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Shark cartilage (SC) and shark liver oil (SLO) treatment for lung damage via formaldehyde (FA) exposure

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Formaldehyde reacts with amino acids in living organisms to form toxic intermediates that cause epithelial cell damage. In past epidemiological studies, a statistically significant relationship was found between FA and leukemia risks and occupational inhalation exposure. As a complementary and alternative medicine (CAM) technique or alternative medicine, shark cartilage (SC) and shark liver oil (SLO) are presented as a new and different alternative source in this study. In this study, the toxic effects of formaldehyde (FA) on lung and the protective effects of SC and SLO against these toxins have been investigated. For the experiment, 40 rats were classified as follows: 4, control group (experiment control); 6, the group that received FA but was not treated (treatment control); 15, the group that was given FA and SC for treatment; and the last 15 were the group that was given FA and SLO for treatment. Negative effects of FA on lung were evaluated biochemically, genetically and pathologically. In terms of therapeutic efficacy, SLO appears to be more effective in improving lung injury on the basis of genetic, pathological and biochemical findings, against to FA administration. The toxic effect of FA in lung and the therapeutic effect of SLO and SC were determined and we believe that our experimental model provided the desired goal and success on the basis of our work.

Keywords: FA, Lung damage, SC, SLO
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In the cells, free radicals occur depending on endogenous and exogenous factors. When free radicals are formed too much, the protective effect of the immune system is insufficient and depending on their accumulation, harmful effects may occur in the metabolism. In order to prevent cellular damage caused by free radicals, antioxidant defense systems develop in the body. Antioxidants inhibit lipid peroxidation and thus cell damage by inhibiting peroxidation chain reactions, which means collecting free radicals.

Fumes, gases, vapors, dusts and other inhaled substances that we breathe in our daily life may show toxic effects to the lungs and other organs. Exposure to such toxic substances may occur in the work environment, at home, in public areas and in other environments. Inhalers such as ammonia, sulfur dioxide, and hydrogen chloride cause rapid irritant damage in the upper respiratory tract (URT). The toxic effects of these are seen in the terminal bronchi and alveoli.

Disadvantageously, individuals who do not experience symptoms of acute URT irritation continue to be present and unconsciously exposed. Formaldehyde (FA) reacts with amino acids in living organisms to form toxic intermediates that cause epithelial cell damage[1].

The effect of FA on lung damage

FA is metabolized to formic acid via formaldehyde dehydrogenase enzyme (FDH) in liver and erythrocytes after ingestion. FA is not stored in the body, it is converted into formic acid and excreted in the body through urine and feces (stool) or by oxidation into carbon dioxide[2]. Formaldehyde is widely used in various industries, including construction, paper product, resin, insulation material, wood, composite, textile, paint, plastic, adhesive and cosmetics. FA is also an indoor air pollutant with furniture, construction materials and chipboard, etc. present everywhere[3].

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Respiratory system toxicity of formaldehyde occurs even in low concentrations. It causes clinical symptoms such as burning sensation in the nose and throat, shortness of breath, cough and wheezing in acute effects. At higher concentrations, pulmonary edema, inflammation and pneumonia develop[4].

Although the specific mechanisms of these findings have not yet been determined, formaldehyde has been reported to cause an inflammatory reaction by indirectly affecting the respiratory epithelium[5]. Among the workers who were exposed to FA, it was reported that the mortality rate of lung cancer was 30% higher.

Alternative treatment and methods (CAM)

In 1998, for the public and the patients' increasing interest in CAM methods, the National Government of the United States and the National Institutes of Health (NIH)established the US National Health Center for Complementary and Alternative Medicine (NCCAM) to conduct research and to make recommendations and guidance on CAM methods.

Patients who are interested in alternative treatment methods, with a different health problem, use many plants, medical treatments or another different CAM technique or related different materials.

There are many ongoing studies to find the relationship between some materials related to sharks and their possible effects on cancer. In this study, the effect of shark cartilage and liver oil on lung injury healing was investigated.

