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Bioactivity of extracts from *Alpinia zerumbet* (Pers.) B.L. Burtt & R.M. Sm. against sinusitis causing bacterial pathogens

Bioatividade de extratos de *Alpinia zerumbet* (Pers.) B.L. Burtt & R.M. Sm. contra patógenos bacterianos causadores de sinusite

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Resumo

Alpinia zerumbet (Pers.) B.L. Burtt & R.M. Sm. tem sido indicada na medicina popular brasileira para tratar reumatismo, dor de cabeça e sinusite. Para avaliar o efeito de extratos e frações de flores de *Alpinia zerumbet* (Pers.) B.L. Burtt & R.M. Sm., a fim de validar cientificamente o uso da planta para o tratamento de sinusite, foi proposto este estudo. Os extratos e frações foram preparados e analisados por ensaios fitoquímicos. A atividade antibacteriana contra 12 patógenos bacterianos associados com a sinusite foi avaliada empregando o método de diluição em ágar. A fração hexânica apresentou o mais amplo espectro de atividade, inibindo 10 das 12 bactérias testadas, especialmente *Porphyromonas gingivalis*, *Fusobacterium nucleatum* e *Fusobacterium necrophorum*. O menor valor de MIC foi observado para a fração em acetato de etila contra *Streptococcus pneumoniae* (32 $\mu g \mu L^{-1}$). Todos os extratos e frações testadas apresentaram atividade contra *Prevotella intermedia*. Por outro lado, nenhum extrato ou fração exibiu efeito antibacteriano contra *Streptococcus agalactiae*. A solução usada na medicina popular brasileira mostrou atividade inibitória contra os micro-organismos isolados a partir de pacientes com sinusite crônica e aguda. Este estudo demonstra promissora atividade antibacteriana de *Alpinia zerumbet* (Pers.) B.L. Burtt & R.M. Sm. contra bactérias responsáveis pela sinusite aguda e crônica e valida o seu uso pela primeira vez.

Palavras-chave: atividade antibacteriana; prospecção fitoquímica; medicina popular; Zingiberaceae.

Abstract

Alpinia zerumbet (Pers.) B.L. Burtt & R.M. Sm. is indicated in Brazilian traditional medicine to treat rheumatism, headache and sinusitis. To evaluate the effect of extracts and fractions of flowers from *Alpinia zerumbet* (Pers.) B.L. Burtt & R.M. Sm. in order to provide a scientific rationale for the use of the plant for the treatment of sinusitis, this study was realized. The extracts and fractions were prepared and analyzed by phytochemical investigation. Antibacterial activity against 12 bacterial pathogens associated with sinusitis was evaluated by employing an agar dilution method. The hexane fraction showed the broader activity spectrum inhibiting 10 out of the 12 tested bacteria specially *Porphyromonas gingivalis*, *Fusobacterium nucleatum*, and *Fusobacterium necrophorum*. The lower MIC value was observed for the ethyl acetate fraction against *Streptococcus pneumoniae* (32 $\mu g \mu L^{-1}$). All extracts and fractions tested expressed activity against *Prevotella intermedia*. On the other hand, none of them exhibited antibacterial effect against *Streptococcus agalactiae*. The solution used in Brazilian traditional medicine showed inhibitory activity against microorganisms isolated from acute and chronic sinusitis patients. This study demonstrates the promising antibacterial activity of *Alpinia zerumbet* (Pers.) B.L. Burtt & R.M. Sm. against bacteria responsible for acute and chronic sinusitis and validates its use for the first time.

Keywords: antibacterial activity; phytochemical investigation; traditional medicine; Zingiberaceae.

