Journal of Pharmacognosy and Phytochemistry

Available online at www.phytojournal.com

E-ISSN: 2278-4136
P-ISSN: 2349-8234
JPP 2019; 8(6): 1206-1209
Received: 20-09-2019
Accepted: 24-10-2019

Samira Zarei
Ph.D., Student, Department of Microbiology, Science, agricultural and new technology college, Islamic Azad University of Shiraz, Iran

Nima Bahador
Associate Professor, Department of microbiology, science, agricultural and new technology college, Islamic Azad University of Shiraz, Iran

Maryam Mirbakhsh
Iranian Fisheries Science Research Institute, Shrimp Research Center, Agricultural Research Education and Extension Organization AREEO, Bushehr, Iran

Mohammad Khalil Pazir
Iranian Fisheries Science Research Institute, Shrimp Research Center, Agricultural Research Education and Extension Organization AREEO, Bushehr, Iran

Corresponding Author:
Samira Zarei
Ph.D., Student, Department of Microbiology, Science Agricultural and New Technology College, Islamic Azad University of Shiraz, Iran

Antibacterial activity of aqueous extract of *Eucalyptus camaldulensis* in different salinity and temperature against *Vibrio harveyi* (PTCC1755) and *Vibrio alginolyticus* (MK641453.1)

Samira Zarei, Nima Bahador, Maryam Mirbakhsh and Mohammad Khalil Pazir

Abstract
Litopenaeus vannamei shrimps are one of the important aqueous proteins resources that has successfully cultured from 1990. Some bacterial diseases like *vibriosis* among *penaeus* shrimps have affected shrimp industry. *Vibrio harveyi* (PTCC1755) and *vibrio algynolyticus* (MK641453.1) bacteria cause *vibriosis* in *Peaeus shrimps*. But over use of antibiotics led to the emergence of drug resistant strains of bacteria and nature cause to find and replace natural therapeutic sources like *eucalyptus camaldulensis* leafs. In this study we determined antibacterial activity of aqueous extract of *eucalyptus camaldulensis* against *vibrio harveyi* (PTCC1755) and *vibrio algynolyticus* (MK641453.1) bacteria tested in different salinity and temperature Results showed that the most antibacterial activity of *eucalyptus camaldulensis* for both *vibrio harveyi* (PTCC1755) and *vibrio algynolyticus* (MK641453.1) was Respectively in 78 ppt at 35 ºC and 60ppt at 35 ºC. According to the results, it can be concluded that the aqueous extract of *eucalyptus camaldulensis* can have an inhibitory effect on vibriosis with *Vibrio harveyi* (PTCC1755) and *Vibrio algynolyticus* (MK641453.1) bacteria.

Keywords: Vibrio harveyi, antibiotic resistant, eucalyptus camaldulensis, eucalyptus camaldulensis, vibrio algynolyticus

Introduction
Aquaculture industry is one of the most important sources of protein in natural marine dietary supplement. There are many problems in this industry to gain economic success. One of the problems that still have remained is low survival rate of breeding species. Janartham reports shows aquaculture and breeding shrimps is one of the fastest grown food industry production with an annual growth of 16.8 percent among the industries. Figure and facts shows economic losses is about 3 billion $ in the word. Aquaculture pathogens caused by disease o shrimps. Aquaculture pathogen could threaten the aquaculture industries and reduce the production per unit area. One of the proposed solutions is enhancing survival improving quality of rearing larvae that will increase production (Hubbard, 2003) [10]. One of these pathogens is *Vibrio harveyi* (IS0PTTC1755) a gram negative, curved rod shape, motile, facultative anaerobe and oxidase positive bacteria that has identified and isolated from seawater (Mirbakhsh, *et al.*, 2014, Wijayati, 2004) [15] [24]. So this is necessary to use natural supplements to fight the bacteria and shrimp bacterial diseade (Jnarthatm *et al.*, 2012) [12].
Vibrionaceae is one of the main bacterial families associated to marine ecosystems that existing as normal and cause secondary *vibriosis* disease under certain condition such as stress, lack of vitamin C, high density, algal toxins and aquatic organisms viral disease. On the other hand indiscrimination of chemical agents, increasing pathogens resistance, so they need to combine new drug more than ever necessary (Forootan, 1391) [9]. Indiscrimination use of antibiotics as stimulus growth, leading the strains becomes resistance to treatment. This is significantly observed in shrmp industry, because large amount of antimicrobial agents have been used in shrimp's culture system because outbreaks of antibiotics resistance strains bacteria in Asian contries (Baticados *et al.*, 1990) [5].
Replacing of antibiotics by using new ways such as natural antibacterial substances bacteria or probiotics (Luis V, *et al.*, 1994) [12]. Researchers noted various sources, especially plants as new drugs. Essences of aromatic plants, is a great group of natural compounds with medicinal properties such as antimicrobial properties. For example eucalyptus plants (Mohammadpour *et al.*, 2013) [18], green tea (Gupta and Saxena, 2010) [17], cloves. This material is ecofriendly and has suggested as a safe and effective therapeutic method (Babayi and *et al.*, 2004; Jucki and

