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Isolation and characterization of aristolactam alkaloids from the stem bark of *Goniothalamus velutinus* (Airy Shaw) and their biological activities

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ABSTRACT

Phytochemical investigation on a crude methanolic bark extract of *Goniothalamus velutinus* resulted in the isolation of four compounds, aristolactam I (**1**), aristolactam BII (**2**), aristolactam AII (**3**) and velutinam (**4**). Compounds (**1**) and (**2**) have previously been isolated from the bark of *G. velutinus* but this is the first report on isolation of compounds (**3**) and (**4**) from this specie. Antibacterial activities of these compounds were tested against Gram-positive bacteria (*Bacillus subtilis*, *Bacillus spizizenii* and *Staphylococcus aureus*) but none of them showed any antibacterial activity under the tested concentrations. These compounds were also tested for anti-biofilm activity against *Klebsiella pneumoniae*, *S. aureus*, *Streptococcus mutans* and *Proteus mirabilis* and among the four compounds only velutinam (**4**) (70 μg/mL) inhibited biofilm formation of both *S. mutans* and *P. mirabilis* up to 41.3 and 78.9%, respectively. Whereas, the compounds aristolactam BII (**2**) (90 μg/mL) and aristolactam AII (**3**) (140 μg/mL) were active against *S. mutans* and inhibited their biofilm formation to 72.8% and 89.9%, correspondingly. In addition aristolactam I (**1**) did not show any inhibition of biofilm against the microorganism used in this study. The anticancer activity of velutinam (**4**) was evaluated against various cell lines including Human lung adenocarcinoma epithelial cells (A549), human embryonic kidney cells (HEK 293) and epidermoid cervical carcinoma cells (CaSki). The MTT antiproliferative assay resulted in IC_{50} values of 21.57 μg/mL, 13.28 μg/mL and 10.97 μg/mL for A549, HEK 293 and CaSki, respectively. Although anticancer activity of velutinam (**4**) has been reported on HeLa and L1210 cell lines, it has never been reported for the cell lines under this study. To our knowledge, this is the first report on anticancer activity of velutinam (**4**) on these cell lines.

1. Introduction

Goniothalamus velutinus belongs to the genus *Goniothalamus* (family: Annonaceae), locally known as "Limpanas hitam or Talipanas hitam," and is being used as a traditional medicine by natives and some ethnics (Kedayan, Iban) of Borneo for treating headache, food poisoning, as well as snake bite remedies. It is also used to induce abortion and as a post-partum remedy (Galappathie et al., 2014; Omar et al., 1992). The stem is also believed to have repellent powers against snakes and wild animals and is believed to protect the house from evil spirits. Light smoke of simmering branches (leaves and stems) is recommended as a remedy for seizures. It is also used as a traditional medicine for the treatment of giddy, injury, diarrhea, aphrodisiac, body pain, cold, stomachache,

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swollen, headache, to maintain body health and as mosquito repellent (Wong, 2008). Phytochemical screening on methanolic extract of *G. velutinus* stem bark and leaf showed the presence of alkaloids, terpenoids, tannins and glycosides (Iqbal et al., 2015). Moreover, acetogenins, styryl lactones, terpenoids, cyanogenic glycoside, aristolactams and other endogenous metabolites have also been isolated from *Goniothalamus* species that showed a wide range of bioactivities which include antibacterial, antimalarial, anti-inflammatory, antitumor, antiviral and anti-larvicidal (Cai et al., 2004; Miliauskas et al., 2004; Priestap, 1985; Wiart, 2007). Previous investigation on *G. velutinus* bark extract revealed the isolation of two flavonoids (pinocombrine, naringinin), one styryl lactone (goniothalamin) and two aristolactams (velutinam and aristolactam BII) (Fasihuddin et al., 2010; Fassihuddin, 2004; Omar et al., 1992).

Aristolactams are a small group of aporphinoid alkaloids containing phenenthrene chromophore that were first isolated from *Aristolochia argentina* (Aristolochiaceae), which is the richest source of this family of alkaloids. Aristolactams are restricted to the families Monimimaceae, Annonaceae, Aristolochiaceae, Menispermaceae, and Piperaceae (Kumar et al., 2003). They are classified as di-, tri-, tetra- and penta-substituted derivatives according to their substitution patterns. With few exceptions, the substituents include methoxy, hydroxy and methylenedioxy groups. Plants containing aristolactam alkaloids have also been used in traditional medicine to treat various diseases. They were known to exhibit many pharmacological activities including cytotoxic, antioxidant, platelets aggregation, neuro-protective and anticancer activities (Chia et al., 2006; Costa et al., 2013; Hongthong et al., 2014; Jung et al., 2009; Urzúa et al., 2013).

This report describes the identification, purification and characterization of four aristolactams isolated from *G. velutinus* crude methanolic extract of stem bark from Brunei Darussalam. Furthermore, this study also investigates the antibacterial activity of all isolated compounds and anticancer activity of compound (**4**).

2. Material and methods

2.1. General experimental procedures

Extensive column chromatography and preparative thin layer chromatography (PTLC) were carried out on silica gel 40 (230–400 mesh) and Polygram Sil N-HR/UV_{254} (Macherey–Nagel), respectively. ^{1}H NMR (600 MHz), ^{13}C NMR (500 MHz), HMBC, HSQC, 2D NMR (^{1}H–^{1}H) COSY and NOESY data were obtained on Bruker Avance NMR spectrophotometer, UV spectra were measured on Thermo scientific Evolution 300 UV spectrophotometer, FTIR spectra were carried out in KBr disc using FTIR-8900 Schimadzu spectrometer, HR-ESI-MS were recorded on AB Sciex Qstar XL MS/MS system and melting point was determined using a Stuart UK melting point apparatus.

