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Research article

Polysaccharide and ethanol extracts of *Anoectochilus formosanus* Hayata: Antioxidant, wound-healing, antibacterial, and cytotoxic activities

Thi-Phuong Nguyen [a], Han N. Phan [a], Thang Duc Do [b], Giap Dang Do [b], Long Hoang Ngo [a], Hoang Dang Khoa Do [a], Khoa Thi Nguyen [a,*]

[a] *NTT Hi-Tech Institute, Nguyen Tat Thanh University, 298-300A Nguyen Tat Thanh Street, Ho Chi Minh City, Viet Nam*
[b] *Department of Plantcell Biotechnology, Institute of Tropical Biology, Vietnam Academy of Science and Technology (VAST), Ho Chi Minh City, Vietnam*

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ABSTRACT

Introduction: Polysaccharide and alcohol extracts of *Anoectochilus formosanus* Hayata have attracted great attention as they exhibit noteworthy properties such as prebiotic and anti-hyperglycemic effects. However, the antioxidant and wound-healing activities of the polysaccharide extract as well as the antibacterial and cytotoxic effects of the ethanol extracts have not been thoroughly uncovered. Therefore, our study investigated these bioactivities of the two extracts prepared from *Anoectochilus formosanus* to broaden understandings of medical benefits of the plant.

Methods: The monosaccharide composition was analyzed by HPAEC-PAD. The antioxidant and wound-healing activities of the polysaccharide extract were evaluated by ABTS and scratch assays, respectively. The broth dilution method was used to determine the antibacterial ability of the ethanol extract. Additionally, the cytotoxic and mechanistic effects of this extract against hepatocellular carcinoma HUH-7 cells was assessed by MTT assay, qRT-PCR and Western blotting methods.

Results: The polysaccharide extract possessed an effective free radical scavenging ability in an ABTS assay (IC_{50} = 44.92 μg/ml). The extract also ameliorated wound recovery in a fibroblast scratch assay. Meanwhile, the ethanol extract was able to inhibit the growth of *Staphylococcus aureus* (MIC = 2500 μg/ml), *Bacillus cereus (*MIC = 2500 μg/ml), *Escherichia coli (*MIC = 2500 μg/ml), and *Pseudomonas aeruginosa* (MIC = 1250 μg/ml). Additionally, it repressed the viability of HUH-7 cells (IC_{50} = 53.44 μg/ml), possibly through upregulating the expression of *caspase 3* (*CASP3*), *CASP8*, and *CASP9* at both mRNA and protein levels.

Conclusion: The polysaccharide extract of *A. formosanus* exhibited the antioxidant and wound-healing properties whereas the ethanol extract showed the antibacterial activity and cytotoxicity against HUH-7 cells. These findings specify notable biological effects of the two extracts which could be of potential use in human healthcare.

* Corresponding author.
E-mail address: khoant@ntt.edu.vn (K.T. Nguyen).

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1. Introduction

Anoectochilus formosanus has long been considered as a valuable folk medicine in Asian countries [[1]]. This perennial "jewel orchid" is commonly used for patients with fever, inflammation, cancer, diabetes, osteoporosis, cardiovascular diseases, and liver disorders [[2]]. Researchers have tried to gain insights into the benefits of *A. formosanus* by investigating the pharmacological properties and mechanisms of various extracts from this plant. Among the noteworthy extracts, the polysaccharide extract was intensively characterized due to its prospects in the complementary and alternative therapies. An indigestible polysaccharide extract from *A. formosanus* increased the growth of *Bifidobacterium* both *in vitro* and *in vivo*, suggesting it has a prebiotic effect [[3]]. A type II arabinoglycan purified from the crude polysaccharide extract was able to reduce tumors and leukopenia in CT26 colon cancer-bearing mice. These results were possibly explained by the effect of polysaccharide on the expression of cytokines, chemokines, costimulatory receptors as well as the number of immune cells [[4,5]].

Another well-studied extract from *A. formosanus* was prepared from the alcohol solvent. The methanol extract, for example, was reported to be capable of scavenging free radicals and reducing the concentration of blood glucose in fructose-induced hyperglycemic mice. Moreover, this extract significantly inhibited the proliferation of the SCC-25 oral cancer cells by decreasing both mRNA and protein levels of programmed death 1-ligand 1 (PD-L1), an important regulator of activated T cell and tumor interaction. This study suggests potential for the methanol extract in the treatment of cancer patients with hyperglycemic condition and *PD-L1* overexpression [[6]].

