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## ***Andrographis paniculata* (Burm.f) Wall. ex Ness: A Potent Antibacterial Plant**

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### **1. Introduction**

Antibacterial agents of plant origin have vast therapeutic potential. They are valuable in the treatment of infectious diseases while simultaneously extenuating many of the side effects that are often associated with synthetic antibacterial agents. The beneficial medicinal effects of plant materials typically result from the combinations of secondary metabolites such as alkaloids, steroids, tannins, phenol compounds, flavonoids and resins fatty acids gums which are capable of producing definite physiological action on body (Paul *et al.*, 2006). Nowadays, multiple drug resistance has developed due to indiscriminate use of commercial antimicrobial drugs commonly used in the treatment of infectious disease. In addition to this problem, antibiotics are sometimes associated with adverse side effects on the host including hypersensitivity, immune-suppression and allergic reactions. This situation forced scientists to search for new antimicrobial substances. Giving the alarming incidence of antibiotic resistance in bacteria of medical importance, there is a constant need for new and effective therapeutic agents. Therefore, there is a need to develop alternative antimicrobial drugs for the treatment of infectious diseases from medicinal plants. 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 productive step to speed up the process of evaluating these potentialities. Moreover, microbiologists and natural product chemists in tropical countries, 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 figuring out the chemical structures 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 (Lene, 1996; Gerald, 2001).

In this context, *Andrographis paniculata* (Burm.f.) Wall. ex Nees., could be a potential source to develop new efficacious antibacterial drugs. *A. paniculata* (Acanthaceae) (King of Bitters) is an annual herbaceous plant and is widely cultivated and traditionally used in Southern Asia, China and some parts of Europe. *A. paniculata* has been effectively used in traditional Asian medicines for centuries. In traditional medicine, *A. paniculata* is widely used to get rid of a body heat, dispel toxins from the body, prevents common cold, upper respiratory tract infections including sinusitis and fever (Gabrielian *et al.*, 2002) and as an antidote against snakes and insects poisons (Samy *et al.*, 2008). *A. paniculata* has been reported to exhibit various mode of biological activities *in vivo* as well as *in vitro* viz., 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), anticancer activity (Li *et al.*, 2007; Geethangili *et al.*, 2008) and antibacterial activity (Leelarasamee *et al.*, 1990; Singha *et al.*, 2003; Zaidan *et al.*, 2005; Xu *et al.*, 2006; Voravuthikunchai *et al.*, 2006; Mishra *et al.*, 2009; Sahalan *et al.*, 2010; Abubacker and Vasanth, 2010; Katakya and Handique, 2010; Parvataneni and Koduru, 2010; Roy *et al.*, 2010; Sule *et al.*, 2011a, 2011b).

## 2. Phytochemical investigations of *A. paniculata*

The characteristic secondary metabolites encountered in *A. paniculata* 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). *A. paniculata* chiefly contains diterpenes, lactones, and flavonoids. Flavonoids mainly exist in the root, but have also been isolated from the leaves. The aerial parts contain alkanes, ketones, and aldehydes. Although it was initially thought that the bitter substance in the leaves was the lactone andrographolide, later investigations revealed that the leaves contained two bitter principles-andrographolide and a compound named kalmeghin. Four lactones-chuanxinlian A (deoxyandrographolide), B (andrographolide), C (neoandrographolide) and D (14-deoxy-11,12-didehydroandrographolide)-were isolated from the aerial parts in China (Chang and But, 1987). A diterpene glucoside (deoxyandrographolide-19- $\beta$ -D-glucoside) has been detected in the leaves (Weiming *et al.*, 1982) and six diterpenoids of the ent-labdane type, two diterpene glucosides and four diterpene dimers (bis-andrographolides A, B, C, and D) have been isolated from aerial parts (Matsuda *et al.*, 1994). Two flavonoids identified as 5,7,2',3'-tetramethoxyflavanone and 5-hydroxy-7,2',3'-trimethoxyflavone were isolated from the whole plant (Koteswara *et al.*, 2004), while 12 new flavonoids and 14 diterpenoids have been reported from the aerial parts (Chen *et al.*, 2006a, 2006b). Two new flavonoid glycosides and a new diterpenoid (andrographic acid) were recently reported (Li *et al.*, 2007), and two new ent-labdane diterpenoid glycosides were also isolated from the aerial parts of *A. paniculata* (Zhou *et al.*, 2008).

