

Research Article



In vitro Cytotoxic Effect of Indole Alkaloids from the Roots of *Kopsia singapurensis* Ridl. against the Human Promyelocytic Leukemia (HL-60) and the Human Cervical Cancer (HeLa) Cells

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Accepted on: 12-02-2015; Finalized on: 31-03-2015.

ABSTRACT

Six indole alkaloids; kopsamine *N*(4)-oxide **1**, kopsifine **2**, rhazinicine **3**, aspidodasycarpine **4**, kopsamine **5** and akuammidine **6**, were isolated from the roots of *Kopsia singapurensis* Ridl. (Apocynaceae). Compound **2** is first time isolated from the roots of *K. singapurensis* Ridl. Those compounds were screened for *in vitro* cytotoxic activity against human cervical cancer (HeLa), human promyelocytic leukemia (HL-60), and the normal mouse fibroblast (NIH/3T3) cell lines by using the MTT assay. Based on comparison with vincristine (control), kopsifine **2** showed very strong cytotoxic effects against HL-60 cells, while, kopsamine **5** exhibited a moderate cytotoxic activity effects against HL-60 cells with CD₅₀ values of 0.9 µg/mL and 6.9 µg/mL, respectively. Akuammidine **6** and rhazinicine **3** exhibited significant cytotoxicity effects against HeLa cells with the CD₅₀ values 2.8 µg/mL and 2.9 µg/mL, respectively. However, aspidodasycarpine **4** showed a moderate cytotoxicity effects against HeLa cells with the CD₅₀ value of 7.5 µg/mL, and kopsamine *N*(4)-oxide **1** was inactive against all these cancer cells. Although kopsifine **2**, rhazinicine **3**, and aspidodasycarpine **4** showed high cytotoxic effects against HL-60 and HeLa cell lines, but they also showed cytotoxic on normal cell (NIH/3T3) with CD₅₀ values of 20.7, 20.8, and 6.4 µg/mL, respectively. Therefore, these three alkaloids are not suggested as cytotoxic agents for these cancer cells. This is the first cytotoxicity report on HL-60, HeLa and NIH/3T3 cell lines for these compounds.

Keywords: Cytotoxicity, Cancer cells, Indole alkaloid, *Kopsia singapurensis* Ridl.

INTRODUCTION

Plants are the extraordinary source for the discovery of new medicinal products in drug development.¹ The plants have a long history of providing an innumerable number of molecules with potential for the treatment of many serious diseases,² which are called as medicinal plants. The term of medicinal plants include a various types of plants used in herbalism and some of these plants have a medicinal activities which commonly used as raw materials for extraction of active substances for synthesis of drugs.³ The ancient civilizations of the Chinese, Indians and North Africans provide written evidence for the use of natural sources for curing various diseases.⁴

The Apocynaceae family including vascular plant has the classification subkingdom Tracheobionta, super division Spermatophyta (division Magnoliophyta), class Magnoliopsida (subclass Asteridae), and order Gentianales, commonly called the dogbane family. There are about 155 genera and 2000 species of five subfamilies; Apocynoideae, Asclepiadoideae, Periplocoideae, Rauvolfieae, and Secamonoideae,⁵ distributed in the tropics and subtropics regions.⁶

The genus *Kopsia* is widely distributed in Southeast Asia,⁷⁻¹¹ it is rich in indole alkaloids,¹²⁻¹³ that is the parent substance of a large number of important compounds that occur in nature.¹⁴ This genus have obtained many new alkaloids.^{11,15} It also produced a large number of biologically active indole alkaloids possessing intriguing

carbon skeletons,^{7-9,11,13-21} including *Kopsia singapurensis* Ridl. This plant is one of 18 species of *Kopsia* which can be found in Malaysia,²²⁻²⁴ it also known as *selada* or white kopsia or Singapore kopsia.^{19,24} In ethnobotany, its roots used to treat poulticing ulcerated noses in tertiary syphilis.^{11,24} Several previous studies also have been reported the cytotoxicity of alkaloids, such as harmalacidine, harmine (*Peganum harmala* seeds) have cytotoxic activity effects against Sp2/O-Ag14 (tumor cell) with IC₅₀ values of 7.96 ± 0.017 and 2.43 ± 0.062 µg/mL, respectively,²⁵ singaporentine A and kopsinic acid (*K. singapurensis* Ridl. leaves) showed cytotoxic activity against P388 murine leukemia cells at IC₅₀ 57µg/ml and 48µg/ml, respectively.¹¹ Now, we report the isolation of indole alkaloids from the roots of this species and their cytotoxicity effects against the human promyelocytic leukemia (HL-60) and the human cervical cancer (HeLa) cells which compared to the normal cell (NIH/3T3).

MATERIALS AND METHODS

General

NMR Spectra were recorded on JEOL ECX 500 MHz, deutriochloroform (CDCl₃) and deutriomethanol (CD₃OD) were used as NMR solvents. The optical rotations (OR) were obtained on Rudolph Research Analytical (Autopol III, Automatic Digital Polarimeter) with tungsten lamp at 25°C and wavelength 589 nm (sodium light) in CH₃OH. UV spectra were obtained in methanol on a Perkin Elmer UV-visible spectrophotometer and the wavelength of the spectrum was recorded in the range of 200 to 400 nm. IR



spectra were recorded by Nicolet 6700 FTIR spectrophotometer in CHCl_3 , and HREIMS was measured on an Agilent 6530 Accurate-Mass Q-TOF LC/MS instrument. The melting point of solid/crystal sample was measured by Stuart melting point apparatus SMP3.

