Piceatannol Enhances the Antitumor Efficacy of Gemcitabine in Human A549 Non-Small Cell Lung Cancer Cells

Bin Xu* and Ze-Zhang Tao†

*Department of Oncology, Wuhan University Renmin Hospital, Wuhan, Hubei, P.R. China
†Department of Otolaryngology-Head and Neck Surgery, Wuhan University Renmin Hospital, Wuhan, Hubei, P.R. China

To enhance the anticancer efficacy of gemcitabine in the treatment of non-small cell lung cancer (NSCLC), the potential synergistic effect of piceatannol on gemcitabine cytotoxicity was investigated in the human NSCLC A459 cell line. The MTT cell viability assay showed that piceatannol significantly enhanced the cytotoxic effects of gemcitabine by lowering the gemcitabine IC_{50} value. Flow cytometry analysis revealed that piceatannol exerted its pharmacological effect mainly by increasing the late apoptotic population. Western blot analysis showed that gemcitabine induced the expression of the proapoptotic proteins Bad and Bak, and pretreatment with piceatannol further increased Bak expression, leading to an increased number of cells undergoing late apoptosis. The findings from this study show that piceatannol can enhance the cytotoxic effects of gemcitabine by enhancing expression of the proapoptotic protein Bak, thereby providing the rational basis for a novel combination strategy for the treatment of NSCLC.

Key words: Piceatannol; Gemcitabine; Non-small cell lung cancer (NSCLC); Combination regimen; Apoptosis

INTRODUCTION

Lung cancer is the leading cause of cancer death worldwide (1), and non-small cell lung cancer (NSCLC) accounts for nearly 80–85% of all the diagnoses of lung cancer. The majority of patients with NSCLC are diagnosed with advanced stage disease, and these patients are usually treated with systemic chemotherapy and/or targeted therapies (2). According to the clinical practice in China, the most commonly used first-line cytotoxic chemotherapy agents are gemcitabine plus platinum (approximately accounting for 27.4%), especially as it relates to the treatment of squamous cell NSCLC (3).

While gemcitabine has well-established clinical efficacy in the treatment of NSCLC, it is also associated with a number of side effects, including flu-like symptoms, fever, fatigue, nausea, vomiting, poor appetite, skin rash, myelosuppression, and pulmonary toxicity in the form of pneumonitis. These various side effects may hinder patient compliance and may have a negative impact on clinical outcome. Moreover, cellular drug resistance, either de novo and/or acquired, is a major issue that usually limits the efficacy of gemcitabine chemotherapy (4,5).

With this in mind, efforts have focused on developing combination strategies to enhance the cytotoxic effects of gemcitabine as well as to prevent and/or overcome the development of cellular drug resistance. Piceatannol is a small molecule initially isolated from the domesticated oilseed *Euphorbia lagascae* (6), and various in vitro and in vivo model systems documented anti-inflammatory (7,8), antihistamine (9), and anticancer activities (6,10–12). In addition, this agent is able to induce apoptosis (13,14), DNA damage (15–17), and exert antioxidant properties (18,19). Additional studies have shown that piceatannol can inhibit serine/threonine protein kinases (20), as well as several tyrosine kinases (PTK), including Syk, Src, Lck, and FAK (21–23). Effects on EGFR protein expression have also been observed with piceatannol treatment in human squamous cell carcinoma A431 cells (24).

Our hypothesis, therefore, is that piceatannol may be able to enhance the cytotoxic effects of gemcitabine in epidermal growth factor receptor tyrosine kinase inhibitor (EGFR-TKI)-resistant NSCLC cells through inhibition of EFGR signaling pathways. In the present study, we used TKI-sensitive human NSCLC cells to investigate the effect of piceatannol and gemcitabine as single agents and as a combination regimen on cell growth proliferation and cell apoptosis. We also evaluated the possible associated molecular mechanisms of the different effects.

