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A Partially Purified Lipid Extracted from *Ruditapes philippinarum* Suppresses Cancer Cell Proliferation

Eun-Kyung Kim, Yon-Suk Kim[1], Jin-Woo Hwang[1], Sang-Ho Moon, Byong-Tae Jeon, and Pyo-Jam Park[1,*]

Division of Food Bio Science and Korea Nokyong Research Center Konkuk University, Chungju, Chungbuk 27478, Korea
[1]Department of Biotechnology, Konkuk University, Chungju, Chungbuk 27478, Korea

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*Corresponding Author
Tel: +82-43-840-3588
Fax: +82-43-852-3616
E-mail: parkpj@kku.ac.kr

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Abstract Anticancer effects of lipids from a clam cultivated in Korea were investigated. Hexane extracts of *Ruditapes philippinarum (R. philippinarum*) exhibited the highest apoptosis rate of 40.9% at 0.5 mg/mL in PC3 cells. Hexane extracts were further separated and purified using TLC. Final lipid compounds were isolated and identified. Monounsaturated fatty acid and polyunsaturated fatty acid levels were significantly (p<0.05) increased in the purified lipid (19.71 and 34.63%), compared with the crude extract (12.96 and 15.56%). Partially purified lipid compounds exhibited an anticancer effect in several cancer cell lines, resulting in apoptosis rates in breast, lung, and liver cancer cells of 37.36, 17.78, 26.56%, respectively, at 30 μg/mL. This is the first report of an anticancer effect of a lipid extracted from *R. philippinarum* cultivated in Korea.

Keywords: *Ruditapes philippinarum*, lipid, apoptosis, anticancer

Introduction

Ruditapes philippinarum (Manila clam)is a marine bivalve mollusc in the family Veneridae. *R. philippinarum* is endemic to East Asian waters (1) and is commonly found on sandy beaches and tidal flats along the coast of East Asia. It is one of the main cultured species of marine shellfish in Korea and the Mediterranean area and is commonly used as food. However, until now, little attention has been paid to lipid compounds of this bivalve. Some reports have investigated the structure and bioactivity of other shellfish. Mytilan, isolated from the mantle of the mussel *Crenomytilus grayanus,* possessed a high immunomodulation activity (2,3). A glycogen polysaccharide extracted from *Perna canaliculus* was found to exhibit an anti-inflammatory activity (4). A heparin-like substance able to bind antithrombin III (ATIII) isolated from the marine clam *Anomalo cardiabrasilian* was found to have a potent anticoagulant activity (5). Study of the lipid composition of *R. philippinarum* has been undertaken in Japan, China, Argentina, and the United States, and other countries (6-9). Many previous studies were related to cultivation conditions and little is known about the mussel cultivated in Korea and anticancer activities.

Cancer chemoprevention is an integral part of a sound strategy in the fight against cancer. Diet-derived compounds are often the focus of study due to perceived safety. The objective of this study was, therefore, to identify and investigate the effects of anticancer components extracted from *R. philippinarum*. Lipid extracts were prepared and anticancer effects of partially purified lipids from *R. philippinarum* against prostate, breast, lung, and liver cancer cells were investigated.

Materials and Methods

Materials Silica gel 60 was obtained from Merck (Darmstadt, Germany). TLC silica gel hard layer fluorescent (HLF) plates were purchased from Analtech, Inc. (Newark, DE, USA), and TLC silica gel 60F254 plates were obtained from Merck. PC3 human prostate cancer cells (No. 21435), MDA-MD-231 human breast cancer cells (No. 30026), A549 human lung cancer cells (No. 10185), and HepG2 human liver cancer cells (No. 88065) were obtained from the Korean Cell Line Bank (Seoul, Korea). Dulbecco's Modified Eagle medium (DMEM), Roswell Park Memorial Institute (RPMI) medium 1640, fetal bovine serum (FBS), and penicillin-streptomycin were obtained from Invitrogen Corporation (Carlsbad, CA, USA). RNase A and Tween-20 were purchased from Novagen (Darmstadt, Germany) and USB (Cleveland, OH, USA), respectively. *R. philippinarum* was purchased from a local aquafarm (Yoesu, Korea) in June of 2012. All other reagents were of the highest commercial grade available.

