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Phytochemical, Antibacterial And Antifungal Activity Of Rhizome From *Anaphyllum Wightii*.Schott Against Clinical Isolates And Plant Pathogens

Manesh Kunjumon[1], ShirlyK Thomas[1], Roshin Elizabeth George[1], VaidyanathalyerThankamani[1]*

***Corresponding author:**

Manesh Kunjumon

[1]Dept. of Biotechnology, University of Kerala,Kariavattom, Thiruvananthapuram 695581, Kerala, India

Abstract

Anaphyllumwightii. Schott is a very rare and endemic threatened plant belonging to the family Areacea. It has been used in the tribal medicine to treat eczema, scabies and as antidote against snakebite.Different solvent extracts of *Anaphyllumwightii.*Schott were prepared and analysed for phytochemical screening by standard protocols. Antimicrobialactivity was measured using well diffusion method and micro dilution broth method against clinical isolates. The n-butanol and methanol extracts of *A. wightii* showedsignificant activity against all of the selected microorganisms. Water extract did not show any activity against any of the organisms except *Klebsiella sp.*The MIC value for leaf n- butanol and methanol were 1.5 mg/ml to 2.5mg/ml and the MBC values were 1.25 to 5 5mg/ml respectively.The antimicrobial activity of rhizome observed in the present study may provide the suggestion that the plant could be a potential source of new and effective antibacterial & antifungal agents. Hence further studies areproposed for the isolation of the antibacterial & antifungal agents from the rhizome extracts of *A. wightii.*

Keywords: *Anaphyllumwightii*, antibacterial, antifungal, Rhizome, Plant pathogen, MIC, MBC

Introduction

The plants are plethora of natural products, such as alkaloids, phenolics, flavonoids and terpenoids or isoprenoidsetcwhich have often been associated with medicinal and pharmacological properties of the plants. These bioactive compounds have assisted as prime molecules for the development of many medical practices and synthetic potential antibiotics. Synthetic antibiotics have sometimes shownantagonistic effects on the host including immune-suppression, hypersensitivity and allergic reactions [1].For this reason there is need to establish and develop antimicrobial drugs from natural origin that are much safer, less expensive and reliable. Plant based antimicrobials represent a vast untapped source for medicines and they provide enormous healing potential. They are effective in the treatment of infectious diseases with fewer side effects that are often associated with synthetic antimicrobials[2].

Anaphyllumwightii. Schott is a very rare and endemic threatened plant belonging to the family Areacea. It is commonly known as 'Keerikizhangu' and used by tribal people in Kerala and Agasthiarmalai Biosphere Reserve, Western Ghats, Tamil Nadu[3,4]. The two different varieties were observed from different localities of Ponmudi, Agusthya peaks, Thenmala, Thattekkad, Periyar, and Wynad in Kerala, India at an altitude 650-1200 meters [4]. The plants are observed growing in groups and singly in cool shady habitat. Two varieties of *A. wightii*are observed - one with broad leaves and other narrow leaves. Tribal communities in Kerala include the kani tribal communities of Kottoor reserve forest, Agasthyavanum, Thiruvanthapuram, [5] and Malapandaram tribes of Achankovil forest of Kollam district[6] use fresh tuber of these plants as antidote against snakebite and as food which makes the characterization of the genus important[7 and 8]. The Kanikkars,a tribal community from Agasthiarmalai Biosphere Reserve, Western Ghats, Tamil Nadu use paste of rhizome from *A wightii* for externalapplies twice a day to treat eczema and scabies.

