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Chemical composition and antifungal and nematicidal activities of the hexanic and methanolic extracts of *Syzygium aromaticum*

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ABSTRACT: In the present study, the fungicidal and nematicidal activities of the hexanic and methanolic extracts of *Syzygium aromaticum* were evaluated. At a concentration of 100 μg/ml, the hexanic extract inhibited 43.70% of the mycelial growth of *Fusarium* sp. at 48 h and 52.90% at 72 h; while at the concentration of 200 μg/ml, it inhibited 54.30% and 60% of the mycelial growth of *Fusarium* sp. at 48 h and at 72 h, respectively. The methanolic extract inhibited 99.87% of the hatching of *Haemonchus contortus* (*H. contortus*) eggs at a concentration of 1.25 mg/ml, with respect to the mortality of J2 larvae of it was 71.11% at the concentration of 1.25 mg/ml. Gas Chromatography coupled to mass spectrometry analysis allowed the identification of 17 compounds in total, of which eugenol, acetyl eugenol and caryophyllene were the most abundant in both hexanic and methanolic extracts. Additionally, hexatriacontane, octacosane, 11, 11-Dimethyl-4,8-dimethylenebicyclo [7.2.0] undecan-3-ol, ledene oxide- (II), and humulene were only present in the methanolic extract, while isoaromadendrene epoxide, 2-naphthalenemethanol, decahydro-α, α, 4a-trimethyl-8-methyle, loxapine *N*-oxide, diepicedrene-1-oxide, aristolochic acid II, and *trans*-isoeugenol were only found in the hexanic extract.

KEYWORDS: phytopathogens, nematodes, eugenol, natural products

INTRODUCTION

Clove (*Syzygium aromaticum*, *S. aromaticum*), an aromatic plant in the Myrtaceae family, is a species originally from Indonesia and currently cultivated in different geographies of the world. Because it is an important source of chemical compounds with remarkable biological properties, cloves have great potential for applications in the pharmaceutical and food industries [1, 2].

For centuries, the spice has been traditionally used for medicinal purposes, which are supported by various studies. Cloves have been reported to have antiviral activity against strains of herpes virus, hepatitis C, and influenza A [3–5]; antimicrobial activity against *Escherichia coli*, *Listeria monocytogenes*, *Pseudomonas aeruginosa*, *Bacillus subtilis*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Proteus vulgaris*, and *Streptococcus suis* [6–8]; antifungal properties against *Trichophytonrum rubella*, *Trichophytonrum mentagrophytes*, *Microsporum canis*, *Fusarium moniliforme*, *Fusarium oxysporum*, *Epidermophyton floccosum*, *Mucor* sp., *Microsporum gypseum*, *Candida albicans*, and *Aspergillus* sp. [9–14]; and antiprotozoal activity against parasites of the genus *Babesia* and *Theileria* [2]. Additionally, the acaricidal activity of clove essential oil has been reported against *Dermatophagoides pteronyssinus* and *Dermatophagoides farina* [15].

These activities are related to the presence of the metabolites: eugenol, carvacrol and eugeniin [16]. It has also been reported that eugenol, the most abundant compound in cloves, has nematicidal activity against *Haemonchus contortus* (*H. contortus*) by inhibiting egg hatching [17], so that extracts of *S. aromaticum* rich in said metabolite can be used to develop alternative control methods against this nematode. Vargas et al [18] reported that the acetone-water extracts of the plants *Lysiloma latisiliquum*, *Laguncularia racemosa*, *Rhizophora mangle*, *Avicennia germinans*, and *Theobroma cacao* (shell and pulp of the seed) affected the hatching of larvae from eggs of *H. contortus* mainly by blocking the hatching process. Likewise, it has been observed that different extracts of these plants have anthelmintic activity against the parasitic nematode of sheep *H. contortus* [19, 20], *Trichinella spiralis* [21], and *Schistosoma mansoni* [22]. Therefore, the objectives of this project were to evaluate the fungicidal and nematicidal activities of the hexanic and methanolic extracts of *S. aromaticum*, as well as the identification of the compounds present in the extracts by gas chro-

matography coupled to mass spectrometry (GC-MS).

MATERIALS AND METHODS

S. aromaticum extracts

Samples of *S. aromaticum* (cloves), weighed approximately 400 g, were commercially acquired in the city of Cuernavaca, Morelos, Mexico in October 2016. Each nail button was split in half and allowed to dry at room temperature for 48 h. Subsequently, from 252 g of dry plant material, organic extracts were obtained by sequential maceration with polarity gradient, using 1.5 l of hexane (hex) and methanol (MeOH), consecutively. The maceration was carried out with constant stirring at 120 rpm and 37 °C for 24 h. After filtration to remove plant material, each sample was concentrated on a rotary evaporator to dryness and dried under vacuum to constant weight. It was kept in closed containers, protected from light and stored in refrigerator until later use.

