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Unveiling the Cytotoxic and Apoptosis-Inducing Abilities of an Edible Bird's Nest Extract in Human Hepatocellular Carcinoma Huh-7 Cells

(Mendedahkan Keupayaan Aruhan Sitotoksik dan Apoptosis Ekstrak Sarang Burung dalam Sel Karsinoma Hepatosel Manusia Huh-7)

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ABSTRACT

Edible bird's nest (EBN) is renowned for diverse pharmacological properties; however, its ability to cause cancer cell death has not been found in recent studies. This study investigates the phytochemical composition of EBN ethanol extract (EEE), the cytotoxic and apoptosis-inducing effects of the extract on human hepatocellular carcinoma Huh-7 cells. Qualitative and quantitative tests showed that proteins and peptides were abundant in the EEE. In the MTT assay, the extract exhibited cytotoxic activity against Huh-7 cells (IC_{50} = 58.57 ± 2.66 µg/mL, 53.15 ± 3.07 µg/mL, and 55.27 ± 1.85 µg/mL at 24 h, 48 h, and 72 h of incubation, respectively). Annexin-V/PI staining suggested that the extract provoked apoptosis in Huh-7 cells by deregulating the expression patterns of apoptotic markers including caspase 3, caspase 8, caspase 9, and survivin. These findings demonstrate the potential of the EEE as a promising anticancer candidate for further studies.

Keywords: Apoptosis; cytotoxicity; edible bird's nest; human hepatocellular carcinoma

ABSTRAK

Sarang burung (EBN) terkenal dengan sifat farmakologi yang pelbagai; walau bagaimanapun, keupayaannya untuk menyebabkan kematian sel kanser masih tidak diketahui. Penyelidikan ini mengkaji komposisi fitokimia ekstrak etanol EBN (EEE) dan kesan aruhan ekstrak terhadap aktiviti sitotoksik dan apoptosis pada sel karsinoma hepatosel manusia Huh-7. Ujian kualitatif dan kuantitatif menunjukkan bahawa protein dan peptida banyak terdapat dalam EEE. Dalam ujian MTT, ekstrak menunjukkan aktiviti sitotoksik terhadap sel Huh-7 (IC_{50} = 58.57 ± 2.66 µg/mL, 53.15 ± 3.07 µg/mL dan 55.27 ± 1.85 µg/mL masing-masing pada 24 jam, 48 jam dan 28 jam eraman). Pewarnaan Annexin-V / PI mencadangkan bahawa ekstrak EEE mencetuskan apoptosis dalam sel Huh-7 dengan menyahkawal atur corak pengekspresan penanda apoptosis termasuk caspase 3, caspase 8, caspase 9 dan survivin. Penemuan ini menunjukkan potensi EEE sebagai calon antikanser yang berpotensi untuk kajian lanjut.

Kata kunci: Apoptosis; karsinoma hepatosel manusia; kesitotoksikan; sarang burung

INTRODUCTION

Edible bird's nest (EBN) is produced from the saliva of swiftlets belonging to two genera, *Aerodramus* and *Collocalia*. Known as 'Caviar of the East', EBN has been considered as one of the most prized tonic delicacy in Asian countries due to its nutraceutical values (Lee et al. 2021). EBN mainly contains mucin glycoproteins composed of proteins (62-63%) and carbohydrates (25.62-27.26%). Minerals (sodium, potassium, calcium, magnesium, phosphorus, and iron, 2.1%) and lipids (0.14-1.28%) are also present in EBN (Marcone 2005). With notable nutrition, EBN is often used as a folk medicine to clear phlegm, alleviate cough, relieve tiredness, and recover from surgery (Lee et al. 2021; Ma & Liu 2012).

Water and digested EBN extracts are widely used to study pharmacological benefits of EBN; however, their cytotoxic effects on cancer cells have not been observed in previous studies. For instance, EBN extracted by hot water or digested by simulated gastro-intestinal enzymes did not exhibit cytotoxic activity against human hepatocellular carcinoma HepG2 cells (Fan et al. 2021; Yida, Imam & Ismail 2014). In other reports, acid-digested EBN extracts promoted the division of human colonic adenocarcinoma (Caco-2) whereas pancreatin-digested crude and water EBN extracts protected human neuroblastoma (SH-SY5Y) cells against neurotoxin-induced apoptosis (Rashed & Nazaimoon 2010; Yew et al. 2014). Although EBN is suggested for cancer treatment due to its immune-enhancing property, the question of whether EBN is able to cause cancer cell death still needs to be solved (Zhao et al. 2016).

