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RESEARCH ARTICLE

EXPLORING ANTIBACTERIAL POTENTIAL OF *ACORUS CALAMUS* RHIZOME AND SHOOT EXTRACT

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Abstract: The present study was carried out to evaluate the *in vitro* antibacterial potential of rhizome and shoot part of *Acorus calamus via* five different extracts namely *n*-hexane, chloroform, ethyl acetate, acetone and methanol. The agar-well diffusion method was used against two-gram positive (*Bacillus cereus* MTCC5981, *Staphylococcus aureus* MTCC96) and three-gram negative (*Escherischia coli* MTCC1697, *Klebsiella pneumoniae* MTCC109, *Pseudomonas aeruginosa* MTCC741) bacterial strains and the findings from broth dilution assay for different plant extracts demonstrated antibacterial properties on the tested strains of bacteria at various degrees, as displayed by their minimum inhibitory concentration (MIC) values. The MIC values for rhizome part of *A. calamus* were in range of 410-835 µg/ml for all the tested bacterial strains, but the significant value was displayed by methanolic extract. For *A. calamus* shoot part, MIC values against the five tested bacterial strains ranged from 415-880 µg/ml, but the acetone extract was found to be the most effective against all bacterial strains as it exhibited the lowest MIC values. The results of present study revealed that rhizome and shoot part of *Acorus calamus* possess antibacterial potential and source of new antibiotics. Therefore, medicinal plants are finding their way into pharmaceuticals, nutraceuticals and food supplements that could be useful in chemotherapy to control infectious diseases.

Keywords: *Acorus calamus,* Antibiotics, Bacterial strains, Minimum inhibitory concentration

INTRODUCTION

Acorus calamus, commonly known as sweet flag, buch or calamus, belonging to family Araceae is a semiaquatic, perennial, aromatic herb with creeping rhizomes. *A. calamus* is a native of Central Asia and Eastern Europe. It is also found in marshy tracts of Kashmir, Shirmaur (Himachal Pradesh), Manipur and in Naga Hills (Umamaheshwari and Rekha, 2018). The rhizomes of *A. calamus* contain aromatic oil that has been used medicinally since ancient times and has been harvested commercially. The rhizomes are considered to possess emetic, aromatic, expectorant, emmenagogue, aphrodisiac, laxative, nauseate, nervine, sedative, stimulant properties and also used for the treatment of epilepsy, mental ailments, schizophrenia, memory disorders, chronic diarrhea, dysentery, hoarseness, flatulence dyspepsia, bronchial catarrh, intermittent fevers, glandular and abdominal tumors (Imam *et al*., 2013). In the Ayurvedic system of medicine, the rhizomes of *A. calamus* are considered to possess diuretic, antispasmodic, carminative and anthelmintic properties. They are also employed for kidney and liver troubles, rheumatism and eczema and help to stop smoking (Rajput *et al*., 2014). In Western herbal medicine, the herb is chiefly employed for digestive problems such as gas, bloating, colic and poor digestive function. Many Native American tribes were familiar with Calamus and it was used as an anesthetic for toothache and headaches. Calamus helps distended and uncomfortable stomachs and headaches associated with weak digestion. Small amounts are thought to reduce stomach acidity, while larger doses increase deficient acid production, it is a good sedative so that the extract is used for epilepsy, insanity and as a tranquillizer. It is an ingredient of any Ayurvedic preparation "Brahmi Bati" (Budhivardhar) which is indicated in epilepsy, coma and hysteria and in cases of mental retardation (Chandra and Prasad, 2017). The tribals in the Garhwal region of the Himalayas take a decoction of the rhizome as a nonalcoholic beverage. The fresh rhizomes are chewed to prevent intoxication from alcohol. The decoction is also given to children for gastroenteritis. The rhizome pieces are tied around the belly for jaundice. It is used as an antipyretic in Meerut region of Uttar Pradesh (Tomar 2007, Tomar 2008 and Tomar 2017). Natives of Tirumala hills also use the rhizomes for the treatment of dental disorders. *A. Calamus* has wonderful power of stimulating and normalizing the appetite. The massage through the dry powder is benefitted in the obesity and further reduces the subcutaneous fatty accumulations (Singh *et al*., 2011). The phytochemical studies have reported the presence of

