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ARTICLE

Antimicrobial activity of *Acorus calamus* (L.) rhizome and leaf extract

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ABSTRACT Antimicrobial activity of *Acorus calamus* rhizome and leaf extracts obtained with different solvents *viz.,* petroleum ether, chloroform, hexane and ethyl acetate was evaluated. Extracts obtained with ethyl acetate among others were found to be highly effective. Rhizomes and leaf ethyl acetate extracts exhibited pronounced antifungal activity with diameter zone of inhibition ranged from 20-28 and 18-25 mm as well as antiyeast activity with diameter zone of inhibition ranged from 22-25 and 20-23 mm, respectively. The minimum inhibitory concentration (MIC) of the rhizome and leaf extracts for antifungal activity measured was 2-4mg/ml, except *Penicillium chrysogenum* whereas against yeasts was relatively higher, 4-5 and 6-8 mg/ml. MIC value for antibacterial activity was comparatively very high ~16-42 mg/ml. In addition, authentic α- and β-asarones were also tested for their antimicrobial potential. Both α- and β-asarones exhibited very strong antimicrobial activities against the fungi and yeasts than those of rhizome and leaf extracts. The study clearly suggested that *A. calamus* rhizomes and leaves must possess active principle α- and β-asarones which is believed to be responsible for their antimicrobial activities. Both rhizomes and leaf extracts, however, had no antibacterial activity except *E. coli*.
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KEY WORDS

Acorus calamus
antimicrobial, asarone
minimum inhibitory concentration
zone of inhibition

Our interest in plants because of their medicinally and pharmacologically important active ingredients is increasing rapidly. Plant produces a plethora of natural products, such as alkaloids, phenolics, flavonoids and terpenoids or isoprenoids which has often been correlated with medicinal and pharmacological properties of the plants. These plant natural products have been studied extensively and their role in several useful biological activities, such as antibacterial, antifungal, antiyeast, insecticides and herbicides have been well documented. Number of plant products with the useful bioactive properties is increasing rapidly as an outcome of several ongoing research programs on investigation of biological activities of a number of plants. These bioactive compounds have served as lead molecules for the development of many synthetic potential antibiotics. *Acorus calamus* (L.) family Araceae is a well known plant in Indian traditional medicines (Mehrotra et al. 2003) for centuries. It is a perennial, semi-aquatic and smelly plant, found in both temperate and sub temperate zones. It grows up to 6 feet tall with sword-shaped leaves, small yellow/green flowers and branched rhizomes (Sabitha et al. 2000). The rhizomes, roots and essential oil distilled from these plant parts have been reported to posses several important biological activities including antifungal (Lee et al. 2004; Lee et al. 2005), antibacterial (McGraw et al. 2002; Phongpaichit et al. 2005), allelopathic (Nawamaki and Kuroyanagi 1996), anticellular and immunosuppressive (Mehrotra et al. 2003). Essential oil of *A. calamus* possesses antigonadal activity in insects (Mathur and Saxena, 1975; Koul et al. 1977a, b; Saxena et al. 1977; Schmidt and Brochers 1981). Aromatic oils obtained by alcoholic extraction of the rhizome are used in the pharmaceutical and oenological industries (Bertea et al. 2005). *A. calamus* whole plant and plant parts *viz.,* roots, rhizomes, leaves and essential oil have been extensively investigated for chemical compositions, previously (Namba 1993; Wang et al 1998; Raina et al. 2003; Venskutonis and Dagilyte 2003). Previous studies on chemical investigations of *A. calamus* have revealed the presence of various compounds, such as asarone (α- and β), caryophyllene, isoasarone, methyl isoeugenol and safrol in rhizome and roots (Namba 1993; Wang et al. 1998). Essential oils also have a very similar chemical composition to that of rhizomes, dominated by α- and β-asarone (Lander and Schreier 1990; Oprean et al. 1998; Raina et al. 2003; Venskutonis and Dagilyte 2003). Both α- and β- asarone identified as the major chemical constituent in roots, rhizomes, leaves and essential oils held responsible for almost all the biological activities of *A. calamus*. Despite the fact that *A. calamus* roots, rhizomes and essential oils possess several useful biological activities, sufficiently not enough efforts have been devoted to study their antifungal and antiyeast properties. In the present study we investigated antimicrobial activities of rhizomes and leaf

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extracts against fungi, yeasts and bacteria (gram negative and positive). Simultaneously, we investigated antimicrobial activities of the active principles α- and β-asarones. The present clearly has suggested that the active principle α- and β-asarones are responsible for the antimicrobial activity of rhizome and leaf extracts.

