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Bioassay Guided Isolation of Antibacterial Compounds from *Andrographis paniculata*

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Abstract: Problem statement: Chronic disease-causing bacteria of medical importance have developed resistance to antibiotics, hence, necessitating distinct and constant need for safe and efficient therapeutic agents. Plants are considered potent candidate for this aim. A way out of reducing antibiotic resistance and adverse effects on host is the employment of antibiotic resistance inhibitors of plant origin. **Approach:** About 5 kg pulverized *Andrographis paniculata* whole plant was macerated with MeOH at room temperature to get 305 g freeze dried MeOH extract. The bioautography of MeOH extract using *Staphylococcus aureus* and *Proteus mirabilis* as indicator organisms revealed the presence of two potent antibacterial compounds. MeOH extract was further fractionated and purified by silica gel column chromatography which led to the isolation of a diterpene lactone and an ent-labdane diterpene glycoside upon crystallization with absolute ethanol. **Results:** Two antibacterial compounds viz., 3-O-β-D-glucosyl-14-deoxyandrographolide and 14-deoxyandrographolide were successfully isolated and characterized. Their structures were exclusively elucidated through spectroscopic methods (UV, IR, ^{1}H- and ^{13}C NMR). **Conclusion:** *A. paniculata* possesses antibacterial activity and could be potential source of a new class of antibiotics that might be useful for infectious disease chemotherapy and control.

Key words: *Andrographis paniculata*, bioassay-guided isolation, antibacterial compounds

INTRODUCTION

Andrographis paniculata (Burm.f.) Wall. ex Nees., (Family-Acanthaceae) (English name-King of Bitters, Local malay name-Hempedu bumi) is an annual herbaceous plant and is extensively cultivated in Southern Asia, China and some parts of Europe. In traditional medicine, *A. paniculata* is widely used to get rid of body heat, dispel toxins from the body, prevent common cold, upper respiratory tract infections including sinusitis and fever (Gabrielian *et al*., 2002) and as an antidote against poisons of snakes and insects (Samy *et al*., 2008). *A. paniculata* has been reported to exhibit various mode of biological activities *in vivo* as well as *in vitro* viz., antibacterial (Singha *et el*., 2003; Mishra *et al*., 2009; Parvataneni and Koduru, 2010; Roy *et al*., 2010; Abubakar *et al*., 2011), antiviral (Wiart *et al*., 2000), anti-inflammatory (Wen *et al*., 2010), antihuman immunodeficiency virus (HIV) (Calabrese *et al*., 2000), immunomodulating/immunostimulatory (Iruretagoyena *et al*., 2005) and anticancer (Li *et al*., 2007; Geethangili *et al*., 2008). The characteristic secondary metabolites encountered in this plant have considerably enhanced its importance in the arena of medicinal plants. It is specifically rated high in therapeutic action in curing liver disorders, common cough and colds in human (Niranjan *et al*., 2010).

Biodiversity is a precious source for modern biotechnology. It is a source which potentially holds innovative and sustainable solutions to a broad range of important problems for modern society. Improved cooperation between the natural product chemists and the microbiologists is a constructive step to speed up the process of evaluating these potentialities. Moreover,

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microbiologists and natural product chemists in tropical countries including Malaysia, with the richest flora and fauna placed right at their door step have a very central position. They are essential for building up international scientific cooperation, with the objective of expanding our understanding of biological and biochemical diversity and based on this bringing forward more biological solutions. The entire process is built on a principle of fairness and equity in sharing of the benefits and respecting the State's sovereign right to its own resources. After structure elucidation of secondary metabolites, it is considered crucial to know how useful these molecules might be in terms of medicinal properties. During the past 40 years, numerous novel compounds have been isolated from different plants and marine organisms and many of these have been reported to have core biological activities, some of which are of interest from the point of view of potential drug development (Gerald, 2001; Houghton, 2001). In this context, *A. paniculata* could be a potential source to develop new efficacious drugs. *A. paniculata* has already been reported for its significant antibacterial potential (Singha *et al*., 2003, Mishra *et al*., 2009; Parvataneni and Koduru, 2010; Roy *et al*., 2010; Abubakar *et al*., 2011). However, no attempt has ever been made to identify and isolate active principles responsible for unleashing its antibacterial activity. Identification and isolation of active principles from *A. paniculata* might prove promising antibacterial agents through foreseeable future endeavors. Hence, this study is a conscientious attempt to identify and isolate pure antibacterial compounds from the methanol extract of the whole plant of *A. paniculata* through bioassay guided isolation method.

