In vitro antimicrobial activity of Alpinia zerumbet and A. purpurata nonpolar fraction of leaf extract

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Abstract

This study aimed to evaluate the in vitro antimicrobial activity of fractions of leaf hydroalcoholic crude extracts: hexane, dichloromethane, ethyl acetate and butanolic of Alpinia zerumbet and A. purpurata by the agar drop diffusion method in order to screen the main compounds involved in antimicrobial activity. Leaves of A. zerumbet (Pers.) B.L. Burtt et R.M. Sm. and A. purpurata (Vieill) K. Schum adult plants were collected and then dried and macerated in 70% ethanol. Leaf extracts were further partitioned using solvents in increasing polarity. Dichloromethane fractions were analyzed by gas chromatography/mass spectrometry (GC/MS). The major compound in the dichloromethane fraction of both species was the hexadecanoic acid (palmitic acid). By drop diffusion assay, the antimicrobial activity of crude extract, as well as fractions of hexane, dichloromethane, ethyl acetate and butanolic, was evaluated against pathogenic bacteria and fungi. No bacteria were inhibited. However, the dichloromethane fraction exhibited promising antifungal activity against the following fungi tested: Cryptococcus neoformans, Fonsecaea pedrosoi, Trichophytoon rubrum, Microsporium canis and M. gypseum.

Keywords: Antifungal activity. Zingiberaceae. Agar drop diffusion. Filamentous fungi. Yeast.

Introduction

The traditional use of species of Zingiberaceae in the treatment of disease, in food and as aromatic is an age-old practice in a large part of Asia and the Americas. Many problems are associated with the use of synthetic antibiotics and pharmaceuticals, exacerbated by the lack of alternative treatments. This has

provoked more interest in plants with antimicrobial properties, including the diverse Zingiberaceous flora which have been tested against microorganisms, such as bacteria, fungi, yeast, and parasitic protists[1-5].

Alpinia zerumbet and *A. purpurata* (Zingiberaceae) are perennial plants widely distributed in subtropical and tropical regions[6]. These species are commonly used for ornamental, medicinal, and aromatic purposes owing to their essential oils. *A. zerumbet* is widely used in folk medicine[7,8] to treat ulcer[9], cardiovascular hypertension[10] and muscular aches and pains[11]. It has been shown that different substances from *A. zerumbet* and *A. purpurata* have pronounced antimicrobial and antioxidant activity, particularly those composed of nonpolar compounds [12-14].

Some fungal and bacterial species cause a broad range of diseases in human and plants, but studies have shown the potential of plant metabolites in combating microorganisms. Plants present a high diversity of bioactive chemicals that are good alternatives for the control of microorganisms in contrast to the use of common fungicides and antibiotics that can result in resistance[15,16]. Resistant strains are common and appear periodically, representing a huge global challenge[17].

Therefore, this study aimed to evaluate the *in vitro* antimicrobial activity of fractions of leaf hydroalcoholic crude extracts: hexane, dichloromethane, ethyl acetate and butanolic of *A. zerumbet* and *A. purpurata* by the agar drop diffusion method in order to screen the main compounds involved in antimicrobial activity.

Material and Methods

Plant material

Leaves of *Alpinia zerumbet* and *A. purpurata* were collected from adult plants in November 2014 on the campus of the Universidade Federal do Rio de Janeiro (Rio de Janeiro, Brazil). Voucher specimens were identified and deposited at the Herbarium of Rio de Janeiro Botanical Garden under accession numbers RB 433485 and RB 433484, respectively.

Plant material extraction and fractionation

Leaves of adult plants were collected in the morning, and then plant material was dried for 3 days at 50ºC and extracted by maceration in 70% ethanol for a week at room temperature (25ºC±2ºC). The total hydroalcoholic extracts were concentrated in a rotational evaporator under reduced pressure at 60ºC, and the residues were resuspended in methanol: water (9:1, v/v). Extracts were submitted to successive partitioning in different solvents of increasing polarity between *n*-hexane, dichloromethane, ethyl acetate and *n*-butanol. The solutions were completely evaporated to give the respective fractions. Each solvent extraction was carried out five times.