Materials and methods

Plant materials

Plants were collected from Horticultural Research Station, Yercaud in Tamil Nadu, India and grown at Herbal Garden, Vellore Institute of Technology University, Vellore, India. The fresh rhizomes and leaves were collected, washed thoroughly. A known amount of rhizome and leaves (100 g each) were kept in an oven at 40°C for drying till constant weight. Rhizome and leaves thus dried were powdered in a mixer grinder.

Extract preparation

A known amount of leaf and rhizome powder (4 g each) was extracted separately with different solvents viz., petroleum ether, chloroform, hexane and ethyl acetate for 24 hrs as per the procedure published previously (Mehrotra et al. 2003). Extracts thus prepared were weighed and stored at 4°C.

TLC analysis

The extracts were subjected to thin layer chromatographic separation. 5-10 µL of extracts were loaded on pre-coated silica gel-G plates along with authentic standard α- and β-asarone (Sigma Aldarich). The plates were developed in a chamber saturated with solvent system toluene: ethyl acetate (v/v, 8:2) described by Oprean et al. (1998). The plates were removed from the chamber, dried and observed under visible and UV light for visualization of the spots. Presence or absence of the α- and β-asarone in the extracts was confirmed by authentic α- and β-asarone.

Antimicrobial screening

Microorganisms

All the microorganisms except *Vibrio cholera* (Clinical isolates) used in the present study were obtained form Microbial Type Culture Collection (MTTC), Institute of Microbial Technology, Chandigarh and National Collection of Industrial Microorganisms (NCIM), Pune, India. Microorganisms used includes bacteria viz., *Escherichia coli* MTCC901, *E. coli* NCIM, *Pseudomonas aeruginosa* MTCC429, *Salmonella parathypi* A MTCC735, *Enterococcus faecalis* MTCC 439, *Staphylococcus aureus* MTCC96 , *Shigella sonnei* MTCC2957fungi viz., *Penicillium chrysogenum* MTCC2725, *Aspergillus niger* MTCC1344, *A. Flavus* MTCC2799, *Microsporum canis* MTCC2820 and yeasts viz., *Cryptococcus gastricus* MTCC1715, *Candida albicans* MTCC3017, *C. albicans* NCIM. Bacterial cultures were maintained in the nutrient agar medium, fungi and yeast in Sabourd Dextrose agar (SDA)

Antibacterial activity

Antibacterial activities of rhizome and leaf extracts were evaluated by agar well diffusion method (Priya and Ganjewala 2007). Simultaneously, antimicrobial activity of α and β-asarones were also investigated. 24 h broth cultures of the bacteria used for the antibacterial assay. A sterile cotton swab dipped into bacterial suspension and evenly streaked over the entire surface of sterile Muller Hinton agar plate to obtain uniform inoculum. Wells were punched on the seeded plates using sterile borer (8 mm). 20 to 200 µL of rhizome and leaf extracts obtained with ethyl acetate, petroleum ether, chloroform and hexane (100 mg extract dissolved in 1 ml of dimethyl sulfoxide, DMSO) were dispensed into each well using sterile micropipette. Streptomycin (30µg/ml) was used as a positive control. The plates were then incubated at 37°C for 18 h and diameter zone of inhibition was measured. The minimum inhibitory concentration (MIC) values were determined by double dilution method (Lorian 1996). The rhizome and leaf extract as well as both α- and β-asarone were serially diluted in double strength of Muller Hinton broth. The broths then inoculated with 100 µl of bacterial culture. The MIC is the highest dilution of extract which shows clear fluid with no developments of turbidity. The absence of growth was confirmed again on solid media. In the second set of experiment α and β-asarone (10 mg/ml DMSO) were used for the antibacterial screening in similar way.

