Received 4 June 2008; Accepted 29 November 2008

Revista Brasileira de Farmacognosia
Brazilian Journal of Pharmacognosy
19(3): 697-701, Jul./Set. 2009

Artigo

Chemical composition of the fractions of leaf oil of *Alpinia zerumbet* (Pers.) B.L. Burtt & R.M. Sm. and antimicrobial activity

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RESUMO: "Composição química de frações do óleo essencial de folhas de *Alpinia zerumbet* (Pers.) B.L. Burtt & R.M. Sm. e atividade antimicrobiana". O óleo essencial de folhas de *Alpinia zerumbet* (Pers.) B.L. Burtt & R.M. Sm., extraído por hidrodestilação, foi analisado por CG/DIC e CG/EM a fim de se determinar sua composição e atividade antimicrobiana. O óleo apresentou um elevado teor de monoterpenos oxigenados (52,5%): terpinen-4-ol, 1,8-cineol e γ-terpineno como principais constituintes. A atividade antimicrobiana do óleo foi analisada pelos testes de difusão em agar e ensaios de bioautografia. Através do teste de difusão em agar, todas as bactérias e fungos testados foram inibidos pelo óleo essencial. Placas preparativas de CCD (cromatografia em camada fina) foram eluídas em hexano: acetato de etila (70:20, v/v), e o óleo foi separado em três frações que foram raspadas, extraídas da sílica e identificadas por CG/EM. A análise bioautográfica permitiu detectar pronunciada inibição de *Cryptococcus neoformans* pelas frações F2 e F3, ambas ricas em monoterpenos oxigenados. A fração F2 apresentou 1,8 cineol (9,6%), linalol (3,7%) e óxido de cariofileno (5,4%), enquanto a fração F3 mostrou principalmente terpinen-4-ol (43,6%) e cerca de 32,7% de sesquiterpenos oxigenados. Os dados obtidos indicam os componentes do óleo de *A. zerumbet* envolvidos na atividade antimicrobiana.

Unitermos: óleo essencial, análise bioautográfica, *Alpinia zerumbet,* planta medicinal, Zingiberaceae.

ABSTRACT: Leaf oil prepared by hydrodistillation of *Alpinia zerumbet* Pers.) B.L. Burtt & R.M. Sm. was analyzed by GC/FID and GC/MS to determine the major compounds and it was also evaluated for antimicrobial activity. The oil presented a high content of oxygenated monoterpenes (52.5%), terpinen-4-ol, 1,8 cineole and γ-terpinene as the major constituents. The antimicrobial activity of leaf oil was tested by drop diffusion and bioauthography methods. Through drop test, all bacteria and fungi tested were inhibited by leaf oil. Preparative TLC (thin-layer chromatography) plates were developed using the mobile phase hexane: ethyl acetate (70:20, v/v) and fractions of the leaf oil were separated in three zones, scraped, extracted from silica and identified by GC/MS. The bioauthography method permitted to verify pronounced inhibition of *Cryptococcus neoformans* by fractions F2 and F3, both rich in oxygenated monoterpenes. The fraction F2 comprised 1,8 cineole (9.6%), linalool (3.7%) and caryophyllene oxide (5.4%), while fraction F3 showed mainly terpinen-4-ol (43.6%) and an amount of 32.7% oxygenated sesquiterpenes. The obtained data may be used to suggest the constituents of *A. zerumbet* leaf oil involved in antimicrobial activity.

Keywords: *Alpinia zerumbet,* Zingiberaceae, essential oil, bioauthography, medicinal plant.

INTRODUCTION

Essential oils have many applications in food, perfumery and pharmaceutical industries (Janssen et al., 1988; Baratta et al., 1998). For a long time, the potential of essential oils as antimicrobial activity have been recognized in several studies (Dorman & Deans, 2000; Ferronatto et al., 2007; Costa et al., 2008).

Leaves, flowers and rhizomes of members of Zingiberaceae family present an appreciable amount of monoterpenes and sesquiterpernes related to antimicrobial activity (Habsah et al., 2000; Oonmetta-Aree et al., 2006). *Alpinia zerumbet* is a perennial plant widely distributed in subtropical and tropical regions and it is very used in folk medicine (Albuquerque & Neves, 2004; Leitão et al., 2009). There are some studies that revealed the use

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of this species to treat ulcer (Hsu, 1987), cardiovascular hypertension (Soares de Moura et al., 2005) and muscular aches and pains (Araújo et al., 2005). It has been shown that essential oils from *A. zerumbet* collected in different places around the world have pronounced antimicrobial activity (Prudent et al., 1993; De Pooter et al., 1995; Wang & Huang, 2005).

