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Evaluation of anti-cancer activity of Geraniin in laryngeal cancer Hep-2 cell line

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Abstract

To investigate the anti-cancer activity, including anti-proliferative, anti-inflammatory, anti-metastatic, and apoptotic effects of Geraniin on the laryngeal cancer human epidermoid carcinoma 2 Hep-2 cell line. The laryngeal cancer Hep-2 cell line was procured from an official institution under a research project. To assess the anti-proliferative properties of Geraniin, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was employed. Various concentrations of Geraniin were administered to the Hep-2 cells for 24 hours, following which its anti-inflammatory, anti-metastatic, and pro-apoptotic effects were evaluated. As a result, different concentrations of Geraniin showed different anti-proliferative roles in the laryngeal cancer Hep-2 cell line. However, the effects of Geraniin on inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α), interleukin-1 beta (IL-1β), interleukin-6 (IL-6), transforming growth factor-beta (TGF-β), and granulocyte-macrophage colony-stimulating factor (GM-CSF), angiogenesis/metastasis biomarker vascular endothelial growth factor (VEGF), and pro-apoptotic indicators such as Caspase 3, 8 and 9 were not statistically different in half lethal concentration 50% (½LC_{50}), LC_{50}, and double LC_{50} ($2LC_{50}$) concentrations than that in LC_0 concentration. Geraniin has an anti-proliferative role in the laryngeal cancer Hep-2 cell line. However, anti-inflammatory, anti-metastatic, and pro-apoptotic effects of Geraniin were not statistically significant. This may be associated with the exposure duration of the cells to Geraniin. This is the first study on this research area, and further studies are needed to evaluate this topic.

Keywords: Geraniin, larynx cancer, cell line, metastasis, cell proliferation

Introduction

Laryngeal cancer is one of the most common malignancies in otorhinolaryngology practice and is associated with psychological distress and poor quality of life despite treatment [1]. Several factors have been implicated in the pathophysiologic basis of laryngeal cancer. For instance, genetic predisposition and environmental carcinogen exposures, bad habits such as smoking and alcohol, and internal and external factors such as inflammation, oxidative stress and hypoxic damage, and viral infections have been identified [2]. The modern treatment of laryngeal cancer is multidisciplinary management where surgery, radiotherapy, chemotherapy, organ preservation and personalized approaches may be preferred [3].

Geraniin is a compound of ellagitannins found in the structure of pomegranate peel. Studies have revealed that Geraniin is associated with multiple protective functions such as inhibition of proliferation and inflammation, activation of autophagy, suppression of oxidative stress and atherosclerosis, and protection against ischemia [4-6]. Although these studies have provided promising findings, considering their experimental in general, it can be said that more investigations including both in vitro and in vivo methods on the effects of Geraniin related to human health are needed.

Cell lines are essential tools in cancer research, and studies on laryngeal cancer have increasingly utilized Hep-2 cell line models over the past 30 years [7]. These models have gained

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popularity due to their ethical and technical advantages, as they provide an in vitro, animal-free, cost-effective, and easily manageable research platform [8].

Up to now, it has been demonstrated that the factors underlying development of laryngeal cancer [9,10] such as proliferation, inflammation, oxidation, and ischemia are also targeted by Geraniin [11,12]. Although many other compounds have been evaluated with laryngeal cancer Hep-2 cell line [9,10], the effects of Geraniin in laryngeal cancer Hep-2 cell line have not been addressed. Accordingly, the purpose of this study was to evaluate the roles of Geraniin in anti-cancer processes, supression of proliferative, inflammatory, and metastatic activity and activation of apoptosis in laryngeal cancer Hep-2 cell line.

Material and Methods

Ethical Approval and Hep-2 Cell Line Procurement

Considering it research content the current study is not one of the trials requiring ethical approval [13]. Ethics committee approval for this study was obtained from the Van Yüzüncü Yıl University (Decision No: E-27552122-604.01.02-162820, Date: 03 February 2022). The Hep-2 laryngeal cancer cell line used in this study was obtained from the cell culture collection of the Turkish Oral and Dental Diseases Institute Cell Bank (Registration No: 92041501).

Cell Culture and Thawing of Frozen Cells

Cell culture was performed in a sterile laminar flow cabinet. First, the chamber was wiped with 70% ethyl alcohol. The materials were exposed to UV light for 15 minutes in the chamber. The chamber was wiped again with 70% ethyl alcohol and the lid of the chamber was closed. The chamber and cell culture room were exposed to UV light for 1 hour. The temperature of the solution that would come into contact with the cells was kept at 37°C and centrifuged at 800-1000 rpm for 5 minutes. Hep-2 laryngeal cancer cell line was stored in liquid nitrogen (-196°C) until the study was performed. Before starting the study, the frozen cells were thawed in a water bath at 37°C for 1-2 minutes (~90 seconds).