A recent study in *Costus speciosus* has shown a stronger cytotoxic potency of the ethanol extract against human hepatocellular carcinoma HepG2 cells over the aqueous extract. This suggested bioactive compounds responsible for the cytoxicity might be dissolved in ethanol more than in water (Gheraibia, Belattar & Abdel-Wahhab 2020). The study inspired us to employ ethanol as the solvent for EBN extraction. The effect of the resultant ethanol extract on human hepatocellular carcinoma Huh-7 cell line, a model to study liver cancer and molecular mechanisms of natural extracts was also investigated in the present work (Krelle, Okoli & Mendz 2013). Our data shed light on an uncharacterized bioactivity of EBN and support further studies to thoroughly understand this pharmacological property.

MATERIALS AND METHODS

PREPARATION OF EBN EXTRACT

Processed EBN collected from specialized houses in Binh Thuan province, Vietnam was kindly provided by Phuoc Tin Development Trading Service Company, Limited, Vietnam. The EBN ethanol extract (designated hereafter as EEE) was prepared using the exhaustive extraction method. In brief, 30 g of dried EBN powder was soaked in 2 L of 85% ethanol for 30 days at room temperature. The extract was concentrated in a rotary evaporator (Labtech, Korea) and then dried in a freeze-dryer (Operon, Korea) until a constant weight of the dried powder was obtained to ensure the complete removal of residual water and ethanol. The dried extract was dissolved in 30% dimethyl sulfoxide (DMSO) (Sigma-Aldrich, Germany) to the final concentration of 20 mg/mL.

DETERMINATION OF ALKALOIDS, PHENOLICS, TERPENOIDS, PROTEINS/PEPTIDES, AND CARBOHYDRATES

The presence of alkaloids, phenolics, and terpenoids in the EEE was detected according to the reported methods (Das et al. 2014; Kancherla et al. 2019; Nguyen et al. 2023). Atropine, gallic acid, and linalool (Sigma-Aldrich, Germany) were applied as positive controls for alkaloids, phenolics, and terpenoids, respectively. Dimethyl sulfoxide (DMSO) (Sigma-Aldrich, Germany) at the concentration of 30% was used as a negative control in each experiment.

In brief, 0.1 mL of Dragendorff' or Bouchardat's reagents was gently dropped into each tube containing 0.2 mL of the test sample (the EEE, atropine, or DMSO). The formation of orange red, and brown precipitates corresponding to Dragendorff's and Bouchardat's tests indicates the presence of alkaloids.

Phenolics were identified with ferric chloride, lead tetraacetate, and Folin-Ciocalteu's tests. For the ferric chloride test, 1 mL of 5% neutral ferric chloride ($FeCl_3$) was added to 0.5 mL of the test sample (the EEE, gallic acid, or DMSO) and the presence of phenolic compounds results in dark blue color. For lead tetraacetate test, 0.1 mL of lead tetraacetate ($Pb(C_2H_3O_2)_4$) was dropped into 0.5 mL of the test sample and precipitate formation confirms the presence of phenolic compounds. For the Folin-Ciocalteu test, 2.5 mL of the reagent and 2 mL of 75 g/L sodium carbonate (Na_2CO_3) were added into 0.5 mL of the test sample and the mixture was incubated in the dark for 30 min. Phenolic compounds are indicated by the resultant dark blue color.

To detect terpenoids in Salkowski's test, 0.5 mL of the test sample (the EEE, linalool, or DMSO) was firstly mixed with 0.2 mL of chloroform and 0.3 mL of concentrated sulphuric acid (H_2SO_4) was then carefully added to form a layer. A reddish brown appearance at the interface indicates the presence of terpenoids.

The absorption spectrum at the wavelength of 190-300 nm of the EEE in this study was first scanned by using the 205 nm protein setting on a Nanodrop One machine (Thermo Scientifics, USA). Bradford's method was used to calculate the approximate concentration of proteins and peptides in the EEE with bovine serum albumin (BSA, Sigma Aldrich, Germany) as a standard (Bradford 1976). To further estimate the molecular weight range of proteins and peptides, Tricine-PAGE was performed as previously described (Jiang et al. 2016). After the run, the gel was stained with Commasie Blue to visualize protein and peptide bands.

To determine the presence of carbohydrates which can be attached to proteins or peptides in the EEE, the Molisch's test was used as previously described (Auwal et al. 2014). Firstly, 0.5 mL of the test sample (the EEE, glucose, starch,

or DMSO) and 0.1 mL of Molisch's reagent were mixed together. After mixing, 0.1 mL of concentrated H_2SO_4 was dropped down the sides of the tube to produce a layer. The formation of a purple red ring at the interface between two layers indicates the presence of carbohydrates.

