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Cell Cycle Regulation and Antioxidant Activity of Psammaplin A, A Natural Phenolic Compound from Marine Sponge

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Abstract – Psammaplin A (PSA), a naturally occurring biophenolic compound has been demonstrated to deliver significant cytotoxicity to many cancer cell lines. In this article, we investigated the effect of PSA on cell cycle progression of lung cancer cells (A549). It was found that PSA could slightly perturb the cell cycle progression of A549 cells and lead to the cell cycle arrest at G2/M phase, indicating PSA might disturb the mitosis process of A549 cells. In addition, inspired by the two phenolic groups in the structure of PSA, the antioxidant activity of it has been evaluated. Although PSA was weak in scavenging the stable free radical 1,1-diphenyl-2-picrylhyrazyl (DPPH), it showed stronger ABTS radical scavenging activity than ascorbic acid in TEAC assay. Furthermore, it was found that PSA could effectively prevent DNA strand scission induced by oxidative stress. These results suggest that PSA have both cell cycle regulation and antioxidant activities. Herein, we suggest that PSA would be a very interesting and promising candidate to be developed as a multi-function drug.
Keywords – psammaplin A, cell cycle arrest, antioxidant, radical scavenger

Introduction

Psammaplin A (PSA) is a natural phenolic compound that was originally isolated in 1987 from the *Psammaplysilla* sponge (Arabshahi *et al.*, 1987). It has been demonstrated that PSA exhibited significant *in vitro* antibacterial activity against both *Staphylococcus aureus* (SA) and methicillinre sistant *Staphylococcus aureus* (MRSA) (Kim *et al.*, 1999). Given the increasingly rapid emergence of multi-drug resistant bacterial strains and corresponding threat to public health, there is significant interest in the development of structurally novel antibacterial agents such as PSA. Furthermore, PSA has been reported to be more potent against fibroblast and lymphocytes than against the bacterial strains (Nicolaou *et al.*, 2001a). A combinatorial library containing simplified analogues of PSA was constructed to study its mechanism of action and potential therapeutic value (Nicolaou *et al.*, 2001b).

Oxidative reactions have been implicated in the development of numerous diseases including atherosclerosis and cancer (Briviba *et al.*, 1994). Oxidation of lipids, proteins, and nucleic acids can result in loss of membrane integrity and function, inactivation of enzymes, modification of lipoproteins, and chemical alteration of DNA. Phenolic compounds are considered as most representative antioxidant agents, which are widely distributed in nature. The functionality of these compounds is expressed through their action as an inhibitor or an activator for a large variety of mammalian enzyme systems, and as metal chelators and scavenger of free oxygen radicals (Russo *et al.*, 2000; Sanchez-Moreno *et al.*, 1999). It is suggested that their antioxidative activity is related to their conjugated rings and hydroxyl groups (Decker, 1995), so it is not a surprise that most of phenolic compounds showed some antioxidative activity. Since there are two phenolic groups in the structure of PSA, we are interested in its antioxidant activity.

As a part of our continuing searches for anticancer and antioxidant natural products from Korean marine sponges, a series of studies on the bioactivity of PSA have been carried out. We previously reported that PSA could inhibit *in vitro* SV40 DNA replication, which might correlate with its cytotoxicity against many cancer cell lines (Jiang *et al.*, 2004). Herein, we describe the cell cycle regulation

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and antioxidant activities of PSA.

Experimental

Materials – A549 (human lung cancer cell line) was obtained from American Type Culture Collection (ATCC). Cell culture materials were all purchased from Gibco Co., USA. Xanthine oxidase was from Sigma Co., USA. Agarose was from Promega Co., USA and pBR322 DNA was from Takara Shuzo Co., Japan. PI/RNase Staining buffer for flow cytometry was from BD Biosciences Pharmingen, USA. DPPH (1,1-diphenyl-2-picrylhydrazyl), Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxy lic acid), and ABTS (2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) were from Sigma Co., USA. All other chemicals were of analytical reagent grade. Stock solution of PSA was prepared in ethanol and kept at −20°C. Further dilutions were made immediately prior to each experiment.

