American Journal of Plant Sciences, 2014, 5, 2869-2877
Published Online September 2014 in SciRes. http://www.scirp.org/journal/ajps
http://dx.doi.org/10.4236/ajps.2014.519302

Scientific Research

Cytotoxic and Anti-Tumour Promoting Activities of Carbazole Alkaloids from Malayan *Murraya koenigii* (L.) Spreng

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Received 1 July 2014; revised 3 August 2014; accepted 22 August 2014

Abstract

***Murraya koenigii* (L.) (Rutaceae) is an indigenous medicinally important herb of Indian origin and now is widely distributed throughout southern Asia. The stem bark, leaves and roots of Malayan *Murraya koenigii* were selected for phytochemical investigation. Eight carbazole alkaloids was isolated and identified using spectroscopic methods including NMR, IR, UV, MS spectra data. Crude extract and isolated compounds from the roots of this plant were screened for cytotoxic activity and antitumor promoting activity. All crude extracts of the roots including the isolated compounds, mahanimbine, mahanine and murrayafoline-A exhibited significant cytotoxic activity against CEM-SS cell line with IC_{50} 3 μg/mL. Girinimbine inhibited EBV-activation in the antitumor promoting assay.**

Keywords

Carbazole, Rutaceae, *Murraya koenigii*, NMR, Cytotoxic

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How to cite this paper: Ahmad, K., Tan, S.-P., Sukari, M.A., Ali, A.M. and Nafiah, M.A. (2014) Cytotoxic and Anti-Tumour Promoting Activities of Carbazole Alkaloids from Malayan *Murraya koenigii* (L.) Spreng. *American Journal of Plant Sciences*, **5**, 2869-2877. http://dx.doi.org/10.4236/ajps.2014.519302

1. Introduction

Murraya koenigii is a member of Rutaceae family and represented by about 150 genera and 1600 species. 14 species of this genus are known [1], but only two species can be found in Malaysia; namely *Murraya koenigii* and *Murraya paniculata* [2] [3]. *Murraya koenigii* is a medicinal important herb of Indian origin. It has been widely used as natural flavouring in curries and sources [3]-[5], and ingredient in traditional medicine formulations [4]-[6]. It has also reported by researchers that carbazole alkaloids possess various biological activities such as antitumor, anti-oxidative, anti-mutagenic, and anti-inflammatory activities [6]-[8].

The aim of the present study was to examine the cytotoxic potential and antitumor promoting activity of crude extract and the isolated carbazole alkaloids present in *M. koenigii*. In our continuing study on leaves, roots and barks of *M. koenigii* (L.) Spreng, have afforded eight carbazole alkaloids; from bark: mahanimbine **1**, girinimbine **2**, murrayacine **3**, murrayazoline **4** and murrayanine **5**; from leaves: mahanimbine **1** and mahanine **6**; and from roots: girinimbine **2** murrayanine **5**, 3-methylcarbazole **7** and murrayafoline-A **8**. Their structures were elucidated by combination of various spectroscopic methods such as 1D and 2D NMR, IR, UV and MS.

2. Experimental

2.1. General Experimental Procedures

Spectra were recorded on the following instruments. The IR spectra were recorded using KBr disc on Perkin Elmer FTIR Spectrophotometer model 1650. UV spectra were recorded on a Shimadzu UV-2100 spectrophotometer. ^{1}H and ^{13}C NMR spectra were obtained on JEOL Spectrometer at 500 MHz with tetramethylsilane (TMS) as the internal standard. Mass spectra were recorded on an AEI-MS 12 instrument. The column chromatography was carried on silica gel (Merck 9385) and Merck silica gel 60 PF_{254} was used for analytical TLC analysis. Analytical Thin Layer Chromatograph (TLC) was performed on commercially available Merck DC-Plasticfolien TLC plastic sheet precoated witih Keiselgel 60 P_{254}.