Antifungal and Antiyeast activity

Antifungal and antiyeast activity assay was performed using well diffusion method. 20-200 µL of the extracts 100 mg/ml DMSO was loaded into wells in SDA plates. Simultaneously, amphotericin B (50 µg/ml) was also loaded as a positive control. The plates were then incubated at room temperature for 2-4 days and diameter zone of inhibition was measured. The MIC values were determined for rhizome and leaves extract as well as both α- and β-asarone by following previous method using Sabourd dextrose broth.

Results

Antimicrobial activities of rhizome and leaves extracts

Our preliminary study of antibacterial assay revealed that only ethyl acetate extracts of rhizome and leaf exhibited strong inhibitory action against the microorganisms tested, however, extracts in other solvents did not show any activities. Antimicrobial potentials of the extracts were evaluated

Table 1. Antimicrobial activity of rhizome and leaf ethyl acetate extracts. 200µL extracts (100mg/ml DMSO) were used in the antimicrobial screening.

Microorganism	Zone of inhibition (mm)			MIC (mg/ml)	
	Rhizome	Leaves	Streptomycin (30µg/ml)	Rhizome	Leaves
Bacteria (Gram negative)					
Escherichia coli MTCC901	20	18	21	16	18
Escherichia coli NCIM	25	22	21	42	42
Pseudomonas aeruginosa MTCC 429	R	R	17	ND	ND
Salmonella parathypi A MTCC 735	R	R	22	ND	ND
Shigella sonnei MTCC2957	R	R	18	ND	ND
Vibrio cholera	R	R	18	ND	ND
Bacteria (Gram positive)					
Enterococcus faecalis MTCC 439	R	R	15	ND	ND
Staphylococcus aureus MTCC 96	R	R	16	ND	ND
Fungi			Amphotericin B (50µg/ml)		
Penicillium chrysogenum MTCC2725	22	20	18	32	32
Aspergillus niger MTCC 1344	25	20	21	2	2
Aspergillus Flavus MTCC2799	28	25	18	3	4
Microsporum canis MTCC 2820	20	18	19	4	4
Yeast					
Cryptococcus gastricus MTCC1715	22	20	18	5	6
Candida albicans MTCC 3017	25	23	22	4	8
Candida albicans NCIM	23	22	21	4	8

R = resistant; ND = Not determined

by measuring the diameter zones of inhibition (mm) as well as minimum inhibitory concentration (MIC). Further, the study revealed that ethyl acetate extract of rhizome and leaves exhibited strong activity against fungi and yeasts but not the bacteria tested (Table 1). Only *Escherichia coli* (MTCC901 and NCIM) was found to be highly sensitive to both rhizomes and leaves extracts. Rest of the bacteria gram negative as well as positive was found to be resistant to rhizome and leaves extract. Rhizome and leaves extract showed marked antifungal activity against *Aspergillus niger* (MTCC 1344), *A. Flavus* (MTCC2799), *Microsporum canis* (MTCC 2820) except *Penicillium chrysogenum*, (MTCC2725) with zone of inhibition (ZI) ranged 20-28 and 18-25 mm, respectively along with MIC value 2-4 mg/ml for each rhizome and leaves extract. *Aspergillus flavus* among other was found to be most sensitive both to the rhizome and leaf ethyl acetate extract (Table 1). Rhizome and leaf extracts also displayed significant antiyeast activity against *Cryptococcus gastricus* (MTCC1715), *Candida albicans* (MTCC 3017 and NCIM) with diameter zone of inhibition 22-25 and 20-23 mm, respectively along with MIC value ranged 4-5 mg/ml for rhizome extract and 6-8 mg/ml for leaves extract. Both rhizome and leaf extracts were not effective against bacteria tested except *E. coli*. however rhizome (MIC 16-42 mg/ml) and leaf extracts (18-42 mg/ml) has shown considerable antibacterial activity against *Escherichia coli* (MTCC901 and NCIM) with dimeter zone of inhibition 20-25 and 18-22 mm, respectively.

