Survey on some ectoparasitic infestation in *Sparus aurata* in mariculture and trials to treatment with an herbal drug

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Abstract

In the present study, seventy sea bream (*Sparus aurata*) were collected from private fish farms in Damietta governorate between October 2016 to Septmber 2017. The clinical manifestations of examined fish were observed and recorded. Parasitic examination of fish revealed infection with two protozoa genera including*: Trichodina*, and *Amyloodinum* with total prevalence rates 34.3%and 17.1%, respectively. Infestation with monogenetic trematode from the genus *Furnestinia* with prevalence rate of 35.7%, as well as one genus of crustacean: *Caligus* with prevalence rate of 21.4% has also been demonstrated. Efficacy of an essential oils product (Vaccium 210) was tested in vitro against *Trichodina* and *Furnestinia.* Also in vivo efficacy was investigated by bathing of naturally infested *Sparus aurata* suffered from mixed infestation of a *Trichodina* and *Furnestinia* at variable Vaccium 210 concentrations and exposure times. Complete eradication of both parasites from all infested *Sparus aurata* was achieved by 10 ppm / 1 hour. Vaccium 210 was not toxic to *Sparus aurata* fish and hat a lethal concentration 50 (LC_{50}) 93.6ppt / 96hr.

Keywords: *Sparus aurata*, Vaccium210, treatment, in vitro, in vivo,*Furnestinia* , *Trichodina*.

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1. Introduction

Fisheries and aquaculture are highly important sources of nutrition, income and livelihoods for millions of people all over the world (FAO, 2016). In Egypt, aquaculture constitute a realistic solution for reducing the gap between fish production and consumption (Soliman and Yacout, 2016). Marine aquaculture represents about 14.5 percent of the total aquaculture in Egypt. About eighty one percent of Egyptian marine aquaculture is located in Damietta Governorate, the other governorates like Port Said, Suez, and Alexandria share the remaining production (Wally, 2016). In the Mediterranean region, Gilthead Sea bream (*Sparus aurata L.)* is considered as one of the most important commercial fish species that has been firstly intensively cultured (Prestinicola et al., 2013). Intensive aquaculture systems are usually associated with the use of artificial diets to feed the high stock density, as well as application of large amounts of antibiotics, pesticides and disinfectants to control mortality and economic losses at outbreaks (Valladão et al., 2015). Of the most important problems confronting intensive aquaculture systems, parasitic infections possess significant impact on fish and shellfish aquaculture production are parasitic infections that result in high economic impact due to high losses in stock as well as the high costs of treatment, prevention and control measurements (Shinn et al., 2015). Trichodiniasis is one of highly significant ectoparasitic disease affecting aquaculture manifested in growth retardation and immune interference leading to inefficient vaccination, as well as chronic mortalities (Ihwan et al., 2016). Also, Monogenean infestation in marine aquaculture is of high economic significance due to severe stock losses as well as treatment costs, in which chemicals with harmful side effects on both fish and human health have been used (Zoral et al., 2017). Nowadays, an increasing attention is forwarded to the utilization of plant products as an alternative for chemotherapy in control and treatment of diseases in different animal production sectors including fish aquaculture. Beside their effects as immunstimulant against parasitic infestations and microbial infections, plant products revealed additional positive effects like stimulating appetite and improving weight gain (Reverter et al., 2014). Of these medicinal plants are *Origanum vulgare* (oregano) and *Thymus vulgaris* (thyme) which are shrubs distributed in Mediterranean and Asia (Santoro et al., 2007). *Origanum* exhibited different degrees of protection against myxosporean infections in gilthead and sharpsnout sea bream under experimental circumstances (Athanassopoulou et al., 2004a, Athanassopoulou et al., 2004b). Furthermore, the extracts of *Capsicum frutescens* have potential effect in the control and treatment of Ichthyophthiriasis in fish aquaculture beside its role as anti-bacterial and antiviral agent (Cichewicz and Thorpe, 1996, Ling et al., 2012). The present study was

carried out to investigate the prevalence and seasonal variation of external parasitic infestations in *Sparus aurata*. The efficiency of herbal extract new commercial product (Vaccium 210) as anti-parasitic treatment against *Trichodina* and *Furnestinia* infesting *Sparus aurata* was also evaluated. The dose that could completely eradicate the parasites, as well as safety margin of the drug for fish, has been also calculated.

