

BIOASSAY GUIDED ISOLATION OF AN ANTIDERMATOPHYTIC ACTIVE CONSTITUENT FROM THE STEM BARK OF *ENTADA SPIRALIS* RIDL.

(Pengasingan komponen aktif antiderma secara berperingkat dari kulit pokok
Entada spiralis Ridl.)

Aiza Harun^{1*}, Siti Zaiton Mat So'ad², Norazian Mohd Hassan²

¹Faculty of Applied Sciences,
Universiti Teknologi MARA Pahang, 26400 Bandar Tun Razak Jengka, Pahang, Malaysia

²Kulliyah of Pharmacy,
International Islamic University Malaysia, Kuantan Campus, Kuantan, Pahang, Malaysia

*Corresponding author: aizaharun@pahang.uitm.edu.my

Received: 23 November 2014; Accepted: 27 June 2015

Abstract

Entada spiralis Ridl. (Leguminosae) is a liana or woody climber that grows in the wild in Malaysia and is locally known as 'Beluru' or 'Sintok'. The isolation and characterization of the chemical constituent from an active fraction have been carried out since no previous study has determined any active components from the stem bark. Our previous study had revealed methanol extract of *E. spiralis* stem bark exhibited promising antifungal activity against three dermatophytes strains, namely *Trichophyton mentagrophytes* ATCC 9533, *Trichophyton tonsurans* ATCC 28942 and *Microsporum gypseum* ATCC 24102 that cause skin infection. This study was performed to elucidate the structure of active constituent known as ester saponin from the active fraction of *E. spiralis* stem bark. The fractions were prepared using fractionation process and repeated antifungal test was conducted to identify the most active fraction. The structure elucidation of this compound was based on spectroscopic data (¹H, ¹³C NMR, HMQC, HMBC and DEPT135) and comparison with literature. On the basis of spectroscopic analysis, the compound was identified as 28- α ,L-rhamnopyranosyl-18,21,22-trihydroxy-12-en-29-(2-acetylamino- β -D-glucopyranosyl) triterpene ester. The current study provides important baseline information for the use of *E. spiralis* stem bark for the treatment of skin infection caused by the microorganisms investigated in this study.

Keywords: antifungal activity, dermatophytes, *entada spiralis*, leguminosae, terpenoid

Abstrak

Entada spiralis Ridl. (Leguminosae) merupakan sejenis pokok memanjat yang tumbuh meliar di Malaysia. Ia dikenali juga dengan nama 'Beluru' atau 'Sintok'. Pemencilan dan pengenalpastian komponen kimia yang aktif telah dilakukan dalam kajian ini memandangkan tiada kajian terdahulu mengenai pengasingan bahan aktif dari kulit batang pokok *E. spiralis*. Kajian terdahulu hanya melaporkan ekstrak metanol mampu merencatkan pertumbuhan tiga jenis kulat penyakit kulit iaitu *Trichophyton mentagrophytes* ATCC 9533, *Trichophyton tonsurans* ATCC 28942 and *Microsporum gypseum* ATCC 24102. Oleh itu kajian ini dijalankan untuk menentukan struktur molekul dan penamaan komponen aktif dari kulit pokok terhadap kulat penyakit kulit dengan menggunakan kaedah spektroskopi (¹H, ¹³C NMR, HMQC, HMBC dan DEPT135) serta perbandingan dengan kajian terdahulu. Melalui analisis spektroskopi komponen aktif tersebut dikenal pasti sebagai 28- α ,L-rhamnopyranosyl-18,21,22-trihydroxy-12-en-29-(2-acetylamino- β -D-glucopyranosyl) ester triterpena. Penemuan ini boleh dijadikan maklumat asas dalam rawatan penyembuhan penyakit kulit.