MATERIALS AND METHODS

Collection of plant material: About 15 kg fresh whole plant of *A. paniculata* was procured from the botanical garden of Forest Research Institute of Malaysia (FRIM), Kuala Lumpur, Malaysia, during the month of April, 2009. The plant was identified by Dr. Richard Chung Cheng Kong (Ph.D., Taxonomist, FRIM). The voucher specimen (NMPC-Q25) has been deposited in the Herbarium, Faculty of Pharmacy, IIUM, Kuantan, Pahang DM, Malaysia for future references.

Preparation of methanol (MEOH) extract: The fresh whole plant (15 kg) of *A. paniculata* was cleaned and dried in a protech laboratory air dryer (LDD-720) at 40°C for 7 days and pulverized to powdered form (5.6 kg, 37.33%) using Fritsch Universal Cutting Mill-PULVERISETTE 19-Germany. It was then stored in a desiccator at 2°C until further use. The air dried powder of whole plant (5 kg) of *A. paniculata* was extracted by macerating in double distilled methanol (20.0 L) at room temperature for 24 h, filtered and evaporated under reduced pressure. The whole process was repeated thrice to ensure maximum yield of methanol soluble compounds from the plant powder. Each time, filtrate was evaporated under reduced pressure (Buchi Rotary Evaporator, R-210) and combined. The dark blackish green residue so obtained was further freeze dried to yield 305 g (6.1%) MeOH extract and was stored at 2°C in a labeled sterile bottle until further antibacterial evaluation and isolation of antibacterial compounds.

Source of microorganism: *Staphylococcus aureus* (IMR S-277), *Streptococcus pyogenes* (IMR S-526), *Micrococcus luteus* (IMR B-7), *Proteus mirabilis* (IMR P-74) and *Pseudomonas aeruginosa* (IMR P-84) were purchased directly from the Institute for Medical Research (IMR), Kuala Lumpur, Malaysia. The bacterial stock cultures were maintained on nutrient agar slants prior to use.

Preparation of standard bacterial suspensions: The average number of viable, *S. aureus*, *S.* pyogenes, *M. luteus*, *P. mirabilis* and *P. aeruginosa* organisms per mL of the stock suspensions was determined by means of the surface viable counting technique (Hedges, 2002). About 10^7-10^8 CFU/mL was used. Each time, a fresh stock suspension was prepared; the experimental conditions were maintained constant so that suspensions with very close viable counts could be obtained successfully.

***In vitro* antibacterial activity test for MeOH extract:** The cup-plate agar diffusion method was adopted according to Kokoska *et al*. (2002) to assess the antibacterial activity of the MeOH extract. 0.6 mL of standardized bacterial stock suspensions corresponding to 10^7-10^8 CFU/mL was thoroughly mixed with 60 mL of sterile nutrient agar. 20 mL of the inoculated nutrient agar were distributed into sterile labeled Petri dishes. The agar was left to set at room temperature and in each of these plates, 3 cups 6 mm in diameter were punched using a sterile cork borer allowing at least 30 mm between adjacent wells and the agar discs were removed. Fixed volumes of the plant extract (1000, 500-250 μg mL^{-1}) were then introduced into each wells using microtiter pipette and allowed to diffuse at room temperature for 2 h. In separate wells, 30 μg each of gentamicin and vancomycin were added as positive controls whereas 10% DMSO was taken as negative control. The plates were then incubated in the upright position at 37°C for 24 h. Three replicates were carried

out for the extract against each of the test organism. After incubation the diameter of the results and growth inhibition zones were measured, averaged and the mean values were recorded.

Determination of Minimum Inhibitory Concentration (MIC): Micro broth dilution method was used for the determination of MIC values for each plant extract showing antibacterial activity against test pathogens (EUCAST, 2003; Jana *et al*., 2004). Serial dilutions of the extracts were carried out in 10% DMSO (which had no inhibitory activity against test microorganisms) to make 500 μg mL^{-1} final concentration, this was then two fold serially diluted by adding to the broth media in a 96-wells microtiter plates to obtain 250, 125, 62.5, 31.3, 15.6 and 7.81 μg mL^{-1}. Thereafter, 100 μL inoculum (10^8 CFU mL^{-1}) was added to each well. Bacterial suspensions were used as negative control, while broth containing standard drug (vancomycin and gentamicin) were used separately as positive controls. The microtiter plates were incubated at 37°C for 24 h. Each extract was assayed in duplicate; one was kept for incubation while the other was kept at 4°C for comparing the turbidity in the wells of microtiter plate. The MIC values were taken as the lowest concentration of the extracts in the well of the microtiter plate that showed no turbidity after incubation. The turbidity of the wells in the microtiter plate was interpreted as visible growth of microorganisms.

