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Reflexin A, a new indole alkaloid from *Rauvolfia reflexa* induces apoptosis against colon cancer cells

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**ABSTRACT**

One new indole alkaloid, reflexin A (1), and two known indoles, macusine B (2) and vinorine (3), were isolated from the bark of *Rauvolfia reflexa*. Their structures were elucidated by 1D and 2D NMR, UV, IR, and MS spectroscopic analyses. Compound 1 displayed anticancer activity against HCT-116 colon cancer cells with an IC\(_{50}\) value of 30.24 ± 0.75 \(\mu\)M. The results implied that the newly isolated 1 induced apoptosis in HCT-116 cells, suggesting its possible role as an anticancer agent. In vivo acute toxicity study was performed on compound 1 to evaluate its safety profile.

1. Introduction

Colorectal cancer is the third most common type of cancer in both males and females. In the United States only, 1.2 million cases with a history of colorectal cancer
were estimated in 2012. Despite noteworthy progress in surgery, chemotherapy, colostomy, radiation and monoclonal antibody therapy, the 5-year survival rate after colorectal cancer eradication is still approximately 64.3%. This rate decreases to 11.7% when the cancer has metastasized and spread to distant organs [1]. Moreover, the survivors of aggressive colorectal cancer suffer from bowel dysfunction with a dramatic decline in their quality of life [2]. Thus, there is abundant interest in the search for novel chemopreventive agents that have more long-term effectiveness and that incur minimal side effects and toxicities [3]. In recent years, the development of cancer treatment strategies that employ natural agents for the suppression of cancer progression has been recognized as a potential field in decreasing cancer incidence [4]. This potential is highlighted by the involvement of natural products in the progress of about 70% of the anticancer drugs between 1980 and 2006 [5]. Nevertheless, numerous plant species, such as *Rauvolfia reflexa*, still need to be scientifically examined to further explore their active components for medicinal applications. *Rauvolfia reflexa* is widely distributed in tropical regions. It is traditionally used as an antidote for poisons and is believed, mainly in Tanzania, to relieve strong plasmodial spasms occurring with malaria by taking a decoction of the leaves [6]. *R. reflexa* is belonging to the Apocynaceae family which comprises about 120 species, mostly evergreen shrubs [7]. The chemistry of the *Rauvolfa* species has been studied expansively; however, less attention was paid to the bioactivity of the isolated compounds [8]. In our previous study, we investigated the *R. reflexa* species and isolated bioactive compounds from the leaves that showed activity on the acetylcholinesterase and butyrylcholinesterase enzymes. Thus, the isolated compounds were suggested to have anti-Alzheimer effects, according to the inhibitory-guided isolation [9]. In this study, we were interested in examining the bioactivity of *R. reflexa* bark. One new indole alkaloid, reflexin A, and two known compounds (Figure 1) were isolated from the bark methanol extract and its corresponding fractions, and the apoptotic effect and underlying mechanism of action were evaluated on HCT-116 colon cancer cells.

