Apis mellifera propolis enhances apoptosis and invasion inhibition in head and neck cancer cells

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Background. Propolis is a resinous product accumulated from several plant sources which possess a wide range of therapeutic properties including anti-cancer activities. However, the role of honeybee-produced propolis on head and neck squamous carcinoma (HNSCC) is not well understood. This study aimed to investigate the effects of *Apis mellifera* propolis on apoptosis and invasiveness activity in HNSCC cell lines. **Methods.** Ethyl acetate extract of propolis (EAEP) was prepared from *A. mellifera* beehives by liquid-liquid extraction technique. High-performance liquid chromatography coupled with electrospray ionization-time of flight-mass spectrometry (HPLC-ESI-TOF-MS) were used to determine the flavonoids in EAEP. Isogenic HNSCC cell lines derived from primary (HN30 and HN4) and metastatic site (HN31 and HN12) were used in this study. Cytotoxicity, apoptosis, invasion, and MMP activity of EAEP for HNSCC cells were determined using MTT assays, flow cytometry, Matrigel invasion assay, and gelatinase zymography, respectively. **Results.** We found that EAEP exhibited cytotoxic activity and induced apoptosis in HNSCC cell lines. Furthermore, EAEP significantly decreased HNSCC cell invasion by reducing MMP-2 and MMP-9 activity. Two flavonoids, galangin and apigenin, were identified in EAEP by HPLC-ESI-TOF-MS. The results suggest that EAEP promoted apoptotic and exerts anti-invasion potential through inhibition of MMP-2 and MMP-9 activity in HNSCC cell lines. It is possible that the inhibitory effects as such were attributed to the biological activities of galangin and apigenin.

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Abstract

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Results. We found that EAEP exhibited cytotoxic activity and induced apoptosis in HNSCC cell lines. Furthermore, EAEP significantly decreased HNSCC cell invasion by reducing MMP-2 and MMP-9 activity. Two flavonoids, galangin and apigenin, were identified in EAEP by HPLC-ESI-TOF-MS. The results suggest that EAEP promoted apoptotic and exerts anti-invasion potential through inhibition of MMP-2 and MMP-9 activity in HNSCC cell lines. It is possible that the inhibitory effects as such were attributed to the biological activities of galangin and apigenin.

Introduction

Head and neck squamous cell carcinoma (HNSCC) is the seventh most common cancer worldwide and the highest occurring cancer observed in southern Asia. Currently, the common form of treatment for HNSCC involves in surgical operation, combined with chemotherapy and/or radiation therapy (Chow 2020; Schwartz & Hayes 2020). Similar to other tumors, proliferation, invasion and metastasis are the critical processes that indicate HNSCC aggressiveness (Chan et al. 2016; Wolf & Claudio 2014). Evading apoptosis is one of mechanism supporting cancer survival in the extreme microenvironment (Raudenska et al. 2021). The invasion and metastasis are driven by matrix metalloproteinases (MMPs). MMP-2 and MMP-9 are the key enzymes which destroy the basement membrane and degrade the extracellular matrix, leading to tumor invasion (Koontongkaew 2013). Thus, more effective treatments aim to trigger apoptosis in cancer cells for local and metastatic HNSCC are challenged (Khan et al. 2012).

Propolis, or bee glue, is a natural resinous material collected by honey bees from various tree buds to seal cracks in the hive and protects the hive against bacterial and fungal infections (Calegari et al. 2017). Propolis have been used in traditional medicine in many countries. More than 300 chemical compounds have been identified from propolis in different geographic regions (Drescher et al. 2019; Xuan et al. 2016) including flavonoids, terpenes, phenolic acid, cinnamic acid, caffeic acid and several esters (Funakoshi-Tago et al. 2016; Jaiswal et al. 1997; Kocot et al. 2018). Propolis has a wide range of pharmaceutical properties, including antimicrobial (Al-Ani et al. 2018; Chen et al. 2018), anti-inflammatory, antioxidant (Kocot et al. 2018), anti-angiogenic activities (Iqbal et al. 2019) and anti-cancer (Badr et al. 2011; Frozza et al. 2017; Sawicka et al. 2012) . The crude extracts of propolis have been reported on cytotoxic activities against various cancer cell lines such as human prostate cancer cells (DU145 and PC-3)(Li et al. 2007), cervix adenocarcinoma cells (HeLa) (Barbaric et al. 2011), human laryngeal epidermoid carcinoma cells (Hep-2) (Frozza et al. 2017), human colorectal adenocarcinoma cells (HT-29), human breast adenocarcinoma cells (MCF-7), human epithelial colorectal adenocarcinoma cells (Caco-2), and murine melanoma cell lines (B16F1) (Choudhari et al. 2013).

However, it has been reported that the biological and pharmacological activities of propolis depend on its chemical composition, geographical zone, plant sources, and seasons (Devequi-Nunes et al. 2018; Omar et al. 2017; Siheri et al. 2016). Propolis extracts from *Apis mellifera* beehives in Thailand have been reported on anti-proliferative and cytotoxic activities against cancer cell lines derived from human breast carcinoma (BT474), human hepatocellular carcinoma (Hep-G2), gastric carcinoma (KATO-III) and colon adenocarcinoma (SW620) (Teerasripreecha et al. 2012). Moreover, the propolis extract from *Trigona sirindhornae* exhibited cytotoxic effects against HNSCC cells (Utispan et al. 2017). However, a few studies on the effect of Thai *A. mellifera* propolis extract on HNSCC cell lines have been reported. This study aims to investigate cytotoxicity, apoptosis and anti-invasive activities of ethyl acetate extract from Thai *A. mellifera* propolis on primary and metastatic HNSCC cell lines.

Materials and Methods

Chemicals

Apigenin, galangin, caffeic acid, ferulic acid, rutin, quercetin and naringenin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Acetonitrile (HPLC grade) was purchased from RCI Labscan (Bangkok, Thailand). Hexane, ethyl acetate, ethanol, methanol and formic acid (analytical grade) were purchased from Merck (Darmstadt, Germany).

Preparation of ethanol extract of propolis (EEP)

The propolis sample from the native Thai bee species *A. mellifera* was obtained in November of 2017 in Loei province, northeastern Thailand. The sample was stored in a desiccator and kept in the dark at 4°C until processed. Raw propolis (5.27g) was cut into small pieces and stirred with 100 ml of 95% (v/v) ethanol (EtOH) at 100 rpm at room temperature for 48 hours in dark condition. Next the insoluble portion was separated by filtration through Whatman filter paper No. 2 (Whatman Inc, Piscataway, NJ, USA). In order to increase the extract yield, this procedure was repeated three times on the same sample. The resulting filtrates were pooled and dried in a rotatory evaporator at 40ºC and

175 mbar (Rotovapor R-215, BUCHI Labortechnik, AG, Switzerland). Then the ethanolic extract of propolis (EEP, 4.32 g) with viscous appearance was obtained.

