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The herbal supplement induces G2 arrest and apoptosis in A549 cells in vitro

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Abstract

In treating cancer, an important health problem, alternative treatment approaches with herbal content continue to be investigated. The study was carried out to determine an herbal supplement's cytotoxic effect and anticancer potential on lung cancer cells. Human lung adenocarcinoma A549 cells were treated with the herbal supplement and cisplatin. The supplement's growth inhibitory and anticancer potential were determined by MTT assay, Annexin V–PI apoptosis assay, cell cycle analysis, and real-time PCR analysis of the apoptosis and cell cycle-related genes. The herbal supplement was determined to have significant growth-inhibitory effects on A549 cells. Results of the apoptosis and cell cycle analyses showed that the supplement caused a significant increase in the rate of late apoptotic cells and induced G2 arrest. Gene expression analysis results showed that pro-apoptotic BAK1, BAX, APAF1, and cell cycle inhibitor CDKN1A significantly increased mRNA levels without altering BCL2 and TP53 expressions in A549 cells. Our experiments have shown that the herbal supplement has a potential anticancer effect on the lung cancer A549 cell line through growth inhibition and apoptosis. However, more in vitro studies and in vivo experiments are needed.

Keywords: herbal supplement, cancer, apoptosis, cell cycle, antiproliferative

1. Introduction

Cancer continues to be an important health problem worldwide. Although there has been an increase in the survival rate for most cancer types in recent years, it has been reported that this rate is lower for lung cancer than for other cancers due to its advanced-stage diagnosis (1). Lung cancer is the second leading cause of cancer-related mortality worldwide, with more than 1.6 million patients diagnosed with lung cancer each year. Most lung cancer patients are diagnosed at an advanced stage and have been associated with an overall survival of about five years. Despite advances in lung cancer treatment, no effective treatment has yet been developed (2). Although the chemotherapy regimen is an effective therapeutic approach in cancer treatment (3, 4), it is known to cause serious side effects such as neutropenia (5, 6). It has also been reported that chemotherapy agents have cytotoxic effects on cancer cells as well as similar toxic effects on healthy cells (7). This negatively affects the quality of life of cancer patients, causes severe side effects, and may even cause non-cancer deaths. However, alternative approaches to cancer treatment are also being developed, and various studies have been reported on the effect of different herbal products on cancer (8-10).

Using supportive herbal formulas is quite common among cancer patients. It is believed that herbal therapies will generally cause fewer side effects for individuals and are effective in obtaining more successful results in the treatment (11). A natural nutrient mixture containing lysine, proline, arginine, ascorbic acid, and green tea extract has been shown to suppress lung carcinoma xenografts in mice (9). Similarly, it has been reported that a rich nutritional supplement containing lysine, proline, arginine, ascorbic acid, green tea extract, N-acetyl cysteine, selenium, copper, and manganese has preventive potential on the development of ureteral lung cancer in mice (12). With this, the search for alternative treatments for cancer treatment, especially lung cancer, continues. In this study, the cytotoxic effect of a commercially available herbal supplement on lung cancer cell lines was investigated. The commercially available product that we refer to as the Diverse Mixture of Herbs (DMH) has been reported that various plants such as Commipharis myrha (Myrrh), Curcuma zedoaria (Cedvar), Elettaria cardamomum (Cardamom), Olea europaea (Olive), Ernyngium (Bullthorn), Illicuim verum (Star anise), Cinnamomum verum (Tarcin), Mysristica and Crocus sativus (Saffron)are prepared by soaking in ethyl alcohol. In addition, as a result of the chemical analysis, it was determined that the product contains YM-53601, L-Arginine, Betaine, Benzanthrone, Lupanine, Germanaism B, Sarpangine, 4-Hydroxycoumarin, Amigdalin, Phenylacetic Acid, Derrustone, Ichtynone, a-Asaron, Visnadin, 16-Fenoxy-o -tetranor PGE2; Osthol, Anthralin, n-Pentadecylamine, 2-Methoxyxanthone, 13-Docosenamide, Sparfloxacin and Triphenylphospha (13). The anti-tumor potentials of YM-53601 and osthol in the content of the product have been supported by various studies (14, 15). In addition, it has been reported that this herbal mixture contains a high concentration of amygdalin compared to other

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components, and amygdalin, a natural glycoside product, inhibits the proliferation of some cancer cells and is important in alternative cancer treatment (16-18). Again, the results of the study performed on lung cancer cell lines revealed the potential therapeutic efficacy of amygdalin for lung cancer by inhibiting the proliferation and migration of cancer cells (19).

