Ethanolic extract of Ya-nang (*Tiliacora triandra*) leaf powder induces apoptosis in cholangiocarcinoma cell lines via induction of hyperacetylation and inhibition of growth signaling

Arunta Samankul[1], Gulsiri Senawong[1], Prasan Swatsitang[1], Banchob Sripa[2], Chanokbhorn Phaosiri[3], Somdej Ka-nokmedhakul[3] and Thanaset Senawong[1]

[1]Department of Biochemistry, Faculty of Science, Khon Kaen University, Khon Kaen 40002, Thailand
[2]Department of Pathology, Faculty of Medicine, Khon Kaen University, Khon Kaen 40002, Thailand
[3]Department of Chemistry, Faculty of Science, Khon Kaen University, Khon Kaen 40002, Thailand

Corresponding Author:
Thanaset Senawong[1]
Department of Biochemistry, Faculty of Science, Khon Kaen University, Khon Kaen 40002, Thailand
Email address: sthanaset@kku.ac.th

Abstract

Objective: To develop alternative medicine for reducing the undesired side effects of chemotherapy in CCA patients, the anticancer activity of *Tiliacora triandra* leaf powder ethanolic (TLPE) extract against cholangiocarcinoma cell lines was investigated.
Methods: Antiproliferation was studied using the MTT assay while apoptosis induction and cell cycle arrest were analyzed by flow cytometry. The levels of key proteins and phenolic acid content were analyzed by western blotting and reversed phase HPLC, respectively.
Results: TLPE extract inhibited CCA cell growth in a dose- and time-dependent manner, with IC_{50} values of 7.86 ± 0.05 µg/ml for KKU-M213B cells and 8.59 ± 0.36 µg/ml for KKU-100 cells at an exposure time of 72 h. TLPE extract inhibited the growth of CCA cell lines by inducing apoptosis of both cell lines and causing an increased population of KKU-100 cells at the G0/G1 phase. TLPE extract up-regulated Ac-H3 but down-regulated p-ERK, p53, Bax, CDK4 and Bcl2 expressions in KKU-M213B cells. TLPE extract up-regulated Ac-H3, p21 and Bax but down-regulated p-ERK, p53, CDK4 and Bcl2 expressions in KKU-100 cells. Additionally, phenolic acids including *p*-hydroxybenzoic, vanillic, syringic, *p*-coumaric, ferulic and sinapinic acids were identified.
Conclusion: These results suggest the possibility of developing *T. triandra* leaf powder ethanolic extract as a chemotherapeutic or chemoprevention agent for cholangiocarcinoma.

KEYWORDS: anticancer effects; cholangiocarcinoma; phenolic acids; *Tiliacora triandra*

Introduction

43
44   Cholangiocarcinoma (CCA) is a bile duct cancer and the second most common primary liver
45   neoplasm after hepatocellular carcinoma (Tyson and El-Serag; Bergquist and von Seth, 2011).
46   The previous report shows that northeastern Thailand has the highest incidence of CCA in the
47   world (Jongsuksuntigul and Imsomboon, 2003). The infection of *Opisthorchis viverrini* has been
48   classified as a risk factor for this disease (Miwa et al., 2014). CCAs are heterogeneous biliary
49   epithelial tumors that are classified into intrahepatic (iCCA), perihilar (pCCA), and distal
50   (dCCA) subtypes based on their anatomic location within the biliary tree. Especially in the recent
51   decade, the incidence of iCCA has been increasing. Unfortunately, the 5-year survival rate for
52   CCA remains less than 10% (Bertuccio et al., 2019; Everhart and Ruhl, 2009).
53   Most CCA patients are diagnosed in the late stages; thus, chemotherapy is very important for
54   the treatment of CCA patients. Chemotherapy is also used as adjuvant chemotherapy in
55   combination with surgery and may be used as neoadjuvant chemotherapy before surgery or
56   radiation. The current chemotherapeutic drugs for cholangiocarcinoma are 5-fluorouracil,
57   gemcitabine, and cisplatin (Valle et al., 2010); however, drug resistance, toxicity, and drug cost
58   remain the significant limitations to the clinical use of these drugs. Therefore, herbal medicine is
59   an alternative for CCA patients in developing countries, where medicinal herbs are being
60   increasingly recognized as complementary treatments for cancer [8,9] (Aras et al., 2014; Shukla,
61   2007).
62   Ya-nang (*Tiliacora triandra*) is classified as a member of the Menispermaceae family. It is a
63   member of a genus of flowering plants native to Southeast Asia, especially in northeast Thailand
64   and the Lao People's Democratic Republic (LPDR) (Singthong et al., 2009). In Thailand and
65   Laos, the leaves of *T. triandra* are extracted with water by rubbing them with hands to produce a
66   product called Nam Ya-Nang which is used as a cooking ingredient for bamboo shoot soup and
67   bamboo shoot salad. In addition, *T. triandra* is used as traditional medicine to cure illnesses such
68   as fever and malaria, and it is also used as an anti-pyretic drug, detoxication agent, anti-
69   inflammation agent, anti-cancer agent, anti-bacterial agent, immune modulator, and antioxidant
70   (Rattana et al., 2010). Phytochemicals of *T. triandra* such as tiliacorinine, 2'-nor-tiliacorinine,
71   tiliacorine and 13'-bromo-tiliacorinine were isolated from the root, which contained many
72   different types of four bisbenzylisoquinoline alkaloids (Sureram et al., 2012). Moreover, the
73   compound oxoanolobine was isolated from the *T. triandra* leaves (Rattana et al., 2016). *T.
74   triandra* leaves exhibited high phenolic content (Singthong et al., 2014; Phunchago et al., 2015),
75   flavonoid (Rattana et al., 2010), polysaccharide gum, which were identified by FTIR detection as
76   xylan (Singthong et al., 2009) and fatty acids including phytol, 1-cyclohexenylacetic acid,
77   oleamide, oleic acid, palmitic acid, neophytadiene 5-hydroxymethyl-2-furancarboxaldehyde, 2,6-
78   dimethyl-3 (methoxymethyl)-benzoquinone (Chaveerach et al., 2016), hexadecanoic,
79   octadecanoic and (Z)-6 octadecenoic acids (Kaewpiboon et al 2014). In this study, phenolic acids
80   such as *p*-hydroxybenzoic, vanillic, syringic, *p*-coumaric, ferulic and sinapinic acids were
81   identified from the *T. triandra* leaves. The ethanolic extract of *T. triandra* leaf, which contains
82   these phenolic acids was demonstrated to possess anticancer activity against cholangiocarcinoma
83   cell lines.
84