Liquid-liquid partitioning

EEP was fractioned by means of liquid-liquid partitioning. For that purpose, the EEP (4.32 g) was dissolved in 100 ml methanol and then partitioned with hexane (3 x 50 ml). The combined hexane extract was then rotatory evaporated at 40°C and 335 mbar to yield hexane extract of propolis (HEP, 0.98 mg). The methanol portion was evaporated at 40ºC and 337 mbar. Next the methanol extract was dissolved in 100 ml of distilled water and submitted to liquid-liquid partitioning with ethyl acetate (3 x 50 ml). Solid masses were obtained for ethyl acetate extract (EAEP, 1.92 g) and aqueous extract (AEP, 0.12 g) after total evaporation of solvents.

Most of the substances found in propolis were obtained in polar organic solvents such as ethanol, methanol and ethyl acetate (Sambou et al. 2020). Solvents like ethyl acetate are used in extraction processes because of its chemical and biological functions such as medium polarity and minimum cell toxicity. Biphasic actions of this solvent enables it to be used to extract both polar and non-polar compounds (Mandal et al. 2015). Therefore only EAEP was used in the present study. Before use, the EAEP extract was dissolved in dimethyl sulfoxide (DMSO) and placed in a freezer (-30ºC) until use.

Cell culture

Two pairs of isogenetic HNSCC cell lines representing both primary and metastatic disease from the same patient were first established at Wayne State University by Ensley J. (Cardinali et al. 1995) who collaborated with the researcher in the National Institute of Dental and Craniofacial Research under supervision of Gutkind S. Gutkind S. provided the cell lines as a gift to Koontongkaew S. HN30 and HN31 cells were obtained from primary pharynx lesions and lymph node metastases (T3N1M0), respectively. HN4 and HN12 cells were obtained from primary tongue lesions and lymph node metastases (T4N1M0), respectively. They were maintained in Dulbecco's Modified Eagle's Medium (DMEM) (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 µg/ml streptomycin (Invitrogen) and an anti-fungal agent. The

cells were cultured in a 37°C humidified 5% CO_2 atmosphere. They were passaged with 0.25% trypsin-EDTA when reaching 70-80% confluence.

Cell viability-MTT assay

The cytotoxicity of EAEP was estimated using the methyl thiazotetrazolium (MTT) assay as previously described (Utispan et al. 2020). HNSCC cells were seeded onto 96-well plates at a density of 2,000 cells/well. The cells were treated with serum-free DMEM with 0.1% dimethyl sulphoxides (DMSO) (vehicle control) or EAEP (0.10-0.40 mg/ml) at 37°C for 72h. After the exposure period, the media were removed, and the cells were washed with phosphate-buffed saline, followed by incubation with 0.5 mg/ml of MTT in culture media for an additional for 4 h. The blue formazan crystals of viable cells were dissolved and measured spectrophotometrically at 570 nm. Cell viability was calculated as a percentage of that of the control (untreated) cells. Each concentration of EAEP was independently assayed three times with three technical replicates. According to ISO 10993-5, cell viability above 80% were considered as non-cytotoxic; within 80%-60% weak; 60%-40% moderate and below 40% strong cytotoxicity, respectively (International Organization for Standardization ISO 10993-5 2009).

Apoptosis assay

In order to verify that the effect of the studied extracts on the growth inhibition of HNSCC cells was related to apoptosis, analysis of the apoptosis and necrotic cells was performed using annexin-V-fluorescein isothiocyanate (FITC) and propidium iodide (PI) staining. The cells were seeded onto 6-well plates and allowed to attach for 24 h. After which the cells were treated with EAEP at the weak cytotoxic doses (cell viability of 60% - 80%) to each cell line for 24h. The concentration of 0.2 mg/ml EAEP was used for HN12, HN30 and HN31 whereas HN4 cells were treated with 0.3 mg/ml EAEP. After this, the cells were washed twice with PBS solution and detached by 0.25% trypsin, washed with PBS and resuspended in ice cold binding buffer. The apoptotic cells were assessed using BD Annexin V FITC Assay (BD Biosciences, San Jose, CA, USA). Ten thousand events were analyzed in a flow cytometer (Cytoflex®, Beckman Coulter, Indianapolis, IN, USA).

The percent of viable, apoptotic and necrotic cells were evaluated by CytExpert Software (Beckman Coulter).

Invasion assay

The modified Boyden chamber (Neuro Probe, Gaithersburg, MD, USA) assay used for analysis of cell invasion is based on a chamber with two medium filled compartments as previously described (Albini et al. 1987). Matrigel, a reconstituted basement membrane gel (BD Bioscience) was applied to polycarbonate membrane filters (13 mm-diameter, 8.0 μm pore size, Whatman). The filter was placed above the lower chamber, which contained serum-free DMEM with o.1% bovine serum albumin (BSA, Sigma). HN4, HN12, HN30 or HN31 cells (1×10^5 cells) were resuspended in 0.1 mg/ml EAEP which diluted in DMEM containing 0.1% BSA and seeded to the top of the chamber. After a 5 h incubation in a 37°C and 5% CO_2 incubator, the filters were fixed with 0.5% crystal violet in 25% methanol for 10 min. The invaded cells on the lower surface of the filters were counted under a microscope at 400× magnification. Cell counting was performed by two investigators. Five randomly selected fields were counted per filter in each group, and the counts were averaged.

Conditioned medium preparation and zymography

HN4, HN12, HN30 and HN31 cells ($1x10^6$ cells) were cultured in 6-well plates and incubated at 37°C for 24 h. After incubation, wells were washed with PBS and treated with 0.1 mg/ml EAEP which diluted in DMEM containing 0.1% BSA for 48 h. The cells cultured in DMEM containing 0.1% BSA were used as control. Conditioned medium (CM) was collected and centrifuged at 1,000 g and 4°C for 10 min to eliminate cells and debris. The CM was stored at -80°C until used. Total protein in CM was estimated using the Pierce™ BCA protein assay kit (Thermo Fisher Scientific, Waltham, MA, USA).

MMP-2 and MMP-9 activities in CM were measured using gelatin zymography as previously described (Koontongkaew et al. 2009). For gelatin zymography, the collected media were subjected to 0.2% gelatin (bloom 300, Sigma)-10% SDS polyacrylamide gel electrophoresis (PAGE) to determine MMP-2 and MMP-9. After SDS-PAGE, the gels were washed in 2.5% Triton X-100 for 30 min and incubated with renaturing buffer (50 mM

Tris, pH 8, 5 mM $CaCl_2$ and 10^{-6} M $ZnCl_2$) for 20-24 h. Next, the gels were stained with 0.5% Coomassie blue G250 in 30% methanol, 10% glacial acetic acid for 30 min and destained in the same solution without Coomassie blue. Gelatinolytic bands were quantified using the Gene Tools software (Syngene, Frederick, MD, USA). Three independent experiments were performed.