Stationary phase of column chromatography (CC) used Silica Gel 60 (0.063-0.200 mm), 70-230 mesh ASTM (MERCK 1.07734) and Silica Gel 60 (0.040-0.063 mm), 230-400 mesh ASTM (MERCK 1.09385.1000). Thin layer chromatography (TLC) used Silica Gel 60 F₂₅₄ (MERCK 1.05554.0001) as the stationary phase on the flat aluminium plate, and Silica Gel 60 PF₂₅₄ containing gypsum (MERCK 1.07749.1000) on the flat glass plate for preparative TLC (PTLC).

Plant Materials

The roots of *K. singapurensis* Ridl. were collected from Kluang, Johor (Malaysia) and the identification was confirmed by Mr. Teo Leong Eng, University of Malaya. A voucher specimen (KL 5334) was deposited at the Herbarium of the Department of Chemistry, University of Malaya, Kuala Lumpur, Malaysia.

Extraction and Isolation

The air-dried roots (1 Kg) of *K. singapurensis* Ridl. were ground and extracted exhaustively with hexane by soxhlet extractor for 72 hours. The extract solution was filtered and evaporated by rotary evaporator. The residue was dried and immersed with ammonia 25% solution and left for 2 hours. They were then extracted exhaustively with dichloromethane (CH_2Cl_2), the CH_2Cl_2 crude (12.85 g) yielded after solvent evaporated. Then it was dissolved in hexane for three days to give crude which dissolved in hexane (3.58 g) and the residue which is not dissolved in hexane, CH_2Cl_2 crude extract (9.27 g). The CH_2Cl_2 crude extract (8.5 g) was subjected to column chromatography (CC) over silica gel. The solvent system used for CC was hexane, CH_2Cl_2 , and MeOH with increasing polarity. The fractions having same spots were grouped into a series of fractions (monitored by TLC) and yielded 22 fractions. Fraction B and C (7-11) were combined and then continued separately by extensive CC. Fraction B1 (1-130) and B8 (230-240) then purified by PTLC to give kopsamine N(4)-oxide **1**, kopsifine **2**, and rhazinicine **3**. The combining of fraction D and E (12-17) also continued by extensive CC and obtained aspidodasycarpine **4**, while fraction D3 (3-13) and D10 (54-104) then purified by PTLC to get kopsamine **5** and akuammidine **6**, respectively.

Cell Lines

The cytotoxic activity was treated against human promyelocytic leukemia (HL-60) (suspension cell), human cervical cancer (HeLa), and the normal mouse fibroblast (NIH/3T3) cell lines (adherent cells). All cells were recognized from the American Type Cell Collection, ATCC (Manassas, VA, USA). Medium without compound used as negative control and the positive control used vincristine as comparison. The cells were cultured using RPMI 1640

culturing media (Sigma, St. Louis, USA) and maintained at 37 °C in 5% CO_2 atmosphere.²⁶ The cells were counted using hemocytometer.

MTT Cytotoxicity Assay

For the suspension cells: A volume of 100 μL of complete growth RPMI medium was added into each well in 96-wells flat bottom microtiter plate (Nunclon, USA) except for first row. Then 100 μL compounds or vincristine sulphate solution (95–105%, Sigma, USA) at 60 $\mu\text{g}/\text{mL}$ was added into wells in triplicate and serially diluted to give variations in concentration. A volume of 100 μL of 1×10^5 cells/mL HL-60 or HeLa or NIH/3T3 cells were seeded into each well and incubated for 72 hours in a humidified 5% CO_2 incubator at 37 °C.

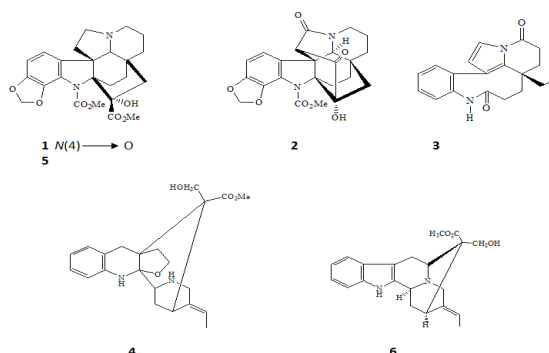
For the adherent cells: The cells were trypsinized and examine the cells using an inverted microscope to ensure that all cells are detached and floating. Cells seeded in 96-wells plate at 1×10^5 cells/mL. After an overnight incubation to allow cell attachment, cells were then added medium and compounds in different variation concentrations and incubated at 37°C, 5% CO_2 for 72 hours.

After 72 hours incubation, a volume of 20 μL of MTT Dye solution (5 mg/mL) was then added into each well (suspension or adherent cells) designated for the MTT Dye test and appropriate control and incubated for 4 hours. The culture medium was removed and 100.0 μL of 100% DMSO solution were added to each well to solubilise the formazan formed. The absorbances of each plate were recorded at 570 nm using ELISA plate reader with reference at 630 nm wavelength (Infinite M200, Tecan, Switzerland).²⁶ The percentage cell viability and concentration of compounds were plotted to get graph and determine the CD_{50} from it.

RESULTS AND DISCUSSION

Alkaloids from the Roots of *K. singapurensis* Ridl.

Investigation of alkaloidal contents of the roots of *K. singapurensis* Ridl. have provided six indole alkaloids; kopsamine N(4)-oxide **1**, kopsifine **2**, and rhazinicine **3**, aspidodasycarpine **4**, kopsamine **5** and akuammidine **6**. Their structures were elucidated by a combination of spectroscopic techniques such as 1D-NMR (^1H , ^{13}C , and DEPT-135°), 2D-NMR (^1H - ^1H COSY, HMQC, and HMBC), UV, IR, OR, and MS.



