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Bactericidal, fungicidal and anthelmintic activities of *Alstonia scholaris* bark extracts

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Abstract

Plant based medicines are effective against many human infectious diseases either by paralysing or killing the pathogen. In the present study, petroleum ether, chloroform, ethanol and aqueous extracts of *Alstonia scholaris* bark were screened for their bactericidal, fungicidal and anthelmintic properties. Antibacterial activity revealed that chloroform extract at the 20 mg/ml showed significant antibacterial effect. Nevertheless, petroleum ether, ethanol and aqueous extracts also showed antibacterial effect against *E. coli* and *S. dyscentreae*, but less effective than chloroform extract. All the extracts were not as potent as the standard drug ciprofloxacin. Fungicidal activity revealed that among all the test extracts, ethanol extract at 20 mg/ml showed significant fungicidal effect against *Rhizopus*. . Interestingly, petroleum ether and ethanol extracts at 20 mg/ml showed more significant fungicidal action when compared to standard drug sulphamethoxazole. Anthelmintic activity of *A. scholaris* extracts was carried out at four different concentrations *viz.,* 2.5, 5.0, 10.0 and 20.0 mg/ml to evaluate their effect in inducing paralysis and death in *Pheretima posthuma*. Anthelmintic activity revealed that petroleum ether extract at 20 mg/ml induced paralysis in worms within 12 min and death within 25.33 min. However, chloroform, ethanol and aqueous extracts at 20 mg/ml also showed significant anthelmintic activity. Among all the extracts of *A. scholaris,* chloroform extract was most potent at concentration 20 mg/ml but less efective than standard drug albendazole. This investigation revealed that all the extracts of *A. scholaris* showed efficient bactericidal, fungicidal and anthelmintic activity against the test pathogens indicating the medicinal property of *A. scholaris*.

Keywords: Bactericidal activity; Fungicidal activity; Anthelmintic activity; *Alstonia scholaris*; Apocynaceae.

Introduction

India is well known for its richness of medicinal plants and their use in Ayurveda system of medicine which has documented several therapeutically potent plants and their application in treating human diseases individually or in combination. About 80% of the world's population depends upon plants as a primary health care remedy [1]. In India, several medicinal plants are known to be used by the traditional medicinal practitioners for the treatment of various ailments in human and animals. The presence of phytochemicals such as alkaloids, tannins, triterpenoids, steroids, glycosides in the medicinal plants extract supports their traditional uses as a therapeutically potent plant. Nowadays the renaissance of herbal based medicine has taken over the modern synthetic medicine because synthetic medicine is usually associated with side effects and remains expensive, which cannot be afforded by the morbid people of the developing countries inluding India. Thus the need for an alternative cure for various ailments is in demand [2]. It has been reported that nearly 7,000 – 8,000 medicinal plant species are being used by the traditional medicinal practitioners to treat different diseases [3, 4].

Alstonia scholaris Linn. (family - Apocynaceae) is commonly known as Saptaparna or devil tree. This tree is commonly found in South India, West Bengal and Himalayas. It mainly grows in deciduous and evergreen forests and also in plain lands. Reports indicate that the bark extracts of this tree is traditionally used as an antiperiodic, febrifuge and astringent [5].The bark is bitter, astringent, acrid, thermogenic, digestive & laxative[6].In Ayurveda, it is reported that the bark of *A. scholaris* soaked in water overnight can reduce the blood glucose level [7]. Many investigators have screened the therapeutic property of this tree *viz., in vivo* antimalarial activity [8]; anticancer [9].antimicrobial [10]. Antidiarrhoeal [11]. immunomodulatory [12]. anti-fertility [13]. developmental toxicity [14]. anticancer activity [15]. anti-tussive, anti-asthmatic and expectorant activity [16]. antidiabetic and antihyperlipidemic activity [17]; anti-inflammatory and analgesic activity [18].