Due to the significant importance of the polysaccharide and alcohol extracts of *A. formosanus*, we investigated rarely characterized bioactivities of these two extracts. Ethanol was used as a solvent in the alcohol extract so that two extracts, polysaccharide and ethanol, could be co-prepared in an extraction procedure. In our study, the polysaccharide extract (APE) exhibited antioxidant activity and promoted the wound-healing process, suggesting potential applications of this polysaccharide extract in antioxidant and wound-healing therapies. Meanwhile, the ethanol extract of *A. formosanus* (AEE) repressed the growth of *Staphylococcus aureus*, *Bacillus cereus, Escherichia coli, Pseudomonas aeruginosa* as well as the viability of hepatocellular carcinoma HUH-7 cells. The AEE is likely to trigger HUH-7 cell death by increasing the transcript and protein levels of apoptotic genes including *caspase 3* (*CASP3*), *caspase 8* (*CASP8*), and *caspase 9* (*CASP9*). These results would serve as a basis for further studies in determining compounds of the ethanol extract and mechanisms underlying antibacterial and cytotoxic activities.

2. Material and methods

2.1. Preparation of polysaccharide and ethanol extracts

A. formosanus plants were genetically identified at Biotechnology Center of Ho Chi Minh City, Vietnam by sequencing the internal transcribed spacer 1 (ITS1) and ITS2 in the polycistronic rRNA precursor transcript [[7,8]]. The plants were *in vitro* cultured and a voucher specimen (number AF19) was deposited at Plant Cell Technology Lab, Institute of Tropical Biology, Vietnam. The polysaccharide and ethanol extracts from tissue-cultured plants were prepared as previously described with some modifications [[9,10]]. In brief, the freeze-dried powder of *A. formosanus* was submerged with absolute ethanol at room temperature for 2 days. The ethanol-soluble fraction was lyophilized to obtain the ethanol extract, designed here as AEE. The ethanol-insoluble residues were subsequently extracted twice with hot water at 60 °C for 6 h each time. The combined extracts were concentrated in a rotary evaporator followed by the precipitation with ethanol. The precipitate was washed with ethanol, air dried and dissolved in water. The obtained solution was deproteinized by $CaCl_2$ solids with the final concentration 5% (w/v). Steps of ethanol precipitation, wash, air dry and water dissolution were repeated in order to collect the crude polysaccharide extract, designed here as APE. Protein content in the APE was measured by the Bradford method.

2.2. Determination of monosaccharide composition in the polysaccharide extract

The APE was hydrolyzed in 4 M trifluoroacetic acid (TFA) at 70 °C for 48 h and then neutralized by 4 M NaOH. The mixture was diluted with distilled deionized water and centrifuged at 16000 g at 10 °C for 10 min. The supernatant was dissolved in water and analyzed by high-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) [[11]]. The monosaccharide composition was identified using a Dionex ion chromatography ICS 3000 (Sunnyvale, USA) with a Carbopac PA20 analytical column (3 × 150 mm, P/N060142, Dionex Corp., USA) and a Carbopac PA20 guard column (3 × 30 mm, P/N 060,144, Dionex Corp., USA). The column temperature was set at 30 °C. Monosaccharides including glucose, arabinose, galactose, fructose, xylose, sorbitol, mannose, fucose, rhamnose, sucrose, maltose, and lactose (Sigma-Aldrich, Germany) were used as standards. Deionized water and 200 mM NaOH were used as solvents. The isocratic eluent at 10 mM NaOH was run for 30 min and followed by 10 min of 200 mM NaOH solution. The flow rate was constantly set at 0.4 ml/min. The data was recorded and analyzed by Chromeleon 6.8. Total sugar content was calculated from the peak areas of all identified monosaccharides in the HPAEC-PAD chromatogram. Glucose was used to establish standard curve to quantify total sugar content.

2.3. Antioxidant assay

The 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) (Sigma-Aldrich, Germany) assay to determine the free radical scavenging activity of an extract was conducted as previously described [[12]]. In our experiment, diluted

concentrations of the APE were assessed their anti-oxidant activities by measuring the absorbance at 734 nm in an UV-VIS spectrometer (Genway, USA). Ascorbic acid (Sigma-Aldrich, Germany) was used as positive control. The percentage of ABTS-scavenging activity was calculated as follows:

$$\text{ABTS-scavenging activity } (\%) = [A_0 - (A_1 - A_{Blank})]/A_0 \times 100 \quad (1)$$

Where, A_{Blank} is the absorbance of the extract without ABTS, A_0 is the absorbance of sample with ABTS at time 0, and A_1 is the absorbance of sample with ABTS after 30 min of incubation.

The IC_{50} value of the sample (the concentration of sample required to inhibit 50% of the ABTS free radicals) was calculated by GraphPad Prism software.

2.4. Wound-healing scratch assay

The scratch assay for human dermal fibroblasts (HDFa) was performed as previously described [[13]]. Fibroblasts were seeded into a 12-well plate at cell density of 5×10^5 cell/ml and incubated with 5% CO_2 at 37 °C for 24 h. Scratches were made by pipette tips and the cells were then treated with APE at the concentrations of 0, 25, 50, and 100 μg/ml, in which distilled deionized water (negative control) was indicated as 0 μg/ml of APE. The plate was kept with 5% CO_2 at 37 °C. Images were taken at time points 0 h, 12 h and 24 h. The areas of scratches were measured by Image J software. Wound-healing abilities of the samples at 12 h and 24 h were calculated as follows:

$$\text{Wound closure } (\%) = 100\% \times (S_0 - S_t)/ S_0$$

Where, S_0 is the scratch area of the samples at 0 h, S_t is the scratch area of the samples at 12 h or 24 h.