2. Materials and methods

2.1. Fish:

2. 1 .1. Fish for parasitic survey:

A total number of 70 sea bream *(Sparus aurata*) (*S. aurata*) with an average body weight 200± 20 g and an average length 18± 2 cm , were collected alive from private fish farms in Diba triangle in Port Said -Damietta way. Involved farms were sampled over different year seasons. The collected fish were transported in large tanks filled with water of the same sources and supplied with battery air pumps and were directly examined. Investigation of collected fish samples was carried out in Fish Diseases Department Lab., at Animal Health Research Institute, Dokki, Giza, Egypt. The clinical signs and postmortem changes were recorded.

2. 1 .2. Fish for LC_{50} test:

A total number of 50 live and apparently healthy *S. aurata* were collected from private fish farm (mean body weight 60±5 g).

2. 1. 3. Fish for in vivo treatment:

A total number of 50 *S. aurata* from a naturally infested fish with *Trichodina sp*. protozoa and monogenetic trematode *Furnestinia* were caught. Collected fish had an average body weight of 150 ±20 g and an average length of 14 ±2 cm. They were collected from a private fish farm in Diba triangle in Damietta Governorate. Fish were acclimatized to laboratory conditions at a water temperature 25 ±1 C º for one week before starting the experimental infection.

2. 2. Clinical and post-mortem examination:

External examination of fish samples were performed and the clinical abnormalities and post-mortem changes were recorded according to Noga (2010).

2. 3. Parasitological examination and identification:

The *S. aurata* were examined externally with naked eye and with aid of magnifying glass for detection of parasitism according to Noga (1996). Mucous smears were immediately prepared

from the skin and fins with the aid of microscopic slides and covers. Then they were examined under stereo microscope at X 10, 40 and 100 magnification for investigation of external parasites. Fish were euthanized and gills were carefully removed and placed in separate petri dish containing sterilized marine water to remove any excess gill mucus, Afterwards they were examined for parasitic infection under stereo microscope. Detected parasites were collected, examined, fixed and stained and identified according to Lucky (1977) ;Paperna and Laurencin (1979) ;Lom (1995) and Paperna (1996).

2. 4. Vaccium210:

Vaccium 210 is a commercial Preparation of herbal extracts origanum oil (Carvacrol and Thymol) and Capsicum oil, produced by Slant Kim Company, Germany.

2. 5. Experiment 1: In vitro effect of Vaccium210 on *Trichodina sp.:*

The experiment was applied according to the method described by Ling et al. (2012) and Fridman et al. (2014). Infested Fish with *Trichodina sp.* were anaesthetised in 0.025% clove oil (Kildea et al., 2004). Mucus scraps from body surface were collected in small petri dish where it was examined under stereo microscope to confirm parasitic existence and parasite livability. Afterwards, 20 ±1 parasites were transferred in each well of 96 well plate containing 0.5 mL filtered marine water from same water source filtered through a filter with a pore size of <20 micrometers (µm) prior to use in a test (EPA, 2016). Parasite movement was then investigated using a stereo microscope to ensure parasite survival. Wells with parasites were exposed to the following set of Vaccium210 concentrations: 0, 2.5, 5, 7.5, 10, 12.5 and 15 ppt.. The time was defined as zero and Parasites were observed every 5 min until all parasites were dead. The data were derived from the mean value of three replicates.

2. 6. Experiment 2: In vitro effect of Vaccium210 on monogenean:

Infested Fish with *Furnestinia* were anaesthetised in 0.025% clove oil (Kildea et al., 2004) followed by pithing. Areas of gill filaments with minimum of three parasites were cut off using a scalpel. Each clipped filaments were transferred to separate well of 96 well plate containing 0.5 mL filtered marine water. Parasites were observed for movement using a dissecting microscope to ensure parasite survival. Wells with parasites were exposed to the following set of Vaccium210 concentrations: 0, 2.5, 5, 7.5, 10, 12.5 and 15 ppt**.** The time was defined as zero and Parasite survival was observed every 5 min until complete eradication. Detached and Non-motile parasites that did not respond to gentle water current were considered dead. The data were derived from the mean value of three replicates.