The purpose of this study was to evaluate the *in vitro* antimicrobial activity of essential oil of *A. zerumbet* by drop agar diffusion and bioauthography methods screening the main compounds involved.

MATERIAL AND METHODS

Plant material

Leaves of *A. zerumbet* were collected in August, in the Universidade Federal do Rio de Janeiro (Rio de Janeiro, Brazil), from adult plants. Voucher specimen was identified and is deposited at the Herbarium of Rio de Janeiro Botanical Garden, under accession number RB 433485.

Leaf oil extraction and analysis

Fresh leaves were cut and subjected to hydrodistillation for 3 h using a Clevenger-type apparatus, mean yielding 0.44% (w/v). Analytical GC/FID was carried out on a Varian Star 3400 gas chromatograph fitted with a DB-5/MS column (30 m × 0.25 mm, film thickness 0.25 μm) and equipped with flame ionization detection (FID). Temperature was programmed from 60-270 ºC at 3 °C/min. Sample injection of 1 μL was performed at 270 oC, splitless mode. Hydrogen was used as carrier gas, at linear velocity of 1 mL/min. GC/MS analysis were performed using a Shimadzu Model GC MS-QP 5000 apparatus under the following conditions: column, DB-5/MS fused silica capillary column (30 m x 0.25 mm, film thickness 0.25 μm); carrier gas, helium at 1 mL/min; injection of 1 μL; injector temperature, 270 °C; interface 200 oC; column temperature, 60-270 ºC at 3 °C/min; mass spectra, 70 eV.

Identification of components

The identification of the constituents was achieved by comparison of Retention Indices (RI) calculated for all volatile contents using a homologous series of *n*-alkanes (C_9-C_{26}) recorded under same operating conditions, comparison of MS data and their GC with those of standard samples held in the computer library search National Institute of Standards and Technology (NIST) and literature (Adams, 1989).

Separation of leaf oil fractions by TLC

Preparative TLC (thin-layer chromatography) plates were developed with the objective of isolating the components of leaf oil that inhibited the growth of microorganism tested. Leaf oil was applied homogeneously as a line, along one edge of the TLC plate (20 x 20 cm) and placed in the TLC chamber contained the mobile phase hexane: ethyl acetate (70:20, v/v). After running, the TLC plate was examined under UV light to observe the zones. A solution contained anisaldehyde, acetic acid, methanol and H_2SO_4 (0.05; 1; 8.5; 0.5 mL) was sprayed on the first 5 cm of the plate which was heated until the zones were revealed. Three zones were visualized on the TLC plate. The zones were scraped from the plates, the substances were eluted from the silica with ethanol (silica:ethanol 1:3, v/v). After extraction each fraction was distributed into five eppendorfs and concentrated at speed vacuum centrifugation. Then the sobrenadants of each fraction was grouped, weighted and resuspended in ethanol to a final volume of 60 μL. The three fractions (F1, F2 and F3) were identified by GC/MS.

Antimicrobial assay

The antimicrobial assay was carried out by the drop agar diffusion method described by Hili et al. (1997). The microorganisms tested were the bacteria *Escherichia coli* (Gram -), *Staphylococcus aureus* MRSA (Gram +) and *Staphylococcus epidermidis* (Gram +), and the fungi *Cryptococcus neoformans* T444 and *Candida albicans*. Microorganisms (2 x 10^5 cells) were spread over Petri plates containing BHI solid medium (Brain Heart Infusion). After 10 min, a 10 μL drop of the essential oil, diluted 1:1 with Tween 80 (0.5% in water), was placed in the center of each plate. Plates were incubated at 37 °C for 24 h to seven days, depends on the microorganism tested. The diameter of inhibition zone (mm) was measured after incubation period. For test the antimicrobial activity of fractions F1, F2 and F3, it was carried out the drop test.

TLC-bioauthography assay

Antimicrobial activity tests were also carried out by bioauthography method on thin-layer chromatography (TLC) plates. An aliquot of 10 μL of essential oil were applied gradually on quadruplicate TLC plates, and hexane:ethyl acetate (70:20, v/v) was used as eluent, for 2 h. The first plate was sprayed with a solution contained anisaldehyde, acetic acid, methanol and H_2SO_4 (0.05; 1; 8.5; 0.5 mL) and the others plates were submitted to antimicrobial assay against the *C. neoformans, S. aureus* and *E. coli*. This test was repeated twice.

