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Study and comparison of antibacterial activities of extracts of *Zataria multiflora* and *Teucrium polium* on *Penibacillus alvei*

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ABSTRACT. In this study, the antibacterial activity of ethanol and methanol extracts of *Zataria multiflora* and *Teucrium polium* was determined against *Paenibacillus alvei* by disc diffusion method. *Paenibacillus alvei* is one secondary bacterium for the European foulbrood disease in honey bee.Minimum inhibitory concentration and minimum bactericidal concentration were determined by using the serial dilution method. For this, *Z. multiflora* and *T. polium* are collected from different areas of Iran then they are dried and extracted in lab. The antibacterial effect of alcoholic extracts of *Z. multiflora* and *T. polium* was lower than usual standard antibiotics ($P<0.01$), but the ethanol and methanol extracts of *Z. multiflora* at a concentration of 60mg/ml, have inhibitory and lethal effects on *P. alvei.* Also, 100mg/ml concentrations of ethanol extract of *T. polium* has inhibitory and lethal effects on this bacterium. But, the no one of used concentrations of its methanol extract has inhibitory and lethal effects. Results indicated that used extracts of *Z. multiflora* have the higher antibacterial effects than extract of *T. polium* on *Paenibacillus alvei*. It can be concluded that regarding the high antibacterial power of *Z. multiflora*, it is necessity to work on how they can be used in control and treatment of bacterial honey bee diseases.

Keywords: Paenibacillus alvei, Zataria multiflora, Teucrium polium, antibacterial effects, honeybee,

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INTRODUCTION

Honeybee belongs to Hymenoptera order, Apidae family and *Apis mellifera* species. The most important act that honeybees do is pollinate, in addition they produce honey, wax, polen, royal jelly, venom and propolis. Moreover, in the most flowered plants, 90% of the pollination has been done by bees (Bartomeus et al., 2014).

The European foulbrood is one of diseases which threaten the bee breeding industry especially in Iran (Shahrestani, 2015). This disease kills honeybee larvae, which could cause significant damage to the beekeeping industry. The causative agent of EFB is *Melissococcus pluton.* However, *Paenibacillus alvei* was initially thought to be the causative agent of EFB due to its isolation from affected larvae (Forsgren, 2010). The presence of P. alvei is used as an indicator of EFB as its growth produces a characteristic odour. *Paenibacillus alvei* is a gram-positive, saprophytic, aerobic, and spore-forming bacterium which causes secondary infections in already infected larvae. *P. alvei* is frequently the first indication of the presence of *M. pluton* and is almost always isolated together with the primary aetiological agent (Forsgren, 2010).

Although, the use of antibiotics is not permitted by European regulations since the year 2000, several antibiotics are still legally used against bacterial diseases of honeybees in many third countries as Iran. But due to being unaffected on the spore of mentioned bacterium, there is no final treatment. On the other hand, the remaining of pharmaceutical materials in the products of honeybees causes the dangers for the consumers of these products (Molino et al., 2011). Therefore, some effective solutions and low risk medicines should be replaced concerning the health of man and bees.

Some plants have incredible effects in treatment of infectious diseases such as antibiotics. Plants are rich in an extensive variety of secondary metabolites such as tannins, alkaloids and flavonoids which have been found to have antimicrobial properties, in vitro (Lewis and Ausubel, 2006). There are several reports about the antimicrobial activity of herbal extractions. According to the World Health Organization (WHO), medicinal plants would be the best source to obtain a variety of drugs and active compounds (Al Akeel et al., 2014).

Two of the plants that have been studied antibacterial properties in previous reports, are *Zataria multiflora* and *Teucrium polium*.

Zataria is a genus of flowering plant in the Lamiaceae family, first described in 1876. It contains only one known species, *Zataria multiflora*, native to south western Asia (Iran, Afghanistan, Pakistan, and Kashmir) (Manikandan et al., 2012).

Z. multiflora with the vernacular name of Avishan-e-Shirazi in Iran is used traditionally in foods as a flavor ingredient. Also, it is used as antiseptic, anesthetic, and antispasmodic, over the centuries (Hosseinzadeh et al., 2000; Ramezani et al., 2005).

The antibacterial activity of *Z. multiflora* has been shown against a number of Gram-positive and Gram-negative bacteria (Moshafi et al., 2007; Saleem et al., 2004; Abbasgholizadeh et al., 2008; Ettehad and Arab, 2007)

Teucrium polium, belong to the Lamiaceae family, is a sub-shrub and herb native to the western Mediterranean region (Albania, Spain, France, Algeria, Morocco, Tunisia). In traditional Persian medicine, *T. polium* (locally called 'kalpooreh') is used as an anti-hypertensive, anti-bacterial, carminative, anti-nociceptive, anti-inflammatory, anti-diarrhea, anti-diabetes and anti-convulsant agent. Today, the researchers investigate these properties, scientifically (Mohammadpour et al., 2015).

