Original Research Article

Pomolic acid inhibits proliferation of human lung carcinoma cells via induction of apoptosis and suppression of cell migration and invasion

Lixin Yang[1], Ying Guo[2], Qian Hao[3], Danqing Li[1], Zhian Qiao[1], Yanli Cui[1], Jing Li[4]*

[1]Department of Radiation Oncology, Xingtai People's Hospital, Xingtai, Hebei 054000, [2]Department of Oncology, People's Hospital of Ningxia Hui Autonomous Region, Yinchuan, Ningxia 750002, [3]Department of Tuberculosis, The Second Hospital of Xingtai, Xingtai, Hebei 054001, [4]Department of Radiation Oncology, The Fourth Hospital of Hebei Medical University, Shijiazhuang, Hebei 050011, China

*For correspondence: **Email:** lijing_527@163.com; **Tel/Fax:** +86-31186077634

Abstract

Purpose: To investigate the anti-proliferative effect of pomolic acid on lung cancer cells (A549), and the underlying mechanism.
Methods: The viability of pomolic acid-treated A549 cells was determined by MTT and colony formation assays. Cell colony formation was monitored with acridine orange/ethidium bromide (AO/EB) staining. Protein expressions of Bax and Bcl-2 were assayed by western blotting.
Results: Pomolic acid suppressed the growth of A549 cells, with an half-maximal inhibitory concentration of (IC_{50}) of 10 µM (p < 0.05). However, the IC_{50} of pomolic acid for normal BEAS-2B cells was 80 µM. Pomolic acid also decreased colony formation of A549 cells. At 20 µM, the percentage of A549 colonies decreased to 14 % of control. The dose-dependent cytotoxicity of pomolic acid against A549 cells was mediated via induction of apoptosis and oxidative stress. Pomolic acid treatment enhanced the expression of Bax and decreased the expression of Bcl-2 in A549 cells. Moreover, pomolic acid inhibited the migration and invasion in A549 cells in a dose-dependent manner (p < 0.05).
Conclusion: These results indicate the potent anticancer effect of pomolic acid against human lung cancer cells. Thus, pomolic acid has promising potential as a lead molecule for the development of chemotherapy.

Keywords: Triterpenes, Pomolic acid, Lung cancer, Apoptosis, Caspase, Cell cycle

INTRODUCTION

In recent years, lung cancer treatment and diagnosis have been the focus of cancer research because of high mortality associated with lung cancer [1]. The Chinese Centre for Disease Control and Prevention has reported that lung cancer has become the third foremost cause of death in China, after strokes and ischemic heart disease [2]. In China, between 1990 and 2017, lung cancer-associated years of

life lost (YLLs) and mortality (age-standardized) increased by 12.6 and 28.2 %, respectively.

Notwithstanding the increased frequencies of lung cancer, China has continued to improve 5-year survival by 1.3 % each year, and the current value stands at 19.7 % [3]. The characteristics of lung cancer in patients primarily determine the treatment methodology to be followed. So far, different treatments have been implemented such as targeted therapy, sensitization of drugs and combination therapy [1]. However, there is need for the development of progressively more novel diagnostic and treatment methods through research, so as to overcome lung cancer.

Quite a few drugs used in chemotherapy act by promoting apoptosis of cancer cells [4]. The process of apoptosis goes through a sequence of biochemical reactions and morphological changes leading to cell breakdown and DNA fragmentation. Apoptosis is activated by initiator proteins (caspase-8 and caspase-9) either on signal transmission from death receptors present on plasma membrane (extrinsic apoptosis), or by mitochondria-mediated stress signals due to changes in mitochondrial membrane potential (MMP). Mitochondrial activation of apoptosis initiates the activity of effector caspases [5]. Moreover, Bcl-2 family proteins including Bcl-2 (anti-apoptotic) and Bax (pro-apoptotic) are important regulators of apoptosis. Chemotherapeutic drugs not only promote apoptosis but also initiate several death mechanisms in cancer cells, e.g., production of reactive oxygen species (ROS) [6].