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glycosides, flavonoids, saponins, tannins, polyphenolic compounds, mucilage, volatile oil and bitter principle. The plant has been reported for the presence of glucoside, alkaloid and essential oil containing calamen, clamenol, calameon, asarone and sesquiterpenes. It also contains a bitter glycoside named acorine along with eugenol, pinene and camphene (Imam *et al*., 2013). Apart from this content plant also contain asamyl alcohol, methyleugenol, α- pinene, acoretine, lucenin, acoradin, calamusenone, asaronaldehyde, acorenone, calamenone, shyobunone, starch and tannine. These natural products from plants have been studied extensively, and their role in several useful biological activities, such as antibacterial, antifungal, antiyeast, insecticides and herbicides, have been well documented. The present study, therefore, undertaken to determine the antibacterial activity of *Acorus calamus.*

MATERIALS AND METHODS

Preparation of plant extracts

Plant material was collected from the herbal garden of SKUAST-J, Main Campus Chatha, Jammu, in the month of May, 2022. Collected plants were washed thoroughly, dried at room temperature under shade. The material was thin sliced and dried under shade. The dried plant material was pulverized in grinder. 50 gm finely powdered samples were weighed into 500 ml screw-capped reagent bottles and subjected to maceration for 72 h at room temperature using *n*-hexane, chloroform, ethyl acetate, acetone and methanol as extraction solvent with occasional shaking. Contents of the bottle were squeezed through muslin cloth and the filtrate was filtered further using filter paper. The filtrate was then concentrated under reduced pressure at 45 ºC in rotary evaporator (Rotavapor R-200 Buchi, Switzerland) under reduced pressure to give residues in different amounts. Extracts were stored in refrigerator at 4 ^{o}C for further use. The yield of the extraction was calculated from the formula:

$$\text{Total extraction yield} = \frac{Weight\ of\ extract}{Weight\ of\ dry\ sample} \times 100$$

Bacterial strains used in the present study

The study utilized two-gram positive bacterial strains namely *Bacillus cereus* MTCC5981and *Staphylococcus aureus* MTCC96, as well as three gram negative strains, *Escherichia coli* MTCC1697, *Klebsiella pneumoniae* MTCC109 and *Pseudomonas aeruginosa* MTCC741. These strains were procured from IMTECH Chandigarh. The bacterial cultures were preserved in nutrient agar slants and stored at 4 ^{0}C. These cultures were used as stock cultures.

Preparation of inoculums

The chosen bacterial strains were introduced into a nutrient broth and incubated for 24 hours at a temperature of 37 ^{0}C. The optical density of the resulting active bacterial cultures was adjusted to match the 0.5 Macfarland standard. This was achieved by diluting the cultures with nutrient broth. The turbidity of the cultures was then determined by measuring the absorbance at 600 nm using a spectrophotometer.

Agar-well diffusion method

The antimicrobial activity of the extracts was evaluated qualitatively using the agar - well diffusion method, as described by Feyza *et al*., 2009, with some modifications. The sterilized nutrient agar (20 ml) was poured into sterile petri plates and allowed to solidify undisturbed in a sterile laminar air flow chamber. Once solidified, the petri plates were uniformly inoculated with 100 µl of a bacterial suspension (10^9 CFU / ml) using a sterile glass spreader. The plates were then left undisturbed for a few minutes to allow the bacterial suspension to be absorbed into the nutrient agar. Wells with a diameter of 6 mm were aseptically created in the agar plates and 20 µl of the extracts (from a 100 mg/ml stock solution in DMSO) were added to each well. Chloramphenicol (10 µl) was added to a well as a positive control to test the bacteria's sensitivity and 10 µl of DMSO was added to the wells as a negative control. The plates were then left at room temperature for a few minutes inside the sterile laminar air flow chamber to allow for proper diffusion of the extracts from their respective wells into the media. Following this, the plates were incubated at 37 ^{0}C for 24 hours. After the incubation period, the zones of inhibition were measured. This method allows for a qualitative assessment of the antimicrobial activity of the extracts.

Determination of minimum inhibitory concentration (MIC)

The minimum inhibitory concentration (MIC) was ascertained following the method outlined by Oke *et al*., 2009. This involved using the broth dilution method to evaluate the individual MICs of the extracts against selected bacterial strains. All tests were conducted using nutrient broth. A stock solution was prepared using 100 mg/ml of the extracts, which was then filtered using a 0.22 m filter disc. Subsequently, different dilutions of the extracts were prepared by taking appropriate volumes from the stock solution. Specifically, 100 µl of different extracts (100 mg/ml) were diluted with nutrient broth to create different concentrations using the serial dilution technique. Each dilution of the extract (100 µl) was transferred to sterilized microfuge tubes containing 90 µl of nutrient broth. A working bacterial suspension (10^6 CFU/ml) of 10 µl was then added to each dilution of the extracts, resulting in a final volume of 200 µl in the microfuge tubes. The positive control tubes contained only broth and inoculum, with no extract, while the negative control tubes contained only DMSO and inoculum. The tubes were then incubated at 37 ^{0}C for 24 hours. Post-incubation, 40 µl of a 0.4 mg/ml solution of 2,4-iodophenyl-3,4-nitrophenyl-5-phenyl tetrazolium

chloride (INT) violet was added to each tube and further incubated for 30 minutes at 37 ^{0}C. The tubes were then inspected for any color change. The development of a pink color, indicative of dye reduction, signaled bacterial growth. The lowest concentration of the extract at which no color change of the dye was observed (or no bacterial growth was detected) was deemed the MIC for that specific extract.

