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Antibacterial activity of water extracts and essential oils of various aromatic plants against *Paenibacillus larvae*, the causative agent of American Foulbrood

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ABSTRACT

Vegetal water extracts, namely the water remaining after hydro-distillation and decoctions, and essential oils of 10 plant species were tested 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 the study. The water remaining after hydro-distillation showed the highest antibacterial activities, the growth of almost all the *P. larvae* strains tested was inhibited by these extracts. Regarding the plants tested, *E. cinerea* and *M. verticillata* were the plant species with the highest biological activity with 100% efficacy (all its extracts inhibited the growth of all *P. larvae* strains). Essential oils were less active for the inhibition of *P. larvae* growth.

1. Introduction

American Foulbrood (AFB) is a common bacterial disease of the honey bee (*Apis mellifera*) brood. AFB is produced by the spore-forming bacterium *Paenibacillus larvae.* Larvae are infected by ingesting spores within the larval food provided by adult worker bees. Diseased larvae will ultimately die from infection when sporulation occurs and then will transmit spores throughout the hive. The disease can kill the colony as spores become widespread unless colonies demonstrate resistance, either physiological or behavioral (Bastos et al., 2008). However, although some honey bees possess inherent mechanisms of resistance, high levels of spores produce clinical infections that inevitably leads to the demise of colonies (Thompson et al., 2007).

AFB is considered to be the most serious illness plaguing apiculture today and is nearly cosmopolitan distribution. AFB is difficult to manage for apiculturists because the pathogen produces environmentally stable spores which are very virulent, as well as resistant to heat, to desiccation and to chemical disinfectants (Thompson et al., 2007). Thus, AFB can be considered as a global threat to apiculture.

The burn of the AFB affected combs is used in most of the European Community countries. However, a common strategy employed in some other countries for the prevention and treatment of AFB affected colonies is the use of antibiotics (Antúnez et al., 2008). Sulfathiazole was used in the 1940s; but the persistence of residues in harvested honey resulted in its discontinuation for use in beekeeping. Oxytetracycline hydrochloride has been used for decades to control AFB. There is no Maximum Residue Limit of tetracyclines established for honey according to the European Community regulations (Mutinelli, 2003). Nevertheless, tetracycline resistant strains were recently identified in USA, Canada and Argentina (Reynaldi et al., 2008) rising many concerns among the scientific community because of the possibility of their worldwide spreading. Other antibiotics, such as tylosin and lincomycin, have also been successfully used to control AFB, but concerns still remain regarding the emergence of resistant strains or the residues that they may leave in hive products. Thus, the development of new strategies to treat AFB infected honey bee colonies is necessary.

Plants, herbs, spices and their derived essential oils (EOs: which are volatile, natural, complex compounds characterized by a strong odor and are formed by aromatic plants as secondary metabolites (Bakkali et al., 2008)) or isolated compounds are known to retard or inhibit the growth of bacteria, yeast and moulds (Hayouni et al., 2008). Herbal aqueous extracts, either in the form of infusions or as decoctions, have been used since ancient times as remedies form many human and animal diseases (Kaufman et al., 2006). In this context, some natural remedies to treat AFB honey bee larvae infections have been tested. Thus, essential oils (Albo et al., 2003 and references therein; Calderone et al., 1994; Fuselli et al., 2006, 2008; Gende et al., 2008), propolis extracts (Antúnez

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et al., 2008), as well as biocontrol agents extracted from antagonic aerobic spore-forming bacteria isolated from honey samples and other apiarian sources (Alippi and Reynaldi, 2006) or extracts of fungal strains isolated from pollen collected from beehives (Gallardo et al., 2004) have been tested. Moreover, various reports of *in vitro* experiments showed that decoctions and EOs with antibacterial activity against microorganisms responsible of bee diseases were harmless both to men and the honey bee (Colin et al., 1989; Floris and Carta, 1990; Di Mayuga and Keer-García, 1991). Although fairly good results were informed in all these studies, somewhat complicated procedures were used to obtain these extracts. Thus, we undertook an investigation of the antibacterial activity of some simpler water extracts of plants (namely the water remaining after hydro-distillation and decoctions) and essential oils against *P. larvae* growth.

The aim of the present work was to investigate the antibacterial activity of aqueous extracts from 10 plants growing in Córdoba (Argentina) against *P. larvae*. Thus, the essential oils, the water remaining after hydro-distillation, as well as the decoctions of *Achyrocline satureioides, Chenopodium ambrosioide, Eucalyptus cinerea, Gnaphalium gaudichaudianum, Lippia turbinata, Marrubium vulgare, Minthostachys verticillata, Origanum vulgare, Tagetes minuta* and *Thymus vulgaris* were tested. Some of these species showed antibacterial activity when tested against bacteria, fungi and yeasts (De Feo et al., 1998; Primo et al., 2001; Calvo et al., 2006; Oussalah et al., 2007; Shekarforoush et al., 2007; Schmidt-Lebuhn, 2008; Svetaz et al., 2010) but less information about their antibacterial activity against *P. larvae* was found in our bibliographic searches. Therefore, we decided to study the antibacterial activity of these vegetal species against *P. larvae*. Bacterial strains isolated from infected colonies were used in the study.

