

Identification of the Major Fungitoxic Component of Cinnamon Bark Oil

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ABSTRACT

The study was done to identify the most active fungitoxic component of cinnamon bark (*Cinnamomum zeylanicum*) oil that can be used as a marker for standardization of cinnamon extract or oil based natural preservative of stored seeds. *Aspergillus flavus* and *A. ruber* were used as test fungi. The hexane extracted crude oil and the hydro-distilled essential oil from cinnamon bark had complete growth inhibition concentration (CGIC) of 300 and 100 $\mu\text{l/l}$, respectively. Both oils produced three fractions on preparative thin layer silica-gel chromatography plates. The fraction-2 of either oil was the largest and most active, with CGIC of 200 $\mu\text{l/l}$, but the fungitoxicity was also retained in the other two fractions. The fraction-1 and 3 of the crude oil reduced growth of both the fungal species by 65%, and those of distilled oil by 45% at 200 $\mu\text{l/l}$. The CGIC of these fractions from both the sources was above 500 $\mu\text{l/l}$. The gas chromatography and mass spectrometry (GC-MS) of the fraction-2 of the hexane extract revealed that it contained 61% cinnamaldehyde, 29% cinnamic acid, and two minor unidentified compounds in the proportion of 4% and 6%. The GC-MS of the fraction-2 of the distilled oil revealed that it contained 99.1% cinnamaldehyde and 0.9% of an unidentified compound. The CGIC of synthetic cinnamaldehyde was 300 $\mu\text{l/l}$ and that of cinnamic acid above 500 $\mu\text{l/l}$. The 1:1 mixture of cinnamaldehyde and cinnamic acid had CGIC of 500 $\mu\text{l/l}$. The data revealed that cinnamaldehyde was the major fungitoxic component of hexane extract and the distilled essential oil of cinnamon bark, while other components have additive or synergistic effects on total fungitoxicity. It is suggested that the natural seed preservative based on cinnamon oil can be standardized against cinnamaldehyde.

Additional keywords: essential oils, antifungal activity, fungitoxicity, active compounds, cinnamon oil, seed preservative.

RESUMO

Identificação do principal composto fungitóxico de óleo de casca de canela

O objetivo deste estudo foi identificar o mais ativo componente fungitóxico de óleo de casca de canela (*Cinnamomum zeylanicum*), que pode ser usado como marcador para padronização de um conservante natural, baseado no óleo de canela, para sementes armazenadas. Os dois fungos de armazenamento, *Aspergillus flavus* e *A. ruber* foram usados como fungos-teste. O extrato de hexano e o óleo obtido por meio de hidro-destilação de casca de canela, tiveram concentração de inibição completa de crescimento (CICC) de 300 e 100 $\mu\text{l/l}$, respectivamente. A cromatografia preparativa de camada delgada de ambos os óleos produziu três frações. Em ambos os óleos a fração-2 foi a maior e mais ativa com CICC de 200 $\mu\text{l/l}$. A fungitoxidez também foi encontrada nas outras duas frações de ambos os óleos. As frações 1 e 3 do óleo extraído com hexano reduziram o crescimento de ambas as espécies de *Aspergillus* em 65%, e do óleo destilado em 45%, com CICC maior que 500 $\mu\text{l/l}$. A cromatografia gasosa-espectrometria de massa revelou que frações-2 de óleo hexânico continha 61% de cinamaldeído, 29% de ácido cinâmico e dois compostos não identificados nas proporção de 4 e 6%. A fração-2 do óleo destilado continha 99,1% de cinamaldeído e 0,1% de um composto não identificado. O CICC de cinamaldeído sintético foi de 300 $\mu\text{l/l}$ e de ácido cinâmico 500 $\mu\text{l/l}$. Os dados revelam que cinamaldeído é o composto principal com atividade antifúngica tanto no óleo extraído com hexano quanto no óleo destilado da casca de canela. Os outros componentes parecem ter efeito aditivo ou sinérgico na atividade fungitóxica total. O conservante natural de sementes baseado no óleo ou extrato de canela pode ser padronizado utilizando cinamaldeído como marcador.

