

# Cytotoxicity and genotoxicity evaluations of oleic acid and conjugated linoleic acid

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## ABSTRACT

**Background and Aims:** Oleic acid (OLA) and conjugated linoleic acid (CLA) occur in dairy products and meats and are also widespread at lower levels in many other foodstuffs. It is known that OLA and CLA are very bioactive compounds with substantially anti-carcinogenic effects. The objective of this study was to evaluate the cytotoxic potentials of OLA and CLA which were tested against cancerous and non-cancerous cell lines and to determine their genotoxicity.

**Methods:** The cytotoxic activities of OLA and CLA against to cancer cell lines (U-87-MG, A549, MCF-7, CaCo-2, HeLa and PC-3) and a control cell line (HEK293) were assessed by MTT assay. Ames MPF™ mutagenicity assay on 4 strains (TA98, TA100, TA 1535 and TA 1537) of *Salmonella typhimurium* was used for genotoxicity determination.

**Results:** CLA showed cytotoxic activity on PC-3 cells, while OLA was created on A549 and PC-3 cell lines with the IC<sub>50</sub> of 20 nM and 15 nM, respectively. No cytotoxic activity was observed on MCF-7, HeLa, U-87-MG, and CaCo-2 cells with the administered doses of OLA and CLA. It has been proved that OLA and CLA are characterized by a high cytotoxic activity towards cancer cells, as observed in the cell line test. There was no evidence for a mutagenic effect of OLA and CLA in the Ames test, with or without metabolic activation (S9) against *Salmonella typhimurium* strains.

**Conclusion:** These *in vitro* test results indicate that these fatty acids can be considered a beneficial dietary supplement for enhancing anti-cancer therapy.

**Keywords:** Fatty acid, Conjugated linoleic acid, Oleic acid, Cytotoxicity, Mutagenicity

## INTRODUCTION

Oilseed crops that contain fat, protein, carbohydrates, minerals and vitamins have an important place in human and animal health (Liu, Johnson, Blacksaw, Hossoin, & Gan, 2019). Safflower, which is a popular medicinal oilseed plant, belongs to the family of *Asteraceae* (Zhang et al., 2019). Recently, the importance and beneficial health effects of safflower have been shown in various studies (Martinez, Sosa, Higa, Fornes, & Capobianco, 2012; Khalid et al., 2017). It is a rich source of unsaturated fatty acids, including monounsaturated and polyunsaturated fats. It is a famous and widely used traditional Chinese medicine that has been verified to have anti-inflammatory, antioxidant, and cytotoxic activities (Yanli, Jian, Zhenyun, Peipei, & Kan, 2018). Safflower plants are used in feed, biodiesel industry, in various fields such as phytoremediation and it are able to grow under different climatic conditions

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(Madan, Mudgal, Mishra, Srivastava, & Singh, 2011). Plant oils are generally composed of unsaturated fatty acids, oleic, linoleic and mainly saturated fatty acids, palmitic and stearic acids (Aydeniz, Güneser, & Yılmaz, 2014). However, the distribution of these fatty acids varies based on the type of plant species. Safflower oil is composed of 6–8% palmitic, 2–3% stearic, 16–20% oleic and 71–75% linoleic acids and exhibits the highest linoleic acid content among all the commercial oils (Aydeniz et al., 2014). The quality of oil is determined by the ratio of fatty acids content. The total unsaturated fatty acid level is very high in plant oil, which is very important for human health. Linoleic acid (Omega-6) is a fatty acid with 18 carbons and contains a double bond at 10<sup>th</sup> and 12<sup>th</sup> positions in the cis configuration (Woodcock, Salvatore, Freeman, & Schopfer, 2021). Dairy and meat products contain linoleic acid, and it is often used in chemical and animal feed industries. As a result of microbial biohydrogenation, the digestive tract of ruminant animals naturally produces Conjugated linoleic acid (CLA).