Address correspondence to Professor Ze-Zhang Tao, Wuhan University Renmin Hospital, No. 99 Zhang-zhidong Road, Wuhan, Hubei 430060, P.R. China. E-mail: 353196085@qq.com; taozezhang@163.com

MATERIALS AND METHODS

Drugs

Piceatannol was obtained from Abcam PLC (Cambridge, UK) and was dissolved in dimethyl sulfoxide (DMSO) to a stock concentration of 100 mmol/L. Gemcitabine was purchased as a commercial product from the pharmacy at the Renmin Hospital of Wuhan University (Wuhan, China) and was dissolved in DMSO at 100 mmol/L, as stock solution. Drugs were stored at −20°C and subsequently diluted with culture medium prior to use.

Cell Lines

The human NSCLC cell line A549 (EGFR-TKI-resistant) was purchased from the American Type Culture Collection (Manassas, VA, USA). A549 cells were cultured in RPMI-1640 medium (Hyclone, Logan, UT, USA), supplemented with 10% heat-inactivated fetal bovine serum (Hyclone), penicillin (100 U/ml), and streptomycin (100 U/ml) at 37°C in a 5% CO_2 atmosphere, then harvested with trypsin-EDTA when cells reached exponential growth.

MTT Cell Viability Assay

Cells were seeded in 96-well culture plates, in which the number of A549 cells was 3.5×10^3/well. Cells were pretreated with varying concentrations of piceatannol (2, 10, or 50 μM) for 12 h to which gemcitabine was then added in a series of concentrations (0, 0.0001, 0.001, 0.01, 0.1, 1, 10, 100, 200, and 400 μM, respectively). Piceatannol was dissolved in DMSO and further diluted with RPMI-1640 medium and sterilized via a 0.22-μm filter. After another 72 h, the treated cells were incubated with 0.5 mg/ml MTT solution for 2 h at 37°C in a humidified atmosphere in the presence of 5% CO_2. The cell plates were centrifuged at 1,000 rpm for 5 min. The supernatant was gently removed from the cell pellet, and DMSO was then added to dissolve the dark blue crystals in each well. The plates were subsequently read on a microplate reader (Multiskan Spectrum; Thermo Scientific) at 570 nm to detect for cell viability.

Apoptosis Measurement by Flow Cytometry

Cells were treated as indicated and then we recollected, washed twice with cold PBS, and centrifuged at $450 \times g$ for 10 min (4°C). After discarding the supernatant, cells were resuspended with binding buffer to which the annexin V/PI reagent was then added according to the kit manufacturer's instructions (Annexin-VFITC/PI Double-Staining Cell Apoptosis Detection Kit; Invitrogen) incubated at 37°C for 30 min. After incubation, cells were washed with binding buffer again and resuspended in PBS, and cells were subjected to flow cytometry (BD Biosciences, San Jose, CA, USA) for apoptosis analysis.

Western Blot

Cells were treated with piceatannol and gemcitabine as single agents and as a combination and then harvested by centrifugation at $450 \times g$ for 10 min at 4°C. Cells were washed with ice-cold PBS solution and scraped in lysis buffer. The lysates were centrifuged at 14,000 rpm for 30 min at 4°C, and the supernatant was collected. Equivalent amounts of protein were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to PVDF membranes. Appropriate primary antibodies to anti-Bad, anti-Bak, anti-Bid, anti-Bcl-2, anti-Bcl-xl purchased from Santa Cruz Biotechnology Inc. (Dallas, TX, USA) and the antibodies against cytochrome c (Cyto-c) and GAPDH obtained from Cell Signaling Technology (Beverly, MA, USA) were used. Proteins were visualized with a HRP-conjugated goat anti-rabbit secondary antibody from Santa Cruz Biotechnology. Specific bands were detected using the enhanced chemiluminescence reagent (ECL; PerkinElmer Life Sciences, Inc., Waltham, MA, USA) on autoradiographic film.

Statistical Analysis

All of the experiments were repeated at least three times. The data are represented as mean±standard deviation (SD) and were analyzed using SPSS software, version 19.0 (SPSS Inc., Chicago, IL, USA). Comparisons of mean values between control and experimental groups were made using Student's t-test. All p values were two-tailed and considered to indicate statistically significant difference when $p < 0.05$.

