In vitro evaluation of anti-epidermoid cancer activity of *Acanthus ebracteatus* protein hydrolysate and their effects on apoptosis and cellular proteins

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Abstract. *Acanthus ebracteatus* Vahl. is commonly consumed with the aim of curing cancer, inflammatory conditions and skin diseases in traditional Thai medicine. It is known to contain various phytochemicals; however, very little is known about the effects of *A. ebracteatus* protein hydrolysate on cancer cells, including its molecular mechanisms. The present study therefore investigated the anti-cancer activity of *A. ebracteatus* protein hydrolysates against epidermoid cancer of the skin cell line A431. Their effects on the apoptosis pathway and expression of proteins involved in the regulation of apoptosis, cell proliferation or cell cycle were also investigated. Crude extract of protein hydrolysate, partially purified peptides and purified peptides extracted from the aerial part of *A. ebracteatus* were administered to the A431 cells. The cytotoxicity effects were then determined using an MTT assay. As a result, *A. ebracteatus* protein hydrolysate significantly inhibited A431 cells with half inhibitory concentration equals to 425.9 ng protein/ml. By performing Annexin V assay, the partially purified peptides of *A. ebracteatus* were demonstrated to enhance the apoptosis pathway. Furthermore, western blot analysis revealed that the partially purified peptides of *A. ebracteatus* increased protein expression levels of RelA (p65) and Cyclin D1 proteins. However, *A. ebracteatus* did not increase the expression levels of p53-serine 15 phosphorylation (Ser15P).

Introduction

Acanthus ebracteatus Vahl. is a shrubby herb commonly found in South Asia. It belongs in the Acanthaceae family and is also known as sea holly or holly mangrove. Whole plants of *A. ebracteatus* have been used as an astringent and expectorant in Ayurveda (1), whereas its leaves and seeds are usually boiled as a treatment for coughs in Malaysia (2). In Thai traditional medicine, the plant is consumed as an anti-inflammatory agent for treating arthritis (3). The entire plant is also boiled and used for treating skin diseases and healing rash (4). For the skin treatment, the combination of ethanol extract of *A. ebracteatus* with collagen was reported to enhance skin angiogenesis and also promoted wound closure in a mouse model (5). Furthermore, hot water extract of *A. ebracteatus* was revealed to inhibit cervical cancer growth and angiogenesis in cell-implanted nude mice (6). However, little is known about the biological effects of protein hydrolysates or peptides extracted from *A. ebracteatus*.

According to a report from the World Health Organization, the incidence rates of both non-melanoma and melanoma skin cancer has been increasing over past years. Approximately 2-3 million cases of non-melanoma skin cancer and ~132,000 cases of melanoma skin cancer are reported worldwide each year (7). Although non-melanoma skin cancer is not always lethal, surgical treatment is often painful and disfiguring. On the other hand, malignant melanoma is a major cause of skin cancer-associated mortality and only early-stage melanomas can be treated with surgery. More advanced types of melanoma skin cancer require other treatments, such as immunotherapy, chemotherapy and radiation therapy. However, these treatments often cause side effects or development of multi-drug resistance (8), thus alternative or complementary treatments from natural products are gaining more attention.

Natural products and compounds derived from plants appear as a potential alternative treatment of cancers as they possess inhibitory effects against various types of human cancer, and can perturb cellular signaling pathways, particularly the activation of apoptosis with minimal effects on normal cells (9-13). The majority of reported phytochemicals are flavonoids, carotenoids, terpenoids, vitamins and certain polyphenoids (8). Nevertheless, proteins or bioactive peptides containing 3-20 amino acid units (14,15) have also been reported as potential cancer treatments due to their

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high target specificity with low toxicity, and they are easy to manipulate (16).

Therefore, the present study aimed to evaluate the anti-non-melanoma skin cancer activity of protein hydrolysate, partially purified peptides and purified peptides extracted from the aerial parts of *A. ebracteatus* using an MTT assay. The effects of plant extracts on the apoptosis pathway were investigated using Annexin V. Finally, the effects of plant extracts on the level of cellular protein expression [p53, p53-serine 15 phosphorylation (Ser15P), RelA (p65) and Cyclin D1] were also determined.

Materials and methods

Plant material. The aerial parts of *A. ebracteatus* in powder form were purchased from a Thai traditional medicine shop (Chao-krom-poe, Bangkok, Thailand).

Preparation of protein hydrolysate. *A. ebracteatus* powder (250 g) was extracted with 0.5% sodium dodecyl sulfate (SDS) (Ajax finechem) for 24 h at 37°C in a shaker (Gallenkamp). The extracts were then centrifuged at 9,100 x g for 30 min at 4°C and protein was precipitated with 80% cold acetone (Avantor) in a ratio of 1:2 (v/v) at -20°C for 24 h. The supernatant was removed via centrifugation at 5,800 x g for 30 min at 4°C. Then, the protein precipitate was dissolved in 0.2 M sodium acetate buffer (Ajax finechem), pH 4.5 and digested with pepsin (Sigma-Aldrich; Merck KGaA) in a ratio of 1:25 (w/w) for 24 h at 37°C followed by heat inactivation for 10 min at 60-70°C. Following this, this protein hydrolysate was centrifuged at 5,800 x g for 30 min at 4°C and pH was adjusted from 6.8 to 7.0 followed by protein filtration through a membrane with a molecular weight cut-off of 3 kDa (GE Healthcare). Finally, the protein content was determined using Bradford assay (Bio-Rad Technologies) according to the manufacturer's protocol.

