JBUON 2020; 25(2): 869-874
ISSN: 1107-0625, online ISSN: 2241-6293 • www.jbuon.com
Email: editorial_office@jbuon.com

ORIGINAL ARTICLE

Herbal extract of *Artemisia maritima* induces selective anti-tumor effects in three human lung carcinoma cells (H1299, NCI-H1437, PC-14) through activating apoptosis-related proteins, G2/M cell cycle arrest and suppression of cell migration

Junqi Tang[1]*, Zhihong Guo[1]*, Yan Wen[2], Suhua Ao[1], Junfeng Wang[1]

[1]Department of Respiratory Medicine, Hospital of Traditional Chinese Medicine, Affiliated to Southwest Medical University, Luzhou 646000, China. [2]Department of Stomatology, Hospital of Traditional Chinese Medicine, Affiliated to Southwest Medical University, Luzhou 646000, China.

*These two authors contributed equally to this work.

Summary

Purpose: *Non-small cell lung cancer (NSCLC) is the leading cause of cancer-related deaths in the United States, accounting for 85% of all diagnosed lung cancers and resulting in over 100,000 deaths per year. The main purpose of the current study was to investigate the antiproliferative effects of ethanolic extract of Artemisia maritima in three human lung cancer cell lines along with studying the effects of the herbal extract on cellular apoptosis, G2/M cell cycle arrest and cell migration.*

Methods: *The CCK-8 assay was used to determine cell viability. Apoptosis was detected by using acridine orange (AO)/ethidium bromide (EB) staining, annexin V/propidium iodide (PI) assay and western blot. The cell migration was determined by wound healing assay while the effects on cell cycle were evaluated by flow cytometry.*

Results: *The results showed that herbal extract of Artemisia maritima decreased the viability of all three cell lines H1299, NCI-H1437, PC-14 dose-dependently with maximum effect on NCI-H1437 cell line. The antiproliferative effects were due to the activation of mitochondrial-dependent apoptotic pathway as seen by fluorescence microscopy which showed chromatin condensation and nuclear fragmentation. This was also associated with increase in Bax and decrease in Bcl-2 levels. Artemisia maritima extract treatment also led to G2/M phase cell cycle arrest along with strong inhibition of cell migration.*

Conclusion: *In conclusion, the results of the current study clearly indicate that Artemisia maritima extract exhibits antiproliferative effects in Nonsmall cell lung cancer cells by triggering apoptosis, cell cycle arrest and inhibition of cell migration.*

Key words: *lung cancer, artemisia maritima, apoptosis, cell migration, cell cycle*

Introduction

Lung cancer is the leading cause of death in both genders. The prevalence and the mortality rates arising from the lung cancer rank highest among all cancers and each year it is projected to rise further. Lung carcinoma has two main types, namely small cell lung carcinoma and non-small cell lung carcinoma (NSCLC). NSCLC attributes to about 80-85% of all lung cancer cases and about

Corresponding author: Yan Wen, BS. Department of Stomatology, Hospital of Traditional Chinese Medicine, Affiliated to Southwest Medical University, No. 182 Chunhui Rd, Longmatan District, Luzhou 646000, China.
Tel/Fax: +86 8302392597, Email: omcogi@163.com
Received: 16/05/2019; Accepted: 06/06/2019

This work by JBUON is licensed under a Creative Commons Attribution 4.0 International License.

more than 70% of the patients already advanced to stage IV at the time of diagnosis [1,2]. In China, lung cancer is the leading cause of cancer-related mortality and its incidence has reached to an all-time high during recent years thus posing a great challenge. Lung cancer has been shown to rely on geographic and gender attributions. Various potential risk factors have been identified which include smoking, asbestos exposure and air pollution [3,4]. Presently, the treatment regimen adopted for NSCLC includes chemotherapy using cisplatin or oxaliplatin in combination with paclitaxel or vinorelbine or gemcitabine. Many patients respond well to this chemotherapy, but in later stages they acquire drug resistance. Additionally, chemotherapy is also associated with various adverse effects affecting the patient quality of life [5-8]. Thus, there is a pressing need for new and effective chemotherapeutic agents required for treating lung carcinoma. The main objective of the current research was to investigate the anticancer effects of herbal extract of *Artemisia maritima* in three human lung cancer cell lines (H1299, H1437, PC-14) along with demonstrating its effects on apoptosis, cell cycle phase distribution and cell migration.