It was indicated that CLA daily supplementation after carcinogen exposure affected linoleic acid metabolites content in rats' serum. Also, a decrease in cancer incidence was shown in CLA supplemented groups (Małgorzata, Agnieszka, Iwona, Hanna, & Andrzej, 2017). Oleic acid (OLA, Omega-9) is a monounsaturated cis-9-octadecenoic acid (Engelbrecht, Schroeter, Haus, & Neubert, 2011). OLA treatment against the starvation might be important to confirm the effect of lipophagy cells (Lee, Ahna, Jang, Ha, & Jung, 2019). In addition, many studies have shown that OLA acted synergistically with cytotoxic drugs by enhancing their antitumor effect (Menendez et al., 200; Carrillo, Cavia, & Alonso-Torre, 2012). In recent years, studies have increased on the type of plants with high ratio of oleic acid and linoleic acid (Cao et al., 2013). Experimental studies of CLA on animals have been shown CLA to be protective against breast cancer (Białek, Zagrodzki, & Tokarz, 2016; Zhang et al., 2018). Studies on rats have shown that CLA has antidiabetic effects by increasing insulin sensitivity (Guiberna et al., 2019). The findings in most studies show that OLA reduces the risk of cancer, especially breast cancer (Simonsen et al., 1998). Therefore, plant derived compounds have been an important source for medicines that treat various diseases. Safflower oil derivatives are also understood to be an important source for cancer studies and phytotherapy (Yang et al., 2018). In this study, we aimed to evaluate the cytotoxic potentials of OLA and CLA, which were tested against cancerous and non-cancerous cell lines, and to determine their genotoxicity.

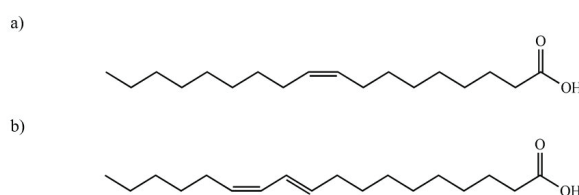
## MATERIAL AND METHODS

### Preparation of oleic acid and conjugated linoleic acid stock solutions

Test substance both 10t-12c CLA (Cas No: 2420-56-6) (Fig.1a.) and OLA (Cas No: 112-80-1) (Fig.1b.) were obtained from Sigma-Aldrich Chemical in liquid form to be restructured with DMSO (Sigma-Aldrich Chemical, CAS No: 67-68-5) as 45  $\mu$ M CLA, 30  $\mu$ M as a stock solution and kept at -20 °C.

### Cell culture and cytotoxicity test

In this study, human cervix adenocarcinoma (HeLa, ATCC<sup>®</sup>CCL-2<sup>™</sup>), human colon colorectal adenocarcinoma



**Figure 1.** Structures of OLA (a) and CLA (b).

(CaCo-2, ATCC-HTB-37), human glioblastoma–astrocytoma (U-87 MG, ATCC<sup>®</sup>HTB-14<sup>™</sup>), human breast adenocarcinoma (MCF7, ATCC<sup>®</sup>HTB-22<sup>™</sup>), human lung adenocarcinoma (A549, ATCC<sup>®</sup>CCL-185<sup>™</sup>) and human prostate cancer (PC3, ATCC CRL-1435<sup>™</sup>) were used as cancer cell lines while human embryonic kidney cells (HEK293, ATCC-CRL-1573) were used as a non-cancerous cell line. Cells were maintained in DMEM/F12-Dulbecco's Modified Eagle's Medium with FBS (%), L-glutamine (2 mM), Pen-strep at 37 °C in a CO<sub>2</sub> incubator. ATCC-formulated Eagle's Minimum Essential Medium was used for the HEK293 (Embryonic kidney, ATCC<sup>®</sup>CRL-1573<sup>™</sup>) cell line. A U.V/ visible spectrophotometer (Thermo Scientific, Germany) was used to measure the optical density. The cultured cells (1x 10<sup>5</sup> cells/mL) were treated with different doses (0.3 nM–30 nM dose range for OLA and 0.45  $\mu$ M–45  $\mu$ M dose range for CLA) and incubated for 48 hours at 37 °C. After the incubation period, the viability percentage of the test substance was calculated using equation

$$\% \text{ Viable cells} = \left[ \frac{\text{(The treated cells absorbance)} - \text{(The blank absorbance)}}{\text{(The blank absorbance)}} \right] \times 100$$

Cytotoxicity of OLA and CLA were fitted to a sigmoidal curve and a 4-parameter logistic model was used to calculate the inhibition of the cells (Mosmann, 1983).

In morphological investigations, the cells which were treated with different concentrations of agents and control cells were examined for morphological changes by inverted microscope. All experiments were done in triplicate.

