

Communication

Cytotoxicity of Two Triterpenoids from *Nigella glandulifera*

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Abstract: During an investigation of antitumor substances from *Nigella glandulifera* Freyn et Sint. (Ranunculaceae) the cytotoxicity of two oleanane triterpene saponins isolated from the seeds of this species, kalopanaxsaponins A and I, was evaluated against HepG2, drug resistant HepG2 (R-HepG2) (two hepatocyte cell lines) and primary cultured normal mouse hepatocytes. Evident cytotoxic activities were observed. Morphological observations and cell cycle analysis suggest that these compounds inhibit the proliferation of hepatoma by inducing apoptosis and consequently kalopanaxsaponins A and I may be potential therapeutic agents for the treatment of parental and drug resistant hepatoma.

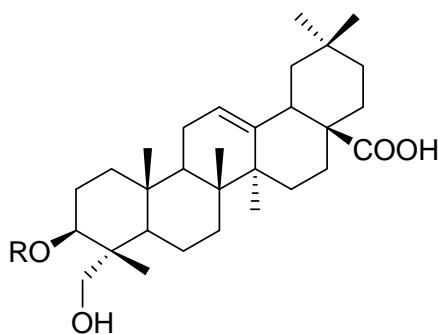
Keywords: *Nigella glandulifera*, oleanane type saponins, cytotoxicity, HepG2, apoptosis

Introduction

The genus *Nigella* (Ranunculaceae) consists of about 20 species, three of which - *Nigella glandulifera* Freyn et Sint, *Nigella sativa* and *Nigella damascena* L. are used in traditional medicine. Their seeds, commonly added in many food preparations of the Uyghur ethnic group, possess diuretic, analgesic, spasmolytic, galactagogue and bronchodilator effects and have been used for a long time in the treatment of oedema, urinary calculus and bronchial asthma. Flavone glycosides, triterpenoids and alkaloids are the main constituents in the *Nigella* genus [1-5]. *Nigella glandulifera* Freyn et Sint. is an erect annual herbaceous plant widely distributed in the west of China. The whole herb has been used as a folk remedy for treatment of colds, cough and insomnia. Previous phytochemical study on the seeds of this plant revealed the presence of several compounds, including a new alkaloid, nigeglanine [6], which is only the third reported naturally occurring product with an indazole ring after nigelicine and nigellidine [7,8].

Kalopanaxsaponin A (3-O-[L-rhamnopyranosyl-(1→2)- α -L-arabinopyranosyl]-hederagenin, **1**) and kalopanaxsaponin I (3-O-[β -D-xylopyranosyl(1→3)- α -L-rhamnopyranosyl-(1→2)- α -L-arabinopyranosyl]-hederagenin, **2**) (Figure 1) are important oleanane triterpene saponins previously found in *Kalopanax pictus* [9,10]. Their isolation for the first time from the seeds of *Nigella glandulifera* has been described recently [11]. Compounds **1** and **2** have been reported to display cytotoxicity in J82, T24, Colon26 and 3LL cancer cells, with IC_{50} values ranging from 1.1-16.5 μ M [12]. In addition, kalopanaxsaponin A significantly reduced the LL/2 tumor weight of mice at a *i.p* dose of 5-10 mg and apparently prolonged the life span of mice bearing Colon 26 and 3LL Lewis lung carcinomas at an *i.p* dose of 15 mg [2,12]. However, there are no reports on their activities towards drug resistant tumor and normal cells and the underlying mechanisms of cytotoxic activity of these two saponins remains unclear.

Figure 1. The structures of compounds **1** and **2**.



Compound

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- | | |
|----------|--|
| 1 | 3-O-[L-rhamnopyranosyl-(1→2)- α -L-arabinopyranosyl]- |
| 2 | 3-O-[β -D-xylopyranosyl(1→3)- α -L-rhamnopyranosyl-(1→2)- α -L-arabinopyranosyl]- |

In the present study, the cytotoxicity of kalopanaxsaponins A and I was evaluated on HepG2, drug resistant HepG2 (R-HepG2) (two hepatocyte cell lines) and primary cultured normal mouse hepatocytes, in order to explore their potential in parental and drug resistant hepatoma therapy.