Preparation of lipid extracts from *R. philippinarum* *R. philippinarum* was lyophilized for 5 days using a freeze-dryer (TFD; IlShinBioBase, Dongduchun, Korea). The freeze-dried *R. philippinarum* was

Table 1. Recovery rates for stages of *R. philippinarum* purification

Fraction	Hexane extracts	1st TLC (200×75 mm)	2nd TLC (200×75 mm)
Recovery rate[1)] (%)	100.0	5.45	2.69

[1)](collected mass/initial mass)*100

suspended in methanol, chloroform, hexane, methanol:chloroform, and methanol:hexane separately for 24 h. The extracts were vacuum-dried for 5 h using a speed vacuum concentrator (Savant ISS110; Thermo Fisher Scientific, Waltham, MA, USA) and stored at −20°C until use. Recovery rates of each lipid extract from *R. philippinarum* are shown in Table 1.

Open column chromatography using silica gel Twenty mL of a hexane extract (g/mL) was loaded onto a silica gel column (30×400 mm), equilibrated with 3 times column volumes of elution buffer, and eluted using hexane/diethyl ether/acetic acid (80:20:2, v/v/v). Fractions of 50 mL were collected, then applied to silica gel plates (silica gel HLF).

Purification of lipids for determination of anticancer-effects Hexane extracts exhibited the highest anticancer activities with the highest apoptosis rate on the cancer cells and were subjected to purification. Concentrated lipid samples (200 μL) of hexane extracts were applied to silica gel plates (silica gel HLF) and eluted using hexane/diethyl ether/acetic acid (80:20:2, v/v/v) following the method described by McPhee *et al*. (10). Briefly, lipids were visualized by spraying plates with 20% sulfuric acid. Upon illumination with UV light at 254 nm, individual lipid classes were detected as violet spots. After initial TLC, individual spots were scraped off and anticancer effects were determined by apoptosis assay using a flow cytometer. The fraction displaying the highest anticancer activity from the first TLC class was further purified using TLC in the same manner as the first TLC method.

Fatty acid analysis Fatty acid analyses were carried out using 20 mg of dry matter freeze-dried *R. philippinarum*. Fatty acid methyl esters (FAME), obtained using transmethylation of total lipids with a 14% BF3 methanol solution following the method of Morrison and Smith (11) were analyzed on an Agilent 6890 gas-liquid chromatograph (GC) equipped with a SP2560 fused silica capillary column (100 m×0.25 mm) (Agilent Technologies, Santa Clara, CA, USA) and a flame-ionization detector (Agilent Technologies) held at 260°C. The oven temperature was set in programmed mode from 140 to 240°C at a rate of 4°C/min with a final isotherm of 10 min. The carrier gas was nitrogen at 1.0 mL/min. Data were processed using an Agilent Chem Station for the GC System. FAME mixtures were identified as previously described (12).

Cell culture Prostate cancer cells were cultured and maintained in RPMI medium 1640 and breast, lung, and liver cancer cells were cultured and maintained in DMEM supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin, and 10% FBS and maintained at 37°C under a humidified atmosphere with 5% CO_2 (CO_2 incubator; Thermo Fisher Scientific). All treatments were performed at 30% confluence.

Apoptosis analysis For apoptosis analysis, harvested cells were fixed with ethanol (containing 0.5% Tween-20) for 24 h, incubated in the CO_2 incubator with 50 μg/mL propidium iodide (PI) and 1 μg/mL RNase A at 37°C for 30 min, and analyzed using flow cytometry on a FACScan (BD, Franklin Lakes, NJ, USA). Cells belonging to the sub-G1 population were considered apoptotic cells, and the percentage of each phase of the cell cycle was determined.