Inoculation and Propagation of Cells

The complete medium used for cell proliferation was prepared using 0.22 µm porous membrane filters. While preparing the medium, 10% fetal bovine serum, 1% penicillin/streptomycin, and 1% sodium pyruvate were added to the filter according to 1 liter volume. Then, the volume was completed to 1 liter by adding glutamine and high glucose concentration 88% DMEM (Dulbecco's Modified Eagle Medium, Sigma). The medium to be used for thawing, washing, passaging, and cell growth was placed in sterile 50 mL falcon tubes and kept at +4°C until use. The medium prepared for use in 50 mL f alcons was heated in 37°C water for 20-30 minutes before cell inoculation.

Frozen Hep-2 cells brought to the laboratory in cryotubes containing liquid nitrogen were taken to -18°C. After 10 minutes, they were thawed by incubating in a hot water bath at 37°C for 1-2 minutes. The cells in the cryotubes about to be thawed were taken into the prepared flask (5 mL medium in a 15 mL flask). Then, they were immediately centrifuged by gentle pipetting. The medium on top was taken with a pipette without touching the cells. 2 mL of medium was added again and washed a second time by gentle pipetting. The medium remaining on the cells was withdrawn again. The thawed Hep-2 cells, purified in 2 washes with dimethyl sulfoxide (DMSO), were dissolved in 1 mL medium for the last time to be seeded in the flask. Complete medium containing 10% FBS prepared for the first seeding at 37°C was added to a 25 mL vial with a volume of 5 mL. Hep-2 cells prepared by thawing and removing DMSO were slowly added to the prepared vial. The 25 mL vial was placed in an incubator at 37°C and 5% CO2 for the growth of inoculated cells. The medium of the cells was monitored daily and changed every 2 days until the cells reached 80-90% confluence.

Subculturing (Passaging)

Passaging at regular intervals is necessary for the cells to continue to grow. Depending on the number of cells initially added, culture conditions and cell line, the culture becomes confluent again at the end of the incubation period and sub-passaging is repeated. Passaging was performed when the Hep-2 cells used in our study became 80-90% confluent. The solutions used in passaging were prepared as follows:

Stock Phosphate Buffered Saline (PBS) Buffer (10X): 80 g NaCl, 2 g KCl, 11.5 g Na2HPO4.7H2O, 2 g KH2PO4 were dissolved in distilled water to a final volume of 1 L, pH value was adjusted to 7.5, sterilised by autoclaving at 121°C for 20 minutes and stored at room temperature until the study day.

PBS Working Buffer (1X): Before the cell culture study, 10X stock PBS buffer was prepared by diluting 1:10 with sterile distilled water and used on the same day.

1X Trypsin-EDTA Solution: Commercially obtained Trypsin-EDTA was diluted 1:10 with sterile 1X PBS and used in cell passaging.

When Hep-2 laryngeal cancer cells became 80-90% confluent, the culture medium in 25 cm^2 flasks was removed, the cells were washed with 5 mL 1X PBS buffer and 1 mL 1X Trypsin-EDTA solution was added and kept on the cells for 1 minute. The flasks were then kept in an incubator with CO2 until the cells detached from the surface (~2 minutes). After incubation, 5 mL each of complete medium and cells were collected in a falcon tube. The cell suspension with complete medium was centrifuged at 1000 rpm for 3 minutes to precipitate the cells. After the supernatant was precisely removed by vacuum, 1 mL of the complete medium was added to the pellet remaining at the bottom to ensure that the cells were well suspended. A sample of the cell suspension was taken and cell counting was performed. After cell counting, the appropriate number of cells (~$2x10^5$) were transferred to 25 cm^2 flasks and the total medium

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volume was completed to 5 mL. In this study, the passaging ratio in subculturing with cell counting was generally found to be 1:3 [14].

Hep-2 Cell Count and Viability Test

For this purpose, cell counting was performed with Trypan blue, which stains only viable cells. On the counting slide, cells were counted and averaged. The number of viable cells was calculated as formula (n/mL): Number of viable cells = (Average Cell Number) x (10^4) x (Dilution Factor). Accordingly, how many mL of the cell suspension should be taken was calculated and manipulations with the cells were started.

Freezing and Storage of Hep-2 cells

After the cells were washed with 5 mL of PBS, 1 mL of Trypsin-EDTA solution was added to the vials. Trypsin was passed over the cells for 1 min and the vials were kept in the incubator for 2 min. After incubation, the cells were collected in a falcon tube containing 5 mL of DMEM containing 10% FBS and the cell suspension was centrifuged at 400 g for 5 min. Then, the supernatant was removed, leaving 1 mL. The cells were diluted in 1 mL of medium and counted. The cells were diluted with DMEM containing 20% FBS to give a cell count of 2×10^5 per mL and aliquoted into 900 µL cryovials. They were placed on ice and DMSO was added dropwise to 10% of the final volume. The cryovials were mixed with a fingertip while adding DMSO. The cryovials were sealed and kept at +4°C for 1 h, at -20°C for 1 h, and then at -80°C overnight. Cells to be used later were stored in the vapor of a nitrogen tank [14].