2.2. Plant material

Stems of *G. velutinus* were collected from Bukit Panjang in Kampung Kulapis from Brunei Darussalam in February 2013. Two voucher specimens of the plant, each with a reference number EI1, were deposited in the National Herbarium of Brunei Darussalam (BRUN) and the Universiti of Brunei Darussalam Herbarium (UBDH). Bark was removed from collected stems, rinse with distilled water to remove dirt and then cut into small pieces and freeze dried until a constant mass was obtained. Dried samples were ground into a fine powder and stored in desiccators until extracted.

2.3. Extraction and isolation

Stem bark powder (1.0 kg) was extracted with petroleum ether (PE, 5 L), followed by dichloromethane (CH_2Cl_2, 5 L) and methanol (MeOH, 5 L) in a soxhlet apparatus for 10 h each. Subsequently each extract was evaporated to dryness to give 5.82 g (0.58%), 21.62 g (2.16%) and 35.13 g (3.51%) of PE, CH_2Cl_2 and MeOH crude extracts, respectively.

The MeOH crude extract of the stem bark (7.5 g) was fractionated by column chromatography on silica gel (CC), eluted with a gradient system of petroleum ether-diethyl ether (PE:DE) in increasing ratio of 0 to 100% DE, giving eight sub-fractions and yellow crystals deposits in fractions 5 and 6, which were then combined. These deposits (78 mg) were further separated on silica gel CC with an isocratic solvent system of CH_2Cl_2–MeOH (12.5–0.5) to give compound (**4**) as a brownish yellow crystals (20 mg, 0.27% of extract, 0.01% of dry weight bark). Fraction 3 was further separated on silica gel CC, eluted with a gradient system of petroleum ether-diethyl ether in increasing ratio of 0 to 100% diethyl ether to give 25 mg of sub-fraction which was further purified by PTLC using PE–DE (1:2) to give compound (**1**) as a yellow amorphous powder (6 mg, 0.081% of extract, 0.003% dry weight of bark). Purification of combined fractions 4, 5 and 6 (brownish color) by preparative TLC yielded compound (**2**) (25 mg, 0.18% of the extract, 0.012% of the dry weight of bark) and (**3**) (6.1 mg, 0.081% of extract, 0.003% dry weight of bark) as a yellow amorphous powder.

2.4. Media and chemicals

2.4.1. For antibacterial assays

Ethanol, nutrient agar, nutrient broth, Mueller-Hilton broth, Mueller-Hilton agar, sterile petri dishes, sterile cotton swabs, antibiotic discs of penicillin (10 units), tetracycline (30 μg), and erythromycin (15 μg) were from Oxoid Ltd., UK. The standard pure cultures of *Staphylococcus aureus* (BioMérieux, Lyfoculs ATCC#25923), *Bacillus subtilis* (BioMérieux, Lyfoculs ATCC 6633), *Bacillus spizizenii* (Microbiologics, ATCC#6633) were used. $BaCl_2$ and H_2SO_4, were of analytical grade from Merck. Filter paper discs (6 mm) were made from Whatman filter paper #4.

2.4.2. For Anti-biofilm

Klebsiella pneumoniae ATCC 13882, *Proteus mirabilis ATCC* 12453, *Streptococcus mutans* ATCC 25175 and *S. aureus* ATCC 43300 were used for the biofilm inhibition assay. All the microorganisms except *S. mutans* were grown in tryptic soya broth and agar (Oxoid, UK). *S. mutans* were grown and maintained in heart infusion broth (Oxoid, UK) supplemented with 1% sucrose.

2.4.3. Cell lines and culture conditions

Human lung adenocarcinoma epithelial cells (A549) (CCL-185), human embryonic kidney cells (HEK 293) (CRL-1573), epidermoid cervical carcinoma cells (CaSki) (CRL-1550), were obtained from the ATCC (Manassas, VA). These cells were grown in Dulbecco's Modified Eagle Medium (Gibco Life Technologies, USA) supplemented with 10% fetal bovine serum. Cells were incubated at 37 °C in a humidified atmosphere of 5% CO_2.

2.5. Bioassays

2.5.1. Antibacterial assay by disc diffusion method

The disc diffusion method was used to test the activity of all the isolated compounds against selected Gram positive bacteria (Hussain et al., 2011; Sadiq et al., 2015). Briefly, Mueller–Hilton (MH) agar plates were inoculated with test microorganisms taken from broth culture, prepared from an overnight culture and

adjusted to 0.5 McFarland standard turbidity (approx. 10^6 CFU/mL). Then filter paper discs were soaked with 10 μL of compound (**1**) (16 mg/mL), compound (**2**) (25 mg/mL), compound (**3**) (16 mg/mL) and compound (**4**) (25 mg/mL), antibiotic discs and a negative control (dimethyl sulfoxide [DMSO] only) were added to the different regions of the plates and incubated for 24 h at 37 °C. Penicillin (10 units), erythromycin (15 μg) and vancomycin (30 μg) antibiotics were used as positive control against *S. aureus* (*SA*), *B. subtilis* (*BS*) and *B. spizizenii* (*BSp*), respectively. Antibacterial activity was evaluated by measuring the diameter of the zone of inhibition produced by the compounds (**1**–**4**) compared with the positive control antibiotics. For each test microorganism, three replicates were carried out.