Palavras-chave adicionais: óleos essenciais, atividade antifúngica, fungitoxidez, compostos ativos, óleo de canela, conservante sementes.

INTRODUCTION

Fungal deterioration of stored seeds and grains is a chronic problem in the Brazilian storage system because of the warm and humid climate. Under such conditions these

products are colonized by various species of *Aspergillus* leading to deterioration and mycotoxin formation. There is no practical, cost-effective, and non-toxic method available for preventing fungal deterioration of these commodities. Therefore use of non-toxic edible substances to control fungal

deterioration of the stored grains and seeds is highly desirable. Several edible botanical extracts have been reported to have antifungal activity (Ferhout *et al.*, 1999; Mastura *et al.*, 1999; De *et al.*, 1999; Pradeep *et al.*, 2003).

The bark and the leaves of *Cinnamomum* spp. are commonly used as spices in home kitchens and their distilled essential oils or synthetic analogs are used as flavoring agent in the food and beverage industry. Although traditionally known, some recent scientific studies have shown antimicrobial activity of essential oils of *Cinnamomum 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. Antifungal activity was reported for respiratory tract infecting fungi such as *Aspergillus niger* Tieghem, *A. fumigatus* Fres., *A. nidulans* (Eidam) Winter and *A. flavus* Link (Singh *et al.*, 1995). Treating high moisture barley (Idler *et al.*, 1996) or wheat (*Triticum aestivum* L.) grains (Scholz *et al.*, 1999) with essential oil of *C. zeylanicum* protected them from deteriorating fungi and ochratoxin formation. Similar findings were reported for protection of stored maize (*Zea mays* L.) against *A. flavus* (Monte-Belmont & Carvajal, 1998).

The *C. zeylanicum* tree is endemic in Brazil, 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 Paraná 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 findings of Chang *et al.* (2001) showed that such differences might be responsible for the varied antimicrobial activity of the essential oil. While the essential oil obtained from one tree of *C. osmophloeum* showed strong antimicrobial activity, that from another tree did not show any activity. 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

The extract or essential oil of *C. zeylanicum* stem bark is composed of a number of compounds and not all of them appear to have antimicrobial activity. Therefore, for developing a reliable ready-to-use product it is necessary to identify the most active component(s) against which the final product can be a standardized. The following study

was done to identify the most active antifungal fraction of *C. zeylanicum* bark distilled essential oil and the hexane extract.

MATERIALS AND METHODS

Preparation of hexane 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 hexane until all the hexane soluble components were removed. The solvent was evaporated using a rotatory evaporator, under vacuum, at 35 °C. The bark essential oil was obtained by hydro-distillation for 6 h. The distillate was extracted twice with dichloromethane, including water soluble or dispersed components, dried over anhydrous sodium sulfate, and the dichloromethane 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 use.

Fractionation of the hexane extract and the distilled oil

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

Bioassays

The antifungal activity of all the tests materials (hexane extract, distilled oil and their TLC fractions) was determined by the use of "food poisoning technique" (Dhingra & Sinclair, 1995). Each extract or its fraction was tested at concentrations of 50 to 500 µl/l at the intervals of 50 µl/l, to determine the complete growth inhibition concentration (CGIC). The test material was dissolved in methanol in proportion of 1:1, and the required amount was added to the cool molten potato dextrose agar which was then shaken vigorously, and poured into 9-cm diameter culture plates. The medium containing 250 µl/l methanol served as control. The medium in each plate was seeded with a 5-mm water-agar culture disc of *A. flavus* Link or *A. ruber* Link, and incubated at 25 °C for ten days, when

the colony diameter was measured. The growth inhibition was expressed as the percentage of the colony diameter in the control plates. All the bioassays were done in four replicates and repeated.

