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 that 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. The aim of this study was to investigate the effects of *Apis mellifera* propolis on apoptosis and invasiveness in HNSCC cell lines. **Methods.** Ethyl acetate extract of propolis (EAEP) was prepared from *A. mellifera* beehives using liquid-liquid extraction. High-performance liquid chromatography coupled with electrospray ionization-time of flight-mass spectrometry (HPLC-ESI-TOF-MS) was 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. The cytotoxicity, apoptosis, invasion, and MMP activity of EAEP on HNSCC cells were determined using an MTT assay, flow cytometry, Matrigel invasion assay, and gelatinase zymography, respectively. **Results.** We found that EAEP exhibited cytotoxic activity and induced apoptosis in the 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 apoptosis and exerts anti-invasion potential by inhibiting MMP-2 and MMP-9 activity in HNSCC cell lines. These inhibitory effects may be mediated by galangin and apigenin.

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Abstract

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39 possess a wide range of therapeutic properties, including anti-cancer activities. However,

40 the role of honeybee-produced propolis on head and neck squamous carcinoma

41 (HNSCC) is not well understood. The aim of this study was to investigate the effects of

42 *Apis mellifera* propolis on apoptosis and invasiveness in HNSCC cell lines.

43 **Methods.** Ethyl acetate extract of propolis (EAEP) was prepared from *A. mellifera*

44 beehives using liquid-liquid extraction. High-performance liquid chromatography coupled

45 with electrospray ionization-time of flight-mass spectrometry (HPLC-ESI-TOF-MS) was

46 used to determine the flavonoids in EAEP. Isogenic HNSCC cell lines derived from

47 primary (HN30 and HN4) and metastatic site (HN31 and HN12) were used in this study.

48 The cytotoxicity, apoptosis, invasion, and MMP activity of EAEP on HNSCC cells were

49 determined using an MTT assay, flow cytometry, Matrigel invasion assay, and gelatinase

50 zymography, respectively.

51 **Results.** We found that EAEP exhibited cytotoxic activity and induced apoptosis in the

52 HNSCC cell lines. Furthermore, EAEP significantly decreased HNSCC cell invasion by

53 reducing MMP-2 and MMP-9 activity. Two flavonoids, galangin and apigenin, were

54 identified in EAEP by HPLC-ESI-TOF-MS. The results suggest that EAEP promoted

55 apoptosis and exerts anti-invasion potential by inhibiting MMP-2 and MMP-9 activity in

56 HNSCC cell lines. These inhibitory effects may be mediated by galangin and apigenin.

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Introduction

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71   Head and neck squamous cell carcinoma (HNSCC) is the seventh most common

72 cancer worldwide and the highest occurring cancer observed in southern Asia.

73 Currently, the typical HNSCC treatment is surgical removal, combined with

74 chemotherapy and/or radiation therapy (Chow 2020; Schwartz & Hayes 2020). Similar

75 to other tumors, proliferation, invasion, and metastasis are the critical processes that

76 indicate HNSCC aggressiveness (Chan et al. 2016; Wolf & Claudio 2014). Evading

77 apoptosis is one way by which cancer cells survive in an extreme microenvironment

78 (Raudenska et al. 2021). HNSCC invasion and metastasis are driven by matrix

79 metalloproteinase (MMP) activity. MMP-2 and MMP-9 are the key enzymes that destroy

80 the basement membrane and degrade the extracellular matrix, leading to tumor

81 invasion (Koontongkaew 2013). Thus, more effective treatments that trigger apoptosis

82 in cancer cells for local and metastatic HNSCC are needed (Khan et al. 2012).

83   Propolis, or bee glue, is a natural resinous material collected by honey bees from

84 various tree buds to seal cracks in the hive and protect the hive against bacterial and

85 fungal infection (Calegari et al. 2017). Propolis has been used in traditional medicine in

86 many countries. More than 300 chemical compounds have been identified from propolis

87 in different geographic regions (Drescher et al. 2019; Xuan et al. 2016), including

88 flavonoids, terpenes, phenolic acid, cinnamic acid, caffeic acid, and several esters

89 (Funakoshi-Tago et al. 2016; Jaiswal et al. 1997; Kocot et al. 2018). Propolis has a wide

90 range of pharmaceutical properties, including antimicrobial (Al-Ani et al. 2018; Chen et

91 al. 2018), anti-inflammatory, antioxidant (Kocot et al. 2018), anti-angiogenic (Iqbal et al.

92 2019), and anti-cancer (Badr et al. 2011; Frozza et al. 2017; Sawicka et al. 2012)

93 effects. The crude extracts of propolis have demonstrated cytotoxic activity against

94 various cancer cell lines, such as human prostate cancer cells (DU145 and PC-3) (Li et

95 al. 2007), cervix adenocarcinoma cells (HeLa) (Barbaric et al. 2011), human laryngeal

96 epidermoid carcinoma cells (Hep-2) (Frozza et al. 2017), human colorectal

97 adenocarcinoma cells (HT-29), human breast adenocarcinoma cells (MCF-7), human

98 epithelial colorectal adenocarcinoma cells (Caco-2), and murine melanoma cell lines

99 (B16F1) (Choudhari et al. 2013).

100   It has been reported that the biological and pharmacological activities of propolis
101   depend on its chemical composition, geographical zone, plant source, and season
102   (Devequi-Nunes et al. 2018; Omar et al. 2017; Siheri et al. 2016). Propolis extracts from
103   *Apis mellifera* beehives in Thailand have demonstrated anti-proliferative and cytotoxic
104   activity against cancer cell lines derived from human breast carcinoma (BT474), human
105   hepatocellular carcinoma (Hep-G2), gastric carcinoma (KATO-III), and colon
106   adenocarcinoma (SW620) (Teerasripreecha et al. 2012). Moreover, the propolis extract
107   from *Trigona sirindhornae* exhibited cytotoxic effects against HNSCC cells (Utispan et
108   al. 2017). However, there are few studies on the effect of Thai *A. mellifera* propolis
109   extract on HNSCC cell lines. Therefore, the aim of this study was to investigate the
110   cytotoxic, apoptotic, and anti-invasive activity of the ethyl acetate extract from Thai *A.*
111   *mellifera* propolis on primary and metastatic HNSCC cell lines.

112

Materials and Methods

Chemicals

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

120

Ethanol extract of propolis (EEP) preparation

122   The propolis sample from the native Thai bee species *A. mellifera* was obtained in
123   November 2017 in Loei province, northeastern Thailand. The sample was stored in a
124   desiccator and kept in the dark at 4°C until processed. Raw propolis (5.27g) was cut into
125   small pieces and stirred in 100 ml 95% (v/v) ethanol (EtOH) at 100 rpm at room
126   temperature for 48 h in the dark. The insoluble portion was separated by filtration through
127   No. 2 Whatman filter paper (Whatman Inc, Piscataway, NJ, USA). To increase the extract
128   yield, this procedure was repeated three times on the same sample. The resulting filtrates
129   were pooled and dried in a rotatory evaporator at 40°C and 175 mbar (Rotovapor R-215,

130   BUCHI Labortechnik, AG, Switzerland). The ethanol extract of propolis (EEP, 4.32 g) with

131   a viscous appearance was obtained.

