Bulletin of Insectology ***61*** *(1): 1-4, 2008*
ISSN 1721-8861

Antimicrobial activity of cinnamon (*Cinnamomum zeylanicum*) essential oil and its main components against *Paenibacillus larvae* from Argentine

Liesel Brenda GENDE[1,2,3], Ignazio FLORIS[4], Rosalia FRITZ[2], Martin Javier EGUARAS[1,3]
[1]*Departamento de Biología, Universidad Nacional de Mar del Plata, Argentina*
[2]*Departamento de Química, Universidad Nacional de Mar del Plata, Argentina*
[3]*CONICET, Universidad Nacional de Mar del Plata, Argentina*
[4]*Dipartimento Protezione Piante, Sezione Entomologia, Università degli Studi di Sassari, Italy*

Abstract

The physicochemical properties, composition and antimicrobial activity of cinnamon essential oil (*Cinnamomum zeylanicum*) were studied. The bioactivity of this essential oil against *Paenibacillus larvae* was analyzed by means of a combination of *in vitro* techniques, such as the tube dilution method and bioautography, a method employed to localize antibacterial activity on a chromatogram. Cinnamaldehyde and eugenol proved to have antibacterial effects against *P. larvae.* Minimal inhibitory concentration (MIC) and minimal bactericide concentration (MBC) for *C. zeylanicum* essential oil were between 25-100 µg/ml and 125-250 µg/ml, respectively, for all strains. Essential oil and, especially, two of its main components presented inhibitory capacity against strains of *P. larvae*.

Key words: Cinnamon essential oil, cinnamaldehyde, antimicrobial activity, bioautography, *Paenibacillus larvae*.

Introduction

American Foulbrood (AFB) is the most serious and dangerous bacterial disease affecting honeybee brood (*Apis mellifera* L.), the causal agent is *Paenibacillus larvae* (White), which produces highly resistant spores. Sustained efforts have been devoted to controlling this disease, including the use of preventive and curative treatments with antibiotics and other antimicrobial substances. The extensive use of antibiotics has led to an accumulation of residues in beehive products (especially in honey), thereby decreasing their quality and hindering marketing opportunities (Fuselli *et al*., 2005). Therefore, natural antimicrobials and essential oils, above all, have been investigated (Carta and Floris, 1989; Floris *et al*., 1996; Alippi, 1996; Albo, 2003). Essential oils can be distilled from the leaves and branches of cinnamon *Cinnamomum zeylanicum* Breyne, a perennial tree belonging to the Lauraceae family (Alonso, 1998). This investigation focuses on the antimicrobial screening of cinnamon *C. zeylanicum* essential oil against *P. larvae,* using the combination of two different bioassay techniques. The antimicrobial activities were determined and compared by means of techniques such as serial dilution and bioautography agar. The bioautography method aided in the identification of the antimicrobial active compounds. The chemical composition of the cinnamon essential oil was evaluated and compared using techniques such as thin layer chromatography (TLC) and gas chromatography (GC). Gas chromatography/mass spectroscopy (GC/MS) analyses were also performed.

Materials and methods

Cinnamon (*C. zeylanicum*) essential oil, furnished by Cruciani (Rome, Italy) and derived from bark is the same previously employed and analysed by Floris *et al*. (1996).

Thin layer chromatography

Thin layer chromatography (TLC) analysis of essential oil was performed on sílicagel (Kieselgel 60H Merck). The cinnamon oil was applied using an aliquot of 10 µl (using Drummond micro-capillaries) to two TLC plates and developed (93:7 toluene/ethyl acetate). On one plate, the separated compounds were sprayed with sulphuric acid in ethanol, and later with vanillin in ethanol, followed by heating at 110 ºC (Wagner and Bladt, 1996). The other plates were used for the bioautography assay.

Analyses of physicochemical properties

Density at 20 °C, triplicate of 1 ml of essential oil was weighed and the average of the obtained values was calculated (Montes, 1981).

Refraction index was measured according to AOAC official method 921.08, 2003 at 20 (± 0.05) °C with an Abbe refractrometer of total reflection.

Acid index was obtained by titulation with an aqueous solution of NaOH 0.1 N. To do so, 1 g of essential oil was dissolved in ethanol 96%, previously neutralized with NaOH using blue of thymol as indicator. Titulation was completed and the final point was visualized.