2.2. Plant Material

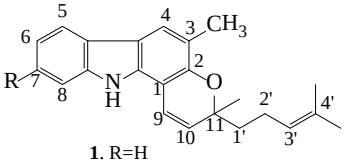
The barks, leaves and roots of *Murraya koenigii* for this study were collected in Banting, Selangor, Malaysia in 1996.

2.3. Extraction and Isolation

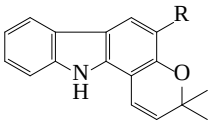
The dried bark (1.1 kg) of *Murraya koenigii* were ground into find powder and extracted continuously soaking for three days with petroluem ether, chloroform and methanol. Each extraction was repeated three times. Then the extract was dried on the rotary evaporator. After removal of the solvents, the crude of petroluem ether (42.2 g), chloroform (40.5 g) and methanol (40.0 g) were obtained. After evaporation of the solvent, 15.0 g of the petroluem ether crude was subjected to column chromatography over silica gel (gradient solvent system; Petroluem ether, $CHCl_3$ and MeOH) yielded five known compounds: mahanimbine (**1**, 60.1 mg), girinimbine (**2**, 70.2 mg), murrayacine (**3**, 4.5 mg), murrayazoline (**4**, 6.3 mg) and murrayanine (**5**, 9.9 mg).

The dried leaves (600 g) of *Murraya koenigii* were ground and extracted exhaustively with petroluem ether followed by chloroform and methanol by soxhlet extractor for 17 hours. After evaporation of the solvent, 16.8 g, 11.2 g and 15.8 g of crude extracts, respectively, were obtained. 15.0 g of petoluem ether crude was subjected to column chromatography over silica gel (gradient solvent system; petroluem ether, chloroform and Methanol) yielded mahanimbine (**1**, 0.21 g). 10.0 g of chloroform crude was subjected over silica gel and yielded mahanine (**6**, 13.1 mg).

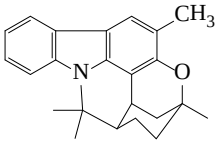
The air-dried and finely grounded roots (1.1 kg) of *Murraya koenigii* were extracted with petroluem ether and methanol for 48 hours for three times. After evaporation of the solvent, 10.0 g of petoluem ether crude was subjected to column chromatography over silica gel (gradient solvent system; petroluem ether, chlorofom and Methanol) yielded four carbazoles: girinimbine (**2**, 0.21 g), murrayanine (**5**, 30.2 mg), 3-methylcarbazole (**7**, 8.3 mg) and murrayafoline-A (**8**, 0.10 g).



1. R=H
6. R=OH



2. R = CH
3. R = CHO



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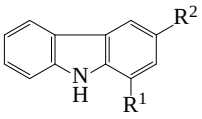


5. $R^1 = OCH_3$, $R^2 = CHO$
7. $R^1 = H$, $R^2 = CH_3$
8. $R^1 = OCH_3$, $R^2 = CH_3$

2.4. Cytotoxic Assay

The CEM-SS cell line (T-lymphoblastic leukimia) was obtained from the Natioanl Institutes Cancer, Frederick, Maryland, USA. The cells were cultured and maintained in growth medium as described by Ali *et al.* [9]. Cytotoxic test were determined by performing the microtitration assay [10]. The tests were performed in 96-well plates, a microtiter plate. Each well was added with 100 µL of varying concentration of crude of *Murraya koenigii* and the isolated carbazoles prepared from the stock solutions by serial dilution in RPM I - 1640 medium. Subsequently, each well was filled with 100 µL of cell suspension in complete growth medium at 2×10^5 cells/mL.

Controls containing only untreated cells were included for each sample. The assay for each concentration of samples were performed in triplicate and the culture plates were incubated at 37˚C with 5% (v/v) CO_2 for three days. The cytotoxicity index used was IC_{50}, which is the concentration that eields 50% inhibition of the cell compared with untreated control. Extracts or isolated compounds which exhibit cytotoxic index (IC_{50}) < 20 µg/mL were considered to possess significant activity.