Production of larvae and eggs of *H. contortus*

At the National Center for Disciplinary Research in Animal Health and Safety (CENID-SAI), a two-month-old male Pelibuey breed sheep (±20 kg) was used as the donor. The animal was kept in a metabolic cage and infected orally with 350 infective larvae per kg of live weight. On the day 21 of the pre-patent period, a McMaster was performed to confirm if the animal was positive for determining the number of eggs per gram feces [23]. The reading was made at 40X under a microscope.

Obtaining *H. contortus* eggs

Fecal samples were collected directly from the rectum of the donor sheep. The samples were later macerated and sieved through a mesh system of different openings (No. 35, 100, 200, and 400) using water. Finally, in a 15 ml test tube, 8 ml of 40% sucrose and 6 ml of the eggs recovered after sieving were added. The mixture was centrifuged at 3500 rpm for 5 min to create a density gradient, and the eggs that were suspended in the ring-shaped phase were recovered. Finally, the eggs were washed with distilled water to remove any stool remains [23, 24].

Obtaining infective larvae of *H. contortus* (L3)

Feces were collected to perform stool cultures to obtain infective larvae. With the help of a basin, the feces were moistened with running water and mixed homogeneously with foam rubber at a ratio of approximately 1:1. The optimum temperature was 28 °C and the optimum pH was 6.5 to 7.5. The stool culture was incubated for seven days. After the incubation period, larvae were recovered using the Baermann funnel technique. The test tubes were left to sit for 24 h until sediment was obtained at the bottom of the test tube, and finally the test tubes were stored at 4 °C until further use. For the elimination of the *H. contortus* (L3) sheath, a 0.187% (10 ml) commercial sodium hypochlorite was used. The larvae were exposed for 7 min, washed four with distilled water, and centrifuged for 1 min at 3500 rpm to remove the sodium hypochlorite. Once the larvae had been washed, the sediment formed in 50 ml Falcón tubes was recovered [23].

Evaluation of antifungal activity

The inhibition of the hexanic and methanolic extracts of *S. aromaticum* against the phytopathogenic fungi: *Alternaria solani*, *Fusarium* sp., and *Phytophtora capsici*, was tested in Potato-Dextrose-Agar (PDA) medium. Initially, PDA medium was prepared, and hexanic and methanolic extracts (100 μg/ml and 200 μg/ml, respectively) were added to the medium. Subsequently, the PDA boxes treated with the extracts and the control (PDA without extract) were each placed with a square of approximately 4 mm^2 of medium with five-day incubation fungus. The squares were placed in the center of the box and incubated at 28 °C for 72 h. To quantify the inhibition effect, the diameter of mycelial growth was measured using a digital vernier at 48 and 72 h after inoculation. Three repetitions were carried out for each test. To obtain the inhibition percentage, the following formula was used: Mycelial inhibition (%) = [1-(Da/Db)] × 100, where Da = the diameter of the growth zone on the treated plates, Db = the diameter of the growth zone on the control plate.

Evaluation of nematicidal activity

In 96-well plates, the effect of the hexanic and methanolic extracts of *S. aromaticum* was evaluated by means of hatching inhibition tests and the mortality of sheathless larvae of *H. contortus*. To perform these bioassays, the eggs and sheathless larvae of *H. contortus* were exposed to different concentrations (0.156, 0.312, 0.625, 1.25 mg/ml) of hexanic and methanolic extracts. Negative (PBS, 4% Tween) and positive controls (Thiabendazole and Levamisole 10 mg/ml) were also placed on each plate. The plates were incubated for 48 h (inhibition of egg hatching) and 72 h (larval mortality). After the confrontation time, it was observed and quantified in a compound microscope (4X and 10X).

Phytochemical identification tests

The qualitative identification of the secondary metabolites (alkaloids, flavonoids, steroids, terpenoids, saponins, and coumarins) present in the extracts that showed biological activity was carried out by turning and precipitation reactions, following the methodologies described by Sasidharan et al [25].

