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Article

Chemical Composition and Antifungal Activity of the Hexane Extract of the Brazilian *Chenopodium ambrosioides* L.

Carolina M. Jardim,[a] Gulab N. Jham,*[,b] Onkar D. Dhingra[b] and Marcelo M. Freire[a]

[a]*Departamento de Química, Universidade Federal de Viçosa, 36570-000 Viçosa-MG, Brazil*

[b]*Departamento de Fitopatologia, Universidade Federal de Viçosa, 36570-000 Viçosa-MG, Brazil*

Extração de *Chenopodium ambrosioides* L. brasileiro revelou um baixo rendimento (0,3%) do óleo essencial (OE), com uma boa atividade contra oito fungos importantes. Visando melhorar a extração de antifúngicos, o extrato hexano foi avaliado. A extração hexânica melhorou o rendimento (1,1%) dos antifúngicos com uma atividade comparável ao OE. As composições químicas dos extratos, bruto e purificado, foram determinadas tentativamente por meio de cromatografia de fase gasosa (índices de retenção de Kováts) e cromatografia de fase gasosa-espectrometria de massas.

Hydroextraction of the Brazilian *Chenopodium ambrosioides* L. produced a very low yield (0.3%) of the essential oil (EO) with a good activity against eight important fungi. Aiming to improve the yield of the antifungals, hexane was evaluated as an extraction solvent. Hexane extraction improved the yield (1.1%) of the antifungals with activity comparable to that of the EO. The chemical compositions of the crude and purified extracts were tentatively determined by gas chromatography (Kováts retention indices) and gas chromatography-mass spectrometry.

Keywords: Brazilian *Chenopodium ambrosioides* L., hexane extract, antifungal activity, chemical composition

Introduction

Species of *Aspergillus, Colletotrichum*, and *Fusarium* are the major causes of post-harvest economic losses of fruit, vegetables and grains in tropical ecosystems. These fungi are presently managed mainly by synthetic fungicides, posing health and environmental hazards. Thus, alternative safer compounds are needed to control these fungi. Although extracts of several edible botanicals are reported to have antifungal activity,[1-7] little work has been done to manage fungal deterioration of stored products by edible plant derived bioactive compounds.[8-10]

Epazote (*Chenopodium ambrosioides* L.) is an herb native to South America, cultivated in sub-tropical and sub-temperate regions, mostly for consumption as leafy vegetable and herb. Because of its pungent flavor, it is traditionally used to season beans and other South American dishes. Its extract and essential oil (EO) are known to have medicinal,[11-14] acaricidal[15,16] and insecticidal[17-19] properties but there are only few reports on its antifungal properties.[20-23] Although a low fungal activity of dichloromethane extracts of epazote was reported,[22,23] neither its chemical composition nor the principal fungitoxic component were reported.

In our previous study, we obtained satisfactory antifungal activity with the Brazilian *C. ambrosioides* EO.[21] However, it's very low yield (0.3% based on fresh weight basis) led us to investigate another extraction solvent. Since the EO contained non polar compounds[21] we have evaluated hexane in this study as it is non polar, inexpensive and widely available. In addition, we determined the fungal activity of the hexane extracts (crude and purified) against eight major postharvest deteriorating fungi, identifying its principal fungitoxic compound along with tentative chemical compositions by gas chromatography (GC) and gas chromatograph-mass spectrometry (GC-MS).

Experimental

Material and reagents

All organic solvents were p.a. grade (Vetec Química Fina, Rio de Janeiro, RJ, Brazil) and were distilled before use. Analytical TLC analysis were conducted on pre-coated silica gel plates (5×10 cm, 0.1 mm thick, 60 GF_{254}) while preparative TLC was conducted on pre-coated silica gel plates (20×20 cm,

*e-mail: gulab@ufv.br

1 mm thick, 60 GF_{254}). TLC plates were purchased in Rio de Janeiro, Brazil from Merck Industries. Disposable Petri dishes (60×15 mm and 90×15 mm) and potato-dextrose agar were obtained from Prolab Rio de Janeiro, RJ, Brazil).