Mitochondria are the key sources of energy generation (ATP synthesis). Thus, they are rich in ROS. Uncontrolled proliferation increases energy demand in cancer cells and causes intracellular stress which elevates mitochondrial functioning and ROS. High levels of ROS production due to application of chemotherapeutic drugs stimulate intrinsic apoptosis via loss of MMP [7]. The MMP loss causes release of cytochrome c into the cytoplasm, thereby initiating pro-apoptotic reaction cascade in the cytoplasm.

Bioactive products from natural sources constitute a pool of novel agents with significant pharmacological applications [8]. Out of these bioactive products, pentacyclic triterpenes have emerged as an interesting group of compounds showing high bioactivity profiles [9]. Pomolic acid is a bioactive pentacyclic triterpene which has been reported to exhibit several medicinal and biological effects [10]. It has been shown to inhibit migration of breast cancer cells [11]. Pomolic acid induced cytotoxic effects against a wide spectrum of neoplastic cells, including leukemia [12]. In this study, the *in vitro* anticancer effect of pomolic acid on lung cancer cells (A549) was studied, and the possible underlying mechanism of action was investigated.

EXPERIMENTAL

Cell culture and conditions

Lung carcinoma A549 cells were bought from American Type Culture Collection (ATCC) (Manassas, VA, United States). The cells were cultured in DMEM supplemented with 10 % (v/v) fetal bovine serum and 100 μg/mL of streptomycin/100 U/mL. This mixture was placed in a controlled incubator with humidified 95 % air and 5 % CO_2 at 37 °C. The medium was replaced after every 3 - 4 days.

Assessment of anti-proliferation activity

The MTT assay was used to determine the effect of pomolic acid on proliferation of A549 cells. The A549 cells were seeded in a 96-well microplate at a density of 5×10^4 cells/well, and left untouched for 24 h for cell attachment. Pomolic acid was added to each well of microplates at varied concentrations (up to 80 μM) for 24 h. After pomolic acid treatment, the incubation medium in each well was replaced with 200 mL newly-prepared medium containing MTT (250 mg/mL). This mixture was incubated for 4 h at 37 °C. Thereafter, loosely adherent A549 cells were washed off by carefully discarding the medium. The formazan crystals formed were solubilized at 37 °C in dimethyl sulphoxide (DMSO; 200 mL) for 15 min. Finally, the absorbance of each formazan solution was read at 492 nm in a Sunrise ElizaReader (Switzerland). Experimental procedures were replicated in triplicate for each individual concentration of pomolic acid.

Clonogenic assay

The A549 cells were seeded for 24 h in 6-well plates in DMEM at a concentration of 500 cells per well. After 24 h of attachment, the DMEM was replaced with fresh medium containing different concentrations of pomolic acid (up to 80 μM), and the cells were incubated for 24 h at 37 °C. The control group received only 0.25 % DMSO. After 24 h, the pomolic acid-containing medium was discarded, and the cells were washed in phosphate buffered saline (PBS). The medium was replaced every 2 days, and the

plates were incubated at 37 °C for two weeks. Subsequently, the cell colonies were fixed in 100 % methanol at -20 °C for 30 min, followed by staining with crystal violet (0.5 %) in 25 % methanol (v/v). The excess dye was removed by washing repeatedly in water. Finally, the cell colonies were dried, and pictures were captured with a Nikon camera.

AO/EB staining

Acridine orange/ethidium bromide (AO/EB) staining kit (Solarbio, Beijing, China) was used to determine pro-apoptotic effect of pomolic acid on A549 cells. The A549 cells seeded in 6-well plates at a density of 1×10^4 cells/mL were incubated for 12 h at 37 °C. Thereafter, the medium was replaced with fresh DMEM containing different concentrations of pomolic acid (up to 80 µM), followed by incubation for a duration of 72 h. After completion of pomolic acid treatment, cells were washed in PBS and treated with freshly prepared 20 µL of AO/EB (v/v in 1 mL PBS) for 5 min. Subsequently, the stained A549 cells were washed continuously to remove excess dye. Finally fluorescent images were captured under a fluorescence microscope (Olympus, Beijing, China).