Antibacterial Activity Index (A_bI): Antibacterial index (A_bI) of MeOH whole plant extract of *A. paniculata* was calculated separately as the average value of zone of inhibition against the Gram-positive and Gram-negative bacteria, respectively (Mbwambo *et al*., 2007).

Bioassay guided isolation: To sterilized 8×4 cm silica gel 60 F_{254} TLC plates (Merck, Germany), 10 μL of MeOH extract was applied as small spots and the plates were developed in Hexane: Acetone (2:1) in duplicate (a TLC plate was used as the bioautogram while the other served as a chromatogram for reference in comparison with the bioautograph). The TLC plates were dried in an oven at 25°C for 7 h to activate the plates by absorbing the moisture content from the plates and removing all residual solvents (Veronica and Scott, 2005).

Bioautography technique: *S. aureus* and *P. mirabilis* were used as the indicator microorganisms for the bioautography of antibacterial compounds from the MeOH extract of *A. paniculata*. 200 μL each from broth cultures of *S. aureus* and *P. mirabilis* (adjusted to 10^8 CFU mL^{-1}) were mixed with 35 mL molten Mueller-Hinton agar (MHA) at 30°C separately. The suspensions of agar and bacteria were spread aseptically onto the already developed TLC plates in square Petri dishes (8×4 cm), allowed for 30 min to solidify and the plates were incubated at 37°C for 24 h. At the end of incubation time, 0.5% *p*-iodonitrotetrazolium violet (INT) was uniformly sprayed over the TLC plates. The active antibacterial compounds in the plant extracts formed clear zones of inhibition on the TLC plates against a deep pink back ground of bacterial growth, allowing the chromatographic Retention factors (Rf) observation by viewing under UV light at 254 nm (Short wave) and 366 nm (Long wave) and comparing with the reference chromatogram (already sprayed with vanillin reagent and heated at 120°C) to note the antibacterial compounds. Vanillin reagent was prepared by dissolving 15 g of vanillin in ethanol (250 mL) and H_2SO_4 (2.5 mL). Vanillin reagent gives different colored spots with different compounds on TLC plate upon heating at 120°C (Rahalison *et al*., 2007).

Identification and isolation of antibacterial compounds (AB-1 and AB-2) from MeOH extract: 100 g MeOH extract was loaded onto column (10×50 cm) packed with silica gel 60 particle size 0.063-0.2 mm (70-230 mesh) (Fluka Chemika) and the column was eluted with ten different concentrations of Hexane: Ethylacetate (9:1-1:9) and finally with Ethylacetate: Methanol (9:1-1:9) solvent systems (with gradual increase in polarity). 190 fractions were obtained and pooled to give a total of 20 (M_1-M_{20}) similar fractions based on their Rf values as indicated by TLC plate analysis in Chloroform: Methanol: Ethylacetate ($CHCl_3$: MeOH: EtOAc) (16:0.8:1.2) solvent system. Antibacterial active fractions M_9-M_{13} and M_{16}-M_{18} as indicated by bioautography of the extract afforded two crystallized compounds which were further purified by using preparative column chromatography on silica gel 60 and eluted with Hexane: Ethylacetate (9:1-1:9) to produce 76 (P_1- P_{76}) fractions. Eluents in test tubes P_1-P_{10}, P_{12}- $P_{21}$and P_{22}-P_{35} upon crystallization with absolute ethanol afforded pure antibacterial compound AB-1 (white crystals, 29 mg, Rf 0.70 ($CHCl_3$: MeOH: EtOAc, 16: 0.8: 1.2). Fractions P_{41}- P_{58} upon crystallization with absolute ethanol afforded pure antibacterial compound AB-2 (colourless crystals, 35 mg, Rf 0.64 ($CHCl_3$: MeOH: EtOAc, 16: 0.8: 1.2). Purity of both antibacterial compounds were further determined through high performance liquid chromatography (HPLC) (Fig. 3-4) and TLC plates in different binary and ternary solvent systems. Structures of both antibacterial compounds were elucidated through the integration of ^{1}H- and ^{13}C NMR spectra and comparison of their physical, chemical and spectral data

was made with the previous reported data of the same compounds.