Cell culture and maintenance. Vero (normal kidney cells, ATCC® CCL-81™) and A431 (skin cancer cells; ATCC® CRL-1555™) cells were cultured in DMEM (Gibco; Invitrogen; Thermo Fisher Scientific, Inc.) supplemented with 10% (v/v) heat inactivated fetal bovine serum (Gibco; Invitrogen; Thermo Fisher Scientific, Inc.) and 1% (v/v) anti-mycotic-antibiotic solution (Gibco; Invitrogen; Thermo Fisher Scientific, Inc.). Both cell lines were cultured in 75 cm² sterile flasks (SPL) at 37°C under 5% CO_2 atmosphere (Heal force).

All cell lines were cultures in 75 cm² sterile tissue culture flasks at 37°C with 5% CO_2 in DMEM supplemented with 10% heat inactivated FBS (10% v/v). Cells were regularly passaged to maintain exponential growth. Basic cell culture techniques were performed as previously described (17). All cells were checked for mycoplasma contamination using PCR and staining with Hoechst 33258. All cell lines were confirmed to be mycoplasma-free cultures.

Cell viability assay (MTT assay). Cell viability was evaluated using MTT assay (Invitrogen; Thermo Fisher Scientific, Inc.) as previously described (18). Briefly, 1.5x10³ cells were seeded in 96 well-microplate (SPL) and incubated under 5% CO_2 at 37°C for 24 h. Cells were them treated with various final concentrations of *A. ebracteatus* protein hydrolysate from 0.00 (water only), 3.33, 33.33, 333.33 and 1,666.67 ng protein/ml. For analyzing the cytotoxicity of the partially purified protein hydrolysate, cells were treated with 425.9 ng protein/ml [the half inhibitory concentration (IC_{50}) value of the *A. ebracteatus* protein hydrolysate against A431] of each fraction. For evaluating the cytotoxicity of the purified peptides, cells were treated with different concentrations of single or combined peptides, ranging from 100-500 μM. Following the addition of protein hydrolysates or peptides, cells were incubated for 5 days without changing the media nor substituting the protein hydrolysates or peptides. Treated cells were then subjected to MTT assay according to the manufacturer's protocol.

High performance liquid chromatography (HPLC) analysis. HPLC analysis with a UV detector (RP-HPLC) was performed to partially purified peptides from *A. ebracteatus* protein hydrolysate. The column employed was an ODS-2 HYPERSIL C18; 250x4.6 mm, 5 μm particle size (Thermo Fisher Scientific, Inc.). A gradient mobile phase of 0-50 min was applied, with HPLC-grade 0.1% trifluoroacetic acid (TFA) (Thermo Fisher Scientific, Inc.), acetonitrile (Thermo Fisher Scientific, Inc.) and water (90:10 v/v). The injected sample volume was 100 μl (17.05 μg protein/ml) and the flow rate was 1.00 ml/min. Detection was performed at 220 nm. Each fraction was collected using a fraction collector (Amersham Pharmacia Biotech). Finally, fractions were lyzed in 50 mM Tris/HCl buffer (Thermo Fisher Scientific, Inc.) and then lyophilized using a freeze dryer (LaboGene).

Liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis. The peptide sequences were identified using LC-MS/MS. Briefly, the peptides were acidified with 0.1% (v/v) formic acid and subjected to an Ultimate 3000 capillary LC system (Dionex). Peptide separation was performed using an Acclaim PepMap RSLC 75 μm x15 cm nanoviper C18 with a 2 μm particle sizes and a 100 Å pore size (Thermo Fisher Scientific, Inc.) at flow rate of 300 nl/min. Mobile phase A consisted of 2% acetonitrile and 0.1% formic acid in HPLC grade water and mobile phase B consisted of 0.1% formic acid in HPLC grade acetonitrile. The instrument was controlled by Hystar software. DataAnalysis™ and Biotools software version 3.2 were used for the data interpretation and *de novo* sequencing.

Peptide synthesis and bioinformatic analysis. Four peptide sequences (Table I) were commercially synthesized by GenScript® for >95% purity. The pI and molecular weight (MW) of each peptide were calculated using a peptide property calculator (19).

Apoptosis study. Cells (1x10⁵ cells) were seeded in 24 well-microplates (SPL) and incubated in the presence of 5% CO_2 at 37°C for 24 h. Cells were then treated with 425.9 ng protein/ml of the partially purified peptide. Cells were incubated in the presence of 5% CO_2 at 37°C for 3 days. Following incubation, cells were washed twice with cold phosphate buffer saline (PBS) and then harvested. For the positive control, cells were induced with 50 μg/ml dihydrochloride hydrate (Sigma-Aldrich; Merck KgaA) for 30 min. Cells were

Table I. Peptide sequences identified from the FR1 of *A. ebracteatus* by using LC-MS/MS.

