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ISSN: 0975-7538
Research Article

Antifungal activity of methanolic fractions of *Alstonia scholaris* against clinical isolates of *Candida* and other *Yeast* species

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ABSTRACT

Secondary metabolites are produced by many plants which are useful to human beings. These metabolites have been known for ages and served as potent antimicrobial agents. *Alstonia scholaris*(AS) is one of the most important plants which in our experiments has demonstrated a promising anti microbial activity. The present study aims at evaluating the anti fungal potential of this plant. Methanol extracts of leaf and bark were used for investigating anti fungal activity against different strains of *Candida* species isolated from clinical specimens. The result of the present study showed that most of the *Candida* sps were sensitive to the butanol and ethyl acetate (EA) fractions while hexane and chloroform showed no activity against the tested strains. However the largest zone of inhibition measured 16 mm for butanol fractions of *Alstonia scholaris* bark against *Candida krusei*. The SDA agar incorporated with the methanol fraction of leaf and bark extract showed complementary result in case of butanol fraction with 100 percent inhibition and almost little inhibition in ethyl acetate fraction. Unlike other fractions, the aqueous fraction showed zero inhibition in both well diffusion method as well as agar dilution method.

Keywords: *Alstonia scholaris; Candida krusei;* antifungal activity

INTRODUCTION

There are about 100,000 species of fungus present in the environment and more than 100 of them are pathogenic in humans (Keeler 1991). Plants and their extracts have been used all over the world since antiquity in folk medicine (Thangadurai 2004), and their use has been supported by the isolation of anti fungals from plants (Fabry et al 1996). Human fungal infections have increased at an alarming rate in the last 20 years, mainly among immunocompromised individuals (Perea et al 2002). New data indicate that the relative proportion of organisms causing nosocomial bloodstream infections has changed over the last decade, with *Candida* species now being firmly established as one of the most frequent agents. Candidaemia is not only associated with a high mortality but also extends the length of hospital stay and increases the costs of medical care. *Candida* species are ubiquitously distributed, they reside on plants and alimentary tracts of mammals and as commensals on human muco-cutaneous membranes (Goncalves et al 2006). Among human gastrointestinal tract isolates 50-70% of total yeast isolates have been identified as *Candida albicans*. Further, frequent isolates are *C. tropicalis*, *C. parapsilosis*, and *C. glabrata*, while *C. kefyr* and *C. guillermondii* are found occasionally (Barberino et al 2006, Colombo et al 2006, and Colvard et al 2006). *Candida albicans* and other yeast species pathogenic to man have become resistant to antifungal agents, in particular triazole compounds, by expressing efflux pumps that reduce drug accumulation, changing the structure or concentration of antifungal target proteins and changing membrane sterol composition. The use of Amphotericin B, known as the gold standard is limited because of its infusion-related reactions and nephro toxicity. This situation highlights the need for the advent of safe, novel and effective antifungal compounds. Antifungal activity of natural extracts and pure compounds can be detected by inhibition of various fungi, yeast or filamentous by samples that are placed in contact with them. The most used methods are Disc diffusion, (Bartner et al 1994, Gulluce et al 2006) agar dilution (Cordell et al 2005, Passos et al 2002, Patwardhan et al 2005, and Souza et al 2003) and tube dilution tests.

Alstonia scholaris, one of the most studied plant of the family Apocynaceae has the medicinal potential in almost all parts from leaves to roots. The leaves are used against beri beri, liver congestion, dropsy and ulcers (Daniel 2006). The bark is a powerful anthelminthic, astringent, antiperiodic and used to treat alimentary disorders (Nadkarni and Nadkarni, 1976). The extracts of flowers, fruits and roots have exhibited powerful

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Received on: 29-02-2012
Revised on: 28-03-2012
Accepted on: 04-03-2012

antimicrobial activity (Antony et al 2012, Thankamani et al 2011, Misra et al 2011). Therefore the present work is an extension to the antimicrobial work and aimed at investigating the anti fungal activity of the methanolic fractions of leaves and bark.

MATERIALS AND METHODS

Collection and Processing of Plant Materials

Alstonia scholaris leaf (ASL)

Fresh mature leaves of *Alstonia scholaris R.Br.* tree were collected during January-February season. They were cleaned, washed in water, dried at room temperature weighed and powdered. Weight of the dry powder was taken.