2.5. Quantification of the total phenolic content in the ethanol extract

The total phenolic content was measured according to the reported method [[14]]. The AEE (0.1 ml) was mixed with Folin Ciocalteu's reagent (Sigma-Aldrich, Germany) and kept at room temperature for 15 min. Then, 0.5 ml of saturated Na_2CO_3 was added and the mixtures were incubated in dark for 30 min. The total phenolic content was determined in an UV-VIS spectrometer (Genway, USA) at the wavelength of 760 nm. Gallic acid (Sigma-Aldrich, Germany) was used as standard reagent. The total phenolic content was expressed in terms of gallic acid equivalent (GAE mg/g of extract).

2.6. Antibacterial test

The antimicrobial ability of the AEE was determined by the minimum inhibitory concentration (MIC) via broth dilution method as previously described [[15]]. Two Gram-positive bacteria, *Staphylococcus aureus* (ATCC 25923) and *Bacillus cereus* (ATCC 10876), and two Gram-negative bacteria, *Escherichia coli* (ATCC 25922) and *Pseudomonas aeruginosa* (ATCC 9027) were used in the assay. Serial dilutions of the AEE were prepared in a microtiter plate containing Mueller Hinton broth (Himedia, India). The bacterial inoculum was added to give a final density of 5×10^6 CFU/mL. Streptomycin sulfate (Sigma-Aldrich, Germany) was used as positive control. The plate was kept at 37 °C for 24 h. Resazurin (Sigma-Aldrich, Germany) was added to the wells and further incubated for 2 h. The MIC was considered as the lowest concentration of the extract that completely inhibits the bacterial growth.

2.7. Cell viability assay

The procedure of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) (MTT) assay was based on a previous study [[16]]. The Hep3B, HUH-6, and HUH-7 cells were seeded into a 96-well plate at 5×10^4 cells per well and incubated with 5% CO_2 at 37 °C. After 24 h, the old medium was replaced by various concentrations of the AEE (0, 3.125, 6.25, 12.5, 25, 50, 100, and 200 μg/ml), doxorubicin (DOX) (Fresenius Kabi, Germany) and dimethyl sulfoxide (DMSO) (Sigma-Aldrich, Germany). After 72 h of treatment, 5 mg/ml of MTT (Sigma-Aldrich, Germany) was added into each well and incubated with 5% CO_2 at 37 °C for 3.5 h. Subsequently, the medium was replaced by DMSO in order to dissolve the crystals. Absorbance of samples was read at 595 nm by an ELISA Reader (BioTek, USA). The percentage of cell viability inhibition (cell death) was estimated by the following formula:

$$\text{Inhibition } (\%) = 100 - [100 \times (\text{ASample} - A_{B1})/(\text{AControl} - A_{B2})]$$

Where, ASample is the absorbance of the sample, A_{B1} is the absorbance of the sample background, and $A_{Control}$ is the absorbance of the control, A_{B2} is the absorbance of the control background.

To define the potential cytotoxicity of the extract, the IC_{50} value was calculated as the concentration that reduces the cell viability by 50%.

2.8. Quantification of mRNA expression

HUH-7 cells were treated with DMSO (negative control) and AEE at the IC_{50} concentration (53.44 μg/ml) for 24 h. Cells subsequently were harvested for total RNA extraction using TriSure (Bioline, USA). RNA samples were converted to cDNA using LunaScript

RT SuperMix Kit (New England BioLabs, USA) after the treatment with Dnase I (New England Biolabs, USA) to remove genomic DNA. Luna Universal qPCR Master Mix (New England Biolabs, USA) was used in the quantitative PCR (qPCR) to assess mRNA levels of *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*), *CASP3, CASP8* and *CASP9*. *GAPDH* was employed as the reference gene to calculate the relative expression of *CASP3, CASP8* and *CASP9* based on the delta threshold cycle method [[17]]. Primer sequences (5'→3') used in the qPCR are as follows: *CASP3*-F: TCGCTTTGTGCCATGCTGAA, *CASP3*-R: ACTCAAATTCTGTTGCCACC, *CASP8*-F: AATGGAACACACTTGGATGC, *CASP8*-R: GCTCTACTGTGCAGTCATCG, *CASP9*-F: TTGAGGACCTTCGACCAGCT, *CASP9*-R: GAPD-CAACGTACCAGGAGCCACTC, *GAPDH*-F: AGCCACATCGCTCAGACAC, and *GAPDH*-R: GCCCAATACGACCAAATCC.