2.5.2. Anti-biofilm activity

Anti-biofilm activities of the extract and pure compounds were determined by microtiter plate crystal violet assay. Briefly, cultures were refreshed in their respected media overnight at 37 °C. Compounds (**1**) and (**4**) were serially diluted in the range of 71–0.14 μg/mL, compound (**2**) was diluted in the range of 90–0.176 μg/mL and compound (**3**) was diluted within the range of 143–0.28 μg/mL in their respective media. 11th and 12th wells served as positive (media plus culture) and negative (only media) controls. In addition 6-amino penicillinic acid was also used as positive control. An inoculum containing 5×10^5 cells was inoculated in all the wells except negative control. After inoculation, plates were incubated at 37 °C for 24 h and were stained for biofilm formation as described previously (O'Toole et al., 1999) with few modifications. Plates were washed with sterile distilled water thrice to remove planktonic bacteria and heat fixed for 30 min. Wells were stained with 0.1% (w/v) crystal violet for 20 min. Stained plates were rewashed and dried. Crystal violet retained by the biofilms was dissolved in 30% (v/v) glacial acetic acid. Absorbance of plates was measured at 590 nm using a micro-titer plate reader (Tecan, USA). Biofilm percent inhibition was calculated by following formula:

$$\%\text{biofilm inhibition} = \{(\text{O.D. in control} - \text{O.D. of test})/\text{O.D. in control}\} \times 100$$

2.5.3. MTT proliferation assay

The antiproliferative activity of the isolated compound (**4**) was determined using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphe nyltetrazolium bromide; thiazolyl blue) assay (Dantu et al., 2012; Yessoufou et al., 2015; Talib and Mahasneh, 2010). Cells were seeded in 96-well plates at a concentration of 1×10^5 cells/mL (100 μL). After an overnight incubation, different concentrations of the compound (**4**) (100 μL) was added to give a final concentration of 0 to 125 μg/mL and then the cells were further incubated for 72 h at 37 °C.

After 72 h, the medium was removed and MTT (200 μL, 0.5 mg/mL) was added to each well and incubated for 4 h at 37 °C. After incubation, MTT was removed and the formazan crystals were solubilized by adding DMSO (150 μL) (Bartlett, 2000; Kolundžić et al., 2016; Siregar and Akbar, 2000).

A stock solution of the compound (**4**) was prepared in DMSO and then diluted with the medium. The final dilution of the compound used for treating the cells contained not more than 0.1% DMSO. The blank contained only medium and the control group contained the same amount of DMSO in medium (0.1%) as in treated cells. The absorbance reading of the blank was subtracted from the control and the treated cells then the% survival was measured using the following formula.

$$\%\text{survival} = [\text{Abs (sample)}/\text{Abs (control)}] \times 100$$

IC_{50} values were calculated by plotting a graph of the log concentration versus% survival.

3. Results and discussion

Compound (**1**) was obtained as a yellow amorphous powder with melting point of 292–293 °C which then decomposed above this melting point. Its R_f was found to be 0.62 in toluene-ethyl acetate (1:1). It has a molecular formula $C_{17}H_{11}NO_4$ determined by HRESIMS 293.0766. It gave *m*/*z* 294.1 $[M+H]^+$, and ESIMSMS scan gave fragments at 279.1, 251.1, 239.1, 221, 193.1 and 167. Its UV spectrum in methanol showed characteristic of the phenanthrene chromophore with absorption at 208, 242,260, 288, 331 and 394 nm. In the FTIR spectrum (KBr), an intense band at 1699 cm^{-1} with the shoulder at 1650 cm^{-1} indicates C=O stretching of lactam, suggesting that the compound (**1**) belongs to lactam group (Chanakul et al., 2011; Omar et al., 1992; Sun et al., 1987).

A complete structure elucidation was carried out by examination of NMR data in DMSO-d_6 (Table 1). The ^1H NMR spectrum revealed five signals of aromatic proton including two singlet at δ 7.66 and 7.36 for uncoupled protons at H-2 and H-9 together with H-5, H-6, H-7 at δ 8.14 (d, J = 8.04 Hz), 7.51 (t, J = 7.9 and 8.0 Hz), 7.20 (d, J = 7.92 Hz) respectively, all showing *ortho* coupling. These assignments were supported by HMBC correlation between H-2 to C-3, C-4, C-10a, CO, H-5 to C-4a, C-4B, C-7 and C-8, H-6 to C-4b, C-7, C-8, H-7 to C-5, C-8, C-8a, H-9 to C-10 and C-10a. Apart from these aromatic protons, one NH proton, one methoxyl group and one singlet for CH_2 also appeared at δ 10.77 (s), 3.98 (s) and 6.47 (d, J = 5.0 Hz), respectively. HMBC correlation of methoxyl group to C-8 also confirmed its assignment to C-8, and methylene protons correlate with C-3 and C-4. Examination of the NOESY spectrum also revealed NOE correlation between the methoxyl ^1H signal at δ 3.98 singlet and the doublet at δ 7.20 (assigned to H-7). Therefore on the basis of spectroscopic data the compound (**1**) was identified as aristolactam I (8-methoxybenzo[f]-1,3-benzo dioxolo[6,5,4-cd]indol-5(6H)-one). Fig. 1 shows the structure of compound (**1**–**4**).

Compound (**2**) was also obtained as a yellow amorphous powder with a melting point of 258–259 °C. R_f value in toluene-ethylacetate (1:1) was 0.54. The molecular formula $C_{17}H_{13}NO_3$ was confirmed by its HRESIMS 279.0973, which gives peak at *m*/*z* 280.1 $[M+H]^+$ and its MSMS scan gave fragments of *m*/*z* $[264.1]^+$, $[236.1]^+$, $[219]^+$. The UV absorption in methanol showed maxima at 242, 260, 277, 288, 331 and 394 nm, which corresponds to the phenenthrene chromophore which was further confirmed by FTIR spectrum showing peaks at 1712 cm^{-1} (C=O) with a shoulder at 1647 cm^{-1}. IR spectrum also revealed NH stretching at 3220 cm^{-1}.

