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RESEARCH ARTICLE

Phytochemical screening and antibacterial activity of *Acorus calamus* Linn. rhizomes

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ABSTRACT

Acorus calamus Linn., a member of the Acoraceae family, is often known as the Vacha (Sanskrit) and Sweet Flag (English). *Acorus calamus* rhizomes contain potential medicinal properties. The present study investigated the phytochemical screening and bioevaluation of crude extract as well as isolated and characterized molecules isolated from *Acorus calamus* rhizomes. Preliminary phytochemical study revealed that the extracts are rich in Alkaloids, Flavonoids, isoflavonoids, carbohydrates etc. The compound α asarone were isolated and characterized from the methanolic crude extract by using different physiochemical properties. The crude extracts as well as isolated and characterized molecule were evaluated at different concentrations against different bacteria (gam positive and gram negative)by using agar well diffusion method. Antioxidant potential of the crude extract and the pure molecule were evaluated by using standard tests. From this study, it was found that the extract as well as the pure molecule are potent in limiting the growth of different bacteria. The most potent were methanolic extract followed by ethyl acetate and ethanol. Thus, the anti-bacterial/ antioxidant properties of the crude extract and the pure compound(s) as reported in this work suggest a promising role of these compound(s), which needs to be precisely evaluated so as to increase their scope in drug discovery research.

Keywords: *Acorus calamus*,Secondary metabolites, NMR, DPPH, α-Asarone

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INTRODUCTION

Current estimates state that a pharmaceutical company spends $1 billion developing a medicine. Targeted therapy and magic pills are exceedingly pricey today (Giffin, 2009). Most people in the world, according to estimates, cannot afford these sophisticated therapies. Safety is a big problem, in the search for a drug that is both inexpensive and effective. Ayurveda is a historic medical practice that dates back over six thousand years to India (Pandey et al., 2013). Ayu is the Sanskrit word for "life'', Ayu means "Life" and Veda means "knowledge or science" Life's science is Ayurveda, enhance longevity and overall health as opposed to treating illness (Agrawal et al., 2017). Three "doshas" have been used to define three different types of primary body constitutions or features (prakriti) i.e Kapha, vata, Pitta and a sickness is caused byany dosha imbalance (Travis,

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2015). Ayurdeva suggests a personalized therapy based on a person's "prakriti" to reestablish balance. One's diet and life style choices can have an impact on their doshas in Sanskrit, which signifies, and a metabolic imbalance may be the root causes of oxidative stress. The *acoraceaous* plant *Acorus calamus* Linn, frequently known as Vacha and Sweet Flag (Joshi, 2016). This plant is common in the north temperate region and is frequently found in subtropical and temperate wetlands. In Ayurvedic medicine, the rhizome of *Acorus calamus* is used to treat a multifariousness of conditions, including fever, asthma, sedatives, and bronchitis (Rajput et al., 2014). According to Derle and Gujar (2011), the *Acorus calamus* has a wide range of pharmacological effects, along with anti-inflammatory, analgesic, and antipyretic (Oh et al., 2004), hypolipidemic (Parab and Mengi, 2002), cellular antagonists and immunosuppressive (Mehrotra et al., 2003), antimicrobial activity (Kim et al., 2011). The oil from leaves and rhizomes have been appeared of *Acorus calamus in india's lower Himalayan region* . α and β asarone are the main compounds detected in the rhizome of *Acorus calamus*, whereas linalool and β asarone are found in leaf extract (Raina et al., 2003). The principal components, including pre-isocalamendiol, isoshyobunone, camphor b, gurjynene, acorenone, cryptoacorone, shyobunone isomer and Z-asarone were found from the rhizomes of the *A.calamus* (Ozcan et al., 2002; Garneau et al., 2008; Wilczewska et al., 2008; Kim et al., 2011).

Plants are known to have active chemical constituents which are used as a drug because they produce of bioactive molecules, most of which probably evolved as chemical defenses against diseases. Bioassays are adaptable for studying of plant extracts (McLaughlin et al., 1998). Extracts from a plants species contains various activity like anticancer, antimicrobial, antibacterial activities. Herbal remedies may be used as treatments of many diseases. Numerous medicinal herbs and spices contain active ingredients. those exhibits antioxidant properties. Oxidative process produces free radicals in foods, drugs and also in living things (Halliwell and Cross, 1994). Antioxidants are those substances, which contains free radical chain inhibition properties. According to earlier research, oxidative stress-related tissue damage can be lessened by using medicinal plants as antioxidants. The research on medicinal plants provides significant evidence in favour of the hypothesis that can have protective effects against oxidative stress in biological systems. Various compounds, which those exhibits strong antioxidant property was also, determined i.e. ascorbic acid, carotenoids and total phenol contents.