2.9. Western blotting

HUH-7 cells were incubated for 48 h after treated with DMSO (negative control) and AEE at the IC_{50} concentration. Subsequently, they were harvested for total protein extraction using RIPA buffer and protein inhibitor cocktail (Sigma-Aldrich, Germany). Total cell lysates were loaded on 15% polyacrylamide gels and transferred onto a nitrocellulose membrane (GE Healthcare, USA) using electrophoresis gel system (Bio-Rad, USA) and semi-dry transfer system (Invitrogen, USA). The blots were first incubated overnight at 4 °C with primary antibodies against caspase 3 (c8487, Sigma-Aldrich, Germany), caspase 8 (c4106, Sigma-Aldrich, Germany), caspase 9 (c4106, Sigma-Aldrich, Germany) and β-actin (A1978, Sigma-Aldrich, Germany). After wash steps, the blots were probed with horseradish peroxidase (HRP)-conjugated secondary antibodies (AP307P and AP308P, Sigma-Aldrich, Germany) and then detected in a G-box imager (Syngene, India) using chemiluminescence detection system (Cytiva, USA). Protein band intensities were analyzed by ImageJ and GraphPad Prism software.

2.10. Statistical analysis

The results for ABTS-scavenging activity, percentage of wound closure, total phenolic content, MIC values, percentage of cell viability inhibition, IC_{50} values, and mRNA/protein relative levels were presented as mean ± SD of biological triplicates. Statistical differences between samples were calculated by one-way ANOVA with Tukey's posttest for wound closure and Student's t-test for mRNA/protein levels in GraphPad Prism version 6.0. Significant values were accepted as $p \leq 0.05$.

3. Results

3.1. Monosaccharide composition of the APE

The APE in our study comprised of approximately 90% polysaccharide and 0.03% protein. HPAEC-PAD analysis showed that sorbitol, arabinose, galactose, glucose, xylose, and fructose were present in the APE. Among identified monosaccharides, glucose contributed up to 94.13% followed by xylose (2.55%) and fructose (2.07%). Arabinose and galactose were found in moderate amounts (0.35% and 0.78%, respectively), whereas sorbitol existed in a low level (0.12%) in the APE (Fig. 1 and Table 1). Glucose, xylose, arabinose and galactose detected in the APE were consistent with previously purified polysaccharides from *A. formosanus* and a related species, *A. roxburghii* [[3,10]]. The presence of these monosaccharides might indicate their importance in the structures and properties of polysaccharides from both orchids.

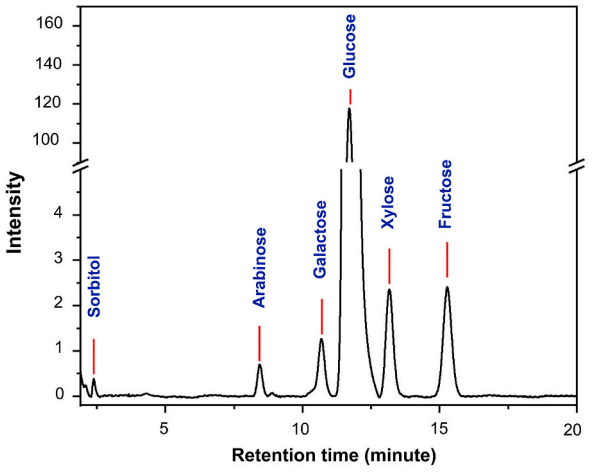

Fig. 1. Monosaccharides identified in the APE by HPAEC-PAD. Monosaccharide derivatives were analyzed using a Dionex ion chromatography ICS 3000 with a Carbopac PA20 analytical column (3 × 150 mm) and a Carbopac PA20 guard column (3 × 30 mm).

Table 1
The chemical composition of the APE. Monosaccharide content was calculated by the percentage of the peak area measured in the HPAEC-PAD chromatogram.

Monosaccharide	Percentage (%)
Sorbitol	0.12
Arabinose	0.35
Galactose	0.78
Glucose	94.13
Xylose	2.55
Fructose	2.07

3.2. Antioxidant activity of the APE

Since polysaccharide extracts can efficiently scavenge free radicals, we investigated this ability in the APE. In the ABTS assay, the APE showed antioxidant activity in a dose-dependent manner in which it was able to scavenge 40% of ABTS free radicals at the concentration of 40 μg/ml and 100% at the concentration of 160 μg/ml (IC_{50} = 44.92 μg/ml) (Table 2 and Supplementary Fig. 1). Although the antioxidant activity of the APE was slightly less effective than that of vitamin C (IC_{50} = 10.80 μg/ml), it is typical to natural polysaccharides [[18,19]]. Our result demonstrated that the APE might be a promising polysaccharide extract in scavenging free radicals for future investigation.

