

Full Length Research Paper

Anticancer activity of ethyl acetate and *n*-butanol extracts from rhizomes of *Agapetes megacarpa* W.W. Smith

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Agapetes megacarpa W.W. Smith, also known as Pratat Doi, is one of the commonly used medicinal herbs in northern Thailand. The water extract of the herb has been used for lactation and body shape-up by gestation women. Toxicity and antitumor activities of this herb have never been reported. The objective of this study was to examine the cytotoxic and antitumor activities of ethyl acetate and *n*-butanol partitioned extracts prepared from the rhizomes of this herb. The breast cancer cell lines, MCF-7 and MDA-MB231 and the lung cancer cell line NCI-H1299 were used. The cells were exposed to serial concentrations of the extracts in dimethylsulfoxide and dissolved in cell culture medium. Cytotoxic and antiproliferative assays were used employing the Sulforhodamine B method. The experiments showed that, none of the extracts expressed acute cytotoxicity to the cancer cells within 24 h. Antiproliferative effect was exhibited with time- and concentration-dependent manner after 5 days of exposure. Apoptotic induction on the cancer cell lines was analyzed by flow cytometry using Annexin-V-FITC/propidium iodide staining. Significant differences of apoptotic percentages were found from the exposed cells to both of the extract partitions when compared with the unexposed control cells. The results implied bioactive apoptotic induction by constituents contained in the more polar solvent partition.

Key words: *Agapetes megacarpa*, Pratat Doi, Thailand, cytotoxicity, antiproliferative, apoptosis, breast cancer, lung cancer, flow cytometry.

INTRODUCTION

Cancer is the second largest cause of death which killed 7.6 million people worldwide in 2005 (Danaei et al., 2005). The number is believed to become 9 million in 2015 and 11.5 million in 2030 (World Health Organization, 2007). There is an increasing interest to research and develop on new anticancer drugs, from both synthetic and natural sources (Mukherjee et al., 2001). Half of the drugs which have been approved recently are from natural sources (Kim and Park, 2002; Newman and

Cragg, 2007). Plants have been extensively used as natural sources to develop anticancer drugs because of their active constituents (Schwartzmann et al., 2002). Medicinal plants in Asian countries play an important role in cancer treatment and indeed, their chemical constituents and derivatives have been utilized for combating cancers over the last half-century (Newman et al., 2003).

The plants in the genus *Agapetes*, which are rich sources of pentacyclic triterpenes (Deng and Chen, 1991; Xuan, 2006), have been used as traditional medicine. The stems of *Agapetes saxicola*, *Agapetes megacarpa* and *Agapetes thailandica* are used for roborant in Thai folk medicine. *Agapetes lobbii* was reported to possess antioxidant properties (Tangkanakul et al., 2006) while

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Agapetes neriifolia is applied as medicinal paste to treat fracture in Chinese people (Xuan, 2006). *Agapetes megacarpa* W.W. Smith, commonly known as Prathat doi belonging to the family Ericaceae, is a medicinal herb in the northern region of Thailand. The water extract of its rhizomes is utilized for gestation woman to increase lactation and also for maintaining the body-shape. However, phytochemical investigations of this species are still rare.

This study was aimed at the evaluation of the cytotoxicity and antiproliferative potential of the ethyl acetate and *n*-butanol extracts of *A. megacarpa* rhizomes. Their cytotoxic activity and antiproliferation were investigated on the non-small cell lung carcinoma cell line, NCI-H1299 and the human breast carcinoma cell lines, MCF-7 and MDA-MB231.

MATERIALS AND METHODS

Plant collection

A. megacarpa (AM) was collected from Doi Phahompok, Chiang Mai Thailand. The plant material was identified by curators of the herbarium at Queen Sirikit Botanic Garden, Chiang Mai. Voucher specimen was deposited to the herbarium with a systemic voucher code of Watthana-1443.

