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Research Article

In-Vitro Antimicrobial Activity and Phytochemical Analysis of *Acorus Calamus* Rhizomes

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ABSTRACT

The above study attempts to evaluate the phytochemical and antimicrobial action of rhizomes of *Acorus calamus* Linn. belongs to the family Acoraceae, with different solvents. Rhizomes are used for different ailments like cough, bronchitis, asthma and for poor digestive functions. Clinically important bacteria viz *Staphylococcus aureus* (ATCC29737), *Escherichia coli* (ATCC2068), *Pseudomonas aeruginosa* (ATCC9027) and *Candida albicans* (ATCC0231) were used. The in-vitro antibacterial activity was performed by Agar well diffusion method. Ethanolic extract of *Acorus calamus* showed the highest zone of inhibition against *S. aureus* and *P. aeruginosa* and against the fungus *C. albicans*. However further studies are needed for their modes of action on bacterial cells.

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INTRODUCTION

It's a well known fact that, there will be variation in clinical results of the crude drugs collected from different regions of the same place. The detailed study of the active ingredients present in the plants helps the scientists to understand the mode of action of that drug. The preliminary phytochemical screening of various medicinal plants was reported by many persons who are working in that field itself [1-5].

Acorus calamus is known for its beneficial and medicinal value in the Asia since long time. It is harvested on commercial scale and mostly cultivated in the Asian region. It is also found indigenously in the marshy tracts of Kashmir, Shirmaur (Himachal Pradesh), Manipur and in Naga Hills of India. *Acorus calamus* is a semi-aquatic, perennial, aromatic herb with creeping rhizomes found nearly marshy places, river banks and lake.

It was also known as "sweet flag", because of its sweet scent and is commonly known as flags in English, since the late fourteenth century [6]. The rhizomes extracts are considered to possess anti-spasmodic, carminative, anthelmintic and inhibits the spontaneous motor activity [7]. Further indications include the loss of consciousness, confusion of the mind and forgetfulness. This traditional Ayurvedic medicine was used to treat memory loss and also used for treatment of epilepsy, anorexia, mental ailments, bronchial catarrh, intermittent fevers, chronic diarrhoea, dysentery, abdominal tumours, kidney and liver illness, rheumatism, sinusitis and eczema.

It is listed as an insecticide, an antifungal agent, an antibacterial agent and as a fish toxin. Further studies revealed that, it also possess CNS depressant activity [8]. It was also used in the treatment of rheumatoid arthritis with pain, swelling and functional disability and for low-grade mentally retarded children [9-13]. The antibacterial and antifungal properties of this fraction contains β -asarone (isoasarone) as a major component and has been subject to considerable studies [14].

In the present study, different solvents like petroleum ether, ethanol and aqueous extracts were used for the *in-vitro* antimicrobial activity and preliminary phytochemical evaluation. This may be used for the development of new tools as antimicrobial agents to control various infectious diseases.

MATERIALS AND METHOD

Collection of plant materials

The rhizomes of *Acorus calamus* were collected randomly from Tropical - Humid regions of Kerala, India. Then the roots were cleaned off and washed thoroughly in water to remove the soil adhered, under the guidance of Ayurvedic experts from Indian Herbs and Pharmaceutics, chempu. Specimens were identified, authenticated with the help of Taxonomist; voucher specimens were prepared and stored for future reference. Plant materials were dried in the dark

at room temperature and stored for future use. The rhizomes were cut into pieces of one inch length and equally partitioned.

Preparation of extract

The rhizomes were crushed in mechanical mortar. Then it was extracted by using Soxhlet apparatus at 37°C for 72 hrs with solvents petroleum ether and ethanol. The aqueous extract was obtained by maceration for 24 hrs. All the above extracts were filtered, concentrated and dried under reduced pressure in a rotary evaporator. The percentage yield for each extract was determined. Extracts were stored in refrigerator (4°C) until further use.