***In vitro* cytotoxicity assay** – A549 cells were maintained in RPMI 1640 supplemented with 10% fetal bovine serum at 37°C (5% CO_2) in a humidified atmosphere. For the cytotoxicity evaluation of PSA, 2×10^3 cells were plated into each well of a 96-well microplate. After the cells were incubated for 24 hr, PSA at various concentrations were added and the plate was incubated further for 48 hr. The cell viability was determined by Cell Counting Kit-8 (Dojindo Laboratories, Japan) (Hideyuki *et al.*, 1999).

Cell cycle arrest assay – A549 cells were harvested by trypsinization and washed with precooled phosphate buffer saline (PBS) *via* centrifugation. The cells were suspended in PBS, fixed with 70% ethanol (v/v) for 1 hr at 4 Samples were washed with PBS and stained with PI/RNase Staining Buffer for 15 min at 4°C The number of cells in different phases of the cell cycle was analyzed using a FACScan Flow cytometer with Modfit software (Becton Dickinson Instruments).

DPPH free radical scavenging activity – The potential antioxidant activity of PSA was assessed on the basis of its scavenging activity of the stable DPPH free radical (Cottelle *et al.*, 1996). Reaction mixtures containing 200 μL of 0.1 mM DPPH-ethanol solution, 90 μL of 50 mM Tris-HCl buffer, and 10 μL of ethanol (as control) or test sample. After 30 min incubation at room temperature, absorbance (517 nm) of the reaction mixtures was measured. The inhibitory ratio (%) was calculated as follows:

[(Absorbance of control-absorbance of test sample)/ absorbance of control] × 100

Measurement of Trolox equivalent antioxidant capacity (TEAC) – The Trolox equivalent antioxidant capacity of the PSA was determined by the method of Re *et al.* (1999). Ascorbic acid was used as an antioxidant reference. ABTS was dissolved in water to a 7 mM concentration. ABTS radical cation (ABTS · $^+$), the blue/green chromophore, was produced by reacting ABTS stock solution with 2.45 mM potassium persulfate and allowing the mixture to stand in the dark at room temperature for 12 hr before use. ABTS · $^+$ solution was diluted with 5 mM PBS to obtain about 0.70(0.02 unit of absorbance at 734 nm. After addition of 1.0 mL of diluted ABTS · $^+$ solution to 10 μL of PSA or Trolox standards in ethanol the absorbance reading was taken at 30°C up to 6 min. Antioxidant activities of PSA and ascorbic acid were expressed as TEAC using the calibration curve plotted with different amounts of Trolox.

Inhibitory effect on hydroxyl radical-induced DNA damage – This assay was done according to the method of Keum *et al.* (2000) with minor modifications. The reaction mixture (30 μL) contained 0.15 μg of pBR322 plasmid DNA, 30 mM H_2O_2, and 10 mM Tris-EDTA buffer (pH 8.0). Various amounts of PSA dissolved in 5 μL of ethanol (final concentrations of PSA were 0.25, 0.5, and 1.0 mM) were added prior to H_2O_2 addition. Hydroxyl radicals were generated by irradiation of the reaction mixtures at a distance of 20 cm with a 12W UV lamp. After incubation at room temperature for 30 min, the reactions were terminated by the addition of a loading buffer (0.25% bromophenol blue tracking dye and 50% sucrose), and the mixtures were then analyzed by 0.8% submarine agarose gel electrophoresis. The gels were stained with ethidium bromide, destained in water, and photographed on a transilluminator.

Results and Discussion

Lung cancer is one of the leading causes of neoplasia death in both men and women, and its incidence is increasing all over the world. The long-term survival rate of lung cancer patients remains unsatisfactory, even when

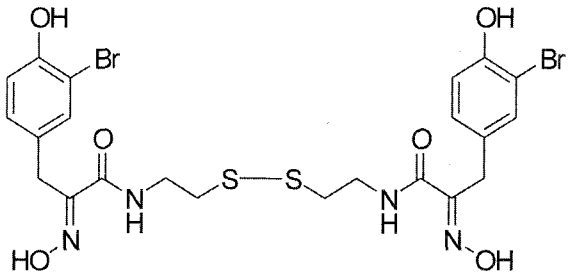

Fig. 1. Chemical structure of PSA.

they undergo complete and potentially curative surgery. Therefore, there is an urgent need for more potent drugs and new strategies aimed to improve lung cancer management. In this article, we invested whether PSA affected the cell cycle distribution of human lung cancer A549 cells. When A549 cells were treated with various concentrations of PSA for 48 hr, PSA inhibited the cell viability dose dependently (Fig. 2A) and IC_{50} value was about 3 μM. Camptothecin, a well-known topo I inhibitor, was used for the positive control and IC_{50} value of this was about 0.15 μM. Although the inhibition of PSA is not as potent as that of camptothecin (Fig. 2B), as a μM inhibitor of cell viability, PSA is still very promising and valuable for further study.