2. Results and discussion

2.1. Chemical interpretation of the alkaloids

Compound 1 was isolated as a brownish solid with an optical rotation of $[\alpha]_{D}^{24} = -31$ (c 0.05, CHCl$_3$). The HRESIMS showed a molecular ion at $m/z$ 413.2662 [M + H]+. The UV spectrum shown wavelengths at 226 and 299 nm, which presents a typical indole chromophore system [10]. In addition, the IR (CHCl$_3$) spectrum showed peaks...
at 1691 cm$^{-1}$ (conjugated carbonyl ester group stretching), 2925–2852 cm$^{-1}$ (C-H stretching), and 3351 cm$^{-1}$ confirming the presence of an NH group. The $^1$H NMR spectrum specified the presence of two overlapped aromatic signals at $\delta$ 6.93 (2H, d, $J = 1.8$ Hz) due to protons on C-9 and C-11, and a singlet at $\delta$ 7.51 assigned to H-17. Another three singlets appeared at $\delta$ 3.79 (3H, s), 3.77 (3H, s) and 3.69 (3H, s), which were attributed to the protons of three methoxyls attached to C-10, C-12 and C-22, respectively. The $^{13}$C NMR spectrum of 1 indicated the presence of 23 carbons, including eight quaternary carbons, seven methines, four methylenes, and four methyl groups based on the DEPT spectrum. The presence of the carbonyl group at C-22 was demonstrated via a correlation between the carbonyl signal at $\delta$ 168.7 and the signal due to H-17 at $\delta$ 7.51 in the HMBC spectrum. The HMBC spectrum also showed correlations between the signals from H-18 to C-19 and C-20, from H-17 to C-16 and C-19, from OMe-10, 12 to C-10, 12 and from OMe-22 to C-22 (Table 1). The relative configuration of 1 was confirmed by the observed $^1$H–$^1$H ROESY experiment. The ROESY spectrum of 1 showed cross-peaks assigned to the dipolar interactions (spatial proximity, Figure 2) of H-14a with H-15 and H-20, H-18 with H-21a and H-20, H-9 with OMe-10, H-11 with OMe -12, and H-3 with H-18. Thus, the structure of the new indole alkaloid was established and named reflexin A (1) (Figure 3).

Two known alkaloids, macusine B (2) [11] and vinorine (3) [8], were also identified based on their spectroscopic data and association with published report.

**Table 1.** $^1$H NMR (acetone-$d_6$, 400 MHz) and $^{13}$C NMR (100 MHz) spectral data of 1 ($\delta$ in ppm, $J$ in Hz).

<table>
<thead>
<tr>
<th>Position</th>
<th>$^1$H-NMR</th>
<th>$^{13}$C-NMR</th>
<th>HMBC ($^J_{CH}$, $^J_{CD}$)</th>
<th>COSY</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>–</td>
<td>133.8</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>3</td>
<td>3.20 dd (10.3, 12.3)</td>
<td>60.4</td>
<td>–</td>
<td>14</td>
</tr>
<tr>
<td>5</td>
<td>2.90</td>
<td>53.5</td>
<td>–</td>
<td>6</td>
</tr>
<tr>
<td>6</td>
<td>2.90</td>
<td>21.9</td>
<td>–</td>
<td>5</td>
</tr>
<tr>
<td>7</td>
<td>–</td>
<td>107.0</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>8</td>
<td>–</td>
<td>131.0</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>9</td>
<td>6.93 d (1.8)</td>
<td>101.3</td>
<td>11, 13</td>
<td>–</td>
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<tr>
<td>10</td>
<td>–</td>
<td>146.9</td>
<td>–</td>
<td>–</td>
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<td>11</td>
<td>6.93 d (1.8)</td>
<td>95.9</td>
<td>8, 10</td>
<td>–</td>
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<td>12</td>
<td>–</td>
<td>145.1</td>
<td>–</td>
<td>–</td>
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<tr>
<td>13</td>
<td>–</td>
<td>120.3</td>
<td>–</td>
<td>–</td>
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<tr>
<td>14</td>
<td>2.30 m</td>
<td>34.2</td>
<td>15</td>
<td>3</td>
</tr>
<tr>
<td>15</td>
<td>1.52 m</td>
<td>31.4</td>
<td>19</td>
<td>14</td>
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<tr>
<td>16</td>
<td>–</td>
<td>110.0</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>17</td>
<td>7.51 s</td>
<td>154.9</td>
<td>19, 16</td>
<td>–</td>
</tr>
<tr>
<td>18</td>
<td>1.38 d (6.8)</td>
<td>17.8</td>
<td>20, 19</td>
<td>19</td>
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<td>19</td>
<td>4.49 m</td>
<td>72.4</td>
<td>–</td>
<td>18, 20</td>
</tr>
<tr>
<td>20</td>
<td>1.70 m</td>
<td>38.5</td>
<td>–</td>
<td>21, 15, 19</td>
</tr>
<tr>
<td>21</td>
<td>2.90</td>
<td>53.5</td>
<td>15, 20</td>
<td>20</td>
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<tr>
<td>22</td>
<td>–</td>
<td>168.7</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>NH</td>
<td>9.80 s</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>OMe -10</td>
<td>3.79 s</td>
<td>56.1</td>
<td>10</td>
<td>–</td>
</tr>
<tr>
<td>OMe -12</td>
<td>3.77 s</td>
<td>56.0</td>
<td>12</td>
<td>–</td>
</tr>
<tr>
<td>OMe -22</td>
<td>3.69 s</td>
<td>50.3</td>
<td>22</td>
<td>–</td>
</tr>
</tbody>
</table>
2.2. Effect of compound 1 on cell proliferation

