

# Cytotoxic Effects of Methanolic Extract of *Ziziphora tenuior* L. on the Growth of the Lung Cancer Cell Line

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*Cytotoxic Effects of Methanolic Extract of Ziziphora tenuior L. on the Growth of the Lung Cancer Cell Line*

*Ziziphora tenuior L.'nin Metanol Ekstresinin Akciğer Kanseri Hücre Hattı Büyümesi Üzerine Sitotoksik Etkileri*

## SUMMARY

Due to the poor coverage of the effects of *Ziziphora tenuior* L. on lung cancer cells in literature, this study was conducted to measure the induction of apoptosis of methanolic extract of *Ziziphora tenuior* L. plant on the A-549 line lung cancer. The cell survival rate was measured by the MTT assay after cell culture in RPMI medium, 10% FBS serum and treatment of cells with different concentrations of the plant extract. The appropriate concentration for Real Time-PCR was selected, and the expression of BAX and Bcl-2 genes was then measured. The survival tests showed that the methanolic extract of *Ziziphora tenuior* L. reduced the growth of A549 cells ( $P < 0.01$ ). However, it did not inhibit the growth of normal HEK cells. Moreover, the results of the Real-Time PCR analysis indicated that the plant extract induces apoptosis of cancerous cells, which is associated with an increase in the expression of the BAX gene. The result of this induction is the higher mortality rate of cells of A549 cell line.

**Key Words:** Lung cancer, *Ziziphora tenuior* L., A549 cells, HEK cells, BAX gene, Bcl2 gene

## ÖZ

Bu çalışma *Ziziphora tenuior* L.'nin akciğer kanseri hücreleri üzerinde etkileri hakkında; bilgi eksikliği nedeniyle, *Ziziphora tenuior* L. bitki ekstresinin akciğer kanseri hücre hattı A-549 üzerindeki apoptozunun indüksiyonunu ölçmek için yapılmıştır. Hücre hayatta kalma oranı, hücre RPMI ortamında, % 10 FBS serumu ve hücrelerin farklı konsantrasyonlardaki bitki ekstre ile muamele edilmesinden sonra MTT analizi ile ölçülmüştür. Real Time-PCR için uygun konsantrasyon seçildi, ve daha sonra BAX ve Bcl-2 genlerinin ekspresyonu ölçüldü. Yapılan hayatta kalma testleri, *Ziziphora tenuior* L. metanol ekstresinin A549 hücrelerinin büyümesini azalttığını gösterdi ( $P < 0.01$ ). Ancak, *Ziziphora tenuior* L.'nin metanol ekstresi normal HEK hücrelerinin büyümesini engellemedi. Ayrıca, Real Time-PCR analizinin sonuçları şunu gösterdi ki bitki ekstresi kanserli hücrelerin apoptozunu indüklemiştir ki bu da BAX geninin ekspresyonunda artış ile ilişkilidir. Bu indüksiyon ile sonucu A549 hücre hattı hücrelerinde daha yüksek ölüm oranı gözlemlenmiştir.

**Anahtar Kelimeler:** Akciğer kanseri, *Ziziphora tenuior* L, A549 hücreleri, Hek hücreleri, BAX geni, Bcl2 geni

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## INTRODUCTION

Nowadays, various cancers are leading causes of death, among which is lung cancer with a mortality rate of 28% (Al-Dabbagh et al., 2018; Bilello et al., 2002). Approximately 75-85 % of lung cancer diagnosis of the non-small cell type (Midthun et al., 1997) with a high mortality rate (Camerlingo et al., 2019) including A549.

Apoptosis is programmed cell death and a natural way of eliminating aged cells in the body. Many anticancer agents induce apoptosis to kill cancer cells. The Bcl-2 protein family involved in the regulation of apoptotic cells death. Bcl-2 is a negative regulator of cell death, whereas BAX promotes cell death and stimulates apoptosis (Pérez-Garijo et al., 2013; Sharifi et al., 2018).

