Investigation of the possibility use of *Zataria multiflora* (Avishan-e Shirazi) essence in control of fungal contamination of cultured shrimp, *Litopenaeus vannamei*

Sharif Rohani M.[1]*; Dashtiannasab A.[2]; Ghaednia B.[2]; Mirbakhsh M.[2]; Yeganeh V.[2]; Vahabnezhad A.[1]

Received: September 2012 Accepted: December 2012

Abstract

This study was conducted to evaluate the possibility of replacing *Z. multiflora* instead of antibiotics and chemicals in shrimp aquaculture industry to protect the environment and prevention of microbial resistance. *In vitro* MIC of *Z. multiflora* essential oil against *Candida albicans* and *Fusarium solani* obtained 0.004% and 0.008% respectively, also MFC of *Z. multiflora* essential oil against *C.albicans* and *F.solani* was 0.004% and 0.04%, respectively. The results showed that 0.0002% dosage of *Z. multiflora* essential oil against total count of fungi in sea water (salinity 39±1ppt) can decrease growth of fungi after 6 h and 0.0004%, 0.0008% and 0.0016% dosages can decrease the total count of fungi in sea water. But very low dosage of *Z. multiflora* essential oil in shrimp *Litopenaeus vannamei* leads to mortality. Lethal dose of *Z. multiflora* essential oil in shrimp postlarvae, juvenile (2±0.2g) and adults (10-12 g) was 0.00050%, 0.00045% and 0.00035%, respectively. The results indicated that *Z. multiflora* have a significant anti-fungal effect but is toxic for *L. vannamei*. We recommend that *Z. multiflora* essential oil can be used in non-living environment, such as disinfecting tanks and equipments.

Keywords: *Zataria multiflora*, Essential oil, Antifungal, Shrimp, *Litopenaeus vannamei*

1-Iranian Fisheries Research Organization, P.O. Box: 14155-6116, Tehran, Iran.

2-Iran Shrimp Research Center, P.O. Box: 1374 Bushehr, Iran

* Corresponding author's email: rohani@ifro.ir

Introduction

Plants were the first therapy to be used in the treatment by man. During the 18th and 19th centuries, scientific and medical knowledge advance, the first, Scientists discovered the active ingredients to dissect a plant and secondly, instead of the purification process of plants, were processed in synthetic and artificial. These drugs were greeted in comparison with herbal medicines due to the high purity, quick efficacy and reduction of long-term health. But in the mid-20th century, the World Health Organization released the report shaker from side effects, dangerous consequences of chemical drugs, in spite of high efficacy may lead to new diseases (Peter et al., 2002).

In 1960, the occurrence of some great disaster in the field of abnormal consequences due to uses chemical drugs and their side effects, many synthetic drugs removed from the pharmacopoeia of the world, and some were advised to use caution. Back to nature and the use of origin herbal medicines and herbal products and their extracts (Herbalism) to treat disease were prevalent universal among developing countries and industrialized societies despite of having a wide range of chemical drugs, most have been referred to the consumption herbal medicines.

Commercial shrimp farming began in the 1970s, and production grew steeply, the total global marketing of farmed shrimp representing a value of 8 billion U.S $ in 2001 (FAO, 2003). The total global production of farmed shrimp reached more than 1.6 million tons in 2002 and the program of global production is somehow which 12-15 % is added to this amount per year (Rosenbery, 2003).

The shrimp farming was successful and this success story continues up to now, as 50% of the world's total shrimp production is attained of shrimp farms, but in the past decade shrimp farming industry are suffered huge economic losses due to disease (Lightner, 2003).

The biggest problem faced by the aquaculture industry worldwide is diseases caused due to various biological and non-biological agents. Most damages have been arising out the viral diseases but the fungal disease in larval and post larval stages are caused one of the most important farm mortality, although official statistics about the severity and prevalence of the disease in shrimp rearing centers has not been published in the world and the country. In recent years evidence is indicating the fact that more than half of the eggs produced in the breeding centers exceed 30 centers in four southern provinces in coastal area of the northern Persian Gulf, Iran may be removed from the production cycle due to several reasons, which one the main factor is a fungal disease.