A complete structure elucidation of compound (**2**) was carried out by NMR spectroscopy (^1H, ^{13}C, DEPT 90, DEPT 135, HMBC, HSQC, J resolve, NOESY, COSY) in DMSO-d_6. ^1H NMR indicates six aromatic proton signals at δ 9.11 (dd, J = 1.1, 5.2, 7.8, Hz, H-5), δ 7.55 (dddd, 1.2, 7.2, 6 and 8.4 Hz, H-6 and H-7) and δ 7.92 (dd, J = 0.6, 8.4, 7.8 Hz, H-8) showing AMX coupling pattern. Two singlets appearing at δ 7.84 and δ 7.12 are assigned to H-2 and H-9 correspondingly. These assignments were well supported by the HMBC correlation between H-2 to C-3, C-4, C-10a, and C=O, H- 5 to C-4a, C-4b and C-7, H-6 and H-7 to C-5, C-7 and C-8, H-8 to C-7 and C-9, and H-9 to C-8, C-8a, C-10 and C-10a (Fig. 2). In addition singlets were also observed for the proton of NH at δ 10.81, and two methoxyl groups at δ 4.03 and δ 4.02. NH proton shows HMBC correlation to C-1, C-10 and C-10a, while methoxy group at position 3 shows correlation with C-3 and methoxyl group proton at position 4 shows correlation with C-4. Examination of NOESY spectra also confirmed the positions of methoxyl groups at 3 and 4,

Table 1
^{1}H and ^{13}C NMR spectral data (δ values) for 1, 2, 3 and 4 in DMSO d_6.

Cpd.	Aristolactam I $C_{17}H_{11}NO_4$		Aristolactam BII $C_{17}H_{11}NO_3$		Aristolactam AII $C_{16}H_{11}NO_3$		Velutinam $C_{17}H_{13}NO_4$	
Assig.	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C
1	–	119.33	–	121.72	–	121.84	–	120.17
2	7.66 s	105.81	7.84 s	110.80	7.59 s	113.38	7.83 s	109.91
3	–	148.94	–	155.32	–	152.23	–	154.20
4	–	147.22	–	150.66	–	148.83	–	150.51
4a	–	111.0	–	135.19	–	121.84	–	121.59
4b		124.02	–	120.19	–	125.98	–	123.94
5	8.14 d *(8.04)*	118.80	9.11 dd *(1.1, 5.2,7.8)*	127,74	9.09 dd *(2, 7.8)*	126.78	8.61 dd *(1.2, 8.2)*	117.95
6	7.51 t *(7.9, 8.0.15.9)*	125.86	7.55 ddd *(1.2, 7.2, 6, 8.4)*	125.72	7.55 dddd *(1.2, 5.8,7.2, 6.3, 2.94, 6.9)*	127.29	7.35 t *(7.9, 8.1)*	125.76
7	7.20 d *(7.92)*	108.38	7.55 dd *(1.2, 7.2, 6, 8.4)*	127.12	7.55 dddd *(1.2, 5.8,7.2, 6.3, 2.94, 6.9)*	125.30	7.06 dd *(1.2, 7.9)*	112.01
8	–	155.32	7.92 d *(0.6, 8.4, 7.8)*	129.28	7.92 dd *(1.2, 7.2, 8.4)*	128.98	–	153.72
8a	–	124.88	–	126.21	–	135.31	–	127.06
9	7.36 s	97.94	7.12 s	104.90	7.08 s	103.89	7.40 s	98.69
10	–	134.77	–	135.08	–	134.84	–	133.88
10a	–	124.99	–	123.52	–	122.31	–	123.29
C=O	–	168.17	–	168.63	–	168.47	–	168.33
N–H	10.77 s	–	10.813 s	–	10.77 s	–	10.77 s	–
3-OCH_3	–	–	4.03 s	57.04	–	–	4.03 s	56.94
4-OCH_3	–	–	4.02 s	60.09	4.01 s	59.46	3.99 s	59.8
8-OCH_3	3.98 s	55.88	–	–	–	–	–	–
3a-CH_2	6.47 s	103.34	–	–	–	–	–	–
3-OH	–	–	–	–	10.30 s	–	–	–
8-OH	–	–	–	–	–	–	10.10 s	–

Coupling constant are (J in Hz) in parenthesis. Assignments were made on the basis of ^{1}H–^{1}H Cosy, ^{1}H–^{1}H NOESY, HMQC, and HMBC pulse sequences, ^{13}C NMR multiplicities were determined by broad band and DEPT experiments, ^{1}H NMR on 600 MHz, ^{13}C NMR on 500 MHz.

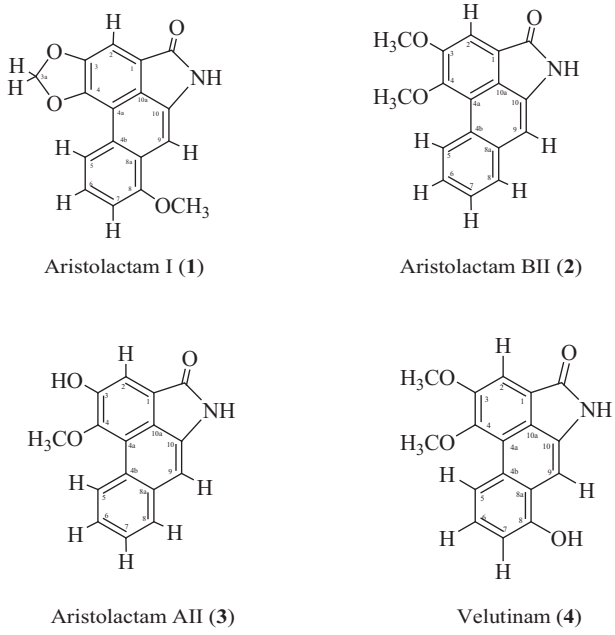

Fig. 1. Structure of compound (**1**–**4**).

showing NOE correlation between methoxyl signal at δ 4.03 and the singlet at δ 7.84 (assigned to H-2) and between the methoxyl signal at δ 4.02 and a doublet at δ 9.11 (H-5). Thus the methoxyl group which resonates at δ 57.04/4.03 ($^{13}C/^{1}H$ resonance) is present at C-3 (δ 155.32), while the other resonating at δ 60.09/4.02 ($^{13}C/^{1}H$) is at C-4 (δ 150.66). The ^{13}C assignment of C-3 and C-4 is further confirmed by literature showing similar assignment in other 3,4-dimethoxyphenanthrene lactam, which proposed that the chemical shift of C-3 is normally downfield relative to C-4 due to the nonplanar nature of methoxyl group at C-4. Upon examination of all these spectral data it becomes obvious that the compound (**2**) is aristolactam BII (10-amino-3, 4-dimethoxy phenanthrene-1-carboxylic acid lactam). Fig. 2 shows the HMBC correlation for compound (**1**–**4**).