Kata kunci: aktivi antikulat, dermatofit, *entada spiralis*, leguminosae, terpenoid

Introduction

Before the emergence of modern medicine, many people throughout the world depended on medicinal herbs to cure various diseases. The high content of chemical compounds in these herbs made them useful in the prevention of microbial invasion. Secondary metabolites produced by plants contain a tremendous source of bioactive compounds such as flavonoid, alkaloid, tannin, saponin, sesquiterpenes, triterpenes, monoterpenes, diterpenes, tetraterpenoid, cardiac glycosides, terpene polymers, isoflavones, coumarins, phenolic acid, lignans, lignin, chalcones and sterols. Scientific interest in these metabolites has increased recently with the search of new therapeutic agents from plant source due to the increasing development of the microorganisms' resistance to most currently used antimicrobial drugs [1].

Bioactive constituents from various types of species of Leguminosae family have been reported to have antimicrobial potential [2 – 6]. *Entada spiralis* Ridl. from the family of Leguminosae is a liana or woody climber that grows in the wild in Malaysia and is known as 'Beluru' or 'Sintok'. The stem bark is traditionally used in scalp treatments and its soapy properties make it suitable as washing agent and body soap. However up until now, there have been no reports on the secondary metabolites and antimicrobial properties of this plant. Previous phytochemical investigation of other *Entada* species have identified the presence of oleanolic acid, echinocystic acid, entagenic acid and acacic acid glycosides [7,8]. The echinocystic acid and acacic acid that were found in *Entada africana* Guill. and Perr were reported to have moderate to high cytotoxic potency [9]. The chemical constituents and the biological activity of *Entada rheedii* Spreng have not been previously investigated but phytochemical studies revealed the presence of saponins [8,9,10], thiomides [11] and phenylacetic derivatives [12,13]. Lately, an investigation revealed that entagenic acid isolated from seed kernels of *E. rheedii* exhibited moderate cytotoxic potency and antioxidant properties [14].

In this present investigation, we report the isolation and structural identification of one triterpenoid ester saponin from the active fraction of *E. spiralis* stem bark.

Materials and Methods

General Experimental Procedures

The ¹H Nuclear Magnetic Resonance (NMR) and ¹³C NMR spectra were recorded in CDCl₃, Fourier Transform NMR (FT-NMR) was carried out using a Cryoprobe on a Bruker Avance 111 600 MHz spectrometer. All chemical shifts (δ) are given in ppm units with reference to tetramethylsilane (TMS) as internal standard. The coupling constant (*J*) values are given in Hz. Electrospray ionization mass spectrometer (ESIMS) was carried out using a Bruker micrOTOF-Q 86 mass spectrometer operating in positive-ion mode. Vacuum liquid chromatography (VLC) was performed using silica gel 60 with a 230-400 mesh particle size (Merck). Thin layer chromatography (TLC) was performed on Kiesegel 60 F₂₅₄ (Merck) aluminium support plates developed using chloroform:methanol system. TLC plates were visualized at UV₃₆₆ and UV₂₅₄.

Extraction and Fractionation

The extraction and fractionation procedures were carried out as previously reported [15].

Antifungal Evaluation

The antifungal activity of fractions was evaluated as previously reported [16].

Results and Discussion

In our previous research work, methanol fraction F1 with the ratio 9:1 (v/v) of chloroform:methanol had been reported to exhibit the strongest antifungal activity against all dermatophytes tested in which terpenoid had been screened to inhibit fungal growth [15,16]. We reported fraction F1 as the most active fraction and hence, it was chosen for the isolation of active component and its structure elucidation.

The antifungal compound from F1 was isolated as a white amorphous powder following chromatographic procedure of the most active fraction of *E. spiralis* stem bark. The inhibitory effect of compound could be observed on the bioautogram of the most active fraction against *T. mentagrophytes* and *T. tonsurans* (Fig. 1). A centrifugal chromatography (Chromatotron) with an elution system of petroleum ether-dichloromethane (40% DCM) was used.