## 3. Literature review on antibacterial studies of *A. paniculata*

*A. paniculata* has been extensively used to treat a variety of conditions of infectious origin in traditional systems of medicine. Modern research has investigated it for antimicrobial activity against various pathogenic and non-pathogenic bacteria. For instance, Leelarasamee *et al.* (1990) reported that crude powder suspended in water had no *in vitro* antibacterial activity against *Salmonella*, *Shigella*, *Escherichia coli*, and *Staphylococcus aureus*, even at a concentration of

25 mg/mL crude powder. Moreover, administration of a single oral dose of powder, up to 6 g, to healthy volunteers in a randomized crossover manner or daily administration of 0.12-24 g/kg body weight to rats for six months also failed to show any *ex vivo* antibacterial activity. A similar conclusion was also reached by Zaidan *et al.* (2005) who found crude aqueous extract of leaves had no activity against *Escherichia coli* or *Klebsiella pneumoniae* but exhibited significant antimicrobial activity against gram positive *S. aureus*, methicillin-resistant *S. aureus* (MRSA), and gram-negative *Pseudomonas aeruginosa*. However, Singha *et al.* (2003) reported significant antibacterial activity of an aqueous extract and attributed it to the combined effect of andrographolides and arabinogalactan proteins. In contrast, Xu *et al.* (2006) investigated the antimicrobial activity using *A. paniculata* methanolic and aqueous extracts and authentic andrographolide against nine human bacterial pathogens. Their results indicated methanolic extracts of *A. paniculata* to be active against only two of the pathogens, while authentic andrographolide did not show any activity. They concluded that the observed antimicrobial activity was due to other active principle(s) present in the extracts that were used in the investigation. The ethanol extract was also reported to be devoid of significant antibacterial activity against enterohemorrhagic strains of *E. coli* (Voravuthikunchai *et al.*, 2006). In another study, Sahalan *et al.* (2010) reported the antibacterial activity of methanol extract of the leaves of *A. paniculata* against *Staphylococcus aureus*, *Bacillus subtilis*, *Streptococcus epidermidis*, *Escherichia coli*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*. Abubacker and Vasanth (2010) reported the antibacterial value of ethanol leaf extract against pathogenic bacteria *Escherichia coli*, *Klebsiella pneumoniae*, *Proteus vulgaris* and *Streptococcus pneumoniae*. Bioactive compound andrographolide was isolated from the leaf. The results revealed that the ethanol leaf extract and andrographolide compound are potent in inhibiting these bacteria and this work highlights that the inhibitory effect is on par with standard antibiotics. Kataky and Handique (2010) reported antimicrobial activity of various organic and aqueous extracts of eight-months old micropropagated plantlets of *A. paniculata* against gram negative (*Klebsiella pneumoniae*, *Escherichia coli*, *Pseudomonas aeruginosa*), gram positive (*Staphylococcus aureus* and *Bacillus subtilis*) bacteria. Among all tested extracts, chloroform extract showed strong inhibitory activity with all the microbes tested. Out of the five microbial test organisms *Staphylococcus aureus* was the most susceptible. The minimal inhibitory concentration (MIC) of the chloroform extract ranged from 15.625 µg/mL to 31.5 µg/mL. Roy *et al.* (2010) reported the antibacterial potential of chloroform extract of the aerial parts of *A. paniculata* against *E. faecalis* (35 mm), followed by *E. cloacae* (30 mm) *P. aeruginosa* (28 mm) and *E. coli* (25 mm). Least inhibition zone was observed against *S. aureus* (15 mm). Though the inhibition zone observed against *S. typhimurium* was only 18 mm, it is noteworthy when comparing it with that of the control result. Out of the 9 pathogenic strains tested, 7 strains showed inhibition zones comparable with that of the control (amikacin) used. The chloroform extract antimicrobial activity seen against all the tested gram-negative opportunistic and pathogenic bacteria is very encouraging and important considering the role of gram-negative bacteria in nosocomial infections leading to increased morbidity and mortality rates. Sule *et al.* (2011a) reported that *A. paniculata* extracts have bactericidal characteristic against most of the Gram positive bacteria and bacteriostatic activity against both Gram negative and Gram positive bacteria.