Spoilage of commercially important food and food products due to molds infestation is a common issue which we face in our daily life, such spoilages accounts for major product loss and is directly

associated with economic loss [19]. Further, consumption of such spoilt food and food products leads fungal infections associated with endotoxemia in human, because most of the molds are known to produce mycotoxins. The most common food spoilage molds are *Aspergillus, Rhizopus, Penicillium, Cladosporium, Mucor* and *Fusarium.* During the past three decades, many new antifungal and antibacterial antibiotics have been released in the market, but it is also a known fact that microbes are gradually developing resistance against the available drugs [20]. Hence the individuals suffering the bacterial or fungal infections by multi drug-resistant strains tend to possess suppressed immunity and consequently end up with death. Hence in view of the above many investigators are screening the antimicrobial potency of medicinal plants to offer safe and effective antimicrobial drugs to the morbid people of the developing countries. Plants generally produce many therapeutically potent bioactive drug molecules which act as fungicides, bactericides, pesticides and cure for many human ailments.

Helminthes are basically classified as eukaryotic endoparasites because they infect, reproduce and live inside the body of humans and/or animals. Many helminthes infections occur in poverty-stricken and developing countries with warm, moist environments with poor sanitary conditions. Helminthes infections are normally infect livestock and live in them by cause life threatening disease to animals and continue to be a major productivity constraint [21, 22]. However, to complete their life cycle they can subsequently be transferred to human through a process called zoonosis and can then cause increased disease prevalence among humans. Helminth infections are most prevalent among the morbid population causing distress and mortality. More than one third of the world's population is infected with helminthes. Although the majority of infections due to helminths are generally restricted to tropical regions and cause enormous hazard to health [23-25]. Anthelmintics are the chemical molecules that are mainly designed and synthesized to paralyse or even kill the helminth or expel them from the body. However, as microbes even helminthes are developing resistance against modern synthetic anthelmintic drugs. Hence, there is an increased demand for alternative new potent drugs against resistant pathogenic helminthes. In this connection many investigator are emphasizing on screening of medicinal plants for their anthelmintic property [26-27]. Plants are well known for rich source of potent phytoconstituents that possess anthelmintic property [28-30]. Many investigators have reported the use of medicinal plants as an anthelmintic agent [31-33].

In the view of the above, this investigation was carried out to screen the bactericidal, fungicidal and anthelmintic property of petroleum ether, chloroform, ethanol and aqueous extracts of *Alstonia scholaris*. Bactericidal activity was carried out against *Staphylococcus aureus*, *Klebsiella pneumoniae, Bacillus cereus, Escherichia coli* and *Shigella dyscentreae* using ciprofloxacin as the standard reference. Fungicidal activity was carried out against *Rhizopus oryzae, Cladosporium* herbarum, Aspergillus niger and Pencillium chrysogenum using sulphamethoxazole as the standard reference. Whereas, anthelmintic activity was carried out against Indian adult earthworm, Pheretima *posthuma* using albendazole as the standard reference to assess action of the *A. scholaris* extracts in paralysing and killing the *Pheretima posthuma* in a dose dependent manner.

Materials and Methods

Plant resource

The fresh bark material of *Alstonia scholaris* was collected from Bangalore, Karnataka, India in the month of March, 2012. The bark material was chopped into small pieces and then shade dried. After complete drying the dried bark material was porously powdered mechanically and was subjected to successive sequential soxhlet extraction using petroleum ether, chloroform, ethanol and water as the solvents.

The air dried coarse powder bark material of the *A. scholaris* was subjected to warm extraction using organic solvents like petroleum ether, chloroform, ethanol and finally with water sucessively by sequential soxhlet extraction. Each time before extracting with next solvent, the marc was air dried and then repacked into the soxhlet apparatus. All the three extracts were allowed for complete evaporation of the solvent on water bath and finally vacuum dried. The yield of crude petroleum ether, chloroform, ethanol and aqueous extract for 1 kg of powdered bark material was 5.0 g, 44.0 g, 96.0 g and 41.0 g respectively.