Alstonia scholaris –Stem Bark

Mature stem bark from woody trunk portion of the same tree was collected. Collected bark was cleaned, washed air dried and weight of the dry powder was taken.

Solvent extraction of samples using Methanol

The finely powdered leaves or stem bark (100 gm) were taken in a 500 ml Soxhlet apparatus and extracted with methanol for 24 hours. After extraction, the methanol extracts were dried free of solvent in a rotary evaporator at low temperature (40 ^{0}C). The dried extracts were tested for anti-microbial and anti-viral activities.

Fractionation of methanol extract and isolation of active fraction

The methanol extracts of the plant powder (leaf and stem bark) showed significant anti-microbial and anti-viral activities and hence fractionation of the extracts was carried out. The methanol extracts of the leaf and stem bark were suspended separately in water (1g extract in 50 ml water) and sequentially extracted with n-hexane (150 ml), chloroform (150 ml), ethyl acetate (150 ml) and butanol (150 ml). All the fractions except water fraction (obtained at the end of fractionation) were dried free of solvents in a rotary evaporator at low temperature (40^{0}C) while aqueous fractions were freeze dried in a lyophilizer. The yield of each fraction was determined (Harborne 1984).

Screening of Extracts for Anti Fungal Activity

Media used for Anti fungal studies

Sabuoraud's Dextrose agar (SDA)

Glucose	40 gm
Peptone	10 gm
Agar	20 gm
Distilled water	1000 ml
pH	5.6

The ingredients were dissolved by subjecting it to a few minutes of steaming. Then filtered through cotton gauze and pH adjusted to 5.4. The media was then dispensed in stock bottles and autoclaved at 115 lbs pressure (121:C) for 15 minutes. Autoclaved medium was then poured into sterile flat bottomed petri plates in a laminar flow hood and allowed to solidify and stored in a cold room (4:C).

Screening for anti fungal activity by well method

SDA plates are allowed to dry for 30 minutes at 37^{0}C before using. 6 wells of 3mm each were cut at equidistant under sterile conditions on the surface of the media, pure cultures of test strains in the log phase and turbidity adjusted were swabbed on the surface of SDA plates under aseptic conditions. 20µl of each extract fraction and 20µl of respective solvents were filled in each well using sterile micro tips and plates were incubated at 37^{0}C for 18 hours. Zones of inhibition found were noted in mm.

Screening of anti fungal activity by incorporation in the medium

Screening for anti fungal activity by incorporating the various methanol fractions in SDA medium poured on to sterile petri plates.

0.5 ml of *Alstonia scholaris* leaf – (Butanol, Ethyl acetate and water) fractions of methanol extracts were incorporated into 10 ml of Sabouraud Dextrose Agar at 60^{0}C, poured into sterile plates and allowed to solidify aseptically to test anti fungal activity. Various clinical strains of *Candida* sp and *Cryptococci* were streaked on these plates along with control plates without extracts and with DMSO being the solvent vehicle. Plates were incubated at 37^{0}C for 24 hours and growth noted. Plates were held for 3 days as *Cryptococci* are a bit slow growing.

RESULTS AND DISCUSSION

Yield : Alstonia scholaris – Leaves: The total weights and that of fractions are given below. Weight of air dried leaf powder was 20 grams and yield of methanol extract from 20grams of leaf powder was 3.480 grams. Yield of sequential extraction with solvents is given in Table 1.

Total weight of fractions = 3.442 grams, loss of 38 milligrams from the total weight of Methanol extract (3.480 grams) disappeared due to the fractionation, drying and transfer procedures.

Stem bark

Yield of Methanol extract was 3.082 grams from 20 grams of dried powder. 3.082 grams of this methanol extract was suspended in 150 ml distilled water (450 ml each- i.e. 1:3 ratio) and subjected to sequential extraction with various solvents such as Hexane, Chloroform, Ethyl acetate and Butanol. The results are given below (Table 2).