Plant material and hydrodistillation

Epazote leaves were harvested from shrubs in Viçosa, Minas Gerais, Brazil, used for EO production[21] and also for hexane extraction. Plants were identified by visual comparison of freshly collected leaves with existing herbalized leaves at the Universidade Federal de Viçosa Herbarium VIC (registration nº 11,762). Immediately after collection, the aerial parts of the plant were transported to the laboratory and the whole leaves used immediately for EO production[21] and hexane extraction. Several 200 g portions of the leaves were extracted with hexane (200 mL) for 12 h with stirring at room temperature. After filtration, the organic phase was collected, dried with anhydrous sodium sulfate; hexane was evaporated in a rotatory evaporator at 30 ºC under reduced pressure, weighed, stored in sealed ampoules at 5 ºC and used within a few days.

Antifungal activity

The antifungal activity of the hexane extract was tested on potato-dextrose agar (PDA) using poison food assay.[24] To obtain a uniform dispersal in PDA, the hexane extract was dissolved in methanol (1:1 v/v), and the mixture was added to cool molten PDA to obtain concentrations of 0.1, 0.2 or 0.3%. The active hexane fraction was tested at 0.1, 0.075, 0.05 and 0.03%. Ten mL of the medium were poured into 9 cm culture plates. The controls consisted of PDA containing an equivalent amount of methanol. *Aspergillus flavus, A. glaucus* and *A. ochraceous* (stored grain and feed deteriorating fungi); *Fusarium semitectum*, *F. oxysporum, C. gloeosporioides* and *A. niger* (post-harvest rot of tropical fruits and vegetables), *C. musae* (banana fruit anthracnose) and were used as test-fungi. The medium in each plate was spot-seeded with the test fungus conidia. Colony diameter was measured on either day 5 or 6 after incubation at 25 ºC. All tests were performed in triplicate. Percentage growth inhibition was calculated by dividing radial growth in the treatment plates by the growth in the control plates and multiplying by 100. The data were analyzed by ANOVA and the means compared by the Tukey test (p = 0.05).

Identification of the fungitoxic fraction

Preparative TLC-bioautography[25] was used to separate the major antifungal fraction of the hexane extract. A 200 mg aliquot of the crude extract was applied on each of the six preparative silica gel TLC plates and developed in dichloromethane:ethyl acetate (9:1 v/v) as the solvent. After solvent evaporation at room temperature, cool molten PDA containing 100 mg L^{-1} streptomycin sulfate and conidia of the respective fungus (10^3 mL^{-1}) was spread over each plate and incubated for six days at 30 ºC in a moisture-saturated plastic box. The plate area without fungal growth was delineated, measured, scrapped and extracted twice by shaking for 2 h with 200 mL of dichloromethane. The mixture was filtered and after dehydration of the filtrate with anhydrous sodium sulfate, the dichloromethane was evaporated in a rotary evaporator, the residue was weighed, redissolved in dichloromethane and analyzed.

Identification of compounds

The compounds in the crude and purified extracts were identified by GC using RIs and GC-MS. The peaks were first identified by GC-MS library system based on similarity indexes (SI). The final identification was based on the best SI (similarity index) and RI fits.[26]

Data were obtained on two instruments. Retention indices data were obtained on a gas chromatograph with a flame ionization detector (Shimadzu, Kyoto, Japan, model GC 17 A), an auto sampler and workstation. Mass spectral data were obtained on a gas chromatograph-mass spectrometer (Shimadzu, Kyoto, Japan, model QP 5000 and software program-Classs-5000, Version.1.2), fitted with an auto sampler, workstation and a database (Wiley 229) with about 350,000 entries. Fused capillary columns (30 m×0.25 mm; film thickness of 0.25 μm) coated with DB-5 stationary phase were purchased from Supelco (Bellefonte, PA, USA). In all cases, the GC oven temperature was programmed from 60 ºC (1 min hold) to 240 ºC (9 min hold) at a rate of 3 ºC min^{-1}. For GC analysis, the injector and detector were maintained at 240 and 250 ºC, respectively. For GC-MS analysis, the injector and the transfer line were maintained at 240 and 250 ºC, respectively. One microliter of the sample dissolved in hexane (10%) was injected by the split mode (10:1) with the split vent being closed for 30 sec. Nitrogen and He were the carrier gases for GC and GC-MS, respectively, at flow rates of 1.33 mL min^{-1}. The mass spectrometer was scanned from *m/z* 40 to 350 in the electron impact mode (70 eV). To obtain representative data, the mass spectra over the entire GC peaks (*ca.* 50 scans) of interest were grouped and subtracted from the grouped mass spectra of the region closest (before or after) to where no compound eluted (*ca.* 50 scans). Only compounds with similarity indexes of 87% or over were considered as positive identifications.