2. 7. Experimental 3: Lethal concentration-50 of Vaccium 210 to *S. aurata*:

The half-lethal concentration (LC_{50}) of drug was conducted to estimate the toxicity of the used drug for treated fish. Fish were divided into 5 groups (n =10 fish) and placed in 40 liter aquaria .The fish were exposed to different concentration of Vaccium210 (control , 30, 60 , 90 and 120 ppt). Aquarium water taken from same fish source was filtered to use in a test .The water temperature were adjusted at 25±2C º in all aquarium and dissolved oxygen in water were measured during experiment with an oxygen meter (cole- parmer Instrument Co., Chicago) and every 24hr thereafter. Any abnormal fish behavior and mortality observations should be recorded at 6, 24, 48 and 96hr. All fish were not fed during the exposure (The LC_{50} of Vaccium210 to *S. aurata* was calculated according to equation according to Hamilton et al. (1977).

$$\mathbf{LC_{50} = LC_{100} - \sum \frac{A \times B}{N}}$$

Where, LC_{50} = Median lethal dose, LC_{100} = Least dose required to kill 100%, A = Dose difference, B = Mean mortality and N = number of fish in group

2. 8. Experimental 4: In vivo Bath treatment:

The protocol of in vivo experiment applied as previously described by Fridman et al. (2014) **.** Efficacy of Vaccium210 was tested using bath treatments of naturally parasitic infestation in fish. Fifty naturally infested sea bream with *Trichodina spp* and *F. echeneis* were divided into 5 groups (n = 10). Each group was placed in separate glass aquaria containing 60 L marine water equipped with aeration. Fish were examined for intensity of infestation before and after treatment. Vaccium210 was added at concentrations of control 0, 2.5, 5, 7.5 and 10 ppt. Fish were examined after 1, 1.5 and 24 hours. parasite load determined Intensity of infection was quantitatively evaluated with the range: (+) 1–5; (++) 6–10; (+++) 11–25; (++++) 26–50; (+++++) 51–100; (++++++) > 100 parasites /microscopic field according to Alvarez-pellitero et al. (1995).

3. Results

3.1. Clinical and post-mortem examinations:

Sampled fish showed variant clinical disease signs including gasping of air, off-food, emaciation, excessive mucous on the body surface, scattered hemorrhagic spots on different body parts as well as congestion of the gills. Some infested fish showed paleness of gills with

excessive mucus, emaciation and mortalities Fig (1). External examination of some fish revealed presence of crustacean attached to skin and fins that could be seen with naked eye.

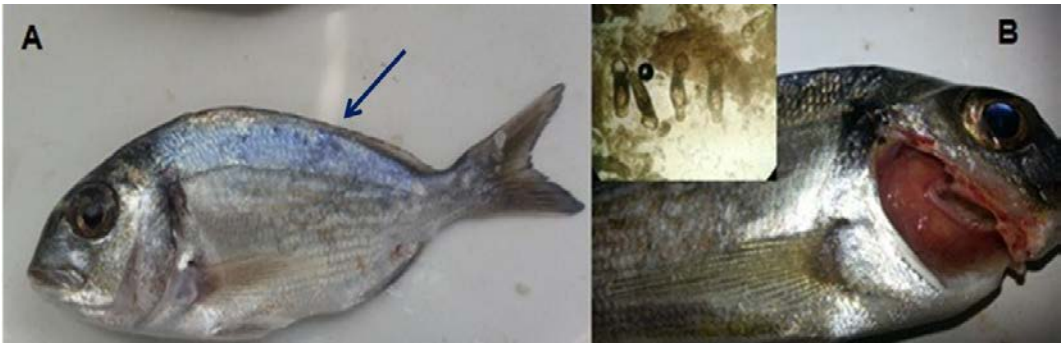


Fig (1): *S. aurata* Naturally infested with *Trichodina spp.* and *F. echeneis* showing emaciation and detached scales arrow. B. *S. aurata* showing paleness of the Gills, wet preparation of gills showing monogenetic trematode *F. echeneis* at the upper corner.