Materials & Methods

85
86   **Chemicals**

87   *Tiliacora triandra* leaf powder was purchased from the Rak Samunpai group, Bangkok, Thailand
88   (https://www.thaiherbbiz.com). Camptothecin, propidium iodide (PI), and all primary and
89   secondary antibodies were obtained as previously described (Saenglee et al., 2016; 2018).
90
91   **Cell lines and culture conditions**
92   The well differentiated (KKU-M213B) and poorly differentiated and drug resistant (KKU-100)
93   adenocarcinoma cell lines were kindly provided by Dr. Banchob Sripa (Khon Kaen University,
94   Khon Kaen, Thailand). The non-cancer cell line (H69; human bile duct epithelial cell) was
95   obtained from Dr. Douglas Jefferson (Tufts University, Boston, MA, USA). Cholangiocarcinoma
96   cells and human bile duct epithelial cells were cultured in suitable conditions as previously
97   described (Saenglee et al., 2018).
98
99   **Sample preparation**
100   *T. triandra* leaf powder ethanolic (TLPE) extract was prepared for testing antiproliferation,
101   induction of cell cycle arrest and apoptosis, regulation of protein expression levels, and total
102   phenolic content. 20 grams of *T. triandra* leaf powder was added in 200 ml of absolute ethanol
103   and stirred for 48 h. The suspension was centrifuged at 6,150 × g, (Centrifuge, Hitachi, R20A2)
104   for 15 minutes. The supernatant was collected and filtered with Whatman No. 4 filter paper. The
105   supernatant was evaporated to 2 ml using a rotary evaporator and finally to dryness under a
106   gentle stream of nitrogen. The ethanolic extract was kept at -20 °C until used.
107
108   **Cell viability**
109   The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was performed
110   to assess cell viability as described in a previous study (Saenglee et al., 2016). Briefly, KKU-
111   M213B, KKU-100 and H69 cells were seeded in each well of 96-well plates (8×10^3 cells/well)
112   and incubated in CO_2 incubator for 24 h. Then, various concentrations of TLPE extract and
113   solvent control (1% of absolute ethanol) were added in each well and incubated for 24, 48 and 72
114   h. Then, the culture medium was replaced with the medium containing 1.2 mM of MTT (100
115   μl/well) and incubated for 2-4 h. The purple formazan crystals were dissolved in DMSO. The
116   absorbance at 550 nm and optical density at 655 nm (the reference wavelength) were measured
117   using a microplate reader (Bio-Rad Laboratories, Hercules, CA, USA). The percentage of cell
118   viability of each well was calculated using the equation: %Cell viability = [Sample Abs (Abs 550
119   - O.D. 655)/ Control Abs (Abs 550 - O.D. 655)] × 100.
120
121   **Analysis of cell cycle phase distribution by flow cytometry**
122   Cell cycle progression was analyzed using propidium iodide staining and flow cytometry as
123   described in a previous study (Saenglee et al., 2018). In brief, KKU-M213B, KKU-100 and H-69
124   cells were seeded at density of 1×10^6 cells/5.5 cm dish for 24 h. The cells were treated with
125   TLPE extract (31.25, 62.5 and 125 μg/ml) and absolute ethanol as a solvent control for 24 h. The
126   treated cells were harvested by trypsinization, centrifuged at 1,754 × g for 3 min, and
127   resuspended in ice-cold 1X PBS with 0.1% glucose. The cells were added with 70% ethanol,
128   incubated at 4 °C for 1 h, and centrifuged at 1,754 × g for 3 min. Cells were resuspended in ice-
129   cold 1X PBS and centrifuged at 1,754 × g for 3 min. Then, the cells were added with 10 mg/ml
130   RNase A and incubated at 37 °C. The cells were stained with 5.0 μl of PI and incubated in the
131   dark at room temperature for 45 min. Cell cycle phases were analyzed by flow cytometry

132 (Becton Dickinson, San Jose, CA, USA) (The service was provided by the Research Instrument
133 Center, Khon Kaen University, Thailand).
134
Analysis of Apoptosis by flow cytometry
136 Apoptosis induction was evaluated by flow cytometry using Annexin V-FITC and PI staining as
137 previously described (Saenglee et al., 2018). In brief, KKU-M213B, KKU-100 and H69 cell
138 lines were cultured in a 5.5-cm dish at a density of 1×10^6 cells/dish for 24 h and treated as
139 described in the analysis of cell cycle phase distribution. Then, cells were harvested, and the cell
140 pellet was rinsed twice with ice-cold PBS. The cell pellet was then resuspended in the Annexin-
141 binding buffer (100 μL) and incubated in the dark with Annexin V-FITC and PI for 15 min at
142 room temperature. Apoptotic cells were analyzed using a BD FACSCantoII Flow Cytometer.
143
Western blot analysis
145 Cells were cultured and treated as described in the analysis of cell cycle phase distribution and
146 apoptosis. The protein levels were detected by western blot as described in a previous study
147 (Saenglee et al., 2018). Briefly, the harvested cells were lysed with RIPA lysis buffer. Total
148 protein samples (30-80 μg) were loaded and separated through 10%-12.5% SDS-PAGE (15 mA
149 for 1.30 h) and then transferred to a polyvinylidene fluoride (PVDF) membrane. After blocking
150 with 5% skim milk for 1 h, the blots were incubated overnight with specific primary antibodies.
151 The following antibodies were used: anti-Bcl2, anti-CDK4, anti-p21, anti-p53, anti-Bax, anti-
152 acetylated H3, anti-pERK1/2 and anti-ERK1/2. The membrane was subsequently incubated with
153 an HRP-conjugated secondary antibody for 2 h at room temperature. The ClarityTM Western
154 ECL substrate (Bio-Rad, CA, USA) was used to develop the protein bands. The X-ray film (GE
155 Healthcare Bio-Sciences, USA) was exposed to the immunoreactive bands.
156
Phenolic acid extraction
158 The 20 grams of *T. triandra* leaf powder were macerated in absolute ethanol at a ratio of 1:10
159 (20 grams *T. triandra* leaf powder: 200 milliliters absolute ethanol). The mixture was stirred for
160 48 h at room temperature in the dark, centrifuged thereafter at 6,150 × g for 15 minutes and
161 filtered through Whatman grade No. 4 filter paper. The supernatant was then processed as
162 previously described (Woranam et al., 2020) to obtain the phenolic acid extract. The phenolic
163 acid extract was dissolved in 50% methanol, filtered through a 0.2 μm syringe filter, and
164 analyzed for the type of phenolic acids using reversed-phase HPLC.
165
HPLC analysis
167 To identify individual phenolic acids in the extract, reversed phase HPLC was used, and the
168 identification was based on matching the spectrum and retention times of phenolic acid standards
169 as described previously (Khaopha et al., 2012). Individual phenolic acids in TLPE extract were
170 analyzed quantitatively by comparison with a standard curve and using m-hydroxybenzaldehyde
171 as the internal standard.
172
Total Phenolic content
174 Total phenolic content was analyzed as previously described (Khaopha et al., 2012) based on the
175 reduction of the Folin-Ciocalteu reagent (phosphomolybdic and phosphotungstic acids) by
176 phenolic groups of phenolic compounds. Briefly, TLPE extract was mixed with Folin-Ciocalteu
177 reagent and 20% Na_2CO_3 and adjusted the final volume to 200 μl by adding distilled water. The

