

RESEARCH ARTICLE

Anticancer Activity of *Acanthopanax trifoliatum* (L) Merr Extracts is Associated with Inhibition of NF- κ B Activity and Decreased Erk1/2 and Akt Phosphorylation

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Abstract

Acanthopanax trifoliatum (L) Merr (AT) is commonly used as an herbal medicine and edible plant in some areas of China and other Asian countries. AT is thought to have anticancer effects, but potential mechanisms remain unknown. To assess the anticancer properties of AT, we exposed prostate cancer cells to AT extracts and assessed cell proliferation and signaling pathways. An ethanol extract of AT was suspended in water followed by sequential extraction with petroleum ether, ethyl acetate and n-butanol. PC-3 cells were treated with different concentrations of each extract and cell viability was determined by the MTT and trypan blue exclusion assays. The ethyl acetate extract of the ethanol extract had a stronger inhibitory effect on growth and a stronger stimulatory effect on apoptosis than any of the other extracts. Mechanistic studies demonstrated that the ethyl acetate extract suppressed the transcriptional activity of NF- κ B, increased the level of caspase-3, and decreased the levels of phospho-Erk1/2 and phospho-Akt. This is the first report on the anticancer activity of AT in cultured human prostate cancer cells. The results suggest that AT can provide a plant-based medicine for the treatment or prevention of prostate cancer.

Keywords: Plant extract - prostate cancer - apoptosis - NF- κ B - HPLC

Asian Pac J Cancer Prev, **15** (21), 9341-9346

Introduction

Prostate cancer is the second leading cause of cancer related deaths in males in western countries (Siegel et al., 2012), but has a relatively low incidence in Asia, particularly in South East Asia (Baade et al., 2009). Early stage prostate cancer requires androgen for growth and thus responds well to androgen deprivation therapy (Loblaw et al., 2007), but tumors become resistant to this therapy as disease progresses (Pilat et al., 1998; So et al., 2005; Schroder, 2008; Chi et al., 2009). Chemotherapy is the primary treatment option for late-stage prostate cancer patients, but this has limited efficacy and serious toxic side effects and the prognosis for these patients is very poor (Gomella et al., 2009; Neri et al., 2009). Therefore, there is a need for novel therapeutic approaches for treating and preventing androgen-independent prostate cancer while minimizing toxic side effects.

Plant-derived natural products are potential sources for developing novel anticancer agents. Naturally occurring compounds in plants have been shown to inhibit multidrug resistance with minimal side effects (Cragg and Newman,

2005; Meiyanto et al., 2012). Furthermore, many existing FDA-approved anticancer agents are of natural origin (Saunders and Wallace, 2010). *Acanthopanax trifoliatum* (L) Merr (AT) is a widely available plant in China that belongs to the *Acanthopanax* species. Plants of this species are commonly used as herbal medicines such as *Radix Acanthopanaxis Senticosol*.

AT is most commonly used as a traditional Chinese medicine and as a food, tea, and shower additive. AT is believed to have anti-inflammatory activity, and we recently observed that AT extract has strong antioxidant and anti-inflammatory effects (unpublished observations). Histological, molecular genetics, and epidemiological studies suggest that inflammation is important for the development of prostate cancer (De Marzo et al., 2007; Hanahan and Weinberg, 2011; Ianni et al., 2013). Thus, the anti-inflammatory activity of AT may provide anticancer benefits for prostate cancer patients. Here we present a study investigating the effects of AT extracts on prostate cancer cells. To our knowledge, this is the first report investigating the anticancer activity of AT against prostate cells

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Materials and Methods

Preparation of AT extracts

AT plants were collected from Jiangmen City in the southwestern part of Guangdong Province, China. Stems and leaves were collected together according to the procedures used for making AT dietary supplements and traditional teas. Plant material was air dried and then ground into a fine powder. The powder (100 g) was boiled with either distilled water (1:10 w/v) or ethanol (1:10 w/v) for 2 hours before filtration. The water extract (WE) was freeze-dried, while the ethanol extract (EE) was vacuum dried, resuspended in distilled water (1:10 w/v) and extracted sequentially with petroleum ether, ethyl acetate and n-butanol (Figure 1). All fractions were stored at 4°C until analyzed. Each fraction was dissolved in dimethyl sulfoxide (DMSO) for functional assays.

