Cinnamomum zeylanicum Blume and *Myristica fragrans* Houtt essential oils as bio-pesticides against the fungus *Guignardia citricarpa*

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The fungus *Guignardia citricarpa* causes serious damage in the orange plantation and lesions in the bark of oranges, lemons, mandarins and their hybrids, resulting in loss of value for the consumer market, causing also premature fruit drop responsible for reducing the production. To collaborate with industry, the scientific community is studying and analysing some properties of the essential oils of spices including the presence of bioactive compounds, other elements and features that have not been fully identified. Some compounds from the essential oil of spices have demonstrated bactericidal and fungicidal properties. In the present study the essential oils obtained by hy drodistillation from *Cinnamomum zeylanicum* Blume (cinnamon) and *Myristica fragrans* Houtt (nutmeg), were tested on the growth of fungal *Guignardia citricarpa* mycelia. The *G. citricarpa* can also, which significantly. The main findings of antifungal activity of the selected essential oils demonstrated that the oils from cinnamon and nutmeg in a concentration of 33.3%, completely inhibited the fungus growth.

Keywords: *Cinnamomum zeylanicum*; *Myristica fragrans*; essential oil; *Guignardia citricarpa*; antifungal activity

1. Introduction

Spices and aromatic herbs are divided into leaves, flowers, bud, seeds bark or dry roots from different plants, and it is possible to define them as a highly flavoured products from vegetal origin that volatize easily when incorporated in small quantities in food products and contribute to their aroma, flavor, color or even to the food preservation [1; 2]. Important spices for the food, pharmaceutical and medicinal industries are *Cinnamomum zeylanicum* Blume (cinnamon) and *Myristica fragrans* Houtt (nutmeg).

Cinnamon is a small tree with 10-15 meters tall which is obtained from the tree inner bark from native species of Sri Lanka and South Asia, primarily distributed between Asia and Australia and then to the rest of the world. Cinnamon species are often used as a spice and has a long history to add flavor in the food; nowadays is also been used as a medicine, bactericide and fungicide [3; 4].

The nutmeg belongs to the family Myristicaceae, The genus Myristica is distributed from India and South-east Asia to North Australia and the Pacific Islands [5]. Nutmeg has its origins in the Spice Islands of Indonesia. It was widely popular in Europe and India for its flavouring, medicinal and fungicide properties. The name nutmeg is derived from the Latin word nux muscatus, meaning 'musky nut'. Two important spices are derived from the fruit – nutmeg and mace. The spice nutmeg is the dried kernel of the seed and mace is the dried aril surrounding it. Both have similar flavour [6].

Cinnamon and nutmeg's unique abilities/properties come from compounds that can be extract from the fresh plant or, more commonly, from their essential oils prepared in the industries. Their composition include alcohols, esters, aldehydes, terpenes, phenols, organic acids and other compounds which have not been fully identified [7; 8; 9].

The "International Standard Organization" (ISO) defines the essential oils as products obtained from parts of plants by hydrodistillation. In general, they are complex mixtures of volatile lipophilic substances, usually odoriferous and liquid. They are originated from the secondary metabolism of plants [10; 11; 12].

The essential oil of these spices have been used as carminative, spasm relieving, general tonic and antiseptic, to low cholesterol and lipids, for gum disease, to stimulate circulation and as anti-inflammatory, and has been also used in pharmaceutical industry, especially for correction of smell and taste of drugs [13; 14], as antiseptic, bactericide, fungicide [15], virucidal, antibiotic, analgesic, sedative, antispasmodic, and local anaesthetic [16].

Furthermore, the Food and Drug Administration listed the essential oils as "Safe-GRAS". GRAS is an acronym for the phrase Generally Recognized As Safe. Under sections 201(s) and 409 of the Federal Food, Drug, and Cosmetic Act, any substance that is intentionally added to food is a food additive, that is subject to premarket review and approval by FDA, unless the substance is generally recognized, among qualified experts, as having been adequately shown to be safe under the conditions of its intended use, or unless the use of the substance is otherwise excluded from the definition of a food additive [17]. So a formulation using the compound could be sprayed like a pesticide, but without the potential for adverse health effects of many insecticides – plus the added bonus of a pleasant and familiar smell [18].

Due to the presence of citrus black spot (CBS) in many countries in the Southern Hemisphere, the European Community and the United States severely limit importation of fresh citrus fruit from those countries where the disease is present [19]. Symptoms on fruit usually develop late in the season, and fruit that are symptomless at harvest may develop CBS during transportation or storage. However, fruit may be severely infected with relatively minor symptoms on foliage. *G. citricarpa* produces ascospores in the decomposing leaf litter on the grove floor [20; 21]. An increasing consumer demand for foods free or with low added synthetic preservatives because they could be toxic to humans, exists [22]. Concomitantly, consumers have also demanded for wholesome and safe food with long shelf lives. These requirements are often contradictory and press on the food industry for progressive removal of chemical preservatives and adoption of natural alternatives such as the use of essential oils [23].