Many medicinal plants and spices are considered to play an important role against microbes and used as antimicrobial agents with novel chemical compounds and their mode of action. Plants produce many compounds, which are able to inhibit and additionally, kill several microorganism alike, *S. aureus, Escherichia coli, Bacillus cereus* and *Micrococcus luteus* that have antibiotic resistance (Friedman et al., 2004). Numerous research teams in the field of ethnopharmacology have emerged over the past several decades as a result of the search for new anti-microbial drugs.

Cancer is uncontrollable and incurable as it can manifest itself in any portion of the body at any moment and at any stage of age. The main cause is an intricate, poorly understood combination between hereditary and environmental factors (Govind and Madhuri, 2011). A huge number of chemicals preventing agents are used in treatment of cancer, but they also exhibit side effects in long-term uses. Although more than 1500 anticancer, drugs are inactive development with over 500 of the drugs under clinical trials, which is a necessary need to produce much efficient and less harmful drugs with low side effects. According to Gordaliza (2007), 60% of therapeutic medications used to treat cancer have been extracted from natural materials. Vinca alkaloids and Podophyllum lignans are some of these of these compounds. Out of these compounds, flavopiridol, isolated from the indian tree *Dysoxylum binectariferum* and the Chinese plant *Indigofera tinctoria* been found to possess anticancer properties with lower toxicity than conventional drugs. Many nations around the world use medicinal plants as a frequent substitute for conventional cancer therapy and in many nations around the world, medicinal plants are a widespread alternative to conventional cancer treatment (Solowey et al., 2014). Currently, anticancer activities have been identified in more than 3000 plants across the globe. About 50% of population relies on plant-derived product which has potential against various cancer cell lines.

The *current* study investigated the rhizome of *Acorus calamus* crude extract for their antibacterial and antioxidant activity and to assess its major compound α asarone by GC-MS, FT-IR and NMR study as a promising future bactericidal agent.

MATERIALS AND METHODS

Collection of plant material

The rhizomes of the *Acorus calamus* were collected from two different districts of Kashmir valley (Ganderbal and Bandipora), of India. Authenticated voucher specimens have been deposited in the herbarium of the institute.

Preparation of extracts

The shade dried underground part (rhizome) of different *Acorus calamus* (2 Kg) was finely ground and soaked in methanol (5L x 2) at room temperature for 30 hrs. The resulting plant material was extracted with ethanol (7.5L x 3) for 40 hrs at room temperature and the resulting extract was concentrated to a gum. The remaining plant material was extracted with ethylacetate (10L x 4) at room temperature for 40 hrs, the resulting extract was reduced to a manageable residue to get gummy material. The extract percentage hexane, methanol and water extract were calculated and recorded. The extracts were stored at 4deg until use.

Preliminary phytochemical screening

Phytochemical screening of methanol extracts of *Acorus calamus* to detect presence of alkaloids, flavonoids, phenols, saponins, glycosides and tannins using standard chemical procedures.

Antioxidant activity

DPPH radical scavenging activity

The free radical scavenging potential of the different plant extract against 2, 2-Diphenyl-1-picryl hydrazyl radical (Sigma-Aldrich) were determined by DPPH assay. The different concentrations of crude extracts were used for evaluating free radical scavenging activity of extracts. Vitamin C, a known antioxidant was used as control. The assay comprised of 1mg/ml of DPPH in methanol, incubated in presence or absence of test material for 15 minutes and read at 517nm in the well plate reader.

Percentage antioxidant activity was calculated as:

$$Antioxidant\ activity\ (\%) = \frac{(Absorbance\ of\ Control - Absorbance\ of\ Test\ sample)}{Absorbance\ of\ Control} \times 100$$

Discoloration of DPPH was taken as the indicator of antioxidant activity.

