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ORIGINAL ARTICLE

Comparison of the Anti-inflammatory Effects of Proanthocyanidin, Quercetin, and Damnacanthal on Benzo(*a*)pyrene Exposed A549 Alveolar Cell Line

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Abstract—Phytochemical compounds are emerging as a new group of anti-inflammatory, antioxidant, and anti-cancer agents that help minimize toxicity in patients with pulmonary diseases. The goal of this study was to investigate the potential curative effects of Quercetin (QC), Damnacanthal (DAM), and Proanthocyanidine (PA) on inflammatory mediators and oxidative stress parameters and to examine the viability of the A549 cell line treated with benzo(*a*)pyrene (BaP) *in vitro*. The A549 cell line was treated with BaP, a BaP/QC combination, a BaP/DAM combination, and BaP/PA combination. Inflammatory markers, oxidative stress parameters, mRNA expression levels of apoptotic and antiapoptotic proteins, and cell viability were assessed, and the results were compared. There were higher levels of lactate dehydrogenase after BaP treatment of A549 cell lines. Interferon-γ level significantly decreased in the QC, DAM, and PA-treated group ($P<0.001$). IL-1β and TNF-α levels significantly decreased after PA and QC treatments ($P<0.001$). Some of the oxidative stress markers (NO, MDA, TOS) and OSI decreased, while antioxidant (GSH) levels increased after treatment with QC, DAM, and PA. The QC and DAM treatments profoundly upregulated apoptotic gene expression and downregulated antiapoptotic gene expression. Viability of QC, DAM, and PA-treated cells was found to be significantly higher in comparison to the control and BaP-treated groups ($p<0.001$). Our results revealed that A549 cell lines treated with BaP-stimulated necrosis produced higher level of inflammatory cytokines and oxidative stress parameters. Treatments with PA, QC, and DAM reduced inflammatory response induced by BaP exposure.

KEY WORDS: inflammation; cell culture; oxidative stress; antioxidants; COPD.

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous environmental cytotoxic and genotoxic compounds found particularly in tobacco. Benzo(*a*)pyrene (BaP) ($C_{20}H_{12}$) is the best known and most studied PAH [1, 2]. In many studies, it has been reported that BaP exposure occurs in both those living in urban areas with air pollution and tobacco users. As a result of this exposure, it has been shown to lead many chronic lung diseases, especially lung cancer and chronic obstructive lung disease (COPD) [1–4]. BaP like other PAHs is converted to highly toxic reactive products with various metabolic activation processes inside the cells [5, 6].

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Epithelial cells of the airways—the first barrier for protection—contact inevitably with a variety of inhaler agents (particles and BaP). In toxicological reactions caused by these malicious agents, respiratory epithelial cells have an important role. Due to the exposure of BaP, increase in the level of inflammatory mediators and oxidative stress were reported in human alveolar epithelial cells (A549) in the literature [2].

In recent years, *in vivo* and *in vitro* studies were conducted to demonstrate favorable effects of many plant-derived extracts and components on treatment of lung cancer and inflammatory processes. These studies were conducted particularly to determine treatment efficacy of plant extracts and their active ingredients that previously determined antioxidants and anticarcinogens [7–9].

In this study, we aimed to investigate and compare the anti-inflammatory, antioxidant, and apoptotic effect of Quercetin (QC), Proanthocyanidin (PA), and Damnacanthal (DAM) on BaP exposed A549 cell line.

METHODS

Major Reactives

We obtained human lung cell lines A549 from Dr. J. Mazella (CNRS, Valbonne, France). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum, and BaP were obtained from Sigma-Aldrich (USA). QC was purchased from Cayman Chemical (USA). PA was purchased from Santa Cruz Biotechnology (Germany). DAM was purchased from Merck Calbiochem (Germany).

Cell Culture and Experimental Procedures

A549 cell line, distal respiratory epithelium (type II pneumocytes), was maintained in DMEM supplemented with 10 % of fetal calf serum (FCS), 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. Prepared cell line was incubated under 5 %CO_2 in atmosphere at 37 °C.

A549 cells were seeded in 75-cm^2 culture flasks and allowed to grow for 1–2 days before experiments [2]. When 70–80 % confluence was achieved, the culture medium was replaced to a new fresh medium containing 20 μM BaP ($C_{20}H_{12}$ >96 % pure) for 48 h [10]. BaP was first dissolved in dimethyl sulfoxide (DMSO) and then added to the culture medium with the final concentration of DMSO less than 0.1 %. The medium was changed daily to maintain the stable concentration of BaP [2, 10].