Results and Discussion

Multi-drug resistance is one of the most serious limitations in chemotherapy and always leads to cancer treatment failure. Meanwhile, lack of selectivity of the active ingredient(s) usually contributes to the many observed side effects. Consequently, there is an urgent need to find new chemical agents that can differentiate between normal and cancerous cells, in order to selectively kill the cancerous cells and drug resistant tumors with reduced toxicity [13]. Therefore, two hepatocyte cell lines (HepG2, R-HepG2) and the primary cultured normal mouse hepatocytes were used to test the cytotoxicity, drug resistance and selectivity of kalopanaxsaponins A and I. Betulinic acid, a selective cytotoxic triterpenoid acid, was used as positive control [14]. Based on our results, kalopanaxsaponins A and I showed significant cytotoxic activities towards all two cell lines and primary cultured normal mouse hepatocytes in a concentration-dependent manner and the IC₅₀ values were derived from the dose effect curves (Table 1). The cytotoxicities of kalopanaxsaponins A and I on R-HepG2 were as effective as on HepG2, but at the same time, the IC₅₀ values towards normal mouse hepatocytes were very low. This indicated that kalopanaxsaponins A and I were effective against the drug resistant HepG2 cell line, but had no selective cytotoxicity towards cancer cells.

Table 1. IC₅₀ values of kalopanaxsaponins A and I on different cell lines and normal hepatocytes

Cells	IC ₅₀ (μM) ^a		
	kalopanaxsaponin A	kalopanaxsaponin I	betulinic acid
HepG2	18.12±1.03	8.95±0.62	13.26±1.57
R-HepG2	13.09±0.94	9.05±0.71	12.02±2.35
Mouse Hepatocytes	2.45±0.23	8.26±0.08	>100

^a Data shown represent the mean±SD of three independent experiments.

Cell death can occur by either of two distinct mechanisms: necrosis (“accidental” cell death) and apoptosis (“programmed” cell death) [15, 16]. Necrosis causes severe inflammation, while apoptosis does not. Most of the cytotoxic antitumor drugs in current use have been shown to induce apoptosis in susceptible cells. Harmlessly disposing of cancer cells is one of the main considerations in chemotherapy [17] and consequently induction of apoptosis is an important strategy in anticancer drug research and development. In our study, treatment with kalopanaxsaponins A and I at concentrations of one third of their IC₅₀ (6 and 3 μM, respectively) for 24 h resulted in significant morphological changes in HepG2 cells (Figure 2), and the changes induced by kalopanaxsaponins A and I were very similar. After treatment with kalopanaxsaponins A and I, chromatin aggregation, nuclear and cytoplasmic condensation and partition of cytoplasm and nucleus into membrane bound-vesicles (apoptotic bodies) occurred in the cells.

Many anticancer agents and DNA-damaging agents arrest the cell cycle at the G₀/G₁, S, or G₂/M phase [18, 19]. After exposure to the tested compound at concentration of one third of their IC₅₀ (6 and 3 μM) respectively for 24 h, the distinct sub-G₁ peak which is recognized as apoptotic fraction was observed on the HepG2 cells treated with kalopanaxsaponins A and I, but no significant change of cell cycle progression was detected (Figure 3). The apoptosis fraction induced by kalopanaxsaponins A and I was 13.61% and 20.81% respectively. While, the apoptosis triggered by positive control betulinic acid was 9.12%. This result confirmed the apoptosis observed in morphological observation.

Figure 2. Morphological changes in HepG2 cell line in response to the triterpene saponins 1 and 2. Photographs were taken under an inverted Leica fluorescence 40×10 microscope. 1) normal HepG2 cells treated with vehicle; 2) kalopanaxsaponin A, 6 μ M for 24 h; 3) kalopanaxsaponin I, 3 μ M for 24 h; 4) betulinic acid, 20 μ M for 24 h.

