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Anticancer effects of saponin and saponin–phospholipid complex of *Panax notoginseng* grown in Vietnam

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ABSTRACT

Objective: To evaluate the antitumor activity both *in vitro* and *in vivo* of saponin–phospholipid complex of *Panax notoginseng*.

Methods: The *in vitro* cytotoxic effect of saponins extract and saponin–phospholipid complex against human lung cancer NCI-H460 and breast cancer cell lines BT474 was examined using MTS assay. For *in vivo* evaluation of antitumor potential, saponin and saponin–phospholipid complex were administered orally in rats induced mammary carcinogenesis by 7,12-dimethylbenz(a)anthracene, for 30 days.

Results: Our data showed that saponin–phospholipid complex had stronger anticancer effect compared to saponin extract. The IC50 values of saponin–phospholipid complex and saponin extract for NCI-H460 cell lines were 28.47 μg/mL and 47.97 μg/mL, respectively and these values for BT474 cells were 53.18 μg/mL and 86.24 μg/mL, respectively. *In vivo* experiments, administration of saponin, saponin–phospholipid complex and paclitaxel (positive control) effectively suppressed 7,12-dimethylbenz(a) anthracene-induced breast cancer evidenced by a decrease in tumor volume, the reduction of lipid peroxidation level and increase in the body weight, and elevated the enzymatic antioxidant activities of superoxide dismutase, catalase, glutathione peroxidase in rat breast tissue.

Conclusions: Our study suggests that saponin extract from *Panax notoginseng* and saponin–phospholipid complex have potential to prevent cancer, especially breast cancer.

1. Introduction

Breast cancer is the most common neoplastic disease in females and is a major cause of death in women. In 2014, it was estimated that approximately 295.24 women and 2.36 men in the United States were newly diagnosed with breast cancer and about 40 cases died each year [1]. Female breast cancer is also reported with the highest frequency in Vietnam.

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Emerging evidence has demonstrated that the main causes of breast mutagenesis and breast carcinogenesis seem to be linked to reactive oxygen species (ROS) [2]. Especially, free radicals are important factor in initiation and progression of tumor [3]. However, organisms have ability to prevent free radical-induced damages with their own antioxidant enzyme system. Also, the antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) can reduce different stages of carcinogenesis [4]. The 7,12-dimethylbenz(a)anthracene (DMBA), a potent carcinogen, has been widely used in model of mammary tumorigenesis [5]. This molecular disrupts the balance of prooxidant-antioxidant, leading to lipid peroxidation process, which is indicated and associated with various cancer diseases, including breast cancer [6].

Panax notoginseng (Burk.) F. H. Chen (*P. notoginseng*) (Araliaceae) is a medicinal plant found in some Asian countries, including Vietnam. *P. notoginseng* has many beneficial effects on the immune system and cardiovascular system. Moreover, its haemostatic, hypolipidemic, hepatoprotective, renoprotective, antioxidant, anti-inflammatory, anti-tumor and estrogen-like activities have been reported [7,8]. It is well known that saponins are mainly active compounds of *P. notoginseng*. Most of them belong to 20(S)-protopanaxadiol and 20(S)-protopanaxatriol structure. Ginsenosides Rg1, Rb1, Rd and notoginsenoside R1 are responsible for the plant's pharmacological activities and are major saponin components in *P. notoginseng* roots [8]. However, saponin has poor intestinal absorption due to their unfavorable physicochemical properties including the large molecular mass, high molecular flexibility, high hydrogen-bonding capacity and poor membrane permeability. Additionally, rapid and extensive biliary excretion is another factor limiting the oral bioavailability of saponins [9]. Different novel delivery systems have been used to improve the membrane permeability and subsequently increase the compound bioavailability. Of those, natural product–phospholipids complex technology has been effectively increased the absorption of medicinal plant extract via oral administration [10]. The natural product–phospholipids complex structures contain the natural active compound bound to phospholipids, such as phosphatidylcholine and phosphatidylethanolamine. Therefore, natural product–phospholipids complex is a lipid compatible molecular complex [11]. In the present study, we extracted saponin from *P. notoginseng* roots and prepared the phospholipid complex of this saponin extract. Its cytotoxic effects against human lung cancer NCI-H460 and breast cancer cell lines BT474 were evaluated. We also investigated the *in vivo* antitumor activity on DMBA-induced breast cancer rat model by evaluating body weight, tumor volume, lipid peroxidation status and antioxidant enzyme activities.