Optic activity $[a]_{20}$ was determined with a circle polarimeter VEB Carl Zeiss Jena, with a solution of 0.02 g/ml of the essential oil samples in ethanol. Readings were made with 2 dm tubes at 20 °C, and rotation was corrected in proportion to dilution (Horwitz, 2003).

Infrared spectroscopy

The IR spectrum of the sample was recorded as a thin liquid film on NaCl windows, 8 scans, 2-cm^{-1} resolution were obtained with FTIR Mattson, model Genesis II spectrophotometer. The spectra were accumulated from x scans measured with a resolution of x cm^{-1} in the range of 500–4000 cm^{-1}.

Antimicrobial assay

Three bacterial strains of *P. larvae* were isolated from the honeycombs of hives undergoing clinical symptoms of American Foulbrood. These hives were located in Mechongué, Cobo and Chapadmalal cities in Buenos Aires province. Isolation and strains identification were made according standard biochemical tests (Alippi, 1992). The pure strains were maintained on MYPGP agar with 15% v/v glycerol until used.

Vegetative cells of *P. larvae* were grown on MYPGP agar for 48 h at 35 ± 0.5 °C, and then suspended in double distilled sterile water. To measure antimicrobial activity with serial dilution method, the concentration was adjusted to 0.5 of Mac Farland scale, and to 1 of Mac Farland scale when applying the bioautography method.

The minimal inhibitory concentration (MIC) was directly assessed by turbidity observation. Essential oil was emulsified with 8% v/v propylene glycol (1-2 propanediol). One millilitre of each stock solution was added to MYT (Mueller-Hinton, yeast extract and thiamine) broth [2.0 g/l extract meat; 17.5 g/l hydrolyzed casein, 1.5 g/l starch, 1.5 % yeast extract and 0.1 mg/l thiamine (autoclaved separately)] and serially diluted (final range 12,5-2000 µg/ml). Microbial biomass suspension was then added to each serial dilution tube with agitation, at room temperature, using a Vortex dispersing tool (Fbr® by Decalab SRL). All sample tubes (as well as positive and negative controls) were incubated at 35 ± 0.5 ºC for 48 h in order to determine MIC values under microaerobic conditions (5-10% of CO2).

Minimal bactericide concentration (MBC) was specified. To that end, known volumes were transferred from MIC negative tubes to MYPGP solid agar (Dingman and Stahly, 1983), and incubated at 35 ± 0.5 ºC under microaerobic conditions for 48 h in order to delimit MBC values. The antimicrobial activities were determined by quintupled analyses for oil and strains.

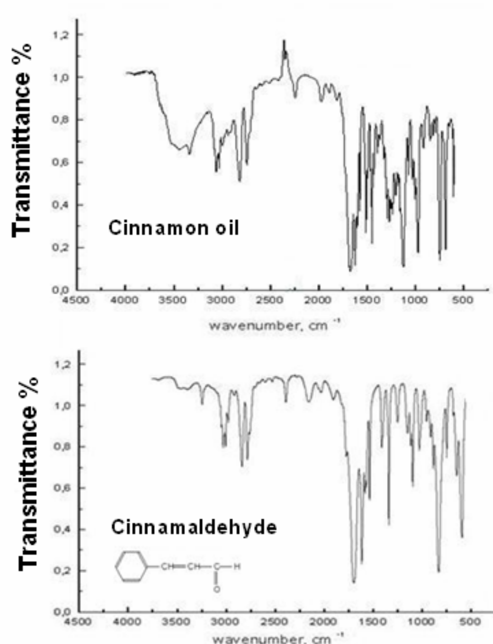

Figure 1. Infrared spectra comparison between cinnamon essential oil and Cinnamaldehyde.

A bioautography technique was employed to define the active constituents (Iskan *et al*., 2002). Strains of *P. larvae* were previously inoculated at 40 ºC into MYPGP agar with a triphenyl tetrazolium chloride solution to 5% w/v in water. 100 ml of the inoculated medium was poured into TLC plates, not being previously revealed; and incubated at 36 ± 1 ºC during 24-48 h under microaerobic conditions. Microbial growth inhibition was determined by measuring the area of the inhibition zones. The inhibition area was calculated as the average of three measurements per TLC plate. For oil and strains, antimicrobial activities by bioautography method were determined by triplicate analyses.