3-O-β-D-glucosyl-14-deoxyandrographolides (AB-1): M.P. 242-244°C, UV λ_{max} MeOH nm: 202. IR (cm^{-1}) ν: 3351, 1732, 165, 899. ^{1}H NMR (600 MHz, in DMSO-*d6*), δ (ppm): 1.25 (o, 1H, C1-CH_2), 1.71 (o, 1H, C1-CH_2), 2.10 (m, 1H, C2-CH_2), 1.98 (m, 1H, C2-CH_2), 3.925 (o, 1H, C3-CH-), 1.3 (m, 1H, C5-CH-), 1.85 (m, 2H, C6-CH_2), 2.4 (m, 2H, C7-CH_2), 3.350 (d, J = 8.4 Hz, 1H, C9-CH-), 1.8 (m, 2H, C11-CH_2), 2.6 (m, 1H, C12-CH_2), 2.3 (m, 1H, C12-CH_2), 7.105 (t, 1H, C14-CH-), 4.779 (brs, 2H, C15-CH_2), 4.874 (brs, 1H, C17-CH_2), 4.594 (brs, 1H, C17-CH_2), 1.007 (brs, 3H, C18-CH_3), 4.036 (d, J = 9.6 Hz, 1H, C19-CH_2), 3.221 (d, J = 9.6, 1H, C19-CH_2), 0.662 (brs, 3H, C20-CH_3), 4.241 (d, J = 7.2 Hz, 1H, C1'-CH-), 3.379 (o, 1H, C2'-CH-), 3.394 (o, 1H, C3'-CH-), 3.570 (o, 1H, C4'-CH-), 3.595 (o, 1H, C5'-CH-), 3.842 (dd, J = 11.4, 4.8 Hz, 2H, C6'-CH_2), "o" denotes overlapping signals; ^{13}C NMR (125.76 MHz, in DMSO*d*6), δ (ppm): 38.19 (C1), 29.72 (C2), 75.04 (C3), 39.56 (C4), 56.44 (C5), 35.97 (C6), 38.44 (C7), 147.30 (C8), 56.18 (C9), 38.89 (C10), 21.72 (C11), 24.46 (C12), 136.02 (C13), 143.84 (C14), 70.11 (C15), 174.33 (C16), 107.08 (C17), 18.93 (C18), 62.61 (C19), 15.41 (C20), 103.10 (C1'), 71.72 (C2'), 72.73 (C3'), 74.03 (C4'), 76.23 (C5'), 70.72 (C6'). From this spectral data and their direct comparison with the previously published spectral data (Zhou *et al*., 2008) of the same compound, AB-1 was unambiguously identified as 3-O-β-D-glucosyl-14-deoxyandrographolide (Fig. 1).

14-deoxyandrographolide (AB-2): M.P. 172-174°C, UV λ_{max} MeOH nm: 223. IR (cm^{-1}) ν: 3367, 1736, 1646, 896. ^{1}H NMR (600 MHz, in DMSO-*d6*), δ (ppm): 1.327 (m, 2H, C1-CH_2), 2.045 (brs, 1H, C2-CH_2), 1.999 (o, 1H, C2-CH_2), 3.353 (o, 1H, C3-CH-), 1.463 (o, 1H, C5-CH-), 2.182 (brs, 1H, C6-CH_2), 1.987 (brd, J = 6 Hz, 1H, C6-CH_2), 2.451 (t, 2H, C7-CH_2), 3.506 (o, 1H, C9-CH-), 2.433 (dd, J = 4.2, 2.4 Hz, 2H, C11-CH_2), 2.557 (dd, J = 12, 7.2 Hz, 2H, C12-CH_2), 7.10 (brs, 1H, C14-CH-), , 4.91 (brs, 2H, C15-CH_2), 4.595 (brs, 1H, C17-CH_2), 4.455 (brs, 1H, C17-CH_2), 1.599 (o, 3H, C18-CH_3), 4.207 (brs, 1H, C19-CH_2), 4.189 (brs, 1H, C19-CH_2), 0.71 (s, 3H, C20-CH_3), "o" denotes overlapping signals; ^{13}C NMR (125.76 MHz, in DMSO*d*6), δ (ppm): 38.93 (C1), 37.74 (C2), 80.50 (C3), 64.13 (C4), 55.21 (C5), 28.22 (C6), 37.06 (C7), 148.94 (C8), 55.96 (C9), 42.96 (C10), 24.88 (C11), 23.76 (C12), 146.64 (C13), 127.83 (C14), 66.31 (C15), 172.17 (C16), 108.86 (C17), 22.67 (C18), 74.61 (C19), 15.16 (C20). From this spectral data and their direct comparison with the previously published data (Poonam *et al*., 2010) of the same compound, AB-2 was unambiguously identified as 14-deoxyandrographolide (Fig. 2).