2. Materials and Methods

2.1. Cell culture

A549 human non-small cell lung cancer cells, a generous gift from Yeditepe University Regenerative Biology Research Group, were cultured in Dulbecco's Modified Eagle Medium - High Glucose (Sigma-Aldrich, USA) (DMEM-H) supplemented with 10% fetal bovine serum (FBS; Gibco), 100 U/mL penicillin-100 µg/mL streptomycin (Sigma Aldrich, USA) and 2.5 µg/ml amphotericin B (Sigma Aldrich, USA) at 37°C in a humidified atmosphere of 5% CO_2.

2.2. Cell viability assay

A549 cells were seeded in 96-well plates in at least three technical replicates at a density of $1x10^4$ per well. After seeding, the cells were incubated in a humid 37°C incubator containing 5% CO_2 to adhere to the surface. Cells were exposed to 4%, 8%, and 32% (v/v) DMH herbal mixture prepared in DMEM-H for 24 hours. The herbal mixture was filtered through a 0.22 µm filter before being prepared in the medium. For vehicle control, ethanol 2% was used as in the herbal mixture. The medium of the control group was also refreshed. After 24 hours of incubation, 10 µl MTT reagent (5 mg/ml stock solution) was added to each well, and cells were incubated in the incubator for 4 hours. Then 100 µl of solubilization solution (0.01 N HCL containing 10% SDS) was added to the wells. After 16 hours, absorbances were measured at 570 nm in a microplate reader.

2.3. Apoptosis assay

To determine the apoptotic effect of the herbal mixture at the concentration selected (8% (v/v)) as a result of the cell viability assay, A549 cells were seeded in 6-well plates at a density of $15x10^4$ per well and incubated for 24h. After the incubation, cells were treated with the herbal mixture for 24 h. Ethanol 2% was used as vehicle control. 20 µM of cisplatin (CDDP), which is close to the therapeutic upper limit cisplatin concentration (approximately 16.6 µM) (20) was used as a positive control. An unstained control was also included to set the acquisition gate for the population of interest. Then, cells were harvested and labeled following the BD Pharmingen™ FITC Annexin V Apoptosis Detection Kit I (BD Biosciences, USA) protocol guidelines. All samples were run on a BD FACSCalibur™ (BD Biosciences, USA) flow cytometer. The results were analyzed using a free online tool Floreada.io (USA).

2.4. Cell cycle analysis

A549 cells were plated in 6-well plates at a seeding density of $15x10^4$ cells/well and incubated for cells to adhere to the surface. Then, the culture medium was aspirated from wells, and cells were washed with 1x PBS. After washing, the starvation medium was added to wells, followed by 24 hours of incubation. Then, the media were replaced with respect to the experimental groups of control, CDDP 20 µM, and DMH %8. Following incubation of the cells for 24 h, A549 cells were stained according to the Cell Cycle Analysis Kit (PromoCell, Germany) instructions. Flow cytometry was performed on BD FACSCalibur™ (BD Biosciences, USA). Output files were analyzed using a third-party software.

2.5. Gene expression analysis

A549 cells were treated with the herbal mixture at a concentration of 8% (v/v) in a 6-well plate for 24 h. 20 µM cisplatin was used as a positive control, and the medium of the control group was renewed. At the end of 24 hours of incubation, cellular RNA isolation was performed by the Quick-RNA™ MiniPrep Kit (Zymo Research, USA) according to the kit's protocol. 5 µg of isolated RNAs were transformed to cDNA using the Protoscript First Strand cDNA Synthesis Kit (New England Biolabs, USA) according to the manufacturer's instructions for use. 120 ng of cDNAs were utilized for each real-time PCR (qPCR) reaction.