It was identified three zones in each plate, then the others plates were cut in three parts and used to the tests. TLC plate part was deposited in the Petri plates, covered with BHI medium and put in the refrigerator. In the following day, the microorganisms were inoculated in separate. The bacteria cultures were incubated at 37 °C

for 24 h and fungi cultures at ambient temperature for two days, after these periods inhibition zones were visualized.

RESULTS AND DISCUSSION

The leaf oil evaluated in this study showed a great percentage of oxygenated monoterpenes (52.5%) (Table 1, Figure 2). The main constituents were terpinen-4-ol, 1,8 cineole and γ-terpinene (Table 1, Figure 1) (Victório, 2008; Victório et al., 2009a). These constituents were found in studies on the essential oil from other samples of *A. zerumbet* (Zoghbi et al., 1999; Ali et al., 2002; Elzaawely et al., 2007; Victório et al., 2009b). Caryophyllene oxide was also found in low amounts (Figure 1). Essential oils from *A. zerumbet* present a great variety of compounds involved in the antimicrobial activity. Terpinen-4-ol and 1,8 cineole have been reported to inhibit several microorganisms (Janssen & Scheffer, 1985; Matasyoh et al., 2007).

Table 1. Chemical composition (%) of the fractions of leaf oil of *Alpinia zerumbet.*

Constituent	RI *calculated*	Relative area (%)		
		F1	F2	F3
1,8 cineole	1038	*	9.6	2.1
linalool	1100	*	3.7	*
trans-sabinene hydrate	1102	4.0	*	*
cis-β-terpineol	1148	6.6	*	*
terpinen-4-ol	1185	7.1	*	43.6
ρ-cymenol	1193	3.2	*	*
α-terpineol	1200	24.2	*	*
bornyl acetate	1288	*	*	13.8
β-caryophyllene	1420	*	*	2.6
α-humulene	1456	*	*	10.7
cubebol	1513	*	*	2.5
caryophyllene oxide	1585	*	5.4	11.3
cubenol	1604	*	*	2.5

RI – retention indices. *Compounds not detected.

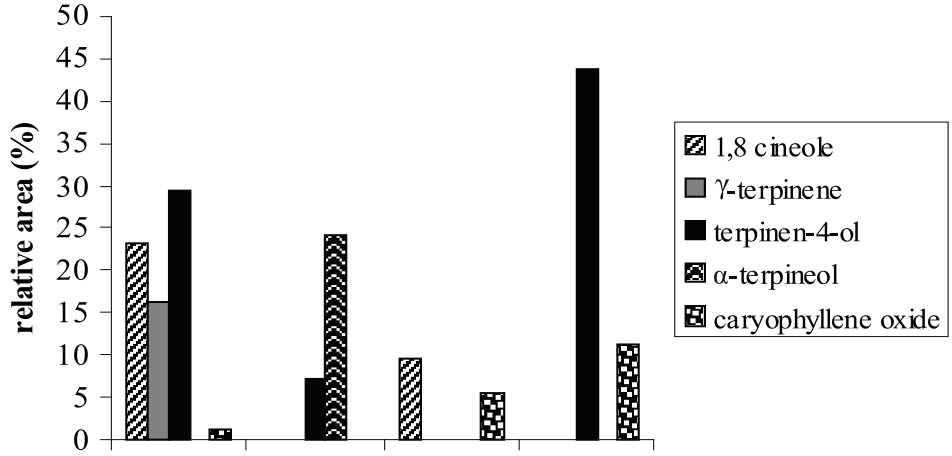

Figure 1. The main monoterpenes and sesquiterpenes of the leaf oil and fractions of *Alpinia zerumbet*.

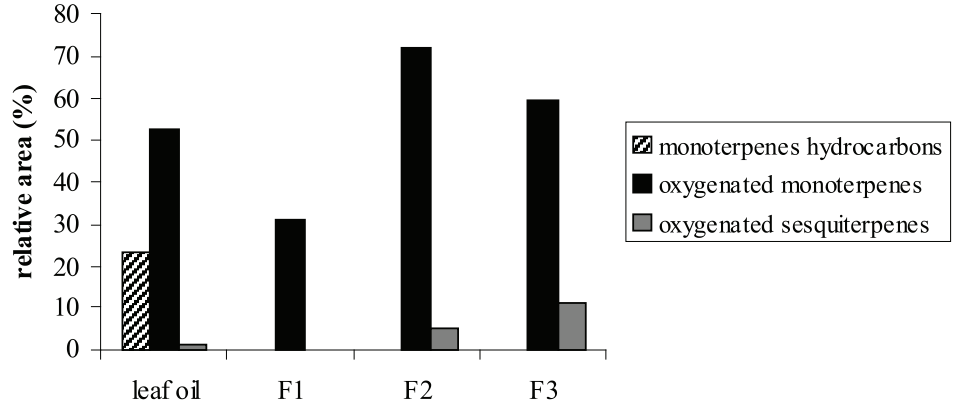

Figure 2. Predominant classes of terpenoids in the leaf oil and fractions of *Alpinia zerumbet.*

