Analysis Of Phytochemical Constituents And Antimicrobial Activities Of Alpinia calcarata Against Clinical Pathogens

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ABSTRACT

To study the antimicrobial activity and phytochemical characterization of essential oil isolated from the rhizome of Alpinia calcarata against pathogenic bacteria and fungi. Fresh rhizomes of Alpinia calcarata were subjected to hydro distillation process to obtain essential oil and characterized by Gas Chromatography- Mass Spectroscopy (GC-MS). The essential oil was evaluated for antibacterial and antifungal activity against ten pathogenic bacteria and seven fungi by the disc diffusion method. GC – MS analysis of the essential oil extracted from the rhizome of Alpinia calcarata contained the derivatives of 2-octanone, camphene, 1,8-cineole, α fenchyl acetate, 2 hexanone, 4 methyl- 2- hexanone and other minor compounds. The antimicrobial activity of the oil showed significant inhibitory activity against the human pathogenic bacteria, no activity was observed against the fungi Aspergillus aculeatus and Fusarium oxysporum. The findings of the present study indicate that the rhizome extract of Alpinia calcarata possess secondary metabolites and potential to develop antimicrobial drugs.

Keywords: Antimicrobial activity, GC/MS, Phytochemistry, Alpinia calcarata

INTRODUCTION

Nature has been a source of medicinal agents for thousands of years. Various medicinal plants have been used for years in daily life to treat disease all over the world [1]. Antibacterial constituents of medicinal plants and their use for the treatment of microbial infections as possible alternatives to synthetic drugs to which many infectious microorganisms have become resistant seem to be very much promising [2]. Different extracts from medicinal plants were tested and some natural products were approved as new antibacterial drugs [3]. The medicinal value of plants lies in some chemical substances that produce a definite physiological action on the human body [4-9]. The most important of these biologically active constituents of plants are alkaloids, flavonoids, tannins and phenolic compounds [10-12].

Alpinia calcarata Zingiberaceae a medicinal plant, used for several pharmaceutical purposes. Drugs from the rhizomes of Alpinia calcarata are used in treatment of rheumatism, bronchial catarrh and asthma. It is also used to stimulate digestion, treating colds and reducing swelling [16]. The present study was aimed to evaluate the phytochemical constituents and antibacterial activity of the rhizome extracts of Alpinia calcarata.

MATERIALS AND METHODS

Plant collection and Oil extraction

Rhizome of Alpinia calcarata was collected from the tropical forest of Bonocaud in the Agasthyamalai Hills of Kerala, India. The fresh rhizomes were shade dried and powdered in a mechanical blender. The powdered rhizome was subjected to hydro-distillation using a modified Clevenger-type glass apparatus for 6 hours for isolation of oils separately. The oil samples were stored at 0°C in air-tight containers after drying them over anhydrous sodium sulfate and filtered before going to GC-MS analysis.

Bacterial isolates

Isolates of bacteria (Bacillus subtilis, Lactobacillus lactis Lactobacillus acidophilus, Staphylococcus aureus, Klebsiella pneumoniae, Acetobacter pasteurianus, Agrobacterium rhizogenes, Bradyrhizobium species, Escherichia coli, Flavobacterium species) and fungi (Aspergillus aculeatus, Aspergillus awomori, Aspergillus niger, Candida albicans, Fusarium oxysporum, Rhodotorula species, Trichoderme virideae) were obtained from the stock culture in Biotechnology laboratory, Malankara Catholic College, Mariagiri. Biochemical tests were performed to re-identify and confirm the identity of the isolates. Fresh plates of the test bacteria were made from the isolate cultures obtained on agar slants. Discrete colonies of fresh cultures of the different bacterial isolates

were then picked and suspended in 5 ml Nutrient broth (NB, Oxoid), in well-labeled sterile Bijou bottles, and incubated for 24 h at 37ºC prior to antimicrobial susceptibility testing.

Determination of antimicrobial activity

Antimicrobial activity of the oil extracts of the plant sample was evaluated by the cup plate agar diffusion method [17]. Bacterial cultures were adjusted to 0.5 McFarland turbidity standards and inoculated onto Mueller Hinton agar (MHA, Oxoid) plates (diameter: 15 cm). A sterile cork borer was used to make a well (6 mm in diameter) on the MHA plates. Aliquots of 100 µl of extract were applied in each of the wells in the culture plates previously seeded with the test organisms. The cultures were incubated at 37oC for 24 h. A well was made in each of the culture plates and filled with 20 µl of 10 mg/ml of ciprofloxacin and streptomycin as positive controls, and sterile filter paper soaked in sterile glycerol served as a negative control. Antimicrobial activity was determined by measuring the zone of inhibition around each well (excluding the diameter of the well). For each extract, three replicate trials were conducted against each organism. Determination of MIC and MBC