2. Materials and methods

2.1. Chemicals and reagents

DMBA, paclitaxel, MTS and phenazine methosulfate were purchased from Sigma–Aldrich, Singapore. Phospholipid, Tris–HCl, $MgCl_2$, ethylene glycol tetraacetic acid, ethylene diamine tetra-acetic acid, leupeptin, pepstatin, phenylmethylsulfonyl fluoride, 1-methyl-2-phenylindole, butylated hydroxytoluene, 1,1,3,3-tetramethoxypropane, pyrogallol, hydro peroxide and dimethyl sulfoxide (DMSO) were obtained from Merck, Germany. Ethanol, ether, *n*-butanol and the other reagents were of analytical grade.

2.2. Preparation of saponin extract from P. notoginseng

The roots of *P. notoginseng* in Lao Cai Province, North Vietnam were collected in October 2014. Then, these samples were further classified and identified by Professor Hai Nguyen Thanh (School of Medicine and Pharmacy, Vietnam National University, Hanoi). A voucher specimen was deposited at the herbarium of School of Medicine and Pharmacy, Vietnam National University.

Total *P. notoginseng* saponins were prepared by a method described previously [12]. The *P. notoginseng* roots were extracted with 80% ethanol (9 L × 3 times) at room temperature for a week. After filtration, the combined ethanol extract was then concentrated to yield a dry residue (840 g). This crude extract was then suspended in H_2O (2 L), partitioned successively with ether (3 × 1.5 L) and *n*-BuOH (3 × 1.5 L), and finally suspended concentrated and dried in vacuum (60 °C) to yield a dry residue (155 g). The dried extract was then applied to D101 macroporous resin column chromatography, washed with water and eluted with ethanol to obtain total *P. notoginseng* saponins extract.

2.3. Preparation of saponin–phospholipid complex

Saponin–phospholipid complex was prepared by mixing *P. notoginseng* saponin extract with phospholipid at a molar ratio of 1:3. The amount of standardized extract of *P. notoginseng* and phospholipid were weighed and taken in a 250-mL round bottom flask, and then 40 mL of dichloromethane was added. The mixture was refluxed at 40 °C for 2 h. The resultant clear solution was evaporated and 50 mL of *n*-hexane was added with continuous stirring. The saponin extract–phospholipid complex was precipitated and the precipitate was filtered and dried under vacuum to remove the traces of solvents. The resultant saponin extract–phospholipid complex (yield 92%, w/w) was kept in an amber colored glass bottle, flushed with nitrogen and stored at room temperature.

2.4. Cytotoxicity assay

The cytotoxic effects of saponin extract and saponin–phospholipid complex on the human lung cancer cell NCI-H460 and human breast cancer cell BT474 were investigated by using the MTS assay. Cells (5000 cells/well) were seeded into 96-well plates and incubated at 37 °C under 5% CO_2 and 95% air overnight to allow attachment onto the wells. All samples (saponin extract, saponin–phospholipid complex, and paclitaxel as positive control) were diluted in DMSO. Saponin extract and saponin–phospholipid complex were added to the wells at final concentration range of 0.25–1000 μg/mL whereas paclitaxel was used at a concentration range of 0.039–5 μg/mL. The maximum concentration of DMSO in culture media was adjusted to 1% (v/v). After incubation at 37 °C in an atmosphere of 5% CO_2 and 95% air for 72 h, 20 μL of MTS was added to each well and after 2–4 h incubation, the absorbance at 490 nm was measured using a 96-well microplate reader. All experiments were performed in triplicate. The percentage of cell viability was calculated using the formula:

Viability (%) = [Absorbance of sample/Absorbance of control] × 100%

The IC_{50} for cell growth was calculated from the equations of the dose–response curves.

