

## INVESTIGATION OF THE CYTOTOXIC EFFECTS OF LAVANDULA STOECHAS ESSENTIAL OILS IN PANCREATIC DUCTAL ADENOCARCINOMA

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**ABSTRACT.** Pancreatic cancer is one of the highest mortality types in the world and it is reported that it ranks 4<sup>th</sup> in the latest data. In recent years, studies on the treatment of the disease has focused on active substances derived from natural products. Lavender plant is known to have many biological activities including cytotoxic effects in many cancer studies. The aim of this study is to investigate the cytotoxic efficacy of essential oils of *Lavandula stoechas* (*L. stoechas*) extracted by water distillation in pancreatic ductal adenocarcinoma. Pancreatic adenocarcinoma cells (AsPC-1) were produced on high glucose DMEM medium and separated into control and *L. stoechas* essential oil applied group (LS). *L. stoechas* essential oil was obtained by water distillation method and applied to cells at different concentrations (0.1, 0.25, 0.5 and 1 µg/ml) for 24 hours. Cell viability tests were performed by MTT method and effective concentration was determined. Expression levels of EGFR and K-ras oncogenes were investigated by real-time quantitative polymerase chain reaction from mRNA samples. Viability tests showed that LS has the effective concentration of 1 µg/ml concentration. In gene expression analysis, it was found that effective concentration of LS downregulates the expression of both EGFR and Kras oncogenes. In the light of all these data, it was concluded that essential oil components of *L. stoechas* could be considered as a potential agent in the regression of PDAK pathogenesis. In order to be able to say this subject in full, in vivo and further studies are needed.

**Keywords:** Pancreatic ductal adenocarcinoma, *L. stoechas*, cytotoxicity, K-ras, EGFR.

### INTRODUCTION

Cancer is a complex disease characterized by uncontrolled proliferation and accumulation as a result of deterioration in the DNA structure of cells [1]. Affected cells also have the ability to spread to other tissues and organs. The synthesis of macromolecules required for cell division, which occurs quite rapidly, occurs with many oncogenic mutations. The molecular mechanisms of carcinogenesis have still not been fully elucidated in studies [2]. While substances such as cigarettes, alcohol consumption, coal dust and benzene cause cancer chemically; mechanical traumas, sun rays, heat and ionizing radiation can cause cancer physically. Agents such as T-cell leukemia virus, *Helicobacter pylori*, and human papilloma virus can cause cancer biologically [1, 3]. In the worldwide, pancreatic cancer is the leading cause of cancer-related deaths. In

pancreatic cancer, whose only curative treatment is surgical resection, resection can be performed in a small number of patients, since most of the cases have locally advanced or metastasized [4].

One of the important oncogenes involved in the formation of pancreatic cancer, as in many cancers, is the Ras gene group. There are three members of the Ras gene family: H-Ras, N-Ras and K-Ras. In pancreatic cancer research, the K-Ras oncogene product and its related signaling pathway are one of the most important topics that have been studied extensively [5].

K-ras gene mutation, which is detected in 20% of all human cancers, is the most common mutation in pancreatic cancer with a rate of 95%. The mutation that occurs in the K-ras gene is a point mutation that usually occurs in the 12th codon, rarely in the 13th and 61st codons [6]. There is also increased expression of epidermal growth factor (EGF) and its receptor (EGFR), other tyrosine kinase receptors and other ligands and transforming growth factor beta (TGF- $\beta$ ) isoforms in pancreatic cancers, especially in pancreatic ductal adenocarcinoma [7].

Lavender is an important member of the *Laminaceae* family. *Lavandula* species are commonly found in the Mediterranean region, France, Spain and Italy [8, 9]. It has been reported that the compounds of different essential oil components such as  $\alpha$ -pipin,  $\alpha$ -terpinone, terpin-4-ol,  $\alpha$ -terpineol, linalyl acetate and linalool found in lavender essential oil have cytotoxic effects in many cancer studies [10,12]. *L. Stoechas*, which is widely seen in the Mediterranean Region, is a hairy, bushy, perennial plant with a height of 45-50 cm. It grows naturally at an altitude of 100-700 meters in the province of Hatay [13]. Due to its antiseptic, wound healing, expectorant, pain reliever, urinary tract infections, and soothing properties in epilepsy and asthma, the medicinal use of *Lavandula Stoechas* is common among the people in Anatolia [14].

Thus, in this study, cytotoxic effects of essential oil components of *lavandula stoechas* was aimed in an *in vitro* pancreatic cancer model.

