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In vitro antimicrobial potential of organic solvent extracts of novel actinomycetes isolated from forest soil

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In vitro **screening of antibacterial and antifungal activities of hexane, chloroform, ethyl acetate, methanol and water extracts of selected promising actinomycetes strains were studied towards Gram-positive, Gram-negative bacteria, dermatophytes and opportunistic pathogens. Crude antimicrobial metabolites were extracted by liquid-liquid extraction and solid-liquid extraction method using hexane, chloroform, ethyl acetate and methanol. The lowest minimum inhibitory concentration (MIC) of the extracts was assessed by the broth micro dilution method. All the extracts obtained from eight strains showed promising activity against tested Gram-positive bacteria. The hexane extracts of strain ERI-1 exhibited activity against *Bacillus subtilis, Staphylococcus aureus* and *Staphylococcus epidermidis* at a concentration of 5 mg/ml. Ethyl acetate extract of strain ERI-4 showed MIC of 5 mg/ml but *S. epidermidis* and *S. aureus. B. subtilis* exhibited activity at 2.5 mg/ml. However, ethyl acetate and lyophilized water extract of strain ERI-3 inhibited the growth of *Trichophyton mentagrophytes, Trichophyton rubrum, Epidermophyton floccosum* and *Scropulariopsis* sp at 10 mg/ml. Overall significant antibacterial and antifungal activities were noted in the ethyl acetate extract of ERI-1 and ERI-3. Methanol extract of ERI-26 exhibited good antibacterial and antifungal activities.**

Key words: Actinomycetes, organic extracts, minimum inhibitory concentration (MIC), antimicrobial activity.

INTRODUCTION

Actinomycetes are Gram-positive bacteria showing a filamentous growth. Actinomycetes are a group of organisms widespread in nature, and play a significant role in the future of biotechnology, because of their importance as producers of vitamins, enzymes, antitumour agents, immune modifying agents and, mainly,

antibiotic compounds (Sofia and Boldi, 2006). According to Maya et al. (2011) number of novel molecules from actinomycetes were discovered. Streptomycetes, the Gram positive filamentous bacteria are widely distributed in a variety of natural and man-made environments, constituting a significant component of the microbial population in most soils. The results of extensive screenings have led to the discovery of about 4,000 antibiotic substances from bacteria and fungi, many of which have been applied in human medicine, veterinary medicine and agriculture (Adinarayana et al., 2007). Most of metabolites are produced from *Streptomyces*. Approximately 75% of metabolites originated from *Streptomyces* genus and at least 5000 documented bioactive compounds are known as being produced by *Streptomyces* genus (Vasanthabharathi et al., 2011). Most *Streptomyces* are used in the production of a diverse array of antibiotics including aminoglycosides, macrolides, β-lactams, peptides, polyenes, polyether, tetracyclines, etc (Augustine et al., 2005). In searching for new antibiotics, number of different bacteria, actinomycetes, Streptomycetes, fungi and algae have been investigated. To prevent exponential emergence of microorganisms from becoming resistant to the clinically available antibiotics already marketed, a periodic replacement of the existing antibiotics is necessary. In the present study, extraction of antimicrobial metabolites from *Streptomyces* and its antimicrobial effects on bacteria and fungi were studied. Previously, nutrients required for the antimicrobial compounds production were optimized under the shake flask condition for the identified *Streptomyces* strains (Arasu et al., 2012; 2013). This study focused on the extraction of antimicrobial metabolites by using different solvents and evaluates minimum inhibitory concentration by broth micro dilution method.

MATERIALS AND METHODS

Chemicals and solvents

Glucose and all other chemicals were obtained from Himedia (India). Organic solvents were procured from Himedia (India).

Antimicrobial compound extraction

Liquid - liquid extraction

Actinomycetes were grown in modified nutrient glucose broth medium for six days. After incubation, the fermentation medium was collected and filtered through Whatman No.1 filter paper. The total culture filtrate 4000 ml was used for the series of solvent extraction by using hexane, chloroform, ethyl acetate and methanol. The low polar to high polar solvent was selected for the organic solvent extraction. Three folds volume of the solvent was mixed thoroughly with the broth by shaking them in 1000 ml capacity separating funnel and allowed to stand for 1 h. The solvents were removed using simple distillation and vacuum rotary evaporator at 40ºC. The extracts were stored at 4ºC until further use. Water extract was lyophilized and the concentrated metabolites were stored at 4ºC until further use.