Table 1: Yield of sequential fractions of Methanol extracts of *Alstonia scholaris* leaf

Extract used in Grams	Solvent used for fractionation	Dry weight of fraction in grams
	Hexane	1.010
	Chloroform	1.310
Methanol extract (3.48g)	Ethylacetate	0.209
	Butanol	0.512
	Water	0.401

Table 2: Yield of sequential fractions of Methanol extracts of *Alstonia scholaris* Stem bark

Extract used in Grams	Solvent used for fractionation	Dry weight of fraction in grams
	Hexane	0.403
	Chloroform	0.612
Methanol extract (3.082 gm)	Ethylacetate	0.216
	Butanol	0.902
	Water	0.909

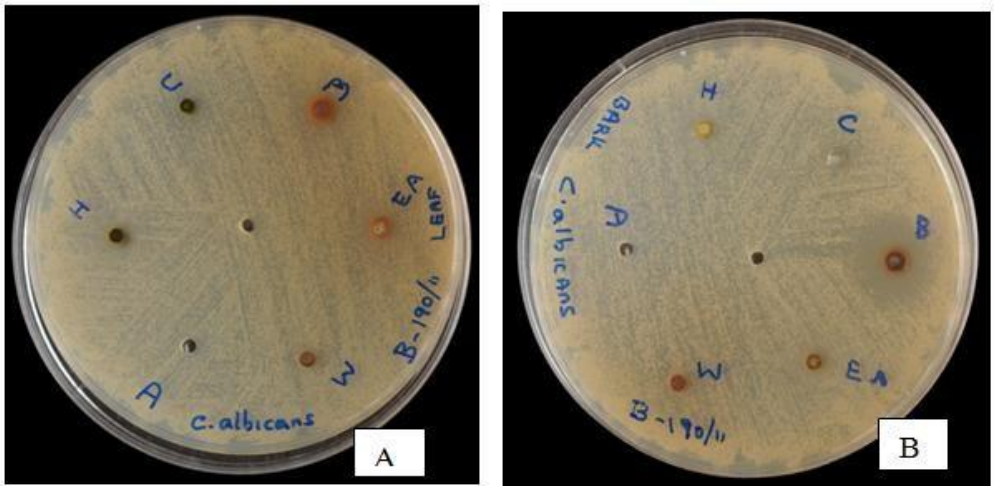

Figure 1: *C. albicans* tested against various fractions of Methanol extracts of *Alstonia scholaris* leaf (A) and bark (B) - well method (20µl/well). Abbreviations: Hexane (H) fraction. Chloroform (C) fraction, Butanol fraction(B), Ethylacetate (EA) fraction. Aqueous fraction (W), DMSO control

Anti Fungal Testing

The result of anti fungal activity of methanolic extract of leaves and bark by well diffusion method are presented in Table 3. In the well method various fractions of *Alstonia scholaris* leaf and bark Methanol extracts dissolved in DMSO were incorporated and Butanol and Ethyl acetate fractions have shown anti fungal activity in most of the *Candida* strains tested. The zone of inhibition is measured in mm and the largest zone of inhibition was seen with *Candida krusei* against *A. scholaris* bark –butanol fraction (Table 3 & Figure 1). The fractions of Methanol extracts of *Alstonia scholaris* leaf were dissolved in DMSO and from the gradation of growth on control plates (SDA) and plates with 0.5 ml fractions there was no inhibition of yeasts by DMSO nonspecifically. (Table 4) There was almost 100% inhibition on the Butanol fraction plate and a very weak growth on the Ethyl acetate fraction plate while there was hardly any inhibition on the aqueous fraction plate. *Cryptococcus albidus* was also inhibited by the Butanol and Ethyl acetate fractions. From this it was proved that antifungal properties are mainly in the Butanol fractions and overlapped into the Ethyl acetate fraction too (Figure 2-3). The results obtained for anti fungal activity are comparable to the standard anti fungal agent Amphotericin B.

This definitely proves that there are anti fungal moieties concentrated in the Butanol fraction that inhibited the fungal growth. The antifungal activity demonstrated in vitro supports the use of *Alstonia scholaris* extracts used in folklore medicine. The Methanolic extracts of root and stem bark of another plant belonging to Apocynaceae family – *Tabernaemontana stapfiana* has been reported by Ruttoch et al in 2009 to have anti fungal activity against *Candida albicans*, *Cryptococci neoformans* and *Trichophyton mentagrophytes*. Four Siddha drugs vix Nandi mezhugh, Parangi pattai choornam, Erasa kenthi mezhugu and Vaan mezhugu were found to have anti fungal activity against 14 strains of *Candida albicans* (Suresh et al 1994).