Kopsamine *N*(4)-oxide **1** possesses the aspidofractinine skeleton, it was isolated as a light yellowish amorphous, with $[\alpha]_D^{25}$ -5.00 ($c = 0.02$, CH₃OH). The ¹³C NMR and DEPT-135° spectra showed a total of 24 carbon signals including two carbamate carbonyl functions, which were observed at δ_c 156.7 (C-22) and δ_c 173.1 (C-24), respectively. The IR spectrum revealed the broad band at 3233.8 cm⁻¹ representing a hydroxyl group, 1619.0 cm⁻¹ indicating a carbamate carbonyl function group, and 1441.7 cm⁻¹ corresponding to a nitrogen oxide. This alkaloid have m/z 473.1919 [M+H]⁺ in the mass spectrum, which analyzed for C₂₄H₂₈N₂O₈. The UV spectrum was typical of a dihydroindole chromophore, with absorption maxima at 220.0, 230.0, 252.3, and 291.7, respectively. The ¹H NMR data showed two aromatic proton signals at δ_H 6.99 (*s*, H-9) and δ_H 6.52 (*d*, $J = 7.5$ Hz, H-10). The presence of oxygen attached at *N*(4) of **1** yielded the proton and carbon signals at C-3 and C-5 were shifted to downfield. Therefore, the structure was ensured as **1**.

Kopsifine **2** showed the characteristic UV maxima of a dihydroindole chromophore¹² at 220.0, 230.0, and 290.1 nm and afforded as a light yellowish amorphous, with $[\alpha]_D^{25}$ +95.77 ($c = 0.01$, CH₃OH). The IR spectrum obtained absorptions at 3290.8 cm⁻¹ (OH), 1765.0 cm⁻¹ (C=O), and 1641.2 cm⁻¹ (NC=O). The ¹³C NMR and DEPT-135° spectra showed a total of 23 carbon signals (7 sp² quaternary carbons, 2 sp² methines, 4 sp³ quaternary carbons, 2 sp³ methines, 7 sp³ methylenes, and 1 methyl). The ketonic carbonyl function was formed by bridging C-6 to C-16, it influenced their peaks shifted toward the downfield (δ_c 59.1, C-6 and δ_c 83.3, C-16, respectively). Those positions were also supported by HMBC data which showed correlations of H-6 – C-22 and H-6 – C-16. The presence of methylenedioxy (C-25) substituent was attached at C-11 (δ_c 150.0) and C-12 (δ_c 136.3), was deduced by HMBC spectrum showed that the methylenedioxy ring which established from proton correlation of methylene signals to C-11 and C-12. The HRESIMS spectrum of this compound showed pseudo-molecular ion peak at m/z 439.1506 [M+H]⁺ matched with the molecular formula C₂₃H₂₂N₂O₇. This compound has kopsine skeleton and it was identified as **2**. It was first isolated from the stem bark of *K. dasyrachis* Ridl.¹², and also found in its leaves.²⁷ Besides, it also existed in the stem bark of *K. arborea* Blume.²⁸ Based on those reports, this indole alkaloid is first time isolated from the roots of *K. singaporensis* Ridl.

Rhazinicine **3**, showed the pseudo-molecular ion peak at m/z 309.1597 [M+H]⁺ in the mass spectrum which appropriated with the molecular formula C₁₉H₂₀N₂O₂. The IR spectrum expressed absorption at 3233.8 cm⁻¹ which indicating the presence of NH/OH and 1634.8 cm⁻¹ exhibiting the presence of a lactam carbonyl function group, while the UV spectrum displayed the absorption maximum at 235.0 and 275.6 nm demonstrating the characteristic of rhazinilam-type compound¹² which obtained as a light yellowish amorphous, with $[\alpha]_D^{25}$ -235.74 ($c = 0.02$, CH₃OH). The ¹H NMR spectrum notified a number of important features, including the existence

of ethyl group which indicated by the appearance of a CH₂-19 multiplet at δ_H 1.32 and δ_H 1.47 and a triplet signal of CH₃-18 at δ_H 0.73. Four aromatic proton signals at the unsubstituted indolic NH ring (δ_H 6.61) detected at δ_H 7.41 (*m*, H-9), δ_H 7.41 (*m*, H-10), δ_H 7.37 (*m*, H-11), and δ_H 7.28 (*d*, $J = 7.5$ Hz, H-12). These assignments were also verified by HMBC spectrum and the COSY correlations due to C-9 – C-10 – C-11 – C12 fragment. Two proton signals are belonging to H-14_α and H-14_β, they were observed at δ_H 2.70 (*td*, $J = 8.1$ and 3.5 Hz) and δ_H 2.93 (*ddd*, $J = 17.8$, 13.8, and 5.2 Hz), respectively. They are shifted to upfield since they are adjacent to the electronegative atom, oxygen (O₂), while two set of multiplet proton signals, corresponding to H-16_α and H-16_β were recorded at δ_H 2.11 and δ_H 2.42, they are shielded also caused by oxygen which adjacent to C-16. The ¹³C NMR spectrum indicated the presence of two lactam carbonyls at δ_c 176.5 (C-2) and 168.1 (C-3), their positions were supported by HMBC spectrum which indicated the cross peak between H-14/C-3, H-17/C-2, and H-15/C-3. One of these lactams, C-2 and the fragments of COSY correlations of C-14 – C-15, C-16 – C-17, and C-18 – C19, showed the characteristic of rhazinilam derivative¹². Therefore, it was identified as **3**.

Aspidodasycarpine **4** has the molecular formula C₂₁H₂₄N₂O₄ as deduced by HRESIMS data (m/z 371.1969 [M+H]⁺), it was yielded as dark purple amorphous, with $[\alpha]_D^{25}$ +2.50 ($c = 0.02$, CH₃OH). The IR spectrum displayed absorption at 3243.3 cm⁻¹ indicating the presence of hydroxyl group, while the presence of carbonyl function group was observed at 1638.0 cm⁻¹. The UV spectrum gave maxima absorptions at 239.9 nm and 295.0 nm corresponding to characteristic of an unsubstituted dihydroindole chromophore. The ¹H NMR spectrum also showed the presence of ethylidene group which presented the olefinic proton at δ_H 5.68 (1H, *d*, $J = 6.9$ Hz, H-19) and the associated methyl signal was observed at δ_H 1.72 as a doublet ($J = 6.3$ Hz, H-18). The carbon at position 16 has formed the bridge at C-7 to C-15 and gave new ring which attached the ethanol and methyl ester groups at C-16. Its position has confirmed by HMBC correlations that showed the long-range correlations, for example H-17 with C-15 and C-22, and H-23 with C-22. Based on the all of spectral data, this compound was confirmed as **4**.