#### 4. Objective of current study

*A. paniculata* has already been reported for its significant antibacterial potential by many researchers across the world (Leelarasamee *et al.*, 1990; Singha *et al.*, 2003; Zaidan *et al.*, 2005;

Xu *et al.*, 2006; Voravuthikunchai *et al.*, 2006; Mishra *et al.*, 2009; Sahalan *et al.*, 2010; Abubacker and Vasanth, 2010; Katakya and Handique, 2010; Parvataneni and Koduru, 2010; Roy *et al.*, 2010; Sule *et al.*, 2011a, 2011b). However, no attempt has ever been made to identify and isolate active principles responsible for unleashing its true antibacterial activity. Identification and isolation of active principles from *A. paniculata* might prove promising antibacterial agents through foreseeable future endeavors. Hence, this study was a scrupulous attempt to identify and isolate pure antibacterial compounds from the methanol extract of the whole plant of *A. paniculata* through bioassay guided isolation method.

## 5. Methods

### 5.1 Isolation of antibacterial compounds from *A. paniculata*

#### 5.1.1 Collection of plant material and preparation of methanol (MeOH) extract

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.

The fresh whole plant (15 kg) of *A. paniculata* was cleaned and dried in a protech laboratory air dryer (FDD-720-Malaysia) 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 desiccators 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 labeled sterile bottle until further antibacterial evaluation and isolation of antibacterial compounds.

#### 5.1.2 Source of microorganism and preparation of standard bacterial suspensions

*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 from the Institute for Medical Research (IMR), Kuala Lumpur, Malaysia. The bacterial stock cultures were maintained on nutrient agar slants prior to use. 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 (Omar, 1974). About 10<sup>7</sup>-10<sup>8</sup> 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.

#### 5.1.3 *In vitro* antibacterial activity test for MeOH extract and determination of minimum inhibitory concentration (MIC)

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 and 250  $\mu\text{g mL}^{-1}$ ) were then introduced into each wells using micro titer-pipette and allowed to diffuse at room temperature for two hours. In separate wells, 30  $\mu\text{g}$  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  $37^\circ\text{C}$  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.

Micro broth dilution method was used for the determination of MIC values for each plant extract showing antibacterial activity against test pathogens (NCCLS, 2003). Serial dilutions of the extracts were carried out in 10% DMSO (which had no inhibitory activity against test microorganisms) to make 500  $\mu\text{g mL}^{-1}$  final concentration, this was then two fold serially diluted by adding to the broth media in a 96-wells micro titer plates to obtain 250, 125, 62.5, 31.3, 15.6 and 7.81  $\mu\text{g mL}^{-1}$ . Thereafter, 100  $\mu\text{L}$  inoculum ( $10^8$  CFU/mL) 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 micro titer plates were incubated at  $37^\circ\text{C}$  for 24 h. Each extract was assayed in duplicate; one was kept for incubation while the other was kept at  $4^\circ\text{C}$  for comparing the turbidity in the wells of micro plate. The MIC values were taken as the lowest concentration of the extracts in the well of the micro titer plate that showed no turbidity after incubation. The turbidity of the wells in the micro titer plate was interpreted as visible growth of microorganisms. Antibacterial index ( $A_{\text{bI}}$ ) 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 (Zakaria *et al.*, 2007).

#### 5.1.4 Bioassay guided isolation

To sterilized 8 x 4 cm silica gel 60 F<sub>254</sub> TLC plates (Merck, Germany), 10  $\mu\text{L}$  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  $25^\circ\text{C}$  for 7 h to activate the plates by absorbing the moisture content from the plates and removing all residual solvents (Veronica *et al.*, 2005).