2. Methods and materials

2.1. Microorganisms

P. larvae bacterial strains were provided by Instituto Nacional de Tecnología Agropecuaria (INTA), Estación Experimental Agropecuaria, Balcarce, Argentina (EEA Balcarce, Argentina) or isolated from infected colonies. *P. larvae* strains provided by INTA were named B-IV, B-V and B-BB. The strain named CS was isolated from an infected colony. Isolation and identification were done using previously described techniques (Alippi, 1990). On MYPGP agar (Mueller–Hinton broth, yeast extract, potassium phosphate, glucose and sodium pyruvate), *P. larvae* colonies were small, regular, mostly rough, flat or raised, and whitish to beige colored. They were Gram positive and catalase negative. These microorganisms hydrolyzed gelatin and casein, but they neither hydrolyzed starch nor withstood serial transfers in nutrient broth. The strains of *P. larvae* were stored at −20 °C on MYPGP agar with 20% v/v of glycerol until used.

2.2. Plant species

Ten plant species were collected from southern Córdoba province (Argentina). The plant species collected were *A.* satureioides, *C. ambrosioide*, *E. cinerea*, *G. gaudichaudianum*, *L. turbinata*, *M. vulgare*, *M. verticillata*, *T. minuta*, *T.* vulgaris and *O. vulgare*. A voucher specimen of each vegetal species was stored at the Herbarium of the Faculty of Agronomy and Veterinary, Universidad Nacional de Río Cuarto as file herbarium 4658, 3853, 5319, 985, 546, 514, 507, 1258, 1760 and 1252 respectively. Leaves, fine stems, flowers and/or fruits of the different plant species were collected, cleaned and dried. Dried specimens were stored until they were used for extraction.

2.3. Plant extracts

Decoctions (D) of specimens were obtained by steeping 5.0 g of the aerial parts of the plant in 100 mL of distilled water, for 20 min (Mongelli et al., 1995). Essential oils (EOs) were extracted by hydro-distillation in Clevenger type apparatus with a separated extraction chamber for 2 h (De Feo et al., 1998). The extraction chamber was positioned immediately above the water container. Water remaining after hydro-distillation (WRHD) was collected directly from the water container of the hydro-distillation apparatus. All these products were stored at 4 °C until they were used in the antibacterial activity tests (no longer than 6 months).

2.4. Tests of antibacterial activity

D and WRHD were tested for antibacterial activity according to a published methodology (Mongelli et al., 1995). Briefly, 4 mL of D or WRHD were added to 16 mL of culture medium and homogenized thoroughly. Vegetative cells of each *P. larvae* strain grown in MYPGP agar for 72 h of incubation at 37 °C under microaerobic conditions were suspended in physiological solution and adjusted to 0.5 of Mac Farland scale. The microorganisms were streaked in radial patterns on the MYPGP agar plates, four streaks per plate. Finally, the plates were hatched at 37 °C, during 72 h under microaerobic conditions.

EOs were tested for antibacterial activity according to published methodology (De Feo et al., 1998). Vegetative cells of each *P. larvae* strain grown in MYPGP agar for 72 h of incubation at 37 °C under microaerobic conditions were suspended in physiological solution and adjusted to 0.5 of Mac Farland scale. An aliquot of 0.10 mL of this solution was poured into a Petri dish containing MYPGP agar. This aliquot was spread onto the surface of the agar using a Drigalsky spatel until the microorganisms were absorbed. Then, a 6 mm diameter sterilized paper filter disk, previously embedded with 10 μL of the EO under analysis, was put onto the agar surface, and the plate was incubated at 37 °C during 72 h under microaerobic conditions.

After the incubation period, the zones of growth inhibition of the bacterium were measured with a caliper. All the experiments were carried out in duplicate.

3. Results

The results obtained in the inhibition tests performed with D, EOs and WRHD are summarized in Table 1. It can be seen at first glance that the growth of all the *P. larvae* strains studied in this work was inhibited by some of the aqueous extracts or essential oils employed.

Decoctions of the 10 plant species studied were tested as growth inhibitors for *P. larvae*. Eight out of the 10 decoctions tested inhibited the growth of all the *P. larvae* strains. Only the decoctions of *O. vulgare* and *T. vulgaris* did not show biological activity against bacterial growth of *P. larvae* strains CS and B-IV.