## MATERIALS AND METHODS

### *Cell Culture (in vitro study)*

The human pancreatic adenocarcinoma AS-Pc1 (ATCC-CRL-1682) cell line was used in the study. Cells were grown under sterile incubator conditions with 5% CO<sub>2</sub> and 95% air at 37°C and was produced with high glucose DMEM (Dulbecco's Modified Eagles) medium containing 10% heat-inactivated fetal bovine serum (FBS) and a penicillin/streptomycin/amphotericin B mixture. The cell numbers in the experimental groups were separated with the help of a hemocytometer as 1x10<sup>5</sup> cells/ml for each well in 24 plates. The study consisted of two groups: the group with AS-Pc1 cells (control) and the group in which *Lavandula stoechas* essential oil (LS) was applied to AS-Pc1 cells (experimental group).

### *Cell viability tests*

In the study, cell viability rates were determined by using the Thiazolyl Blue Tetrazolium Bromide (MTT) reagent and following the methodology of Kucukgul and Erdogan [15]. In the application of this method, briefly, different concentrations of LS essential oils (0.1, 0.25, 0.5 and 1  $\mu$ g/ml) were applied to the cells in 24-well medium plates for 24 hours and then, the medium on the cells was removed and replaced with 200

$\mu\text{L}$  of fresh complete medium containing 20  $\mu\text{L}$  of MTT reagent (obtained by dissolving in 5 mg/ml medium). The cells were incubated at 37 °C for 4 hours. Reagent-stained crystals were dissolved in 100  $\mu\text{L}$  of 0.04 M HCL/isopropanol mixture for 15 d. Then, the samples taken into the tubes were centrifuged at 12000 rpm and +4 °C and read and recorded in a microplate ( $\mu\text{Quant}$  ELISA reader) reader at 570 nm.

### ***RNA isolation and qRT-PCR analyzes***

ASPC-1 cells were removed using a scraper and transferred to sterile tubes. Then, 1 ml of RNA isolation reagent (Trizol Reagent, Sigma) was placed in the tubes and RNA isolation was performed after chloroform, isopropyl alcohol and ethyl alcohol processes specified in the protocol, respectively. For the polymerase chain reaction, ready-made commercial syber green probe-labeled kits with a single step quality were used (EurX, PL). For this process, the amount of isolated RNA in the kit protocol, master mix buffer solution, specific primers (designed in Oligomer/Turkey) are collected in 200  $\mu\text{l}$  eppendorf tubes with a strep in accordance with the procedure, and cDNA conversion in the thermal cycle (Bio RAD CFX96) and PCR cycles were provided. Primers used in the study: for K-ras, reverse: 5'GGT CCT GCA CCA GTA ATA TG3', forward: 5'GGC CTG CTG AAA ATG ACT G3'; for EGFR, reverse: 5'CAC ACA GCA AAG CAG AAA CT3', forward: 5'ACC ATC TCA CAA TTG CCA3' and for GAPDH, reverse: 5'-CAT TGA ACT TGC CGT GGG TA-3', forward: 5'-GCTACC AGG GCT GCC TTC T -3'. With this analysis, K-ras and EGFR gene expression levels in RNA samples taken from the experimental groups after the incubation periods of the study were investigated and revealed by optimizing the control gene GAPDH levels. In the study, PCR cycles, cDNA synthesis step at 50 °C for 30 minutes, initial inactivation at 95 °C for 3 minutes, performed as 1 cycle, denaturation was performed at 94 °C for 15 seconds, adhesion was 30 seconds at 57 °C for EGFR gene, Kras and GADPH genes were applied for 45 seconds at 60°C. Chain elongation was performed at 72 °C for 30 seconds. Cycles were performed with 40 cycles repetitively.