Now, In order to prevent the mortality in rearing centers are used a large amount of chemical disinfectants and too many antibiotics which is leaded to increases the Antimicrobial resistance and environmental pollution. Environmental pollutions by organic wastes, remaining food and chemical materials are as well as the concerns of billions of dollars industry because acceding of pharmaceutical materials and chemical pollutants to the cycles of nature and moreover are leaded

to increase problems relating to public health and safety of final consumers for aquaculture products would be irreparable. Also, due to the indiscriminate use of antibiotics, have been reported increases drug resistances of bacterial fish disease all over the world (Tsoumas et al., 1989; Herwing *et al*., 1997; Rahim *et al*., 1998).

The uses of drugs have been studied in shrimp larval rearing industry. Baticados *et al*. (1990) reported Luminous *V. harveyi* and *V. splendidus* species which are separated from the shrimp larvae were resistant to antibiotics erythromycin, kanamycin, penicillin, and streptomycin. Nash et al. (1992) reported most commonly used of ox tetracycline in shrimp hatcheries indicating the development of resistance microbial. To solve this problem, many researchers have investigated the antibiotic properties of medicinal plants (Karunasagar et al., 1994; Abraham et al., 1997; Sahul and Balasubramanian, 2000).The family of Labiatae is generally known for their various effects such as analgesic and anti inflammatory activity (Hernandez-Perez et al., 1995), antioxidant (Cuppett and Hall, 1998), Hepatoprotective (Wasser et al., 1998) and hypoglycemic effects (Hosseinzadeh et al., 2000). *Z. multiflora* belongs to family that is distributed only in Iran, Pakistan and Afghanistan. It is greatly used for medicinal and condimental purposes in these countries. This plant with the vernacular name of Avishan-e-Shirazi in Iran has several traditional uses such as antiseptic, anesthetic and antispasmodic (Zargari, 1990).

The aim of this study was to evaluate the possibility of replacing *Z. multiflora* instead of antibiotics and chemical materials in shrimp farming industry In order to protect the environment and prevention of microbial resistance.

Materials and methods

1. Z. multiflora essence

Essential oil used in the project was produced in pharmaceutical Barij Essence Company.

2. Fungi species

In this research, two species of the fungi *Fusarium solani* and *Candida albicans* PTCC 5027 were used. *Fusarium solani* that previously was isolated of Shrimp Breeding Center in the Bushehr province during a research work in 2005 and were kept in Iran Shrimp Research Center, microbiology laboratories. The *Candida albicans* species and bacteria infectious were prepared from Iran Industrial Research Center.

3. Determination of antifungal effect

Determination of MIC and MFC:

Fungi species were cultured on Sabouraud's dextrose agar (SDA) and incubated at 37˚C for 24 h. Several colonies of each fungi species were collected in 2 ml of sterile PBS to prepare a suspension. The suspension was adjusted to 70% transmittance by a spectrophotometer at 530 nm. This should result in a suspension containing about 1×10^6 cfu per ml.

MIC was carried out according to elsewhere (Murray et al., 1995) method, in summary, a serial dilution of *Z. multiflora* essential oils in dimethylsulfoxide (DMSO) was prepared in SDA tubes. The

solvent (DMSO), at an appropriate dosage was also used as a negative control. A tube was considered as positive control without *Z. multiflora* essential oils and solvents. 20 ml of standardized suspension of different species of fungi were inoculated in to each tube. The tubes were incubated at 30˚C for 24h to 5 days. The lowest *Z. multiflora* essential oils dosage at which tube was no visible growth (was clear) defined as the minimal inhibitory dosage (MIC). For the determination of MFC, a portion of liquid (5µl) from each tube that was clear in color and then was placed on SDA for further incubation at 37 °C for 24 h to 5 days. The lowest dosage that yielded no growth after this sub-culturing was defined as the MFC, with 3 replicates for each experiment.