Shark cartilage (SC) contains some specific molecules including thrombospondin-1, chondromodulin-1, type XVIII-derived endostatin, SPARC (acid and cysteine-rich secreted protein) and type II collagen derivative N-terminal propeptide (PIIBNP)[6]. It was concluded that intestinal absorption of the compounds of cartilage occurred and these compounds in the shark cartilage contain pharmacological activity, therefore, these can be administered orally for strengthening the defense system and for their antiangiogenic effect[7].

Shark liver oil (SLO) was also investigated as an anti-cancer source. Alkylglycerols and squalene are important factors in the fight against infections and cancer. Shark liver oil; includes a large amount of alkylglycerol, squalene and n-3 EFA. Therefore, it can be used in the treatment of cancer, especially for increasing of body resistance in radiotherapy and in the treatment of infectious diseases[8].

With this study, we aimed to investigate toxic effects of systemically administered formaldehyde on the lung and the protective effects of SC (shark cartilage) and SLO (shark liver oil) against these toxic effects at pathological, genetic and biochemical levels.

Materials and Methods

All the applications were made with an ethics committee report no: AKUHADYEK-239-13; taken from Animal Experiments Local Ethics Committee of Afyon Kocatepe University.

Animal material

The supplied 40 rats were grouped as follows:
4 of them: Control group; 6 of them: Formaldehyde applied but not treated; 15 of them: Formaldehyde applied and treated with shark cartilage; the last 15 of them: Formaldehyde applied and shark liver oil treated group.

After the control group was separated from the rats in the whole group, the remaining 10 mg/kg doses of formaldehyde were injected intraperitoneally every other day. After the formaldehyde dose was set to 1cc FA + 9cc SF = 10 cc, from them,1cc was given to each rat. While only FA injections were performed to the first group, the second group was given to SC and to third group was given SLO together with FA by the gavage method every day as well.

At the end of the study, all rats were sacrificed and blood samples were collected from the heart of rats for blood biochemical analysis, tissue samples were taken from the cranial lobe of the right lung for pathological examinations and genetic analysis and storaged at -20°C.

Biochemical test

In oxidative stress studies, generally, malondialdehyde (MDA), superoxide dismutase (SOD), glutathione peroxidase (GPx) and Catalase (CAT) antioxidant enzymes are examined, which is the final product of lipid peroxidation. To determine lipid peroxidation, products in the measurement of oxidative damage biomarkers CAT, SOD and GPx and MDA are examined.

Measurement of plasma CAT, SOD, GPx and MDA levels

These measurements in plasma were performed by Cayman brand catalase; superoxide dismutase; glutathione peroxidase and Thiobarbituric Acid Reactive Substances (TBARS) assay kit (Cayman

Chemical, Ann Arbor, Michigan, USA). Absorbance reading was made using the ChemWell 2910 brand Enzyme-Linked ImmunoSorbent Assay (ELISA) reader device. (Awareness Technology, Inc. Martin Hwy. Palm City, USA). Results were given in nmol/min/mL for CAT; in U / mL for SOD; in nmol/min/mL for GPx; µmol/L for MDA, respectively.

Analysis of biochemical data

In the analysis of the data collected, non-parametric tests were preferred because of the insufficient number of subjects. The Kruskal-Wallis test (k independent samples) was used for the three groups and Mann-Whitney U test was used for the comparison of the two groups. The data obtained from the study were analyzed with SPSS (Statistical Package for Social Sciences) 18.0 program.

Molecular analysis

RNA isolation from tissue

30 mg of tissue were weighed into RNAase free ependorfs. Repeating the reaction using wash buffer 1 and 2, approximately 300 µL of lysis buffer solution and 600 µL of proteinase K solution were used and at the last, the isolated RNAs were kept at -70°C and the process was completed.

Real-Time PCR

For each well, a PCR reaction medium was prepared with 5 µL SYBR green master mix (Promega) and 0.2 µL primer mix (Forward and Reverse, 10 pmol) and real-time PCR was performed following the reaction steps with a Light Cycler Roche 480 device.

