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In-vitro Antidermatophytic Activity of Methanolic Fractions from *Entada spiralis* Ridl. Stem Bark and Its Bioautographic Profile

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ABSTRACT

This study was performed to evaluate the antifungal activities of methanolic fractions from the stem bark of *Entada spiralis* Ridl. against human dermatophytes and yeast-like fungus *in vitro*. Three types of human dermatophyte, *Trichophyton mentagrophytes* ATCC 9533*, Microsporum gypseum* ATCC 24102 and *Trichophyton tonsurans* ATCC 28942, and one yeast-like fungus, *Candida glabrata* ATCC 66032, were tested against the methanolic fractions labelled FA1, FA4 and FA5. *T. mentagrophytes, T. tonsuran* and *M. gypseum* were susceptible to all tested fractions in a concentration-dependent manner whereas *C. glabrata* was resistant. Fraction FA1 at a concentration of 400 mg/mL was found to exhibit the highest antifungal activity with the inhibition zone diameter of 22 mm (*T. mentagrophytes*). This fraction showed a minimum inhibitory concentration MIC of 0.097 mg/mL while the MIC value for the fraction FA4 and fraction FA5 was 3.12 mg/ml and 1.56 mg/ml respectively. Agar overlay bioautography assay results showed that most of the bioactive compounds were found in the fraction FA1. Based on these findings, it can be concluded that the stem bark extracts of *E. spiralis* can be a future source of potent natural antimicrobial drugs for superficial skin diseases.

Keywords: Leguminoceae, *Entada spiralis* antidermatophytic activity, dermatophytes, bioautography

INTRODUCTION

Medicinal plants contain numerous chemicals that can be employed by humans to overcome certain microbial invasions and they have been used as major sources of drug development (Kuete *et al*., 2009). Presently, their uses in traditional medicine have expanded widely and are gaining in popularity. Herbal

medicines have been reported to serve the health needs of about 80 % of the world's population, and are especially used in the vast rural areas of developing countries (WHO, 2001). The plant extracts of herbal medicines and products have been used for the treatment of bacterial, fungal and viral infections (Bruneton, 1999; Cowan, 1999). In past decades, infection caused by dermatophytes has increased considerably. Dermatophytes are parasitic fungi that cause infections of the skin in animals and humans. These include the imperfect fungi of the genera *Epidermophyton, Microsporum* and *T. richophyton.* The infections occur as the fungi are able to obtain nutrients from keratinised material. They do not invade living tissues but colonise the outer layer of the skin. Some of the skin infections caused by *Trichophyton tonsurans, Trichophyton mentagrophytes* and *Microsporum gypseum* are tinea capitis (scalp and hair), tinea corporis (glabrous skin), tinea unguium (nails) and tinea manuum (hand). Since dermatophytes are responsible for serious human pathogenic disorders, work on alternative approaches such as the use of natural antimicrobial agents from plant extracts of herbal medicines to control such pathogens would be beneficial (Bajpai *et al*., 2009).

The antimicrobial potential of plant species of the Leguminoceae family has been reported (Fabry *et al.,* 1998; Doughari, 2006; Mboso *et al.*, 2010; Khattak *et al.*, 2010). Within this family, certain species of the genus *Entada* has also been reported to possess antimicrobial potency. For example, *Entada phaseoloides* has been reported to have been used to treat skin diseases such as eczema, itches and scabies (Ram *et al.*, 2004) While *Entada africana* is used traditionally to treat hepatitis, sores, skin-eruptions, rheumatism, cataracts, fevers, dysentery and promote wound healing (Burkill, 1995). In addition, the stem bark of *Entada spiralis* Ridl., a liana or woody climber that grows in the wild in Malaysia and locally known as 'beluru' or 'sintok', has been traditionally used for generations as a shampoo to clean the scalp and a soap for general cleaning. It has also been used to treat syphilis, insect bites and bloody defecation. Because of its wide usage and since it has not yet been studied in detail by researchers, this plant is now the subject of this study, which set out to investigate the antidermatophytic activity of this plant species against several dermatophytes that cause skin diseases. The results of this study will determine its efficacy as an antifungal agent. In the previous study, we reported that the methanol extract successfully inhibited the growth of *T. mentagrophytes, M. gypseum* and *T. tonsurans* (Harun *et al*., 2011).