Treatment with Quercetin, Damnacanthal, and Proanthocyanidin

After 24 h incubation of A549 cell line with BaP, culture medium containing BaP was removed, and fresh medium containing 10 μM QC (BaP+QC) [11], 50 μM DAM (BaP+DAM) [12], and 50 μg/ml PA (BaP+PA) [8] alone were added into the flasks according to the groups. As control group, A549 cells were incubated with medium containing vehicle (DMSO) at the same concentration. After 48 h incubation with agents, medium was removed and the cells in all four groups were used to perform the experiments described as follows.

Utmost care for sterilization was taken at every stages of the experiment. Bacterial contamination was prevented with Penicillin and Streptomycin combination treatment in cell culture media. All measurements were performed in triplicate and each experiment was repeated three times.

Protein Analysis

Cells from all groups were washed twice with ice-cold phosphate-buffered saline (PBS) and then removed by scraping. The removed cells were lysed in lysis buffer (100 mM NaH_2PO_4, 1 % Triton X-100, 1 M HEPES, and 1 % protease inhibitor cocktail). The homogenate was centrifuged at 13,000*g* at 4 °C for 40 min. The supernatants were collected, and protein concentrations were determined using Bradford method [13]. In this study, cells which were obtained in the same passage were used in all groups.

Cell Viability Measurements Test and MTT Assay

The number of viable A549 cells after treatments was evaluated by the MTT (3-[4,5-methylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide) assay. In summary, A549 cells (2×10^4 cells/well) were seeded in a 24-well plate and kept overnight for attachment. The next day, the medium was replaced with fresh medium with BaP and cells were let to grow for 48 h. After completion of incubation, therapeutic agents were added in each well, and after completion of second incubation, 100 μl of MTT (10 mg/ml, Sigma, USA) was added in each well, followed by 4 h incubation at 37 °C. Later, medium was removed and 150 μl DMSO was added to each well. The plate was then shaken for 10 min in the dark at room temperature. The absorbance value at 490 nm was quantified using a UV/VIS spectrophotometer (T70, PG Instruments). The results were expressed as the percentage of treated cells relative to controls.

Analysis of Lactate Dehydrogenase Activity and Cytokine Production

Lactate dehydrogenase (LDH) activity was measured in the supernatant prior to the BaP application and after 2 h incubation of cells with BaP and combined treatments applied [(BaP+QC), (BAP+DAM), and (BaP+PA)] in accordance with the proposal of manufacturer to examine whether necrotic cell death occurred or not. The effect of QC, DAM, and PA on release of cytokine levels (IFN-γ, IL-1β, TNF-α) in cell lysate was analyzed by ELISA technique. The results were expressed as pg/mg protein.

Measurement of Nitric Oxide Production

Nitric oxide (NO) level in cell culture medium and cell lysate were measured by Giress reaction [14]. Specimens were deproteinized with 75 mmol zinc-sulphate. Total nitrite was measured by a spectrophotometer at 546 nm after alteration of nitrate to nitrite by using vanadium-III-chloride. The values were expressed in μmol/L in culture medium and μmol/g protein in cell lysate.

Evaluation of Glutathione Level

Glutathione (GSH) level in cell lysate was evaluated by the method defined by Buetler *et al.* [15]. The results were expressed as μmol/g protein.

Measurement of Malondialdehyde Level in Cell Lysate

Malondialdehyde (MDA) level was measured by the method described by Yoshioka *et al.* [16]. In this method, the formation of a pink color under the acidic condition upon the reaction of MDA and thiobarbituric acid is essential. The absorbance values were read on a spectrophotometer at 535 nm. After computation of the values, the results were implied as nmol/g protein.

Analysis of Total Oxidant Status, Total Antioxidant Status, and Oxidative Stress Index

Total oxidant status (TOS) of cell lysate was measured by using automated colorimetric method described by Erel *et al.* [17]. The results were expressed as μmol H_2O_2 Eq/g protein. Total antioxidant status (TAS) of cell lysate was determined by another automated direct colorimetric measurement method described Erel *et al.* [18]. Results were expressed as mmol Trolox equivalent/g protein. Oxidative stress index (OSI) which indicates the degree of oxidative stress was calculated *via* a formula described by Esen *et al.* [19] as follows:

$$\mathrm{OSI(arbitrary\,unit)} = \mathrm{TOS}\left(\mu\mathrm{mol\,H_2O_2Eq}/\mathrm{g\,protein}\right)/\mathrm{TAS}\left(\mathrm{mmol\,Trolox\,Eq}/\mathrm{g\,protein}\right) \times 100$$