ASSESSMENT OF CELL VIABILITY BY THE 3-(4,5-DIMETHYLTHIAZOL-2-YL)-2,5-DIPHENYL TETRAZOLIUM BROMIDE) (MTT) ASSAY

The MTT assay evaluating cellular metabolic activity is used as an indicator of viable, metabolically active cells (Kumar, Nagarajan & Uchil 2018). The assay was conducted following a previous study with some modifications (Meerloo, Kaspers & Cloos 2011). The hepatocellular carcinoma Huh-7 cells at the concentration of 5×10^4 cells per well were seeded into a 96-well plate and incubated at 37 ºC with 5% CO_2 for 24 h. The medium was then replaced by different concentrations of the EEE (200, 100, 50, 25, 12.5, and 3.125 µg/mL), doxorubicin (DOX, Fresenius Kabi, Germany) (50, 25, 12.5, 6.25, 3.125, 1.5625, and 0.78 µg/mL) as positive control and DMSO (0.3%, 0.15%, 0.075%, 0.038%, 0.009%) as negative control. After incubation of 24 h, 48 h, and 72 h, MTT (Sigma-Aldrich, Germany) was added into each well to the final concentration of 0.5 mg/mL and incubated at 37 ºC for 3.5 h. The formazan crystals formed by the interaction between MTT and living (metabolically active) cells were dissolved in DMSO. Absorbance of samples was read at 570 nm by an ELISA Reader (BioTek, USA). The inhibition percentage of cell viability (cell death) was estimated by the following formula:

$$\text{Inhibition (\%)} = 100 - [100(A_{Sample} - A_{B1})/(A_{Control} - A_{B2})]$$

where A_{Sample} is the absorbance of the sample; A_{B1} is the absorbance of the sample background; $A_{Control}$ is the absorbance of control (DMSO); and A_{B2} is the absorbance of DMSO background. The percentage of inhibition was presented as mean ± SD of three independent experiments.

The cytotoxic potential of the extract was defined as an IC_{50} value (the concentration that reduces the cell viability by 50%). The values of IC_{50} and R-squared (R^2, coefficient of determination, a statistical measure of the goodness of fit between the experimental data and the regression model) were calculated by GraphPad Prism software.

ANNEXIN V/PI STAINING

Annexin V and propidium idodie (PI) labeling was used to evaluate viable, apoptotic, late apoptotic/necrotic, and early necrotic cells (Khanavi et al. 2010; Pagliara et al. 2015). In our study, Huh-7 cells were seeded in a 24-well plate at the concentration of 5×10^5 cells per well and incubated at 37 ºC with 5% CO_2. The EEE, DOX (positive control), and DMSO (negative control) were added to the wells to the final concentrations of 55 µg/mL, 5 µg/mL, and 0.083%, respectively. After incubation, Annexin V/PI staining was performed according to the manufacturer's instruction (Roche, Sigma Aldrich, Germany). Briefly, the treated cells were gently washed in phosphate buffered saline (PBS, pH 7.0) to remove the old medium. The cells were subsequently covered with Annexin-V-FLUOS labeling solution (300 µL/well) and incubated at 25 °C for 10 min. After replacing the fluorescent solution by PBS, the images were taken under a fluorescence inverted microscope (Nikon Eclipse Ts2R, Japan). Cells negative with both Annexin V (green) and PI (red) were considered as living (Annexin V-/PI-) cells while cells positive with both of them were considered as late apoptotic/necrotic (Annexin V+/PI+) cells. Cells only positive with Annexin V or PI were considered as early apoptotic (Annexin V+/PI-) or early necrotic (Annexin V-/PI+) cells (Khanavi et al. 2010; Pagliara et al. 2015). Cell counts from images were carried out by ImageJ software and at least 100 cells were analyzed. The number of early necrotic (Annexin V-/PI+) cells was negligible in both treatment and control samples. Therefore, this cell type was not presented in the result.