Undoubtedly, using medicinal herbs has been the oldest human approach to the treatment of diseases (Ghasemi Pirbalouti et al., 2013). Besides, returning to herbal and natural remedies is a rapid growth due to the clarification of the adverse effects of chemical drugs. A new approach has been started over the past decades to study medicinal plants and to investigate their physiological and pharmacological effects. Lastly, medicinal plants are also an essential source of new chemicals with strong therapeutic effects. Some plants like *Croton tiglium* L. (Kowalczyk et al., 2019) and *Menyanthes trifoliata* L. (Li et al., 2016) have anticancer effects on A549 cells via BAX/Bcl-2 pathway.

*Z. tenuior* is a herbaceous plant, belongs to the Lamiales family of Asteridae subclass (Ganjali et al., 2016). It is annual with a short stem height of 5 to 15 cm. It has narrow, sharp, and short knitted leaves. Its flowers are light purple or purple. The geographical distribution of this plant is Iran, Turkey, Russia, Turkmenistan, Afghanistan, Pakistan, Caucasus, and Siberia (Rechinger, 1982). The plant was used in traditional medicine for the treatment of fever, menstrual cycle symptoms and stomach tones (Ghorbani Ranjbar et al., 2016) and closely aligned to it in modern medicine the treatment of gastrointestinal and respiratory disorders (Dehkordi et al., 2014; Naghibiet al., 2005).

It possesses many other remedial effects, including being antibacterial, antifungal, and antioxidant (Dakah et al., 2014). It is also a natural remedy for intestinal disinfection, and tonsillitis (Antonsson et al., 2000; Boise et al., 1993; Ghasemi Pirbalouti et al., 2013). Due to the existence of pulegone as one of its main active chemical components whose analgesic and anti-inflammatory effects have been well documented (de Souse et al., 2007). It has other applications in illnesses like dysentery, diarrhea, gut inflammation, cough, and bladder stones. The hydroalcoholic extract of *Z. tenuior* is useful against the reprotoxic effects of formaldehyde in male mice (Hassanpour et al., 2018).

Apart from pulegone, phytochemical analysis of *Z. tenuior* essential oil reveals other major components like limonene, thymol and menthone (Amirkhosravi et al., 2012). Owing to the therapeutic effects of this plant and its use in traditional medicine, as well as the absence of reports, having been working on its cytotoxic effects plus BAX and Bcl-2 genes expression on cells A-549 lung cancer, this study aimed to investigate the effect of methanolic extract of *Z. tenuior* on the previously mentioned cancer cells, as well as induction of its apoptotic effect on cells and the expression of the genes involved in the process, BAX and Bcl-2.

## MATERIALS AND METHODS

### Extraction

In the first stage, the *Z. tenuior* plant was collected from the city of Esfarain (North Khorasan Province, Iran), in the spring of 2015, selected samples were approved by the Herbarium Department of the Islamic Azad University of Tonekabon branch (Tonekabon, Mazandaran Province), and its herbarium code is Kaku1315.

Collected samples were thoroughly washed several times in clean water, and then leaves were dried for four days at room temperature of 30°C after complete removal of water. The methanol extract of the leaves were done by Soxhlet apparatus. The extract was condensed using a rotary evaporate distillation apparatus

and was kept in sterile plates inside the refrigerator at 4 °C away from heat and light until the cell culture experiments were carried out.

### Cell culture

In this study, the human lung cancer cell line A549 purchased from the cell bank of the Razi Institute of Karaj, Iran (NCBI code: C137) has been investigated. For normal control, HEKcell line (NCBI code: C497) was used as a healthy cell. Cell passaging was applied in RPMI medium (ThermoFisher Scientific, CA, USA, Gibco) for A549 cells and DMEM medium (ThermoFisher Scientific, CA, USA, Gibco) for HEKcells with 10% fetal bovine serum (FBS), 100 units/mL penicillin and 100µg/mL streptomycin in an incubator at 37 °C using sufficient humidity and 5% carbon dioxide. To perform several tests, when the cells reached at least 70% of cell growth, they were detached by trypsin-ethylene diamine tetraacetic acid (EDTA) (ThermoFisher Scientific, CA, USA, Gibco) from the bottom of the flask and centrifuged for five minutes at 1500 rpm. The cellular deposition was prepared in 1mL culture medium in suspension form, and the percentage of cell survival in the cell suspension was measured by mixing the equal ratio of trypan blue (Sigma Aldrich, USA) with the hemocytometer slide using the optical microscope. After ensuring that cell contamination has been properly avoided, testing samples were collected using the cells with viability higher than 90% (Aydemir et al., 2015).