Methods

Cell lines, culture conditions and cell viability assay

The H1299, NCI-H1437, PC-14 lung cancer cells were procured from the Department of Biochemistry and Biology, Chinese Academy of Sciences, Shanghai, China. The cells were maintained in Dulbecco's modified Eagle's medium (Gibco, USA) at 37°C with 95% humidity and 5% CO_2. The cell proliferation was determined by CCK-8 (Cell Counting Kit-8) assay obtained from Dojindo Laboratories, Kumamoto, Japan. The H1299, H1437, PC-14 lung cancer cell lines were initially treated separately with 0, 2.5, 5, 10, 20, 40, 80, 160 and 320 μg/ml of *Artemisia maritima* extract after which 30 μl CCK-8 were slowly added to 96-well culture plates. The plates were then incubated for 24 h at 37°C. The absorbance was measured at 450 nm wavelength using a microplate reader (BioTek Instruments, USA). Cell cytotoxicity was calculated from the observed absorbance.

Acridine orange/ethidium bromide (AO/EB) staining and Annexin V/PI staining assay for evaluating apoptosis

The NCI-H1437 human lung cancer cells at a cell density of 1×10^6 cells/ml were placed into 6-well plates and cultured for 24 h. The cells were treated with various concentrations (0, 6, 12 and 24 μg/ml) of *Artemisia maritima* extract and incubated for 24 h. Afterwards, 30 μl of cell culture were placed onto glass slides and stained