QUANTIFICATION OF mRNA EXPRESSION

The mRNA expression level was quantified by quantitative reverse transcription PCR (qRT-PCR). Huh-7 cells were seeded into a 6-well plate at the concentration of 1×10^6 cells per well and incubated with 5% CO_2 at 37 ºC. The cells were treated with the EEE and DMSO (negative control) at the final concentration of 55 µg/mL and 0.083%, respectively, and then harvested for total RNA extraction by TriSure reagent (Bioline, USA). After being treated with DNase I (New England Biolabs, USA), RNA samples were converted to cDNAs by LunaScript RT SuperMix Kit (New England BioLabs, USA). The mRNA levels of *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*), *CASP3, CASP8, CASP9,* and *survivin* were assessed using Luna Universal qPCR Master Mix (New England Biolabs, USA). *GAPDH* was used as the reference gene to calculate the relative expression of *CASP3, CASP8, CASP9* and *survivin* based on the delta threshold cycle method (Schmittgen & Livak 2008). Primer sequences (5'→3') used in the qPCR are as follows: *CASP3*-F: TCGCTTTGTGCCATGCTGAA, *CASP3*-R: ACTCAAATTCTGTTGCCACC, *CASP8*-F: AATGGAACACACTTGGAT, *CASP8*-R: GCTCTACTGTGCAGTCATCG, *CASP9*-F: TTGAGGACCTTCGACCAGCT, *CASP9*-R:

GAPDCAACGTACCAGGAGCCACTC, *survivin-F*: TTGGCGGTCTAAACTTAGCG; *survivin-R*: *CCTCGGCCAATCCGCTC*; *GAPDH*-F: AGCCACATCGCTCAGACAC, and *GAPDH*-R: GCCCAATACGACCAAATCC. Primers for survivin were designed in this study, the other primers were used as previously described in Nguyen et al. (2023).

WESTERN BLOTTING

Huh-7 cells were seeded into a 6-well plate at the concentration of 5×10^5 cells per well and incubated at 37 ºC with 5% CO_2. The EEE and DMSO (negative control) at the final concentrations of 55 µg/mL and 0.083% were added to wells. After incubation, cells were subsequently harvested and extracted in a lysis buffer containing radioimmunoprecipitation assay (RIPA) buffer and protein inhibitor cocktail (Sigma-Aldrich, Germany). Total cell lysates were loaded onto a 15% polyacrylamide gel and transferred onto a nitrocellulose membrane (GE Healthcare, USA) using an electrophoresis gel system (Bio-Rad, USA) and a semi-dry transfer system (Invitrogen, USA). The blotting membranes were first blocked with 1% (w/v) skim milk in phosphate buffered saline with Tween 20 (PBS-T) (Sigma Aldrich, Germany) for 1 h at room temperature. Subsequently, the membranes were incubated with primary antibodies against caspase 3 (c8487, Sigma-Aldrich, Germany), caspase 8 (c4106, Sigma-Aldrich, Germany), caspase 9 (c4106, Sigma-Aldrich, Germany), survivin (ab76424, Santa Cruz, United Kingdom), and β-actin (A1978, Sigma-Aldrich, Germany) at 4 ºC overnight. The dilution factor was *1:500 for the antibodies against caspase 3 and caspase 9 and 1:1000 for the other antibodies. After washing* 5 times with PBST-T, the membranes were probed with diluted HRP-conjugated secondary antibodies (AP307P and AP308P, Sigma-Aldrich, Germany) (1:10000 v/v) for 1 h at room temperature. The membranes were then washed 5 times with PBS-T, followed by treated with chemiluminescence reagent (ECL, Cytiva, USA). The images were detected in a G-box imager (Syngene, India) and protein band intensity was analyzed by ImageJ and GraphPad Prism softwares.

STATISTICAL ANALYSIS

All experiments were performed in three independent experiments. Data were presented as mean ± SD. Statistical evaluation between samples was calculated by Student's t-test for mRNA/protein levels and cell population analysis in Annexin V/PI staining experiment. Significant differences were presented as * for $p\leq0.05$, ** for $p\leq0.01$, and *** for $p\leq0.001$.

RESULTS AND DISCUSSION

ANALYSIS OF ALKALOIDS, PHENOLICS, TERPENOIDS, PROTEINS/PEPTIDES, AND CARBOHYDRATES IN THE EEE

Previous studies showed that terpenoids and phenolic compounds were found in EBN (Permatasari et al. 2023; Quek et al. 2018). Additionally, alkaloids can be synthesized in a few animal species (Braekman, Daloze & Pasteels 1998). As these phytochemical compounds can be alcohol-soluble, their presence were determined in the extract (Truong et al. 2019). Qualitative tests showed that terpenoids and phenolic compounds were undetectable in the EEE. Alkaloids did not generate positive precipitates in the Bouchardat's and Dragendorff's assays, suggesting the ignorable amount of alkaloid components in the EEE (Supplementary Table 1).