Cell culture and reagents

PC-3 cells, a commonly used human prostate cancer cell line in chemotherapeutic studies, were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA) PC-3 cells were maintained in RPMI-1640 culture medium, supplemented with 10% FBS, penicillin (100 units/ml)-streptomycin (0.1 mg/ml) (Gibco, Grand Island NY).

Cytotoxic activity assay

PC-3 cells were seeded in 96-well dishes at a density of 1.5×10^3 cells/well and incubated at 37°C for 24 hours (h). The cells were then treated with various concentrations (10~160 µg/ml) of each AT extracts for 72h. To measure cell viability, the MTT assay was performed as described earlier (Wei et al., 2012). The effect of different AT extracts on growth was assessed as percent cell growth as compared to the DMSO-treated cells. The cytotoxic effects of the AT extracts was measured by the trypan blue exclusion assay as previously described (Hansson et al., 2005) using a hemocytometer under a light microscope (Nikon Optiphot). For all treatments, the DMSO concentration did not exceed 0.1%, and no effects on cell growth were observed at this solvent concentration.

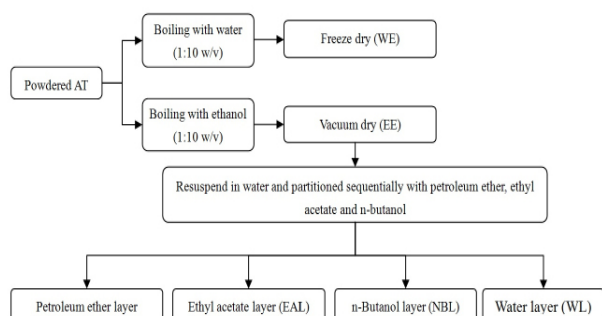


Figure 1. Extraction Procedure. *Acanthopanax trifoliatum* (L) Merr (AT) was air dried and then ground into a fine powder. The powder (100g) was added to boiling distilled water (1:10 w/v) or boiling ethanol (1:10 w/v) for 2h before filtration. The water extract (WE) was freeze-dried while the ethanol extract (EE) was vacuum dried, resuspended in water and extracted sequentially with petroleum ether, ethyl acetate and n-butanol. Fractions were obtained after removal of solvent, and stored for 3-4 days at 4°C until analysis

Assessment of apoptotic cells by morphology and caspase-3 activation

Apoptosis following AT extract treatment was determined by morphologic assessment of propidium iodide stained cells, as described previously (Zheng et al., 2004). Briefly, PC-3 cells were seeded at a density of 0.2×10^5 cells/ml in 35-mm tissue culture dishes and incubated for 24h. The cells were then treated with AT extracts (80 µg/ml) or DMSO (1 µL/ml final concentration) for 96h. The cells were fixed with methanol/acetone (1:1) for 10 min and stained with propidium iodide (1 µg/ml) for 10 min. Stained cells were then visualized under a fluorescent microscope to identify apoptotic cells. A minimum of 200 cells were counted to determine the percentage of apoptotic cells in each sample. Caspase-3 activation was measured using an EnzoLyte AMC Caspase-3 Assay Fluorimetric kit (AnaSpec, Fremont, CA, USA) following the manufacturer's instructions (Wei et al., 2012). Fluorescence intensity was measured using a Tecan Infinite M200 plate reader (Tecan US Inc., Durham, NC, USA).