Rhizomes extraction

The fresh rhizomes of AM were collected, washed cleaned with tap water, cut into small pieces and weighted (2,186 g) before air-dried at 23 to 32°C for 5 to 7 days to get a consistent weight. The dried material was then, ground into a powder by an electric-grinder. The powder (773 g) was extracted with methanol using Soxhlet apparatus at 64.7°C. After evaporation of the methanol, the extract (113 g) was suspended in water and partitioned with ethyl acetate and *n*-butanol, respectively. Removal of the solvents from each fraction by an electrical evaporator and then freeze-drying yielded the partitioned extracts of ethyl acetate (AM-E, 18.46 g) and *n*-butanol (AM-N, 17.20 g). The partitioned extracts were stored at -20°C until needed.

Preparation of exposed solution

Concentrated stock solutions were prepared by adding a known weight of the partitioned extract to a known volume of dimethyl sulfoxide (DMSO). From each stock solution, 5 to 6 serially diluted working solutions were prepared. The exposed solutions were then, prepared prior to use by adding 1% of the working solutions to the known volume of appropriate medium to each cell type. The control solution contained 1% DMSO (v/v) in-well or the same volume of culture medium. The concentration of DMSO at 1% is non-toxic for cell viability.

Cell lines and culture

Human breast cancer cell line, MCF-7, was retrieved from a frozen stock of the Human and Animal Cell Technology Research Unit, Faculty of Science, Chiang Mai University. This cell line was continuously maintained from the stock cultured at the National

Cancer Research Institute, Bangkok, Thailand. Another human breast cancer cell line, MDA-MB231 and the human lung cancer cell line, NCI-H1299, were purchased from the American Type Culture Collection (ATCC, USA). The culture media, DMEM, Leibovitz L-15 and RPMI-1640 (all from Gibco/Invitrogen, USA) were used for MCF-7, MDA-MB231 and NCI-H1299, respectively. The culture media were supplemented with 10% fetal bovine serum (FBS) (Gibco/Invitrogen, USA) and 100 ng/ml of penicillin and streptomycin (Sigma, USA). Cells in culture flasks (Nunc, USA) were incubated in the standard atmosphere of 95% relative humidity at 37°C and 5% CO₂.

The cytotoxicity assay

The cytotoxicity assay was performed by using the Sulforhodamine B (SRB) (Sigma, USA) colorimetric method on 96 wells culture-plate (Nunc, USA) to assess growth inhibition according to Vanicha and Kirtikara (2006). Briefly, the cell suspensions at 5,000 cells/well in 100 µl media were transferred into 2 sets of 3 plates. The media in a set of three wells basis on each plate was added with 100 µl of a dilution of the exposed mixture of AM-E or AM-N (in DMSO) and consequently, incubated for another 24 h. The exposed concentrations of the partitioned extracts were 5, 10, 20, 40, 60, 80 and 100 µg/ml. At the end of the exposed time, cells in each well were fixed by addition of 100 µl of cold (4°C) 10% (w/v) trichloroacetic acid (TCA) into the growth medium. Each plate was incubated at 4°C for 1 h before gently washed five times with tap water to remove TCA, the growth medium and dead cells. Plates were allowed to dry in air and to each well were added 50 µl of 0.057% (v/v) SRB dye in 1% acetic acid in deionized water and allowed to stand for 30 min at room temperature. At the end of the staining period, unbound SRB was removed by washing four times with 1% of an acetic acid solution. The plate was air-dried and 150 µl of 10 mM aqueous Tris base buffer of pH 10.5 was added to each well to dissolve the cell-bound dye. The plate was then shaken for 15 to 30 min on a gyratory shaker and the optical density (OD) was read at 510 nm in a microplate reader; control wells were used as blanks. Inhibition concentration at 50% of cell population or IC₅₀ was calculated by using Priprobit program version 1.63 (Sakuma, 1998).

The antiproliferation assay

The antiproliferation assay aimed to study the basic pharmacological activities of the extracts. The experiments were carried out by using the SRB colorimetric method as mentioned earlier for the cytotoxicity assay except for the variation of exposed concentrations and exposed durations of the extract to the cells. Five plates of each cell line were prepared. On each plate, cells were exposed to a set of 6 serial concentrations of each extract varying between 10 to 100 µg/ml. These serial concentrations were used according to the value of the IC₅₀ cytotoxic assay of each extract. The plates were incubated in standard CO₂ atmosphere and were taken each to the SRB assay at the end duration of 24, 48, 72, 96 and 120 h. The study was carried out by plotting the mean value of percentage cell proliferation of the exposed cells to the controls against the extract concentrations.