Since EBN is rich in proteins and peptides, it is possible that they can be dissolved in ethanol solvent (Wang et al. 2012). As a result, the measurement of ultraviolet absorbance at the wavelength of 205 nm was applied to determine their abundancy in the EEE (Anthis & Clore 2013). An initial scan at the spectral wavelength of 190-300 nm demonstrated a strong absorption at 205 nm and a high concentration of proteins and peptides (~20 mg/mL) (Figure 1(A)). The estimated concentration of proteins and peptides by Bradford's assay (19.89 ± 0.2 mg/mL) was also almost equal to the final concentration of the prepared EEE (20 mg/mL), suggesting the dominance of proteins and peptides in the extract (Table 1). This was also confirmed by a thick band with the molecular weight of 1.5-10 kDa and other bands at various ranges of molecular weight in Tricine-PAGE analysis (Figure 1(B)). This result implies that the EEE is comprised of a considerable amount of proteins and peptides which may be significant contributors to the EEE bioactivities. Proteins and peptides can be attached by carbohydrate chains to form glycoproteins and glycopeptides in EBN (Shim et al. 2016). However, the negative result of the Molisch's test indicates that carbohydrates might be absent in the EEE or below the detection range of the test (Supplementary Table 1).

THE CYTOTOXIC ACTIVITY OF THE EEE AGAINST HUMAN HEPATOCELLULAR CARCINOMA HUH-7 CELL LINES

As natural extracts with cytotoxic activity against cancer cells can be promising candidates for the development of anticancer agents, we examined if the EEE possesses this property by using Huh-7 cell line as a model. In the MTT assay, at the concentration of 100 µg/mL, approximately 70% of Huh-7 cells were killed by the extract treatment at the incubation period of 24 h, 48 h, and 72 h, suggesting the cytotoxic effect of the extract on Huh-7 cells (Figure 2).

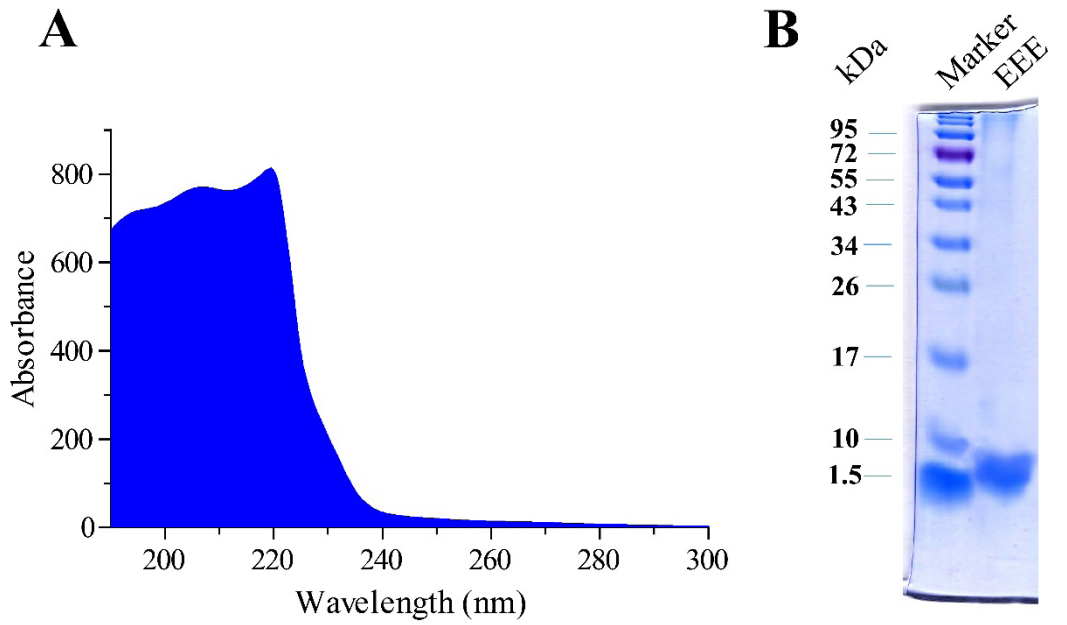

FIGURE 1. A considerable amount of proteins and peptides were present in the EEE. (A) An absorption spectrum of the EEE and (B) Tricine-PAGE separation of proteins and peptides in the EEE

TABLE 1. The concentrations of peptide and protein quantified by the absorbance at 205 nm and Bradford's assay, respectively

Quantification method	A_{205}	Bradford's assay
Peptide (mg/mL)	~ 20	-
Protein (mg/mL)	-	19.89 ± 0.2