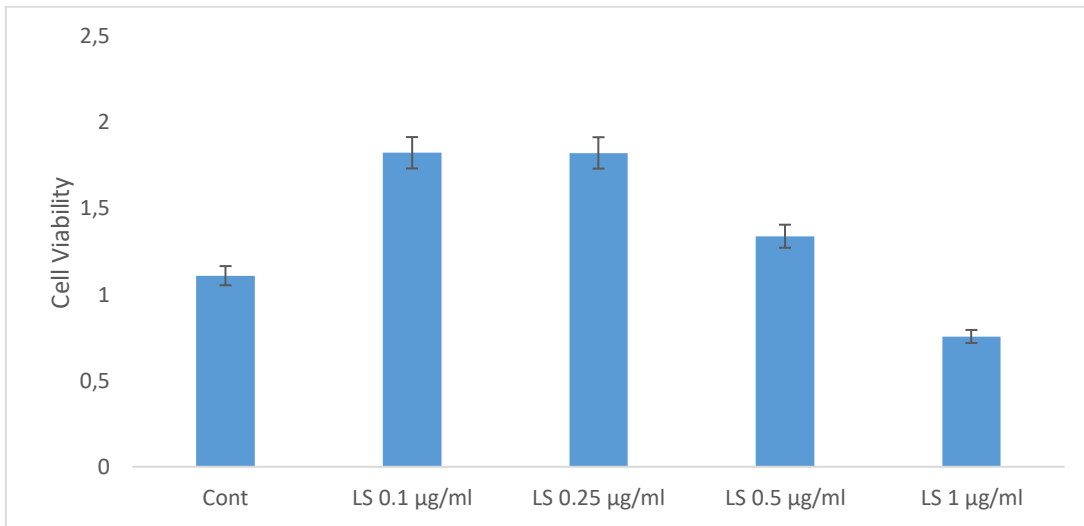
### ***Extraction of L. Stoechas Essential Oils***

*L. Stoechas* specimens were collected from the flora in the high mountain foothills of central Hatay, between September and October, when they were just beginning to bloom. The essential oils were obtained by the "Water Distillation Method" (Hydrodistillation). With this method, approximately 1 kg of dried lavender plant in a moisture-free environment was crushed with a "clavenger" device and placed in the distillation device, and then enough water was added to cover it. In the externally heated system, the evaporated water and oil were condensed by passing through the cooler and collected in the collection container. The dark preservation container containing the essential oils obtained was kept at +4 °C throughout the study.

## **RESULTS AND DISCUSSION**

### ***Viability results***

Pancreatic ductal adenocarcinoma cells were exposed for 1 day by applying essential oils obtained from *L. stoechas* at different concentrations (0.1, 0.25, 0.5 and 1  $\mu\text{g/ml}$ ). At the end of the period, the viability levels of the cells in the trial wells were analyzed by the MTT method.