The alcoholic extract of the rhizome from *A.wightii*has shown a significant anthelmintic activity at high concentration of 200 mg/ml [9]. The rhizome extract of *A.wightii* had significant membrane stabilization(Antiinflammatory activity)and antioxidant activity [10 and 11].Methanol extract of the rhizomes of *A.wightii* efficiently inhibited both alpha amylase and alpha glucosidase enzymes in vitro in a dose dependent manner [12]. *A. wightii* had an anti-diabetic effect in alloxan induced diabetic rats and the effect was equivalent to that of reference drug Glibenclamide. K [13].Antimicrobial activityhas not been reported in*A.wightii.* Hence, in the present study an attempt has been made to evaluate the preliminary phytochemical analysis and antimicrobial activity of crude extracts prepared from the rhizome of *A. wightii*against clinically important bacterial,fungal strains and plant pathogens.

Materials and Methods

Collection of sample and identification

Anaphyllumwightii was collected from near the Jersey Farm, Vithura, Thiruvanthapuram District Kerala and India. Rhizome was washed with distilled water, andexcess water was removed and air

dried for two weeks at room temperature. The sample was finely powdered in a blender, weighed and stored in dry polythene bags at 4◦C prior to antibacterial and antioxidant analysis.

Extract preparation

The dry powdered material was subjected to successive organic solvent extraction by refluxing in the soxhlet apparatus each for 12 hours. The solvents used were non polar to polar consisting of hexane, n-butanol, methanol and water. Each fraction was collected when no further elution of compounds was observed. The collected extracts were subject to distillation and drying in incubator .The dried extracts were stored in sterile containers in the refrigerator till further analysis.

Phytochemical analysis

Standard methods used for identification of a mixture of phytochemicals were followed by qualitative chemical test to obtain information regarding the nature of constituents present in crude extract.The various extracts of different parts of *A.wightii*were analyzed for the presence of carbohydrates, alkaloids, phenols and tannins, flavonoids,saponins, steroids, triterpenoids and coumarins[14].

Microorganisms

The bacterial cultures used were obtained from the Collections of clinical isolates maintained at Department of Biotechnology, University of Kerala and plant pathogens collected from Central Tuber Crops Research Institute (CTCRI)Thiruvanathapuram , Kerala, India. The clinical isolates consisted of *Shigella*sp. *Staphylococcus aureus, Pseudomonas aeruginoisa, Escherichia coli, Klebsiella*sp,*Salmonella typhi* and *Salmonella paratyphi.* Stock cultures were maintained at 4^0C on slopes of nutrient agar. A pure single colony grown on an agar plate was transferred to 5ml of peptone water and incubated for 2 hours at 37^0C. The fungal strains such as *Penicilliummarneffei, Cryptococcus*sp, *Candida* sp,*Curvlaria*sp,*Pencillium*sp,*Epidermoshyton*sp,*Microsporum*sp,*Fusarium*sp,*Aspergillusflavus, Aspergilluniger, Rhizopus*sp., and *Aspergillusfumigatus* from among human pathogens and *Sclerotiarolfrii, Phytopthorapalmivora, Phytopthoracolocaceae, Collectotriumsp* plant pathogens were used to test the inhibition activity of the extracts. Stock cultures were maintained at 4^0C on slopes of Sabouraud Dextrose Agar (S.D.A).

Antibacterial activity

The well diffusion method was used to evaluate the antimicrobial activity [15]. Nutrient agar (Hi-Media -Mumbai) plates were prepared and wells of 8mm diameter were cut using a sterile borer. 100µl of each of the prepared culture of test bacteria was placed on the nutrient agar. The inoculum was swabbed uniformly over the entire agar surface and allowed to dry for 5 minutes. 80µl (500mg/ml) of various extracts dissolved in DMSO were loaded into the wells. Streptomycin (0.125mg/ml) was taken as positive control.Plates were incubated at 37^0C for 24 hrs. For each bacterial strain, pure solvent was used as control. At the end of the incubation period, inhibition zones formed around the well were measured.