Compound (**3**) was obtained as a pale yellow powder with melting point 271–272 °C and R_f value in toluene–ethylacetate (1:1) was 0.41. Its molecular formula $C_{16}H_{11}N0_3$ was deduced from HRESIMS 265.0817, having m/z 266.1 $[M+H]^+$. The fragmentation patterns of the compound were 251.1, 223.1, 195.1 and 167.1 which was similar to that reported in the literature for aristolactam-AII (Sun et al., 1987; Urzúa et al., 2013). Its UV spectrum in methanol shows λ_{max} at 246, 299 and 405 nm, which correspond to phenenthrene chromophore. The IR spectrum shows peak similar to compounds (**1**) and (**2**), giving peak at 1670 cm^{-1} (C=O) with a shoulder at 1614 cm^{-1} (C=C stretching of aromatic ring). In addition a broad peak of OH at 3419 cm^{-1} which is overlapping NH region, –C–H stretch of methyl group at 2921 cm^{-1} was also observed with other peaks.

A complete structure elucidation of compound (**3**) was carried out by NMR spectroscopy in DMSO-d_6. The ^{13}C NMR chemical shifts suggested that 14 of the 16 carbons were aromatic, one acyl carbon (δ 168.47) and one methoxyl group (δ 59.46). The ^{1}H NMR signals showed the presence of six aromatic protons, two singlet uncoupled protons at δ 7.59 (assigned to H-2) and at δ 7.08 (assigned to H-9), and four aromatic protons at H-5 δ 9.09 (dd, J = 2, 7.8 Hz), H-6 and H-7 at δ 7.55 (dddd, J = 1.2, 5.8, 7.2, 6.3, 2.9, 6.9 Hz) and H-8 at δ 7.92 (dd, J = 1.2, 7.2 and 8.4 Hz) showing AMX coupling pattern. This assignment was furthered confirmed by HMBC correlations showing correlation of H-2 to C-1, C-3, C-4, C-10a, C = 0, H-5 to C-4b, C-6, C-8a, H-6 to C-5, H-7 to C-8, H-8 to C-6, C-7 and C-9, H-9 to C-4a, C-4b, C-8a, C-10. Furthermore three singlets appear in ^{1}H NMR, showing NH proton at δ 10.77, methoxyl proton at δ 4.01 and OH proton signal at δ 10.30. The hydroxyl group (OH) was assigned at C-3 on the basis of ^{13}C chemical shift at C-2 which in the presence of OH group gives upfield resonance at δ 113.38 due to its shielding effect. The location of a

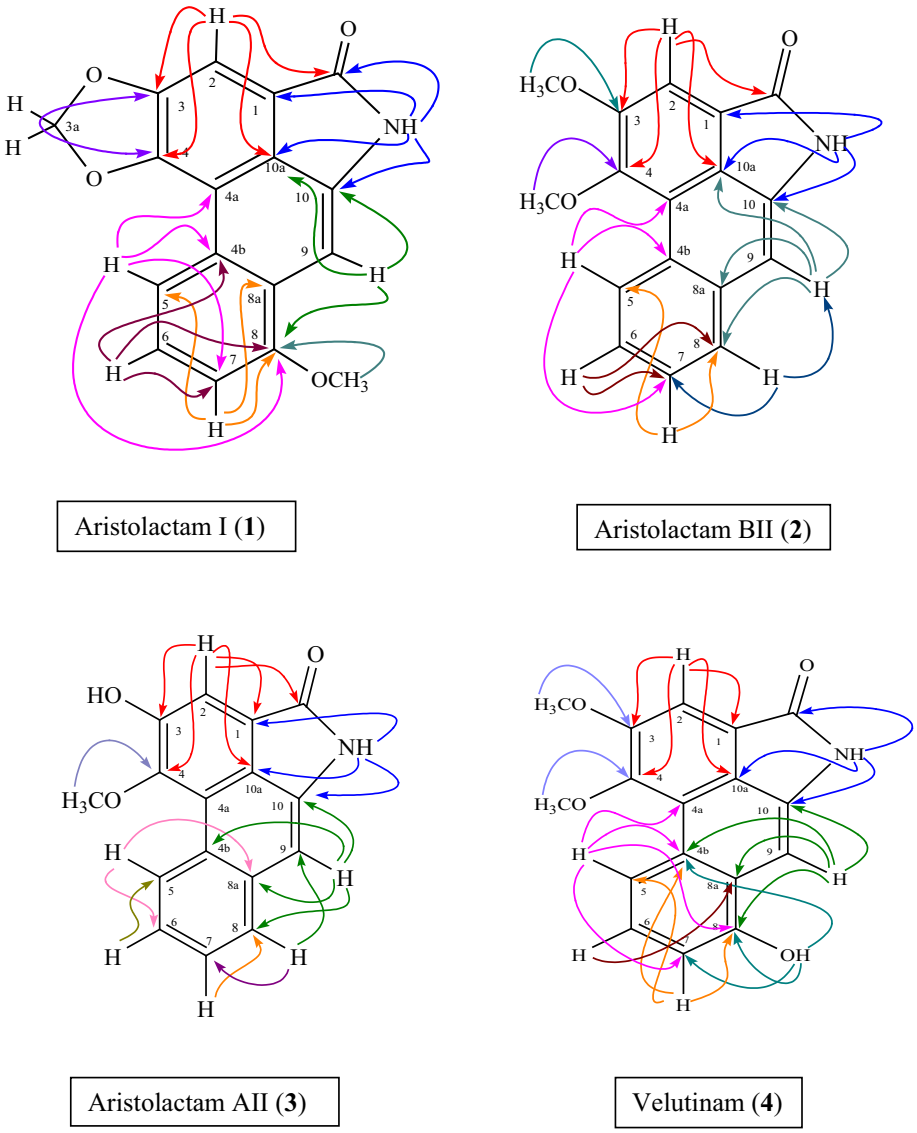

Fig. 2. HMBC correlations for compounds (**1**–**4**).