### Evaluation of cytotoxicity of the *Ziziphora tenuior* L. plant by MTT assay

To evaluate the effect of *Z. tenuior* extract on growth and proliferation of cancerous lung cells, MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (Sigma, Germany) colorimetric method was used. This method is a mitochondrial metabolic test based on the breakdown of tetrazolium salt by the mitochondrial succinate dehydrogenase enzyme of living cells. In order to conduct positive control observation, cells were treated with colchicine in a process within which, 100 µL culture medium containing five to eight thousand of each cell type was separately

placed in each well of a 96-well plate. After 24 hours of incubating, the concentrations of 200, 150, 100, 50, 10, 1, 0.1 µg/mL of the extract were diluted with PBS buffer and added to the cells and incubated over 24, 48 and 72 hours. Subsequently, the colchicine compound was added to each separate microplate of the cultivation of A549 and HEKcells, which were incubated for 24, 48 and 72 hours. After the above mentioned time, twenty µL tetrazolium with five mg/mL concentration was added to each well of the plate and incubated for 3 hours in the dark. Afterward, the media containing MTT was carefully removed, and 150 µL diluted DMSO solution was added to each well of the plate to dissolve the purplish formazan (Li et al., 2016; Lu et al., 2015). Following the incubation for 15 minutes at room temperature, the optical absorption of each well was read using an ELISA device in wave length of 490-630 nm (Lu et al., 2015).

The results are reported as the percentage of cell survival against the concentration of the extract. The percentage is calculated as follows, where all stages of the test were repeated three times (Bendale et al., 2017).

- Cell viability rate =  $100 \times (\text{optical density of test} / \text{optical density of control})$

Using the absorbance readings in the A549 and HEKcell lines, the survival rate was measured after exposing the extract of the plant and the colchicine as a positive control and the completion of the MTT test. IC<sub>50</sub> (the concentration at which a particular drug inhibits 50% of cell growth *in vitro*) was estimated by the nonlinear regression equation of cell growth versus the concentration derivative.

### RNA extraction and C-DNA synthesis

RT-PCR provides a quick, diverse, and extremely sensitive way to examine the expression of the desired gene. It can also provide semi-quantitative information about the amount of gene expression. The basis of RT-PCR is a reverse transcriptase enzyme, RNA-dependent DNA polymerase, and its ability to construct complementary DNA strands based on the template mRNA. First, A549 and HEKcells were cultured in 25

mL flasks with appropriate culture medium and 10% calf serum, and after complete cell growth for a count of four to six million cells, the selected doses of the extract obtained from the MTT test, 1 and 50 µg/mL were added to the flasks and incubated for 24, 48 and 72 hours (each dose was added to three flasks). After a while, the supernatant containing the culture medium and the extract was removed. The cells were washed once with PBS buffer prior to carrying out the trypsinization process. Subsequently, the cell suspension was provided. The steps for extracting RNA are as follows (using Cinnapure KIT from Sinaclone Company).

The cell suspension prepared with the culture medium, was centrifuged for 5 minutes at about 3000 rpm. The supernatant was then discharged and mixed with the PBS buffer. Then the solution was centrifuged for 5 minutes at the same rotational speed.

After emptying the overcoat, 400 λ of lysis solution was added, and the solution was vortexed for a minute. To ensure the homogeneity of the solution, it was emptied and refilled consecutively ten times in the container using a syringe with 20 gauge injection needle. Three hundred λ of precipitation solution was added and transferred to the pillar and centrifuged for a minute at 12000 rpm. Similarly, 400 λ solution of washing buffer-I was added and again centrifuged at 12000 rpm.

After previous steps, 400 λ washing buffer-II was

added, and the solution was then centrifuged one minute at the same rate.