HPLC-ESI-TOF-MS analysis of EAEP

High-performance liquid chromatography coupled with electrospray ionization-time of flight-mass spectrometry (HPLC-ESI-TOF-MS) was used to investigate selected phenolic acids and flavonoids in EAEP. EAEP was prepared at 5 mg/ml in ethanol and filtered through a 0.45 µm membrane filter. For compound identification, seven standard polyphenolic compounds (apigenin, galangin, caffeic acid, ferulic acid, rutin, quercetin and naringenin) were dissolved in methanol (10 ppm). Analyses were conducted in an UltiMate® 3000 system (Thermo Fisher Scientific, DionexSoftron GmbH, Dornierstr. 4, Germany) with a reverse phase column (C18 analysis column, 2.1 mm x 150 mm and 3 µm particle size, Thermo Fisher Scientific, Sunnyvale, CA, USA) at a temperature of 40 °C. The injection volume for all samples was 5 µl. The mobile phase consists of solvent (A) 0.1% (v/v) formic acid in water, and solvent (B) acetonitrile, which were previously degassed and filtered. The gradient program for the HPLC was as follows: 0–1 min, 5% B; 1–40 min 5-55% B; 41–50 min 55-95% B; 50.1-55 min 5% B, and the flow rate was 0.3 ml/min.

The separated components from the HPLC system were applied to mass to charge ratio (m/z) analysis using an ESI-TOF-MS system. ESI-TOF-MS was carried out using a time of flight mass spectrometer (micrOTOF-Q-II, Bruker Daltonik, Bremen, Germany). An electrostray ion source (ESI) was used in negative ion mode (ESI–) with following settings: capillary voltage 3 kV, nebulizer gas pressure 2.0 bar, dry gas temperature 200°C, dry gas flow rate 8.0 L/min. Spectra were collected from m/z 50 – 1000 Da. Bruker Compass Data Analysis 4.0 software (Bruker Daltonik) was used for recording and processing the data. The phenolic and flavonoid content in EAEP was determined by interpolation with a calibration curve constructed with the standard solutions of selected polyphenols. The determination was performed 3 times.

Statistical analysis

Data analyses were performed using GraphPad Prism 7.04 software (GraphPad Software, La Jolla, CA, USA). All results are expressed as means and standard error of the mean (SEM) values from three independent experiments. *P* value ≤ 0.05 is considered to be statistically significant by using one-way ANOVA followed by Dunnett's multiple comparison.

Results

Cytotoxic assessment of EAEP on HNSCC cell lines

At first, cytotoxic effect of EAEP was evaluated using MTT assay. As shown in figures 1A-D, the EAEP (0.2 mg/ml) significantly decreased viability of HN30 and HN12 compared with the control in dose-dependent manner whereas HN4 and HN31 significantly reduced in cell viability at the concentrations of 0.25 and 0.1 mg/ml, respectively in a dose-dependent manner. The IC_{50} value of EAEP for HN30, HN31, HN4 and HN12 were 0.19, 0.16, 0.31 and 0.14 mg/ml, respectively. However, according to ISO 10993-5 non-cytotoxic dose (cell viability above 80%) for HN12, HN30 and HN31 was 0.1 mg/ml EAEP whereas 0.2 mg/ml EAEP was not toxic to HN4. Therefore, non-cytotoxic concentration at 0.1 mg/ml was selected to treat with HNSCC cell lines and evaluate their invasion and MMPs activity in the subsequent experiments. The weak cytotoxic dose (cell viability of 60-80%) was used for apoptosis assay.

Apoptotic effects of EAEP

In view of the above-mentioned effect of EAEP on the HNSCC cell viability, we considered apoptosis as an underlying mechanism. Flow cytometry was applied to quantify the apoptotic, alive and necrotic cells. HN12, HN30 and HN31 were exposed to EAEP at 0.2 mg/ml. HN4 cells were treated with 0.3 mg/ ml of EAEP. The apoptotic effects of EAEP on HNSCC cells are given in figure 2. We found that EAEP induced 42.42%, 44.01%, 43.28%, and 13.08% apoptosis in HN30, HN31, HN4 and HN12, respectively.

EAEP decreased HNSCC invasion

Furthermore, HNSCC cell invasion using Boyden chamber system was used for a functional assay. We found that invasion of HNSCC down-regulated after EAEP treatment for 5 h. EAEP at the non-toxic concentration (0.1 mg/ml) decreased the invasiveness of HN30, HN31, HN4 and HN12 cells by approximately 51%, 67%, 37%, and 56%, respectively, compared with control (Figs. 3A and 3B).

EAEP reduced MMPs activities of HNSCC cells

To further confirm whether MMPs are down-regulated in EAEP treated HNSCC cells. The cell culture medium of HNSCC cells was assayed for MMP-2 and MMP-9 activities after treatment with EAEP for 48 h. Zymographic data showed that EAEP inhibited the MMP-2 and MMP-9 activity of HN30, HN31, HN4 and HN12 cells (Fig. 4A). Quantitative analysis of MMP activity showed that at non-toxic concentration (0.1 mg/ml), EAEP significantly reduced MMP-2 activity of HN30 and HN31 cells by 64% and 27%, respectively as shown in figure 4B. However, MMP-2 activities in EAEP-treated HN4 and HN12 and control cells were not significantly different. In contrast, EAEP (0.1 mg/ml) significantly decreased MMP-9 activity in HN30, HN31, HN4 and HN12 cells to approximately 51%, 32%, 18% and 21%, respectively.

HPLC-ESI-TOF-MS analysis of EAEP

HPLC-ESI-TOF-MS was used to analyze the profiles of phenolics and flavonoids of EAEP. Commercially available polyphenolic compounds were used as standards in this determination. At first, baseline calibration of HPLC system was performed using the sample solvent (Fig. 5A). Although very minor peaks were inevitably present, only two compounds were detected in measurable quantity. The EAEP chromatograms demonstrated peaks 1 and 2 with retention times that corresponded to those of apigenin (24.7 min) and galangin (32.9 min), respectively (Figs. 5B and 5C). Furthermore, HPLC-ESI-TOF-MS parameters were optimized and used to profile EAEP. The selected 2 compounds in EAEP were putatively identified by comparison to the database (Table 1). The results revealed that compound 1 and 2 were apigenin and galangin, respectively. The amount and chemical structures of apigenin and galangin were shown in figure 6.

The concentration of these phenolic compound in EAEP was estimated by interpolation with a calibration curve constructed with standard solutions of apigenin and galangin. The quantitative determination revealed that the amount (mean ± SD) of apigenin and galangin in the EAEP was found to be 149.0 ± 7.07 µg/g and 628 .66±16.42 µg/g, respectively.