A retention factor R_f 0.64 was obtained for this compound after being developed with thin layer chromatography (TLC) technique using a binary solvent system of chloroform:methanol (30:1,V/V). The purple colour on TLC chromatogram of the compound after spraying with vanillin/ H_2SO_4 reagent was identified as terpenoid.

The ESIMS (positive-ion mode) data exhibited a molecular ion peak at m/z 911.6931 $[M+H]^+$ (MW 909) consistent with the molecular formula $C_{47}H_{75}NO_{16}$. The fragmentation of ion peak at m/z 563.3628 $[(M+H)-348]^+$ corresponded to the successive loss of one unit of rhamnose (Rha) and one unit of N-acetylglucosamine (GlcNAc). Other significant fragmented ion peaks were observed at m/z 413.2580, 301.1353, and 149.0214. The mass fragmentation was illustrated in Fig. 2. The infrared spectrum showed a broad absorption band indicative of a hydroxyl group at 3400 cm^{-1} , as well as strong absorption due to a carbonyl group at 1619.31 cm^{-1} . The carbon bonded oxygen showed absorption at 1111.81 cm^{-1} .

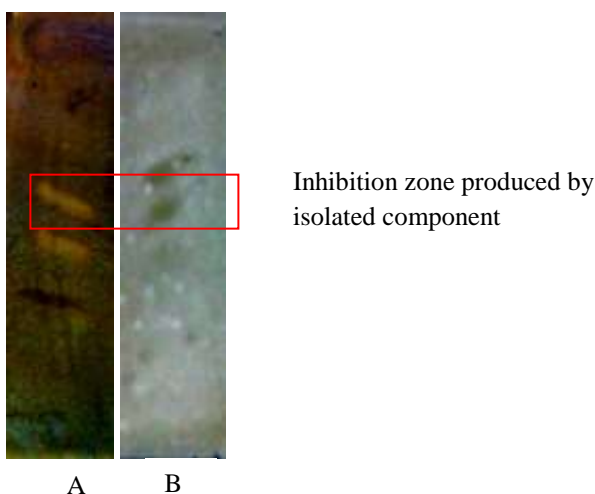


Figure 1. The agar overlay thin layer chromatography (TLC) bioautographic profile of most active fraction of *E. spiralis* stem bark. A: Zone of inhibition caused by compound against *T. tonsurans*; B: Zone of inhibition caused by compound against *T. mentagrophytes*

Extensive analysis of one-dimensional (1D) and two-dimensional (2D) NMR spectra indicated the presence of nine tertiary methyl groups at δ_H 0.89, 1.18, 1.19, 1.28, 1.32, 1.57, 1.64, 2.03 and 2.04 in aglycone molecule. The chemical shifts at δ_H 0.89 and at δ_H 1.18 were in agreement with the chemical shifts of methyl groups at δ_H 0.88 and δ_H 1.18 triterpenoid saponins from *Cylicodiscus gabunensis* (of the family Mimosaceae) that was reported previously [17]. An olefinic proton at δ_H 5.35 was coupled to a carbon at δ_C 130.04 (C-12), consistent with the previous finding regarding the same chemical shift of an olefinic proton of triterpenoid saponins [17]. Two oxymethine protons were shown at chemical shift δ_H 4.15 and δ_H 4.30, and one methylene at chemical shift δ_H at 2.30.

In ^{13}C NMR, each peak represents a carbon atom in a different environment within the molecule. The absorption peaks of the carbon methyl groups of aglycone were observed at δ_C 22.65, 22.69, 24.49, 27.19, 27.24, 28.99, 29.14, 29.36 and 29.51. In this case, there were nine different environments for carbon methyl. The chemical shift at δ_C 28.99 was quite similar to the chemical shift at δ_C 28.7 that was reported previously [14] and the chemical shift at δ_C 24.49 was similar to δ_C 24.9 [18]. The three different peaks at δ_C 62.12, δ_C 65.06 and δ_C 68.88 were due to the carbon singly bonded to oxygen. The absorption at δ_C 68.88 was in agreement with the absorption at δ_C 68.2 of triterpene glycoside of *Stryphnodendron fissuratum* (of the family Leguminosae [19]). The two peaks at δ_C 129.73 and δ_C 130.04 corresponded to the carbon-carbon double bond in the structure and the other two peaks at δ_C 172.91 and δ_C 173.36 were due to the carbon in the carbon carbonyl of an ester bond.