Samples of dichloromethane fractions were dissolved at 1 mg/500 μL and filtered. GC analyses were performed to identify volatile compounds. 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) equipped with flame ionization detection (FID). Temperature was programmed from 60ºC to 290ºC at 5°C/min. Sample injection of 1 μL was performed at 270ºC, splitless mode. Hydrogen was used as the carrier gas at linear velocity of 1 mL/min. GC/MS analyses were performed using a Shimadzu Model GC MS-QP 5000 apparatus under the

137

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following conditions: 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; injector temperature, 270°C; ion-source temperature 280°C; column temperature, 60ºC to 290ºC at 5°C/min. Mass spectra were obtained by electron impact ionization (EI) at a scan rate of 0.5 scans/s and fragments from 40 to 500 Da. The identification of the major constituents was confirmed by comparison of their retention time (RT) and mass spectra with those from the NIST database, Wiley Libraries, Adams[18], and the literature.

Agar drop diffusion method

The antimicrobial assay was carried out by the agar drop diffusion method described by Hili et al.[19]. The microorganisms tested included bacteria, as follows: Gram-negative *Escherichia coli*, Gram-positive, methicillin-resistant *Staphylococcus aureus (*MRSA), *Enterococcus faecalis*, *Streptococcus mutans*, and *Lactobacillus casei*, as well as the following fungi: *Cryptococcus neoformans* T444, *Candida albicans*, *Fonsecaea pedrosoi*, *Trichophyton rubrum*, *Microsporum canis* and *M. gypseum*. Microorganisms (2 x 10^5 cells) were spread over an agar plate surface containing Brain Heart Infusion (BHI) agar medium for bacteria, Sabouraud Dextrose Agar medium for yeast, and Potato Dextrose Agar for filamentous fungi, respectively. For antimicrobial activity, crude extracts were tested, along with hexane, dichloromethane, ethyl acetate and *n*-butanol fractions. As standard, the flavonoid rutin (Merck®) was used by its previously identification in extracts of *A. zerumbet* and *A. purpurata*[20,21]. The nonpolar samples were initially solubilized in DMSO (50 mg/ml) and diluted in sterile distilled water 1:3 (1 part of the sample in DMSO + 2 parts of sterile distilled water) before being dropped onto the surface of inoculated medium. The polar samples were solubilized directly in sterile water (50 mg/ml) and used in the test. Tests were carried out by application of a 10 μL drop of the samples placed in the center of each plate. The final concentration was 50 mg/ml for those solubilized in water and 16.6 mg/ml for those solubilized in DMSO. The antimicrobial activity of the extracts against the bacteria and fungi was indicated by the inhibition zone diameter (cm) around the point where each sample drop was placed on inoculated medium surface. Hexane, ethyl acetate and butanolic fractions did not show any antimicrobial activity. All tests were performed under sterile conditions in duplicate and repeated three times. Ten μL of the antibiotic ciprofloxacin (Cipro) (1 mg/ml) and the antifungal amphotericin B (1 mg/mL) were used as positive controls, and DMSO: sterile distilled water (1:3) was used as negative control. Plates were incubated at 37ºC for 24 h for bacterial samples or 25ºC from 48 h to seven days for fungi, depending of the microorganism tested. The diameter of inhibition zone (cm) was measured after each incubation period.

Results and Discussion

Different groups of compounds were identified, such as hydrocarbons, fatty acids, ester and alcohol (**TABLE 1**). *A. zerumbet* and *A. purpurata* leaf extracts contain palmitic acid (*n*-Hexadecanoic acid), as the main component, and other fatty acids, such as stearic acid, nonadecanoic acid and oleic acid. Fatty acids are distributed in natural fats and dietary oils, and studies show their antibacterial and antifungal properties[22]. Fatty acids can act as anionic surfactants and have antibacterial and antifungal properties at low pH, rendering the cell membrane more permeable to these compounds[23,24]. Besides palmitic and stearic acids, oleic acid found in dichloromethane extracts of *A. purpurata* has been used to control cucumber powdery mildew and is also known to have potential antibacterial and antifungal activity[22].

138

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TABLE 1: The main chemical compounds of the dichloromethane fractions of *Alpinia zerumbet* and *A. purpurata* by GC/MS.