The β-actin gene was selected as a house-keeping gene. The PCR protocol for this gene as; consisted of 45 cycles of denaturation at 95°C for 10 s, followed by 55°C for 30 s annealing and 72°C for 25 s to allow extension and amplification of the target sequence.

For molecular analysis, *β-actin* (as house keeping gene), *P53*, *Tnf-α*, *ATF6*, *ATF4*, *CHOP*, *GRP78* and *EDEM1* genes were used. *CHOP*, *EDEM1*, *Tnf-α* and *P53* genes act as tumor supressor genes. *ATF6*, *ATF4* and *GRP78* have a carcinogenic effect.

Data analysis

The analysis was performed using the 465-510 channel of the Light Cycler 480 device. Using the values obtained by the relative quantitation analysis (Target gene/reference gene), the change rates of the mRNA expression levels of the target genes were calculated by calculating the $2^{-\Delta\Delta Ct}$ method[9] and results were given as a graph. In the calculation, ΔΔCt= (Ct target gene-Ct App) subject group (Ct target gene-Ct App) control group formula was used.

Primers were designed from https://www.ncbi.nlm.nih.gov/gene/?term=*beta+actin* +and+rattus; term *P53*+and+rattus; term *Tnf-α*+ and +rattus; term *ATF6*+ and +rattus; term *ATF4*+ and +rattus;term *CHOP*+ and +rattus; term *GRP78*+ and +rattus;term *EDEM1*+ and +rattus sites[10]. Primers that used in this study and related informations are presented in Table 1.

Pathological analysis

Lung and intestinal tissues were used. Tissues were transferred to tissue tracking cassettes by trimming. The tissue was passed through an alcohol series and xylene with tissue tracking device (Leica TP 1020)

Table 1 — Primers[11]

GENES	F 5' 3'	R 5' 3'	SOURCE *Rattus norvegicus*
β-actin:	GAGGGAAATCGTGCGTGACAT	ACATCTGCTGGAAGGTGGACA	NC_005111.4 Amplicon length: 452 bp
P53	CGGAGGTCGTGAGACGCTG	CACATGTACTTGTAGTGGATGGTG	NC_ 005109.4 Amplicon length: 220 bp
Tnf-α	AGCCAGGCAGGTTCCGTCCCTC	TTACTGTGCCCACCAGCCGAC	NC_ 005119.4 Amplicon length: 358 bp
ATF6	TCCTCGGTCAGTGGACTCTTA	CTTGGGCTGAATTGAAGGTTTTG	NM_ 007348 Amplicon length: 283 bp
ATF4	TGGCTGGCTGTGGATGG	TCCCGGAGAAGGCATCCT	NM_ 001675 Amplicon length: 300 bp
CHOP	AGAACCAGCAGAGGTCACAA	TCTTCCTCCTCTTCCTGA	NM_ 001195053.1 Amplicon length: 250 bp
GRP78	GGTGGATCACAAGGTCAAGAG	CTACCACGCCCAGCTAATTT	NM_ 005347.4 Amplicon length: 310 bp
EDEM1	AGGTAGGGCTGAGTGATTACC	GGCACTAGAATAGGAGCTGGA	NM_ 014674.2 Amplicon length: 240 bp

and blocked in the paraffin. 6-8-µthick sections were obtained by Rotary microtome (Leica RM 2155). All sections were stained usinghematoxylin eosin (HE) stain. Zeiss ICC 5 camera was evaluated by ZEN imaging software in a light microscope (Zeiss Axiolab.A1) and microscopic images were taken.

Statistical analysis

For the analysis of the collected data, independent samples t-test was applied for the variables containing two groups. ANOVA analysis was applied to compare the difference between the groups. One-way analysis of variance (one-way ANOVA) was used to compare the average of all groups. Results are expressed as mean ± standard deviation. The Kolmogorov Smirnov test was used to test whether the continuous variables were normally distributed and $p < 0.05$ was determined as the significance level. The data obtained from the study were analyzed using SPSS (Statistical Package for Social Sciences) 18.0 program.

