In Vitro Antibacterial Activity of Methanol Extract of *Acorus calamus* and *Shorea robusta* Alone and in Combination

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Abstract

Combining antibiotics with plant extracts offers a promising approach for the development of novel antimicrobials effective against drug resistant bacteria. In the current study combinations of Acorus calamus and Shorea robusta extracts with antibiotic were used against drug-resistant strain of Staphylococcus aureus and Pseudomonas aeruginosa. A methanol extract of A. calamus and Shorea robusta showed extraction yield of 3%, w/w and 6.5%, w/w respectively. The present investigation showed presence of polyphenolics and flavonoid based major phytochemical constituents with bioactivities. Staphylococcus aureus was completely inhibited by the A. calamus extracts with MIC values of 10mg/ml and for S. robusta the MIC value was 12.5 mg/ml. When A. calamus and S. robusta both were used together a synergistic effect was observed and the MIC value obtained was lower, 7.5 mg/ml. For Pseudomonas aeruginosa when treated with A. calamus extracts, MIC obtained was 12.5 mg/ml and with S. robusta extract MIC value was 17.5 mg/ml. When both plant extracts were used in combination, synergism was observed with MIC value of 7.5 mg/ml. The combination of the plant extract with ciprofloxacin showed enhanced effectiveness against S. aureus and P. aeruginosa.

Key words*: Acorus calamus, Shorea robusta, Staphylococcus aureus, Pseudomonas aeruginosa ciprofloxacin.*

1: Introduction

In recent years, the emergence of multidrug-resistant strains of *Staphylococci* and *Pseudomonas* has presented a significant challenge as a nosocomial (hospital-acquired) pathogen. These strains have developed resistance to almost all antibiotics currently available for clinical use, making infections caused by them extremely difficult to treat effectively. The significant rise in the demand for herbal medicine over the past two decades

has highlighted the necessity to guarantee the quality, safety, and effectiveness of herbal drugs. Plants have been used by humans for centuries to treat a variety of ailments. Even today, approximately 80% of the global population relies on traditional medicines for their physical and mental health needs, primarily due to the cost and potential side effects of Western pharmaceuticals, as well as limited access to healthcare facilities. Rural areas in many developing countries continue to depend on traditional medicine as their primary source of healthcare, integrating it into their daily lives. Recent studies have discovered that plant antimicrobials can enhance the effectiveness of standard drugs when used together [1].

Although these plant antimicrobials may not have inherent antimicrobial properties, their concurrent use with standard drugs enhances their efficacy. *Acorus calamus* is a perennial wetland plant native to Europe, Asia, and North America. It is also known as sweet flag, calamus, and sweet grass. The plant has long, thin stems with sword-shaped leaves and fragrant, yellow-green flowers [2]. It has been used for a variety of purposes, including as a spice, a medicine, and a source of oil [3]. *Acorus calamus* plant has been used in traditional medicine to treat a variety of ailments, including digestive issues, headaches, fever, and inflammation [4]. It is also used as a natural remedy for anxiety, depression, and insomnia [5]. Additionally, it is believed to have anti-bacterial and anti-fungal properties, making it a popular ingredient in natural skin care products. *Acorus calamus* oil is used in aromatherapy to help improve focus and concentration. The herb has also been used as a flavoring agent in food and beverages. Studies have shown that extracts from the plant have strong antibacterial effects against a variety of bacteria, including *Staphylococcus aureus*, *Escherichia coli*, and *Pseudomonas aeruginosa* [6]. The plant's essential oils have also been found to be effective against bacteria, fungi, and viruses. Additionally, research has found that the plant's compounds can inhibit the growth of drug-resistant bacteria and reduce the risk of infection [7]. In addition, studies have revealed that the plant has antifungal activity against organisms such as *Candida albicans* and *Aspergillus fumigatus* [8].