2.5. Anti-Tumor Promoting Activity Assay

2.5.1. Treatment of Cells with Samples

Dividing Raji cells/mL in log phase at a density of 5.5×10^5 cells/mL were incubated with 5 µL plant extracts and isolated compounds at various concentrations (1 to 32 µg/mL) in the presence of 10 µL of PMA (5 µM) and 6 µL of sodium-n-butyrate (0.5 µM). The cells were incubated for 48 hours in a ninidified incubator at 37 C with 5% CO_2 in air. The negative controls consisted of untreated Raji cells treated with the combination of 10 µL of PMA (5 µM) and 6 µL of sodium-n-butyrate (0.5 µM).The positive controls consisted of Raji cells treated with the combination of 10 µL of PMA (5 µM) and 6 µL of sodium-n-butyrate (0.5 µM).

2.5.2. Epstein-Barr Virus Activation Assay

The inhibition of Epstein-Barr virus (EBR) activation was used to evaluate in vitro antitumor promoting activity [11]. Raji cells were grown in 10 µL of RPMI 1640 suplemented with 10% fetal calf serum. The cells (5.5×10^5 cells/mL) were maintained in 10 mL of RPMI 1640 medium suplemented witih 10% fetal calf serum containing sodium-n-butyrate (0.5 µM), phorbol 12-myristate 13-acatate (5 µM) and test extract (5 µL) at 37˚C under 5% CO_2 for 48 hours using 24 wells plate. After 48 hours, the viability of cells was checked using tryphan blue dye exclusion method. Only those with viability more than 90% were used for this assay. The cells were transferred into appendorf tube and spun down at 1000 rpm for 10 minutes by using a bench centrifuge. The supernatant the cell pellet 1 mL of PBS was added into wash cells and repeated twice. After the second washing, the cells were being spun down at 1000 rpm for 10 minutes. The supernatant was removed and a drop of the cell suspension was added on to a 12 wells test Teflon coated slide (Cooke, USA).

The distribution of the cells was examined under the inverted microscope in order to make sure the drop is confluent and not over dripping. If the spread of the cells was not satisfactory, the dilution of the cells was altered. The slides with the drops of cells were dried in a laminar flow hood. The slides were then fixed in cold

acetone for 10 minutes. Cold acetone was obtained by keeping it in −20˚C overnight before fixing the cells. Then, the slides were dried at room temperature and stored in slide boxed at −20˚C for a short period.

Antibody obtained from Nasopharryngeal carcinoma (NPC) patients was used to cover the whole well and incubated at 37˚C for 40 minutes. To avoid drying, the cells were incubated by placing the slides on a wet tissue with cover. After 40 minutes, the slides were washed with Phosphate buffer saline (PBS) twice by dipping in PBS with gentle agitation on a platform shaker for 10 minutes each. Excess PBS was removed using tissue paper by avoiding touching the cells.

Second antibody FITC-conjugated IgG was added by covering the wells. The conjugate used in this study was anti-human IgG flourescein isothiocyanate-conjugated (FITC) (Sigma, USA). Again, the cells were incubated for 40 minutes at 37 C by putting in a box with water. Then, excess antibody was removed by washing with PBS twice. The slides were dripped in PBS with gentle agitation on platform shaker for 10 minutes each. PBS was removed using tissue paper and avoid touching the cells. Prior checking under UV microscope, 50% glycerol was dropped on the slip (Germany). The slides were then ready for examined under fluorescent microscope.

3. Results and Discussions

Mahanimbine ($C_{23}H_{25}NO$) 1: white needles, with melting point 93˚C - 95˚C; UV (MeOH) λ_{max} 238, 288 nm; IR (KBr disc) λ_{max} 3338 (N-H), 3312, 2964, 1646 and 1156 (C-O) cm^{-1}; GC-MS *m/z* 331 (M^+). ^1H and ^{13}C NMR see **Table 1** and **Table 2**.