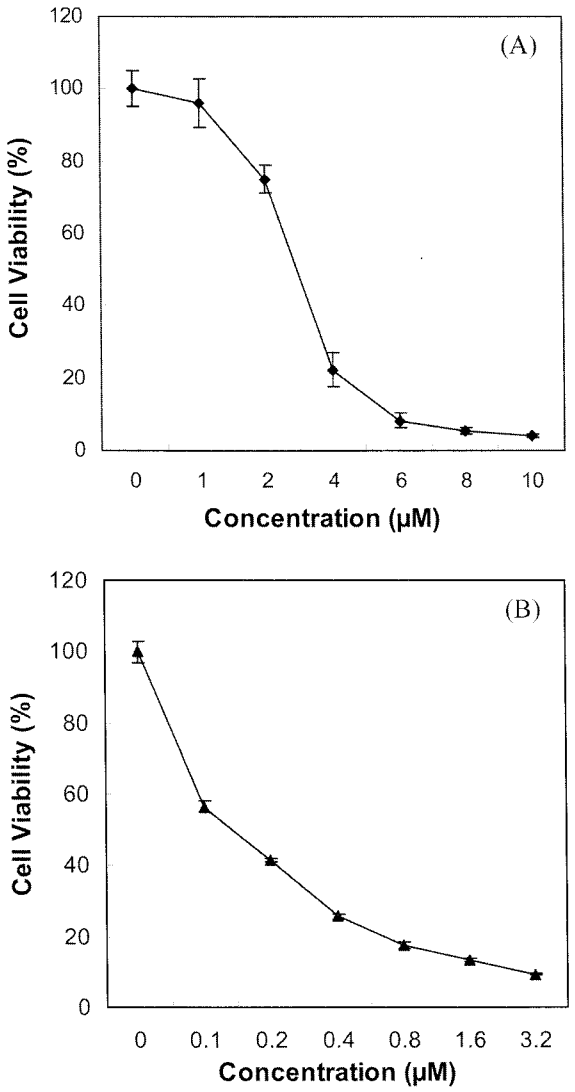

Fig. 2. Effects of PSA on the viability of lung cancer cell line A549. A549 cells were treated with various concentrations of PSA (A) for 48 hr. Relative cell viability was determined by WST-8 and 1-Methoxy PMS and is shown as a percentage of living cells. Camptothecin (B) was used as a positive control. Data are shown as means±SD of three independent experiments.

Flow cytometry analysis showed that PSA could perturbed cell cycle in a time-dependent course (Fig. 3). After 12 hr treatment, the population of the cells at the G2/M phase increased from 6.97% to 13.05%, which was occurred by decreasing both the G0/G1 cell population and S phase cell population. However, this increase of cell population in G2/M phase was not significant even when we extended the treating time of PSA to 24 or 48 hr (data not shown). We ruled out the possibility of random errors since the population increase at G2/M phase exhibited a time-dependent manner over a range. These data suggest that PSA do not disturb the cell cycle progression of A549 to a significant extent, which leaves the agent's primary target still be defined. PSA has been reported to exhibit weak inhibition of a number of enzymes including DNA gyrase, topoisomerase II, farnesyl protein transferase, and leucina aminopeptidase (Nicolaou *et al.*, 2001a). Recent findings indicate that PSA and its analogues are potent DNA methyltransferase and histone deacetylase inhibitors (Pina *et al.*, 2003). However, in the absence of definitive evidence of the primary target of PSA at the present time, we can only speculate as to through which type of mechanism this compound act. Given the distinct disulfide bridge in the structure of PSA, we suggest that the cytotoxicity of PSA might involve specific interaction of the molecule with a particular cellular target(s), leading to covalent modification of biological target via nucleophilic attack of a cysteine thiol, resulting in the formation of a mixed disulfide. Of course, we could not yet rule out the possibility of some nonspecific toxicity before further biochemical investigations aimed at identifying the biological target(s) of PSA are warranted.

