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The impact of caffeic acid phenethyl ester, chrysin and ethanolic extracts of propolis on *PLD1* gene expression in AGS cell line

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ABSTRACT

Introduction: Up-regulation of phospholipase D (PLD) is functionally related to tumorigenesis and oncogenic signals. Chrysin, caffeic acid phenethyl ester (CAPE) and ethanolic extract of propolis (EEP) are three safe compounds which have been shown to possess antiproliferative, antioxidant, anti-inflammatory and antineoplastic properties. In this study, the effects of these three compounds on *PLD1* gene expression were examined in AGS cell line.

Methods: After determining the appropriate concentrations of EEP, CAPE, and chrysin, AGS cells were cultured in mediums with proper ratios of the compounds. AGS cells were maintained in exponential growth and the culture mediums refreshed every other day. Finally, after extracting total RNA from AGS cells, real-time PCR was performed to evaluate the mRNA expression of *PLD1* in the presence of each compound.

Results: CAPE decreased the mRNA level of *PLD1* gene in a dose-dependent manner. A solution with 30 µM concentration of CAPE was the effective dose in comparison to control group as well as 15 and 20 µM concentrations of the compound, whereas no changes were observed in the presence of EEP and chrysin.

Conclusion: Taken together, the results of the study indicated that CAPE might exert its anti-neoplastic effect by targeting *PLD1* expression in AGS cell line.

Implication for health policy/practice/research/medical education:
Caffeic acid phenethyl ester (CAPE) can effectively inhibit cell proliferation in gastric cancer cell line and might be useful in the treatment of patients with gastric cancer.

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Introduction

On the molecular level, various factors can have effects on incidence and progression of cancers, including environmental factors, epigenetics and changes in specific genes expression (1-3). In many cancers, chemotherapy resistance is a common phenomenon and the major limitation in their treatment (4). Therefore, finding new ways and approaches to improve the life quality of patients with cancer or treating them, is quite crucial. Dietary supplements or alternatives to medication have become increasingly popular and the main focus to improve nature-based compounds with chemopreventive properties (5, 6). Chrysin, caffeic acid phenethyl ester (CAPE) and ethanolic extract of propolis (EEP) are three safe compounds and potential adjuvant therapies for patients with advanced cancers (7). CAPE is a polyphenolic compound which has influence on some of the cellular processes, including cell survival and proliferation (8). Chrysin and EEP are two active compounds that can be found in honey. These three compounds are considered to possess antitumor properties (7). It has been shown that they have influence on some important cell activities such as proliferation, apoptosis, and viability, in some cancer cell lines. They can exert their anticancer effects through several signaling pathways and genes (9, 10). Phospholipase D (PLD) is a member of phospholipase enzymes superfamily with two

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major isoforms, including PLD1and PLD2. Phospholipase D1 catalyzes the hydrolysis of phosphatidylcholin which leads to production of phosphatidic acid and free choline. *PLD1* gene and its protein product become activated in some cancer cell lines, such as gastric cancer (11). Studies in glioma cells confirmed that the overexpression of PLD increases invasion via elevating *MMP-2* gene transcription through NF-kB mediated signaling pathways (12). In the present study, by using real time PCR, the effects of CAPE, EEP, and chrysin compounds on *PLD1* gene expression were analyzed in human gastric cancer cell line (AGS cell line).

Materials and methods

Chemicals and reagents

Trypsin-EDTA, DMEM, fetal bovine serum (FBS), and penicillin-streptomycin were purchased from Gibco Co (Invitrogen, Carlsbad, CA, USA). DMSO, CAPE and chrysin were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Ethanolic extract of propolis (EEP) obtained from fresh propolis samples collected from Dinaran area (Iran) (13). Other materials and chemical reagents used to conduct this study were purchased from laboratory reagents providers in Iran. MTT test was previously carried out to determine the concentration of the compounds in which the cells viability was normal and had no effect on the cytotoxicity assays (13, 14). The test reagents were dissolved to prepare three stock solutions in DMSO for each compound (CAPE 100 µM, chrysin 100 µM, and EEP 100 µg/mL), and stored in aliquots at -20°C. They were diluted with fresh culture medium before use and added to the micro-plates at specific concentrations.

Cell line and cell culture conditions

AGS cell line was purchased from Royan Institute, Tehran, Iran. The cells were maintained at exponential growth at 37°C, in DMEM, supplemented with heat inactivated 10% FBS and exposure to 5% CO2. Moreover, penicillin and streptomycin 1% were added to the culture medium. The culture medium of the cells renewed every second day.

Cell growth assay

The growth characteristics of the AGS cell lines were previously described by Amini-Sarteshnizi et al. (13, 14).