132

Liquid-liquid partitioning

134   EEP was fractioned using liquid-liquid partitioning. The EEP (4.32 g) was dissolved

135   in 100 ml methanol and then partitioned with hexane (3 x 50 ml). The combined hexane

136   extract was then rotatory evaporated at 40°C and 335 mbar to yield the hexane extract of

137   propolis (0.98 mg). The methanol portion was evaporated at 40ºC and 337 mbar. The

138   methanol extract was dissolved in 100 ml distilled water and underwent liquid-liquid

139   partitioning with ethyl acetate (3 x 50 ml). After the ethyl acetate was evaporated under

140   reduced pressure on a rotary evaporator, the residual solvent was removed by drying

141   under vacuum (Edwards RV12 rotary vane vacuum pump, Bolton, England) at room

142   temperature for 16 h. The residual ethyl acetate in EAEP was determined by Nuclear

143   Magnetic Resonance (NMR) Spectroscopy. The crude extract was dissolved in DMSO-

144   d_6 (Sigma-Aldrich, St. Louis, MO, USA) and the ^{1}H-NMR spectrum was recorded on a

145   Bruker ASCEND 400 FT-NMR spectrometer (Bruker, Faellanden, Switzerland) operating

146   at 400 MHz. The chemical shifts are shown as parts per million (ppm). Finally, solid

147   masses were obtained for the ethyl acetate extract (EAEP, 1.92 g) and aqueous extract

148   (0.12 g) after complete solvent evaporation.

149   Most of the substances found in propolis were obtained in polar organic solvents,

150   including ethanol, methanol, and ethyl acetate (Sambou et al. 2020). Solvents, e.g. ethyl

151   acetate, are used in extraction processes because of its chemical and biological

152   functions, such as its medium polarity (polarity index = 4.3) (Synder 1974). The biphasic

153   action of this solvent enables it to be used to extract both polar and non-polar compounds

154   (Mandal et al. 2015). Ethyl acetate is classified as a class 3 solvent with low toxic potential

155   (Abarca-Vargas et al. 2016; Aru et al. 2019). Furthermore, previous studies demonstrated

156   that the ethyl acetate extracts of many medicinal plants were not cytotoxic to normal cells

157   (El Khalki et al. 2018; Jain et al. 2011; Seklic et al. 2018). Therefore, only EAEP was used

158   in the present study. Before use, the EAEP extract was dissolved in dimethyl sulfoxide

159   (DMSO) and placed in a freezer (-30ºC) until use.

160

Cell culture

Two pairs of isogenetic HNSCC cell lines representing 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 a researcher at the National Institute of Dental and Craniofacial Research under the supervision of Gutkind S. Gutkind S. provided the cell lines as a gift to Koontongkaew S. The HN30 and HN31 cells were obtained from primary pharynx lesions and lymph node metastases (T3N1M0), respectively. The HN4 and HN12 cells were obtained from primary tongue lesions and lymph node metastases (T4N1M0), respectively. The cells 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. The cells were passaged with 0.25% trypsin-EDTA when reaching 70–80% confluence.

Cell viability-MTT assay

The cytotoxicity of EAEP on the HNSCC cell lines was investigated using the methyl thiazotetrazolium (MTT) as previously described (Utispan et al. 2020). Briefly, the incubation periods for the MTT assay were 24, 48, or 72 h. However, tumor cells are very heterogenous and their doubling times vary. Therefore, a 24 h incubation period was enough if the doubling time was ~16–24 h. However, for certain cancer cell lines, a 24 h incubation period with a given test substance is too short to demonstrate a significant effect on cell viability (Abdul Latif et al. 2019; Alizadeh-Navaei et al. 2016; Guo et al. 2015). Therefore, in the present study, a 72 h incubation period was used to assess cell toxicity. The HNSCC cells were seeded in 96-well plates at a density of 2,000 cells/well. The cells were treated with serum-free DMEM with 0.1% DMSO (vehicle control) or EAEP (0.10–0.40 mg/ml) at 37°C for 72 h. After the exposure period, the media was removed, and the cells were washed with phosphate-buffered saline (PBS), and incubated with 0.5 mg/ml MTT (Sigma) in culture media for 4 h. The purple formazan crystals of the viable cells were dissolved and measured at 570 nm by a microplate reader (Tecan, Salzburg, Austria). Cell viability was calculated as a percentage of that of the control (untreated) cells. Each EAEP concentration was independently assayed three times with three

192 technical replicates. Based on ISO 10993-5, cell viability above 80% was considered non-
193 cytotoxic, between 80%–60% weak, 60%–40% moderate, and below 40% strong
194 cytotoxicity, respectively (International Organization for Standardization ISO 10993-5
195 2009).

196

Apoptosis assay

198 To verify that the effect of the studied extracts on the growth inhibition of HNSCC
199 cells was related to apoptosis, the apoptosis and necrotic cells were analyzed using
200 annexin-V- fluorescein isothiocyanate (FITC) and propidium iodide (PI) staining. The cells
201 were seeded in 6-well plates and allowed to attach for 24 h. The cells were then treated
202 with EAEP at the weak cytotoxic dose (cell viability of 60%–80%) for 24 h. The HN12,
203 HN30, and HN31 cells were treated with 0.2 mg/ml EAEP, whereas the HN4 cells were
204 treated with 0.3 mg/ml EAEP. The cells were washed twice with PBS and detached by
205 0.25% trypsin, washed with PBS and resuspended in ice cold binding buffer. The
206 apoptotic cells were assessed using the BD Annexin V FITC Assay (BD Biosciences, San
207 Jose, CA, USA). Ten thousand events were analyzed in a flow cytometer (Cytoflex®,
208 Beckman Coulter, Indianapolis, IN, USA). The percent viable, apoptotic and necrotic cells
209 were determined by CytExpert Software (Beckman Coulter).

210

Invasion assay

212 The modified Boyden chemotaxis chamber (Neuro Probe, Gaithersburg, MD,
213 USA) assay used for the cell invasion analysis is based on a chamber with two medium
214 filled compartments as previously described (Albini et al. 1987). Matrigel, a reconstituted
215 basement membrane gel (BD Bioscience, San Jose, CA)) was applied to a polycarbonate
216 membrane filter (13 mm-diameter, 8.0 µm pore size, Whatman). The filter was placed
217 above the lower chamber that contained serum-free DMEM with 0.1% bovine serum
218 albumin (BSA, Sigma). HN4, HN12, HN30, or HN31 cells (1×10^5 cells) were resuspended
219 in 0.1 mg/ml EAEP diluted in DMEM containing 0.1% BSA and seeded into the upper well
220 of the chamber. The cells were incubated for 5 h, then the filters were fixed and stained
221 with crystal violet for 10 min. The invaded cells were counted by two investigators using

222 a microscope at 400x magnification. The invaded cell counts were averaged from five
223 randomly selected fields.

224

Conditioned medium preparation and zymography

226   HN4, HN12, HN30, or HN31 cells (1×10^6 cells) were cultured in 6-well plates and
227 incubated at 37°C for 24 h. After incubation, the wells were washed with PBS and treated
228 with 0.1 mg/ml EAEP diluted in DMEM containing 0.1% BSA for 48 h. Cells cultured in
229 DMEM containing 0.1% BSA were used as control. The conditioned medium (CM) was
230 collected as previously described in Utispan et al., 2020 (Utispan et al. 2020).

231   The MMP-2 and MMP-9 activity in the CM were measured using gelatin
232 zymography as previously described (Koontongkaew et al. 2009). The clear bands in the
233 gel were identified as degrading activity of MMPs. Images of the stained gels and the
234 gelatinolytic band quantification were processed using the G:BOX gel documentation
235 system (Syngene, Frederick, MD, USA) and Gene Tools software (Syngene),
236 respectively. Three independent experiments were performed.