As shown in Table 2, the indole alkaloid exhibited cytotoxic effects against cancer cell lines. At the tested concentrations (1.5–100 µg/ml), compound 1 showed cytotoxic effects against two breast cancer cell lines (MCF-7 and MDA-MB-231) and HT-29 colon cancer cells. However, compound 1 elicited the strongest effect on HCT-116 cells, with IC50 values of 30.24 ± 0.75, 21.60 ± 0.83, and 16.06 ± 0.55 μM after 24, 48, and 72 h, respectively. Meanwhile, the 5-FU positive control demonstrated an IC50 value of 21.29 ± 0.25 μM against HCT-116 cells after 24 h. Normal human CCD-841 cancer cells and WRL-68 hepatic cells were not distinctly affected by reflexin A. Therefore, the MTT assay results suggest that the suppressive effect of compound 1 is

Figure 2. Important 1H-1H ROESY correlations for reflexin A (1).

Figure 3. Selected (—) COSY and (→) HMBC correlation of reflexin A (1).
selective for HCT-116 cancer cells. Amongst all three isolated compounds, compound 1 showed the most potent cytotoxic effects against HCT-116 cells; therefore, the possible mechanism of action of its antiproliferative effect was evaluated.

2.3. Apoptotic effect of compound 1

The morphological changes of HCT-116 cells treated with compound 1 were observed under a fluorescent microscope. At 24 h, membrane blebbing and cytoplasmic shrinkage were noted, and small apoptotic bodies were discernible at 48 and 72 h, as observed and compared to the control in Figure 4. Early apoptosis was observed using intervening AO within the fragmented DNA under bright green fluorescence, whereas the presence of living healthy cells was monitored by the visualization of green intact nuclei. Moderate apoptosis in the form of nuclear chromatin condensation and blebbing was noticed prior to the late stage apoptosis and secondary necrosis, which was indicated by the reddish-orange color of the denatured DNA. Fluorescent microscopy subsequently showed the binding of AO as an apparent marker of apoptosis in a time-dependent manner.

2.4. Compound 1 induced early and late apoptosis

Distinctive characteristics of apoptosis morphology were observed in HCT-116 cells after treatment with reflexin A. The morphological characterizations of apoptosis were accompanied by numerous biochemical modifications, such as protein cleavage, DNA breakdown and protein cross-linking. The translocation of the cell surface marker phosphatidylserine on the outer membrane is a critical biochemical modification during early apoptosis [12]. Therefore, we applied an Annexin V-FITC probe to examine the externalization of phosphatidylserine (PS) in colon cancer cells. The recombinant phosphatidylserine-binding protein Annexin V has a high affinity for externalized PS, which is employed to detect early apoptosis [13]. In contrast, PI is used to detect late apoptosis and necrosis. Analysis of the fluorescence intensity revealed the quantity of necrotic (Annexin V negative and PI positive), late apoptotic (both Annexin V and PI positive), early apoptotic (Annexin V positive and PI negative) and viable (both Annexin V and PI negative) cells [14]. As illustrated in Figure 5, the number of treated cells that faced early apoptosis elevated after 24 and