methoxyl group at C-4 was corroborated by the NOESY spectrum which shows correlation between $OC\underline{H}_3$ and H-5. The ^1H–^1H COSY and NOE correlations were also in support of structure (**3**). The COSY and NOESY showed correlations between long range H-1 to H-5, H-6 with H-5 and H-8, H-7 to H-8 and H-9, and interestingly NOESY spectrum also showed correlation of H-5 with all proton of the compound. On the basis of these spectral data, compound (**3**) is identified as aristolactam AII (3-hydroxy-4-methoxy-10-aminophe nanthrene-1-carboxylic acid 1, 10-lactam). Fig. 3 shows NOESY and COSY correlation of compounds (**1**–**4**).

Compound (**4**) was isolated as brownish yellow needles with melting point of 269–270 °C which then decompose and R_f value in toluene–ethylacetate (1:1) was 0.40. Its molecular formula $C_{17}H_{13}NO_4$ was derived from HRESIMS 296.0842, mass spectrum displayed peak at *m*/*z* 296.1 $[M+H]^+$ while ESIMSMS showed ion fragmentations at *m*/*z* at 280.1, 264.1, 252.1, 235.1, 206.1, 196.1. Its UV spectrum was almost similar to compound (**3**) showing maxima at 247, 298 and 405 nm suggesting the presence of phenanthrene group. This was supported by FTIR spectrum showing an intense peak at 1674 cm^{-1} (C=O) with a shoulder at 1614 cm^{-1}. In addition it also shows NH band at 3298 cm^{-1} with OH band around 3400 cm^{-1}.

Analysis of NMR data in DMSO-d_6 suggested highly aromatized molecule as ^1H NMR shows the three proton signals showing coupling (*ortho*), assigned to H-5 (δ 8.61, dd, J = 1.2, 8.2 Hz), H-6 (δ 7.35, t, J = 7.9, 8.1 Hz) and H-7 (δ 7.06, dd, J = 1.2, 7.9 Hz) and two singlets assigned to H-2 (δ 7.84) and H-9 (δ 7.41). In addition one singlet each for OH at δ 10.08, NH δ 10.76 and methoxyl proton at δ 4.04 and δ 3.99 was also observed. The ^{13}C spectrum revealed two methoxyl peaks at δ 56.94 and δ 59.80, one C=O peak at δ 168.33 with 14 aromatic carbon peaks. Assignment of methoxyl group at C-3 and C-4 was done by analyzing NOESY spectrum, the methoxy which resonated at δ 56.94/4.03 ppm showed correlation with H-2 was assigned to C-3, and the one that resonated at δ 59.8/3.99 showed correlation with H-5 was assigned to C-4, which was further confirmed by HMBC correlation of δ 56.94/4.03 OCH_3 to C-3 and δ 59.8/3.99 OCH_3 to C-4. By analyzing ^1H and ^{13}C spectra, OH group was assigned at C-8 because of presence of OH group at C-8 caused the values of C-7, C-9 and H-7 to move upfield (shielding effect) to δ 112.10, 98.69 and 7.06 respectively, and value of C-8 to downfield at δ 153.72. HMBC correlation was also in agreement with these assignments showing correlations of H-2 to C-1, C-3, C-4, C-10a, C=O, H-5 to C-4a, C-4b, C-7, C-8, C-6 to C-8, C-8a, C-7 to C-5, C-8, C-4b, C-9 to C-4b, C-8a, C-10, NH to