RESULTS

Piceatannol Enhances the Cytotoxicity of Gemcitabine

Using the MTT assay, treatment with 400 μM gemcitabine alone for 72 h resulted in growth inhibition of nearly 51% of A549 cells. The growth inhibitor effects of 400 μM gemcitabine were significantly higher when cells were treated in the presence of 2, 10, and 50 μM piceatannol, respectively ($p < 0.05$) (Fig. 1). In addition, the IC_{50} values of gemcitabine combined with different concentrations (2, 10, and 50 μM) of piceatannol were 0.071±0.013, 0.069±0.02, and 0.132±0.09 μM, while the IC_{50} value of gemcitabine alone was 391.2±2.7 μM, which was significantly higher than the former ones ($p < 0.05$), suggesting a potent synergistic cytotoxic effect of the combination treatment.

Piceatannol Increased Gemcitabine-Induced Late Apoptosis in A549 Cells

As shown in Figure 2A, the survival rate of cells treated with 1 μM gemcitabine alone was nearly 70%, while that of cells treated by 1 μM gemcitabine combined
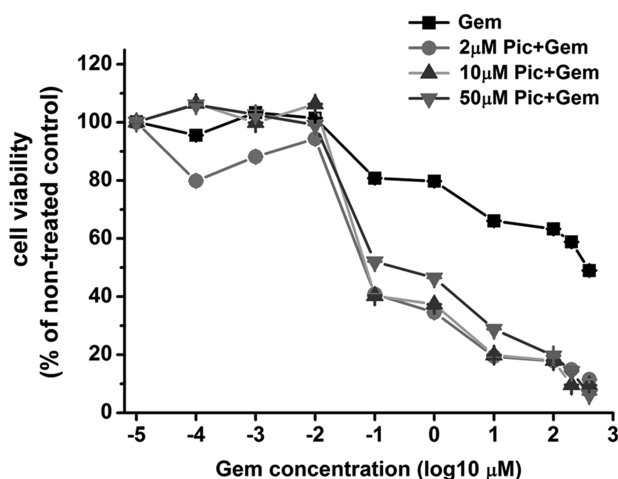
Figure 1. Piceatannol elevates the anticancer efficacy of gemcitabine significantly. Cells were pretreated with 2, 10, or 50 μM piceatannol for 12 h and then treated with different concentrations of gemcitabine (0, 0.0001, 0.001, 0.01, 0.1, 1, 10, 100, 200, and 400 μM) for an additional 72 h. Cell viability was determined using the MTT assay. The data shown are the mean ± SD. All data are representative of three independent experiments.

with 2 μM piceatannol pretreatment reduced to around 35% ($p < 0.01$). However, piceatannol treatment alone did not induce cell death, which was consistent with the cell viability assay (Fig. 1), showing that piceatannol as a single agent did not exert its own cytotoxic effects at any of the concentrations tested (2, 10, and 50 μM; data not shown).

Cells subjected to flow cytometry were classified as two different populations, annexin V single-positive population and annexin V/PI double-positive population, the first group being considered as early apoptotic, while the second group was considered as late apoptotic. After 72 h, even 6.1% of nontreated cells in the control group were observed to be in the early apoptosis stage. However, cells treated with piceatannol alone exhibited little evidence of apoptosis, suggesting a potential cytoprotective effect of piceatannol on tumor cells (Fig. 2B). The early apoptotic population in cells treated with gemcitabine alone or with gemcitabine plus piceatannol was not significantly different, at 18.4% and 13.0%, respectively ($p > 0.05$). However, a significantly larger late apoptotic cell population was observed in cells treated with the combination of gemcitabine plus piceatannol (42.5%), while that late apoptotic cell population in the gemcitabine alone group was only 7.0% ($p < 0.001$).