<table>
<thead>
<tr>
<th>Cell Lines</th>
<th>IC50 (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCT-116</td>
<td>30.24 ± 0.75</td>
</tr>
<tr>
<td>MCF-7</td>
<td>85.80 ± 1.24</td>
</tr>
<tr>
<td>HT-29</td>
<td>107.11 ± 2.31</td>
</tr>
<tr>
<td>MDA-MB-231</td>
<td>146.67 ± 3.48</td>
</tr>
<tr>
<td>HepG2</td>
<td>207.08 ± 4.25</td>
</tr>
<tr>
<td>A549</td>
<td>232.28 ± 4.46</td>
</tr>
<tr>
<td>WRL-68</td>
<td>&gt;100</td>
</tr>
<tr>
<td>CCD-841</td>
<td>&gt;100</td>
</tr>
</tbody>
</table>
48 h of incubation with reflexin A. After 24, 48, and 72 h of incubation, the population of late apoptotic cells increased to approximately 25%. The findings obtained from fluorescence detection together with the externalization of PS, suggested the induction of apoptosis by reflexin A.

2.5. Compound 1 induced G1 cell cycle arrest

Cancer is widely known to be a cell cycle disease. Thus, one of the most prevalent alterations during the occurrence of cancer is the deregulation of the cell cycle. The highly ordered cell cycle process contains four distinct phases and involves multiple checkpoints that regulate DNA integrity, extracellular growth signals and cell size [15]. Cell cycle progression depends on the negative regulators, the cyclin-dependent kinase inhibitors (CKIs), and the accelerators or positive regulators, the cyclin-dependent kinases (CKIs). Abnormal suppression of negative regulators and
activation of accelerators can lead to tumor development [16]. Thus, the activation of cell cycle arrest in cancer cells has become an effective cancer treatment strategy. In the current study, we evaluated the cell cycle progression in HCT-116 cells treated with compound 1 using flow cytometry analysis. As shown in Figure 6, significant G1 phase arrest was induced in a time-dependent manner because the number of HCT-116 cells increased significantly from 55.74% (control) to 63.71% after 24 h of

**Figure 5.** Time-dependent induction of early and late apoptosis by reflexin A as indicated by the percentage of early apoptotic, late apoptotic and necrotic cells after 24, 48 and 72 h of treatment. The data are shown as the mean ± SEM. * represents significant difference ($p < .05$) compared with the control.
2.6. Compound 1 induced caspase-3/7, caspase-8 and caspase-9 activation

The apoptotic machinery, including the two main extrinsic and intrinsic pathways, can be activated by a variety of stimuli that lead to the activation of the caspases [17]. The activation of these cysteine proteases is followed by a cascade of events that lead to the ultimate demise of the apoptotic cell. The key caspases in the activation of the extrinsic and intrinsic pathways are the initiator caspases, caspase-8 and caspase-9, respectively [18]. Activated initiator caspases activate executioner or effector caspases, specifically caspase-3, caspase-6 and caspase-7. The aforementioned biochemical and morphological changes in apoptotic cells are triggered and induced by effector caspases [19]. The activation of caspase-3/7, caspase-8 and caspase-9 was measured to determine the pathway involved in the induction of apoptosis in HCT-116 cells by reflexin A. As indicated in Figure 7, compound 1 induced significant activation of caspase-3/7 and caspase-9 after 24 h of treatment and was associated with the activation of caspase-8 after 48 h. These data implied the involvement of the intrinsic pathway in apoptosis induction, accompanied by the activation of the extrinsic pathway during the later stage of treatment.

2.7. Migration and invasion effects of compound 1 on the HCT-116 cells

Cell migration is considered to be a vital step towards metastasis and tumor invasion. Cancer cells can migrate in different ways based on the degree of differentiation and cell type [20]. The movement of cancer cells within tissues via their own motility is a
Figure 7. Relative luminescence time-dependent expression of (A) caspase-3/7, (B) caspase-8 and (C) caspase-9 in HCT-116 cells treated with reflexin A. Caspase-3/7 and -9 were significantly activated after 24 h of treatment. Moreover, caspase-8 was activated after 24 h of treatment. The data are shown as the mean ± SEM. * represents significant difference (p < .05) compared with the control.