Drugs and chemicals

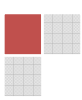
The standard drug Albendazole (Zentel (400mg), GSK Pharmaceuticals Ltd. Baddi, India), Sulphamethoxazole (SEPMAX Double strength (960 mg) GlaxoSmithKline Pharmaceuticals Ltd, Andrhra Pradesh, India), Ciprofloxacin (Ciplox-250, Cipla Ltd., Kumrek, Rangpo, Sikkim, India), petroleum ether and chloroform (SDFine Chem Ltd, Mumbai, India), Ethanol (Hong Yang Chemical Corporation, China).

Extract preparation for anthelmintic activity

The crude petroleum ether, chloroform, ethanol and aqueous extracts were stored in the dessicator until further use. All the extracts were dissolved in 0.5% DMSO. Standard drug albendazole was dissolved in normal saline and was used for evaluation for anthelmintic activity. 0.5% DMSO in normal saline was used as a control.

Earthworms collection

Healthy adult Indian earthworms (*Pheretima posthuma*) were collected from vermi-composting division, The Indo-American Hybrid Seeds, Bangalore. Earthworms from moist soil were washed with normal saline and then were used for the study. The earthworm of 3-5 cm in length and 0.1-0.2 cm in width were used for all the experimental protocol due to its anatomical and



physiological resemblance with the intestinal roundworm parasites of human beings [34, 35].

Anthelmintic activity

Anthelmintic activity of *A. scholaris* was carried out as per the method reported by Ajaiyeoba *et al.,* [36] with minor modifications. Because earthworms are easily available, they have been widely used for the initial *in vitro* evaluation of anthelmintic compounds [37-41]. 20 ml of each formulation at five different concentrations of crude petroleum ether, chloroform, ethanol and aqueous extracts (25, 50, 75, 100 and 125 mg/ 20ml in normal saline) with each concentration in a separate dish. All the extracts and the standard drug solution were freshly prepared just before starting the experiments. Earth worms were released in each plate and observed for time taken for paralysis and death. The mean of time taken for paralysis (in min) was noted; similarly time for death of worms (in min) was recorded after ascertaining that worms lost their motility followed by fading away of their body color [42]. Albendazole (50 mg/ 20ml) was used as reference standard [43]. This study was carried out in triplicates. The result of anthelmintic activity is depicted in Table 1.

Source of microorganisms for bactericidal and fungicidal studies

The test bacterial strains *viz., Staphylococcus aureus*, *Klebsiella pneumoniae, Bacillus cereus, Escherichia coli* and *Shigella dyscentreae* were collected from Bangalore Institute of Oncology (HCG), Bangalore, India. The test fungal strains comprised of *Rhizopus oryzae, Cladosporium herbarum, Aspergillus niger* and *Pencillium chrysogenum.*

Assay of bactericidal activity

Antibacterial activities of the extracts were studied by agar well diffusion method [44]. Test cultures of the bacterial pathogens were prepared by transferring a loop full of bacteria from nutrient agar slants into Mueller Hinton broth and incubated at 37±1 C. Lawn cultures of the test pathogens were prepared by swabbing sterile Mueller Hinton agar plates with 24 hrs old bacterial broth. Wells were punched with a sterile cork borer and 100 µl of the extracts was added to each well. Controls were maintained with respective solvents. Ciprofloxacin (5 mg/ml) were used as standard antibiotics. Following incubation at 37 C for 24 hrs, diameters of the zones of inhibition was measured in millimeter and documented.

Assay of fungicidal activity

Antifungal activities of the extracts were studied by agar well diffusion method. Suspensions of fungal pathogens were prepared by transferring a loop full of fungi from Sabouraud Dextrose agar slants into Sabouraud Dextrose broth. Lawn cultures of the test pathogens were prepared by swabbing sterile Sabouraud Dextrose agar plates with the fungal spore suspensions. Wells were punched with a sterile cork borer and 100 µl of the extracts was added to each well. Controls were maintained with respective solvents. Sulphamethoxazole (5 mg/ml) was used as the standard antifungal reference. Following incubation at 27 C for 72 hrs, diameters of the zones of inhibition was measured in millimeter and documented [45].