Results

Biochemical results

In view of the results in Table 2, the toxic effects of FA on lung were compared. When the FA, FA + SC and FA + SLO values were compared, a statistically significant difference was found between CAT, GPX and SOD values ($p<0.05$). There was no difference in MDA value ($p>0.05$).

In terms of antioxidant effects, we observed the following:, in FA application, an increase was observed in the values of 3 enzymes (CAT, SOD and GPx) compared to the control and for both SC and SLO administration as treatments, all these 3 enzymes levels decreased compared to only FA administration, and it was seen that it approached to the control group data. If we compared in itself, the SLO administration was associated with a significant decrease in the level of these 3 enzymes against to the FA application group. Against to the toxic effective FA application used, these enzymes initiate and increase the defense; It is thought that there is a significant decrease in the level of antioxidant enzymes with therapeutically given cartilage and SLO applications, and this is thought to be supressed by the decrease in the need for defense mechanisms due to the success of the substances used in the treatment. If a comparison is made in terms of treatment, it is thought that SLO can provide more effective treatment by lowering the values more than SC, thus the need for defense mechanisms is further reduced.

When we examined the MDA values, which is among the important biochemical parameters, we observed that in the treated rats, the FA value first increased due to lipid peroxidation and then these values decreased with the damage repair that started after SC and SLO application. However, these obtained values were slightly higher than in the control group. From this it can be said that SC and SLO have achieved a certain degree of success in ameliorating the damage caused by FA implementation. In terms of decreasing MDA values, SC and SLO showed the same success.

According to Table 3, there was no statistically significant difference between the control group and the FA-applied group on the basis of biochemical MDA and GPX values ($p>0.05$). However, there was a significant difference between SOD and CAT values ($p<0.05$). Compared to control; There was a significant decrease in the values of SOD and CAT antioxidant defense system enzymes with the treatment applied against the FA application; It is thought that the defense criteria values increase in the FA application by triggering the defense of these enzymes in the toxic effective FA application.

It is observed that there is a significant decrease in the levels of SOD and CAT antioxidant enzymes with cartilage treatment and therapeutically given SLO applications, and this is thought to occur with the decrease in the need for defense mechanisms due to the success of the substances. If we compare the treatment effect, it is thought that SLO is more effective by decreasing the values compared to SC in

Table 2 — FA; FA + SC ve FA + SLO groups toxic and antioxidant effects comparison to control[11]

	N	Mean	1.quartile	3.quartile	P
CAT	19	560.465	188.75	673.542	0*
GPX	19	336.507	66.575	582.61	0.007*
MDA	19	41.318	26.225	44.3	0.125
SOD	19	45.001	3.29	15.4	0.001*

p>0.05

Table 3 — Comparison of the groups containing FA with the control on the basis of the treatment[11]

	N	Mean	1.Quartile	3.Quartile	P
CAT	22	560.465	188.75	673.542	0.006*
MDA	22	41.318	26.225	44.3	0.138
SOD	22	45.001	3.29	15.4	0.019*
GPX	22	336.507	66.575	582.61	0.138

p>0.05.

terms of treatment, thus decreasing the need for defense mechanisms.

The Figure 1 shows the increased MDA values in the group FA due to oxidative damage. The Figure 2 and 3 show, there is a significant difference between FA and control on the basis of CAT and GPX values. The increasing value shows that the defense mechanism has become active despite the FA application.

Molecular results

In Figure 4, it is seen that the activity of the *CHOP* and *EDEM1* genes decreased with the use of FA. Herein, it is thought that tumor suppressor genes are adversely affected due to the destruction of lung tissue caused by FA application.