Essential oils were obtained from *A. satureioides, Ch. ambrosioide, E. cinerea, M. verticillata, O. vulgare, T. minuta* and *T. vulgaris.* The EOs of *E. cinerea* and *M. verticillata* showed biological activity as growth inhibitors against all the *P. larvae* strains studied. *E. cinerea* produced inhibition zones diameters in the range between 9.0 mm and 19.0 mm while *M. verticillata* produced inhibition zones diameters in the range between 10.0 mm and 26.0 mm. The growth of three *P. larvae* strains (CS, B-IV and B-V) was inhibited by *T. minuta,* with inhibition zones diameters of 34.0 mm, 59.0 mm and 58.0 mm, respectively. The EO of *A. satureioides* inhibited the growth of 2 *P. larvae* strains, showing inhibition zone diameters of 32.5 mm on strain CS and of 20.0 mm on strain B-IV. The EO

Table 1
Inhibition of the growth of different *P. larvae* strains by decoctions, essential oils and waters remaining after steam distillation.

Vegetal species	Fraction	*Paenibacillus larvae* strain			
		CS	B-IV	B-V	B-BB
Achyrocline satureioides	D	I	I	I	I
	EO	32.5 ± 2.1	20.0 ± 1.7	NI	NI
	WRSD	I	I	I	I
Chenopodium ambrosioide	D	I	I	I	I
	EO	NI	NI	NI	NI
	WRSD	–	–	–	–
Eucalyptus cinerea	D	I	I	I	I
	EO	16.0 ± 1.9	9.0 ± 2.2	16.0 ± 1.7	19.0 ± 2.1
	WRSD	I	I	I	I
Gnaphalium gaudichaudianum	D	I	I	I	I
	EO	–	–	–	–
	WRSD	–	–	–	–
Lippia turbinata	D	I	I	I	I
	EO	–	–	–	–
	WRSD	I	I	I	I
Marrubium vulgare	D	I	I	I	I
	EO	–	–	–	–
	WRSD	I	I	I	I
Minthostachys verticillata	D	I	I	I	I
	EO	14.5 ± 1.6	10.0 ± 2.2	26.0 ± 1.9	19.0 ± 2.3
	WRSD	I	I	I	I
Origanum vulgare	D	NI	NI	I	I
	EO	NI	NI	15.0 ± 1.9	NI
	WRSD	NI	NI	I	I
Tagetes minuta	D	I	I	I	I
	EO	34.0 ± 2.2	59.0 ± 1.9	58.0 ± 1.8	NI
	WRSD	I	I	I	I
Thymus vulgaris	D	NI	NI	I	I
	EO	NI	NI	NI	NI
	WRSD	I	I	I	I

D: decoction; EO: essential oil; WRSD: water remaining after steam distillation; I: inhibition; NI: no inhibition; numbers represent the average diameter (in mm) ± SD of the inhibition zone (three repetitions); – means not tested.

of *O. vulgare* only inhibited the growth of *P. larvae* strain B-V with an inhibition zone diameter of 15.0 mm. The EOs of *Ch. ambrosioide* and *T. vulgaris* did not show biological activity as growth inhibitors on any of the *P. larvae* strain in this study.

The waters remaining after hydro-distillation of eight plant species were tested as growth inhibitor against *P. larvae*. Almost all the WRHDs were active against the growth of every *P. larvae* strain (Table 1). Only the WRHD of *O. vulgare* seemed less active against the growth of *P. larvae*, it merely inhibited the growth of *P. larvae* strains B-V and B-BB.

4. Discussion

Comparing the diversity of biological activity of the different extracts (D and WRHD) and EOs of the various plant species tested, it can be observed that WRHD was the most active extract to inhibit *P. larvae* growth, followed closely by D. The EOs of the studied species showed the lesser activity. These results may not be entirely surprising considering that a greater variety of chemical compounds could be included in the aqueous extracts while EOs are mainly composed of terpenes, terpenoids and aromatic compounds (Bakkali et al., 2008). However, it has to be considered that the results of antibacterial activity in this work were obtained by two different methods (disk diffusion and agar dilution methods) which may not be directly comparable, though they might be used as a lead to continue the search of active substances in the extracts.

The WRHD samples were active against the growth of *P. larvae* in 94% of the tests (30 out of 32), while D samples were active in 90% of the growth tests (30 out of 36) and only 50% of the growth tests (14 out of 28) were inhibited by EOs. WRHD is normally discarded after the hydro-distillation procedure. Thus, the biological activity found in this study could bring up an unsuspected use for this byproduct. In this way, the WRHD fraction could be reserved for apiaries sanitation while EOs could be used for other purposes (as for cooking or fragrances) giving its lower biological activity. However, it is worth noting that there still remains a long way to the implementation of WRHD fractions *in vivo*. Toxicity studies of WRHDs fractions on honey bees as well as WRHD delivery and/or application methods should be first analyzed.

