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Anti-*Staphylococcus aureus* and Anti-yeast activity of *Streptomyces* species isolated from rhizosphere soil of Sahyadri Science College, Shivamogga, Karnataka

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Abstract
The present study was undertaken to investigate Anti-*Staphylococcus aureus* and Anti-yeast activity of ethyl acetate extracts from six *Streptomyces* species (SSC-MB-01 to SSC-MB-06) isolated previously from rhizosphere soil of Sahyadri Science College campus, Shivamogga. Antibacterial activity was performed by agar well diffusion assay against four isolates of *Staphylococcus aureus* (resistant to methicillin) recovered previously from burn patients. Anti-yeast activity was checked against two yeasts *viz., Candida albicans* and *Cryptococcus neoformans* by agar well diffusion assay. Extracts of all *Streptomyces* species have shown inhibition of yeasts and clinical isolates of *S. aureus*. Among six isolates, SSC-MB-04 caused highest inhibition of *S. aureus* isolates. Among yeasts, higher susceptibility was recorded in case of *C. neoformans*. The *Streptomyces* species from the campus soil have shown to be promising against *S. aureus* and human pathogenic yeasts. Further studies on separation of bioactive principles from the extracts and determination of their bioactivities are under progress.

Keywords: *Streptomyces*, Agar well diffusion, *Staphylococcus aureus*, *Cryptococcus neoformans*, *Candida albicans*

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1. INTRODUCTION

Soil is an important ecological niche inhabited by a number of organisms and many of which are useful as they produce biologically active natural products, including clinically important antibiotics. Streptomycetes (Order Actinomycetales, Family Streptomycetaceae) are Gram-positive, filamentous bacteria (similar to fungi). The morphological differentiation of *Streptomyces* involves the formation of hyphae that can differentiate into a chain of uninucleated spores by the formation of septa at regular intervals. This process requires a specialized and coordinated metabolism. *Streptomyces* are one of the most important and abundant microbial genera in soil and constitute major portion of the total population of soil actinomycetes. Soil streptomycetes are saprophytic and are known to play significant roles in biotransformation and biodegradation. In addition, these also produce a wide variety of biologically active compounds. The biosynthesis of secondary metabolites has been investigated thoroughly by biochemical and genetic analysis and many different pathways have been characterized. The *Streptomyces* species have the ability to produce bioactive metabolites such as antimicrobials, antivirals, anthelmintics, insecticides, herbicides, antitumor agents, enzyme inhibitory agents, antihypertensives, antioxidants, immunosuppressive, plant growth promotory and other agents. The majority of known antibiotics (>70%) are produced by streptomycetes. Many streptomycetes produce more than one antibiotic and also possess resistance to multiple antibiotics[1-6].

Sahyadri Science College (Autonomous) is a constituent college of Kuvempu University and is located in Bangalore-Honnavar Road, Vidyanagara, Shivamogga, Karnataka, India. The college campus has rich vegetation represented by a variety of herbs, shrubs and trees. In a previous study, we have shown inhibitory activity of six *Streptomyces* species recovered from the rhizosphere soil sample of Sahyadri Science College (Autonomous) campus against a panel of Gram positive and Gram negative bacteria[7]. In continuation of our previous work, in this study we report anti-*Staphylococcus aureus* (against clinical isolates of methicillin resistant *S. aureus*) and anti-yeast activity (against *Candida albicans* and *Cryptococcus neoformans*) of ethyl acetate extracts from these six *Streptomyces* species.