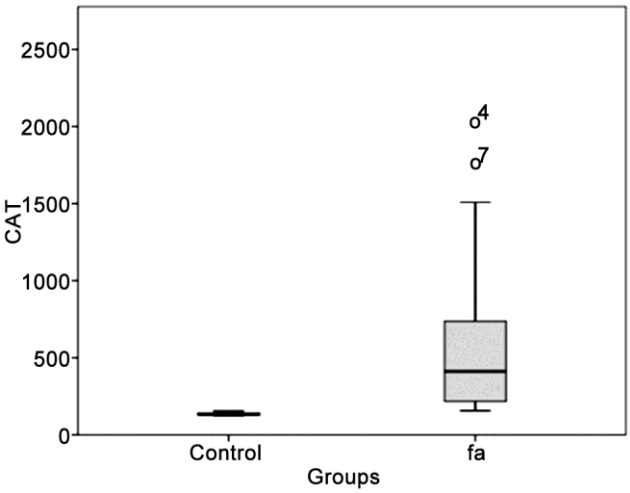

Fig. 2 — Figure showing the CAT values

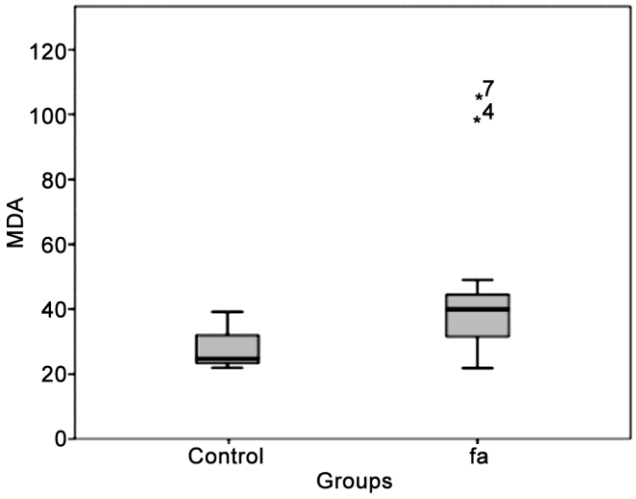

Fig. 1 — MDA values of FA and control group.

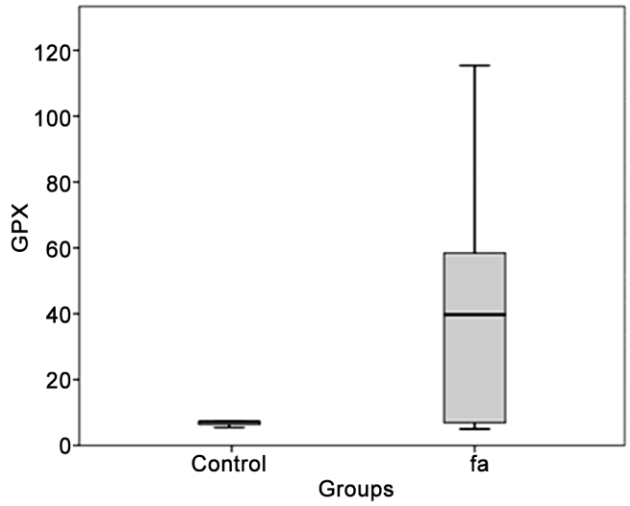

Fig. 3 — Figure showing the GPX values of FA and control group.