237

HPLC-ESI-TOF-MS analysis of EAEP

239   High-performance liquid chromatography coupled with electrospray ionization-time
240 of flight-mass spectrometry (HPLC-ESI-TOF-MS) was used to investigate selected
241 phenolic acids and flavonoids in EAEP. EAEP was prepared at 5 mg/ml in ethanol and
242 filtered through a 0.45 µm membrane filter. Although a large number of natural
243 compounds have been reported in propolis, we focused on specific substances in propolis
244 that exhibit anti-cancer activity (Anjum et al. 2019), Thus, we investigated the presence
245 of apigenin, galangin, caffeic acid, ferulic acid, rutin, quercetin, and naringenin in EAEP.
246 To identify the compounds, seven standard polyphenolic compounds (apigenin, galangin,
247 caffeic acid, ferulic acid, rutin, quercetin and naringenin) were dissolved in methanol (10
248 ppm). The analyses were conducted in an UltiMate® 3000 system (Thermo Fisher
249 Scientific, DionexSoftron GmbH, Dornierstr. 4, Germany) with a reverse phase column
250 (C18 analysis column, 2.1 mm x 150 mm and 3 µm particle size, Thermo Fisher Scientific,
251 Sunnyvale, CA, USA) at 40°C. The injection volume for all samples was 5 µl. The mobile
252 phase consisted of solvent (A) 0.1% (v/v) formic acid in water, and solvent (B) acetonitrile,

253   which had been degassed and filtered. The gradient program for the HPLC was: 0–1 min,

254   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

255   0.3 ml/min.

256   The separated components from the HPLC system were subjected to mass to

257   charge ratio (m/z) analysis using an ESI-TOF-MS system. ESI-TOF-MS was carried out

258   using a time of flight mass spectrometer (micrOTOF-Q-II, Bruker Daltonik, Bremen,

259   Germany). An electrostray ion source (ESI) was used in negative ion mode (ESI−) with

260   the following settings: capillary voltage 3 kV, nebulizer gas pressure 2.0 bar, dry gas

261   temperature 200°C, and dry gas flow rate 8.0 L/min. Spectra were collected from m/z 50

262   – 1000 Da. Bruker Compass Data Analysis 4.0 software (Bruker Daltonik) was used for

263   recording and processing the data. The phenolic and flavonoid content in EAEP was

264   determined by interpolation with a calibration curve constructed using the standard

265   solutions of selected polyphenols. The determination was performed 3 times.

266

267   **Statistical analysis**

268   Data analyses were performed using GraphPad Prism 7.04 software (GraphPad

269   Software, La Jolla, CA, USA). All results are expressed as means and standard error of

270   the mean (SEM) values from three independent experiments. $P \leq 0.05$ was considered

271   statistically significant using one-way ANOVA followed by Dunnett's multiple comparison.

272

273   **Results**

274   **Cytotoxic assessment of EAEP on HNSCC cell lines**

275   The cytotoxic effect of EAEP was evaluated using an MTT assay. The EAEP (0.2

276   mg/ml) significantly decreased the HN30 and HN12 cell viability compared with the control

277   in a dose-dependent manner, whereas the HN4 and HN31 cells demonstrated

278   significantly reduced cell viability at 0.25 and 0.1 mg/ml EAEP, respectively in a dose-

279   dependent manner (Fig. 1A-D). The IC_{50} value of EAEP for the HN30, HN31, HN4, and

280   HN12 cells were 0.19, 0.16, 0.31, and 0.14 mg/ml, respectively. However, according to

281   ISO 10993-5, the non-cytotoxic dose (cell viability above 80%) for the HN12, HN30, and

282   HN31 cells was 0.1 mg/ml EAEP, whereas 0.2 mg/ml EAEP was not toxic to the HN4

283   cells. Therefore, the non-cytotoxic concentration at 0.1 mg/ml was selected to treat the

284   HNSCC cell lines and evaluate their invasion and MMP activity in the subsequent

285   experiments. The weak cytotoxic dose (cell viability of 60–80%) was used for the

286   apoptosis assay.

287

288   **Apoptotic effects of EAEP**

289   Based on the effect of EAEP on HNSCC cell viability, we hypothesized that

290   apoptosis was an underlying mechanism. Flow cytometry was used to quantify the

291   apoptotic, alive, and necrotic cells. The HN12, HN30, and HN31 cells were exposed to

292   0.2 mg/ml EAEP. The HN4 cells were treated with 0.3 mg/ml EAEP. We found that EAEP

293   induced 42.42%, 44.01%, 43.28%, and 13.08% apoptosis in the HN30, HN31, HN4, and

294   HN12, cells respectively (Fig. 2).

295

296   **EAEP decreased HNSCC invasion**

297   HNSCC cell invasion using a Boyden chamber system was used as a functional

298   assay. We found that HNSCC invasion was down-regulated after EAEP treatment for 5

299   h. EAEP at the non-toxic concentration (0.1 mg/ml) decreased the invasiveness of the

300   HN30, HN31, HN4, and HN12 cells by approximately 51%, 67%, 37%, and 56%,

301   respectively, compared with control (Figs. 3A and 3B).

302

303   **EAEP reduced the MMP activity of HNSCC cells**

304   To determine whether MMPs were down-regulated in EAEP-treated HNSCC cells,

305   the cell culture medium of the HNSCC cells was assayed for MMP-2 and MMP-9 activity

306   after treatment with EAEP for 48 h. The zymographic data demonstrated that EAEP

307   inhibited the MMP-2 and MMP-9 activity of the HN30, HN31, HN4, and HN12 cells (Fig.

308   4A). Quantitative analysis of the MMP activity revealed that at the non-toxic concentration

309   (0.1 mg/ml), EAEP significantly reduced the MMP-2 activity of the HN30 and HN31 cells

310   by 64% and 27%, respectively (Fig. 4B). However, the MMP-2 activity in the EAEP-

311   treated HN4 and HN12 and control cells was not significantly different. In contrast, 0.1

312   mg/ml EAEP significantly decreased MMP-9 activity in the HN30, HN31, HN4, and HN12

313   cells to approximately 51%, 32%, 18%, and 21%, respectively.

314

HPLC-ESI-TOF-MS analysis of EAEP

316   HPLC-ESI-TOF-MS was used to analyze the profiles of the phenolics and

317   flavonoids ofin EAEP. Commercially available polyphenolic compounds were used as

318   standards in this determination. Baseline calibration of the HPLC system was performed

319   using the sample solvent (Fig. 5A). Although very minor peaks were present, only two

320   compounds were detected in a measurable quantity. The EAEP chromatograms

321   demonstrated peaks 1 and 2 with retention times that corresponded to those of apigenin

322   (24.7 min) and galangin (32.9 min), respectively (Figs. 5B and 5C). The HPLC-ESI-TOF-

323   MS parameters were optimized and used to profile EAEP. The selected 2 compounds in

324   EAEP were putatively identified by comparison to the database (Table 1). The results

325   revealed that compound 1 and 2 were apigenin and galangin, respectively. The amount

326   and chemical structures of apigenin and galangin are shown in Figure 6. The

327   concentration of these phenolic compounds in EAEP was estimated by interpolation with

328   a calibration curve constructed with standard solutions of apigenin and galangin. The

329   quantitative determination revealed that the amount (mean $\pm$ SD) of apigenin and

330   galangin in the EAEP was 149.0 $\pm$ 7.07 µg/g and 628.66 $\pm$ 16.42 µg/g, respectively.

