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Isolation, purification and identification of the anti-diabetic components from *Cinnamomum zeylanicum* and *Cinnamomum cassia* bark oil extracts

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KEYWORDS

Gas chromatography, Mass Spectrophotometry, Cinnamaldehyde, Cinnamic acid, *C. cassia* & *C. zeylanicm*

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ABSTRACT

The bark and the leaves *Cinnamomum sp* are commonly used as spices in home kitchens and their distilled essential oils are used as flavouring agent in the food & beverage industries. The chemical analysis of the oils from both the sources revealed that the active oil contained 74% cinnamaldehyde, compared to only 8.3% in the inactive oil. Cinnamon has been reported to have remarkable pharmacological effects in the treatment of type II diabetes and insulin resistance. However the plant material used in the study was mostly from *C. cassia* and only few of them are truly from *C. zeylanicum.* Three types of distinguishing method for cinnamon barks have been described: Microscopy, Mucilage content determination and Thin layer chromatography (TLC). The preparatory TLC fractionation of ether, extracted from *C.cassia* and *C. zeylanicum* yielded 3 fraction representing 58.5% of the crude extract. Similar to the ether extract the preparative TLC of the distilled oil also yielded 3 fraction with a combined yield of 97.5% of the crude distilled oil. The Gas Chromatography/Mass spectrophotometry (GS/MS) of the fraction 2 of ether extract, yielded 4 peaks, with the relative intensity of 4, 6, 61,6 and 29%. The first and second most intense peaks were identified as cinnamaldehyde and cinnamic acid repectively. The remaining two peaks with relative intensity of 6% or less could not be identified. The GC/MS of the fraction 2 of the distilled oil yielded only one peak with the relative intensity of 99.1% which was identified as cinnamaldehyde.

Introduction

The bark and the leaves of C*innamomum spp.* are commonly used as spices in home kitchens and their distilled essential oils or synthetic analogs are used as flavouring agent in the food and beverage industry. Although traditionally known, some recent scientific studies have shown antimicrobial activity of essential oils of C*innamomum cassia* presl., *C. osmophloeum* kaneh. And *C. zeylanicum* Blume (Tiwari & Tiwari, 1997; Ferhout *et al.* 1999; Mastura *et al.* 1999; De *et al.,* 1999; Chang *et al.,* 2001). Quattara *et al.* (1997) reported the inhibitory effect of *C.zeylanicum* essential oil on meat deteriorating organisms.

The *C. zeylanicum* tree is endemic in India, and our initial exploratory studies have shown that hexane extract of *C. zeylanicum* bark protected high moisture soybean (*Glycine max L)* and wheat grains from storage fungi (unpublished data).

The chemical composition of the essential oil or hexane extract of *C. zeylanicum* is not well known, and there appears to be very high variability depending upon the year, climate, production area and the tree chemotype (Koketsu *et al.,* 1997; Jirovetz *et al.,* 1998; Mallavarapu *et al.,* 2000; Raina *et al.,* 2001). The essential oil of bark from trees grown in the State of Kerala was found to have cinnamaldehyde constituting on average, 55% of the total oil. However, there were considerable difference among the individual trees (Koketsu *et al.,* 1997). The chemical analysis of the oils from both the sources revealed that the active oil contained 74% cinnamaldehyde, compared to only 8.3% in the inactive oil. Such variations of the antimicrobial activity and chemical composition of essential oil obtained from different trees can be a stumbling block for developing a reliable product for use.

It is necessary to identify the most active component(s) against which the final product can be standardized. The following study was done to identify the most active components *C. zeylanicum* bark distilled essential oil and the hexane extract.

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 alsoperformed.

Material and Methods

Preparation of Ether Extract And Distilled Oil

Locally produced Cinnamon bark was purchased from the local spice store. All the chemical and solvents used in the study were analytical grade and distilled before use. Cinnamon bark was ground to pass through a 1-mm screen and the powder obtained was extracted at room temperature by constant percolation with ether until all the ether soluble components were removed. The solvent was evaporated using a rotator evaporator, under vacuum, at 35°C. The bark essential oil was obtained by hydro-distillation for 6h. The distillate was extracted twice with ether, including water soluble or dispersed components, dried over anhydrous sodium sulphate, and the ether was evaporated using the rotary evaporator under vacuum. The hexane extract and the distilled oil were stored in airtight screw capped vials at-10°C until used Budarri (1989), Lsman (2001).