Molecular Analysis (Total RNA Isolation and mRNA Expression Levels of Genes by Real-Time Reverse Transcription-Polymerase Chain Reaction (RT-PCR))

Molecular analyses were performed as described by Ulasli *et al.* [10]. A549 cells were seeded in 25 cm^2 culture flasks and were allowed to grow for 1–2 days before the analyses were performed. Cells were collected and washed with phosphate-buffered saline (PBS) after completion of incubations. Total RNA was isolated by RNAeasy kit (QIAGEN), and cDNA was generated with a First Strand cDNA Synthesis kit (Thermo Scientific) at a total volume of 20 μl in accordance with the manufacturer's instructions. Real-time quantitative PCR was performed in a Strategene Mx3005P QPCR system (USA). Expression levels of target gene were normalized to the housekeeping gene β-actin ($^{\Delta}$Ct). Gene expression values were then calculated based on the $^{\Delta\Delta}$Ct method using the equation: $RQ=2^{-\Delta\Delta Ct}$. PCR amplification was performed with Maxima SYBR Green/ROXqPCR Master Mix (Thermo Scientific). The primer sequences are described in Table 1. Each assay was performed in triplicate and repeated three times.

Statistical Analysis

The statistical analyses of experimental data were performed by using SPSS (Statistical Package for Social Science for Windows, Version 18.0; Chicago, IL, USA). The normality of distributions of all variables was investigated by using Shapiro-Wilk test. The results were expressed as mean±standard deviation (SD) or median value (min-max range) according to distribution. One-way ANOVA or Kruskall-Wallis test was used to compare continuous parameters according to distribution among study groups. When an overall significance was present, pairwise post hoc tests were performed. The *P* value less than 0.05 was accepted as significance level.

Table 1. Oligonucleotide Primer Sequences and PCR Programs

Transcripts	Primer sequences	PCR programs	Cycles
Bax	F-5′cgcctcactcaccatctggaa3′ R-5′cctcaagaccactcttcccca3′	Initial: 95 °C-10 min; 94 °C-1 m/58 °C-1 m/72 °C-1 m	35
Bcl-2	F-5′gaggggctacgagtgggatgc3′ R-5′ggaggagaagatgcccggtgc3′	Initial: 95 °C-10 min; 94 °C-1 m/62 °C-1 m/72 °C-1 m	35
Bcl-xL	F-5′cacatcaccccagggacagca3′ R-5′aaaggccacaatgcgaccccca3′	Initial: 95 °C-10 min; 94 °C-1 m/61 °C-1 m/72 °C-1 m	40
IKK1	F-5′gctacagaagagcccctatgga3′ R-5′agatcaatggcacgctgttcc3′	Initial: 95 °C-10 min; 94 °C-1 m/57 °C-1 m/72 °C-1 m	35
TRAIL-R1	F-5′gagaagtccctgcaccacgac3′ R-5′ccggaaagttcctggtttgcac3′	Initial: 95 °C-10 min; 94 °C-1 m/59 °C-1 m/72 °C-1 m	35
TRAIL-R2	F-5′tccttacctgaaaggcatctgc3′ R-5′gtcgttgtgagcttctgtcca3′	Initial: 95 °C-10 min; 94 °C-1 m/57 °C-1 m/72 °C-1 m	35
CYCLIN-D1	F-5′atgctggaggtctgcgaggaa3′ R-5′cgacaggaagcggtccaggta3′	Initial: 95 °C-10 min; 94 °C-1 m/60 °C-1 m/72 °C-1 m	35
P21	F-5′ccgtgagcgatggaacttcgac3′ R-5′tgggaaggtagagcttgggca3′	Initial: 95 °C-10 min; 94 °C-1 m/60 °C-1 m/72 °C-1 m	35
NFKB	F-5′ggtgcggctcatgtttacagc3′ R-5′gcgtctgataccacgggttcc3′	Initial: 95 °C-10 min; 94 °C-1 m/59 °C-1 m/72 °C-1 m	35
β-Actin	F-5′caccccagccatgtacgttgc3′ R-5′ccggagtccatcacgatgcca3′	Initial: 95 °C-10 min; 94 °C-1 m/61 °C-1 m/72 °C-1 m	35

RESULTS

Inflammatory Mediators

In groups which were treated with QC, DAM, and PA, IFN-γ levels were significantly decreased when compared with BaP exposed A549 cell line ($P<0.001$). QC and PA-treated cells had significantly lower IL-1β and TNF-α level when compared with BaP-exposed cells ($P<0.001$). IL-1β level was similar in DAM-treated cells and only BaP-exposed cells (Table 2).