Apoptosis detection by Annexin V-FITC/propidium iodide staining

Annexin V staining for apoptosis detection was performed as previously described by Van et al. (1996). In brief, 10⁵ cells/ml were seeded in 12 wells plates (corning) for 24 h. The exposed

Table 1. Cytotoxic activity of extracts from *A. megacarpa* on the human breast cancer cell lines, MCF-7 and MDA-MB-231, and the human lung cancer cell line, NCI-H1299.

Cell line	IC ₅₀ (µg/ml) ^a	
	AM-E ^b	AM-N ^c
MCF-7	447.83	340.73
MDA-MB231	373.91	256.01
NCI-H1299	91.53	74.54

^a IC₅₀, inhibition concentration at 50% effective level; ^bAM-E, ethyl acetate partition; ^cAM-N, *n*-butanol partition.

concentrations of each extract were prepared at 40 and 80 µg/ml and with 72 h exposure duration. At the end of incubation, cells were trypsinized and collected from each well into a 15 ml conical centrifuge tube and washed twice with 3 ml of the phosphate buffer saline (PBS) and centrifuged at 200 g. Cell pellet was resuspended in binding buffer (10 mM HEPES pH 7.4, 140 mM NaCl and 2.5 mM CaCl₂) containing Annexin V-FITC/propidium iodide (Invitrogen Carlsbad, CA, USA), and was incubated in the dark for 15 min at room temperature. The binding buffer was added to the stained cells and cells were analyzed immediately by FACS (BD Biosciences, San Jose, CA, USA) analysis. At least 10,000 counts were recorded in each analysis. Cells positive to Annexin V-FITC stained were considered as apoptotic cells.

Statistical analysis

All the experiments were carried out on triplicate basis and were presented as mean and standard deviation. Analysis of variance was performed by one way ANOVA, followed by Duncan test for pair wise comparison. The P-values less than 0.05 were considered to be statistically significant.

RESULTS AND DISCUSSION

The results of the cytotoxicity assay at 24 h of the exposed cells to the extracts are shown in Table 1. The criteria of cytotoxic activity for the crude extracts, as previously described by the American National Cancer Institute (NCI-USA), is an IC₅₀ < 30 µg/ml (Suffness and Pezzuto, 1990). The extracts from *A. megacarpa* in this study expressed a non-cytotoxic activity to both of the breast cancer cell lines, MCF-7 and MDA-MB231 with the IC₅₀ > 250 µg/ml. Selectively, very weak cytotoxic activities to the lung cancer cells, NCI-H1299, was expressed by both of the extracts, (AM-E and AM-N), at the IC₅₀ = 91.5 and 74.5 µg/ml, respectively. The IC₅₀ values in this experiment confirmed the non-toxic effects of *A. megacarpa* on the herbal remedies application by the northern Thai indigenous medicinal practitioners (Kornwongwan, 2006). Although, this herb has been used for a long time among the hill tribe people, there is no recorded data for clinical studies or for cytotoxic test against any cancer cell line.

The antiproliferative activity of the extracts on the cell lines was evaluated. Both partitioned extracts of the ethyl acetate (AM-E) (Figure 1) and the *n*-butanol (AM-N) (Figure 2), exhibited the antiproliferative activities to the cells in a time-and concentration-dependent manner. Time-dependent effects of AM-E on the breast cancer cells, MCF-7 and MDA-MB231 (Figure 1ab, respectively) were uniquely greater at 96 and 120 h than those on 24, 48 and 72 h. The effective time-dependent manner of AM-E was shown at 48 h and later was higher at 24 h on the lung cancer, NCI-H1299 (Figure 1c). The concentration-dependence of AM-E on the breast cancer cell, MCF-7 (Figure 1a), was notably shown at ≥ 10 µg/ml, while on MDA-MB231 it was at ≥ 40 µg/ml. On the lung cancer cells of NCI-H1299, the concentration effect of AM-E was clearly shown at ≥ 40 µg/ml (Figure 1c). Antiproliferative activity of the *n*-butanol partitioned extracts (AM-N) is shown in Figure 2. For the breast cancer cells, MCF-7 and MDA-MB231, the time-dependent activity of AM-N was exhibited at ≥ 72 h (Figure 2a) and ≥ 96 h (Figure 2b), respectively. At ≥ 48 h, the antiproliferation activity was observed on the lung cancer cells, NCI-H1299 (Figure 2c). The concentration-dependent manner of AM-N on the breast cancer cells, MCF-7, was not very clear (Figure 2a), while on MDA-MB231 (Figure 2b) as well as on the lung cancer cells, NCI-H1299 (Figure 2c) it was at ≥ 30 µg/ml.