Solid - liquid extraction

A metabolite which could not extract from fermented broth was extracted by this method. The spore suspensions of the culture were inoculated on Modified Nutrient Glucose Agar (MNGA) media and incubated at 28ºC for six days. Then, agar media with cultures were taken into a 500 ml flask containing 100 ml of methanol and kept in shaker for 2 h at 200 rpm. Then the suspension was centrifuged at 8000 rpm, 10 min to separate the organic phase and extracted twice. The methanol phase was concentrated by using vaccum at 35^0 C. The extracts were stored at 4ºC until further use.

Test organisms

The reference strains used in this study was procured from American type culture collection (ATCC) and Microbial type culture collection Chandigarh, India (MTCC). The following microorganisms were used to test minimum inhibitory concentration of the extracts: Gram-positive (*Staphylococcus aureus* ATCC 25923, *Staphylococcus epidermidis* MTCC 3615, *Bacillus subtilis* MTCC 441 and *Enterococcus faecalis* ATCC 29212) and Gram-negative (*Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Klebsiella pneumoniae* ATCC 15380, *Proteus vulgaris* MTCC 1771, *Erwinia* sp MTCC 2760, *Vibrio fischer*i MTCC 1738 and *Salmonella typhi* MTCC 733).

Minimum inhibitory concentration

The minimum inhibitory concentration (MIC) was performed according to the standard reference method (NCCLS, 1999). The extracts were dissolved in water + 2% dimethyl sulfoxide (DMSO). The initial concentration of extract was 10 to 0.156 mg/ml. The initial test concentration was serially diluted twofold. Each well was inoculated with 5 µl of suspension containing 10^8 CFU/ml of bacteria. The antibacterial agent streptomycin was included in the assays as positive controls. The plates were incubated 24 h at 37°C. After incubation 5 µl of testing broth was placed on the sterile MHA plates for bacteria and incubated at respective temperature. The MIC for bacteria was determined as the lowest concentration of the extracts inhibiting the visual growth of the test cultures on the agar plate. Three replications were maintained.

Fungal strains

The fungal strains were procured from Microbial type culture collection Chandigarh, India (MTCC), and Christian Medical College, Vellore, India. The following fungi were used for experiments: *Trichophyton rubrum* MTCC 296, *T. rubrum* 57/01, *T.*

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mentagrophytes 66/01, *T. simii* 110/02, *Epidermophyton floccosum* 73/01, *Scopulariopsis* sp. 101/01 *Aspergillus niger* MTCC 1344, *Botyritis cinerea, Curvularia lunata* 46/01, and *Candida albicans* MTCC 227.

Preparation of fungal spore

The filamentous fungi were grown on Sabouraud Dextrose Agar (SDA) slants at 28°C for 10 days and the spores were collected using sterile doubled distilled water and homogenized. Yeast was grown on Sabouraud Dextrose Broth (SDB) at 28°C for 48 h.

Antifungal assays

The antifungal activity was performed according to the standard reference method (NCCLS, 1999). The extracts were dissolved in water+2% dimethyl sulfoxide (DMSO). The initial concentration of extract was 10 mg/ml. The initial test concentration was serially diluted twofold. Each well was inoculated with 5 µl of suspension containing 10^4 spore/ml of fungi. The antifungal agent Fluconazole was included in the assays as positive controls; the plates were incubated for 24 h up to 9 days at 27°C for dermatophytic strains. MIC was defined as the lowest extract concentration, showing no visible fungal growth after incubation time. Experiment was carried out in triplicates.

RESULTS

Five different extracts from eight strains of actinomycetes were screened against four Gram-positive and eight Gram-negative bacteria; among them three strains extracts exhibited good activity against tested microbes (Table 1). Some extract had a significant activity for Gram-positive bacteria but not on Gram-negative bacteria. Ethyl acetate extracts of all the strains showed activity against more than four different bacteria.