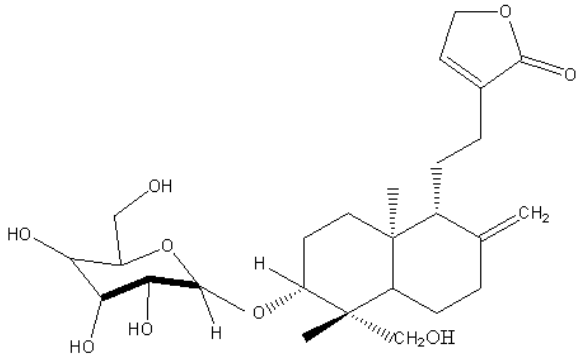


Fig. 1: Structure of AB-1 (3-O-β-D-glucosyl-14-deoxyandrographolide) based on ^{1}H- and ^{13}C NMR spectra

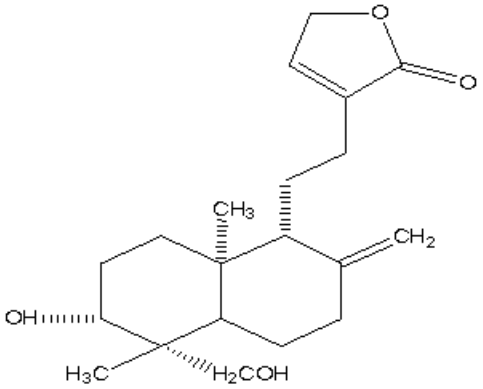


Fig. 2: Structure of AB-2 (14-deoxyandrographolide) based on ^{1}H- and ^{13}C NMR spectra

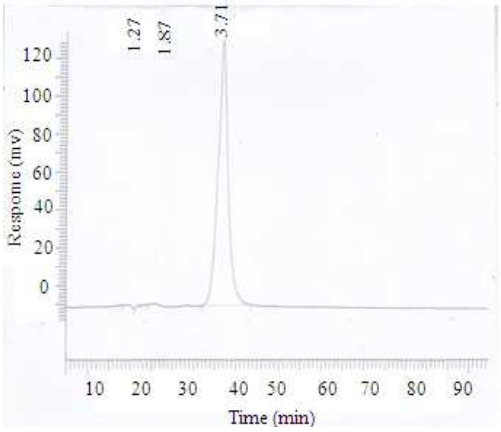


Fig. 3: HPLC analysis of compound AB-1 from the MeOH extract of *Andrographis paniculata*. Mobile phase: Water: Acetonitrile: Methanol (11:6:3) at Wave length: 223 nm, Flow rate: 1mL min^{-1} and Injection volume: 1µL

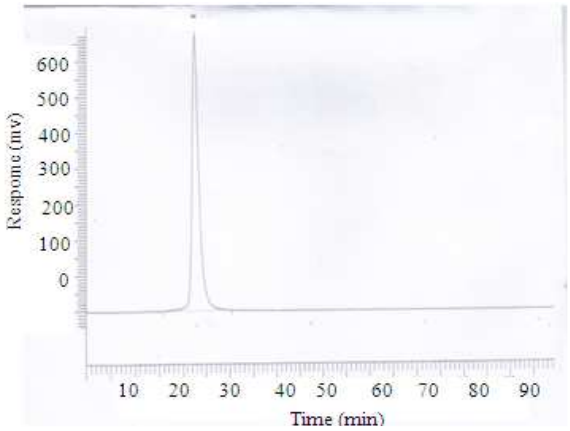


Fig. 4: HPLC analysis of compound AB-2 from the MeOH extract of *Andrographis paniculata*. Mobile phase: Water: Acetonitrile: Methanol (11:6:3) at Wave length: 214 nm, Flow rate: 1mL/min and Injection volume: 1µL