The same process was repeated using buffer-II. After being centrifuged for two minutes, the column was placed in a new microtube where 50 λ of RNase-Free distilled water; previously heated to 55 °C; was added to it. The solution was kept at 55 ° for 3-5 minutes, then to extract the RNA, it was centrifuged for 12 minutes at 12000 rpm.

The procedure for preparing the RNA primer medium is fairly simple. 0.2 µL microtubes were used in which we placed seven µL of extracted RNA, one µL of oligo(dt) primer, one µL of random hexamer primer, one µL of dNTP and ten µL of distilled water. The tubes were then placed in the PCR device, and the process was carried out by setting it upon “synthesis c-DNA” mode (Paul-Samojedny et al., 2005).

**Real-time PCR**

As previously mentioned, the examined genes are BAX, Bcl-2 and the housekeeping gene (used for the Beta Actin gene). 12.5 µL of Sybergreen, one µL of synthesized cDNA (from different doses), one µL of the forward primer of mentioned genes separately, one µL of the reverse primer of the genes (Table 1) mentioned above (9.5 µL of distilled water was added so that the final volume reached 20 µL). Forty-five cycles are performed on the real-time PCR machine. Eventually, the analysis and calculation were performed with the REST software (Paul-Samojedny et al., 2005).

**Table 1.** A sequence of Primers for used genes (prepared by Primer-BLAST Software)

Gene	Sequence	Size	Temperature
BAX	Forward: CCT GTG CAC CAA GGT GCC GGA ACT	24 mer	68.4
	Reverse: CCA CCC TGG TCT TGG ATC CAG CCC		67.7
Bcl-2	Forward: TTG TGG CCT TCT TTG AGT TCG GTG		62.8
	Reverse: GGT GCC GGT TCA GGT ACT CAG TCA		65.2
B-Actin	Forward: GCA CCA CAC CTT CTA CAA TG	60	
	Reverse: TGC TTG CTG ATC CAC ATC TG	60	

## RESULTS

### The effect of methanolic extract of *Ziziphora tenuior* L. on the growth of A549 and HEK cells at 24-48-72 hours

A549 and HEK cells were treated with 200, 150, 100, 50, 10, 1 and 0.1 µg/mL *Z. tenuior* L. extract for 24, 48, 72 h respectively. MTT assay showed that various doses of *Z. tenuior* exert a significant inhibitory effect on the proliferation of A549 cells, whereas these doses did not affect HEK cell growth. In addition, the plant extract inhibited A549 cell proliferation compared to the control group (0 µg/mL).

After 24 hours, the percentage of live A549 cells having been exposed to different concentrations of *Z. tenuior* extract reduced significantly. Tested doses were proved to be effective in lowering A549 cells ( $p < 0.05$ ) except for doses of 200, 150 and 100 µg/mL as can be seen in Figure 1.

After 48 hours, there has been a marked difference between the control group (0 concentration) and A549 treated groups with *Z. tenuior* extract ( $p < 0.05$ ). All doses of *Z. tenuior* extract inhibited the growth of A549 cancer cells in 48 hours. Furthermore, none of

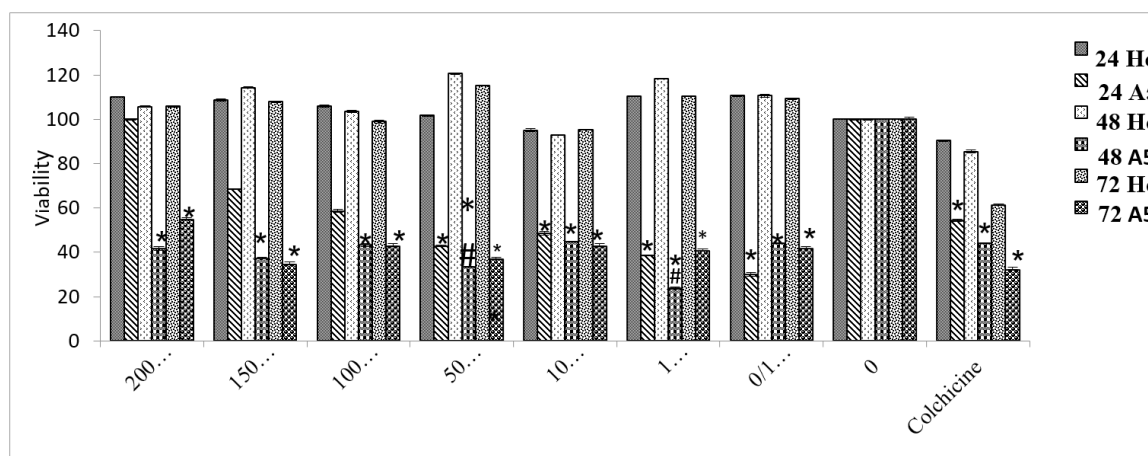
them affected HEK cells.