Statistical analysis

The data were analyzed statistically using least significant differences (LSD) test at the $p \leq 0.05$ probability level. For each measurement, three replicates of samples were subjected to one way analysis of variance (ANOVA) and significant differences between samples were determined using R studio software (Version, 2018).

Table 1. Antibacterial activity of *Acorus calamus* rhizome part

Extract	Conc. (µg/ml)	Inhibition zone diameter (mm)				
		Gram positive bacteria		Gram negative bacteria		
		Bacillus cereus	***Staphylococcus aureus***	***Pseudomonas aeruginosa***	***Escherichia coli***	***Klebsiella pneumoniae***
n-Hexane	20 µg	ND	ND	ND	ND	ND
Chloroform	20 µg	ND	ND	ND	ND	2.00±0.01
Ethyl acetate	20 µg	6.00±0.16	7.00±0.23	ND	2.00±0.12	15.00±0.21
Acetone	20 µg	10.00±0.05	10.00±0.15	12.00±0.08	17.00±0.04	8.00±0.11
Methanol	20 µg	17.08±0.26	18.00±0.23	18.00±0.25	18.00±0.15	16.00±0.27
Chloramphenicol	10 µg	18.00±0.14	21.00±0.16	20.00±0.12	20.00±0.28	18.00±0.22
DMSO	10 µl	ND	ND	ND	ND	ND

Data is Mean ± S.D. of three replicates

ND: activity not determined at tested concentration

Table 2. Minimum Inhibitory concentration of *Acorus calamus* rhizome part against bacterial strains

Extracts	MIC (µg/ml)				
	Bacillus cereus	***Staphylococcus aureus***	***Pseudomonas aeruginosa***	***Escherichia coli***	***Klebsiella pneumoniae***
n-Hexane	ND	ND	ND	ND	ND
Chloroform	ND	ND	ND	ND	730
Ethyl acetate	645	645	ND	835	530
Acetone	420	410	465	515	425
Methanol	410	415	465	410	465
Chloramphenicol	1.50	2.00	2.00	2.00	1.50

ND: activity not determined at tested concentration

Table 3. Antibacterial activity of *Acorus calamus* shoot part

Extract	Conc. (µg/ml)	Inhibition zone diameter (mm)				
		Gram positive bacteria		Gram negative bacteria		
		Bacillus cereus	***Staphylococcus aureus***	***Pseudomonas aeruginosa***	***Escherichia coli***	***Klebsiella pneumoniae***
n-Hexane	20 µg	ND	ND	ND	ND	ND
Chloroform	20 µg	ND	2.00±0.05	ND	2.00±0.11	ND
Ethyl acetate	20 µg	18.00±0.11	10.00±0.16	09.00±0.22	13.00±0.64	12.00±0.37
Acetone	20 µg	12.00±0.26	14.00±0.43	ND	20.00±0.29	17.00±0.01
Methanol	20 µg	10.00±0.31	19.00±0.45	10.00±0.25	21.00±0.53	16.00±0.25
Chloramphenicol	10 µg	26.00±0.17	26.00±0.29	24.00±0.14	25.00±0.03	25.00±0.19
DMSO	10 µl	ND	ND	ND	ND	ND