HNRNPL was used as the housekeeping gene, and the whole primer sequences are shown in Table 1. qPCR reaction mixes were prepared using Maxima SYBR Green/ROX qPCR Master Mix (Thermo Fisher Scientific, USA). The reactions were run in a qPCR instrument (Applied Biosystems™ 7500 Fast Real-Time PCR System (Thermo Fisher Scientific, USA)). Cq values were analyzed using DataAssist™ v3.01 software (Thermo Fisher Scientific, USA). Then, the gene expression data were log2 transformed (Log2RQ) for normalization.

Table1. Human primer sequences used for qPCR

Gene Symbol	Primer Sequences (5'-3')	Reference
APAF1	F: GCCAAGCAGGAGGTCGATAATG R: GACCATCCTCAGAAAAGCAGGC	(25)
BAK1	F: TTACCGCCATCAGCAGGAACAG R: GGAACTCTGAGTCATAGCGTCG	(26)
BAX	F: TCAGGATGCGTCCACCAAGAAG R: TGTGTCCACGGCGGCAATCATC	(25)
BCL2	F: ATCGCCCTGTGGATGACTGAGT R: GCCAGGAGAAATCAAACAGAGGC	(27)
CDKN1A	F: AGGTGGACCTGGAGACTCTCAG R: TCCTCTTGGAGAAGATCAGCCG	(25)
CYCS	F: ACCTTCCATCTTGGCTAGTTGTG R: ATCGCTTGAGCCTGGGAAATAG	(28)
HNRNPL	F: GTGTGGTGGAAGCAGACCTTGT R: CAAACTCCACCAGTGCTTGTCTC	(29)
TP53	F: CCTCAGCATCTTATCCGAGTGG R: TGGATGGTGGTACAGTCAGAGC	(25)

2.6. Statistical analysis

All statistical calculations were conducted using Prism software, v7 (GraphPad Software Inc., USA). While one-way ANOVA, with Tukey test, was performed in the cell viability and apoptosis analyses, two-way ANOVA followed by Tukey test was used for the gene expression analysis and p values were given in GraphPad Style. The graphical representations of the results were prepared using GraphPad Prism v7 ModFit LT™ v5 was also used to visualize the cell cycle analysis results in addition to GraphPad Prism v7

3. Results

3.1. Herbal mixture causes the cytotoxic effect on A549 cells

The cytotoxic effect of the herbal supplement mixture on A549 cells was determined by an MTT assay. Cells were exposed to DMH at different concentrations (4%, 8%, and 32%) for 24 hours. The effective and relatively low concentration of the applied product was determined. Although no statistically significant change was observed in cell viability at the DMH concentration of 4%, cell viability was significantly decreased by DMH treatment at concentrations of 8% and 32% after 24 hours ($p < 0.0001$(****)) (Fig. 1A). The relatively low dose DMH concentration of 8% was further compared with the vehicle control, and approximately 55% inhibition in cell viability was observed ($p < 0.0001$(****)) (Fig. 1).

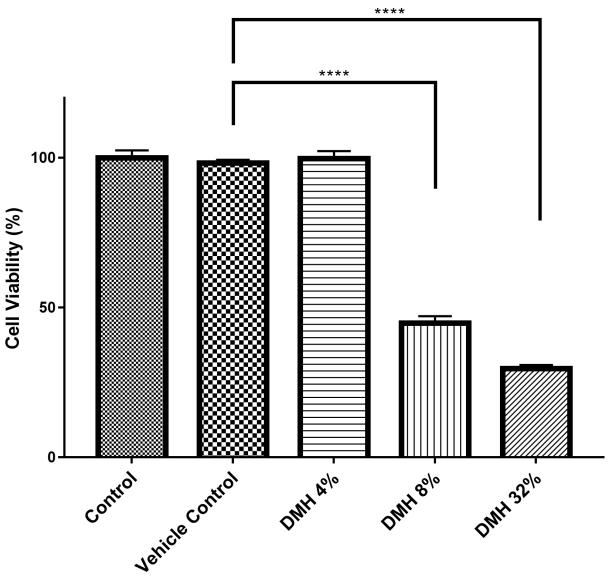

Fig. 1. MTT assay results of cytotoxic effect of DMH (the Diverse Mixture of Herbs) on A549 cells at different concentrations ($p < 0.0001$(****))

