Comparative evaluation of free radical scavenging activities and cytotoxicity of various solvent fractions of Sandong *Sageretia thea* (Osbeck) M.C. Johnst. branches

Gyeong-A Ko¹, Moa Son², and Somi Kim Cho^{1,2,3,*}

¹Faculty of Biotechnology, College of Applied Life Sciences, SARI, Jeju National University, Jeju 63243, Korea

²School of Biomaterials Science and Technology, College of Applied Life Sciences, SARI, Jeju National University, Jeju 63243, Korea

³Subtropical Tropical Organism Gene Bank, Jeju National University, Jeju 63243, Korea

Received March 7, 2016
Revised September 8, 2016
Accepted October 12, 2016
Published online December 31, 2016

*Corresponding Author
Tel: +82-64-754-3348
Fax: +82-64-756-3351
E-mail: phd.kim.somi@gmail.com;
somikim@jeju.ac.kr

pISSN 1226-7708
eISSN 2092-6456

© KoSFoST and Springer 2016

Abstract *Sageretia thea* (Osbeck) M.C. Johnst., an evergreen tender shrub of the Rhamnaceae, has been used as folk medicine and in traditional tea. To the best of our knowledge, this is the first study that investigates the free radical scavenging activity and cell cytotoxicity of *S. thea* branches. The ethyl acetate fraction and *n*-butanol fraction were identified to be rich in phenolic compounds exhibiting impressive antioxidant activity after stepwise partitioning of solvent fractions of the methanol extracts of *S. thea* branches (MB). Among the different human cancer cells tested, maximum cytotoxicity was found in MDA-MB-231 human breast cancer cells with MB, chloroform, ethyl acetate, *n*-butanol and water fraction. A significant ($p < 0.05$) correlation between antioxidant activities and cytotoxicity exists in each fraction. These results suggest the branches of *S. thea* can be a valuable source of antioxidants, and they can serve as natural anticancer constituents in nutraceutical and pharmaceutical applications.

Keywords: antioxidant, cytotoxicity, gas chromatography–mass spectrometry, human breast cancer cell, *Sageretia thea* (Osbeck) M.C. Johnst

Introduction

S. thea (Osbeck) M.C. Johnst., locally known as “Sangdong,” is a shrub belonging to the Rhamnaceae family and grows along the seashore (1). In Korea, the leaves have been used in traditional tea and medicine for treatment of cold and fever (1,2). *S. thea* is also a traditional Chinese medicine for hepatitis (2). Studies regarding the medical properties of *S. thea* leaves have been published, which include their antioxidant activity (1,2) and inhibitory activity on HIV-1 protease (3); However, to date, no report on the cell cytotoxicity and its relation with antioxidant activity of *S. thea* has been found in the literature.

Cancer is currently a significant chronic disease that leads to an increase in the death worldwide (4). Cancer can occur due to reactive oxygen species (ROS), which damage the DNA, proteins, and lipids, ultimately leading to mutations that transform normal cells into cancer cells. ROS are produced by endogenous and exogenous stimuli (5). Cells maintain the redox equilibrium through the endogenous antioxidant defense systems, which are balanced between the generation and scavenging of ROS (6). Failure of apoptosis and increased rate of cell survival occurs due to DNA damage and subsequently results in the tumorigenesis (7). Because

in vivo and *in vitro* studies demonstrated that excessive intracellular ROS affect an early event of cancer initiation and progression, several antioxidants are used in therapeutic agents to prevent cancer development via decreasing the intracellular ROS levels (8). Antioxidants can suppress the propagation of cancer cells through defense mechanisms that resist oxidative stress (9).

Effective synthetic antioxidants, including butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT), are generally used in food processing industries; however, these synthetics are suspected of being responsible for toxicities and carcinogenesis (10). Thus, new and safe antioxidants from natural sources must be identified to replace these synthetic antioxidants. Previous studies reveal that many plant have great antioxidant activity and can be used as potential sources of safe antioxidants (11,12). The antioxidant effect of plant products is associated with the antiproliferative activity of cancer cells. Many plants with antioxidant properties are used as valuable therapeutic and preventive agents for different carcinoma cell lines induced by free radicals (13). In particular, quercetin, a plant-derived antioxidant found in many fruits and vegetables, is one of the most potent free radical scavengers. The proliferation and survival of cancer cells may be prevented by long-term exposure to quercetin (8,14). Curcumin, a natural polyphenol derived from the

rhizome of turmeric, also shows strong antioxidant activities, antiproliferative activities, and antimetastasis (15).

The purpose of this study was to evaluate the antioxidant capacity and cytotoxicity of *S. thea* on human cancer cells. In addition, the correlation between the antioxidant activities and cytotoxicity of *S. thea* was investigated along with chemical composition profiling by gas chromatography–mass spectrometry (GC–MS) analysis. To the best of our knowledge, this is the first research report on the antioxidant activities, cell cytotoxicity, and chemical composition of different solvent extracts of *S. thea* branches. Our results show that branches exhibit higher extraction yield and antioxidant activities than leaves; this result that may strongly enhance the utilization of *S. thea*.

