INTRODUCTION
Breast cancer is the most common malignancy in women throughout the world, accounting for 18% of all female cancers, and there are approximately 600,000 annual deaths worldwide (Kumar et al., 2013). Approximately 76% of all breast tumors have been categorized as invasive breast cancers (Sathya et al., 2010). Surgery, radiotherapy and chemotherapy remain the primary option for the treatment of BC. The chemotherapy of BC leads to an emerging drug resistance and tumor relapse, and post-treatment toxicity limits their use in the clinic; these hurdles facilitate BC as the number one killer in women (Sathya et al., 2010; Crown et al., 2012). Due to high mortality and the associated side effects of chemotherapy and/or radiotherapy, cancer patients often seek alternative forms of therapies such as natural or herbal medicines (Sadagopan et al., 2015; Zheng et al., 2016). This has led to an increased interest and active search for novel anticancer agents from natural sources. Historically, many potent anticancer agents such as vincristine, vinblastine, paclitaxel, etoposide, camptothecin, topotecan, and doxorubicin were derived from plants (Bhanot et al., 2011; Yao et al., 2016). Vinblastine, vincristine, vinorelbine, vindesine, Taxol, etoposide, topotecan and irinotecan are well used in clinical practice as combinational therapy for many malignancies (Mann, 2002; Newman and Cragg, 2012).

Apoptosis is a form of cell death, a goal of cancer treatment, and is characterized by cell shrinkage, plasma membrane blebbing, and chromatin condensation, which are associated with cleavage of DNA into ladders (Matsuura et al., 2016). However, in response to some effective therapeutic treatments, a decreased ability to undergo apoptosis occurred in human malignant tumor cells (Dabrowska et al., 2016). Therefore, the further development of agents that can induce or enhance the extent of apoptosis seems to be a promising strategy in the treatment of cancer. On the other hand, many agents target the cell cycle and its signaling proteins to inhibit the proliferation of cancer cells (El-Naa et al., 2016; Zhu et al.,...
in 1 ml of ice-cold 70% ethanol overnight at −80°C. Fixed cells
were harvested and spun down for 5 min at 2,000 rpm.

Cytotoxicity assay
This study was aimed to determine the cytotoxic effect of PA on MCF-7 human breast cancer cells in vitro. In addition, the mechanism of action of PA related to its cytotoxicity was elucidated through the detection of cell cycle-related proteins and the apoptotic pathway.

MATERIALS AND METHODS

Cell lines and cell culture
MCF-7, MCF-10A and T47D breast cancer cells were obtained from the American Type of Cell culture (ATCC, VA, USA), and the cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) and RPMI medium, respectively, supplemented with 10% heat-inactivated fetal bovine serum (FBS) and penicillin/streptomycin (100 U/mL) and 3.7 mg/mL of NaHCO₃ at 37°C in 5% CO₂.

Cytotoxicity assay
The cytotoxicity of PA was procured from Hangzhou Dayangchem Co. Ltd (Zhejiang, China) and was analyzed by the MTT assay. Briefly, 1×10⁴ cells/well were seeded in a 96-well plate and were incubated at 37°C in 5% CO₂. After 24 h, the cells were treated with different concentrations (2.5, 5, 7.5, 10, 15, 20 and 30 µM) of PA. PA was dissolved in 0.01% DMSO, and it was used as the vehicle control. The cells were incubated for 24 and 48 h at 37°C. Subsequently, 50 µl/well of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, 2 mg/ml) was added and incubated for 2 h. After the incubation, the media was discarded, and 100 µl of DMSO was replaced into each well to dissolve the formazan crystal. The colorimetric assay was quantified at a wavelength of 570 nm using a Chameleon V microplate reader (Hidex, Turku, Finland). The anti-proliferation activity of PA was expressed as the absorbance at 570 nm and was calculated.

