

In vitro Study of the Effect of *Melissa Officinalis* Aqueous Lemon Balm Extract on *Aeromonas Hydrophila* Causative Hemorrhagic Septicemia Disease in *Oncorhynchus Mykiss*

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ABSTRACT

Nowadays by becoming cognizant of the effects of antimicrobial herbs is increasing according to the use of these plants in the world. Mentioned treatment method cause to reduce the side effects and prevention of bacterial resistance to antibiotics. In this regard, the main focus of the paper is centered on *Melissa officinalis* aqueous Lemon balm extract antibacterial effect of lamiaceae family on *Aeromonas hydrophila* factor in vitro condition and it cause hemorrhagic septicemia disease in *Oncorhynchus mykiss*. *A. hydrophila* of rainbow trout with septicemia symptoms were studied after isolation and culturing in different environments in order to definitive diagnosis by using biochemical and molecular (PCR) tests. In order to determine extract antibacterial power; we have used broth micro-dilution (liquid dilution), minimum bactericide concentration (MBC) and minimum inhibition concentration (MIC) standard methods. Then, MIC concentration was used in MULLER Hinton agar by creating wells and it was compared zone diameter of bacterial growth inhibition. Results showed that *M. Officinalis* aqueous lemon balm extract with MIC amount is equal to 2.5 mg/ml and MBC amount is equal to 5 mg/ml. Results demonstrated the positive effect of the extract on bacteria and in order to use it as a medicine, treatment and prevention of fish disease, accomplishment of further clinical research is required.

Key words: *Aeromonas hydrophila*, minimum bacterial concentration (MBC), Inhibitory concentration- bacteria- *Melissa officinalis*.

INTRODUCTION

Herbs are as strong reservoirs of secondary metabolites and indeed they are effective resources of many pharmaceutical materials which contain active ingredient in one or some of their organs. It is less than 1 percent of dry weight of plant and it has organisms. It isn't known exactly when people commenced to use herbs as medicine as well. Of course, information about medicinal and pharmaceutical properties of plants were available since ancient time at last they were available till the contemporary generations. To date this effort continues and now it is progressing in very fast pace. (Burt, 2004; Tajkarimet.al, 2010).

According to the number of chemical compounds in plant extracts, it is impossible to a consider single mechanism for their antibacterial effects; but they will have multiple targets in cells. These Mechanisms aren't acted separately, but some of them are affected by others. (Burt, 2004). One of the most important property of essential oils and their components is hydrophobic property which leads to penetration of the materials to bacterial cell membrane lipids and mitochondrial which is the main reason that causes of creating disorder in themselves structures and further permeability. This led to exit and leakage of ions and other cell contents. Although the withdrawal of limited quantities of this material is tolerable for bacteria;

but it has an impact on viability and withdrawal of bulky amounts of cell contents or withdrawal of vital ions molecules cause cell death. (Burt, 2004). Generally, when phenolic compounds level is higher in essence; their antibacterial properties against food pathogens will be more. These compounds include carvacrol, eugenol and thymole. Probably, the impact mechanism of these compounds as the other phenolic compounds are as follows: disorder in cytoplasmic membrane, disruption the proton motive force and electric current, coagulation of cell contents. (Burt, 2004; Tajkarimet.al, 2010). Also the chemical structure of an essential oil has impact on its mechanism. The importance of hydroxyl groups presence in the phenolic compounds such as carvacrol and thymole was approved. The relative position of the phenolic hydroxyl group in phenolic ring doesn't have much impact in the antibacterial effect. (Burt, 2004). *Melissa officinalis* is one of the oldest and most vogue herbs. It belongs to *Lamiaceae* family and it is also called lemon balm. Numerous studies have been done on this plant and its various properties were proved. The most common therapeutic properties of lemon balm are its (*Melissa officinalis*) Sedative property, antioxidant, antispasmodic, carminative; antibacterial, anti viral and anti-inflammatory cases. (Koch. Heitzman et.al, 1984; Lamaison et.al , 1991). Moreover, The effect of this herb studied on known on the nervous system revealed that *Melissa officinalis* reduces symptoms of neurological disorders. Such as stress, anxiety and irritability (Bisset et. Al 2001). This herb protects nervous cells and eradicates free radicals. (Lopez et al 2009) and because of increasing of increasing valerinan quality effect on sleep. (Lopez, et.l 2009). Aqueous and methanol extracts of mentioned herb are mono -amine- oxidase enzyme (MAO) inhibitors and in this case, the effect of methanol is stronger , so it is deduced that the mentioned herb has antidepressant effects. (Lopez et al, 2009). *Aeromonas hydrophila* is a factor that cause bacterial septicemia disease. The bacteria usually exists in the fishes digestive system and it is considered as an opportunistic pathogen. Outbreaks of disease is related to changes in environmental conditions such as stress, congestion , sudden temperature changes, fish transportation , poor water quality and high level of nitrite and carbon dioxide. In turkey *Aeromonas*