- *Effect of Z. multiflora* essential oil on the total number of fungi in natural environments*:*

To study the effects of *Z. multiflora* essential oils in natural environment, salt water shrimp farms was transferred to the laboratory under sterile conditions with salinity 40 ppt, temperature in 29 ºC and pH=8.1 .The cultured shrimp farms were treated with different dosages of *Z. multiflora* essential oil (0, 20, 40, 80, 160, 200, 400, 800, 1600 ppm) with three replicates. One ml was taken of treatments at different time (0, 6, 12 , 24, 48 h) and transferred to Sabouraud dextrose agar, during the incubation at 30°C for 48 to 72h, for further counting of total number of fungal colonies .

4. *Shrimp species examined*

To achieve health and lethal dose of *Z. multiflora* essential oil and its impact on growth and survival of white shrimp (*Litopenaeus vannamei*), the different size of shrimps consist of larval and post-larval, juvenile (2±0.2 g) and adult (10 to 12 g) stages. All stages of shrimp were collected from the Iran Shrimp Research Center stations which located in Bandargah and Helleh area, Bushehr province.

5. *Determine the maximum tolerable dosage*

In order to determine the health of *Z. multiflora* essential oil dosage for different stages in growth of *P. vannamei*, after three days of preparing halls and establishment of white leg shrimp in 100 liter tanks, different weight of white shrimp were treated with different dosages of *Z. multiflora* essential oil (0.001% to 0.1%) with 3 replicates and each replicate were used for 30 individuals of shrimp in larval and post-larval stages and 10 individuals in adult and juvenile for 48h. Due to the volatility of the oil extraction and remain constant of dosage to over time each 8h shrimp were transferred to the new dissolved and For was uniformed stress conditions in the control shrimp were passed to the tank containing fresh water every 8h.

During the experiment water temperature was between 28-30°C, Salinity was 39±1 ppt and acidity (pH) was 7.9-8.2 Mortality rates was recorded at different times (1, 2, 3, 6, 12, 24).

6. *Statistical analysis*

One-way analysis of variance (ANOVA) was analyzed on all the data and statistical differences of individually were evaluated by Tukey test. A p-value of less than 0.05 was statistically considered significant.