The MIC was determined by broth dilution method. The multidrug resistant Bacillus subtilis, Stapylococcus aureus and Escherichia coli were used for determination of MIC and MBC. The 96 well microtitre plates were filled with 0.1 mL of varying concentration of active fractions prepared in Muller Hinton Broth with culture was added to it. The microtitre plates were incubated at 37ºC for 18 hrs. One row served as positive control (antibiotics) and one as negative control (methanol). After incubation, the OD was read at 610 nm in an ELISA reader. For measuring MBC, irrespectively all the MIC cultures were plated on Muller Hinton Agar and incubated at 37ºC for 24 hrs. A reduction in the number of viable colonies compared with the culture of the initial inoculum was noted. The ratio of MBC/MIC was calculated as an index of bacetriostatic and bactericidal.

Identification of Bioactive Compound Present in Sponge by GC-MS Analysis

Mass spectrometry analysis was performed on a Shimadzu GC 17A QP 5000 MS coupled with a mass detector, fitted non – polar DB-5 (Di phenyl Di methyl siloxane). Capillary column of length 25m X 0.25mm Id. GC – MS operation conditions used are initial temperature 60ºC – 300ºC with the injection temperature at 260°C and detector temperature at 300ºC. The injection volume was 0.1µl with helium gas as carrier at the flow rate of 0.6 ml per minute. Relative Retention times (RRts) of constituents were determined using C5 – C30 straight chain alkenes as standards. Individual constituents of the extract were identified by WILEY11 and NIST database matching by comparison of mass spectra with published data and by comparison of their RRts.

RESULTS

Rhizome of Alpinia calcarata was collected from the tropical forest of Bonocaud in the Agasthyamalai Hills of Kerala, India. The present study mainly focuses on the bioactive screening of the rhizome of Alpina calcarata against some of the pathogens causing human infectious diseases.

Antimicrobial activity of oil extract

The antimicrobial activity of essential oil extract from Alpinia calcarata rhizome was tested against ten pathogenic bacteria and seven fungi. In terms of antibacterial activity, the essential oil showed remarkable antibacterial activity with zone of inhibition of 15mm each against E. coli and Bacillus subtilis, followed by Klebsiella pneumoniae (12mm), Acetobacter pasteurianus (10mm) and Agrobacterium rhizogenes (11mm). Three bacteria Lactobacillus lactis, Lactobacillus acidophilus and Staphylococcus aureus displayed the inhibition zone of 8mm each and Bradyrhizobium species Flavobacterium species showed each 7mm of inhibitory activity against the essential oil isolated from the rhizome of Alpinia calcarata.

In order to find out the antifungal activity of chemicals present in the rhizome of Alpinia calcarata seven species of fungus were tested. Of these, Aspergillus aculeatus, and Aspergillus awomori exposed the maximum and minimum inhibitory zones of 10mm and 7mm respectively. Aspergillus niger, Candida albican, Fusarium oxysporum, Rhodotorula species and Trichoderme virideae showed the inhibitory zone of 12mm each (Table 1). The overall inhibitory effect of Alpinia calcarata extract revealed the better activity against the pathogenic bacteria than fungus.

MIC and MBC

The active antimicrobial extracts of Alpinia calcarata were tested against Bacillus subtilis, Stapylococcus aureus and Escherichia coli in order to determine the MIC and MBC. The 7µl dilution of crude extract of Alpinia calcarata showed minimum inhibitory concentration (in OD) against Bacillus subtilis (0.006nm), Staphylococcus aereus (0.004nm) and Escherichia coli (0.002 nm). The optical densities of all the tubes were detected at 520nm by using nutrient broth as suitable blank. Minimum bactericidal concentrations of oil extract against Bacillus subtilis, Stapylococcus aureus and E.coli were determined (Table 2, Figure 1).

Identification of Bioactive Compound from Sponge by GC-MS Analysis

The GC-MS analysis of the essential oil extracted from the Alpinia calcarata rhizome showed different bioactive compounds. The chromatogram of extract was presented in the Figure 1. The analysis showed the presence of 6 different compounds (2-octanone, camphene, 1,8-cineole, α fenchyl acetate, 2 hexanone and 4 methyl- 2- hexanone) (Table 3, Figure 2).

DISCUSSION

Medicinal plants have been used for the treatment and prevention of infectious diseases has attracted the attention of scientists worldwide [18]. Extraction of bioactive compounds from medicinal plants permits the demonstration of their physiological activity. It also facilitates pharmacology study leading to the synthesis of more potent drugs for meeting demand for effective and safe use. In the present study, the plant collected from Western Ghats was identified according to their taxonomical characters as Alpinia calcarata belongs to the family Zingiberaceae. Pharmacological activities of oil extracted from the members of the family Zingiberaceae, which exhibit, antimicrobial [19], anti-inflammatory [20], anti-hyperlipidaemic [21], hepatoprotective and immunomodulatory [22] and cytotoxic [23] activities.