Considering the antifungal potential of essential oils, the purpose of this study was to identify the active compounds present in the essential oils of cinnamon and nutmeg spices and to analyse the antifungal activity of these essential oils against the citrus black spot (CBS). *Guignardia citricarpa* has becoming a serious widespread problem for citrus production in Brazil and many countries that produce any kind of citrus fruit [24; 25]. This fungus attacks on the mature fruit and is characterized as hard spot, false melanose, freckle spot, virulent spot and also described as a cracked spot type [26].

2. Materials and methods

2.1 Samples

Cinnamomum zeylanicum Blume (cinnamon) and *Myristica fragrans* Houtt (nutmeg) were donated by HiKari Industry and Commerce LTDA – BRAZIL.

2.2 Hydrodistillation

The hydrodistillation was performed using 50g of dry cinnamon or nutmeg (depending on the test to be done) and placed in a round-bottom flask with 500mL of boiling distilled water.

The contents were distilled for 3 hours to ensure the total extraction of the essential oil. Most of the essential oil does not mix well with water in the liquid phase so, after condensation, they were separated by decantation in the end of the hydrodistillation.

2.3 Fungal Isolation and Identification

The fungal colonies of *Guignardia citricarpa* (black spot) donated by the Biomedical Sciences Institute II - ICB, University of São Paulo - USP, were isolated and identified as [27].

For the isolation and identification of the fungus, fragments about 5 mm^2 were removed from fruits with hard spot symptoms and immersed in 70% ethanol for 1 min, then in a 1:3 v/v mixture of sodium hypochlorite: sterile water for 3 min and rinsed in sterile water for 30s.

After drying of the material on sterilised filter paper, the fragments were placed aseptically in Petri dishes containing oatmeal (OA) media and potato dextrose agar (PDA) (Oxoid) with chloranphenicol (0.01% w/v). Plates were incubated for 7 to 10 days at 25ºC. After incubation, typical positive G. colonies were identified and prepared to the antifungal test [24; 28].

2.4 Antifungal test preparation

Following to isolation and identification, the antifungal assays were carried out in vitro according two authors [29; 30]. Briefly, in the control samples, 4 mycelium fragments of the fungus (G. citricarpa) were placed aseptically in a sterilized Petri dish (triplicate) containing 10 mL of PDA (common microbiological growth media).

To verify the antifungal potential of the essential oils, 4 mycelium fragments of the fungus were placed in a sterilized Petri dish (triplicate) containing 10 mL of PDA mixed with 5 mL of Cinnamon or nutmeg essential oil to obtain the desired concentration of 33.3% of essential oil.

All Petri dish were incubated for 10 days at 25ºC. The antifungal potential was determined by measured the radial growth.

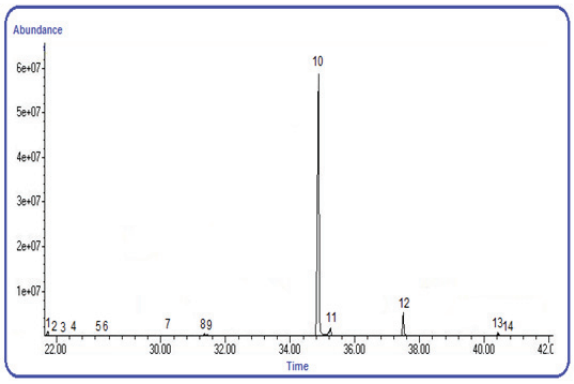
3. Results and discussion

3.1 Identification of cinnamon and nutmeg essential oils compounds

Figure 1 show the compounds which have been identified in the cinnamon essential oil and its respective chromatogram. The main compounds are cinnamaldehyde, eugenol, cinnamyl acetate, α-pinene and other less expressive ones. The most abundant compound is cinnamaldehyde (88.39%).