3.2. The apoptotic induction of the herbal mixture on A549 cells is greater than that of cisplatin.

Annexin V-PI staining followed by flow cytometry was performed to investigate the apoptotic effect of DMH on A549 cells. Flow cytometric dot plot diagrams of one replicate of every sample are represented in Figure 2A. In the A549 vehicle control group, the proportion of mean viable cells was detected at 89.33%, mean early apoptotic cells at 1.36%, mean late apoptotic cells at 4.22%, and mean necrotic cells at 5.09%. The percentage of mean viable, early apoptotic, late apoptotic, and necrotic A549 cells in the DMH 8% treatment group were 38.34%, 11.01%, 36.18%, and 14.48%, respectively. Compared to vehicle control, DMH induced a significant increase in early, late apoptotic, and necrotic cells ($p = 0.0004$ (***), $p < 0.0001$ (****), and $p = 0.0005$ (***), respectively) (Fig. 2.).

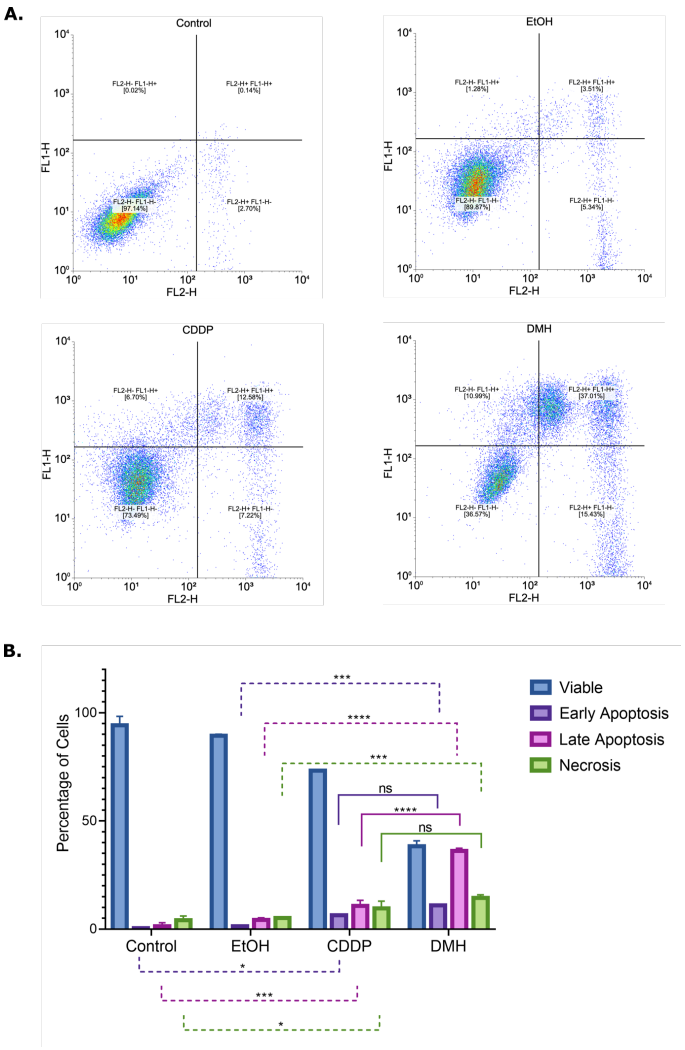

Fig. 2. Flow cytometric apoptosis analysis in A549 cells treated with EtOH 2%, CCDP 20 µM, or DMH 8% for 24 hours. (A) Representative dot plots of Annexin V (y-axis) vs PI (x-axis). Viable cells are shown in the lower left quadrant, cells in the upper left quadrant represent early apoptotic cells, late apoptotic cells are shown in the upper right quadrant, and cells in the lower right quadrant represent necrotic cells. (B) Percentage of viable, early apoptotic, late apoptotic, and necrotic cells. Asterisks show statistically significant differences between the vehicle control (EtOH) and DMH treatment group. ***, ****, *** indicate Tukey's corrected p values of 0.0004, 0.0001, and $p = 0.0005$, respectively

3.3. DMH induces G2 phase cell cycle arrest

The effect of DMH on the cell cycle was analyzed using flow cytometry. While cisplatin at 20 µM, which we used as a positive control in the study, caused the cells to accumulate in the G1 phase (Figure 3B), DMH at an 8% concentration induced G2 phase arrest in A549 cells dramatically after 24 hours (Fig. 3).