Generally, kopsamine **5** is similar all respects to kopsamine *N*(4)-oxide **1**. Kopsamine **5** was isolated as a light yellowish amorphous, with $[\alpha]_D^{25}$ -15.99 ($c = 0.01$, CH₃OH), possessing the aspidofractinine skeleton except the deprivation of oxygen attached at *N*(4) thus in accordance with the molecular formula C₂₄H₂₈N₂O₇ due to m/z 457.1977 [M+H]⁺, the pseudo-molecular ion peak which was showed by HRESIMS spectrum. The IR spectrum showed a broad band at 3249.6 cm⁻¹ demonstrating the presence of OH and 1631.7 cm⁻¹ indicating the presence of carbamate carbonyl functional group. The UV spectrum revealed absorption characteristic of dihydroindole chromophore (220.0,



230.5, 248.1, and 288.3 nm). The ^{13}C NMR spectrum showed the presence of two carbamate carbonyl functions, they were recorded at δ_{C} 156.3 (C-22) and δ_{C} 173.1 (C-24), respectively.

The HMBC spectrum supported their positions which showed the correlation from between H-23 with C-22 and H-25 with C-24. The presence of methylenedioxy substituent at δ_{C} 148.4 (C-11) and 135.5 (C-12) was displayed by the ^{13}C NMR spectrum and supported by HMBC spectrum which showed the correlation from methylene signal (δ_{C} 100.4) to C-11 and C-12. Therefore, the structure was identified as **5**.

Akuamidine **6** was isolated as colorless crystals (m.p. 250–252 °C), with $[\alpha]_{\text{D}}^{25} +14.79$ ($c = 0.03$, CH_3OH). The ^{13}C NMR spectral data contained 21 signals. The IR spectrum showed the presence of NH/OH group at 3246.4 cm^{-1} and carbonyl functional group at 1631.7 cm^{-1} , while the signal at 1429.0 cm^{-1} suggesting an ethanol group.

The UV spectrum displayed the maxima absorption at 280.0 nm which was characteristic of a dihydroindole chromophore. The pseudo-molecular ion peak was showed by HRESIMS data at m/z 353.1858 $[\text{M}+\text{H}]^+$, which was analyzed for $\text{C}_{21}\text{H}_{24}\text{N}_2\text{O}_3$. There are five aromatic proton signals detected in the ^1H NMR spectrum, four of them were appeared at δ_{H} 7.35 (d , $J = 8.0$ Hz, H-9), δ_{H} 6.94 (d , $J = 8.0$ Hz, H-10), δ_{H} 7.02 (t , $J = 6.9$ Hz, H-11), δ_{H} 7.25 (d , $J = 8.1$ Hz, H-12) which demonstrated the existence of an unsubstituted dihydroindole.

This spectrum also showed the existence of a deshielded doublet of doublet signal at δ_{H} 5.43 ($J = 13.8$ and 6.9 Hz, H-19) assigned to the olefinic of the ethylidene group and the associated methyl signal at δ_{H} 1.66 (d , $J = 6.7$ Hz, H-18) in the upfield. One methoxyl was observed at δ_{H} 2.92 (H-23) as a singlet. The unusual shielded signal value in the chemical shift of methoxyl was caused by anisotropic interactions from the benzene ring, a high electron density area.

A carbonyl functional group exhibited at δ_{C} 173.8 (C-22), was observed as methyl ester group wherein the methyl (methoxyl) was conformed at δ_{C} 50.6 (C-23) which showed by the ^{13}C NMR spectrum. The examination of the spectral data led to the identification of this compound as **6**.

Cytotoxic Effects

Cytotoxicity is the degree to which an agent has specific destructive action on certain cells. A lot of studies have been conducted to look for cytotoxic treatment in humans, mostly using the compounds, especially alkaloids from the plants.

The cytotoxic effects of six alkaloids isolated from the roots of *K. singapurensis* Ridl. were tested against human cervical cancer HeLa, human promyelocytic leukemia HL-60, and the normal mouse fibroblast NIH/3T3 cell lines in vitro using MTT assay for 72 hours treatment with the different concentrations. Each concentration of the

samples was assayed in triplicate. The positive cytotoxic control was using vincristine (VCR), the commercial drug for the treatment of leukemia and multiple myeloma.²⁶ Those several alkaloids were assumed to possess a very strong cytotoxic activity towards NIH/3T3, HeLa and HL-60 cells. Compounds which obtained the CD_{50} value of 10–25 $\mu\text{g}/\text{mL}$ were considered to be weak in cytotoxicity, the CD_{50} value between 5 and 10 $\mu\text{g}/\text{mL}$ were classified as moderately active, while compounds with the CD_{50} value of less than 5 $\mu\text{g}/\text{mL}$ were considered very active.²⁶

Table 1 showed that the CD_{50} values, describing the concentrations level of cytotoxicity of the compounds toward 50 % of cell viability, of each cell lines. Those are displayed in Figure 1 (HL-60 cell), Figure 2 (HeLa cell), and Figure 3 (NIH/3T3 cell), respectively. Alkaloid **2** showed a very strong cytotoxicity, while **5** exhibited a moderate cytotoxic activity effects against HL-60 cell with the CD_{50} values of 0.9 $\mu\text{g}/\text{mL}$ and 6.9 $\mu\text{g}/\text{mL}$, respectively. It could be seen in Figure 1.