2. MATERIALS AND METHODS

Streptomyces species

Six *Streptomyces* species recovered previously from rhizosphere soil of *Jatropa curcas* of Sahyadri Science College (Autonomous) campus, Shivamogga-577203, Karnataka, India were used in this study. The isolates were identified as *Streptomyces* based on cultural, microscopic and biochemical characteristics[7]. The cultural characteristics *viz.,* color of aerial mycelium and substrate mycelium and production of diffusible pigments were studied on different media *viz.,* Yeast Extract-Malt Extract Agar (yeast extract 4g; malt extract 10g; dextrose 4g; agar 15g; distilled water 1000ml), Tryptone Yeast Extract Agar (tryptone 5g; yeast extract 3g; agar 15g; distilled water 1000ml), Inorganic Salts-Starch Agar (soluble starch 10g; K_2HPO_4 1g; $MgSO_4.7H_2O$ 1g; $CaCO_3$ 2g; NaCl 1g; $(NH_4)_2SO_4$ 2g; agar 15g; distilled water 1000ml), Oat Meal Agar (Oat meal 20g; trace salt solution 1ml; agar 15g; distilled water 1000ml), Nutrient Agar (peptone 5g; beef extract 3g; NaCl 5g; agar 15g; distilled water 1000ml), Starch Casein Nitrate Agar (soluble starch 10g; KH_2PO_4 2g; KNO_3 2g; NaCl 2g; casein 0.3g; $MgSO_4.7H_2O$ 0.05g; $CaCO_3$ 0.02g; $FeSO_4.7H_2O$ 0.01g; agar 15g; distilled water 1000ml), Kenknight and Munnaier's Medium (dextrose 1g; K_2HPO_4 0.1g; $NaNO_3$ 0.1g; KCl 0.1g; $MgSO_4.7H_2O$ 0.1g; agar 15g; distilled water 1000ml) and Actinomycetes Isolation Agar (sodium caseinate 2g; L-asparagine 0.1g; sodium propionate 4g; K_2HPO_4 0.5g; $MgSO_4.7H_2O$ 0.1g; $FeSO_4.7H_2O$ 0.001g; agar 15g; distilled water 1000ml). Trace salts solution (100ml) contained 1g $FeSO_4.7H_2O$, 1g $MnCl_2$ and 1g $ZnSO_4$.

Fermentation and extraction

250ml Erlenmeyer flasks containing 150ml sterile Starch casein nitrate broth (soluble starch 10g; potassium phosphate dibasic 2g; potassium nitrate 2g; sodium chloride 2g; casein 0.3g; $MgSO_4.7H_2O$ 0.05g; $CaCO_3$ 0.02g; $FeSO_4.7H_2O$ 0.01g; distilled water 1000ml) medium were aseptically inoculated with the spore suspension of well sporulated *Streptomyces* species and incubated aerobically at 30°C for 10 days. After incubation, the contents in the flasks were aseptically filtered through sterile Whatman No. 1 filter papers. The culture filtrates were centrifuged and the supernatant obtained were subjected for solvent extraction in separation funnel and extracted using ethyl acetate. Equal volume (1:1) of supernatant and ethyl acetate were taken in a separation funnel and agitated for about 30 minutes. Solvent layer was separated and the supernatant was twice extracted with ethyl acetate. The ethyl acetate layers were pooled and evaporated to dryness at 40°C[6,7].

Anti-*Staphylococcus aureus* activity of ethyl acetate extracts

Four strains of *Staphylococcus aureus* (*Sa*-01 to *Sa*-04) recovered previously from burn patients and displaying resistance against methicillin were screened for their susceptibility to ethyl acetate extracts

(obtained from the fermentation broth of *Streptomyces* isolates) by agar well diffusion assay. The bacterial isolates were grown in sterile Nutrient broth (peptone 5g; beef extract 3g; NaCl 5g; distilled water 1000ml) tubes overnight at 37°C. The broth cultures were swabbed uniformly on sterile Nutrient agar plates using sterile cotton swabs. Wells of 6mm diameter were punched in the inoculated plates. Ethyl acetate extracts (5mg/ml of 25% dimethyl sufloxide [DMSO]), tetracycline (1mg/ml of sterile distilled water) and DMSO (25%) were filled in respectively labeled wells. The plates were incubated at 37°C for 24 hours in upright position. The zones of inhibition formed around the wells were measured using a ruler[6].

Anti-yeast activity of ethyl acetate extracts

The anti-yeast efficacy of ethyl acetate extracts obtained from the fermentation broth of *Streptomyces* isolates was tested against two yeasts *viz., Candida albicans* NCIM-3466 and *Cryptococcus neoformans* NCIM-3378 by agar well diffusion assay. The yeasts were grown in sterile Sabouraud's dextrose broth (peptone 10g; dextrose 40g; distilled water 1000ml) tubes at 37°C for 48 hours. The broth cultures of yeasts were swabbed uniformly on sterile Sabouraud's dextrose agar (peptone 10g; dextrose 40g; agar 15g; distilled water 1000ml) plates using sterile cotton swabs. Wells of 6mm diameter were punched in the inoculated plates. Ethyl acetate extracts (5mg/ml of 25% DMSO), fluconazole (1mg/ml of sterile distilled water) and DMSO (25%) were filled in respectively labeled wells. The plates were incubated at 37°C for 48 hours in upright position. The zones of inhibition formed around the wells were measured using a ruler[6].