Compounds	% of compounds	Retention time
1 - α-Pinene	1,03 ± 0,09	21.10
2 - Canfene	0,29 ± 0,05	21.86
3 - Benzaldehyde	0,15 ± 0,04	22.38
4 - β-Pinene	0,35 ± 0,08	23.11
5 - Limonene	0,23 ± 0,09	25.27
6 - Eucalyptol	0,64 ± 0,04	25.45
7 - Propanal benzene	0,38 ± 0,03	30.65
8 - Borneol	0,39 ± 0,06	31.19
9 - Methyl benzofuran	0,44 ± 0,11	31.35
10- Cinnamaldehyde	88,39 ± 0,06	34.87
11 - Bornyl acetate	1,02 ± 0,04	35.18
12 - Eugenol	4,21 ± 0,08	37.49
13 - Cinnamyl acetate	2,34 ± 0,09	40.46
14 - Benzopyrene	0,08 ± 0,04	40.61

a)



b)

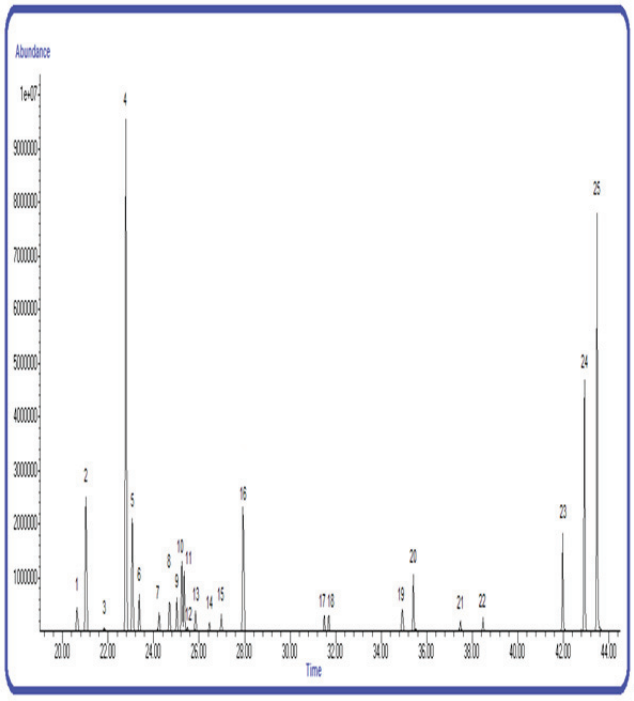
Fig. 1 Volatile compounds identified in the cinnamon essential oil: a) compounds identification including their percentage and respective retention time. b) GC-MS chromatogram.

These results about the compounds and the major one are in agreement with those reported by other authors [31], where they founded the cinnamaldehyde with approximately 70%. They also identified other compounds that we found in this study, with few variations in their amounts. These differences in the concentration of the compounds in plants of the same species can occur due to variations in its geographical location, harvest time, weather conditions, cultivation management, age of the plant and storage conditions [32].

Figure 2 shows twenty-five different compounds identified in the nutmeg essential oil and the respective chromatogram.

Compounds	% of compounds	Retention time
1 - α - tujene	1,40 ± 0,08	20.63
2 - α - Pinene	8,18 ± 0,07	21.02
3 - Canfene	0,16 ± 0,11	21.81
4 - Sabinene	27,03 ± 0,09	22.78
5 - β - pinene	6,02 ± 0,13	23.06
6 - β - myrcene	1,59 ± 0,07	23.36
7 - α - phellandrene	0,65 ± 0,06	24.24
8 - α - terpinene	1,96 ± 0,1	24.69
9 - p - cymene	1,36 ± 0,08	25.01
10 - Limonene	2,95 ± 0,06	25.23
11 - β - phellandrene	2,34 ± 0,08	25.33
12 - Eucalyptol	0,18 ± 0,12	25.42
13 - 3 - Carene	3,12 ± 0,08	25.80
14 - γ - terpinene	0,73 ± 0,09	26.43
15 - Terpinene	1,31 ± 0,06	26.97
16 - Geraniol	8,85 ± 0,13	27.99
17 - Terpinenol - 4	0,11 ± 0,15	31.41
18 - α - terpineol	0,15 ± 0,08	31.55
19 - Bornyl acetate	0,23 ± 0,07	35.16
20 - Safrole	2,23 ± 0,09	35.40
21 - Eugenol	0,36 ± 0,11	37.47
22 - Copaene	0,47 ± 0,05	38.47
23 - Methyl isoeugenol	3,57 ± 0,07	41.96
24 - Myristicin	9,50 ± 0,07	42.92
25 - Elemicin	15,56 ± 0,06	43.47

a)



b)

Fig. 2 Volatile compounds identified in the nutmeg essential oil: a) compounds identification and their percentage and retention time. b) GC-MS chromatogram.

The major compound identified in the nutmeg essential oil was sabinene with 27.03% (Figure 2a. Comparing both essential oils it is visible that cinnamon oil (Figure 1) is dominated by one bioactive compound (cinnamaldeyde) while nutmeg oil composition contains some representative different bioactive compounds such as elemicin (15.56%), myristicin (9.50%) and α-pinene (8.18%) (Figure 2).

The composition of nutmeg essential oil is similar of those previously reported by other authors [33; 34; 35]. These authors also identified sabinene as the major compound; however, the relative amount of compounds was slightly

different. The geographic location, harvest time, weather and storage conditions may influence in the chemical composition of the same species of plant [32].