Ferrous ion chelating activity

Chelating activity was assessed using the technique outlined by Xie et al. (2008). 0.05 mL of 2mM $FeCl_2$ solution and 1.85 mL of double distilled water were used to premix the various sample concentrations (10, 20, 40, 60, 80 and 100%) then 0.1 mL of a

5mM ferrozine solution was added and thoroughly mixed. After the combination stood at room temperature for 10 minutes, the absorbance at 562 nm was measured.

Antimicrobial assay of crude extracts

Test pathogens

Gram-positive and Gram negative human pathogens with authenticated cultures include *Staphylococcus aureus*, *Enterococcus faecalis* and Gram-negative bacteria such as *Streptococcus pneumonia*, *Salmonella typhi*, *Escherichia coli* and *Pseudomonas aeurignosa* were procured from Department of Microbiology Sher-i-Kashmir Institute of Medical Sciences, Soura, Srinagar, Jammu and Kashmir, India.

Antibacterial activity

The antibacterial activity of *Acorus calamus* plant extracts was determined by agar well diffusion method adopted by (Parekh and Chanda, 2007). Micro-organisms were grown overnight at 37°C in Mueller- Hinton Broth. Ten microlitres (10µL) of standardized inoculum (0.5 Mac- Farland) of each test bacterium was inoculated on molten Mueller-Hinton agar, homogenized and poured into sterile Petri dishes. The Petri dishes were allowed to solidify inside the laminar hood. Extracts were made in Dimethyl sulphoxide (DMSO). Standard antibiotic ampicillin was used as positive control and DMSO alone as negative control. The plates were then incubated at 37 ± 1°C for 24h. The zone of inhibition was measured with the help of standard scale.

Determination of the minimum inhibitory concentration (MIC) for bacteria

Minimum inhibitory concentration of the methanolic crude *Acorus calamus extract* of rhizomes and isolated compound was utilizing a modified resazurin microtitre plate test, in Mueller Hinton Broth (MHB) as reported by Sarker et al. (2007) and Teh et al. (2013). Sterile 50 µl of MHB for bacteria were put one into each of 96-wells of a sterile microtitre plate. The crude extract/compounds were mixed in 10% of DMSO to form 2 to 20% stock solutions, respectively. The first well received 50µl of crude extract and an isolated chemical. After thoroughly combining the broth and crude extracts, 50 ml of the combination was added to the second well, and the serial dilution procedure was used to achieve a two fold dilution to get concentrations each of 200 to 6.25 g/ml (for the crude) and 500 to 15.625 g/ml (for the active ingredient). Ten µl of the resazurin indicator solution were summed to each well. When the bacterial suspension had reached an accumulation of roughly 5×10^5 cfu/mL, 10L of it was added to each well. Each plate contained a set of controls: a column containing all solutions other than crude extracts; a column containing MHB 10 µl exception of the bacterial solution and a 10% DMSO solution column that serves as a negative control. All other bacterial strains were cultured on the plates for 24 hours at 37°C.The colors change was then visually evaluated after incubation, color shifts from purple to pink(or colorless) indicated the growth. The MIC value (CLSI) was determined to be the lowest concentration at which the color changed.

Isolation of antibacterial compound from methanolic crude extract of *Acorus calamus* rhizomes

Column chromatography was carried out on silica gel (Qualigens, 60-120 and 120-240 mesh) for isolation of Secondary metabolite. The progress of chromatogrphic run was monitored using TLC plates (Qualigens). Thin layer chromatographic (TLC) plates were viewed with ultraviolet light at 254 nm for fluorescence quenching spots and at 366 nm for fluorescent spots. Cerric sulphate, sulphuric acid, Iodine or FeCl3 (Qualigens) were used visualize non-UV spots. The purity of compound were further

identified by GC-MS and FT-IR.

Characterization of isolated molecules.

Melting points were determined in glass capillary tubes using Buchi B-545 melting point apparatus. Optical rotations were measured in Jasco DIP-360 digital polarimeter. Ultraviolet (UV) spectra's were recorded in methanol in nm on Specard S 100. 1H NMR and 13C NMR were run on 200 and 500 MHz Bruker Daltonics spectrometers respectively. The chemical shifts (δ) are reported in ppm relative to tetrametylsilane (TMS) as internal standard and coupling constants were measured in Hz.