### Bacterial reverse mutation test (Ames test)

An Ames MPF<sup>™</sup> mutagenicity assay (Xenometrix Inc. Switzerland) was conducted on 4 strains of *Salmonella typhimurium*, tester strains TA98, TA100, TA 1535 and TA 1537 according to OECD Guideline 471. TA98 and TA 1537 strains are used for the detection of frameshift mutations and TA100 and TA1535 for base pair substitutions. Mice liver post-mitochondrial (S9) fraction was used for metabolic activation. The 30% S9 mix contained cofactors such as phosphate buffer pH 7.4, MgC<sub>2</sub>, KCl, Glucose-6-phosphate, NADP and NaH<sub>2</sub>PO<sub>4</sub> buffer. Reference negative control (0.5% DMSO) and strain-specific positive control were tested in all *Salmonella* strains (Maron & Ames, 1983). In the study, a 96-well dilution microplate was used for dilution of the test substances (6 doses) and 24-well microplates were used for bacterial exposure to the test substance during the process. For the detection of mutagenic activity of exposure, 384-well microplates were used. The first dilutions were performed in 96-well microplates for the test substance and were transferred to 24-well exposure microplates. The bacteria culture was incubated at 37°C, 250 rpm. After the incubation period, the cytotoxic dose of the compounds was evaluated. The lowest dose showing cy-

toxicity was chosen as the highest concentration in the test. According to the manufacturer's instructions, appropriate solutions for OLA and CLA were prepared (0.8, 4, 20, 100, 500, 2000 µg/mL). After 90 minutes incubation, 2.8 mL of indicator broth was added to each well of a 24-well exposure microplate. The exposure culture was transferred from the 24-well microplate to 384-well microplates. These plates were incubated at 37°C for 2 days. Then the microplate was removed from the incubator for counting. Raw data interpretation and calculation were performed according to manufacturer's instructions.

An Ames MPF™ Mutagenicity Assay kit is available for strain-specific positive control chemicals. The following positive controls were used in assessing the performance of the Ames assays (Table 1).

Strains	without S9		with S9	
		Concentration		Concentration
<b>TA 98</b>	2-Nitrofluorene	2 µg/mL	2-Aminoanthracene	25 µg/mL
<b>TA 100</b>	4-Nitroquinoline-N-oxide	0.1 µg/mL	2-Aminoanthracene	62.5 µg/mL
<b>TA 1535</b>	N <sup>4</sup> -aminocytidine	100 µg/mL	2-Aminoanthracene	125 µg/mL
<b>TA 1537</b>	9-aminoacridine	15 µg/mL	2-Aminoanthracene	125 µg/mL

### Statistical analysis

Statistical analyses were performed by using SPSS for Windows 10.0 and GraphPad Prism 7 statistical analysis programs. The results were compared according to the control group using the Student's-t Test. Values were expressed as mean ± SD. A value of  $p < 0.05$  was considered statistically significant.

## RESULTS

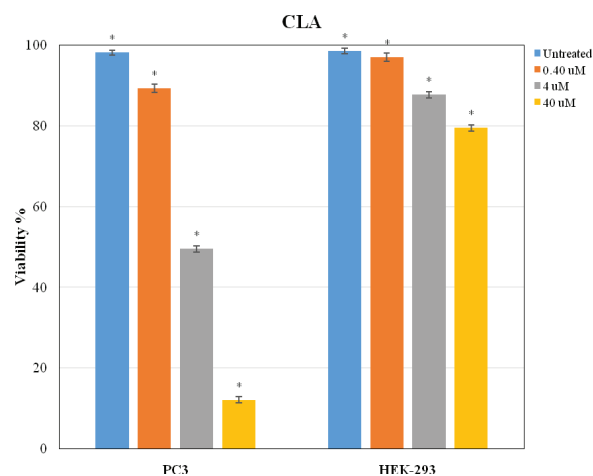
### Cell culture and cytotoxicity test

Depending on the exposure to compounds, each cell line showed differences in IC<sub>50</sub> values (Table 2). No cytotoxic activity on MCF-7, HeLa, U-87-MG, and CaCo-2 cells was observed with the administered doses of OLA and CLA. The OLA IC<sub>50</sub> values were found to be 20 nM and 15 nM for the 48 h treatment on A549 and PC3 cells, respectively. The IC<sub>50</sub> values for CLA were determined to be 27 µM and 38 µM on PC3 and HEK-293 cells (Fig. 2,3).