Potential Role of Bcl-2 Family Members

Western blot analysis revealed that the expression level of both the antiapoptotic (Bcl-2 and Bcl-xl) and proapoptotic (Bid, Bad, and Bak) proteins was higher in cells treated with gemcitabine alone when compared to those in control, nontreated group (Fig. 3). When compared with the gemcitabine-alone group, the level of expression of the Bak and Bid proteins was significantly increased in cells treated with the combination of gemcitabine plus piceatannol. In contrast, the protein expressions of Bad, Bcl-2, and Bcl-xl were unchanged upon addition of piceatannol (Fig. 3). In addition, we investigated the
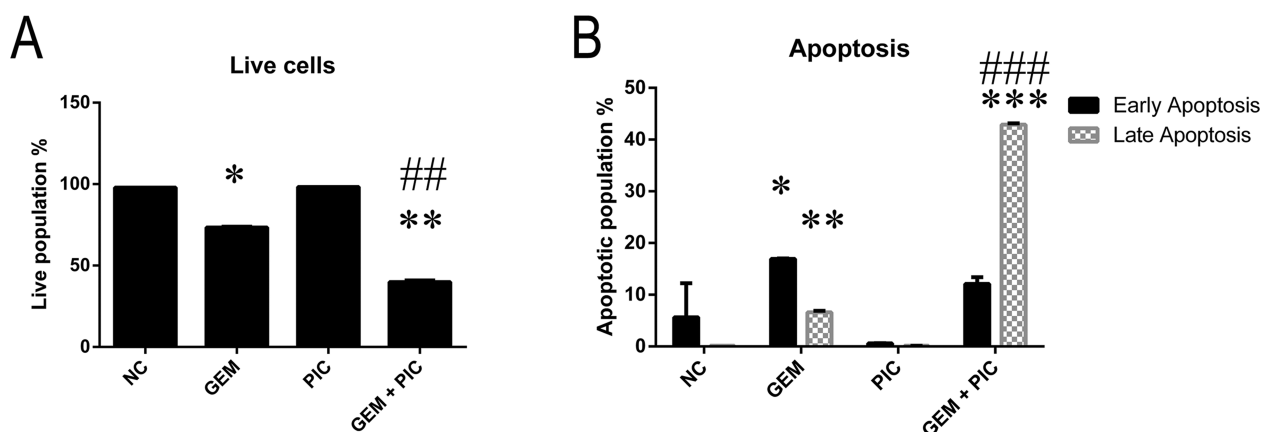
Figure 2. Piceatannol enhances gemcitabine-associated late apoptotic cells. Cells were first treated with 2 μM piceatannol for 12 h and then treated with 1 μM gemcitabine for an additional 72 h. Live cells (annexin V⁻, PI⁻), early apoptotic cells (annexin V⁺, PI⁻), and late apoptotic cells (annexin V⁺, PI⁺) were detected and analyzed by flow cytometry. The data presented in (A) and (B) are the mean ± SD. *$p < 0.05$, **$p < 0.01$, ***$p < 0.005$ relative to nontreatment control, and ##$p < 0.01$, ###$p < 0.005$ relative to gemcitabine treatment group. The data presented are representative of at least three independent experiments that were performed. NC, nontreatment control group; GEM, gemcitabine treatment group; PIC, piceatannol treatment group; GEM + PIC, gemcitabine and piceatannol treatment group.

expression of the executor of mitochondrial apoptosis pathway, cytochrome c, and found that piceatannol did not alter gemcitabine-induced cytochrome c levels.

DISCUSSION

Currently, research efforts have focused on the identification of novel potential targets of response of gemcitabine as well as to identify novel agents with which to combine gemcitabine chemotherapy. With respect to this latter point, Zhao and colleagues have studied the combination of dihydroartemisinin plus gemcitabine (25), while Li et al. have investigated the multi-kinase inhibitor sorafenib in combination with gemcitabine in EGFR-TKI-sensitive and EGFR-TKI-resistant human lung cancer cell lines (26). In this report, we have investigated the potential effect of a novel small molecule piceatannol on the cytotoxicity of gemcitabine using the human NSCLC A549 cells. We have shown that piceatannol was very effective in enhancing the cytotoxic effects of gemcitabine in A549 cells as highlighted by a significant nearly 2,000-fold reduction in the IC_{50} of gemcitabine on A549 cancer cells from 391.2 μM (alone) to less than 0.2 μM (combination). Moreover, the combination treatment resulted in a significant increase in the late apoptotic cell population, when compared to gemcitabine treatment alone.