Peptide name	Sequence	pI	Molecular weight (g.mol⁻¹)
AE1	KVGNAADRV	9.9	929.03
AE2	NDLSGDNSVRW	3.71	1,262.29
AE3	VLNPNLEPPPNP	1.1	1,300.46
AE4	WAGEEVKAPHW	5.26	1,309.43

FR1, fraction number 1; LC-MS/MS, liquid chromatography tandem mass spectrometry.

Figure 1. Cytotoxic effects of protein hydrolysate from *A. ebracteatus* on A431 cells. (A) MTT result. (B) The IC_{50} of *A. ebracteatus* protein hydrolysate calculated by GraphPad QuickCalcs (GraphPad software). The data were presented from two independent experiments with 8 replicates. **$P<0.001$; ***$P<0.0001$ when compared with control cells without treatment.

then stained using Annexin V and dead cells assay kit (Merck Millipore) in the dark for 15 min. Finally, cells were analyzed using a flow cytometer (Merck Millipore).

Western blot analysis. Cells (1×10^5 cells) were seeded in 24-well microplates and incubated at 5% CO_2, 37°C for 24 h. Cells were then treated with 425.9 ng protein/ml of the partially purified peptides and incubated in the presence of 5% CO_2, 37°C for 5 days. Protein extraction was performed using passive lysis buffer (PLB) (Promega), and the protein concentration was determined using a Bradford assay. For the immunoblotting step, the protein was firstly separated using NuPAGE® Bis-Tris gel (Invitrogen; Thermo Fisher Scientific, Inc.) and transferred on to PVDF membrane (Merck Millipore). Then, the membrane was blocked with 5% skimmed milk in TBS-T for 1 h. Next, the membrane was washed with TBS-T buffer and then incubated overnight using primary antibodies that were diluted in 0.01% BSA in TBS-T. The dilution ratios for anti-p53 (Invitrogen; Thermo Fisher Scientific, Inc.), anti-RelA (p65), anti-Cyclin D1 (Santa Cruz Biotechnology) were equal to 1:1,000 (v/v); for anti-alpha-tubulin (Invitrogen; Thermo Fisher Scientific, Inc.) was equal to 1:1,500 (v/v); and anti-Ser15P was equal to 1:300 (Abcam). The membrane was then washed and incubated with horseradish peroxidase-conjugated secondary antibodies (Abcam) for 1 h. Finally, the ECL substrate (GE Healthcare) was added onto a membrane and analyzed using a molecular imager (Bio-Rad Technologies).

Statistical analysis. An unpaired Student's t-test was performed using GraphPad QuickCalcs (GraphPad software). Unless any statistically significant differences between the mean of three or more independent groups were compared, one-way ANOVA with Dunnett's T3 analysis was performed using IBM SPSS software. $P<0.05$ was considered to indicate a statistically significant result.

Results

Protein hydrolysate of A. ebracteatus significantly inhibits the cell viability of non-melanoma skin cancer cells in a dose dependent manner. Natural bioactive peptides are primarily released from their precursor proteins by digestive enzymes during gastrointestinal digestion (14). Therefore, the production of bioactive peptides in the present study mimicked the human digestive system in the body by digesting the extracted lysates with the enzyme pepsin. The MTT assay was then performed to evaluate the anti-non-melanoma skin cancer activity of *A. ebracteatus* protein hydrolysate. The non-melanoma skin cancer cells (A431) were treated with various concentrations of protein hydrolysate ranging from 0-1,666.7 ng protein/ml. Notably, Vero cells, an epithelial cell line from African green monkeys were used to represent normal cells in the present study. As a result, *A. ebracteatus* protein hydrolysate significantly inhibited the cell viability of A431 in a dose dependent manner (Fig. 1A). By treating cells with 333.3 ng protein/ml, the cell viability of A431 was only $47.5 \pm 10.8\%$ ($P<0.001$) without affecting the Vero cells (Fig. 1A). The IC_{50} of *A. ebracteatus* protein hydrolysate against A431 cells calculated by GraphPad was equal to 425.9 ng protein/ml (Fig. 1B). Furthermore, *A. ebracteatus* protein hydrolysate was revealed to induce anticancer activities against a board range of cancer cell lines (Fig. S1).

Subsequently, partially purified peptides of the *A. ebracteatus* protein hydrolysate were then investigated in order to identify bioactive fractions.