331

Detection of residual solvent in EAEP

333   The final product extract should not contain any residual solvent to ensure that the

334   biological activities observed were not due to ethyl acetate in the test fraction. The

335   residual solvents were evaporated under reduced pressure on a rotatory evaporator,

336   followed by residual solvent removal in a vacuum dryer for 16 h. Further, ^{1}H-NMR analysis

337   of the ethyl acetate confirmed the absence of residual ethyl acetate in the EAEP. Three

338   sets of protons were responsible for the three signals in the ^{1}H-NMR spectra of ethyl

339   acetate (Fig. 7A). The two H_b protons in ethyl acetate split the H_c signal into a triplet at

340   approximately δ 1.70 ppm, and the three H_c protons split the Hb signal into a quartet at

341   approximately δ 4.02 ppm. There was an unsplit single peak at approximately δ 1.98 ppm

342   that corresponds to the acetyl (H_a) protons. The ^{1}H-NMR spectra were consistent with a

343   previous report (Fulmer et al. 2010). The ^{1}H-NMR spectrum of EAEP did not exhibit

344   signals identifying ethyl acetate (Fig. 7B). Therefore, the ^{1}H-NMR spectra indicated that

345   the EAEP extract was not contaminated with ethyl acetate. This confirms that the

346   bioactive compounds in EAEP were responsible for the cytotoxicity, apoptosis, cell

347   invasion, and MMP activity of HNSCC cell lines in our studies.

348

Discussion

350   The main objective of the present study was to evaluate the anti-cancer effect

351   and establish the underlying mechanisms of Thai propolis on HNSCC cells. This study

352   investigated, for the first time, the anticancer effect of the ethyl acetate extract of

353   propolis (EAEP) from Thai *A. mellifera* on primary and metastatic HNSCC cell lines. We

354   found that EAEP demonstrated a dose-dependent cytotoxic effect and caused

355   apoptosis in the HN30, HN31, HN4, and HN12 cell lines. Our findings agree with

356   previous studies demonstrating the anti-cancer effects of propolis obtained from many

357   countries. Brazilian propolis extract inhibits cell growth and induces apoptotic

358   mechanisms in human prostate carcinoma (DU145 and PC-3 cells) (Li et al. 2007).

359   Similarly, a propolis extract from Turkey inhibited cell proliferation and induced

360   apoptosis and cell cycle arrest in breast cancer (MCF7), lung cancer (A549) and gastric

361   cancer (HGC27) (Aru et al. 2019; Misir et al. 2020).

362   We analyzed standards of apigenin, galangin, caffeic acid, ferulic acid, rutin,

363   quercetin and naringenin, however; only apigenin and galantine were observed in our

364   propolis samples. This finding demonstrated that propolis extracts differ qualitatively and

365   quantitatively regarding phenolic acids and flavonoids (Anjum et al. 2019). Apigenin

366   (Swanson et al. 2014; Yan et al. 2017; Zhu et al. 2016) and galangin (Seyhan et al. 2019;

367   Yang et al. 2018; Zhu et al. 2014) have demonstrated anticancer activity against various

368   cancer cell lines and they might be involved in the cytotoxic effect on HNSCC cell lines.

369   These results suggest that the cytotoxic activities of phenolic compounds depends on

370   their chemical structure, especially the total number of hydroxyl groups in their molecules

371   (Czyzewska et al. 2016). Here, we demonstrated the inhibition of HNSCC cell proliferation

372   by propolis extracts could occur, at least partially, through apoptosis. Apigenin and

373   galangin may play an important role in the EAEP cytotoxic effect on HNSCC cell lines.

374 The mechanism by which EAEP caused apoptosis in HNSCC cells is unclear. These
375 phenolic compounds may induce apoptosis in HNSCC cells by decreasing the expression
376 of antiapoptotic proteins and increasing the expression of proapoptotic proteins (Zhu et
377 al. 2014). EAEP might modulate the caspase-3 and AKT signaling pathways (Wang &
378 Tang 2017). However, the synergistic effects of polyphenols in the propolis extract might
379 be responsible for their cytotoxicity (Czyzewska et al. 2016) .

380   Invasion and migration are considered important hallmarks of malignant tumors.
381 MMP-2 and MMP-9 are enzymes that play an important role in basement membrane
382 degradation, which is the first step in the invasion and metastasis of cancer cells
383 (Koontongkaew, 2013). In the present study, we investigated the effects of EAEP on cell
384 invasion by focusing on the activity of MMP-2 and MMP-9 in HNSCC cell lines. Notably,
385 EAEP significantly decreased the invasion of stage III, HN30 and HN31, cells by inhibiting
386 MMP-2 and MMP-9 activity. However, the extract reduced invasion of stage IV, HN4 and
387 HN12, cells by attenuating only MMP-9 activity. Previous studies found that apigenin
388 inhibited the invasion and migration of human metastatic cancer cell lines by reducing
389 MMP-9 expression by suppressing the p38 MAPK signaling pathways (Noh et al. 2010).
390 Moreover, galangin reduces MMP-9 expression and cell migration in human
391 neuroblastoma cell lines (Yang et al. 2018) and human fibrosarcoma cells (Choi et al.
392 2015). Based on these and our findings, we hypothesize that apigenin and galangin in
393 EAEP may be a key factor in inhibiting invasive HNSCC cells.

394   Previous studies have reported the cytotoxic and anti-invasiveness concentrations
395 of apigenin and galangin in the range 0.01–0.1 and 5–30 µg/ml, respectively (Seyhan et
396 al. 2019; Swanson et al. 2014). In the present study we obtained 1,920 mg EAEP and
397 found approximately 286.08 and 1,207 µg apigenin and galangin, respectively, in the
398 extract. We estimated that the effective dose of EAEP contains maximum concentrations
399 of 0.045 and 0.188 µg/ml of apigenin and galangin respectively. These results imply that
400 apigenin and galangin are at least two candidate compounds that exhibit anticancer
401 effects on HNSCC cells. Based on the results of the present study, other compounds in
402 EAEP should be identified and their synergistic effect investigated.

403   One limitation of our study is that we did not compare the biological activities of
404 EAEP between HNSCC cells and normal cells. Therefore, it is difficult to specify that the
405 EAEP activities are specific to cancer cells. To clarify these observations, further studies
406 are needed to investigate the anticancer activities in counterpart normal cells. Additional
407 in vivo studies should be undertaken to determine the efficacy of EAEP and its related
408 compounds in inhibiting tumor progression.
409

Conclusions

In conclusion, the present study revealed that EAEP from Thai *A. mellifera* has a dose-dependent cytotoxic effect and induces apoptosis of HNSCC. The EAEP inhibited the invasion of primary and metastatic HNSCC cells by inhibiting MMP-2 and MMP-9 expression. Apigenin and galangin were identified in the EAEP. These two flavonoids may contribute to the anti-cancer activities of EAEP. Therefore, 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 the anticancer properties of EAEP *in vitro* and *in vivo*.

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628   **Figures and Legends**

629   **Figure 1 Cytotoxic evaluation of EAEP on HNSCC cells measured by MTT assay.**

630   The EAEP at various concentrations were used to treat (A) HN30, (B) HN31, (C) HN4,

631   and (D) HN12 cells for 72 h. Bars represent means±SEM of three independent

632   experiments (n=3). *$P < 0.05$ compared with the control.

633

634   **Figure 2 Effect of EAEP on HNSCC cells, after treatment for 24 h, evaluated by flow**

635   **cytometry.** (A) Dot plots of apoptosis assay with Annexin V-FITC (X-axis)/ 7-AAD-PE (Y-

636   axis). (B) Percentage of apoptotic HN30, HN31, HN4, and HN12 cells treated with 0.2,

637   0.2, 0.3, and 0.2 mg/ml EAEP, respectively for 24 h. Bars represent means±SEM of three

638   independent experiments (n=3). *$P < 0.05$ compared with the control.