3. RESULTS :

Media	Characteristic	SSC-MB-01	SSC-MB-02	SSC-MB-03	SSC-MB-04	SSC-MB-05	SSC-MB-06
SCNA	AM	Pale yellow	Grey	Orange	Grey	White	White
	SM	Brown	Brown	Reddish orange	Light brown	Brown	Pale yellow
	DP	Brown	-	-	Brown	Brown	-
YEMEA	AM	Light brown	Light brown	Light brown	Brown	Light brown	Yellowish brown
	SM	Brown	Light brown	Light brown	Light brown	Brown	Yellow
	DP	-	-	-	-	-	-
TYEA	AM	White	Grey	Grey	White	Grey	Grey
	SM	Light brown	Brown	Light grey	White	Brown	Yellow
	DP	Light brown	Light brown	-	-	Brown	-
ISSA	AM	Brownish yellow	Light grey	Light grey	Grey	Pale yellow	Whitish yellow
	SM	Yellow	Yellow	Pink	Grey	Light grey	Light yellow
	DP	-	-	-	-	-	-
OMA	AM	Cream	Grey	Grey	Grey	Grey	Dark grey
	SM	Creamish pink	Brown	Light grey	Green	Brown	Light grey
	DP	-	Brown	-	Light green	Brown	-
NA	AM	Light grey	Light grey	White	White	Grey	Grey
	SM	Light brown	Grey	Light yellow	Pale yellow	Light grey	Grey
	DP	Brown	-	-	-	Light brown	-
AIA	AM	White	Grey	Whitish grey	Light grey	Grey	White
	SM	Light brown	Light grey	Grey	Greyish white	White	White
	DP	-	-	-	-	-	-
KMM	AM	Whitish pink	Dark grey	Light grey	Grey	Whitish brown	White
	SM	Pink	Brown	Grey	Greyish green	Brown	Light grey
	DP	-	Brown	-	-	Light brown	-

Table 1: Cultural characteristics of Streptomyces species on various media

Table 1 shows cultural characteristics of six *Streptomyces* species on various media *viz.,* Yeast Extract-Malt Extract Agar (YEMEA), Tryptone Yeast Extract Agar (TYEA), Inorganic Salts-Starch Agar (ISSA), Oat Meal Agar (OMA), Nutrient Agar (NA), Starch Casein Nitrate Agar (SCNA), Kenknight and Munnaier's Medium (KMM) and Actinomycetes Isolation Agar (AIA). The cultures of the isolates have shown variations in diffusible pigment (DP) production and colorations of substrate mycelia (SM) and aerial mycelia (AM).

Inhibitory efficacy of ethyl acetate extracts of *Streptomyces* species against clinical strains of *S. aureus* is shown in Table 2. Extracts of all isolates displayed inhibition of all test bacteria with zones of inhibition ranging 0.8 to 4.1cm. Out of six *Streptomyces* species,

marked inhibitory effect was observed in case of ethyl acetate extract of isolate SSC-MB-04 as it caused inhibition of test bacteria to higher extent and the inhibition produced was higher than that of standard antibiotic. Least inhibitory activity was observed in case of isolate SSC-MB-06. DMSO was not found to cause inhibition of test bacteria.

Treatment	Zone of inhibition in cm			
	Sa-01	*Sa-02*	*Sa-03*	*Sa-04*
SSC-MB-01	1.2	1.1	1.2	1.0
SSC-MB-02	1.1	1.0	1.0	0.8
SSC-MB-03	1.1	0.8	1.1	1.2
SSC-MB-04	4.1	3.2	3.4	3.2
SSC-MB-05	1.3	0.9	1.2	1.1
SSC-MB-06	1.0	0.8	0.8	1.0
Tetracycline	3.1	2.7	2.4	3.1
DMSO	0.0	0.0	0.0	0.0

Table 2: Anti-*Staphylococcus aureus* activity of extracts of *Streptomyces* species

Table 3 shows anti-yeast activity of ethyl acetate extracts of *Streptomyces* species. Among yeasts, susceptibility was recorded higher in case of *C. neoformans* when compared to *C. albicans*. Marked antifungal activity was shown by *Streptomyces* species SSC-MB-02 and SSC-MB-04. Inhibition of yeasts by fluconazole was higher than that of ethyl acetate extracts. Here also, *C. neoformans* was inhibited to higher extent (with zone of inhibition ranging between 3.0 to 3.5cm) when compared to *C. albicans*. Extract of all isolates displayed similar inhibition of *C. albicans*. There was no inhibition of test fungi by DMSO.