Introduction

Sinusitis is defined as an inflammation of the tissues lining one or more of the paranasal sinuses consequently to an upper respiratory tract infection, an allergic reaction, or autoimmune problems. Infectious sinusitis is a highly prevalent respiratory condition associated predominantly with viruses. However, bacterial sinusitis usually causes more severe symptoms and lasts longer than viral sinusitis (DeMuri and Wald, 2012). The most common bacteria isolated from pediatric and adult patients with acute sinusitis are *Streptococcus pneumoniae*, *Haemophilus influenza*, *Moraxella catarrhalis* and *Streptococcus pyogenes*. *Staphylococcus aureus* and anaerobic bacteria (*Prevotella*, *Porphyromonas*, *Fusobacterium*, and *Peptostreptococcus*) are the main microorganisms recovered from chronic sinusitis patients (Brook, 2011).

Sinusitis has also important implications from a societal perspective. In the United States, the annual treatment cost associated with the disease has been estimated at $ 3.5 - 5.8 billion, including $ 1.8 billion for children less than 12 years of age (Abzug, 2014). Conventional medical treatment of sinusitis includes antibacterials and corticosteroids, with adjunctive care involving decongestants and antihistamines (Helms and Miller, 2006).

Antimicrobial resistance has become a global concern, mainly due to the increasing rates of multiresistant bacterial pathogens consequently to the haphazard use of commercial antibacterial drugs commonly employed for the treatment of infectious diseases. Medicinal plants offer the most suitable alternative in the search for herbal and new antibacterial compounds of natural origin (Panda, 2014). As an example recently Noundou and collaborators (2014) reported the antibacterial activity of the extracts of roots, stems, and leaves of *Alchornea floribunda* Müll. Arg. a plant commonly found throughout Central, Western, Eastern, and

Southern Africa, used to treat urinary, respiratory, and intestinal problems.

The growing use of phytotherapy as an integrating medical practice in several countries has been remarkable. The use of medicinal plants in Brazil is facilitated by plant diversity and low cost associated with therapeutics, which has called the attention of health assistance programs and professionals (Santos et al., 2011).

Based on this context, *Alpinia zerumbet* (Pers.) B.L. Burtt & R.M. Sm. (Zingiberaceae) has considerable value. The genus *Alpinia* originated in East Asia, but it is currently cultivated in various regions, as a particular consequence of its ornamental and therapeutic value (Victório, 2011). *Alpinia zerumbet* (Pers.) B.L. Burtt & R.M. Sm. also known as *A. speciosa* (Blume) D. Dietr is native of tropical China, Japan, India, Cambodia, Thailand, Vietnam, and Malaysia. It is widely cultivated and distributed in most tropical and semi-tropical areas including the United States, Peru, and Brazil (Thenmozhi, Sureshikumar and Venugopalan, 2011). According to Victório (2011) the rhizomes of members of this species arrived in Brazil by accident, having been mixed in the sand that served as ballasts for the Portuguese caravels returning from the Indies.

Alpinia zerumbet (Pers.) B.L. Burtt & R.M. Sm. is among the most popular medicinal plants in different regions of Brazil, and its use has been suggested by Brazil's Unified Health System (UHS, in Brazil SUS – Sistema Único de Saúde (Brazil, 2009). The peasants in the Ribeirão Preto municipality (São Paulo state, Brazil) use the plant to treat rheumatism and heart disease (Victório, 2011). In Recife (Pernambuco, Northeastern Brazil), where *Alpinia zerumbet* (Pers.) B.L. Burtt & R.M. Sm. is called ''colonia'', it is used for the treatment of sinusitis (Albuquerque et al., 2007). The antibacterial activities of crude extracts prepared from flowers of *Alpinia zerumbet* (Pers.) B.L. Burtt & R.M. Sm. by using solvents with a large range of polarities against pathogens related to sinusitis was evaluated for the first time in order to validate the use of *Alpinia zerumbet* (Pers.) B.L. Burtt & R.M. Sm. in traditional treatments for the disease.