Nuclear factor- κ B (NF- κ B)-dependent reporter gene expression assay

NF- κ B transcriptional activity was measured as described previously (Zheng et al., 2008). Following transduction of the reporter construct, a single stable clone, PC-3/N, was generated for use in the present study. Briefly, PC-3/N cells were seeded at a density of 0.2×10^5 cells/ml and incubated for 24h. PC-3/N cells were then treated with AT extracts for 24h, and the NF- κ B-luciferase activity was measured using a luciferase assay kit (Promega, Madison, WI, U.S.A) (Zheng et al., 2008). Luciferase activity was normalized against the sample protein concentration as determined using the Bio-Rad protein assay kit (Bio-Rad, Hercules, CA, USA). Luciferase activity was expressed as a percentage of the luciferase activity in DMSO treated control cells.

Western blotting

For biochemical analysis, PC-3 cells were seeded at a density of 1×10^5 cells/ml of medium and incubated at 37°C for 24h, then cells were treated with different AT extracts (80 µg/ml) or DMSO (1 µL/ml) as a negative control for 24h. After treatment the cells were washed with ice-cold phosphate buffered saline (PBS) and lysed with lysis buffer (10 mM Tris-HCl (pH 7.4), 50 mM sodium chloride, 30 mM sodium pyrophosphate, 50 mM sodium fluoride, 100 µM sodium orthovanadate, 2 mM iodoacetic acid, 5 mM ZnCl₂, 1 mM phenylmethylsulfonyl fluoride, and 0.5% Triton X-100). Erk 1/2 and Akt expression were measured by western blot analysis with anti-phosphorylated-Erk1/2 antibodies (#4376, Cell Signaling Technology, Danvers, MA, USA) and anti-phosphorylated-Akt antibodies, respectively (#4501, Cell Signaling Technology, Danvers, MA, USA).

β-Actin was used as a loading control to normalize protein levels. Expression was detected with fluorochrome-conjugated secondary antibody (Santa Cruz Biotechnology Inc., USA) using the Odyssey infrared imaging system (LiCor, Biosciences, Lincoln, NE, USA).

HPLC analysis of AT extracts

Prior to HPLC analysis, 50 mg portions of extract were dissolved in 25 mL methanol and filtered through 0.45 μ m filters. The injection volume was 10 μ L. Elution of AT compounds was detected by monitoring the eluate at 254 nm.

Statistical analysis

Results were expressed as the mean \pm standard error of the mean (SEM). The Analysis of Variance (ANOVA) method with the Tukey-Kramer multiple comparison test (Hsu, 1996) was used for the comparison of number of viable and apoptotic cells among different treatment groups at the end of the treatment.

Results

HPLC fingerprinting analysis of AT extracts

The HPLC fingerprint profile standardized with known marker compounds served as standards for comparisons with subsequent preparations of AT extracts. The HPLC chromatogram of EE was found to contain constituents with major peaks at 18.73 min, 26.52 min and 28.22 min. The presence of chlorogenic acid in EE at RT 18.73 min was confirmed by comparing its retention time and UV spectra with that of the reference standard chlorogenic acid. Additional characterization of the peak by mass spectrometry confirmed the peak as chlorogenic acid (data not presented). The major peak at RT around 26.5 min in EAT had the same mobility and the same UV absorption spectra (not shown) as isochlorogenic acid A (C25H24O12). The identity of this compound was further verified by Q-TOF mass spectrometry (data not presented). Results of the HPLC analysis of PEL, EAL and NBL showed that each extract produced a distinct fingerprint profile (Figure 2) that may be used for standardizing the preparation of the each extracts.

Inhibitory effects of AT extracts on the growth of cultured human prostate cancer cells

To assess the effects of AT extracts on the growth of human prostate cancer cells, we assessed cell viability