Phytochemical Screening

The dried extracts were analyzed for various phytoconstituents like carbohydrates, tannins, amino acids, alkaloids, terpenoids, steroids, glycosides, saponins, and flavonoids by the following procedure [16, 17]. All the chemicals and reagents used were of analytical grade [15, 16].

TEST FOR CARBOHYDRATES

Molisch's Test: Treat 1 mL of test solution with few drops of alcoholic α -naphthol. Add 0.2 mL of concentrated sulphuric acid slowly along the sides of the test tube. Purple to violet colour ring appears at the junction.

Fehling's Test: Equal volume of Fehling's A (Copper sulphate in distilled water) and Fehling's B (Potassium tartarate and Sodium hydroxide in distilled water) reagents are mixed and few drops of sample is added and boiled, a brick red precipitate of cuprous oxide forms, if reducing sugars are present [17, 18].

TESTS FOR TANNINS

Ferric Chloride Test: To the test solution few drops of ferric chloride test reagent were added. An intense green, purple, blue or black colour developed was taken as an evidence for the presence of tannins [17, 18].

Lead Acetate Test: To the test solution, a few drops of 10% lead acetate were added. Precipitate were formed, indicate the presence of tannins [17, 18].

TESTS FOR AMINOACIDS

Millon's Test: To the test solution, add about 2 mL of millon's reagent (Mercuric nitrate in nitric acid containing traces of nitrous acid), white precipitate appears, which in turns red upon gentle heating [17, 18].

Ninhydrin Test: Amino acids and proteins when boiled with few drops of 5% solution of Ninhydrin, violet colour appears [17, 18].

TEST FOR ALKALOIDS

Mayer's Test: Alkaloids give cream colour precipitate with Mayer's reagent (Potassium mercuric iodide solution) [17, 18].

Dragendorff's Test: Alkaloids give reddish brown precipitate with Dragendorff's reagent (Potassium bismuth iodide solution) [17, 18].

TEST FOR STEROLS AND TERPENOIDS

Liebermann-Burchard Test: Extract treated with few drops of acetic anhydride, boil and cool, concentrated sulphuric acid is added along the side of test tube, shows brown ring at the junction of two layers and the upper layer turns green which shows the presence of sterols and formation of deep red colour indicate the presence of terpenoids.

Salkowski's Test: Treat extract in chloroform with few drops of concentrated sulphuric acid, shake well and allow to stand for some time, red colour appears in the lower layer indicate the presence of sterols and formation of yellow colour in the lower layer indicate the presence of terpenoids.

TEST FOR GLYCOSIDE

Keller Killiani Test (Cardiac glycosides): To 0.5 g of plant extract, add 0.4 mL of glacial acetic acid containing a trace amount of ferric chloride. Transfer to a small test tube; add carefully 0.5 mL of concentrated sulphuric acid along the sides of the test tube, blue colour appears in the acetic acid layer, indicate the presence of cardiac glycosides [17, 18].

Borntrager's Test (Anthraquinone Glycosides): 0.5 g of the plant extract was shaken with benzene and organic layer got separated and half of its own volume of 10% ammonia solution added. A pink, red or violet colouration in the ammoniacal phase indicate the presence of anthraquinone glycosides [17, 18].

TEST FOR SAPONINS

Forth Test: A pinch of the dried powdered plant was added to 2-3 mL of distilled water. The mixture was shaken vigorously. Formation of foam indicates the presence of saponin.

TEST FOR FLAVANODIS

Shinoda Test (Magnesium hydrochloride reduction test):

To the test solution add few fragments of magnesium ribbon and add concentrated hydrochloric acid drop wise. pink scarlet, crimson red or occasionally green to blue colour appears after few minutes.

DETERMINATION OF ANTIMICROBIAL ACTION

Microorganisms used

The test organisms used were *Staphylococcus aureus* (ATCC29737), *Escherichia coli* (ATCC2068), *Pseudomonas aeruginosa* (ATCC9027), and *Candida albicans* (ATCC10231). Microorganisms were maintained at 4°C on nutrient agar slants.