Shorea robusta, is commonly known as the Sal tree. It belongs to the dipterocarp family and is native to India, Bangladesh, Sri Lanka, Nepal, Bhutan, Thailand, and Myanmar. The tree is a large evergreen, reaching heights of up to 40 meters high and a trunk diameter of 1–1.7 meters. It also has many cultural, religious, and medicinal uses. The fruits of the tree have reportedly been used in the making of alcoholic beverages. The tree is considered sacred by Hindus and can often be found near temples. It is also used in Ayurvedic medicines for treating various skin diseases, including wound healing. In one study, an alcohol extract of *Shorea robusta* bark was found to be effective in the treatment of cutaneous wounds in rats. It was observed that treatment with *Shorea robusta* extract accelerated wound healing and decreased inflammation [9]. This suggests that the extract may improve wound closure. Phytotherapy, which involves using plant-based treatments, offers various advantages through these synergistic interactions. These benefits include increased efficiency, reduced side effects, improved stability or bioavailability of the active agents, and achieving therapeutic effects with smaller doses compared to synthetic medications. In the current study the plants extracts from *Acorus calamus* and *Shorea robusta* were assessed for their antibacterial potential against gram positive and gram negative clinical isolates, when used individually and in combination.

Materials and Methods

2.1: Plant material

Acorus calamus rhizome was collected from Pathare fields in Ratnagiri, Maharashtra. *Shorea robusta* was obtained from the Bastar district forest, which is located in the southern portion of Chhattisgarh and has an area of 4029.98 km [10]. The plants were harvested using standard method as per the the C.C.R.A.S guidelines [11, 12]. Both plant materials were authenticated from Blatter Herbarium, Department of Botany, St. Xavier's College, Mumbai, India. The raw rhizomes and resins were washed thoroughly and 100g each were kept at room temperature for drying. Dried rhizomes and resins were powdered in a mixer grinder. The powdered materials were stored in airtight polythene bags until use.

2.2: Preparation of extracts

The extract was prepared by taking 50 grams of powder of both plant materials in 500 ml of methanol. For four days, this mixture was shaken at 50 rpm on an orbital shaker. After 4 days the mixture was filtered using Whatman filter paper No. 1. The filtrate was allowed to evaporate on a rotary evaporator and extractive values were calculated in terms of percentages. For subsequent use, the extract was preserved in the refrigerator. The stock solution of the extract was prepared by taking 100 milligrams of extract per ml of Dimethyl sulfoxide (DMSO). The stock solution was stored at 4^0C [13].

2.3: Microbial strains

The clinical isolates used in the study were as follows, *Escherichia coli, Pseudomonas aeruginosa, Bacillus subtilis, Klebsiella pneumoniae and Staphylococcus aureus*. All the microbial isolates were routinely sub cultured using nutrient agar and maintained at 4^0C. Prior start of each experiment, the cultures were revived in nutrient broth and grown for 24 hours at 37^0C.

2.4: Phytochemical analysis

In order to determine the chemical components such as alkaloids, tannins, saponins, flavonoids, glycosides, steroids, protein, carbohydrates and terpenoids in the methanolic extract of *Shorea robusta* resin and *Acorus calamus* rhizome, following tests were performed [14].

2.4:1: *Test for alkaloids*

To 1 ml of extract, 1 ml Mayer's reagent was added. Alkaloids were identified with the appearance of yellowish or white precipitate.

2.4:2: *Test for tannins*

To 2 ml of plant extract, 1 ml 10% of alcoholic ferric chloride was added; formation of brownish blue or black color indicated the presence of tannins.

2.4:3: *Test for saponins*

To 2 ml of plant extract, 1ml 1% lead acetate solution was added. The presence of saponins was indicated by the formation of white precipitate.

2.4:4: *Test for flavonoids*

To 2 ml of plant extract, two to three drops of 20% sodium hydroxide were added. To this, a few drops of 70% HCL were added, the initial intense yellow color gradually faded away, indicating the presence of flavonoids.