Materials and Methods

Chemicals The Folin–Ciocalteu phenol reagent, sodium carbonate anhydrous, rutin, gallic acid, 2,2-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), pentadecanoic acid, potassium peroxodisulfate, 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azobis(2-methylpropionamid) dihydrochloride (AAPH), and α -(4-pyridyl-1-oxide)-N-tert-butyl nitron (4-POBN) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Hoechst 33342, dimethyl sulfoxide (DMSO), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) were purchased from Amresco Inc. (Solon, OH, USA). The Roswell Park Memorial Institute (RPMI) medium, Dulbecco's modified Eagle's medium (DMEM), F-12K medium, trypsin/EDTA, fetal bovine serum (FBS), and 100x penicillin/streptomycin solution were purchased from Invitrogen Inc. (Grand Island, NY, USA). Annexin V-FITC Apoptosis Detection Kit-1 was purchased from BD Biosciences (Franklin Lakes, NJ, USA). For GC–MS analysis, high-performance liquid chromatography (HPLC) grade solvents were purchased from Merck Inc. (Darmstadt, Germany). All standard chemicals and reagents were of analytical grade.

Plant materials The *S. thea* leaves and branches were locally collected from Seogwang, Jeju Island, in November 2013. Botanical samples were previously taxonomically identified, and voucher specimens were deposited by Dr. Hui Kim at Medicinal Plants Resources, Mokpo National University (Muan-gun, Jeollanam-do, Korea).

Extraction and fractionation The sample (70 g) was lyophilized for 1 day, pulverized, and extracted with 80% ethanol or methanol at room temperature with sonication (maximum 60 Hz) for 45 min. The extracts were filtered, concentrated with a vacuum rotary evaporator under reduced pressure at 40°C, and lyophilized to afford crude extracts. The concentrated methanol extract of the branches was suspended in water and further fractionated by additional extraction with *n*-hexane, chloroform, ethyl acetate, and *n*-butanol in a stepwise

manner. Each fraction was lyophilized to obtain the powder of the corresponding fractions of the branch extracts, and this powder was dissolved in 20 mg/mL dimethyl sulfoxide (DMSO) and diluted with DMSO to obtain the final required concentrations.

Determination of the total polyphenol and flavonoid contents The total polyphenol content was determined by a slightly modified method of Cheung *et al.* (16). A 125 μ L aliquot of the sample was mixed with 0.5 mL of Folin–Ciocalteu's phenol reagent. After 5 min, 1 mL of 10% (w/v) Na₂CO₃ was added to the mixture. The reaction was performed in dark conditions for 30 min, after which its absorbance was recorded at 725 nm using a microplate reader (Sunrise, Tecan, Salzburg, Austria). Results were expressed as mg gallic acid equivalent (GAE)/g dry weight on the basis of the dried sample. The flavonoid content was measured using a previously developed colorimetric assay (17). The absorbance was measured at 510 nm against the blank (DMSO), and the flavonoid content was expressed as mg rutin equivalents (RE)/g dry weight on the basis of the dried sample.

ABTS radical scavenging activity ABTS radical scavenging measurements were performed according to Gião *et al.* (18). The ABTS radical cation was generated by adding 7 mM ABTS to a 2.45 mM potassium persulfate solution and allowing the mixture to stand in dark conditions at room temperature for 16 h before use. To obtain an absorbance of 0.700 $\pm$ 0.005 at 734 nm measured with a UV 1800 spectrophotometer (Shimadzu, Kyoto, Japan), the stock solution was diluted with distilled water as required. Further, 100 μ L of the sample was added to 900 μ L of this diluted stock solution, and the absorbance was determined after 2 min of initial mixing. The percentage of scavenging activity was calculated as the percentage reduction in absorbance.

Alkyl radical scavenging activity Alkyl radicals were generated by reaction mixtures containing 40 mM AAPH and 40 mM 4-POBN (19). This solution and sample mixture were incubated at 37°C in a water bath for 30 min, and then transferred to 50 μ L Teflon capillary tubes. The radical scavenging activity was measured using a JES-FA200 ESR spectrometer (JEOL, Tokyo, Japan). The measurement conditions were as follows: magnetic field, 336.000 mT; power, 7 mW; sweep time, 30 s; sweep width, 10 mT; frequency, 9.43 GHz; modulation width, 0.2 mT; time constant, 0.03 s. The radical scavenging activities were calculated according to the following formula: (control group-treated group)/(control group) $\times$ 100%. Signal intensity was compared on the basis of the ratio against the magnetic marker (standard of ESR, Mn²⁺ marker) and was represented by relative height ratio.

DPPH radical scavenging activity DPPH radicals were measured using a JES-FA200 ESR spectrometer by a previously described method (20). A 30 μ L sample was added to 30 μ L of 60 μ M DPPH, mixed vigorously, and allowed to stand for 2 min. The solution was

transferred to 50 μ L Teflon capillary tubes and the radical scavenging activity was measured. The measurement conditions were as follows: magnetic field, 336.000 mT; power, 5 mW; sweep time, 30 s; sweep width, 10 mT; frequency, 9.43 GHz; modulation width, 0.8 mT; time constant, 0.03 s. The radical scavenging activities were calculated according to the following formula: (control group-treated group)/(control group) $\times$ 100%. Signal intensity was compared on the basis of the ratio against the magnetic marker (standard of ESR, Mn^{2+} marker) and was represented by relative height ratio.