The antimicrobial activity of oil extracted from Alpinia calcarata could be attributed to the broad spectrum of bioactive chemical compounds. On hydrodistillation of fresh rhizomes, about 0.66% of white coloured, pleasant smelling oil was obtained from Alpinia calcarata. In the present study the essential oil showed remarkable antibacterial activity with zone of inhibition of 15mm each against E. coli and Bacillus subtilis, followed by Klebsiella pneumoniae (12mm), Acetobacter pasteurianus (10mm) and Agrobacterium rhizogenes (11mm). Three bacteria Lactobacillus lactis, Lactobacillus acidophilus and Staphylococcus aureus displayed the inhibition zone of 8mm each and Bradyrhizobium species Flavobacterium species showed each 7mm of inhibitory activity against the essential oil isolated from the rhizome of Alpinia calcarata. In order to find out the antifungal activity of chemicals present in the rhizome of Alpinia calcarata seven species of fungus were tested. Of these, Aspergillus aculeatus and Aspergillus awomori exposed the maximum and minimum inhibitory zones of 10mm and 7mm respectively. Aspergillus niger, Candida albican, Fusarium oxysporum, Rhodotorula species and Trichoderme virideae showed the inhibitory zone of 12mm each. The overall inhibitory effect of Alpinia calcarata extract revealed the better activity against the pathogenic bacteria than fungus. Joji Reddy repoted that the antibacterial activity of the essential oil of Alpinia calcarata [24].

The 7µl dilution of crude extract of Alpinia calcarata showed Minimum inhibitory concentration against E.coli (0.07nm), and Candida albicans (0.08nm). In this study it was observed that the MIC of the active oil extract are lower than the MBC and MFC, suggesting that the oil extract were bacteriostatic at lower concentration and bactericidal at higher concentration [25].

Based on GC/MS analysis the major compound was identified 6 different compounds such as 2-octanone, camphene, 1, 8-cineole, α fenchyl acetate, 2 hexanone and 4 methyl- 2- hexanone. Cineole is an oxygenated monoterpenes. Medicinal application of essential oils of Alpinia calcarata from southern India seems to be of special interest in two cases [26]: (i) external treatment of rheumatic pain zones using these oils in creams or pastes will be effective due to the relatively high content of camphor and camphene, which are known for increasing the blood supply of these zones and therefore anti-inflammatory activities, (ii) inhalation of the oils when suffering from respiratory diseases will furnish positive effects because of the high content of 1,8-cineole and fenchol derivatives with known antiphlogistic, antibacterial and cooling activities.

Plants have rich sources of biologically active metabolites with novel chemical structures, including cytotoxic and anticancer compounds. This investigation reveals a detailed schematic isolation and identification of bioactive compounds from selected plant. This information may help to develop potential purified bioactive compounds in the pharmaceutical industry for the development of drugs. With the advanced molecular biological tools, target-oriented screens have become available that will accelerate the quest for new sponge-derived drugs.

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Figure 1: Minimum bactericidal concentration (MIC) of oil extracts of Alpinia calcarata