RNA extraction and qRT-PCR

Total cellular RNA was extracted from AGS cells treated with increasing doses of EEP, CAPE, and chrysin (10,15,30 µM for CAPE, 20, 30, and 40 µg/mL for EEP, and 15, 20, and 30 µM for chrysin) after 72 hours, using Biozol Total RNA Extraction Reagent kit (Qiagen, Germany). RNA was quantified with a nanodrop 1000 spectrophotometer. The purity of the extracted RNA was demonstrated using 260/280 and 260/230 wavelength ratios, which are commonly in the range of 1.8-2.2. cDNA synthesis was conducted by using moloney murine leukemia virus (MMLV) reverse transcriptase (Fermentas, Vilnius, Lithuania) with oligo-dT primers. qRT-PCR for *PLD1* and glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) transcripts were performed using specific primers. The thermal cycle consisted of an initial denaturation step at 95°C for 10 minutes, followed by 45 amplification cycles consisting of denaturation at 95°C for 15 seconds, annealing at 60°C for 20 seconds, and an extension at 72°C for 25 seconds. The PCR products were isolated by agarose gel electrophoresis, colored with ethidium bromide, and became visible under the ultraviolet light. To evaluate the effects of CAPE, chrysin, and EEP on *PLD1* expression in AGS cells, relative quantitative PCR and comparative Ct methods, using Maxima SYBR Green/Rox QPCR Master Mix (2X) kit (Fermentase), were performed and analyzed on Rotor-Gene 3000 (Corbett Robotics, Australia). All tests were carried out at least in triplicate (100 ng total RNA per well). Template control was run in each experiment. The primer sequence of *PLD1* and *GAPDH* are listed in Table 1. Relative mRNA levels of the target gene in each sample were normalized to its GAPDH content: Ratio= (Etarget) ΔCt target (control−treatment)/ (Ereference) ΔCt reference (control−treatment) (15,16).

Statistical analysis

Data on the cell populations with different treatments and conditions were analyzed using GraphPad Prism software (v5.01, USA) and Kruskal-Wallis test followed by post-hoc comparisons and Dunn's multiple comparison.

Results

Growth characteristics of AGS cell line and optimization of qRT-PCR

The growth characteristics of the AGS cell line were previously described (13,14). Prior to the quantitative analysis, optimization procedures were carried out for qRT-PCR on the EEP, chrysin, and CAPE treated AGS cell lines. Using specific primers for human *PLD1* and

Table 1. The sequence of the designed primers for amplification of the target genes for qRT-PCR

Target gene	Primer sequence	Size of the amplicon
Phospholipase D1 (*PLD1*)	5'-GCTGGGAAAGCGTGACAGTGA-3' 5'-AGCCAAGGACAACCCTAAAGCAG-3'	150bp
glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*)	5'-GTGAAGGTCGGAGTCAAC-3' 5'-GTTGAGGTCAATGAAGGG-3'	112bp

GAPDH genes, electrophoresis of the PCR products was conducted on acrylamide gel which demonstrated single bands with the expected sizes for amplified *PLD1* (150 bp) and *GAPDH* (112 bp) segments. Expression analysis of the genes showed a unique melting curve without any primer dimer for all the inspected genes (Figure 1), which was further confirmed by acrylamide gel separation and staining. Standard curve was plotted for consistent real-time PCR results (Figure 1).

Expression of *PLD1* gene in AGS cell specimens

After optimization of qRT-PCR, the effects of CAPE, chrysin, and EEP on *PLD1* gene expression (mRNA) were investigated. CAPE decreased the mRNA level of *PLD1* gene in a dose-dependent manner, in comparison with the control in the AGS cell line ($P=0.04$) whereas no change was observed in the presence of EEP ($P=0.3$) and chrysin ($P=0.08$) in this cell line (Figure 2).

Discussion

The current study is the first to show the effects of CAPE, chrysin, and EEP on *PLD1* gene regulation in the AGS cell line. According to our results, CAPE (with the concentrations of 30 μM) down-regulated the expression