Cell culture Cancer cell lines, including AGS, a human gastric adenocarcinoma cell line, A549, human lung carcinoma, MDA-MB-231, a human breast adenocarcinoma cell line, and SNU-1 and SNU-16, human gastric carcinoma cell lines, were obtained from the Korean Cell Line Bank (Seoul, Korea). The cell lines were cultured in DMEM, RPMI 1640, or F-12K containing 10% (v/v) heat-inactivated FBS, 100 units/mL penicillin, and 100 μ g/mL streptomycin. The cells were maintained in a humidified incubator at 37°C in a 5% CO₂ atmosphere.

Cell viability assay The effects of *S. thea* on the viability of different cancer cell lines were determined by MTT colorimetric assay according to a previously described protocol (21). Briefly, AGS, A549, and MDA-MB-231 cells (3×10^4 cells/mL) and SNU-1 and SNU-16 cells (5×10^4 cells/mL) were seeded in 96 well culture plates. After overnight incubation, the cells were treated with different concentrations of the different fractions for 24 h. Then, 0.1 mg MTT was added to each well, and the cells were incubated at 37°C for 4 h. The growth medium was carefully removed and DMSO (150 μ L) was added to each well to dissolve the formazan crystals formed. The intensity of the produced formazan was measured immediately at 570 nm using a microplate reader. The percentage of cell viability was calculated as the percentage reduction in absorbance.

Cellular morphology and nuclear fragmentation MDA-MB-231 cells were placed in 60 mm dish at 3×10^4 cells/mL and treated with 100 μ g/mL concentration of fractions. After 24 h, the treated cells were stained with 10 μ M Hoechst 33342 for 10 min at 37°C and observed under a fluorescence microscope (Olympus, Essex, UK). The percentage of apoptotic bodies was calculated according to the following formula: (apoptotic cell number)/(whole cell number) $\times$ 100%.

Cytometric analysis An Annexin V-FITC Apoptosis Detection Kit-1 was used to distinguish apoptosis. The cells were washed with PBS, harvested, diluted in a buffer containing Annexin V and PI, and incubated for 15 min at room temperature. These analyses were performed using a FACS caliber flow cytometer (BD Biosciences). Each sample was analyzed by counting 10,000 cells and the data were analyzed using the Cell Quist Software (BD Biosciences).

Chemical composition by GC–MS GC–MS analysis was conducted using Shimadzu (Model QP-2010; Shimadzu) in the electron impact mode. The ionization voltage was 70 eV and the temperatures of the inlet and interface were 250 and 290°C, respectively. The capillary Rtx-5MS column (30 m length, 0.25 mm i.d., and 0.25 μ M film thickness) was used. The oven temperature was programmed at 60°C (isothermal for 2 min) and then ramped to 250°C at 5°C/min and to 310°C at 8°C/min (isothermal for 12 min). The flow rate was 1 mL/min at 57.4 kPa pressure with Helium and injector volume was a 1 μ L with 1:10 split ratio. Mass range was from m/z 40–500 amu. The MS spectral data were compared with the WILEY9 and NIST05 libraries.

Statistical analysis All experimental results were expressed as mean $\pm$ SD with samples run in triplicate. Analysis of variance (ANOVA) and Pearson's correlation were analyzed using SPSS software (version 18.0; SPSS Inc., Chicago, IL, USA).

Results and Discussion

Determination of antioxidant activities of different *S. thea* extracts

The leaves of *S. thea* are small in size and may not be effectively utilized (22); therefore, the branches were evaluated and compared with the leaves alone on antioxidant capacity. Thus, the methanol extract of branches (MB), ethanol extract of branches (EB), methanol extract of leaves (ML), and ethanol extract of leaves (EL) were prepared. The extraction yields from MB were the highest (20.4%), followed by EB, EL, and ML (19.3, 15.4, and 9.7%, respectively). Phenolic compounds could be the major determinants of the antioxidant capacity of different plants (23). Therefore, the contents of total polyphenol and flavonoid were measured and expressed as mg GAE/g and mg RE/g of extracts, respectively. Among the extracts, MB was the richest extract of *S. thea* in polyphenol and flavonoid with values of 37.2 mg GAE/g and 7.8 mg RE/g, respectively (Table 1). The ABTS, alkyl, and DPPH assays were accessed for comparison with the free radical scavenging activities of *S. thea* extracts. As shown in Table 2, the MB showed IC₅₀ 55.4 μ g/mL of ABTS, IC₅₀ 37.0 μ g/mL of alkyl, and IC₅₀ 8.4 μ g/mL of DPPH radical scavenging activity, respectively. The contents of total polyphenol, flavonoid, and free radical scavenging activities were higher in the MB than in the EB. It was also observed that the branch extracts have higher extraction yields and antioxidant effects than leaves. Thus, the MB was selected to investigate antioxidant activities and cell cytotoxicity.