In vitro identification of partially purified peptides against non-melanoma skin cancer from A. ebracteatus. To partially purify protein hydrolysate and identify fractions that possess anti-epidermoid cancer activities, a reverse phase (RP) HPLC with a UV detector was performed. The chromatogram indicated several major and minor peaks, which were sorted into 19 fractions (Fig. 2A). According to the IC_{50} value of
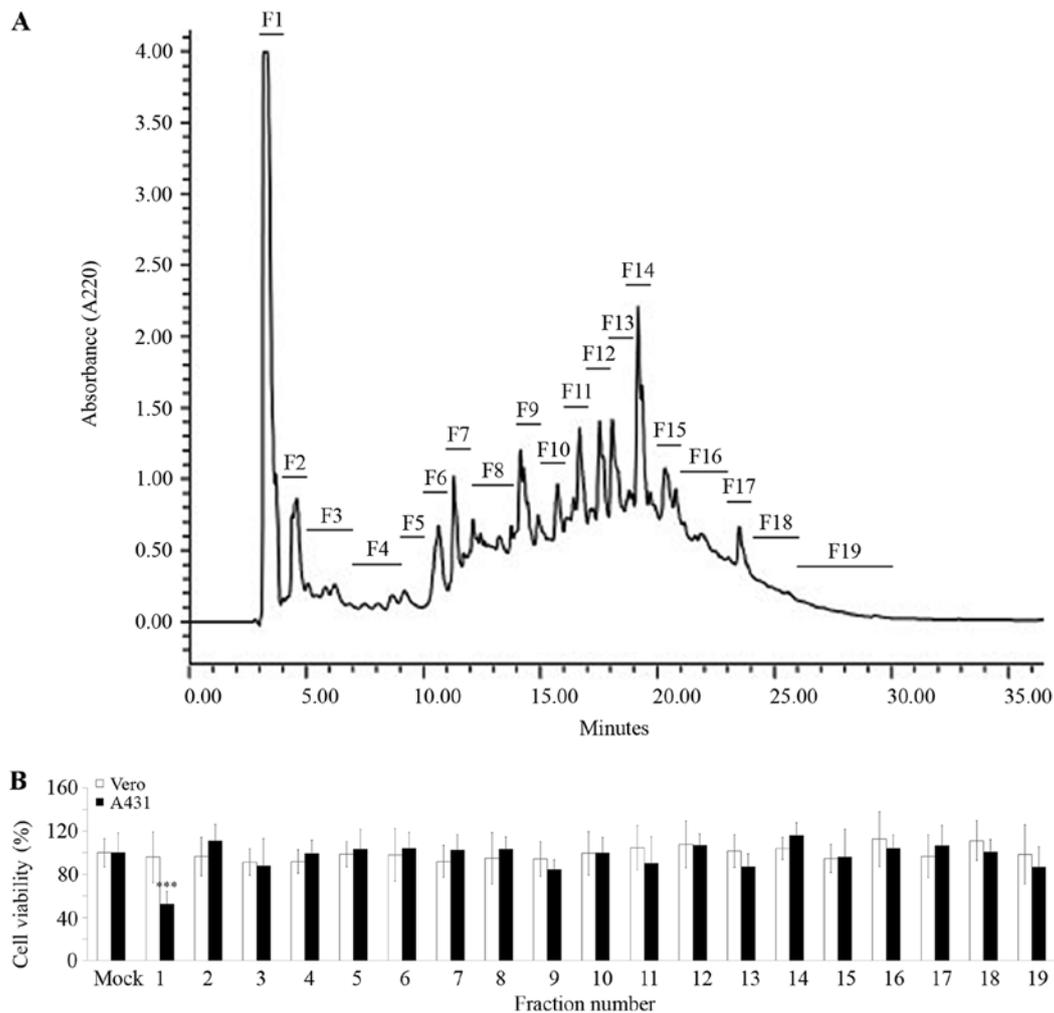
Figure 2. Identification of bioactive fractions from *A. ebracteatus* protein hydrolysate. (A) RP-HPLC chromatogram with detection at 220 nm. Nineteen pooled fractions were labeled and designated as FR1-FR19. (B) The cytotoxic effects of the partially purified protein hydrolysate (FR1-FR19) at 425.9 ng protein/ml on A431. The data were calculated from two independent experiments that performed in quadruplicates. ***P<0.0001 when compared with cells without treatment (Mock). HPLC, high performance liquid chromatography; RP-HPLC, HPLC analysis with a UV detector.

A. ebracteatus protein hydrolysate against A431 cells (Fig. 1B), the concentration at 425.9 ng protein/ml of each fraction was then administered to the A431 cells. After 5 days of incubation, the cells were subjected to an MTT assay. The results demonstrated that the majority of the fractions had no inhibitory effects on the cells (Fig. 2B). Nevertheless, the fraction number 1 (FR1) demonstrated approximately 53±11% inhibition against A431 cell viability (P<0.0001) without affecting the Vero normal cells (Fig. 2B). Therefore, the FR1 was selected for further analysis to identify their potential peptide sequences using LC-MS/MS and *de novo* analyses.

Evaluation of anti-epidermoid cancer activity of obtained peptides from aerial part of A. ebracteatus using A431 cells. The LC-MS/MS and bioinformatic analyses on the FR1 identified four peptide sequences; these were named AE1-AE4, as presented in Table I. Excluding peptide AE1, peptides AE2-AE3 had a molecular weight of ~1,300 g/mol and pI values <7. On the other hand, AE1 was the smallest size about 929 g/mol and had a pI of 9.9.