Minimum inhibitory concentration (MIC)

Determination of the Minimal Inhibitory Concentration (MIC) of antibacterial activities of selected extracts were done using by micro dilution broth method based on National Committee for Clinical Laboratory Standards (2012) [16].Mueller Hinton broth was used for the preparation ofa series of dilutions of extract at final concentrations ranging from 0.312 µg/ml to 40mg/ml. The inoculum of microorganisms was prepared from 24 hours cultures and suspensions were adjusted to 0.5 McFarland standard suspensions. The tubes were distributed into 1000 µL with different concentrations of extract and 100µL inoculum. The control tubes contained only NB and inoculum suspensions.The inoculated tubes were incubated at 37^0 C for 24 hours. The MIC was calculated where in no visible growth of tested microorganism appeared, and were expressed in µg/ml. The tests were conducted in triplicate. The least concentration of each extract showing a clear zone of inhibition was taken as the MIC.

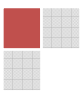
Determination of the Minimal Bactericidal Concentration (MBC)

The minimal bactericidal concentration of the plant extract on the clinical isolates was done according to the method highlighted in National Committee for Clinical Laboratory Standards(2000)[17]. Briefly 5µL was pipetted from the microbe mixture obtained in the determination of MIC stage was streaked out on the nutrient agar at 37^0 C for 24 hours. The least concentration of the extract with no visible growth was taken as the Minimal Bactericidal Concentration.

Antifungal activity

Preparation of the media for fungal culture: Sabouraud Dextrose Agar (SDA) was used as the media for human pathogens and Carrot agar media for plant pathogen testing. The media were sterilized in an autoclave and poured in sterile culture tubes, 1ml in each tube. The slants were kept for sterility check before use. Control tubes were treated with solvents only. Fungal culture were inoculated on SDA and Carrot agar slopes and incubated at room temperature (30-320C). Results were analyzed after a period of one week. The results were compared with Imidazole 100µg/ml of SDA [18].

Results and Discussion



Phytochemical screening

The powdered rhizome sample of *A.wightti* was extracted with various solvents viz. hexane, butanol, methanol and water. These extracts were subjected to qualitative chemical tests and the results are summarized in Table No 1. Phytochemical analysis of various extracts of the rhizome showed the presence of primary metabolites, starch, sugar, proteins carbohydrates, and secondary metabolites like flavonoids, alkaloids, steroids, phenols, saponinsand cumarin. Similar phytochemicals have been reported in various extracts of rhizome from *A.wightii*.[11and 19].Dominic etal[4] has reporteda similar phytochemical except the presence of cumarin in the rhizome of *A.wightii.*

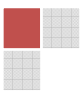
Antibacterial activity

Table No. 2 shows results of the antibacterial activity of the various rhizome extracts from *A wightii* tested against clinical isolates by the well diffusion methods ccompared with standard antibiotic streptomycin (0.125mg/ml) as positive control and DMSO as solvent control [20,21]. Among the extracts n-Butanol and Methanol extracts showed the highest antibacterial activity against all the bacterial strains used with a zone of inhibition rangingfrom12±1-16±1mm.The biggest zone of inhibition of rhizome wasin n-butanol and methanol extracts of *A.wightii* was found against *Shigella, salmonella paratyphi, Pseudomonas aeruginosa* (16±1mm) and least activity was observed in hexane (11±1 mm) and water (11±1.52mm) extracts.The water extracts of rhizome did not exhibit any reasonable activity against any of the clinical isolates except *Klebsiellasp* (11±1.52mm).In this study the organic extracts provided more powerful antimicrobial activity as compared to aqueous extracts. The antibiotic compounds already identified in plants were reportedly aromatic or saturated organic molecules which could easily be solubilized in organic solvents[22].The zone of inhibition of methanol and butanol extracts against *Staphylococcus aureus*and *Pseudomonas aeruginosa*were comparatively more than standard antibiotic streptomycin.Secondary metabolites of plants protect themselves from microorganisms, herbivores and insects, thus antimicrobial effect is expected from plants containing flavonoids, alkaloids, tannins, saponins and tri-terpenoidswhich can be tested against various range of microorganisms[23]. The results obtained from this work revealed that the plants contained phytocompoundswhich are associated with antimicrobial properties in plants [24]. Flavonoids are hydroxylated phenolic substance known to be synthesized by plants in response to microbial infection and it should not be surprising that they have been found in vitro to be effective antimicrobial substances against a wide array of microorganisms[25]. Coumarins are also known to act against Gram positive bacterial and fungal infections which could be attributed to its antimicrobial activity[26].