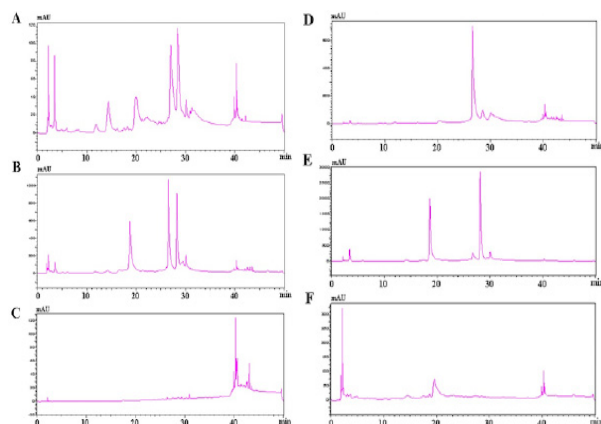


Figure 2. HPLC Chromatograms of AT Extracts. Fifty mg portions of each AT extract was dissolved in 25mL methanol and filtered through 0.45 μ m filters before HPLC analysis. **A)** water extract; **B)** ethanol extract (EE); **C)** petroleum ether layer (PEL); **D)** ethyl acetate layer (EAL); **E)** n-butanol layer (NBL); **F)** remaining water layer (WL) obtained as described in Figure 1

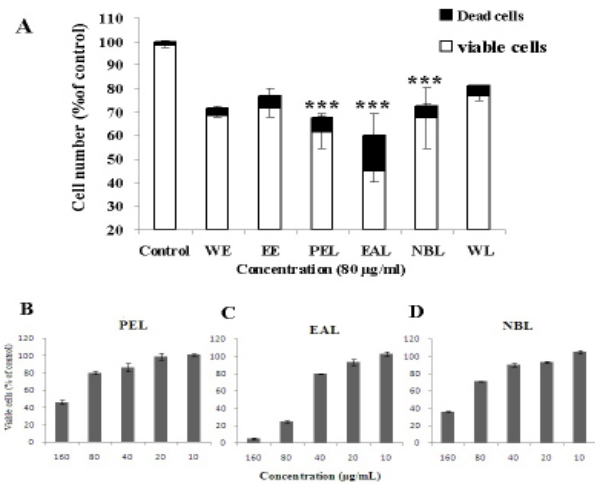


Figure 3. Effects of AT Extracts on Human Prostate Cancer PC-3 Cells. **A)** PC-3 cells were seeded at a density of 0.2×10^5 cells/ml in 35-mm tissue culture dishes and incubated for 24h. The cells were then treated with DMSO (0.1%, final concentration, control) or the indicated AT-extracts (80 μ g/ml in DMSO) for 96h. The number of viable cells after treatment with AT extracts is expressed as percent of control. Both viable and dead cells were determined by a trypan blue exclusion assay. **B-D)** PC-3 cells were seeded at a density of 0.2×10^5 cells/ml in a 96-well plate and incubated for 24h. The cells were then treated with DMSO (0.1%, final concentration, control) or the indicated AT extracts (10-160 μ g/ml in DMSO) for 72h, and the relative cell growth was determined by the MTT assay. Data are expressed as mean \pm SEM of percent of viable cells from triplicate experiments. Differences between AT-extract treated groups and the DMSO-treated (control) group were analyzed by the Tukey-Kramer multiple comparison test. *** $p < 0.001$

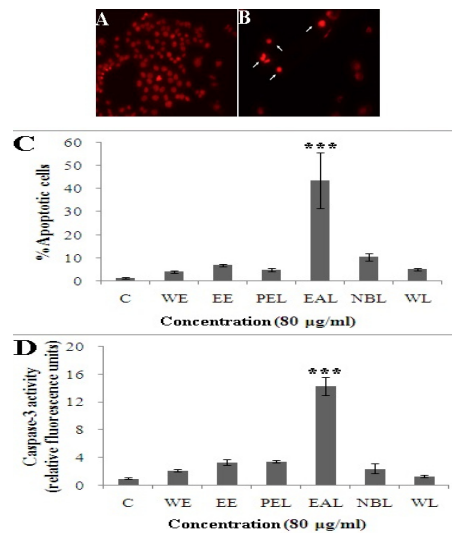


Figure 4. Effects of AT Extracts on Apoptosis of PC-3 Cells. **A-B)** Morphological assessment of propidium iodide stained PC-3 cells after treatment with DMSO (A) or the EAL AT extract (B). Apoptotic cells were determined by morphological assessment using a fluorescence microscope. Arrows indicate apoptotic cells. **C)** Quantification of the percentage of apoptotic cells by morphological assessment. **D)** Quantification of caspase-3 activity as measured by fluorescence intensity in PC-3 cells treated with the indicated AT-extracts. Caspase-3 activity is expressed as arbitrary units relative to the control. Columns are the mean \pm SEM of 3 separate experiments. Differences between AT-extract treated groups and the DMSO-treated (control) group were analyzed by the Tukey-Kramer multiple comparison test. *** $p < 0.001$