A. zerumbet		Constituent	A. purpurata		Constituent
RT*	(%)		RT*	(%)	
22.16	9.8	2-Undecanone,6,10-dimethyl-	18.31	4.1	5,9-Undecadien-2-one,6,10-dimethyl- (geranyl acetone)
23.80	3.2	Octadecane, 1-chloro	20.73	6.2	n.d.
24.78	14.4	n-Hexadecanoic acid (Palmitic acid)	22.14	3.7	2-Undecanone,6,10-dimethyl-
25.33	4.4	Hexadecanoic acid, ethyl ester	23.79	2.0	2-Hexyl-1-octanol
28.65	3.8	Octadecanoic acid (Stearic acid)	24.80	18.6	n-Hexadecanoic acid (Palmitic acid)
29.21	4.6	Nonadecanoic acid	25.33	4.4	Nonadecanoic acid
30.39	4.5	1-Nonadecanol	28.17	2.8	Oleic acid
32.00	2.7	2,5,9-Trimethylfuro[3,2-g]chromen-7-one	28.66	5.8	Octadecanoic acid,2-(2-hydroxyethoxy) ethyl ester (Diethylene glycol monostearate)
39.40	1.31	Diethylene glycol dibutyl ether	30.39	4.3	1-Bromohexadecane
49.09	3.59	Stigmasta-3,5-dien-7-one	31.99	2.2	4,8,12,16-Tetramethyl Pentadecane-4-olide
			40.18	6.6	4-[(4-Decyloxybenzylidene)amino]cinnamic acid 2-methylbutyl ester
			43.09	2.2	5-hydroxy-2-(4-hydroxyphenyl)-3,7-dimethoxychromen-4-one (Kumatakenin)
			49.13	6.4	(3-β,24S)-stigmast-5-en-3-ol
			53.14	0.5<	α-Tocopherol (Vitamin E)

*Retention time (min) according to GC-MS. n.d. – not identified.

The hydroalcoholic extracts, including hexane, dichloromethane, ethyl acetate and butanol fractions, of *A. zerumbet* and *A. purpurata* leaves were investigated for *in vitro* antimicrobial activity (**TABLE 2**). No effects were observed for leaf extracts against bacteria compared with Cipro (positive control), although recent studies show that fatty acids are alternative to conventional antibiotics because act as antibacterial, antibiofilm and antivirulence[25]. Crude extracts and fractions of *A. zerumbet* and *A. purpurata* leaves did act against a wide variety of human pathogenic fungi. In support of antifungal activity, studies have demonstrated the potential of extracts and fractions of different organs of plants of Zingiberaceae: *Alpinia*, *Zingiber*, *Elettaria*, and *Curcuma* species[4]. Recently, Mathew and Victório[5] verified that rhizome methanolic extracts of *Alpinia calcarata* exhibit significant antifungal effects against *Aspergillus flavus*, *A. niger, A. fumigatus*, *Rhizopus stolonifer* and *Candida albicans.* Biological activity against the bacteria tested was not found. However, in studies with different species of Zingiberaceae, crude extracts of rhizome were reported to be active against bacteria, but almost completely inactive against fungi[26].

According to Kochuthressia et al.[27], moderate activity of ethanolic extracts of *A. purpurata* against bacteria was verified. The hydroalcoholic crude extracts of *A. zerumbet* inhibited *T. rubrum* and *M. canis* fungi. *A. purpurata* crude extracts inhibited *C. neoformans*, *T. rubrum* and *M. canis* fungi. Hydroalcoholic extracts and polar fractions, such as ethyl acetate and butanol, of *A. zerumbet* and *A. purpurata* contain high concentrations of phenolic compounds, such as flavonoids[20,21,28], which present antimicrobial properties[29].

139

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However, assays using rutin, a constituent of *A. zerumbet* and *A. purpurata* and known to have antimicrobial properties, did not show any antimicrobial activity. The partition dichloromethane of both species was the most active against fungi. The dichloromethane fraction of *A. zerumbet* exhibited a slight inhibition of *C. albicans*, but a marked inhibition of other fungi, such as *C. neoformans, F. pedrosoi, T. rubrum, M. canis* and *M. gypseum*. The absence of antifungal activity against *C. albicans* by different rhizome extracts of *Alpinia* species was also verified by Habsah et al.[30], different from the findings reported by Mathew and Victório[5] cited above. *A. purpurata* dichloromethane fractions were effective against *C. neoformans, F. pedrosoi, M. canis* and *M. gypseum*. Different from crude extracts, nonpolar fractions have a major concentration of some compounds similar to those found for essential oils, which have shown high antimicrobial activity, indicating that polarity seems to be correlated with biological effects against fungi[13,14]. In the same way, Habsah et al.[26] found the dichloromethane extracts to be much stronger than methanol extracts of rhizomes of species from Zingiberaceae.