Discussion

The main objective of the present study was to evaluate the anti-cancer effect and establish the underlying mechanisms of Thai propolis in HNSCC cells. In this study we investigated, for the first time, the anti-cancer effect of ethyl acetate extract of propolis (EAEP) from Thai *A. mellifera* on primary and metastatic HNSCC cell lines. We found that EAEP showed dose-dependent cytotoxic activity and caused apoptosis in HN30, HN31, HN4 and HN12 cell lines. Our findings are agree with previous showing that anti-cancer effects of propolis obtained from many countries. Brazilian propolis extract inhibits cell growth and induces apoptotic mechanism in human prostate carcinoma (DU145 and PC-3 cells) (Li et al. 2007). Propolis extract from Turkey exhibited inhibition of cell proliferation, apoptotic and cell cycle in breast cancer (MCF7), lung cancer (A549) and gastric cancer (HGC27) (Aru et al., 2019). Turkish propolis extract also exhibited an increase in the apoptosis of MCF-7 (Misir et al. 2020).

Although we analyzed standards of apigenin, galangin, caffeic acid, ferulic acid, rutin, quercetin and naringenin but only apigenin and galantine were observed in our propolis samples. This demonstrated that propolis extracts differed qualitatively and quantitatively regarding phenolic acids and flavonoids (Anjum et al. 2019). Apigenin (Swanson et al. 2014; Yan et al. 2017; Zhu et al. 2016) and galantine (Yang et al. 2018; Zhu et al. 2014) have been reported to show anticancer activity against various cancer cell lines and they might involve in cytotoxic effect on HNSCC cell lines. It was suggested that cytotoxic activities of phenolic compounds depended on their chemical structures especially the total number of hydroxyl groups in their molecules (Czyzewska et al. 2016). Here we demonstrated the the inhibition of HNSCC cell proliferation by propolis extracts could undergo at least partially through apoptosis. Apigenin and galantine may play an important role in cytoxicity as such. We do not know exactly how

EAEP caused apoptosis in HNSCC cells. It is possible that these phenolic compounds induced apoptosis in HNSCC cells with decreased expression of the antiapoptotic proteins and increased expression of proapoptotic proteins (Zhu et al. 2014). EAEP might modulate caspase-3 and AKT signaling pathways (Wang & Tang 2017). However, synergistic effects of polyphenols in the propolis extract might be responsible for their cytotoxicity (Czyzewska et al. 2016).

Invasion and migration are considered as the important hallmarks of malignant tumors. MMP-2 and MMP-9 are enzymes that play an important role in the basement membrane degradation process which is the first step of invasion and metastasis of cancer cells (Koontongkaew, 2013). In our studies, we investigated the effects of EAEP on cell invasion by focusing on the activity of MMP-2 and MMP-9 on HNSCC cell lines. It is interesting that EAEP significantly decreased the invasion of stage III, HN30 and HN31 cells through inhibition of MMP-2 and MMP-9 activities. However, the extract reduced invasion of stage IV, HN4 and HN12 cell by attenuating only MMP-9 activity. Previous studies showed apigenin inhibited invasion and migration abilities of human metastatic cancer cell lines by reducing MMP-9 expression through suppressing the p38 MAPK signaling pathways (Noh et al. 2010). Moreover, galangin reduces MMP-9 expression and cell migration in human neuroblastoma cell lines (Yang et al. 2018) and human fibrosarcoma cells (Choi et al. 2015). We inferred that apigenin and galangin in EAEP may be a key factor in inhibiting invasive HNSCC cells.

Conclusions

In conclusion, present studies revealed that EAEP from Thai *A. mellifera* have shown cytotoxic activity in dose-dependent manner and induces apoptosis of HNSCC. The EAEP inhibited the invasion of primary and metastatic HNSCC cells through inhibition of MMP-2 and MMP-9 expression. Apigenin and galangin, were identified in EAEP. The two flavonoids may contribute to anti-cancer activities of EAEP. As evidence from the above results, the EAEP has the potential to be a powerful candidate in developing preventive agents for cancer metastasis and this beneficial effect may expand future research on anticancer properties of EAEP *in vitro* and *in vivo*.

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Figures and legends

Figure 1 Cytotoxic evaluation of EAEP on HNSCC cells measured by MTT assay. The EAEP in various concentrations were used to treat (A) HN30, (B) HN31, (C) HN4, and (D) HN12 cells for 72 h. Bars represent means±SEM of three independent experiments (n=3). * $P < 0.05$ compared with the control.

Figure 2 Effect of EAEP on HNSCC cells, after treatment for 24 h, evaluated by flow cytometry. (A) Dot plots of apoptosis assay are displayed with Annexin V-FITC (X-axis)/ 7-AAD-PE (Y-axis). (B) Percentage of apoptotic cell HN30, HN31, HN4, and HN12 cells treated with EAEP at concentrations of 0.2, 0.2, 0.3 and 0.2 mg/ml respectively for 24 h. Bars represent means±SEM of three independent experiments (n=3). * $P < 0.05$ compared with the control.

Figure 3 EAEP decreased HNSCC cell invasion. A non-cytotoxic dose of EAEP was used to treat HN30, HN31, HN4 and HN12 cells. (A) Representative images of cells that invaded onto the underside of polycarbonate filters coated with Matrigel membrane from Boyden chemoinvasion assays at 400× magnification under a light microscope. (B) Number of cells of each field was counted under a microscope at 400× magnification. Values are the mean±SEM of three independent experiments (n=3) of. * $P < 0.05$ compared with the control.

Figure 4 EAEP reduced MMP activities. EAEP (0.1 mg/ml) was used to treat the cancer cells for 48 h and MMP activities in conditioned media of (A) HN30 and HN31, and (B) HN4 and HN12 cells were detected using zymography. GeneTools software was used to quantify gelatinolytic bands of (C) MMP-2 and (D) MMP-9 activities. Bars represent means±SEM of three independent experiments (n=3). * $P <0.05$ compared with the control.

581 **Figure 5 HPLC-ESI-TOF-MS analysis of EAEP.** (A) Total ion chromatogram (TIC) of
582 blank solution, (B) EAEP sample and (C) standard compounds [apigenin (Cmpd 1, 24.7
583 min) and galangin (Cmpd 2, 32.9 min)] by negative mode HPLC-ESI-TOF-MS.
584
585 **Figure 6 Concentration and putative structure of compounds in EAEP.** (A)
586 Concentrations of apigenin and galangin in EAEP measured by HPLC-ESI-TOF-MS. (B)
587 The chemical structure of apigenin and galangin.
588

Figure 1

Cytotoxic evaluation of EAEP on HNSCC cells measured by MTT assay.

The EAEP in various concentrations were used to treat (A) HN30, (B) HN31, (C) HN4, and (D) HN12 cells for 72 h. Bars represent means±SEM of three independent experiments (n=3). * $P < 0.05$ compared with the control.