The basis of development of new chemotherapeutic agent lies in the potential chemical constituent present in the form of phytochemicals (Tona et al 1998). Many reports on the findings of the antibacterial, anti fungal

Table 3: Activity of methanolic fractions of leaves and barks of *Alstonia scholaris* against fungal strains by well diffusion method

Test Organisms	Zone of inhibition(mm) of 20µl/well- Solvent extracts(mg)											
	Hexane		Chloroform		Butanol		Ethyl Acetate		Water		Solvent	
	L (5mg)	B (2mg)	L (2mg)	B (2mg)	L (1mg)	B (2mg)	L (1mg)	B (1mg)	L (4mg)	B (2mg)	DMSO (Control)	
C. albicans B190/11	4	-	-	-	6	12	6	4	4	-		-
C. tropicalis E323/11	-	-	-	-	-	4	-	4	-	-		-
C. krusei U 42/11	-	-	-	-	-	16	-	-	-	4		-
C. parapsilosis L25/11	-	-	-	-	-	4	-	4	-	4		-
C. albicans U 611/10	-	-	-	-	-	4	-	4	-	-		-
C. tropicalis U 324/10	-	-	-	-	4	4	4	4	4	-		-
C. albicans U 282/10	-	-	-	-	4	4	4	4	-	4		-
C. albicans U 1138/11	-	-	-	-	-	-	4	4	8	-		-
C. incospica U 379/10-	-	-	-	-	4	4	4	4	8	4		-
C. sphericaa U 441/11	-	-	-	-	-	-	-	-	-	-		-
Kodamaea ohmeri B 1238/10-	-	-	-	-	-	-	-	-	-	-		-
C. pelliculosa B 383/11	-	-	-	-	-	-	-	-	-	-		-
Cryptococci albidus M 337-	-	-	-	-	-	-	-	-	-	-		-
Aspergillus	-	-	-	-	-	-	-	-	-	-		-

- No Activity

activity, anti viral, ant helminthic, anti inflammatory and analgesic property of plants are available in the literature (Al-Ghamdi 2001, Janssen et al 1963, Misra et al 2011, Thankamani et al 2011). Most of the plants have been studied for identifying the active principles responsible for such activities and developing drugs for the therapeutic use to human beings. However not much data is available for the anti fungal property of the plant for their utilization as a commercial drug formulation (Mahesh and Satish, 2008). In the present study, the anti fungal (clinical isolates of yeast and yeast like) activity of *Alstonia scholaris* is reported for the first time. Present investigation showed that different fungal strains showed varied range of susceptibility towards the different plant extracts which in turn vary with solvent and the plant part (Kalyoncu et al., 2006) .

The overall results showed that the most susceptible strain was *C. krusei* which was inhibited most. The inhibition of *Candida sps*. by the plant extracts is well documented in the literature (Parekh and Chanda, 2008). Hexane showed no inhibition against the tested organism in a well diffusion method as comparable to the similar work done by Mathur *et al.* In contrary to this, the hexane extracts of *Berberis aristata* and *Asparagus racemosus* showed antifungal activity against *Aspergillus niger* unlike the present study in which *Aspergillus* was found to be resistant against all the fractions. The reports of plant extracts inhibiting *Aspergillus* is also reported by Bobbarala *et al* in which out of 49 plant tested, methanolic extract of *Grewia arborea* showed maximum activity but *Emblica officinales*, *Heldigordia populipolia*, *Hyptis sueolences*, *Moringa heterophylla*, *Strychnos nuxvomica* and *Vitex negundo* did not exhibit antifungal activity. Though plants extracts with prominent inhibitory effect on *Aspergillus* have been reported, no significant inhibition has been reported in case of *Candida* sps which are severe pathogens. This comprehensive study showing the

Table 4: Anti fungal activity of various fractions of Methanol extract of *Alstonia scholaris* leaf incorporated in Sabourauds Dextrose agar (SDA)

Test Organisms	Test Material Concentration &Growth Density				
	10 ml SDA +0.5 ml DMSO	**10 ml SDA+ 0.5 ml Butanol fraction (150 mg)**	**10 ml SDA +0.5 ml EA fraction (175 mg)**	**10 ml SDA + 0.5 ml water fraction (500 mg)**	**Control DMSO 10 ml SDA**
C. tropicalis U 1143/10	4+	-	1+	4+	4+
C. inconspica U 379/11	4+	-	1+	4+	4+
C. parapsilosis L 25/10	4+	-	1+	4+	4+
C. albicans U 611/10	4+	+/-	1+	4+	4+
C. haemulanii B 594/10	4+	-	1+	4+	4+
Cryptococci albidus M 337/10	2+	-	-	1+	2+

- indicates no activity; 4+ growth means good growth on SDA plates which have no extracts incorporated so it is a comparison of growth inhibition when compared to the good growth seen on the control plate Good growth is seen on SDA plates which have no extracts and on plates which have DMSO incorporated just to show that DMSO used as a solvent also did not inhibit growth non specifically.