Fluorescence microscopy

The production of ROS in pomolic acid-treated A549 cells was monitored via DCF fluorescence. The A549 cells were cultured in white 96-well plates at a concentration of 1×10^4 cells/well and left untouched for 24 h at 37 °C, to allow for attachment. Subsequently, the A549 cells were incubated with different concentrations of pomolic acid (up to 80 µM) for 24 h. The control group was administered 0.25 % DMSO only. After treatment, A549 cells were incubated in the dark at 37 °C with DCFH-DA (10 µM) for 1 h. Finally, the cells were examined under a fluorescence microscope (BZ-9000 Keyence, Osaka, Japan) and images were taken at 485 and 530 nm. The ROS data were calculated as percentage relative to control group.

Matrigel migration and invasion assays

Matrigel migration and invasion assays were used to determine the effect of pomolic acid on migration and invasion of A549 cells. In these assays, the cells were seeded in a 24-well Transwell chamber fitted with a membrane of 8-µM pore size (Corning, Lowell, MA, United States) holding serum free DMEM, at a density of 1.5×10^3 cells/well. Then, the wells were treated with different concentrations of pomolic acid (up to 80 µm). Cells in the control group were treated with 0.25 % DMSO only. The lower compartment of the chamber contained 10 % fetal bovine serum. The cells were incubated for 24 h, after which the pomolic acid-treated A549 cells which migrated/invaded were stained using crystal violet (0.5 %) at -20 °C for 30 min. Finally, images of the migrated/invaded cells were captured under inverted microscopy at x10 magnification.

Western blotting

The A549 cells were seeded and cultured in 12-well plates at a concentration of 1.5×10^5 cells/well, and treated with pomolic acid for 24 h. Thereafter, the pomolic acid-treated A549 cells were lysed with RIPA lysis buffer, and the protein content of each lysate was quantified via BCA assay. Equal amounts of proteins (20-µg samples) from the lysates were separated on 12 % SDS-PAGE, followed by transfer to polyvinylidene difluoride membranes. The membranes were blocked using non-fat powdered milk (5 %) at room temperature for 1 h. Blocking of membranes was followed by incubation overnight at 4 °C with primary antibodies against Bax (1:1,000), Bcl-2 (1:1,000) and caspase-3, caspase-8 and caspase-9 (1:1,000). Then, the membranes were subjected to incubation with HRP-conjugated secondary antibody (1:5,000). Finally, the blots were washed and Clarity™ Western Enhanced Chemiluminescent Substrate (Bio-Rad Laboratories, InC.) was used to develop the bands.

Statistical analysis

Results from at least three independent experiments are presented as mean ± standard deviation (SD). Comparisons among groups were carried out using Student's *t*-test, and differences were analyzed through Dunnett and Tukey multiple comparison test and one-way ANOVA. GraphPad Prism 5 software (version 5.01; La Jolla, CA, United States) was used for all statistical analyses. Values of $p < 0.05$ were taken as indicative of statistically significant differences.

RESULTS

Antiproliferative effect of pomolic acid

Pomolic acid exerted concentration-dependent cytotoxic effect on A549 cells, with an IC_{50} of 10 µM. However, the cytotoxic effect was comparatively lower against normal BEAS-2B cells, as was evident from an IC_{50} of 80 µM (Figure 1 A and B). Likewise, the colony-forming

potential of A549 cells was markedly decreased on treatment with pomolic acid (Figure 2 A). The number of colonies decreased with increase in the concentration of pomolic acid (Figure 2 B). The percent colonies decreased to 14 % at pomolic acid concentration of 20 μM. Hence, pomolic acid exhibited inhibitory effects on proliferation of A549 cells. The results demonstrated strong inhibition of proliferation after treatment for 24 h. The potential of pomolic acid to inhibit proliferation of A549 cells increased significantly with increase in its concentration.
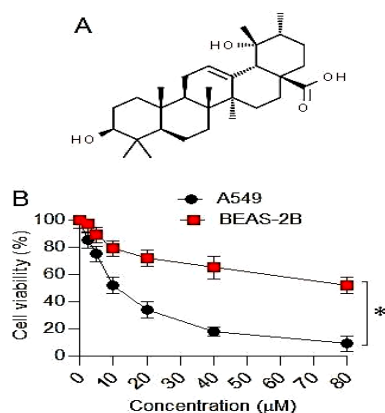