Minimum Inhibitory Concentration (MIC) of Isolated Compounds: The minimum inhibitory concentrations of the isolated compound AB-1 and AB-2 were determined using the agar dilution method following the standard protocol of the European Committee for Antimicrobial Susceptibility Testing (EUCAST, 2003). The compounds were dissolved in 10% DMSO and 2-fold diluted in MHA to obtain 250, 125, 62.5, 31.3, 15.6 and 7.81 µg mL^{-1}. The mixture of the media and compounds were thoroughly mixed and poured onto pre-labeled sterile Petri dishes on a level surface. Additional Petri dishes containing only the growth media were prepared in the same way so as to serve for comparison of growth of the respective bacteria. The plates were then set at room temperature and dried. The suspensions of the respective bacteria (corresponding to 10^8 CFU mL^{-1}) were inoculated onto the series of agar plates. The plates were then incubated at 37°C for 24 h. The experiments were performed in duplicate and MIC values expressed as the lowest concentration of the plant extracts that produced complete suppression of colony of respective bacteria.

Statistical analyses: The experimental results were expressed as mean ± Standard Deviation (STD) of triplicate experiments. Statistical differences between the antibiotics and inhibition zones formed by the plant extracts were detected by Analysis Of Variance (ANOVA) using SPSS 19.0 statistical software (SPSS, Chicago, Illinois, USA) followed by the Tukey test for multiple comparisons between means. P values lower than 0.05 ($p<0.05$) were considered significantly different whereas P values lower than 0.01 ($p<0.01$) were considered highly significant.

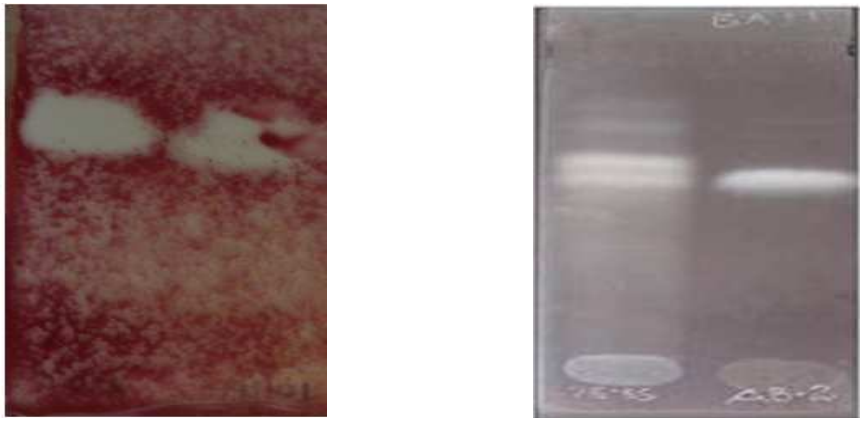
RESULTS

The results of the cup-plate agar diffusion method showed that MeOH extract of the whole plant of *A. paniculata* do possess antibacterial activity against all 5 bacteria taken into account *in vitro* (Table 1). Maximum antibacterial activity was observed against *S. aureus* (19.67 ± 0.76 mm) at 1000 µgmL^{-1} and the lowest activity was detected against *P. aeruginosa* (7.00 ± 1.50 mm) at 250 µgmL^{-1}. MIC values for MeOH extract and isolated compounds are shown in Table 2. MIC of MeOH extract ranged from 125-250 µg mL^{-1} with the highest MIC value exerted by the extract against *S. pyogenes*, *P. mirabilis* and *P. aeruginosa* (250 µg mL^{-1}) and the lowest against *S. aureus* and *M. luteus* (125 µg mL^{-1}). The bioassay-guided isolation of antibacterial compounds from MeOH extract led to the identification and subsequent isolation of an ent-labdane diterpene glycoside (AB-1) and a diterpene lactone (AB-2) as the main active principles. Both compounds were active against *S. aureus* (Fig. 5-6) and *P. mirabilis* which were used as indicator organisms by the bioautography technique on TLC plates forming clear zones against pink background of the living microorganisms when compared to the reference chromatogram. MIC values for both isolated compounds ranged from 15.6-250 µg mL^{-1}. Highest MIC value was exerted by compound AB-1 against *P. aeruginosa* (250 µg mL^{-1}) while the lowest was exerted by compound AB-2 against *S. aureus* (15.6 µg mL^{-1}), however, no activity was exerted by compound AB-1 against *M. luteus* (Table 2). The MeOH extract's antibacterial index (A_bI) was found to be the best against Gram-positive strains tested as compared to the Gram-negative strains with mean inhibition zones of 13.9 mm and 10.4 mm, respectively (Table 3).