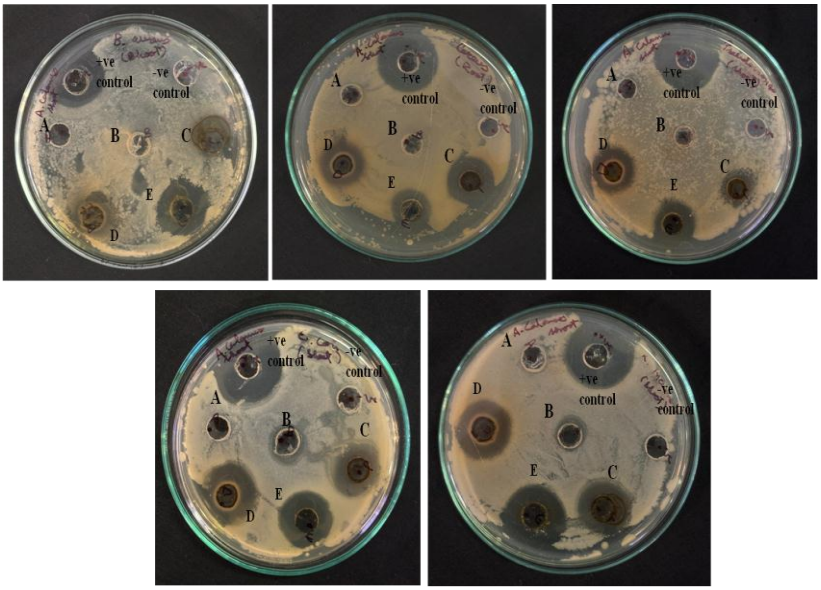
Data is Mean ± S.D. of three replicates

ND: activity not determined at tested concentration

Table 4. Minimum Inhibitory concentration of *Acorus calamus* shoot part against bacterial strains

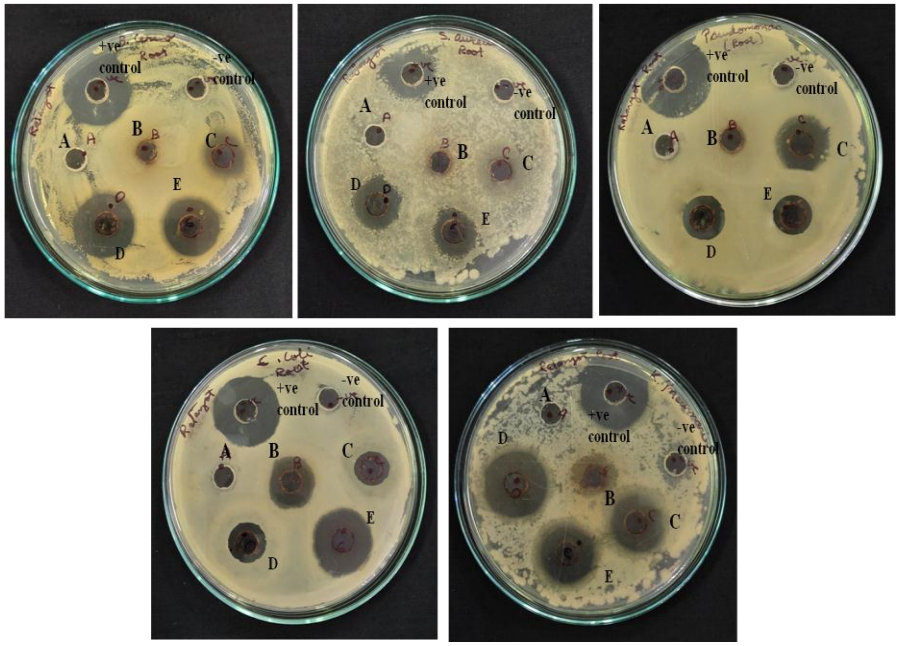
Extracts	MIC (µg/ml)				
	Bacillus cereus	***Staphylococcus aureus***	***Pseudomonas aeruginosa***	***Escherichia coli***	***Klebsiella pneumoniae***
n-Hexane	ND	ND	ND	ND	ND
Chloroform	ND	ND	ND	ND	880
Ethyl acetate	640	650	ND	765	465
Acetone	530	535	460	405	655
Methanol	420	415	415	510	430
Chloramphenicol	1.50	2.00	2.00	2.00	1.50

ND: activity not determined at tested concentration



Antibacterial activity of *Acorus calamus* (rhizome) extracts of different solvents namely (A) n-Hexane, (B) Chloroform, (C) Ethyl acetate, (D) Acetone, (E) Methanol, against five different test bacterial strains 1) *B. cereus*; 2) *S. aureus*; 3) *Pseudomonas*; 4) *E. coli*; 5) *K. pneumonae*

Plate 1



Antibacterial activity of *Acorus calamus* (shoot) extracts of different solvents namely (A) n-Hexane, (B) Chloroform, (C) Ethyl acetate, (D) Acetone, (E) Methanol, against five different test bacterial strains 1) *B. cereus*; 2*) S. aureus*; 3) *Pseudomonas*; 4) *E. coli*; 5) *K. pneumonae*