Gas chromatography coupled to mass spectrometry

Analysis of gas-mass chromatography was performed on a Thermo Scientific brand TRACE GC with an ITQ900 ion trap mass detector (Thermo Electron Corporation, Milan, Italy). The carrier gas was helium with a flow rate of 1 ml/min. The column used was TRACE-5MS (30 m, 0.25 μm of film and 0.25 mm internal diameter). The program used was full scan and started with a temperature of 50 °C which was maintained for 1 min; and, subsequently, a temperature ramp was applied with increments of 7 °C/min up to 300 °C. The 300 °C was maintained for 5 min, the interface temperature was 280 °C, and the mass temperature was 200 °C.

RESULTS

Obtaining extracts

The hexanic extract (27.2 g, 10.8% w/w) was obtained as a dark gray pasty solid, embedded in an oily phase, and exhibited an intense clove odor; while the methanolic extract (7.23 g, 2.% w/w) displayed a very dense liquid in dark brown color and with pungent odor.

Antifungal activity

The methanolic extract in the mycelial growth inhibition test against *A. solani*, *P. capsici*, and *Fusarium* sp. at a concentration of 100 μg/ml did not show any significant effect on growth inhibition at 48 and 72 h. However, the hexanic extract inhibited the mycelial growth of *Fusarium* sp., by 43.70% at 48 h, and 52.90% at 72 h (Table 1). At the concentration of 200 μg/ml, the same behavior of the hexanic extract was observed at 48 h where it inhibited 54.30% of the mycelial growth of *Fusarium* sp. and at 72 h it inhibited 60% (Table 2).

Nematicidal activity

The methanolic extract in the concentrations of 0.625 and 1.25 mg/ml inhibited the hatching of *H. contortus* eggs by 99%, without significant difference with respect to the positive control (Thiabendazole), but significantly different against PBS. In case of the lowest concentration of 0.156 mg/ml, it inhibited 67.95% of the hatching of eggs. While in the hexanic extract, no significant difference was observed in any of the different concentrations evaluated against the hatching of *H. contortus* eggs (Table 3).

In case of the mortality percentages of the hexanic and methanolic extracts against *H. contortus* larvae, the results were shown in Table 4. The highest percentage was 71.11% in the methanolic extract at 1.25 mg/ml. On the other hand, the highest effect observed in the hexanic extract was 29.5% at 1.25 mg/ml (Table 4).

Identification of metabolites in extracts

In the hexanic extract, only the presence of coumarins was identified; while in the methanolic extract, the presence of flavonoids, saponins, and alkaloids was detected.

Identification of compounds by gas chromatography coupled to mass spectrometry

In total, seventeen different chemical compounds were found between sesquiterpenes, hydrocarbons and phenyl propanoids, with a different combination of eleven of them in each extract (Table 5).

DISCUSSION

S. aromaticum is an aromatic plant with abundant phenolic compounds, sesquiterpenes, monoterpenes, and other hydrocarbon compounds. Among these substances, phenolic compounds are the most relevant, and they include flavonoids such as quercetin and kaempferol; phenolic acids, such as gallic, ferulic, and caffeic acids; hydrolyzable tannins derived from gallic and ellagic acid; and hydroxyphenyl allylic compounds such as eugenol and acetyl eugenol. These last two compounds are found in large quantities in the aromatic plant. Other volatile compounds in essential oil are β-caryophyllene, α-humulen, β-pinene, limonene, farnesol, benzaldehyde, 2-heptanone, and ethyl hexanoate [1]. In other aromatic plants like lemongrass, similar volatile compounds including geraniol, geranial, and citral are the main constituents [26]. In the present study, the hexanic and methanolic extracts obtained from the dried clove buds were analyzed by gas chromatography coupled to mass spectrometry (GC-MS). Seventeen different chemical compounds were identified among sesquiterpenes, hydrocarbons, and phenyl propanoids, with a different combination of eleven of them in each extract (Table 5). Eugenol, acetyl eugenol, caryophyllene, caryophyllene oxide, and humulenol II are compounds that were found in both of the extracts in different proportions. In both extracts, eugenol and acetyl eugenol were the most abundant phytochemicals, with 67.28% and 25.29% relative abundance in the methanolic extract and with 74.49% and 7.38% in the hexanic extract, respectively. This proportion observed in the extracts agrees with that reported for the essential oil of *S. aromaticum*, in which around 85 to 92% is eugenol, and between 5 and 15% corresponds to acetyl eugenol [1, 27]. In the methanolic extract, the compounds found in decreasing in abundance were caryophyllene (1.28%), isoaromadendrene epoxide (1.21%), and β-eudesmol (1.02%). Meanwhile, in the hexanic extract, the compounds were caryophyllene (6.13%), hexatriacontane (2.84%) and octacosane (1.30%).