For RIs data, a mixture of linear saturated hydrocarbons and samples were analyzed by GC. The RIs were calculated from the following formula: KI = 100y+100(z–y) X $t_{R(x)}$– $t_{R(y)}$/ $t_{R(z)}$– $t_{R(y),}$ where y and z are the carbon numbers in the hydrocarbons eluting before and after a GC peak of interest, respectively; $t_{R(x)}$ is the retention time of the peak of interest; $t_{R(y),}$ and $t_{R(z)}$ are the retention times of the hydrocarbons eluting before and after a GC peak of interest, respectively.

Results and Discussion

Visual comparison of freshly collected leaves with those of the existing herbalized materialed us to conclude that the plant species was *C. ambrosioides* L.

The hexane extraction yield was 1.1%, based on fresh weight basis and much higher than of the EO yield (0.3%) on a fresh weight basis).[21] Although hexane extraction improved the yield of the antifungals, we had to verify if its antifungal activity was comparable to that of EO. In addition, we wanted to identify the principal fungitoxic component in the hexane extract.

The crude hexane extract at a concentration of 0.3% completely inhibited the growth of all test fungi, except for *C. gloesporioides* and *C. musae*, whose growth was inhibited by 94.3 and 96.4%, respectively (Figure 1A). At a concentration of 0.2%, the growth of *A. glaucus*, *A. niger* and *F. semitectum* was inhibited completely, while other fungi were less inhibited at a concentration of 0.1%. The fungicidal activity of the TLC-fractioned hexane extract was much higher than that of the crude extract. The growth of all test fungi was completely inhibited at a concentration of 0.1%, and even of 0.075% growth inhibition exceeded 60%, although the fungi sensitivity differed significantly. This antifungal activity and differential fungal sensitivity are similar to those reported for its EO.[21]

There are few studies reporting antifungal activity of epazote extracts. The dichloromethane extract inhibited growth of *A. flavus*[22] only by 13% and that of *Tilletia indica*[23] by 40% at the concentration of 0.05%. However, neither its chemical composition nor the principal fungitoxic were reported.

The following percentages of the six compounds were identified in the crude hexane extract by GC and GC-MS (Figure 2, Table 1): α-terpinene (11.2), *p*-cymene (6.0), benzyl alcohol (0.4), (*Z*)-ascaridole (54.0), carvacrol (2.3) and (*E*)-ascaridole (17.3), constituting 91.2% of the extract. In addition 8.8% of the crude hexane extract consisting of nine compounds with relative concentrations of less than 1% could not be identified. The hexane extract composition differed considerably from that of the EO,[21] which reported the following percentages of