Statistical analysis All results were expressed as a mean±standard deviation (SD). Statistical analysis was carried out using the GraphPad PRISM program (GraphPad Software, Inc., San Diego, CA, USA). The paired *t*-test was used for comparisons between crude and purified groups. Multiple group data were analyzed using a one-way analysis

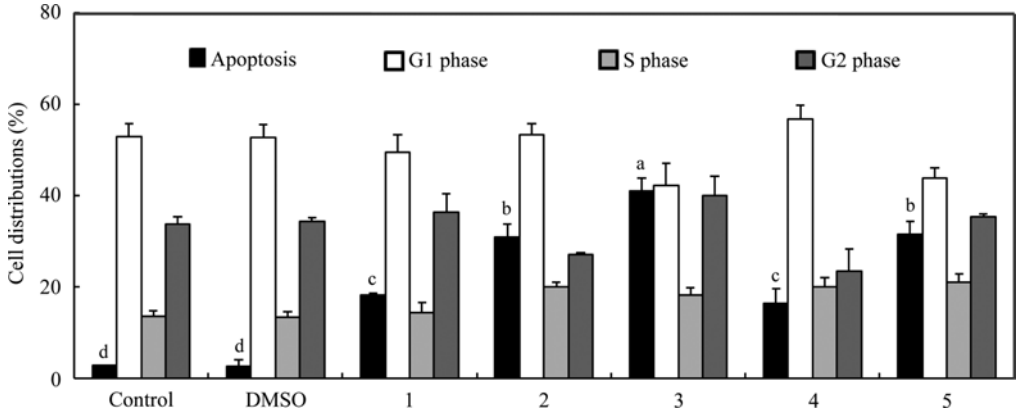

Fig. 1. Anticancer effects of organic solvent extracts from *R. philippinarum* in PC3 cells, a prostate cancer cell line. 1. methanol; 2. chloroform; 3. hexane; 4. methanol/chloroform (1/1), 5. chloroform/hexane (1/1). Cells were treated with 0.5 mg/mL of each organic solvent fraction from *R. philippinarum*. Mean±SD of determinations were reported for triplicate experiments. Lower case letters [a-d] indicate apoptosis values are significantly different at $p<0.05$ based on Bonferroni *post-hoc* testing.