Table No.3 shows results of the minimum inhibitory concentration (MIC) of rhizome extracts from A. wightii against test bacterial strains Salmonella paratyphi, Pseudomonas aeruginosa, Staphylococcus aureusandKlebsiella sp.The MIC value of butanol extract from A. wightiiranged from 1.25 mg/ml (Pseudomonas aeruginosa, Staphylococcus aureusand Klebsiellasp) to 2.5 mg/ml (Salmonella paratyphi). In methanol extract of rhizome, MIC values were 1.25mg/ml. MBC results of n-butanol and methanol extracts of rhizome ranged from 2.5 to 5mg/ml against selected bacteria. The highest value of MBC (5mg/ml) were againstKlebsiellasp.(n-butanol) and Staphylococcus aureus (methanol).

The results showedsignificant antibacterial effects expressed as MIC and MBC of the hexane, butanol and methanol extracts against the 4 strains of clinical isolates. The hexane extracts showed the lowest MIC and MBC compared other root extracts. This result indicates the high efficacy against selected bacteria andhigh MIC and MBC values represent the low activity[27]. The demonstration of antibacterial activity of *A.wightii*against selected clinical isolates may be indicative of the presence of broad spectrum antibiotic like compounds.The antimicrobial activity of various plants belonging to family*Areacea* has been reported by many researchers [28].

Mechanism of antimicrobial activity of such compounds has not been clearly established, but they may possibly interfere with peptidoglycan bacterial cell wall synthesis in the effected organisms [29] inhibiting protein synthesis, interfering with nucleic acid synthesis, breaking the peptide bonds, inhibiting the metabolic pathway,acting as chelating agents, and preventing the utilization of available nutrients by the microorganisms. Saswati et al (2013) [28] reported that many plants from the family *Araceae*possessed significant antibacterial activity against *E.coli, Basillussubtilis* and*Staphylococcusaeruginosa.* This antimicrobial activity could be incited by compounds presents in their composition such as phenolic compounds and/or flavonoids.The antibacterial activity of these compounds are widely demonstrated [36,37 and38]. Flavonoids which are hydroxylated phenolic substances might be exerting antimicrobial activity due to their capability to complex with extracellular and soluble proteins and to complex with bacterial cell wall [31] . Phenolic constituents of plants can bind to bacterial cell wall and inactivation of bacterial enzymes[32]. The results obtained from these studies demonstrate that plants contain bioactive compounds which are connected with antimicrobial properties in plants against bacterial and fungal strains. Most plants contain several compounds like flavonoids, alkaloids, tannins and phenolic compounds with antimicrobial properties for protection against aggressor agents, especially microorganisms.These bioactive compounds are important raw materials for drug production from the medicinal plants [33]. Chanda and Kaneria 2011[34] reported that in plant cell the bioactive compounds are normally accumulated as secondary metabolites but their concentration could vary in different parts of plants. Bioactivecompounds from plants serve as a novel source for infectious disease management as an alternate to synthetic drugs and several phytochemicals have been derived from the plant materials like bark, stem,leaves,roots,fruits,seeds,fruit rind, flowers andwhole plants[35].