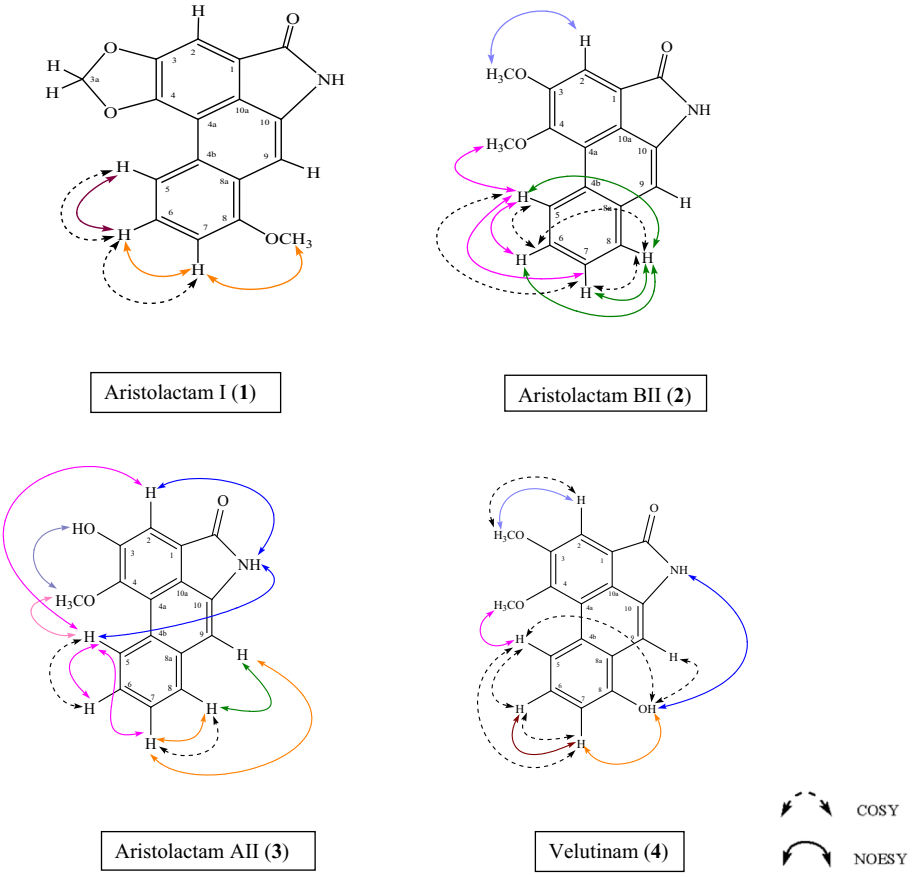

Fig. 3. NOESY and COSY correlations for compounds (**1**–**4**).

C-1, C-10, C-10a, C═O, 2-OCH_3 to C-3, 3-OCH_3 to C-4, and OH to C-4b, C-7, C-8. Omar et al. (1992) also reported the similar NMR pattern but in pyridine-d_6, in which all peaks were moved little downfield. In light of these entire spectral studies, compound (**4**) is identified as velutinam (10-amino-8-hydroxy-3,4-dimethoxyphe nanthrene-1-carboxylic acid lactam). Table 1 shows the summary of NMR spectra of compounds (**1**–**4**).

3.1. Antibacterial study of aristolactams (ALs)

Antibacterial study of isolated compounds showed that none of these were active against the tested bacterial strains, while the crude methanolic extract of stem bark *G. velutinus* showed antibacterial activity against these bacteria, indicating that there may be some other compounds present in the extract responsible for these activities or these compounds may work synergistically and show antibacterial activity. Although Omar et al. (1992) reported that velutinam and aristolactam BII are active against some Gram positive bacteria, their report lacks information on both the tested concentrations and bacterial strains used. In contrast in the present study, aristolactam I (**1**) and aristolactam AII (**3**) were tested at 16 mg/mL and they did not show any antibacterial activity against tested strains of Gram-positive bacteria. Furthermore, similar results were obtained for aristolcatam BII (**2**) and velutinam (**4**) when tested at 25 mg/mL concentration.

3.2. Anti-biofilm study of aristolcatms (ALs)

Compounds (**1**–**4**) were tested for their antibiofilm activity against two Gram negative (*K. pneumoniae* and *P. mirabilis*) and two Gram positive bacteria (*S. aureus* and *S. mutans*). None of the compounds showed antibiofilm activity against *K. pneumoniae* and *S. aureus*. Among the four compounds, only velutinam (**4**) at 70 μg/mL concentration inhibited biofilm formation for both *S. mutans* and *P. mirabilis* option 41.3% and 78.9%, respectively. While the compounds aristolactam BII (**2**) at 90 μg/mL concentration and aristolactam AII (**3**) at 140 μg/mL concentration were active against only *S. mutans* and inhibited their biofilm formation to 72.8% and 89.9% respectively (Table 2). In addition aristolactam I (**1**) did not show any inhibition of biofilm against the microorganisms used in this study. The compounds which inhibited the biofilms might interfere with the adherence of bacteria to the surface, which is the initial step in the formation of biofilm. Biofilm provides microorganisms shelter against antibiotic and host defense, hence these compounds might be used as an alternative to antibiotics or used as an adjuvant with different antimicrobials to treat different bacterial infections. Our result suggests that these compounds are active against *S. mutans*, the primary pathogen of oral cavity (Ahmed et al., 2014). Thus, these compounds may be used as a part of different formulation to inhibit the dental biofilm formation and to cure oral infection by maintaining oral hygiene.

3.3. MTT assay

Due to limited amounts of the isolated compounds, anticancer activity was carried out for velutinam only. Cytotoxicity assay for velutinam was investigated on three different cancer cell lines (A549, CaSki and HEK 293) using the MTT assay following 72 h treatment. The MTT assay is commonly employed in determining cytotoxicity as it offers a quantitative and simple method for evaluating cell population's response to external factors. As shown in

Table 2
Effect of compounds (**1**–**4**) on biofilm formation of bacteria.

Compounds	(μg/mL)	Biofilm % inhibition			
		KP	*SA*	*SM*	*PM*
Aristolctam I (**1**)	70	NA	NA	NA	NA
Aristolctam BII (**2**)	89.6	NA	NA	72.8 ± 1.8	NA
Aristolctam A II (**3**)	140	NA	NA	89.97 ± 2.3	NA
Velutinam (**4**)	70	NA	NA	41.34 ± 0.5	78.98 ± 1.2
6 Amino penicillinic acid	–	67.4 ± 0.5 (35 μg/mL)	73.5 ± 0.04 (82 μg/mL)	29.31 ± 0.84 (70 μg/mL)	89.2 ± 0.08 (35 μg/mL)

NA: Not active.
KP = *Klebsiella pneumoniae*, SA = *Staphylococcus aureus*, SM = *Streptococcus mutans*, PM = *Proteus mirabilis*.