2.4:5: *Test for glycosides*

2.5 ml plant extract was treated with 2 ml of glacial acetic acid and one drop of $FeCl_3$. Presence of glycosides was indicated by the development of brown color ring.

2.4:6: *Test for steroids*

2 ml acetic anhydride was added to 2 ml plant extract, mixed with concentrated sulphuric acid. The color change from violet to blue or green indicated the presence of steroids.

2.4:7: *Test for proteins*

The presence of peptide linkage was determined by development of violet color upon the addition of 1 ml 50% sodium hydroxide and a few drops of 1% copper sulphate to 1 ml of plant extract.

2.4:8: *Test for carbohydrates*

To 1 ml of plant extract, 0.5 ml of Molisch's reagent and 1 ml of concentrated sulfuric acid was added. The mixture was further allowed to stand for two to three minutes. The presence of carbohydrates in the sample extract was observed by the development of red or pale violet color.

2.4:9: *Test for terpenoids*

To 1 ml of plant extract, 0.5 ml of chloroform and a few drops of concentrated sulfuric acid were added. Formation of reddish-brown precipitate indicated the presence of terpenoids.

2.5: Study of antibiogram of the clinical isolates

Antibiotic resistance pattern of the clinical isolates namely, *Escherichia coli*, *Pseudomonas aeruginosa*, *Bacillus subtilis*, *Klebsiella pneumoniae* and *Staphylococcus aureus* was studied using Clinical and Laboratory Standard Institute guidelines. Agar disk diffusion method using Mueller–Hinton (MH) agar was employed and the following antibiotics were tested: Ciprofloxacin, Gentamicin, Kanamycin, Methicillin, and Vancomycin. Clinical isolates were grown in nutrient broth for 18 hours. Using sterile swabs, the culture was spread on the plate. Antibiotic discs were positioned with at least 22 mm between the centers of each disc. The plates were incubated for 24 hours at 37°C. The Kirby Bauer chart was used to evaluate

the millimeter-scale zone diameter [15].

2.6: Determination of Minimum Inhibitory concentration

Minimum Inhibitory Concentration of the plant extracts of *S robusta* and *A calamus* were determined by broth dilution technique. The different concentrations of plant extracts 0.5 mg/ml to 20 mg/ml were mixed with Mueller–Hinton (MH) broth. *Staphylococcus aureus* and *Pseudomonas aeruginosa* were added to the broth as 2% inoculum and the tubes were incubated for 24 hours at 37°C. Using a spectrophotometer set at 600 nm, the growth of the bacterial isolates in the test tubes was detected as turbidity after incubation. The MIC value represents the lowest concentration at which turbidity was not seen.

2.7: Evaluation of antibacterial efficacy by agar-well diffusion method

The antibacterial activity of the combination of plant extracts (*S. robusta* + *A. calamus*) was studied against antibiotic resistant clinical isolates by agar well diffusion method. The isolates were grown in nutrient broth and incubated at 37°C for 24 h. 0.1 ml culture was seeded in 20 ml molten nutrient agar butts, mixed and poured into sterile petri plates and allowed to solidify. Wells with a diameter of 6 mm were punched aseptically with a cork borer on seeded nutrient agar plate. Different concentrations of extracts ranging from 10 mg/mL to 50 mg/mL were added in the wells. The plates were incubated at 37°C for 24 h and the diameter of the zone of inhibition was recorded [16].

2.8: Synergistic activity of plant extracts with antimicrobial agents

Synergistic activity of plant extracts in combination with Ciprofloxacin was determined by using the paper strip diffusion method. The antibacterial activity of methanolic extracts of *Acorus calamus* rhizome and *Shorea robusta* resin and their combination with Ciprofloxacin was tested against *Pseudomonas aeruginosa* and *Staphylococcus aureus*. 0.1 ml overnight grown cultures of above pathogens were swabbed over the nutrient agar plate. The plant extract and antibiotic were added in concentration of 50 µg/ml to sterile distilled water. Sterile filter paper strips were dipped in the plant extract and antibiotic mixture and placed on nutrient agar plate pre swabbed with test pathogen culture. The plates were then incubated for 24 hours at 37 °C before the zones of inhibition surrounding the strips were measured [17].