Fractionation of The Hexane Extract And The Distilled Oil

The ether extract and the distilled oil were fractionated by preparative silica gel thin layer chromatography (TLC) using 20X20 cm silica gel plates (ca. 1mm thick layer, 60GF, Merck). Several TLC plates were used to obtained workable quantities of the fractions. The crude extract or distilled oil (200mg) was applied on each plate, which was then developed with hexane: ethyl acetate (9:1v/v). To visualize the fractions, a small edge of the TLC plate was sprayed with phosphomoliblinin acid followed by 5-min heating with hot air using an electric hair drier. The fractions that did not react where scraped off and transferred to a beaker and extracted with ether two hours with constant stirring. The mixture was filtered and the ether was evaporated in a rotary evaporator under vacuum, weighed and stored at -10°C until use Lawless (2002).

Characterization of Active Fraction

The most active fraction of the ether extract and the distilled oil was characterized by gas chromatography-mass spectrometry (GC-MS), using a JEOL GC-MATE-II system equipped with a data base with 250,000 compounds, auto-sampler, fused silica capillary column coated with HD-5 stationary phase (30 m x 0.25mm; 0.25µm film thickness, J & W. Scientific). The GC oven temperature was raised from 40 to 320°C at the rate of 4°C /min. Helium gas was used as the carrier for all the analysis, and electron ionization mass spectra (70 eV) were recorded by scanning from *m/z* to 500. The injector and transfer line temperatures were maintained at 220°C and 280°C, respectively. One micro litre of each sample was injected in the split mode (100:1) Yang *et al.* 2003), Yang *et al.* (2004).

Bark Anatomy

C. zeylanicum and *C. cassia* microtome sections were taken and stained with the standard techniques from (Prof. P. Jayaraman's lab) and comparative studies were made, about the bark oil locations and comparisons.

Collection Of Specimens

The plant specimens for the proposed study were collected from local Indian traditional medical shop. Care was taken to select healthy plants and normal organs. The required samples of different organs were cut and removed from the plant and fixed in FAA (Formalin-5ml+ Acetic acid-5ml + 70% Ethyl alcohol-90ml). After 24hrs of fixing, the specimens were dehydrated with graded series of tertiary- Butyl alcohol as per the schedule given by **Sass, 1940**. Infiltration of the specimens was carried by gradual addition of paraffin wax (melting point 58-60°C) until TBA solution attained super saturation. The specimens were cast into paraffin blocks.

Sectioning

The paraffin embedded specimens were sectioned with the help of Rotary Microtome**.** The thickness of the sections was 10-12µm. Dewaxing of the sections was by customary procedure (Johansen, 1940). The sections were stained with Toluidine blue as per the method published by O'Brien et al. (1964). Since Toludine blue is a polychromatic stain. The staining results were remarkably good; and some cytochemical reactions were also obtained. The dye rendered pink colour to the cellulose walls, blue to the lignified cells, dark green to suberin, violet to the mucilage, blue to the protein bodies etc. Wherever necessary sections were also stained with safranin and fast-green and IKI (for Starch).

Results

The preparative TLC fractionation of ether extracted from *Cinnamomum zeylanicum* and *Cinnamomum Cassia* yielded three fractions, representing 58.5% (w/w) of the crude extract. The fraction-2 represented 77% of the total yield, which corresponds to 45% of the crude extract. The fractions 1 and 3, each represented 11.5% of the total yield, corresponding to about 6.75% each of the crude extract.

Similar to the ether extract, the preparative TLC of the distilled oil also yielded three fractions, with a combined yield of 977.5% of the crude distilled oil. The freaction-2 represented the 70% of the total yield, which corresponds to 71.8% of the crude distilled soil. The fraction-1 and 3 made up21 and 6.5% of the total yield, corresponding to 21.5 and 6.6%, respectively of the crude distilled oil.

In *Cinnamomum zeylanicum* and *C. Cassia* the IR, and GC-MS results were recorded and identified. In *Cinnamomum zeylanicum* IR, spectrum peaks were recorded with the following readings (3513, 3335, 3061, 2815, 2742, 2242, 1975, 1731,1677, 1626, 1576, 1495, 1450, 1393, 1294, 1250, 1159, 1124,1072, 1007, 973, 844, 748, 689, 605, 583, 499) and *Ci. Cassia* (3484, 3335, 3061, 3028, 2927, 2815, 2742, 2242,1975, 1731, 1679, 1629, 1576, 1513,1495, 1450, 1391, 1294, 1251, 1159, 1124, 1072, 1006, 973, 921, 845, 748,639, 605, 583, 499).