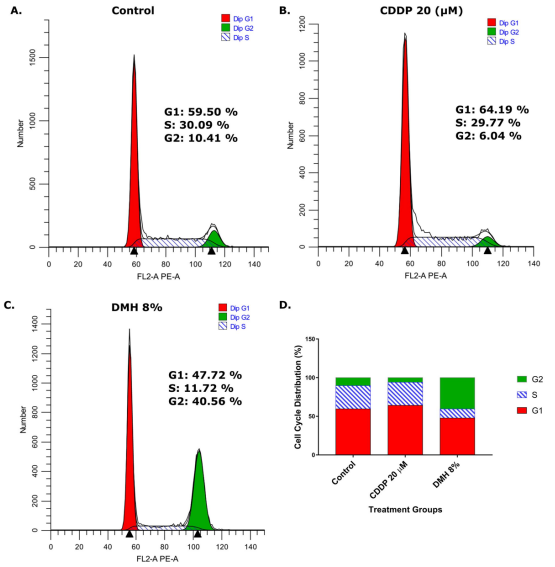

Fig. 3. Cell cycle analysis by flow cytometry in A549 cells. (A-C) Cell cycle histograms of A549 cells in untreated and treated groups. (A) control group; (B) cells treated with CDDP 20 µM or (C) DMH 8% for 24 hours. (D) Cell cycle distributions of the control and experimental groups were summarized in the bar graph

3.4. DMH increases the transcription of pro-apoptotic genes without significant effect on *BCL2* and *TP53* mRNA levels

Quantitative PCR results demonstrated that DMH 8% significantly elevated mRNA levels of pro-apoptotic *BAK1* ($p < 0.0001$), *BAX* ($p = 0.0076$), *APAF1* ($p = 0.0075$), and cell cycle inhibitor *CDKN1A* ($p < 0.0001$) without leading to significant changes in *BCL2* and *TP53* expressions in A549 cells. On the other hand, as expected, 20 µM of cisplatin upregulated the expressions of pro-apoptotic *BAK1*, *BAX*, *APAF1*, and cell cycle-related genes *CDKN1A* and *TP53* while downregulating anti-apoptotic *BCL2* expression significantly (Fig. 4).

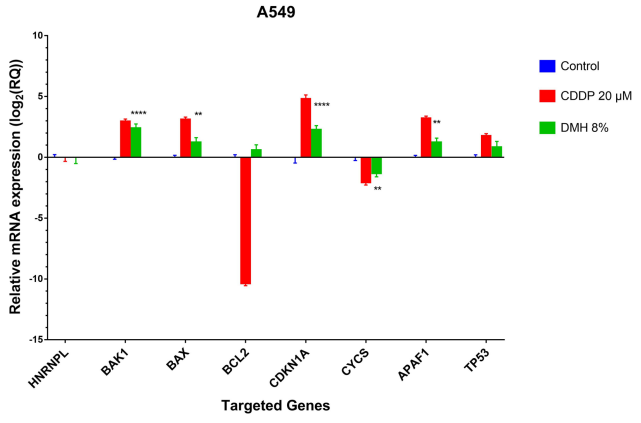

Fig. 4. Mean log2 relative quantity (log2(RQ)) values of gene expressions of apoptosis- and cell cycle-related biomarkers. Relative expression levels of the genes were normalized to *HNRNPL*. Asterisks above the boxplots indicate significant differences between the control and DMH-treated groups. More asterisks imply a higher significance level. Error bars represent standard deviations

4. Discussion

The main treatment approaches for lung cancer are chemotherapy, radiotherapy, and surgery. In addition, immunotherapy and targeted therapies have gained importance in recent years. Although radiotherapy, chemotherapy, and surgery are important in treating early-stage lung cancer patients, there is a risk of cancer recurrence after treatment. Thus, searching for more effective and tolerable treatment alternatives has been going on for many years (21). Numerous studies have shown the anticancer activities of different herbal products used on cancer cells (22, 23). In our study, the cytotoxic effect of the commercially available herbal mixture on A549 cells was observed.

