

Ethanollic extract of *Ocimum sanctum* leaves reduced invasion and matrix metalloproteinase activity of head and neck cancer cell lines

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1 **Abstract**

2 Head and neck squamous cell carcinoma (HNSCC) has a yearly incidence of 600,000
3 cases worldwide with a low survival rate. *Ocimum sanctum* L. or *Ocimum tenuiflorum* L. (Holy
4 basil; Tulsi in Hindi), is a traditional medicine herb that demonstrates numerous effects,
5 including anti-oxidant, anti-microbial, and anti-tumor effects. The aim of this study was to
6 evaluate the anti-invasive effect of *O. sanctum* leaf extract on HNSCC cell lines. Ethanolic
7 extract of *O. sanctum* leaf (EEOS) was prepared and the phenolic compounds were identified
8 using high-performance liquid chromatography-electrospray ionization-time of flight-mass
9 spectrometry. Genetically-matched HNSCC cell lines derived from primary (HN30 and HN4)
10 and metastatic sites (HN31 and HN12) from the same patient were used in this study. The EEOS
11 cytotoxicity to the cell lines was determined using an MTT assay. The invasion and matrix
12 metalloproteinase (MMP)-2 and -9 activity of EEOS-treated cells were tested using a modified
13 Boyden chamber assay and zymography, respectively. We found that EEOS significantly
14 inhibited the invasion and MMP-2 and MMP-9 activity of HN4 and HN12 cells, but not HN30
15 and HN31 cells. Rosmarinic acid, caffeic acid, and apigenin were detected in EEOS. Moreover,
16 rosmarinic acid was found as the major phenolic compound. Therefore, EEOS exerted its anti-
17 invasive effect on HNSCC cells by attenuating MMP activity.

18 **Introduction**

19 Head and neck squamous cell carcinoma (HNSCC) originates in the epithelial cells of the
20 mucosal linings of the oral cavity, oropharynx, larynx, or hypopharynx [1]. HNSCC has an
21 incidence of 600,000 cases per year worldwide, with a 40–50% mortality rate [2]. Similar to
22 other tumors, invasion and metastasis are the critical processes that indicate HNSCC

23 aggressiveness [3]. Matrix metalloproteinase (MMPs) are the key enzymes involved in tumor
24 invasion and metastasis. MMP-2 and MMP-9 destroy the basement membrane and degrade the
25 extracellular matrix, promoting tumor invasion [4]. Although modern medicine has contributed
26 to treating cancers by surgery, chemotherapy, and radiotherapy, these modalities have not
27 significantly changed the survival rate over the past three decades [5]. Thus, more effective
28 treatments for local and metastatic HNSCC are needed.

29 *Ocimum sanctum* Linn. or *Ocimum tenuiflorum* Linn., commonly known as Holy Basil in
30 English or Tulsi in Indian language [6], is a highly potent medicinal herb that is native
31 throughout the eastern tropical countries including Thailand [7, 8]. *O. sanctum* is primarily
32 composed of phytochemicals [9]. The fresh leaves and stem contain several flavonoids and
33 phenolic compounds. Phenolic compounds such as apigenin, rosmarinic acid, cirsilineol,
34 cirsimaritin, isothymusin, and isothymonin have been detected in *O. sanctum* leaf extracts [10].
35 The phytochemicals in this plant varies depending on different growing, harvesting, extraction,
36 and storage conditions [11]. The leaf extracts of *O. sanctum* have numerous medicinal effects
37 such as anti-oxidant [12, 13], wound healing [14], anti-microbial [15] and anti-tumorigenic
38 effects [11]. The ethanol extract of *O. sanctum* leaf (EEOS) has demonstrated anti-tumorigenic
39 effects on several cancer types including gastric cancer [16], pancreatic cancer [17], non-small
40 cell lung cancer [18], and lung cancers [19, 20]. EEOS exhibited a variety of therapeutic effects
41 on tumor cells. EEOS decreased the expression of proteins involved in the proliferation,
42 invasion, and angiogenesis of carcinogen-induced rat gastric carcinoma [16]. Moreover, EEOS
43 inhibited cancer invasion and metastasis. EEOS reduced vascular endothelial growth factor
44 production and MMP-9 activity in metastatic-induced NCI-H460 non-small cell lung cancer cells

45 by inhibiting the phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathway [18]. Similarly,
46 when treated with EEOS, Lewis lung carcinoma cell MMP-9 activity was inhibited [20].

47 This evidence suggests that EEOS has a wide range of activity against tumor cells.
48 However, there is no data concerning the effect of EEOS on HNSCC. We hypothesized that
49 EEOS would reduce HNSCC cell invasion. Therefore, this study evaluated the toxicity and anti-
50 invasive effect of EEOS on primary and metastatic HNSCC cell lines. Moreover, the chemical
51 constituents of the EEOS were identified.

52

53 **Materials and methods**

54 **Chemicals**

55 Caffeic acid, rosmarinic acid, and apigenin were purchased from Sigma-Aldrich (St. Louis,
56 MO). Acetonitrile (HPLC grade), ethanol, methanol, and formic acid (all analytical grade) were
57 purchased from Merck (Darmstadt, Germany).

58 ***O. sanctum* leaf collection**

59 The *O. sanctum* leaves were collected in the Phra Pradaeng district, Samut
60 Prakan province, in central Thailand, during the rainy season. The collected leaves were
61 authenticated for taxonomic identification by The Forest Botany Division, Forest Herbarium-
62 BKF, Thailand.

63 ***O. sanctum* leaf extraction**

64 The *O. sanctum* leaves were dried at room temperature and pulverized using a grinder. The
65 *O. sanctum* powder was soaked in 95% ethanol for two weeks at room temperature. The extract

66 was filtered through a 0.45 nm filter paper and concentrated using a rotary vacuum evaporator
67 (Rotovapor R-215, BUCHI Labortechnik AG, Switzerland). The viscous residue was dried in a
68 vacuum oven at 40°C. The ethanolic extract of *O. sanctum* (EEOS) was stored as a powder at 4°C
69 until used.

70 **Cell culture**

71 Genetically-matched HNSCC cell lines derived from primary and metastatic sites from
72 the same patient were provided by Professor Silvio Gutkind (Moores Cancer Center, Department
73 of Pharmacology, UCSD, CA, USA). The HN30 and HN31 cells were obtained from primary
74 pharynx lesions and lymph node metastases (T3N0M0), respectively. The HN4 and HN12 cells
75 were obtained from primary tongue lesions and lymph node metastases (T4N1M0), respectively
76 [21]. The cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) (Invitrogen,
77 Carlsbad, CA) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 µg/ml
78 streptomycin (Invitrogen), and an anti-fungal agent. The cells were cultured in a 37°C
79 humidified 5% CO₂ atmosphere. The cells were passaged with 0.25% trypsin-EDTA when
80 90–100% confluent. Only cultures with at least 95% cell viability were used in the experiments.