Antioxidant activities of the stepwise partitioned solvent fractions of the MB

The MB was sequentially fractionated on the basis of solvent polarities such as *n*-hexane, chloroform, ethyl acetate, *n*-butanol, and water. As shown in Table 1 and 2, the contents of total polyphenol and flavonoid and the free radical scavenging activities

Table 1. The extraction yield, total polyphenol content, and total flavonoid content of different extracts from the *S. thea*¹⁾

Extract	EL ²⁾	EB	ML	MB	HF	CF	EF	BF	WF
Extraction yield (%)	15.44	19.33	9.69	20.43	1.75	3.83	7.74	14.56	55.74
Total polyphenol (mg GAE/g) ³⁾	23.9±2.0	28.3±4.3	26.4±0.8	37.2±4.5	9.3±6.4	11.9±3.1	57.9±6.4	40.8±6.1	35.8±7.6
Total flavonoid (mg RE/g)	6.1±0.5	6.9±0.5	6.1±0.8	7.8±0.5	ND ⁴⁾	4.5±0.4	9.8±0.6	9.8±0.5	7.6±1.0

¹⁾Values are mean±SD (n=3).
²⁾EL, ethanol extract of leaves alone; EB, ethanol extract of branches; ML, methanol extract of leaves alone; MB, methanol extract of branches; HF, *n*-hexane fraction of MB; CF, chloroform fraction of MB; EF, ethyl acetate fraction of MB; BF, *n*-butanol fraction of MB; WF, water fraction of MB
³⁾GAE, gallic acid equivalents; RE, rutin equivalents
⁴⁾ND, Not detected

Table 2. Antioxidant activities (IC₅₀ µg/mL) of the methanol extract and stepwise partitioned solvent fractions from the *S. thea* branches¹⁾

Extract	EL ²⁾	EB	ML	MB	HF	CF	EF	BF	WF
ABTS assay	84.4±6.6	64.2±2.1	78.1±10.5	55.4±5.1	>100	>100	35.0±3.8	46.4±2.1	59.6±4.6
Alkyl assay	52.5±7.2	52.6±7.6	52.1±11.3	37.0±6.7	>50	>50	17.8±4.7	14.6±4.6	43.0±2.3
DPPH assay	18.2±1.5	14.0±0.9	15.3±1.0	8.4±0.9	>10	>10	4.8±1.2	7.0±0.7	8.1±0.2

¹⁾The half maximal inhibitory concentration on radical scavenging activity. Values are mean±SD (n=3).
²⁾EL, ethanol extract of leaves alone; EB, ethanol extract of branches; ML, methanol extract of leaves alone; MB, methanol extract of branches; HF, *n*-hexane fraction of MB; CF, chloroform fraction of MB; EF, ethyl acetate fraction of MB; BF, *n*-butanol fraction of MB; WF, water fraction of MB

Fig. 1. Effects of the methanol extract of branches and stepwise partitioned solvent fractions from *S. thea* branches on the viability of several different cancer cells. MTT assay was performed to evaluate the cytotoxicity of *S. thea* on different cancer cells. The cells were treated for 24 h with indicated concentrations of methanol extracts and its fractions. (A) the methanol extract of branches (MB); (B) the *n*-hexane fraction of MB; (C) the chloroform fraction of MB; (D) the ethyl acetate fraction of MB; (E) the *n*-butanol fraction of MB; (F) the water fraction of MB. Each value is expressed as mean±SD (n=3). The asterisk indicates a significant difference in comparing different cell lines (*p<0.01). Cell lines: ●, AGS; ○, A549; ▼, MDA-MB-231; △, SNU-1; ■, SNU-16.

Fig. 2. Induction of apoptosis by the methanol extract of branches and stepwise partitioned solvent fractions on MDA-MB-231 cells. (A) Cells were treated with 100 µg/mL concentration of methanol extract and its fractions for 24 h, stained with Hoechst 33342, and observed under the fluorescence microscope. (B) Graph for quantification of the number of apoptotic bodies. (C) Cells were treated with 50 µg/mL for 24 h. The treated cells were harvested and stained with annexin V and PI according to the instructions of the company that supplied the kit. The methanol extract of branches (MB); *n*-hexane fraction of MB (HF); chloroform fraction of MB (CF); ethyl acetate fraction of MB (EF); *n*-butanol fraction of MB (BF); water fraction of MB (WF). All data are the mean±SD (*n*=3). The asterisk indicates a significant difference in comparing different cell lines (**p*<0.01).

were accessed with the MB and stepwise partitioned solvent fractions. From Table 1, it can be observed that total polyphenol and flavonoid contents were the highest in the ethyl acetate fraction (EF) as 57.9 mg GAE/g and 9.8 mg RE/g respectively, and the *n*-butanol fraction (BF) had the next highest content (40.8 mg GAE/g and 9.8 mg RE/g, respectively) followed by MB, water fraction (WF), chloroform fraction (CF), and *n*-hexane fraction (HF). A similar tendency was observed in radical scavenging activities and total polyphenol and flavonoid contents, the EF and BF presenting the best activities in ABTS (IC_{50} 35.0 and 46.4 µg/mL, respectively), alkyl (IC_{50} 17.8 and 14.6 µg/mL, respectively), and DPPH radical scavenging (IC_{50} 4.8 and 7.0 µg/mL, respectively; Table 2). These results indicate that the EF and BF constituents may contribute to free radical scavenging and effectively prevent cancer formation or progression. Previous studies have shown that fractions with antioxidant properties helped in preventing cancer (24,25).