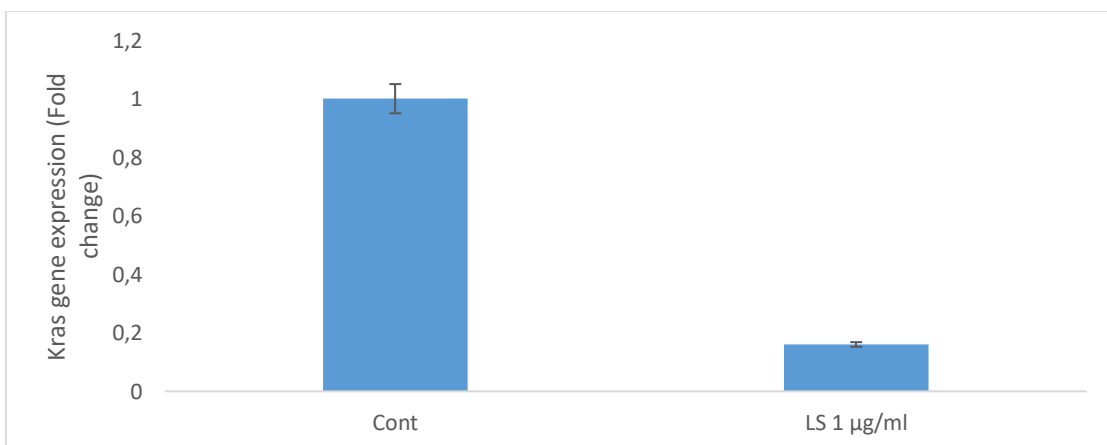


**Fig. 1.** Effects of different doses of *L. Stoechas* essential oils on cell viability (24 hours).

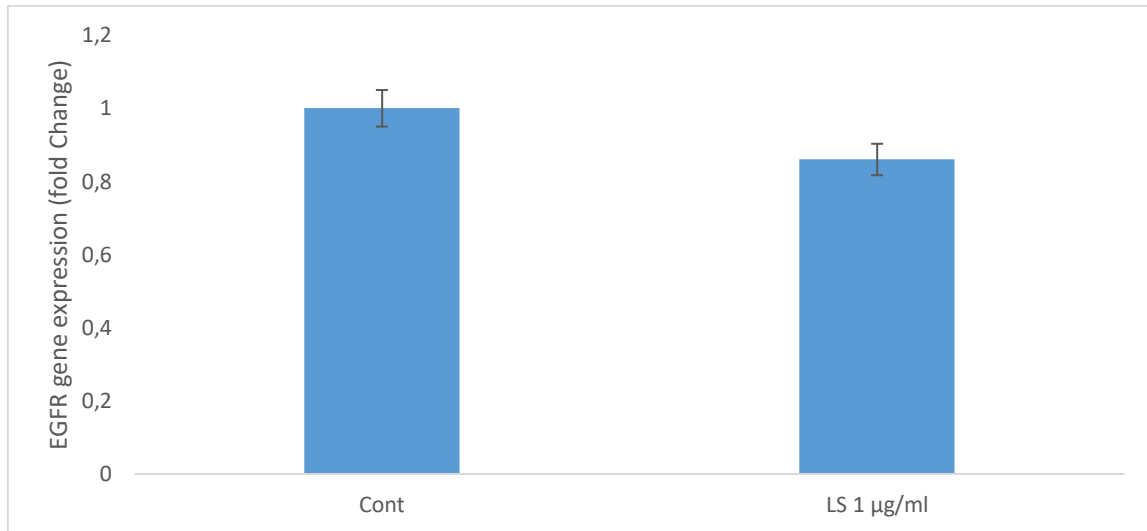
According to the results of the analysis, viability caused an increase in cells at the levels of 64%, 64% and 20% at the concentrations of 0.1, 0.25 and 0.5 µg/ml of LS, respectively, compared to the control group. However, as a result of the application of LS to the cells at a concentration of 1 µg/ml, it was determined that the cell density decreased by 32% compared to the control group. This concentration was chosen as the effective dose for subsequent studies (n=5).

### **Gene expression analysis results**

RNA isolation was performed from the experimental groups following the application of LS to adenocarcinoma cells at a concentration of 1 µg/ml for 24 hours. Afterwards, the expressions of EGFR and Kras genes were analyzed by using single step Syber Green kit and real-time polymerase chain reaction method. In the study, the average of the results of 3 independent samples was taken and the standard errors were given according to the percentages.



**Fig. 2.** Kras gene expression levels in the groups after 24 hours of administration



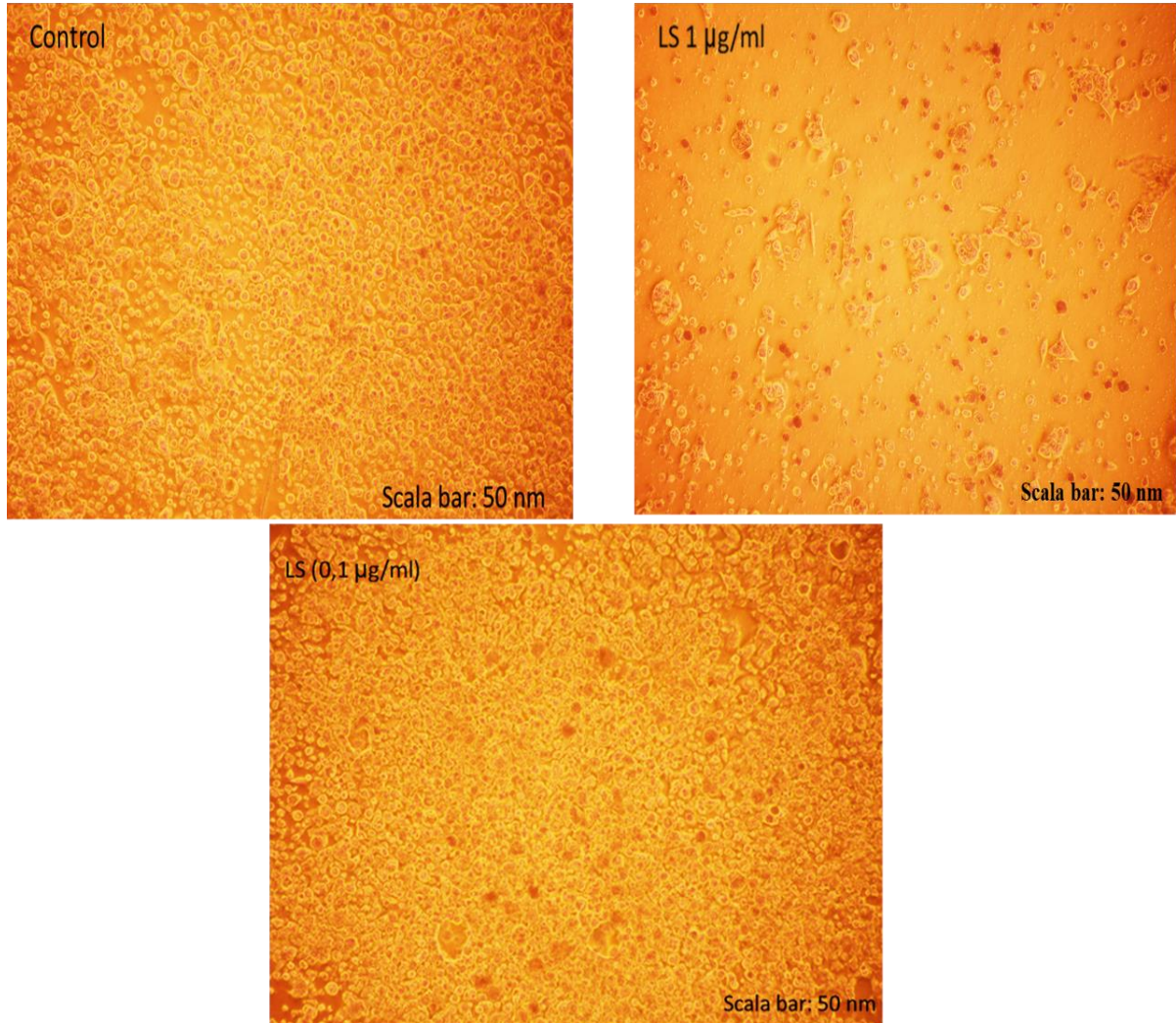
**Fig. 3.** EGFR gene expression levels in the experimental groups after 1 day of incubation