Figure 8. Reflexin A suppressed colon cancer cell migration. HCT-116 cells were seeded in 6-well plates. Confluent cells were wounded and imaged (0 h). (A) After treating HCT-116 cells with reflexin A at the IC_{50} concentration, the cells were incubated at 37 °C for 24, 48 and 72 h and photographed. (B) The treated cells of HCT-116 with 0.1% vehicle DMSO (control) significantly migrated within 72 h. (C) A representative bar chart of images revealed that reflexin A significantly suppressed HCT-116 migration. (D) Reflexin A suppressed HCT-116 cell invasion. The percentage of invasion was determined as the percentage of HCT-116 cells that had invaded the Matrigel inserts vs. the total HCT-116 cells that had migrated through the control inserts. The data are shown as the mean ± SEM. * represents significant difference (p < .05) compared with the control.

major obstacle in cancer treatment. Thus, in addition to the available therapeutic strategies, the suppression of this process in cancer cells can significantly elevate the survival rate of cancer patients [21]. The development of potential anticancer agents
with suppression against cell migration and invasion can lead to a suitable therapeutic strategy. In this work, the scratch test effect of compound 1 was investigated in HCT-116 cells, which are highly metastatic [22]. The results indicated that compound 1 markedly suppressed the migration of HCT-116 cells. Furthermore, as shown in Figure 8, quantitative analysis revealed a more than 40% reduction in scratch test in HCT-116 cells compared to the control. The invasion effect of compound 1 was analyzed by using the chamber assay and by measuring the number of HCT-116 cells that migrated through the barrier. This result revealed that compound 1 significantly suppressed the invasion of HCT-116 cells. The invasion of HCT-116 cells was reduced to approximately 50% after 72 h of treatment.

2.8. Safety of compound 1

The results of acute toxicity confirmed that compound 1 has no toxicity and shows the safety of this compound. Throughout of the animal monitoring within 14 days, no sign of mortality or toxicity was monitored in any dose of compound 1. The safety of compound 1 was confirmed with the histological slides of the kidney and liver (Figure 9).

3. Experimental

3.1. General experimental procedures

The IR spectra were measured by Fourier transform infrared (FT-IR) spectroscopy using a Perkin-Elmer RX 1 spectrometer (Massachusetts, USA) for the 4000-400 cm⁻¹ frequency range. Jasco P-1020 polarimeter (Pennsylvania, USA) was used to measure optical rotation and silica gel 40 (40–63 μm) ASTM (Merck 9385) was used for Column Chromatography. Thin Layer Chromatography (TLC) was done with silica gel 40 F₂₅₄ (Merck). 1D NMR and 2D NMR spectra were determined in acetone-d₆ (JEOL JNM-FX400, Tokyo, Japan) and the UV spectra were measured on a.

Figure 9. Liver and kidney histological sections. H & E stain of liver and kidney did not show any sign of toxicity after treatment with 1 in low dose 2 g/kg (A) and high dose 5 g/kg (B) compared to the vehicle control 10% Tween-20 (C).
spectrophotometer (Shimadzu UV-160A, Kyoto, Japan) using MeOH as the solvent. The MS data were obtained with an Agilent 6530 (California, USA). Preparative HPLC was conducted using a Waters (Massachusetts, USA) instrument connected to a C-18 Phenomenex column (250 × 21.2 mm, 5 μm, California, USA) with a PDA detector.

3.2. Planta materials

The barks of *Rauvolfia reflexa* were collected from Kelantan, Malaysia in July 2009 and identified by botanist, Teo Leong. A voucher sample (KL 4900) was placed at the Chemistry Department’s herbarium at the University of Malaya.