It has been reported that eugenol is the main bioactive compound in both extracts and the essential oil of cloves [1]. Although the antimicrobial activ-

Table 1 Inhibition of the hexanic and methanolic extracts (100 μg/ml) in the three phytopathogenic fungi.

	% Inhibition					
	48 h			72 h		
Extract	*A. solani*	*P. capsici*	*Fusarium* sp.	*A. solani*	*P. capsici*	*Fusarium* sp.
Methanol	10.30	8.50	8.57	14.58	10.45	5.17
Hexanic	12.60	0.0	43.70	5.90	3.60	52.90

Table 2 Inhibition of the hexanic and methanolic extracts (200 μg/ml) in the three phytopathogenic fungi.

	% Inhibition					
	48 h			72 h		
Extract	*A. solani*	*P. capsici*	*Fusarium* sp.	*A. solani*	*P. capsici*	*Fusarium* sp.
Methanol	14.90	3.23	3.88	12.95	9.09	0.0
Hexanic	10.30	4.70	54.30	1.40	0.0	60.00

Table 3 Effect on the hatching of *H. contortus* eggs by different extracts of *S. aromaticum*.

Concentration	Inhibition of egg hatching (%) ± SD	
(mg/ml)	MeOH	Hex
0.156	67.95 ± 14.20^{b}	8.16 ± 1.30^{a}
0.312	83.09 ± 5.60^{bc}	7.07 ± 3.56^{a}
0.625	99.44 ± 0.61^{c}	4.86 ± 4.44^{a}
1.25	99.87 ± 0.23^{c}	0.91 ± 0.85^{a}
PBS	6.31 ± 4.95^{a}	–
Tween 4%	–	9.42 ± 5.79^{b}
Thiabendazole	100 ± 0^{c}	100 ± 0^{c}

MeOH: Methanolic extract; Hex: Hexanic extract; SD: Standard deviation. Equal letters in the same column indicate that the values do not differ statistically, according to Tukey's test ($p < 0.05$).

Table 4 Mortality of infecting sheathless larvae of *H. contortus* due to the effect of different extracts of *S. aromaticum*.

Concentration	Larval mortality (%) ± SD	
(mg/ml)	MeOH	Hex
0.156	4.68 ± 4.87^{ab}	3.97 ± 1.10^{a}
0.312	18.09 ± 0.80^{ab}	14.01 ± 1.22^{ab}
0.625	26.31 ± 20.36^{b}	27.37 ± 10.43^{b}
1.25	71.11 ± 5.98^{c}	29.50 ± 12.30^{b}
PBS	0 ± 0^{a}	–
Tween 4%	–	0 ± 0^{a}
Levamisole	100 ± 0^{d}	100 ± 0^{c}

MeOH: Methanolic extract; Hex: Hexanic extract; SD: Standard deviation. Equal letters in the same column indicate that the values do not differ statistically, according to Tukey's test ($p < 0.05$).

ity of essential oils and crude clove extracts depends on their chemical constitution, the antimicrobial action observed in *S. aromaticum* is mainly attributed to eugenol. Eugenol is present in various aromatic plants including *Pimenta dioica*, *Ocimum basilicum* and *Cinnamomum tamala*; however, *S. aromaticum* constitutes the main plant source [28]. In studies by Rana et al [29] against the fungi of the genera *Mucor*, *Microsporum*, *Fusarium*, *Aspergillus*, and *Trichophytum*, eugenol was the main compound responsible for the antifungal activity due to the lytic effect observed on mycelium and spores. Also, Kaur et al [30] reported that the essential oil of *S. aromaticum* had a moderate antifungal potential against *Fusarium moniliforme*, *Helminthosporium oryzae*, and *Rhizoctonia solani*; while when eugenol benzoate essential oil component was evaluated, it was more effective against *F. moniliforme* and *H. oryzae*. The same effect was observed when eugenol acetate was tested against *R. solani*. Anthelmintic activity of some polar clove extracts has also been reported against *H. contortus* [20, 31, 32]. Meyer et al [33] reported the nematicidal activity of clove oil against *Meloidogyne incognita* by reducing the hatching of eggs and J2 larvae. The methanolic extract showed greater activity in the inhibition of the egg hatching test, from the lowest concentration evaluated; while the hexanic extract did not show any activity against hatching in any of the concentrations evaluated. On the other hand, in the case of the mortality of *H. contortus* larvae, the greatest effect was observed in the methanolic extract; while in the hexanic extract, the effect was low at the highest concentration evaluated. Previous studies have shown that methanolic extracts could extract a greater variety of compounds that affect the hatching of *H. contortus* [34].