Researches using natural sources against microorganisms which cause damage to human health are very important considering the increasing to resistance to antibiotics by them. The leaf oil of *A. zerumbet* inhibited all microorganisms tested (Table 2). The pronounced inhibition of *C. neoformans* (30 mm) using drop test was confirmed by TLC-bioauthography assay. Similar inhibition zone was found to E. coli and *C. albicans* tested with stem oil from *A. zerumbet* collected in Egypt that revealed the same major constituents: terpinen-4-ol (16%), 1,8 cineole (11.5%) and γ-terpinene (8.2%) (De Pooter, 1995). Results showed that monoterpenes hydrocarbons contributed very little to the antimicrobial activity (Figure 2). Low antimicrobial activity of monoterpenes hydrocarbons have been verified by other studies, the reason may be related to free hydroxyl group from the alcohols, to the limited hydrogen capacity or water solubility (Griffin et al., 1999; Griffin et al., 2000). In the current study, oxygenated monoterpenes were the main constituents responsible for the antimicrobial activity (Figure 2).

Table 2. Inhibitory activity of *Alpinia zerumbet* leaf oil by the drop agar diffusion method.

Microorganisms	Inhibition zone diameter (mm)
Bacteria	
Escherichia coli	12
Staphylococcus aureus MRSA	12
Staphylococcus epidermidis	13
Fungi	
Cryptococcus neoformans T444	30
Candida albicans	14

TLC of essential oil showed three zones: F1, F2 and F3. The fractions F2 and F3 were active against *C. neoformans*. Bioauthography revealed a large zone containing oxygenated monoterpenes and oxygenated sesquiterpenes that inhibited the growth of *C. neoformans*. Previous studies demonstrated that antifungal activity may be associated with the oxygenated terpenes (Knobloch et al., 1987; Jansen et al., 1987). The fraction F1 did not inhibit *C. neoformans, S. aureus* and *E. coli*, however its major constituent α-terpineol (24.2%) has been reported to inhibit the growth of bacteria and fungi (Carson & Riley, 1995; Hammer et al., 2003). The compounds identified for fraction F2 were 1,8 cineole, linalool and caryophyllene oxide were already described for their antimicrobial activity. Linalool has a wide range of activity spectrum (Knobloch et al., 1987) and caryophyllene oxide was reported for its efficient activity against bacteria and fungi (Yang et al., 1999; Matasyoh et al., 2007). The fraction F3 possessed the highest percentage of terpinen-4-ol (43.6%) and oxygenated monoterpenes (Table 1, Figure 2). Furthermore, bornyl acetate, α-humulene and caryophyllene oxide were the main sesquiterpenes identified in the fraction F3, corresponding to the amount of 43.4% (Table 1). Bornyl acetate and α-humulene are known for their antimicrobial properties (Dorman & Deans, 2000; Pichette et al., 2006). The combination of bornyl acetate and α-humulene could be responsible for the wide zone of inhibition verified to fraction F3. Comparing fractions F2 and F3, it can be suggested that oxygenated monoterpenes and oxygenated sesquiterpenes had a significant influence on *C. neoformans* inhibition. Terpinen-4-ol, 1,8 cineole and caryophyllene oxide were the main antimicrobial agents cited in other studies (Carson & Riley, 1995; Matasyoh et al., 2007).

TLC-bioauthography method permitted to identify and evaluate the main compounds of leaf oil fractions of *A. zerumbet* with antimicrobial activity.

ACKNOWLEDGEMENTS

C. P. Victório acknowledges the PhD fellowship and financial support from CAPES/PROEX. The authors are especially thankful to Mrs. Maria Cristina de Holanda, Núcleo de Pesquisas de Produtos Naturais/UFRJ for recording the GC/MS spectra and to MSc Davi Oliveira e Silva for his help and assistance in the microbiological experiments.

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