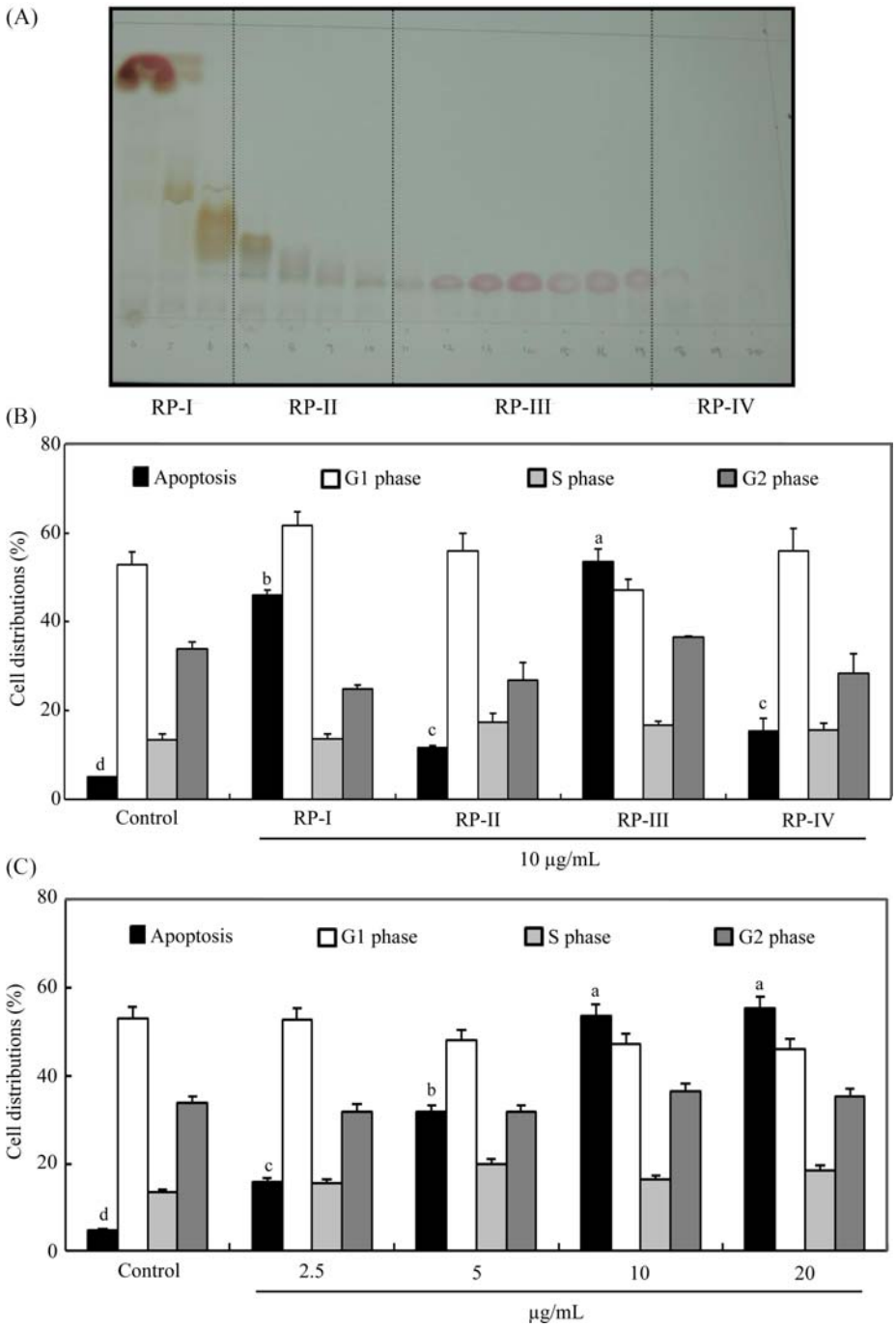

Fig. 2. Anticancer effects of hexane extracts from *R. philippinarum*in PC3 cells. (A) The first TLC of hexane extracts from *R. philippinarum*; (B) Anticancer effects of first TLC fractions from *R. philippinarum* in PC3 cells. (C) Anticancer effects of fraction RP-III in PC3 cells. Mean±SD were determined for triplicate experiments. Lower case letters $^{a\text{-}d}$indicate values are significantly different at $p<0.05$ based on Bonferroni *post-hoc* testing.

of variance (ANOVA), followed by Bonferroni *post-hoc* testing.

Results and Discussion

Analysis of *R. philippinarum* PC3 cell apoptosis Regulation of cell growth involves a homeostatic balance between stimulatory and inhibitory signals. Negative growth control due to tumor suppressor genes, differentiation factors, and programmed cell death (apoptosis) are commonly targeted mechanisms exploited for strategies in treatment of malignancies and other diseases. Apoptosis is a highly attractive and widely studied area in the search for effective agents for treatment of human cancers. Many *in vivo* and *in vitro* studies published in recent years have suggested that chemotherapeutic

Table 2. Fatty acid components of the partially purified anticancer-effective lipid RP-III-a from *R. philippinarum*