2.5. Animals

A total of 65 female Sprague–Dawley rats (45-day old, 180–210 g) were purchased from Charly-River Company (USA). All experimental procedures were reviewed and approved by the Ethical Committee of the Vietnam National University, Hanoi. Procedures were performed according to the guidelines of School of Medicine and Pharmacy, Vietnam National University, Hanoi on the ethical use of animals. Rats were maintained in standard conditions [a good ventilation room, (28.0 ± 0.5) °C, (55 ± 5)% relative humidity and 12 h light/dark cycles]. Rats were housed in cage and given with a standard (Zeigler, USA) diet *ad libitum* before use.

2.6. DMBA treatment

After 5 days of acclimatization, rats were randomly divided into seven tested groups of 10 animals and one control group of 5 animals. Treated rats were injected subcutaneously into the mammary gland with a DMBA dose of 25 mg/kg body weight with interval 1 week for 30 days. Rats were palpated weekly to check for tumor appearance and tumor size. The treatment of each group was as follow: the rats in Group I (normal control) were fed with normal diet; the rats in Group II (DMBA) were fed with normal diet without any treatment; the rats in Group III (paclitaxel) were given paclitaxel (33 mg/kg body weight) intragastrically by gavaging for 30 days; the rats in Group IV (Sap 50) were given saponin extract (50 mg/kg body weight) intragastrically by gavaging for 30 days; the rats in Group V (Sap 150) were given saponin extract (150 mg/kg body weight) intragastrically by gavaging for 30 days; the rats in Group VI (Phyt 150) were given saponin–phospholipid complex (150 mg/kg body weight) intragastrically by gavaging for 30 days (the amount of saponin in Phyt 150 saponin–phospholipid complex was 50 mg) and the rats in Group VII (Phyt 450) were given saponin–phospholipid complex (450 mg/kg body weight) intragastrically by gavaging for 30 days (the amount of saponin in Phyt 150 saponin–phospholipid complex was 150 mg).

All rats were weighted daily. All changes in tumor volume and body weight were recorded. Tumor volume was calculated using the formula:

$$V = 0.5 \times D \times R^2$$

where, V is tumor volume (mm^3), D is tumor length (mm) and R is tumor width (mm).

Tumor inhibition was calculated at the final day of experimental using the formula:

$$\text{Inhibition (\%)} = (A - B)/A \times 100$$

where, A is tumor size of DMBA group and B is tumor size of treated group.

On Day 30, rats in all groups were sacrificed by cervical dislocation. All breast tissues were resected, washed in 0.9% NaCl and frozen rapidly at −80 °C. Frozen tissues were defrosted, weighted and homogenized in ice-cold lysis buffer, containing 50 mmol/L Tris–HCl (pH 7.5), 8 mmol/L $MgCl_2$, 5 mmol/L ethylene glycol tetraacetic acid, 0.5 mmol/L ethylene diamine tetraacetic acid, 0.01 mg/mL leupeptin, 0.01 mg/mL pepstatin, 0.01 mg/mL aprotinin, 1 mmol/L phenylmethylsulfonyl fluoride and 250 mmol/L NaCl. Homogenates were then centrifuged at 10020 r/min at 4 °C. The supernatants were collected and stored until use at −80 °C. Protein concentration was determined by Noble and Bailey's method [13].

2.7. Lipid peroxidation assay

Lipid peroxidation assay was performed by determining the reaction of malonaldehyde with two molecules of 1-methyl-2-phenylindole at 45 °C as described previously [14]. The reaction mixture consisted of 0.64 mL of 10.3 mmol/L 1-methyl-2-phenylindole, 0.2 mL of sample and 10 μL of 2 μg/mL butylated hydroxytoluene. After vigorously mixing, 0.15 mL of 37% v/v HCl was added. The mixture was incubated at 45 °C for 45 min and centrifuged at 9147 r/min. Cleared supernatant absorbance was recorded at 586 nm. A calibration curve prepared from 1,1,3,3-tetramethoxypropane (Sigma–Aldrich, Singapore) was used for calculation. Peroxidized lipids were expressed as nmol malondialdehyde (MDA) equivalents/mg of protein.

2.8. SOD activity determination

SOD activity was determined as described previously [14]. This method is based on the capacity of SOD to inhibit the autoxidation of pyrogallol. Each assay was measured in triplicate.