Girinimbine ($C_{18}H_{17}NO$) 2: colourless crystal, with melting point 171˚C - 173˚C; UV (MeOH) λ_{max} 237 and 287 nm; IR (KBr disc) λ_{max} 3338 (N-H), 1121 (C-O), 742 and 690 cm^{-1}; GC-MS *m/z* 263 (M^+). ^1H and ^{13}C NMR see **Table 1** and **Table 2**.

Murrayacine ($C_{18}H_{15}NO_2$) 3: greenish needles, with melting point 240˚C - 242˚C; UV (MeOH) λ_{max} 237 and 288 nm; IR (KBr disc) λ_{max} 3222 (N-H), 1188 (C-O) and 1614 cm^{-1}; GC-MS *m/z* 277 (M^+). ^1H and ^{13}C NMR see **Table 1** and **Table 2**.

Murrayazoline ($C_{23}H_{25}NO$) 4: yellow crystal, with melting point 265˚C - 267˚C; UV (MeOH) λ_{max} 235 and 287 nm; IR (KBr disc) λ_{max} 2936 (C-H stretching) and 1150 (C-O) cm^{-1}; GC-MS *m/z* 331 (M^+). ^1H and ^{13}C NMR see **Table 1** and **Table 2**.

Murrayanine ($C_{14}H_{11}NO_2$) 5: white needles, with melting point 170˚C - 172˚C; UV (MeOH) λ_{max} 238, 273 and 340 nm; IR (KBr disc) λ_{max} 3167 (N-H) and 1661 (C=O) and 1160 (C-O) cm^{-1}; GC-MS *m/z* 225 (M^+). ^1H and ^{13}C NMR see **Table 1** and **Table 2**.

Mahanine ($C_{23}H_{25}NO_2$) 6: white needles, with melting point 91˚C - 92˚C; UV (MeOH) λ_{max} 235 and 287 nm; IR (KBr disc) λ_{max} 3417 (NH/OH) and 1113 (C-O) cm^{-1}; GC-MS *m/z* 347 (M^+). ^1H and ^{13}C NMR see **Table 1** and **Table 2**.

3-Methylcarbazole ($C_{13}H_{11}N$) 7: white solid, with melting point 205˚C - 208˚C; UV (MeOH) λ_{max} 235 and 287 nm; IR (KBr disc) λ_{max} 3407 (NH/OH) and 1607 cm^{-1}; GC-MS *m/z* 181 (M^+). ^1H and ^{13}C NMR see **Table 1** and **Table 2**.

Murrayafoline-A ($C_{23}H_{25}NO_2$) 8: dark brown viscous oil; UV (MeOH) λ_{max} 237 and 287 nm; IR (KBr disc) λ_{max} 3420 (NH/OH) and 1137 (C-O) cm^{-1}; GC-MS *m/z* 211 (M^+). ^1H and ^{13}C NMR see **Table 1** and **Table 2**.

The bark, leaves and roots of *M. koenigii* were extracted with petroluem ether, chloroform and methanol. The fractionation of the petroluem ether and chloroform extracts followed by column chromatography and TLC yield 8 carbazole alkaloids which were identified as mahanimbine **1**, girinimbine **2**, murrayacine **3**, murrayazoline **4**, murrayanine **5**, mahanine **6**, 3-methylcarbazole **7** and murrayafoline-A **8** by spectroscopic methods (**Figure 1**). All compounds have showed the similar spectroscopic features with the carbazole alkaloids published in the literature.

Crude extract of the roots and isolated compounds of *M. koenigii* were screened for cytotoxic activity and antitumor promoting activity. **Table 3** showed the inhibition concentration (IC_{50}) of the tested crude extracts and isolated pure compounds that exhibited 50% of T-lymphoblastic leukimia cells (CEM-SS) as compared to the untreated control for 72 hours. All tested samples exhibited significant cytotoxic effects towards CEM-SS cells with IC_{50} of 3 μg/mL, except girinimbine, in which IC_{50} of 30 μg/mL.