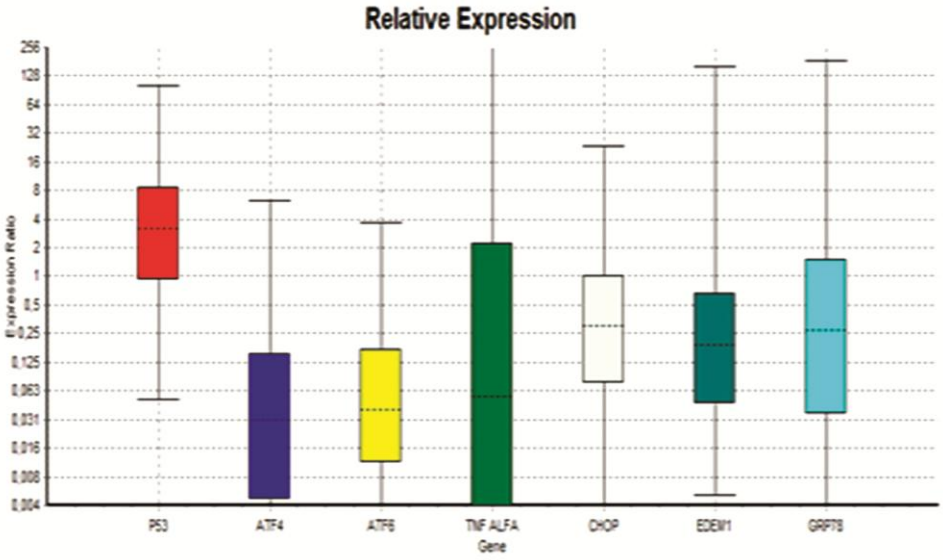

Fig. 4 — FA -Molecular analysis Figure

It is seen that GRP78 gene activity has decreased in FA + SC (Fig. 5). It is thought that SC used for therapeutic purposes acts by suppressing GRP78 gene expression for carcinogenic effect.

The activity of ATF4, ATF6, GRP78 genes, i.e., the carcinogenic effect, is seen to be decreased (Fig. 6). In contrast, the expression of the CHOP gene has been found to increase, indicating that it is found to provide protection against damage.

Compared with the therapeutic efficacy of SC and SLO in the FA group, it is observed that SLO was more successful. There was no significant difference in the activities of other genes.

Pathological result

The sample in E2 belongs to the control group. No chemical substances were given during the working period in this group. From the result lung tissue looked normal. The sample in F5 was lung tissue from a rat belonging to the FA administration group in which we tried to induce lung injury (Fig. 7). Severe emphysema developed at the end of the study. Arrows indicate severe emphysematous areas.

The sample tissue in G4 belongs to the group that was treated with SC after administration of FA. The areas with severe emphysema in this tissue were regressed into slightly severe emphysema areas. Mild severe emphysema with mild severe cuffing, moderate-to-severe interalveolar thickening and multifocal mononuclear cell infiltrations are seen. Arrows indicate slightly severe emphysema areas. The sample tissue in H2 belongs to the group tried to be treated with SLO after the administration of FA. It is seen that areas with severe emphysema in this