Antimicrobial activities of the α and β-asarone

Both α- and β-asarone exhibited significant and comparable antimicrobial activities (zone of inhibition) to that of rhizome and leaf extracts but with relatively less MIC values (Table 2). Overall the study showed that β- asarone had relatively higher antimicrobial activity than the α-asarone. For instance, β-asarone displayed with MIC value 0.5-8 mg/ml a high antifungal activity (ZI, 20-29 mm) whereas α-asarone displayed almost equal antifungal activity (ZI, 19-27 mm) but with high MIC value 2-8 mg/ml. Trends of antiyeast activity obtained for α- and β-asarone was almost similar to that of antifungal activity except slight alteration in the ZI and MIC values. Both α- and β-asarone did not display antibacterial activity except *E. coli* (MTCC901 and NCIM).

Discussion

Several previous and recent studies have described many important biological activities, particularly antimicrobial of *A. calamus* roots, rhizome and essential oils (Grosvenor et al. 1995; Mungkornasawakul 2000; MacGaw et al. 2002; Rani et al. 2003; Phongpaichit et al. 2005). Although our present study only further strengthened and supported previously published reports on useful biological properties of the *A. calamus*, for the first time we attempted to investigate antimicrobial properties of the leaves derived active ingredients. Overall analysis of the results obtained here has clearly

Table 2. Antimicrobial activity of α-and β-asarone. 200 µL of α and β-asarone (10 mg/ml DMSO) were used in the antimicrobial screening.

Microorganism	Zone of inhibition (mm)			MIC(mg/ml)	
	α-asarone	β-asarone	Streptomycin (30µg/ml)	α-asarone	β-asarone
Bacteria (Gram negative)					
Escherichia coli MTCC901	19	22	21	16	8
Escherichia coli NCIM	20	24	21	32	16
Pseudomonas aeruginosa MTCC 429	R	R	17	ND	ND
Salmonella parathypi A MTCC 735	R	R	22	ND	ND
Shigella sonnei MTCC2957	R	R	18	ND	ND
Vibrio cholera	R	R	18	ND	ND
Bacteria (Gram positive)					
Enterococcus faecalis MTCC 439	R	R	15	ND	ND
Staphylococcus aureus MTCC 96	R	R	16	ND	ND
Fungi			Amphotericin B (50 µg/ml)		
Penicillium chrysogenum MTCC2725	24	22	18	8	4
Aspergillus niger MTCC 1344	22	26	21	2	0.5
Aspergillus Flavis MTCC2799	27	29	18	3	0.5
Microsporum canis MTCC 2820	19	20	19	4	8
Yeast					
Cryptococcus gastricus MTCC1715	24	26	18	5	6
Candida albicans MTCC 3017	26	28	22	2	0.5
Candida albicans NCIM	22	23	21	2	0.5