The MB and stepwise partitioned solvent fractions induce apoptosis in MDA-MB-231 cells The cytotoxicity effect of the MB and stepwise partitioned solvent fractions on different human cancer cell lines (AGS, A549, MDA-MB-231, SNU-1, and SNU-16 cells) were evaluated by MTT assays (Fig. 1). The MB and stepwise partitioned solvent fractions induced significant increase in cytotoxicity of the MDA-MB-231 cells in a concentration-dependent manner. The cell viability against MDA-MB-231 cells were observed to be 42.7% of MB, 52.1% of EF, 49.0% of BF, and 46.8% of WF, whereas 78.4% of HF and 72.9% of CF at a concentration of 100 µg/mL after a 24 h incubation. Interestingly, the fractions with higher cytotoxicity, such as MB, EF, BF, and WF, exhibited the highest antioxidant activities among the fractions. Apoptotic cell death is characterized by distinct features such as cell shrinkage, blebbing, nuclear condensation, and fragmentation (26). To evaluate the effect of these fractions on apoptotic cell death, MDA-MB-231 cells were treated with the

Table 3. Compounds identified in the methanol extract of *S. thea* branches and the stepwise partitioned solvent fractions using GC–MS

No.	RT ¹⁾ (min)	Compounds ²⁾	Area (%) ³⁾					
			MB ⁴⁾	HF	CF	EF	BF	WF
1	10.489	2-Hydroxy-3-methyl-4-pyrone	1.43	ND ⁵⁾	ND	ND	ND	ND
2	11.377	3,3,5,5-Tetramethyl-1,2-cyclopentanedione	5.73	ND	ND	ND	ND	ND
3	12.362	3,5-Dihydroxy-6-methyl-2,3-dihydro-4H-pyran-4-one	4.29	ND	ND	ND	ND	ND
4	13.584	Isonicotinic acid-β-D2	1.83	ND	ND	ND	ND	ND
5	17.183	Dodecanol	ND	ND	2.31	ND	ND	7.77
6	20.858	Acetoveratrone	ND	ND	ND	ND	ND	1.78
7	24.010	2,5-Dimethoxy-4-methylbenzaldehyde	1.81	ND	ND	ND	ND	ND
8	25.385	Tetradecyl acrylate	ND	3.07	13.49	1.82	4.52	18.95
9	30.062	Neophytadiene	1.24	0.24	ND	ND	ND	ND
10	30.790	6,10,14-Trimethyl-2-pentadecanone	ND	1.59	0.26	ND	ND	ND
11	32.676	Palmitic acid	4.16	ND	ND	ND	ND	ND
12	33.430	Methyl palmitoleate	ND	ND	1.09	ND	ND	ND
13	33.561	Methyl palmitate	ND	2.88	8.59	1.14	3.58	1.83
14	34.854	Pentadecanoic acid	ND	ND	0.91	10.98	23.43	5.81
15	36.473	Dodecyl 2-nitrophenyl ether	ND	1.11	7.91	3.02	11.12	20.89
16	38.046	Margaric acid	ND	ND	ND	0.33	1.27	ND
17	38.468	Galactopyranosid	2.56	ND	ND	ND	ND	ND
18	38.575	Isobutyl caprylate	1.14	ND	ND	ND	ND	ND
19	39.039	Methyl linoleate	3.48	2.15	2.19	ND	ND	ND
20	39.219	Methyl oleate	ND	ND	ND	0.96	3.55	ND
21	39.290	Ethyl linoleate	0.70	5.50	13.32	0.16	ND	ND
22	39.701	Phytol	5.84	15.23	1.47	ND	ND	ND
23	40.021	Methyl stearate	ND	1.08	3.38	0.31	ND	0.78
24	40.491	Olealdehyde	ND	ND	ND	2.06	ND	ND
25	40.523	9,12,15-octadecatrien-1-ol	ND	ND	ND	ND	10.19	ND
26	41.173	Stearic acid	ND	ND	ND	1.78	6.35	1.48
27	42.436	2-Palmitoylglycerol	1.61	ND	ND	ND	ND	ND
28	46.797	6-Hexyltetrahydro-2H-pyran-2-one	ND	3.28	ND	ND	ND	ND
29	52.066	2,6-dimethyl-N-(2-methyl-α-phenylbenzyl)aniline	ND	1.93	ND	ND	ND	ND
30	52.070	3-Nitrophthalic acid	ND	ND	ND	1.85	ND	ND
31	52.429	1-Propene, 3-(2-cyclopentenyl)-2-methyl-1,1-diphenyl-	ND	1.71	ND	ND	ND	ND
32	52.577	Clionasterol	3.18	ND	ND	ND	ND	0.56
33	52.673	Dimethyl 3,3-thiodipropionate	ND	ND	1.02	ND	ND	3.52
34	57.018	Friedelin	ND	ND	22.63	ND	ND	ND
35	58.139	Oleamide	1.24	1.08	1.96	0.62	3.94	6.96
36	59.004	1,1,6,6-Tetramethylspiro[4.4]nonan-2-one	ND	ND	ND	ND	1.04	ND
37	59.101	Lycopersene	ND	2.46	ND	ND	ND	ND
38	59.732	δ-Tocopherol	ND	3.91	ND	ND	ND	ND
39	60.943	1,2,3,5-Tetraisopropylcyclohexane	ND	1.01	ND	ND	ND	ND
40	61.223	Solanesol	ND	1.02	ND	ND	ND	ND
41	61.285	Octadecyl 3,5-di-t-butyl-4-hydroxyhydrocinnamate	ND	ND	6.63	0.44	ND	0.52
42	61.560	Dilauryl thiodipropionate	35.40	ND	ND	1.68	16.60	25.50
43	61.660	Icosamethylcyclodecasiloxane	ND	ND	ND	3.64	2.51	ND
44	61.875	1-Hydroxymethyl-4,5,7-tir-T-butyl-1,4-epo	ND	ND	3.38	ND	ND	ND
45	63.541	2-Benzyloxy-2-methylhept-3-ene	ND	1.00	ND	ND	ND	ND
46	64.280	(24R,25S)-Aplysterylacetate	ND	ND	ND	2.17	ND	ND
47	64.818	α-Tocopherol-β-D-mannoside	14.64	13.69	1.12	ND	0.99	ND
48	65.575	Ethyltrimethyllead	ND	ND	ND	ND	1.59	ND
49	65.925	Silicone grease	ND	ND	0.27	ND	1.07	ND
50	65.985	1,1,3,3,5,5-Hexamethyl-cyclohexasiloxane	ND	ND	ND	ND	1.37	ND
51	66.040	Chlorthiophos oxygen analog	ND	ND	ND	ND	1.60	ND
52	66.140	(5Z,7Z)-1,2,3,4,6a,6b,9,10,11,12,12a,12b-Dodecahydrocycloocta[3,4]cyclobuta[1,2][8]annulene	ND	ND	ND	ND	1.44	ND
53	67.906	β-sitosterol	ND	9.52	2.54	59.49	3.82	ND