Materials and methods

Plant material

The flowers of Alpinia zerumbet (Pers.) B.L. Burtt & R.M. Sm. were purchased from Company of Natural Plants and Flowers, Natureza São Francisco LTDA, Itaúna municipality, Minas Gerais state, Southeastern Brazil, in september 2013. The identification of the plant material was confirmed by comparing with INCT Herbarium - Virtual Herbarium of the Flora and Fungi (INCT, 2013), and with a voucher specimen (BHCB 2290) deposited in the BHCB Herbarium, ICB, UFMG, Belo Horizonte, Brazil.

Preparation of extracts and fractions

The plant material was dried, finely ground into powder, and extracted for 120 h (hours) with methanol at room temperature (75 g plant material/1 L solvent). After extraction, the methanol was concentrated using a rotary evaporator and dried in a fume hood, providing crude methanol extract (ME). Approximately, 1/3 of the extract was sequentially partitioned with hexane, dichloromethane, ethyl acetate, and butanol. The fractions obtained from this partition were concentrated using a rotary evaporator until the total removal of the solvent, providing fractions in hexane (HF), dichloromethane (DF), ethyl acetate (AF), and butanol (BF), according to the methodology proposed by Filho and Yunes (1998) with modifications. The solution used in Brazilian traditional medicine for treating sinusitis patients was also prepared. The flowers were dried, extracted with ethanol 92 % at room temperature, concentrated

using a rotary evaporator, and dried in a fume hood, providing the alcoholic extract (AE).

Phytochemical investigation

The screening of chemical constituents (steroids, triterpenes, saponins, alkaloids and flavonoids) of the extracts and fractions was performed using chemical methods, according to the methodology previously suggested by Wagner and Bladt (2001). The chemical characterization was based on the addition of specific reagents to decoction aliquots and observing the changes in solution color or precipitate formation. The following experiments were performed: characterization of flavonoids (cyaniding reaction) and saponins (the rate of spume) and testing for the presence of triterpenes, steroids (Liebermann-Burchard reaction), and alkaloids (precipitation reactions with Dragendorff) (Wagner and Bladt, 2001).

Bacterial strains

The bacterial pathogens Porphyromonas gingivalis FDC 381, Prevotella intermedia ATCC 25611, Peptostreptococcus anaerobius ATCC 27337, Fusobacterium nucleatum ATCC 10953, Fusobacterium necrophorum ATCC 25586, Pseudomonas aeruginosa ATCC 27853, Staphylococcus aureus ATCC 33591, Streptococcus agalactiae (clinical sample), Streptococcus pyogenes ATCC 19615, Streptococcus pneumoniae (clinical sample), α-hemolytic Streptococcus (clinical sample), and Moraxella catarrhalis (clinical sample) were used as indicator of biological activity of tested extracts and fractions.

Antibacterial activity of extracts and fractions against sinusitis-causing bacterial pathogens

Antibacterial activity of the extract ME and its derived fractions HF, DF and AF was determined by employing an agar dilution method (CLSI, 2013). This quantitative assay allows the determination of the minimum inhibitory concentration of each tested substance. Stock solutions of the specified extracts (5120 μgmL^{-1}) were prepared in 10% dimethyl sulfoxide (DMSO)/water. The solutions were then added to melted solid culture media in order to obtain concentrations ranging from 512 to 0.25 μgmL^{-1}.

Bacterial inoculate were adjusted to match the 0.5 McFarland turbidity standard and spotted onto the agar surface. To confirm the viability of the organisms, two control plates containing culture media without any extract were included in every assay. One of them was the first plate to be inoculated and the other one corresponded to the last plate. Additionally, another control consisting of culture media added with different concentrations of chloramphenicol was also employed. It was also confirmed that the final concentration of DMSO did not inhibit bacterial growth.