The Bcl-2 family of proteins plays a critical role in the regulation of apoptosis (27,28), and mitochondrial signaling has been shown to mediate late apoptosis and cell death (29). With this in mind, we investigated the potential role of the Bcl-2 family in mediating the enhanced effects of piceatannol on gemcitabine-induced cell death. Our studies have shown that piceatannol treatment results in a marked induction of gemcitabine-induced late apoptosis in A549 cancer cells and that this is associated with a marked increase in expression of the proapoptotic Bcl-2 family members, Bak and Bid. These proteins usually work through activation and release of cytochrome c from the mitochondria with subsequent activation of downstream caspases to execute the apoptotic process. However, somewhat to our surprise, we did not observe alterations in cytochrome c expression with the combination of piceatannol and gemcitabine. Further studies are planned to investigate the downstream mechanism(s) by which piceatannol is able to enhance the gemcitabine-associated apoptosis.

In conclusion, we have shown that the novel small molecule piceatannol is able to significantly enhance the cytotoxic and apoptotic effects of gemcitabine in the human A549 NSCLC cell line. This cell line has particular biological relevance as it is a well-established EGFR-TKI-resistant cell line. Given the much higher incidence of EGFR mutations in the Asian patient population with NSCLC (up to 60%) when compared to the Western population (30%), our results also have direct
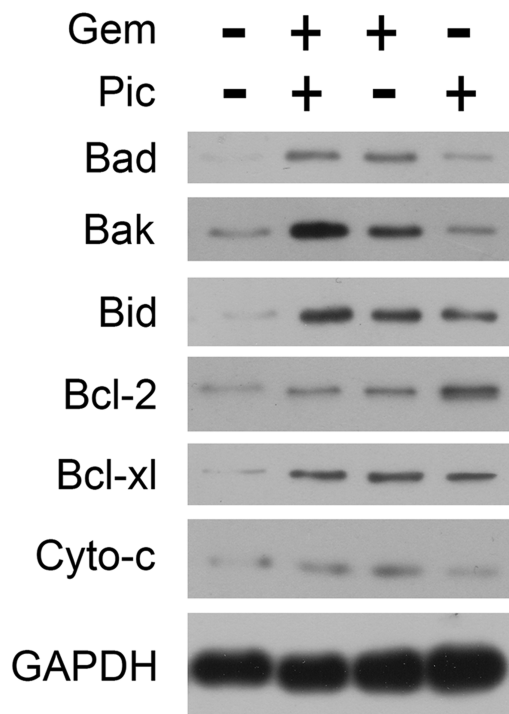
Figure 3. Piceatannol enhances gemcitabine's anticancer efficacy by regulating Bcl-2 family. Cells were first treated with 2 μM piceatannol for 12 h and then treated with 1 μM gemcitabine for an additional 72 h. The expression of Bcl-2 family proteins was detected by Western blot assay. The level of expression of GAPDH protein was used to control for loading. The Western blot is representative of at least three independent experiments that were performed. GEM, gemcitabine treatment group; PIC, piceatannol treatment group.

clinical relevance. Our future studies will further elucidate the direct mechanisms by which piceatannol is able to enhance gemcitabine's anticancer activity as well as to investigate the cytotoxic and apoptotic effects of this novel combination in EGFR mutant NSCLC cell lines.