3.3. Wound-healing activity of the APE

Skin healing is a prominent property of the polysaccharide extract as it can be applied in the design of dressings or ointments to treat skin wounds [[20]]. Various studies showed that plant polysaccharide extracts could promote wound recovery both *in vitro* and *in vivo*. Polysaccharides from *Bletilla striata*, for example, supported L929 cells to proliferate and migrate in the wound-healing process [[21]]. Therefore, it is in our interest to study whether the APE possesses a similar effect. In a scratch assay on human fibroblasts, the area of wound closure was moderately ameliorated on the plate treated with all of the tested concentrations (25, 50, and 100 μg/ml) as compared to that on the plate treated with 0 μg/ml of APE after 12 h. Remarkably, 50 and 100 μg/ml of APE were capable of recovering wounds approximately 1.8 and 1.6 folds, respectively, higher than control after 24 h of supplementation, suggesting 50 μg/ml of APE is likely a suitable concentration in this assay (Fig. 2A and B). These results indicate another attractive activity of the APE which could inspire further studies for the underlying mechanism and pharmaceutical application.

3.4. Total phenolic content in the AEE

We first determined the total phenolic content in the AEE since phenolic compounds exhibit a broad range of pharmacological properties such as antioxidant, antimicrobial, anti-tumor, and anti-inflammatory activities [[22]]. Our analysis showed that the AEE contained a significant amount of phenolic compounds (11.78 mgGAE/g), suggesting it has promising bioactivities against bacteria and cancer cells.

3.5. Antibacterial activities of the AEE

As the polar extracts of *A. roxburghii*, a species close to *A. formosanus*, showed the antibacterial activity against *Escherichia coli*, *Bacillus subtilis*, and *Bacillus thuringensis*, it is valuable to know if the AEE could exhibit similar activities [[23]]. In our study, *S. aureus*, *B. cereus*, *E. coli*, and *P. aeruginosa* were selected for the antibacterial assay. It was observed that *P. aeruginosa* was the most susceptible strain to the AEE (MIC = 1250 μg/ml). The other bacteria strains (*S. aureus*, *B. cereus*, and *E. coli*) were also moderately inhibited by the AEE treatment (MIC = 2500 μg/ml) (Table 3). These results imply the inhibitory potential of the AEE against various Gram-positive and -negative bacteria and further studies are required to investigate phytochemicals responsible for the antibacterial activity of the AEE.

Table 2
IC_{50} values of APE and ascorbic acid (vitamin C) in the ATBS assay. Ascorbic acid was used as positive control.

Samples	IC_{50} ((μg/ml)
APE	44.92 ± 2.27
Ascorbic acid (vitamin C)	10.80 ± 0.07

APE: *A. formosanus* polysaccharide extract. IC50: half-maximal inhibitory concentration.