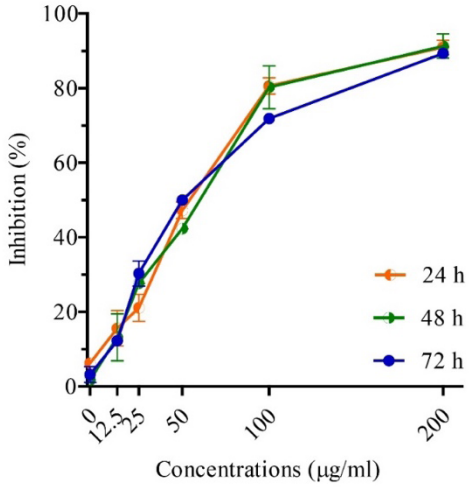

FIGURE 2. The EEE exhibited cytotoxic effect on Huh-7 cells. The percentage of inhibition was presented as mean ± SD of three independent experiments. EEE: EBN ethanol extract

TABLE 2. IC_{50} values of the EEE and DOX against Huh-7 cells

Time treatment	24 h		48 h		72 h	
Sample	EEE	DOX	EEE	DOX	EEE	DOX
IC_{50} (µg/mL)	58.57 ± 2.66	5.51 ± 0.32	53.15 ± 3.07	5.10 ± 0.03	55.27 ± 1.85	4.61 ± 0.14
R^2	0.9702	0.9955	0.9753	0.9996	0.9968	0.9989

Each value was presented as mean ± SD of three independent experiments. EEE: EBN ethanol extract. DOX: doxorubicin. IC_{50}: half-maximal inhibitory concentration. R^2: coefficient of determination, curve fit

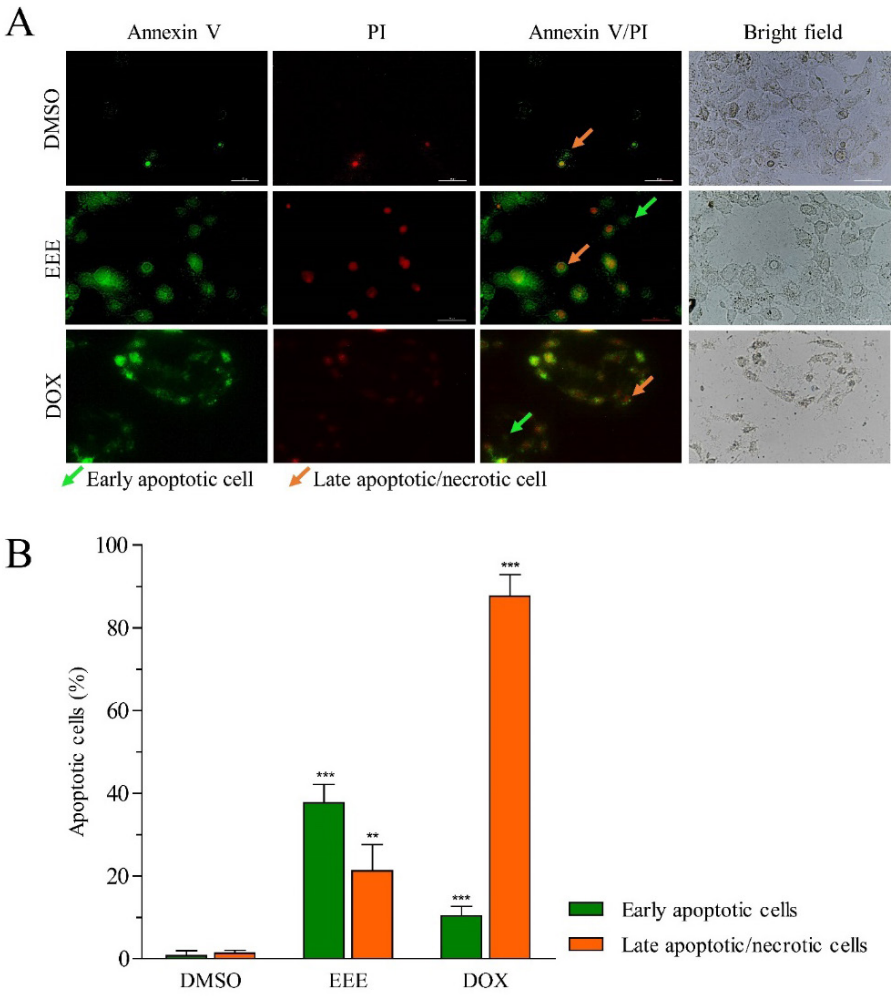

FIGURE 3. The EEE induced apoptosis in Huh-7 cells. (A) Fluorescent images showing Annexin V/PI- stained Huh-7 cells upon the treatment with EEE, DMSO (negative control), and DOX (positive control). (B) Quantification of early-apoptotic (Annexin V+/PI-) and late-apoptotic/necrotic (Annexin V+/PI+) cells. Green and orrange arrows indicate early-apoptotic (Annexin V+/PI-) and late-apoptotic/necrotic (Annexin V+/PI+) cells, respectively. Scale bars: 50 µm. Each data bar was presented as mean ± SD of three independent experiments. Statistical differences between DMSO and EEE/DOX-treated samples were calculated by Student's t-test (**, $p \leq 0.01$; ***, $p \leq 0.001$)