In *Cinnamomum zeylanicum* GC-MS results were recorded with the following readings 3.60, 3.98, 4.09, 4.18, 4.22, 4.86, 4.85, 5.07, 5.22, 5.43, 5. 56, 5.77, 5.92, 6.42, 6.76, 6.84, 7.1, 7.11, 7.47, 7.62, 7.7, 8.01, 8.09, 8.22, 8.5, 8.59, 8.67, 8.68, 8.99, 9.63, 10.07, 10.55, 10.86, 11.55, 11.61. in *C. Cassia* GC-MS results were observed with the following readings 5.33, 5.82, 6.35, 6.38, 6.42, 6.61, 7.02, 7.15, 7.34, 7.46, 7.55, 7.69, 7.84, 7.99, 8.06, 8.68, 8.77.

The GC-MS of the fraction-2 of the ether extract yielded four peaks, with relative intensity of 4, 61, 6 and 29%. The first and second most intense peaks were identified as cinnamaldehyde and cinnamic acid, respectively. The remaining two peaks with relative intensity of 6% or less could not be identified. The GC/MS of the fraction-2 of the distilled oil yielded only one peak with a relative intensity of 99.1%, which was identified as cinnamaldehyde.

For studying the stomatal morphology, venation pattern and trichome distribution, paradermal sections (sections taken parallel to the surface of leaf) as well as clearing of leaf with 5% sodium hydroxide or epidermal peeling by partial maceration employing Jeffrey's maceration fluid (Sass, 1940) were prepared. Glycerine mounted temporary preparations were made for macerated/cleared materials. Powdered materials of different parts were cleared with NaOH and mounted in glycerine medium after staining. Different cell component were studied and measure.

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Relative composition of inner bark oils from *Cinnamomum zeylanicum*

	Relative retention time	Source			
		Sigma	Chemanex	Volanka	Gunasena
Benzyl benzoate	1.97	0.005	0.018	0.033	0.107
3-Caryophyllene oxide	1.56	0.005	0.012	0.024	0.028
2-Methoxycinna maldehyde	1.51	Trace	0.010	0.006	0.020
Eugenol acetate	1.43			0.013	0.062
a-Humulene	1.30	0.002	0.009	0.012	0.028
Cinnamyl acetate	1.27	0.002	0.058	0.074	0.148
3-Caryophyllene	1.24	0.014	0.049	0.061	0.155
Eugenol	1.13	0.072	0.090	0.478	1.005
Safrole	1.02			0.010	0.016
a's-Cirmamaldehyde	0.90	0.004	0.008	0.006	0.006
a-Terpineol	0.83	Trace	0.011	0.013	0.027
Linalool	0.66	0.018	0.065	0.097	0.150
p-Phellandrene	0.57	Trace	0.039	0.018	0.050
Limonene	0.56	0.022	0.012	0.011	0.020
Cymene	0.55	0.005	0.035	0.048	0.060
a-Pinene	0.43	0.017	0.012	0.011	0.019

Relative to the area response and retention time (18.71min) of transcinnamaldehyde

Microphotographs

Microscopic descriptions of tissues are supplemented with micrographs wherever necessary. Photographs of different magnifications were taken with Nikon lab photo 2 microscopic Unit. For normal observations bright field was used. For the study of crystals, starch grains and lignified cells, polarized light was employed. Since these structures have birefringent property, under polarized light they appear bright against dark background. Magnifications of the figures are indicated by the scale-bars. Descriptive terms of the anatomical features are as given in the standard Anatomy books (Esau, 1964).