Previously, compound **5** has been reported that it inhibited cell growth of the resistant strain (KB/VJ300) significantly with IC_{50} of 5.6 $\mu\text{g}/\text{mL}$.²⁹ Figure 2 and Figure 3 were also showed cytotoxic activity the compounds against HeLa and the normal cells. Alkaloid **6** and **3** exhibited significant cytotoxicity effects against HeLa cell with the CD_{50} values 2.8 $\mu\text{g}/\text{mL}$ and 2.9 $\mu\text{g}/\text{mL}$, respectively. Besides, **4** showed a moderate cytotoxicity effects against HeLa cell with the CD_{50} value of 7.5 $\mu\text{g}/\text{mL}$, while **1** is inactive on all tested cells ($\text{CD}_{50} > 60\text{ }\mu\text{g}/\text{mL}$). In 2007, Subramaniam³⁰ also have reported that **3** also show appreciable cytotoxicity against KB/VJ300 with IC_{50} of 2.5 $\mu\text{g}/\text{mL}$. Although **2**, **3**, and **4** showed high cytotoxic effects but they also showed cytotoxic to the normal cell (NIH/3T3) with CD_{50} values of 20.7, 20.8, and 6.4 $\mu\text{g}/\text{mL}$, respectively. Therefore, in this case, these three alkaloids are not suggested as cytotoxic agents for these cancer cells. Prior to this, there have been no reports of cytotoxic activity of these indole alkaloids against HL-60 and HeLa cell lines compared to the normal mouse fibroblast NIH/3T3 cell line.

Table 1: The cytotoxic effects of isolated alkaloids from *K. Singapurensis* Ridl.

Alkaloids	CD_{50} , $\mu\text{g}/\text{mL}$		
	NIH/3T3*	HL-60**	HeLa***
Kopsamine <i>N</i> (4)-Oxide 1	> 60	> 60	> 60
Kopsifine 2	20.7	0.9	36.5
Rhazinicine 3	20.8	> 60	2.9
Aspidodasycarpine 4	6.4	> 60	7.5
Kopsamine 5	> 60	6.9	> 60
Akuamidine 6	> 60	30.2	2.8
Vincristine (control)	> 60	1.8	0.4

* The normal mouse fibroblast cells

** The human promyelocytic leukemia cells

*** The human cervical cancer cells



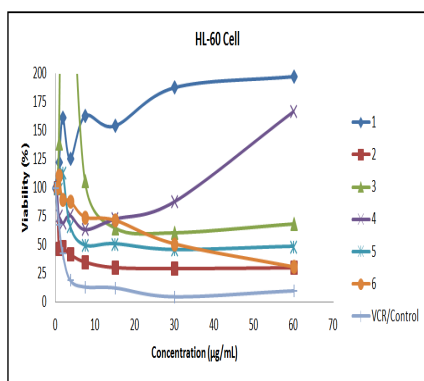


Figure 1: The cytotoxic effects of the isolated alkaloids from *K. Singaporensis* Ridl. against HL-60 cell line.

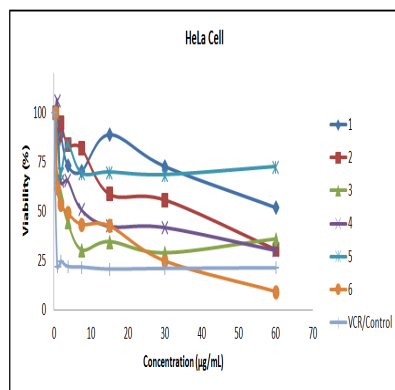


Figure 2: The cytotoxic effects of the isolated alkaloids from *K. Singaporensis* Ridl. against HeLa cell line.

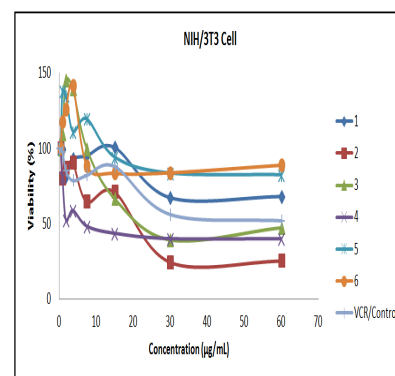


Figure 3: The cytotoxic effects of the isolated alkaloids from *K. Singaporensis* Ridl. against NIH/3T3 cell line.