Time-and concentration-dependent manner of the extract activities reflects the logical pharmacokinetics and pharmacodynamics on the cancer cells (Lees et al., 2004; Hsieh and Korfmacher, 2006). This is normally indicated in the cellular uptake across membrane and the metabolic disturbance within the cells (Le Coutre et al., 2004). These cellular pathways of activities are concerned with necessary signaling transduction through cytosol and nucleoplasm. The study of drug response and development of drug response model using these cell lines is the key to determine safety and hazardous levels and dosages of the extracts to which the cells are exposed (Sheiner et al., 1977, 1979).

Growth inhibition of the cancer cells due to the apoptotic induction was determined by using Annexin V-FITC/propidium iodide staining assay and was analyzed by flow cytometry. Investigation was carried out on both partitioned extracts (AM-E and AM-N) each at the exposed concentrations of 40 and 80 µg/ml for 72 h. The results indicated that, both partitions significantly induced apoptosis by concentration-dependent manner on all the investigated cell lines (Figure 3). The percentages of apoptotic cells induced by the two partitioned extracts were shown at the equivalent concentration. The effect of the AM-E expressed was significantly lesser than that of the AM-N in all the cancer cell lines. Apoptotic cells induced by AM-E of the equivalent concentrations were shown with similar percentage in all the cancer cell lines,