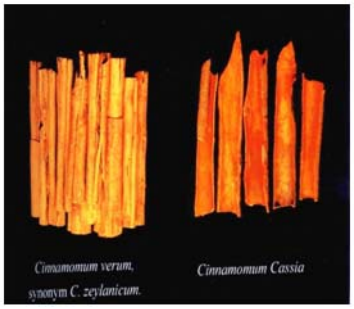

Fig 1a: Barks of Cinnamomum zeylanicum & Cinnamomum cassia

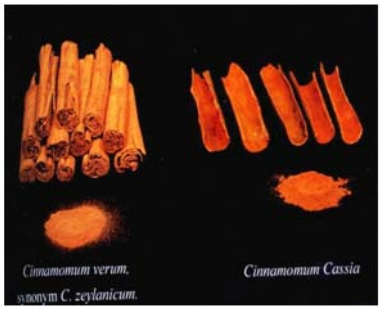

Fig 1b: Powdered Barks of C. zeylanicum & C. cassia

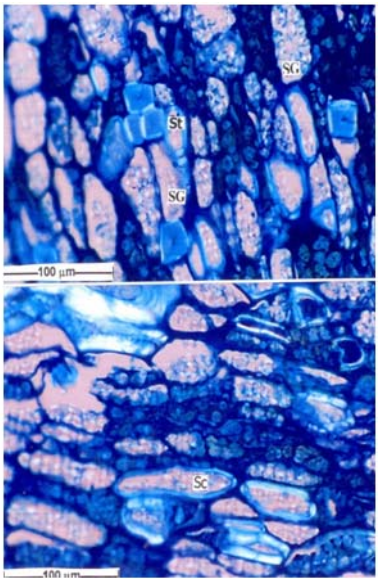

Fig 2: Distribution of Crystals, Sclereids and Starch grains in C. cassia
SG-Starch Grains, SC-Sclereid

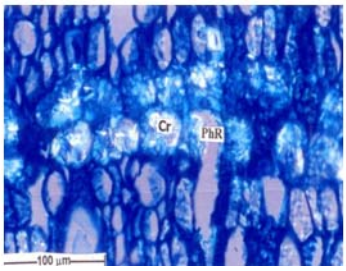

Fig 3: Needle Crystals in the Phloem ray (*c. cassia)*
Cr-Crystals, PhR-Phloem Ray

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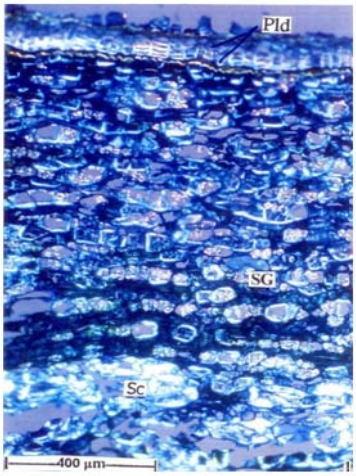

Fig 4: T.S. of bark Outer Sector showing distribution of Starch Grains & Sclereids in *C. cassia*
Pld-Phelloderm, SG-Starch Grains, SC-Sclereid

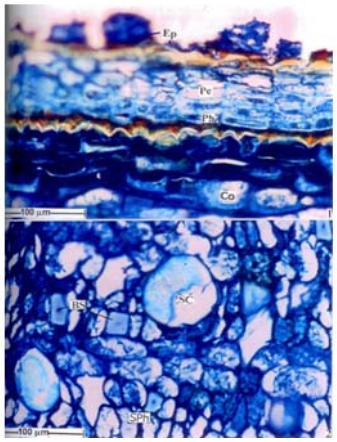

Fig 5: T.S. of bark through periderm and Secondary Phloem
BS-Brachy Sclereids, Co-Cortex, EP-Epidermis, Pe-Periderm, Ph-Phellam, SC-Sclereid, SPh-Secondary Phloe

Fig 6: Structure of the C. cassia bark, (a) Periderm and Cortex, (b) Cortex with Sclereid, (c) Secondary Phloem with Phloem Ray
OB.S-Brachy Sclereids, Co-Cortex, OC-Outer Cortex, Pe-Periderm, PhR-Phloem Ray, SC-Sclereids, SPh-Secondary Phloem

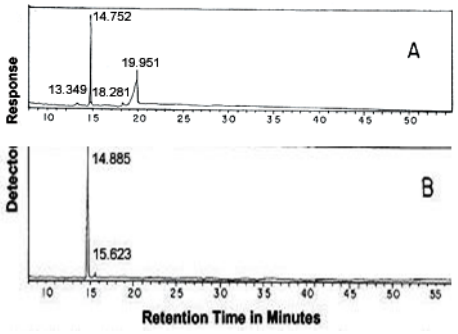

Fig 7: Gas chromatographic analysis of A) *Cinnamom zeylanicum* and B) *Cinnamom cassia*