Statistical analysis

The data of bioactivity evaluations were expressed as mean ± S.E.M of three replicas in each group. The statistical analysis was carried out using one way ANOVA followed by Tukey's t-test. The difference in values at $P< 0.01$ was considered as statistically significant. The analysis of variance (ANOVA) was performed using ezANOVA (version 0.98) software to determine the mean and standard error of paralysis and death time of the earthworms; and zone of inhibition for bactericidal and fungicidal activity.

Results and Discussion

The bark of *Alstonia scholaris* is medicinally used as an astringent, tonic, anthelmintic, antiperiodic and febrifuge [5]. Recent report indicates that the plant has got bronchovasodilator activity [46]. However, only few reports are available antibacterial, antifungal and anthelmintic activity of this medicinal plant. Therefore, the aim of the present work is to evaluate these properties of *Alstonia scholaris* stem bark extract.

Traditional medicines are effective against many diseases and are based on their age-old use in folklore system of medicine. Natural products of plant origin with potent bioactivity and therapeutic properties play an important role in treatment of various human ailments and pathology.

Initially, *A. scholaris* bark material was sequentially extracted using petroleum ether, chloroform, ethanol and water as the solvent system. In continuation with our interest, this investigation on evaluating the anthelmintic property of *Alstonia scholaris* was carried out.

The crude extracts of *A. scholaris* were tested at different concentrations *viz.,* 2.5 mg/ml (50 mg/ 20ml), 5 mg/ml (100 mg/ 20ml), 10 mg/ml (200 mg/ 20ml) and 20 mg/ml (400 mg/ 20ml). Among the various concentrations of petroleum ether extract, concentration at 20 mg/ml showed significant anthelmintic activity by inducing paralysis in *Pheretima posthuma* within 12 min and death within 25.33 min. Similarly, chloroform, ethanol and aqueous extracts also showed pronounced anthelmintic activity at 20 mg/ml concentration with paralysis time of 5.67, 17.33 and 72.33 min respectively, and death time of 24.67, 24.67 and 163.33 min respectively. However, among all the extracts of *A. scholaris* screened for their anthelmintic property, chloroform extract was most potent at concentration 20 mg/ml (Table 1). This investigation revealed that all the extracts of *A. scholaris* showed efficient anthelmithic activity against *Pheretima posthuma* indicating that *A. scholaris* is a potent anthelmintic plant.

Table 1: *In vitro* anthelmintic activity of chloroform and methanol extracts of *A. scholaris* against *Pheretima posthuma.*

Test samples	Concentration (mg/20ml)	Paralysis Time (min)	Death Time (min)
Control (Normal Saline)	(0.9% NaCl)	334.67 ± 17.02	386.33 ± 13.96
Petroleum ether extract of *A. scholaris*	50	26.67 ± 1.45**	31.33 ± 2.91**
	100	24.67 ± 2.03**	29.67 ± 1.76**
	200	16.67 ± 1.45**	27.33 ± 1.76**
	400	12.00 ± 1.53**	25.33 ± 0.88**
Chloroform extract of *A. scholaris*	50	31.33 ± 3.18**	49.67 ± 4.91**
	100	28.67 ± 2.73**	42.33 ± 2.6**
	200	14.67 ± 1.45**	25.67 ± 2.91**
	400	5.67 ± 1.76**	24.67 ± 2.33**
Alcoholic extract of *A. scholaris*	50	36.67 ± 3.76**	124.33 ± 6.89**
	100	39.33 ± 3.18**	63.33 ± 4.48**
	200	22.67 ± 2.33**	26.33 ± 3.18**
	400	17.33 ± 0.88**	24.67 ± 2.33**
Aqueous extract of *A. scholaris*	50	ND	ND
	100	122 ± 12.49**	210.67 ± 7.13**
	200	170.33 ± 6.69**	228.67 ± 12.99**
	400	72.33 ± 4.33**	163.33 ± 8.84**
Albendazole	1.0	85.33 ± 14.84**	176 ± 28.01**

Values are the mean ± S.E.M. of three earthworms. Symbols represent statistical significance.
* P < 0.05, ** P < 0.01, as compared to compared to control group, ND: Not Defined.