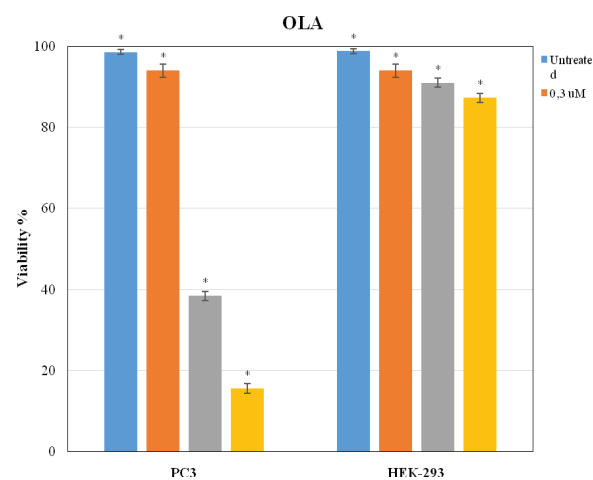
After the post-treatment of the agents, the morphological changes were observed on PC3, A549 and HEK293 cells (Fig 4). OLA was shown to have a cytotoxic effect at 30 nM doses

**Table 2. IC<sub>50</sub> values of OLA and CLA.**

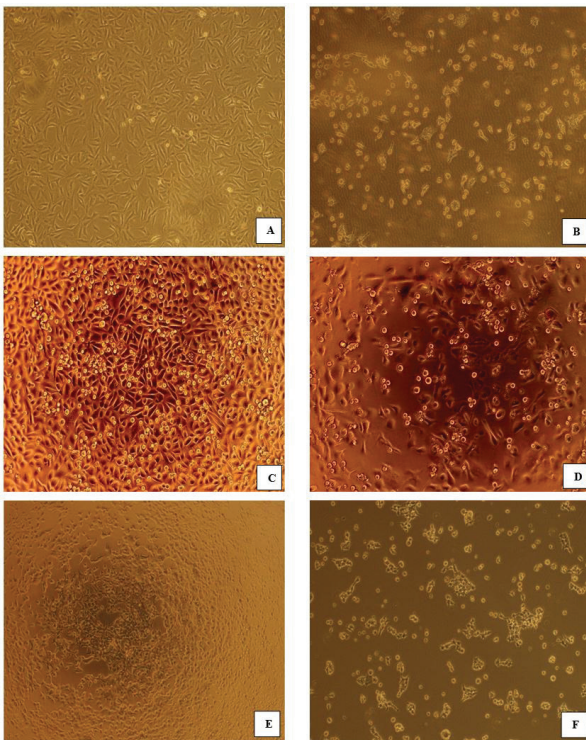
Cell Lines	IC <sub>50</sub> Values	
	OLA	CLA
<b>A549</b>	20 nM	> 45 µM
<b>U-87-MG</b>	> 30 nM	> 45 µM
<b>MCF-7</b>	> 30 nM	> 45 µM
<b>CaCo-2</b>	> 30 nM	> 45 µM
<b>HeLa</b>	> 30 nM	> 45 µM
<b>PC-3</b>	15 nM	27 µM
<b>HEK-293</b>	> 30 nM	38 µM



**Figure 2.** Cytotoxic effects of CLA on PC-3 and HEK-293 cells after 48 h exposure. All test substances were reconstituted with DMSO, which was evaluated as a control. Control was exposed only to vehicles which were 100% viable. Data are expressed as mean ± SD.



**Figure 3.** Cytotoxic effects of OLA on PC-3 and HEK-293 cells after 48 h exposure. All test substances were reconstituted with DMSO, which was evaluated as a control. Control was exposed only to vehicles which were 100% viable. Data are expressed as mean ± SD.



**Figure 4.** Morphological characterization cell lines. A. A549 untreated control cells; B. OLA treated A549 cells (30 nM); C. PC3 untreated control cells; D. OLA treated PC3 cells (30 nM); E. HEK-293 untreated control cells; F. CLA treated HEK-293 cells (45 μM).