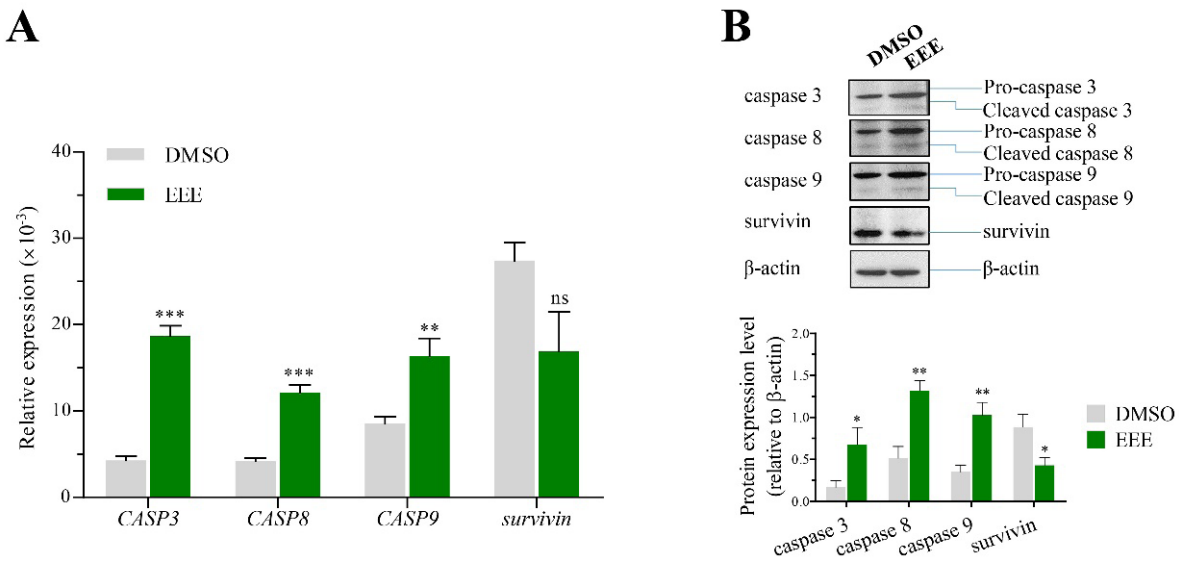

FIGURE 4. The EEE regulated the expression patterns of apoptotic markers. A) Quantification of the relative mRNA levels of *CASP3, CASP8*, *CASP9*, and *survivin* in the Huh-7 cells treated with EEE and DMSO and B) Quantification of protein levels of caspase 3, caspase 8, caspase 9 and survivin in the Huh-7 cells upon the treatment with DMSO and EEE (55 µg/mL). *GADPH* was used as a reference gene to normalize mRNA levels. β-actin was used as a loading control and reference protein to normalize protein levels. Each data bar was presented as mean ± SD of three independent experiments. Statistical differences between DMSO and EEE-treated samples were calculated by Student's t-test (ns: non-significant; **, $p \leq 0.01$; ***, $p \leq 0.001$)

R^2 values of 0.9702 (24 h), 0.9753 (48 h), and 0.9968 (72 h) indicated that the extract significantly caused Huh-7 cell death in a concentration-dependent manner (Table 2).

Huh-7 cells were also treated with the chemotherapeutic drug DOX as a positive control. The drug had IC_{50} values of 5.51 µg/mL, 5.10 µg/mL, and 4.61 µg/mL at the incubation period of 24 h, 48 h, and 72 h, respectively. Meanwhile, the IC_{50} values of the EEE were found to be 58.57 µg/mL (24 h), 53.15 µg/mL (48 h), and 55.27 µg/mL (72 h) (Table 2). Since similar IC_{50} values of the EEE were obtained at all test incubation period, a representative concentration (55 µg/mL) was selected for further experiments.