Cell cycle analysis
The effect of PA on cell cycle analysis was performed using propidium iodide (PI). Briefly, cells were seeded in a 25-cm² flask, and then 60% confluent cells were treated with PA (10, 20 and 20 µM) or without PA for 24 h. Following incubation, the cells were harvested and spun down for 5 min at 2,000 rpm. The supernatant was removed, and the cells were then fixed in 1 ml of ice-cold 70% ethanol overnight at −80°C. Fixed cells were washed with 1 ml of 1X PBS and were stained in 500 µl of PI containing 5 µg/ml DNase-free RNase for 30 min at room temperature in total darkness. The DNA content of the cells was analyzed by flow cytometry. The percentages of G0-G1, S and G2-M cells were then calculated using fluorescence-activated cell sorting (FACS) software (BD Biosciences, CA, USA).

Analysis of apoptosis by annexin V-FITC
Briefly, MCF-7 cells were plated in chamber slides at the concentration of 1×10⁵ and were allowed to attach overnight. Next, the cells were treated with PA at three concentrations for 24 h. After the incubation, the slides were washed with PBS and then were incubated with ice-cold methanol-acetone (1:1) at −20°C for 10 min. After air drying, the cells were washed two times with cold PBS and then were incubated with Annexin V-FITC conjugate (Santa Cruz Biotechnology Inc., TX, USA) for 30 min in the dark. After washing with PBS, the cells were incubated with 4',6-diamidino-2-phenylindole (DAPI; 1:5000 v/v; Invitrogen, Carlsbad, CA, USA) to stain the nucleus for 10 min. After incubation, the cells were washed with PBS, and the slides were mounted, coverslipped and visualized under a FSX100 fluorescence microscope (Olympus, Tokyo, Japan). Similarly, a portion of the cells will be analyzed for annexin V-FITC expression using a flow cytometer (BD FACS Count™ Flow Cytometer, CA, USA).

Isolation of mitochondria for cytochrome c determination
To study the cytochrome c protein level of mitochondria, the instructions issued by Mitochondria Isolation Kit for Cultured Cells were followed (Thermo Scientific, Rockford, IL, USA). The protein content was measured from the supernatants that contain the mitochondrial fraction. All the wells were loaded with 25 µg of protein.

Western blot analysis
Briefly, 1×10⁶ cells/flask were seeded and treated with PA in a dose-dependent manner for 24 h. Later, the cells were collected and lysed in RIPA buffer supplemented with a 10-µl protease inhibitor cocktail, sodium orthovanadate, and PMSF (Santa Cruz, USA). The lysate was stored at −80°C until further use. A 40-µg protein sample was resolved on 10% SDS-PAGE and was transferred to an Immun®-Blot polyvinylidene difluoride (PVDF) membrane (BIO-RAD, CA, USA). The membrane was blocked in 5% BSA for 1 h at room temperature following an overnight incubation at 4°C with the following primary antibodies: anti-Cyclin D1, anti-CDK4, anti-cytochrome C, anti-Bax, anti-Bcl-2, anti-p21, anti-p27 and mouse anti-β-actin (Cell Signaling Technology, USA) antibodies. Membranes were washed with 1× TBS-T prior to incubating with HRP-conjugated goat anti-mouse or anti-rabbit secondary antibodies for 2 h at room temperature. The membranes were washed 3
times with 1× TBS-T to remove excess antibodies before the protein-antibody complex was detected with Amersham ECL prime western blotting detection reagent (GE Healthcare, IL, USA).

**Statistical analysis**

Statistical analysis was processed according to conventional procedures using the Statistical Program for Social Sciences (SPSS) software for Windows (NY, USA), Version 20.0 (Post hoc, Turkey’s test). A *p*-value <0.05 was considered to be statistically significant.