178 mixture was incubated at 50 °C for 2 h, and then the absorbance was measured at 750 nm using a
179 spectrophotometer. Total phenolic content was calculated and reported as mg gallic acid
180 equivalent (GAE) per gram dry weight.
181
182 **Statistical analysis**
183 Data were expressed as the mean ± standard deviation (SD) from three independent experiments.
184 The significant differences between solvent control and treatments were analyzed as previously
185 described (Saenglee et al., 2018).
186

187 # Results
188 **Antiproliferative activity of TLPE extract against CCA cell lines**
189 Anti-proliferative activity of TLPE extract against cholangiocarcinoma cell lines (KKU-100 and
190 KKU-M213B cells) and non-tumorigenic biliary epithelial cells (H69 cells), was investigated
191 using the MTT assay. The results were shown as percentages of cell viability and IC_{50} values.
192 The TLPE extract had a dose- and time-dependent anti-proliferative effect against KKU-M213B,
193 KKU-100 and H69 cells (Fig. 1). KKU-M213B cell line was the most sensitive to the TLPE
194 extract. TLPE extract significantly inhibited the proliferation of KKU-M213B cells with IC_{50}
195 values of 90.50 ± 9.62, 9.09 ± 0.15 and 7.86 ± 0.05 µg/ml at exposure times of 24, 48 and 72 h,
196 respectively (Fig. 1A-B). TLPE extract significantly inhibited the proliferation of KKU-100 cells
197 with IC_{50} values of 115.32 ± 9.53, 13.67 ± 1.44 and 8.59 ± 0.36 µg/ml at exposure times of 24,
198 48 and 72 h, respectively (Fig. 1C-D). In addition, the cytotoxicity of TLPE extract in non-
199 tumorigenic biliary epithelial cells (H69 cells) was investigated in comparison with the cancer
200 cell lines. TLPE extract exhibited less toxicity to non-cancer H69 cells with IC_{50} values of
201 143.39 ± 8.52, 14.75 ± 0.60 and 11.95 ± 0.60 µg/ml at exposure times of 24, 48 and 72 h,
202 respectively (Fig. 1E-F).
203

204 **TLPE extract induces cell cycle arrest in CCA cell lines**
205 In this study, the cholangiocarcinoma cell lines (KKU-M213B, KKU-100) and non-tumorigenic
206 biliary epithelial cells (H69) were treated with TLPE extract at 31.25, 62.50 and 125.00 µg/ml
207 for 24 h. KKU-M213B cells were treated with TLPE extract at 31.25, 62.50 and 125.00 µg/ml
208 for 24 h but did not show accumulation of cells at any cell cycle phase. However, a significant
209 increase in the cell population in the SubG1 phase was observed up to 12.3%, 17.1% and 19.7%,
210 respectively (Fig. 2A-B). KKU-100 cells treated with TLPE extract at 31.25, 62.50 and 125.00
211 µg/ml for 24 h induced cell cycle arrest at the G0/G1 phase (Fig. 2C-D). The cell populations in
212 the G0/G1 phase were significantly increased up to 82.9%, 84.2% and 84.6% when treated with
213 TLPE extract at 31.25, 62.50 and 125.00 µg/ml, respectively. In addition, a significant increase
214 in the cell population in the SubG1 phase was observed up to 1.3%, 1.7% and 7.3%, respectively
215 (Fig. 2C-D). H69 cells treated with TLPE extract at 31.25, 62.50 and 125.00 µg/ml for 24 h
216 showed no accumulation of cells at any cell cycle phase (Fig. 2E-F). However, a significant
217 increase of the SubG1 population in H69 cells was observed up to 7.4%, 8.5% and 9.6%,
218 respectively (Fig. 2E-F).
219

220 **TLPE extract induces apoptosis in CCA cell lines**
221 Apoptosis induction was determined by Annexin V-FITC and propidium iodide (PI) staining.
222 The stained cells were analyzed by flow cytometry. KKU-M213B cells treated with TLPE
223 extract at 31.25, 62.50 and 125.00 µg/ml exhibited the increased percentages of early apoptotic

224 cells accounted for at 12.1%, 15.6% and 25.8%, respectively (Fig. 3A-B). TLPE extract
225 stimulated apoptosis induction in KKU-M213B cells in a dose-dependent manner for 24 h. In
226 KKU-100 cells, early apoptotic cell populations were significantly increased to 10.5%, 11.7%
227 and 17.8%, respectively (Fig. 3C-D). The late apoptotic cell populations were 0.6%, 0.1% and
228 0.2%, respectively. In the non-cancer H69 cells, early apoptotic cell populations were slightly
229 increased to 4.9%, 6.4% and 7.3% (Fig. 3E-F), respectively.