Oxidative Stress Parameters

In cells treated with QC, DAM, and PA, oxidative stress parameters were lower than only BaP-exposed cells. Oxidative stress index (OSI) was significantly lower in PA-treated cells than all other cells. Glutathione level was significantly higher in PA treated group ($p<0.001$). Oxidative stress results were outlined in Table 3.

mRNA Expression Levels of Genes in A549 Cells

QC and DAM upregulated TRAIL receptor 1 (TRAIL-R1) and TRAIL receptor 2 (TRAIL-R2) expressions. PA

Table 2. The Levels of Proinflammatory Cytokines (in cell lysates) and LDH Activity (in medium)

Groups	IFN-γ (pg/mg protein)	IL-1β (pg/mg protein)	TNF-α (pg/mg protein)	LDH (U/L)
BaP	0.52±0.02	136.71±24.68	7.57±1.33	43.00±2.00
QC	0.31±0.02 ‡	80.52±8.53 †	4.19±2.08†	37.00±1.73 †
DAM	0.37±0.02‡	135.47±26.24	6.39±1.79	32.67±0.58†
PA	0.14±0.02‡	65.18±17.00 ‡	3.19±0.60 †	31.33±3.05†

All experiments were performed in triplicate, and data was expressed as mean±SD for all parameters. One-way analysis of variance (ANOVA) with Bonferroni corrections

IFN-γ interferon gamma, *IL-1β* Interleukin-1 beta, *LDH* lactate dehydrogenase, *TNF-α* tumor necrosis factor alpha, *BaP* benzo(*a*)pyrene, *QC* Quercetin, *DAM* Damnacanthal, *PA* Proanthocyanidin

† Significantly different compared to benzo(*a*)pyrene treated group at $P<0.05$

‡ Significantly different compared to benzo(*a*)pyrene treated group at $P<0.001$

Table 3. Oxidative Stress Parameters of All Study Groups

Groups	GSH[a] (μmol/g protein)	NO- medium[a] (μmol/L)	NO- lysate[a] (μmol/g protein)	MDA[a] (nmol/g protein)	TAS[a] (mmol Trolox equivalent/g protein)	TOS[a] (μmol H_2O_2 Eq/g protein)	OSI[b]
BaP	24.38±6.86	7.83±0.85	1.47±0.41	7.53±0.46	0.52±0.16	7.04±0.87	12.97 (9.91–23.00)
QC	24.10±1.33	9.24±1.85	1.19±0.37	5.30±0.25 ‡	0.19±0.11 †	3.98±0.28‡	22.12 (11.15–90.20)
DAM	24.07±3.04	5.63±1.02	1.14±0.21†	6.38±0.12 ‡	0.47±0.18	6.37±1.07	13.10 (8.56–29.04)
PA	60.22±2.70 ‡	7.07±1.28	0.82±0.17†	2.45±0.27 ‡	0.34±0.05	1.91±0.53 ‡	4.81 (3.83–8.79) †

All experiments were performed in triplicate, and the values reported as mean±SD or median (min.-max) according to the distribution status
GSH glutathione, *MDA* malondialdehyde, *NO* nitric oxide, *OSI* oxidative stress index, *TAS* total antioxidant status, *TOS* total oxidant status, *BaP* benzo(*a*)pyrene, *QC* Quercetin, *DAM* Damnacanthal, *PA* Proanthocyanidin
[a] One-way analysis of variance (ANOVA) with Bonferroni corrections
[b] Kruskall-Wallis Test and pairwise post hoc test with Mann-Whitney *U* test
† Significantly different compared to benzo(*a*)pyrene treated group at $P<0.05$
‡ Significantly different compared to benzo(*a*)pyrene treated group at $P<0.001$

upregulated TRAIL-R1 expression. PA and QC upregulated Bax gene expression. DAM downregulated Bax gene expression. QC and DAM upregulated p21 gene expression. All agents (QC, DAM, and PA) decreased the expression of cyclin D1 gene expression. mRNA expression levels of genes in A549 cells are presented in Table 4.

Cell Viability

Cell viability was investigated and compared among groups (Fig. 1). Viability of BaP-exposed cells was significantly decreased when compared with control ($P<0.05$). Significantly increased cell viability was seen after QC, DAM, and PA treatment when compared with BaP exposure.

