microbiological results. The treated animals had minimal histopathological changes and lower bacterial loads in the organs examined. These results suggested that the essential oils have potential value as an additional or supporting treatment in gastrointestinal inflammations. This correlated well with some biological effects of essential oil such as its antiseptic properties against a range of microbial agents and, recently, its anti-inflammatory potential, both in vitro and *in vivo* Takarada *et al*. (2004). These results encourage the use of essential oils as substitute for antibiotics and can be added to the animal feeds in very low concentrations without any harmful effect.

5. CONCLUSION

Aeromonas species was isolated more frequently from goats (21.7%) than from other animal groups sampled and isolated more frequently from well water (38.2%) than chlorinated supplies (23.0%). The most potent antibiotics against *Aeromonas* species isolated in this study were ceftriaxone, ceftazidime, cefotaxime, cefepime. Clove, Olive and Peppermint oil exhibited a wide spectrum of antimicrobial activity.

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