MATERIALS AND METHODS

Plant materials and fractionation

The stem bark of *E. spiralis* was collected from the forest at Tasik Chini, Pahang and authenticated by a plant botanist from Universiti Kebangsaan Malaysia (voucher specimen-KMS -5228). About 3 kg of finely powdered dry stem bark of the plant was macerated in 9 L of petroleum ether for 3 days, and this process was repeated 3 times. The petroleum ether extract was filtered and evaporated to dryness. The remaining stem bark was further macerated in chloroform and methanol sequentially in a similar manner. All extracts were stored at 4 ^{0}C prior to use. The methanol extract was chosen as the target extract as it exhibited moderate activity against tested dermatophytes (Harun *et al*., 2011). Thus, the screening of antifungal substances was studied in the methanol extract.

The mixture of methanol extract and silica gel (Merck, Germany) (ratio1:3) was dissolved in methanol and heated in a water bath to dryness to form a methanol extract-silica gel powder. Part of the methanol extract-silica gel powder (30 g) was subjected to vacuum liquid chromatography (VLC) on silica gel (200-430 mesh, 100 g) eluting with gradient systems of chloroform-methanol (1L for each gradient system). Fractions of 200 mL that were collected consecutively and combined on the basis of their thin layer chromatography (TLC) profiles were evaporated to dryness. The fractionation process afforded eleven main fractions and only fraction FA1 (0.15 g, eluted with chloroform-methanol 9:1), fraction FA4 (0.95 g, eluted with chloroform-methanol 6:4) and fraction FA5 (0.54 g, eluted with chloroform-methanol 5:5) fractions were further investigated. Fraction FA1, FA4 and FA5 were chosen based on their solubility in chloroform-methanol. Fractions obtained from other eluting solvents in which the composition of methanol was higher than that of chloroform were discarded because of poor solubility.

Microorganisms

The microorganisms used in this study were three dermatophytes (*Trichophyton mentagrophytes* ATCC 9533, *Trichophyton tonsurans* ATCC 28942, *Microsporum gypseum* ATCC 24102) and one non-dermatophyte (*Candida glabrata* ATCC 66032). The stock cultures of dermatophytes were maintained by monthly subculturing in Saubaroud Dextrose Agar (Merck, Germany) (SDA) at room temperature.

Preparation of test solution and disc

A stock solution of 400 mg/ml of fraction was prepared by dissolving 0.4 g of fraction in 1 mL of methanol and serially diluting it to achieve a concentration of 200 mg/mL, 100 mg/mL and 50 mg/mL. Sterile filter paper discs (Whatman AA disc, 6mm, England) were impregnated with 20 mL of fractions of each concentration and allowed to dry at room temperature (Chandrasekaran & Venkatesalu, 2004; Prasad *et al*., 2004). All the discs were stored at -5 ^{0}C prior to use.

Fungal suspension

The dermatophytes were subcultured in SDA and incubated at room temperature for 7-14 days. The mycelia was scraped aseptically, crushed and macerated thoroughly in sterile distilled water. The fungal suspension was standardised spectrophotometrically to an absorbance of 0.600 at 450 nm using a UV-VIS spectrophotometer (Secoman, France) which corresponded to 0.5 - 2.5 x 10^3 cells/ml. It was used as an inoculum for antifungal susceptibility testing (Pankajalakhsmi *et al.,* 1995; Prasad *et al.,* 2004, Chandarasekaran & Venkatesalu, 2004).

Antifungal assay

Antifungal activity was evaluated using the disc diffusion agar method (Bauer *et al.,* 1966) and broth microdilution. Test plates were prepared by pouring 20 mL of sterile molten SDA into petri dishes after which the agar was allowed to solidify. A sterile cotton swab was dipped

into the standardised fungal suspension and the solidified agar was then uniformly swabbed. The impregnated paper discs were applied and incubated for 48-168 hr (4-7 days) for *Candida* and the dermatophytes respectively. The antifungal activity of the fractions was determined by measuring the clear zones of growth inhibition around the paper discs. Nystatin (100 *u*g/ disc) (Oxoid, England) was used as standard reference. The solvent and empty discs were used as a negative control. The clear zone around the discs indicated that the inhibition was fungicidal while the absence of a clear zone showed fungistatic properties. All assays were carried out in triplicate.