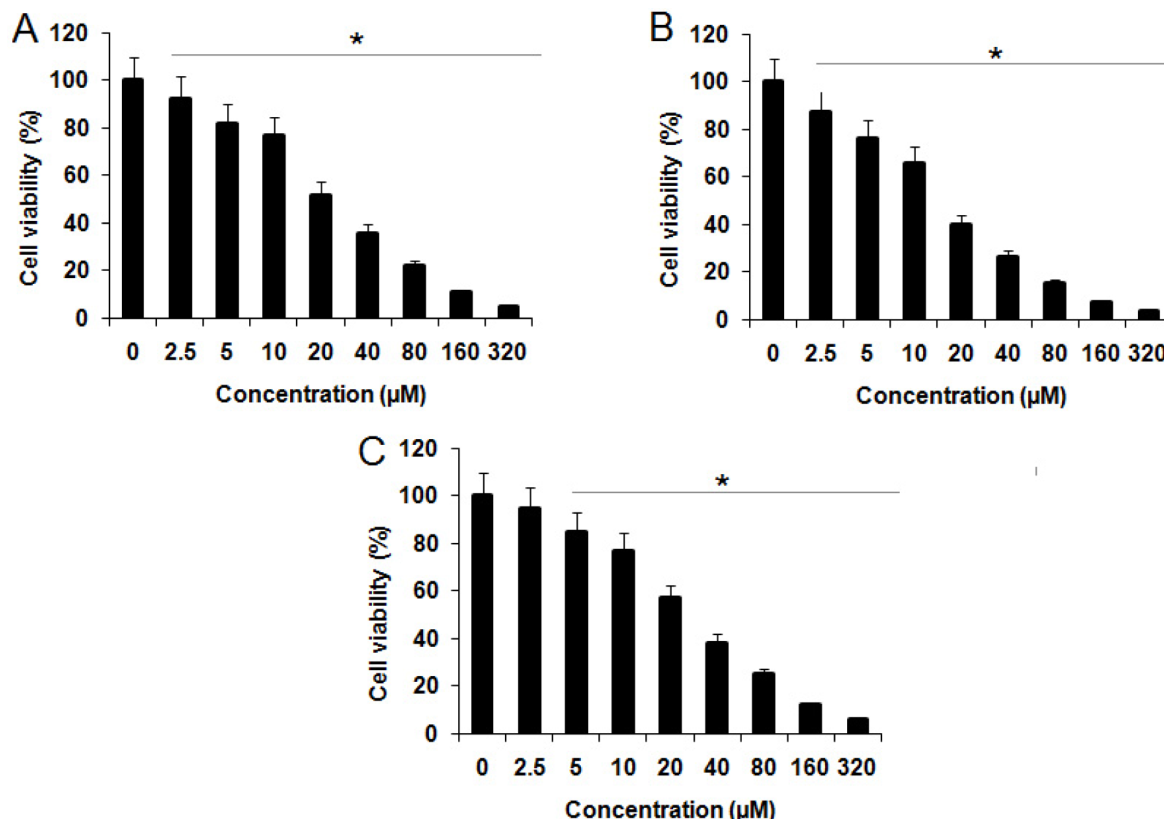

Figure 1. Cell viability assay showing the inhibitory effects of herbal extract of *Artemisia maritima* on the viability of the three human lung carcinoma cells. Figure **1A** shows the cytotoxic effects on PC-14 cells, Figure **1B** shows cytotoxic effects on NCI-H1437 cells, while Figure **1C** shows cytotoxicity in H1299 lung carcinoma cells. The experiments were performed in triplicate and presented as mean ± SD (*p<0.05).

with AO/EB independently (10 µl). The slides were then cover-slipped and examined under fluorescence microscope (Nikon Instruments Inc., NY, USA) for evaluating apoptotic changes. Annexin V/PI staining was carried out as reported in the literature [9].

Flow cytometry for cell cycle analysis

The cultured NCI-H1437 human lung cancer cells were initially treated with 0, 6, 12 and 24 µg/ml of *Artemisia maritima* extract for 24 h at 37°C. The cells were then washed with phosphate buffered saline (PBS). Afterwards, the NCI-H1437 cells were stained with PI and the distribution of the cells in cell cycle phases was assessed by FACS flow cytometer (FACSCalibur; BD Biosciences).

In vitro wound healing assay

Artemisia maritima extract-treated cells were cultured till 85% confluence. This was followed by removal of the Dulbecco's modified Eagle's medium and subsequent washing with PBS. Afterwards, a wound was scratched with a sterile pipette tip and a picture was taken. The plates were then incubated for about 24 h at 37°C and then a picture was taken again under an inverted microscope.

Western blot analysis

The NCI-H1437 human lung cancer cells were originally washed with ice-cold PBS and then lysed in RIPA lysis buffer encompassing the protease inhibitor. Around 50 µg of proteins from each cell culture were then loaded on the SDS-PAGE. The protein contents were measured using a bicinchoninic acid (BCA) protein quantitative kit (Boster, China). The gels were then transferred to nitrocellulose membranes and treated with primary antibody at 4°C for 12 h. Thereafter, the membranes were incubated with HRP-conjugated secondary antibody for 60 min at 37°C. Enhanced chemi-luminescence reagent was used to visualise the protein bands. As a final point, the signal was detected by Odyssey Infrared Imaging System (LI-COR, USA). Actin was used as control for normalization.

Statistics

The experiments were done in triplicate. The values presented are the mean of three experiments ± SD. $^*p<0.05$, $^{**}p<0.01$ and $^{***}p<0.001$ were considered statistically significant. Student's t-test using GraphPad prism 7 software was employed for statistical analyses.

Results

Artemisia maritima extract induced cytotoxicity in all three lung cancer lines

The growth inhibitory effects of *Artemisia maritima* extract were seen on three lung cancer cell lines (H1299, NCI-H1437, PC-14) by CCK-8 assay at concentrations ranging from 0 to 320 µg/ml. *Artemisia maritima* extract was shown to suppress the viability of all three tested lung cancer cell lines, exerting maximum toxicity in NCI-H1437 cells. The cytotoxicity induced by the extract was found to be concentration-dependent and is shown in Figure 1A, B and C. Figure 1B shows the cytotoxicity induced by the herbal extract in NCI-H1437 lung cancer cell line while as Figure 1A and Figure 1C show the effects on PC-14 and H1299 cell lines respectively.