2.9. CAT activity determination

CAT activity was measured in triplicate by monitoring the disappearance of H_2O_2 at 240 nm as described previously [14]. Each assay was measured in triplicate.

2.10. GPx activity determination

GPx activity was measured with a coupled enzyme assay as described previously [14]. Each assay was measured in triplicate.

2.11. Statistical analysis

All data are expressed as mean ± SD. One-way ANOVA was used to determine significance among groups. Statistical significance was set at $P < 0.05$.

3. Results

3.1. Cytotoxicity test

The *in vitro* cytotoxicity of saponin extract, saponin–phospholipid complex and paclitaxel against two cancer cell lines was evaluated by MTS assay. The results were shown in Table 1. Saponin extract showed mild cytotoxicity against NCI-H460 and cancer cell lines BT474 with IC_{50} values of 47.97 and 86.24 μg/mL, respectively. Saponin–phospholipid complex showed stronger cytotoxicity compared to saponin extract against the NCI-H460 and cancer cell lines BT474 with IC_{50}

Table 1
The cytotoxicity of saponin extract, saponin–phospholipid complex and paclitaxel on human lung cancer (NCI-H460) and breast cancer cell lines (BT474) (μg/mL).

Treatments	IC_{50}	
	Human lung cancer cell line NCI-H460	Human breast cancer cell line BT474
Saponin extract	47.97 ± 1.03	86.24 ± 1.45
Saponin–phospholipids complex	28.47 ± 0.67	53.18 ± 1.14
Paclitaxel	0.51 ± 0.09	0.52 ± 0.07

Data were showed as mean ± SD of IC_{50} of three independent experiments.

values of 28.47 and 53.18 μg/mL, respectively. This higher cytotoxicity could be explained by saponin–phospholipid complex which had more lipophilic property than saponin extract. Then, it could have easily gone through the membrane cell to provide more potent cytotoxicity for cancer cells.

3.2. Anti-tumor activity

Table 2 shows the body weight and tumor volume of rats in each group. Comparing to control group, there was a significant decrease in the body weight of rats in DMBA group ($P < 0.05$). Rats in paclitaxel and Phyt 450 treated-groups showed significant increase in their body weight ($P < 0.05$) when compared with DMBA group. Sap 50, Sap 150 and Phyt 150 treated-groups tended to have higher body weight than DMBA group, but not statistically significant. The tumor volume of rats in DMBA group was significantly less than the group treated with paclitaxel, Sap 150, Phyt 150 and Phyt 450 ($P < 0.05$). However, rats in Sap 50 group tended to have lower tumor volume than those in DMBA group. Paclitaxel reduced tumor volume more than 50%, which was higher than any other compounds used in this study. Comparison between Sap 50 and Phyt 150, which both contained about 50 mg saponin compounds, Phyt 150 had two fold higher in percentage of tumor reduction. For Sap 150 and Phyt 450, which both contained about 150 mg saponin compounds, slightly reduction of tumor volume (%) was observed.

3.3. Lipid peroxidation

The levels of lipid peroxidation product (MDA) from the rat breast tissues in the studied groups were shown in Table 3. A significant increase in MDA level was observed in the DMBA group when compared with control group ($P < 0.05$). A significant decrease in MDA levels was observed in group of paclitaxel, Sap 150, Phyt 150 and Phyt 450 ($P < 0.05$). MDA level in rats of Sap 50 group tended to decrease but it was not significantly different.

Table 2
The effect of saponin extract, saponin–phospholipid complex and paclitaxel on the body weight and tumor volume of rats.

Treatment groups	Body weight (g)	Tumor volume (mm^3)	Reduction of tumor (%)
Normal control	243.5 ± 15.4	–	–
DMBA	168.5 ± 13.6*	41.5 ± 2.8	
Paclitaxel	225.7 ± 14.3#	19.4 ± 1.9#	53.25
Sap 50	189.4 ± 14.1	37.3 ± 2.1	10.12
Sap 150	205.3 ± 10.7	31.5 ± 3.4#	24.09
Phyt 150	195.9 ± 15.2	33.4 ± 2.7#	19.52
Phyt 450	210.4 ± 9.5#	27.6 ± 2.5#	28.67

Values were expressed as mean ± SD. *: Significant difference compared with the control group, $P < 0.05$ ($n = 10$); #: Significant difference compared with the DMBA group, $P < 0.05$.