639

640   **Figure 3 EAEP decreased HNSCC cell invasion.** A non-cytotoxic dose of EAEP was

641   used to treat HN30, HN31, HN4, and HN12 cells. (A) Representative images of cells that

642   invaded onto the underside of polycarbonate filters coated with Matrigel membrane from

643   Boyden chemoinvasion assays at 400× magnification using a light microscope. (B) The

644   number of cells of each field was counted using a microscope at 400× magnification.

645   Values are the mean±SEM of three independent experiments (n=3) of. *$P < 0.05$

646   compared with the control.

647

648   **Figure 4 EAEP reduced MMP activities.** EAEP (0.1 mg/ml) was used to treat the cancer

649   cells for 48 h and MMP activity in the conditioned media of (A) HN30 and HN31, and (B)

650   HN4 and HN12 cells were detected using zymography. GeneTools software was used to

651   quantify gelatinolytic bands of (C) MMP-2 and (D) MMP-9 activity. Bars represent

652   means±SEM of three independent experiments (n=3). *$P < 0.05$ compared with the

653   control.

654

655   **Figure 5 HPLC-ESI-TOF-MS analysis of EAEP.** (A) Total ion chromatogram (TIC) of

656   blank solution, (B) EAEP sample, and (C) standard compounds [apigenin (Cmpd 1, 24.7

657   min) and galangin (Cmpd 2, 32.9 min)] by negative mode HPLC-ESI-TOF-MS.

658   **Figure 6 Concentration and putative structure of the compounds in EAEP.** (A)

659   Concentrations of apigenin and galangin in EAEP measured by HPLC-ESI-TOF-MS. (B)

660   The chemical structure of apigenin and galangin.

661

662   **Figure 7 ^{1}H-NMR spectra of ethyl acetate and EAEP**. (A) ^{1}H-NMR spectra (400 MHz)

663   of ethyl acetate in DMSO-d_6. Three sets of protons are responsible for the three signals

664   in the ^{1}H-NMR spectra of ethyl acetate. The signals at approximately δ 1.98, 4.02, and

665   1.70 ppm correspond to the H_a, H_b, and H_c hydrogens, respectively. (B) The ^{1}H-NMR

666   spectra (400 MHz) of EAEP in DMSO-d_6 did not exhibit signals identifying ethyl acetate.

667

Figure 1

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

The EAEP at 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.

A

B

C

D

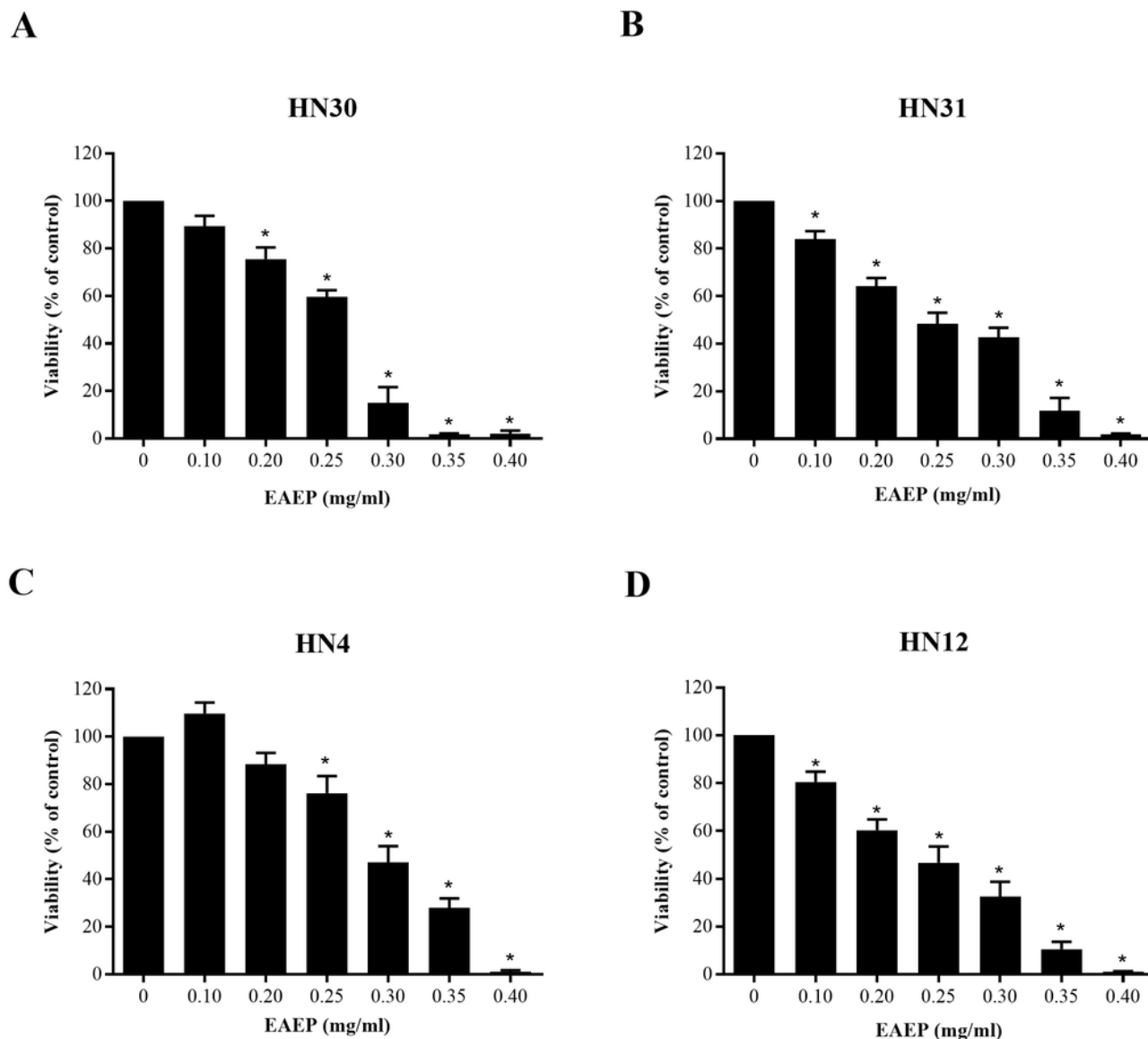
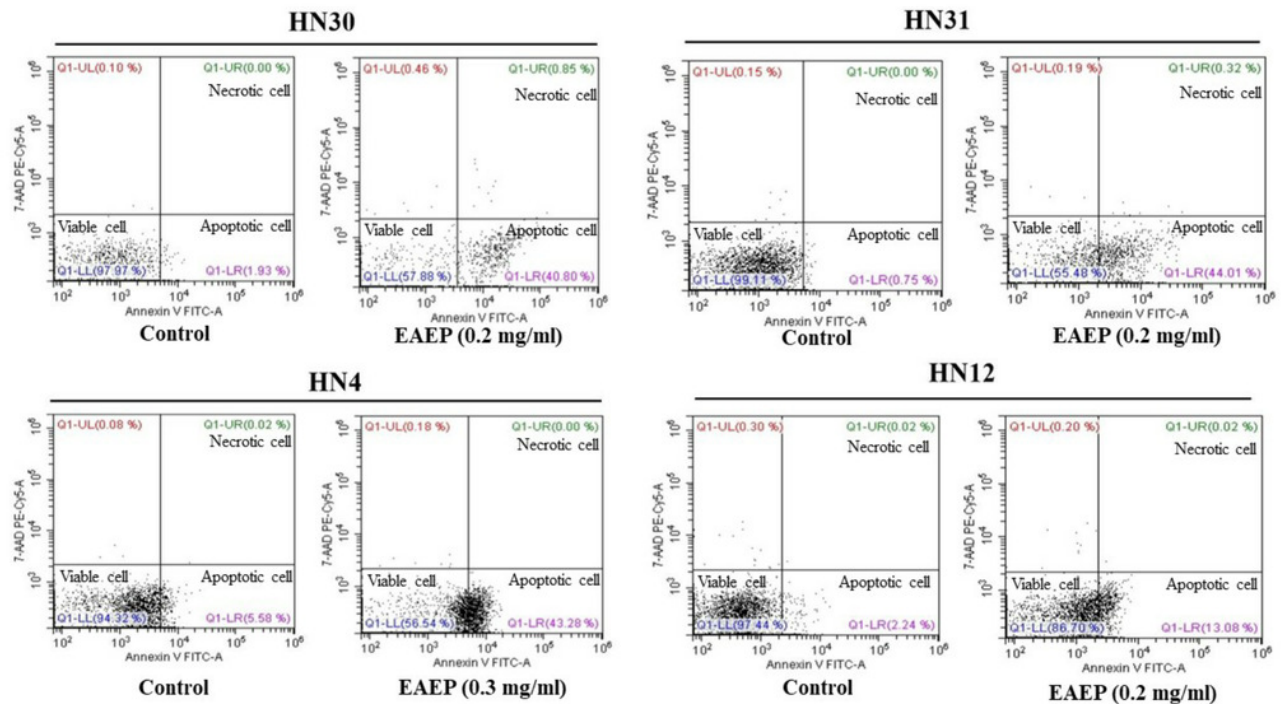