Compound AB-1 gave positive Legal and Kedde test, suggesting the presence of an α, β-unsaturated lactone in the compound. The ^{1}H- and ^{13}C-NMR spectra of AB-1 revealed signals due to a β-glucopyranosyl group [δ_H 4.241 (d, J = 7.2 Hz, 1H)] and δ_C 103.10, 71.72, 72.73, 74.03, 76.23 and 70.72 and the characteristic signals for the double bond containing one hydrogen at carbon 14 in γ-lactone ring were observed at δ 7.105 (t, 1H) in ^{1}HNMR as well as in ^{13}C at δ 143.84, respectively, which corresponds to the 3-O-β-D-glucosyl-14-deoxyandrographolide (Zhou *et al*., 2008) (Fig. 1). AB-2 was also found to be positive for the Legal and Kedde reactions, suggesting the presence of an α, β-unsaturated lactone in the molecule.

(a) (b)

Fig. 5: Bioautography of AB-1 (3-O-β-D-glucosyl-14-deoxyandrographolide) against *S. aureus*. (a) Referenced chromatogram sprayed with vanillin/H_2SO_4 spray reagent. (b) Bioautogram against *S. aureus*



(a) (b)

Fig. 6: Bioautography of AB-2 (14-deoxyandrographolide) against *S. aureus*. (a) Bioautogram against *S. aureus*. (b) Referenced chromatogram viewed under UV light

Table 1: Antibacterial activity of MeOH extract of *A. paniculata* whole plant. Numbers indicate the mean diameters of inhibition of triplicate experiments ± standard deviation (SD)

	Zones of Inhibition (mm)				
Bacterial strains	Plant extracts	1000 µg mL^{-1}	500 µg mL^{-1}	250 µg mL^{-1}	Antibiotics (30 µg $disc^{-1}$)
Gram positive strains					
S. aureus					Vancomycin
(IMR S-277)	MeOH	19.67±0.76*	18.00±0.50	14.00±1.00**	17.00±1.05
M. luteus					
(IMR B-7)	MeOH	18.50±0.58	15.00±0.89*	13.00±0.58**	19.00±0.50
S. pyogenes					
(IMR S-526)	MeOH	16.00±0.58*	13.00±0.74	10.67±1.15**	14.50±1.00
Gram negative strains					Gentamicin
P. mirabilis					
IMR P-76	MeOH	14.50±0.58**	14.00±0.58**	10.50±1.00**	21.33±0.89
P. aeruginosa					
IMR P-84	MeOH	11.50±0.50**	09.00±1.26**	07.00±1.50**	18.50±0.50

Comparison with Antibiotics; **: P < 0.01 highly significant; *: P < 0.05 significant difference-No Activity

Table 2: Minimum inhibitory concentrations (MIC) of the MeOH extract of *A. paniculata* whole plant and isolated compounds against bacterial strains

	MIC (μg mL^{-1})		
		Compounds	
	MeOH extract	AB-1	AB-2
Gram positive			
S. aureus	125	62.5	15.6
M. luteus	125	N/A	125
S. pyogenes	250	125	62.5
Gram negative			
P. mirabilis	250	125	125
P. aeruginosa	250	250	250

*NA: No Activity

Table 3: Antibacterial activity indexes (A_bI) of MeOH extract of *A. paniculata* whole plant.

Bacterial strains	Activity index (mm) MeOH extract
Gram positive	
S. aureus, M. luteus, S. pyogenes	13.9
Gram negative	
P. mirabilis, P. aeruginosa	10.4

The characteristic NMR spectral data indicated that compound AB-2 was a labdane-type diterpene with α, β-unsaturated γ-lactone. In the ^{1}H-NMR spectrum of AB-2, two methyl singlets were observed at δ 0.71 and 1.599, respectively. The characteristic exocyclic methylene protons for AB-2 diterpenoids were observed at δ 4.595 (brs, 1H) and 4.455 (brs, 1H) in ^{1}HNMR as well as at δ 108.86 in ^{13}C, respectively. The ^{1}H- and ^{13}C-NMR (in $CDCl_3$) spectra of AB-2 suggested a diterpenoid compound with a structure similar to that of 14-deoxyandrographolide (Poonam *et al*., 2010) (Fig. 2).