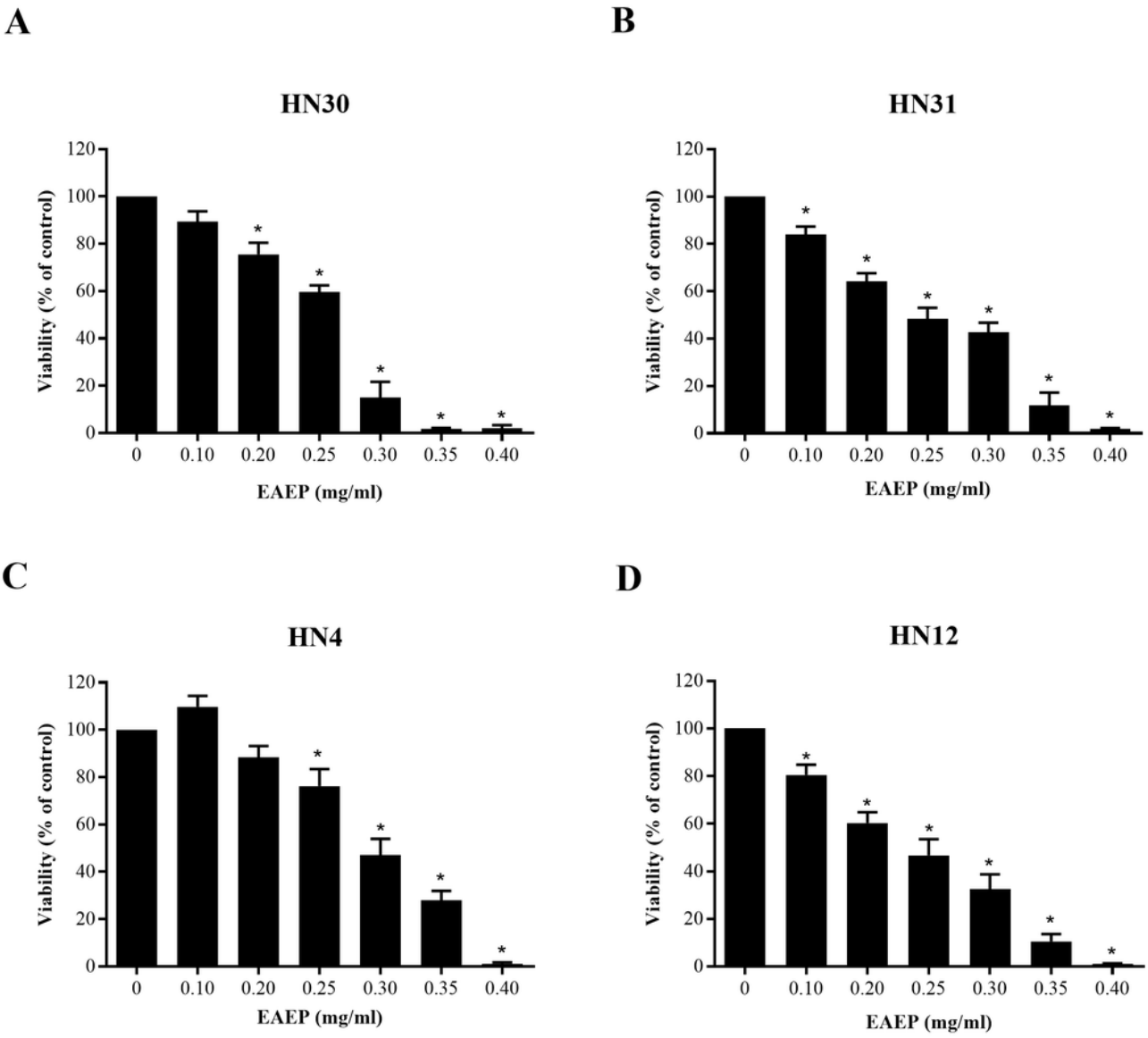
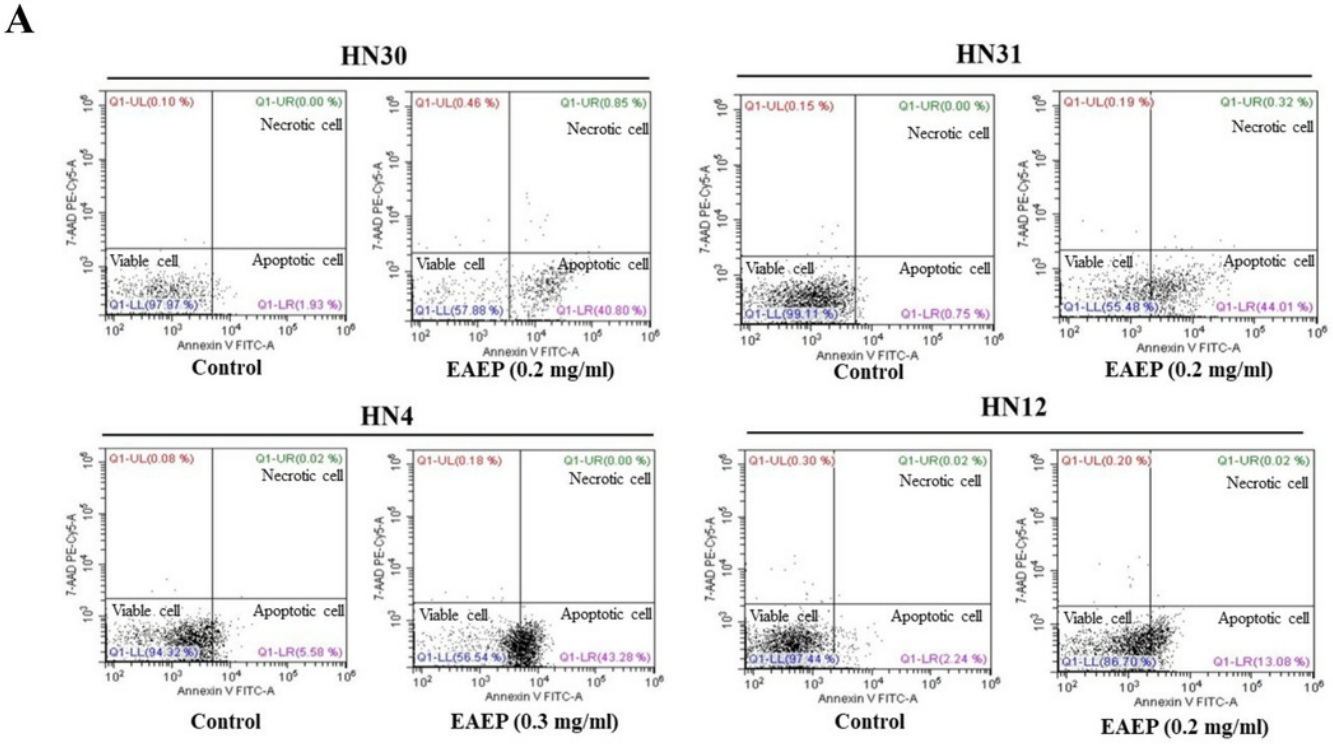


Figure 2

Effect of EAEP on HNSCC cells, after treatment for 24 h, evaluated by flow cytometry.