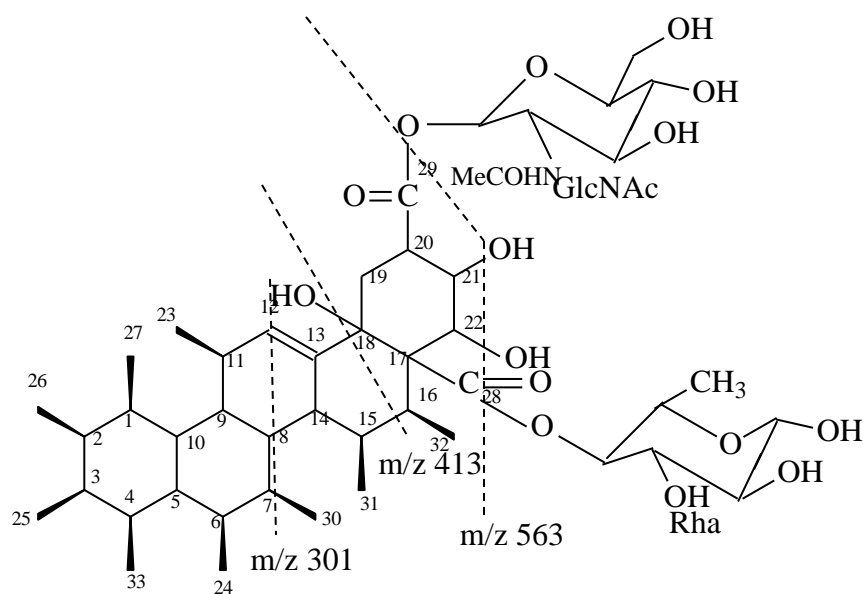


Figure 2. Mass fragmentation of ester saponin from the stem bark of *E. spiralis*

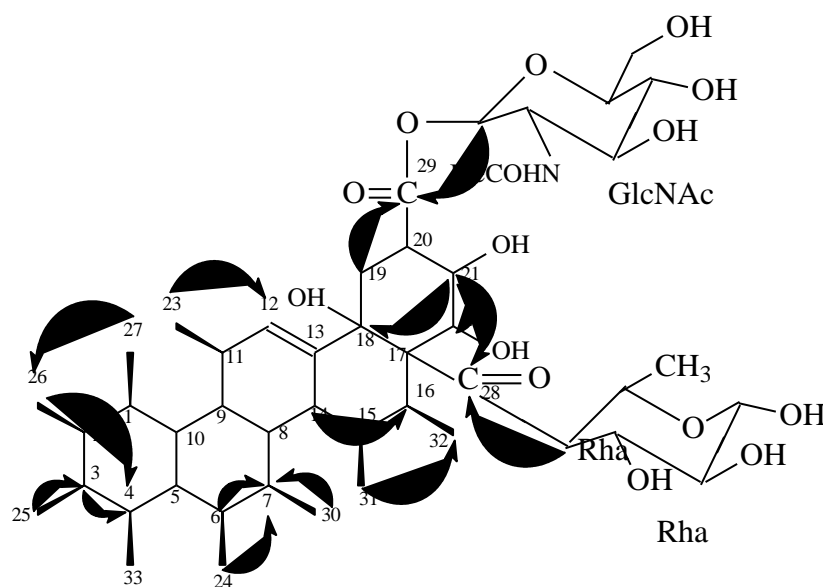


Figure 3. Key HMBC correlation of ester saponin from the stem bark of *E. spiralis*