The extract AE and the fraction BF were tested by using an overlay method (Booth, Johnson and Wilkins, 1977). An aliquot of 10 µL of pure AE and BF (5210 μgmL^{-1}) were dropped onto the surface of Tryptic Soy Agar. After allowing the surface of the agar plates to dry, semisolid medium added with each tested bacterial culture was poured onto the plate. Ethanol 92 % employed to obtain the extract AE was also tested and showed no effect on bacterial strains.

Information regarding culture media, bacterial inoculates, and incubation conditions are described in the **TABLE 1**. Both tests were performed in duplicate.

TABLE 1. Cultivation conditions and bacterial inoculate employed for antibacterial activity testing of extracts and fractions obtained from flowers of *Alpinia zerumbet* (Pers.) B.L. Burtt & R.M. Sm.

Bacterial strains	Antibacterial activity testing			Incubation conditions
	Agar dilution (MIC[a])	Overlay method		
		Media[b]	Inoculate	
Porphyromonas gingivalis, *Prevotella intermedia*, *Peptostreptococcus anaerobius*, *Fusobacterium nucleatum* and *Fusobacterium necrophorum*	BA[c]	BHI-S[d]	150 µL	Anaerobiosis 37 °C, 48 h
Pseudomonas aeruginosa and *Staphylococcus aureus*	MH[e]	TSB[f]	10 µL	Aerobiosis, 37 °C, 24 h
Streptococcus spp. And *Moraxella catarrhalis*	MH-S[g]	BHI-S	50 µL	Candle jar, 37 °C, 48 h

[a], minimum inhibitory concentration; [b], semisolid media (0.7 % w/v agar); [c], Brucella Agar (Difco, Sparks, MD, USA) supplemented with 0.05 mg⁄mL hemin, 0.01 mgmL^{-1} menadione, and 5 % sheep blood; [d], Brain Heart Infusion (Difco) supplemented with 0.5 % yeast extract (Difco), 0.05 mgmL^{-1} hemin (Inlab, São Paulo, SP, Brazil), and 0.01 mg⁄mL menadione (Inlab); [e], Mueller Hinton Agar (Difco); [f], Tryptic Soy Broth (Difco); [g], Mueller Hinton Agar (Difco) supplemented with 5 % sheep blood.

Results and Discussion

Antimicrobial resistance is one of the most serious health threats worldwide with unpredictable consequences. The spread of drug resistance among clinically relevant bacteria emphasizes the need for searching for alternative antimicrobial agents. The main advantage of natural products is that crude extracts contain a mixture of compounds like phenols, acids, esters, aldehydes etc. differently from synthetic antimicrobial drugs that contain a single active principle. This feature makes the development of resistance unlike to occur (Rao et al., 2010).

The phytochemical screening of the extracts obtained from *Alpinia zerumbet* (Pers.) B.L. Burtt & R.M. Sm. flowers employing methanol and ethanol and of the fractions originated from the partition of ME revealed the presence of five classes of secondary metabolites as depicted in the **TABLE 2.** Triterpenoids were the most commonly found compounds, detected in all but one fraction. On the other hand, steroids and saponins were observed in only one extract/fraction each. AF and AE, the extract employed in Brazilian traditional medicine showed the higher diverse composition represented by three secondary metabolites groups. Both of them presented triterpenoids and flavonoids. In addition, alkaloids and saponins were also observed in AF and AE, respectively.

TABLE 2. Groups of secondary metabolites in the extracts and fractions of flowers from *Alpinia zerumbet* (Pers.) B.L. Burtt & R.M. Sm.

Secondary metabolites	Extracts its fractions					
	AE	ME	HF	DF	AF	BF
Steroids	-	-	+	-	-	-
Triterpenoids	+	+	-	+	+	+
Saponins	+	-	-	-	-	-
Flavonoids	+	-	-	-	+	+
Alkaloids	-	+	-	-	+	-

[a], alcoholic extract; [b], methanol extract; [c], hexane fraction; [d], dichloromethane; [e], ethyl acetate fraction; [f], butanol fraction.