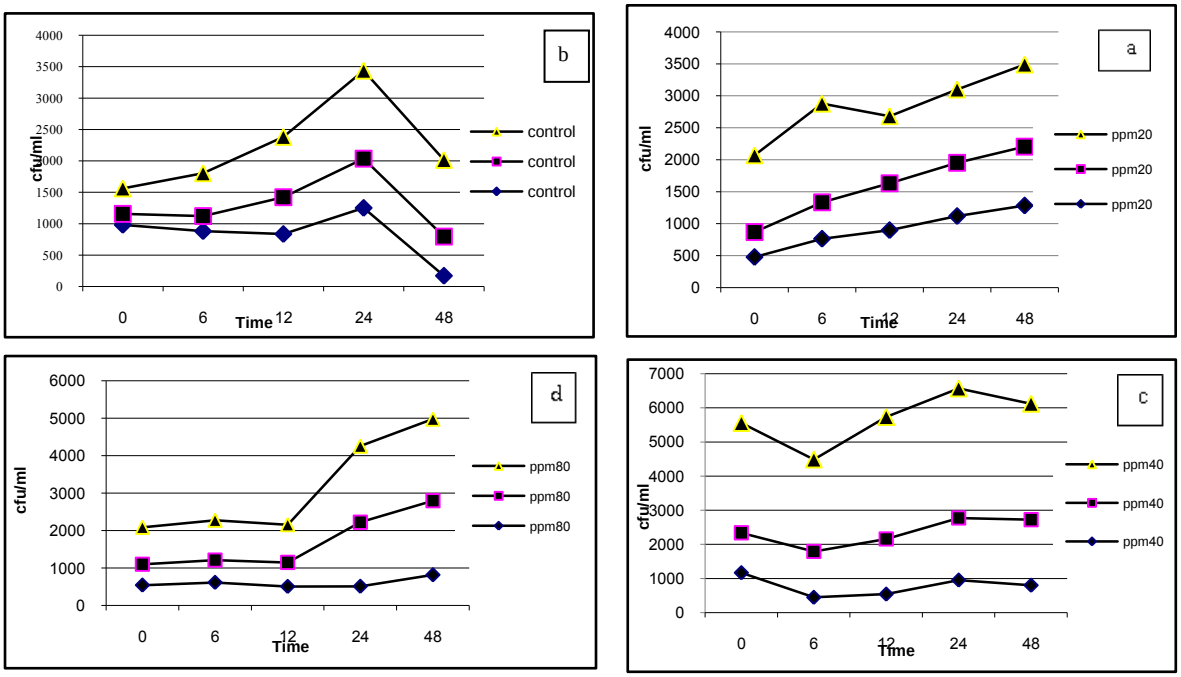
Results

As shown in Table 1, results showed that dosage of 0.004% or 400ppm for *Candida albicans* and dosage of 0.008% (or 800 ppm) for the *Fusarium solani* had obvious inhibitory rate.

Table 1: The results of MIC (%) for the two fungi using different dosages of *Z. multiflora* essential oil

	Negative control	Positive control	0.0008%	0.004%	0.008 %	0.040 %	0.080 %	0.400 %	0.800 %	4%	8 %
Candida albicans	-	+	+	+	+	-	-	-	-	-	-
Fusarium solani	-	+	+	+	+	+	-	-	-	-	-

Our result revealed after plate culture, in fungus *Candida albicans* at the same dosage of 0.004 % or minimum inhibitory dosages (MIC) occurred inhibition of fungal growth over a dosage range and to obtain the MFC. But for *Fusarium solani*, the MFC value estimated 0.04% or 4000 ppm. Figure 1 illustrated the different dosages of *Z. multiflora* essential oil. The dosages including 400, 800, 1600 ppm demonstrate to inhibit the growth of fungi after 6 h in the water ponds (Fig. 1 h, i, g) and these dosages compared to the other groups and the control group was effective in inhibiting fungal growth ($P<0.05$).