The apoptotic mechanism is important in the control of cell number in healthy cells and is known to be impaired in cancer cells (24). Apoptosis induction is one of the most significant hallmarks of cytotoxic antitumor agents. Many natural herbal compounds act by inducing apoptotic pathways in cancer cells that escape apoptosis through various mechanisms in cancer cells. Various studies have shown that many patients struggling with cancer often use herbs or herbal products as supportive treatments. It is also known that some chemotherapeutic drugs, such as paclitaxel are used as plant-derived anticancer agents (25). In this study, it was observed that the herbal mixture DMH caused a significant increase, especially in the rate of late apoptotic cells, inducing cell death. Important regulatory mechanisms of the cell cycle are checkpoints. The occurrence of cell cycle arrest during cell division represents the difficulty in repairing damage and error during cell division and is a targeted outcome in cancer therapy. Cell cycle arrest in the G2/M phase indicates that intracellular DNA damage is challenging to repair, and many studies have shown that different herbal products induce G2/M arrest in cancer cells (26-28). Our cell cycle analysis results, which we carried out within the scope of the study, showed that DMH induced G2 arrest in A549 cells in conformity with the literature.

The gene expression analysis results demonstrated that DMH significantly increased the mRNA levels of pro-apoptotic *BAK1*, *BAX*, *APAF1*, and cell cycle inhibitor *CDKN1A* without changing *BCL2* and *TP53* expressions in A549 cells. Induction of p21 (*CDKN1A*) in the absence of p53 has been demonstrated in the studies of distinct p53-mutant mouse and human cancer types (29, 30). There has been no reported decrease in Bax and Apaf-1 expression levels compared to wild-type in p53-null hematopoietic cancer cells. Furthermore, apoptosis can be induced independently of p53-dependent transcription, as shown in studies on some human and mouse cancer cell lines (31). In another study, it has been demonstrated in a gastric cancer cell line that Bak overexpression, independent of p53, promotes apoptosis (32). One of the important mechanisms in the apoptotic pathway is the formation of apoptosomes associated with the intrinsic pathway. In the intrinsic pathway, Cytochrome c binds to and activates Apaf-1. Then, with the addition of ATP, a complex called the apoptosome is formed (33). In the mechanism of apoptosis, it has been reported that Cytochrome c will induce apoptosis in any cell to which it is delivered (34). In our study, a statistically significant increase in the expression level of

CYCS was observed in the DMH administered group, in line with the increase in the rate of apoptosis.

Considering all these data, we concluded that DMH treatment on A549 cells caused G2 cell cycle arrest by increasing *CDKN1A* expression and subsequently elevated *BAX*, *BAK1*, and *APAF1* expressions, leading to an anti-apoptotic *BCL2* threshold resulting from *TP53* stabilization to be exceeded, thus induced late apoptosis.

Conflict of interest

The authors declared no conflict of interest.

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Authors' contributions

Concept: M.Y., Design: M.Y., C.G., Data Collection or Processing: M.Y., C.G., Analysis or Interpretation: C.G., Literature Search: M.Y., C.G., Writing: M.Y., C.G.

Ethical Statement

Ethics committee permission is not required fort his study.