Figure 2

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

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

A

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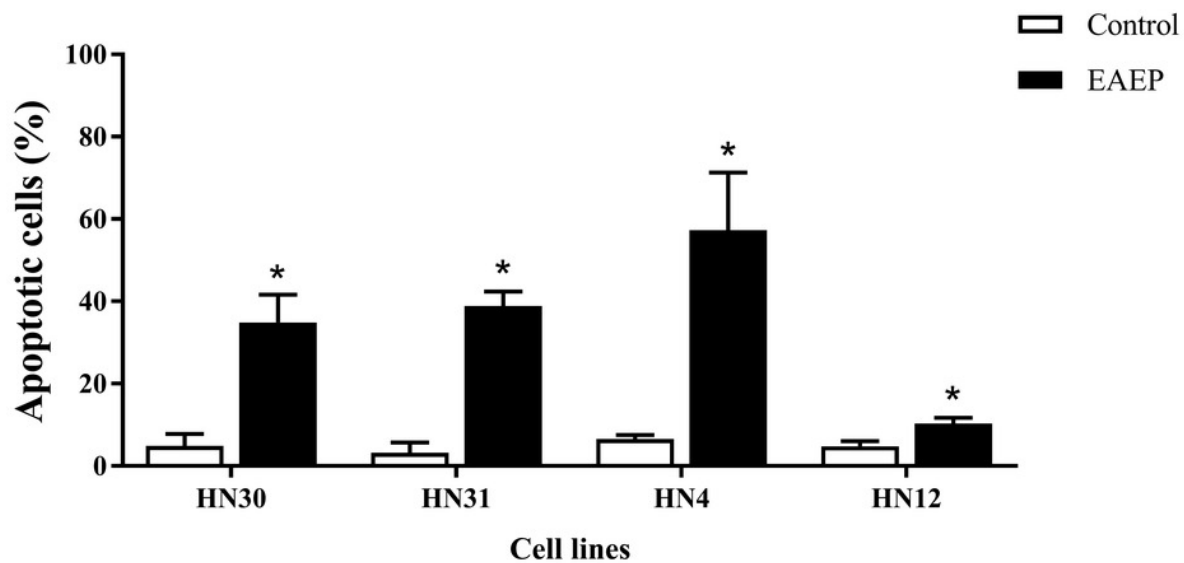


Figure 3

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 using a light microscope. (B) The number of cells of each field was counted using a microscope at 400× magnification. Values are the mean±SEM of three independent experiments (n=3) of. *P < 0.05 compared with the control.

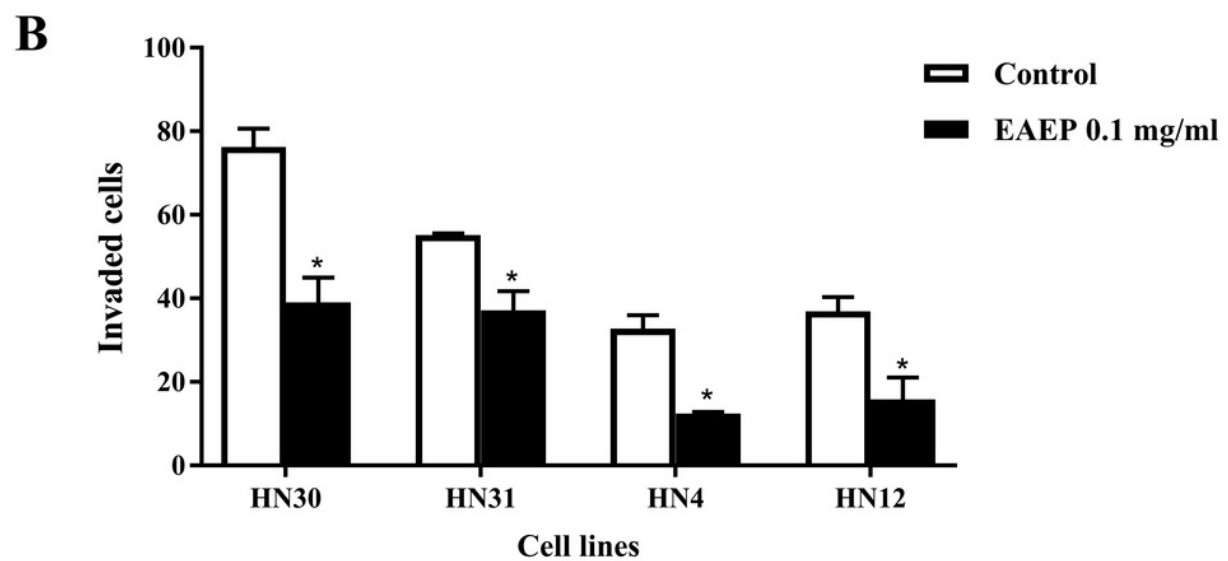
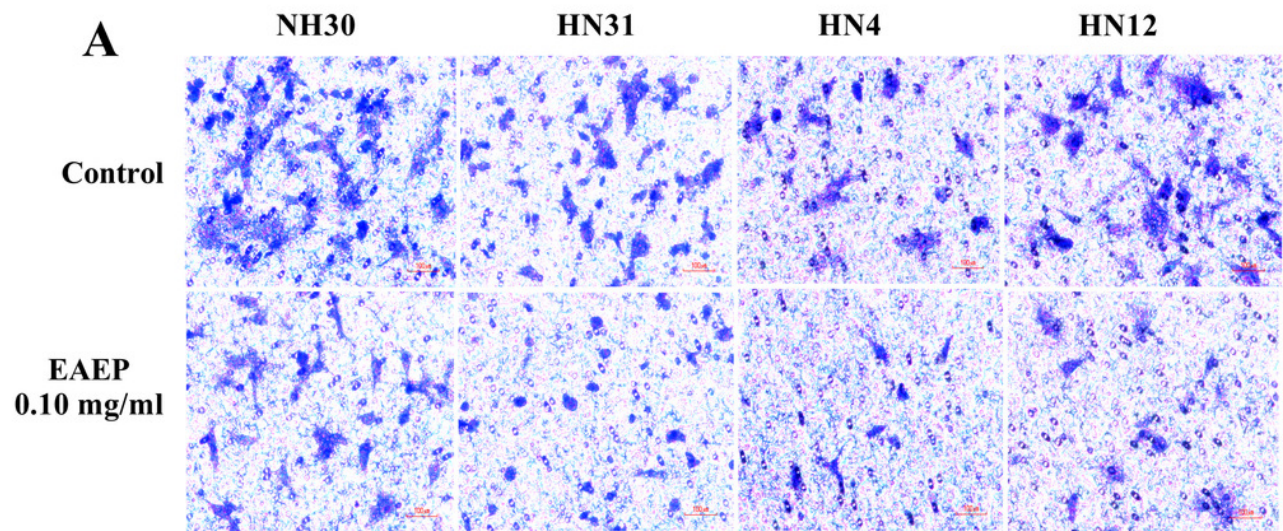