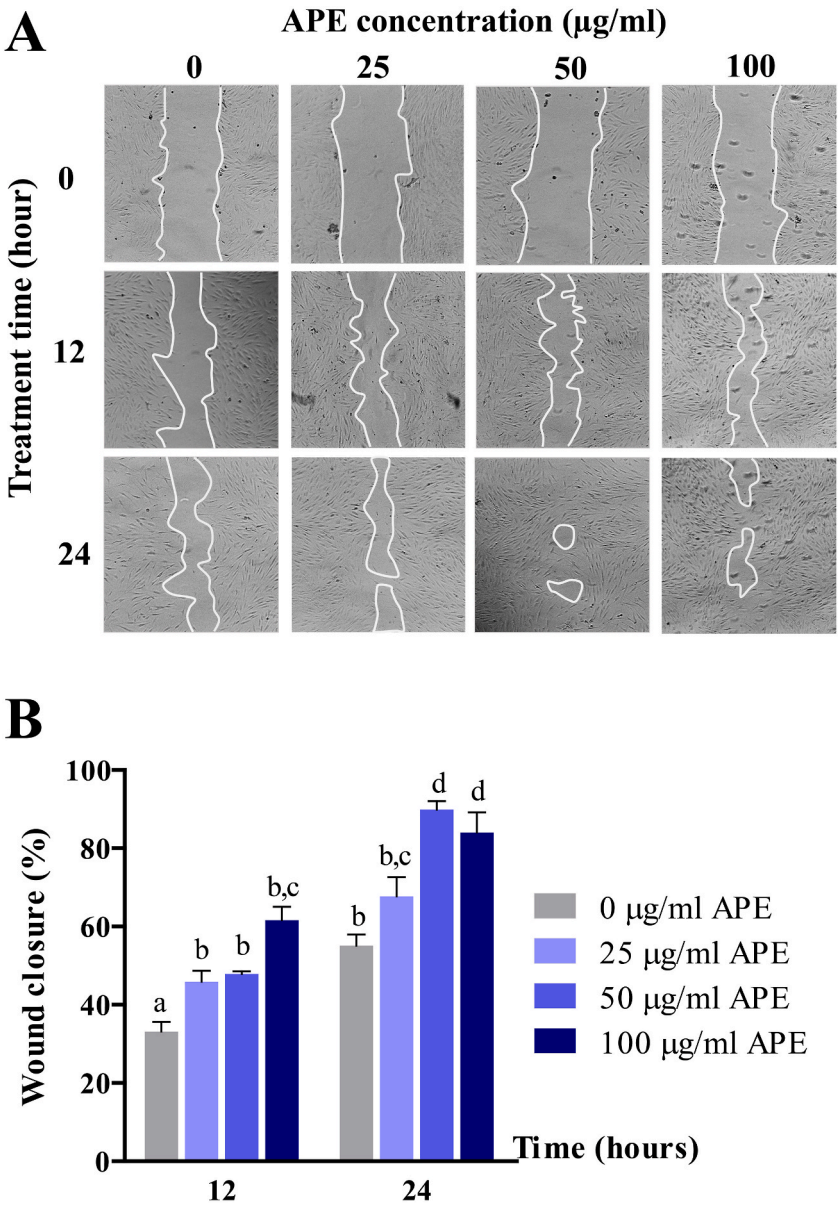

Fig. 2. The APE exhibited wound-healing activity *in vitro*. (A) Images of wound closure in human dermal fibroblasts treated with 0, 25, 50, and 100 μg/ml of APE at 0, 12, and 24 h. (B) Quantification of wound closure (%) at 12 and 24 h. Statistical significance was indicated by different letters.

Table 3
MIC values of AEE and streptomycin against *S. aureus*, *B. cereus*, *E. coli*, and *P. aeruginosa*. Streptomycin was used as positive control.

Bacterial strains	MIC (μg/ml)	
	AEE	Streptomycin
E. coli	2500 ± 0	6.25 ± 0
P. aeruginosa	1250 ± 0	6.25 ± 0
S. aureus	2500 ± 0	6.25 ± 0
B. cereus	2500 ± 0	1.5625 ± 0

AEE: *A. formosanus* ethanol extract. MIC: minimum inhibitory concentration.

3.6. Cytotoxic activities of the AEE against HUH-7 cells

We next investigated the potential cytotoxicity of the AEE against hepatocellular carcinoma HUH-7 cells since the cell line is considered as a model system to study liver cancer and the molecular pathways underlying the cytotoxic effect of an extract [[24]]. The viability of the cancer cells was suppressed by the AEE treatment in a concentration-dependent manner. At the concentration of 50 μg/ml, the AEE was able to cause approximately 40% of cell death, whereas it was 70% at the concentration of 200 μg/ml (IC_{50} = 53.44 μg/ml) (Fig. 3A and Supplementary Table 1). It was reported that the methanol extract of *A. formosanus* increased the transcript expression of several apoptotic activators in the SCC25 oral cancer cells [[6]]. Therefore, we determined the mRNA and protein levels of several *caspase* genes when HUH-7 cells were treated with AEE at the IC_{50} concentration. It was shown that *CASP3*, *CASP8*, and *CASP9* mRNA levels were upregulated after the AEE treatment (Fig. 3B). Consistent with the mRNA levels, the protein levels produced by these genes were also increased in the AEE-treated cells (Fig. 4). These results suggest a possible function of the AEE in promoting apoptosis in HUH-7 cells.