230

231 **TLPE extract modulates expression of proteins involved in apoptosis and cell cycle arrest**
232 **in CCA cell lines**
233 The above results showed that TLPE extract induced apoptosis in both KKU-M213B (Fig. 3A-B)
234 and KKU-100 (Fig. 3C-D) cells, respectively. Moreover, TLPE extract induced cell cycle arrest
235 at G0/G1 in KKU-100 (Fig. 2C-D) cells. To understand the molecular mechanisms by which
236 TLPE extract induces apoptosis and cell cycle arrest in CCA cell lines, the expression levels of
237 Bcl2 (anti-apoptotic protein), Bax (pro-apoptotic protein), p53 (tumor suppressor protein) and
238 cell cycle-related proteins (p21; inhibitor of cyclin dependent kinases, CDK4; the progression
239 through the G1 phase), were examined. TLPE extract decreased the level of p53 in KKU-M213B
240 and KKU-100 cells when compared to the control (solvent control) (Fig. 4). TLPE extract also
241 caused a decrease in the level of the anti-apoptotic protein Bcl2 in both cancer cell lines when
242 tested at higher concentrations (Fig. 4A-B, 4D-E). The amount of the pro-apoptotic protein Bax
243 was decreased in KKU-M213B cells but increased in KKU-100 cells. The relative ratio of
244 Bax/Bcl2 was significantly increased at the high concentration treatments in both KKU-M313B
245 and KKU-100 cells (Fig. 4C,4F).
246   TLPE extract caused a decrease in a protein level of CDK4 in both KKU-M213B (Fig. 4A-B)
247 and KKU-100 cells (Fig. 4D-E) but did not affect the amount of p21 in KKU-M213B cells (Fig.
248 4A-B). In KKU-100 cells, the protein level of p21 was increased, which was related to the
249 induction of the cell cycle arrest at G1 phase in KKU-100 cells (Fig. 2C-D). Ethanol extracts
250 from most parts of the plant often contain *p*-coumaric acid and several phenolic acids that act as
251 histone deacetylase inhibitors (HDAC inhibitors) (Saenglee et al., 2016). In this study, *p*-
252 coumaric and sinapinic acids were the most predominant phenolic acids that were identified in
253 the TLPE extract (Fig. 5 and Table 1). Therefore, we tested whether the TLPE extract could
254 inhibit the activity of histone deacetylases in CCA cells. As shown in Figure 4, the increased
255 acetylated histone H3 protein was observed both in KKU-M213B and KKU-100 cells, indicating
256 that the TLPE extract possessed HDAC inhibitory activity.
257   In addition, the p-ERK1/2 levels were significantly reduced in both CCA cell lines at the
258 concentrations tested (Fig. 4A-B, 4D-E), indicating that inhibition of the ERK1/2 signaling
259 pathway may be implicated in TLPE extract-induced apoptosis in cholangiocarcinoma cells.

260 **Total phenolic content and type of phenolic acids in TLPE extract**
261 The total phenolic content in TLPE extract was 47.67 ± 6.77 mg gallic acid equivalent/g of dry
262 extract. Figure 5 shows typical chromatograms of phenolic acid standards (Fig. 5A) and the
263 TLPE extract (Fig. 5B). Six phenolic acids including *p*-hydroxybenzoic, vanillic, syringic, *p*-
264 coumaric, ferulic and sinapinic acids were identified in TLPE extract with *p*-coumaric and
265 sinapinic acids being the most pre-dominant (Table 1).
266

267 # Discussion
268 The MTT results revealed that TLPE extract inhibited proliferation of both CCA cell lines in a
269 dose- and time-dependent manner. In addition, non-tumorigenic biliary epithelial (H69) cells

270   exhibited the lowest sensitivity to TLPE extract, indicating somewhat greater specificity to CCA
271   cell lines of the extract. Similarly, *T. triandra* leaf powder methanolic extract has been shown to
272   inhibit the growth of hepatocellular carcinoma cells (IC_{50} = 0.41 mg/ml) but is less toxic to white
273   blood cells (Lymphocytes) (IC_{50} = 10 mg/mL) (Chaveerach et al., 2016). *T. triandra* leaf was
274   previously extracted by various solvents (petroleum ether, dichloromethane, ethyl acetate,
275   methanol, and water) and was also investigated for its ability to inhibit the growth of oral (KB),
276   lungs (NCI-H187) and breast (MCF7) cancer cells (Rattana et al., 2016). Methanol and water
277   extracts of *T. triandra* leaf powder had the potential for growth inhibition of NCI-H187, KB and
278   NCI-H187 cells (IC_{50} < 50 µg/ml). In addition, *T. triandra* leaf powder ether, dichloromethane
279   and ethyl acetate extracts inhibited the growth of many cancer cells (IC_{50} > 50 µg/ml) (Rattana et
280   al., 2016).
281   Recently, tiliacorinine was isolated from the root and stem of *T. triandra*, inhibiting
282   proliferation of cholangiocarcinoma cell lines (KKU-055, KKU-213, KKU-214 and KKU-100)
283   with IC_{50} values of 4.50-7.00 µM (Janeklang et al., 2014). This evidence showed that the
284   anticancer effect of *T. triandra* leaf might be related to the solvent used for extraction and the
285   type of cancer cells. However, *T. triandra* leaf powder ethanolic (TLPE) extract has not been
286   studied previously on cholangiocarcinoma cell lines. The present study demonstrated that TLPE
287   extract induced apoptosis in two cholangiocarcinoma cell lines (KKU-M213B and KKU-100
288   cells). The toxicity of TLPE extract in non-tumorigenic biliary epithelial cells (H69 cells) was
289   also investigated and less toxicity was found in this cell line compared to CCA cells. In the
290   previous study, tiliacorinine isolated from *T. triandra*, induced apoptosis and caspase activity in
291   cholangiocarcinoma (KKU-M213B, KKU-100) cell lines (Janeklang et al., 2014). In this study,
292   natural HDAC inhibitors including *p*-coumaric, ferulic and sinapinic acids were identified in
293   TLPE extract (Fig. 5 and Table 1). In addition, western blot results demonstrated that TLPE
294   extract caused an increase in acetylated histone H3 protein content in both KKU-M213B and
295   KKU-100 cells (Fig. 4A-B, 4D-E). Similarly, the compounds with HDAC inhibitory activity
296   caused hyperacetylation with a more relaxed chromatin structure (Fang, 2005; Mork et al.,
297   2005).
298   The level of p53 protein was decreased in both types of cancer cells, indicating that the
299   mechanism of stimulating apoptosis in cholangiocarcinoma cells may be p53-independent
300   (Hiebert et al., 1995). Furthermore, the level of Bcl2 was decreased when the cells were treated
301   with TLPE extract (Fig. 4). The reduction of the anti-apoptotic protein Bcl2, causes a release of
302   cytochrome c from the mitochondrial membrane leading to the activation of apoptosis in the
303   intrinsic pathway (Nahle et al., 2002). TLPE extract could induce cell cycle arrest at the G0/G1
304   phase in KKU-100 cells. TLPE extract caused the increased p21 protein levels in KKU-100 cells.
305   p21 is an inhibitor of the cell cycle that can inhibit CDK4 activity causing the cell cycle arrest.
306   However, it was found that TLPE extract caused a decrease in CDK4 protein levels in both
307   KKU-M213B and KKU-100 cells (Fig. 4A-B, 4D-E). In KKU-100 cells, CDK4 protein levels
308   were decreased which may be related to the cell cycle arrest at G1 phase. In KKU-M213B cells,
309   the CDK4 protein level was slightly reduced, but there was no cell cycle arrest in the G1 phase.
310   Moreover, the level of p21 protein was not increased in KKU-M213B cells. It has been reported
311   that p21 down-regulation inhibits apoptosis by binding with procaspase 3 and blocking the
312   protease cleavage site (Suzuki et al., 1999). In addition, we hypothesized that TLPE extract may
313   cause an increase in the level of pro-apoptotic proteins such as Bax protein. In this study, TLPE
314   extract caused a decrease in the level of Bax protein in KKU-M213B cells but an increase in
315   KKU-100 cells (Fig. 4A-B, 4D-E). However, the relative ratio of Bax/Bcl2 was significantly