The antimicrobial activity of phenolic compounds such as eugenol is attributed to the presence of a hydroxyl group bound to a system of delocalized electrons. Ultee et al [35] showed that compounds with a hydroxyl group attached to a benzene ring have antimicrobial activity, while analogous compounds without the OH group or in the absence of the benzene ring had no activity. On the other hand, in a subsequent study against *Salmonella typhi*, it was shown that eugenol

Table 5 Chemical composition of the hexanic and methanolic extracts of *S. aromaticum* determined by GC-MS.

Compound		Relative percentage	
No.	Name	Methanolic extract	Hexanic Extract
1	Eugenol	67.28	74.49
2	Acetyl eugenol (4-Allyl-2-methoxyphenyl acetate)	25.29	7.38
3	Caryophyllene	1.28	6.13
4	Caryophyllene oxide	0.77	0.75
5	Humulenol II	0.61	0.70
6	Hexatriacontane	–	2.84
7	Octacosane	–	1.30
8	Isoaromadendrene epoxide	1.21	–
9	11,11-Dimethyl-4,8-dimethylenebicyclo [7.2.0] undecan-3-ol	–	1.17
10	Ledene oxide-(II)	–	1.06
11	γ-Sitosterol	–	1.05
12	2-Naphthalenemethanol, decahydro-α,α,4a-trimethyl-8-methyle	1.02	-
13	Humulene	–	0.85
14	Loxapine *N*-oxide	0.72	–
15	Diepicedrene-1-oxide	0.66	–
16	Aristolochic acid II	0.62	–
17	*trans*-Isoeugenol	0.55	–
Total identified compounds		100	97.72

exerts a disruptive action on the cytoplasmic membrane of the cell causing an increased permeability and leakage of intracellular content [36]. Thus, the overwhelming evidence on the antimicrobial activity of eugenol makes it possible to consider that the activity observed in this work is due to this compound.

It has been proven that the wide spectrum of antibacterial activity of eugenol can be enhanced in the presence of other substances. For example, the combined treatment of cinnamaldehyde and eugenol showed synergistic efficacy against *E. coli* [37]; while the combination of eugenol with carvacrol showed antibacterial activity against *E. coli* and *L. monocytogenes* [38]. In this aspect, it should be considered that the effectiveness of the complete extracts may be greater than the activity of the compounds separately due to a synergistic effect; however, this is an aspect that must be evaluated in detail.

Additional studies would be needed to compare the antifungal and nematicidal activities of eugenol and acetyl eugenol with those of the hexanic and methanolic extracts, and to determine if the activity observed in this study can be attributed to such compounds or if minor compounds have an important role in the activity of the extracts.

Of the compounds identified by means of the GC-MS, it is interestingly observed that the compounds shared by both extracts are eugenol, caryophyllene, 3-allyl-6-methoxyphenyl acetate, humulenol-II, and caryophyllene oxide. These compounds have also been identified by gas-liquid chromatography analysis in clove oil [33]. The variation in isolated secondary compounds is due to the effect of the solvents used, as previously reported [18, 34]. In addition, it is important to highlight that other compounds were identified, which could possibly be what affecting the interaction of synergies or antagonists of the bioactivity of the molecules.

Analyzing the activity of the compounds individually, the eugenol (reported in the two extracts) anthelmintic activity has been reported against the hatching of *H. contortus* eggs [17]. This compound has been extensively evaluated for its pharmacokinetics and has been reported with antibacterial, antifungal, analgesic activity, among others. Additionally, one of the compounds that stands out from the analysis of GC-MS is the aristolochic acid II present in the methanolic extract, which has been reported to have a toxic effect harmful to human health [39]. The derivatives of aristolochic acid II and their bioactivity have been studied; however, the anthelmintic activity has not been evaluated. Therefore, these two molecules, eugenol and aristolochic acid II, may possibly be responsible for the anthelmintic effect that was observed with the methanolic extract. Most of the compounds identified in the hexanic and methanolic extracts of *S. aromaticum* have been reported with bioactivity in human health, such as a nerve conduction blocker [40].

CONCLUSION

The hexanic extract of *S. aromaticum* has antifungal activity against *Fusarium* sp., while the methanolic extract has nematicidal activity against the hatching of eggs and mortality of sheathless larvae of *H. contortus*. In the hexanic and methanolic extracts, eugenol and acetyl eugenol were identified by GC-MS as the most abundant phytochemicals. Other compounds have not been reported their nematicidal and antifungal activity.

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