At 72 hours, there is a significant difference among all groups with the control group ( $p < 0.05$ ). All doses have been effective in reducing the A549 cell count with no meaningful effect on HEK cells death (Figure 1).

The normal HEK cell's survival rate was high once being exposed to the plant extract, which means that normal cells are not affected by the extract. Moreover, the longer the treatment time of the cells, the fewer changes were observed with the plant extract.

HEK healthy cells survival rate at exposure to 50 µg/mL and 1 µg/mL extract of the *Z. tenuior* showed that the extract does not have a destructive effect on the survival for the HEK cells (Figure 1); however, these doses have an inhibitory effect on the cells of the A549 in comparison to control group. In addition, these doses of the plant extract were more effective than colchicine to inhibit A549 cell viability (Figure 1).

Furthermore, IC<sub>50</sub> values for A549 cells were 9.06, 6.32 and 6.48 µg/mL, and for HEK cells 41.90, 34.82 and 21.31 µg/mL, respectively in 24, 48 and 72 hours for both cases.



**Figure 1:** The effect of different concentrations of *Z. tenuior* extract on A549 cancer cells and HEK healthy cells at 24, 48, 72 hours

\*  $P < 0.05$  difference between *Z. tenuior* extract received groups or colchicine group and control group (concentration 0).

#  $P < 0.05$  difference between *Z. tenuior* extract received groups and the colchicine group.

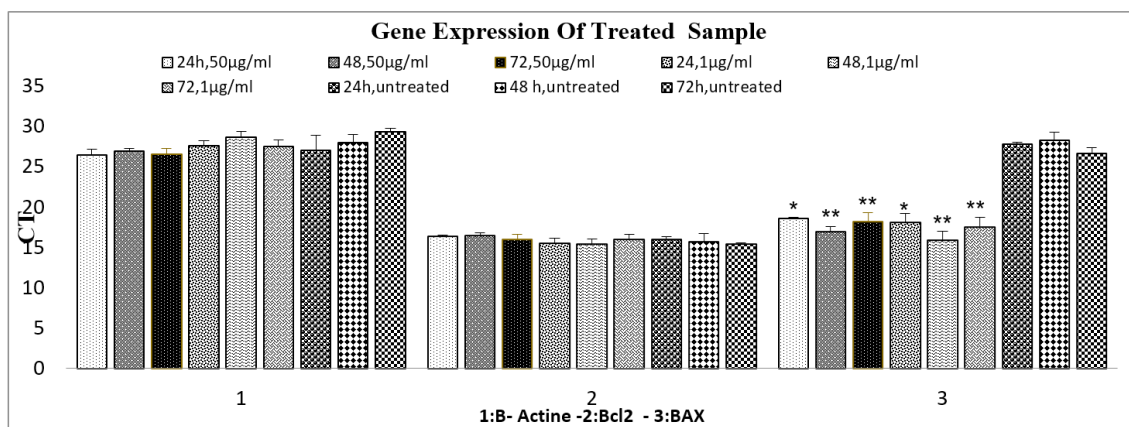
**The effect of methanol extract of *Z.tenuior* on BAX and Bcl2 genes expression**

Regarding the more effective doses to inhibit A549 cells growth in comparison to control and colchicine groups, doses of 50 µg/mL and 1 µg/mL were selected for molecular analysis. A549 cells were exposed to 50 µg/mL and 1 µg/mL concentrations of *Z.tenuior* for 48 hours. The mRNA expression level of BAX and Bcl-2 were measured by real-time PCR.