R = resistant; ND = Not determined

showed that rhizome predominantly possess bioactivities (antifungal and antiyeast) than any other plant parts such as leaf that has less bioactive effects (Fig. 1). Although both rhizome and leaf extracts demonstrated substantial antifungal and antiyeast activities, they did not show any antibacterial activity except that of *E. coli*. Previously, De et al. (1999) in his report on antimicrobial activities of *A. calamus* has described lack of antibacterial activity while recently Phongpaichit et al. (2005) have observe very less antibacterial activity in his study on antimicrobial properties of *A. calamus* rhizome. Our results of lack of antibacterial activities hence are in full agreement with those reported by De et al. (1999) and Phongpaichit et al. (2005). Although, there are several published reports are also available on antibacterial activity of *A. calamus extracts* (Grosvenor et al. 1995; MacGaw et al. 2002; Rani et al. 2003). A recent study by Phongpaichit et al. (2005) has revealed strong antimicrobial (antifungal and antiyeast) properties of the crude methanol extracts of *A. calamus rhizome*. In their study they found that methanol extract of the rhizome exhibited high activity against filamentous fungi, *Trichophyton rubrum, Microsporum* gypseum, and *Penicillium marneffei* with IC50 values of 0.2, 0.2 and 0.4 mg/ml, respectively. However, it showed moderate activity against yeasts: *Candida albicans*, *Cryptococcus neoformans* and *Saccharomyces cerevisiae* (MIC 0.1-1 mg/ml) and low activity against bacteria (MIC 5-10 mg/ml). Results presented here on the antimicrobial activities of the rhizome and leaf extracts completely matches with those reported by Phongpaichit et al. (2005). Nevertheless, there are slight differences in the MIC and zone of inhibition values. Also, there are reports available suggesting that antimicrobial properties of the plant parts or whole plants vary with the type of solvent used to prepare the extracts from respective plant parts. Extracts of *A. calamus* rhizomes obtained with dichloromethane and ethanol (Mungkornasawakul 2000; Thirach et al. 2003) has been reported to exhibit substantial antifungal activity. The ethanol extract of *A. calamus* inhibited clinical isolates of *C. albicans*. Therefore from several previous studies it has become clear that the differences in the effectiveness (MIC value) could be due to the solvents used for extraction of active ingredients from the concerned plant parts or climatic and geographical differences. The sensitivity of the microorganisms to the rhizome and leaf extract could be due to morphological as well as

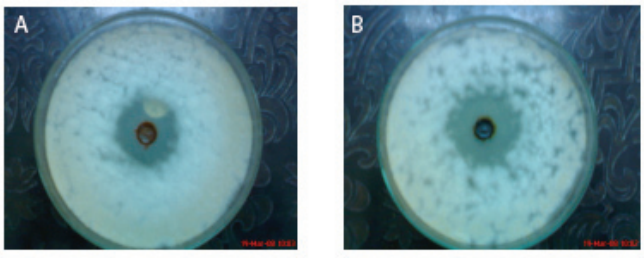

Figure 1. Well diffusion assay of rhizome [A] and leaves [B] extracts exhibiting antifungal activity against *Aspergillus flavis*.

difference in the cell wall constitution of the microorganisms tested. Opposite to the previous studies, ethyl acetate in the present study has been found to be a best solvent for extraction of the active ingredient (α- and β-asarone) from rhizome and leaves among other solvents. Though, solvents such as methanol, ethanol and hexane used in most of the previous studies were also found to be suitable for extraction of active ingredients. It is well established that the α- and β-asarones found in leaf, roots and rhizome tissues are responsible for almost all of the antimicrobial activities of the *A. calamus* (MacGaw et al. 2002). Leet et al. (2007) have recently investigated fungicidal property of *A. gramineus* rhizome (methanol extract) against phytopathogenic fungi apart from human pathogenic microorganisms. They attributed these antifungal activities to the α-asarone and asaronaldehyde present in *A. gramineus*. In our study, we confirmed the presence of α- and β-asarones in *A. calamus* rhizome and leaf extracts by thin layer chromatography (TLC). Antimicrobial activities of *A. calamus* reported here thus could be attributed to the α- and β-asarones found in the rhizome and leaf extracts. To substantiate these results, we investigated the antimicrobial activity of authentic α- and β-asarones. Both α- and β-asarones demonstrated strong antifungal and antiyeast activities but not the antibacterial activity. These results clearly suggested that the antimicrobial activity of the rhizome and leaf extracts was due to the presence of α- and β-asarones. Nevertheless, both α- and β-asarones have comparatively higher microbial growth inhibition potential than the rhizome and leaf extracts because of their purity. Finally, when antimicrobial activities of the extracts of the respective plant parts and pure α- and β-asarones were compared to those of standard streptomycin and amphotericin B it revealed that pure α- and β-asarones possess significant antimicrobial activity while rhizome and leaf extracts possess only considerable antimicrobial activity. However, purification of the crude extract will enhance the antimicrobial activity of the concerned plant parts. Currently biosynthesis of α- and β-asarones along with their bioactive potential is being studied in our laboratory.

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