According to real-time polymerase chain reaction analysis; It was found that LS down-regulated K-ras gene expression by 84% compared to the control group (n=3). It was also determined that LS down-regulated the EGFR gene expression level by 14% compared to the control group (n=3).

### **Cell Images**

As a result of the application of LS essential oils to pancreatic adenocarcinomas for 1 day, cell images were visualized with an invert microscope (Olympus CK40, JP) with a 10x objective. In the photographic images taken from the study, it was revealed that while the effects of LS at low concentrations were proliferative, high concentrations (1 µg/ml) had a cytotoxic effect compared to the control group.

In the study, it was observed that the morphology of the control cells had the appearance of normal pancreatic ductal adenocarcinoma, as well as a homogeneous distribution at the bottom of the feeder. As a result of the addition of 0.1 µg/ml of LS to the cells, it was observed that the cell density increased while the cells preserved their morphology. LS was added to adenocarcinomas at a concentration of 1 µg/ml and incubated for 24 hours. At the end of the period, it was observed that the cell density decreased significantly, and the cell morphologies differed significantly in the microscopic images of the cells.



**Fig. 4.** Cell images in the control and LS (0,1 µg/ml and 1 µg/ml) applied groups (24 h)

Pancreatic adenocarcinomas took the 10th place among cancer-related deaths in developed countries. It has been reported to cause an average of over 40000 deaths in Europe and approximately 30000 deaths in the United States. Diagnosis of pancreatic cancer is very difficult due to its anatomical location. It is known that the chemotherapeutic agents currently used in the treatment of the disease are insufficient and have many side effects. For these reasons, researches in treatment have focused on alternatives consisting of natural products. Phytotherapy has been used in the treatment of various diseases throughout history. Today, it also finds wide usage areas in cancer treatment. The lavender plant belongs to the widely distributed *Lamiaceae* family. Due to its many pleasant scents, lavender oils have been used in many cosmetic products as well as in traditional medicine for hundreds of years [16]. The main components of lavender oil are linalool, linalyl acetate, 1,8-cineole (eucaptol), c-ocimene (cis and trans-), terpinen-4-ol, and camphor [17]. Önel et al., [18], in the GS-MS content analysis of *L. stoechas* plant obtained from the Hatay flora, reported that this plant contains intensely

Fenchone (46.12%), Eucalyptol (1,8-cineole-16.13%), linalool (11.32%) and terpinen 4-ol (3.83%).