Table 3
The effect of saponin extract, saponin–phospholipid complex and paclitaxel on lipid peroxidation and antioxidant enzymes in rat breast tissue.

Treatment groups	MDA (nmol/mg protein)	SOD (IU/min/mg protein)	CAT (IU/min/mg protein)	GPx (IU/min/mg protein)
Normal control	0.45 ± 0.13	6.10 ± 0.70	86.45 ± 3.36	6.41 ± 0.30
DMBA	1.95 ± 0.12*	1.50 ± 0.40*	27.26 ± 2.14*	2.14 ± 0.09*
Paclitaxel	0.93 ± 0.11#	3.60 ± 0.70#	55.34 ± 3.58#	4.57 ± 0.12#
Sap 50	1.75 ± 0.21	2.20 ± 0.30	32.12 ± 2.19	2.89 ± 0.23
Sap 150	1.38 ± 0.14#	2.80 ± 0.20#	40.12 ± 3.51#	3.33 ± 0.15#
Phyt 150	1.45 ± 0.18#	2.60 ± 0.40	36.14 ± 2.76	3.15 ± 0.21
Phyt 450	1.24 ± 0.16#	3.00 ± 0.60#	45.26 ± 3.54#	4.16 ± 0.17#

Values were expressed as mean ± SD. *: Significant difference compared with the control group, $P < 0.05$ ($n = 10$); #: Significant difference compared with the DMBA group, $P < 0.05$.

3.4. Biochemical analysis

Activities of several antioxidant enzymes including CAT, SOD and GPx in the breast tissue of control and experimental animals were reported in Table 3. Rats in DMBA group showed a significant lower in the activities of these antioxidants compared with control group ($P < 0.05$). Groups treated with paclitaxel, Sap 150 and Phyt 450 showed significant higher levels of CAT, SOD and GPx compared with DMBA group ($P < 0.05$). These enzyme activities tended to increase in Sap 50 and Phyt 150 groups compared with DMBA group.

4. Discussion

Recent studies have reported the cytotoxicity and anti-tumor activity of saponins extracted from *P. notoginseng*. The mechanism action may relate to the accumulation of cells in G1 or S phase of cell cycle and apoptosis [15]. In this study, we showed that saponin extract has mild cytotoxicity and saponin–phospholipid complex had strong cytotoxicity towards the NCI-H460 and cancer cell lines BT474 (Table 1). Our data were supported by the study of Park *et al.* [16]. They showed that the water extract of *P. notoginseng* inhibited the growth and induced apoptosis in A549 and in NCI-H460 human lung carcinoma cells. The proposed mechanism of *P. notoginseng* may involve in up-regulation of pro-apoptotic Bax, down-regulation of anti-apoptotic Bcl-2 expression, loss of mitochondrial membrane potential, activation of proteolytic of caspases and dephosphorylation of the Akt signaling pathway [17]. In this study, we showed that saponin–phospholipid complex had stronger cytotoxicity than saponin extract. This result may be explained due to saponin–phospholipid complex is more compatibility to lipid than saponin extract. Therefore, it can be able to transport from a hydrophilic environment into the hydrophobic environment of the enterocyte cell membrane and

from there into the cell [18]. Xie *et al.* indicated that *P. notoginseng* extract possesses significant antiproliferative activities in human breast carcinoma MCF-7 cell lines [19]. They also identified that ginsenoside Rb1 is the responsible chemical constituent of antiproliferation effects on the MCF-7 cells [19].

The present investigation reveals that saponin extract and saponin–phospholipid complex exhibited potential anticancer activity on DMBA-induced mammary tumors in rats (Table 2). Our results showed that in all the treated groups, the body weight was slightly increased and the tumor volume was decreased compared with DMBA group (Table 2). Moreover, the percentage of tumor reduction in these groups was statistically significant compared with DMBA-group ($P < 0.05$). Our data are agreed with previous study of Park *et al.* who showed that water extract of *P. notoginseng* had potential capacity to inhibit the growth of solid tumors induced by NCI-H460 in mice [16]. They reported that the tumor weight and volume had decreased and the mean survival time of mice was also increased [16].