Discussion

Plant essential oils have potential as natural products for organisms control because some of them are selective, often biodegrade to nontoxic products, and have little or no harmful effects on nontarget organisms (Veal, 1996, Barton, Hadfield-law, 2000 and Mumcuoglu *et al.,* 2002). They can be applied to humans in the same way as other conventional pesticides. They also provide useful information on resistance management because certain plant extracts or phytochemicals can be highly effective against insecticide-resistant insect pests (Lindquist, Adams, Hall, Adams, 1990. Laboratory and greenhouse valuations of Margosan-O against bifenthrin-resistant and – susceptible greenhouse whiteflies, *Trialeurodes vaporarium* (Homoptera: Aleyrodidae). Proc. the USDA neem workshop, USDA-ARS 86, Beltsville, pp. 91-99; Ahn et al.,1997). In addition, some plant essential oils or their constituents have been proposed as an alternative of the commonly used synthetic pediculicides, because they were exempted from toxicity data requirements by the US Environment Protection Agency as minimum risk pesticides under the Federal Insecticide, Fungicide, and Rodenticide Act (US EPA. 1996). Furthermore, plant essential oils are widely available and some are relatively inexpensive compared with plant extracts (Isman, 2001). Adulticidal activity against lice has been reported for some essential oils such as aniseed, cinnamon leaf, thyme red, tea tree, and nutmeg oils (Veal, 1996); anise and ylang ylang oils (Mumcuoglu et al., 2002); and cade, cardamone Ceylon, clove bud, eucalyptus, marjoram, myrtle, pennyroyal, rosewood, and sage oils (Yang et al., 2004). Ovicidal activity has been also reported in clove bud and leaf oils (yang et al., 2003) and *Eucalyptus globules* leaf oil (Yang et al., 2004). In the present study, *C.zeylanicum* bark essential oils exhibited potent adulticidal and ovicidal activities against *P.h . capitis.*

Various compounds, including phenolics, terpenoids, and alkaloids, exist in plant essential oils and jointly or independently they contribute to bioefficacy such as insecticidal, ovicidal, repellent, and antifeeding activities against various insect species (Isman, 2000 and Isman, 2001). Much effort has been focused on the determination of the distribution, nature, and practical use of plant essential oil-derived chemical substances that have insecticidal activities. Naturally occurring adulticidal and ovicidal compounds against lice include eugenol and methyl salicylate (Yang et al., 2003); and α- pinene, ß-pinene, (ε) – pinocaveol, γ-terpinene, and 1- α-terpineol (Yang et al, 2004). In the current study, the pediculicidal constituents of *C. zeylanicum* bark essential oil were identified as benzaldehyde, (ε)- cinnamaldehyde, and linalool by GC-MS analysis. Of the compounds used, benzaldehyde, linalool, and salicylaldehyde were found to be more potent adult pediculicides than either d-phenothrin or pyrethrum. Since the louse population used appeared to be susceptible to natural pyrethrum and synthetic pyrethroids as determined by genotyping analysis of sodium channel (Yang et al, 2004 and Yang et al, 2004).A survey on *Pediculus capitis* (Anoplura: Pediculidae) infestation in primary school children in Korea (2002) and development of natural pediculucides for mass control. Ph.D dissertation, Chonbuk National University, Jeonju, Republic of Korea), relatively lower

potency of d – phenothrin and pyrethrum was not likely due to its intrinsic resistance to the insecticides. Additionally, benzaldehyde, benzyl cinnamate, cinnamaldehyde, and salicylaldehyde were highly effective ovicides against *P.h. capitis.*

Elucidation of mode of action natural insecticidal products and insecticides is of practical importance for insect control because it may give useful information on the most appropriate formulation, delivery means and resistance management. Volatile compounds of many plant extracts and essential oils consist of alkanes, alcohols, aldehydes and terpeniods, particularly monoterpeniods (Coats et al., 1991). They exhibit fumigant activity against P.h. capitis. Fumigant toxicity against female *P.h.capitis* has been reported with eugenol and methyl salicylate (Yang et al, 2003); and 1, 8-cineole, α- pinene, ß-pinene, (ε) – pinocaveol (Yang et al, 2004). In the present study, benzaldehyde, linalool, and salicylaldehyde were more effective against female *P.h. capitis* in closed containers than in open ones. These results indicate that the mode of delivery of the essential oil and compounds was likely by vapour action via the respiratory system, although the exact mode of action remains unknown.

Results of this indicate that *C. zeylanicum* bark essential oil and test compounds described could be useful as insect control fumigants or ovicides for *P.h. capitis* adults and eggs. Benzaldehyde (LD_{50} orally, 1300 mg/kg rat), Cinnamaldehyde (LD_{50} orally, 2220 mg/kg rat), linalool (LD_{50} orally, 2790 mg/kg rat), and salicylaldehyde (LD_{50} orally, 520 mg/kg rat) have low acute toxicity to mammals (Budavari et al, 1989). For the practical use of *Cinnnamomum* bark essential oil and test compounds as novel fumigants to proceed, further research is required on the safety issues materials for human health. Other areas requiring attention are pediculicide mode of action and formulations to improve potency and stability and to reduce costs.

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 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 700µg/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 vtro 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 **LC50** (median lethal concentration) has been tested on bees to specify its optimum concentration in apiaries studies, so asnot 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 as resistancee factors, resulting for the indiscriminate use of antibiotics.

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