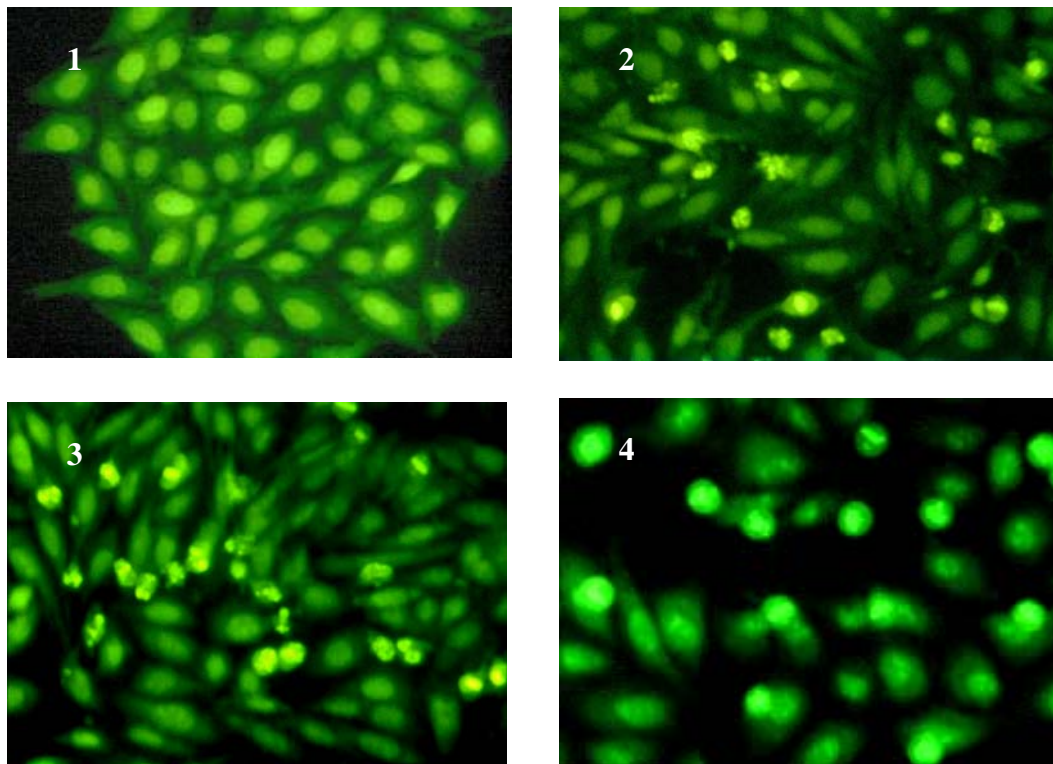
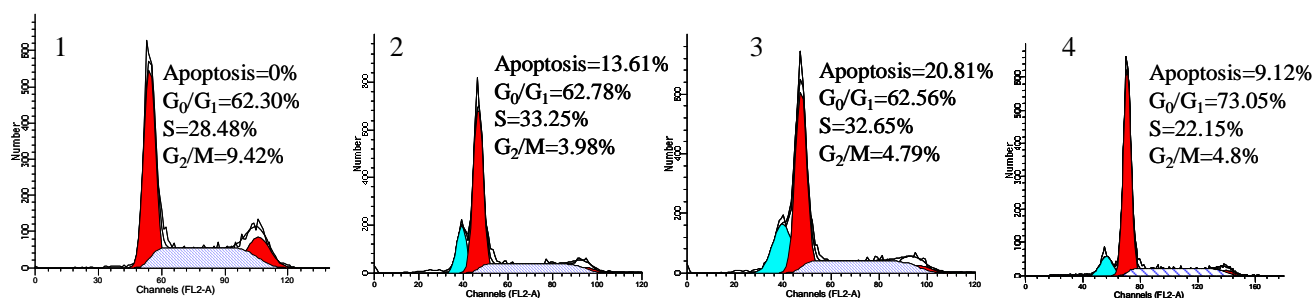


Figure 3. Flow cytometry analysis of HepG2 cells treated with two saponins. 1) HepG2 cells treated with vehicle; 2) kalopanaxsaponin A, 6 μ M for 24 h; 3) kalopanaxsaponin I, 3 μ M for 24 h; 4) betulinic acid, 20 μ M for 24 h. Data are the results of one experiment representative of three.