Characterization of active fraction

The most active fraction of the hexane extract and the distilled oil was characterized by gas chromatography-mass spectrometry (GC-MS), using a Shimadzu QP5000 system equipped with a data base with 250,000 compounds, auto-sampler, fused silica capillary column coated with DB-1 stationary phase (30 m x 0.25mm; 0.25 μ m 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* 29 to 500. The injector and transfer line temperatures were maintained at 220 °C and 280 °C, respectively. One microliter of each sample was injected in the split mode (100:1).

RESULTS AND DISCUSSION

The hexane extract and the distilled oil showed CGIC of 300 μ l/l and 100 μ l/l for both species of *Aspergillus*, which is much lower than the reported 2000 μ l/l (Mishra *et al.*, 1992) and 400 μ l/l (Tiwari & Tiwari, 1997).

The preparative TLC fractionation of hexane extract 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. The fraction-2 was most active against both the test fungi with CGIC of 200 μ l/l, which is 33% less than the CGIC of the crude extract. The fractions-1 and 3 reduced growth by an average of 65% at 200 μ l/l. The CGIC of either of these fractions was higher than 500 μ l/l, since the growth was reduced only by 90% at this concentration. The lower CGIC of fraction-2 compared to the crude extract suggests that it contained the major antifungal component of the hexane extract of *C. zeylanicum* bark. The other two fractions also contained antifungal components of lower activity.

Similar to the hexane extract, the preparative TLC of the distilled oil also yielded three fractions, with a combined yield of 97.5% of the crude distilled oil. The fraction-2 represented the 70% of the total yield, which corresponds to 71.8% of the crude distilled oil. The fraction-1 and 3 made up 21 and 6.5% of the total yield, corresponding to 21.5 and 6.6%, respectively of the crude distilled oil. Similar to the hexane extract, the fraction-2 was most active against both the test fungi with CGIC of 200 μ l/l, which is twice the CGIC of the crude distilled oil. This behavior of the distilled oil and its fraction-2 is similar to the crude hexane extract. At 200 μ l/l both, the fraction-1 and 3 reduced fungal growth by an average of 43%, which is considerably

lower than the corresponding fractions of the hexane extract. Like fractions-1 and 3 of the hexane extract the CGIC of these fractions was also above 500 μ l/l as the growth was reduced only by 74%, which is also lower than the same fractions of hexane extract. These data show that the major antifungal component of the distilled oil, similar to the hexane extract, was retained in the fraction-2.

The GC/MS of the fraction-2 of the hexane 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 (Figure 1A). 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 (Figure 1B).

Since the most fungitoxic fraction-2 of the hexane extract consisted mainly of cinnamaldehyde and cinnamic acid, and only the former was found in the same fraction of distilled oil, antifungal activity of the synthetic analog of cinnamaldehyde and cinnamic acid was tested separately and in 1: 1 mixture. The synthetic cinnamaldehyde had CGIC of 300 μ l/l, which is 33% higher than the fraction-2 of distilled oil, which contained 99.1% cinnamaldehyde, and also of hexane extract which contained 61% of cinnamaldehyde. At 300 μ l/l the synthetic cinnamic acid reduced growth only by 26%, and its CGIC appears to be much above 500 μ l/l since the growth at this concentration was reduced only by 36%. On the other hand, CGIC of 1:1 mixture of these compounds was 500 μ l/l, which is also much higher than that of the crude hexane extract and crude distilled oil and their corresponding fractions-2. The similarity of CGIC value of the fractions-2 of the hexane extract which contained about 61% cinnamaldehyde and 29% cinnamic acid, and of the fraction-2 of the distilled oil, which contained 99.1% cinnamaldehyde, was rather intriguing and could not be explained by the data from this