Figure 1: (A) Chemical structure of pomolic acid, **(B)** Proliferations of A549 and BEAS-2B cells post-pomolic acid treatment. *$P < 0.05$, vs control
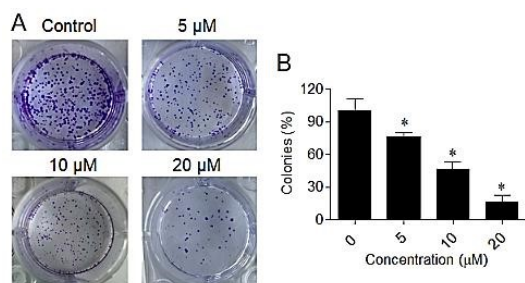

Figure 2: (A) Pomolic acid inhibited the formation of A549 cell colonies. **(B)** Quantitative evaluation of cell colonies in pomolic acid-treated A549 cells, relative to untreated cells. The data are shown as mean ± SD of triplicate experiments. *$P < 0.05$, vs control

Apoptotic effects of pomolic acid

To estimate apoptotic effects of pomolic acid on A549 cells, AO/EB staining was used. The results showed that pomolic acid induced apoptosis in A549 cells (Figure 3 A). Moreover, the percentage of apoptotic A549 cells increased with increase in the concentration of pomolic acid (Figure 3 B). The percentage growth of apoptotic cells was enhanced with increase in concentration of pomolic acid. Western blotting analysis showed that pomolic

acid increased the expression of Bax and decreased the expression of Bcl-2, thereby further confirming the induction of apoptosis (Figure 4). The results show up-regulated expressions of caspase and Bax proteins in A549 cells post-pomolic acid treatment. The expression of Bcl-2 protein was strongly targeted and reduced by pomolic acid.
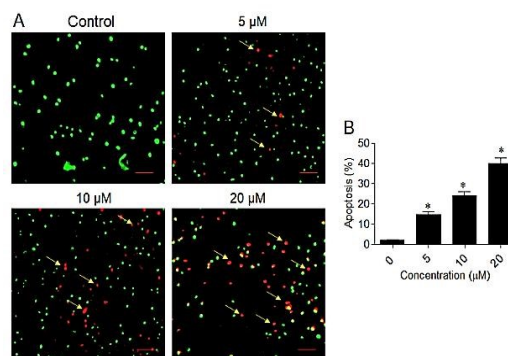

Figure 3: (A) Effect of pomolic acid on apoptosis of A549 cells. The green fluorescence of AO denotes the number of viable cells, while the red fluorescence of EB denotes the apoptotic/dead cells. **(B)** Quantitative evaluation of apoptotic cells in pomolic acid-treated group, relative to untreated cells. Arrows signify apoptotic/dead cells with red fluorescence. The data are results of triplicate experiments shown as mean ± SD. *$P < 0.05$, vs control
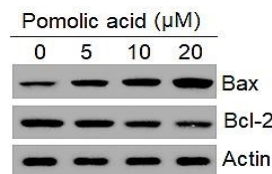

Figure 4: Expressions of biomarkers of apoptosis, as assayed via western blotting. Data are results of triplicate experiments

Pomolic acid induced oxidative stress

As shown in Figure 5A, pomolic acid induced robust DCF fluorescence in A549 cells, when compared to controls. This increased fluorescence revealed enhanced ROS accumulation in A549 cells. The DCF-fluorescence intensity indicates rise in ROS production in pomolic acid-treated A549 cells. The levels of ROS were increased in A549 cells in a dose-dependent manner (Figure 5 B). The fluorescence intensity was increased with increase in pomolic acid concentration.