In many cases, excessive oxidation stress is strongly associated with aging process and certain degenerative diseases including various cancers. Among the possible causes of cancer, damage to DNA and other molecular molecules by reactive oxygen species, ranks high as a major culprit in the onset and development of the disease. In clinic, many cancer patients who are undergoing therapy take antioxidant supplements in an effort to alleviate treatment toxicity and improve long-term outcome (Borek, 2004). However, the antioxidant supplements may interfere with the mechanism of therapy, causing diminished treatment effect and protecting of tumor tissue (Block, 2004). Therefore, we have a great interest to look for both anticancer and antioxidant compounds from nature. It was found that many natural products have both these two functions (Ju *et al.*, 2004). For example, resveratrol in

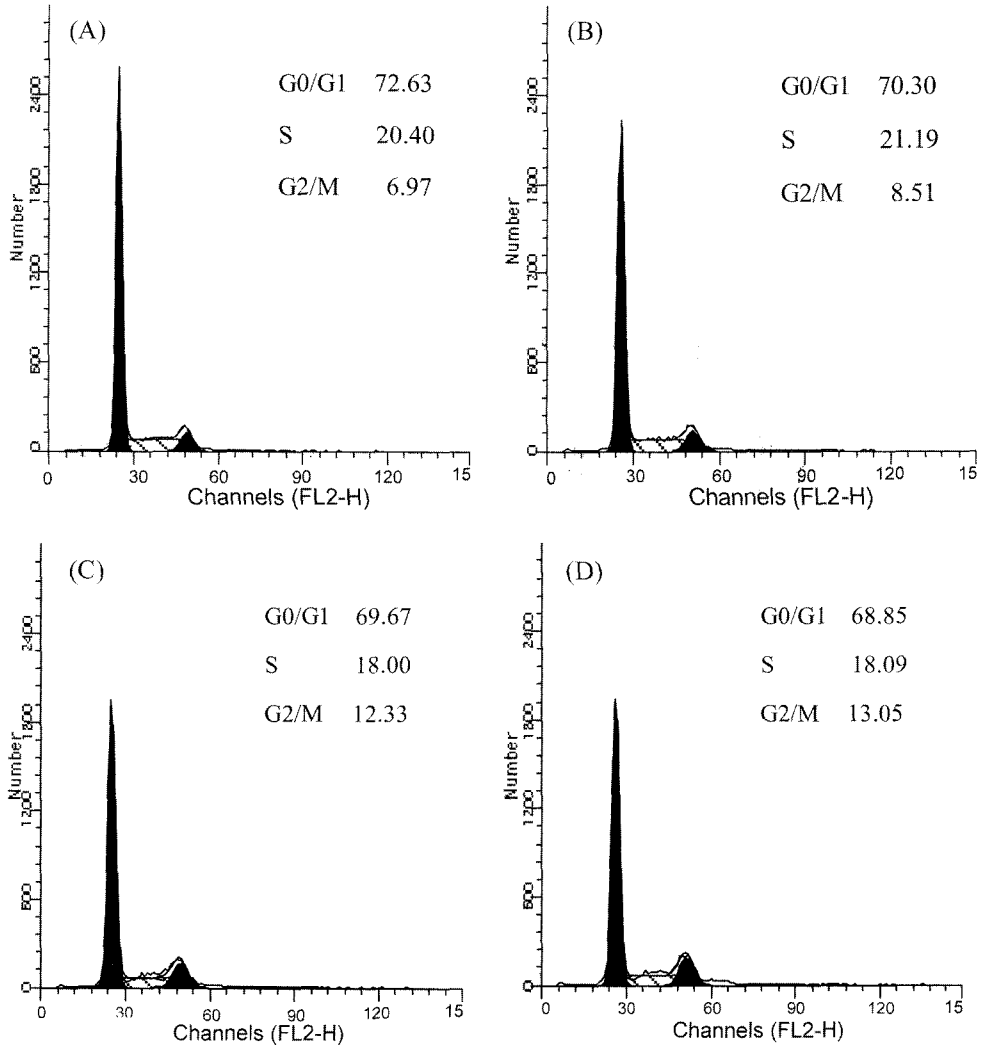

Fig. 3. Effect of PSA on cell cycle progression of A549 cells. A549 cells were treated with 4 μM PSA for (A) 0, (B) 4, (C) 8, and (D) 12 hr. DNA content was analyzed by flow cytometry with PI staining. The cell cycle distribution was calculated as the percentage of cells containing G0/G1, S, and G2/M phase. Data are representative of three independent experiments.