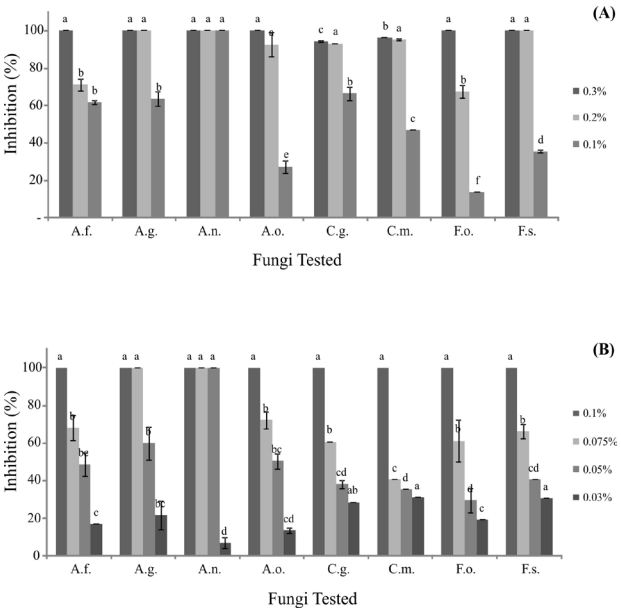


Figure 1. Percent radial growth inhibition by (A) the crude hexane extract of *Chenopodium ambrosioides* and (B) by its purified antifungal fraction at 25 °C. Mean of three replications. For each concentration, the histograms of different fungi, headed by the same letter do not differ at (p = 0.05). The bars represent the standard deviation within the treatment. *A.f.* = *Aspergillus flavus, A.g.* = *A. glaucus, A.n.* = *A. niger*, *A.o.*= *A. ochraceous, C.g.* = *Colletotrichum gloesporioides, C.m.*= *C. musae,* F.o. = *Fusarium oxysporum* and *F.s.* = *F. semitec*

thirteen compounds: α-terpinene (0.9), *p*-cymene (2.0), benzyl alcohol (0.3), *p*-cresol (0.3), *p*-mentha-1,3,8-triene (0.2), *p*-cimen-8-ol (0.6), α-terpineol (0.5), (*Z*)-ascaridole (61.4), piperitone (0.9), carvacrol (3.9), (*E*)-ascaridole (18.6), (*E*)-piperitol acetate (0.5) and (*Z*)-carvyl acetate (0.3). Different compositions of epazote EO have been reported worldwide[27-33] including in southern Brazil.[34] In the Brazilian study the epazote EO composition was very different from ours[21] with the following % being reported: limonene (29.6), mycerne (19) and β-pinene (3.6). These compounds were not detected in our study. In addition, 30.6% of the components could not be identified while we identified 90.4% of the volatile compounds.

TLC-bioautographic fractionation of the crude hexane extract to isolate and identify the most active antifungal constituent yielded only one band (R_f = 0.89, longitudinal width 5 cm), without fungal growth. The GC and GC-MS analysis of this fraction revealed the following percentage composition (Table 1): α-terpinene (0.4), *p*-cymene (1.0), (*Z*)-ascaridole (95.6) and (*E*)-ascaridole (3.0). This composition differs considerably from that of the purified EO,[21] which presented the following percentage composition: (*Z*)-ascaridole (44.4), (*E*)-ascaridole (30.2) and *p*-cymene (25.4). This fraction at 0.1% completely inhibited growth of all test fungi, but at 0.05% the inhibition of *A. flavus, A. niger* and *C. gloesporioides* was lower, and

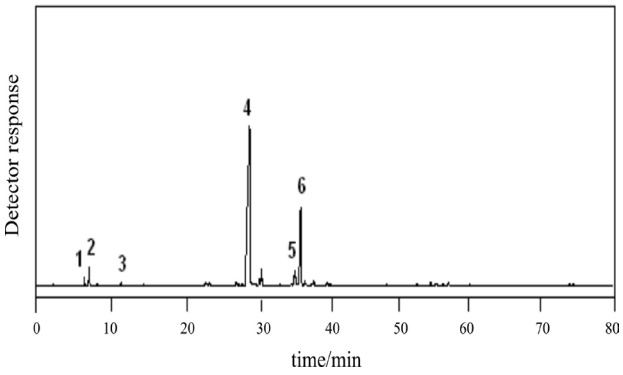


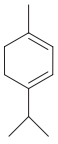
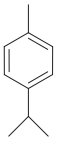
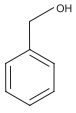
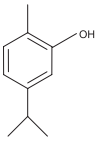
Figure 2. Reconstructed gas chromatogram obtained on analyses of a crude *Chenopodium ambrosioides* hexane extract. Chromatography conditions: oven temperature programmed from 60 °C (1 min hold) to 240 °C (9 min hold) at 3 °C min^{-1}; carrier gas, He (1.33 mL min^{-1}), injector and the transfer line were maintained at 240 and 250 °C, respectively, 70 eV; scanning range (*m/z*), 40-350 with a fused silica capillary column coated with the DB-5 stationary phase. Peaks 1-6 were identified as α-terpinene, *p*-cymene, benzyl alcohol, (*Z*)-ascaridole, carvacrol and (*E*)-ascaridole, respectively.