3.3. Extraction and isolation

The bark (1 kg) was exhaustively extracted with 4 L of hexane for 48 h to take out nonpolar organic compounds. The extract was then evaporated on a rotary evaporator, and successively re-extracted with methanol (2 × 7 L). After solvent removal, a crude methanol extract (20 g) was obtained. The presence of alkaloids was confirmed by Mayer’s reagent, which yielded white precipitate [23]. The methanol crude extract (10 g) was passed through a silica gel column by using ethyl acetate-methanol (C₄H₈O₂/MeOH, 100:0 to 0:100) as a mobile phase to give 7 fractions, A₁-A₇, based on TLC analysis. Fraction A₇ (1.2 g) was chromatographed over silica gel and eluted with ethyl acetate – methanol (100:0 to 0:100) to yield four sub fractions, A₇a-A₇d. Separation of fraction A₇c (0.35 g) by preparative HPLC (50–100% ACN-H₂O, detection at 210 nm, 7 ml/min) successfully yielded compound 1 (5 mg, 0.0005%). Fraction A₄ (1.3 g) was chromatographed over silica gel and eluted with ethyl acetate-methanol (100:0 to 0:100) to give five sub fractions, A₄a-A₄e. Fractions A₄b and A₄c were combined (0.51 g) and purified by a PTLC (CH₂Cl₂/MeOH, 87:13 v/v) to yield compounds 2 (8 mg, 0.0008%) and 3 (7 mg, 0.0007%).

3.3.1. Compound 1

Brownish amorphous; [α]D²⁴ -31 (c 0.05, CHCl₃); IR (CHCl₃) νmax 1691, 2852–2925,3351 cm⁻¹; UV (MeOH) 226, 299 nm; ¹H (acetone-d₆, 400 MHz) and ¹³C (acetone-d₆, 100 MHz) NMR spectral data see Table 1; HRESIMS: m/z 413.2662 [M + H]⁺ (calcd for C₂₃H₂₉N₂O₅, 413.2076).

3.4. Cell culture and MTT assay

All cell lines were procured from the American Type Cell Collection (ATCC, Manassas, VA, USA). The cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM, Sigma) with 10% fetal bovine serum supplement (FBS, Sigma) at 37 °C with 5% CO₂. The negative control for each assay was prepared by adding 0.1% DMSO (vehicle) to untreated medium. MTT assay was used to measure the cytotoxicity values of the isolated compounds against different cell lines. Briefly, the cells were treated with variable concentrations of the indole alkaloids in 96-well plates and
incubated overnight. After 24 h of incubation, each well was supplemented with MTT solution (5 mg/ml, 20 μl, Sigma, Missouri, USA) and the plate was incubated for 3 h. To dissolve the formazan crystals, the cell media was replaced by DMSO. The optical density was measured using a microplate reader (Hidex, Turku, Finland) at 570 nm wavelength. The antiproliferative potential of the compounds was defined as an IC50 value. Because compound 1 showed the lowest IC50 value against HCT-116 colon cancer cells, we used HCT-116 cells to continue this study. In the MTT assay, the positive standard used was 5-fluorouracil (5-FU) (Sigma).

3.5. Acridine orange/propidium iodide double staining assay

Acridine orange (AO) and propidium iodide (PI) double staining assays were used to quantify the apoptotic morphological changes and were performed according to the standard procedure. Briefly, the cultures were performed in a 25 ml flask and incubated at 37 °C. The HCT-116 cells were plated at a concentration of 5 × 10⁴ cells/ml and treated with compound 1 at the IC50 dose. The colon cancer cells were washed stained with fluorescent dyes and examined using a BX51 Olympus fluorescent microscope (Tokyo, Japan).

3.6. Annexin V-FITC assay

The Annexin V-FITC assay was accomplished using the commercially available BD Biosciences kit (San Jose, CA, USA). HCT-116 cells (1 × 10⁶ cells/mL) were seeded in 60-mm² culture dishes and supplemented with compound 1 at the IC50 concentration. After 24, 48 and 72 h of incubation, the harvested cells were centrifuged and washed with PBS, followed by suspension in Annexin V binding buffer. Then, Annexin V-FITC and PI were employed to stain the cells according to the vendor’s protocol. PI was applied to look out for late apoptosis and necrosis, while Annexin V was applied to detect early and late apoptosis. The fluorescence intensity was analyzed using the BD FACSCanto II flow cytometer (BD Biosciences), and the apoptotic and necrotic cell populations were measured using the ModFit LT software (Verity Software House, Inc., Topsham, ME, USA).