grapes and other food products has been shown to protect cells from oxidative damage and cell death and to prevent carcinogenesis in a murine model (Clement *et al.*, 1998). Epigallocatechin-3-galate (EGCG) in green tea has been reported to scavenge free radicals and to inhibit carcinogen-induced tumors in the skin, lung, forestomach and colon of rodents (Stoner, *et al.*, 1995). So far, there is no any report about the antioxidant activity of PSA. Inspired by the two phenolic groups in the structure of PSA, a series of antioxidant assays were carried out to investigate the antioxidant activity of PSA, which might partly contribute to its anticancer activity.

It is well known that free radicals are one of the causes of several diseases, such as Parkinson's disease (Adams *et al.*, 1991), coronary heart disease (Hertog *et al.*, 1993), and cancer (Cerutti, 1994). To evaluate the radical scavenging activity of PSA, two different assay systems, namely, the DPPH and TEAC assays were performed and a well-known antioxidant, ascorbic acid was used as a reference. It is generally accepted that electron-donating ability of chemical substances results in their antioxidant activity toward lipid oxidation. DPPH radical-scavenging assay is one of the short methods for investigation of the hydrogen donating potency (Blois, 1958). In this assay, antioxidants present as free radical scavengers should pair up with the stable DPPH free radical forming 1,1-dipheny-2-picrylhydrazine. In Table 1, PSA showed very weak free-radical scavenging activity, which slowly increased with concentration over a range of 0.01 to 1.0 mM. Ascorbic acid was used as a reference in this test. At 0.05 mM, ascorbic

Table 1. Percent inhibition of DPPH radical scavenging effects of PSA and ascorbic acid as positive control

Sample \ Conc.(mM)	0.01	0.03	0.05	0.25	0.5	1.0
PSA	2.08 ± 1.36	5.10 ± 2.15	5.86 ± 1.23	9.07 ± 2.44	20.04 ± 2.19	27.35±4.03
Ascorbic acid	28.54 ± 3.12	51.98 ± 3.04	81.28 ± 2.01	83.55 ± 2.35	85.07 ± 2.48	87.90±3.01

Data are shown as means±SD of three independent experiments.

acid almost reached its inhibition plateau while PSA just showed about 6% inhibition. These values suggest that PSA has weak free radical scavenge activity which is not comparable to that of ascorbic acid. However, PSA showed a significant antioxidant activity superior to ascorbic acid in the TEAC assay. The TEAC assay is based on the scavenging of the ABTS radical (ABTS $\cdot^{+}$) and converting it into a colorless product. The assay was first carried out on Trolox, the water-soluble α-tocopherol analogue, which serves as a standard. The results of the assay were expressed relative to Trolox in terms of TEAC. Since TEAC is a measurement of the effective antioxidant activity of test compound, a higher TEAC value would imply greater antioxidant activity of the sample.

As shown in Fig. 4, PSA exhibited the ABTS radical-scavenging activity in a concentration-dependent manner over a range of 0.15 to 2.5 mM. Furthermore, in comparison with ascorbic acid, PSA had higher TEAC values over this range. At the same time point, 1.25 mM of PSA had the TEAC value of 2.66 while ascorbic acid just had the value of 1.31. These values suggest that PSA showed stronger antioxidant activity than ascorbic acid in the TEAC assay. It is puzzling that PSA had very weak antioxidant activity in DPPH assay while exhibited such a strong antioxidant activity in TEAC assay. Now we just roughly contribute this discrepancy to the structure of PSA. Since it has been demonstrated that the TEAC value is significantly correlated with the content of total phenolic compounds, the number and position of hydroxyl group (OH) (Lien *et al.*, 1999), it is not a surprise that PSA which has two phenolic groups in its structure has potent ABTS radical-scavenging activity. In addition, reduction potentials of samples may change as a result of solvent effects and pH which can influence the state of protonation or deprotonation of the hydroxyl radical. Therefore the antioxidant activity may differ significantly between model systems depending on where these factors place the reduction potentials of the hydroxyl moieties related to that of the oxidizing radical, or transition metal ion, used in the system (Mitchell *et al.*, 1998).