Antifungal activity

The data pertaining to theantifungal potential of different extracts of *A.wightti* rhizome are presented in Table 3& 4. The n-Butanol, methanol and water extracts of *A. wightii* strongly inhibited the growth of fungi including pathogenic strains of human and plant fungi. The n-butanol extract of rhizome showed inhibition against of the growth of pathogenic fungus except *Sclerotiarolfrii* (Table No.4). The methanol extract showed inhibition of the growth of selected 10 human pathogenic fungi and minimal growth in *Aspergillusfumigatus*. It is notable that the methanol extract showed inhibition of *Phytophthorapalmivora*. The water extract showed no inhibition of human and plant pathogenic fungi.The results obtained from these studies determine that various parts of plants contained bioactive compounds which possess antimicrobial properties in plants against external bacterial and fungal strains.

Table No.1 Preliminary screening for phytochemicals of rhizome extracts from*A, wightii*

SL.No	Name of the compound	Name of the extracts			
		n-Hex	n-But	Met	Wat
1	Carbohydrate	+	+	+	+
2	Protein & fat	+	+	+	-
3	Alkaloids	-	+	+	-
4	Phenolic Compounds	-	+	+	-
5	Terpenoid	+	+	+	-
6	Flavonoid	+	+	+	+
7	Coumarins	-	+	+	-
8	Steroids	+	+	+	+

(+) Presence; (-) Absence)

Table No.2 Antibacterial activityof n-hexane, n-butanol, methanol and water extracts of Rhizome from *A.wightii*(500mg/ml).

Sl. No	Name of bacteria	Zone of inhibition in mm (diameter)					
		CONT	DMSO	HEX	BUT	MET	WAT
1	*Proteus sp*	16±0.57	0±0.0	7±0.57	15±1.15	15±1	0±0.0
2	*Shigellasp*	0±0.0	0±0.0	10±1052	16±1	15±0.57	0±0.0
3	*Salmonella paratyphi*	16±1.5	0±0.0	0±0.0	15±1.52	15±1	0±0.0
4	*Pseudomonas aeruginosa.*	14±1.52	0±0.0	11±1	15±0.57	16±1	0±0.0
5	*Klebsiellasp*	16±1.52	0±0.0	0±0.0	15±0.57	15±0.577	11±1.52
6	*Escherichia coli*	19±1	0±0.0	0±0.0	12±1	14±1.15	0±0.0
7	*Salmonella typhi*	18±0.577	0±0.0	0±0.0	15±0.95	14±0.52	0±0.0
8	*Staphylococcus aureus*	16±1.15	0±0.0	0±0.0	0±0.0	15±0.64	0±0.0

All the values are mean ± SD; SD: standard deviation
Positive control: Streptomycin (0.125mg/ml) and Negative control: DMSO