Fatty acids		Content (%)	
Compound	Molecular formula	Crude	Purified
Palmitic acid	C16:0	14.48	29.65
Palmitoleic acid	C16:1	3.06	19.71
Stearic acid	C18:0	8.61	15.82
Eicosapentaenoic acid	C20:5	0.357	14.10
Docosahexaenoic acid	C22:6	9.93	20.15
SFAs[1)]		44.41	45.47
MUFAs[2)]		12.96	19.71*[4)]
PUFAs[3)]		15.56	34.625**

[1)]Saturated fatty acids
[2)]Monounsaturated fatty acids
[3)]Polyunsaturated fatty acids
[4)]*(p<0.05) and **(p<0.01)are significantly different based on a paired t-test comparing crude and purified groups.

agents can induce apoptotic cell death in different cancer cells (13,14). For this reason, the anticancer activities of organic solvent extracts were evaluated using flow cytometry for an ability to induce apoptosis in this study. The highest anticancer activity was observed in hexane extracts from *R. philippinarum*, exhibiting an apoptosis rate of 40.9% at 0.5 mg/mL in human prostate cancer cells PC3 (Fig. 1). Therefore, hexane extracts were subjected to further study.

Purification of anticancer lipids from *R. philippinarum* For purification of anticancer-effective lipids, hexane extracts of *R. philippinarum* were subjected to TLC. The 4 individual fractions of RP-I, RP-II, RP-III, and RP-IV were detected (Fig. 2A). The highest anticancer activity was observed in RP-III (52.9%) (Fig. 2B) in a dose-dependent manner (Fig. 2C). Therefore, the RP-III fraction was subjected to further study. The RP-III fraction was subjected to TLC and 2 individual fractions of RP-III-a and RP-III-b were detected (Fig. 3A). The highest anticancer activity was observed in RP-III-a (75.8%) (Fig. 3B) in a dose-dependent manner (Fig. 3C). Therefore, RP-III-a was subjected to analysis of fatty acid components.

RP-III-a was composed of palmitic (29.65%), palmitoleic (19.71%), stearic (15.82%), eicosapentaenoic (EPA) (14.10%), and docosa-hexaenoic fatty acids (DHA) (20.15%) (Table 2). There was no change between before and after purification in the concentrations of saturated fatty acids (SFAs) in RP-III-a, while levels of mono-unsaturated fatty acid (MUFAs) and polyunsaturated fatty acid (PUFAs) were increased significantly (p<0.05). Thus, partially purified lipids possessed more concentrated MUFAs and PUFAs than raw material, potentially resulting in positive effects on the body.

Apoptosis analysis of *R. philippinarum* in MDA-MB 231, A549, and HepG2 cells Breast cancer is the fastest-growing cancer in Korean women due to a Westernized lifestyle and accelerated rapid socioeconomic development (15). Lung cancer is the most common cause of death from malignancy and contributes substantially to the global burden of disease, as highlighted in 2008 by the World Health Organization (16). Each year in Korea, more than 16,000 new cases of lung cancer are diagnosed, accounting for 12.1% of all cancers during the period from 2003-2005 (17). Liver cancer is one of the most common malignant tumors and surgical removal and chemotherapy have been mainstays of liver cancer treatment. However, conventional treatments have not improved the survival rate over the past 2 decades. Thus, apoptosis rates of these critical cancer cells were investigated. Hexane extracts from *R. philippinarum* were used to treat cancer cells, resulting in apoptosis rates in breast, lung, and liver cancer cells of 43.5, 23.5, 33.5%, respectively, at 30 μg/mL in a dose-dependent manner (Fig. 4).

Dietary fat has been observed to affect gene expression, in addition to a role as an important source of energy, and exerts effects on membrane lipid compositions (18). In mammals, expression of many genes is modulated by fatty acids in both a positive and negative manner, leading to pronounced changes in metabolism, cell differentiation, and growth (19,20).

Fatty acids (FAs) are major components of biological cell membranes, playing important roles in intracellular signaling and as precursors for ligands that bind to nuclear receptors. FAs are chemically classified as saturated and unsaturated (monounsaturated and polyunsaturated) and molecular structures affect biological activities (21).