References

1. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2019. CA: a cancer journal for clinicians. 2019;69(1):7-34.
2. Baharuddin P, Satar N, Fakiruddin KS, Zakaria N, Lim MN, Yusoff NM, et al. Curcumin improves the efficacy of cisplatin by targeting cancer stem-like cells through p21 and cyclin D1-mediated tumour cell inhibition in non-small cell lung cancer cell lines. Oncology reports. 2016;35(1):13-25.
3. Cooper S, Spiro SG. Small cell lung cancer: treatment review. Respirology. 2006;11(3):241-8.
4. Yang S, Zhang Z, Wang Q. Emerging therapies for small cell lung cancer. Journal of hematology & oncology. 2019;12:1-11.
5. Smagurauskaite G, Mahale J, Brown K, Thomas AL, Howells LM. New paradigms to assess consequences of long-term, low-dose curcumin exposure in lung cancer cells. Molecules. 2020;25(2):366.
6. Cao X, Ganti AK, Stinchcombe T, Wong ML, Ho JC, Shen C, et al. Predicting risk of chemotherapy-induced severe neutropenia: a pooled analysis in individual patients data with advanced lung cancer. Lung Cancer. 2020;141:14-20.
7. Zulkapli R, Abdul Razak F, Zain RB. Vitamin E (α-Tocopherol) exhibits antitumour activity on Oral squamous carcinoma cells ORL-48. Integrative Cancer Therapies. 2017;16(3):414-25.
8. Buhrmann C, Yazdi M, Dezfouli AB, Sahraneshin FS, Ebrahimi SM, Ghaffari SH, et al. Significant decrease in the viability and tumor stem cell marker expression in tumor cell lines treated with curcumin. Journal of Herbal Medicine. 2020;22:100339.
9. Waheed Roomi M, Ivanov V, Kalinovsky T, Niedzwiecki A, Rath M. In vivo and in vitro antitumor effect of a unique nutrient mixture on lung cancer cell line A-549. Experimental lung research. 2006;32(9):441-53.
10. Roomi MW, Kalinovsky T, Niedzwiecki A, Rath M. Modulation of u-PA, MMPs and their inhibitors by a novel nutrient mixture in human lung cancer and mesothelioma cell lines. International Journal of Oncology. 2013;42(6):1883-9.
11. Olaku O, White JD. Herbal therapy use by cancer patients: a literature review on case reports. European journal of cancer. 2011;47(4):508-14.
12. Roomi MW, Roomi NW, Kalinovsky T, Rath M, Niedzwiecki A. Chemopreventive effect of a novel nutrient mixture on lung tumorigenesis induced by urethane in male A/J mice. Tumori Journal. 2009;95(4):508-13.
13. Saydam F, Nalkiran H, Ozkaya S, Dirican A. Analysis of Bioactive Components of Dr. M. Healing® As a Mixed Herbal Product J Alt Med Res. 2018;3(1):102.
14. Ugawa T, Kakuta H, Moritani H, Matsuda K, Ishihara T, Yamaguchi M, et al. YM-53601, a novel squalene synthase inhibitor, reduces plasma cholesterol and triglyceride levels in several animal species. British journal of pharmacology. 2000;131(1):63-70.
15. Shokoohinia Y, Jafari F, Mohammadi Z, Bazvandi L, Hosseinzadeh L, Chow N, et al. Potential anticancer properties of osthol: A comprehensive mechanistic review. Nutrients. 2018;10(1):36.
16. Chen Y, Ma J, Wang F, Hu J, Cui A, Wei C, et al. Amygdalin induces apoptosis in human cervical cancer cell line HeLa cells. Immunopharmacology and immunotoxicology. 2013;35(1):43-51.
17. Makarević J, Rutz J, Juengel E, Kaulfuss S, Reiter M, Tsaur I, et al. Amygdalin blocks bladder cancer cell growth in vitro by diminishing cyclin A and cdk2. PloS one. 2014;9(8):e105590.
18. Park H-J, Yoon S-H, Han L-S, Zheng L-T, Jung K-H, Uhm Y-K, et al. Amygdalin inhibits genes related to cell cycle in SNU-C4 human colon cancer cells. World journal of gastroenterology: WJG. 2005;11(33):5156.
19. Qian L, Xie B, Wang Y, Qian J. Amygdalin-mediated inhibition of non-small cell lung cancer cell invasion in vitro. International journal of clinical and experimental pathology. 2015;8(5):5363.
20. Charlier C, Kintz P, Dubois N, Plomteux G. Fatal overdosage with cisplatin. Journal of analytical toxicology. 2004;28(2):138-40.
21. Wen T, Song L, Hua S. Perspectives and controversies regarding the use of natural products for the treatment of lung cancer. Cancer Medicine. 2021;10(7):2396-422.
22. Chen Y-L, Lin S-Z, Chang J-Y, Cheng Y-L, Tsai N-M, Chen S-P, et al. In vitro and in vivo studies of a novel potential anticancer agent of isochaihulactone on human lung cancer A549 cells. Biochemical Pharmacology. 2006;72(3):308-19.
23. Li W-Y, Chan S-W, Guo D-J, Chung M-K, Leung T-Y, Yu PH-F. Water extract of Rheum officinale Baill. induces apoptosis in human lung adenocarcinoma A549 and human breast cancer MCF-7 cell lines. Journal of ethnopharmacology. 2009;124(2):251-6.
24. Hu W, Kavanagh JJ. Anticancer therapy targeting the apoptotic pathway. The lancet oncology. 2003;4(12):721-9.
25. Safarzadeh E, Shotorbani SS, Baradaran B. Herbal medicine as inducers of apoptosis in cancer treatment. Advanced pharmaceutical bulletin. 2014;4(Suppl 1):421.
26. Pan Z, Zhang X, Yu P, Chen X, Lu P, Li M, et al. Cinobufagin induces cell cycle arrest at the G2/M phase and promotes apoptosis in malignant melanoma cells. Frontiers in Oncology. 2019;9:853.
27. Stan SD, Zeng Y, Singh SV. Ayurvedic medicine constituent withaferin a causes G2 and M phase cell cycle arrest in human