In the broth microdilution assay, the fraction was two-fold serially diluted using an appropriated solvent in a 96-well microtiter plate to give a final volume of 200 *u*L with a final concentration ranging from 50 mg/ml to 0.024 mg/mL. The plates were incubated at room temperature for 2-5 days. To indicate fungal growth, 20 *u*L 0.5% (w/v) of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Merck, Germany) solution was added to each well and incubated for 2 hrs. The dark-blue colour that emerged revealed microbial growth whereas the colour yellow showed inhibition of microbial growth. The lowest concentration of fraction that produced a yellow colour in a microtiter well would give the minimum inhibitory concentration (MIC) value of the fraction. Appropriate controls such as solvent used to dissolve fractions, Saubaroud Dextrose Broth (SDB) with inoculum, SDB alone and nystatin were also used in the assay. The MIC was defined as the minimum concentration of the samples that inhibited a visible growth of the tested microbes.

Agar overlay bioautography assay

The thin layer chromatography (TLC) of each fraction was performed on sterile 10 cm x 10 cm commercial aluminium sheets silica gel $60F_{254}$ of layer thickness 0.2 mm (Merck, Germany). Two TLC plates of each fraction were developed in the suitable developing solvent system and one of them was used as a reference chromatogram. The chromatogram was placed in sterile square petri dishes after the complete removal of solvent. The inoculum concentration was measured and adjusted to achieve 0.5 - 2.5 x 10^3 cells/ml by diluting with sterile agar at 40^0C. Twenty mililitres of inoculated molten agar were immediately distributed on each chromatogram to form a thin layer of agar and was then allowed to solidify. The plates were incubated at room temperature for 2-5 days. The areas where active compounds of fraction were located were detected as a clear zone against the background of living fungal mycelia. The reference chromatograms were observed under UV lights at 254 nm and 366 nm and analysed after spraying with vanillin/sulfuric acid reagent.

RESULTS AND DISCUSSION

The results of the disc diffusion agar method indicated that all fractions possessed antifungal activity against all tested dermatophytes in a concentration-dependent manner except for *Candida glabrata* (Table 1). Fraction FA1 had a statistically bigger inhibition zone compared to the fraction FA4 and the fraction FA5. Therefore, fraction FA1 was found to be the most effective fraction with the highest inhibition zone diameter of 22 mm against *T. mentagrophytes*. All fractions displayed lower activity against all tested dermatophytes at concentration of 50

mg/mL. The standard antifungal drug test showed *T. tonsurans* was the most sensitive towards nystatin at 100 μg/disc with an inhibition zone of 42.5 mm.

The results from this current study revealed the scientific basis of the traditional usage of *E. spiralis*. The results were comparable with previous reports where methanol extract from *Psoralea corylifolia* (Leguminoceae) suppressed the growth of *T. mentagrophytes* and *M. gypseum* with inhibition zones of 28 mm and 25 mm respectively (Prasad *et al.,* 2004). Likewise, the ethanol extract of *E. rheedei* (Leguminoceae) was reported to exhibit moderate activity against fungi such as *Candida albicans* (Ram *et al.,* 2004). Currently, Mutai *et al.,* (2009) found the number and the nature of substituents of triterpene responsible for the antimicrobial activity of the *Acacia mellifera* extract (Leguminoceae) against *S. aureus* and *T. mentagrophytes*. The inhibition of the microbial growth could depend on the presence of hydroxyl groups and the nature of conformation in the triterpene molecule. Thus, these factors could also be the reason for the effectiveness of fraction FA1 since terpenoid compounds were found in this fraction.

The MIC of all tested fractions against dermatophytes in the broth microdilution assay is given in Table 2. Fraction FA1 showed MIC of 0.097 mg/mL for *T. mentagrophytes*, 3.125 mg/mL for *T. tonsurans* and 0.195 mg/mL for *M. gypseum*. For the fraction FA4, the MIC was determined as 3.125 mg/mL for *T. mentagrophytes* and *T. tonsurans* and 6.25 mg/mL for *M. gypseum*. Fraction FA5 showed MIC value of 1.56 mg/mL, 0.78 mg/mL and 3.125 mg/mL for *T. mentagrophytes, T. tonsurans* and *M. gypseum*, respectively. The standard antibiotic nystatin (100μg/disc) showed MIC of 0.0019 mg/mL against *T. mentagrophytes*, 0.031 mg/mL against *T. tonsurans* and 0.25 mg/mL against *M. gypseum*.