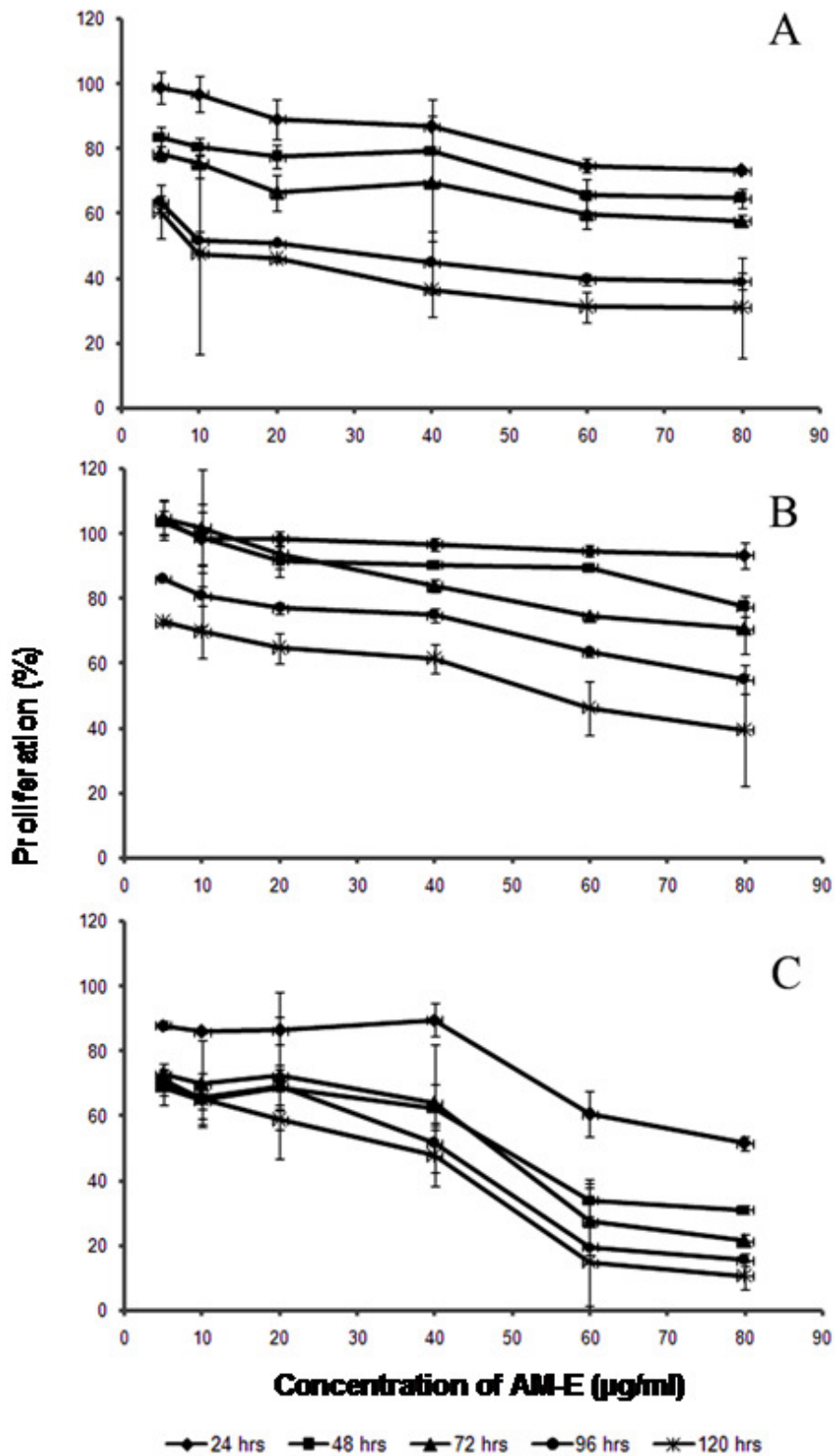


Figure 1. Antiproliferative effect of the ethyl acetate partition of *A. megacarpa* extract (AM-E) on the breast cancer cells, MCF-7 (A) and MDA-MD-231 (B) and the lung cancer cells, NCI-H1299 (C). The mean values of triplicates are performed with standard deviation bar.