(A) Dot plots of apoptosis assay are displayed with Annexin V-FITC (X-axis)/ 7-AAD-PE (Y-axis). (B) Percentage of apoptotic cell HN30, HN31, HN4, and HN12 cells treated with EAEP at concentrations of 0.2, 0.2, 0.3 and 0.2 mg/ml respectively for 24 h. Bars represent means±SEM of three independent experiments (n=3). * $P < 0.05$ compared with the control.


A
HN30
Q1-UL(0.10 %)
Q1-UR(0.00 %)
Necrotic cell
Viable cell
Apoptotic cell
Q1-LL(97.97 %)
Q1-LR(1.93 %)
7-AAD PE-Cy5-A
Annexin V FITC-A
Control
Q1-UL(0.46 %)
Q1-UR(0.85 %)
Q1-LL(57.88 %)
Q1-LR(40.80 %)
EAEP (0.2 mg/ml)
HN31
Q1-UL(0.15 %)
Q1-UR(0.00 %)
Q1-LL(99.11 %)
Q1-LR(0.75 %)
Control
Q1-UL(0.19 %)
Q1-UR(0.32 %)
Q1-LL(55.48 %)
Q1-LR(44.01 %)
EAEP (0.2 mg/ml)
HN4
Q1-UL(0.08 %)
Q1-UR(0.02 %)
Q1-LL(94.32 %)
Q1-LR(5.58 %)
Control
Q1-UL(0.18 %)
Q1-UR(0.00 %)
Q1-LL(56.54 %)
Q1-LR(43.28 %)
EAEP (0.3 mg/ml)
HN12
Q1-UL(0.30 %)
Q1-UR(0.02 %)
Q1-LL(97.44 %)
Q1-LR(2.24 %)
Control
Q1-UL(0.20 %)
Q1-UR(0.02 %)
Q1-LL(86.70 %)
Q1-LR(13.08 %)
EAEP (0.2 mg/ml)

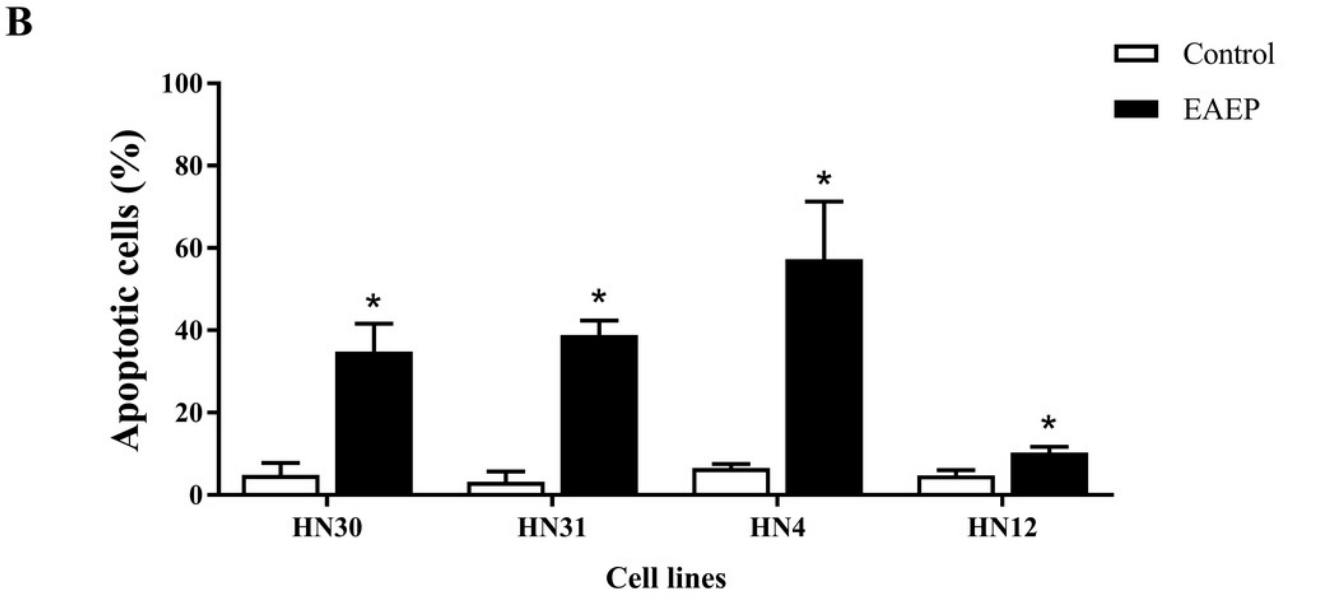

B
Control
EAEP
Apoptotic cells (%)
100
80
60
40
20
0
*
HN30
HN31
HN4
HN12
Cell lines

Figure 3

EAEP decreased HNSCC cell invasion.

A non-cytotoxic dose of EAEP was used to treat HN30, HN31, HN4 and HN12 cells. (A) Representative images of cells that invaded onto the underside of polycarbonate filters coated with Matrigel membrane from Boyden chemoinvasion assays at 400× magnification under a light microscope. (B) Number of cells of each field was counted under a microscope at 400× magnification. Values are the mean±SEM of three independent experiments (n=3) of. * $P < 0.05$ compared with the control.