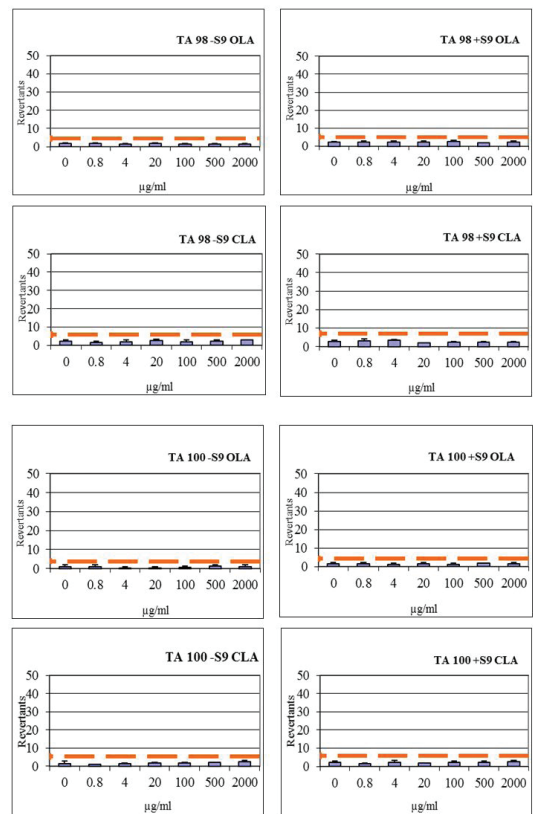
on PC3 and A549 cancer cell lines. CLA was effective at a 38 μM dose on HEK293 cells. There was a clear difference, such as various morphological abnormalities, between the untreated control cells and treated cells. The cells lost their normal appearance when compared with untreated control cells.

### Bacterial reverse mutation test

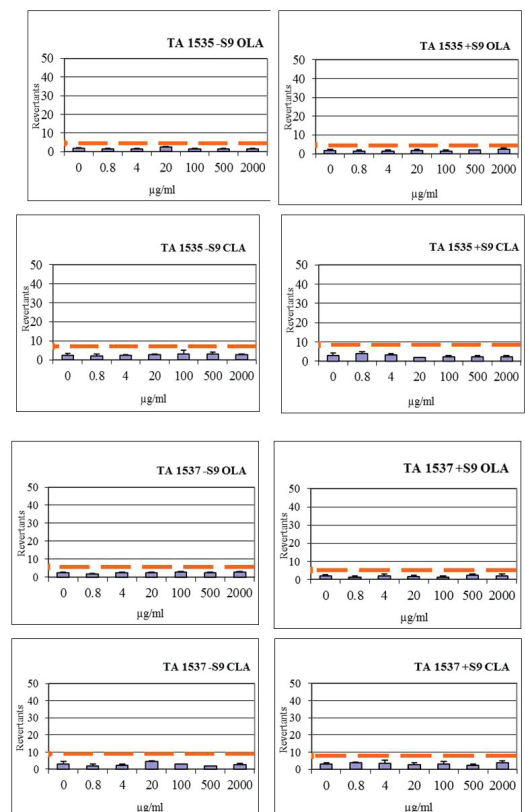
In the Ames assay, the mean number of positive yellow wells per 6 doses was calculated from the triplicates and the fold increases above the baseline were determined for each dose of OLA and CLA. According to the results no mutagenic evidence was determined for the maximum dose of 2000 μg/mL of OLA and CLA (Fig 5-6).

### DISCUSSION

Safflower plants contain medical and biologically essential flavonoids, alkaloids, steroids, and polysaccharide compounds such as fatty acids. These compounds form the basis of the therapeutic efficacy of the plant (Khalid et al., 2017). An increasing trend for safflower and its production has been observed over the previous few years, as evident from the increase of crop land at the rate of 4.9% per annum. Due to the importance of alternative and complementary medicine in the treatment of many diseases, safflower and its products, which are traditional herbal products, have started to be studied intensively (Bae, Kim, Lee, Kim, & Son, 2015; Guner, Kizilsahin, Nalbantsoy & Karabay Yavasoglu, 2020). Therefore, in this study, the anti-cancer potential of OLA and CLA, which are bioactive



**Figure 5.** Mutagenicity test results of OLA and CLA with *Salmonella typhimurium* TA 98 and TA 100 strains.



**Figure 6.** Mutagenicity test results of OLA and CLA with *Salmonella typhimurium* TA 1535 and TA 1537 strains.



components of the safflower plant, was investigated. Investigating of biological effects of these bioactive components will contribute to the conversion into pharmaceutical forms of these structures for therapeutic purposes in the future.