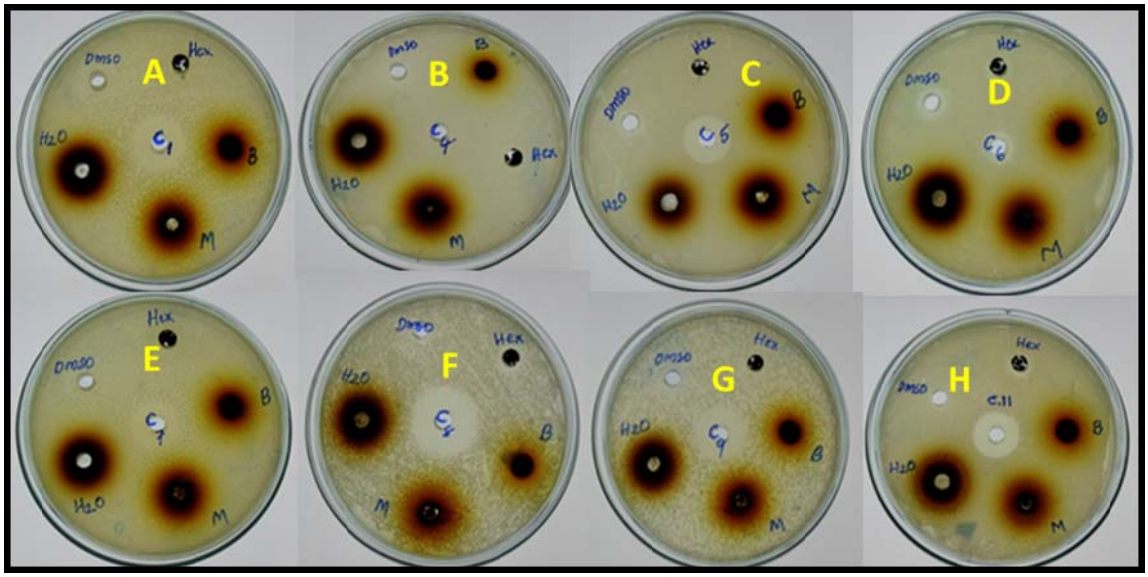


Plate No.1 Inhibition activity of rhizome extracts from *A. wightii*.Schottagainst

(A) *Proteus* sp, (B) *Shigella*sp (C) *Salmonella paratyphi*,(D) *Pseudomonas aeruginosa*(E) *Klebsiella*sp, (F) *Escherichia Coli,* (G) *Salmonella typhi,* (H) *Staphylococcus aureus.*

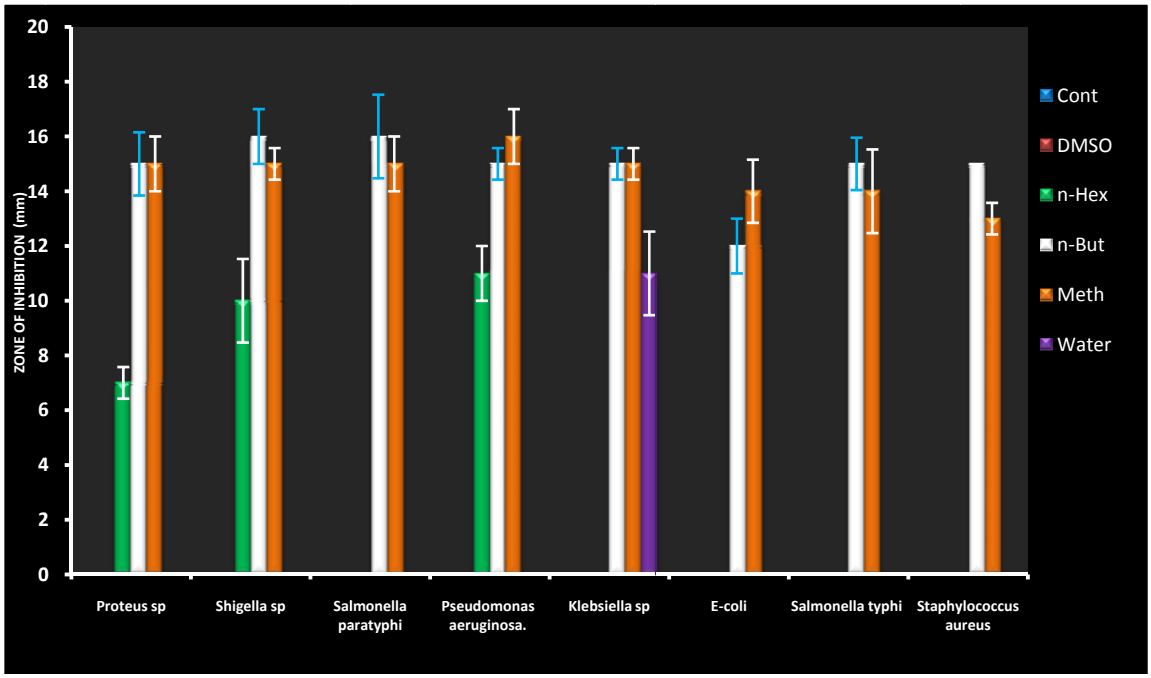


Figure. No 1: Figure showing comparative antimicrobial activity of different solvent extract from *A. Wightii* .Schott.rhizome.