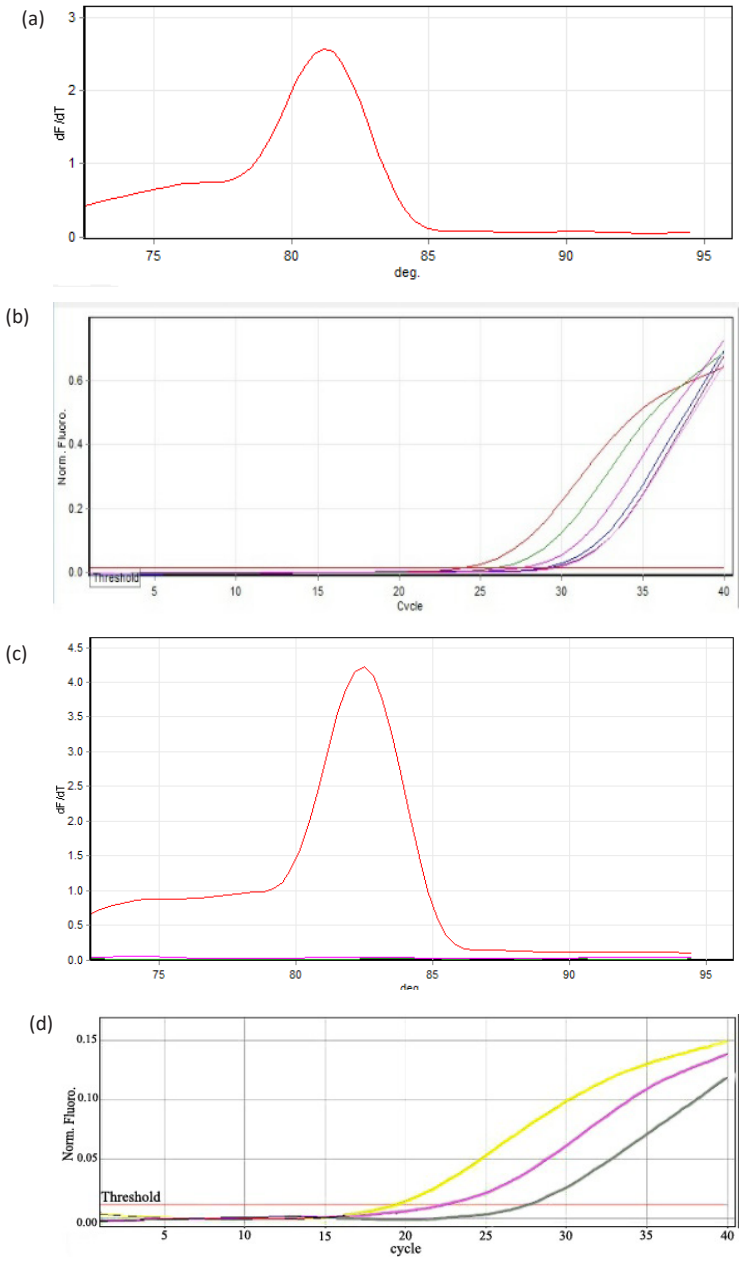

Figure 1. Optimization of quantitative RT-PCR (qRT-PCR). A unique melting curve without primer dimers showing specific amplification of glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) (a) and phospholipase D1 (*PLD1*) (c) on qRT-PCR. Standard curves of a cDNA dilution series for determination of amplification efficiencies of *PLD1* (b) and *GAPDH* (d).

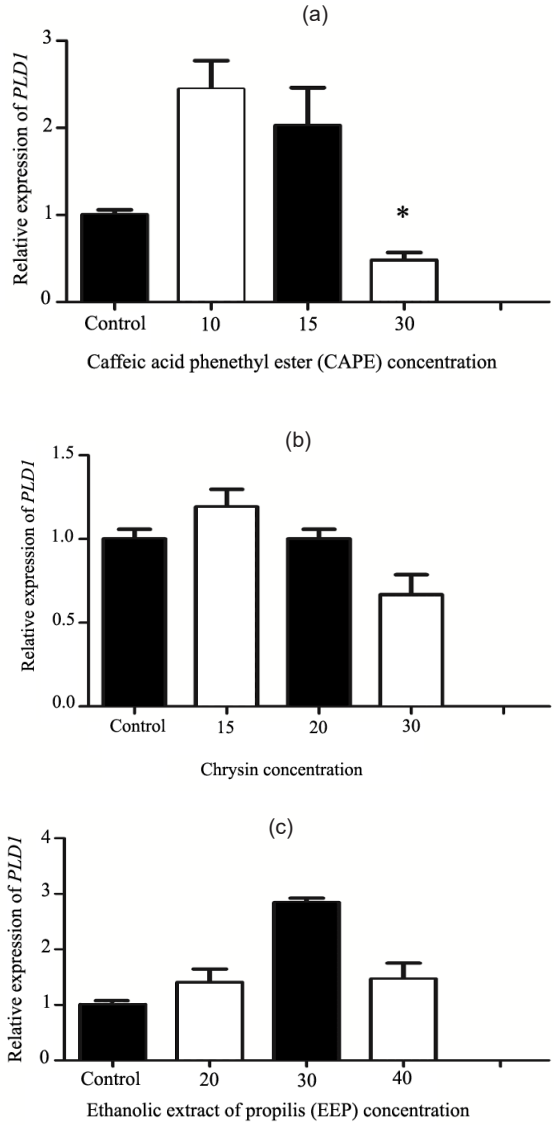

Figure 2. Effects of caffeic acid phenethyl ester (CAPE) (a), chrysin (b), and ethanolic extract of propolis (EEP) (c) on expression of PLD1 gene in AGS cell lines.
Data are expressed as mean ± standard deviation; * $P < 0.05$ compared with the corresponding value of control group.