that of other fungi was similar to that reported for EO at the same concentration. These results are quite significant as *Aspergillus*, one of the most common fungal species that can produce mycotoxins in food and feeds, is a serious concern for human and animal health.[35,36]

Conclusions

Besides being operationally simpler than hydroextraction, hexane extraction of *C. ambrosioides* produced a higher yield. The antifungal activity of the hexane extract was comparable to that of the EO. This study has reported for the first time the wide spectrum antifungal activity of the hexane extract and that (*Z*)-ascaridole appeared to be the major antifungal component. However, further detailed studies are needed to confirm the identity of (*Z*)-ascaridole by more reliable techniques (NMR) and to conduct bioassays.

Table 1. Compositions of the crude hexane extract and the principal fungitoxic fraction of the Brazilian *Chenopodium ambrosioides* (identification based on SI-Similarity Index, Kováts retention indices-RI and gas chromatography-mass spectrometry)

		Crude hexane extract*		Principal fungitoxic fraction		RI	Structure
Peak No. (Figure 2)	SI	Compound	%	Compound	%		
1	96	α-terpinene	11.2	α-terpinene	0.4	1019	
2	95	*p*-cymene	6.0	*p*-cymene	1.0	1026	
3	93	benzyl alcohol	0.4	N.D.	N.D.	1031	
4	87	(*Z*)-ascaridole	54.0	(*Z*)-ascaridole	95.6	1247	
5	93	carvacol	2.3	N.D.	N.D.	1287	
6	89	(*E*)-ascaridole	17.3	(*E*)-ascaridole	3.0	1305	
Total			91.2		100		

*In addition, nine unidentified compounds with relative concentrations of less than 1%, were also present. SI-similarity index (similarity between the actual mass spectra of the compounds and those stored in the mass spectral database). N.D.: Not detected.

Supplementary Information

Supplementary data are available free of charge at http://jbcs.sbq.org.br, as a PDF file.

Acknowledgments

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[a]*Departamento de Química, Universidade Federal de Viçosa, 36570-000 Viçosa-MG, Brazil*

[b]*Departamento de Fitopatologia, Universidade Federal de Viçosa, 36570-000 Viçosa-MG, Brazil*

Mass spectra of compounds tentatively identified by gas chromatography (Figure 2) (Kováts retention indices) and gas chromatography-mass spectrometry. Also presented are the SI-similarity index between the actual mass spectra of the compounds and those stored in the mass spectral database.

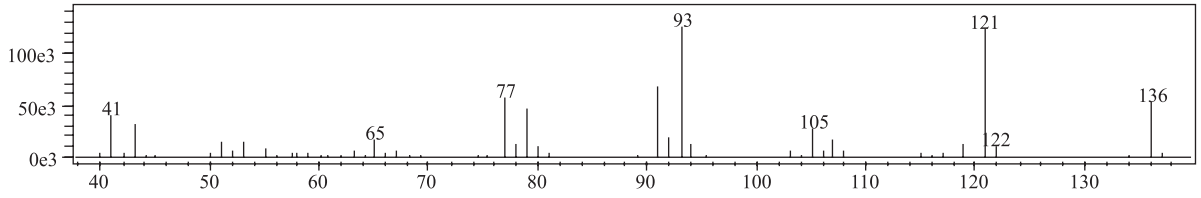

Figure S1. Mass spectra of peak number 1, α-terpinene (SI=96%).

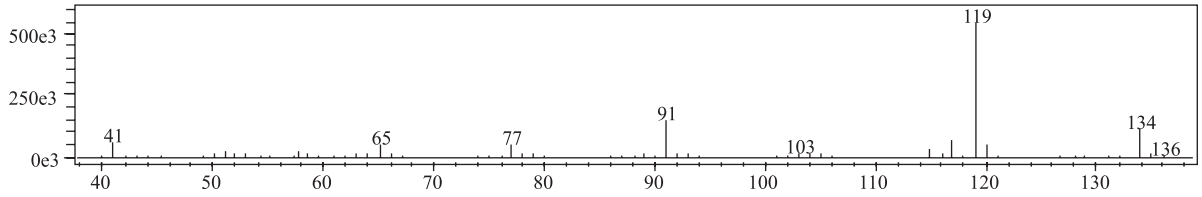

Figure S2. Mass spectra of peak number 2, p-cymene (SI=95%).