3.2. Parasitological examination:

Macroscopical examination of isolated parasites from fish proved infestation with 2 genera of protozoan including; *Trichodina spp.* and *Amyloodinum ocellatum* (*A. ocellatum*) with a total prevalence rate of 34.3% and17.1% respectively. However, infestation with monogenea *Furnestinia echeneis* (*F. echeneis*) and *Caligus minimus* (*C. minimus*) (crustacean) was recorded in the examined fish with rates of 35.7% and 21.4%, respectively. Mixed infestation of *Trichodina spp.* and *F. echeneis* was detected in 21.4% of collected samples. While mixed infestation of *Trichodina spp.* and *C. minimus* was 8.6% of collected samples table (1) and Fig (2).

Table (1): Total prevalence of external parasites recovered from examined *S. aurata*

Parasites	No. of examined Fish	No. of infested Fish	%
Trichodina spp.	70	24	34.3
Amyloodinum ocellatum		12	17.1
Furnestinia echeneis		25	35.7
Caligus minimus.		15	21.4
Mixed infestation *Trichodina spp.* and *Furnestinia echeneis*		15	21.4
Mixed infestation *Trichodina spp.* and *C. minimus.*		6	8.6

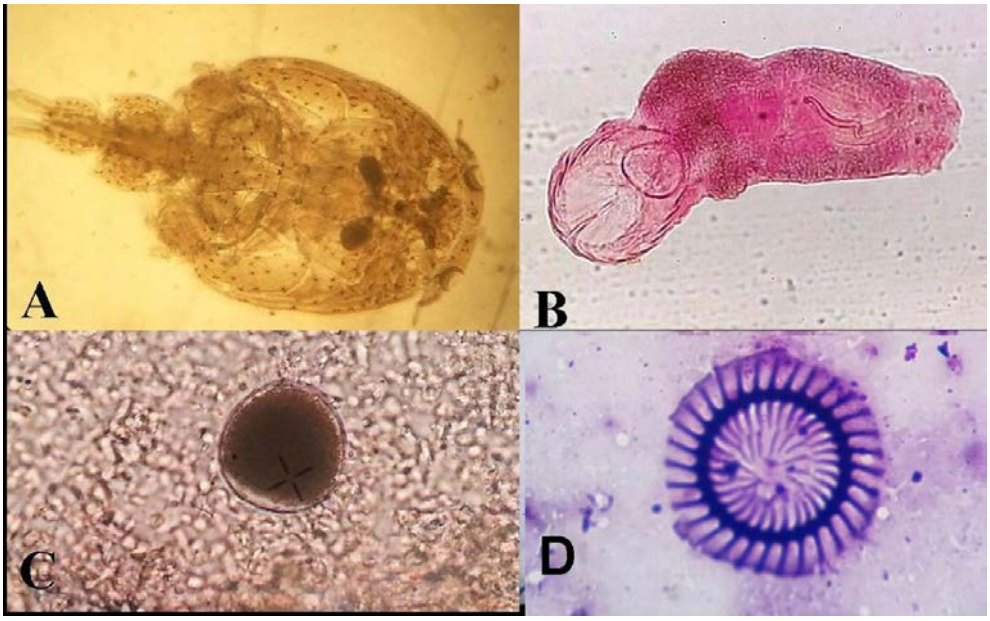


Fig. (2): A- *C. minimus* wet preparation(X10), B- *F. echeneis* Stained with carmine(X40) .C – *A.ocellatum* wet preparation(X40), D - *Trichodina spp.* stained with Giemsa stain (X100).

3.3. Seasonal prevalence of external parasites:

Seasonal prevalence in collected *S. aurata* was investigated as illustrated in Fig. (3). The highest prevalence of *Trichodina spp.* was in winter (45.0%) followed by autumn (35.0%). The highest infestation rate of *A. ocellatum* was shown in autumn (25%), *F. echeneis* showed highest infection rate in spring (60%) followed by summer (53.3%), while the lowest infestation rate in case of *C. minimus* recorded in winter (5.0%). Mixed infestation of *Trichodina spp.* and *F. echeneis* was recovered with highest rate in spring (33.3%) followed by autumn (20.0%). Mixed infestation of *Trichodina spp.* and *C. minimus* disappeared during summer.