Treatment	Zone of inhibition in cm	
	C. albicans	*C. neoformans*
SSC-MB-01	0.8	3.2
SSC-MB-02	0.8	3.5
SSC-MB-03	0.8	3.4
SSC-MB-04	0.8	3.5
SSC-MB-05	0.8	3.0
SSC-MB-06	0.8	3.2
Fluconazole	3.2	4.1
DMSO	0.0	0.0

Table 3: Anti-yeast activity of extracts of *Streptomyces* species

4. DISCUSSION

Candida albicans and *Cryptococcus neoformans* are among the two most common etiological agents of opportunistic fungal infections. The infections caused by these fungi accounts for majority of nosocomial fungal infections and these causes disease predominantly in immunocompromised patients. These fungi frequently cause of fatal mycotic infections among AIDS patients. The clinical manifestation of Candidiasis and Cryptococcal meningoencephalitis is usually incurable in immunocompromised patients despite antifungal therapy as both fungi are found to be resistant to most commonly used antifungal agents such as Azoles, Amphotericin B etc.[8,9,10].

In the present study, the ethyl acetate extracts of *Streptomyces* species have shown to inhibit two human pathogenic fungi *viz., C. albicans* and *C. neoformans*. It was observed that the inhibition of *C. neoformans* was higher than that of *C. albicans*. Similar result was obtained in an earlier study by Gautham *et al*.[11] where majority of *Streptomyces* isolates showed high inhibitory activity against *C. neoformans* than *C. albicans*. However, in a previous study, Kekuda *et al*.[6] observed higher susceptibility of *C. albicans* to extract than *C. neoformans*. Shobha and Onkarappa[12] observed higher inhibition of *C. albicans* by butanol extract of *Streptomyces* isolates when compared to *C. neoformans.* A non-polyene antibiotic extracted from *Streptomyces albidoflavus* PU 23 was found to possess similar inhibitory effect against *C. albicans* and *C. neoformans*[13]. Methicillin-resistant *Staphylococcus aureus* (MRSA) was once restricted to hospital environments is now common in community also. MRSA is one of the leading causes of infections and the infections caused by MRSA limit treatment options, since these strains are resistant to the entire class of β-lactams and other antibiotics such as Clindamycin and Erythromycin. Strains have even become resistant to Vancomycin which was used for definitive therapy[14]. Burn wounds are among the most suitable sites for multiplication of bacteria. These wounds are more persistent richer sources of infection than surgical wounds, mainly due to the larger area involved and longer duration of patient stay in hospital[15]. Burn patients with extensive injuries are particularly susceptible to infection by MRSA. Burn units in hospitals have become major reservoir for MRSA which spread quickly in hospital environment. Hence, MRSA is considered as an important nosocomial pathogen causing out breaks of infection in burn patients[16].

In the present study, we have evaluated antibacterial activity of ethyl acetate extracts obtained from fermentation broths of six *Streptomyces* species against MRSA isolates. Marked inhibitory effect was observed in case of ethyl acetate extract of isolate SSC-MB-04. The inhibition caused by extract of this isolate was higher than that of standard antibiotic. Actinomycetes are promising as their crude extracts and purified components from them have shown to be effective against drug resistant microorganisms. In a previous study of Yoo *et al*.[17], the culture broth and a purified substance Laidlomycin from *Streptomyces* sp. CS684 have shown to possess antibacterial activity against MRSA and vancomycin resistant enterococci. The inhibitory activity of laidlomycin was higher than that of oxacillin and vancomycin**.** Malik *et al*.[18] isolated a protein from the fermented culture of *Streptomyces fulvissimus* and found its significant inhibitory efficacy against MRSA strains. Cyslabdan, produced by *Streptomyces* sp. K04-0144 was found to potentiate imipenem activity against MRSA[19]**.** A molecule A2,

isolated from a mangrove symbiont *Streptomyces* sp. PVRK-1 was shown to possess inhibitory activity against MRSA[20]. Three new depsipeptides, fijimycins A-C isolated from the fermentation broth of a *Streptomyces* sp. CNS-575 were shown to possess significant *in vitro* antibacterial activity against three MRSA strains[21]. Ethyl acetate extract of *Streptomyces* sp. strain BCNU 1001 was found to be effective against MRSA[22].

5. CONCLUSION

Ethyl acetate extracts of six *Streptomyces* species isolated from rhizosphere soil of college campus have shown inhibitory activity against human pathogenic yeasts and MRSA strains. The inhibitory activity could be attributed to the presence of bioactive components present in the solvent extracts. Further studies are under progress to isolate active principles from the solvent extracts and to determine their bioactivities.

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