81 **MTT assay**

82 Cells (2,000 cells/well/100µl) were seeded in 96-well plates and incubated in a 37°C
83 humidified 5% CO₂ atmosphere. The cells were treated with 0.05, 0.1, 0.2, 0.4, or 0.8 mg/ml
84 EEOS diluted in growth medium. Cells in growth medium served as control. After a 72 h
85 incubation, the amount of viable cells in each treatment group were determined using
86 thiazolylblue tetrazolium bromide (MTT, Sigma). The medium was removed, 150 µl of fresh
87 medium was added, followed by adding 50 µl/well of 2 mg/ml MTT solution. The plates were

88 incubated for 4 h at 37°C in a 5% CO₂ incubator. The precipitated formazan crystals were
89 solubilized in DMSO (200 µl/well). The absorbance of the resulting solution was measured at
90 570 nm by a microplate reader (Tecan trading, Austria) and converted to percent viable cells
91 compared with control. Cell viability (%) was determined as follows: cell viability (%) =
92 $(\text{mean Abs}_{570_{\text{treated cells}}} - \text{mean Abs}_{570_{\text{blank}}}) / (\text{mean Abs}_{570_{\text{control cells}}} - \text{mean Abs}_{570_{\text{blank}}}) \times 100$.
93 Three independent experiments were performed.

94 **Invasion assay**

95 To evaluate cell invasion, an in vitro assay for cell invasion through Matrigel was
96 performed using a blind-well Boyden chemotaxis chamber (Neuro Probe, Gaithersburg, MD) as
97 previously described [22]. Briefly, the upper surface of 13 µm pore polycarbonate filters (Fisher
98 Scientific, Canada) was coated with Matrigel, a reconstituted basement membrane gel (Corning,
99 Tewksbury, MA) and placed between the upper and lower well plates of a blind-well
100 Boyden chemotaxis chamber. Growth medium was used as a source of chemoattractants in the
101 lower chamber. HNSCC cells (8×10^4 cells) were resuspended in 0.4 mg/ml EEOS diluted in
102 DMEM containing 0.1% BSA and were seeded into the upper well of the chamber. Cells treated
103 with DMEM containing 0.1% BSA served as control. After 5 h incubation in a 37°C and 5%
104 CO₂ atmosphere incubator, the non-migrating cells on the upper surface of the filter were wiped
105 off with a cotton bud. The filters were fixed with 0.5% crystal violet in 25% methanol for
106 10 min. The invaded cells on the lower surface of the filters were counted under a microscope at
107 400× magnification. Cell counting was performed by two investigators. Five randomly selected
108 fields were counted per filter in each group, and the counts were averaged. Three independent
109 experiments were performed.

110 **Conditioned medium preparation and zymography**

111 HNSCC cells (2×10^6 cells) were cultured in 6-well plates and incubated at 37°C for 24 h.
112 After incubation, the wells were washed with PBS and treated with 0.4 mg/ml EEOS diluted in
113 DMEM containing 0.1% BSA for 48 h. Cells cultured in DMEM containing 0.1% BSA were
114 used as control. Conditioned medium (CM) was collected and centrifuged at 1,000 g and 4°C for
115 10 min. The CM was stored at -80°C until used. Total protein in the CM was estimated using the
116 Pierce™ BCA protein assay kit (Thermo Fisher Scientific, Waltham, MA).

117 MMP-2 and MMP-9 activity in the CM were measured using gelatin zymography as
118 previously described [23]. Briefly, gelatin (bloom 300, Sigma) was added to a 10% acrylamide
119 separating gel at a final concentration of 0.2%. Samples containing equal amounts of total
120 protein were mixed with non-reducing sample buffer and added to the gel. Following
121 electrophoresis, the gels were washed in 2.5% Triton X-100 for 30 min at 37°C. The gels were
122 incubated at 37°C overnight in developing buffer. The gels were stained with 0.5% Coomassie
123 blue G250 in a 30% methanol and 10% glacial acetic acid solution for 30 min and destained in
124 the same solution without Coomassie blue. The gelatin-degrading enzymes were identified as
125 clear bands against the blue background of the stained gel. Images of the stained gels were
126 captured under illumination using a G:BOX gel documentation system (Syngene, Frederick,
127 MD). The gelatinolytic bands were quantified using GeneTools software (Syngene, Frederick,
128 MD). Three independent experiments were performed.

129 **HPLC-ESI-TOF-MS analysis of EEOS**

130 The *O. sanctum* extract was chemically analyzed using high-performance liquid
131 chromatography coupled with electrospray ionization-time of flight-mass spectrometry (HPLC-
132 ESI-TOF-MS). EEOS (1 mg/ml) was filtered through a 0.45 µm membrane filter and injected into

133 an HPLC system (UltiMate® 3000 system, Thermo Fisher Scientific, Sunnyvale, CA). Caffeic
134 acid, rosmarinic acid, and apigenin standards were prepared (10, 50, 100, 150, 200, and 250
135 µg/ml). HPLC was performed using a reverse phase column (Symmetry C18 analysis column, 2.1
136 mm x 150 mm, and 5 µm particle size). A gradient elution was performed with a mobile phase of
137 0.1% formic acid (Component A) and 0.1% acetonitrile (Component B). Elution was performed at
138 a 0.3 ml/min flow rate. The injection volume was 5 µl and the column temperature was 40°C. The
139 components that were separated by the HPLC system were subjected to mass to charge ratio (m/z)
140 analysis using a ESI-TOF-Ms system. ESI-TOF-MS was performed using a time of flight mass
141 spectrometer (micrOTOF-Q-II, Bruker Daltonics, Germany). The ESI system negative-ion mode
142 was used to generate m/z in a range 50–1000. The optimized mass spectrometric conditions were
143 gas temperature (200°C), drying gas flow rate (8 l/min), nebulizer gas pressure (2 bar), and the
144 capillary potential was 3000 V. Quantitative determination of the EEOS phenolic components was
145 performed using a standard calibration curve. The data was analyzed using DataAnalysis 4.0
146 software (Bruker Daltonics, Germany).

147 **Statistical analysis**

148 The results are presented as means and standard error of the mean (SEM). Statistical
149 analysis was performed using one-way ANOVA followed by the Tukey's multiple comparisons
150 test with Prism GraphPad 7.0 (GraphPad Software, La Jolla, CA). The significance level was set
151 at 0.05.

152 **Results**

153 **Cytotoxic assessment of EEOS on the HNSCC cell lines**

154 The cytotoxic effect of EEOS on the HNSCC cell lines was evaluated using an MTT
155 assay (Fig 1). EEOS (0.8 mg/ml) significantly decreased the HN30, HN31, HN4, and HN12 cell
156 viability to approximately 40%, 53%, 52%, and 40%, respectively, of that of their controls ($P <$
157 0.05). Whereas, 0.05, 0.1, 0.2, and 0.4 mg/ml EEOS were non-toxic to the cell lines. Therefore,
158 the HNSCC cell lines were treated with 0.4 mg/ml EEOS and their invasion and MMP-2 and-9
159 activity were evaluated.