Multiple molecular mechanisms have been implicated in palmitic acid-associated apoptosis (22). Hardy *et al*. (23), in a study of saturated fatty acid palmitate induction of apoptosis in the MDA-MB-231 breast cancer cell line, reported that mitochondria and cardiolipin play critical roles in palmitate-induced apoptosis in breast cancer cells. It is widely recognized that linolenic acid, EPA, DHA, and other PUFAs have antitumor effects (24,25). PUFAs inhibit cancer-cell growth, induce apoptosis, and increase the efficiency of chemotherapeutic drugs (26). EPA has significant effects on cell growth and proliferation, and triggers apoptosis in a number of cell types (27,28). Several mechanisms, including modulation of membrane properties, eicosanoid formation, and free radical generation, have been postulated to account for the anticancer activities of PUFAs (29). Increased peroxidation of PUFAs is one of the possible mechanisms involved (30). Previous study has demonstrated that oxidative stress plays a central role in fatty acid-induced cell death, whether directly or as a feedback mechanism (31,32). Thus, the mechanism of apoptosis induced by EPA in several cancer cell types may be partly due to lipid peroxidation. DHA was reported to be the most potent fatty acid for enhancement of doxorubicin efficacy inbreast cancer cell lines (33). DHA, with 6 double bonds, is prone to oxidation. Therefore, increased membrane unsaturation index values resulting from DHA supplementation provides more targets for ROS generated during doxorubicin metabolism.

In vivo and *in vitro* studies of the influence of DHA supplementation have reported higher rates of peroxidation and oxidative stress resulting from ROS attack, demonstrated by an increase in