This result suggests that the less polar compounds present in the dichloromethane extracts contributed towards increased biological activity compared to the polar compounds contained in hydroalcoholic extracts, ethyl acetate and butanol fractions. Nonpolar constituents, such as curcuminoids, kava pyrones, and gingerols, isolated from plants of Zingiberaceae have been reported to possess antifungal, antioxidant, insecticidal, and anti-inflammatory activities[26,30,31]. On the contrary, previous studies of antimicrobial activity of rhizomes and leaves of *A. purpurata* showed that the ethanolic extracts of rhizome is more effective than nonpolar extracts for the inhibition of *Enterobacter aerogenes*, *Bacillus cereus*, *Enterococcus faecalis*, *Staphylococcus aureus, Salmonela typhi*, *E. coli*, *Aspergillus niger*, *A. flavus*, *A. fumigatus* and *C. albicans*[27]. In addition, some studies have shown palmitic acid (*n*-Hexadecanoic acid) and others fatty acids with an antifungal action against the yeast of *C. albicans*[32], in despite of founds results. Besides that, antifungal activities of palmitic acid against some phytophatogenic and others fungal strains have also been described[33,34]. This substance has been devoted to act specially in the spore germination inhibition of a large amount of fungi[35]. According to these observations, it is possible to speculate the contribution of *n*-Hexadecanoic acid in the antifungal microbial activity of leaves from *A. zerumbet* and *A. purpurata*.

TABLE 2: Antimicrobial activity of *Alpinia zerumbet* and *A. purpurata* leaf extracts by the agar drop diffusion method.

	*Alpinia zerumbet*					*Alpinia purpurata*					Control	
**Inhibition zone diameter (cm)**
	CE*	HEX	DCM	EA	BUT	CE*	HEX	DCM	EA	BUT	Cipro	Amph
Bacteria												
S. aureus (MRSA)	-	-	-	-	-	-	-	-	-	-	2.5	-
S. mutans	-	-	-	-	-	-	-	-	-	-	2.0	-
E. coli	-	-	-	-	-	-	-	-	-	-	3.0	-
E. faecalis	-	-	-	-	-	-	-	-	-	-	1.2	-
L. casei	-	-	-	-	-	-	-	-	-	-	2.5	-
Yeast												
C. albicans	-	-	**	-	-	-	-	**	-	-	-	1.6

C. neoformans	-	-	0.9	-	-	0.8	-	0.8	-	0.8	-	1.6
Filamentous fungi											
F. pedrosoi	-	-	1.0	-	-	-	-	1.0	-	-	-	1.6
T. rubrum	1.0	-	1.0	-	-	1.0	-	-	-	-	-	1.3
M. canis	0.6	-	1.0	0.7	-	0.5	0.9	0.9	-	-	-	1.1
M. gypseum	-	-	1.0	-	-	-	-	0.9	-	-	-	0.9

*Ethanol 70%. **Slight inhibition. *Candida albicans*, *Cryptococcus neoformans* T444, *Fonsecaea pedrosoi, Trichophytoon rubrum, Microsporium canis* and *M. gypseum*. Samples tested: crude extract (CE), fractions: n-hexane (HEX), dichlorometane (DCM), ethyl acetate (Cipro), Amphotericin B (Amph). Negative control for all microorganisms tested indicated its respective growth in the absence of test sample applied.

Conclusion

In the current study, nonpolar fractions from leaf extracts evidenced a positive antifungal activity against *C. neoformans* T444, *F. pedrosoi*, *T. rubrum*, *M. canis* and *M. gypseum*. These results provide additional data in support of using products from dichloromethane fractions of *A. zerumbet* and *A. purpurata* as an alternative against yeast and filamentous fungi. Such findings further support the use of plant metabolites as alternatives. However, more studies must be carried out to isolate metabolites from dichloromethane fractions (nonpolar) and, thus, to determine if crude plant extracts have greater *in vitro* antifungal activity than isolated constituents at an equivalent dose.

Acknowledgements

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141

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142

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143

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