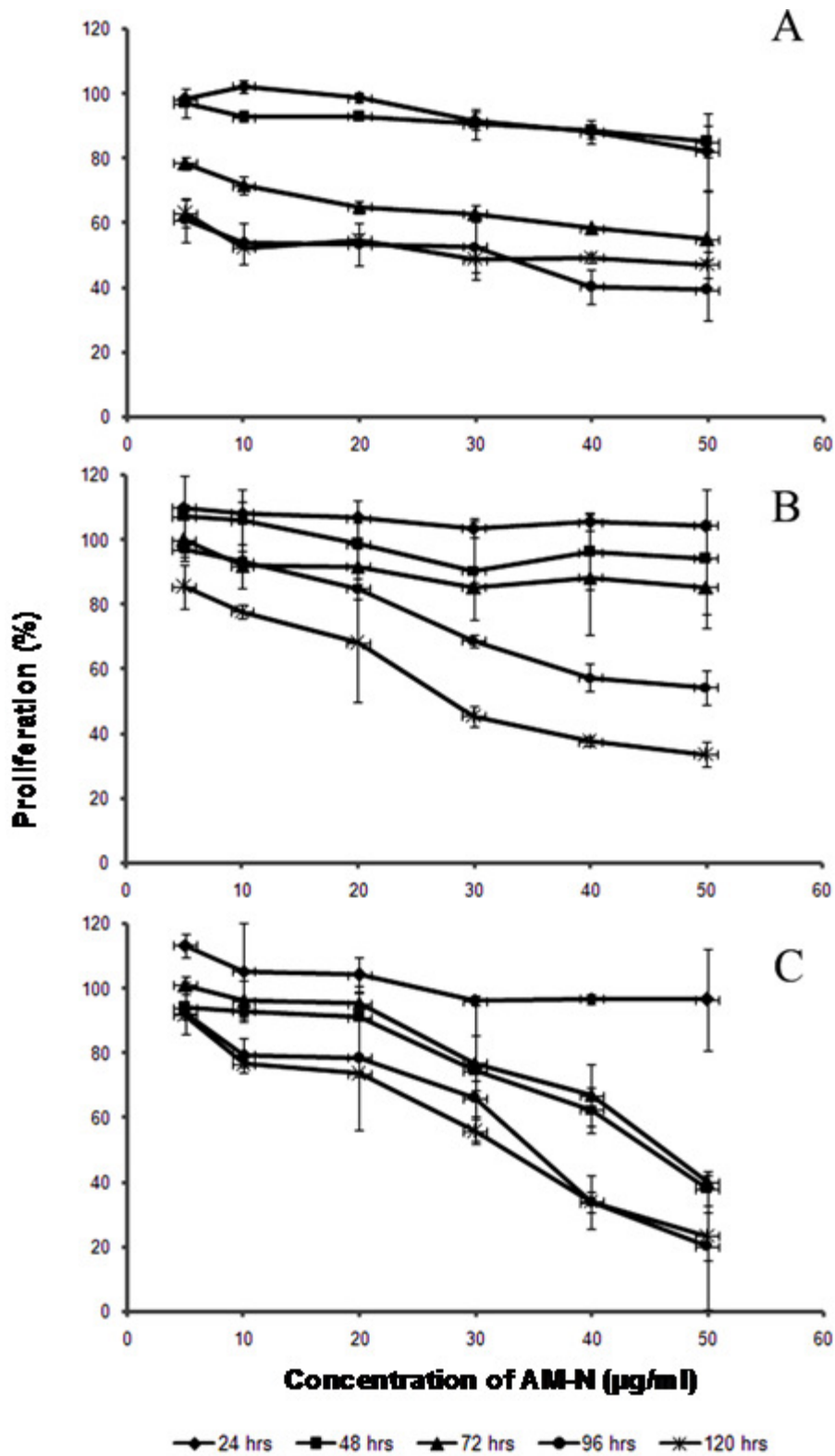


Figure 2. Antiproliferative effect of the *n*-butanol partition of *A. megacarpa* extract, AM-N, on the breast cancer cells, MCF-7 (A) and MDA-MD-231 (B) and the lung cancer cells, NCI-H1299 (C). The mean values of triplicates are shown with standard deviation bar.