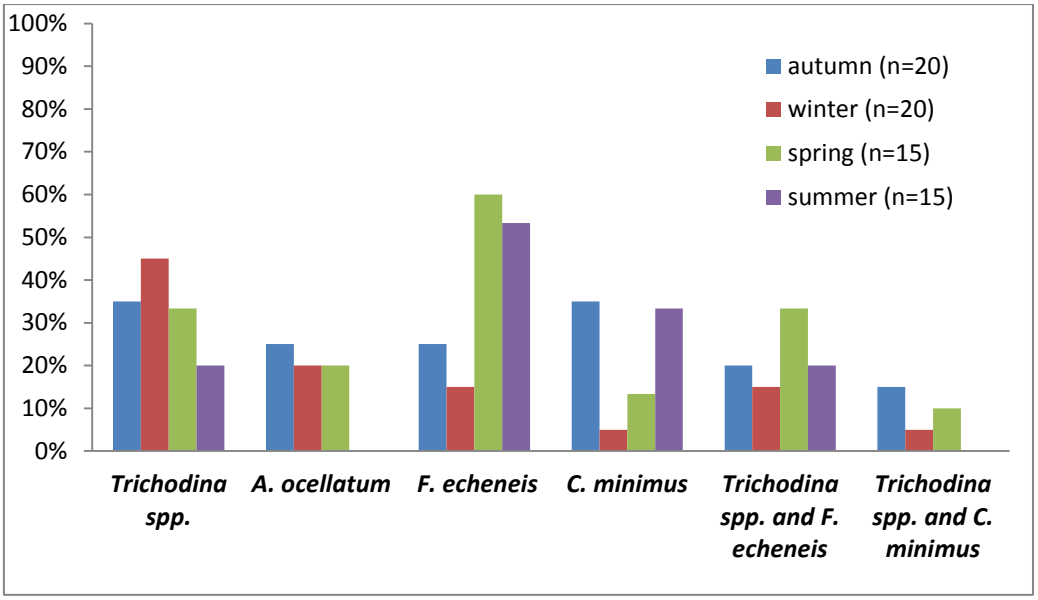
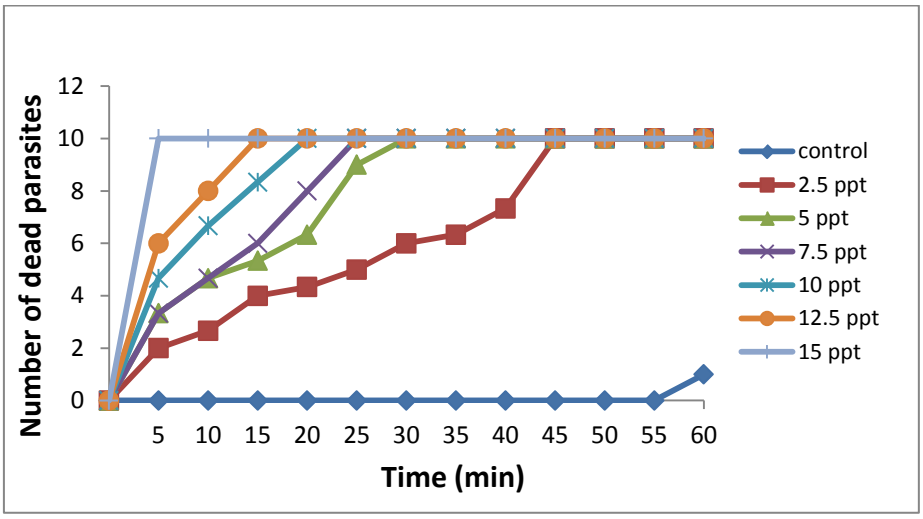


Fig. (3) Seasonal prevalence of external parasites recovered from examined *S aurata*.