Physical and Spectral Data of Isolated Indole Alkaloids

Kopsamine N(4)-oxide 1

Light yellowish amorphous; Molecular formula: $C_{24}H_{28}N_2O_8$; $[\alpha]_D^{25}$ -5.00 ($c = 0.02$, CH_3OH); UV λ_{max} (MeOH), nm (log ϵ): 220.0 (6.49), 230.0 (6.51), 252.3 (6.55), 291.7 (6.62); IR V_{max} ($CHCl_3$), cm^{-1} : 3233.8, 1619.0, 1441.7; Mass spectrum, m/z : 473.1919 $[M+H]^+$; 1H NMR (500 MHz, $CDCl_3$), δ ppm: 3.59 (1H, td , $J = 13.2$ and 5.2 Hz, H-3 $_{\alpha}$), 3.83 (1H, m , H-3 $_{\beta}$), 3.44 (1H, t , $J = 10.3$ Hz, H-5), 3.83 (1H, m , H-5 $_{\beta}$), 2.28 (1H, dd , $J = 14.9$ and 7.5 Hz, H-6 $_{\alpha}$), 2.45 (1H, m , H-6 $_{\beta}$), 8.14 (1H, d , $J = 8.6$ Hz, H-9), 6.56 (1H, d , 8.1 Hz, H-10), 1.89 (1H, m , H-14 $_{\alpha}$), 1.89 (1H, m , H-14 $_{\beta}$), 1.46 (1H, s , H-15 $_{\alpha}$), 1.68 (1H, d , $J = 13.2$ Hz, H-15 $_{\beta}$), 1.43 (1H, s , H-17 $_{\alpha}$), 3.02 (1H, dd , $J = 16.1$ and 2.9 Hz, H-17 $_{\beta}$), 1.60 (1H, m , H-18 $_{\alpha}$), 2.45 (1H, m , H-18 $_{\beta}$), 1.35 (1H, d , $J = 12.6$ Hz, H-19 $_{\alpha}$), 1.89 (1H, m , H-19 $_{\beta}$), 3.66 (1H, s , H-21), 3.90 (1H, s , H-23- $N-CO_2CH_3$), 3.79 (1H, s , H-25- CO_2CH_3), 5.87 (1H, d , $J = 1.7$ Hz, H-26 $_{\alpha}$ - OCH_2O), 5.92 (1H, d , $J = 1.8$ Hz, H-26 $_{\beta}$ - OCH_2O), 7.31 (1H, s , O-H); ^{13}C NMR (125 MHz, $CDCl_3$), δ ppm: 74.0 (C-2), 65.8 (C-3), 65.6 (C-5), 34.4 (C-6), 59.0 (C-7), 133.1 (C-8), 119.5 (C-9), 104.8 (C-10), 148.8 (C-11), 133.7 (C-12), 123.3 (C-13), 19.6 (C-14), 33.6 (C-15), 73.9 (C-16), 40.8 (C-17), 23.0 (C-18), 33.0 (C-19), 34.3 (C-20), 84.9 (C-21), 156.7 (C-22, $N-CO_2CH_3$), 53.6 (C-23, $N-CO_2CH_3$), 173.1 (C-24, CO_2CH_3), 53.2 (C-25, CO_2CH_3), 100.5 (C-26, OCH_2O).

Kopsifine 2

Light yellowish amorphous; Molecular formula: $C_{23}H_{22}N_2O_7$; $[\alpha]_D^{25}$ +95.77 ($c = 0.01$, CH_3OH); UV λ_{max} (MeOH), nm (log ϵ): 220.0 (6.45), 230.0 (6.47), 290.1 (6.57); IR V_{max} ($CHCl_3$), cm^{-1} : 3290.8, 1765.0, 1641.2; Mass spectrum, m/z : 439.1506 $[M+H]^+$; 1H NMR (500 MHz, $CDCl_3$), δ ppm: 2.87 (1H, dd , $J = 12.6$ and 4.0 Hz, H-3 $_{\alpha}$), 4.23 (1H, dd , $J = 13.8$ and 5.2 Hz, H-3 $_{\beta}$), 2.91 (1H, s , H-6), 6.71 (1H, d , $J = 8.0$ Hz, H-9), 6.64 (1H, d , $J = 6.3$ Hz, H-10), 1.51 (1H, m , H-14 $_{\alpha}$), 1.63 (1H, m , H-14 $_{\beta}$), 1.59 (1H, brd , $J = 2.3$ Hz, H-15 $_{\alpha}$), 1.63 (1H, m , H-15 $_{\beta}$), 1.59 (1H, brd , $J = 2.3$ Hz, H-17 $_{\alpha}$), 2.14 (1H, dd , $J = 15.5$ and 4.0 Hz, H-17 $_{\beta}$), 1.70 (1H, m , H-18 $_{\alpha}$), 2.57 (1H, ddd , $J = 13.8$, 12.1 , and 4.0 Hz,

H-18 $_{\beta}$), 1.45 (1H, m , H-19 $_{\alpha}$), 1.79 (1H, ddd , $J = 17.8$, 12.6 , and 4.6 Hz, H-19 $_{\beta}$), 3.60 (1H, d , $J = 1.2$ Hz, H-21), 3.81 (3H, s , H-24- $N-CO_2CH_3$), 5.93 (1H, d , $J = 1.2$ Hz, H-25 $_{\alpha}$ - OCH_2O), 5.96 (1H, d , $J = 1.2$ Hz, H-25 $_{\beta}$ - OCH_2O), 7.07 (1H, s , O-H); ^{13}C NMR (125 MHz, $CDCl_3$), δ ppm: 75.1 (C-2), 40.8 (C-3), 164.4 (C-5), 59.1 (C-6), 52.7 (C-7), 128.7 (C-8), 115.2 (C-9), 105.3 (C-10), 150.0 (C-11), 136.3 (C-12), 123.9 (C-13), 19.4 (C-14), 32.7 (C-15), 83.3 (C-16), 39.4 (C-17), 18.9 (C-18), 32.0 (C-19), 33.0 (C-20), 65.5 (C-21), 202.4 (C-22), 155.4 (C-23, $N-CO_2CH_3$), 54.0 (C-24, $N-CO_2CH_3$), 101.0 (C-25, OCH_2O).