Figure 4

EAEP reduced MMP activities.

EAEP (0.1 mg/ml) was used to treat the cancer cells for 48 h and MMP activity in the 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 activity. Bars represent means±SEM of three independent experiments (n=3). *P <0.05 compared with the control.

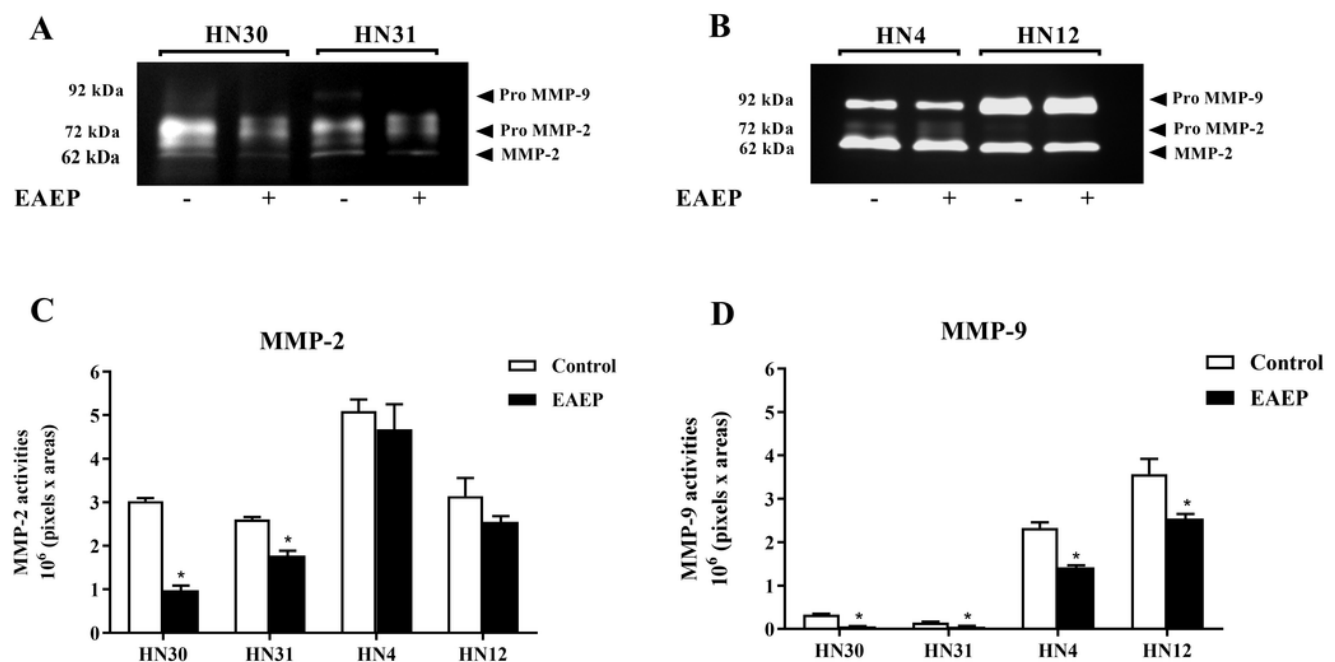



Figure 5

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.

A

Intens.
x10⁵

Blank:BPC-

B

Intens.
x10⁵

EAEP (5000 ppm):BPC-

Cmpd 2, 33.0 min

Cmpd 1, 24.8 min

C

Intens.
x10⁵

standard (10 ppm):BPC-

Cmpd 1. Apigenin
Cmpd 2. Galangin

Cmpd 2, 32.9 min

Cmpd 1, 24.7 min

Time [min]