Compared with the untreated group *Z.tenuior* extract reduced the CT count of BAX gene while it did not cause any meaningful change in CT count of Bcl-2.

The percentage of expression of the Bcl-2 gene is higher than that of BAX gene, as shown in the CT Figure, and the lower the CT count, the greater the gene expression.

Comparison of gene expression in samples A-549 treated with *Z. tenuior* showed no meaningful difference between untreated and treated groups of A-549 cancer cells with a plant extract Bcl2. The CT level obtained for BAX gene in treated groups was lower than untreated groups, leading to a higher percentage of gene expression and apoptosis. In other words, the death of cancerous cells treated with the plant has increased (Figure 2).



**Figure 2.** Comparison of gene expression in A-549 treated samples with *Z. tenuior* extract and untreated cells: Treatment of A-549 cancer cells with *Z.tenuior* plant extract at doses of 50 µg/mL and 1 µg/mL suggests the amount of CT obtained for BAX gene is less, resulting in a higher percentage of gene expression and more apoptosis.

\* P<0.05 difference between *Z.tenuior* extract received groups and untreated group.

\*\* P<0.01 difference between *Z.tenuior* extract received groups and untreated group.

**DISCUSSION**

Lung cancer is by far a leading cause of cancer death worldwide and the prevalence of this malignancy is increasing in most countries around the world. Radon gas, air pollution, stress, lifestyle factors such as diet and smoking are mentioned among the causes associated with the ascending incidence of cancer. Studies have shown that the consumption of food containing antioxidants is effective in preventing and reducing the risk of cancer (Alvanja, 2002; Parsa,

2012). Despite the use of therapeutic approaches such as surgery, chemotherapy, and radiotherapy, cancer mortality is still high in patients indicating the inadequacy of these therapeutic approaches. Also, the destructive effects of chemotherapy and radiation therapy on normal cells are among other disadvantages of these therapeutic procedures (Chabner et al.,1992). Moreover, nowadays, pharmacists and physicians are focusing more on plant origin compounds to discover new drugs for the treatment of diseases owing to

their abundance, affordability, and fewer side effects (Deshpande et al., 2008). Therefore, concerning the mentioned cases, the tendency and attention to the use of natural treatments and anticancer drugs have increased dramatically in recent years. A case in point regarding the medicinal herbs is the family of Lamiaceae, among which *Z. tenuior* has been investigated in this study.

Medicinal plants are a great source of natural antioxidants which are used to improve health. *Rhazya stricta* Decne is higher in antioxidants and has more active antiproliferation than *Trigonella foenum-graecum* L. and *Cassia acutifolia* Delile in cancer cells (Al-Dabbagh et al., 2018). *Z. tenuior* is an aromatic plant that has strong antioxidant activity (Dakah, 2017) which could be linked to an antiproliferative effect on A549 cancer cells.

The anticancer property of *Z. tenuior* could be associated with its essential oil being highly rich in the organic compound pulegone. Bearing in mind that most monoterpenes compounds, including pulegone are anticancer agents (Andrade et al., 2015). *Z. tenuior* essential oil has, in fact, the highest percentage of pulegone in comparison to the other members of its family (71.2 to 85 % pulegone, 5.1 to 7.8 % limonene monoterpenes) (Ghasemi Pirbalouti et al., 2013). Because of the poor coverage of *Z. tenuior* effects on lung cancer cells in literature, this study was conducted where the effect of methanol extract of *Z. tenuior* on the proliferation of lung cancer cells of A-549 group was investigated and the expression of BAX and Bcl-2 genes in the apoptotic process of these cancer cells was evaluated. The results of this study revealed that the treatment of A549 cancer cells with methanol extract of *Z. tenuior* decreases the proliferation of these cells (Figure 1) and inducing apoptosis (Figure 2). However, this extract did not inhibit the growth of normal HEK cells (Figure 1). IC<sub>50</sub> values of HEK cells were higher than A549 cells causing a higher toxicity of the extract in A549 cancer cells compare with HEK cells.