**RESULTS**

**PA inhibits the cell proliferation of MCF-7 and T47D breast cancer cells**

The viability of MCF-7 and T47D breast cancer cells treated with various concentrations of PA was measured using the MTT assay. Additionally, to check whether PA has any adverse effects on non-tumorigenic cells, we tested using MCF-10A breast cells. As shown in Fig. 1, PA could cause cytotoxicity towards the cells in a dose-dependent manner at 24 and 48 h. The data suggest concentration-dependent inhibition in MCF-7 cells with an IC_{50} value of 15 µM at 24 h and 11.5 µM at the 48 h time point. In the case of T47D cells, the IC_{50} was 17.5 µM at 24 h and 14.5 µM at 48 h. Notably, PA treatment had no effect on the proliferation of the non-tumorigenic MCF-10A cells.

**PA induces cell cycle arrest in the G0/G1 phase**

Flow cytometric analysis of the cell cycle distribution of MCF-7 breast cancer cells treated with PA for 24 h was performed. Fig. 2 shows the relative percentages of MCF-7 cells in each phase of the cell cycle following treatment. We noticed an increase in the mean percentage of cells in the G0/G1 phase of the cell cycle from 53.4 ± 2.3% (control) to 67.2 ± 3.5% (20 µM). G0/G1 phase cell cycle arrest was accompanied by a decrease in the percentage of cells in the S and G2/M phases of the cell cycle. The FACS results showed that PA inhibited the proliferation of MCF-7 cells during the G0/G1 phase.

**PA deregulates the expression of cell cycle-related proteins**

To understand the mechanism by which PA induces cell cycle arrest in MCF-7 cells, the expression of cell cycle-related proteins were examined using western blotting (Fig. 3A). We observed a dose-dependent increase in the expression of cell cycle-inhibitory proteins (p21 and p27) and decrease in cell cycle-promoting proteins (CDK4 and Cyclin D1), respectively, after 24 h of treatment. The quantification of the respective blots was represented in Fig. 3B and 3C.

**PA induces apoptosis in MCF-7 breast cancer cells**

As shown in Fig. 4A, treatment with PA resulted in an increase in the cell population that was positive for annexin V-FITC staining. Starting from 15 to 20 µM at the 24 h time point, MCF-7 cells undergo apoptosis as detected by the green fluorescence of annexin V-FITC when compared with that of the untreated control cells. Hence, we confirmed that PA induced apoptosis as evidenced by the increased phosphatidyl serine (PS) exposure. To further confirm apoptosis, we analyzed annexin V-FITC/PI expression by flow cytometry (Fig. 4B). The MCF-7 cells were treated with PA at the doses of 10, 15 and 20 µM. The control cells exhibited a lower percentage of cells in early and late apoptosis (3.1% and 9.4%, respectively), and PA dose dependently increased annexin V expression. The percentages of early and late apoptosis for 10
Incubation of MCF-7 cells with PA showed decreased expression of apoptotic Bcl-2 by western blotting (Fig. 4C-4E). We also analyzed the expression of M were 9.3% and 4.1% and 17.4%, respectively, those for 20 \( \mu M \) were 3.4 and 13.2%, respectively, those for 10 \( \mu M \) were 4.1% and 17.4%, respectively, and those for 20 \( \mu M \) were 9.3% and 32.1%, respectively. We also checked the expression of mitochondrial cytochrome c, pro-apoptotic Bax and anti-apoptotic Bcl-2 expression by western blotting (Fig. 4C-4E). Incubation of MCF-7 cells with PA showed decreased expression of Bcl-2 and increased expression of mitochondrial cytochrome c and Bax.

Caspases are the key enzymes in the cell that can be activated either by auto-catalytic processing or by another caspase (Bell and Megeney, 2017). Generally, these proteases can be classified into two groups: 'initiator' caspases, e.g., caspase-8 and -9 that have long pro-domains at the NH2-termini and can be activated by another caspase or by activated effector caspases. Once activated by apoptotic stimuli, caspases contribute to the morphological and biochemical changes of apoptosis (Dabrowska et al., 2016; Duclos et al., 2017). Initially, we checked the activity of caspase-3 in MCF-7 cells and noticed no activity of caspase 3 (data not shown). We observed a significant \( p<0.05 \) increase in the activities of caspase-7, 8 and -9 following the incremental dose of PA, demonstrating a dose-dependent manner (Fig. 5). We assumed that PA-induced apoptosis is mediated by the activation of caspases 7, 8 and 9.