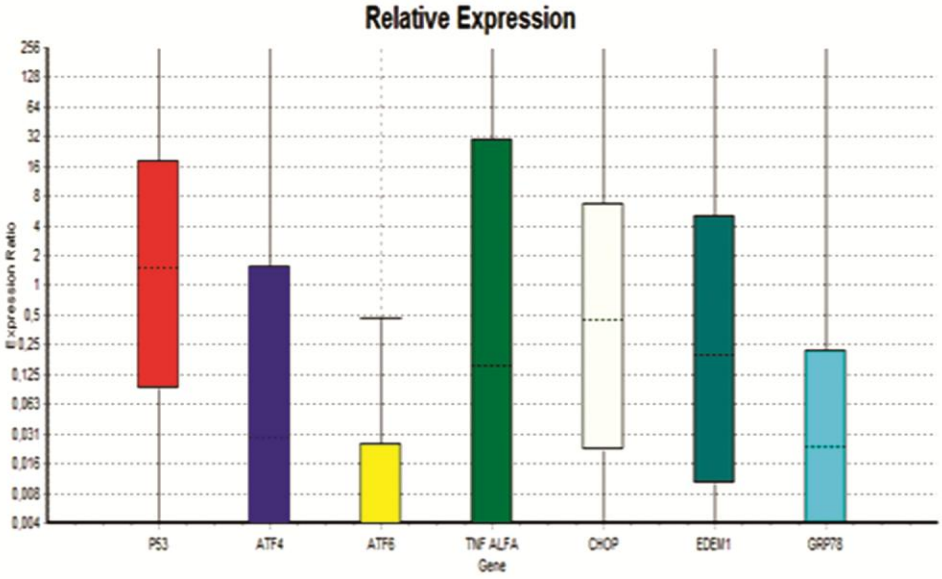

Fig. 5 — FA+SC -Molecular analysis Figure

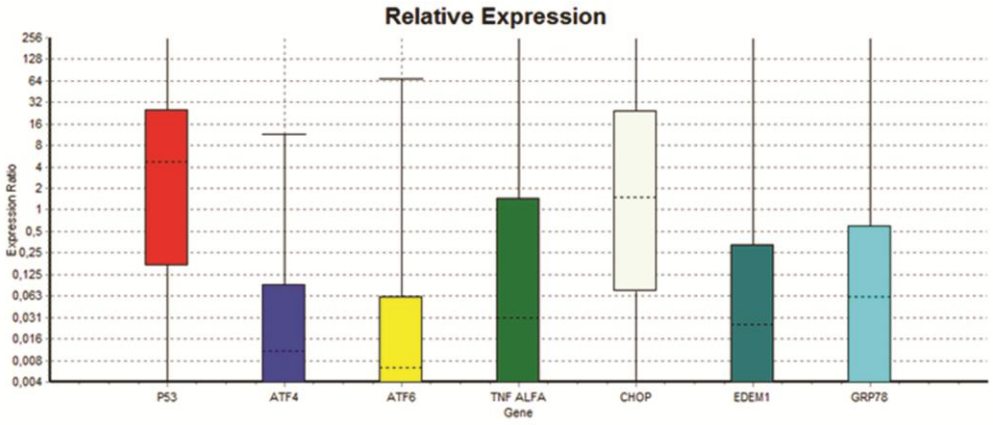

Fig. 6 — FA+SLO -Molecular analysis Figure

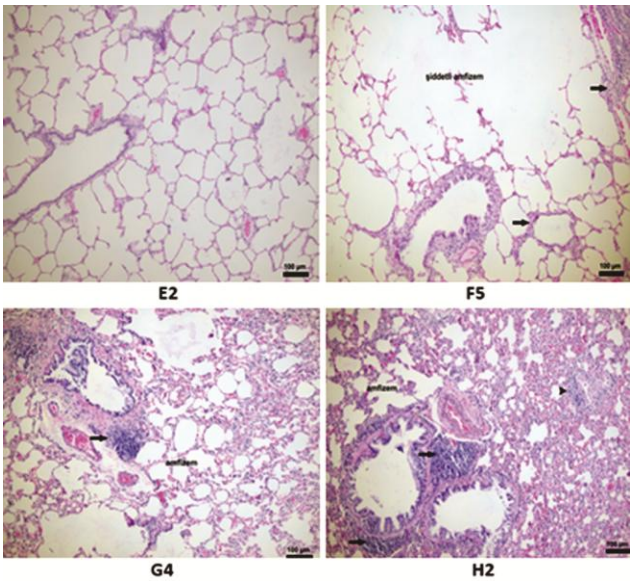

Fig. 7 — Pathology Results of Rats with Pulmonary Damage caused by FA

Table 4 — Scoring Table for Pulmonary injury caused by FA[11]

	Emphysema	Cuffing	Interalveolar Thickening	Interstitial pneumonia
Control	2	2	0	0
FA	3	0	0	2
FA + SLO	2	1	0	0
FA + SC	1	1	2	0

tissue have regressed to the areas with mild severe emphysema. Arrows indicate moderate severe emphysema areas.

SCORING

0. None 1. Mild Severe 2. Moderate 3. Severe 4. Very Severe (Table 4)

Discussion

In an experimental study of isolated rat hepatocytes, even low concentrations of FA have been reported to cause oxidative damage[12]. FA's role in enhancing the destruction was confirmed by our study as well. In another study, it was determined that CAT activity was decreased and SOD activity increased in liver tissue by administering FA by inhalation[13]. In other study, male rats were administered 10% formaldehyde for 14 days as well as 25 mg/kg dose of melatonin as a treatment and FA exposure of the prefrontal cortex, resulting in the occurrence of oxidative damage and it was determined that this damage could be prevented with melatonin application. The study reports and results of the effects of FA are in line with our study results[14]. Akyürek[15] *et al.* conducted a study on the use of alternative therapy in lung cancer patients and found that 63% of the patients were using alternative treatment. Among these patients, 59% usednettles or its seeds, 23% used multivitamins and 11% used SC. The results of the study are consistent with our experimental model to support the protective effect of SC[15].