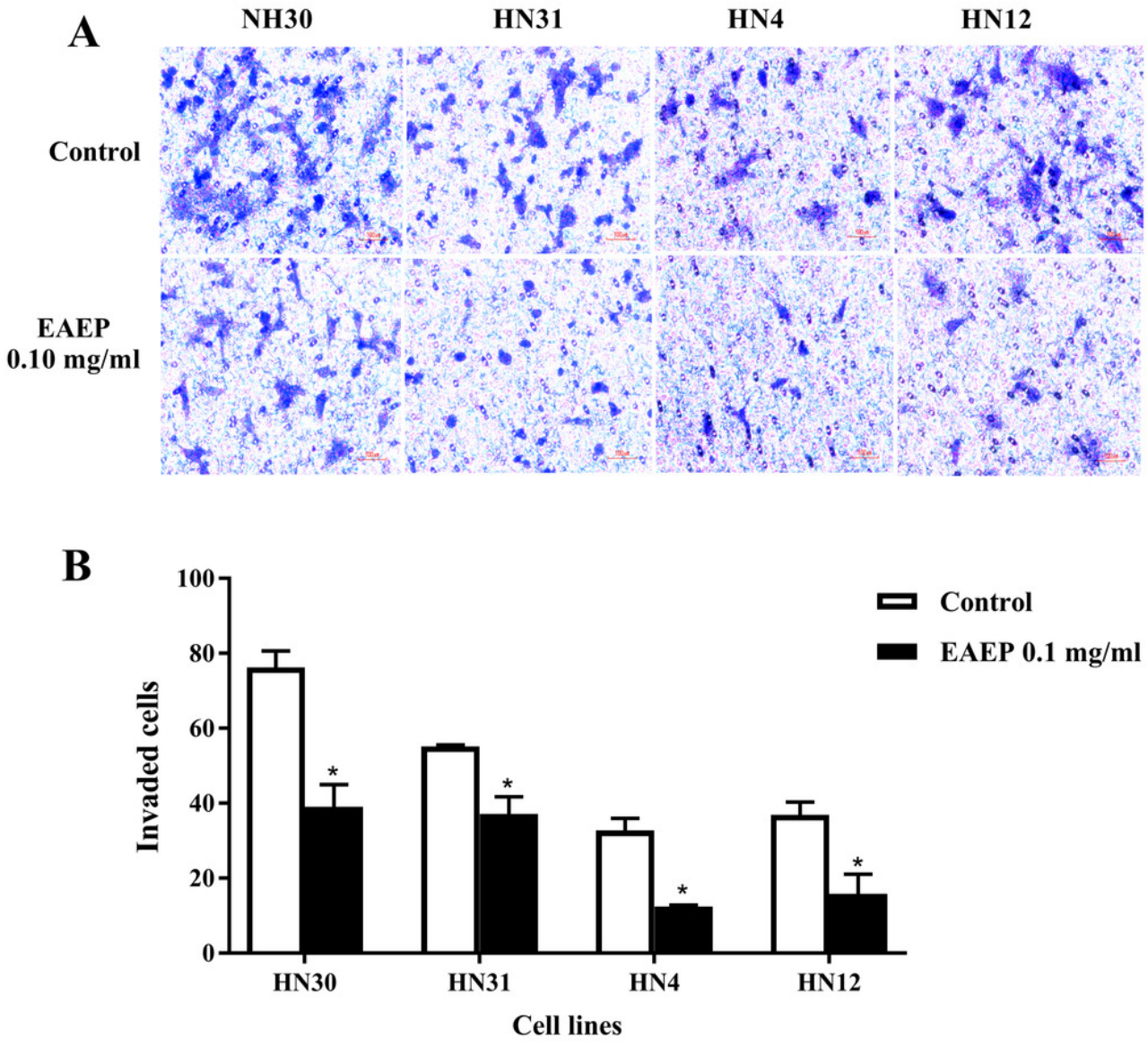

A
NH30
HN31
HN4
HN12
Control
EAEP
0.10 mg/ml
B
Control
EAEP 0.1 mg/ml
Invaded cells
100
80
60
40
20
0
*
HN30
HN31
HN4
HN12
Cell lines

Figure 4

EAEP reduced MMP activities.

EAEP (0.1 mg/ml) was used to treat the cancer cells for 48 h and MMP activities in conditioned media of (A) HN30 and HN31, and (B) HN4 and HN12 cells were detected using zymography. GeneTools software was used to quantify gelatinolytic bands of (C) MMP-2 and (D) MMP-9 activities. Bars represent means±SEM of three independent experiments (n=3). * P <0.05 compared with the control.

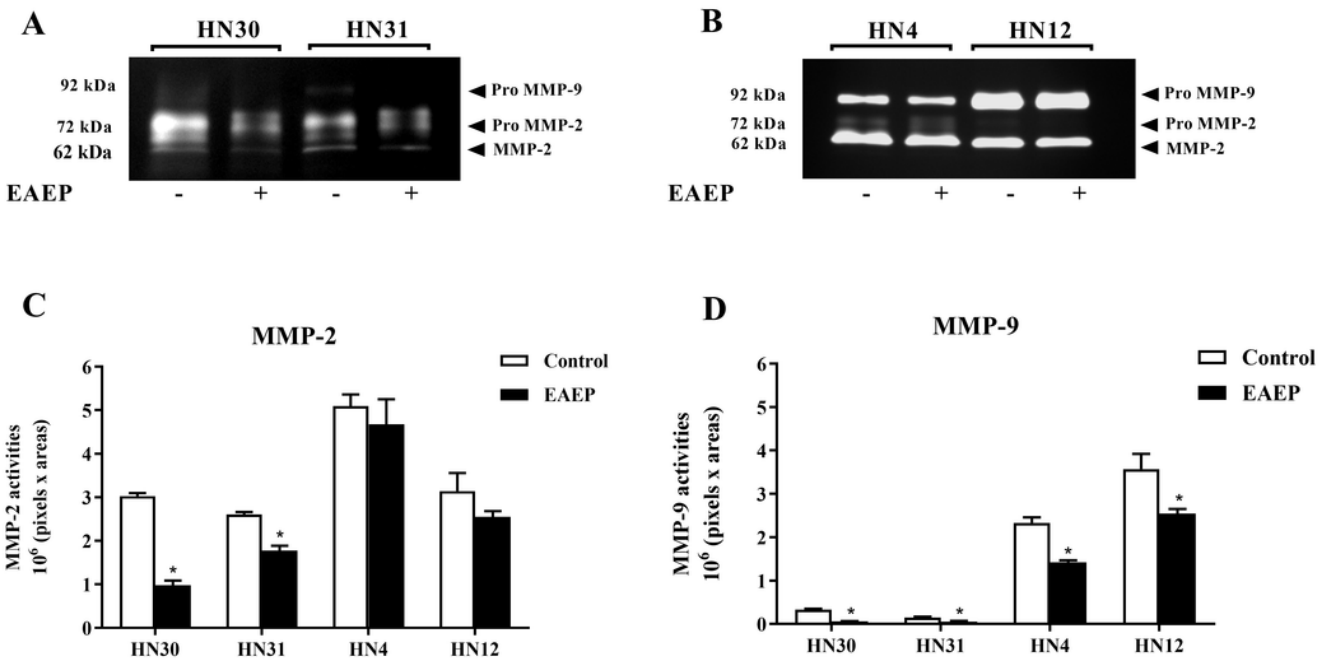


Figure 5

HPLC-ESI-TOF-MS analysis of EAEP.

(A) Total ion chromatogram (TIC) of blank solution, (B) EAEP sample and (C) standard compounds [apigenin (Cmpd 1, 24.7 min) and galangin (Cmpd 2, 32.9 min)] by negative mode HPLC-ESI-TOF-MS.