**DISCUSSION**

For a therapeutic agent to be truly effective, it should be toxic to tumor cells without affecting normal cells. From the literature, it appears that natural products fulfill this criterion. Many natural products are reported to have anti-tumor effects by multiple mechanisms. We checked the cytotoxic effect of PA on MCF-7, T47D (human breast cancer) and MCF-10A (breast non-tumor) cells. The IC50 of PA in MCF-7 cells displayed an IC50 value of 15 \( \mu M \) at 24 h and 11.5 \( \mu M \) at 48 h. In the case of T47D cells, the IC50 was 17.5 \( \mu M \) at 24 h and 14.5 at 48 h. It is noteworthy that PA treatment does not affect the proliferation of MCF-10A cells. We further analyzed the effect of PA on the cell cycle arrest and apoptosis-inducing property in MCF-7 human breast cancer cells.

Cell cycle progression is orchestrated by a complex network of interactions among proteins, including cyclins, CDKs, E3 ubiquitin ligase complexes, CDK activating kinase, CDC25 phosphatases and CDK inhibitors (Diaz-Moralli et al., 2013). To explore the mechanisms by which PA induces cell cycle arrest in MCF-7 cells at the G0/G1 phase, western blot analysis was used to assess the modulation of the cell cycle regulatory proteins. The results showed that PA treatment resulted in decreased expression of cyclin D1 and CDK4 and increased expression of p21Cip1 and p27, explaining the G0/G1-phase arrest observed. Cyclin D1 and CDK4 are specific G0/G1-phase regulatory proteins (Diaz-Moralli et al., 2013). Previously, Yun et al. (2006) reported that PA isolated from Kaempferia pandurata induces cell cycle arrest in androgen-independent PC3
and DU145 human prostate cancer cells. Additionally, it (i) induces p21WAF1/Cip1 and p27Kip1, (ii) downregulates cdk2, 4 and 6 and (iii) decreases cyclins D1 and E (Yun et al., 2006). Thus, we confirmed that PA-induced G0/G1 phase arrest was mediated through the modulation of p21 and p27.

Inter-nucleosomal DNA fragmentation is one of the hallmarks of apoptosis. Because the low-molecular-weight DNA fragments are extracted during cell staining in aqueous solutions, apoptotic cells can be identified on DNA content frequency histograms as cells with fractional ("sub-G1") DNA content (Kajstura et al., 2007). We analyzed the population of cells of the sub-G1 phase in untreated and dose-dependent PA-treated MCF-7 cells. We found that the sub-G1 content in the untreated control population was 1.17 ± 0.11 and those of PA-treated (10, 15 and 20 µM) cells were 1.84 ± 0.18, 2.62 ± 0.21 and 4.52 ± 0.28, respectively. We assumed that the increase in PA treatment leads to the DNA fragmentation in MCF-7 cells that was confirmed as the cell population in the sub-G1 phase.

Activation of the intrinsic apoptotic pathway is regulated by the Bcl-2 family of proteins. The Bcl-2 family proteins play vital roles in the regulation of cell death mechanisms (Adams and Cory, 1998; Cory et al., 2003). Anti-apoptotic Bcl-2 family members (Bcl-2, Bcl-xL) can block these mitochondrial events, whereas pro-apoptotic Bcl-2 family members (Bax, Bak, Bad) can trigger these changes. It is well known that caspases play central roles in the terminal execution of apoptosis induced by various stimulations (Salvesen and Riedl, 2008). To understand the induction mechanism of apoptosis by PA, we examined the expression levels of Bcl-2 and Bax by immunoblot analysis. The treatment of MCF-7 cells with PA resulted in a marked decrease in Bcl-2 protein levels in a dose-dependent manner (Fig. 4C). By contrast, the mitochondrial cytochrome c and Bax protein expression levels were increased compared with that in untreated control cells.