REFERENCES

1. Jemal, A.; Siegel, R.; Xu, J.; Ward, E. Cancer statistics. 2010. Cancer J. Clin. 60:277–300; 2010.
2. Bareschino, M. A.; Schettino, C.; Rossi, A.; Maione, P.; Sacco, P. C.; Zeppa, R.; Gridell, C. Treatment of advanced non-small cell lung cancer. J. Thorac. Dis. 3:122–133; 2011.
3. Xue, C.; Hu, Z.; Jiang, W.; Zhao, Y.; Xu, F.; Huang, Y.; Zhao, H.; Wu, J.; Zhang, Y.; Zhao, L.; Zhang, J.; Chen, L.; Zhang, L. National survey of the medical treatment status for non-small cell lung cancer (NSCLC) in China. Lung Cancer 77:371–375; 2012.
4. Gallelli, L.; Nardi, M.; Prantera, T.; Barbera, S.; Raffaele, M.; Arminio, D.; Pirritano, D.; Colosimo, M.; Maselli, R.;

Pelaia, G.; DeGregorio, P.; DeSarro, G. B. Retrospective analysis of adverse drug reactions induced by gemcitabine treatment in patients with non-small cell lung cancer. Pharmacol. Res. 49:259–263; 2004.

5. Zhou, J.; Chen, Z. M.; Malysa, A.; Li, X.; Oliveira, P.; Zhang, Y.; Bepler, G. A kinome screen identifies checkpoint kinase 1 (CHK1) as a sensitizer for RRM1-dependent gemcitabine efficacy. PLoS One 8:e58091; 2013.

6. Ferrigni, N. R.; McLaughlin, J. L.; Powell, R. G.; Smith, C. R. Jr. Use of potato disc and brine shrimp bioassays to detect activity and isolate piceatannol as the antileukemic principle from the seeds of Euphorbia lagascae. J. Nat. Prod. 47:347–352; 1984.

7. Ashikawa, K.; Majumdar, S.; Banerjee, S.; Bharti, A. C.; Shishodia, S.; Aggarwal, B. B. Piceatannol inhibits TNF-induced NF-kappaB activation and NF-kappaB-mediated gene expression through suppression of IkappaB alpha kinase and p65 phosphorylation. J. Immunol. 169:6490–6497; 2002.

8. Dang, O.; Navarro, L.; David, M. Inhibition of lipopolysaccharide-induced interferon regulatory factor 3 activation and protection from septic shock by hydroxystilbenes. Shock 21:470–475; 2004.

9. Matsuda, H.; Tewtrakul, S.; Morikawa, T.; Yoshikawa, M. Anti-allergic activity of stilbenes from Korean rhubarb (Rheum undulatum L.): Structure requirements for inhibition of antigen-induced degranulation and their effects on the release of TNF-alpha and IL-4 in RBL-2H3 cells. Bioorg. Med. Chem. 12:4871–4876; 2004.

10. Tsang, F.; Fred Wong, W. S. Inhibitors of tyrosine kinase signaling cascade attenuated antigen challenge of guinea-pig airways in vitro. Am. J. Respir. Crit. Care Med. 162:126–133; 2000.

11. Wieder, T.; Prokop, A.; Bagci, B.; Essmann, F.; Bernicke, D.; Schulze-Osthoff, K.; Dörken, B.; Schmalz, H. G.; Daniel, P. T.; Henze, G. Piceatannol, a hydroxylated analog of the chemopreventive agent resveratrol, is a potent inducer of apoptosis in the lymphoma cell line BJAB and in primary, leukemic lymphoblasts. Leukemia 15:1735–1742; 2001.

12. Barton, B. E.; Karras, J. G.; Murphy, T. F.; Barton, A.; Huang, H. F. Signal transducer and activator of transcription3 (STAT3) activation in prostate cancer: Direct STAT3 inhibition induces apoptosis in prostate cancer lines. Mol. Cancer Ther. 3:11–20; 2004.

13. Gledhill, J. R.; Walker, J. E. Inhibition sites in F1-ATPase from bovine heart mitochondria. Biochem. J. 386:591–598; 2005.

14. Zheng, J.; Ramirez, V. D. Piceatannol, a stilbene phytochemical, inhibits mitochondrial F0F1-ATPase activity by targeting the F1 complex. Biochem. Biophys. Res. Commun. 261:499–503; 1999.

15. Azmi, A. S.; Bhat, S. H.; Hadi, S. M. Resveratrol-Cu(II) induced DNA breakage in human peripheral lymphocytes: Implications for anticancer properties. FEBS Lett. 579:3131–3135; 2005.