DMBA is a carcinogen which can produce free radical and generate oxidative stress to produce deleterious effects by starting lipid peroxidation [20]. It has been used to induce the mammary carcinogenesis in animals in many studies [20,21]. In our study, the administration of DMBA in rats was accompanied by significant increase in lipid peroxidation and decrease in the activities of antioxidant enzymes. Our results were similar to several previous studies. Selamoglu has shown that DMBA induced significant decrease in the levels of GPx, CAT, glutathione reductase activities of erythrocyte and total glutathione level and increase in MDA levels in adult female Wistar rats [22]. Padmavathi *et al.* also showed an increase in the extent of lipid peroxidation and a decrease in the activities of SOD, CAT, GPx and non-enzymic antioxidants (reduced glutathione, vitamin C and vitamin E) levels on DMBA-induced mammary carcinogenesis in rats [23].

It is well known that O_2^-, H_2O_2 and •OH play an important role in carcinogenesis. SOD, CAT and GPx are principally antioxidant enzymes, scavenging free radical, preventing lipid peroxidation and protecting cellular and molecular against ROS damages [2]. The biochemical function of SOD is to convert O_2^- to O_2 and H_2O_2. Then H_2O_2 is further converted to water and O_2 by CAT. GPx reduces lipid hydroperoxides to their corresponding alcohols and reduces free H_2O_2 to water. Previous study has shown that the antioxidant defense system was altered in cancerous breast tissues [2]. Our study has demonstrated that saponin extract from *P. notoginseng* and saponin–phospholipid complex could increase the CAT's, SOD's, GPx's activities and at the same time decrease the level of lipid peroxidation. In the present study, antioxidant enzyme activities of CAT, SOD and GPx were significantly high in Phyt 450 and Sap 150 treated-groups compared with the DMBA group. Our data agreed with the study of Han *et al.* [24]. They revealed that the extracts of *P. notoginseng* could reduce the oxidative stress on anti-myocardial ischemia injuries *in vivo* by decreasing MDA level and elevating the activities of SOD and GPx [24]. Ginsenoside Rd, one of the main compounds in *P. notoginseng* saponins, has a potential neuroprotective agent for cerebral ischemic injury by increasing L-glutathione content and improving antioxidant activity of CAT, SOD and GPx in hippocampal neurons [25]. Ginsenoside Rd also reduced the intracellular ROS level, decreased MDA level and enhanced the activities of SOD and GPx on H_2O_2-induced cytotoxicity PC12 cell line [26]. In the present study, Sap 50 and Phyt 150 (each contained 50 mg saponin) treated-groups showed slightly increase in the activities of SOD, CAT and GPx, but they are not significantly different. However, when the doses of saponin extract and saponin–phospholipid complex were increased to 150 mg/kg body weight and 450 mg/kg body weight, respectively (Sap 150 group and Phyt 450 group contained 150 mg saponin each), strong differences in these antioxidant activities were found. Our findings indicated that the same amount of saponin content and saponin–phospholipid complex had stronger antioxidant enzyme activities than saponin extract.

In conclusion, the present study reveals that saponin extracted from the roots of *P. notoginseng* has anti-tumor activity on human lung cancer (NCI-H460) and human breast cancer cell lines (BT474). Our data suggest that the administration of saponin extract at a dose of 150 mg/kg body weight or saponin–phospholipid complex at 450 mg/kg body weight (each of them contained 150 mg saponin) significantly decreases the tumor progression on DMBA-induced breast cancer rats and increases the levels of antioxidant enzymes including SOD, CAT and GPx. Our results showed that saponin–phospholipids complex has potential to be used as a delivery system for saponin extracted from the roots of *P. notoginseng*. Development of natural product–phospholipids complex as a delivery system of medicinal plant extracts should be further studied in pharmaceutical industry.

Conflict of interest statement

We declare that we have no conflict of interest.

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