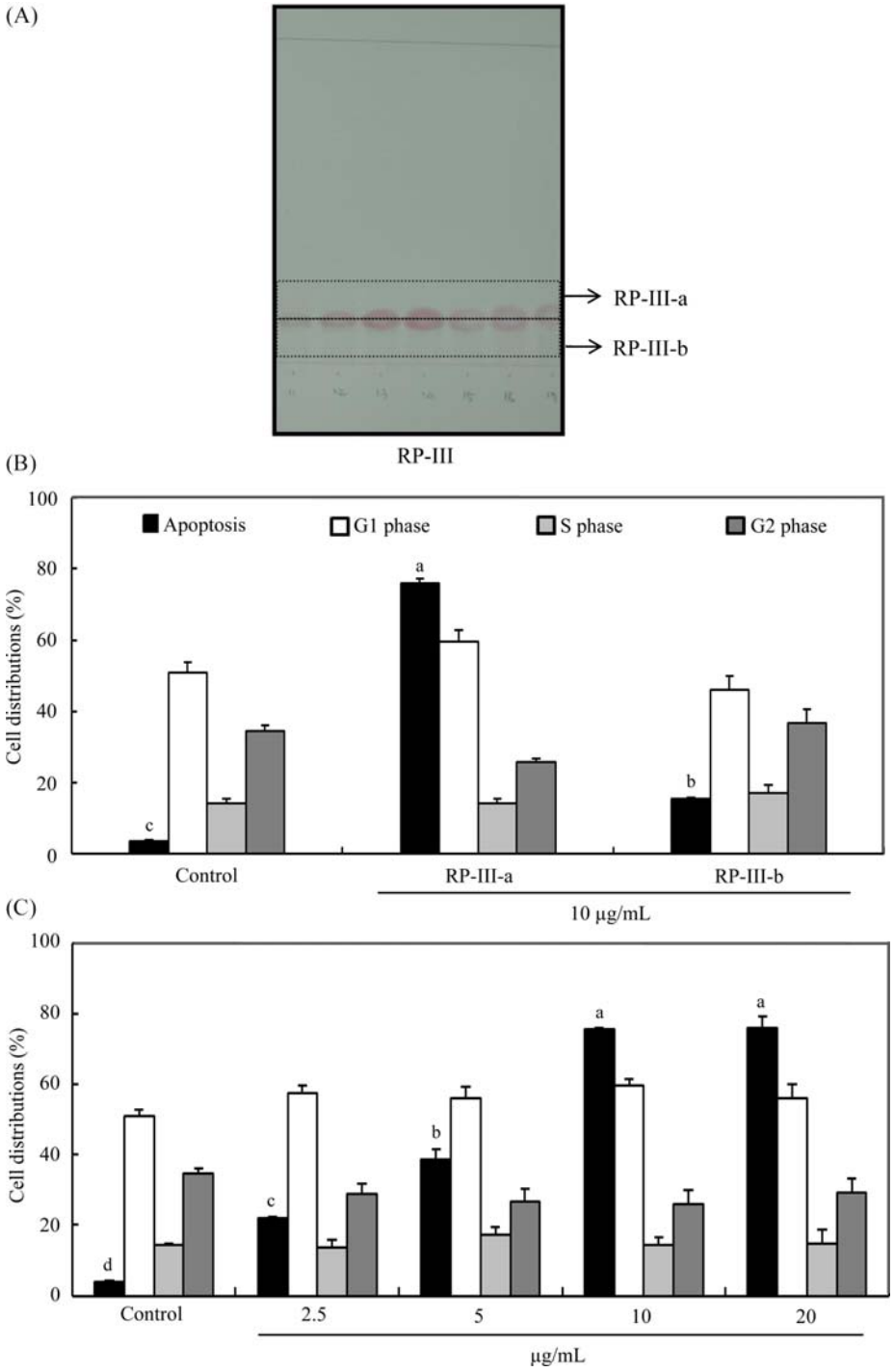

Fig. 3. Anticancer effects of hexane extracts from *R. philippinarum* in PC3 cells. (A) Second TLC of hexane extracts from *R. philippinarum*; (B) Anticancer effects of second TLC fractions from *R. philippinarum* in PC3 cells. (C) Anticancer effects of fraction RP-III-a in PC3 cells. Mean±SD determined from triplicate experiments. Lower case letters $^{a-d}$indicate values are significantly different at $p<0.05$ based on Bonferroni *post-hoc* testing.

thiobarbituric acid-reactive substance, conjugated diene, and or malondialdehyde levels, and a decrease in levels of antioxidant vitamins (34). Hydroperoxide and aldehyde products of lipid peroxidation have been implicated in the cytotoxic process and in increased drug efficacy (35). Thus, conditions favoring increased lipid peroxidation in response to doxorubicin would lead to a higher level of drug activity. Therefore, the antioxidant status appears to be a crucial determinant for the sensitivity of tumors to ROS-generating anticancer therapies. Hexane extracts from Korean-cultivated *R. philippinarum* probably have substantial anticancer properties related to lipid peroxidation and concentrated PUFAs.