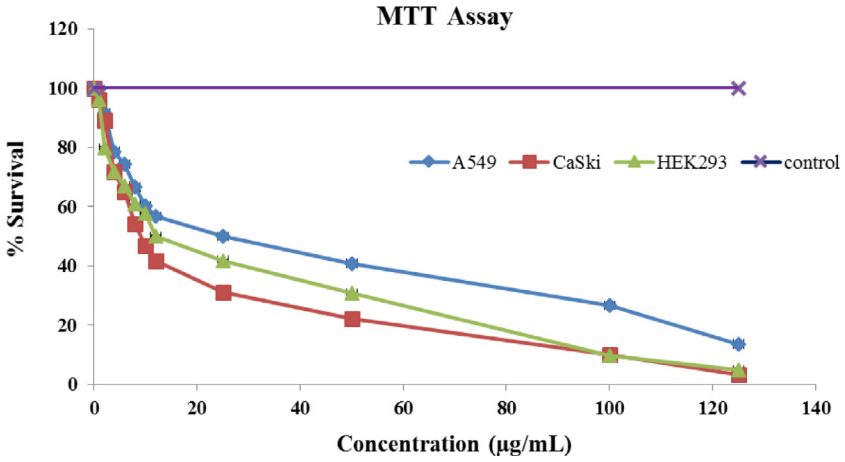

Fig. 4. Antiproliferative activity of velutinam against different cell lines determined by the MTT assay.

Table 3
MTT proliferation assay (IC_{50}) of velutinam (**4**).

Cell lines	IC_{50} (μg/mL)
A549	21.57 ± 0.22
CaSki	10.97 ± 0.03
HEK293	13.28 ± 0.12

±Standard error.

Fig. 4, this compound inhibits cell growth in a dose dependent manner.

Moreover, the cytotoxic activity (IC_{50}) of the compound is summarized in Table 3 which shows that velutinam is more cytotoxic against Caski having IC_{50} value of 10.97 μg/mL than the other two cell lines. It is reported that velutinam is cytotoxic against HeLa (human cervical carcinoma) and L1210 (murine leukaemia cell line) with IC_{50} of 0.39 mg/mL and IC_{50} 1.16 mg/mL, respectively (Omar et al., 1992), but no reports have been found on its anticancer activity against A549, CaSki and HEK 293. This is the first report describing its antitumor activity towards these cell lines.

Velutinam (**4**) is an aristolactam type alkaloid and aristolactams are known to have antitumor activity against many cancer cell lines. For example Aristolactam BII had an IC_{50} of 3.92 mg/mL and 3.77 mg/mL against L1210 and HeLa, respectively (Omar et al., 1992), enterocarpam III, and stigmalactam exhibit significant cytotoxicites against human colon adenocarcinoma (HCT15) cell lines with IC_{50} of 1.68 and 1.32 μM, respectively (Nayyatip et al., 2012). Aristolactam AII showed cytotoxicity against PE388 and KB cells at IC_{50} 11.71 and 0.72 μg/mL (Hongthong et al., 2014). 3, 5-Dihydroxy-2,4-dimethoxyaristolactam, aristolactam BI, goniopedaline, griffithinam showed cytotoxicity, 2.13, 11.18, 2.59, 13.28 μg/mL against P-388 (murine lymphocytic leukemia), respectively (Chanakul et al., 2011).

Traditional plants containing aristolactams have been used in folk medicine in East Asia and China. These aristolactams have distinctive biological activities including anticancer activity against a broad range of mammalian cancer cell lines (Choi et al., 2009), antioxidant and antifungal (Tabopda et al., 2008), antiplatlets aggregation (Chen et al., 2004), neuroprotective (Kim et al., 2004), activities. In addition, neurological disorders, especially Parkinson's disease, have been treated by the administration of the aristolactam, talisacanine (Kumar et al., 2003). Furthermore, they also possess antimalarial activity (Levrier et al., 2013). Even though aristolactam structure is closely related to aristolochic acid, but it is non mutagenic when tested against several bacterial strains. In contrast aristolochic acid has been shown to be mutagenic. It has been reported that for cytotoxic activity the substitution on carbons 2, 3, and 4 (ring B), 5, 6, 7 (ring D) and 9 on ring C, influence the activity. For example, multi methoxyl group substitution on rings B, C, and D showed significant potent anticancer activity in a broad array of mammalian cancer cell lines, while substitution of methyl group on ring A nitrogen tends to decrease activity. Although aristolactams (ALs) were known to have antitumor activities, but the mechanism of their action is not clear. However, Li et al. (2009) reported the antitumor mechanism of aristolactam AIII in which cell-based assays indicated that aristolactam AIII could inhibit proliferation of HeLa, A549, HGC, and HCT-8/V cells, induce mitotic arrest at the G2/M phase with spindle abnormalities and promote apoptosis (Li et al., 2009).

4. Conclusion

In this study, four compounds have been isolated from the stem bark of *G. velutinus*, out of which two compounds, aristolactam BII (**2**) and velutinam (**4**), have already been reported from the stem bark of *G. velutinus*. Aristolactam AII (**3**) is being reported first time

in this study, previously they were reported from other *Goniothalamus* species (*G. cheliensis*, *G. griffithii*, *G. sesquipedalis* and *G. tenuifolius*) but not from *G. velutinus*. To the best of our knowledge this is the first report of aristolactam I (**1**) from *Goniothalmus* genus, which has been previously reported from other Aristolochia species. However, these compounds did not show antibacterial activity at concentration of 16 mg/mL (160 μg/disc) for aristolactam I (**1**) and aristolactam AII (**3**), and at 25 mg/mL (250 μg/disc) for aristolactam BII (**2**) and velutinam (**4**) against tested strains of Gram positive bacteria. Hpwever, velutinam exhibited anticancer properties and showed cytotoxicity against A549, CaSki and HEK293. Furthermore, these compounds, except aristolactam I (**1**), exhibit a good anti-biofilm activity against two bacteria *S. mutans*, and *P. mirabilis* and can be a good anti-biofilm agent. In conclusion, our result suggests that these compounds are active against *S. mutans*, the primary pathogen of oral cavity. Thus, they could be used as a part of different formulation to inhibit the dental biofilm formation and to cure oral infection by maintaining oral hygiene. Also, they could be good addition to the pharmaceutical industry for preparing drugs for anticancer and anti-biofilm.

Conflict of interest

The authors declare that they have no competing interests.

Acknowledgments

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