hydrophila infection has been reported for eel and crocodiles and grass carp. (Nielsen et al 2001, Janda et al , 1996, Agniswar et al, 2012). This bacteria is in gram-negative , motile and opportunistic forms which can cause to bacterial hemorrhagic septicemia (BHS) in fishes . (Chu et al, 2005). The release of protease, hemolysin and cytolytic toxins are linked to the pathogenesis of these bacteria. (Hu. et al, 2012). *Aeromonas* are mainly found in fresh waters and they are compatible with different environmental conditions such as temperature, salinity and turbidity. (Hazen, et al, 1987). According to results, to fill the knowledge gap related to the effect of Aqueous extract of herb on fish bacterial disease, the main aim of research is to investigate the lemon balm aqueous extract anti- bacterial effect of lemon balm on pathogenic *Aeromonas hydrophila* bacteria in rainbow trouts which is conducted in vitro condition for *Oncorhynchus mykiss*.

MATERIALS AND METHODS

Bacteriological examination

For investigation of the patient fishes, the common diagnostic methods were used and the separated bacteria were identified using biochemical and molecular methods. In autopsy, most of the findings for naked-eye , were ascites and visceral congestion. In bacterial culture for whole of patient fishes, we seen severe contamination with *Aeromonas hydrophila* bacteria. So, bacterial species was confirmed with PCR method and it was investigated by biochemical methods. To study the anti- bacterial effects, a new 24 culture was prepared. Before 24 hours of examination ; reserve stock culture medium was inoculated to nutrient agar culture; and incubated at 37°C for 24 hours. Then, the colonies on the surface level of the culture environment were washed with normal saline solution and bacterial suspension was diluted with 5 ml of normal saline and uniformity suspension of the bacteria was obtained. Tubes were placed in incubator for 30 minutes at 37 in order to create turbidity similar to half Mac Farland standard tubes. So that, in this case, the concentration of bacteria was about 1×10^3 cfu/ml. (Soltaninejad et al, 2010). After preparation of suspensions and using of swap sterile and dipping it into the bacterial suspension,

all levels of Mueller Hilton agar culture in parallel lines and vertical, horizontal and diagonal directions and the entire surface of the plate was covered with a uniform microbial layer. Sterile blank discs cultured via dispenser and they were placed at the proper intervals with 3 iterations. 10 ml of pure extract was poured on sterile discs; then we returned plate and incubated bacterial cultures at 37 °C for 24 hours in aerobic conditions. After the elapse of the necessary time, we put plates on a dark surface which doesn't reflect light. In the presence of light, results of calipers diameter were measured by using Koulis and also they were recorded in millimeter scale. (Khosravani *et al.*, 2004) In order to determine anti-bacterial activity; erythromycin and doxycycline antibiotics have been used on bacteria culture medium and the rest of process was similar to the extract. In order to conduct quantity experiments and determine minimum inhibition concentration (MIC) and MBC in lemon balm extract (7.5 mg/ml concentration) was prepared in Mueller Hilton broth medium. So, for each of concentration per ml of liquid medium 1 CFU/ml active bacteria was added. Furthermore, we have used positive control (culture medium including erythromycin, doxycycline and bacteria without extract) and negative control (culture medium without bacteria). At last the tubes were incubated for 24 hours at 37 °C and then the result was read. In the last case, we didn't observe any turbidity for dilute form (inhibition) and it was considered as MIC. All of tubes without turbidity were cultured on Mueller Hinton agar. After 24 hours of incubation at 37 °C, the last extract concentration was considered as MBC which it was able to annihilate of 99 percent initial live bacteria (Khosrovani *et al.*, 2004). For data analysis, analysis of variance (ANOVA) was used by considering treatment effect as a fixed effect. The test method was completely random with 3 repeats for each treatment. Average compare in this research included difference between inhibition diameter for various concentrations of the extract which they were calculated with Tukey method. Also, mean difference of inhibition diameter for various concentration of the extract were done by using t-test. (Motlagh, 2014). For bacterial study, 30 samples were collected from rainbow trout's liver, spleen, blood, kidney and heart in sterile condition with mentioned signs and they were

cultured in nutrient agar. Prepared culture mediums were kept at 25 °C inside incubator. To identify the bacterial colonies grown and they were harvested in culture medium and also they were stained. Another biochemical diagnostic experiments such as oxidase, catalase, motile experiment and sugar consumption were done which showed in the table 1.