*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  $\mu\text{L}$  each from broth cultures of *S. aureus* and *P. mirabilis* (adjusted to  $10^8$  CFU/mL) were mixed with 35 mL molten Mueller-Hinton agar (MHA) at  $30^\circ\text{C}$  separately. The suspensions of agar and bacteria were spread aseptically onto the already developed TLC plates in square Petri dishes (8 x 4 cm), allowed for 30 mins to solidify and the plates were incubated at  $37^\circ\text{C}$  for 24 h. At the end of incubation time, 0.5% *p*-Iodonitrotetrazolium Violet (INT) was sprayed on the plates for 5 mins. 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 (R.f.) 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<sub>2</sub>SO<sub>4</sub> (2.5 mL). Vanillin reagent gives different colored spots with different compounds on TLC plate upon heating at 120°C (Rahalison *et al.*, 2007).

### 5.1.5 Identification and isolation of antibacterial compounds from MeOH extract

100 g MeOH extract was loaded onto column (10 x 50 cm) packed with silica gel 60 particle size 0.063-0.2 mm (70-230 mesh) (Fluka Chemika) as stationary phase, the extracts as the mobile phase 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<sub>1</sub>-M<sub>20</sub>) similar fractions based on their R.f. values as indicated by TLC plate analysis in chloroform:methanol:ethylacetate (CHCl<sub>3</sub>:MeOH:EtOAc; 16:0.8:1.2) solvent system. Antibacterial active fractions M<sub>9</sub>-M<sub>13</sub> and M<sub>16</sub>-M<sub>18</sub> 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<sub>1</sub>- P<sub>76</sub>) fractions. Eluents in test tubes P<sub>1</sub>- P<sub>10</sub>, P<sub>12</sub>- P<sub>21</sub>, and P<sub>22</sub>- P<sub>35</sub> upon crystallization with absolute ethanol afforded pure antibacterial compound AB-1 (white crystals, 29 mg, R.f. 0.70-CHCl<sub>3</sub>:MeOH:EtOAc, 16: 0.8: 1.2). Fractions P<sub>41</sub>- P<sub>58</sub> upon crystallization with absolute ethanol afforded pure antibacterial compound AB-2 (colourless crystals, 35 mg, R.f. 0.64 (CHCl<sub>3</sub>:MeOH:EtOAc; 16: 0.8: 1.2), respectively. The purity of both antibacterial compounds were further determined through high performance liquid chromatography (HPLC) and running TLC plates in different binary and ternary solvent systems. Structures of both antibacterial compounds were elucidated through the integration of <sup>1</sup>H- and <sup>13</sup>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<sub>max</sub> MeOH nm: 202. IR (cm<sup>-1</sup>) v: 3351, 1732, 165, 899. <sup>1</sup>H NMR (600 MHz, in DMSO-*d*<sub>6</sub>), δ (ppm): 1.25 (o, 1H, C1-CH<sub>2</sub>), 1.71 (o, 1H, C1-CH<sub>2</sub>), 2.10 (m, 1H, C2-CH<sub>2</sub>), 1.98 (m, 1H, C2-CH<sub>2</sub>), 3.92 (o, 1H, C3-CH-), 1.3 (m, 1H, C5-CH-), 1.85 (m, 2H, C6-CH<sub>2</sub>), 2.4 (m, 2H, C7-CH<sub>2</sub>), 3.35 (d, J=8.4 Hz, 1H, C9-CH-), 1.8 (m, 2H, C11-CH<sub>2</sub>), 2.6 (m, 1H, C12-CH<sub>2</sub>), 2.3 (m, 1H, C12-CH<sub>2</sub>), 7.10 (t, 1H, C14-CH-), 4.77 (brs, 2H, C15-CH<sub>2</sub>), 4.87 (brs, 1H, C17-CH<sub>2</sub>), 4.59 (brs, 1H, C17-CH<sub>2</sub>), 1.007 (brs, 3H, C18-CH<sub>3</sub>), 4.03 (d, J=9.6 Hz, 1H, C19-CH<sub>2</sub>), 3.22 (d, J=9.6, 1H, C19-CH<sub>2</sub>), 0.66 (brs, 3H, C20-CH<sub>3</sub>), 4.24 (d, J=7.2 Hz, 1H, C1'-CH-), 3.37 (o, 1H, C2'-CH-), 3.39 (o, 1H, C3'-CH-), 3.57 (o, 1H, C4'-CH-), 3.59 (o, 1H, C5'-CH-), 3.84 (dd, J=11.4, 4.8 Hz, 2H, C6'-CH<sub>2</sub>), "o" denotes overlapping signals; <sup>13</sup>C NMR (125.76 MHz, in DMSO-*d*<sub>6</sub>), 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. 3).