DISCUSSION

Antibiotics provide the main basis for the therapy of chronic bacterial infections. However, the high genetic variability of bacteria enables them to rapidly evade the action of antibiotics by developing antibiotic resistance. As resistance becomes more common, there becomes a greater need for alternative treatments. However despite a push for new antibiotic therapies there has been a continued decline in the number of newly approved drugs (Bachi, 2002; Nagi *et al*., 2010). According to the World Health Report on infectious diseases 2000, overcoming antibiotic resistance is the major issue of the WHO for the next millennium. Hence, the last decade witnessed an increase in the investigations on plants as a source of human disease management (Paul *et al*., 2006). *A. paniculata* is common throughout Southeast Asia and India and is extensively used by traditional healers for the treatment of a wide variety of ailments (Coon and Ernst, 2004). The antibacterial activity of *A. paniculata* extracts are well known (Singha *et al*., 2003; Mishra *et al*., 2009; Parvataneni and Koduru, 2010; Roy *et al*., 2010; Abubakar *et al*., 2011). Whilst many studies have isolated and characterized *A. paniculata* compounds, no study has ever determined the antimicrobial activity of isolated compounds so far. In the present experiment, the MeOH extract of the whole plant of *A. paniculata* showed broad spectrum antibacterial activity. 3-O-β-D-glucosyl-14-deoxyandrographolide and 14-deoxyandrographolide were isolated as active principles, which may serve as lead for the development of new pharmaceuticals that might address the unmet therapeutic needs. The obvious fields where the natural product chemist can harvest benefits from a cooperation with the microbiologists are development of bioassay for efficient monitoring of isolation and purification of new compounds; bioassay fingerprinting to help early de-selection of known compounds (hereby supplementing the chemical data and giving additional avenues for tapping into the computerized data bases); activity spectrum to help de-selecting the very toxic compounds; obtaining a sharper focus in the natural product chemistry work on biologically active compounds. Novel and potentially useful may be of more interest than to go exclusively for just novelty (Houghton, 2001). Bio-autography provides more information about plant compounds requires a smaller weight of sample and can be used for the bioassay-guided isolation of biological active compounds, simplifying the process of the identification and isolation of the active compounds (Rahalison *et al*., 2007).

The antibacterial activity measured by the cup-plate agar diffusion method was more pronounced on the Gram-positive bacteria (*S. aureus*, *M. luteus* and *S. pyogenes*) than the Gram-negative bacteria (*P. mirabilis* and *P. aeruginosa*). Gram-positive bacteria were more susceptible to growth inhibition by MeOH extract of *A. paniculata* whole plant. The greater susceptibility of Gram-positive bacteria has been previously reported for South American (Paz *et al*., 1995), African (Kudi *et al*., 1999) and Australian (Palombo and Semple, 2001) plant extracts. Susceptibility differences between Gram-positive and Gram-negative bacteria may be due to cell wall structural differences between these classes of bacteria. Gram-negative bacteria have an outer phospholipid membrane carrying the structural lipopolysaccharide components. This makes the cell wall impermeable to antimicrobial chemical substances.

The Gram-positive bacteria tested were more susceptible to the plant extracts because it is well known that all Gram-positive bacteria have an outer peptidoglycan layer which is not an effective permeability barrier. The cell walls of Gram-negative organisms are more complex in lay out than the Gram-positive ones acting as a diffusion barrier and making them less susceptible to the antimicrobial agents than are Gram-positive bacteria (Nikaido, 2003). In the present study, after the first chromatography of the MeOH extract of the whole plant of *A. paniculata* on a silica gel column, the antibacterial activity of the collected fractions were tested against *S. aureus* and *P. mirabilis* using bio-autography on a TLC plate. This revealed that all the fractions except nine were active against *S. aureus* and *P. mirabilis*. Isolation of these compounds in pure form was achieved by repeated washing of the crystalline matter off the green coloring material with toluene and repeated recrystallization with absolute ethanol and final washing of the crystals with cold methanol. The purity of the sample at every stage of recrystallization was monitored through TLC and HPLC.

CONCLUSION

The TLC bioautography-guided strategy was used to separate the antibacterial compounds from the MeOH plant extract. Two antibacterial compounds were successfully isolated from MeOH extract of the whole plant of *A. paniculata* for the first time. The isolated 3-O-β-D-glucosyl-14-deoxyandrographolide and 14-deoxyandrographolide demonstrated significant antibacterial activities against the selected microbial strains. Quantitative HPLC and TLC analysis confirmed that these isolated compounds are predominant components in whole plant MeOH extract, indicating their significant contribution to the overall antibacterial activity. Further investigation of the activities of these compounds and their potential use in the treatment of bacterial diseases are still warranted. This is the first report on the isolation of antibacterial components through bioassay-guided isolation from *A. paniculata*.

Conflict of interest: None declared.

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