Several reports on different *Alpinia zerumbet* (Pers.) B.L. Burtt & R.M. Sm. extracts have been previously published. A phytochemical investigation of aqueous extract of leaves from the plant revealed the presence of flavonoids (Mpalantinos et al., 1998). According to Zoghbi and collaborators (1999), the major component of the essential oil from the leaves and flowers of *Alpinia zerumbet* (Pers.) B.L. Burtt & R.M. Sm. was identified as terpinene-4-ol. The leaves also showed limonene (25.1%) and terpinene (17.4%) and in the flowers 1,8-cineole (23.1%) and sabinene (14.5%) were also identified. The terpenes 1,8-cineole, camphor, borneol, and methyl cinnamate were reported as main constituents of essential oils of *Alpinia zerumbet* (Pers.) B.L. Burtt & R.M. Sm. flowers, whereas the major components of seed oils were alpha-cadinol, T-muurolol, alpha-terpinenol, delta-cadinene, and terpinene-4-ol. The analysis of phenolic composition indicated that *p*-hydroxybenzoic acid, syringic acid, and ferulic acid predominated in the ethyl acetate extract of plant flowers, while p-hydroxybenzoic acid, syringic acid, and vanillin were the major phenolics detected in the seeds (Elzaawely, Xuan and Tawata, 2007). The decoction of aerial parts of *Alpinia zerumbet* (Pers.) B.L. Burtt & R.M. Sm. showed the presence of flavonoids (Macedo et al., 2012). Steroids were found in the acetone extract from rhizomes, stems, leaves, flowers, pericarps, and seeds of the plant (Chompoo et al., 2012). To the best of our knowledge, alkaloids and triterpenoids have not been detected in *Alpinia zerumbet* (Pers.) B.L. Burtt & R.M. Sm. yet.

Sinusitis is a highly prevalent respiratory disease that frequently presents infectious origin. Considering that the *Alpinia zerumbet* (Pers.) B.L. Burtt & R.M. Sm. alcoholic extract is employed to treat sinusitis patients, the antibacterial activity of two extracts and the derived fractions obtained from plant flowers against 12 bacteria were evaluated. The microorganisms selected for the study are associated with the etiopathogenesis of acute and chronic sinusitis in both pediatric and adult patients. Two different methods were selected taking into account the characteristics and availability of the material. The agar dilution method was used whenever possible. This assay is more informative since it generates quantitative data. The fraction BF could not be tested by this technique due to the small amount available. Also the determination of MIC was also not possible for AE since the extract was tested used as it is prepared in the traditional medicine without any quantitation procedure.

Data regarding the susceptibility testing of tested sinusitis-causing bacteria are presented in the **TABLE 3**. The lower MIC value was observed for a polar fraction, AF (32 µg/mL against *S. pneumoniae*). In this result, among the non-polar fractions, the lower MIC value was observed for HF (64 µg/mL against *P. gingivalis*, *F. nucleatum*, and *F. necrophorum*). This fraction inhibited 10 out of the 12 tested strains, showing the broader antibacterial activity spectrum which demonstrates that overall HF was the most

active fraction. ME, DF, and AF exhibited activity against eight bacterial strains each. The fraction BF, tested by the overlay method, showed activity against only four bacterial strains each. The same result was observed for the extract employed in the Brazilian traditional medicine. AE exhibited activity against *P. intermedia*, *P. aeruginosa*, α-hemolytic *Streptococcus*, and the *M. catarrhalis*, agents of acute and chronic sinusitis. It should be highlighted that *S. agalactiae* was not inhibited by any of the tested extracts and fractions obtained from flowers of *Alpinia zerumbet* (Pers.) B.L. Burtt & R.M. Sm. On the contrary, *P. intermedia* showed susceptibility to all of them.

TABLE 3. Results of the antibacterial activity testing of extracts and fractions obtained from flowers of *Alpinia zerumbet* (Pers.) B.L. Burtt & R.M. Sm.