*14-deoxyandrographolide* (AB-2): M.P. 172-174°C, UV  $\lambda_{\max}$  MeOH nm: 223. IR (cm<sup>-1</sup>)  $\nu$ : 3367, 1736, 1646, 896. <sup>1</sup>H NMR (600 MHz, in DMSO-*d*<sub>6</sub>),  $\delta$  (ppm): 1.32 (m, 2H, C1-CH<sub>2</sub>), 2.04 (brs, 1H, C2-CH<sub>2</sub>), 1.99 (o, 1H, C2-CH<sub>2</sub>), 3.35 (o, 1H, C3-CH-), 1.46 (o, 1H, C5-CH-), 2.18 (brs, 1H, C6-CH<sub>2</sub>), 1.98 (brd, J=6 Hz, 1H, C6-CH<sub>2</sub>), 2.45 (t, 2H, C7-CH<sub>2</sub>), 3.50 (o, 1H, C9-CH-), 2.43 (dd, J= 4.2, 2.4 Hz, 2H, C11-CH<sub>2</sub>), 2.55 (dd, J= 12, 7.2 Hz, 2H, C12-CH<sub>2</sub>), 7.10 (brs, 1H, C14-CH-), 4.91 (brs, 2H, C15-CH<sub>2</sub>), 4.59 (brs, 1H, C17-CH<sub>2</sub>), 4.45 (brs, 1H, C17-CH<sub>2</sub>), 1.59 (o, 3H, C18-CH<sub>3</sub>), 4.20 (brs, 1H, C19-CH<sub>2</sub>), 4.18 (brs, 1H, C19-CH<sub>2</sub>), 0.71 (s, 3H, C20-CH<sub>3</sub>), "o" denotes overlapping signals; <sup>13</sup>C NMR (125.76 MHz, in DMSO-*d*<sub>6</sub>),  $\delta$  (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. 4).

#### 5.1.6 Minimum inhibitory concentration (MIC) of isolated compounds

The minimum inhibitory concentrations of the isolated compounds 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  $\mu\text{g mL}^{-1}$ . 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<sup>8</sup> CFU/mL) 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.

#### 5.1.7 Statistical analyses

The experimental results were expressed as mean  $\pm$  standard deviation (STD) of triplicate experiments. Statistical differences between the antibiotics and inhibition zones formed by the plant extract 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.

### 6. Results

The results of the cup-plate agar diffusion method revealed that MeOH extract of *A. paniculata* whole plant extract do possess antibacterial activity against all 5 bacteria taken into consideration *in vitro* (Table 1). Maximum antibacterial activity was observed against *S. aureus* (19.67  $\pm$  0.76 mm) at 1000  $\mu\text{g mL}^{-1}$  and the least activity was detected against *P. aeruginosa* (7.00  $\pm$  1.50 mm) at 250  $\mu\text{g mL}^{-1}$ , respectively.



Bacterial strains	Plant Extract	Zones of Inhibition (mm)			Antibiotics (30 µg)
		1000 µg/ml	500 µg/ml	250 µg/ml	
Gram Positive Strains					Vancomycin
<i>S. aureus</i> (IMR S-277)	MeOH	19.67 ± 0.76*	18.00 ± 0.50	14.00 ±1.00**	17.00 ± 1.05
<i>M. luteus</i> (IMR B-7)	MeOH	18.50 ± 0.58	15.00 ± 0.89*	13.00 ±0.58**	19.00 ± 0.50
<i>S. pyogenes</i> (IMR S-526)	MeOH	16.00 ± 0.58*	13.00 ± 0.74	10.67 ±1.15**	14.50 ± 1.00
Gram Negative Strains					Gentamicin
<i>P. mirabilis</i> IMR P-76	MeOH	14.50±0.58**	14.00±0.58**	10.50 ±1.00**	21.33 ± 0.89
<i>P. aeruginosa</i> IMR P-84	MeOH	11.50±0.50**	9.00±1.26**	7.00±1.50**	18.50 ± 0.50