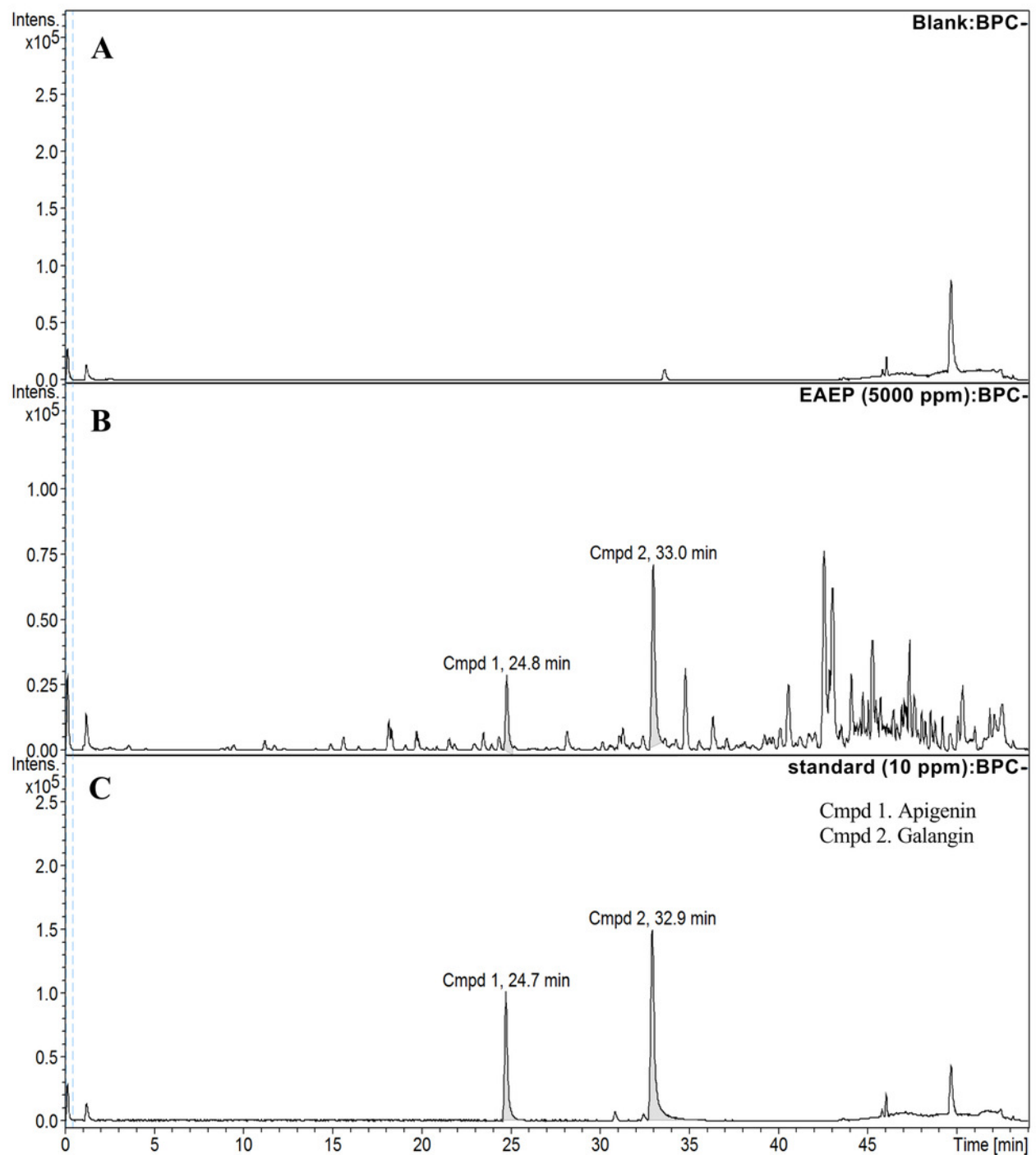



Figure 6

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.

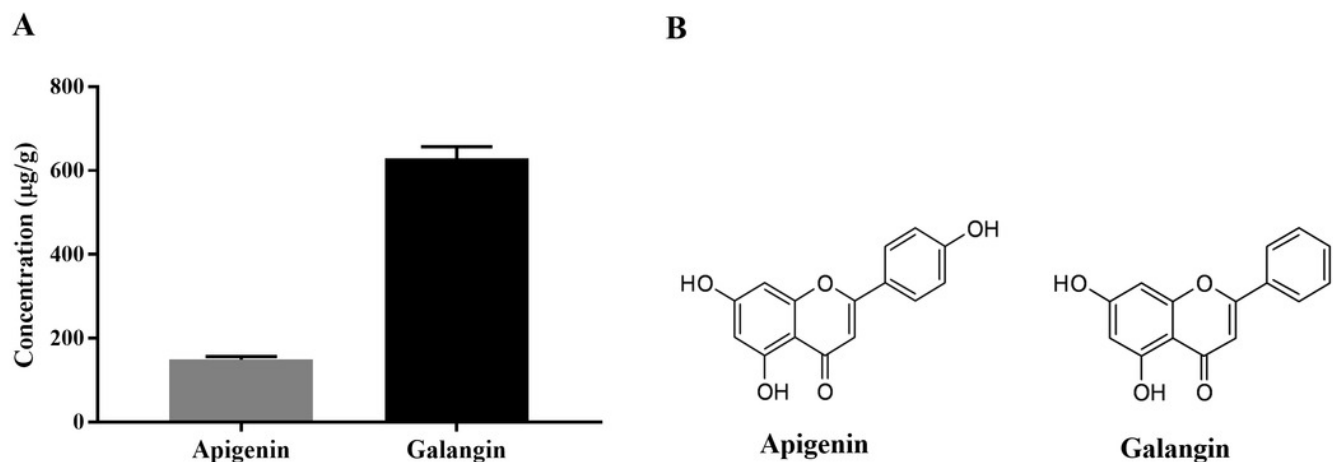
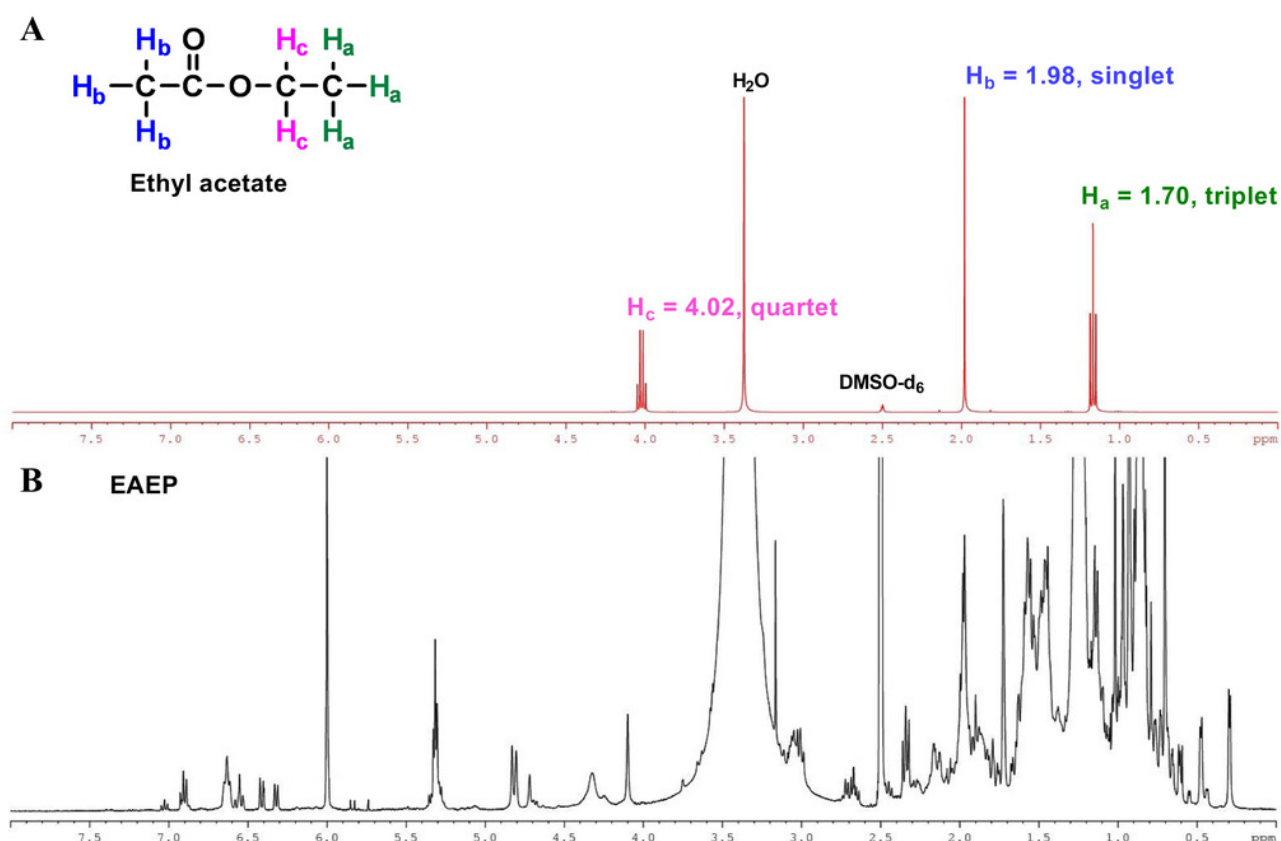



Figure 7

NMR spectrum of ethyl acetate

(A) ^{1}H-NMR spectra (400 MHz) of ethyl acetate in DMSO-d$_6$. Three sets of protons are responsible for the three signals in the ^{1}H-NMR spectra of ethyl acetate. The signals at approximately δ 1.98, 4.02, and 1.70 ppm correspond to the H$_a$, H$_b$, and H$_c$ hydrogens, respectively. (B) The ^{1}H-NMR spectra (400 MHz) of EAEP in DMSO-d$_6$ did not exhibit signals identifying ethyl acetate.

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

1   **Table 1:**

2   **Retention time, calculated and detected masses, calculated formula, concentration and**

3   **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