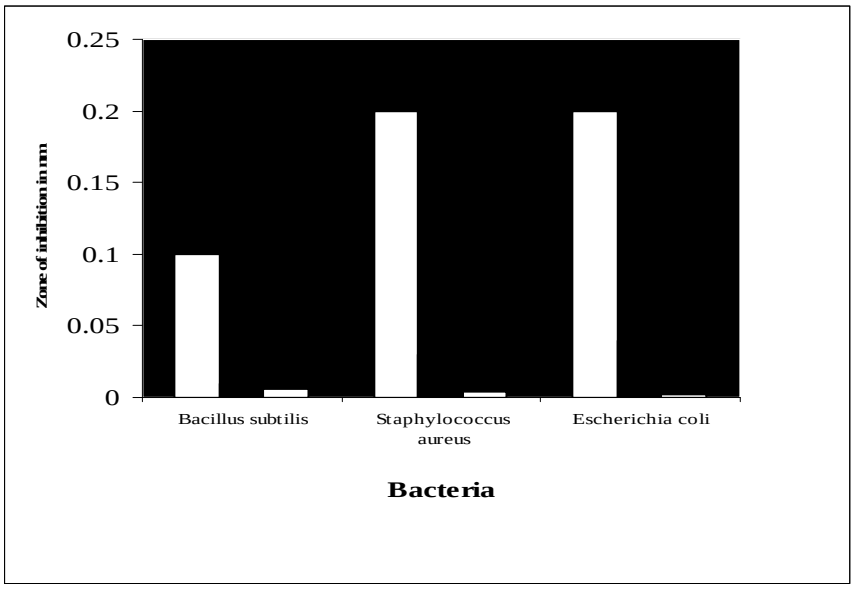

Figure 2: Gas chromatogram of ethyl acetate crude extract of Alpinia calcarata

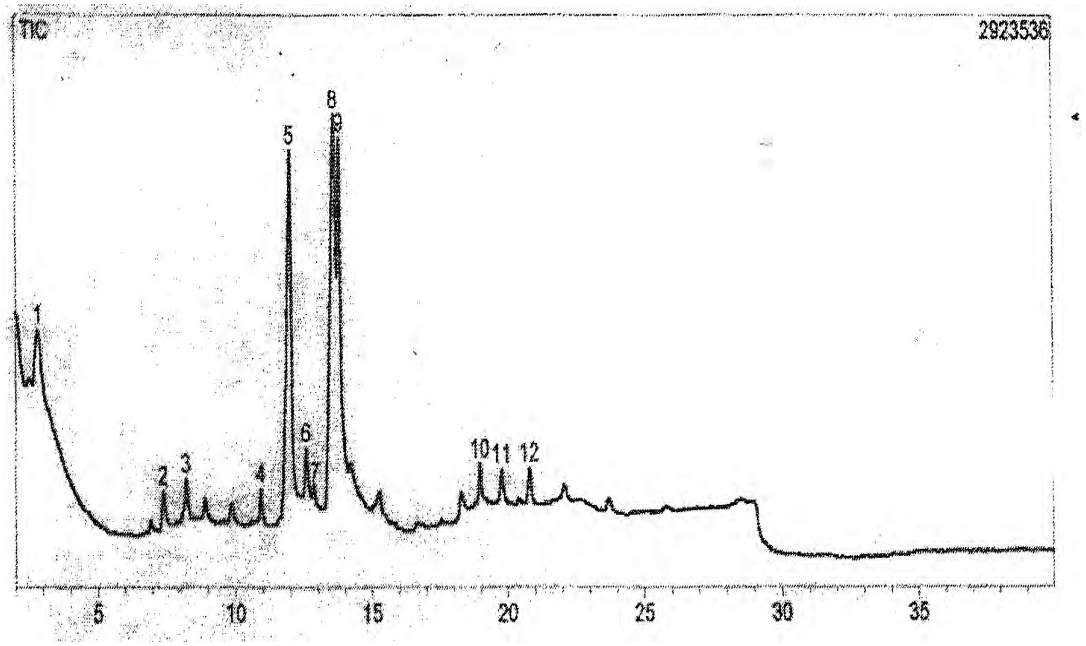

Table 1: Antimicrobial activity of Alpinia calcarata oil against pathogenic bacteria and fungi

SI. No.	***Microorganisms***	***Zone of inhibition (mm)***
	Bacteria	
1	*Bacillus subtilis*	*15*
2	*Lactobacillus lactis*	*8*
3	*Lactobacillus acidophilus*	*8*
4	*Staphylococcus aureus*	*8*
5	*Klebsiella pneumoniae*	*12*
6	*Acetobacter pasteurianus*	*10*
7	*Agrobacterium rhizogenes*	*11*
8	*Bradyrhizobium species*	*7*
9	*Escherichia coli*	*15*
10	*Flavobacterium*	*7*
	Fungi	
1	*Aspergillus aculeatus*	*10*
2	*Aspergillus awomori*	*7*
3	*Aspergillus niger*	*12*
4	*Candida albicans*	*12*
5	*Fusarium oxysporum*	*12*
6	*Rhodotorula species*	*12*
7	*Trichoderme virideae*	*12*

Table 2: Minimum bactericidal concentration (MIC) of oil extracts of *Alpinia calcarata*

Microorganisms	**Zone of inhibition in nm**		
	5	**6**	**7**
Bacillus subtilis	0.1	0.01	0.006
Staphylococcus aureus	0.2	0.03	0.004
Escherichia coli	0.2	0.04	0.002

Table 3: Identification of bioactive compounds in Alpinia calcarata by GC-MS analysis

Number of peaks	**Retention time (min)**	**Compounds**	**Abundance (%)**
1	2.86	2-Octanone	6.27
2	7.389	β-pinene	1.83
3	8.220	(E)-methyl cinnamate	1.63
4	10.939	Guaiol	1.32
5	12.008	1,8-cineole	27.13
6	12.594	2-Hexanone	2.43
7	12.900	Borneol	0.71
8	13.604	α fenchyl acetate	33.46
9	13.792	Camphene	20.61
10	18.976	Elemol	1.52
11	19.781	Bornyl acetate	1.42
12	20.797	4 mathyl-2-hexanone	1.67