Anti-cell migration and invasion effects of pomolic acid

Matrigel migration and invasion assays were

used to determine the effect of pomolic acid on the migration and invasion of A549 cells. As shown in Figure 6, the migration of A549 cells was decreased in the treated group, when compared to controls. The A549 cells were treated with indicated concentrations of pomolic acid, and Matrigel migration assay was used to study its effects on migration. The pictures of migrated cells were captured through inverted microscopy at x10 magnification. Consistently, the invasiveness of A549 cells was reduced in a concentration-reliant manner, as presented in Figure 7. The A549 cells were treated with indicated concentrations of pomolic acid, and Matrigel invasion assay was used to study its effect on invasion. The pictures of invasive cells were captured through inverted microscopy at x10 magnification.\
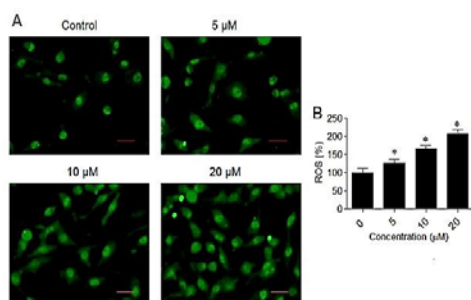

Figure 5: (A) Results of DCFH-DA staining assay showing enhanced DCF-fluorescence intensity in the pomolic acid-treated group, relative to control. **(B)** Quantitative evaluation of ROS in pomolic acid-treated group, relative to untreated cells. The data are results of triplicate experiments shown as mean ± SD. *$P < 0.05$, vs control
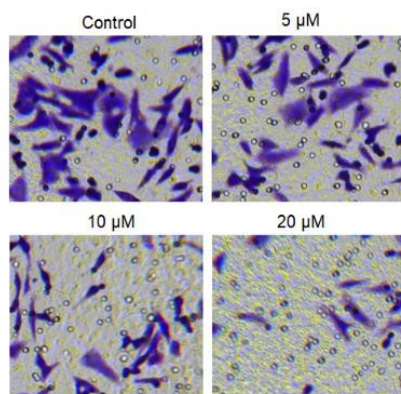

Figure 6: Pomolic acid inhibited the migration of A549 cells, relative to untreated controls. Data are results of triplicate experiments

DISCUSSION

The identification of novel bioactive plant-derived compounds has attracted the interest of
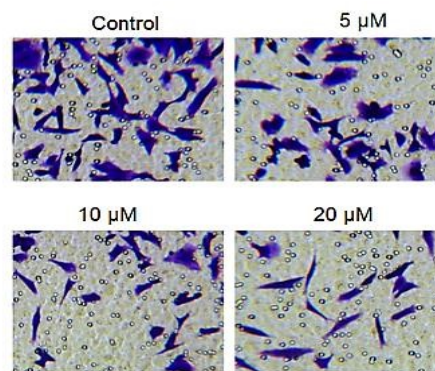

Figure 7: Effect of pomolic acid on the invasion of A549 cells. Data are results of triplicate experiments

researchers for their utilization in the management of human cancers. These compounds have been found to be harmful to normal cells, but with higher cytotoxic effects on cancer cells [13]. Natural products promote inhibition of cancer through different molecular mechanisms, including apoptosis, cell cycle inhibition, ROS elevation, and autophagy [6]. Despite advances in lung cancer research and development of effective treatment methods, the overall 5-year survival for cancer patients remains a major concern. Besides, lung cancer recurrence and metastasis, and hazardous side effects of chemotherapeutics pose huge threats to lung cancer patients. Therefore, novel chemotherapeutic agents are required for effective management of lung cancer. In this study, the anticancer effect of pomolic acid on A549 lung cancer cells, and the underlying molecular mechanism, were studied.