Bacterial strains	**Plant extractions, fractions and control**						
	ME[a,b]	**HF**[a,c]	**DF**[a,d]	**AF**[a,e]	**CM**[a,f]	**AE**[g,h]	**BF**[g,i]
Porphyromonas gingivalis	256[j]	64	-[k]	-	1	-	+[l]
Prevotella intermedia	512	128	256	256	1	+	+
Peptostreptococcus anaerobius	512	128	256	256	2	-	+
Fusobacterium nucleatum	512	64	512	512	1	-	-
Fusobacterium necrophorum	-	64	-	512	1	-	-
Pseudomonas aeruginosa	-	-	-	-	256	+	+
Staphylococcus aureus	256	512	256	128	4	-	-
Streptococcus agalactiae	-	-	-	-	2	-	-
Streptococcus pyogenes	-	256	512	-	2	-	-
Streptococcus pneumoniae	128	128	128	32	2	-	-
α-hemolytic *Streptococcus*	512	128	256	256	2	+	-
Moraxella catarrhalis	512	128	256	256	0.5	+	-

[a], agar dilution method; [b], methanol extract; [c], hexane fraction; [d], dichloromethane; [e], ethyl acetate fraction; [f], chloramphenicol; [g], overlay method; [h], alcoholic extract; [i], butanol fraction;.[j], minimum inhibitory concentration (μgmL^{-1}); [k], absence of activity; [l], antibacterial activity.

Correa, Lima and Costa (2010) published a survey of investigations on *Alpinia zerumbet* (Pers.) B.L. Burtt & R.M. Sm. conducted from 1987 to 2008. They reported the expression of activity against several bacteria by extracts of different polarities obtained from leaves and rhizomes of the plant. Results from qualitative and quantitative assays were presented. The hexane, chloroform, acetone, methanol, and hydroalcoholic extracts were tested against eight bacteria; *S. aureus*, *Bacillus subtilis*, *Enterococcus faecalis*, *Micrococcus luteus*, *Escherichia coli*, *P. aeruginosa*, *Serratia marcescens*, and *Mycobacterium smegmatis*. The chloroform extract of the rhizome showed good activity against *E. faecalis*. Also significant activities were reported for the acetone extract of the rhizome against *S. aureus*, *B. subtilis*, *E. faecalis*, and *M. luteus*. Halos exceeding 20 mm were obtained for all of them and MIC value was 500 μgmL^{-1} for *E. faecalis* and 125 μgmL^{-1} for the other three microorganisms.

This paper reports, for the first time, the bioactivity of extracts and fractions from *Alpinia zerumbet* (Pers.) B.L. Burtt & R.M. Sm. flowers against sinusitis-causing bacterial pathogens. The overall data generated, demonstrate that the antimicrobial activity of the two extracts and the derived fractions cover a wide range of bacteria associated with the etiopathogeny of the disease, indicating that the plant may be an important source for alternative antimicrobials. The results provide a scientific rationale for the use of *Alpinia zerumbet* (Pers.) B.L. Burtt & R.M. Sm. to treat sinusitis.

Conclusion

The antibacterial activity of *Alpinia zerumbet* (Pers.) B.L. Burtt & R.M. Sm. against sinusitis-associated bacterial pathogens contributes to validate its use as a traditional treatment for the disease. In general all the extracts and fractions obtained from plant flowers exhibited a broad activity range inhibiting several bacterial agents of sinusitis. Five secondary metabolites have been detected in the extracts and fractions and up to three metabolites groups have been observed in each extract/fraction. Data generated suggest that more than one of these metabolites group expresses antibacterial activity that may explain the different activity spectrum of the extracts and fractions obtained. Taken together, the results indicate the potentiality of *Alpinia zerumbet* (Pers.) B.L. Burtt & R.M. Sm. as a source of antimicrobial substances against clinically relevant bacteria.

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