breast cancer cells. Nutrition and cancer. 2008;60(S1):51-60.

28. Sundarraj S, Thangam R, Sreevani V, Kaveri K, Gunasekaran P, Achiraman S, et al. γ-Sitosterol from Acacia nilotica L. induces G2/M cell cycle arrest and apoptosis through c-Myc suppression in MCF-7 and A549 cells. Journal of ethnopharmacology. 2012;141(3):803-9.

29. Zamble DB, Jacks T, Lippard SJ. p53-Dependent and-independent responses to cisplatin in mouse testicular teratocarcinoma cells. Proceedings of the National Academy of Sciences. 1998;95(11):6163-8.

30. Matsuda T, Kato T, Kiyotani K, Tarhan YE, Saloura V, Chung S, et al. p53-independent p21 induction by MELK inhibition. Oncotarget. 2017;8(35):57938.

31. Ho T, Tan BX, Lane D. How the other half lives: what p53 does when it is not being a transcription factor. International Journal of Molecular Sciences. 2019;21(1):13.

32. Tong Q-S, Zheng L-D, Wang L, Liu J, Qian W. BAK overexpression mediates p53-independent apoptosis inducing effects on human gastric cancer cells. BMC cancer. 2004;4(1):1-8.

33. Atagün G, Zafer E, ;Gürkanlı İ. Apoptoziste mitokondrinin rolü. Türk Bilimsel Derlemeler Dergisi. 2011(2):9-53.

34. Saxena M, Delgado Y, Sharma RK, Sharma S, Guzman SLPDL, Tinoco AD, et al. Inducing cell death in vitro in cancer cells by targeted delivery of cytochrome c via a transferrin conjugate. PLoS One. 2018;13(4):e0195542.

35. Roh JI, Lee J, Sung YH, Oh J, Hyeon DY, Kim Y, et al. Impaired AKT signaling and lung tumorigenesis by PIERCE1 ablation in KRAS-mutant non-small cell lung cancer. Oncogene. 2020;39(36):5876-87. Epub 2020/07/31. doi: 10.1038/s41388-020-01399-5. PubMed PMID: 32728173; PubMed Central PMCID: PMCPMC7471098.

36. Mologni L, Brussolo S, Ceccon M, Gambacorti-Passerini C. Synergistic effects of combined Wnt/KRAS inhibition in colorectal cancer cells. PloS one. 2012;7(12):e51449.

37. Bednarz-Misa I, Fortuna P, Diakowska D, Jamrozik N, Krzystek-Korpacka M. Distinct Local and Systemic Molecular Signatures in the Esophageal and Gastric Cancers: Possible Therapy Targets and Biomarkers for Gastric Cancer. Int J Mol Sci. 2020;21(12). Epub 2020/07/08. doi: 10.3390/ijms21124509. PubMed PMID: 32630408; PubMed Central PMCID: PMCPMC7349922.

38. Khosroushahi AY, Naderi-Manesh H, Yeganeh H, Barar J, Omidi Y. Novel water-soluble polyurethane nanomicelles for cancer chemotherapy: physicochemical characterization and cellular activities. J Nanobiotechnology. 2012;10:2. Epub 2012/01/10. doi: 10.1186/1477-3155-10-2. PubMed PMID: 22221539; PubMed Central PMCID: PMCPMC3286383.

39. Wu Q, Zhang H, Yang D, Min Q, Wang Y, Zhang W, et al. The m6A-induced lncRNA CASC8 promotes proliferation and chemoresistance via upregulation of hnRNPL in esophageal squamous cell carcinoma. International Journal of Biological Sciences. 2022;18(13):4824-36.