Comparison with antibiotics  
\*\*P < 0.01 highly significant  
\*P < 0.05 significant difference  
- No Activity

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

Bioautography of MeOH extract revealed two prominent spots on the bioautogram against *S. aureus* and *P. mirabilis* which were used as an indicator organism and consequently led to the identification and subsequent isolation of two antibacterial compounds viz., 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. 1 & 2) and *P. mirabilis* which were used as indicator organisms in the bioautography technique on TLC plates forming clear zones against pink background of the living microorganisms when compared to the reference chromatogram.

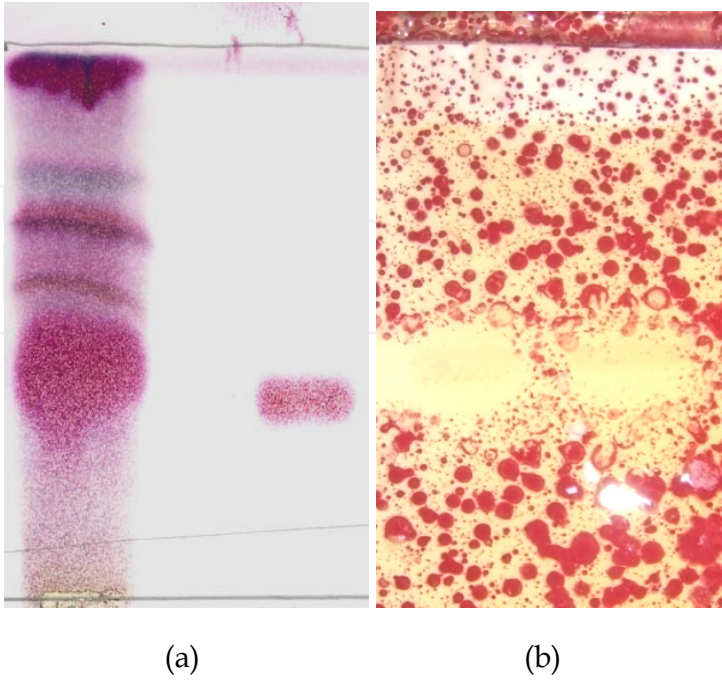


Fig. 1. Bioautography of AB-1 against *S. aureus*. (a) Referenced chromatogram sprayed with vanillin/ $\text{H}_2\text{SO}_4$  spray reagent. (b) Bioautogram against *S. aureus*.