The Heteronuclear Single Quantum Coherence (HSQC) analysis showed the direct interaction of a proton within its carbon. The data depicted all methyl, methylene and methine protons were coupled directly with the carbon and the hydroxyl protons at C-21 (δ_c 62.12) and C-22 (δ_c 65.06). However, there was no direct coupling between the proton

and carbon bonds at C-13 (δ_C 129.73), C-17 (δ_C 37.40), C-18 (δ_C 68.88), C-28 (δ_C 172.91) and C-29 (δ_C 173.76). Fig. 3 shows the Heteronuclear Multiple Bond Correlations (HMBC) analysis of the compound. The two-bond correlations could be observed between methyl proton at δ_H 0.89 (C-25) and carbon at δ_C 39.38 (C-3), between the methine proton at δ_H 1.28 (C-3) and carbon at δ_C 30.06 (C-4), between the proton at δ_H 1.28 (C-3) and proton at δ_H 1.13 (C-6) and carbon at δ_C 29.39 (C-7) and between the proton at δ_H 4.30 (C-22) and the carbon at δ_C 62.21 (C-21). The three-bond correlations were depicted between the methylene proton at δ_H 2.30 (C-19) and the carbon carbonyl at δ_C 173.76 (C-29), between the proton at δ_H 1.16 (C-14) and the carbon at δ_C 29.72 (C-16), between the methyl proton at δ_H 2.03 (C-24) and carbon at δ_C 29.39 (C-7) and between the methyl proton at δ_H 2.04 (C-23) and carbon double bond at δ_C 130.03 (C-12). The four-bond correlations could be seen between the methyl proton at δ_H 1.32 (C-26) and the carbon at δ_C 30.06 (C-4), between the proton at δ_H 4.15 (C-21) and the carbon at δ_C 68.88 (C-18), between the proton at δ_H 1.64 (C-31) and the carbon at δ_C 29.14 (C-32), between the proton at δ_H 1.57 (C-27) and the carbon at δ_C 22.69 (C-26) and between the oxymethine proton at δ_H 4.15 (C-21) and the carbonyl carbon at δ_C 172.91 (C-28).

The HMBC spectrum also displayed two sugar anomeric protons at δ_H 4.32 and at δ_H 5.28, which corresponded to the location of the sugar moieties of GlcNAc and Rha attached at δ_C 173.76 and δ_C 172.91 respectively. However, the two sugars were not attached to each other as the HMBC data did not record any correlation between them. From the chemical shift value observed for C-28 (δ_C 172.91) and C-29 (δ_C 173.76), it was suggested that the compound was a bidesmosidic glycoside with sugar linkages via an ester bond at both C-28 and C-29). Therefore, for the extensive analysis of the NMR data (1H , ^{13}C NMR, Distortionless Enhancement Polarization Transfer (DEPT), Correlation Spectroscopy (COSY), HSQC and HMBC) and mass spectrometry, this antifungal compound showed similar structure as a pentacyclic triterpene glycoside with a molecular formula of $C_{47}H_{75}NO_{16}$. The structure was identified as 28- α ,L-rhamnopyranosyl-18,21,22-trihydroxy-12-en-29-(2-acetylaminob- β -D-glucopyranosyl) triterpene ester. The proton and carbon chemical shifts and the full structure of ester saponin are shown in Table 1 and Fig. 4 respectively.