Table 3. Continued

No.	RT ¹⁾ (min)	Compounds ²⁾	Area (%) ³⁾					
			MB ⁴⁾	HF	CF	EF	BF	WF
54	68.688	Norolean-12-ene	ND	2.27	ND	ND	ND	ND
55	69.080	N-[3-(2-methoxycarbonylphenoxy)propyl]3,4-dihydroxyphenylacetamide	ND	ND	ND	7.32	ND	ND
56	69.185	Hop-22(29)-ene	ND	1.01	ND	ND	ND	ND
57	69.272	2,2-Dibenzylethanol	ND	ND	1.10	ND	ND	ND
58	69.572	Lupeol acetate	1.28	7.66	ND	ND	ND	ND
Total			91.56	84.40	95.57	99.77	99.98	96.35

¹⁾Retention time²⁾Compounds tentatively identified on the basis of parent molecular ions, retention times, retention indices, and elution order, as well as the fragmented spectra compared with the literature.³⁾Peak area relative to the total peak area %.⁴⁾MB, methanol extract of branches; HF, *n*-hexane fraction of MB; CF, chloroform fraction of MB; EF, ethyl acetate fraction of MB; BF, *n*-butanol fraction of MB; WF, water fraction of MB⁵⁾ND, Not detected

fractions, stained with Hoechst 33342, and then observed under fluorescence microscopy. After treatment with the MB and its fractions at 100 µg/mL concentration for 24 h, a significant increase in apoptotic cells was observed in the MB (15.6%), EF (12.3%), BF (19.4%), and WF (9.0%), whereas HF and CF showed few apoptotic cells (Fig. 2A and 2B).

Furthermore, flow cytometric analysis through annexin V/PI double staining showed that MB and its fractions increased the proportion of annexin V-positive cells (Fig. 2C). The percentage of total apoptotic cells increase from 4.5% of control to 52.2% of BF, 51.7% of MB, 44.5% of EF, 39.8% of HF, 37.5% of WF, and 9.2% of CF. These results suggest that the MB and its fractions inhibit MDA-MB-231 cell proliferation by apoptosis and that *S. thea* may serve as therapeutic resources for treating the human breast cancer.

GC–MS analysis of the MB and stepwise partitioned solvent fractions The MB and stepwise partitioned solvent fractions were analyzed using GC–MS. The GC–MS analysis revealed total 18 compounds in MB, 23 compounds in HF, 20 compounds in CF, 18 compounds in EF, 19 compounds in BF, and 13 compounds in WF, respectively (Table 3). The main constituent of the MB, HF, CF, EF, BF, and WF was identified as dilauryl thiodipropionate (DLTDP), phytol, friedelin, β-sitosterol, pentadecanoic acid, and DLTDP, respectively. Interestingly, MB, BF, and WF contained DLTDP, which is known to possess antioxidant activities (35.4, 16.6, and 25.5%, respectively) (27).

Moreover, pentadecanoic acid, which has been reported to have antioxidant activity (28) and anticarcinogenic effects on cancer cells (29), was detected in the EF (11.0%), BF (23.4%), and WF (5.81%). The cytotoxicity of EF, BF, WF could be due to pentadecanoic acid, whereas the cytotoxicity of HF on different cancer cells may be attributed to phytol, which has been reported to induce apoptosis in different human cancer cell lines (30). EF had relatively less DLTDP (1.7%), but β-sitosterol was identified as the most abundant compound (59.5%), which has been known to have antioxidant activity (31). Thus, the antioxidant activity of *S. thea* branches could be derived from DLTDP, pentadecanoic acid, and β-sitosterol as well as other polyphenols.