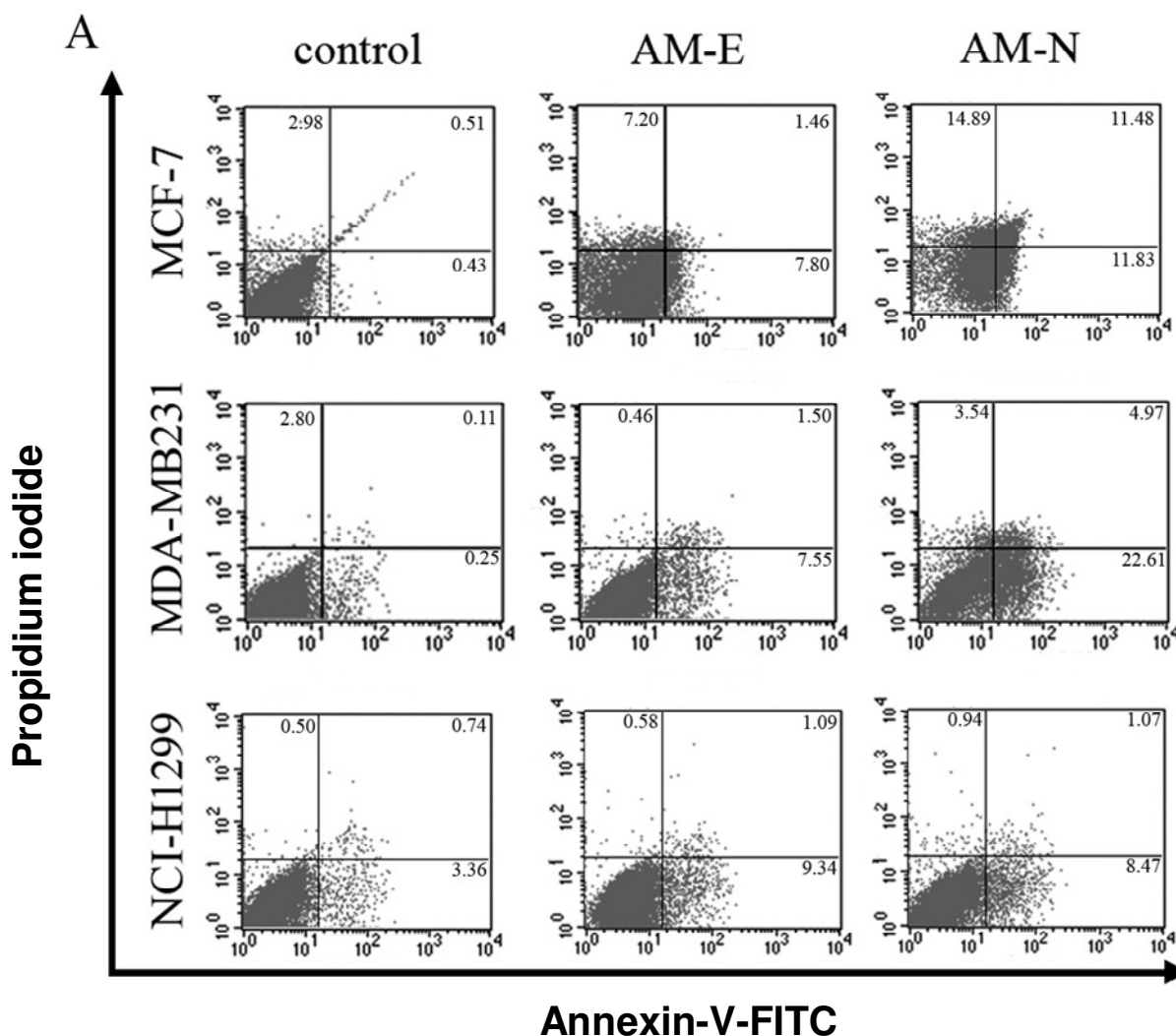


Figure 3. Apoptotic activities of the ethyl acetate partition (AM-E) and the *n*-butanol partition (AM-N), presented by flow cytometric dot-plot examples (A) and the mean percentage value of apoptotic cells (B to D) with standard deviation bars of triplicates. The concentration of the extracts were indicated in number (40 and 80 μ g/ml) after the abbreviated name of partitions. Control groups were unexposed cells to the extract (1% DMSO). The small alphabets a-d, on the graph show statistical significant difference ($p \leq 0.05$).

while the AM-N induced more breast cancer cells (MCF-7 and MDA-MB231), than the lung cancer cells (NCI-H1299). This suggests that *A. megacarpa* was more active and selective for the induction of apoptosis on the human breast cancer cells than the human lung cancer cells.

The overall figures of the investigated activities of the two partitioned extracts of *A. megacarpa* were considered. The ethyl acetate partition (AM-E), expressed less effects than the *n*-butanol partition (AM-N), in all the studied activities. This indicates that, the active constituents obtained by the more polar solvent (*n*-butanol) in the extraction processes were responsible for the anticancer properties in this herb. The biological selective

activity of any compound might depend on the type of chemical composition and the concentration of active constituents as well as the types and developmental stages of the cancer (Lee et al., 2004). The different activities of the extracts to the two breast cancer cells may be due to the biological nature of the cell lines (Liu et al., 2001). MCF-7, the estrogen receptor possessing cells (ER+), expressed was less sensitive to the extract than the non-possessing estrogen receptor cells, MDA-MB-231. The activities of the extracts in breast cancer cells are probably evoked by the ER-mediated genomic pathway and the non-ER-mediated mechanisms (Zierau et al., 2002). This may also be because of the various physico-chemical properties of any individual component