Another species of *Ziziphora* genus is *Ziziphora clinopodioides* Lam that has anticancer effects on AGS

gastric cancer cells. Aqueous extract of its aerial parts induced 50 % cell growth inhibition with 2.356, 1.779 and 1.674 mg/mL in 24, 48 and 72 hours. *Z. clinopodioides* significantly decreased AGS cell viability in doses of 5, 2 and 1 mg/mL in 48 and 72 hours (Ghazanfari et al., 2013). In our study IC<sub>50</sub> values against A549 cells were 9.06, 6.32 and 6.48 µg/mL, in 24, 48 and 72 hours and the values of 50 µg/mL and 1 µg/mL were more effective to inhibit A549 cell viability. According to the results, *Z. tenuior* extract has more cytotoxic effects than *Z. clinopodioides*.

The study has shown *Scutellaria barbata* D. Don ethanolic extract has antitumor activity in A549 cell line (Yin et al., 2004).

Ethanol extract of aerial part of *Adenosoma bracteosum* Bonati has anticancer activity on human large cell lung carcinoma (NCI-H460) and hepatocellular carcinoma (HepG2) cell lines (Nguyen et al., 2020). In another study, *S. barbata* ethanol extract inhibited A549 cell growth with IC<sub>50</sub> of 0.21 mg/mL (Yin et al., 2004). In our study, methanol extract of *Z. tenuior* also induced the death of A549 lung cancer cells with IC<sub>50</sub> values of 9.06 µg/mL in 24 hours. The IC<sub>50</sub> values of ethanol extract of *A. bracteosum* on HepG2 and NCI-H460 was 39.15 ± 0.61 and 30.31 ± 1.60 µg/mL. The anticancer mechanisms of *S. barbata* extract include cell apoptosis and cytotoxic effects plus the downregulation of CD209 related to dendritic cell (Yin et al., 2004) The mechanism of *A. bracteosum* extract was the attenuation of mitochondrial membrane potential and inducing the activation of caspase-3 in both human lung and liver cancer cells (Nguyen et al., 2020). In our study, the mechanism of the extract was induction of BAX gene expression. Comparing this study and previous mentioned studies shows that cytotoxicity of *Z. tenuior* was higher and had different mechanism. Apoptosis is a highly regulated process that plays a significant role in maintaining homeostasis in multi-cell organisms (Cory et al., 2002). Previous studies have shown that apoptosis is controlled by many external and intracellular factors, among which the balance between Bcl-2 (inhibitor of

apoptosis) (Karakas et al., 2014) and BAX (inducer of apoptosis) has been identified (Kowalczyk et al., 2019) as the most important determinant of the cell's fate in response to the extracellular stimulus (Boise et al., 1993). It was shown that BAX gene expression altered in non-small cell lung cancer compared to normal tissue (Porebska et al., 2006).

The results of our study indicate that *Z. tenuior* extract increases the expression of the BAX gene mRNA considerably ( $p < 0.01$ ). One of the anticancer mechanisms of the extract achieves by up-regulation of BAX gene. BAX gene leads to the release of cytochrome C and activates apoptosis (Antonsson et al., 2000).

Our study hence argues that the extract of this plant possesses antitumor effects and has a novel inhibitory effect on the A549 class through apoptosis that is one of anticancer properties of medicinal plant extract.

#### CONCLUSION

As a summary, the results of this study confirmed the apoptotic properties of *Ziziphora tenuior* L. and the effectiveness of this herb against the lung cancer cell line A549. Naturalness, low cost, and public access to this plant are among its benefits. According to the results, *Z. tenuior* methanolic extract showed an inhibitory effect on the cells of the A549 in all doses. Moreover, the extract was more effective than the colchicine group in comparison to the control group in the doses of 50  $\mu\text{g/mL}$  and 1  $\mu\text{g/mL}$ . In addition, the extract induced BAX gene expression in A549 lung cancer cells *in vitro*. It seems that the use of this herbal drug may reduce the incidence of lung cancer and its side effects. This, however, needs more research, especially under *in vivo* conditions.

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#### CONFLICT OF INTEREST

The authors declare that no conflict of interest exists

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