RESULTS AND DISCUSSION

Appropriate phytochemical tests were employed to investigate the phytochemical constituents present in the rhizomes of *Acorus calamus*. The preliminary phytochemical enumerated that the extracts are rich in alkaloids, sugars, flavonoids, saponins, proteins etc, as shown in Table 1. The plant material tested negative for the Glycosides. From the preliminary phytochemical study of the crude extracts, it is deciphered that *Acorus calamus* is the richest source of flavonoids, isoflavonoids and phenolics, the biologically and pharmacologically active constituents.

Table.1: Preliminary phytochemical analysis of *Acorus calamus* Rhizomes

Secondary metabolites	Ethanol	Methanol	Ethyl acetate
Alkaloids	+	-	-
Flavonoids	-	-	+
Glycosides	-	-	-
Resin	++	+	++
Tannins	-	+++	+
Steroids	-	+++	-
Saponins	-	++	-
Carbohydrates	+	+++	-
Proteins	-	-	-

(+++) Very highly present, (++) highly present, (+) present, (-) absent

The most essential class among these secondary metabolites are those of flavanoids and isoflavanoids-the entities known to have varied medicinal properties ranging from anti-bacterial or anti-inflammatory to anti-cancer activities.

DPPH scavenging activity of *Acorus calamus* rhizomes

The antioxidant activity of methanolic crude extract of *A. calamus* rhizomes was examined and the different concentrations of *A. calamus* extract showed the significant results as compared to the reference (Fig 1 and Table 2). *A. calamus* rhizomes have been demonstrated that it contains significant dosage dependent anti-oxidant activity. The methanol crude extract showed 62% highest activity at the concentration of 100 mg/mL whereas; the least activity was shown at the concentration of 10 mg/mL. The above activity can be attributed to redox properties of phenolic or flavonoid compounds present in the extracts, which play an important role in absorbing and neutralizing free radicals and hence terminating the free radical chain reaction

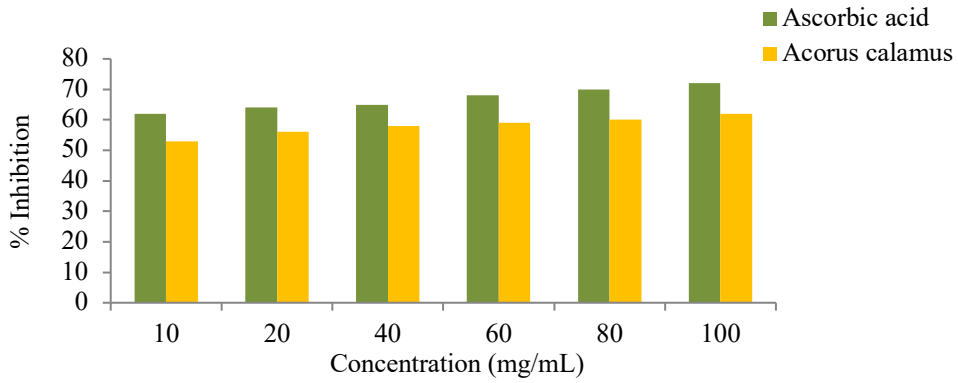

Fig.1. DPPH Scavenging activity of *Acorus calamus*

Table.2 .DPPH Scavenging activity of *Acorus calamus* rhizome extract

Concentration (µg/mL)	DPPH free radical scavenging activity of ascorbic acid (%)	DPPH free radical scavenging activity of *Acorus calamus*
10	62.14%	53.24%
20	64.26%	56.31%
40	65.92%	58.17%
60	68.35%	59.12%
80	70.00%	60.52%
100	72.27%	62.52%

Ferrous ion-chelating assay

The capacity of chelate ferrous ions of various crude extracts of *Acorus calamus* rhizomes are shown in Fig.2. The highest ferrous ion chelating ability was 72.5% whereas at the concentration of 10 mg/mL, it showed a least activity.