Activity against Gram-positive bacteria

All the extracts from eight strains showed activity against tested Gram positive bacteria. The hexane extracts of ERI-1 exhibited activity against *B. subtilis, S. aureus* and *S. epidermidis* at a concentration of 5 mg/ml. Chloroform and methanol extract did not show antibacterial activity. Lyophilized water extract showed MIC of 5 mg/ml for *S. aureus, S. epidermidis* and *E. faecalis*. *B. subtilis* exhibited MIC of 2.5 mg/ml. Ethyl acetate extract showed MIC of 5 mg/ml to *B. subtilis, S. aureus, S. epidermidis* and *E. faecalis*. Hexane extracts of ERI-3 showed MIC of 5 mg/ml to *S. epidermidis* and *B. subtilis* for 10 mg/ml. Chloroform extract did not exhibit any activity against Gram positive bacteria. Ethyl acetate extract showed MIC of 10 mg/ml for *B. subtilis, S. aureus* and *E. faecalis*. For *S. epidermidis* growth was inhibited at a concentration of 2.5 mg/ml. Lyophilized water extract exhibited MIC of 5 mg/ml to *B. subtilis* and *S. aureus. E. faecalis* and *S. epidermidis* revealed MIC of 10 and 2.5 mg/ml respectively in the lyophilized water extract.

Hexane and ethyl acetate of ERI-26 did not show any activity against both Gram positive and Gram negative bacteria. The chloroform extract showed MIC of 10 mg/ml to all the tested bacteria. Methanol extract exhibited MIC of 2.5 mg/ml to *B. subtilis, S. epidermidis* and *E. faecalis.* Lyophilized water extract exhibited MIC of 5 mg/ml to *E. faecalis* and *B. subtilis. S. epidermidis* and *S. aureus* showed MIC of 10 mg/ml. Ethyl acetate extract of ERI-4 showed MIC of 5 mg/ml to *S. epidermidis* and *S. aureus. B. subtilis* exhibited MIC of 2.5 mg/ml for ethyl acetate extract and *S. aureus* revealed 10 mg/ml. Hexane, methanol and lyophilized water extract did not show any activity. Chloroform extracts of ATSI-13 showed MIC of 10 mg/ml to all the tested bacteria. Ethyl acetate extract exhibited MIC of 5 mg/ml. Hexane, methanol and lyophilized water extract of ATSI-13 did not show any activity against bacteria (Table 1).

Activity against Gram-negative bacteria

Most of the actinomycetes extracts did not inhibit the growth of Gram negative bacteria. Ethyl acetate extract of ERI-1 inhibited the growth of *P. aeruginosa.* Hexane, ethyl acetate and lyophilized water extract showed MIC of 5 mg/ml to *E. coli. P. aeruginosa, K. pneumoniae, Xanthomonas* sp, *Erwinia* showed MIC of 10 mg/ml to ethyl acetate and lyophilized water extracts. Ethyl acetate extract showed MIC of 10 mg/ml to *S.typhi* and *V. fischeri*. Hexane, chloroform, ethyl acetate and lyophilized water extract did not show any activity against *P. vulgaris.* Lyophilized water extracts of ERI-3 showed MIC of 10 mg/ml to all the tested bacteria. Ethyl acetate extract also showed MIC of 10 mg/ml to all the tested bacteria except *S. typhi, V. fischeri* and *P.vulgaris.* Hexane and chloroform extract of ERI-3 did not show any antibacterial activity. The methanol extract of ERI-26 exhibited MIC of 5 mg/ml to *E. coli, P. aeruginosa* and *K. pneumoniae. Xanthomonas* sp., *Erwinia, S. typhi, V. fischer*i, *P. vulgaris* showed MIC of 10 mg/ml. Lyophilized water extract showed MIC of 5 mg/ml to *E.coli.* Hexane, chloroform and ethyl acetate extracts of ERI-26 did not reveal any activity against tested bacteria.

Antifungal activity

Results of antifungal activity were summarized in Table 2. All the 8 strains showed varying degrees of antifungal activity. Most of the extracts inhibited more than four fungal strains. From the evaluation we found that ethyl acetate extracts inhibited the growth of fungus. Hexane and methanol extracts also nearly showed the same level

Table 1. Minimum inhibitory concentration of crude extracts obtained from selected actinomycetes against bacteria.