Pentadecanoic acid induces apoptosis in MDA-MB-231 cells The effect of different concentrations of pentadecanoic acid on the growth of MDA-MB-231 cells was examined using an MTT assay. As shown Fig. 3A, pentadecanoic acid reduced the viability of MDA-MB-231 cells in a concentration-dependent manner. To determine whether cell cytotoxicity of pentadecanoic acid was attributable to apoptosis, the morphology of MDA-MB-231 cells treated with different concentrations of pentadecanoic acid was examined by Hoechst 33342 staining. As shown in Fig. 3B, after treatment with pentadecanoic acid, MDA-MB-231 cells showed condensed chromatin and apoptotic bodies (arrows) from 1.1% in control cells to 31.3% in the cells treated with 500 µM pentadecanoic acid (Fig. 3C). These

Table 4. Pearson's correlation coefficients of total polyphenol and flavonoid content, antioxidant activities, and cytotoxicity in *S. thea* branches

	TPC ¹⁾	TFC	ABTS	Alkyl	DPPH	Cytotoxicity
TPC	1					
TFC	0.900** ²⁾	1				
ABTS	0.981**	0.950**	1			
Alkyl	0.941**	0.942**	0.985**	1		
DPPH	0.981**	0.957**	0.994**	0.964**	1	
Cytotoxicity	0.812*	0.869*	0.890*	0.891*	0.880*	1

¹⁾TPC, total polyphenol content; TFC, total flavonoid content²⁾Significant at **p*<0.05 and ***p*<0.01.

Fig. 3. Effect of pentadecanoic acid on cell viability and morphological changes in MDA-MB-231 cells. (A) MTT assay was performed to evaluate the cytotoxicity of pentadecanoic acid on MDA-MB-231 cells for 24 h. (B) Cells were treated with pentadecanoic acid at different concentrations for 24 h, stained with Hoechst 33342, and observed under the fluorescence microscope. Arrows indicate cells with condensed and fragmented chromatin suggesting the apoptotic bodies. (C) Graph for quantification of the number of apoptotic bodies. All data are the mean $\pm$ SD ($n=3$). The asterisk indicates a significant difference in comparing control (* $p<0.01$).

results suggest that pentadecanoic acid induces apoptosis in MDA-MB-231 cells and contributes to the cytotoxicity of *S. thea*.

Correlations between total polyphenol and flavonoid content with antioxidant activities or cytotoxicity Previous studies have reported on a correlation of phenolic content with antioxidant activities (24,32). As shown in Table 4, there were significantly high correlations between polyphenol content and antioxidant activities, such as ABTS

($R^2=0.981$), alkyl ($R^2=0.941$), and DPPH ($R^2=0.981$) assays. Similarly, the correlations between flavonoid content and ABTS, alkyl, and DPPH assays were substantially positive with correlation coefficients of 0.950, 0.942, and 0.957, respectively. Moreover, the cytotoxicity of MDA-MB-231 cells highly correlated to polyphenol ($R^2=0.812$) and flavonoid contents ($R^2=0.869$). Results indicated that polyphenol and flavonoid contents of *S. thea* correlated with antioxidant activities or cytotoxicity in human breast cancer cells.

In conclusion, the branches of *S. thea* showed considerable antioxidant activities and cytotoxicity in human breast cancer cells. Therefore, they can be a natural source of antioxidants and a therapeutic resources for human breast cancer. The correlation of total polyphenol and flavonoid contents with antioxidant activities or cytotoxicity significantly ($p<0.05$) exists in each solvent fraction of the branches of *S. thea*.

Acknowledgments This research was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Science, ICT & Future Planning (2016R1A2B4016005).

Disclosure The authors declare no conflict of interest.

References

1. Chung SK, Kim YC, Takaya Y, Terashima K, Niwa M. Novel flavonol glycoside, 7-O-methyl meansitrin, from *Sageretia theezans* and its antioxidant effect. *J. Agr. Food Chem.* 52: 4664-4668 (2004)
2. Chung SK, Chen CY, Blumberg JB. Flavonoid-rich fraction from *Sageretia theezans* leaves scavenges reactive oxygen radical species and increases the resistance of low-density lipoprotein to oxidation. *J. Med. Food* 12: 1310-1315 (2009)
3. Park JC, Hur JM, Park JG, Hatano T, Yoshida T, Miyashiro H, Min BS, Hattori M. Inhibitory effects of Korean medicinal plants and camelliatannin H from *Camellia japonica* on human immunodeficiency virus type 1 protease. *Phytother. Res.* 16: 422-426 (2002)
4. Li F, Li S, Li HB, Deng GF, Ling WH, Wu S, Xu XR, Chen F. Antiproliferative activity of peels, pulps and seeds of 61 fruits. *J. Funct. Foods* 5: 1298-1309 (2013)
5. Ziech D, Franco R, Georgakikas AG, Georgakila S, Malamou-Mitsi V, Schoneveld O, Pappa A, Panayiotidis MI. The role of reactive oxygen species and oxidative stress in environmental carcinogenesis and biomarker development. *Chem-Biol. Interact.* 188: 334-339 (2010)
6. Garis D, Skiada V, Barbouti A. Redox signaling and cancer: the role of "labile" iron. *Cancer Lett.* 266: 21-29 (2008)
7. Jambunathan S, Bangarusamy D, Padma PR, Sundaravadivelu S. Cytotoxic activity of the methanolic extract of leaves and rhizomes of *Curcuma amada* Roxb against breast cancer cell lines. *Asian Pac. J. Trop. Med.* 7: S405-S409 (2014)
8. Fuchs-Tarlovsky V. Role of antioxidants in cancer therapy. *Nutrition* 29: 15-21 (2013)
9. Kim HY, Lee SH, Hwang IG, Woo KS, Kim KJ, Lee MJ, Kim DJ, Kim TJ, Lee J, Jeong HS. Antioxidant and antiproliferation activities of winter cereal crops before and after germination. *Food Sci. Biotechnol.* 22: 181-186 (2013)
10. Gomaa EZ. *In vitro* antioxidant, antimicrobial, and antitumor activities of bitter almond and sweet apricot (*Prunus armeniaca* L.) kernels. *Food Sci. Biotechnol.* 22: 455-463 (2013)
11. Hwang YJ, Lee EJ, Kim HR, Hwang KA. *In vitro* antioxidant and anticancer effects of solvent fractions from *Prunella vulgaris* var. *lilacina*. *BMC Complement. Altern. Med.* 13: 310 (2013)
12. Chua MT, Tung YT, Chang ST. Antioxidant activities of ethanolic extracts from the twigs of *Cinnamomum osmophloeum*. *Bioresource Technol.* 99: 1918-1925 (2008)
13. Tauhehn J, Dosekocil I, Caffi C, Lulekal E, Marsik P, Havlik J, Damme PV,