These peptides were synthesized and their anti-epidermoid cancer activities were evaluated using an MTT assay with

A431 cells. Subsequently, A431 cells were treated with single or combined peptides ranging from 100-500 μM. After 5 days of incubation, the cells were subjected to the MTT assay. The results revealed that each single peptide had no cytotoxicity effects on either A431 or Vero cells (Fig. 3A-D). Nevertheless, 500 μM of the combined treatment with the 4 peptides (in the same ratio, 125 μM of each peptide) revealed ~35 and 50% inhibition against Vero and A431 cells, respectively. (Fig. 3E). These results implied that the mixture of the 4 peptides provided synergistic cytotoxic effects against the cultured cells. Further MTT assays were then performed and the IC_{50} of the combined peptides against A431 cells was equal to 482.8 μM (Fig. 3F). Despite this IC_{50} demonstrating no cytotoxic effect against the normal cells (Fig. 3F), the value was considerably high. Subsequently, the partially purified FR1 (Fig. 2B) was considered and investigated to identify its effect on the apoptosis pathway.

Partially purified protein hydrolysate extracted from A. ebracteatus significantly induces the apoptosis pathway. To investigate the effects of partially purified peptides on the apoptosis pathway, A431 cells were treated with 425.9 ng
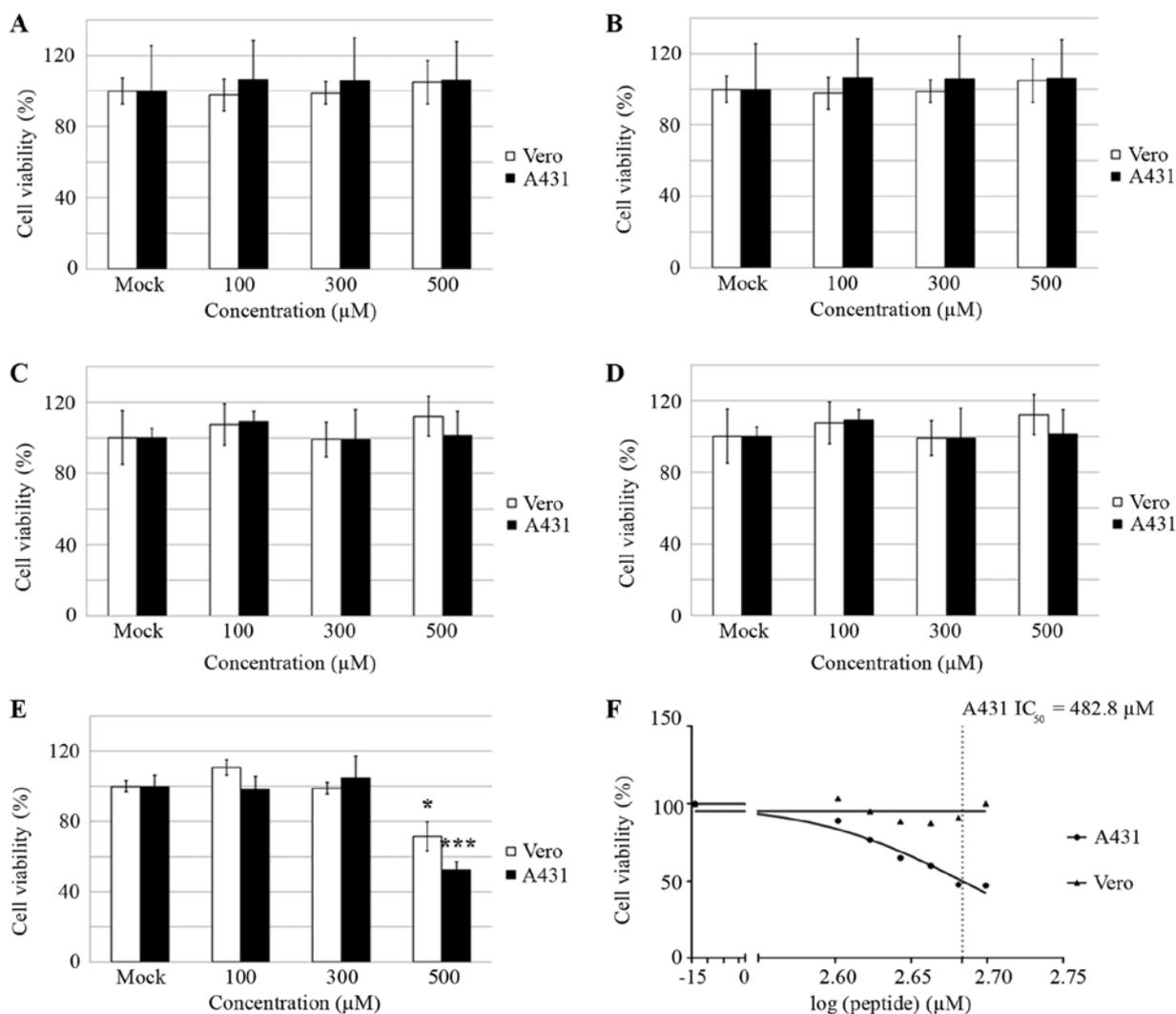
Figure 3. Effects of *A. ebracteatus* peptides on the cell viability of A431 cells using MTT assay. (A) AE1 peptide. (B) AE 2 peptide. (C) AE 3 peptide. (D) AE 4 peptide. (E) Mixed all peptides (same ratio). (F) The IC_{50} of combined peptides. ***$P<0.0001$; *$P<0.001$ when compared with cells without treatment (Mock). IC_{50}, the half inhibitory concentration.