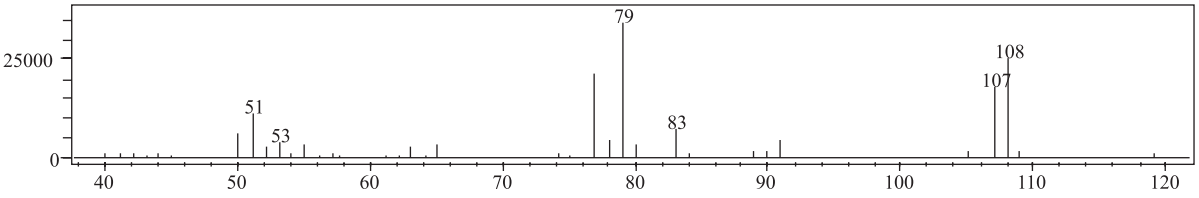

Figure S3. Mass spectra of peak number 3, benzyl alcohol (SI=93%).

*e-mail: gulab@ufv.br

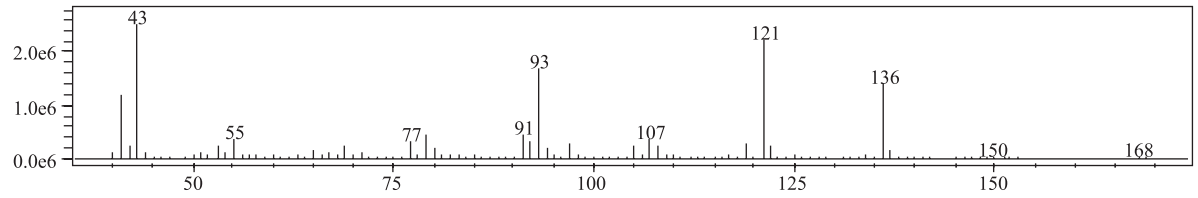

Figure S4. Mass spectra of peak number 4, (Z)-ascaridole (SI=87%).

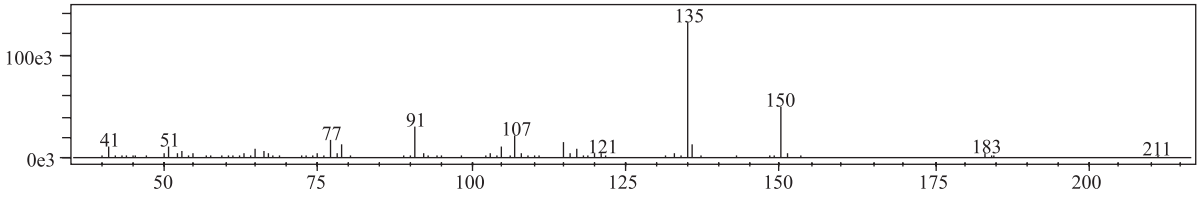

Figure S5. Mass spectra of peak number 5, carvacol (SI=93%).

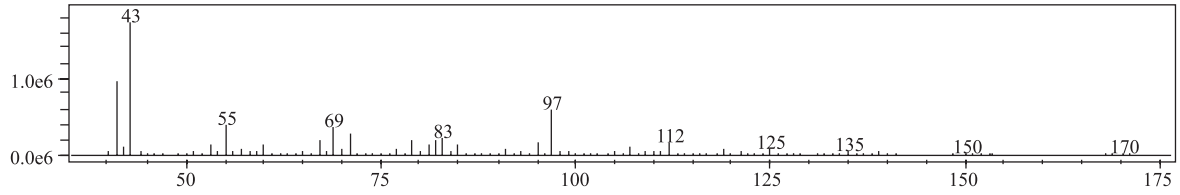

Figure S6. Mass spectra of peak number 6, (E)-ascaridole (SI=89%).