- Kokoska L. *In vitro* antioxidant and anti-proliferative activity of Ethiopian medicinal plant extracts. *Ind. Crop. Prod.* 74: 671-679 (2015)
14. Boots AW, Haenen GR, Bast A. Health effects of quercetin: From antioxidant to nutraceutical. *Eur. J. Pharmacol.* 585: 325-337 (2008)
 15. Radomska-Leoniewska DM, Hevelke A, Skopiński P, Bała B, Józwiak J, Rokicki D, Skopińska-Różewska E, Białoszewska A. Reactive oxygen species and synthetic antioxidants as angiogenesis modulator: Clinical implications. *Pharmacol. Rep.* 68: 462-471 (2016)
 16. Cheung LM, Cheung PCK, Ooi VEC. Antioxidant activity and total phenolics of edible mushroom extracts. *Food Chem.* 81: 249-255 (2003)
 17. Zhishen J, Mengcheng T, Jianming W. The determination of flavonoid contents in mulberry and their scavenging effects on superoxide radicals. *Food Chem.* 64: 555-559 (1999)
 18. Gíão MS, González-Sanjósé ML, Rivero-Pérez MD, Pereira CI, Pintado ME, Malcata FX. Infusions of Portuguese medicinal plants: Dependence of final antioxidant capacity and phenol content on extraction features. *J. Sci. Food Agr.* 87: 2638-2647 (2007)
 19. Hassimotto NM, Genovese MI, Lajolo FM. Antioxidant activity of dietary fruits, vegetables, and commercial frozen fruit pulps. *J. Agr. Food Chem.* 53: 2928-2935 (2005)
 20. Nanjo F, Goto K, Seto R, Suzuki M, Sakai M, Hara Y. Scavenging effects of tea catechins and their derivatives on 1,1-diphenyl-2-picrylhydrazyl radical. *Free Radical Bio. Med.* 21: 895-902 (1996)
 21. Carmichael J, DeGraff WG, Gazdar AF, Minna JD, Mitchell JB. Evaluation of a tetrazolium-based semiautomated colorimetric assay: Assessment of chemosensitivity testing. *Cancer Res.* 47: 936-942 (1987)
 22. Chen Y, Chou P. *Flora of China*. Vol. 12. pp. 137. In: Rhamnaceae. Chen Y, Schirarend C (eds). MBG Press, St. Louis, MO, USA (2007)
 23. Robards K, Prenzler PD, Tucker G, Swatsitang P, Glover W. Phenolic compounds and their role in oxidative processes in fruits. *Food Chem.* 66: 401-436 (1999)
 24. Cha MN, Jun HI, Lee WJ, Kim MJ, Kim MK, Kim YS. Chemical composition and antioxidant activity of Korean cactus (*Opuntia humifusa*) fruit. *Food Sci. Biotechnol.* 22: 523-529 (2013)
 25. Bai Y, Li D, Zhou T, Qin N, Li Z, Yu Z, Hua H. Coumarins from the roots of *Angelica dahurica* with antioxidant and antiproliferative activities. *J. Funct. Foods* 20: 453-462 (2016)
 26. Anh TT, Krishnamoorthy K, Song YW, Cho SK, Kim SJ. Toxicity of nano molybdenum trioxide toward invasive breast cancer cells. *ACS Appl. Mater. Inter.* 6: 2980-2986 (2013)
 27. Karahadian C, Lindsay RC. Evaluation of the mechanism of dilauryl thiodipropionate antioxidant activity. *J. Am. Oil Chem. Soc.* 65: 1159-1165 (1988)
 28. Henry GE, Momin RA, Nair MG, Dewitt DL. Antioxidant and cyclooxygenase activities of fatty acids found in food. *J. Agr. Food Chem.* 50: 2231-2234 (2002)
 29. Jenkins B, West JA, Koulman A. A review of odd-chain fatty acid metabolism and the role of pentadecanoic acid (C15:0) and heptadecanoic acid (C17:0) in health and disease. *Molecules* 20: 2425-2444 (2015)
 30. Pejčin B, Kojic V, Bogdanovic G. An insight into the cytotoxic activity of phytol at *in vitro* conditions. *Nat. Prod. Res.* 28: 2053-2056 (2014)
 31. Moreno JJ. Effect of olive oil minor components on oxidative stress and arachidonic acid mobilization and metabolism by macrophages RAW 264.7. *Free Radical Bio. Med.* 35: 1073-1081 (2003)
 32. Eberhardt MV, Lee CY, Liu RH. Nutrition: Antioxidant activity of fresh apples. *Nature* 405: 903-904 (2000)