3: Results and discussion

3.1: Plant material and extraction

The collected plant specimen was identified as *Acorus calamus* and *Shorea robusta* by Dr. Rajendra Shinde, Curator, Blatter Herbarium, St. Xavier's College, Mumbai, India. The specimen sample was deposited in Blatter Herbarium with specimen no 2574. The extraction yields from *A. calamus* and *S. robusta* showed maximum extraction yield in methanol 3%, w/w and 6.5%, w/w respectively.

3.2: Preliminary Phytochemical analysis

The phytochemical screening showed that *Acorus calamus* rhizome contained alkaloids, tannins, saponins, flavonoids, steroids, carbohydrates and proteins. However the extract didn't show the presence of glycosides. Similar results were obtained in earlier study by Pawar et al [11]. *Shorea robusta* extract showed presence of all the above mentioned phytochemicals including glycosides, the results are represented in Table 1. It contained significant amount of tannins, flavonoids and steroids. These components are pharmacologically active phytochemicals and contribute to antimicrobial activity.

Table 1: Qualitative estimation of phytochemicals in methanolic extract of *Acorus calamus* and *Shorea robusta*

Phytochemical Constituent	Solvent extracts and phytochemical results	
	Methanolic extract of *Acorus calamus*	Methanolic extract of *Shorea robusta*
Alkaloids	+++	++
Tannins	++	+++
Saponins	+++	++
Flavonoids	+	+++
Glycosides	–	++
Steroids	++	+++
Carbohydrates	+	+
Proteins	+	+

Keys:- (+): *Present in low concentration*;
(--): *Not present*;
(++): *Present in moderately high concentration*;
(+++): *Present in very high concentration*

3.3: Antibiotic susceptibility test

Standard antibiotic discs impregnated with 10 and 30 ug/ml of following antibiotics were used; Ciprofloxacin, Gentamicin, Kanamycin, Methicillin and Vancomycin. The assessment of antibacterial activity was based on the measurement of inhibition zones formed around the discs. The antibiotic susceptibility testing of the five isolates namely *Escherichia coli, Pseudomonas aeruginosa, Bacillus subtilis, Klebsiella pneumoniae* and *Staphylococcus aureus* was carried out by disk diffusion method. All isolates showed

resistance to more than two antibiotics. Isolates were found to be resistant to Ciprofloxacin, Gentamicin, Kanamycin, Methicillin and Vancomycin. Gram positive strains showed resistance against Ciprofloxacin, Gentamicin, Kanamycin (10 & 30 mcg/ml). *Staphylococcus aureus* was resistant to multiple antibiotics including Ciprofloxacin, Vancomycin, Methicillin and Gentamicin. *Staphylococcus aureus* was a clinical burn wound isolate. It was observed that 50% of strains were found to be resistant against Ciprofloxacin, Gentamicin, Kanamycin, Methicillin and Vancomycin. The results are represented in Table 2.

Table 2: Antibiotic susceptibility pattern of clinical isolates

Isolates	Antibiotics									
	Ciprofloxacin mcg/ml		Kanamycin mcg/ml		Vancomycin mcg/ml		Methicillin mcg/ml		Gentamicin mcg/ml	
	10	30	10	30	10	30	10	30	10	30
S. aureus	R	R	I	S	R	R	I	S	R	R
B. subtilis	I	S	R	R	I	S	R	R	I	S
P. aeruginosa	I	S	R	R	R	R	I	S	R	R
K. pneumoniae	R	R	R	R	S	S	I	S	R	R
E. coli	R	R	I	S	R	R	I	S	R	R