Artemisia maritima induced programmed cell death in NCI-H1437 lung cancer cells

Since NCI-H1437 cells were most sensitive to the extract treatment, further experiments were conducted on this cell line to verify whether the extract could induce apoptotic cell death. This was examined by fluorescence microscopy followed by flow cytometry. The results of AO/EB staining are shown in Figure 2 and reveal that the number of cells with orange/yellow fluorescence (apoptotic cells) increased considerably with increasing *Artemisia maritima* extract dosage. Untreated control cells only showed green fluorescence, indicating no signs of apoptosis. Finally, flow cytometry measurements computed the percentage of cells which had undergone apoptosis at each tested dose of the herbal extract. Increasing doses of the extract (0, 6, 12 and 24 µg/ml) increased the percentage of apoptotic cells from 6.34% in the control group to 17.61, 26.57 and 31.55%, respectively (Figure 3). Apoptosis was finally confirmed by carrying out western blot analysis which showed that the extract of this molecule led to increased expression in the Bax and decrease expression of Bcl-2 (Figure 4).

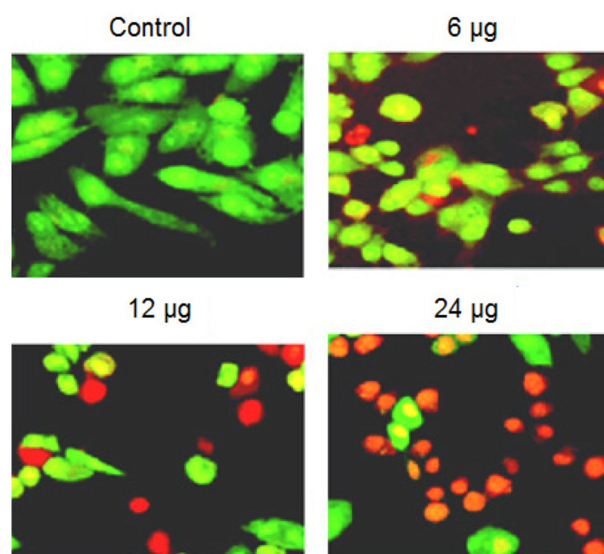

Figure 2. Fluorescence microscopy using acridine orange/ethidium bromide (AO/EB) staining used to evaluate the apoptotic effects of herbal extract of *Artemisia maritima* in human NCI-H1437 lung cancer cells. The percentage of apoptotic cells (yellow and orange fluorescence) increased concentration-dependently. The experiments were performed in triplicate.

Artemisia maritima extract led to G2/M phase cell cycle arrest in NCI-H1437 lung cancer cells

The growth inhibitory effects of *Artemisia maritima* extract were further perceived to be mediated through inhibiting cell cycle phase distribution at the G2/M phase. The results obtained from flow cytometric measurements indicated that *Artemisia maritima* extract led to G2/M cell cycle arrest in a dose-dependent manner. With increase in the *Artemisia maritima* extract dosage, the NCI-H1437 lung cancer cells in G2/M phase were also seen to increase and at 24 μg/ml dose 61.2% of the cells were witnessed to lie in G2/M phase of the cell cycle (Figure 5). Western blot analysis further indicated that the herbal extract of this molecule could alter the expression of cell cycle-regulating proteins like cyclin-B1 as it was seen in this experiment that the extract led to a significant decrease in the expression of cyclin-B1 in a dose-dependent manner (Figure 6).

Artemisia maritima extract led to suppression of cell migration in NCI-H1437 cells

Finally, *in vitro* wound healing assay was carried out to check whether the herbal extract of *Artemisia maritima* could induce inhibition of cancer cell migration in NCI-H1437 lung cancer cells. The results which are shown in Figure 7 indicate that the herbal extract of the molecule at 12 μg/ml led to significant cell migration inhibition. Further, western blot assay confirmed the results as it was seen that the herbal extract could decrease dose-dependently the expression of matrix metalloproteinase-9 (MMP-9) (Figure 8). It has been reported earlier that matrix metalloproteinase-9 protein expression has a key role to play in cell migration. If this expression could be decreased, this could ultimately lead to inhibition of cell migration.