160 **EEOS decreased metastatic HNSCC invasion**

161 We found that HN30 and HN31 cell line invasion was not significantly inhibited by the
162 non-toxic dose of EEOS (0.4 mg/ml) compared with control ($P > 0.05$) (Figs 2A and B). In
163 contrast, 0.4 mg/ml EEOS significantly inhibited the invasion of the HN4 and HN12 cell lines by
164 approximately 30% compared with control ($P < 0.05$) (Figs 2C and D).

165 **EEOS reduced MMP-2 and -9 activity of HNSCC cell lines**

166 MMP-2 and MMP-9 activity was detected and quantified as gelatinolytic bands and
167 arbitrary number of intensity, respectively. We found that 0.4 mg/ml EEOS treatment did not
168 alter MMP-2 and MMP-9 activity of HN30 and HN31 cells (Fig 3A). However, MMP-2 and
169 MMP-9 activity of HN4 and HN12 cells were downregulated when treated with EEOS (Fig 3B).
170 Quantitative analysis of MMP activity revealed that the MMP-2 and MMP-9 activity in EEOS-
171 treated HN30 and HN31 cells and control cells were not significantly different ($P > 0.05$) (Figs
172 3C and D). Differently, 0.4 mg/ml EEOS significantly reduced HN4 and HN12 cell MMP-2

173 activity to approximately 65% and 71%, respectively, of that of the control cells ($P < 0.05$). In
174 addition, 0.4 mg/ml EEOS significantly reduced the MMP-9 activity of the HN4 and HN12 cells
175 to approximately 44% and 85%, respectively, of that of the control cells ($P < 0.05$) (Figs 3C and
176 D).

177 **HPLC analysis of EEOS**

178 The HPLC retention times of caffeic acid, rosmarinic acid, and apigenin standards were
179 determined (Fig 4A). The EEOS chromatograms demonstrated peaks 1, 2, and 3 with retention
180 times that corresponded to those of the caffeic acid, rosmarinic acid, and apigenin standards,
181 respectively (Fig 4B).

182 **Structural identification of the compounds in EEOS**

183 HPLC-ESI-MS parameters were optimized and used to profile the EEOS. The selected 3
184 compounds in EEOS were putatively identified by comparison to the database (Table 1).
185 Moreover, structures of the putative compounds were drawn by comparison to the known
186 structure of standard compounds. The result revealed that compounds 1, 2, and 3 were caffeic
187 acid, rosmarinic acid, and apigenin, respectively (Figs 5A and A). Quantification of the EEOS
188 caffeic acid, rosmarinic acid, and apigenin indicated that rosmarinic acid was the major phenolic
189 component (Fig 5C).

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195 **Table 1. HPLC-ESI-MS analysis of EEOS.** Elucidation of empirical formulas and putative
196 identification of each compound.

Compounds	Putative identification	Retention time (min)	Empirical formula	Theoretical <i>m/z</i>	Experimental <i>m/z</i>
1	Caffeic acid	9.40	C ₉ H ₈ O ₄	179.0349	179.0355
2	Rosmarinic acid	27.66	C ₁₈ H ₁₆ O ₈	359.0772	359.0764
3	Apigenin	46.63	C ₁₅ H ₁₀ O ₅	269.0455	269.0449

197

198 Discussion

199 The present study investigated the effects of crude *O. sanctum* leaf extract on HNSCC
200 invasiveness. EEOS cytotoxicity on the HNSCC cell lines was evaluated to determine the
201 concentration to be used in subsequent experiments. A previous study reported that 0.2 mg/ml
202 EEOS demonstrated a significant cytotoxic effect on a lung cancer cell line (A549) and mouse
203 Lewis lung carcinoma cells [19]. However, 0.2 mg/ml EEOS was non-toxic to NCI-H460 non-
204 small cell lung cancer cells [18]. Our findings indicated that 0.05, 0.1, 0.2, and 0.4 mg/ml EEOS
205 did not reduce HNSCC cell viability whereas, 0.8 mg/ml EEOS was toxic to the cell lines
206 evaluated. Thus, the cytotoxic effect of EEOS may be cell type-dependent.

207 MMP-2 and MMP-9 activity is important in initiating tumor invasion [4]. Several studies
208 have reported the expression and role of MMP-2 and MMP-9 in HNSCC aggressiveness [24-27].
209 We evaluated the effect of EEOS on HNSCC invasion and MMP activity. We found that EEOS
210 decreased HN4 and HN12 cell invasion by attenuating MMP-2 and MMP-9 activity. In contrast,
211 there was no significant change in HN30 and HN31 cell invasion or MMP activity after EEOS

212 treatment. Our results indicated that EEOS regulation of HNSCC invasiveness may be stage-
213 dependent. These results imply that EEOS may have a potent role in inhibiting the invasiveness
214 of a stage IV tumor. A study demonstrated that EEOS reduced the metastatic activity of Lewis
215 lung carcinoma cell-injected mice [20]. They found that EEOS inhibited cancer invasion and
216 MMP-9 activity, but not that of MMP-2. Another group confirmed that EEOS reduced MMP-9
217 and urokinase plasminogen activator activity in non-small cell lung cancer cells (NCI-H460) by
218 inhibiting the PI3K/Akt signalling pathway [18]. MMP-9 production in HNSCC cell is induced
219 through various signalling pathways, including epidermal growth factor receptor (EGFR),
220 mitogen-activated kinase (MAPK) and PI3K/Akt [26, 28]. This suggests that EEOS may regulate
221 MMP-2 and MMP-9 activity by targeting EGFR, MAPK, or PI3K/Akt pathways, leading to
222 decreased HNSCC invasion.

223 The chemical composition of EEOS has been reported [29]. They found that EEOS was
224 composed of several phenolic compounds and flavonoids, including rosmarinic acid and
225 apigenin. Our results confirmed that the EEOS used in the present study contained rosmarinic
226 acid, apigenin, and caffeic acid. Moreover, rosmarinic acid was the major phenolic component in
227 our EEOS. Rosmarinic acid inhibited colon cancer invasion [30] colorectal cancer metastasis
228 [31] by inhibiting MMP-2 and MMP-9 activity. These findings imply that the rosmarinic acid in
229 EEOS may be a key factor in suppressing HNSCC cell invasion and MMP activity. Future
230 studies should investigate the mechanism of rosmarinic acid and the other EEOS-derived
231 components in suppressing HNSCC aggressiveness.

232 **Conclusions**

233 Taken together, the present study demonstrated the cytotoxic and anti-tumorigenic effects
234 of EEOS on HNSCC invasion and MMP-2 and MMP-9 activity. Interestingly, EEOS selectively
235 regulated the invasion and MMP activity of HN4 and HN12 cells that were derived from stage
236 IV tumors. We showed that the phenolic compounds rosmarinic acid, caffeic acid, and apigenin
237 were present in EEOS. Moreover, rosmarinic acid was found as a major phenolic component.
238 These results suggest that EEOS may be used as an alternative therapeutic agent in clinical
239 research. However, the anti-tumorigenic mechanisms of the active compounds in EEOS require
240 further investigation.