The lavender plant has been reported to have many biological activities including antibacterial [19], ascaricidal [20], antifungal [21], eliminating the side effects of chemotherapeutic drugs [22], hepatoprotective and renoprotective [23]. It has also been reported to have cytotoxic effects in many cancer studies [10-12]. According to the data obtained in the study, we determined that LS essential oil showed strong cytotoxic activity on PDAK cells. Similar to our findings, Gören et al., [8] used LS essential oils *in vitro* for KB human epidermoid carcinoma, BC1 human breast cancer, LU1 human lung cancer, COL-2 human colon cancer, P-388 mouse leukemia cancer and LNCaP hormone-dependent human prostate cancer cells and they reported that it showed cytotoxic activities in these cell lines. However, in the same study, it was also reported that the effects of *L. Stoechas* essential oils on ASK rat glioma cells were limited. Again, similar to our findings, Shapira et al., [24] investigated the cytotoxic activity of Terpinen-4-ol on different gastric cancer cell lines in their *in vitro* study and reported that it showed a dose-dependent high cytotoxic activity especially in colon and pancreatic cancer lines. Researchers have emphasized that this effect triggers apoptosis but does not affect necrosis. In addition to these, Wu et al., [25] demonstrated the anticancer activity of Terpinen-4-ol by inducing apoptosis in large cell human lung cancer models in their *in vivo* and *in vitro* study. Nakayama et al., [26] reported that Terpinen-4-ol showed cytotoxic activity with caspase 3/7 activation and increase in mitochondrial reactive oxygen species in xenograph *in vivo* mouse colorectal cancer model. Zu et al., [10] investigated the cytotoxic activities of different essential oils, including lavender, in their *in vitro* study, and showed that they showed strong cytotoxic activity especially on prostate cancer cells. In the same study, it was stated that these essential oils have limited effects on lung and breast cancer cell lines. Jardak et al., [27] reported that rosemary essential oil showed significant cytotoxic activity on mcf7 breast cancer. Researchers stated that the cytotoxic activity is due to polyvalent active substances such as 1,8-cineole and camphor, which are also found in the content of *L. stoechas*. In addition, Moteki et al., [28] reported that 1,8-cineole triggered apoptosis in 2 different leukemia cancer lines, but was not effective in gastric cancer cell lines. Murata et al., [29] also reported that Eucalyptol showed cytotoxic activity with a strong apoptotic effect on cancer cells in xenograft colon cancer models.

In our study, we found that the K-ras and EGFR gene expressions, which are important biomarkers of carcinogenesis, were significantly down-regulated in pancreatic cancer cells treated with *L. stoechas* essential oils. Although H-Ras and N-Ras gene mutations are very rarely detected in pancreatic cancer, it is reported that K-Ras gene mutation is the most common mutation detected in pancreatic cancer with a rate of 95%. Kras regulates the functions of the cell such as development, proliferation and differentiation. It has been shown that the increase in “Epidermal Growth Factor (EGF)”, “Epidermal Growth Factor Receptor (EGFR)”, TGF- $\alpha$  and amphiregulin levels seen in pancreatic cancer cases is associated with decreased life span [5]. Ligands belonging to the EGF family (TGF- $\alpha$ , Heparin-linked EGF-like growth factor (HB-EGF) betacellulin and amphiregulin) are expressed at high levels in PDAK and are significantly effective in the development of PDAK in cancer cells [7]. EGFR; as a result of the increase in the frequency of K-ras and Smad 4 mutations and TGF- $\beta$  in the extracellular matrix, it is important in the formation of metastasis and angiogenesis and epithelial mesenchymal communication in tumorigenesis, and is effective in many complex cell pathways [30-

32]. Similar to our study, Shapira et al., [24] reported that Terpinen-4-ol, which is also found in *Lavandula* species, inhibits the activation of the promoter that triggers the anti-EGFR therapy-resistant K-ras mutation in colorectal cancer lines.

## CONCLUSION

Essential oils from *L. stoechas* were found to cause significant cancer cell death in a human pancreatic adenocarcinoma model. In order to demonstrate this efficacy of LS, K-ras and EGFR genes, which are responsible for the pathogenesis of pancreatic adenocarcinoma, were examined, and it was observed that LS extract down-regulated the expression of these genes and a significant suppression in the expression of the K-ras gene in particular. According to all literature information and the results of this study, it was concluded that LS may be a potential alternative in the treatment of pancreatic cancer. It is thought that the data obtained in the study can be used as a source for future comprehensive studies on what kind of changes LS causes in the cellular mechanisms of pancreatic cancer.

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**Authorship Contributions.** Concept: A.K., Design: A.K., Data Collection or Processing: Ö.K., A.K., Analysis or Interpretation: Ö.K., A.K., Literature Search: Ö.K., Writing: Ö.K., A.K.

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