TABLE 1

In vitro Antifungal Activity of FA1, FA4 and FA5 Fractions of Methanol Extract of *E. spiralis* Stem Bark Against Tested Dermatophytes

Fractions	Concentration (mg/ml)	Inhibition zone (mm) [a]			
		TM	TT	MG	CG
FA1	50	10±0	9 ±0	-	-
	100	14.50±2.12	13±1	12±0	-
	200	19±0	13.50±3.54	18±0	-
	400	22.0±1.41[*]	17.33±1.53[*]	19.50±0.71[*]	-
FA4	50	-	-	-	-
	100	-	8.67±1.15	-	-
	200	10±0	10.33±0.58	9.0±1.73	-
	400	11±0	14.0±0	11.0±1.73	-
FA5	50	-	-	-	-
	100	8.0±0	8.67±1.15	8.67±0.58	-
	200	10.0±0	10.67±0.58	10.0±1	-
	400	12.0±0	12.67±1.53	10.0±1	-
Nystatin		21±0	42.50±0.71	21±0	24.50±0.71

-, No activity; TM, *Trichophyton mentagrophytes*; TT, *Trichophyton tonsurans*; MG, *Microsporum gypseum*; CG, *Candida glabrata*; ±, Standard deviation (SD); [a] Mean of three replicates; Statistical significance was determined using paired *t*-test. Differences were considered significant at (P<0.05); *, larger inhibition zone compared to FA4 and FA5

TABLE 2
MIC (mg/mL) of FA1, FA4 and FA5 Fractions of Methanol Extract of *E. spiralis* Stem Bark Against Tested Dermatophytes

Dermatophytes	Minimum inhibitory concentration (mg/ml)			
	FA1	FA4	FA5	Nystatin (*ug*/mL)
Trichophyton mentagrophytes	0.097	3.125	1.56	0.0019
Trichophyton tonsurans	3.125	3.125	0.78	0.031
Microsporum gypseum	0.195	6.25	3.125	0.25

Based on MIC determination, it was found that fraction FA1 possessed strong inhibitory activity against *T. mentagrophytes* and *M. gypseum* but only weak activity against *T. tonsurans*, which was not consistent with the results of the disc diffusion assay. Uneven fungal plating on the surface of the agar could have been one reason for this inconsistency. Fraction FA4 showed weak inhibition against all tested dermatophytes while fraction FA5 showed moderate activity against *T. mentagrophytes* and *T. tonsurans* and weak inhibition against *M. gypseum*. The broth microdilution assay results of fraction FA4 and FA5 seemed to be consistent with the disc diffusion assay results. Generally, the disc diffusion assay is a semi-quantitative assay which is employed to screen the fractions against susceptible fungal strains and may produce equivocal results in quantitative determination. In the broth microdilution assay, the antidermatophytic activity was evaluated quantitatively by means of the MIC values which established the fungal susceptibility. Hence, MIC results confirmed the effectiveness of fraction FA1.

The MIC results were comparable to MIC results of the active compounds from *P. corylifolia*, which showed MIC of 0.0625 mg/mL for *T. mentagrophytes* and 0.125 mg/mL for *M. gypseum* (Prasad *et al*., 2004). Significantly, the MIC determination provided an important guideline for choosing an appropriate effective concentration of a dermatophyte-inhibiting substance. The suppression of a dermatophyte's growth might be due to interference by the active constituents of the fractions; this is supported by Ibrahim and Osman, 1995.