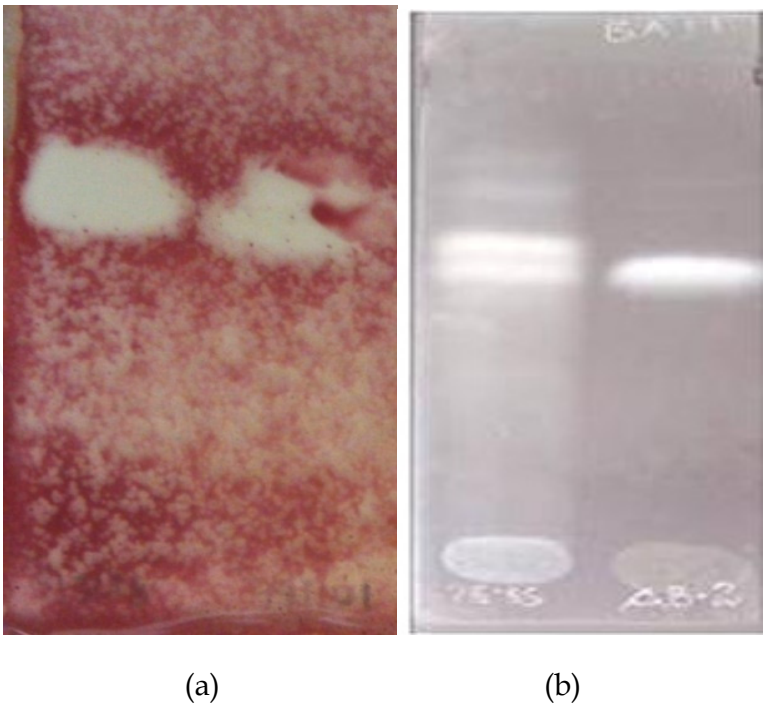


Fig. 2. Bioautography of AB-2 against *S. aureus*. (a) Bioautogram against *S. aureus*. (b) Referenced chromatogram viewed under UV light.

Minimum inhibitory concentration (MIC) values for MeOH extract and isolated compounds are shown in Table 2. MIC of MeOH extract ranged from 125-250 µgmL<sup>-1</sup> with the highest MIC value exerted by the extract against *S. pyogenes*, *P. mirabilis* and *P. aeruginosa* (250 µgmL<sup>-1</sup>) and the least against *S. aureus* and *M. luteus* (125 µgmL<sup>-1</sup>). MIC values for both isolated compounds ranged from 15.6-250 µgmL<sup>-1</sup>. Highest MIC value was exerted by compound AB-1 against *P. aeruginosa* (250 µgmL<sup>-1</sup>) while the least was exerted by compound AB-2 against *S. aureus* (15.6 µgmL<sup>-1</sup>), however, no activity was exerted by compound AB-1 against *M. luteus* (Table 2). The MeOH extract's antibacterial index (A<sub>b</sub>I) was 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).

	MIC (µg/ml)		
	MeOH	Compounds	
	Extract	AB-1	AB-2
<b>Gram Positive</b>			
<i>S. aureus</i>	125	62.5	15.6
<i>M. luteus</i>	125	N/A	125
<i>S. pyogenes</i>	250	125	62.5
<b>Gram Negative</b>			
<i>P. mirabilis</i>	250	125	125
<i>P. aeruginosa</i>	250	250	250

\*NA-no activity

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

Strains	Activity Index (mm)
	MeOH Extract
Gram Positive <i>S. aureus</i> , <i>M. luteus</i> , <i>S. pyogenes</i>	13.9
Gram Negative <i>P. mirabilis</i> , <i>P. aeruginosa</i>	10.4

Table 3. Antibacterial activity indexes (A<sub>b</sub>I) of MeOH extract of the whole plant of *A. paniculata*

Compound AB-1 gave positive Legal and Kedde test, suggesting the presence of an α, β-unsaturated lactone in the compound. The <sup>1</sup>H- and <sup>13</sup>C-NMR spectra of AB-1 revealed signals due to a β-glucopyranosyl group [δ<sub>H</sub> 4.24 (d, J=7.2 Hz, 1H)] and δ<sub>C</sub> 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  $\gamma$ -lactone ring were observed at  $\delta$  7.10 (t, 1H) in  $^1\text{H}$ NMR as well as in  $^{13}\text{C}$  at  $\delta$  143.84, respectively, which corresponds to the 3-O- $\beta$ -D-glucosyl-14-deoxyandrographolide (Zhou *et al.*, 2008) (Fig. 3).

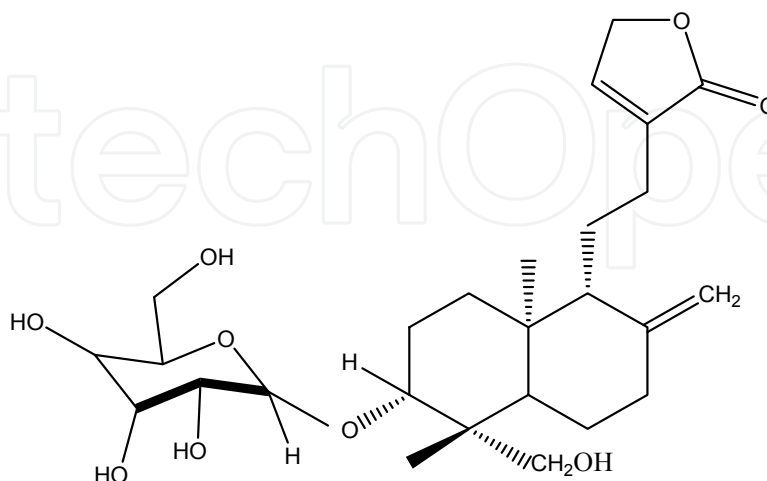


Fig. 3. Structure of AB-1 (3-O- $\beta$ -D-glucosyl-14-deoxyandrographolide) based on  $^1\text{H}$ - and  $^{13}\text{C}$  NMR spectra