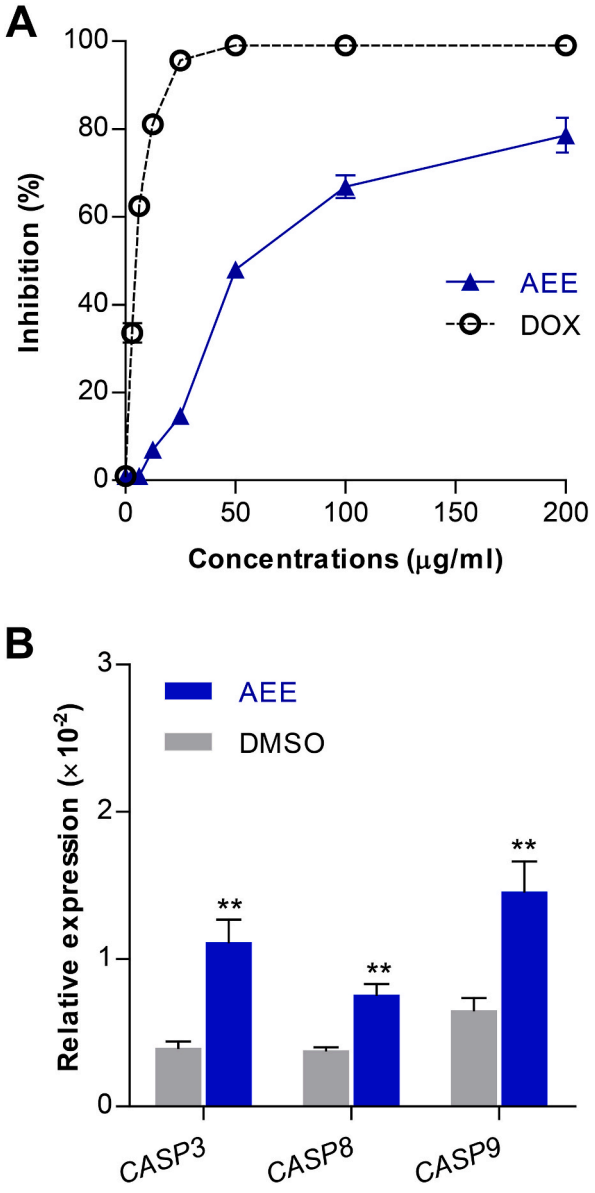

Fig. 3. The AEE inhibited the viability of hepatocellular carcinoma HUH-7 cells by increasing the mRNA expression levels of *CASP3, CASP8* and *CASP9*. (A) The cell viability inhibition (%) of various AEE concentrations against HUH-7 cells in the MTT assay. (B) Quantification of the relative mRNA levels of *CASP3, CASP8* and *CASP9* in the HUH-7 cells treated with DMSO and AEE. The mRNA levels were indicated relative to *GADPH* expression. Statistical significance, **: $p \leq 0.01$.

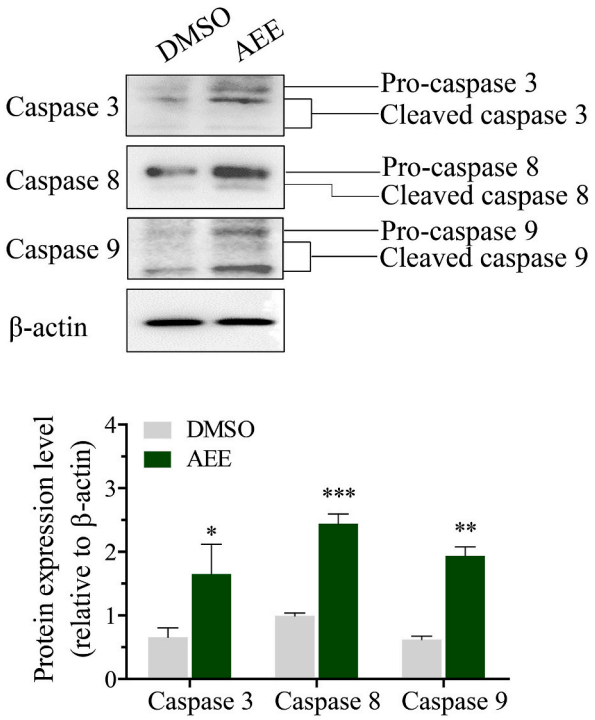

Fig. 4. The AEE elevated the protein levels of caspase 3, caspase 8 and caspase 9 in HUH-7 cells. Cancer cells were treated with DMSO or AEE at the IC_{50} concentration for 48 h. β-actin was used as loading control. The protein levels of caspase 3, caspase 8 and caspase 9 in the AEE-treated cells were indicated relative to β-actin. The band intensities were quantified by ImageJ software. Statistical significance, *: $p \leq 0.05$; **: $p \leq 0.01$; ***: $p \leq 0.001$. Images of original Western blots are shown in Supplementary Fig. 3.

4. Discussion

The polysaccharide extract of *A. formosanus* exhibited *in vitro* antioxidant activity in our study. It was proposed that structure characteristics of the polysaccharide including chemical composition, molecular weight, type of branches, glycoside linkages, and conjugates influence the free radical scavenging capacity, reducing power and chelating ability on ferrous iron [[25]]. The polysaccharide extract obtained in this study might have structural features facilitating the antioxidant activity. Another factor, the freeze-drying method, which was used for our polysaccharide extraction procedure could also enhance the antioxidant property since this method was considered as an effective method for generating high free radical-scavenging polysaccharides [[25]].

Wound-recovering ability is a newly characterized bioactivity of the polysaccharide extract from *A. formosanus*. The result of the *in vitro* scratch assay revealed that the APE might shorten the duration of the wound-healing process by accelerating cell proliferation and migration at the site of injury. It was reported that the enhancing activity of a polysaccharide extract is possibly rooted from its antioxidant activity as the reduction of free radical levels in the inflammatory phase of wound repair helps to prevent cell and tissue damage. Additionally, the polysaccharide extract might alter the expression of transforming growth factor β (TGF-β), vascular endothelial growth factor (VEGF), collagen and cytokines to hasten the injury recovery [[26]]. It is necessary to elucidate which mechanisms were used by the APE to ameliorate the wound-healing process.

Plant-derived alcohol extracts can inhibit the growth of various Gram-positive and -negative bacteria as they contain high amounts of antibacterial compounds such as polyphenols and alkaloids. For example, methanol and ethanol extracts of hawthorn *Crataegus pinnatifida,* rich in flavonoid and polyphenol contents, showed effective antibacterial activities against *Staphylococcus aureus*, *Bacillus subtilis*, *Listeria monocytogenes*, *Escherichia coli*, *Salmonella typhimurium*, *Shigella dysenteriae*, and *Pseudomonas aeruginosa*. The action mechanism of the extracts on *S. aureus* was proven via the damaging of cell membrane permeability and integrity, decreasing the activity of intracellular enzyme ATPase, and by inducing apoptosis [[27]]. Since polyphenols were present in a substantial amount in our ethanol extract of *A. formosanus*, we can speculate that these phenolic compounds might be bioactive components functioning in the antibacterial effect of the AEE. The exact phytochemicals and underlying mechanisms need to be investigated in further studies.