Strain	Extract	MIC (mg/ml)											
		B. s	***S. a***	***S. e***	***E. f***	***E. c***	***P. a***	***K. p***	***X***	***Er.***	***S. t***	***V. f***	***P. v***
	He	5	5	5	10	5	-	-	-	-	-	-	-
	Cl	-	-	-	-	-	-	-	-	-	-	-	-
ERI-1	Ea	5	5	2.5	5	5	10	10	10	10	10	10	-
	Me	-	-	-	-	-	-	-	-	-	-	-	
	Ly	2.5	5	5	5	5	10	10	10	10	-	-	-
	He	10	10	5	-	-	-	-	-	-	-	-	-
	Cl	-	-	-	-	-	-	-	-	-	-	-	-
ERI-3	Ea	10	10	5	10	10	10	10	10	10	-	-	-
	Ly	5	5	2.5	10	10	10	10	10	10	10	10	-
	He	-	-	-	-	-	-	-	-	-	-	-	-
	Cl	10	10	10	10	-	-	-	-	-	-	-	-
ERI-26	Ea	-	-	-	-	-	-	-	-	-	-	-	-
	Me	2.5	5	2.5	2.5	5	5	5	10	10	10	10	10
	Ly	5	10	10	5	5	10	10	10	10	10	-	-
	He	-	-	-	-	-	-	-	-	-	-	-	-
	Cl	10	-	-	-	-	10	10	10	-	-	-	-
ERI-4	Ea	2.5	10	5	5	10	5	10	10	-	-	-	-
	Me	-	-	-	-	-	-	-	-	-	-	-	-
	Ly	-	-	-	-	-	-	-	-	-	-	-	-
	He	-	-	-	-	-	-	-	-	-	-	-	-
	Cl	10	10	10	-	-	-	-	-	-	-	-	-
ATS1-13	Ea	5	5	5	5	-	-	-	-	-	-	-	-
	Me	-	-	-	-	-	-	-	-	-	-	-	-
	Ly	-	-	-	-	-	-	-	-	-	-	-	-
	He	5	5	5	2.5	-	-	-	-	-	-	-	-
	Cl	-	-	5	5	-	-	-	-	-	-	-	-
AMW-17	Ea	5	5	2.5	5	10	-	-	-	-	-	-	-
	Me	-	-	-	-	-	-	-	-	-	-	-	-
	Ly	2	20	2.5	-	-	-	-	-	-	-	-	-
	He	10	10	5	10	10	-	-	-	-	-	-	-
	Cl	-	-	-	-	-	-	-	-	-	-	-	-
BMS-4	Ea	-	-	-	-	-	-	-	-	-	-	-	-
	Me	-	-	-	-	-	-	-	-	-	-	-	-
	Ly	10	10	10	10	10	-	-	-	-	-	-	-
	He	5	5	2.5	5	10	10	10	10	10	-	10	-
	Cl	-	-	-	-	-	-	-	-	-	-	-	-
AMW-23	Ea	5	5	10	10	-	-	10	10	10	-	-	-
	Me	-	-	-	-	-	-	-	-	-	-	-	-
	Ly	5	5	5	5	5	10	10	-	-	-	-	-

He - Hexane; Cl -Chloroform, Ea -Ethyl acetate; Me -Methanol, Ly - Lypholized water extract *B.s - B. subtilis; S.a - S. aureus ;S.e - S. epidermidis ;E.f - E. faecalis; E.c - E.coli*; *P.a - P. aeruginosa; K.p - K. pneumoniae; X.sp - Xanthomonas* sp.; *E.*sp - *Erwinia* sp;*S.t - S.typhi* ; *V.f - V. fischer*i ;*P.v - P. Vulgaris.*

Table 2. Minimum inhibitory concentration of crude extracts obtained from selected actinomycetes against fungi.