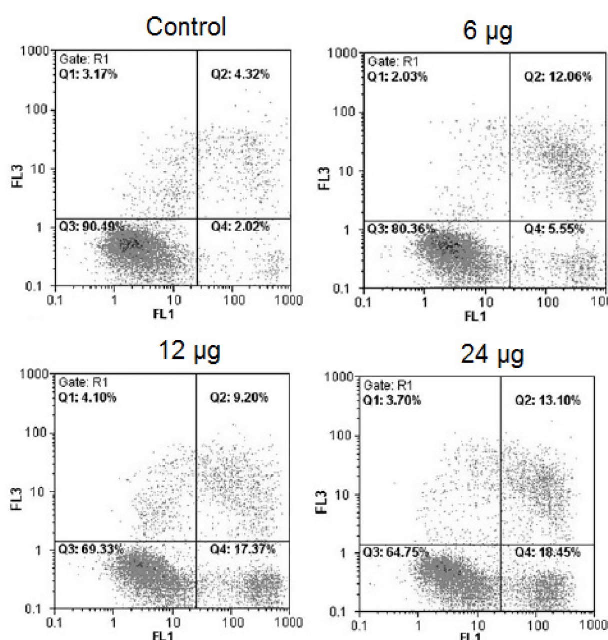

Figure 3. Annexin-V/PI staining to quantitatively measure how much apoptosis was induced by herbal extract of *Artemisia maritima*. Increasing doses of the extract (0, 6, 12 and 24 μg/ml) increased the percentage of apoptotic cells from 6.34% in the control group to 17.61, 26.57 and 31.55%, respectively.

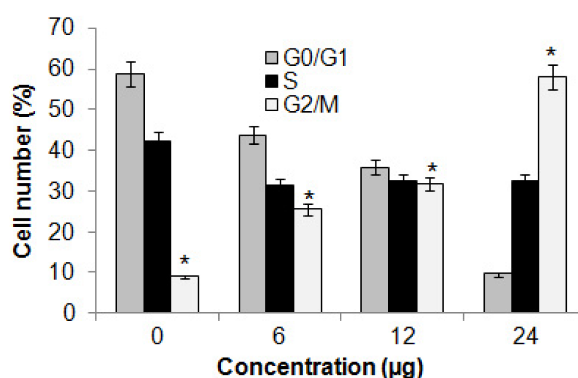

Figure 5. Impact of herbal extract of *Artemisia maritima* on the cell cycle phase distribution in human NCI-H1437 lung cancer cells using flow cytometry and presented as mean±SD (*p<0.05). With increase in the *Artemisia maritima* extract dosage, the NCI-H1437 cells in the G2/M phase were seen to increase and at 24 mg/ml dose 61.2% were in the G2/M phase of the cell cycle. The experiments were performed in triplicate.

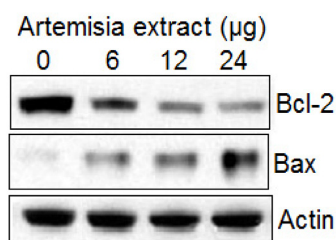

Figure 4. Assessment of the effect of herbal extract of *Artemisia maritima* on the expression levels of Bax and Bcl-2 (apoptosis-related proteins) using western blot method. Increasing doses of *Artemisia maritima* extract led to decrease in the expression of Bcl-2 (anti-apoptotic) and to increase of Bax. The experiments were performed in triplicate.