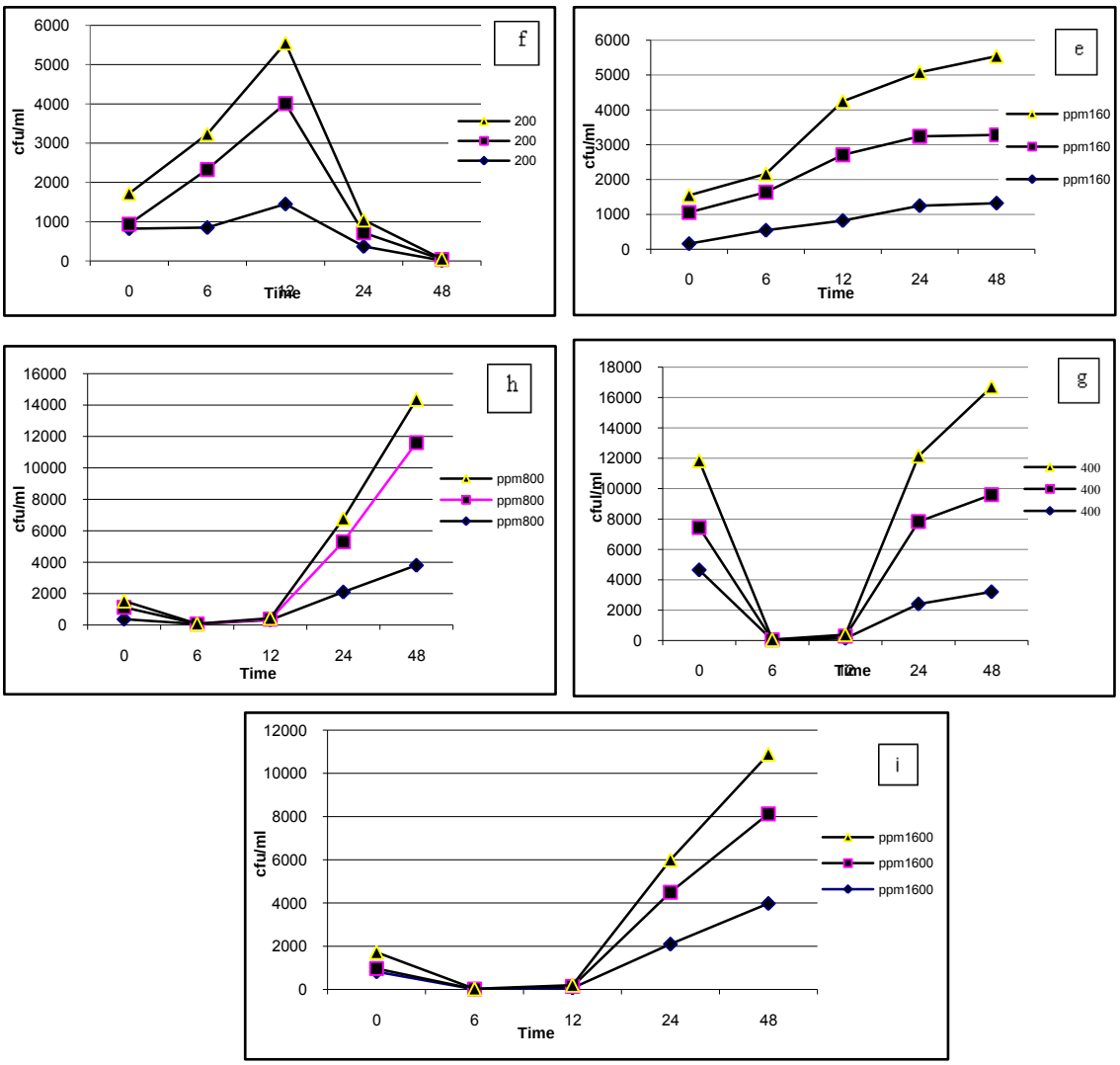


Figure 1: Changes of total number of fungi in the presence of different dosages of *Z. multiflora* essential oil (a-i).

Figures 2, 3, and 4 showed the survival post larval, Juvenile and adult shrimp exposed to different dosages of *Z. multiflora* essential oil. The dosages of 60, 80 and 100 ppm are revealed total mortality for all groups and at all developmental stages of shrimps after 48 h which show the significant difference between the control group and the other groups ($P<0.05$). On the other hand, post-larvae groups that exposed to dosages in 50 ppm of *Z. multiflora* essential oil (Fig. 2), also showed the significant difference between the control group and the other groups ($P<0.05$). Therefore the maximum dosage could be tolerated by post-larvae of *L. vannamei* was about 45 ppm, which was not observed significant mortality after 48 h. The maximum tolerable dosages are estimated for Juvenile and adult white leg shrimp, 40 and 30 ppm, respectively (Figs. 3, 4).