Plate 2

RESULTS AND DISCUSSION

The antibacterial activity of *A. calamus* rhizome part was assessed against both gram-positive and gram-negative bacteria, with significant variations observed across different extracts. For gram-positive bacteria, *Bacillus cereus* showed the highest inhibition zone with the methanol extract (17.08±0.26 mm) whereas lowest inhibition for *B. cereus* was recorded with the ethyl acetate extract, measuring 6.00±0.16 mm. No antibacterial activity was detected for the *n*-hexane and chloroform extracts at the tested concentration. Similarly, for *S. aureus*, the methanol extract again demonstrated the highest inhibition at 18.00±0.23 mm, whereas the lowest inhibition was noted with the ethyl acetate extract at 7.00±0.23 mm, with no activity detected for the *n*-hexane and chloroform extracts. Regarding gram-negative bacteria, the results varied significantly. The methanol extract consistently showed high inhibition for *P. aeruginosa, E. coli* and *K. pneumoniae* with inhibition zones of 18.00±0.25 mm, 18.00±0.15 mm and 16.00±0.27 mm, respectively. The lowest inhibition was observed with the chloroform extract against *K. pneumoniae* (2.00±0.01 mm). No activity was detected for the *n*-hexane extract across all gram-negative bacteria tested (Table 1, Plate 1).The MIC values for rhizome part of *A. calamus* were in range of 410-835 µg/ml for all the tested bacterial strains. The significant value of MIC was displayed in methanolic extract (Table 2).

In case of *A. calamus* shoot part, extracts prepared from five different solvents exhibited varying inhibition zones. The *n*-hexane and chloroform extracts did not show any inhibitory activity against *B. cereus*. However, the ethyl acetate extract exhibited significant antibacterial activity against *B. cereus* with an inhibition zone of 18.00±0.11 mm, the highest among the extracts tested followed by acetone (inhibition zone 12.00±0.26 mm), methanol extract had the lowest activity among the effective extracts (inhibition zone 10.00±0.31 mm). The *n*-hexane extract was also inactive against bacterial strain *S. aureus*. The chloroform extract showed minimal activity with inhibition zone of 2.00±0.05 mm. The methanol extract showed the highest activity among the extracts, with an inhibition zone of 19.00±0.45 mm followed by acetone (14.00±0.43 mm) and ethyl acetate extract (10.00±0.16 mm). The shoot extracts of *A. calamus* had varied effects on *P. aeruginosa*. The *n*-hexane, acetone and chloroform extracts showed no activity. The ethyl acetate extract exhibited some activity, with an inhibition zone of 9.00±0.22 mm, while the methanol extract displayed similar activity with 10.00±0.25 mm inhibition zone. For *E. coli*, the *n*-hexane extract displayed no activity. The chloroform extract showed minimal activity with a small inhibition zone of 2.00±0.11 mm < ethyl acetate (13.00±0.64 mm) < acetone (20.00±0.29 mm) < methanol extract (21.00±0.53 mm). Further, in case of *K. pneumoniae* the zone inhibition was in the order: ethyl acetate (12.00±0.37 mm) < methanol (17.00±0.01 mm) < acetone (16.00±0.25 mm) while no activity was observed in chloroform and *n*-hexane (Table 3, Plate 2). For *A. calamus* shoot part, MIC values against the five tested bacterial strains ranged from 415-880 µg/ml. The acetone extract was found to be the most effective against all bacterial strains as it exhibited the lowest MIC values (Table 4).

Antimicrobial assays from plant extracts are conducted to identify and evaluate the potential of natural compounds to inhibit or kill pathogenic microorganisms. This is crucial for discovering new, effective antimicrobial agents, particularly in the face of increasing antibiotic resistance. Plant extracts offer a diverse array of bioactive compounds, such as phenolics, flavonoids and alkaloids, which have demonstrated significant antimicrobial properties. These natural compounds can provide alternatives or supplements to conventional antibiotics, helping to combat resistant strains and reduce side effects associated with synthetic drugs (Vaou *et al*., 2021). Evaluating antimicrobial activity also supports the validation and scientific basis for the traditional use of medicinal plants in treating infections, potentially leading to the development of new therapeutic agents (Eloff, 2019).The current investigation sought to determine the antibacterial activity of *A. calamus* extracts against five test bacterial strains *viz.*, *Escherichia coli, Klebsiella pneumoniae, Pseudomonas, Bacillus cereus and Staphylococcus aureus.* The inhibitory effects of the plant extracts were assessed using both quantitative (broth dilution test) and qualitative (agar well diffusion) techniques. The different mechanisms of action through which phytochemicals can exert antimicrobial activities include inhibition of the activity of enzymes and toxins, damage of the bacterial membrane, suppression of virulence factors, formation of biofilm and inhibition of protein synthesis (Ugboko *et al*., 2020).

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