3.4. Experiment 1: In vitro effect of Vaccium210 on protozoan

The in vitro results of Vaccium 210 efficacy testing on *Trichodina* revealed that the time required for killing all *Trichodina* parasites at lowest concentration (2.5 ppt) was 45 minutes. However, at the highest concentration (15 ppt) it required 5 minutes to kill all parasites. Parasites in control wells were observed for up to 1 h after the last result of lowest concentration (Fig. 4).



Fig(4) cumulative number of dead parasites (*Trichodina spp.*) from in vitro treatments with varying concentrations of Vaccium 210 in different time period.

3.5. Experiment 2: In vitro effect of Vaccium210 on monogenean

The results of Vaccium210 efficacy on monogenean parasites *F. echeneis* showed that the time needed for killing all parasites in each well correlated to concentration. With 2.5 ppt concentration, the time was 60 min, while the 15 ppt concentration required only 15 min (Fig5).

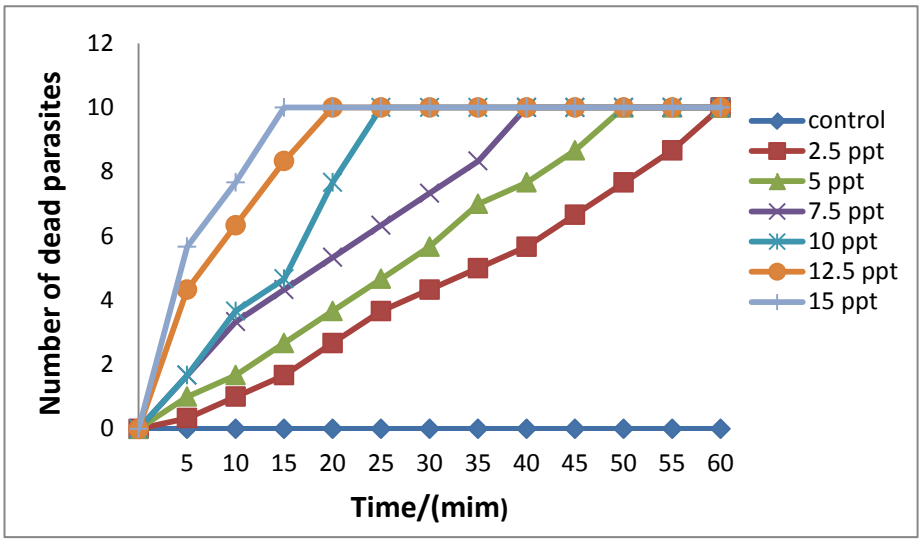


Fig (5) cumulative number of dead parasites (*F. echeneis*) from in vitro treatments with varying concentrations of Vaccium 210 in different time period.

3.6. Vaccium 210 Lethal concentration-50 (LC_{50})

The results of toxicity tests (LC_{50}) of Vaccium 210 to sea bream appeared to be 93.6 ppt after 96 hrs under observation (Table 2).

Table (2) The Lethal concentration-50 of Vaccium 210 to *S. aurata.*

Exposure Dose ppt	Fish No.	Difference between Dose	NO. of Dead Fish			Oxygen conc. ppm		
			24hrs	48hrs	96hrs	24hrs	48hrs	96hrs
Control	10	0	0	0	0	7.5	7.2	7.2
30	10	30	1	0	0	6.5	6.3	6
60	10	30	1	1	0	6.0	5.5	5.5
90	10	30	3	2	0	2.4	2.2	2.0
120	10	30	8	2	-	1.5	1.0	-

3.6.1. Measurements of dissolved oxygen concentration:

The dissolved oxygen level in LC_{50} test at 25 ± 2°C resulted that fish showed erratic behavior at concentration > 60ppt. Dissolved oxygen level showed little differences from those of control at drug concentration level <60 ppt for 96hr and sever depletion in oxygen at drug concentration> 90ppt.