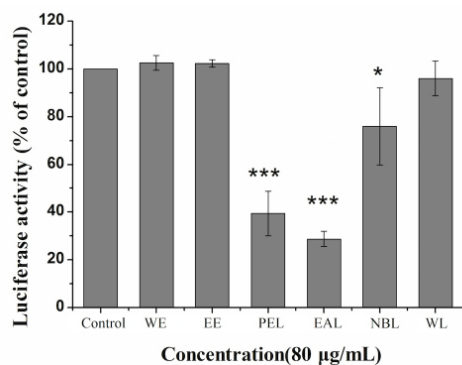


Figure 5. Effects of AT Extracts on NF-κB Transcriptional Activity in PC-3/N Cells. Quantification of NF-κB luciferase reporter activity in PC-3/N cells after treatment with AT extracts. Cells were seeded at a density of 0.2×10^5 cells/ml of medium in 35mm culture dishes and incubated for 24h. The cells were treated with 0.1% DMSO final concentration (Control) or with the indicated AT extracts (80 μg/ml in DMSO) for 24h. NF-κB transcriptional activity was measured by a luciferase activity assay as described in the Materials and Methods section. Differences between AT-extract treated groups and the DMSO-treated (control) group were analyzed by the Tukey-Kramer multiple comparison test. * $p < 0.05$; ** $p < 0.01$

after treatment with each AT extract. PC-3 cells were treated with PEL, EAL, and NBL AT extracts for 72h, and viability was assessed using MTT and trypan blue exclusion assays. We found that EAL had the strongest inhibitory effect on the growth of PC-3 cells, followed by the PEL and NBL extracts (Figure 3A). All three extracts inhibited cell growth in a dose-dependent manner (Figure 3B-D). The other extracts (WE, EE, NBL and WL) had only a small inhibitory effect on the viability of PC-3 cells that was not dose-dependent (data not shown).

Effects of AT extracts on apoptosis in PC-3 cells

Given the strong inhibitory effect of EAL on PC-3 cell viability, we investigated whether this extract promoted apoptosis in PC-3 cells. Treatment of PC-3 cells with EAL resulted in apoptosis, as determined by morphological assessment (Figure 4A-B). Treatment of PC-3 cells with 80 μM EAL resulted in a 43% increase in morphologically distinct apoptotic cells, compared to only 10% in NBL treated cells (Figure 4C). Equivalent doses of WE, EE, PEL or WL alone caused little or no increase in apoptosis. We further assessed apoptosis induction in PC-3 cells using the caspase-3 activation assay. Consistent with the morphological assessment, EAL stimulated caspase-3 activity more dramatically than other extracts, suggesting that this extract efficiently stimulates apoptosis in PC-3 cells (Figure 4D).

Effects of AT extracts on NF-κB activity

To investigate the mechanisms by which AT extracts stimulate apoptosis in PC-3 cells, we assessed the activity of NF-κB, an important regulator of cell growth and apoptosis, in AT extract treated cells. NF-κB transcriptional activity was assessed using a luciferase reporter gene expression assay in PC-3/N cells. After treatment with each AT extract, luciferase activity was