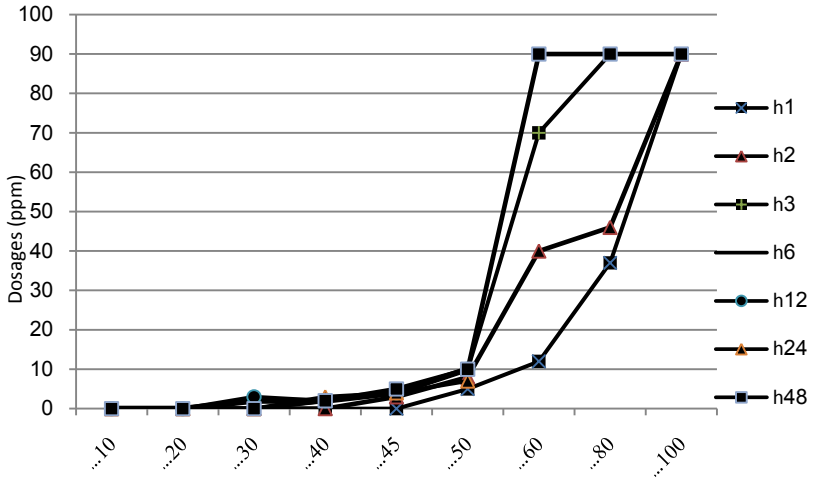


Figure 2: The mortality of post-larvae stage of *L. vannamei* after exposure to different dosages of *Z. multiflora* essential oil

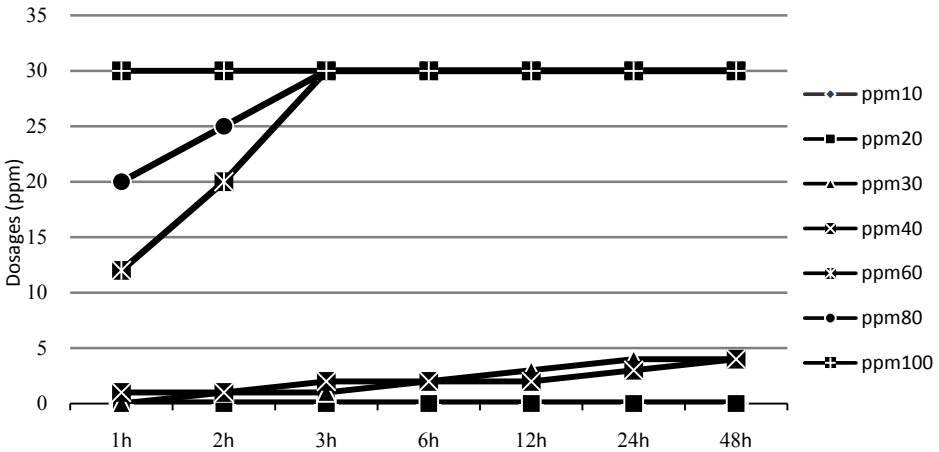


Figure 3: The mortality of Juvenile stage of *L. vannamei* (2g) after exposure to different dosages of *Z. multiflora* essential oil

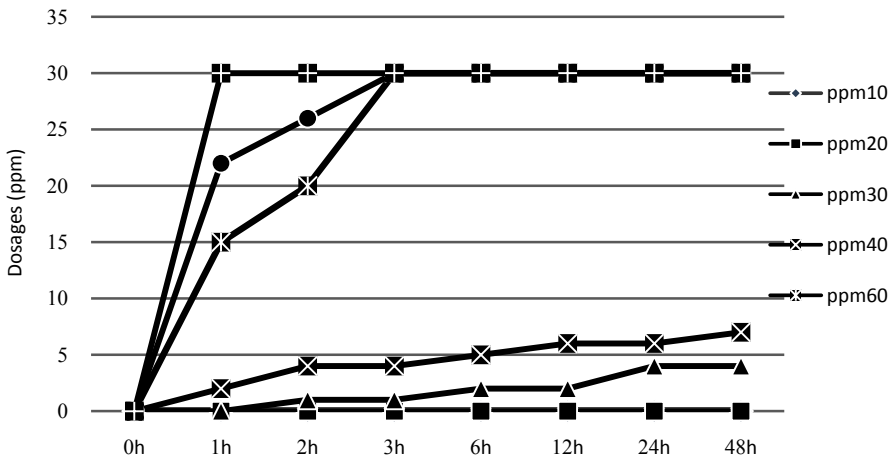


Figure 4: The mortality of adult stage of *L. vannamei* (10-12g) after exposure to different dosages of *Z. multiflora* essential oil