Strain	Extract	MIC (mg/ml)									
		T. m	***E. f***	***T. s***	***C. l***	***A. n***	***B. c***	***T. r 296***	***T. r 57***	***Scro***	***C. a***
	He	-	-	-	10	5	10	-	-	-	5
	Cl	-	-	-	-	-	-	-	-	-	-
ERI-1	Ea	10	10	10	10	2.5	10	10	10	10	2.5
	Me	10	10	10	10	10	5	-	-	-	5
	Ly	10	10	10	5	5	5	10	10	-	2.5
	He	10	-	-	10	10	-	-	-	-	10
	Cl	-	-	-	-	-	-	-	-	-	-
ERI-3	Ea	10	10	10	5	2.5	10	10	10	10	5
	Ly	10	10	5	5	5	5	10	10	10	10
	He	-	-	-	-	-	-	-	-	-	-
	Cl	-	-	-	-	-	-	-	-	-	-
ERI-26	Ea	10	10	10	10	-	-	-	-	-	-
	Me	10	10	10	5	2.5	10	10	10	10	1.25
	Ly	10	5	10	10	10	-	-	-	-	-
	He	-	-	-	-	-	-	-	-	-	10
	Cl	-	-	-	-	-	-	-	-	-	10
ERI-4	Ea	-	-	-	-	-	-	-	-	-	5
	Me	-	-	-	-	-	-	-	-	-	10
	Ly	10	10	10	5	5	5	10	10	10	5
	He	-	-	-	-	-	-	-	-	-	-
	Cl	-	-	-	-	-	-	-	-	-	-
ATS1-13	Ea	-	-	-	-	-	-	-	-	-	-
	Me	-	-	-	-	-	-	-	-	-	-
	Ly	-	-	-	-	-	-	-	-	-	-
	He	-	-	-	-	-	-	-	-	-	
	Cl	-	-	-	-	-	-	-	-	-	-
AMW-17	Ea	10	10	10	10	10	-	-	10	10	5
	Me	-	-	-	-	-	-	-	-	-	-
	Ly	10	10	-	10	5	10	-	-	-	5
	He	-	-	-	-	-	-	-	-	-	-
	Cl	-	-	-	-	-	-	-	-	-	-
BMS-4	Ea	-	-	-	-	-	-	-	-	-	-
	Me	-	-	-	-	-	-	-	-	-	-
	Ly	10	10	-	10	10	10	-	-	-	10
	He	-	-	-	-	-	-	-	-	-	-
	Cl	10	-	-	10	5	5	-	-	-	5
AMW-23	Ea	10	10	-	10	10	10	-	-	-	10
	Me	-	-	-	-	-	-	-	-	-	-
	Ly	10	10	10	10	5	-	-	-	-	5

He -Hexane; Cl -Chloroform, Ea - Ethyl acetate; Me - Methanol, Ly - Lypholized water extract. *T.m - T. mentagrophytes*; *E.f - Epidermophyton floccosum*, T.s. - *T. simii; C.l - Curvularia lunata*, *A.n - Aspergillus niger* ; *B.c -Botrytis cinerea; T.r- Trichophyton rubrum*, Scro - *Scropulariopsis* sp.; *C.a- Candida albicans* MTCC 227.

of inhibition against fungal growth. Chloroform extracts showed minimum antifungal activity. Ethyl acetate, methanol and lyophilized water extracts of ERI-1 inhibited the growth of *T. rubrum, T. mentagrophytes* and *T. simii* at MIC values of 10 mg/ml. In addition ethyl acetate and lyophilized water extract inhibited growth of *T. rubrum* (296, 57), *B. cinerea* and *Scropulariopsis* sp. at 10 mg/ml and *C. albicans*, *A. niger* at 2.5 mg/ml. Methanol and lyophilized water extract inhibited the growth of *B. cinerea* at MIC of 5 mg/ml and *C. albicans* showed MIC of 2.5 mg/ml. Hexane extract of ERI-1 inhibited the growth of *A. niger* and *C. albicans* at MIC of 5 mg/ml. Chloroform extract did not show antifungal activity against tested fungi. Hexane extract inhibited the growth of *C. lunata* and *B. cinerea* at MIC of 10 mg/ml also *A. niger* growth was inhibited at 5 mg/ml concentration (Table 2).