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366

Figure legends

Fig 1. Cytotoxic evaluation of EEOS on HNSCC cells measured by MTT assay. EEOS in a range of concentrations were used to treat HN30 (A), HN31 (B), HN4 (C), and HN12 (D) cells for 72 h. Bars represent means \pm SEM (n=3). * indicates $p < 0.05$ compared with control.

Fig 2. EEOS decreased HNSCC cell invasion. A non-cytotoxic dose of EEOS was used to treat HN30 (A), HN31 (B), HN4 (C), and HN12 (D) cells and evaluated cell invasion. Bars represent means \pm SEM (n=3). * indicates $p < 0.05$ compared with control.

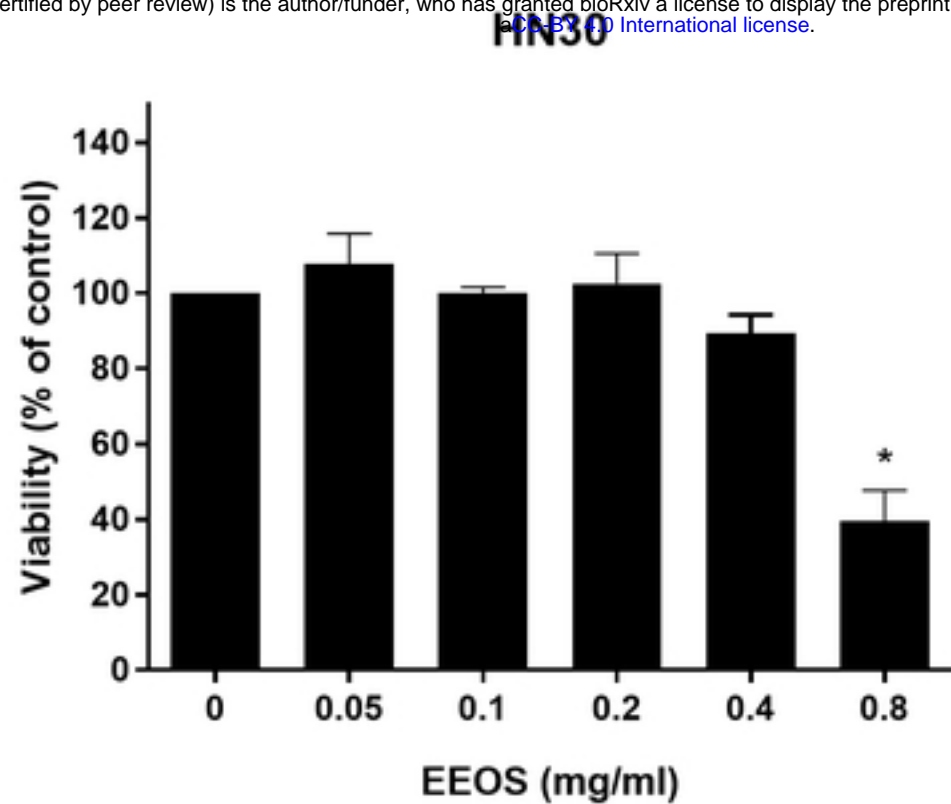
Fig 3. EEOS reduced MMP activity. The HNSCC cell lines were treated with 0.4 mg/ml EEOS for 48 h and the MMP activity in the conditioned media of HN30 and HN31 cells (A), and HN4 and HN12 cells (B) were detected using zymography. GeneTools software was used to quantify the gelatinolytic bands of MMP-2 (C) and MMP-9 (D) activity. Bars represent means \pm SEM (n=3). * indicates $p < 0.05$ compared with the control.

Fig 4. HPLC analysis of EEOS. Chromatogram of standard phenolic compounds (A) and the compounds detected in EEOS (B) are shown.

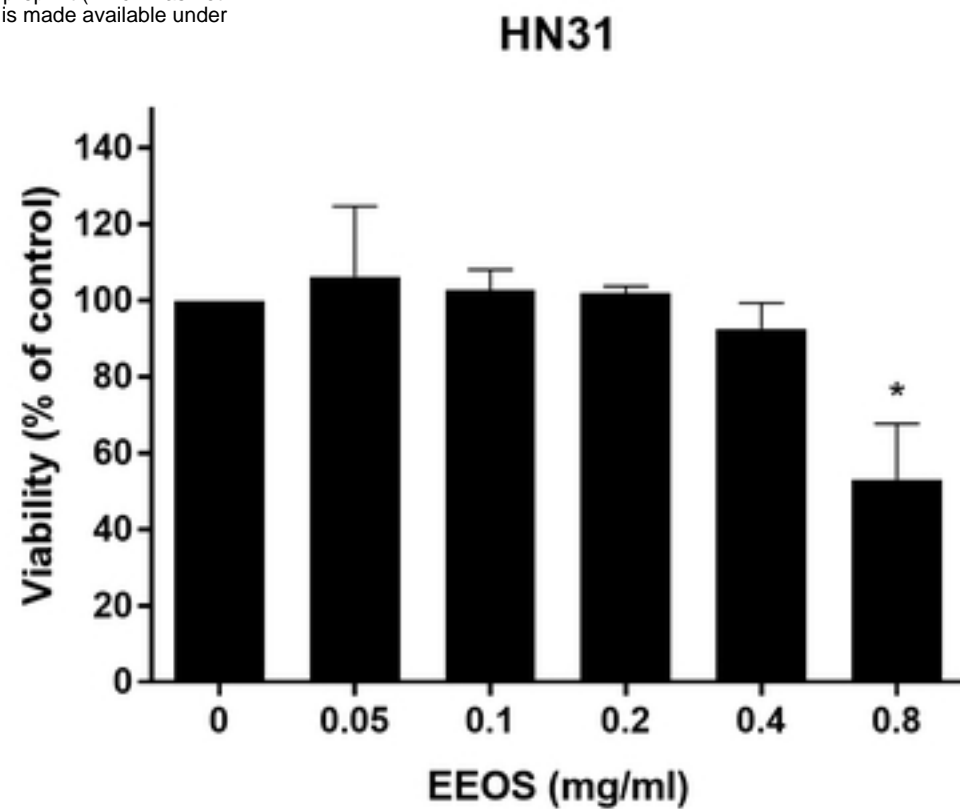
Fig 5. ESI-TOF-MS analysis of the compounds in EEOS. The structures of standard phenolic compounds (A) and the compounds in EEOS (B) analyzed from HPLC were identified. Quantification of the compounds in EEOS (C).

