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Short communication. Inhibition of *Paenibacillus larvae* subsp *larvae* by the essential oils of two wild plants and their emulsifying agents

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Abstract

In honey bees (*Apis mellifera* L.), American foulbrood (AFB) is caused by the infection of the larvae and pupae with the bacterium *Paenibacillus larvae* subsp. *larvae*. The antibacterial effects of the essential oils of wild camomile (*Tagetes minuta* L.) and Andean thyme (*Acantholippia seriphioides* A. Gray) against different strains of *P. larvae* subsp. *larvae* were evaluated *in vitro*. The possible inhibitory effects of two emulsifiers of these oils (propylene glycol and soybean lecithin) were also assessed. Significant differences in antibacterial action were seen between the oils and emulsifiers ($P < 0.01$). Andean thyme had the strongest antibacterial effect; the minimum inhibitory and minimum bactericide concentrations were 200-250 mg l^{-1} and 300 mg l^{-1} respectively. An additional inhibitory effect was seen when propylene glycol was used as an emulsifier; no such extra effect was seen when soybean lecithin was employed.

Additional key words: *Acantholippia seriphioides,* bacteria, honey bee, propylene glycol, soybean lecithin, *Tagetes minuta*.

Resumen

Nota corta. Evaluación de aceites esenciales de dos plantas silvestres y sus emulsificantes utilizados en tests de inhibición de *Paenibacillus larvae* subsp. *larvae*

La patología conocida como loque americana, que afecta a los estadios de larva y pupa de abejas (*Apis mellifera* L.), es causada por la bacteria *Paenibacillus larvae* subsp. *larvae*. Se evaluó el efecto antibacteriano *in vitro* de los aceites esenciales de manzanilla silvestre (*Tagetes minuta* L.) y tomillo andino (*Acantholippia seriphioides* A. Gray) frente a diferentes cepas de *P. larvae* subsp. *larvae*, así como también el posible efecto inhibitorio adicional del propilenglicol y la lecitina de soja, utilizados como emulsionantes de los aceites esenciales. Los resultados de la evaluación *in vitro* indican diferencias significativas entre los dos aceites esenciales emulsionados ($P < 0{,}01$). El tomillo andino presentó el mayor efecto inhibitorio con valores de concentración inhibitoria mínima y concentración bactericida mínima, entre 200-250 mg l^{-1} y 300 mg l^{-1} respectivamente. Se observó un efecto inhibitorio adicional producido por la utilización de propilenglicol como emulsionante y la ausencia de dicho efecto para el caso de la lecitina de soja.

Palabras clave adicionales: abeja melífera, *Acantholippia seriphioides,* bacterias, lecitina de soja, loque americana, propilenglicol, *Tagetes minuta.*

American foulbrood (AFB), caused by the bacterium *Paenibacillus larvae* subsp. *larvae* is one of the main problems for beekeepers worldwide. Much effort has gone into controlling this disease, including the use of preventive and curative treatments with antibiotics and other antimicrobial substances. Unfortunately, the extensive use of antibiotics leads to an accumulation of residues in beehive products (especially in honey), decreasing their quality and making their marketing more difficult.

Many authors have worked on preventive and curative treatments for AFB (Gochnauer, 1970; Machova *et al.,*

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1992) while others have developed methods of control. A range of substances of natural origin have been found to show antimicrobial activity against *P. larvae* subsp *larvae*, such as the essential oils of cinnamon (Floris and Carta, 1990), savory, thyme, lemon grass, oregano, eucalyptus, peppermint, rosemary, lavandin, basil and others (Alippi *et al.,* 1996; Floris *et al.*, 1996; Albo *et al.*, 2001, 2003).

Tagetes minuta L., commonly called wild camomile, *chinchilla*, *suico* or *chilca*, etc. (Retamar, 1982), is a herbaceous plant belonging to the family Compositae; a native of Central America, it grows as a wild species in the north of Argentina. The antimicrobial activity of wild camomile on the spores and vegetative cells of *Clostridium botulinum* and *Bacillus cereus* has previously been demonstrated (Chaibi *et al*., 1997). *T. minuta* oil can be effective in the control of coleoptera (Weaver *et al*., 1997), mosquitoes and other insects (Wells *et al*., 1992), helminthes (Oduor-Owino and Waudo, 1994), bacteria (Tereschuk *et al*., 1997) and fungi (Zygadlo *et al*., 1994). A certain toxic effect is also seen when *Varroa destructor* mites are pulverized with low concentration solutions of this oil (Ruffinengo *et al.,* 2001).

Acantholippia seriphioides A. Gray*,* usually called Andean thyme although it is a native of the Western Mediterranean, is an aromatic, perennial sub-bush belonging to the Verbenaceae. In Argentina it grows wild, although it is also cultivated in the Northwest, the San Luis and Córdoba regions, and in the northwest of the Buenos Aires province. The literature contains no references on the use of this species to control AFB.