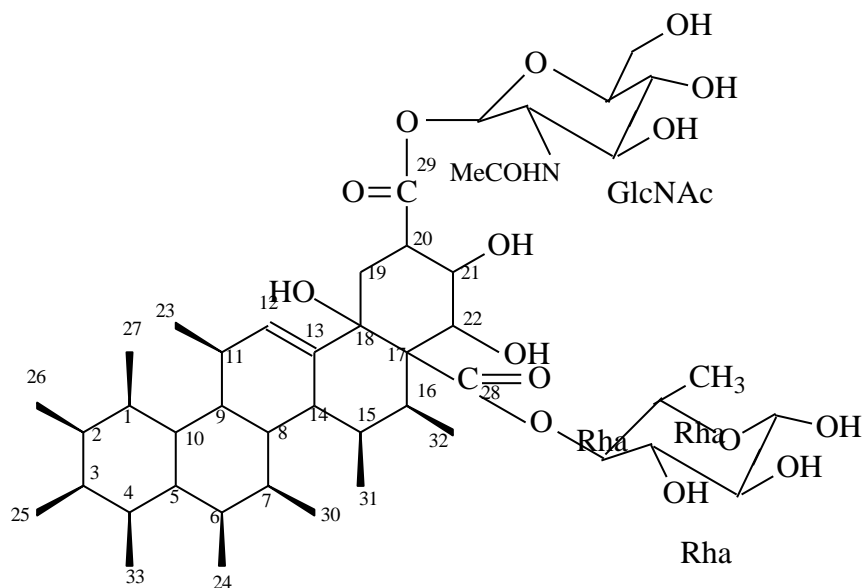


Figure 4. Chemical structure of ester saponin from the stem bark of *E. spiralis*

Table 1. ¹H NMR (600 MHz) and ¹³C NMR (600 MHz) data of ester saponin in CDCl₃.

Position	DEPT	¹³ C (δ)	¹ H δ, mult,J (in Hz)	HMBC
1	CH	37.11	1.14, m	
2	CH	24.88	1.24, m	
3	CH	39.38	1.28, m	C4
4	CH	30.06	2.08, m	
5	CH	31.95	1.02, m	
6	CH	34.06	1.13, m	C7
7	CH	29.39	2.09, m	
8	CH	27.11	2.07, m	
9	CH	29.30	1.56, m	
10	CH	24.49	1.58, m	
11	CH	37.46	1.04, m	
12	CH	130.04	5.35, m	
13	C	129.73	-	
14	CH	29.20	1.16, m	C16
15	CH	28.00	1.10, m	
16	CH	29.72	1.01, m	
17	C	37.40	-	
18	C(OH)	68.88	-	
19	CH ₂	32.79	2.30, m	C29
20	CH	29.55	1.55, m	
21	CH(OH)	62.12	4.15, m	C18, C28
22	CH(OH)	65.06	4.30, m	C21
23	CH ₃	27.24	2.04, m	
24	CH ₃	27.19	2.03, m	C7
25	CH ₃	22.65	0.89, m	C3
26	CH ₃	22.69	1.32, m	C4
27	CH ₃	24.49	1.57, m	C26
28	C	172.91	-	
29	C	173.76	-	
30	CH ₃	29.51	1.28, m	C7
31	CH ₃	28.99	1.64, m	C32
32	CH ₃	29.14	1.19, m	
33	CH ₃	29.36	1.18, m	
GlcNAc				
1	CH		4.32, m	C29
Rha				
1	CH		5.28, m	C28

According to a previous report, the known antimicrobial mechanisms associated with each chemical group in which the isolated compounds belong may explain their antimicrobial potency [20]. Membrane disruption could be suggested as one of the possible mechanisms of action of the compound. Previous investigation found that inhibition of microbial growth was depended on hydroxyl substituents group [21]. This might also explain the inhibitory effect of *E. spiralis* stem bark against dermatophytes, as hydroxyl groups were also found in this

compound. The presence of sugar molecules in the structure reduced the hydrophobicity and led to the loss of amphiphatic features [22]. The results were also consistent with previous investigation where triterpenoid saponins with hederagenin or oleanolic acid as aglycone, had been found to possess antifungal activity against *Microsporium canis* and *Trichophyton mentagrophytes* [23,24,25]. To the best of our knowledge, this is the first reported isolation of ester saponin from the stem bark *E. spiralis*.