316   increased in both KKU-M213B and KKU-100 cells (Fig. 4C, 4F), which was corresponding to
317   an increased apoptosis at a higher concentration of TLPE extract treatment (Fig. 3A-B). Due to
318   the reduced Bax protein levels in KKU-M213B cells, it has been reported that Bax down-
319   regulation involves p53 mutations (Beerheide et al., 1999).
320

Conclusions

322   TLPE extract inhibited the growth of CCA cell lines. KKU-M213B cells were more sensitive to
323   TLPE extract than KKU-100 cells. In addition, TLPE extract was less toxic to non-tumorigenic
324   biliary epithelial cells (H69) than to both cancer cell lines. TLPE extract inhibited both human
325   cholangiocarcinoma cell lines via apoptosis induction. Induction of cell cycle arrest was
326   observed at the G0/G1 phase only in KKU-100 cells. Apoptosis and cell cycle arrest inductions
327   were confirmed by the levels of protein expression involved. Moreover, the analysis of the type
328   of phenolic acids in TLPE extract revealed 6 phenolic acids: 1) *p*-hydroxybenzoic acid, 2)
329   vanillic acid, 3) syringic acid, 4) *p*-coumaric acid, 5) ferulic acid and 6) sinapinic acid.
330   Accordingly, TLPE extract has potential for development as an alternative medicine for human
331   cholangiocarcinoma treatment.
332

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336

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428

429 **Figures**

430 **Figure 1. The effect of TLPE extract on the proliferation of CCA cells.** KKU-M213B (A, B),
431 KKU-100 (C, D) and H69 (D, E) cells were treated with TLPE extract of 31.25-500 µg/ml for 24
432 h and 3.75-30 µg/ml for 48 and 72 h. Antiproliferative activity was determined using MTT assay
433 according to manufacturer protocol. Cell viabilities are expressed as percentages of the solvent
434 control, which was defined as 100% cell viability. The IC_{50} values are indicated as the means $\pm$
435 S.D. from three independent experiments.
436

437   **Figure 2. Histograms display the DNA content distribution of CCA cells.** KKU-M213B (A),
438   KKU-100 (C) and H69 (E) cell cycle profiles after treated with TLPE extract. Cells treated with
439   1% EtOH (v/v), 31.25, 62.50 and 125.00 μg/ml of TLPE extract were stained with propidium
440   iodide (PI) and analyzed by flow cytometry. Bar graphs represent the summarized data on cell
441   cycle distribution of KKU-M213B (B), KKU-100 (D) and H69 (F) cells. The percentages of cells
442   are presented as means ± S.D. from two independent experiments performed in duplicate. The
443   asterisk "*" indicates a significant increase when compared with sol-vent control treatments (p <
444   0.05).
445
446   **Figure 3. Apoptotic death analysis by Flow cytometry.** Representative dot plots display the
447   apoptotic death of the cells treated with the indicated concentrations in KKU-M213B (A), KKU-
448   100 (C) and H69 (E) cells treated with TLPE extract. After 24 h-treatment, cells labeled with
449   Annexin V-FITC and Propidium iodide (PI) were analyzed by flow cytometry to determine the
450   percentage of cells displaying an increase in early (panels Q4) and late (panels Q2) apoptosis. The
451   cells treated with camptothecin (15 mg/mL) and absolute ethanol (1%; v/v) were used as positive
452   and negative controls, respectively. Bar graphs show the summarized data on KKU-M213B (B),
453   KKU-100 (D) and H69 (F) cells from two independent experiments performed in duplicate.
454   Graphs represent the percentages of early and late apoptotic cells with means ± S.D. The symbol
455   "*" indicates a significant difference (p < 0.05).
456
457   **Figure 4. The effect of TLPE extract on cell cycle and apoptosis regulatory proteins in CCA**
458   **cells.** KKU-M213B (A) and KKU-100 (DC) cells were treated with absolute ethanol as control,
459   and TLPE extract at 31.25, 62.5 and 125 μg/ml for 24h. After 24 h, the cells were harvested, and
460   proteins were extracted and separated on SDS-PAGE. Expression of p53, p21, CDK4, Bax, Ac-
461   H3, Bcl2 and p-ERK were detected by immunoblotting. Total ERK was used as a loading control
462   in western blot analysis. The intensity of protein bands was quantitated by densitometric analysis.
463   The bar graphs represent the relative fold of protein expression in KKU-M213B (B), and KKU-
464   100 (E) cells compared with internal control. The relative ratio of Bax/Bcl2 was calculated and
465   found to be significant for the high concentration treatments in both KKU-M213B (C) and KKU-
466   100 (F) cells.
467
468   **Figure 5. HPLC chromatograms of the phenolic acids.** Standard phenolic acids (A) and TLPE
469   extract (B) :1, gallic acid; 2, protocatechuic acid; 3, *p*-hydroxybenzoic acid; 4, vanillic acid; 5,
470   caffeic acid; 6, *m*-hydroxybenzaldehyde; 7, syringic acid; 8, *p*-coumaric acid; 9, ferulic acid and
471   10, sinapinic acid.