A

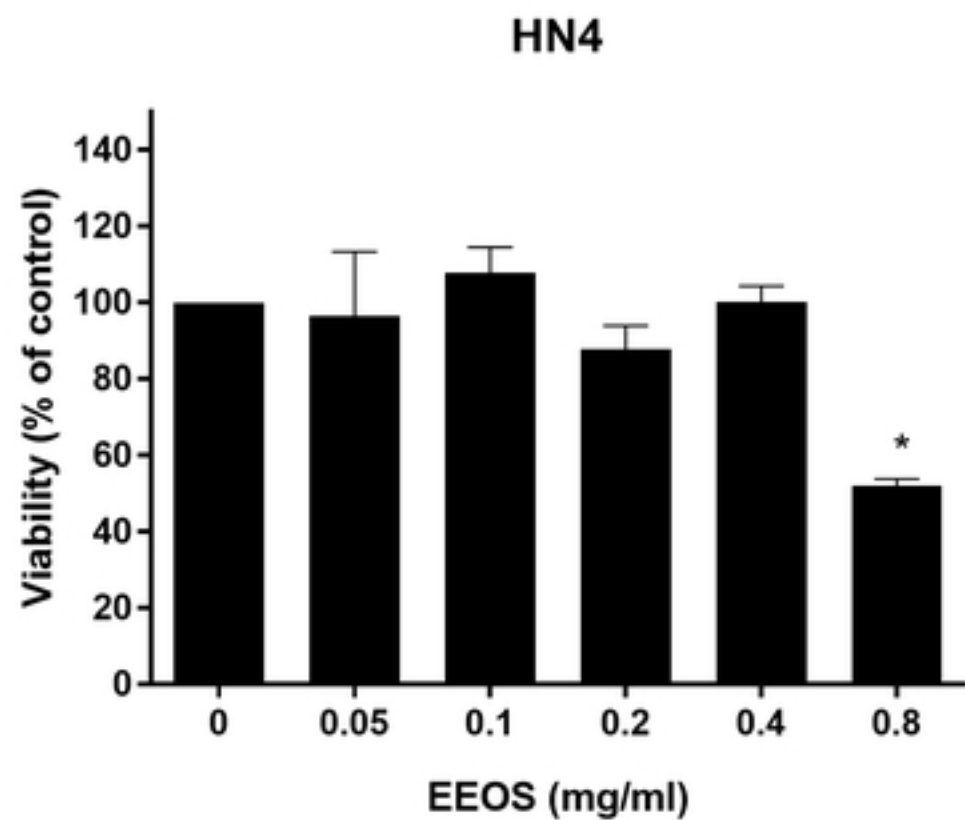
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B