Inoculum

The microorganisms were inoculated into soybean casein broth (SBCB) and incubated at $35 \pm 2^\circ\text{C}$ for 4 hr. The resultant turbid suspension was diluted with SBCB to match with 1 McFarland turbidity standard. This level of turbidity is equivalent to approximately 3.0×10^8 cfu/mL.

Agar diffusion assay

Modified agar well diffusion method was employed [19]. Mueller-Hinton agar plates were inoculated by streaking the swab over the entire sterile agar surface. This procedure was repeated by streaking 2 more times, rotating the plate approximately 60° each time to ensure even distribution of the inoculum. As a final step, the rim of the agar was also swabbed. After allowing the inoculum to dry at room temperature, 6 mm diameter wells were bored in the agar. Each extract was checked for antimicrobial activity by introducing 100 μL of 4000 $\mu\text{g/mL}$ concentration into triplicate wells. Simultaneously, gentamicin sulphate (*S. aureus*, *P. aeruginosa*, and *E. coli*), and nystatin (*C. albicans*) were used as positive controls at a concentration of 1.0 $\mu\text{g/mL}$. The dilution medium for the positive controls was sterile distilled water, ethanol and petroleum ether. The plates were allowed to stand at room temperature for 1hr for the extract to diffuse into the agar plate. Then they were incubated at $35 \pm 2^\circ\text{C}$ for 24 hr, except *C. Albicans* which was incubated at $29 \pm 2^\circ\text{C}$.

DETERMINATION OF MINIMAL INHIBITORY CONCENTRATION (MIC), MINIMAL BACTERICIDAL CONCENTRATION (MBC) AND MINIMAL FUNGICIDAL CONCENTRATION (MFC)

The MIC was determined by micro-broth dilution method. The reconstituted drug was serially diluted two fold in Mueller-Hinton broth (Oxoid) medium. Duplicate tubes of dilution ranging from 0.025 mg/mL to 25.6 mg/mL were inoculated with 5×10^5 cells (cfu) of the test bacterial strain and cultures incubated at 37°C for 18 hr. MIC was taken as the highest dilution (least concentration) of the drug showing no detectable growth. MBC and MFC were determined by sub-culturing the test dilution in a fresh drug-free solid medium and incubating further for 18-24 hr. The highest dilution that yielded no single bacterial and fungal colony on a solid medium was taken as MBC and MFC respectively.

RESULT AND DISCUSSION

In higher plants, the presence of antibacterial substances was well established. As in the case of other systems of medicines viz, Unani and Ayurvedic, Phytomedicines can be used for the treatment of diseases or as a base for the development of a medicine, a natural blueprint for the development of a drug [20]. Some of the investigated plants didn't show antibacterial activity. That doesn't mean the absence of bioactive constituents or it was an inactive plant. Active compound(s) may be present in insufficient quantities in the crude extracts [21]. Lack of activity can thus only be proven by using large doses. Alternatively, if the active principle is present in high quantities, there could be other constituents exerting antagonistic effects or negating the positive effects of the bioactive agents.

The results of preliminary phytochemical screening from Table No.1 shows that extracts of rhizome of *Acorus calamus* revealed the presence of Carbohydrates, Tannins, Amino acids, Alkaloids, Sterols, Terpenoids, Glycosides, Saponins and Flavanoids. It should be noted that the ethanol extracts give more positive tests for different chemical constituents. The traditional healers make use of water primarily as a solvent but this study has shown that the ethanol extract of rhizome of *Acorus calamus* contains more of the pharmacologically active secondary metabolites. This is because most of these secondary metabolites being organic in nature are soluble in ethanol. The result of antimicrobial susceptibility assay from Table No.2 shows promising evidence for the antimicrobial effects of rhizome of *Acorus calamus* against bacterial (*S. aureus*, *P. aeruginosa*, *E. coli*) and fungal (*C. albicans*) pathogens. Methanolic extract of rhizome of *Acorus calamus* showed maximum inhibition 0.9 mm against *C. Albicans*, 11mm against *Staphylococcus aureus*. It also shows inhibition of 12.3 mm against *P. aeruginosa* and 8.6 mm against *Escherichia coli*. From the above findings, it may be suggested that rhizome of *Acorus calamus* have highest antimicrobial properties.