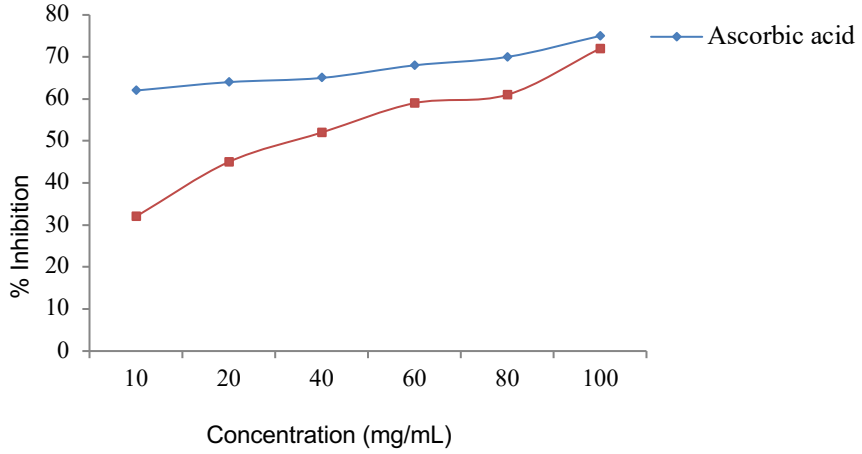

Fig. 2: Ferrous ion chelating activity of *Acorus calamus* rhizomes

Antibacterial activity

The *Acorus calamus* solvent extracts shows antibacterial activity. The highest activity was shown by methanol extract in contrast to the ethanol and ethyl acetate while as no such activity was shown against *S. Pneumonia* (Table 3). The antibacterial activity. Showed dose dependent response.

Table 3: Antibacterial potential of different extracts of *Acorus calamus* rhizomes

Microorganism (Bacteria)	Different concentration of crude extract from *Acorus calamus* rhizomes									Antibiotic
	25 mg/mL	50 mg/mL	100 mg/mL	25 mg/L	50 mg/L	100 mg/L	25 mg/L	50 mg/mL	100 mg/mL	Ampicillin (10µg/mL)
	Inhibition Zone by Methanolic extract (mm)			Inhibition zone by Ethanol extract (mm)			Inhibition zone by Ethyl acetate extract (mm)			Inhibition zone by antibiotic (mm)
E. coli	7.2± 0.23	8.8±0.17	9.7± 0.22	7.4± 0.17	8.7± 0.49	9.2± 0.76	17.2±0.23	19.8±0.01	22.7± 0.73	23.4 ± 1.6
E. faecalis	6.7± 1.56	8.5±0.11	11.5± 0.76	7.1± 0.18	8.2±0.24	9.1±0.18	8.8± 0.56	9.2± 0.81	9.9± 0.34	22.5±0.14
S. typhi	6.7± 2.12	7.2±0.16	8.6± 0.18	7.2± 0.11	7.8± 0.39	8.8±0.27	7.2± 0.21	8.2± 0.12	8.9±0.39	12.9±0.49
S. Pneumonia	0	0	0	0	0	0	0	0	0	16.1 ±0.62
P. aeurignosa	9.3±0.12	11.1±0.24	14.5±0.12	8.4±0.45	9.2±0.24	10.3±0.16	7.6±0.12	9.4±0.24	10.2±0.12	22.2±0.24
S. aureus	11.4±0.12	15.3±0.45	19.2±0.78	8.3±0.12	9.5±0.24	11.8±0.14	7.8±0.12	8.7±0.24	9.7±0.12	18.5±0.2

The different extracts inhibited the growth of not only gram-positive bacteria but also gram-negative bacteria, which most often show resistance to different antibacterial agents. These results demonstrate that crude extracts of *Acorus calamus* contain potent anti-bactericidal agents. The antibacterial action of different *Acorus calamus* extracts may be attributed to the presence of a variety of secondary metabolites including flavonoids, isoflavonoids and phenols which have already been reported to exert antibacterial effect.

Minimum Inhibitory Concentration

The Minimal Inhibitory Concentration (MIC) of the methanol extract was lower against *E. faecalis, S. typhi* and *E. coli* (Table 4). Therefore, the methanolic extract of *Acorus calamus* rhizomes was chosen to carry out bio-assay guided fractionation for the isolation of bioactive compound(s). The activity directed fractionation of methanolic extract yielded oily yellow compound α-asarone.