Conclusions

Kalopanaxsaponins A and I might be potential anticancer agents towards either common or drug resistant cancers, after further structure modification to enhance their selective cytotoxicity on tumor cells. They exert their cytotoxic activity through apoptosis.

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Experimental

General

3-O-[L-Rhamnopyranosyl-(1→2)-α-L-arabinopyranosyl] hederagenin (kalopanaxsaponin A, **1**) and 3-O-[β-D-xylopyranosyl(1→3)-α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranosyl] hederagenin (kalopanaxsaponin I, **2**) were obtained from oil free seeds of *N. glandulifera* collected from Ürümuqi in Xinjiang, Uyghur Autonomous Region, China, in February 2002. A voucher specimen (HB-02-0325) was deposited at the Institute of Medicinal Plant Development, Chinese Academy of Medical Sciences and Peking Union Medical College. The saponins were isolated and characterized as previously described [11].

Cell culture and drug treatment

HepG2 (ATCC) cells were maintained in RPMI 1640 (Gibco) containing 10% FBS (Gibco), sodium bicarbonate (2 mg/mL,) penicillin sodium salt (100 µg/mL) and streptomycin sulfate (100 µg/mL). R-HepG2 (City University of Hong Kong) cells were maintained in the presence of 1.2 µM doxorubicin (Sigma). Cells were grown to 60% confluence, trypsinized with 0.25% trypsin/2 mM EDTA, and plated for experimental use. In all experiments, cells were grown in RPMI-1640 medium with 10% FBS for 24 h prior to treatment. Mouse hepatocytes were isolated from normal Kunming mice (Experimental Animal Center of Zhongshan Medical University) by the enzymatic perfusion technique as we have described previously [20]. The viability of mouse hepatocytes was about 80% tested with Trypan blue. Compounds were dissolved in DMSO at a concentration of 100mM, and diluted in tissue culture medium before use.

Cytotoxicity assay

Cells were seeded in 96 well tissue culture plates and treated with the saponins or vehicle (0.1% DMSO) at various concentrations and incubated for 48 h followed by MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay [21]. Betulinic acid was used as positive control. Briefly, IC₅₀ values for the two saponins and betulinic acid on different cell lines and the primary cultured normal mouse hepatocytes were obtained from the concentration-effect curves.

Morphological observations

HepG2 cells were cultured in 3.5 cm dishes. The tested compounds were added to the medium at a concentration of one third of their IC₅₀ values (6 and 3 µM, respectively). A vehicle treated sample and betulinic acid were used as negative and positive controls separately. After treatment, all the cultures

were incubated at 37°C, 5% CO₂ for 24 h. Photographs were taken under an inverted Leica fluorescence 40×10 microscope after AO (acridine orange)/EB (ethidium bromide) staining [22].

Flow cytometry analysis

Flow cytometry was used to elucidate the cytotoxicity of the two saponins. HepG2 cells were treated with the compounds at concentration of one third of their IC₅₀ values (6 and 3 μM, respectively) for 24 h. A vehicle (0.1% DMSO) treated sample and betulinic acid were used as negative and positive controls respectively. Cells were collected and fixed in 70% cold (-20°C) ethanol overnight. After washing twice with PBS, cells were resuspended in PBS containing 1% FCS (vertex). RNA in the fixed cells was digested using RNase A (0.5 mg/mL) at 37°C for 1 h. Finally, the cells were stained by propidium iodide (PI, 2.5 μg/mL). The DNA content of cells was then analyzed with a FACSCalibur instrument (Becton-Dickinson).

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Sample Availability: Available from the authors.