In the search for potential anticancer agents from fruits, *Carissa carandas* (in karon) and *Syzygium cumini* (jamun) seed parts were used against nine human cancer cell lines including lung cancer lines. Even at lower concentrations, both seeds were observed to be cytotoxic *in vitro*, particularly against lung cancer cells. It was emphasized that it is especially important to develop anticancer agents in the treatment of lung cancer and to reveal effective herbal sources and active ingredients for use bycancer patients. Likewise, SC and SLO, which are also used in our experimental model, are important and have a similar positive therapeutic effect against lung damage, which can be defined as the onset of cancer, as components that can be counted in natural and alternative therapy[16].

Angiogenesis (vascularization) is an important mechanism in terms of growth, development, wound healing and care. However, due to excessive angiogenesis at a certain stage of development, it can cause pathological disorders such as cancer, diabetic blindness, age-related macular degeneration and rheumatoid arthritis. In this study, aqueous extract of different ethnomedicinal plants parts such as *Butea monosperma* (Kamarkas), *Dioscorea hispida* (Beychandi), *Myristica fragrans* (Nutmeg) and *Mesua ferrea* (Nagkesar) were searched for modulation of angiogenesis. In the findings, *M. fragrans* and *D. hispida* inhibited angiogenesis. *M. ferrea* revealed its potential as antiangiogenic material by inhibiting the vascularization by reducing the number of blood vessels as well. Similarly, in our study, compounds of cartilage have also shown an antiangiogenic effect, which has importance in lung damage as an onset of cancer development[17].

SLO contains low levels of polyunsaturated fatty acids (EFA) and has been shown to play an important role in promoting immunosuppression against bacterial and fungal infections and cancer[18]. Coal dust from respiration causes lung diseases, especially in mine workers. MDA, NO, XO levels increase, and *IL-6* and *TNF-α* expression occur in lung tissue and

erythrocytes. It has been reported that Erdostein inhibits damage to lung tissue by increasing antioxidant enzyme levels and decreasing myeloperoxidase activity[19].

It has been supported by many studies that these compounds in shark cartilage, where cartilage-derived compounds are absorbed by the intestine, contain pharmacological activity and can be administered orally[8]. Similarly, in our experimental model, SC was used orally, and the positive therapeutic effect against the damage that we can describe as the cancer onset was observed.

In another study, it was stated that SLO is a natural source of alkylglycerols and can be used at doses of 100 mg 3 times a day without side effects. In the same study, it was stated that alkylglycerols could be used as an alternative source of immune system support in complementary therapy[20]. In this study, it was determined that SLO could be used as an antioxidant and our experimental model reached the same result.

Conclusions

Negative effects of FA on lung tissue were evaluated biochemically, genetically and pathologically. In terms of therapeutic efficacy, according to the results of biochemical, pathological and genetic analyses, despite the negative impact of FA application, SLO appears to be more successful in healing lung damage. It can be stated that the experimental model we have created provides the desired goal and success in this direction. However, more comprehensive studies are needed to reveal treatment options and/or preventable ways.

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Conflict of interest

The authors whose names are listed certify that they have No affiliations with or involvement in any organization or entity with any financial interest (such as honoraria; educational grants; participation in speakers' bureaus; membership, employment, consultancies, stock ownership, or other equity interest; and expert testimony or patent-licensing arrangements), or non-financial interest (such as personal or professional relationships, affiliations, knowledge or beliefs) in the subject matter or materials discussed in this manuscript. Our reserarch don't have any financial or personal matters that may pose a conflict of interest.

Authors' contribution

All authors approve the content of the manuscript and have contributed significantly to research involved or the writing of the manuscript. MDA: conceive and design the project and study, interpret the data, EA: analyze the data and carryout the study, FB: pathological analysis and AV: biochemical analysis.

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