16. Cavalieri, E. L.; Li, K. M.; Balu, N.; Saeed, M.; Devanesan, P.; Higginbotham, S.; Zhao, J.; Gross, M. L.; Rogan, E. G. Catecholortho-quinones: The electrophilic compounds that formed purinating DNA adducts and could initiate cancer and other diseases. Carcinogenesis 23:1071–1077; 2002.

17. Hirakawa, K.; Oikawa, S.; Hiraku, Y.; Hirosawa, I.; Kawanishi, S. Catechol and hydroquinone have different redox properties responsible for their differential DNA-damaging ability. Chem. Res. Toxicol. 15:76–82; 2002.

18. Ovesna, Z.; Kozics, K.; Bader, Y.; Saiko, P.; Handler, N.; Erker, T.; Szekeres, T. Antioxidant activity of resveratrol, piceatannol and 3,3',4,4',5,5'-hexahydroxy-trans-stilbene in three leukemia cell lines. Oncol. Rep. 16:617–624; 2006.

19. Waffo Teguo, P.; Fauconneau, B.; Deffieux, G.; Huguet, F; Vercauteren, J.; Merillon, J. M. Isolation, identification, and antioxidant activity of three stilbene glucosides newly extracted from Vitis vinifera cell cultures. J. Nat. Prod. 61:655–657; 1998.

20. Wang, B. H.; Lu, Z. X.; Polya, G. M. Inhibition of eukaryote serine/threonine-specific protein kinases by piceatannol. Planta Med. 64:195–199; 1998.

21. Geahlen, R. L.; McLaughlin, J. L. Piceatannol (3,4,3',5'-tetrahydroxy-trans-stilbene) is a naturally occurring protein-tyrosine kinase inhibitor. Biochem. Biophys. Res. Commun. 165:241–245; 1989.

22. Thakkar, K.; Geahlen, R. L.; Cushman, M. Synthesis and protein-tyrosine kinase inhibitory activity of polyhydroxylated stilbene analogues of piceatannol. J. Med. Chem. 36:2950–2955; 1993.

23. Law, D.A.; Nannizzi-Alaimo, L.; Ministri, K; Hughes, P. E.; Forsyth, J.; Turner M.; Shattil, S. J.; Ginsberg, M. H.; Tybulewicz, V. L.; Phillips, D. R. Genetic and pharmacological analyses of Syk function in alpha IIb beta3 signaling in platelets. Blood 93:2645–2652; 1999.

24. Klimowicz, A. C.; Bisson, S. A.; Hans, K.; Long, E. M.; Hansen, H. C.; Robbins, S. M. The phytochemical piceatannol induces the loss of CBL and CBL-associated proteins. Mol. Cancer Ther. 8:602–614; 2009.

25. Zhao, C; Gao, W.; Chen, T. Synergistic induction of apoptosis in A549 cells by dihydroartemisinin and gemcitabine. Apoptosis 19:668–681; 2014.

26. Li, J.; Pan, Y. Y.; Zhang, Y. Synergistic interaction between sorafenib and gemcitabine in EGFR-TKI-sensitive and EGFR-TKI-resistant human lung cancer cell lines. Oncol. Lett. 5:440–446; 2013.

27. Siddiqui, W. A.; Ahad, A.; Ahsan, H. The mystery of BCL2 family: Bcl-2 proteins and apoptosis: An update. Arch. Toxicol. 89:289–317; 2015.

28. Ola, M. S.; Nawaz, M.; Ahsan, H. Role of Bcl-2 family proteins and caspases in the regulation of apoptosis. Mol. Cell. Biochem. 351:41–58; 2011.

29. Ogura, A.; Oowada, S.; Kon, Y.; Hirayama, A.; Yasui, H.; Meike, S.; Kobayashi, S.; Kuwabara, M.; Inanami, O. Redox regulation in radiation-induced cytochrome c release from mitochondria of human lung carcinoma A549 cells. Cancer Lett. 8:277:64–71; 2009