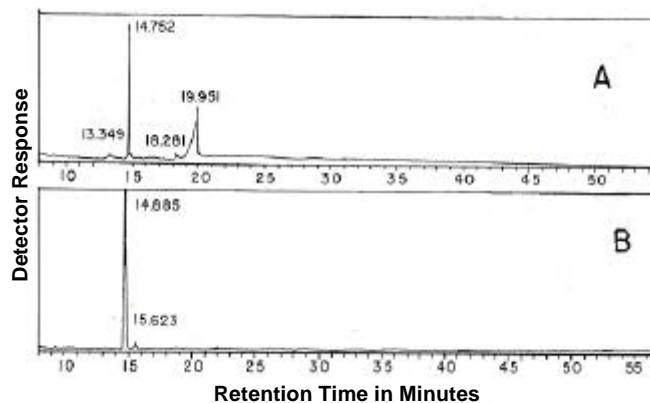


FIG. 1 - A total ion chromatogram obtained by gas chromatography-mass spectrometry (GC-MS) analysis of the most active fraction-2 from preparative silica gel thin layer chromatography of the hexane extract (A) and the distilled essential oil (B).

study. However, these data clearly suggest that although the cinnamaldehyde is the major fungitoxic component of the *C. zeylanicum* bark distilled oil, and cinnamic acid present in the hexane extract may act as a secondary component, total fungitoxic activity of either hexane extract or the distilled oil involves others components whose presence may be additive or synergistic to the activity of these two components.

The lower CGIC of the fraction-2 of the distilled oil or the hexane extract compared to the synthetic cinnamaldehyde is similar to that reported by Chang *et al.* (2001) who showed that antibacterial activity of synthetic cinnamaldehyde was 50% less than that of the *C. osmophloeum* leaf distilled oil containing 77% cinnamaldehyde. The composition of *C. zeylanicum* bark distilled oil is known to differ considerably, according to region, year, climate and the tree chemotype. Chalchat & Valade (2000) reported that *C. zeylanicum* bark distilled oil from Madagascar consisted predominantly of cinnamaldehyde and camphor, while Nath *et al.* (1996) found benzyl benzoate constituting 85% of the oil distilled from tree bark grown in northeast India, and the GC/MS revealed seventeen components. On the other hand, Jirovetz *et al.* (1998) reported eugenol as the major (85%) component of bark distilled oil from Cameroon. The bark essential oil from Paraná was found to have about 55% cinnamaldehyde (Koketsu *et al.*, 1992). Such large differences in the chemical compositions of the distilled oil may explain the large difference of the CGIC for fungal growth obtained in this study and by other workers (Mishra *et al.* 1992; Tiwari & Tiwari, 1997). This study, however, did not aim at determining the total composition of distilled or hexane extracted oil from Brazilian *C. zeylanicum*, but to identify the most active fungitoxic compound that can be used as standardizing marker for product development. It is possible that benzyl benzoate may have some toxic activity, and it separated from the crude extract or oil to the fraction 1 or 3. Ross (1976) reported eugenol, known for its antimicrobial activity, as one of the major components of bark distilled oil along with cinnamaldehyde, which constituted 50 to 70% of the total essential oil. Eugenol, if present in the extracts, may have separated as indicated by fungicidal activity of fraction 1 and 3, although at a much lower level. Due to the large variation in the chemical composition of *C. zeylanicum* bark oil it is rather difficult to compare the findings of this study to those of the others. On the other hand, since in most studies cinnamaldehyde was found to constitute more than 50% of the total essential oil, it would be safe to assume that this compound is the major fungitoxic component of the *C. zeylanicum* oil. According to Chang *et al.* (2001), cinnamaldehyde has good antibacterial activity with CGIC ranging from 250 to 500 $\mu\text{l/l}$ depending upon the bacterial species, while the cinnamic acid showed CGIC of 1000 $\mu\text{l/l}$, which is two to four times higher than that of cinnamaldehyde. These results corroborate the findings of this study where cinnamaldehyde showed much higher

fungitoxic activity than the cinnamic acid.

In conclusion this study shows that non-toxic seed and grain preservatives can be developed by sourcing the raw material from high cinnamaldehyde chemotype trees, and both the distilled oil or hexane extracted oil may be equally useful for the final product which should be standardized against cinnamaldehyde.

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