3.7. Experimental 4: In vivo Bath treatment:

Results showed correlation between Vaccium 210 concentration and efficacy. The concentration of 10 ppm Vaccium 210 was the most effective and could eradicate both *Trichodina sp* and *F. echeneis* after one hour. Also, 5 ppm concentration could eradicate both parasites after 1.5 hour of exposure. However, a concentration of 2.5 ppm required 24 hrs to kill both parasites as showed in Table (3).

Table (3): Efficacy of Vaccium210 bath treatment on *Trichodina spp* .and *F. echeneis* mixed infestation in *S. aurata*

Conc. (ppt)	Time	Degree of *Trichodina spp* intensity.	Degree of *F. echeneis* intensity.
Control	Before treatment	++++	++++
	30 min.	++++	++++
	1hour	++++	++++
	1.5 hours	++++	++++
	24 hours	++++	++++
2.5	Before treatment	++++	++++
	30 min.	++++	++++
	1hour	+++	+++
	1.5 hours	+	++
	24 hours	-	-
5	Before treatment	++++	++++
	30 min.	++	+++
	1hour	+	++
	1.5 hours	-	-
7.5	Before treatment	++++	++++
	30 min.	+	++
	1hour	-	+
	1.5 hours	-	-
10	Before treatment	++++	++++
	30 min.	+	+
	1hour	-	-

N. B. : The number of fish in each group is 10
(+) 1–5; (++) 6–10; (+++) 11–25; (++++) 26–50 parasites /microscopic field
(-) : infection with parasites is negative

4. Discussion

Fisheries and cultured fish represent important sources for human food and protein in different countries all over the world including the Mediterranean countries. The increasing intensive aquaculture is often accompanied with different problems (Athanassopoulou et al., 2009). Of these problems are the parasitic diseases that cause high economic losses in fish farms due to mortalities and or retardation of normal growth rate of fish, in addition to treatment costs (El-Galil and Aboelhadid, 2012). In the present study, the investigated *S. aurata* suffered from gasping of air, decreases in appetite and demonstrated emaciation, excessive mucous on the body surface, scattered hemorrhagic spots on different parts of the body, congestion/paleness of the gills with excessive mucus and mortalities. Our parasitic examination proved infestation with *Trichodina spp.* and *A. ocellatum* with a total prevalence rate of 34.3% and17.1% respectively. Similar clinical picture has been previously reported in cases with external protozoa infestations with *Trichodina spp., Epistylis spp. and Chilodonella sp.* (Marzouk et al., 2012) and (Bahri, 2012) who isolated *Trichodina sp. ,Cryptocaryon-like* species, *Amyloodinium ocellatum sp. , Ichthyobodo sp.* from gilthead sea bream with similar clinical picture. Through the present study, monogenetic trematode *F. echeneis* was isolated from the gills of *S. aurata* with a mean occurrence rate 35.7%. This occurrence rate showed variation between different year seasons, where it was highest in spring (60 %) followed by summer (53.3%) then autumn (25%) and winter (15%). These findings agree with other records (Heba et al., 2012) that described higher parasitic infestation rates in spring and summer compared to winter and autumn. However, our findings do not agree with other reports (Mahmoud et al., 2014) that described higher rate of infestation with *F. echeneis* in summer. Concerning the *caligus minimus,* the total prevalence through the present study was 21.4%, where the highest prevalence was in autumn (35 %) followed by summer (33.4%) then spring (13.3%) and winter (5%). These results partially agree with the findings of others (Eissa et al., 2012) and (Hassanin, 2016) who found the highest prevalence in summer followed by autumn then spring and winter. Also, our results differed from reports of others (El-Lamie 2007) who recorded that the highest prevalence was in winter (81.3%) followed by spring (78.6%) then autumn (66.6%) and summer (53%). Actually, there is no longer any doubt that the earth's climate is changing that induces reasonable influences on parasitism and diseases in marine ecosystem. Consequently, a relation between parasitic infestation and temperature of water explains the seasonal variation in the rate of infestation which differ from parasites to another according to optimum temperature for its life cycle (Marcogliese, 2008).

On the other hand and for long years, chemical preparations were the main choice for treatment of parasitic infestation in aquaculture either by immersive or bath treatment. Actually, most of these chemical drugs are associated with serious problems like low efficacy, harmful effects on fish, environment and human health (Schelkle et al., 2009). Recently, there is an increasing trend for using herbal extracts for controlling diseases in different animal production sectors including aquaculture (Valladão et al., 2015).