Antibacterial activity

Chloroform extract of *A. scholaris* at the concentration 20 mg/ml showed significant antibacterial effect by inhibiting *E. coli* and *S. dyscentreae* cell growth, as revealed by 26.0 ± 0.58 mm and 20.67 ± 0.67 mm zone of inhibition respectively, when compared to other extracts. Nevertheless, petroleum ether, ethanol and aqueous extract also showed antibacterial effect against *E. coli* and *S. dyscentreae*, but were less effective than chloroform extract. Where as, in case of *S. aureus* ethanol extract at 20 mg/kg revealed to be most effective in inhibiting the bacterial growth with 21.67 ± 0.33 mm zone of inhibition. However, petroleum ether and chloroform showed antibacterial effect against *S. aureus,* but aqueous extract was completely ineffective against *S. aureus*. All the extracts of *A. scholaris* efficiently inhibited the growth of *Bacillus cereus.* Among them ethanol extract was most potent which showed 18.67 ± 0.33 mm zone of inhibition. Interestingly, all the extracts of *A. scholaris* were ineffective against *Klebsiella pneumonia.* However, all the extracts were less potent than the standard drug ciprofloxacin in their bactericidal action against all the pathogens under study (Table 2).

Fungicidal activity

Among all the four extracts of *A. scholaris,* ethanol extract at 20 mg/ml showed significant fungicidal effect against *Rhizopus oryzae* with 21.67 ± 0.67 mm zone of inhibition. However, petroleum ether, chloroform and aqueous extracts at 20 mg/ml also exhibited fungicidal property against *Rhizopus oryzae* with 20.67 ± 0.67, 14.33 ± 0.33 and 14.00 ± 0.58 mm zone of inhibition respectively. Interestingly, petroleum ether and ethanol extracts at 20 mg/ml were more potent than the standard drug sulphamethoxazole (19.00 ± 1.15).

All the extracts of *A. scholaris* at the concentrations and the standard drug sulphamethoxazole did not show any fungicidal effect against *Cladosporium herbarum.* Chloroform extract showed better inhibition of *Aspergillus niger* and *Penicillium chrysogenum* 21.67 ± 0.88 and 20.00 ± 0.58 respectively, when compared to petroleum ether and ethanol extracts. But aqueous extract was impotent against *Aspergillus niger.* Petroleum ether and aqueous extracts showed fungicidal effect against *Penicillium chrysogenum* only at 20 mg/ml concentration, below this concentration both the extract revealed to be impotent. In case of *Aspergillus niger* and *Penicillium chrysogenum* standard drug showed better inhibition when compared to all the test extracts of *A. scholaris* (Table 3).

Table 2: *In vitro* bactericidal activity of petroleum ether, chloroform, ethanol and aqueous extracts of *Alstonia scholaris* against *Staphylococcus aureus*, *Klebsiella pneumoniae, Bacillus cereus, Escherichia coli* and *Shigella dyscentreae.*