This paper mainly aims to determine the sensitivity value of *P. alvei* to alcoholic extracts (ethanol and methanol) of *Zataria multiflora* and *Teucrium polium*. Also, calculate the minimally inhibitory concentration (MIC) of *P. alvei* growth by the means of these two elements.

MATERIAL AND METHODS

Preparation of Zataria multiflora and Teucrium polium extracts

It has been specified that biologic activities of a sample depend on the applied methodology to prepare the desired extract. The most common used materials to provide the extract in biologic methods are ethanol and methanol.

The leaf *T. polium* and *Z. multiflora* were collected in the different region of Iran. The taxonomic identification of these plans was done by herbarium in Graduate University of Advanced Technology in

Kerman. Aerial parts of the plants dried at dark place and room temperature. Samples were ground to a fine powder and transferred into glass container and preserved until extraction procedure was performed in the laboratory. Then, 25g of these powders were separately mixed with 250ml alcohol (ethanol and methanol) and shaken by a rotator (150 rounds in 1min) at room temperature for 48 hr (Van Wyk and Wink, 2004). Afterwards, the alcoholic extract was filtered by the Whatman filter paper No.1 and the alcohol was evaporated by the use of a rotary device; finally, pure alcoholic extract was achieved. These extract of plans should be completely dried within 48 hours at 40 °C then, for providing the required concentrations; the certain weight of each dried extract was dissolved in appropriate volume of ethanol and methanol in order to obtain the desired concentrations.

Preparation of appropriate concentrations of used bacteria

Paenibacillus alvei strain NRS662, was prepared in culture medium of nutrient broth of Iranian Razi Institute. It was put in the incubator shaker at 37 °C for 48 hours in order to make the bacterial vaccine. After the growth, the bacteria were cultured in the agar nutrient medium. Afterwards, several colonies from the plates were added to Mueller-Hinton broth by the help of a sterilized loop. The inoculums of microorganisms were standardized according to the 0.5 McFarland standards which correspond to 108 colony-forming units. In next stage, 500 micro liters of resultant standard bacterial vaccine were cultured by a sterilized loop in the solid agar Mueller-Hilton culture medium.

Disk diffusion

After getting different concentrations of desired extracts (serial doubling dilutions of the extracts were prepared in a 96-well microtiter plate ranged from 10.0 mg/ml to 100.0 mg/mL), Whatman filter papers No. 2 were cut by a puncher and saturated with filter sterilized plant extracts at the prepared concentrations for 2 hours. Then, they were allowed to dry at 37°C for 5 hours. The two discs prepared in the same condition with only the corresponding volume of ethanol and methanol (1ml), were used as negative control. Each of the discs was placed on culture mediums and then the plates were incubated at 37 °C for 18 hours. The free zone around each disc indicates an area with no bacteria growth. Zones of inhibition were measured in mm.

In addition, a disc of Tetracycline (used antibiotics in the industrial beehives) was provided with a specific concentration in order to cure European foulbrood was used as positive control. All the experiment stages were repeated three times for each sample and every inhibitory zone diameter was measured by the digital caliper.

MBC (minimum bactericidal concentration) and MIC (minimum inhibitory concentration) determination

Determination of the MIC was carried out using the macro broth dilution method as recommended by the Clinical and Laboratory Standards Institute using Mueller-Hinton broth as the test medium. Overnight cultures of bacteria were diluted to yield a final concentration of 5×105 CFU/ml. The reconstituted extracts were serially diluted in two-fold in MHB medium to obtain various concentrations of the stock (10-100 mg/ml) and were assayed against the test bacteria. In the following, 1 ml of standardized inoculum (5×105CFU/ml) was added to 1ml of each extract concentration. Then, all tubes were incubated at 37°C for 18h and MIC was defined as the lowest concentration that was able to inhibit bacterial growth. Three control tubes were maintained for each test batch. These included tube containing extract and growth medium, tube containing the growth medium and inoculums, and tube containing the inoculum and standard antibiotic.

MBC values were determined using sub-culturing 150 µl of bacterial suspension from the MIC tubes into MHA plates and then incubated at 37°C for 18 h. After incubation, the concentration at which no growth was seen was recorded as the MBC.

Statistical analysis

To measure the diameter of inhibition zone, data were statistically analyzed using ANOVA method and SAS software (version 9.1) and means comparison has been done by Tukey method and proc GLM, also Pearson correlation between concentration of

Table 1. Average zones of inhibition of a standard antibiotic, Z. multiflora and T. polium ethanol and methanol extracts tested against Paenibacillus alvei cultures (mm).