Our results showed for the first time that the EBN ethanol extract was able to cause cell death in Huh-7 cell line. Although the specific compounds underlying this ability have not been identified in this study, a high amount of proteins and particularly peptides suggests their possible contribution to this property. Indeed, proteins and peptides derived from animal sources have been demonstrated for their cytotoxic prospects against cancer cells (Serna et al. 2018; Wang et al. 2017). For example, lactoferrin, an iron-binding glycoprotein, causes apoptosis in human leukemia HL-60 cells whereas peptide fractions from the hydrolysate of dark tuna muscle displays an anti-proliferative activity against breast cancer MCF-7 cells (Hsu, Li-Chan & Jao 2011; Roy et al. 2002). Therefore, it would be interesting to isolate proteins or peptides responsible for the cytotoxicity of EBN ethanol extract in further work.

INDUCTION OF APOPTOSIS IN HUH-7 CELLS BY THE EEE TREATMENT

Natural extracts capable of triggering apoptosis in cancer cells have great potentials in the treatment of cancer (Wang et al. 2018). It is of our interest to see if the EEE also uses the apoptotic pathway for its cytotoxicity in Huh-7 cells. We first performed Annexin V/PI staining of Huh-7 cells with the treatment of the EEE (55 µg/mL), DOX (postive control), and DMSO (negative control). In the EEE-treated sample, about 40% of cells were detected undergoing early apoptosis (Annexin V+/PI-) and 22% of cells were in late apoptosis/necrosis stage (Annexin V+/PI+). The exposure with DOX, a well-known apoptosis-inducing drug, resulted in approximately 10% of early apoptotic cells and 90% of late/necrotic cells (Lee, Lau & Ng 2002). Meanwhile, both early apoptotic and late apoptotic/necrotic cells accounted for less than 1% in the DMSO negative control (Figure 3(A) and 3(B)). Our result showed that the apoptotic cells were remarkably increased in the EEE-treated sample compared to the negative control, suggesting the ability of the EEE in inducing apoptosis in Huh-7 cells.

To further confirm the participation of the EEE in apoptosis, the expression patterns of several key pro- and anti-apoptotic markers following the EEE and DMSO treatment were examined by qRT-PCR and Western blotting methods. As shown in Figure 4, the mRNA level of *CASP3* in the presence of the EEE was 4 folds higher than that in the control. Consistently, the protein level of caspase-3 in the EEE-treated Huh-7 cells was approximately twice as high as the DMSO-treated sample. Similar results at mRNA and protein levels were also observed for another two pro-apoptotic genes, *CASP8* and *CASP9*. On the other hand, the transcript level of an apoptotic repressor, *survivin*, was not significantly altered by the EEE treatment. However, its protein level was decreased upon treatment with the EEE, implying that the EEE may have an effect on survivin at the post-transcriptional level (Figure 4(A) and 4(B)). Taken together, these results reflect the ability of the EEE in inducing apoptosis in Huh-7 cells, which is likely through regulating the expression pattern of markers in the apoptotic pathway.

Previous studies have demonstrated that animal extracts are capable of causing cancer cell death via triggering apoptotic pathway. For instance, the ethanol extract of sea cucumber, *Holothuria atra*, induced apoptosis in human breast ductal carcinoma T47D cells whereas the ethanol extract of *Dysidea avara* elicited apoptotic effect on leukemia K562, myeloma H929, and HeLa cells (Ciftci et al. 2020; Nursid, Marraskuranto & Chasanah 2019). The EBN ethanol extract in our study provoked apoptosis-inducing ability via the deregulation of apoptotic markers including caspase-3, caspase-8, caspase-9, and survivin in Huh-7 cells, making itself as an encouraging extract in the list of animal-derived anticancer candidates. Although our research has reached its aims, several limitations including the apoptosis-inducing activity of the EBN ethanol extract in other cancer cell lines, its cytotoxicity against normal cells, and anticancer properties *in vivo* should be noted. It is important to evaluate these aspects by valid analyses in further studies.

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SUPPLEMENTARY TABLE 1. Preliminary analysis of alkaloids, phenolics, flavonoids, terpenoids, and carbohydrates in the EEE

Compounds	Test/Reagent	Positive expression	Inference
Alkaloids	Bouchadat	Precipitate	-
	Dragendorff	Orange red precipitate	-
	Mayers	White precipitate	*
Phenolics	Ferric chloride	Dark blue color	-
	Lead tetraacetate	Precipitate formation	-
	Folin-Ciocalteu	Dark blue color	-
Flavonoids	Alkaline reagent	Deep yellow color appeared when adding NaOH and then colorless when adding HCl	-
	Shinod's test	Pink color	-
Terpenoids	Salkowski's test	Reddish brown color	-
Carbohydrates	Molisch's test	Purple red ring at the interface	-

- : negative, * : very weak