Pomolic acid is a pentacyclic triterpene which exerts highly-rated anti-proliferative effect on different human cancers, including breast cancer, glioblastoma and melanoma [14]. In this study, it was found that pomolic acid induced dose- and time-dependent antiproliferative effects on A549 cells. Therefore, the findings are consistent with previous studies which reported anti-proliferative effects of pomolic acid.

Apoptosis is a key mechanism in regulation of homeostasis, and it is one of the primary targets of chemotherapy. Its downregulation is often associated with the development of chronic diseases, including cancer [5]. In this study, it was found that pomolic acid induced apoptotic cell death in A549 cells. Moreover, the expressions of apoptosis biomarker proteins were determined through western blotting, and it was observed that pomolic acid enhanced Bax expression in A549 cells. The pro-apoptotic protein Bax is localized in the cytosol, a feature

which helps it to associate with membrane-bound Bcl-2 to form heterodimer which inhibits the stabilization of cytoplasmic pro-apoptotic proteins and ultimately suppresses apoptosis. However, the dissociation of this heterodimer takes place due to several pro-apoptosis signals which aid the movement of Bax to mitochondria membrane, thereby stimulating apoptosis [15]. Caspases are considered as initiators and effectors of apoptosis. The increased levels of these factors in pomolic acid-treated A549 cells indicated onset of apoptosis. The results of the present study are similar to those reported by Li & Yan [16].

Mitochondria are cellular organelles of high importance in regulation of tumorigenesis, apoptosis and drug tolerance. Several studies have reported that the mitochondrial signal transduction pathway plays important roles in cellular metabolism and apoptosis [6]. Excessive production of ROS in mitochondria results in the depletion of MMP which enhances the permeability of mitochondrial membrane. The enhanced permeability of membranes results in release of cytochrome c into the cytoplasm where it induces the caspase cascade, and ultimately apoptosis. Previous studies have reported that pomolic acid enhanced the production of ROS in several human cancer cells including human glioma cells [17]. In the present study, it was found that pomolic acid enhanced ROS production in a dose-reliant manner, and hence promoted apoptosis.

The migration and invasion of cancer cells are initial stages of the metastasis of cancer cells. Several natural products exhibit anti-cell migration and invasion effects [17]. Pomolic acid has also been shown to exhibit anti-migration and anti-invasion potential against breast cancer cells [18]. This study showed that pomolic acid inhbited migration and invasion of lung cancer cells. Pomolic acid induced anticancer effects on A549 lung cancer cells through a mechanism involving ROS-mediated mitochondrial apoptosis and cell cycle arrest. Moreover, pomolic acid inhibited the migration and invasion of A549 cells.

CONCLUSION

Pomolic acid suppresses the proliferation of lung cancer cells via induction of ROS-mediated apoptosis. Moreover, it inhibits the migration and invasion of lung cancer cells. These results suggest that pomolic acid may have promising potential as a lead molecule for the development of lung cancer chemotherapy.

DECLARATIONS

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

Open Access

This is an Open Access article that uses a funding model which does not charge readers or their institutions for access and distributed under the terms of the Creative Commons Attribution License (http://creativecommons.org/licenses/by/4.0) and the Budapest Open Access Initiative (http://www.budapestopenaccessinitiative.org/read), which permit unrestricted use, distribution, and reproduction in any medium, provided the original work is properly credited.

REFERENCES

1. Cheng B, Xiong S, Li C, Liang H, Zhao Y, Li J, Shi J, Ou L, Chen Z, Liang P, et al. An annual review of the remarkable advances in lung cancer clinical research in 2019. J Thorac Dis 2020; 12(3): 1056.

2. Zhou M, Wang H, Zeng X, Yin P, Zhu J, Chen W, Li X, Wang L, Wang L, Liu Y, et al. Mortality, morbidity, and risk factors in China and its provinces, 1990-2017: a systematic analysis for the Global Burden of Disease Study 2017. Lancet 2019; 394: 1145-1158.