3.2 Antifungal test – Control samples

Figure 3 shows the colonies of *Guignardia citricarpa* in a PDA medium, these are the normal aspect of the test without the fungal colonies growth and were named control samples.

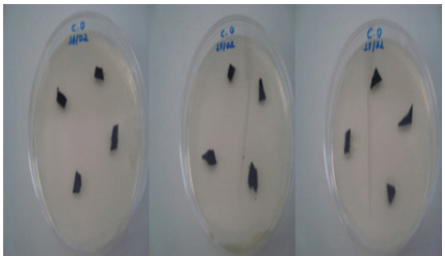


Fig. 3 Control samples containing PDA medium in a sterilized Petri dish inoculated with 4 mould spores of *Guignardia citricarpa* colonies.

Figure 4 shows the colonies of *G. citricarpa* in the same PDA medium of the control samples (Fig. 3), but incubated for 10 days; It is clear the difference between the control samples and the positive growth samples.

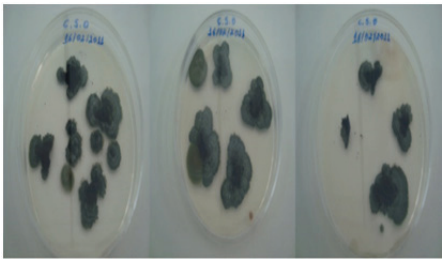


Fig. 4 Positive fungal growth control samples containing PDA medium in a sterilized Petri dish inoculated with 4 mould spores of Guignardia citricarpa colonies and incubated for 10 days at 25ºC.

3.3 Antifungal test – cinnamon essential oil

The figure 5 shows the samples containing PDA medium mixed with cinnamon essential oil in a 33% of concentration and inoculated with colonies of mould spores of G. citricarpa and thus, when the samples in figure 6 were compared with control samples (fig. 3) it was possible to verify that they had the same size, indicating that the essential oil of nutmeg was effective to control the fungus growth.

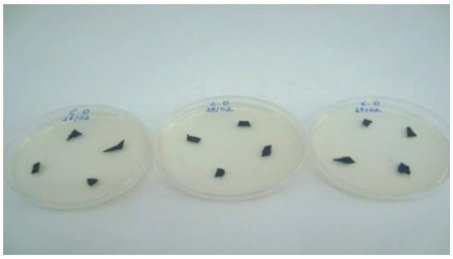


Fig. 5 Samples containing cinnamon essential oil in a 33,3% oil concentration and the fungus G. citricarpa incubated for 10 days.

The literature describes the biological activity of a wide range of essential oils from plants against several fungal phytopathogens [36; 37]. The activity of cinnamon essential oil may be related to the action of cinnamaldehyde and eugenol [36]. There are studies [31; 38] that reported the cinnamaldehyde as the major active compound respecting antifungal properties.

3.4 Antifungal test – nutmeg essential oil

Figure 6 shows the samples containing PDA medium mixed with nutmeg essential oil in a 33% of concentration inoculated with colonies of mould spores of *G. citricarpa*; it was possible to verify after 10 days of incubation that the nutmeg essential oil completely inhibited the growth of fungus *G. citricarpa*, keeping the colonies with the initial size of the control samples (Fig. 3).

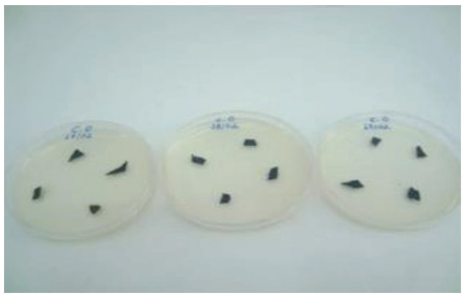


Fig. 6 Samples containing nutmeg essential oil in a 33.3% oil concentration and the fungus *G. citricarpa* incubated for 10 days.

Nutmeg is well known for its intense use in culinary; however some authors claim that the essential oil has antifungal properties [39; 40; 41]. According to study [42], the compounds responsible for the antifungal activity are pinene, camphene, sabinene, copene, eugenol and isoeugenol. However, other authors [43] questioned the action of one isolated compound and propose the possibility of interactions between the compounds, synergistic effects.

4. Conclusions

This study reports the activity of the essential oils of cinnamon and nutmeg against the fungus *G. citricarpa*. The next step is to prove their effectiveness and to establish the mycotoxic activity. "In vivo" studies are also important to be performed to determine the optimal climatic conditions; temperature, humidity and wind power on essential oils plant production. After, it will be possible the use of these essential oils in a commercial scale against the fungus *G. citricarpa*.

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