In summary, a purified fraction from hexane extracts of *R.*

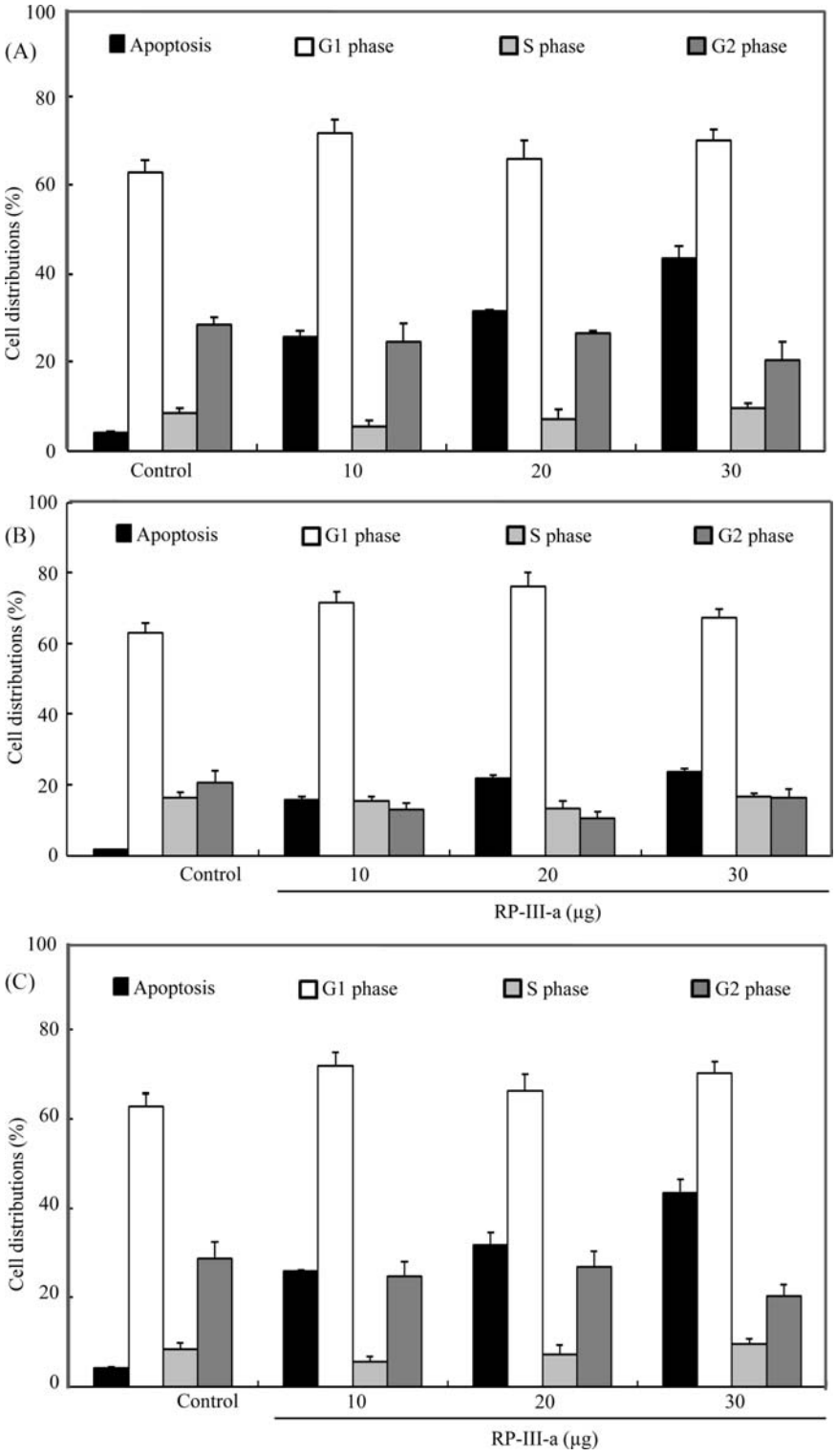

Fig. 4. Anticancer effects of RP-III-a from *R. philippinarum* in MDA-MB-231 breast cancer cells (A), A549 lung cancer cells (B), HepG2 liver cancer cells (C). Means±SD of determinations were reported for triplicate experiments.

philippinarum designated RP-III-a was found to contain high levels of palmitic, palmitoleic, stearic, eicosapentaenoic, and docosahexaenoic acids. RP-III-a exhibited a strong anticancer effect. RP-III-a increased apoptosis in human prostate, breast, lung, and liver cancer cells, but did not increase apoptosis in normal liver cells (data not shown). RP-III-a can effectively inhibit tumor growth based on induction of cancer cell apoptosis. Although the mechanism for induction of apoptosis in tumor cells is not known and needs further investigation,

RP-III-a is a potential chemotheraputic agent.

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Disclosure The authors declare no conflict of interest.

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