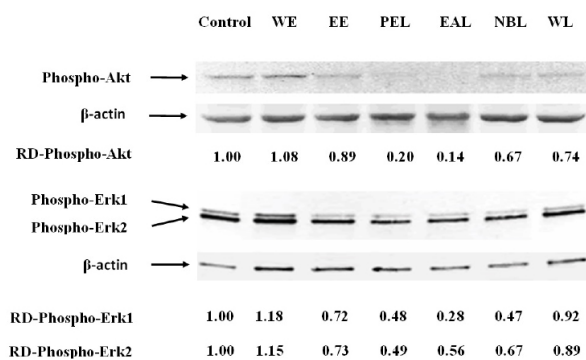


Figure 6. Inhibitory Effects of AT Extracts on Activation of Akt and Erk1/2. Western blot analysis of phospho-Akt, phospho Erk 1/2, and β-actin in PC-3 cells treated with DMSO (Control) or the indicated AT extracts. Cells were seeded at a density of 1×10^5 cells/ml and incubated for 24h, then treated with AT extracts (80 μg/ml) for 24h. β-actin was used as a loading control. The data is representative of three experiments

measured to determine NF-κB activity levels. Using this assay, we found that only the EAL and PEL fractions had a strong inhibitory effect on NF-κB transcriptional activity (Figure 5). This result suggests that EAL may promote apoptosis through inhibition of NF-κB activity in PC-3 cells.

Effects of AT extracts on the levels of phospho-Akt and phospho-Erk 1/2 in PC-3 cells

Activation of Akt and Erk 1/2 plays a role in the survival and growth of prostate cancer cells (Kaur et al., 2008; Bhargavan et al., 2012; Ye et al., 2012). Thus, we assessed Akt and Erk 1/2 activity in PC-3 by western blot analysis of Akt and Erk 1/2 phosphorylation. PC-3 cells were treated with DMSO (0.1% final concentration) or different AT extracts (80 μg/ml) in DMSO for 24h, and the levels of phospho-Akt, phospho-Erk 1/2, and β-actin in whole cell lysates were determined by Western blot. Treatment of PC-3 cells with EAL and PEL caused a strong decrease in Akt phosphorylation, while the other AT extracts had a small or negligible effect Akt phosphorylation (Figure 6). Similarly, EAL treatment resulted in a strong decrease in Erk 1/2 phosphorylation, while PEL and NBL also inhibited Erk 1/2 activity to a lesser degree (Figure 6).

Discussion

Here, we demonstrated for the first time that AT extracts can suppress the growth of the PC-3 human prostate cancer cell line. Among the AT extracts, EAL was the most potent inhibitor of PC-3 cell growth and stimulator of apoptosis. AT is commonly used as an herbal medicine in China and in other Asian countries and is also consumed as a vegetable in areas of southern China such as Guangdong Province. Although some species of the AT genus have anti-inflammatory activity (Chi, 1997; Loi, 2000), there have been no studies investigating the anti-cancer activities of AT extracts. Our results suggest that AT extracts, in particular the EAL extract can exert anti-cancer activities in cultured prostate cancer cells by promoting

apoptosis. Our current results may lay the foundation for further studies to determine the mechanisms of action by which AT extracts exert anti-cancer effects on tumor cells.

Since many bioactive constituents in plants are lipophilic, we prepared an ethanol extract (EE) of AT. The EE was further extracted sequentially with petroleum ether, ethyl acetate and n-butanol as described in Figure 1. These extracts, along with the remaining water layer (WL) fractions were analyzed for their effects on cell growth and apoptosis. The EE had a modest inhibitory effect on the growth of PC-3 cells, similar to the WE fraction (Figure 3), but the effect was not dose-dependent (data not presented). Among the different fractions of EE, the EAL extract was the most potent stimulator of apoptosis, while the other fractions had little to no effect on apoptosis rates. HPLC analysis showed that EAL, WE and EE all had a major peak at RT around 26.52 min which was either small (NBL) or absent (PEL and WL) in other fractions which had no effect on cell growth (Figure 2). Furthermore, the concentration of this component was highest in the EAL, consistent with the more higher potency of this extract. The major peak at RT around 26.5 min in EAT had the same mobility and UV absorption spectra as isochlorogenic acid A (C₂₅H₂₄O₁₂) (data not shown). The identity of this compound was further verified by Q TOF mass spectrometry. Since EAL had the strongest stimulatory effect on apoptosis, isochlorogenic acid A is likely the apoptotic AT constituent. However, further studies using preparative HPLC are needed to identify the specific AT-derived compounds responsible for inducing apoptosis.