Statistical analysis

MIC and MBC data obtained from cinnamon essential oil were comparatively analysed using Fisher exact test, specially suited for small samples, and then employed to estimate significant differences (P<0.05) between bacterial strains. With regard to inhibition zones analysis, Pearson correlation was determined using the PROC GLM of SAS procedure (SAS, 2000).

Results

Among the 22 compounds identified by means of GC and CG/ MS analyses (Floris *et al*., 1996), cinnamaldehyde (79.3% w/w) and eugenol (11.9% w/w) are the main components, and some terpenic compounds, such us α− pinene and caryophyllene in a lesser proportion.

Physicochemical properties values were as follows: a density of 1.061 g/ml at 20 ºC, a refraction index of 1.6073, an optic activity $[a]_{20}$ of 12.5º (C=0.02 g/ml), and an acid index of 14.87 mg KOH/g oil.

The infrared spectroscopy spectrum displayed characteristic bands corresponding to aromatic CH bonds, between 3000 cm^{-1} and 3100 cm^{-1}; to CH alquenes, between 3020 cm^{-1} and 3080 cm^{-1}; to C=C, between 1640 cm^{-1} and 1680 cm^{-1}; and to the aldehydes C=O group between 1690 cm^{-1} and 1760 cm^{-1}. The spectra similarity of the cinnamon essential oil and a cinnamaldehyde (SDBSWeb at http://www.aist.go.jp/RIODB/SDBS) are shown in figure 1.

Results of MIC and MBC for the cinnamon oil against the three bacterial strains are shown in table 1. Relationships between MIC values and bacterial strains (Chapadmalal, Mechongué and Cobo) (P<0.05) were found, but no correlation between the three strains (P<0.05) for MBC values was evidenced.

Table 1. Minimal inhibitory concentration (MIC) and minimal bactericial concentration (MBC) values of cinnamon essential oil for three bacterial strains of *P. larvae* (Chapadmalal, Mechongué and Cobo).

Strains	Chapadmalal	Mechongué	Cobo
MIC values in (mg /l)	25-50	50	50-100
MBC values in (mg /l)	125-150	150-200	150-200

The antimicrobial activities were determined by quintupled analyses for oil and strains.

The bioautography assay was applied to strains of *P. larvae*. The bioautography technique allows to evaluate the minimum concentrations of the compound responsible for the biological activity being observed the growth inhibition areas at first sight (Marquez *et al*., 2003). This experiment showed clear inhibition zones corresponding to cinnamaldehyde (3-phenyl-2-propenal) and eugenol (2-methoxy-4-propenyl-phenol) after separation on TLC. On silica gel thin-layer chromatograms, cinnamon essential oil was separated into two bioautographic spots with different Rfs (0.67; 0.35) which showed activity against *P. larvae*. No active bands corresponding to Rfs between 0.05 and 0.21 related to the region of terpenic alcohols were observed. Figure 2 displays chromatogram bands and coinciding zones of growth inhibition on the bioautography plate. Moreover, table 2 shows the results obtained for these two components in the tests of antimicrobial activity against *P. larvae* strains from three different geographical origins. For cinnamaldehyde compound, significant differences were detected for Chapadmalal strain in relation to Mechongué and Cobo strains (P<0.05); however, regarding eugenol values of inhibition area, the three strains showed no significant differences (P<0.05).

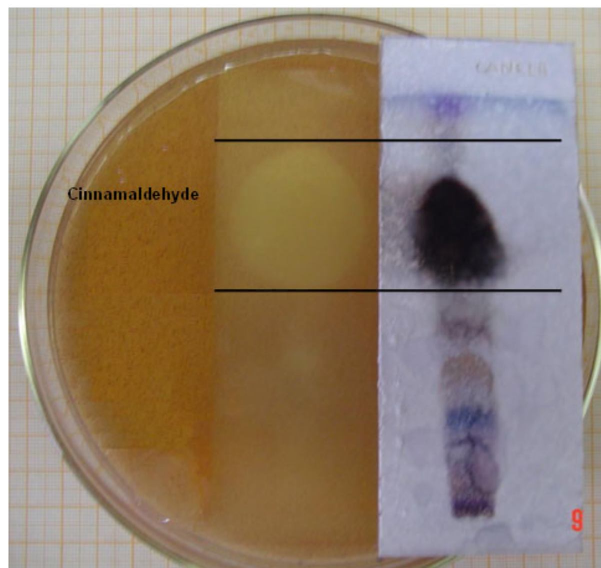

Figure 2. Bioauthography of Chapadmalal strain and chromatography on a cinnamon plate.
(In colour at www.bulletinofinsectology.org).