Khazaei, 2010; Mohammadi and *et al*., 2013) [4] [9] [16]. Among this material the essence oil and herbal extract of *eucalyptus camaldulensis* leaves can be cited. This plant belongs to the *Myrataceae* family that grows in tropical and subtropical earth and is rich of *polyphenols*, *terpenoides*, *cineol* (Ayepola and *et al*., 2008) [3].

In this paper we have studied the effect of *eucalyptus camaldulensis* extract in preventing bacterial contamination with *Vibrionaceae* family and due to the specific ecological conditions of shrimp breeding areas in the south of Iran that have specific climate and distinguish it from other countries, the biological activity of the plant extracts in different temperature, salinity was checked out.

Materials and methods

Preparation of plant materials and extract:

Fresh leaves of *eucalyptus camaldulensis* was collected from Bushehr agricultural college. The aqueous extraction was prepared by soxhlet (Adenyi and Ayepola. 2008) [3] and rotary method (shagal and *et al*. 2012) [17].

Soxhlet extract was prepared according to the Adenyi and Ayepola (2008) [3] method. In this case 25gr of dried powder of *eucalyptus camaldulensis* leaves were placed in the filter paper, then 250 ml of distilled water added to flask and heated (65 ºC) for 8 hours. Water extract was filtered into clean bottle glass and kept in refrigerator.

Rotary extract was prepared by shagal and *et al.* (2012) [17]. Water soxhlet extract placed in rotary system and worked for 2 hours in 40 ºC and kept in glass bottle in refrigerator.

Study of Retention of antibacterial effect of extract in different salinity

For aqueous extract of *eucalyptus camaldulensis*, 6 falcons with 3 repetitions were considered. Initially sterile sea water mixed with sterile distilled, and salt solutions of different concentration were 44, 50, 60 and 78 ppt in volume 5 prepared with a ratio of 1:1 was added to the tubes of *eucalyptus camaldulensis* extract and final salt solution was measured by refract meter. After 30 minutes harvested from treatments and according to the well diffusion method the on Muller Hinton agar the effect of antimicrobial extracted substances from the under study bacteria was evaluated. The diameter of inhibition for each treatment and its repetition was measured and recorded by caliper.

Study of Retention of antibacterial effect of extract in different temperature

In order To determine the effects of *Eucalyptus camaldulensis* extract on the study bacteria during temperature (at 35 ºC, 40 ºC, 45 ºC, 55 ºC, 65 ºC, 75 ºC, 85 ºC, 100 ºC), extracts was monitored and its antibacterial properties studied by well diffusion method on Muller Hinton agar, also inhibition zone was measured and recorded by caliper.

Results

Antibacterial effect of extract in different salinity on *vibrio harveyi* and *vibrio algynolyticus* bacteria

The results showed that despite the higher level of biological activity of *Eucalyptus camaldulensis* extracts So far on Vibrio bacteria *Vibrio Harveyi* to *vibrio algynolyticus* on salinity of 44ppt no significant difference was observed ($P>0.05$). it wasWhile the amount of biological activity of *Eucalyptus camaldulensis* extracts in the salinity of 50 ppt on *Vibrio Harveyi* was significantly higher than the biological activity of aqueous extract of the *Vibrio alginolyticus* bacterium ($P<0.05$). But despite the higher biological activity of *Eucalyptus camaldulensis* extracts on *Vibrio Harveyi* in salinity degrees of 60 and 78 ppt compared to the biological activity of extracts So far *Eucalyptus camaldulensis* on *Vibrio alginolyticus*, no significant difference was observed ($P>0.05$).