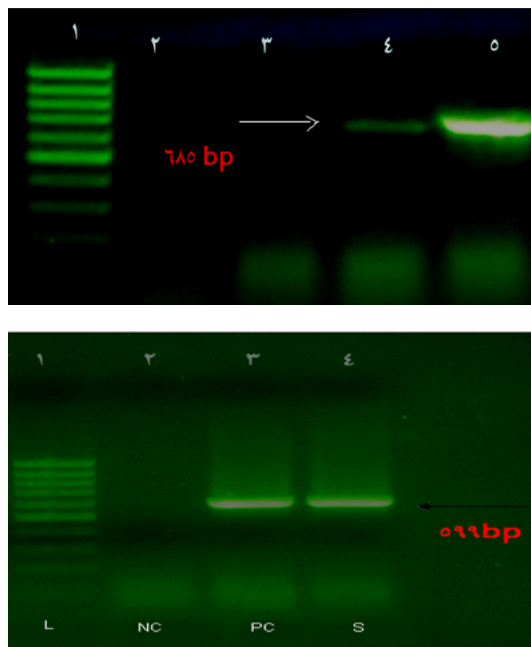
Molecular diagnostic test (PCR) was used for recognition of pathogenic factor in bacteria species. For separation and extraction of bacteria DNA, we have used boil and centrifuge method. Molecular diagnostic operation was done by using of *Aeromonas* sp. 16S rRNA gene primer. All of PCR stages were accomplished in 0.2 ml sterile microtubes without DNase in EPENDORF thermocycler device. In this case, in every microtube of 12.5 microliters master mix include Tag, Dntp, MgCl₂, enzymes; 8.5 microliters DEPC water, 1 microliters of each primer and 2 microliters of extracted DNA and in order to reach final volume; 25 microliters have been used. At last 8 microliters of PCR was driven in agar gel at 100 volt for 1 hour which include 1.5 percent safe salt in bands in document gel device. (Nilsen *et al.*, 2001). For DNA electrophoresis, we used gene ladder with 100bp weight. If the band is visible with 599 bp molecular weight, it is *Aeromonas* species and the band with 685bp molecular weight is *Hydrophila* species.

RESULTS

Bacterial culture result after 24 hours inside incubator at 25 °C showed the existence of many small and yellow colonies with +4 intensity. Bacteria are negative -gram in painting stage and according to table 1 biochemical test results were consistent with *Aeromonas*. *Hydrophila* features and molecular method proved this finding (figures 1 and 2). The result of research were 14mm for diameter without corona creation which it showed meaningful difference in comparison of another groups. ($p < 0.05$). The most and lowest effects showed by doxycycline and erythromycin respectively. Comparison of MIC and MBC showed that lemon balm extract with 2.5 and 5 mg/ml had more impact than the other groups after doxycycline.

Table 1: *A. hydrophila* biochemical diagnostic test for investigated samples

<i>A. hydrophila</i>	Biochemical diagnostic test
Gram negative <i>bacillus</i>	Stain
+	Motility
-	Endol
-	Citrate
-	H ₂ S
-	Lactose
-	Urea
+	Catalase
-	Vp(Voges-Proskauer)
+	Oxidase
-	Lysine decarboxylase
+	Glucose
-	Arabinose
-	Saccharose
-	Manitol
+	β hemolysis
+	Growth in Blood Agar
+	Growth in MacConkey Agar

**Fig. 1: PCR molecular diagnostic test****Table 2: Comparison of development of inhibition zone diameter (mm) in lemon balm extract and control groups**

Disk diffusion lemon balm mm	herb extract erythromycine mm	positive control doxycycline mm	Negative control Sterile distilled	watermm
Aeromonas	14	10	16	0
Hydrophila				

Table 3: Comparison lemon balm antibacterial effect and controlling groups against *A. hydrophila*

Test group	herb extract positive control				Negative control			
	lemon balm		erythromycine doxycycline		Sterile distilled water			
Aeromonas	MIC mg/ml	MBC mg/ml	MIC mg/ml	MBC mg/ml	MIC mg/ml	MBC mg/ml	MIC mg/m	MBC mg/ml
Hydrophila	2.5	5	5	10	1.25	2.5	-	-

DISCUSSION

In 2004, Mimica duki *et al.* investigated antioxidant and bacterial activity of lemon balm essential oil against 13 bacterias and 6 mold species. Suitable effective results of this essence

were supposed by dosage. Most antimicrobial and antifungal activity was observed against *T. shigella* and *Trichophyton* fungal species. In 2005, Depasqua and *et al.* showed that mentioned essential is effective against lacto coccus, anthrococcus, pseudomonase and staphylococcus aureus at

concentrations which they are more than one percent and also antibacterial effect relate to dosage. *Melissa officinalis* (lemon balm extract) investigated on streptococcus sanguis salmonella enteritidis, streptococcus mutans, staphylococcus aureus, vibrio anguillarum, aeromonas hydrophila, yersinia ruckeri, pseudomonas aeruginosa, escherichia coli, corynebacterium parvum, listeria spp, lacto coccus garvieae and flavobacterium psychrophilum. Also interaction of this extract studied on anaerobic bacteria and periodontal aerobic flavobacterium such as ; porphyromonas, veillonella parvul, capnocytophaga gingivalis, shigella sonnei, s.enterica, actinomyces

odontolyticus, peptostrep tococcus micros, eilenella corrodens. (mimica- Dukic et al, 2003, frideman et al , 2004; Di Pasqua et al ; 2005). Many researchers studied herb extracts on aeromonas hydrophila and this research is the first case for determining lemon balm antibacterial effect and it's aqueous extract on aeromonas hydrophila causative hemorrhagic septicemia disease in oncorhynchus mykiss which showed its suitable effect on bacteria controlling in comparison with another antibiotics. We need to do more researches for investigation of pharmaceutical and clinical effects of extract against aeromonas hydrophila.

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