Ethyl acetate and lyophilized water extract of ERI-3 inhibited the growth of *T. mentagrophytes T. rubrum, E. floccosum* and *Scropulariopsis* sp at MIC of 10 mg/ml. Ethyl acetate extract of ERI-3 showed MIC of 2.5 mg/ml to *A. niger* and also 5 mg/ml to *C. albicans.* Hexane extract inhibited the growth of *T. rubrum, C. lunata, C. albicans* and *A .niger* with MIC of 10 mg/ml. Lyophilized water extract revealed MIC of 10 mg/ml to *T. simii, C. lunata, A. niger* and *B. cinerea.* Chloroform extract of ERI-3 did not show activity against tested fungi. The methanol extract of ERI-26 showed activity against all the tested fungi. *C. albicans* growth was inhibited by methanol extract of ERI-26 with MIC of 2.5 mg/ml followed by *A. niger* at 5 mg/ml. *C. lunata* growth was inhibited at 5 mg/ml. *T. rubrum, T. mentagrophytes, T. simii, B. cinerea, T. rubrum* (296, 57) and *Scropulariopsis* sp growth were inhibited at 10 mg/ml. Hexane and chloroform extracts of ERI-26 did not inhibit the growth of tested fungi. Minimum Inhibitory Concentration of all the solvent extracts obtained from ERI-4 recorded as10 mg/ml for *C. albicans* except ethyl acetate. Lyophilized water extract showed MIC of 10 mg/ml for *T. rubrum, E. floccosum, T. simii, T. mentagrophytes* and *Scropulariopsis* sp. *C. albicans*, *C. lunata, A. niger* and *B. cinerea* showed MIC at 5 mg/ml. None of the tested fungus was inhibited by extracts obtained from ATS1-13. Hexane and chloroform extracts of AMW-17 did not show activity against tested fungi. Ethyl acetate and lyophilized water extract exhibited MIC of 10 mg/ml to *T. rubrum, C. lunata*, *B. cinerea* and *T. mentagrophytes.* Ethyl acetate extract inhibited the growth of *T. rubrum* (57) and *Scropulariopsis* sp at MIC of 10 mg/ml. Lyophilized water extract recorded MIC of 5 mg/ml to *A. niger* and *C. albicans.*

DISCUSSION

In the present study, five different extracts of eight actinomycetes were tested for antimicrobial activity. All the isolates were grown in this medium for 96 to 120 h at 30^{o}C for antimicrobial compound production. The spent medium was used for extraction of antimicrobial metabolites. Forar et al. (2008) used sucrose, $(NH_4)_2SO_4$ and yeast extract as a fermentation medium for new actinomycete strain SK4-6. This organism exhibited strong activity against bacteria including methicillin resistant *S. aureus* and *M. luteus*, in addition to the causative agents of Candidiasis and Aspergillosis diseases.

Hexane extract of ERI-1, ERI-3, AMW-17, BMS-4 and AMW-23 showed antibacterial activity against Gram-positive bacteria and Gram-negative bacteria. Hexane extract of ERI-1 showed MIC of 5 mg/ml for *B. subtilis*, *S. aureus* and *S. epidermidis.* Hexane extract of ERI-3 showed MIC of 5 mg/ml for *S. aureus.* However Forar et al. (2007) used a range of solvents like petroleum ether, n-hexane, chloroform, diethyl ether, ethyl acetate, butyl acetate, benzene, n-butanol and ethanol to extract the antimicrobial metabolite from actinomycetes strain RAF-10. They reported that n-hexane, chloroform and diethyl ether were poor solvent for antibiotic extraction and n-butanol was good for the extraction of active compounds. Sumitha and Philip (2006) extracted antimicrobial metabolite from actinomycetes by ethyl acetate, Hexane and 1-butanol. The extracts were found to be active against *Vibrios*. Hexane extract of S26 showed inhibition against *V. alginolyticus*. Chloroform extract of ERI-26 showed activity against *B. subtilis, S. aureus, S. epidermidis* and *E. faecalis*. Moustafa et al. (2006) isolated meroparamycin from the fermented broth of *Streptomyces* sp. by chloroform as solvent. Jaime et al. (1991) and Thangadurai et al. (2004) used ethyl acetate, chloroform and hexane for the extraction of antimicrobial metabolites from bacteria. Three organic solvents of differing polarity were used subsequently to extract the active principle from the 15 actinomycetes.

Conclusion

The results of the present work indicated that the isolates of actinomycetes possess antibacterial and antifungal properties underlining the importance of the actinomycetes in the discovery of new bioactive compounds. We found significant antibacterial and antifungal activity in the ethyl acetate extract of isolates ERI-1 and ERI-3. Methanol extract of ERI-26 exhibited good antibacterial and antifungal activity.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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