Discussion

The results indicated that *Zataria multiflora* have a significant anti-fungal effect which can attributed to compounds such as carvacrol and thymol (Shaffiee and Javidnia, 1997; Ali et al., 2000). The antifungal activities in the present research have been previously reported by other researchers (Sardari et al., 1998; Sharif Rohani, 2004; Rasooli et al., 2006). The results also indicated that the effective dosage for aquatic fungi in marine ecosystems is higher than other fungi. This is being even more about for many aquatic fungi lethal dosage so that the growth inhibitory dosage (MIC) for the *Candida albicans* was 0.004% (400 ppm) but *Fusarium solani* was 0.008% equal to 800 ppm. The results also showed that the essential oil has a good antifungal effect *in vivo* condition (Fig. 1a to i). The 200 ppm dosage inhibit growth of fungi after 24 h and 400,800,1600 ppm inhibit fungi growth after 6 h. Sharif Rohani (2004) reported that the value of MIC was 160 ppm for *Fusarium solani* because they were isolated from a source other than seawater.

The minimum fungicidal dosage (MFC) estimated for *Candida albicans and Fusarium solani* 0.004% (400 ppm) and 0.04% (4000 ppm), respectively which indicated 10 times higher. The obtained results in our research showed *L. vannamei* was more sensitive at different growth stages to *Z. multiflora* essential oil therefore dosages was obtained much lower than the effective dosage for fungi. Phenolics, polysaccharides, proteoglycans and flavonoids components have a major role in preventing or controlling infectious microbes. In aquaculture, there has been limited work performed against the fungal pathogens related to fish or shrimp diseases. Ethanolic, methanolic and hexane extracts of *Ocimum basilicum* have been good effects in reduce the fungal infection in tilapia eggs (Chitmanat et al., 2005).

The main reason was leaded high mortality in white leg shrimps *L. vannamei* due to existence of aromatic compounds in *Z. multiflora* essential oil thus reduced oxygen in the water. If *L. vannamei* transferred to water without *Z. multiflora* essential oil, they will return to normal status.

As an overall conclusion *Z. multiflora* essential oil have performed to inhibit the growth of pathogenic fungi and eliminate these fungi in abiotic condition, disinfecting equipment and there is limitation in application if we have shrimp species in the environment.

Acknowledgments

We thank Prof. Tooraj Valinassab for valuable suggestions that considerably improved the manuscript.

References

Abraham, T., Manley, J. R., Palaniappan, R. and Devendran, K., 1997. Pathogenicity and

antibiotic sensitivity of luminous *Vibrio harveyi* isolated from diseased penaeid shrimp. *Journal of Aquaculture in the Tropics*, 12 (1): 1– 8.

Ali, M. S., Saleem, M., Ali, Z. and Ahmad, V. U., 2000. Chemistry of *Zataria multixora* (Lamiaceae). *Phytochemistery, 55*, 933–936.

Baticados, M. C. L., Lavilla-Pitigo, C. R., Cruz-Lacicerda, E. R., Delapena, L. D. and Sunaz, N. A., 1990. Studies on the chemical control of luminous bacteria *Vibrio harveyi* and *V. splendidus* isolated from diseased *Penaeus monodon* larvae and rearing water. Disease of Aquatic Organisms. 9, 133 – 139.

Chitmanat C, Tongdonmuan K, Khanom P, Pachontis P. and Nunsong, W., 2005. Antiparasitic, antibacterial, and antifungal activities derived from a terminalia catappa solution against some tilapia (*Oreochromis niloticus*) pathogens. Acta Hortic 678:179–182.

Cuppett, S. L. and Hall, C. A.III., 1998. Antioxidant activity of Labitae. Advances in Food and Nutrition Research. 42:245-271.

FAO, 2003. Yearbook of fishery statistics, vol. 98/1& 2. Food and Agricultural Organization of the United Nations, ISBN 92-5-104986-6 Rome.

Hernandez-Perez JA, Garcia-Alvardo MA, Trystram G, Heyd B., 2004. Neural networks for the heat and mass transfer prediction during .Drying of cassava and mango. Innov. *Food Science Emergency Technology*. 5.64-57

Herwing, R. P., Gary, J. P. and Weston, D.P., 1997. Antibiotic resistant bacteria in surfacial sediments near Salmon net cage farms in Puget Sound, Washington. *Aquaculture* 14, 263–283.