Rhazinicine 3

Light yellowish amorphous; Molecular formula: $C_{19}H_{20}N_2O_2$; $[\alpha]_D^{25}$ -235.74 ($c = 0.02$, CH_3OH); UV λ_{max} (MeOH), nm (log ϵ): 235.0 (6.38), 275.6 (6.45); IR V_{max} ($CHCl_3$), cm^{-1} : 3233.8, 1634.8; Mass spectrum, m/z : 309.1597 $[M+H]^+$; 1H NMR (500 MHz, $CDCl_3$), δ ppm: 6.61 (1H, s , $N(1)-H$), 7.43 (1H, d , $J = 1.7$ Hz, H-5), 5.93 (1H, d , $J = 3.5$ Hz, H-6), 7.41 (1H, m , H-9), 7.41 (1H, m , H-10), 7.37 (1H, m , H-11), 7.28 (1H, d , $J = 7.5$ Hz, H-12), 2.70 (1H, td , $J = 8.1$ and 3.5 Hz, H-14 $_{\alpha}$), 2.93 (1H, ddd , $J = 17.8$, 13.8 , and 5.2 Hz, H-14 $_{\beta}$), 1.74 (1H, td , $J = 8.0$ and 3.5 Hz, H-15 $_{\alpha}$), 2.11 (1H, m , H-15 $_{\beta}$), 2.11 (1H, m , H-16 $_{\alpha}$), 2.42 (1H, m , H-16 $_{\beta}$), 1.59 (1H, m , H-17 $_{\alpha}$), 2.42 (1H, m , H-17 $_{\beta}$), 0.73 (3H, t , $J = 7.5$ Hz, H-18), 1.32 (1H, m , H-19 $_{\alpha}$), 1.47 (1H, m , H-19 $_{\beta}$); ^{13}C NMR (125 MHz, $CDCl_3$), δ ppm: 176.5 (C-2), 168.1 (C-3), 116.8 (C-5), 114.7 (C-6), 122.0 (C-7), 137.2 (C-8), 130.4 (C-9), 129.1 (C-10), 128.0 (C-11), 127.7 (C-12), 137.2 (C-13), 29.1 (C-14), 31.9 (C-15), 28.3 (C-16), 33.7 (C-17), 8.1 (C-18), 29.8 (C-19), 38.4 (C-20), 133.4 (C-21).

Aspidodasycarpine 4

Dark purple amorphous; Molecular formula: $C_{21}H_{24}N_2O_4$; $[\alpha]_D^{25}$ +2.50 ($c = 0.02$, CH_3OH); UV λ_{max} (MeOH), nm (log ϵ): 239.9 (6.47), 295.0 (6.56); IR V_{max} ($CHCl_3$), cm^{-1} : 3243.3, 1638.0; Mass spectrum, m/z : 371.1969 $[M+H]^+$; 1H NMR (500 MHz, $CDCl_3$), δ ppm: 4.36 (1H, s , H-3), 3.43 (1H, d , $J = 7.5$ Hz, H-5 $_{\alpha}$), 4.20 (1H, s , H-5 $_{\beta}$), 2.50 (1H, m , H-6 $_{\alpha}$), 2.62 (1H, d , $J = 9.2$ Hz, H-6 $_{\beta}$), 7.41 (1H, d , $J = 7.5$ Hz, H-9), 6.77 (1H, t , $J = 7.5$ Hz, H-10), 7.04 (1H, t , $J = 7.4$ Hz, H-11), 6.56

(1H, *d*, *J* = 7.5 Hz, H-12), 2.17 (1H, *d*, *J* = 1.2 Hz, H-14_α), 2.17 (1H, *d*, *J* = 1.2 Hz, H-14_β), 3.70 (1H, *s*, H-15), 3.15 (1H, *d*, *J* = 11.5 Hz, H-17_α), 3.60 (1H, *d*, *J* = 11.5 Hz, H-17_β), 1.72 (3H, *d*, *J* = 6.3 Hz, H-18), 5.68 (1H, *d*, *J* = 6.9 Hz, H-19), 3.80 (2H, *d*, *J* = 4.1 Hz, H-21_α), 3.80 (2H, *d*, *J* = 4.1 Hz, H-21_β), 3.77 (3H, *s*, H-23-CO₂CH₃); ¹³C NMR (125 MHz, CDCl₃), δ ppm : 101.2 (C-2), 51.8 (C-3), 68.2 (C-5), 39.7 (C-6), 56.8 (C-7), 129.1 (C-8), 126.2 (C-9), 119.5 (C-10), 129.2 (C-11), 109.4 (C-12), 147.9 (C-13), 24.5 (C-14), 29.8 (C-15), 57.3 (C-16), 66.4 (C-17), 13.8 (C-18), 129.6 (C-19), 128.2 (C-20), 46.8 (C-21), 173.7 (C-22, CO₂CH₃), 52.0 (C-23, CO₂CH₃).

Kopsamine 5

Light yellowish amorphous; Molecular formula: C₂₄H₂₈N₂O₇; [α]_D²⁵ -15.99 (c = 0.01, CH₃OH); UV λ_{max} (MeOH), nm (log ε): 220.0 (6.60), 230.5 (6.62), 248.1 (6.66), 288.3 (6.72); IR V_{max} (CHCl₃), cm⁻¹: 3249.6, 1631.7; Mass spectrum, *m/z*: 457.1977 [M+H]⁺; ¹H NMR (500 MHz, CDCl₃), δ ppm: 2.93 (1H, *m*, H-3_α), 3.10 (1H, *m*, H-3_β), 3.04 (1H, *dd*, *J* = 13.8 and 2.9 Hz, H-5_α), 3.10 (1H, *m*, H-5_β), 1.68 (1H, *m*, H-6_α), 2.10 (1H, *m*, H-6_β), 6.99 (1H, *s*, H-9), 6.52 (1H, *d*, *J* = 7.5 Hz, H-10), 1.28 (1H, *m*, H-14_α), 1.83 (1H, *m*, H-14_β), 1.28 (1H, *m*, H-15_α), 1.68 (1H, *m*, H-15_β), 1.41 (1H, *d*, *J* = 14.9 Hz, H-17_α), 2.93 (1H, *m*, H-17_β), 1.51 (1H, *m*, H-18_α), 2.35 (1H, *t*, *J* = 11.5 Hz, H-18_β), 1.12 (1H, *t*, *J* = 13.8 Hz, H-19_α), 1.68 (1H, *m*, H-19_β), 2.93 (1H, *m*, H-21), 3.87 (3H, *s*, H-23-N-CO₂CH₃), 3.75 (3H, *s*, H-25-CO₂CH₃), 5.87 (1H, *d*, *J* = 1.8 Hz, H-26_α-OCH₂O), 5.89 (1H, *d*, *J* = 1.2 Hz, H-26_β-OCH₂O); ¹³C NMR (125 MHz, CDCl₃), δ ppm: 74.5 (C-2), 47.6 (C-3), 50.3 (C-5), 37.1 (C-6), 57.6 (C-7), 134.2 (C-8), 114.9 (C-9), 104.1 (C-10), 148.4 (C-11), 135.5 (C-12), 123.3 (C-13), 17.3 (C-14), 35.5 (C-15), 74.7 (C-16), 41.6 (C-17), 23.9 (C-18), 32.2 (C-19), 32.4 (C-20), 68.2 (C-21), 156.3 (C-22, N-CO₂CH₃), 53.3 (C-23, N-CO₂CH₃), 173.1 (C-24, CO₂CH₃), 52.6 (C-25, CO₂CH₃), 100.4 (C-26, OCH₂O).