3. Zeng H, Chen W, Zheng R, Zhang S, Ji JS, Zou X, Xia C, Sun K, Yang Z, Li H, et al. Changing cancer survival in China during 2003-15: a pooled analysis of 17 population-based cancer registries. Lancet Glob Health 2018; 6: e555-67.

4. Kamal A, Faazil S, Malik MS. Apoptosis-inducing agents: a patent review (2010–2013). Expert Opin Ther Pat 2014; 24(3): 339-354.

5. Igney FH, Krammer PH. Death and anti-death: Tumour resistance to apoptosis. Nat Rev Cancer 2002; 2: 277-288.

6. Trachootham D, Alexandre J, Huang P. Targeting cancer cells by ROS-mediated mechanisms: A radical therapeutic approach? Nat Rev Drug Discov 2009; 8: 579-591.

7. Choi EO, Park C, Hwang HJ, Hong SH, Kim GY, Cho EJ, Kim WJ, Choi YH. Baicalein induces apoptosis via ROS-dependent activation of caspases in human bladder cancer 5637 cells. Int J Oncol 2016; 49(3): 1009-1018.

8. Wali AF, Majid S, Rasool S, Shehada SB, Abdulkareem SK, Firdous A, Beigh S, Shakeel S, Mushtaq S, Akbar I,

et al. Natural products against cancer: Review on phytochemicals from marine sources in preventing cancer. Saudi Pharm J 2019; 27(6): 767-777.

9. *Salvador JAR, Moreira VM, Gonçalves BMF, Leal AS, Jing Y. Ursane-type pentacyclic triterpenoids as useful platforms to discover anticancer drugs. Nat Prod Rep 2012; 29: 1463-1479.*

10. *Fernandes J, Weinlich R, Castilho RO, Amarante-Mendes GP, Gattass CR. Pomolic acid may overcome multidrug resistance mediated by overexpression of anti-apoptotic Bcl-2 proteins. Cancer Lett 2007; 245: 315-320.*

11. *Park JH, Cho YY, Yoon SW, Park B. Suppression of MMP-9 and FAK expression by pomolic acid via blocking of NF-κB/ERK/mTOR signaling pathways in growth factor-stimulated human breast cancer cells. Int J Oncol 2016; 49: 1230-1240.*

12. *Fernandes J, Castilho RO, da Costa MR, Wagner-Souza K, Coelho Kaplan MA, Gattass CR. Pentacyclic triterpenes from Chrysobalanaceae species: Cytotoxicity on multidrug resistant and sensitive leukemia cell lines. Cancer Lett 2003; 190: 165-169.*

13. *Li JW, Vederas JC. Drug discovery and natural products: end of an era or an endless frontier? Science 2009; 325(5937): 161-165.*

14. *Guimarães LP, Rocha GD, Queiroz RM, Martins CA, Takiya CM, Gattass CR. Pomolic acid induces apoptosis and inhibits multidrug resistance protein MRP1 and migration in glioblastoma cells. Oncol Rep 2017; 38(4): 2525-2534.*

15. *Dewson G, Kluck RM. Mechanisms by which Bak and Bax permeabilise mitochondria during apoptosis. J Cell Sci 2009; 122: 2801–2808.*

16. *Li TH, Yan HX. Antitumor-and apoptosis-inducing effects of pomolic acid against SK-MEL-2 human malignant melanoma cells are mediated via inhibition of cell migration and sub-G1 cell cycle arrest. Mol Med Rep 2018; 17(1): 1035-40.*

17. *Frolova TS, Lipeeva AV, Baev DS, Tsepilov YA, Sinitsyna OI. Apoptosis as the basic mechanism of cytotoxic action of ursolic and pomolic acids in glioma cells. Mol Biol 2017; 51(5): 705-711.*

18. *Park JH, Yoon J, Park B. Pomolic acid suppresses HIF1α/VEGF-mediated angiogenesis by targeting p38-MAPK and mTOR signaling cascades. Phytomedicine 2016; 23(14): 1716-1726.*