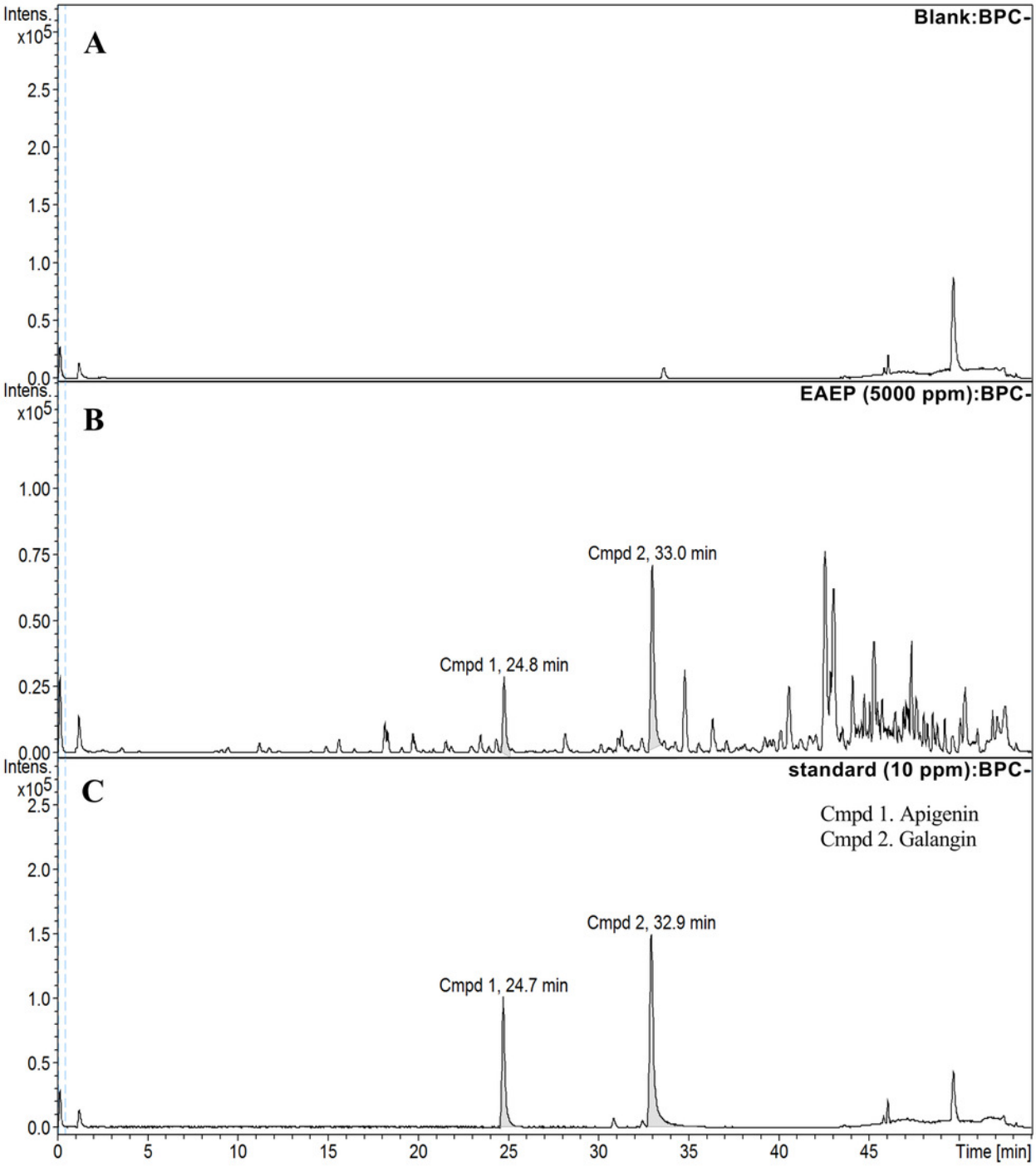

Intens.
x10^5
A
Blank:BPC-
2.5
2.0
1.5
1.0
0.5
0.0
Intens.
x10^5
B
EAEP (5000 ppm):BPC-
1.00
0.75
0.50
0.25
0.00
Cmpd 2, 33.0 min
Cmpd 1, 24.8 min
Intens.
x10^5
C
standard (10 ppm):BPC-
Cmpd 1. Apigenin
Cmpd 2. Galangin
2.5
2.0
1.5
1.0
0.5
0.0
Cmpd 2, 32.9 min
Cmpd 1, 24.7 min
0
5
10
15
20
25
30
35
40
45
Time [min]

Figure 6

Concentration and putative structure of compounds in EAEP.

(A) Concentrations of apigenin and galangin in EAEP measured by HPLC-ESI-TOF-MS. (B) The chemical structure of apigenin and galangin.

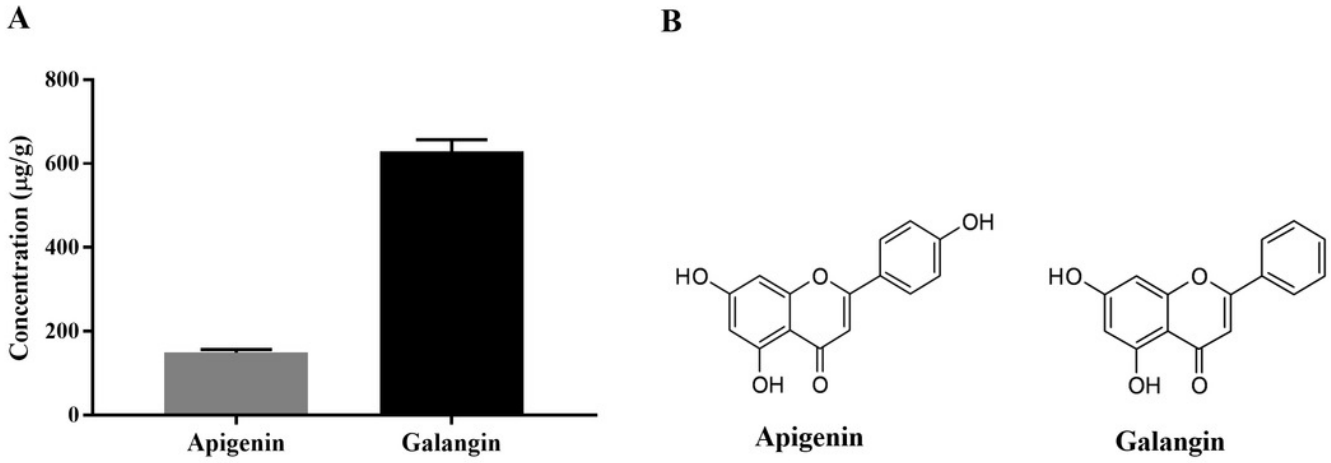


Table 1(on next page)

Retention time, calculated and detected masses, calculated formula, concentration and putative identification of the two compounds in EAEP analyzed by HPLC-ESI-TOF-MS.

Table 1:

Retention time, calculated and detected masses, calculated formula, concentration and putative identification of the two compounds in EAEP analyzed by HPLC-ESI-TOF-MS

Peak	Retention time (min)	Calculated mass $[M-H]^-$ (m/z)	Detected mass $[M-H]^-$ (m/z)	Calculated formula $[M-H]^-$	Concentration (μg/g)	Putative Identification
Cmpd 1	24.8	269.0455	269.0465	$C_{15}H_9O_5$	149.0	Apigenin
Cmpd 2	33.0	269.0455	269.0471	$C_{15}H_9O_5$	628.6	Galangin