AB-2 was also found to be positive for the Legal and Kedde reactions, suggesting the presence of an  $\alpha$ ,  $\beta$ -unsaturated lactone in the molecule. The characteristic NMR spectral data indicated that compound AB-2 was a labdane-type diterpene with  $\alpha$ ,  $\beta$ -unsaturated  $\gamma$ -lactone. In the  $^1\text{H}$ -NMR spectrum of AB-2, two methyl singlets were observed at  $\delta$  0.71 and 1.59, respectively. The characteristic exocyclic methylene protons for AB-2 diterpenoids were observed at  $\delta$  4.59 (brs, 1H) and 4.45 (brs, 1H) in  $^1\text{H}$ NMR as well as at  $\delta$  108.86 in  $^{13}\text{C}$ , respectively. The  $^1\text{H}$ - and  $^{13}\text{C}$ -NMR (in  $\text{CDCl}_3$ ) spectra of AB-2 suggested a diterpenoid compound with a structure similar to that of 14-deoxyandrographolide (Poonam *et al.*, 2010) (Fig. 4).

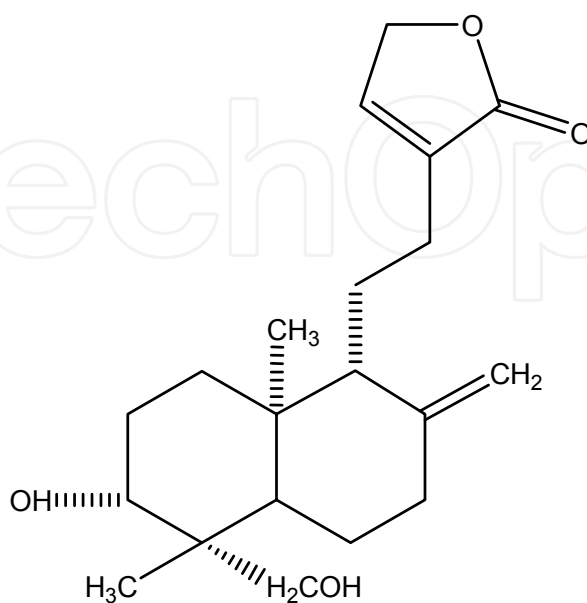


Fig. 4. Structure of AB-2 (14-deoxyandrographolide) based on  $^1\text{H}$ - and  $^{13}\text{C}$  NMR spectra.



## 7. Discussion

Antibiotics offer the core basis for the effective therapy of chronic bacterial infections. However, the high genetic variability of bacteria enables them to rapidly elude 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 (Bächi, 2002). According to the World Health Organization (WHO) report on infectious diseases in 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 (Leelarasamee *et al.*, 1990; Singha *et al.*, 2003; Zaidan *et al.*, 2005; Xu *et al.*, 2006; Voravuthikunchai *et al.*, 2006; Mishra *et al.*, 2009; Sahalan *et al.*, 2010; Abubacker and Vasanth, 2010; Katakya and Handique, 2010; Parvataneni and Koduru, 2010; Roy *et al.*, 2010; Sule *et al.*, 2011a, 2011b). 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- $\beta$ -D-glycosyl-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 to cure chronic bacterial infections effectively. 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 (Lene, 1996). 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 prominent 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 the most 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 very 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.

## 8. Conclusion

The thin layer chromatography bioautography-guided strategy was successfully used to isolate two antibacterial compounds from MeOH extract of *A. paniculata* whole plant for the first time. The 3-O- $\beta$ -D-glycosyl-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 predominate 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 sought.

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