Hosseinzadeh, H., Ramezani, M. and Salmani, G. H., 2000. Antinociceptive, anti-inflammatory and acute toxicity effects of *Zataria multiflora* Boiss extracts in mice and rats. *Journal of Ethnopharmacology*, 73: 379–385.

Karunasagar, I., Pai, R. and Malathi, G. R., 1994. Mass mortality of *P. monodon* larvae due to antibiotic resistant *Vibrio harveyi* infection. *Aquaculture*, 128: 203– 209.

Lightner, D. V., 2003. Exclusion of specific pathogens for disease prevention in a penaeid shrimp biosecurity program. pp. 81-116 *in*: Lee, C.-S, and O'Bryen, P.J. (eds.) Biosecurity in Aquaculture Production Systems: Exclusion of Pathogens and Other Undesirables.

The World Aquaculture Society, Baton Rouge, LA, USA.

Murray, P. R., Baron, E. J., Pfaller, M. A., Tenover, F. C. and Yolke, R. H., 1995. Manual of Clinical Microbiology, 6th ed. ASM,Washington, DC.

Nash, G., Nithimathahoke, C., Tungmandi, C., Arkarjamon, A. and Rumathaversub, P., 1992. Vibriosis and its control in pond reared *P. monodon* in Thailand. In: Shariff, M., Subasinghe, R.P., Arthur, J.R. (Eds.), Diseases in Asian Aquaculture: I. Fish Health Section. Asian Fisheries Society, Manila, Philippines, 143– 155P.

Peter, A. G. M., Smet, D. and Pharm, D., 2002. Herbal remedies. *England Journal of Medicine* 347:2046–2056P.

Rahim, Z., Sanyal, S. C., Aziz, K. M. S., Huz, M. I. and Chowdhury, A. A., 1998. Isolation of enterotoxigenic, haemolytic and antibiotic resistant *Aermonas hydrophila* strains from infected fish in Bangladesh. *Applied and Environmental Microbiology*. 48: 865– 867P**.**

Rasooli, I., Rezaei, M. B. and Allameh, A., 2006. Growth inhibition and morphological alterations of *Aspergillus niger* by essential oils from *Thymus eriocalyx* and *Thymus x-porlok. Food Control*; 17: 359 – 64P.

Sahul, A. S. and Balasubramanian, G., 2000. Antibiotic resistance in bacteria isolated from *Artemia nauplii* and efficacy of formaldehyde to control bacterial load. *Aquaculture*, 183: 195– 205P.

Sardari, S., Amin, G., Micetich, R. G. 1998. Phytopharmaceuticals, part 1. Antifungal Activity of selected Iranian and Canadian plant . *Pharmaceu Biology*; 36: 180-188.

Shafiee, A. and Javidnia, K., 1997. Composition of essential oil of *Zataria multixora*. *Planta Medicine, 63*, 371–372.

Sharififar, F., Moshafi, M. H., Mansouri S. H., Khodashenas M. and Khoshnoodi, M., 2007. *In vitro* evaluation of antibacterial and antioxidant activities of the essential oil and methanol extract of endemic *Zataria multiflora Boiss. Food Control*. 18, 800–805.

Sharif Rohani, M., 2004. Evaluation of antifungal effects of some plants escences as a possible replacement condition farm under with Malachite green in rainbow trout egg. A thesis for degree of Ph.D of Aquatic Animal Health and Disease. Tehran University.

Tsoumas, J. V., Alderman, D. J. and Rodgers, C. J., 1989. *Aeromonas salmonicida*, development of resistance to quinolone antimicrobials. *Journal of Fish Diseases*. 12, 493–507.

Zargari, A., 1990. Medicinal Plants, Tehran University Press, 4th Edn, Tehran. 1–57 pp.