of *PLD1* gene and subsequently inhibited the proliferation of gastric cancer cell lines. CAPE is a potent antioxidant agent isolated from honeybee hive propolis. Several studies have shown that CAPE has the potential for the treatment of several cancers (17). The compound has already been reported to suppress the cell proliferation of prostate cancer cell lines (18). A study showed that CAPE down-regulated *PLD1* expression at the transcriptional level and suppressed the proliferation of glioma cells (19). Accordingly, the inhibition of *PLD1* activity may occur via direct binding of the catechol moiety in CAPE to cysteine 837 in the PLD1 (19,20). Therefore, this study confirmed the evidence on the down-regulation of *PLD1* gene by CAPE. The phospholipase superfamily is defined by a conserved catalytic site and a common transphosphatidylation activity at phosphodiester bonds. Studies have reported that PLD1 promoted RAS and ERK activation and transformation in epithelial cells via interaction with PEA-1S (20). Another study demonstrated that PLD1 was required for NF-kB activation, due to inducing the activation and expression of *MMP2*, and promoted the invasion of the glioma cells (21). Rebamipide down-regulates the expression and enzymatic activity of PLD1 and inhibits the proliferation of gastric cancer cells. A study reported that rebamipide inhibited the transactivation of NF-kB in the AGS and MKN-1 gastric cancer cells. Such PLD1 suppression may be due to the inhibition of NF-kB activity by rebamipide (22).

Chrysin, a naturally occurring flavone, which is found in honey and plant extracts, has been shown to possess anticancer properties (23). Chrysin exerts its effects through the selective modulation of multiple cell signaling pathways which are related to the survival, inflammation and metastasis of cancer cells. Chrysin is able to kill cancer cells of several histotypes including hematologic, lung, colon, liver, and breast cancers as well as glioblastoma. Chrysin was found to significantly sensitize the TNF-alpha-induced apoptosis in human colorectal cancer cell line HCT-116, human nasopharyngeal carcinoma cell line CNE-1, and human liver cancer cell line HEPG2; in which such sensitization is closely related to inhibitory effect on NF-kB activation (24). On the other hand, NF-kB binds to and activates the *PLD1* promoter; therefore, chrysin is likely to exert its anticancer activity through *PLD1* down-regulation (19). EEP is one of the sources of phenolic acids and flavonoids. Several mechanisms were offered to explain the overall cancer-preventive and antitumor effects of propolis and its phenolic components (25). A study reported that EEP inhibited the growth and induced apoptosis in HeLa cancer cell line in a dose-dependent manner (25). In the present study, however, no correlation between EEP or chrysin and *PLD1* gene expression was observed. It can be hypothesized that EEP and chrysin may exert their effects on the protein activity of PLD1. PEA-1S, a binding partner of PLD1, promotes H-RAS-mediated cell transformation via the enhanced expression and activation of *PLD1* (20). It is likely that PEA-1S inhibits the effects of EEP and chrysin.

Conclusion

The results of this study indicated that CAPE might be associated with anti-cancer outcome by targeting *PLD1* in the AGS cell line. This study showed that CAPE influenced on *PLD1* gene expression and down-regulated its expression in a dose-dependent manner. Therefore, it can be proposed that we may be able to use CAPE as an adjuvant therapy for gastric cancer. However, we

recommend further research to evaluate PLD1 protein activity, so that more definite arguments can be made on the effects of the CAPE on *PLD1* expression. Regarding chrysin and EEP, although no association between them and *PLD1* expression was observed, research on the effect of these two compounds on the translation and activity of the *PLD1* protein may show promising results and enable us to introduce more effective therapies for different cancers.

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Authors' contributions

HJG: Experimental design, collection and/or assembly of data, data analysis, interpretation, and manuscript writing. AJ: Data analysis, interpretation, and manuscript writing. NAS: Data analysis and interpretation. HT: Conception and design, financial support, data analysis, interpretation, manuscript writing, and final approval of the manuscript.

Conflict of interests

Authors declare no conflict of interests.

Ethical considerations

This study was only performed on commercially available cell lines and no human specimens or animal models were examined. The study was approved by the Research Ethics Committee of the Shahrekord University of Medical Sciences (Approval code: 94-1-6)

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