Furthermore, to illustrate the protective effect of PSA on DNA strand scission, in this study the pBR322 DNA cleavage by hydroxyl radical generated by UV photolysis

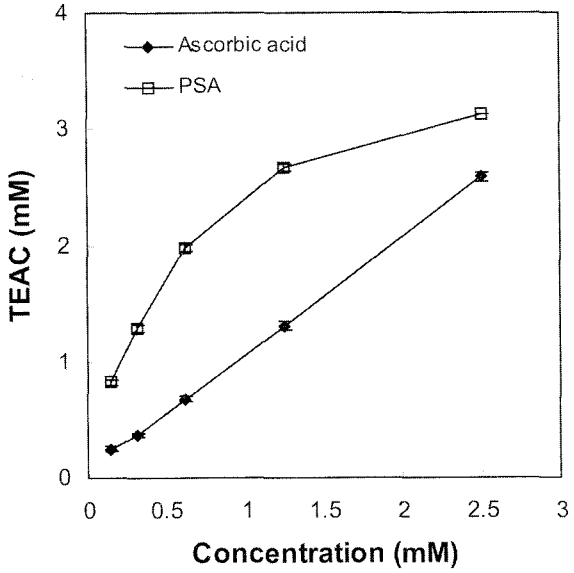

Fig. 4. Antioxidant capacity of PSA as determined by TEAC assay. Ascorbic acid was used as a reference. Data are shown as means±SD of three independent experiments.

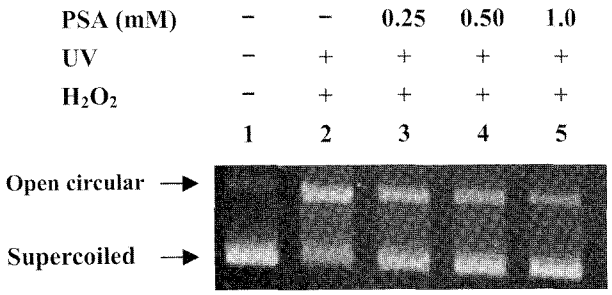

Fig. 5. Protective effect of PSA on DNA strand scission induced by H_2O_2 and UV. Lane 1, native supercoiled DNA without any treatment; Lane 2, DNA treated with H_2O_2 + UV irradiation; Lane 3-5, DNA samples treated with H_2O_2 + UV irradiation in the presence of 0.25, 0.5, 1.0 mM of PSA. Data are representative of three independent experiments.

of H_2O_2 was measured by agarose gel electrophoresis. In Fig. 5, treating the native DNA with both H_2O_2 and UV caused more than 90% conversion of the DNA from a supercoiled DNA to an open circular form (lane 2), whereas H_2O_2 or UV illumination alone did not cause DNA strand cleavage (data not shown). However, PSA was able to reduce the DNA strand scissions in a dose

dependent manner (lane 3-5). A detectable effect on the prevention of DNA cleavage of PSA was found at 0.25 mM (lane 3). At a dose of 1.0 mM (lane 5), the supercoiled pBR322 DNA was nearly totally protected, in comparison to native DNA (lane 1). This result indicates that PSA could effectively reduce the oxidation stress resulting from H_2O_2 or its product, hydroxyl radicals.

It is known that cancer process is a result of imbalance between cell growth and cell death. Endogenous and exogenous factors that influence DNA damage, cell growth, and cell death contribute to carcinogenesis. Experimental evidence supports an important role of reactive oxygen species in the cancer process. Increase in reactive oxygen in the cell through either physiological modification or through chemical carcinogen exposure, contribute to the carcinogenesis processes. We have found in this paper that PSA has significant antioxidant activity especially in scavenging ABTS radical and preventing DNA strand scission from oxidation stress. It is not clear now whether the antioxidant activity contributes to its anticancer effect because of lacking definite evidence. In order to clarify this question, further studies should be carried out. However, this is first report about the antioxidant activity of PSA, which gives some new information about this unique compound. Herein, we suggest that PSA would be a very interesting and promising candidate to be developed as a multi-function drug.

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