DISCUSSION

In this study, we observed significantly higher activity of LDH after BaP exposure in A549 cells when compared with controls. Additionally, we observed significant decrease in cell viability in BaP-exposed cells. When these findings and increased inflammatory and oxidative stress levels were taken into account together, we supposed that *in vitro* BaP exposure in A549 cells results in a trend to cell death causing necrosis with a higher inflammatory process. After application of protective agents, increased cell viability, marked antiinflammatory, and antioxidant activity were observed, especially in PA and QC-treated cell lines.

Benzo(*a*)pyrene (BaP) is a prototype of PAH family. They are formed as a result of incomplete combustion of organic substances (such as biomass fuel or waste materials). Additionally, BaP is also found in tobacco smoke [20, 21]. BaP is an environmental pollutant, and it is known that it is associated with airway inflammation and damage in smokers [22, 23]. BaP exposure results in decreased cellular antioxidant level (glutathione, GSH) and increased oxidative stress [24]. In our study, increased inflammatory mediators and oxidative stress parameters were observed in BaP-exposed cell line when compared to control.

Table 4. mRNA Expression Levels of Genes in A549 Cells

Groups	mRNA expression levels of genes (fold increase +/ decrease −)								
	BAX	BCL-2	BCL-XL	IKK1	TRAIL-R2	NF-κB	P21	CYCLIN-D1	TRAIL-R1
BaP[a]	(−) 1.1	(−) 2.0	(−) 2.3	(−) 1.74	(−) 1.41	(−) 1.11	(+) 1.33	(−) 2.5	(−) 1.15
QC[b]	(+) 1.2	(−) 1.41	(+) 2.0	(−) 1.2	(+) 1.24	(−) 1.14	(+) 1.31	(−) 1.2	(+) 1.07
DAM[b]	(−) 1.02	(−) 1.3	(+) 1.77	(+) 1.01	(+) 1.28	(+) 1.05	(+) 1.74	(−) 1.07	(+) 1.1
PA[b]	(+) 2.3	(+) 53.44	(+) 2.82	(+) 12.46	(−) 4.6	(+) 3.31	(−) 2.82	(−) 1.54	(+) 5.3

QC Quercetin, *DAM* Damnacanthal, *PA* Proanthocyanidin
[a] According to the control group
[b] According to the benzo(*a*)pyrene (BaP) group

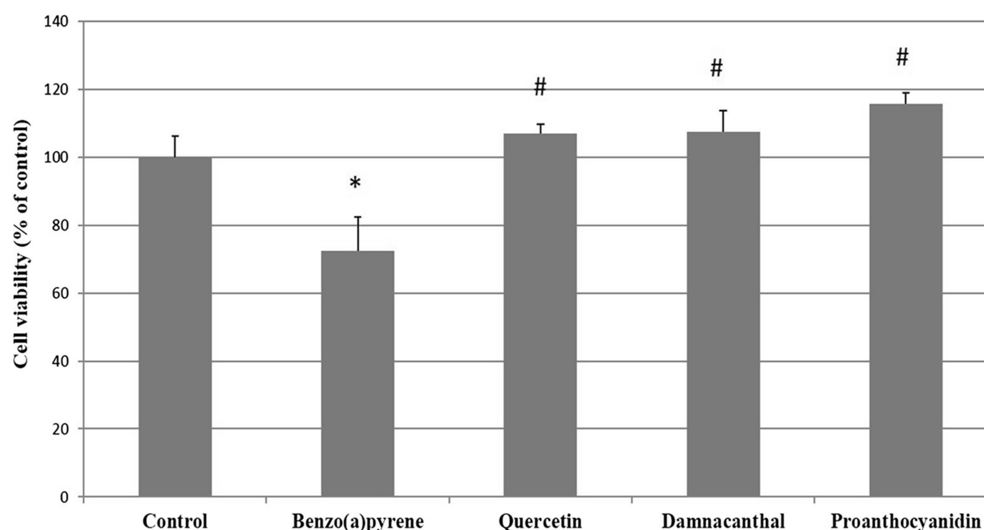

Fig. 1. Cell viability of A549 cells after treatment with benzo(*a*)pyrene (BaP), BaP + Quercetin (QC), BaP + Damnacanthal (DAM), BaP + Proanthocyanidin (PA). Cell viability was quantified by a MTT assay 72 h after exposure to BaP, following a 24-h treatment with other drugs. Significantly decreased in cell viability was seen after treatment with BaP alone when compared to control. QC, DAM, and PA showed significant protection against BaP-induced cell death/necrosis with significant increase in cell viability. *$P<0.05$ compared to the control group, and $^{\#}p<0.001$ compared to the BaP group. The graph is representative of at least three independent experiments.