Components of the apoptosis signaling cascade, including caspases (Boatright and Salvesen, 2003; Philchenkov et al., 2004) and triggers and regulators such as Fas ligand 45 and Bcl-2 family members, are among the most promising targets for pharmacological modulation of cell death (Bouillet and
Strasser, S., D’Halleux, E. M., Leemans, S. M., Lambech, D. and Struys, M. (2002) Apoptosis and cancer: mechanisms of inactivation and new treatment modalities. *Nat. Rev. Cancer* **2**, 43-87.

Neuman, D. J. and Cragg, G. M. (2012) Natural products as sources of new drugs over the 30 years from 1981 to 2010. *J. Nat. Prod.* **75**, 311-335.

Philek, A., Zavelevich, M., Kroczań, T. and Los, M. (2004) Caspases and cancer: mechanisms of inactivation and new treatment modalities. *Exp. Oncol.* **26**, 82-97.

Rukhui, Y., Han, S., Yong, D. and Hwang, J. K. (2010) In vitro antibacterial activity of panduratin A against enterococci clinical isolates. *Bioll. Pharm. Bull.* **33**, 1489-1493.

Sadogopan, S. K. A., Mohabii, N., Loi, C. Y., Hasani-rounah, M., Pandurangan, A. K., Arya, A., Karimian, H. and Mustafa, M. R. (2015) Forkhead Box Transcription Factor (FOXO3a) mediates the cytotoxic activity of vermodalin in vitro and inhibits the breast tumor cell survival. *Cell Death Differ.* **22**, vi56-vi65.

Dabrowska, C., Li, M. and Fan, Y. (2012) Emerging targeted therapies in triple-negative breast cancer. *Ann. Oncol.* **23**, vi56-vi65.

Cheah, S.-C., Appleton, D. R., Lee, S.-T., Lam, M.-L., Hadi, A. H. A. and Mustafa, M. R. (2011) Panduratin A inhibits the growth of A549 cells through induction of apoptosis and inhibition of NF-κB translocation. *Molecules* **16**, 2583-2596.

Cohen, G. M. (1997) Caspases: the executors of apoptosis. *Biochem. J.* **326**, 1-16.

Cory, S., Huang, D. C. and Adams, J. M. (2003) The Bcl-2 family: roles in cell survival and oncogenesis. *Oncogene* **22**, 8590-8607.

Crowe, J., O’Shaughnessy, J. and Gullo, G. (2012) Emerging targeted therapies in triple-negative breast cancer. *Ann. Oncol.* **23**, vi56-vi65.

Gadownik, Y. and Aziz, A. (2005) Caspases and cancer: mechanisms of inactivation and new treatment modalities. *Exp. Oncol.* **27**, 89-112.

El-Naa, M. M., Ottman, M. and Younes, S. (2016) Sildenafil potentiates the antitumor activity of cisplatin by induction of apoptosis and inhibition of proliferation and angiogenesis. *Drug Des. Devel. Ther.* **10**, 3661-3672.

Kajstura, M., Halicka, H. D., Pryjma, J. and Darzynkisewicz, Z. (2007) Discontinuous fragmentation of nuclear DNA during apoptosis revealed by discrete sub-G1 peaks on DNA content histograms. *Cytometry A* **71**, 125-131.

Kiri, C., Jones, G. P., Record, I. R. and McIntosh, G. H. (2007) Anticancer properties of panduratin A isolated from Boesenbergia pandurata (Zingiberaceae). *J. Nat. Med.* **61**, 131-137.