Table 1 Phenolic acid compositions of *T. triandra* ethanolic extract

[a]Results are expressed as means ± SD of three determinations.

1 **Table 1 Phenolic acid compositions of *T. triandra* ethanolic extract**

Sample	Phenolic acids (mg/g of dry extract)[a]					
	p-Hydroxybenzoic acid	Vanillic acid	Syringic acid	*p*-Coumaric acid	Ferulic acid	Sinapinic acid
TLPE	2.36±0.84	2.56±0.13	2.22±0.10	4.16±0.11	3.26±0.16	6.16±0.31

2 [a]Results are expressed as means ± SD of three determinations.

Figure 1

The effect of TLPE extract on the proliferation of CCA cells.

KKU-M213B (A, B), KKU-100 (C, D) and H69 (D, E) cells were treated with TLPE extract of 31.25-500 µg/ml for 24 h and 3.75-30 µg/ml for 48 and 72 h. Antiproliferative activity was determined using MTT assay according to manufacturer protocol. Cell viabilities are expressed as percentages of the solvent control, which was defined as 100% cell viability. The IC_{50} values are indicated as the means ± S.D. from three independent experiments.

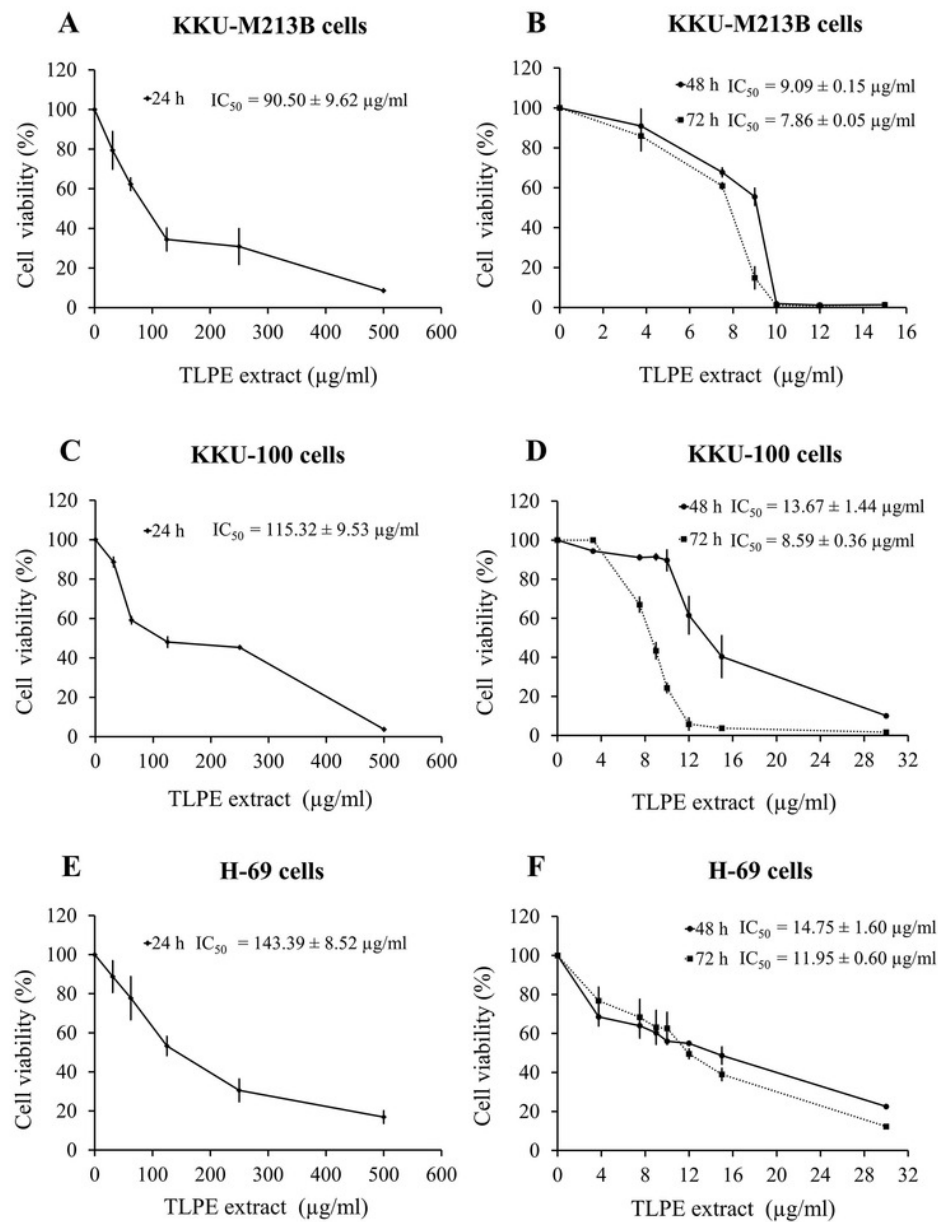



Figure 2

Histograms display the DNA content distribution of CCA cells.

KKU-M213B (A), KKU-100 (C) and H69 (E) cell cycle profiles after treated with TLPE extract. Cells treated with 1% EtOH (v/v), 31.25, 62.50 and 125.00 µg/ml of TLPE extract were stained with propidium iodide (PI) and analyzed by flow cytometry. Bar graphs represent the summarized data on cell cycle distribution of KKU-M213B (B), KKU-100 (D) and H69 (F) cells. The percentages of cells are presented as means ± S.D. from two independent experiments performed in duplicate. The asterisk "*" indicates a significant increase when compared with sol-vent control treatments (p < 0.05).