Bioautography is an assay used to facilitate the isolation of antimicrobial active compounds from natural products. It is considered to be the most efficacious technique for the detection of antimicrobial compounds as it allows the localisation of the activity even in a complex matrix and, therefore, permits a target-directed isolation of the active constituent (Rahalison *et al*., 1991). Usually, the active compounds can be seen as clear spots against the background of growing fungi. The results for the TLC agar overlay bioautography of fraction FA1, FA4 and FA5 are shown in Table 3. Generally, the results revealed the presence of antidermatophytic active compounds in all fractions according to the clear inhibition zones on bioautograms. Fraction FA1 was found to contain antidermatophytic active compounds that inhibited the growth of all tested dermatophytes, whereas active compounds that had been screened in the fraction FA4 inhibited the growth of *T. mentagrophytes* and fraction FA5 inhibited *T. tonsurans*. The results of the antifungal bioautographic profiles of fraction FA1 presented in Table 4 revealed that the inhibitory effect was due to the presence of terpenoid compounds as detected by spraying the reference chromatogram with vanillin/sulfuric acid reagent (Fig.1). The bioautogram of B, C and D in fig.1 displayed the location of the inhibition zone against *M.*

gypseum, *T. mentagrophytes* and *T. tosurans* respectively, while the reference chromatogram A showed various terpenoid compounds found in the fraction FA1. The terpenoid compounds labelled as 1, 2 and 3 are the responsible antidermatophytic compounds of the fraction. The clear inhibition zone detected on bioautogram B was due to the presence of terpenoid compound 3, while the clear inhibition zone on bioautogram C resulted from the presence of terpenoid compound 1 and 3. The clear inhibition zone on bioautogram D was caused by the presence of terpenoid compound 1 and 2. Therefore, the results were very encouraging as the fraction FA1 was verified as containing antidermatophytic substances. This is consistent with previous investigations reporting that triterpenoid saponins present as major compounds such as echinocystic acid, entagenic acid, acacic acid, homogentistic acid and diterpene kolavenol in *Entada sp* act as bioactive compounds (Freiburghaus *et al*., 1998; Cioffi *et al*., 2006; Nzowa *et al*., 2010). Although no phytochemical investigation has been recorded for *E. spiralis* to date, it is possible that such bioactive compounds may be responsible for the antidermatophytic properties of this plant.
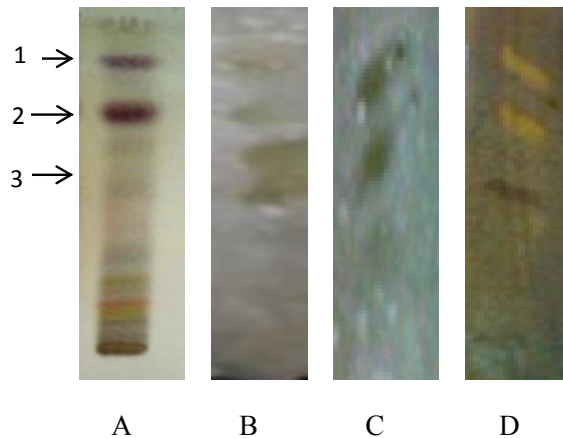

Fig.1: TLC bioautography profiles of FA1 fraction. A. Chromatogram after spraying with vanillin/H2SO4 B. Bioautogram against *M. gypseum* C. Bioautogram against *T.mentagrophytes* D. Bioautogram against *T. tonsurans* (after exposure to iodine vapour)

TABLE 3
Agar Overlay Bioautography of FA1, FA4 and FA5 Fractions of Methanol Extract of *E. spiralis* Stem Bark Against Tested Dermatophytes

Fraction	*T.mentagrophytes*	*T. tonsurans*	*M. gypseum*
FA1	√	√	√
FA4	√	x	x
FA5	x	√	x

√ = Clear zone ; x = Absence of clear zone

TABLE 4
Antifungal Bioautographic Profile of Fraction FA1 of *E. spiralis* Stem Bark

Labelled Susceptible Compound	UV_{254} light	UV_{366} light	Vanillin/H_2SO_4	Comment	fungal
1	Black+	Blue+	Purple+++	Terpenoid	*TT, TM*
2	Black+	Blue+++	Purple+++	Terpenoid	*TT, MG*
3	Black+	Blue+++	Grey++	Terpenoid	*MG, TM*

Intensity of colour: +++ high, ++ definite, + slight

CONCLUSION

Entada spiralis is a promising antidermatophytic species because of significant inhibitory activity of its fractions on dermatophytic proliferation. We believe that fraction FA1 has great potential to treat skin infections, and we have provided important scientific support for the traditional use of the stem bark of *E. spiralis*. The results also can be considered as a new finding since no antidermatophytic studies against dermatophytes have been reported for this species previously. For further work, the isolation of natural antifungal compounds need to be carried out in order to identify the active compounds responsible.

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