Table No.3 Minimum inhibitory concentration (MIC) of n-butanol and methanol extracts of rhizome from *A. wightii*.Schott.

Sl.No	Organism and solvent extract	Concentration (mg/ml)							
		40	20	10	5	2.5	1.25	0.625	0.312
	n--Butanol Extract								
1	*Salmonella paratyphi*	-	-	-	-	-	+	+	+
2	*Pseudomonas aeruginosa*	-	-	-	-	-	-	+	+
3	*Klebsiella*sp	-	-	-	-	-	-	+	+
4	*Staphylococcus aureus*	-	-	-	-	-	-	+	+
	Methanol extract								
1	*Salmonella paratyphi*	-	-	-	-	-	-	+	+
2	*Pseudomonas aeruginosa*	-	-	-	-	-	-	+	+
3	*Klebsiella*sp	-	-	-	-	-	-	+	+
4	*Staphylococcus aureus*	-	-	-	-	-	-	+	+

(-) Inhibition of organism, (+) Growth of organism

Table No.4 Minimal Bactericidal Concentration (MBC) of n-butanol and methanol extracts of rhizome from *A. wightii*.

SL.No	Test Microorganism and solvent extract	Concentration (mg/ml)							
		40	20	10	5	2.5	1.25	0.625	0.312
	n--Butanol Extract								
1	*Salmonella paratyphi*	-	-	-	-	-	+	+	+
2	*Pseudomonas aeruginosa*	-	-	-	-	-	+	+	+
3	*Klebsiella*sp	-	-	-	-	+	+	+	+
4	*Staphylococcus aureus*	-	-	-	-	-	+	+	+
	Methanol extract								
1	*Salmonella paratyphi*	-	-	-	-	-	+	+	+
2	*Pseudomonas aeruginosa*	-	-	-	-	-	+	+	+
3	*Klebsiella*sp	-	-	-	-	-	+	+	+
4	*Staphylococcus aureus*	-	-	-	-	+	+	+	+

(-)

Inhibition of organism, (+) Growth of organism.

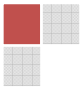


Table No. 5 Activity of solvent crude extracts of rhizome from *A. wightii* against fungal strains.

Sl. No	Name of the fungus	Rhizome solvent extracts (500mg /ml)			
		n-Hexane	N-Butanol	Methanol	Water
1	*Penicilliummarneffei*	-	-	-	+
2	*Cryptococus*	-	-	-	+
3	*Candida*	-	-	-	+
4	*Penicillium*sp	-	-	-	+
5	*Epidermophyton*	-	-	-	+
6	*Microsporum*	-	-	-	+
7	*Fusarium*	-	-	-	+
8	*Aspergillusflavus*	-	-	-	+
9	*Aspergillusniger*	-	-	-	+
10	*Rhizopus*sp	-	-	-	+
11	*Aspergillusfumigatus*	-	-	-	+

(-) Inhibition of organism, (+) Growth of organism

Table No.6 Activity of solvent crude extracts from rhizome of *A. wightii* against plant pathogens.

Sl .No	Name of the fungus	Solvent extracts (500mg/ml)			
		n-Hex	N-But	Meth	Wat
1	*Sclerotiarolfrii*	-	-	-	+
2	*Phytopthorapalmivora*	+	+	+	+
3	*Phytopthoracolocaceae*	-	-	-	+
4	*Collectotrium*sp	-	-	+	+

(-) Inhibition of organism, (+) Growth of organism

Conclusion

From this study we concluded that this plant has a wide range of medicinal-antimicrobial activity. It has also been demonstrated that herbal medicine can be effective to combat pathogenicmicroorganisms. Using different purification methods, these antimicrobial compounds can be further purified and studied.

Acknowledgement

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