Discussion and conclusions

Data about the essential oil composition obtained with GC and GC/MS are in accordance with those reported by Floris *et al*. (1996) and Wagner and Bladt (1996).

Physicochemical properties were analyzed with the aim of establishing the quality, purity and chemical stability of the essential oil. All physicochemical properties obtained ranged within the prospective values, except for the acid index which was higher, if compared with the results by Montes (1981) and Retamar (1982). The reason for this could be that the oil applied in this case had been distilled years before the trial was conducted.

Comparing infrared spectrum of cinnamon essential oil and the individual spectra of the main compounds, i.e., cinnamaldehyde and eugenol (database of SDBS) superimposed, both spectra are notoriously similar. This could result from the high content of cinnamaldehyde as well as from the presence of eugenol in the essential oil analyzed.

Against the different strains under analysis, cinnamon (*C. zeylanicum)* yielded good antimicrobial activity comparable to that reported thyme and lemon grass oils, and greater than other essential oils such as rosemary, whose MIC values were of 700 µg/ml, and oregano oils whose MIC values ranged from 250 to 450 µg/ml (Alippi, 1996).

Bioautography was used to detect qualitative antibacterial activity. Since this method is visual, the stability of the compounds on the plate can be easily verified (Dhar *et al*., 2004). The results obtained in this work suggest that cinnamon (*C. zeylanicum*) essential oil and two of its main components, cinnamaldehyde and eugenol, presented inhibitory activity against three strains of *P. larvae* of different geographical origins. Therefore, the present experience, supported by previous studies (Carta and Floris, 1989; Floris *et al*., 1996; Carpana *et al*., 1996; Floris, 2001) where antimicrobial properties of cinnamon oil against *P. larvae* were proved in vitro and *in vivo*, promotes the use of its main compounds for the AFB management, allowing a most precise dosage of the active component. This work represents the first part of a research being conducted, in which the concentration and main compounds of the essential oil inhibiting bacterial growth were studied. Recently, *in vitro* LC_{50} (median lethal concentration) has

Table 2. Antimicrobial activities by bioautography method expressed in cm for wide, high and area of bacterial inhibition.

Strain	compound	wide	sd	high	sd	area	sd
Chapadmalal	cinnamaldehyde	3.43 a	0.04	3.10 a	0.05	8.36 a	0.15
	eugenol	1.03 b	0.04	0.57 b	0.05	0.46 b	0.15
Cobo	cinnamaldehyde	1.97 c	0.04	1.57 c	0.05	2.42 c	0.15
	eugenol	1.00 b	0.04	0.57 b	0.05	0.45 b	0.15
Mechongue	cinnamaldehyde	1.97 c	0.04	1.90 d	0.05	2.94 c	0.15
	eugenol	1.00 b	0.04	0.50 b	0.05	0.39 b	0.15

Letters indicate significances inside lines (Fisher exact test, p<0.05) for the same column and compound. Area = $\pi \times r_1 \times r_2$ (r_1= wide, r_2 = high).

been tested on bees to specify its optimum concentration in apiaries studies, so as not to cause toxicological risks to bees and not to overcome taste threshold in honey (Bogdanov *et al*., 1999) as well as other undesirable effects such us resistance factors, resulting for the indiscriminate use of antibiotics.

Acknowledgements

The authors would like to thank Dr Gabriel Gutkind (UBA) for his orientation in bioautography technique and Dr Diana Fasce for IR advice. Also Claudia Faverin and Adriana Cano for the assistance in providing the statistical analysis and some anonymous reviewers. This work was supported by UNMDP, ANPCyT and CONICET.

References

ALBO G., HENNING C., RINGUELET J., REYNALDI F., GIUST M., ALIPPI A., 2003.- Evaluation of some essential oils for the control and prevention of American foulbrood disease in honey bees.- *Apidologie*, 34: 417-437.

ALDICARA J. R., 1976.- Essential oils, p. 262. In: *Encyclopedia of Chemical Processing and Design* (MCKETTA J. J., CUNNINGHAM W. A., Eds).- Marcel Dekker, New York, USA.