To investigate the mechanisms by which AT extracts inhibit cell growth and promoted apoptosis in PC-3 cells, we analyzed cell-proliferation signaling pathways after treatment with each extract. NF- κ B can regulate of proliferation and apoptosis in a variety of cells including prostate cancer cells (Paule et al., 2007; Karin, 2009; Sambantham et al., 2013). Moreover, NF- κ B is commonly activated in invasive prostate cancer (Lessard et al., 2003; Ross et al., 2004; Shukla et al., 2004) and of NF- κ B-responsive genes are commonly associated with prostate cancer progression (Shukla et al., 2004). Thus, NF- κ B provides an attractive therapeutic target for the treatment of prostate cancer. We found that AT extracts inhibited the transcriptional activity of NF- κ B, inhibition NF- κ B by each AT extract correlated with its inhibition of cell growth. In particular, EAL was a potent inhibitor of both NF- κ B and cell growth, as well as a strong inducer of apoptosis. A large number of studies have shown that the LPS-TLR4/NF- κ B signaling pathway regulates the inflammatory response. This is consistent with our findings, in which AT extracts with anti-inflammatory properties had an inhibitory effect on PC-3 cell proliferation.

In addition to inhibiting NF- κ B, EAL decreased phosphor-Erk 1/2 expression, as assessed by Erk 1/2 phosphorylation analysis. Other AT extracts had small to moderate effects for decreasing the level of phospho-Erk1/2 with the exception of WE, which had no effect relative to controls. Erk 1/2 activity is generally associated with mitogenesis and suppression of apoptosis (McCubrey

et al., 2007; Junttila et al., 2008), and constitutive activation of Erk has been observed in prostate cancer (Gioeli et al., 1999; Uzgare and Isaacs, 2005). Erk 1/2 can activate a number of transcription factors, including NF- κ B, to regulate gene expression (Raman et al., 2007; Kook et al., 2011). Thus, EAL may suppress PC-3 cell growth by downregulating Erk 1/2 and subsequent pro-survival signaling through NF- κ B.

In addition to Erk 1/2 suppression, we found that the EAL also suppressed Akt activity in PC-3 cells. Akt is a serine-threonine protein kinase that regulates cell proliferation and survival in a wide variety of cancer types, including prostate cancers (Sarker et al., 2009; Wegiel et al., 2010). Advanced prostate cancers frequently have elevated levels of phospho-Akt, and Akt signaling appears to be critical to prostate cancer cell survival and proliferation (Antonarakis et al., 2010). Treatment with NBL and PEL extracts also decreased Akt phosphorylation while WE, EE and wL had little or no effect, suggesting that AT may suppress Akt activity through one or more constituents shared among these extracts. Earlier studies indicated that some cytokines induced activation of NF- κ B through Erk1/2 and Akt pathways (Maeda and Omata, 2008). Thus, it is possible that AT, and the EAL extract in particular, may be able to suppress prostate cancer cell growth through an Erk/Akt dependent pathway.

In summary, we demonstrate that AT extracts inhibit cell growth and induce apoptosis in cultured prostate cancer cells. Among the AT extracts tested, EAL was the most potent inhibitor of growth and stimulator of apoptosis. The ability of AT extracts to suppress prostate cancer cell growth was associated with their ability to inhibit NF- κ B, Erk 1/2 and Akt activity. These results suggest that the AT, in particular the EAL extract, could provide a novel therapy for prostate cancer patients. Further studies are needed to determine the *in vivo* effects of these extracts in animal models in order to better evaluate the therapeutic potential of AT.

Acknowledgements

This work was supported by funds from a leadership grant From China awarded to AHC, Guangdong Natural Science Foundation, China (S2012040007488).

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