C



D

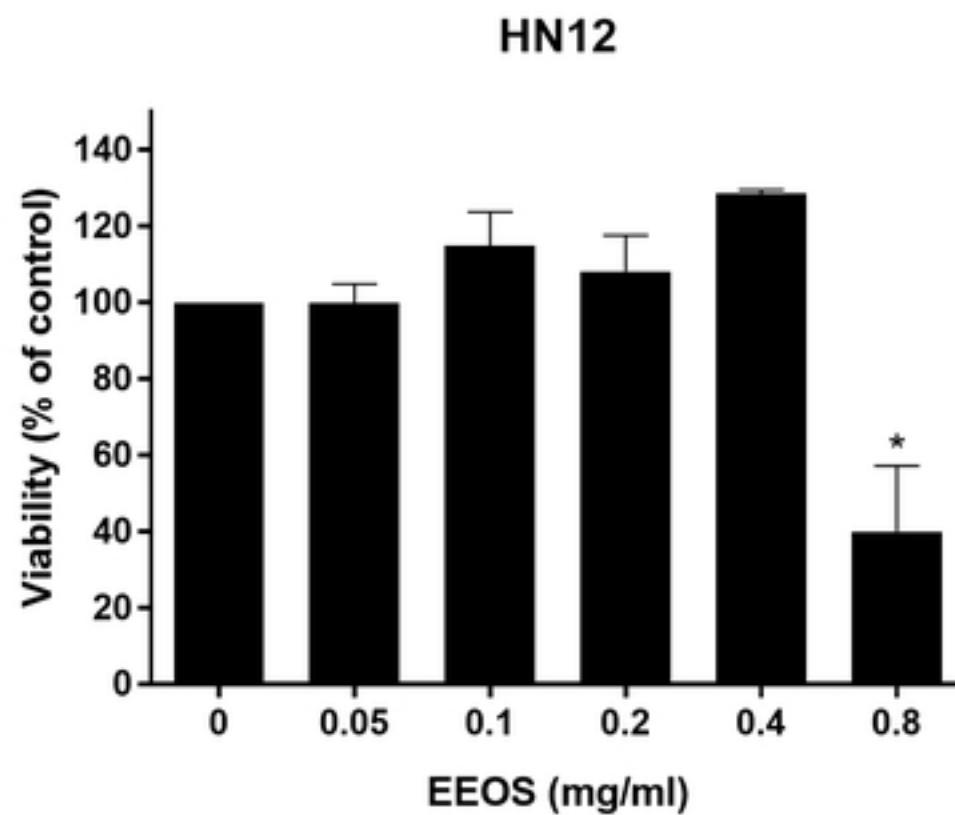


Fig 1

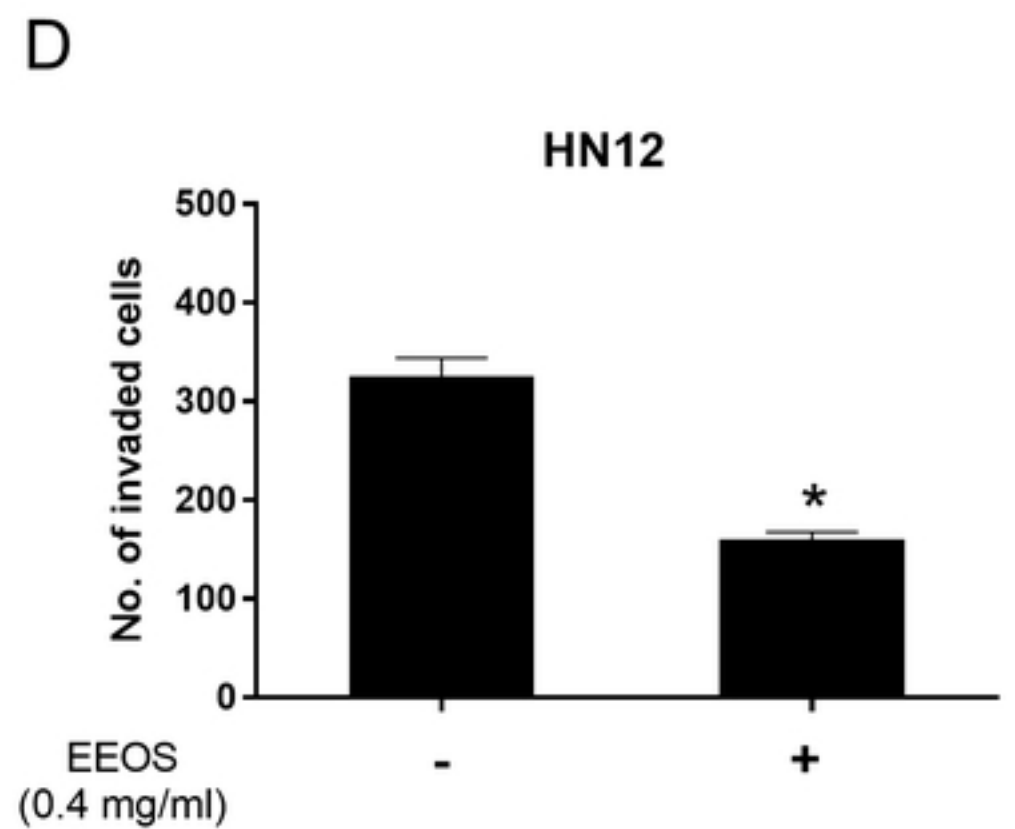
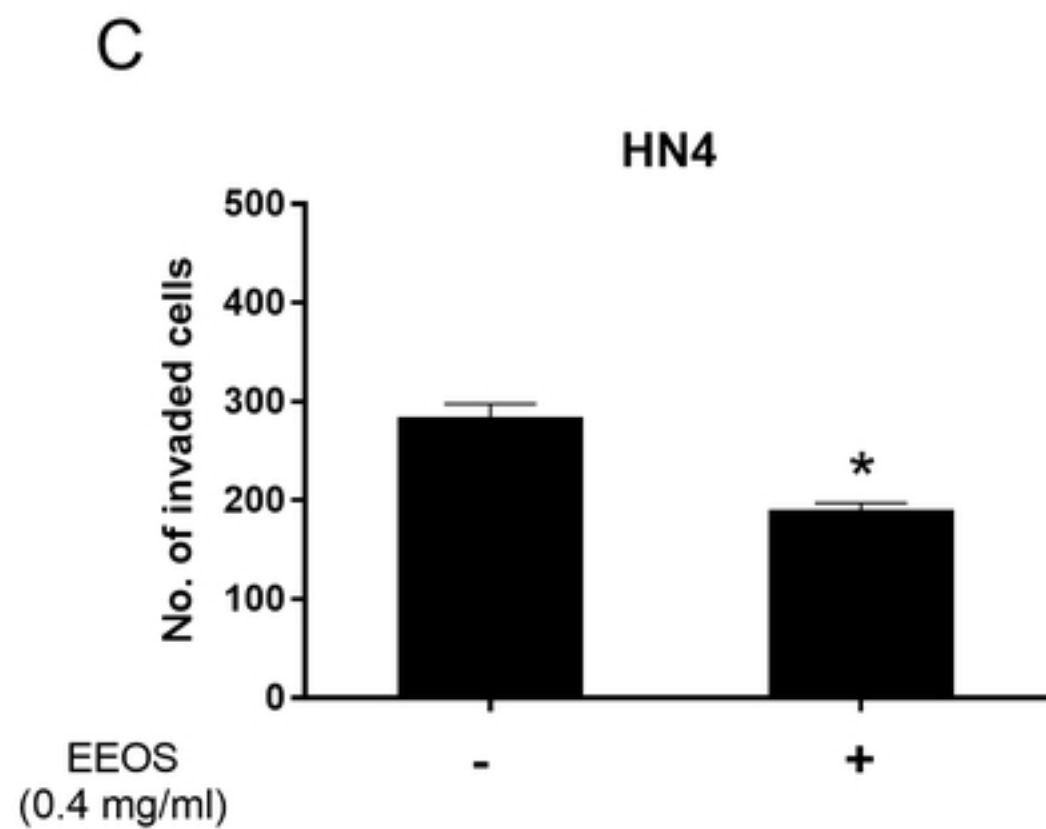
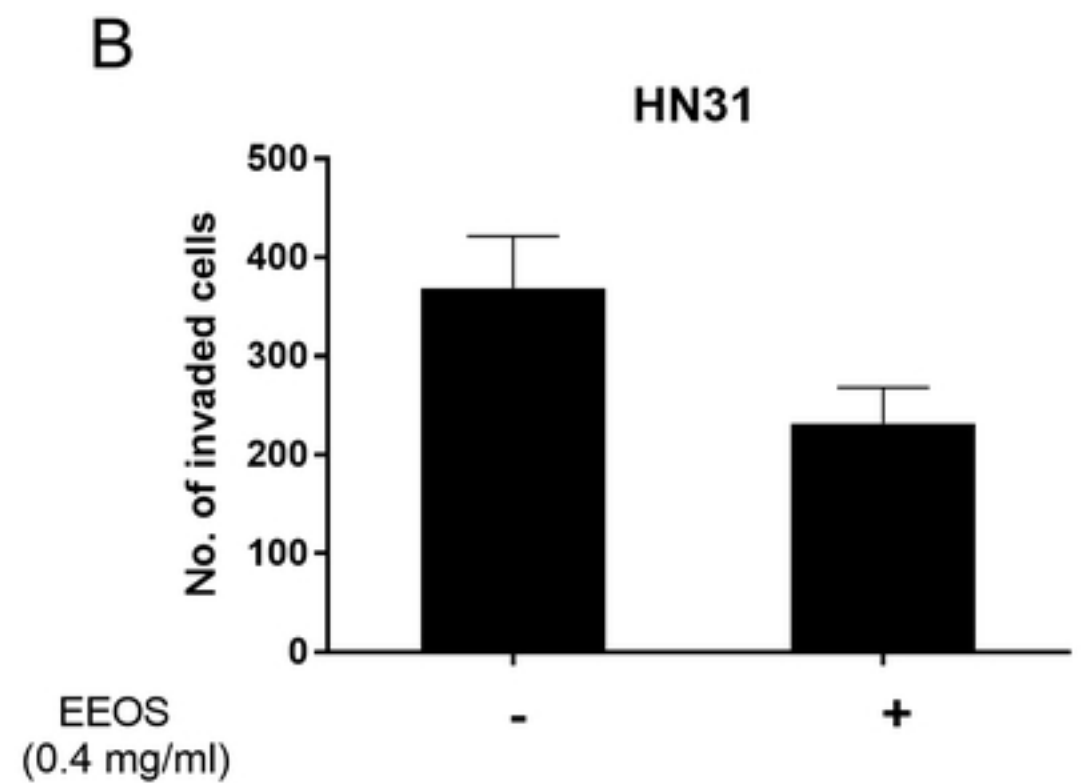
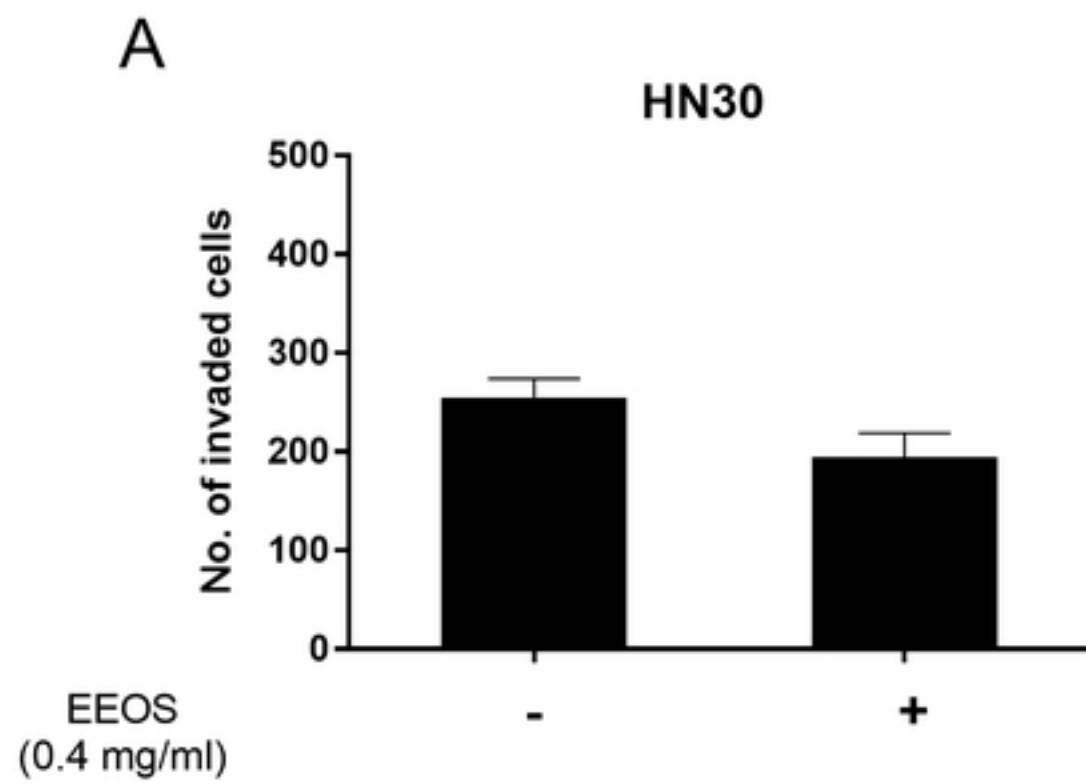