3.7. Cell cycle assay

HCT-116 cells (5 × 10⁴ cells/ml) were supplemented with compound 1 at the IC50 concentration. After 24, 48, and 72 h of incubation, the treated cells were fixed using 95% cold ethanol. Then, PI dye (50 μL, 10 mg/ml) was used to stain the treated cells for 1 h at 37 °C. Moreover, RNaseA (10 mg/mL) was also employed to exclude the possibility that PI bound to RNA molecules. The DNA content of the stained cells was determined using the BD FACSCanto II flow cytometer.
3.8. Caspase activation assay

We used commercial kits (Caspase-Glo-3/7, Caspase-Glo-8 and Caspase-Glo-9 assays, Promega Corporation, Madison, WI, USA) to determine activation of the caspases. HCT-116 cells (2 \times 10^4 cells/well) were treated with compound 1 at the IC_{50} concentration in a white-walled 96-well plate for 3, 6, 12, 24, and 48 h, while untreated cells acted as the control. Then, the treated HCT-116 cells were supplemented with Caspase-Glo Reagent-3/7, Reagent-8, Reagent-9 (50 \mu l) and incubated for 30 min in the dark, according to manufacturer protocol. The activities of the caspases were determined using a microplate reader, Infinite M200PRO (Tecan, Männedorf, Switzerland).

3.9. Cell migration and invasion assays

The suppressive potential of compound 1 on the migration of cancer cells was determined by seeding HCT-116 cells in a 6-well plate for 24 h. After treatment with compound 1 at the IC_{50} concentration, the cells were wounded using a 200 \mu l pipette tip. Photomicrographs were taken at different times using an Olympus BX51 microscope. According to manufacturer protocol, the Cultrex 96-well membrane invasion assay kit (Trevigen, Inc., Gaithersburg, MD, USA) was employed to perform invasion analysis. Briefly, the 96-well unit containing 8 \mu m polycarbonate nucleopore filters (Corning Inc., Corning, NY, USA) was coated with the basement membrane extract coating solution (100 \mu l) at 37°C for 4 h. The upper compartment was filled with HCT-116 cells (2 \times 10^5 cells/ml) in DMEM medium while the lower compartment filled with just the medium. After treatment with compound 1 at the IC_{50} concentration for 24, 48, and 72 h, the noninvading cells were then removed by a cotton swab. Finally, the invasion of treated HCT-116 cells to the lower surface was measured using the Infinite M200PRO microplate reader.

3.10. Animals

A total of 30 Female Sprague Dawley® strain rats (180–250 g) were bought from Animal house, University of Malaya, Malaysia. All the animals were kept in a temperature controlled room (~24°C) and were supplied with standard rat pellets and water ad libitum. The animal experiment was proved by Ethical committee, University of Malaya (FAR/21/02/2012/MF).

3.11. Acute toxicity

The experiment was approved by the institutional Ethics Committee in University of Malaya (Ethic #: FAR/21/02/2012/MF).

The 6–8 weeks rats weighing 180 g were obtained from the animal house facility, University of Malaya. An over-all of 18 male rats were divided into three different groups placed in three different cages. The first group were labeled as a vehicle control group Tween-20 10% weight/volume (VC); 5 ml/kg, the second group low dose of compound 1 with 2 g/kg (LD) and the third group with 5 g/kg of compound 1
All rats fasted a day before doing the experiment and allowed to access water only. Then compound 1 was fed to the animal based on respective dosage and groups, then monitored in two weeks for any indication of toxicity. Then histological parameters were analyzed after sacrificing the animals.

### 3.12. Statistical analysis

The data of at least three individual experiments are presented as the mean ± the SEM. The data were analyzed by Prism software (GraphPad Software, CA, USA). The experimental data were analyzed using one-way analysis of variance followed by Tukey’s post hoc test. Comparisons with $p$-values less than .05 were considered significant (*).

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### Disclosure statement

No potential conflict of interest was reported by the authors.

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### References