Table 4: MIC of methanolic extract

Bacterial strains	*Acorus calamus*
	MIC (%)
S. aureus	20
E. faecalis	8
S. Pneumonia	20
P. aeurignosa	20
S. typhi	8
E. coli	8

Determination of structure

The Mass spectrum provided molecular weight (ion)- 208.00 and retention time (RT) 6.60- suggesting molecular formula $C_{12}H_{16}O_3$.Based on spectral studies, the isolated molecule was identified as Asarone (Fig.3).The strong absorption was visible in the IR spectra at 1709 cm^{-1} which indicated the determination of olefinic group (Fig 4.) and rest are at 3431, 2930, 2871, 1709,1510, 1378, 1175, 1120,1032, 929cm^{-1}.

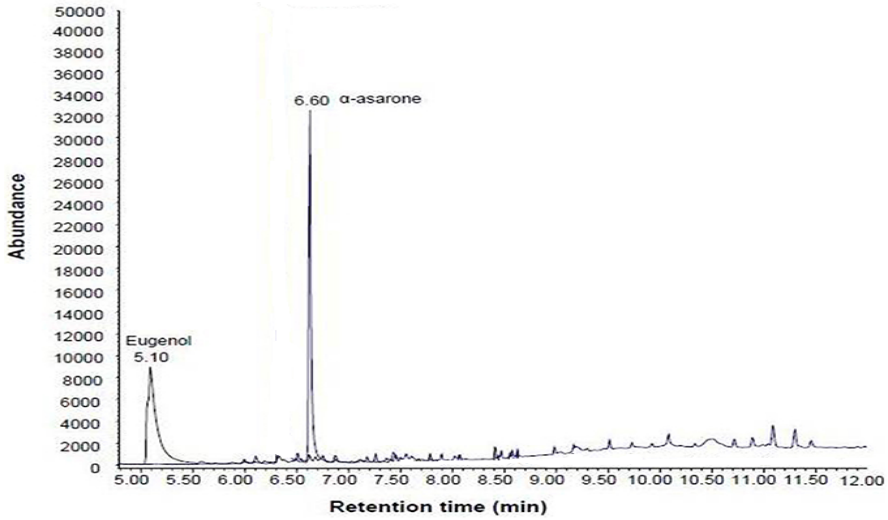

Fig 3: GC-MS analysis of *A. sarone*

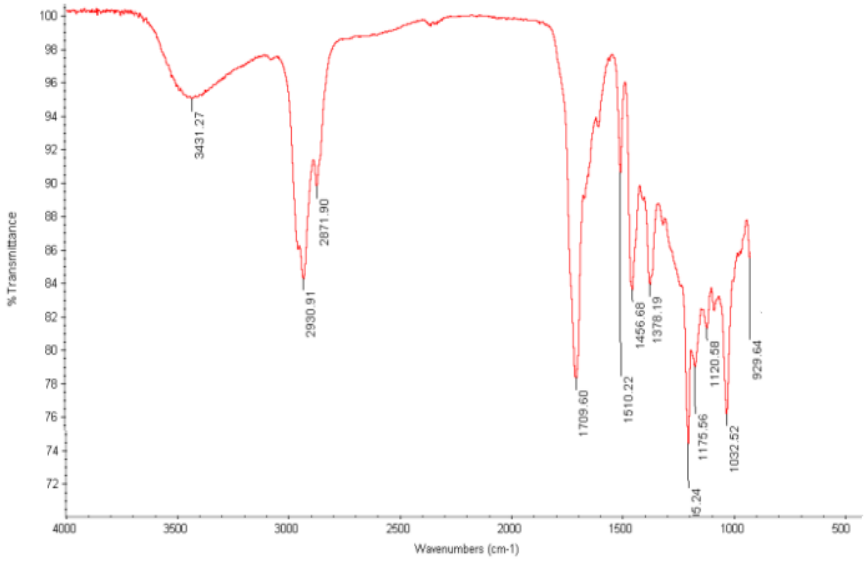

Fig 4: FTIR spectrum of isolated compound (asarone)

CONCLUSION

The phytochemical and bio-evaluation studies showed that the plant is rich source of secondary metabolites that too of Flavonoids and isoflavonoids having tremendous pharmacological potential. Our results demonstrate that methanolic extract of *Acarus Calamus* as well as isolated and characterized molecule exhibits antibacterial activity against both gram positive as well as gram-negative bacteria. The potent antioxidant nature of the extract adds to its anticarcinogenic potential suggesting a promising role in development of anticancer therapeutics in future. However, these findings warrant extensive studies to evaluate mechanistic action of antiproliferative and antioxidant principles.

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