ALIPPI A. M., 1992.- Detección de *Bacillus larvae* en poblaciones mixtas de esporas bacterianas a partir de restos larvales.- *Microbiología SEM*, 8: 115-118.

ALIPPI A. M., RINGUELET J. A., CERIMELE E. L., RE M. S., HENNING C. P., 1996.- Antimicrobial activity of some essential oils against *Paenibacillus larvae*, the causal agent of American foulbrood disease.- *Journal of Herbs, Spices and Medicinal Plants*, 4 (2): 9-16.

ALONSO J., 1998.- Canelo, pp. 334-336. In: *Tratado de fitomedicina. Bases clínicas y farmacológicas*.- ISIS Ediciones SRL, Buenos Aires, Argentina.

BOGDANOV S., KILCHENMANN V., FLURI P., BÜHLER U., LAVANCHY P., 1999.- Influence of organic acids and components of essential oils on honey taste.- *American Bee Journal*, 139: 61-63.

CARPANA E., CREMASCO S., BAGGIO A., CAPOLONGO F., MUTINELLI F., 1996.- Profilassi e controllo della peste americana in alcune regioni italiane.- *Apicoltore Moderno*, 87: 11-16.

CARTA C., FLORIS I., 1989.- Prospettive di controllo della peste americana delle api con oli essenziali. Prove preliminari.- *Atti della Società Italiana Ecologia*, 8: 183-187.

DHAR P., AYALA U., ANDARGE E., MORISSEAU S., 2004.- Study of the structural changes on the antimicrobial activity of [3.1.1]-bicyclics.- *The Journal of essential oil research*, 16: 612-615.

DINGMAN D. W, STAHLY D. P., 1983.- Medium promoting sporulation of *Bacillus larvae* and metabolism of medium components.- *Applied and Environmental Microbiology*, 46: 860-869.

FLORIS I., 2001.- *Controllo delle malattie delle api con l'impiego di oli essenziali*. - Regione Autonoma della Sardegna. Assessorato agricoltura e reforma agro-pastorale. 3esse, Serramanna (CA), Italy.

FLORIS I., CARTA C., MORETTI M. D. C., 1996.- Activites in vitro de plusieurs huiles essentielles sur *Bacillus larvae* White et essai au rucher.- *Apidologie*, 27 (2): 111-119.

FUSELLI S. R., GENDE L. B., GARCÍA DE LA ROSA S. B., EGUARAS M. J., FRITZ R., 2005. - Inhibition of *Paenibacillus larvae* subsp *larvae* by the essential oils of two wild plants and their emulsifying agents.- *Spanish Journal of Agricultural Research*, 3 (2): 220-224.

HORWITZ W., 2003.- *Official methods of analysis of AOAC*. 17th ed., Current through revision 2.- AOAC Inc., Arlington, USA.

ISKAN G., KIRIMER N., KÜRKCÜOOGLU M., HÜSNÜ K., DEMIRCI F., 2002.- Antimicrobial screning of *Mentha piperita* essential oils.- *Journal of Agricultural and Food Chemistry*, 50: 3943-3946.

MARQUEZ B., CARDENAS A., MORALES C., VERDE STAR M., 2003.- Identificación de compuestos de *Melia azedarach*, *Syzgium aromaticum* y *Cinnamomum zeylanicum* con efecto inhibitorio sobre bacterias y hongos.- *Ciencia UANL*, 6 (3): 333-338.

MONTES A., 1981.- *Bromatología*, tomo l.- Ed. EUDEBA, Universitaria de Buenos Aires, Buenos Aires, Argentina.

RETAMAR J. A., 1982.- Especies herbáceas anuales o plurianuales aromáticas, pp. 1-301. In: *Aceites esenciales de especies vegetales diversas: sus posibilidades químicas*.- IPNAYS, Santa Fé, Argentina.

SAS, 2000.- SAS OnlineDoc (rtm). Version 8, Copyright (c)2000, SAS Institute Inc., Cary, NC, USA.

WAGNER H., BLADT S., 1996.- Essential Oil Drugs, pp. 5-49. In: *Plant drug analysis. A thin layer chromatography atlas*.- Springer, Berlin, Germany.

Corresponding author: Ignazio FLORIS (ifloris@uniss.it), Dipartimento Protezione Piante – Sezione Entomologia, Università degli Studi di Sassari, via E. De Nicola, 07100 Sassari, Italy.

Received June 21, 2007. Accepted October 11, 2007.