Antibacteris / bacteri	Ethanol *Z. multiflora* extract	Methanol *Z. multiflora* extract	Ethanol *T. polium* extract	Methanol *T. polium* extract	Sterilized distilled water	Ethanol 96%	Methanol 96%	Standard antibiotic
Paenibacillus alvei	8.612±0.961^{b}	8.505±0.680^{b}	7.77±0.714^{bc}	6.954±0.311^{c}	*	*	*	18.07^{a}

*No growth zone. In each row, mean with the same uppercase letter is not significantly different at 5% level

the extracts and diameters of inhibition zones were calculated by proc CORR.

RESULTS

The antibacterial effect of alcoholic extracts of plants *Z. multiflora* and *T. polium* were observed against P. alvei, but it was lower than Tetracycline (P<0.01) (Table 1).

As it has been shown in Table 2, the diameter of free zone which inhibits the growth of bacteria had a direct relationship with the amount of *Z. multiflora* and *T. polium* existing in the discs. The correlations between concentration of the extracts and diameters of inhibition zones were 0.98 and 0.96 for *Z. multiflora* and *T. polium* extracts, respectively. The diameters of inhibition zones of the extracts dilutions were increased with respect to the concentration of the extracts (Pmax=0.044 and Pmin=0.001).

In order to determine the MIC and MBC values, two fold serial dilutions from concentration of 10 mg/mL to 100 mg/mL were used. Both MIC and MBC values for Z. multiflora and T. polium against P. alvei were equal to 60 and 100 mg/ml respectively.

DISCUSSION

Antibacterial effect of Z. multiflora

Today, increased resistance to antimicrobial agents has spread (Saga and Yamaguchi, 2009). So, researchers begin to evaluate the effect of plants and their active compounds for finding new and relatively low-risk compounds from different natural plants (Raskin et al., 2002). On the other hand comparing the antibacterial effect of these plants is important for choosing the most appropriate ones. In this study, the effect of alcoholic extract of two species of Labiatae family against *P. alvei* were investigated and compared.

Results indicate that the *Z. multiflora* plant extract have considerable antibacterial effects against *P. alvei* in vitro, but we do not know if the extract of this plant can control EFB in field experiments (in vivo). This research has shown that the antibacterial effect of alcoholic extract of *Z. multiflora* has been increased by the increased concentration.

The *Z. multiflora* is a plant of genus Thymus; the plants of this genus contain many phytochemical substances including terpenoids and phenolics (Fazeli et al., 2007; Guillen et al., 1998; Fatemi et al., 2012). Two of the most important secondary compounds of this genera are fairly known; volatile oils and phenolic compounds. On the other hand the most pharmacological effects of Thymus genus are because of these two classes of compounds (Simeon de Bouchberg et al., 1976; Stahl-Biskup and Saez, 2002). Among terpenoids, the phenolic terpenes; thymol and carvacrol, rank highest in importance (Shafizade, 2002). Studies have shown that these phenolic compounds, especially carvacrol (a main constituent of Avishan-e-Shirazi essential oils) have a high antibacterial effect (Nejad Ebrahimi et al., 2008; Baydar et al., 2004). Moreover, it is possible that low-concentration components or interaction between some of the constituents are responsible for the antibacterial effects (Stanković Nemanja et al., 2011).

Antibacterial effect of T. polium

T. polium is one of the most important medicinal plants that extensively used in traditional medicine in Iran. The previous researches have shown that ethanolic and methanolic extracts of *T. polium* aerial parts are effective against both gram positive and gram negative bacteria. However, studies have indicated that hydroalcoholic extract of *T. polium* is more effective in positive gram bacteria

Table 2. Average zones of inhibition in different Concentrations of *Z. multiflora* and *T. polium* extracts against *Paenibacillus alvei* cultures

Antibacterial materials Concentration (mg/mL)	Ethanol *Z. multiflora* extract (mm)	Metanol *Z. multiflora* extract (mm)	Ethanol *T. polium* extract (mm)	Methanol *T. polium* extract (mm)
10	6.8^{iA}	6.5^{hB}	6^{hC}	6.5^{fB}
20	7.2^{hA}	6.75^{ghB}	6^{hD}	6.5^{fC}
30	7.5^{gA}	7.06^{gB}	6^{hD}	6.5^{fC}
40	8^{fA}	7.57^{fA}	6.57^{gB}	6.78^{eB}
50	8.2^{fA}	8.09^{eA}	7.73^{fB}	6.85^{deC}
60	8.5^{eA}	8.7^{dA}	8.13^{eB}	6.97^{dC}
70	9^{dB}	9.16^{cA}	8.76^{dC}	7^{dD}
80	9.32^{cB}	9.8^{bA}	9^{cC}	7.18^{cD}
90	10.28^{bA}	10.08^{bA}	9.2^{bB}	7.52^{bC}
100	11.32^{aA}	11.34^{aA}	10.31^{aB}	7.74^{aC}

Note: In each column, mean with the same lowercase letter is not significantly different at 5% level. In each row, mean with the same uppercase letter is not significantly different at 5% level

in comparison with gram-negative ones due to the difference of cellular wall structure or other genetic factors of gram-positive and gram-negative bacteria (Darabpour et al., 2010).