protein/ml of the FR1 and incubated for 3 days rather than 5 days to ensure that enough cell population were presented for testing the apoptotic assay. After 3 days of incubation, cells defined as positive control were treated with 50 µg/ml dihydrochloride hydrate, and apoptosis inducer. All cells were then stained using the Muse® Annexin V and Dead Cell kit that uses a single reagent and two stains to reliably stain and differentiate live, dead and apoptotic cells. The assay relies on the binding of fluorescently labeled Annexin V to phosphatidylserine (PS) molecules, which translocate to the outer surface of the cell membrane upon the onset of apoptosis (20). 7-Amino-Actinomycin D (7-AAD) is a fluorescence indicator that is used as a dead cell marker. It also binds selectively to GC regions of DNA (21). Following staining, apoptotic cells were analyzed using a flow cytometer.

The result from the mock analysis revealed that the majority of the A431 cells were alive (~90%) with 5% of naturally occurring late apoptotic cells and 1% of naturally occurring early apoptotic cells and 1% of cell dead (Fig. 4A and D). The addition of the apoptotic inducer increased the number of late apoptotic cells by 3 fold. However, the apoptotic inducer

only significantly increased numbers of late apoptotic cells (Fig. 4B and D) whereas the effects of FR1 increased numbers of early apoptotic cells by 2 fold, and moderately increased late apoptotic cells by ~1.5 fold when compared with mock (Fig. 4C and D). Therefore, these results suggested that the FR1 contains bioactive compounds that may inhibit the viability of cancer cells by promoting the apoptosis pathway in A431 cells. Subsequently, the effects FR1 on the protein expression of p53, Ser15P, NF-κB (RelA/p65) and cyclin D1 were further investigated.

Effects of partially purified protein hydrolysate extracted from A. ebracteatus on protein expression of p53, NF-κB and cyclin D1. A431 cells are reported to carry a p53 mutation at codon 273 (p53-R273H) (22,23). This R273H mutation affected the DNA-binding ability of p53, thus, p53-R273 could not act as a transcription factor (24). Furthermore, p53-R273H has been reported to induce drug resistance (25). Therefore, whether the FR1 could restore p53 transcriptional activity in A431 cells or not was further investigated via assessing the protein expression levels of p53 and Ser15P. Furthermore,
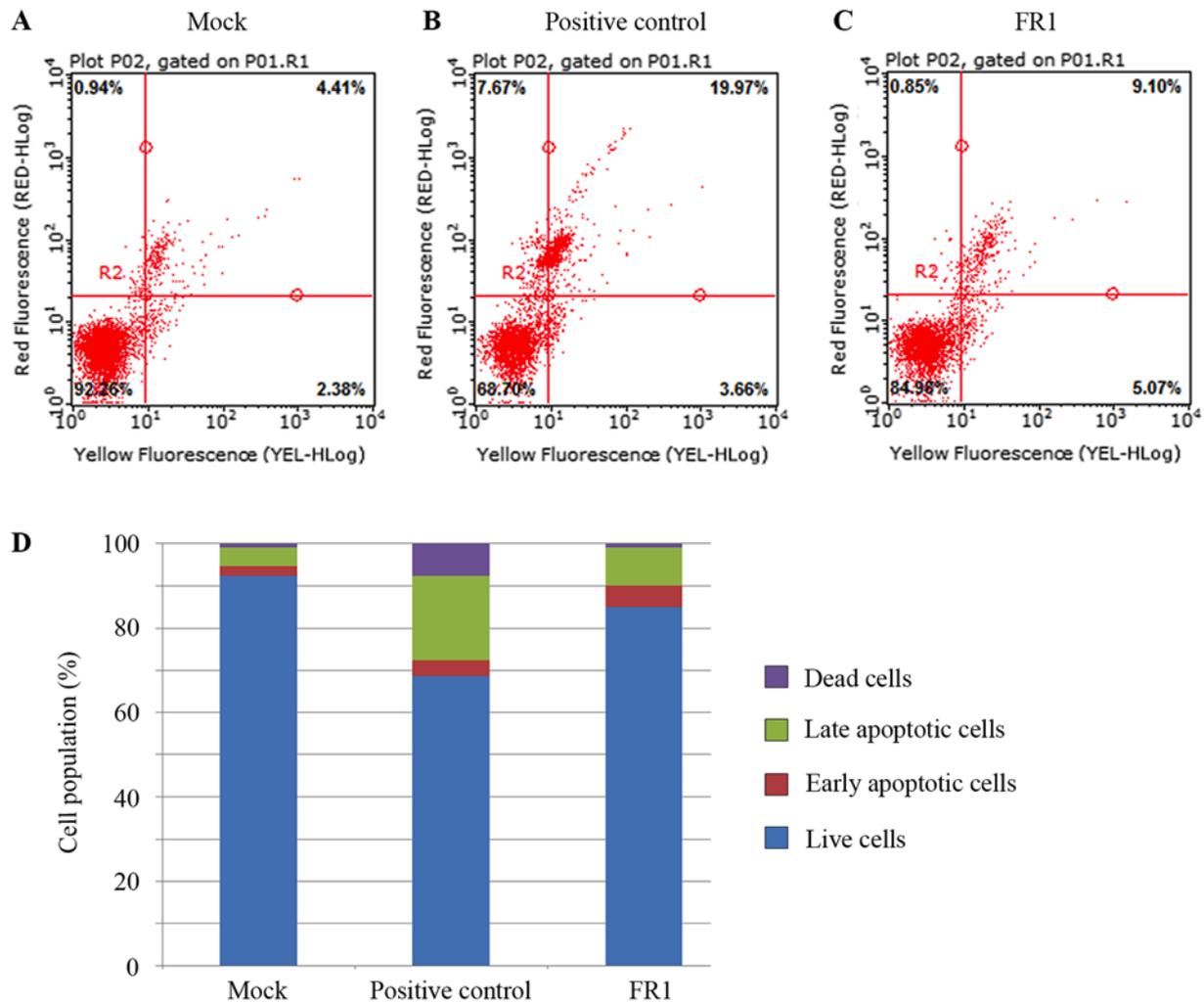
Figure 4. Effects of partially purified *A. ebracteatus* protein hydrolysates on the apoptosis pathway. (A-C) Apoptosis assay analyzed by flow cytometry, with apoptotic and dead cells stained with yellow fluorescence (Annexin V) and red fluorescence (7-AAD). (A) Mock. (B) Positive control was cells treated by 50 *µ*g/ml dihydrochloride hydrate and (C) Cell treated with FR1. (D) A bar graph illustrated results from the apoptosis analysis in (A-C). FR1, fraction number 1.