In the *in vitro* anticancer efficacy study, cells exposed to the compounds showed different LD<sub>50</sub> values. These results thought that different cell lines are affected by different doses of the compounds. In addition to this, another reason for the difference in results was considered to be use of different isomers of the compounds. Moreover, differences in the proliferation activities of cancer cell lines have led to change in the results. According to the results of MTT assay on the U-87 MG cell line, OLA and CLA have not shown any cytotoxic effect. Also, CLA at 45 µM dose was also exhibited similar effects against A549, MCF-7, and CaCo-2 HeLa cell lines. Nevertheless, in a study evaluating the effects of 5 different purified isoforms of CLA and a mixture of CLA isoforms on estrogen receptor positive (ER+) breast cancer cells (MCF-7), it was revealed that CLA (t10, c12) isomer showed dose dependent inhibition on the MCF-7 cell line (Tanmahasamut, Hendry, & Sidell, 2004). The observed effect is thought to be related to administration as a mixture and the application dose. In our study, CLA showed cytotoxic activity on PC-3 prostate cancer cells after 48 hours of exposure with an IC<sub>50</sub> value of 27 µM. These findings were similar to other studies on this subject (Cohen, Zhao, Pittman, & Scimeca, 2003; Palombo, Ganguly, Bistriani, Menard, 2002). Also our results exhibited that OLA was found effective on A549 and PC-3 cancer cell lines with the IC<sub>50</sub> of 20 nM and 15 µM, respectively. Although anti-proliferative activities using different doses of OLA were determined on mouse lung carcinoma, LLC cells and human prostate cancer cells (PC-3) in many *in vitro* studies (Hughes-Fulford, Chen, & Tjandrawianata, 2004; Kritchevsky, 2002), there was no data on its anti-proliferative activity on A549 cells in the literature. In this study, we determined the cytotoxicity of OLA on A549 cells for the first time. However, the administered OLA and CLA concentrations on HeLa, CaCo-2, MCF-7, and HEK293 cell lines also did not present any anti-proliferative activity. In the literature, it was confirmed that there was no toxic effect of the compounds on control cells HEK293. In this study, cytotoxic effects of OLA and CLA were screened on many cancer cell lines for the first time.

In genotoxicity evaluation of OLA and CLA, according to Ames MPFTm assay *S.typhimurium* TA 98, TA 100, TA 1535, and TA 1537 strains and S9 fraction were used. According to the assay, a substance with mutagenic effect must be show a statistically significant difference for at least 1 dose. The substance should be compared with the control group and it should increase the number of revertant colonies more than 2 times. In this study, safflower oil derivatives at even a 2000 mg/mL concentration, which is the maximum dose, according to OECD, did not cause mutation effects on TA 98, TA 100, TA 1535, and TA 1537 strains with/without S9 fraction. Similarly our findings, in the anti-mutagenic effect studies conducted with many isomers of CLA originating in different foods, no mutagenic effect was observed at the application doses of the isomers (Kritchevsky, 2002). However, there was no study of mutagenic activity related to OLA in the literature. Thus, this is the first genotoxic potential evaluation of oleic acid.

## CONCLUSION

In conclusion, OLA and CLA, which are derivatives of safflower, are important oil components for biologic activity. According to our results, these components can be considered as beneficial dietary supplement for supporting anti-cancer therapy without side effects.

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**Author Contributions:** Conception/Design of Study- N.Ü.K.Y., Ç.K.Y., B.B., A.N.; Data Acquisition- S.K., N.Ü.K.Y.; Data Analysis/Interpretation- S.K., N.Ü.K.Y.; Drafting Manuscript- Ç.K.K., N.Ü.K.Y.; Critical Revision of Manuscript- N.Ü.K.Y., Ç.K.Y., B.B., A.N.; Final Approval and Accountability- S.K., Ç.K.Y., B.B., A.N., N.Ü.K.Y.

**Conflict of Interest:** The authors have no conflict of interest to declare.

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