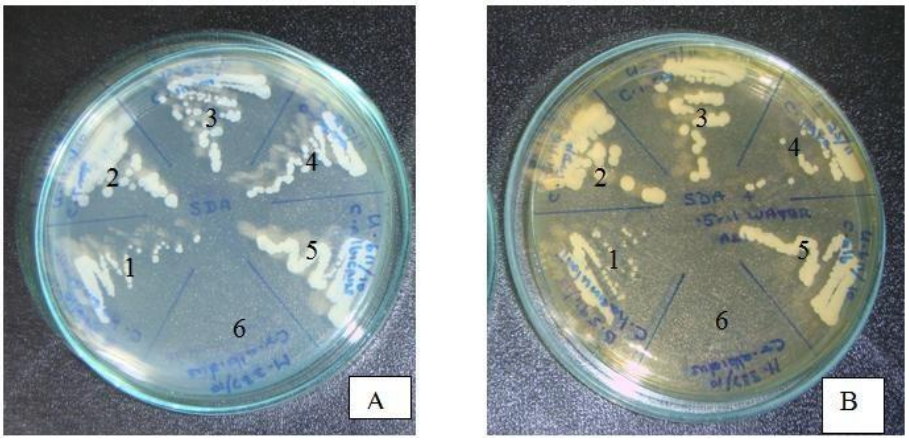

Figure 2: SDA Plate as control(A) and SDA plate with Water(500 mg) fraction(B) of AS Leaf incorporated. List of strains tested: (order as shown in Table 4) 1.*Candida tropicalis* 2.*Candida inconspica* 3. *Candida parapsilosis* 4. *Candida albicans* 5. *Candida haemulanii* 6.*Cryptococci albidus*

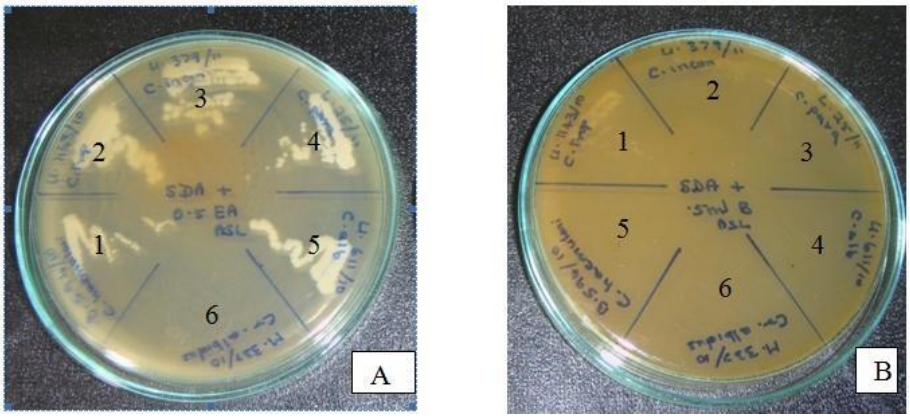

Figure 3: SDA plate with EA(175 mg) fraction(A) and Butanol(150 mg)fraction(B) of AS Leaf incorporated. List of strains tested: (order as shown in Table 4) 1.*Candida tropicalis* 2. *Candida inconspica* 3. *Candida parapsilosis* 4.*Candida albicans* 5.*Candida haemulanii* 6.*Cryptococci albidus*

inhibition of *Candida sps* can be validated further based on the present scientific investigation which supports the use of this plant as a potential anti fungal agent against infections by these pathogens.

CONCLUSION

The methanol fractions of plant extracts used showed significant anti fungal activity against *Candida* sps but no activity against *Aspergillus* in well diffusion method. The present evaluation of the anti fungal property therefore offers a scientific basis for the use of this plant as suitable anti fungal agent against a range of pathogens but further investigation against the broader range of pathogens is certainly required to identify the active ingredients. But to understand the mechanisms of action of these agents, more detailed chemical structure elucidation of the bioactive components followed by therapeutic investigations and toxicological assessment are required.

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