Bioflavonoid Quercetin (QC) (3,3′,4′,5,7-pentahydroxyflavone) is a polyphenolic compound found in some fruits and vegetable. It has been shown that QC has chemopreventive activity during carcinogenesis and also has cytotoxic activity against various *in vitro* and *in vivo* tumor cells [7, 25]. Furthermore, in the previous studies, it has been reported that QC has protective activity on pulmonary toxicity and oxidative stress [26, 27]. Additionally, QC has beneficial effect on lipid peroxidation and reducing pulmonary damage occurred after CCL_4^- and paraquate [28–30]. In a recent study, Verma *et al.* [31] reported that QC ameliorates the development of bleomycin-induced pulmonary fibrosis in rats. In the present study, we observed a significant decrease in inflammatory mediators (IFN-γ, IL-1β, and TNF-α) and oxidative stress parameters (especially TAS and MDA level) when compared with BaP-exposed cell line.

Proanthocyanidin (PA) is a biologically active polyphenolic bioflavonoid that was derived from many plants. Generally, PA is used as an enriched grape seed extract in traditional herbal medicine. In the literature, protective effects of PA on oxidative stress were demonstrated [32–34]. PA has a cleaning effect on reactive oxygen substances (ROS), inhibitor effect on ischemia-reperfusion injury, and anti-inflammatory and inhibitory effects on oxidative stress produced by pesticides. In addition to these crucial functions, antioxidant, vasodilator, antithrombotic, cardioprotective, and anticancer effects of PA also reported in the literature [32–34]. Agackiran *et al.* [34] reported that PA has a protective and anti-inflammatory effect in bleomycin-induced lung fibrosis model in rats. In the present study, we showed that PA has a statistically significant effect on reducing the level of inflammatory mediators (IFN-γ, IL-1β, and TNF-α) and oxidative stress indicators (GSH, NO, MDA, TOS, and oxidative stress index (OSI)) when compared with only BaP-exposed A549 cell line. These findings suggest that PA has a significant positive effect on oxidative stress and inflammation in A549 alveolar epithelial cells exposed to BaP.

Damnacanthal (DAM), an anthraquinone compound, is extracted from the roots of *Morinda citrifolia* L. (noni), which has been used for traditional therapy in several chronic diseases. DAM is an activity inhibitor of tyrosine kinase. Kinases control cell division and mediate transduction and intracellular and extracellular processing of signals. These kinases are involved in oncogenesis. Impaired function of tyrosine kinase leads to the development of non-small cell lung cancer [35, 36]. Additionally, anti-inflammatory effect of DAM has been reported in the literature [37]. In this study, IFN-γ level was significantly decreased after DAM treatment. Additionally, in our study, statistically significant reduction in NO and MDA levels were found in cells treated with DAM. Besides, in the present study, DAM showed its anti-inflammatory effect by increasing NF-κB gene expression which was previously reported by Nualsanit *et al.* [37].

When we consider the effects of therapeutic agents on apoptotic pathways in cell culture model, it has been shown that QC upregulated the apoptotic gene expression (Bax, p21, TRAIL-R1, and TRAIL-R2) and downregulated the antiapoptotic gene expression (bcl-2, NF-κB, IKK1, cyclin-D1). Meanwhile, DAM upregulated the apoptotic gene expression especially TRAIL-R1, TRAIL-R2, and p21 and decreased the expression of bcl-2 and cyclin-D1. Nevertheless, it has been shown that PA showed its apoptotic activity with upregulation of bax and TRAIL-R1 gene expression. Therefore, we suggest that QC and DAM show their protective effect on the cancer cells triggered by BaP by influencing apoptotic pathway. Nevertheless, further studies are warranted to understand their anticancer activity.

In conclusion, in this cell culture model with A549 alveolar epithelial cells exposed to BaP, we demonstrated that both PA and QC had higher anti-inflammatory and antioxidant activity than DAM. We concluded that although this is an *in vitro* study, these results will shed light on the further *in vivo* studies. Therefore, we suggest that PA and QC may be used in prevention and treatment of inflammatory lung diseases caused by smoking in the near future.

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COMPLIANCE WITH ETHICAL STANDARDS

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