A KKU-M213B cells

TLPE extract

Solvent control 31.25 µg/ml 62.50 µg/ml 125.00 µg/ml

	G0/G1	78.6%
	S	4.2%
	G2/M	10.0%
	Sub G1	7.2%

	G0/G1	77.4%
	S	2.9%
	G2/M	5.8%
	Sub G1	12.3%

	G0/G1	77.3%
	S	2.9%
	G2/M	2.7%
	Sub G1	17.1%

	G0/G1	67.9%
	S	5.5%
	G2/M	6.8%
	Sub G1	19.7%

B KKU-M213B cells

C KKU-100 cells

TLPE extract

Solvent control 31.25 µg/ml 62.50 µg/ml 125.00 µg/ml

	G_0/G_1	72.0%
	S	12.2%
	G_2/M	14.4%
	Sub G_1	0.8%

	G_0/G_1	82.9%
	S	7.9%
	G_2/M	7.5%
	Sub G_1	1.3%

	G_0/G_1	84.2%
	S	6.4%
	G_2/M	7.5%
	Sub G_1	1.7%

	G_0/G_1	84.6%
	S	4.0%
	G_2/M	4.2%
	Sub G_1	7.3%

D KKU-100 cells

E H-69 cells

TLPE extract

Solvent control 31.25 µg/ml 62.50 µg/ml 125.00 µg/ml

	G_0/G_1	46.3%
	S	14.9%
	G_2/M	31.6%
	Sub G_1	3.7%

	G_0/G_1	48.4%
	S	15.0%
	G_2/M	26.9%
	Sub G_1	7.4%

	G_0/G_1	51.5%
	S	14.5%
	G_2/M	23.7%
	Sub G_1	8.5%

	G_0/G_1	49.1%
	S	15.3%
	G_2/M	24.1%
	Sub G_1	9.6%

F H69 cells

Count number

Intensity of Propidium iodide (PI)

Figure 3

Apoptotic death analysis by Flow cytometry.

Representative dot plots display the apoptotic death of the cells treated with the indicated concentrations in KKU-M213B (A), KKU-100 (C) and H69 (E) cells treated with TLPE extract. After 24 h-treatment, cells labeled with Annexin V-FITC and Propidium iodide (PI) were analyzed by flow cytometry to determine the percentage of cells displaying an increase in early (panels Q4) and late (panels Q2) apoptosis. The cells treated with camptothecin (15 mg/mL) and absolute ethanol (1%; v/v) were used as positive and negative controls, respectively. Bar graphs show the summarized data on KKU-M213B (B), KKU-100 (D) and H69 (F) cells from two independent experiments performed in duplicate. Graphs represent the percentages of early and late apoptotic cells with means ± S.D. The symbol "*" indicates a significant difference (p < 0.05).

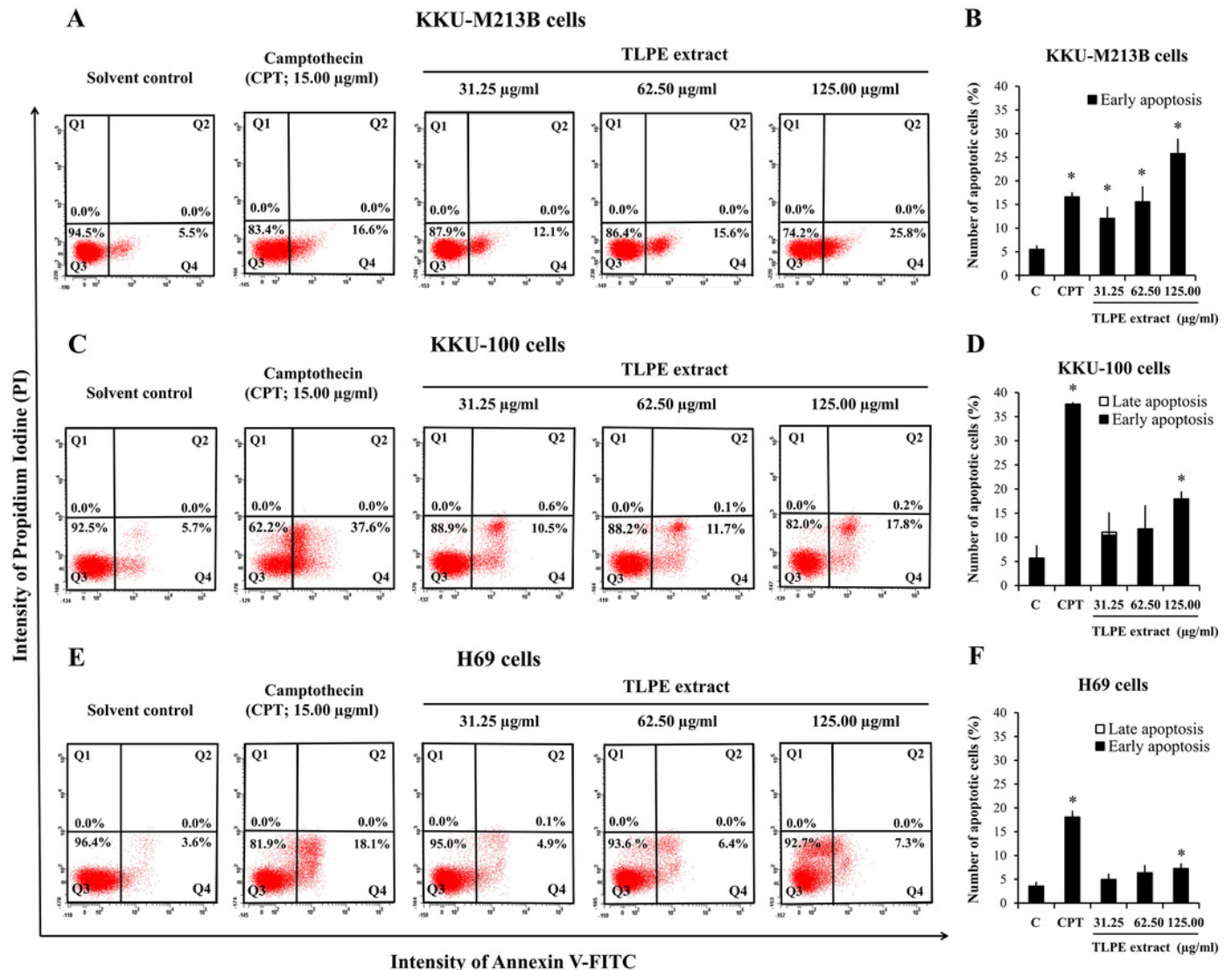
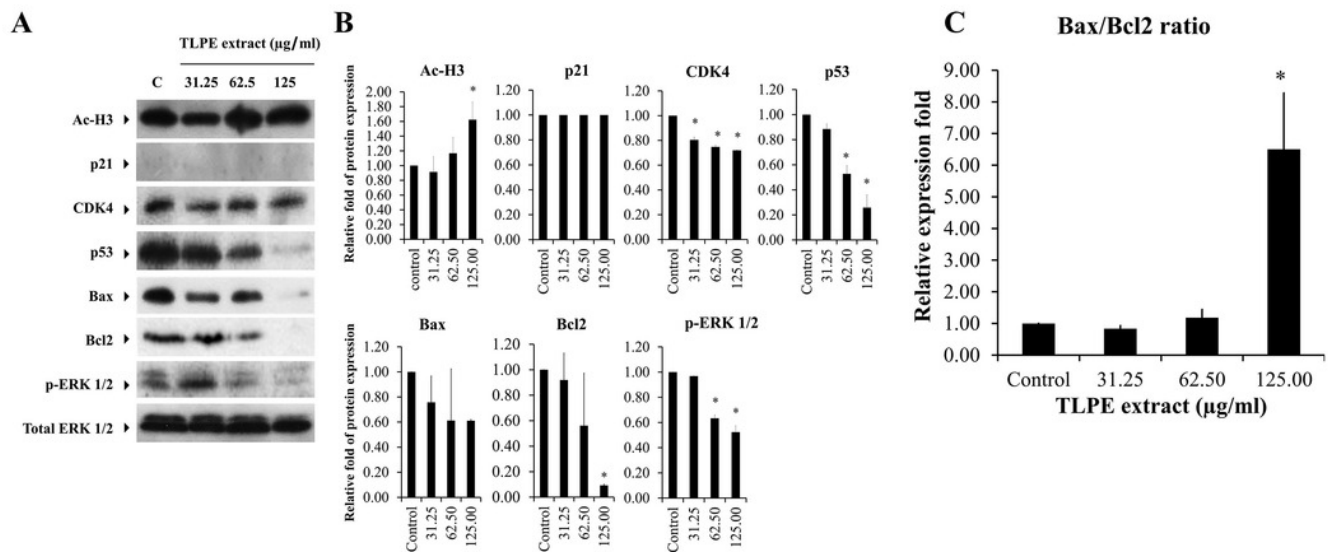