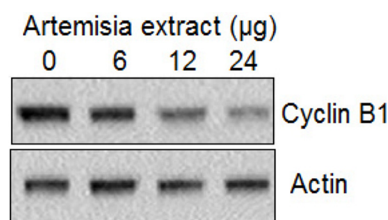

Figure 6. Assessment of the effect of herbal extract of *Artemisia maritima* on the expression levels of cyclin-B1 (cell cycle-related protein) employing western blot assay. *Artemisia maritima* could inhibit cyclin B1 protein expression which is a key factor in cell cycle regulation. The experiments were performed in triplicate.

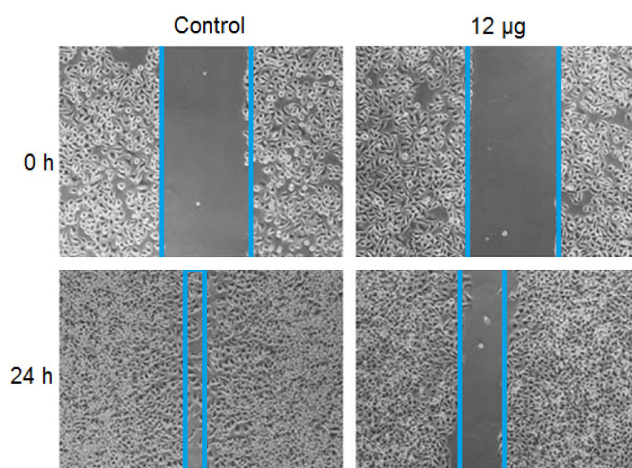

Figure 7. *In vitro* wound healing assay for the determination of cell migration inhibition effects induced by the herbal extract of *Artemisia maritima*. The experiments were performed in triplicate. *Artemisia maritima* extract could significantly reduce the cancer cell migration.

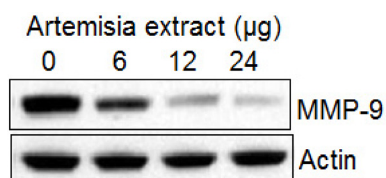

Figure 8. Assessment of the effect of herbal extract of *Artemisia maritima* on the expression levels of MMP-9 (cell migration-related protein) employing western blot assay. The extract led to reduction of the expression of MMD-9 dose-dependently. The experiments were performed in triplicate.

Discussion

Natural products which are derived either from plants or microbial sources have played crucial roles in treating a range of human diseases including some very dangerous disorders like cancer and infection. A number of natural products have been isolated which have been shown to exhibit anticancer efficacy and in fact have been used in the clinic for the last 5-6 decades. Plant-derived natural products play a major role in drug discovery keeping in view the increasing number of new and novel drugs which are currently in different phases of clinical development [10]. Many of these naturally occurring secondary metabolites have the tendency to induce apoptosis. Apoptosis is a well-controlled biochemical process which plays a crucial role in tissue homeostasis by eradicating the damaged cells out of the body. The apoptosis process is one of the most well studied biochemical processes primarily because of its role in normal as well as in processes which cause numerous pathological conditions including carcinomas. The apoptotic process is characterized by some unique morphological as well as biochemical signposts which can indicate whether the process of apoptosis has occurred or not. These changes include stimulation of numerous caspases including caspase 3 and 9, stimulation of Bax protein and inhibition of Bcl-2 protein, DNA fragmentation and protein degradation. Morphological changes include cellular contraction and membrane blebbing. Caspase activation is regarded as the most characteristic feature of apoptosis which has a role in slicing various important cell proteins that ultimately lead to nuclear degradation [11-13]. In the current study *Artemisia maritima* extract was shown to induce cytotoxicity in three human lung cancer cell lines (H1299, NCI-H1437, PC-14) and was also shown to suppress the viability of all three tested lung cancer cells exerting maximum toxicity in NCI-H1437 cells. Further using fluorescence microscopy and flow cytometry, it was shown that the extract of this molecule could induce potent apoptotic effects. The results of AO/EB staining showed that the number of apoptotic cells increased considerably with increasing *Artemisia maritima* extract dosage. Untreated control cells showed no signs of apoptosis. Flow cytometry showed that increasing doses of the extract (0, 6, 12 and 24 μg/ml) increased the percentage of apoptotic cells from 6.34% in the control group to 17.61, 26.57 and 31.55%, respectively. Finally, western blot assay confirmed the results in which Bax expression was shown to increase while Bcl-2 expression was shown to decrease as the dose of *Artemisia maritima* extract increased. *Artemisia maritima* extract was also seen to induce G2/M phase cell cycle arrest along with inhibition of cell migration. Herbal extract could decrease the expression of matrix metalloproteinase-9 (MMP-9) dose-dependently. It has been reported earlier that MMP-9 protein expression has a key role to play in cell migration. If this expression could be decreased, this could ultimately lead to inhibition of cell migration [14-17].