Fig 2

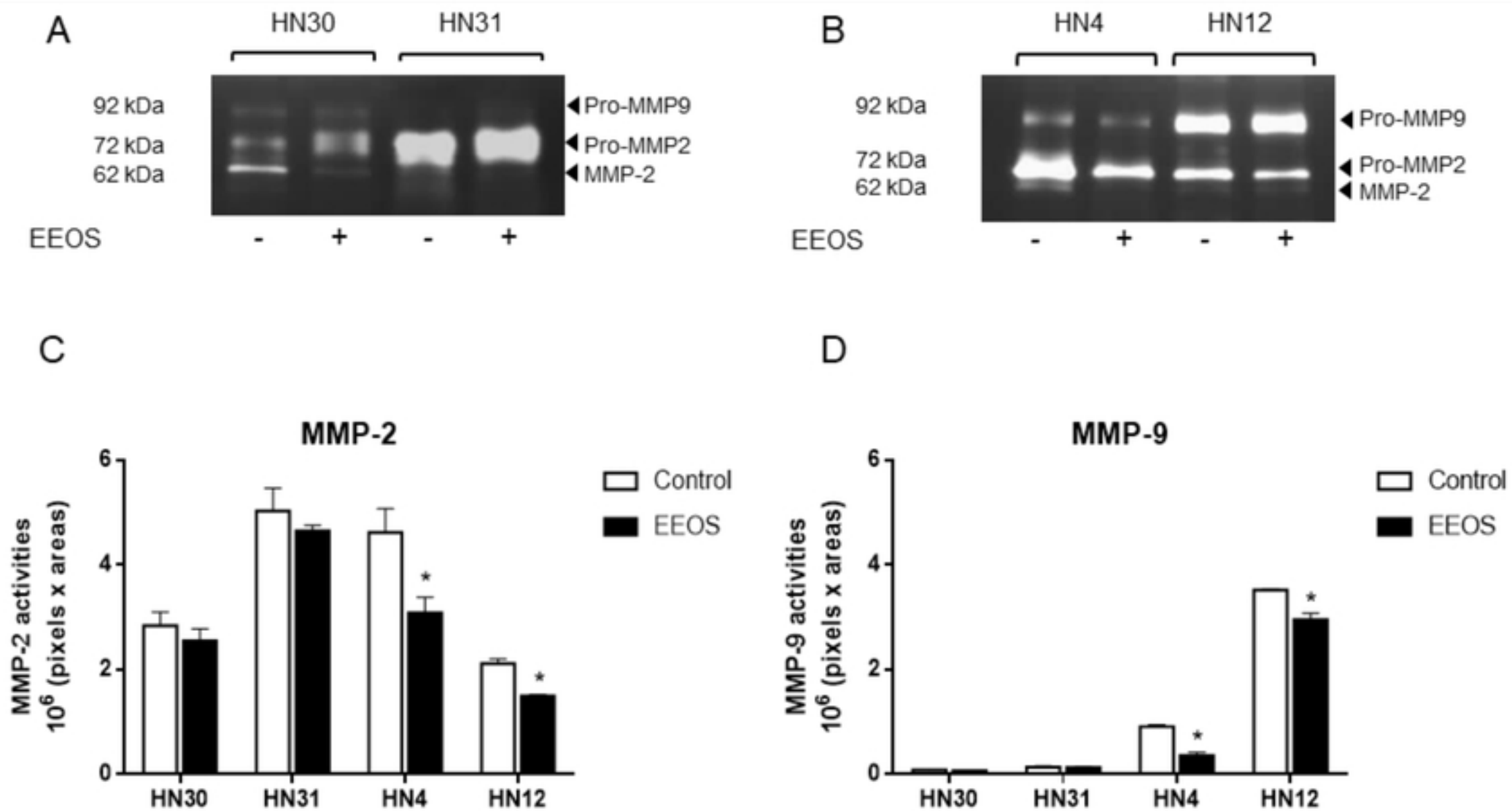
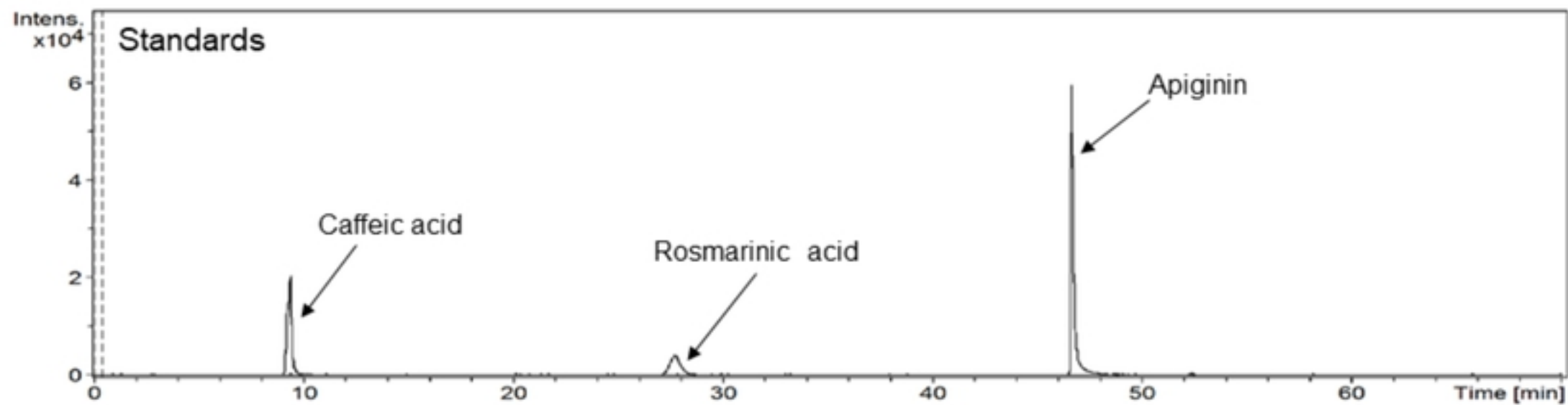
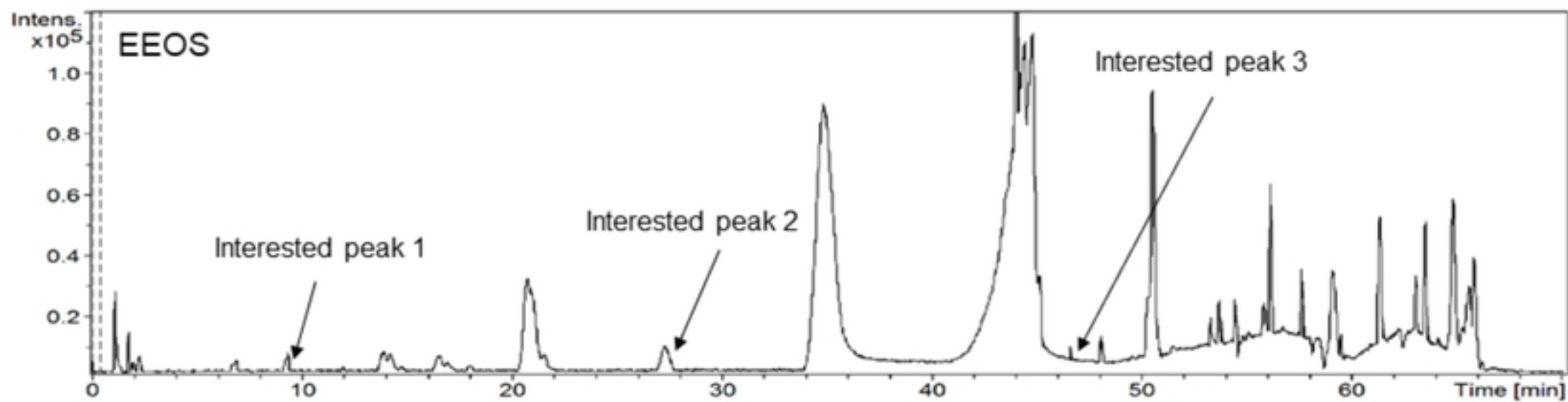