Figure 4

The effect of TLPE extract on cell cycle and apoptosis regulatory proteins in CCA cells.

KKU-M213B (A) and KKU-100 (DC) cells were treated with absolute ethanol as control, and TLPE extract at 31.25, 62.5 and 125 µg/ml for 24h. After 24 h, the cells were harvested, and proteins were extracted and separated on SDS-PAGE. Expression of p53, p21, CDK4, Bax, Ac-H3, Bcl2 and p-ERK were detected by immunoblotting. Total ERK was used as a loading control in western blot analysis. The intensity of protein bands was quantitated by densitometric analysis. The bar graphs represent the relative fold of protein expression in KKU-M213B (B), and KKU-100 (E) cells compared with internal control. The relative ratio of Bax/Bcl2 was calculated and found to be significant for the high concentration treatments in both KKU-M213B (C) and KKU-100 (F) cells.

KKU-M213B cells

KKU-100 cells

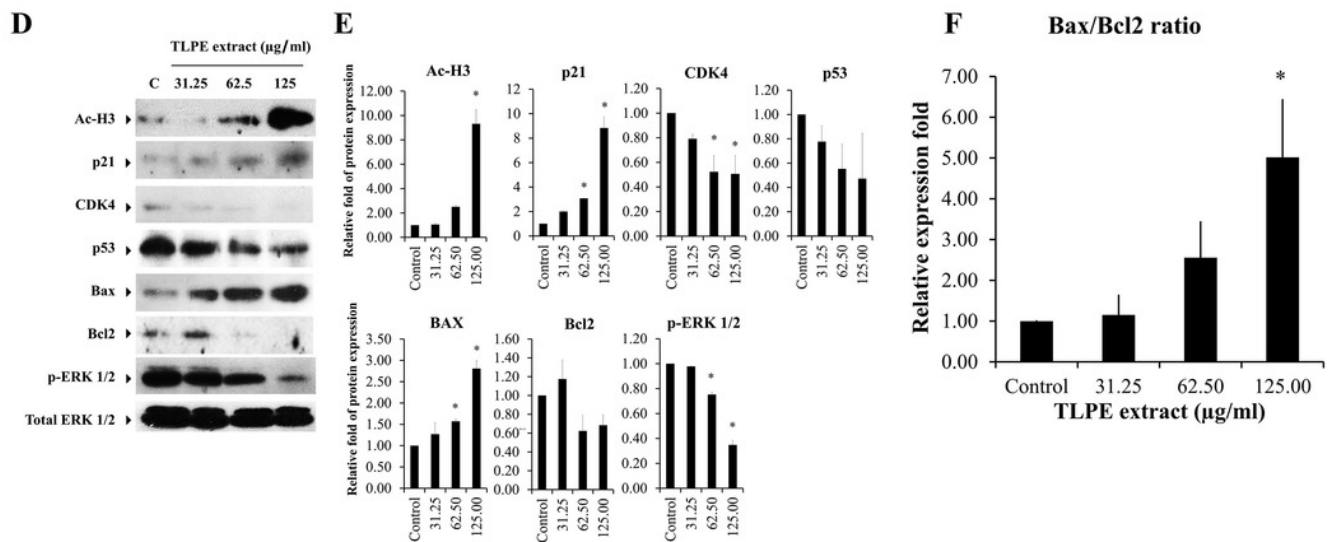



Figure 5

HPLC chromatograms of the phenolic acids.

Standard phenolic acids (A) and TLPE extract (B) :1, gallic acid; 2, protocatechuic acid; 3, *p*-hydroxybenzoic acid; 4, vanillic acid; 5, caffeic acid; 6, *m*-hydroxybenzaldehyde; 7, syringic acid; 8, *p*-coumaric acid; 9, ferulic acid and 10, sinapinic acid.