the effects of partially purified peptide FR1 were also determined via the protein expression levels of another important transcription factor, RelA (p65), which is involved in the regulation of cell apoptosis, the cell cycle and carcinogen transformation (26). Finally, the effects of FR on the protein expression of cyclin D1, an important regulator of cell cycle progression (27), was also investigated.

The results from the western blot analysis indicated that partially purified peptide FR1 from *A. ebracteatus* may increase the expression of p53. On the other hand, FR1 decreased the protein expression of RelA (p65) and cyclin D1 (Fig. 5A and B). Ser15P was barely detected in mock as its expression is often very low in carrying p53 mutants such as cancer cells (28-30); however, FR1 had no effect on the expression levels of Ser15P (Fig. 5A and B).

Discussion

Previously, the stem of *A. ebracteatus* was indicated to contain bioactive polysaccharides such as galactose, 3-O-methylgalactose and arabinose, which could strongly

affect the complement system (31). In addition, the results from the present study suggested that the protein hydrolysate of *A. ebracteatus* (<3 kDa) (Fig. 1) and partially purified protein hydrolysate, FR1, possessed anti-skin cancer activity (Fig. 2). New peptides derived from aerial part of *A. ebracteatus* were also identified (Table I). Although a single peptide could not provide potent cytotoxic effects on the epidermoid cancer cell line, it was demonstrated that the combined peptides resulted in the synergistic effects against cell viability of A431 cells (Fig. 3E and F). Similarly, previous studies have reported that the combination of bioactive peptides displayed a synergism of biological activities. For example, the effects of the combined two antimicrobial peptides, magainin-2 and PGLa were synergistic against *E. coli* and human melanoma cells (32). Co-administration therapy between pro-apoptosis peptide (kla-TAT) and the cationic anticancer peptide (HPRP-Al) also drastically increased anticancer activities against cancer cell lines (33). In the present study it was hypothesized that the synergistic effects from mixtures of several polypeptides, oligopeptides and free amino acids are crucial for the anticancer activity of *A. ebracteatus*. Notably, temperatures ranging from
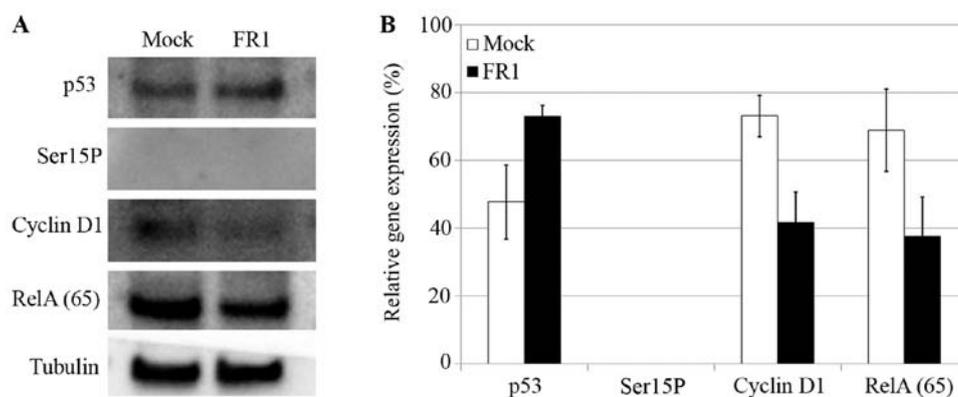
Figure 5. Western blot analysis demonstrating the effects of partially purified *A. ebracteatus* protein hydrolysates on the expression levels of cellular proteins, p53, cyclin D1 and NF-κB. (A) Western blot analysis. (B) Relative protein expression (%) calculated using the ImageJ program. The quantitative data was calculated from four independent experiments. Mock indicates A431 cells without treatment, whereas FR1 represents A431 cells treated with FR1. FR1, fraction number 1; Ser15P, p53-serine 15 phosphorylation.