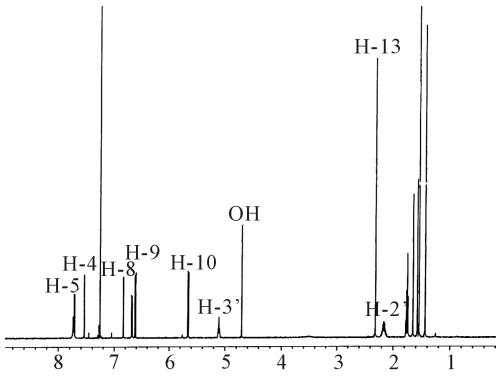
For the *in vitro* antitumor promoting activity, all crude extracts and the isolated pure compounds exhibited 100% of inhibition rate. However, most of the samples were cytotoxic towards Raji cells except girinimbine which gave 70.7% viability (**Table 4**).

Table 1. ^{1}H NMR [500 MHz, δH (J Hz)] of **1 - 8** in $CDCl_3$.

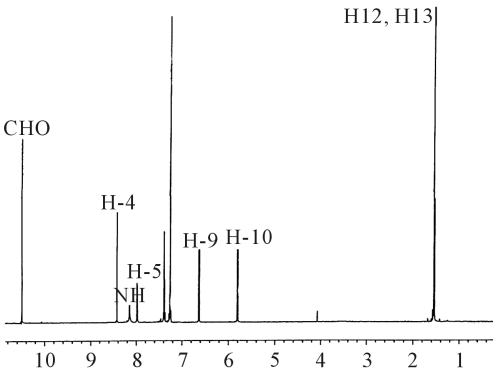
Position	δH, J Hz							
	1	2	3	4	5	6	7	8
NH	7.84 (s)	7.84 (s)	8.14 (s)		8.62	7.74 (s)	7.94 (s)	8.11 (s)
1							7.40 (d, 7.91 Hz)	
1a								
2					7.46 (s)		7.32 (d, 8.41 Hz)	6.68 (s)
3								
4	7.66 (s)	7.67 (s)	8.42 (s)	7.48 (s)	8.14 (s)	7.54 (s)	7.87 (s)	7.45 (s)
4a								
5	7.90 (d, 7.33 Hz)	7.91 (d, 7.08 Hz)	7.98 (d, 7.81 Hz)	7.89 (d, 7.08 Hz)	8.11 (d, 7.93 Hz)	7.71 (d, 8.24 Hz)	8.04 (d, 7.91 Hz)	7.98 (d, 7.81 Hz)
5a								
6	7.17 (ddd, 8.6, 7.08, 0.97 Hz)	7.18 (ddd, 8.6, 7.08, 0.98 Hz)	7.25 (ddd, 8.24, 7.32, 0.91 Hz)	7.14 (t, 7.45 Hz)	7.32 (t, 7.93 Hz)	6.67 (dd, 8.24, 2.14 Hz)	7.21 (m)	7.15 (m)
7	7.29 (ddd, 8.06, 7.08, 0.97 Hz)	7.30 (ddd, 8.06, 7.08, 0.98 Hz)	7.38 (ddd, 8.45, 7.32, 1.22 Hz)	7.24 (t, 7.08 Hz)	7.51 (t, 8.24 Hz)		7.21 (m)	7.15 (m)
8	7.36 (d, 7.81 Hz)	7.36 (d, 8.06 Hz)	7.39 (d, 7.08 Hz)	7.46 (d, 10.50 Hz)	7.49 (d, 7.93 Hz)	6.83 (d, 2.14 Hz)	7.40 (d, 7.91 Hz)	7.31 (d, 6.35 Hz)
8a								
9	6.63 (d, 9.76 Hz)	6.61 (d, 9.77 Hz)	6.63 (d, 9.77 Hz)	3.30 (d, 4.64 Hz)		6.61 (d, 10.00 Hz)		
10	5.65 (d, 9.76 Hz)	5.69 (d, 9.77 Hz)	5.80 (d, 9.77 Hz)	1.45 (m)		5.65 (d, 10.00 Hz)		
11								
11-Me'	1.45 (s)	1.48 (s)	1.56 (s)	1.28 (s)		1.43 (s)		
11-Me"		1.48 (s)	1.56 (s)					
3-Me	2.33 (s)	2.33 (s)		2.32 (s)		2.31 (s)	2.53 (s)	2.49 (s)
3-CHO			10.50 (s)		10.05 (s)			
1-OMe					4.06 (s)			3.89 (s)
1'	1.76 (t, 8.42 Hz)			1.45 (m)		1.75 (t, 8.39 Hz)		
2'	2.15 (m)			1.45 (m)		2.16 (m)		
3'	5.11 (t, 7.20 Hz)			1.45 (m)		5.11 (t, 7.17 Hz)		
4'								
5'	1.58 (s)					1.54 (s)		
6'	1.65 (s)					1.65 (s)		
4'-Me'				1.90 (s)				
4'-Me"				1.90 (s)				
7-OH						4.70 (s)		