Kumar, H., Kim, I.-S., More, S. V., Kim, B.-W., Bahk, Y.-Y. and Choi, D.-K. (2013) Gastrodin protects apoptotic dopaminergic neurons in a toxin-induced Parkinson’s disease model. *Evid. Based Complement. Alternat. Med.* **2013**, 1322-1326.

Liu, Z., Lu, H., Jiang, Z., Pastuszyn, A. and Hu, C. A. (2005) Apoptotic caspases in pro-apoptotic Bcl-2 homology 3-only protein, induces mitochondria-mediated apoptosis in cancer cells. *Mol. Cancer Res.* **3**, 21-31.

Mann, J. (2002) Natural products in cancer chemotherapy: past, present and future. *Nat. Rev. Cancer* **2**, 143-148.

Matsuura, K., Canfield, K., Feng, W. and Kurokawa, M. (2016) Metabolic regulation of apoptosis in cancer. *Int. Rev. Cell Mol. Biol.* **327**, 43-87.

Newman, D. J. and Cragg, G. M. (2012) Natural products as sources of new drugs over the 30 years from 1981 to 2010. *J. Nat. Prod.* **75**, 311-335.

REFERENCES
Sathyas, S., Sudhagar, S., Priya, M. V., Raja, R. B., Muthusamy, V. S., Devaraj, S. N. and Lakshmi, B. S. (2010) β-hydroxy lup-20(29)-ene-27,28-dioic acid dimethyl ester, a novel natural product from Plumbago zeylanica inhibits the proliferation and migration of MDA-MB-231 cells. *Chem. Biol. Interact.* **188**, 412-420.

Sohn, J. H., Han, K. L., Lee, S. H. and Hwang, J. K. (2005) Protective effects of panduratin A against oxidative damage of tert-butyhydroperoxide in human HepG2 cells. *Biol. Pharm. Bull.* **28**, 1083-1086.

Yanti, Rukayadi, Y., Lee, K. H. and Hwang, J. K. (2009) Activity of panduratin A isolated from Kaempferia pandurata Roxb. against multispecies oral biofilms in vitro. *J. Oral. Sci.* **51**, 87-95.

Yao, C. J., Chow, J. M., Yang, C. M., Kuo, H. C., Chang, C. L., Lee, H. L., Lai, I. C., Chuang, S. E. and Lai, G. M. (2016) Chinese herbal mixture, tien-hsien liquid, induces G2/M cycle arrest and radiosensitivity in MCF-7 human breast cancer cells through mechanisms involving DNMT1 and Rad51 downregulation. *Evid. Based Complement. Alternat. Med.* **2016**, 3251046.

Ye, L., Yan, C. and Schor, N. F. (2001) Apoptosis in the absence of caspase 3. *Oncogene* **20**, 6570.

Yun, J.-M., Kweon, M.-H., Kwon, H., Hwang, J.-K. and Mukhtar, H. (2006) Induction of apoptosis and cell cycle arrest by a chalcone panduratin A isolated from Kaempferia pandurata in androgen-independent human prostate cancer cells PC3 and DU145. *Carcinogenesis* **27**, 1454-1464.

Yun, J. M., Kwon, H. and Hwang, J. K. (2003) In vitro anti-inflammatory activity of panduratin A isolated from Kaempferia pandurata in RAW264.7 cells. *Planta Med.* **69**, 1102-1108.

Zheng, W., Han, S., Jiang, S., Pang, L., Li, X., Liu, X., Cao, M. and Li, P. (2016) Multiple effects of Xihuang pill aqueous extract on the Hs578T triple-negative breast cancer cell line. *Biomed. Rep.* **5**, 569-586.

Zhu, X., Wang, K., Zhang, K., Zhang, T., Yin, Y. and Xu, F. (2016) Ziyuglycoside I inhibits the proliferation of MDA-MB-231 breast carcinoma cells through inducing p53-mediated G2/M cell cycle arrest and intrinsic/extrinsic apoptosis. *Int. J. Mol. Sci.* **17**, E1903.