Table 1: Biological activity values (mean ± standard deviation) of aqueous extract of Eucalyptus camaldulensis on Vibrio Harveyi and Vibrio algynolyticus in different salinity levels (non-identical letters in each column indicate significance and the same letters indicate no significance with the level 95% confidence).

Bacteria	44 ppt	50 ppt	60 ppt	78 ppt
Vibrio harveyi	***a***74/95±2/20	***a***96/95±6/70	***a***105/64±4/87	***a***120/30±3/11
Vibrio alginolyticus	***a***45/79±1/01	***b***49/11±1/38	***a***71/36±3/78	***a***52/98±2/10

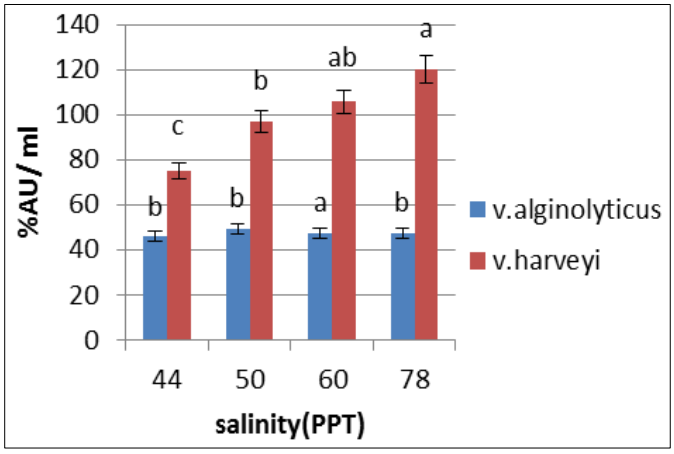

Fig 1: Biological activity values (mean ± SD) of aqueous extract of *Eucalyptus camaldolensis* leaf on Vibrio *harveyi* and vibrio *alginolyticus* at different salinity levels (non-identical letters indicating significant and similar letters not significant with confidence level).

Antibacterial effect of extract in different temperatures on *vibrio harveyi* and *vibrio alginolyticus* bacteria

The results showed that the maximum and minimum level of biological activity of the aqueous extract of *Eucalyptus camaldulensis* leaves on *Vibrio Harveyi* bacterium at different temperatures was at 35 °C with 133. 06% and at 100 °C with 72 / 93%. On the other hand, it was observed that although the biological activity of the aqueous extract of *Eucalyptus camaldulensis* leaves at 35 °C was higher than 40, 45, 55, 65 and 75 °C, but no significant differences were observed ($P>$ 0.05). At the same time, the percentage of biological activity at 35 °C was significantly higher than the values obtained at 85 °C and 100 °C ($P<$ 0.05). On the other hand, it was observed that despite the higher percentage of biological activity at 85 °C in comparison with the temperature of 100 °C, No significant statistical differences were observed ($P>0.05$). The maximum and minimum percentage of biological activity of aqueous extract of *Eucalyptus camaldulensis* leaves on *Vibrio alginolyticus* bacterium was observed at 35 °C with 139.66% and 100 °C with 99.43%.

results showed that despite the higher percentage of biological activity at 35 °C than the temperature of 40 °C, no significant difference was found (P>0.05), while this value was significantly higher than the values obtained At temperatures 45, 55, 65, 75, 85 and 100 degrees Celsius (P< 0.05). On the other hand, it was observed that despite the higher percentage of biological activity at 40 °C temperature, there was no significant statistical difference between the 45, 55, 65, 75 and 85 temperatures (P>0.05), but the obtained values Was significantly higher than biochemical percentages at 100 °C (P< 0.05) (Figure 3-10).