Table 2. ^{13}C NMR [125 MHz, δC (J Hz)] of **1 - 8** in $CDCl_3$.

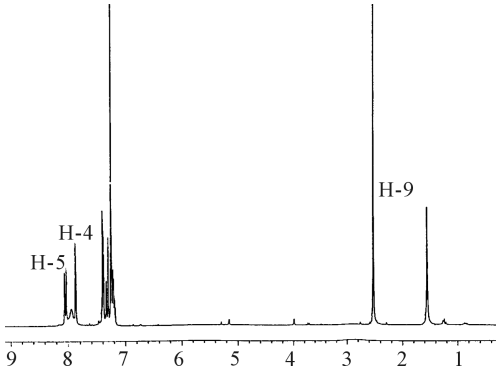
Position	δC							
	1	2	3	4	5	6	7	8
1	104.2	104.4	103.6	107.3	146.1	104.2	127.1	145.3
1a	138.4	139.4	139.7	142.4	139.4	140.7	139.8	129.4
2	149.9	149.8	154.3	155.0	103.5	149.0	119.2	107.6
3	116.6	116.7	117.7	113.9	130.2	116.7	123.2	127.9
4	121.2	121.1	119.4	113.4	120.4	120.4	120.2	112.5
4a	118.4	118.6	118.6	118.4	123.6	118.2	123.5	123.4
5	119.4	119.5	119.8	119.8	120.7	120.0	120.2	120.4
5a	123.9	123.9	123.6	127.3	123.6	118.3	128.7	124.3
6	119.3	119.3	120.5	119.2	120.7	117.5	110.2	119.0
7	124.2	124.2	125.5	122.6	111.5	153.5	125.6	110.9
8	110.3	110.4	110.2	119.1	126.6	96.9	110.5	125.4
8a	134.8	134.8	139.6	140.6	134.0	134.7	137.7	139.4
9	117.5	117.2	115.6	29.2		108.3		
10	128.5	129.4	129.6	28.3		128.7		
11	78.1	75.8	76.2	76.1		78.0		
11-Me'	25.8	27.6	27.5	22.9		25.8		
11-Me"		27.6	27.5					
3-Me	16.1	16.1		15.4		16.0	21.4	21.9
3-CHO			188.7		191.8			
1-OMe					55.8			55.4
1'	40.7			48.6		40.7		
2'	22.7			36.1		22.7		
3'	124.1			21.8		124.2		
4'	131.7			60.4		131.6		
5'	17.6					17.6		
6'	25.7					25.6		
4'-Me'				30.1				
4'-Me"				36.8				