Keys : S- sensitive, R- resistant, I- intermediate

3.4: Minimum Inhibitory concentration

The minimum inhibitory concentration was determined using a range of 0.5 to 20 mg/ml (Table 3). MIC was determined by broth dilution method as the lowest concentration that completely inhibited bacterial growth after 24 hr of incubation at 37^0C. Growth of *Staphylococcus aureus* was completely inhibited by the *A. calamus* extracts at MIC values of 10mg/ml and for *S. robusta* the MIC value was 12.5 mg/ml. When *A. calamus* and *S. robusta* both were used together a synergistic effect was observed and the MIC value obtained was lower, 7.5 mg/ml. Similar trend was observed in the case of *Pseudomonas aeruginosa.*. When treated with *A. calamus* extracts, MIC obtained was 12.5 mg/ml and with *S. robusta* extract MIC value was 17.5 mg/ml. When both plant extracts were used in combination, synergism was observed with MIC value of 7.5 mg/ml.

Table 3: Determination of Minimum Inhibitory Concentration (MIC) against *Staphylococcus aureus* and *Pseudomonas aeruginosa*

Plant Extract	Concentration mg/ml									
	0.5	1.0	2.5	5	7.5	10	12.5	15	17.5	20
S. aureus										
A. calamus	+	+	+	+	+	-	-	-	-	-
S. robusta	+	+	+	+	+	+	-	-	-	-
S. robusta + *A.calamus*	+	+	+	+	-	-	-	-	-	-
P. aeruginosa										
A.calamus	+	+	+	+	+	+	-	-	-	-
S. robusta	+	+	+	+	+	+	+	+	-	-
S. robusta + *A.calamus*	+	+	+	+	-	-	-	-	-	-

3.5: Antibacterial activity of *Acorus calamus* and *Shorea robusta* extracts

The antibacterial activities of *Acorus calamus* and *Shorea robusta* extract individually as well as combination were checked at 10, 30, 50 mg/ml concentrations against five clinical isolates namely, *Escherichia coli*, *Pseudomonas aeruginosa*, *Bacillus subtilis*, *klebsiella pneumoniae* and *Staphylococcus aureus.* The methanol extract of *A. calamus* rhizome showed strong inhibition (21-26 mm) at 50 mg/ml concentration. At 10 mg/ml concentration all the isolates showed inhibition zone in the range of 15-18mm. The zone of inhibition increased with increase in concentration of plant extract.

The methanol extract of *S. robusta* resin was found to be effective in inhibiting all the clinical isolates. It showed strong inhibition (19-23 mm) at 50 mg/ml concentration. At 10 mg/ml concentration, the zone of inhibition ranged from 14 to 15 mm.

However when both plant extracts were used together in combination they were even more effective, indicating a synergistic effect. Zones ranged from 28 to 33 mm at 50mg/ml and 20-25mm at 10 mg/ml concentration. In the present study, the growth of all pathogenic

bacteria was remarkably inhibited by methanolic extract of *A. calamus* rhizome and *S. robusta* resin and which was significantly similar to standard Ciprofloxacin antibiotic. The results are represented in the table 4.

Table 4: Antibacterial activity of methanol extract of *A. calamus* and *S. robusta* against clinical isolates

Isolates	Zone of Inhibition (mm)								
	Shorea robusta mg/ml			***Acorus calamus*** mg/ml			***S. robusta + A. calamus*** mg/ml		
	10	30	50	10	30	50	10	30	50
S.aureus	14	17	22	16	20	24	22	26	30
B.subtilis	15	18	22	17	21	23	21	24	28
P.aeruginosa	14	18	23	18	22	26	25	29	33
K.pneumoniae	13	15	19	15	18	21	20	24	28
E.coli	13	16	20	16	19	23	22	26	30