The essential oils of wild camomile and Andean thyme contain different compounds that might be related to their antimicrobial activities. β-ocimene is the main component of wild camomile oil (62.8%), and thymol the major component of Andean thyme oil (29.2%) (M. Ponzi, UNSL, Argentina, pers comm).

Evaluating the antimicrobial activity of essential oils is difficult because of their volatility, their insolubility in water and complex chemistry. Factors such as the assay technique, growth medium, micro-organism characteristics and the oil itself are important factors that need to be taken into account in any attempt (Janssen *et al.,* 1987; Paxton, 1991; Cowan, 1999; Hammer *et al*., 1999).

Propylene glycol (1,2-propanediol) is commonly used as an emulsifier since it is immiscible in common oils. Moreover, it is used as inhibitor of fermentation and mould growth, and in mist form it can be used to disinfect the air (The Merck Index, 1996). Lecithins are frequently used in the food industry as emulsifiers, viscosity regulators and dispersing agents: soybean lecithin promotes oil/water emulsions (Pan *et al*., 2002).

The aim of this work was to compare the antibacterial activity of the essential oils distilled from wild camomile and Andean thyme against different strains of *P. larvae* subsp. *larvae*, and to evaluate the possible additional effect of the two emulsifiers mentioned above.

Essential oils were extracted from the flowers of *T. minuta* and *A. seriphioides* [collected in Fraga, Province of San Luis (Argentina) during February/March 2004]. Steam distillation was performed for 4 h using stainless steel equipment, according to the Aldicara method (1976). The distilled oils were preserved at –20ºC, dissolved in dimethyl sulphoxide and filtered before use.

Three strains of *P. larvae* subsp. *larvae* were isolated from the honeycombs of hives showing clinical symptoms of AFB. These hives were located at Miramar (38°15'S-57°50'W), Balcarce (37°52'S-58°15'W) and Vidal (37°27'S-57°44'W), localities in the south of the Province of Buenos Aires.

Bacterial strains were isolated and identified using the techniques of Gordon *et al*. (1973) and Alippi (1991, 1992). Cultures were confirmed according to biochemical and physiological tests (Jelinski, 1985) and through the use of API CH50 kits (Api System, BioMérieux S.A., Marcy l'Étoile, FR). *P. larvae* subsp. *larvae* were stored at –20ºC on MYPGP agar (Mueller-Hinton yeast extract-glucose-sodium pyruvate) with 20% v v^{-1} glycerol until use.

Vegetative cells of *P. larvae* subsp. *larvae* were grown on MYPGP agar for 48 h at 35 ± 0.5°C and then suspended in double distilled sterile water and standardized to a turbidity of about 10^7-10^8 cells ml^{-1} (FDA, 1998). This concentration corresponds to an absorbance of 0.258 at 620 nm as measured using a Bausch and Lomb spectrophotometer (Spectronic 20, USA).

Emulsions of the essential oils (both *T. minuta* and *A. seriphioides*) were made by mixing them in sterile, double distilled water with 1% (v v^{-1}) propylene glycol (1-2 propanediol, Merck), or in sterile double distilled water with 1% (w v^{-1}) soybean lecithin (granulated). Both types of emulsion were prepared with vortex agitation.

Bacterial serial suspensions were cultivated in Mueller-Hinton broth (2.0 g l^{-1} meat extract, 17.5 g l^{-1}

hydrolysed casein and 1.5 g l^{-1} starch). This medium is appropriate for the growth of both aerobic and facultative anaerobic microorganisms, it shows no sulphonamide antagonism, and is routinely used to evaluate the sensitivity or resistance of bacteria to different substances (Merck, 1994; NCCLS, 1999). A stock solution was then made of each of the emulsified essential oils. One millilitre of each was added to Muller-Hinton broth and serially diluted (final range 50-1500 mg l^{-1}) Microbial biomass suspensions (0.5 ml) were then added to each serial dilution tube (at room temperature) using a Vortex dispersing tool (Fbr® by Decalab SRL) with agitation. All sample tubes (as well as positive and negative controls) were incubated at 35 ± 0.5°C for 48 h to determine the minimal inhibitory concentration (MIC). This value is the lowest concentration of an antimicrobial agent capable of inhibiting bacterial growth (the last dilution of a series in which there is no growth of microorganisms) (Lenettte *et al.,* 1987).

Known volumes were transferred from MIC-negative tubes onto MYPGP solid agar (Dingman and Stahly, 1983) amended with 9 µg ml^{-1} nalidixic acid (Alippi, 1992), and incubated at 35 ± 0.5°C for 48 h to determine the minimal bactericide concentration (MBC). This is the lowest concentration of an antimicrobial agent capable of killing 99.9% of the initial biomass (the last dilution of a series for which no colonies are produced) (García Damiano, 1991).