60-70°C were used for inactivating pepsin activity. This high temperature is likely to denature the majority of temperature sensitive polypeptides or oligopeptides. Therefore, it was proposed that FR1 contains oligopeptides that are relatively stable at temperatures around 60-70°C.

In addition, p53 is a protein that serves an important role in several cellular mechanisms. Elevation of the p53 protein can promote apoptosis in cancer cells. In the p53 signaling pathway, p53 can regulate downstream targets resulting in inhibition of cyclin D1, which leads to inhibition of cell cycle progression (34). In particular, A431 cells carry mutant p53 with a missense mutation (R273H) at a hotspot for DNA binding (23). Mutant versions of p53, such as p53-R273H, have been revealed to lose transcriptional activity, but maintain the ability to enhance tumor progression, metastasis and drug resistance (35,36). Therefore, restoration of p53 transcriptional activity and depletion of mutant p53 may be potential strategies to combat different types of cancer. For example, styrylquin-azoline compound (CP-31398) could induce mitochondrial translocation of mutant p53-R273H in A431 cells, resulting in cytochrome *c* release and the induction of apoptosis (37). On the other hand, geldanamycin or Hsp90 inhibitors may promote the degradation of different varieties of p53 mutants, such as p53-R175H, p53-L194F and p53-R273H (38). In the present study, partially purified peptide FR1 demonstrated the ability to induce apoptosis (Fig. 4). Although FR1 could increase expression levels of p53, it could not upregulate the expression of Ser15P (Fig. 5). Therefore, it was hypothesized that FR1 could induce apoptosis through other proteins, such as NF-κB and associated family members.

In cancer cells, NF-κB is also known as a transcriptional factor, which plays an important role in cell proliferation, cell survival, the cell cycle and cell death (39). Thus, down-regulation of NF-κB may inhibit proliferation and promote apoptosis of cancer cells (40,41). A number of studies have demonstrated that natural products from plants could alter the protein expression levels of NF-κB. For example, crude extracts of *C. formosum* demonstrated the activation of protein expression of p53 and downregulation of NF-κB and cyclin D1 proteins (42). Partially purified peptides from *Gloriosa superba* rhizome could significantly inhibit the cell

viability of colon cancer cell line SW620, by inducing apoptosis signaling via upregulation of p53 and downregulation of (43). In the present study, FR1 decreased expression levels of RelA (p65) (Fig. 5), which is important for NF-κB-mediated gene transactivation (26). Beg *et al* (44) reported that RelA (p65)-deficient mice die during embryonic development due to apoptosis of hepatocytes. It was therefore proposed that FR1 is likely to induce apoptosis via the downregulation of NF-κB and also leads to the depletion of cyclin D1 expression (Fig. 5) as NF-κB promotes the cell-cycle transition from the G_1 to S phase, and cyclin D1 is a downstream signaling molecule of NF-κB (26). Nevertheless, investigation on protein expression of nuclear or chromatin-bound NF-κB should be performed in the future studies because it is a better indicator of NF-κB-mediated transcription.

In conclusion, the present study demonstrated that the partially purified peptide FR1 from aerial parts of *A. ebracteatus* possessed anti-epidermoid cancer activity. FR1 induced apoptosis via the downregulation of RelA (p65) protein expression resulting in decreased cyclin D1 expression and upregulation of p53, but not Ser15P. Therefore, FR1 has the potential for further development as a therapeutic mechanism against anti-non-melanoma skin cancer.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

AK carried out all experiments and drafted the manuscript. DJ performed western blot analysis and revised the manuscript. OR performed LC-MS/MS, analyzed and identified peptide sequences and also drafted the manuscript. NPTT conceived the idea, designed and coordinated the study and prepared the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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