The diverse vegetal species studied showed differences in their biological activity against *P. larvae* growth. Thus, *E. cinerea* and *M. verticillata* were the plant species with the highest biological activity with 100% efficacy (all its extracts inhibited the growth of all *P. larvae* strains). The extracts of *T. minuta* also showed high inhibition efficacy (91%) for the growth of the *P. larvae* strains. Only the growth of *P. larvae* strain B-BB was not inhibited by the EO of *T. minuta*. *A. satureioides* was another species whose extracts showed biological activity as inhibitor of *P. larvae* growth (83% efficacy). Finally, *O. vulgare* and *T. vulgaris* were the plant species whose extracts were less active as growth inhibitors for *P. larvae*. *O. vulgare* showed only 42% efficacy, while *T. vulgaris* showed 50% efficacy.

Eucalyptus species are largely known because of their production of insecticidal and medicinal essential oils. *Eucalyptus* leaf essential oils have been used in the treatment of lung diseases, as expectorant and cough stimulants. They have also been reported to possess antitubercular properties (Oyedeji et al., 1999). Thus, *Eucalyptus* oils are commonly used for the production of cough medicines, and the production of *Eucalyptus* WRHD fractions is important. On the other hand, *M. verticillata*, known as "peperina" in Argentina, is also recognized as an aromatic and medicinal plant and is widely distributed in the mountains of the Córdoba province. It's essential oil is frequently used as a natural insecticide

by the local farmers of Los Andes, from Venezuela to Argentina (Cantín et al., 2001), although it may also possess antifungal activity (Primo et al., 2001). The essential oils of both *Eucalyptus* and *Mintosctachys* species are also efficient as inhibitors for the growth of filamentous fungi and yeasts. They were also effective for the control of many food contaminating microorganisms (Tassou et al., 1995). Therefore, the biological activity of the WRHD fractions found in this study adds extra value to these species extracts as they could be used to prevent *P. larvae* outbreaks in place of antibiotics.

T. minuta is a vegetal species growing in many mountain regions of South-America. Vulgarly known as "suico" in Argentina, it is recognized as efficient against many stomach disorders. Oil extracted from various parts of *T. minuta* L. (Mexican marigold) (Asteraceae) are used in the Tropics as a dressing for livestock to control blowfly (Broussalis et al., 1999). *A. satureioides* popularly known as "marcela" has been used in natural medicine for many years in Argentina, Uruguay, Brazil and Paraguay. Decoctions or infusions of this plant are traditionally used for gastrointestinal disorders as an emmenagogue and menstrual regulator and as sedative and antispasmodic (Rivera et al., 2004). The repellent properties of *A. satureioides* EO against *Ae. aegypti* mosquitoes has been recently reported (Gillij et al., 2008), and it appears to be associated with the presence of monoterpenoids and sesquiterpenes. The essential oil of *O. vulgare*, or its major components, has shown antimicrobial activity against diverse microorganisms (Faleiro et al., 2005). Essential oils derived from *Thymus* species (Lamiacae family) have been found to possess significant antifungal, insecticidal, and antimicrobial activities (Chorianopoulos et al., 2004). These properties depend greatly on their chemical compositions and are mainly attributed to their contents in carvacrol and thymol. Thus, the antibacterial activity of the aqueous extracts of all these species, found in our screening against *P. larvae* growth, could also be useful to add extra commercial value to these medicinal plants.

Although the general belief is that herbs are safer than pharmaceuticals because they are natural, the fact is that many remedies obtained from herbs are neither completely safe nor poisonous. Thus, toxicity studies of the extracts on the honeybee have to be performed. Acute oral toxicity tests (Albo et al., 2003; Reynaldi et al., 2008) and lethal concentration studies (Antúnez et al., 2008) should be considered before the field experiments are performed.

5. Conclusions

Water remaining after hydro-distillation, decoctions and essential oils of *A. satureioides*, *C. ambrosioide*, *E. cinerea*, *G. gaudichaudianum*, *L. turbinata*, *M. vulgare*, *M. verticillata*, *O. vulgare*, *T.* minuta and *T.* vulgaris tested active as growth inhibitors of *P. larvae*. WRHDs were the extracts with the highest antibacterial activity, while EOs showed the lesser activity. WRHD of *E.* cinerea and *M.* verticillata showed the highest antibacterial activity with 100% efficacy (all its extracts inhibited the growth of all *P. larvae* strains). Considering that some of the compounds in the different extracts may be harmful to honeybees, further studies are thus needed not only to elucidate the compound/s with biological activity but also to test their toxicity.

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