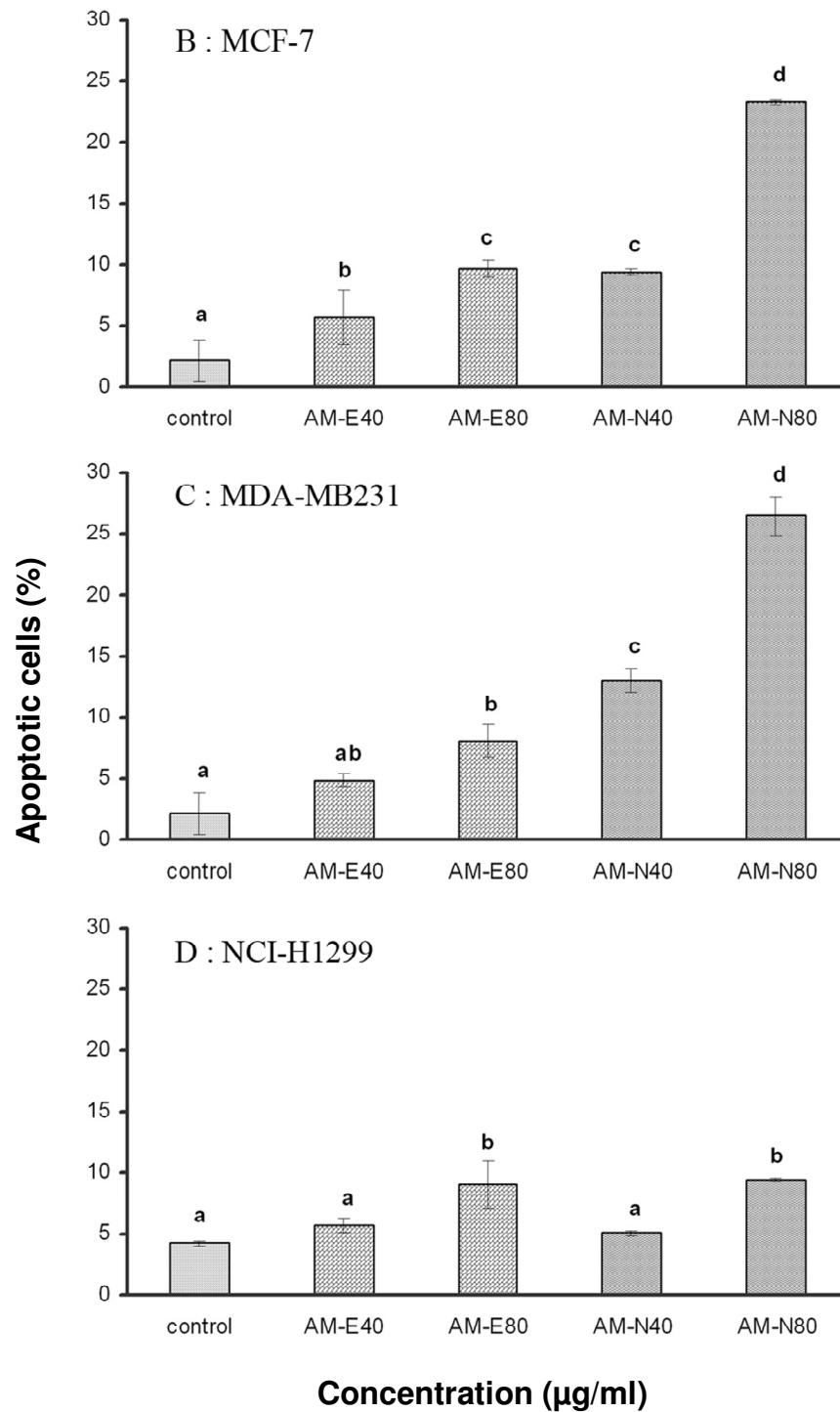


Figure 3. Contd.

of the extracts to the cells (Liske et al., 2002).

Although, some species of the herb in the genus *Agapetes* have been actually investigated, no specific active compounds have been reported so far on *A.*

megacarpa. Several constituent have been identified from *A. obouata* (Chen et al., 1990), *A. hosseana* (Deng and Chen, 1991) and *A. neriifolia* (Xuan, 2006). Among those, the anticancer activities for β -sitosterol (Awad et

al., 2003; Park et al., 2007; Zhao et al., 2009), friedelanol (Kundu et al., 2000) and friedelin (Monkodkaew et al., 2009; Ding et al., 2010) have been shown. Those compounds were reported as cytotoxic to some cancer cells *in vitro*. Some of the compounds were from the non-polar constituents which differed from the activities of the extract from the polar fractions of this experiment. The results from this study, however, indicated for the first time, the apoptosis induction activities by the extracts from *A. megacarpa*. Further investigation is needed to identify the highly active compounds from this herb.

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