Conclusion

In conclusion, it can be clearly suggested that *Artemisia maritima* herbal extract induces anticancer effects in NSCLC cells by inducing programmed cell death, G2/M phase cell cycle arrest and inhibition of cell migration.

Conflict of interests

The authors declare no conflict of interests.

References

1. Mori S, Ito F, Usami N et al. p53 apoptotic pathway molecules are frequently and simultaneously altered in Nonsmall cell lung carcinoma. Cancer 2003;100:1673-82.
2. American Cancer Society, Cancer Facts & Figures. Atlanta, GA: American Cancer Society; 2005.
3. McIntyre A, Ganti AK. Lung cancer-A global perspective. J Surg Oncol 2017;115:550-4.
4. Martin-Sanchez JC, Lunet N, Gonzalez-Marron A et al Projections in Breast and Lung Cancer Mortality among Women: A Bayesian Analysis of 52 Countries Worldwide. Cancer Res 2018;78:4436-42.
5. Zhang C, Leighl NB, Wu YL, Zhong WZ. Emerging therapies for non-small cell lung cancer. J Hematol Oncol 2019;12:45.
6. Chamberlain MC, Baik CS, Gadi VK, Shailender B, Chow LQM. Systemic therapy of brain metastases: non–small cell lung cancer, breast cancer, and melanoma. Neurooncology 2017;19:i1-i24.
7. Chan BA, Hughes BGM. Targeted therapy for non-small cell lung cancer: current standards and the promise of the future. Transl Lung Cancer Res 2015;4:36-54.
8. Shtivelman E, Hensing T, Simon GR et al. Molecular pathways and therapeutic targets in lung cancer. Oncotarget 2014;5:1392-1433.
9. Hammill AK, Uhr JW, Scheuermann RH. Annexin V staining due to loss of membrane asymmetry can be reversible and precede commitment to apoptotic death. Exper Cell Res 1999;251:16-21.
10. Rayan A, Raiyn J, Falah M. Nature is the best source of anticancer drugs: Indexing natural products for their anticancer bioactivity. PLoS One 2017;12:e0187925.
11. Ghobrial IM, Witzig TE, Adjei AA. Targeting apoptosis pathways in cancer therapy. CA Cancer J Clin 2005;55:178-94.
12. Ouyang L, Shi Z, Zhao S et al. Programmed cell death pathways in cancer: a review of apoptosis, autophagy and programmed necrosis. Cell Prolif 2012;45:487-98.
13. Lavrik IN, Golks A, Krammer PH. Caspases: pharmacological manipulation of cell death. J Clin Invest 2005;115:2665-72.
14. Dufour A, Zucker S, Sampson NS, Kuscu C, Cao J. Role of matrix metalloproteinase-9 dimers in cell migration: design of inhibitory peptides. J Biol Chem 2010;285:35944-56.
15. Zarrabi K, Dufour A, Li J et al. Inhibition of matrix metalloproteinase 14 (MMP-14)-mediated cancer cell migration. J Biol Chem 2011;286:33167-77.
16. Dufour A, Sampson NS, Li J et al. Small-molecule anticancer compounds selectively target the hemopexin domain of matrix metalloproteinase-9. Cancer Res 2011;71:4977-88.
17. Swetha R, Gayen C, Kumar D, Singh TD, Modi G, Singh SK. Biomolecular basis of matrix metalloproteinase-9 activity. Future Med Chem 2018;10:1093-1112.