Previous studies showed that the methanol extract of *A. formosanus* at the concentration 1 mg/ml inhibited more than 82% of human oral cancer SCC-25 cell proliferation. As tumor cells overexpress *PD-L1* to escape from T-cell cytotoxicity, the extract prevented this immune inhibitory effect by suppressing the mRNA level and ultimately the protein level of PD-L1. Moreover, the expression of inflammatory genes (*cyclooxydase 2* (*COX-2*), *tumor necrosis factor-α* (*TNF-α*)), proliferative genes (*Cyclin D1* (*CCND1*), *c-Myc*, and *matrix metalloproteinase-1* (*MMP-1*)) were decreased, whereas pro-apoptotic genes (*Bcl-2 associated agonist of cell dead* (*BAD*) and *caspase 2* (*CASP2*)) were increased in SCC-25 cells treated with the methanol extract [[6]]. In our study, the transcript levels of *caspase*

genes, including *CASP3, CASP8* and *CASP9,* were upregulated in the AEE-treated HUH-7 cells. Their protein levels were consistently elevated in these cells. It is interesting to study if the expression patterns of inflammatory and proliferative genes are also altered by the AEE treatment in HUH-7 cells since these effects were previously observed for the methanol extract in SCC-25 oral cancer cells [[6]]. The inhibition of cell viability by the AEE was also observed in two other hepatocellular carcinoma cells, Hep3B and HUH-6 (Supplementary Fig. 2 and Table 1), suggesting the effect of the AEE on various liver cancer cell lines.

Various strategies have been studied for the application of polysaccharide and ethanol extracts from plants, including films, hydrogels, and even oral administration [[28]]. Films with outstanding physicochemical and biological properties are prepared by molding these extracts with different scaffolds. For example, a compact and homogenous film composed of fenugreek (*Trigonella foenum-graecum*)-derived polysaccharide and poly(vinyl alcohol) (PVA) displayed strong antioxidant activity *in vitro* and improved wound recovery in rat burn model [[29]]. The combinations of PVA and ethanol extracts from flowers of the lavender plant (*Lavandula angustifolia*), leaves of the peppermint plant (*Mentha piperita*), hemp plant (*Cannabis sativa*), verbena plant (*Verbena officinalis*) and sage plant (*Salvia officinalis folium*) generated biomaterials with significant antibacterial effects on *S. aureus* and *E. coli* [[30]]. A cellulolic nanofiber film incorporated with the ethanol extract of mangosteen *Garcinia mangostana* peel showed enhanced cytotoxic activity against B16F10 melanoma and MCF-7 breast cancer cells, suggesting its application potential in cancer therapy [[31]]. Another efficient method has been developed using plant-sourced extracts and hydrogel scaffolds. A hydrogel composite based on the polysaccharide extract from Egyptian oat grains (*Avena sativa*) and hydroxyethylcellulose (HEC) showed healing activity in rats equivalent to a conventional product (Mebo® ointment) [[32]]. A mixture of the ethanol extract from *Epilobium angustifolium* and HEC also produced a topical hydrogel capable of inhibiting the growth of various Gram-negative and -positive bacteria [[33]]. With all of these promising strategies, the clinical application of *A. formosanus* extracts in our study needs to be properly evaluated in the future.

Taken together, this study indicated rarely characterized bioactivities of two extracts of *A. formosanus*. The polysaccharide extract showed *in vitro* antioxidant and wound-healing activities while the ethanol extract exhibited antibacterial property on several Gram-positive and -negative bacteria and cytotoxic effect on hepatocellular carcinoma HUH-7 cells. These results suggest great beneficial uses of the two extracts in complementary and alternative therapies since only a few plants have been reported to possess wound-healing, antibacterial and anticancer activities. In addition, *A. formosanus* is very well-known in traditional medicine in Asian countries. Therefore, the application of the extracts from this plant may offer great attraction to consumers similar to the extracts from Lingzhi mushroom (*Ganoderma lucidum*) [[34,35]].

Declarations

Author contribution statement

Thi-Phuong Nguyen: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper. Han N. Phan: Performed the experiments; Analyzed and interpreted the data. Thang Duc Do: Performed the experiments; Contributed reagents, materials, analysis tools or data. Giap Dang Do, Long Hoang Ngo, Hoang Dang Khoa Do: Contributed reagents, materials, analysis tools or data. Khoa Thi Nguyen: Conceived and designed the experiments; Wrote the paper.

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Data availability statement

Data included in article/supp. Material/referenced in article.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e13559.

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