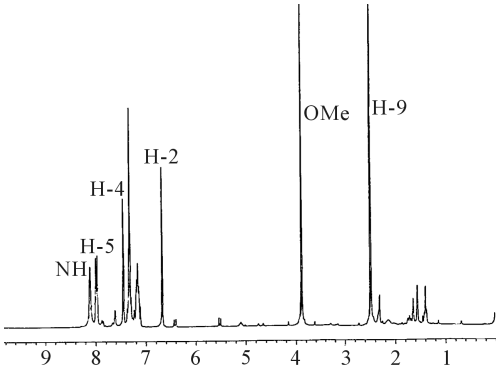
Mahanimbine **1**



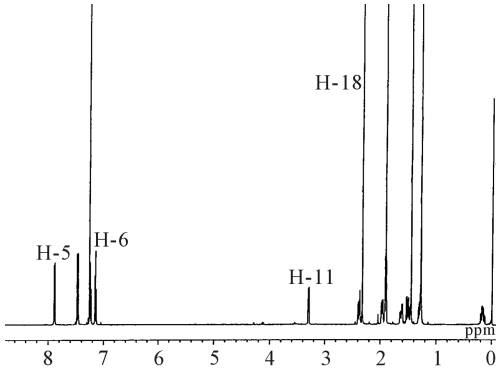
Girinimbine **2**



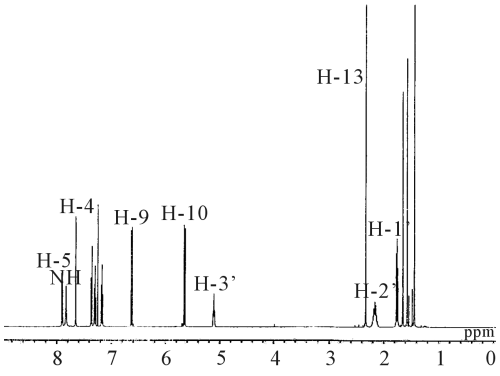
Murrayacine **3**



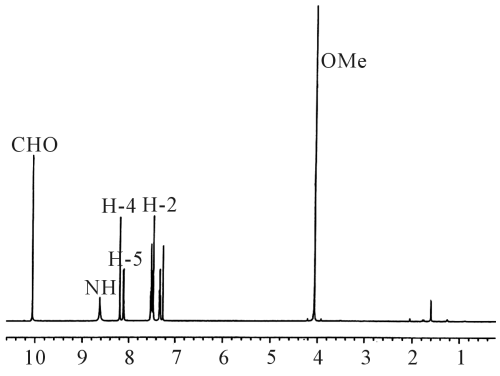
Murrayazoline **4**



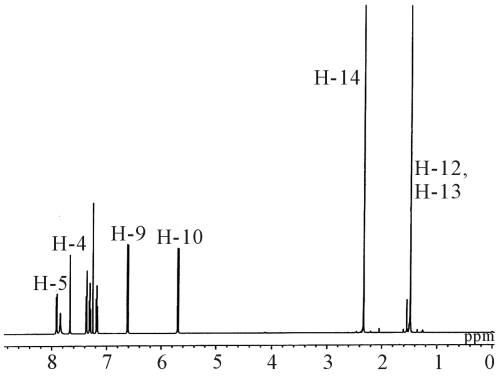
Murrayanine **5**



Mahanine **6**



3-Methylcarbazole **7**



Murrayafoline-A **8**

Figure 1. ^{1}H NMR Spectra of **1** - **8**.

Table 3. Cytotoxic activity of crude extracts and isolated pure compounds from the roots of *M. koenigii* on CEM-SS cell line.

Samples	IC_{50} (μg/mL)
Petroluem ether crude	3
Chloroform crude	3
Methanol crude	3
Mahanimbine **1**	3
Girinimbine **2**	30
Mahanine **6**	3
Murrayafoline-A **8**	3

Table 4. *In vitro* antitumor promoting activity and Raji cell viability.

Samples	Viability (%)	Inhibition Rate (%)
Petroluem ether crude	2.4	100
Chloroform crude	0	100
Methanol crude	2.2	100
Mahanimbine **1**	0	100
Girinimbine **2**	70.7	100
Mahanine **6**	0.7	100
Murrayafoline-A **8**	0	100

Acknowledgements

We gratefully acknowledge the technical support provided by University Pendidikan Sultan Idris and University Putra Malaysia.

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