Fig 3

A**B****Fig 4**

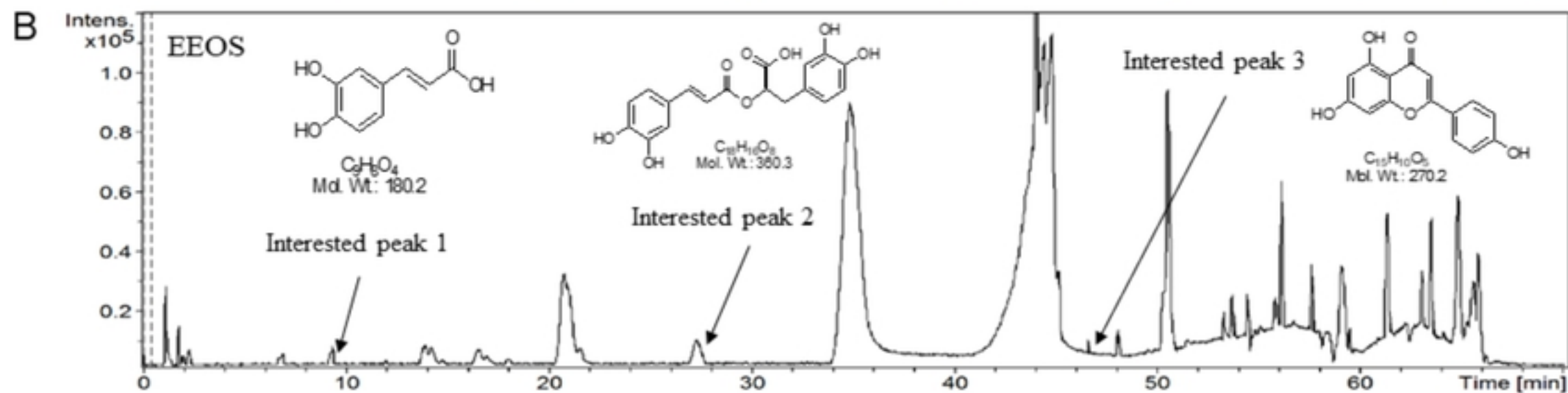
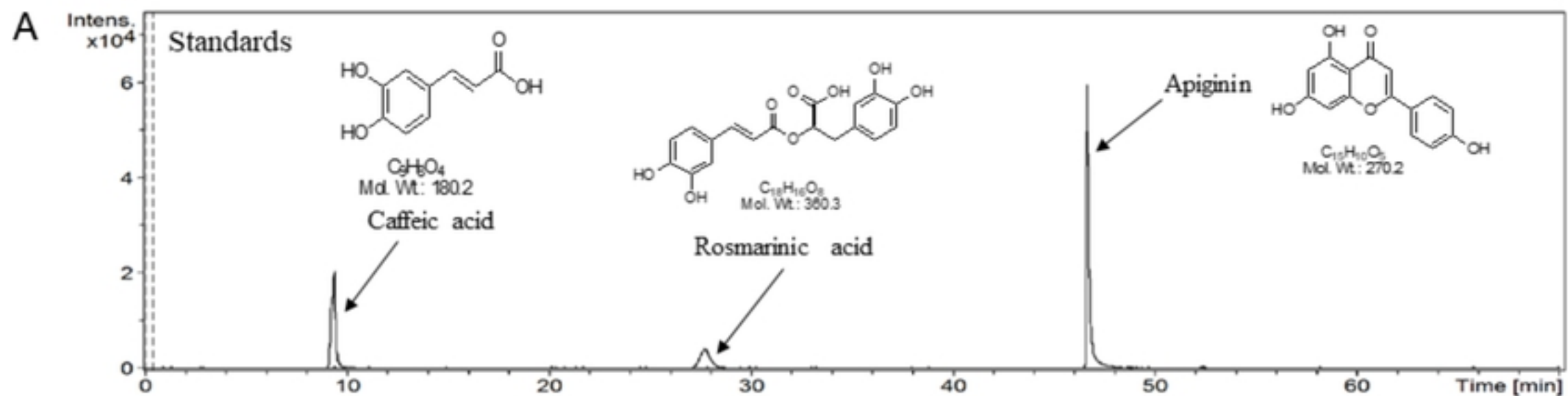


Fig 5