Conclusion

The ester saponin isolated from the most active fraction of *E. spiralis* stem bark was identified as 28- α ,L-rhamnopyranosyl-18,21,22-trihydroxy-12-en-29-(2-acetylamino- β -D-glucopyranosyl) triterpene ester. The presence of a hydroxyl group is believed to enhance the antidermatophytic properties of *E. spiralis* stem bark. It is hoped that this investigation will give a new insight into the search for potent natural drugs, and also aid in the development of a standard reference for the efficacy analysis on antimicrobial properties against skin-disease-causing microbes.

Acknowledgement

This work was supported by a Science Fund grant from MOSTI (project no: 06-01-08-SF0074). The author wish to express gratitude to Kulliyah of Pharmacy IIUM, Malaysia, UiTM Kampus Khazanah Alam Pahang, Malaysia and Mr. Mohd Zahid Mohd Yusof from Universiti Kebangsaan Malaysia for carrying out NMR experiments. The author also wishes to express gratitude to Dr. Deni Susanti from Kulliyah of Science IIUM, Malaysia for guiding in structure elucidation confirmation

References

1. Mbosso, E.J.T., Ngouela, S., Nguedia, J.C.A., Beng, V.P., Rohmer, M. and Tsamo, E. (2010). In vitro antimicrobial activity of extracts and compounds of some selected medicinal plants from Cameroon. *Journal of Ethnopharmacology* 128 (2): 476-481.
2. Prasad, N.R., Anandi, C., Balasubranium S. and Pugalendi, K.V.(2004). Antidermatophytic activity of extracts from *Psoralea corylifolia* (Fabaceae) correlated with the presence of flavonoid compound. *Journal of Ethnopharmacology* 9 (1): 21-24.
3. Silva, G.M., Teles, H.L., Zanardi, L.M., Marx Young, M.C., Eberlin, M.N., Hadad, R., Pfenning, L.H., Costa-Neto, C.M., Castro-Gamboa, I., Da Silva Bolzani, V. and Araujo, A.R.(2006). Cadinane sesquiterpenoids of *Phomopsis cassiae*, an endophytic fungus associated with *Cassia spectabilis* (Leguminosae). *Phytochemistry* 67 (14): 1964-1969.
4. Doughari, J.H. (2006). Antimicrobial activity of *Tamarindus indica* Linn. *Tropical Journal of Pharmaceutical Research* 5(2): 597-603.
5. Duraipandiyar, V. and Ignacimuthu, S. (2007). Antibacterial and antifungal activity of *Cassia fistula* L.: An ethnomedicinal plant. *Journal of Ethnopharmacology* 112 (3): 590-594.
6. Khattak, K.F. and Simpson, T.J. (2010) Effect of gamma irradiation on the antimicrobial and free radical scavenging activities of *Glycyrrhiza glabra* root. *Radiation Physics and Chemistry* (79): 507-512.
7. Liu, W.C., Kugelmann, M., Wilson, R.A., and Rao, K.V. (1972). A Crystalline Saponin With Anti-tumor Activity from *Entada phaseoloides*. *Phytochemistry* 11 (1): 171-173.
8. Okada, Y., Shibata, S., Javellana A.M.J. and Kamo, O. (1988). Entada saponin (ES) II and IV, from the bark of *Entada phaseoloides*. *Chemical and Pharmaceutical Bulletin*, (36): 1264-1269.
9. Cioffi, G., Dal Piaz, F., De Caprariis, P., Sanego, R., Marzocco, S., Autore, G. and De Tommasi, N. (2006). Antiproliferative triterpene saponins from *Entada africana*. *Journal of Natural Products* 69 (9): 1323-1329.
10. Taponjdjou, A.L., Miyamoto, T., Mirjolet, J.F., Guilbaud, N., and Lacaille-Dubois, M.A. (2005). Pursaethosides A-E, Triterpene saponins from *Entada pursaetha*. *Journal of Natural Products* 68 (8): 1185-1190.
11. Ikegami, F., Sekine, T., Duangteraprecha, S., Matsushita, N., Matsuda, N., Ruangrunsi, N. and Murakoshi, I. (1989). A sulphur-containing amide from *Entada phaseoloides*. *Phytochemistry*, (28): 881-882.
12. Barua, A.K., Chakrabarty, M., Datta, P.K. and Ray, S., (1988). Phaseoloidin, a homogentistic acid glucoside from *Entada phaseoloides*. *Phytochemistry* 27 (10): 3259-3261.
13. Dai, J., Kardono, L.B.S., Tsauro, S., Padmawinata, K., Pezzuto, J.M. and Kinghorn, A.D. (1991). Phenylacetic acid derivatives and a thiomide glycosides from *Entada phaseoloides*. *Phytochemistry* (30): 3749-3752.
14. Nzowa, L.K., Barboni, L., Teponno, R.B., Ricciutelli, M., Lupidi, G., Quassinti, L., Bramucci, M. and Taponjdjou, L.A. (2010). Rheediosides A and B, two antiproliferative and antioxidant triterpene saponins from *Entada rheedii*. *Phytochemistry* 71 (2): 254-261.