The MIC, MBC and MFC values of the ethanol extract from the rhizome of *Acorus calamus* are shown in Table No.3. Thus MIC, MBC and MFC assay are capable of verifying that the compound has antimicrobial activity and that it gives reliable indication of the concentration of drug required to inhibit the growth of microorganisms. The MIC values of ethanol extracts against *Staphylococcus aureus* (ATCC29737), *Escherichia coli* (ATCC2068) *Pseudomonas aeruginosa* (ATCC9027), *Candida albicans* (ATCC10231) were, respectively, 0.1563, 0.3125, 0.3125 and 0.0781 mcg/mL. The MIC values of the extract (0.0391mg/mL) against both the organisms *Staphylococcus aureus* (ATCC29737), *Pseudomonas aeruginosa* (ATCC9027), were the same. The MBC values of ethanolic extract against *Staphylococcus aureus* (ATCC29737), *Escherichia coli* (ATCC2068), *Pseudomonas aeruginosa* (ATCC9027), were, respectively, 0.0781, 0.1563 and 0.1563 mcg/mL. Whereas, the MFC value of ethanolic extract against *Candida albicans* (ATCC10231) was 0.0391 mcg/mL. This scientific study has revealed that, *Acorus calamus* rhizome has the potential to provide an alternative for modern medicinal products as well as cosmetics and skin care products.

Table 1: Phytochemical analysis of rhizomes of *Acorus calamus* extracts

S.No	Phytochemicals Test	Petroleum ether	Ethanol	Aqueous
1	Carbohydrates	-	+	+
2	Tannins	+	+	+
3	Amino acids	-	+	-
4	Alkaloids	+	+	-
5	Sterols and Terpenoids	+	-	+
6	Glycosides	+	+	+
7	Saponins	-	+	+
8	Flavanoids	+	+	+

Notes: + = Present ; - = Absent

Table 2: Antimicrobial activity of extracts of *Acorus calamus* rhizomes

Plant Extract	ZONE OF INHIBITION in mm			
	<i>Staphylococcus aureus</i>	<i>Escherichia coli</i>	<i>Pseudomonas aeruginosa</i>	<i>Candida albicans</i>
Pet. Ether	8.6 ± 0	0.0	8.6 ± 0	4 ± 0
Ethanol	11 ± 0	8.6 ± 0	12.3 ± 0	9 ± 0
Aqueous	10.1 ± 0	7.6 ± 0	11.3 ± 0	8 ± 0
Gentamycin (1µg/mL)	22 ± 0	21 ± 0	23 ± 0	NT
Nystatin (1µg/mL)	NT	NT	NT	16±0
PE, ET, AQ	-	-	-	-

Table 3: Minimum Inhibitory Concentration, Minimum Bactericidal Concentration & Minimum Fungicidal Concentration Values of ethanol extracts of *Acorus calamus* rhizomes

Staphylococcus aureus (ATCC29737)		Escherichia coli (ATCC206)		Pseudomonas aeruginosa (ATCC9027)		Candida albicans (ATCC10231)		Control	
MI C	M BC	MI C	M BC	MI C	M BC	MI C	M FC	+	-
0.1 563	0.0 781	0.3 125	0.1 563	0.3 125	0.1 563	0.0 781	0.0 391		

CONCLUSION

From the study it was found out that various bioactive constituents like Alkaloids, Flavanoids, Glycosides etc were present. The MIC, MBC and MFC values shows that, the compound has marked Anti-fungal and Anti-bacterial activity in terms of inhibition of fungal and bacterial growth *in-vitro*. It can be concluded that the potential of *Acorus calamus* can be used for the preparation of pharmaceutical formulations.

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