The antibacterial activity of the *T. polium* extract might be due to its richer in the phenols and flavonoids Compounds (Chedia et al., 2013). It is known that polyphenols are bioactive molecules. These biological activities are related to the molecules structures; by their hydroxyl groups or by phenolic ring, phenolics compounds have capacity to link with proteins and bacterial membrane to form complexes (Zongo et al., 2011). The antibacterial compounds of plants may inhibit bacterial growth by different mechanisms. Therefore, these plants may have a significant clinical value in treatment of resistant bacterial strains (Sarac and Ugur, 2007).

Antibacterial properties of ethanolic and methanolic extracts of *T. polium* against some of clinical pathogens were studied by Darabpour et al. (2010), the minimum inhibitory concentration (MIC) against *Staphylococcus aureus* and *Salmonella typhi* was 40mg/mL and *Bordetella bronchiseptica* and *Bacillus anthracis* was 10 mg/mL. The minimum bactericidal concentration (MBC) against *Bacillus anthracis* was 10 mg/mL while against other species were not found (>200 mg/mL).

Mahboubi et al. (2014) have investigated the antibacterial effect of *Zataria multiflora* against four foodborne and four other bacteria including *Staphylococcus aureus, Shigella dysenteriae, Salmonella typhimurium, Escherichia coli, Staphylococcus epidermidis, Bacillus subtilis* and *Pseudomona aeruginosa*. This study showed that Z. multiflora was effective with MIC values between 0.78-3.125 mg/mL against all of the bactria.

Haider et al. (2014) have studied antibacterial activity of northern Ontario medicinal plant extracts. In their study, the antibacterial activity (in vitro) of the leaf or flower extracts of Anaphalis margaritacea *L., Grindelia squarrosa* (Pursh), *Apocynum androsaemifolium L., Arctostaphylos uva-ursi L., Cornus canadensis L.* and *Xanthium strumarium L.* (medicinal plants) was analyzed through the hole-plate diffusion, minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) assays against *Escherichia coli, Aeromonas caviae, Paenibacillus alvei, Micrococcus luteus, Mycobacterium avium* subsp. avium and *Bacillus cereus* bacteria. The leaf and flower extracts of *Anap.* margaritacea and *G. squarrosa* have a significant antibacterial activity against all the bacteria tested, with inhibition of *A. caviae*, *P. alvei* and *M. luteus* within 1-12 h of incubation at MBC.

In present research, the antibacterial effects of ethanol and methanol extracts of *Z. multiflora, T. polium* and standard antibiotic concerning *P. alvei* in honeybees were compared. Results have shown that there is a meaningful difference between the effects of used extracts and common antibiotic on P. alvei ($P<0.01$). As well, the ethanol and methanol extracts of *Z. multiflora* were significantly higher than ethanol and methanol extracts of *T. polium* ($P<0.01$). However, four extracts have significant antibacterial activities on the used bacteria.

In previous studied, it has been demonstrated that regarding European foulbrood, tetracycline antibiotics had stronger effects on European and American foulbrood diseases (Mutinelli, 2003). The water extract of 10 plant species were tested by González and Marioli (2010) as inhibitors for the growth of *Paenibacillus larvae*, the causative agent of American Foulbrood. *Achyrocline satureioides, Chenopodium ambrosioide, Eucalyptus cinerea, Gnaphalium gaudichaudianum, Lippia turbinata, Marrubium vulgare, Minthostachys verticillata, Origanum vulgare, Tagetes minuta* and *Thymus vulgaris* were included in their study. Results showed the growth of almost all the *P. larvae* strains tested was inhibited by these extracts. However, no research has been carried to study antibacterial effect of plant extracts against EFB or AFB in field experiments.

CONCLUSION

Finally, this research results indicated that ethanol and methanol extracts of *Z. multiflora, T. polium* have the antibacterial effects on *Paenibacillus alvei* under laboratory conditions, and if more studies are conducted, the main active substance can be achieved by the means of these products. By the way, antibacterial effect of plant extracts may be different under field experiments.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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