15. Harun, A., Mat So'ad, S.Z., Mohd Hassan, N. and Che Mohd Ramli, N.K. (2011) In-vitro antifungal activity of *Entada spiralis* Ridl. crude extract against dermatophytes of superficial skin disease. *Revelation and Science* 1(1): 57-61.
16. Harun, A., Mat So'ad, S.Z., Mohd Hassan, N. and Che Mohd Ramli, N.K. (2014). In-vitro Antidermatophytic Activity of Methanolic Fractions from *Entada spiralis* Ridl. Stem Bark and Its Bioautographic Profile. *Pertanika Journal Science & Technology* 22 (1): 113 – 121.
17. Tene, M., Chabert, P., Note, O., Kenla, T.J.N., Tane, P. and Lobstein, A. (2011). Triterpenoid saponins from *Cylicodiscus gabunensis*. *Phytochemistry* 4 (2): 89-92.
18. Miyase, T., Melek, F.R., Ghaly, N.S., Warashina, T., El-Kady, M. and Nabil, M. (2010). Echinocystic acid 3, 16-O bisglycosides from *Albizia procera*. *Phytochemistry* 71 (11): 1375-1380.
19. Yokosuka A, Kawakami S, Haraguchi M and Mimaki Y. (2011). Seven new triterpene glycosides from the pericarps of *Stryphnodendron fissuratum*. *Phytochemistry Letters* 4 (3): 259-266.
20. Cowan, M.M. (1999). Plants product as antimicrobial agents. *Clinical Microbiology Review* 12: 564-582.
21. Mutai, C., Bii, C., Vagias, C., Abatis, D. and Roussis, V. (2009). Antimicrobial activity of *Acacia mellifera* extracts and lupane triterpenes. *Journal of Ethnopharmacology* 123 (1): 143-148.
22. Ming, D.M., Hillhouse, B.J., Guns, E.S., Eberding, A., Xie, S., Vimalanathan, S. and Towers, G.H.N. (2005). Bioactive Compounds from *Rhodiola rosea* (Crassulaceae). *Phytother Res*, 19: 740-743.
23. Du, Z., Zhu, N., Ze-ren-Wang-Mu, N. and Shen, Y. (2003) Two new antifungal saponins from Tibetan herbal medicine *Clematis tangutica*. *Planta Medica* 69: 547-551.
24. Lee, M.W., Kim, S. and Hahn, D.R. (2001) Antifungal activity of modified hederagenin glycosides from the leaves of *Kalopanax pictum* var.chinese. *Biological and Pharmaceutical Bulletin* 24: 718-719.
25. Tsuzuki, J.K., Svidzinski, T.I.E. and Shinobu, C.S. (2007). Antifungal activity of the extracts and saponins from *Sapindus saponaria* L. *Annals of the Brazilian Academy of Sciences*, 79 (4): 577-583.