Akuammidine 6

Colorless crystals; Molecular formula: C₂₁H₂₄N₂O₃, m.p. 250–252 °C; [α]_D²⁵ +14.79 (c = 0.03, CH₃OH); UV λ_{max} (MeOH), nm (log ε): 280.0 (6.47); IR V_{max} (CHCl₃), cm⁻¹: 3246.4, 1631.7, 1429.0; Mass spectrum, *m/z*: 353.1858 [M+H]⁺; ¹H NMR (500 MHz, CD₃OD), δ ppm: 4.21 (1H, *d*, *J* = 9.8 Hz, H-3), 2.80 (1H, *s*, H-5), 2.80 (1H, *s*, H-6_α), 3.39 (1H, *dd*, *J* = 18.4 and 4.6 Hz, H-6_β), 7.35 (1H, *d*, *J* = 8.0 Hz, H-9), 6.94 (1H, *d*, *J* = 8.0 Hz, H-10), 7.02 (1H, *t*, *J* = 6.9 Hz, H-11), 7.25 (1H, *d*, *J* = 8.1 Hz, H-12), 1.88 (1H, *t*, *J* = 10.9 Hz, H-14_α), 2.67 (1H, *dd*, *J* = 13.2 and 2.3 Hz, H-14_β), 3.23 (1H, *d*, *J* = 2.3 Hz, H-15), 3.63 (1H, *d*, *J* = 9.8 Hz, H-17_α), 3.75 (1H, *d*, *J* = 9.8 Hz, H-17_β), 1.66 (3H, *d*, *J* = 6.7 Hz, H-18), 5.43 (1H, *dd*, *J* = 13.8 and 6.9 Hz, H-19), 3.49 (1H, *d*, *J* = 17.2 Hz, H-21_α), 3.63 (1H, *d*, *J* = 9.8 Hz, H-21_β), 2.92 (3H, *s*, H-23-CO₂CH₃); ¹³C NMR (125 MHz, CD₃OD), δ ppm: 137.4 (C-2), 50.9 (C-3), 58.0 (C-5), 24.0 (C-6), 105.1 (C-7), 126.9 (C-8), 117.6 (C-9), 118.7 (C-10), 121.1 (C-11), 111.0 (C-12), 137.6 (C-13), 29.1 (C-14), 29.3 (C-15), 51.6 (C-16), 67.9 (C-17), 12.3 (C-18), 117.2 (C-19), 136.8 (C-20), 55.1 (C-21), 173.8 (C-22, CO₂CH₃), 50.6 (C-23, CO₂CH₃).

CONCLUSION

Six known indole alkaloids have obtained from the roots of *K. singapurensis* Ridl. which are kopsamine *N*(4)-oxide **1**, kopsifine **2**, and rhazinicine **3**, aspidodasycarpine **4**, kopsamine **5** and akuammidine **6**.

Their structural elucidation was carried out by using spectroscopic methods such as 1D-NMR (¹H, ¹³C, and DEPT-135°) and 2D-NMR (COSY, HMQC, and HMBC), ultraviolet (UV), infrared (IR) and mass spectrometry (MS). The isolated compounds were then screened for cytotoxic activities on human cervical cancer (HeLa), human promyelocytic leukemia (HL-60), and the normal mouse fibroblast (NIH/3T3) cell lines by using the MTT assay. Alkaloid **2** exhibited significant cytotoxicity effects against HL-60 cell line with CD₅₀ values of 0.9 µg/mL. While, **3** and **4** showed very strong and moderate activities against HeLa cell line with CD₅₀ values of 2.9 µg/mL and 7.5 µg/mL, respectively. Besides, these three indole alkaloids also displayed cytotoxicity effects against the normal mouse fibroblast (NIH/3T3) cell line with CD₅₀ values of 20.7, 20.8, and 6.4 µg/mL, respectively. In this case, it defined that those compounds were not recommended for the cancer treatment, because they were cytotoxic against the normal cells, although they have cytotoxicity effects against HeLa and HL-60 cells. However, alkaloid **5** was considered to possess a moderately active cytotoxic activity towards HL-60 cell line with CD₅₀ values of 6.9 µg/mL. While, compound **6** showed strong cytotoxic activity against HeLa cell line with CD₅₀ values of 2.8 µg/mL, and **1** was inactive against both of cancer cells (HeLa and HL-60 cell lines) with CD₅₀ values of >60 µg/mL. In this case, this is the first report of cytotoxicity of these indole alkaloids against human cervical cancer HeLa, human promyelocytic leukemia HL-60, and the normal mouse fibroblast NIH/3T3 cell lines.

This is the first report of cytotoxicity of these indole alkaloids on cancer cells (HL-60 and HeLa) which is compared to the normal cell (NIH/3T3).

Acknowledgement: We would like to thank the Universiti Pendidikan Sultan Idris, University of Malaya and Universiti Sultan Zainal Abidin Terengganu for the technical supports, and also UM Research Collaborative Grant Scheme (CG032-2013).

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Source of Support: Nil, Conflict of Interest: None.