In the present study, the in vitro efficiency of vaccium 210 (a herbal essential oil based product) was tested. Vaccium 210 is used in poultry sector as immunstimulant with some anti-infectious activities, however little is known about its efficacy against fish infectious agents, including parasitic infections. In the present study, the efficacy of vaccium 210 against *Trichodina Sp.* and *F. echeneis* was tested, where it showed efficacy against both parasites. Results showed also a correlation between the concentration of the drug and the exposure time to treatment, where higher concentration of drug required less time for killing the parasite. High concentration (15 ppt) required 5 and 15 minutes to kill *Trichodina Sp*. and *F. echeneis* respectively, while low concentration (2.5 ppm) of the drug could eradicate the *Trichodina Sp*. and *F. echeneis* after 45 minutes and 60 minutes respectively. Similarly, *Origanum vulgare* L. (oregano) and *Thymus vulgaris* L. (thyme) were effective against the *Trypanosoma brucei*, *Trypanosoma cruzi* and *Leishmania major* (Mikus et al., 2000, Santoro et al., 2007)

Actually, the mode of action and mechanism of essential oils as anti-infectious agents has not been fully studied. Since the essential oils are complex mixtures of several compounds, some reports assume that their antimicrobial activity is not attributable to one specific mechanism but through several mechanisms and targets in the infectious microorganism (Skandamis and Nychas, 2001). Some studies suggested that anti-infectious activity for essential oils is due to the phenolic compounds, such as thymol and carvacrol. These compounds change the fluidity of organism's membranes resulting in leakage of radicals, calcium ions, cytochrome C, and proteins. The abnormal permeability of mitochondrial outer and inner membranes results in cell death by necrosis and apoptosis (Armstrong, 2006).

On the other side, determination of the lethal concentration-50 (LC_{50}) for new products in fish species before its application as a treatment is very important. LD_{50} defines the medicinal dosage which is effective for treatment but nontoxic for the fish. Our findings showed that the acute bioassays LC_{50} of the drug in *S. aurata* were 96.3 ppt / 96 hours. The mortality of fish increased when the dissolved Oxygen decreased. This might be due to negative correlation between oxygen depletion and drug decomposition. Our findings concur with others

(Lambert et al., 2001) who suggested that high concentrations of thymol and carvacrol led to increased chemical turbidity due to the insolubility of the oils. Our findings also support previous records (Malheiros et al., 2016) that found the essential oil of *Mentha piperita* has a toxic activity against monogenea infestation in vitro, while pathological changes in the tissues of the gill of *Arapaima gigas* fingerlings at higher dose (LC_{100}) were detected through in vivo evaluation of its toxicity.

Through the present study, eradication of *Trichodina Sp. and F. echeneis* infesting *S. aurata* was demonstrated with 2.5 ppt drug concentration (after 24 hrs of exposure) and with 10 ppt (after 1hour). This result concur with previous records (Athanassopoulou et al., 2004a, Athanassopoulou et al., 2004b, Karagounim et al., 2005) that reported anti protozoal activity for *Origanum* oil against *Myxobolus sp.* in *S. aurata* and *Puntazzo puntazzo.* Similarly, other findings (Ling et al., 2012) reported that aqueous extract of *Capsicum frutescens* had potential in vitro and in vivo effect against *Ichthyophthirius multifiliis* theronts. Also, the essential oil of *Thymus vulgaris* was found to be effective against larvae of *Anisakis* larvae (Giarratana et al., 2014).

5. Conclusion

The present study revealed that parasitic infestation is still a serious problem for marine aquaculture. Variant parasites *Trichodina spp , Amyloodinum ocellatum, Furnestinia echeneis* and *Caligus minimus* were isolated from naturally infected fish farms. Vaccium 210 seemed to be a promising anti-parasitic agent againest *Trichodina spp.* and *F. echeneis* infestation in *S. aurata*, however futher investigations for its application with other fish parasites and fish species should be considered.

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