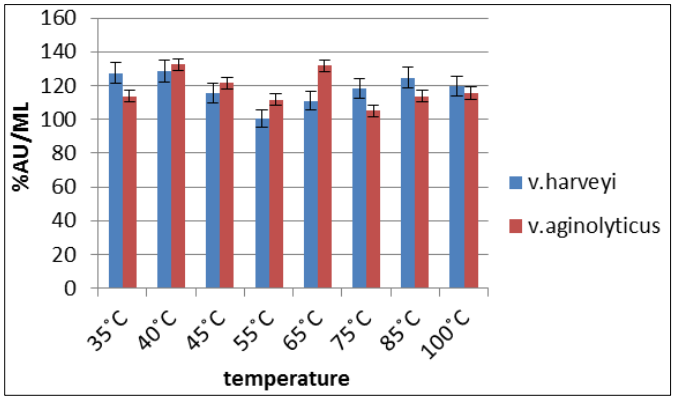

Fig 2: Biological activity of *Eucalyptus Camaldolensis* Leaf Extract at Different Temperatures on *Vibrio Harveyi* and *vibrio alginolyticus* Bacteria

Discussion

With the respect to Reproduction and development of shrimp farming industry over the past twenty years, the risk of outbreak of some disease has threatened this industry. One of the most important pathogenic agents is bacterial agents belong to Vibrionaceae family that cause vibriosis diseases in Penaeidae shrimps and cause reducing the growth and survival of hatchery and shrimp farms (Saulnie *et al*.., 2000) [18]. The accurate identification and control factors have been one of the main concerns in shrimp breeding and proliferation canters. Considering the results of chemical and molecular tests obtained two species of bacteria respectively *vibrio harveyi* (IS01PTTC1755), (Mirbakhsh *et al.*, 2013) [15] and *Vibrio alginolyticus* (MK641453.1), (Mirbakhsh *et al.*, 2014) [15]. Identified and recorded in the world gene bank. Today, various ways, including use of Chemical disinfectants and broad-spectrum antibiotics such as oxy tetracycline are used for controlling and management of the mentioned factors, thus, because of drug resistance and stay this material in shrimp´s tissue use of this material has prohibited (Noriega-Orozco *et al*., 2007; Mohamadi *et al*., 2013) [16, 13]. The use of eco-friendly materials such as extracts, essential oils and flavors from the leaves of plants As a safe and effective therapeutic method has been suggested (Babyi *et al*., 2004; Jucki and Khazaei, 2010; Mohammadi *et al*., 2013) [4, 9, 16]. Also these compounds have antibacterial, antifungal, antiviral and antioxidant is able to stimulate the immune system and prevent the growth of pathogens (adebola *et al*., 1999; Takasaki *et al*., 2009; Alizadeh Behbehani *et al*., 2013) [1, 19, 2]. Among these materials extracted from the leaves of herbs and essential oils of Eucalyptus species camaldulensis can be cited that is Which is a rich source of polyphenols; Terpenoides, cineol and bases compounds (the ayepola *et al*., 2008) [3].

In order to determine the biological activity of two bacterial strains, Vibrio Harveyi and Vibrio alginolyticus were used in different conditions. The results showed that the maximum biological activity of the aqueous extract of Eucalyptus camaldulensis leaves in both species of vibrio has shown at low temperatures, so that with increasing temperature, this activity gradually decreases, but it can be noted that the increase The temperature, breaking some of the chemical bonds and destroying some of the extracted compounds, reduce the amount of biological activity and antibacterial effects of the aqueous extract (Giles *et al.*, 2009) [6].

The salinity of the *Vibrio harveyi* bacteria used in this study is noteworthy that this species is able to create more virulent in saline (Kannapiran *et al.*, 2009; Selven and Philip, 2013) [9]. On the other hand, due to the high salt-tolerant by *eucalyptus camaldulensis* plant it could grow easily in dry saline areas. Hence, in this study it was observed that the amount of salinity of *eucalyptus camaldulensis* water extracted plant is 22 ppt. Therefore, given that the most virulent bacteria Vibrio activities at salinities are above 35ppt, but the results of the biological activity of different salinity levels suggests that with the increase in salinity biological activity of aqueous extract of this plant will increase. Also, given that the bacterial strain used in the study are able to create more virulent in high salinity levels, however, no significant differences in the biological activity of aqueous extracts of both species of Vibrio bacteria were observed at different salinity levels. However, due to the salt water shrimp farms in Bushehr province is in the range of 55-45ppt, uses of this extract at these culturing environments can be effective.

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