	Concentration mg/ml	*Klebsiella pneumoniae*	*Bacillus cereus*	*Shigella dyscentreae*	*Staphylococcus aureus*	*Escherichia coli*
Petroleum-ether extract of *Alstonia scholaris*						
	5	ND	10.33 ± 0.33	11.33 ± 0.33	10.67 ± 0.33	ND
	10	ND	13.67 ± 0.33	13.33 ± 0.33	11.67 ± 0.33	ND
	15	ND	16.0 ± 0.58	13.33 ± 0.33	15.67 ± 0.67	11 ± 0.58
	20	ND	17.33 ± 0.33	14.0 ± 0.58	17.67 ± 0.33	12 ± 0.58
Chloroform extract of *Alstonia scholaris*						
	5	ND	16.67 ± 0.67	16.33 ± 0.33	14.33 ± 0.33	15.0 ± 0.58
	10	ND	17.00 ± 0.0	17.67 ± 0.33	18.33 ± 0.33	18.0 ± 0.58
	15	ND	18.33 ± 0.33	19.67 ± 0.67	20.33 ± 0.33	19.33 ±0.33
	20	ND	18.33 ± 0.33	20.67 ± 0.67	20.67 ± 0.33	26.0 ± 0.58
Ethanol extract of *Alstonia scholaris*						
	5	ND	15.67 ± 0.33	14.67 ± 0.33	14.0 ± 0.0	17.67 ± 0.33
	10	ND	19.0 ± 0.58	15.0 ± 0.58	16.33 ± 0.33	18.0 ± 0.0
	15	ND	18.67 ± 0.33	20.33 ± 0.33	19.33 ± 0.33	20.67 ± 0.33
	20	ND	18.67 ± 0.33	19.33 ± 0.33	21.67 ± 0.33	22.0 ± 0.0
Aqueous extract of *Alstonia scholaris*						
	5	ND	ND	ND	ND	ND
	10	ND	12.33 ± 0.33	11.67 ± 0.33	ND	14.33 ± 0.33
	15	ND	12.0 ± 0.0	13.67 ± 0.33	ND	11.33 ± 0.33
	20	ND	14.67 ± 0.33	13.67 ± 0.33	ND	16.67 ± 0.33
Standard Drug Ciprofloxacin						
	5	31.0 ± 0.0	51.0 ± 0.0	46.33 ± 0.33	45.67 ± 0.33	40.0 ± 1.0

Table 3: *In vitro* fungicidal activity of petroleum ether, chloroform, ethanol and aqueous extracts of *Alstonia scholaris* against *Rhizopus oryzae, Cladosporium herbarum, Aspergillus niger* and *Pencillium chrysogenum.*

	Concentration mg/ml	*R. oryzae*	*C. herbarum*	*A. niger*	*P. chrysogenum*
Petroleum-ether extract of *Alstonia scholaris*					
	5	16.67 ± 0.33	ND	10.33 ± 0.33	ND
	10	17.33 ± 0.33	ND	14.33 ± 0.33	ND
	15	20.00 ± 0.58	ND	15.67 ± 0.33	ND
	20	20.67 ± 0.67	ND	17.33 ± 0.33	12.67 ± 0.33
Chloroform extract of *Alstonia scholaris*					
	5	13.67 ± 0.33	ND	11.67 ± 0.33	13.33±0.33
	10	14.67 ± 0.33	ND	13.00 ± 0.58	14.33 ±0.67
	15	14.67 ± 0.33	ND	16.00 ± 0.58	14.00 ± 0.58
	20	14.33 ± 0.33	ND	21.67 ± 0.88	20.00 ± 0.58
Ethanol extract of *Alstonia scholaris*					
	5	13.67 ± 0.67	ND	11.67 ± 0.67	12.00 ± 0.58
	10	15.33 ± 1.45	ND	12.33 ± 0.88	16.33 ± 0.88
	15	19.00 ± 0.58	ND	13.67 ± 1.20	14.67 ± 0.88
	20	21.67 ± 0.67	ND	15.33 ± 0.88	16.67 ± 0.33
Aqueous extract of *Alstonia scholaris*					
	5	11.33 ± 0.88	ND	ND	ND
	10	12.33 ± 0.88	ND	ND	ND
	15	12.33 ± 0.33	ND	ND	ND
	20	14.00 ± 0.58	ND	ND	12.00 ± 0.58
Standard Drug sulphamethoxazole					
	5	19.00 ± 1.15	ND	24.33 ± 0.67	31.00 ± 1.00

Values are the mean ± S.E.M. of triplicates of bactericidal activity. Symbols represent statistical significance. * P < 0.05, ** P < 0.01, as compared to compared to control group, ND: Not Defined.

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