Positive and negative controls for the propylene glycol and soybean lecithin were established; Mueller-Hinton broth and MYPGP agar controls were also used. All MIC and MBC tests were performed in triplicate.

The MIC and MBC data obtained with each essential oil/emulsifier were compared by two-way ANOVA. This measured the differences between the bacterial strains, the treatment data, and their interaction. Significance was assessed using the Student t test.

Table 1 shows the mean MIC and MBC values for the three strains of *P. larvae* subsp. *larvae* in the presence of the essential oils and both the propylene glycol and soybean lecithin emulsifiers. For the essential oil of *T. minuta*, the MIC values were 700-800 mg l^{-1} when propylene glycol was used as the emulsifier, and 800-900 mg l^{-1} when soybean lecithin was used. The MBC values were between 900-1000 mg l^{-1} with propylene glycol and between 900-1100 mg l^{-1} with soybean lecithin. For the essential oil of *A. seriphioides*, the MIC values were 200 mg l^{-1} when propylene glycol was the emulsifier, and 200-250 mg l^{-1} when soybean lecithin was used. The MBC values observed were the same for both the propylene glycol and soybean lecithin emulsifiers: 300 mg l^{-1}.

No significant differences were found in the MIC for the different bacterial strains (Balcarce, Miramar and Vidal) ($P < 0.05$), although very significant differences were seen between the essential oils with the different emulsifiers ($P < 0.01$). The same was seen for the MBC results. When propylene glycol was used as the emulsifier, smaller concentrations of both essential oils were required to achieve bacterial inhibition.

Alippi *et al.* (2001) determined that the highest antibacterial activity *in vitro* against *P. larvae* subsp. *larvae* was provided by the essential oils of lemon grass [*Cymbopogon citrates* (DC.) Stapf], thyme (*Thymus vulgaris* L.) and wild camomile, with MIC values of between 50-100, 100-150 and 500-650 mg l^{-1} respectively.

In the present work, the MIC values for Andean thyme with both emulsifiers were 200-250 mg l^{-1} —a slightly inferior inhibitory action than that observed by Alippi *et al.* (1996) when using propylene glycol as an emulsifier of thyme oil. However, these differences could be due to the different percentages of thymol present in the two oils— 29.2% in Andean thyme and 39.9% in thyme. Thymol is a highly effective antibacterial agent against *P. larvae* subsp. *Larvae*, and is

Table 1. Mean ± SD of MIC and MBC for the essential oils of *T. minuta* (A) and *A. seriphioides* (B) when emulsified with propylene glycol or soybean lecithin and used against different strains of *P. larvae* subsp *larvae*

Essential oils	Strain from Balcarce hive		Strain from Vidal hive		Strain from Miramar hive	
	A	B	A	B	A	B
MIC 1% v v^{-1} propylene glycol	700.0 ± 0.0	200.0 ± 0.0	700.0 ± 0.0	200.0 ± 0.0	733.3 ± 57.7	200.0 ± 0.0
MBC	933.3 ± 57.7	300.0 ± 0.0	900.0 ± 0.0	300.0 ± 0.0	966.7 ± 57.7	300.0 ± 0.0
MIC 1% w v^{-1} soybean lecithin	800.0 ± 0.0	233.3 ± 28.8	833.3 ± 57.7	250.0 ± 0.0	900.0 ± 0.0	233.0 ± 28.9
MBC	966.7 ± 57.7	300.0 ± 0.0	966.7 ± 57.7	300.0 ± 0.0	966.7 ± 115.5	300.0 ± 0.0

Concentrations are expressed in mg l^{-1}.

reported to have antibacterial activity against *Salmonella typhimurium* and *Staphylococcus aureus* (Juven *et al*., 1994). The present MIC values for the essential oil of wild camomile were 700-800 mg l^{-1} using propylene glycol, and 800-900 mg l^{-1} using soybean lecithin — a slightly inferior effect than that obtained by Alippi *et al.* (1996) for the same oil when using propylene glycol as an emulsifier. These differences might be attributed to differences between the composition of the oils, the harvest time, or local climatic and environmental conditions (Janssen *et al.,* 1987; Sivropoulou *et al.,* 1995; Hammer *et al*., 1999).

The present results show that Andean thyme oil is more effective against *P. larvae* subsp. *larvae* than is wild camomile oil. Many papers deal with the composition of essential oils and their antimicrobial actions, but few authors have related them to any additional effect that might be gained from emulsifier agents. This work shows that the soybean lecithin provides no additional inhibitory effect to the oils studied, whereas propylene glycol does show such an effect. Since it has no potentiating effect, soybean lecithin could be used as a natural emulsifier when testing the antimicrobial activities of natural essential oils.

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