3.6: Synergistic activity of plant extracts with antimicrobial agents

By using the paper strip diffusion method as described by Hemaiswarya et al, the synergistic antibacterial activity of methanolic extracts of *Acorus calamus* rhizome and *Shorea robusta* resin and their combination with Ciprofloxacin was tested against *Pseudomonas aeruginosa* and *Staphylococcus aureus*. The diluted extracts (30 mg/ml) and antibiotic solution (10 ug/ml) mentioned above were applied to sterile paper strips as an impregnation. Zone of inhibitions were calculated in accordance with standards. Synergism was assumed when the zones of combination treatments were greater than the zones containing plant extract and the corresponding antibiotic individually. *Pseudomonas aeruginosa* and *Staphylococcus aureus* were synergistically inhibited by the combination of Ciprofloxacin and *Acorus calamus* extract and also with the combination of Ciprofloxacin and *Shorea robusta*. The zone of inhibition was indicative of a synergistic interaction Table 5, 6 and 7. The findings of this study are consistent with previous reports that have demonstrated the ability of certain plant extracts to enhance the effectiveness of antimicrobial drugs against bacteria. Similar results were obtained by Saquib and Cha et al with a synergistic activity in combinations of herbal materials and antibiotics [18, 19].

Table 5: Synergistic activity of *Shorea robusta* in combination with Ciprofloxacin.

Bacteria	Zone of Inhibition (mm)			Outcomes
	Ciprofloxacin	*S.robusta*	Ciprofloxacin and *S.robusta*	
S. aureus *P.aeruginosa*	12 16	15 22	23 26	Synergism Synergism

Table 6: Synergistic activity of *Acorus calamus* in combination with Ciprofloxacin.

Bacteria	Zone of Inhibition (mm)			Outcomes
	Ciprofloxacin	*A.calamus*	Ciprofloxacin and *A.calamus*	
S. aureus *P.aeruginosa*	12 16	19 22	26 29	Synergism Synergism

Table 7: Synergistic activity of *Acorus calamus* and *Shorea robusta* in combination with Ciprofloxacin.

Bacteria	Zone of Inhibition (mm)			Outcomes
	Ciprofloxacin	*A.calamus* and S.*robusta*	Ciprofloxacin + (*A.calamus* and S.*robusta*)	
S. aureus *P.aeruginosa*	12 16	26 28	30 34	Synergism Synergism

4: Conclusion

The increasing prevalence of bacteria that are resistant to multiple drugs and existing antibiotics poses a significant problem, leading to the failure of infection treatments and higher mortality rates. Urgent action is required to develop new antibacterial substances or compounds that can hinder resistance mechanisms and improve treatment efficacy against these resistant strains. Combining multiple antibacterial agents, known as antibacterial combinations, is a crucial strategy to overcome multidrug-resistant organisms. *S. aureus*, and

P. aeruginosa are widely acknowledged as a significant contributor to infections in humans, both in community settings and hospitals.

The observed synergistic interaction between *A. calamus* and *S .robusta* extracts and the tested antibiotic holds potential for valuable clinical applications in treating infections caused by *S. aureus* and *P. aeruginosa*. *A. calamus* contains asarone as one of its main components. It is a type of phenylpropene, which is a class of aromatic compounds and plays a major role in antimicrobial activity. The utilization of plant extracts in conjunction with antibiotics presents an important opportunity for the development of a novel strategy in resistance-modifying agents. This approach carries a reduced risk of enhancing bacterial resistance compared to the effects of single-component antibiotics. The inclusion of diverse bioactive compounds within extracts makes it considerably more difficult for microbes to adapt and acquire resistance, as opposed to the use of antibiotics containing only a single active constituent. Due to the low risk of bacterial resistance development associated with the use of extracts unlike single-component antibiotics, it offers a sustainable strategy to combat against multidrug resistant bacteria. The extracts contain mixtures of various bioactive compounds, making it challenging for microbes to adapt and develop resistance.

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