Preparation of stock and working solutions: Firstly, daily working solutions were prepared for Geraniin at different concentrations [15].

Geraniin stock solution (1 mM) (MW: 952.65 g/mol): An aliquot of DMEM was taken into a sterile falcon tube, 9.526 mg Geraniin was added, DMEM was added to a total volume of 10 mL and vortexed.

Preparation of Geraniin solutions at different concentrations: Starting from 1 mM stock Geraniin solution, 1, 2, 4, 8, 16, 32, 32, 64, 128, 256 and 512 µM Geraniin solutions were prepared by dilution with DMEM using serial dilution method.

Cell Viability Tests

Anti-proliferative activity was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method [16] as follows: 100 µL of the cell suspension prepared (2×10^4 cells/mL) and transferred to each well of 96-well cell culture plates. Then Geraniin at concentrations of 1, 2, 4, 8, 16, 32, 64, 128, 256, and 512 µM was added to the cells and incubated at 37°C. At the end of 24 hours incubation period, 20 µL MTT dye (5 mg/mL) was added to each well and the cells were incubated at 37°C for 2-4 hours. At the end of this period, MTT dye was removed from the cells and 200 µL DMSO was added to each well and incubated for 10 minutes. The colour change was determined at a wavelength of 540 nm in an ELISA plate reader. The viability rates were expressed as percentages and the viability rates of cells not treated with Geraniin were accepted as 100% [17,18]. As a result of MTT method, Geraniin doses that reduced cell viability by 50% compared to the control group were accepted as LC_{50} doses. In order to provide the necessary samples for analyses, ½LC_{50}, LC_{50} and 2LC_{50} doses were used together with LC_0 as Geraniin doses. After the lethal doses were determined as a result of MTT method, experimental groups were formed. In the experimental model to determine the effects of Geraniin on inflammatory, metastatic, and apoptotic activities in Hep-2 cells, LC_0, ½LC_{50}, LC_{50} and 2LC_{50} doses were applied to the cells separately.

Enzyme-Linked Immunosorbent Assay (Elisa) Studies

After the incubations, Trypsin (1 mL 0.25%) was added to the flasks. Thus, the cells were removed from the flask floor and then released with light tapping movements after a very short time. The suspensions were collected by pipette and pellet was obtained by centrifugation at 25°C, 800 rpm for 5 minutes in capped tubes. After the cell pellet was washed with PBS, cell lysis buffer was added. Lysis buffer was prepared as follows. In 200 mL PBS buffer (pH 7.4), 1% Triton-X-100, 8% (40 mL) protease inhibitor cocktail calculated according to the final volume of 500 mL was added. The volume of the final lysis buffer was completed to 500 mL with PBS buffer. A homogenous mixture was formed by shaking well. The prepared lysis buffer was added to the cell pellet obtained from the flask in a volume of 500 mL and pipetted. Then it was sonicated for 20 seconds and waited for 40 seconds. As a result of sonication, intracellular fluids were allowed to pass into the lysis buffer. Proteins that did not dissolve in the lysis buffer despite all applications were precipitated by centrifugation at 8500 rpm at 4°C for 10 minutes. The supernatants obtained in this way were used as cell lysate for biochemical analyses. TNF-α, IL-1β, IL-6, TGF-β, GM-CSF, VEGF, Caspase 3, 8, and 9 levels were measured using human specific ELISA kits and microplate reader according to the manufacturer's instructions.

Statistical Analysis

Statistical analyses of the study were performed using the SPSS (version 22.0) statistical package program. Statistical analyses were conducted to examine the effects of Geraniin on cell viability rates, pro-inflammatory cytokines, growth factors, and caspases. Accordingly, statistical comparisons were made between different concentrations of Geraniin. Statistical comparisons were done using analysis of variance (ANOVA) test. After ANOVA, Tukey's multiple comparison test was used to determine differences. In the calculations, the level of statistical significance was taken as $p<0.05$.

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Results

Figure 1 schematizes the effect of Geraniin on cell viability: After incubation (24 hours), the LC_{50} concentration of Geraniin on Hep-2 cells was found to be 254.69 µM. In this context, 0 µM Geraniin for LC_0 concentration, 127.35 µM Geraniin for ½LC_{50} concentration, 254.69 µM Geraniin for LC_{50} concentration, and 509.38 µM Geraniin for 2LC_{50} concentration were applied to Hep-2 cells. Figure 2 schematizes the effect of Geraniin on proinflammatory cytokines. Panels: (A) TNF-α; (B) IL-1β; (C) IL-6; (D); TGF-β levels were not statistically different ($p>0.05$). Figure 3 schematizes the effect of Geraniin of Geraniin on growth factors. Panels: (A) GM-CSF; (B) VEGF levels were not statistically different ($p>0.05$). Figure 4 schematizes the effect of Geraniin of Geraniin on on caspases. Panels: (A) Caspase-3; (B) Caspase-8; (C) Caspase-9 levels were not statistically different ($p>0.05$).

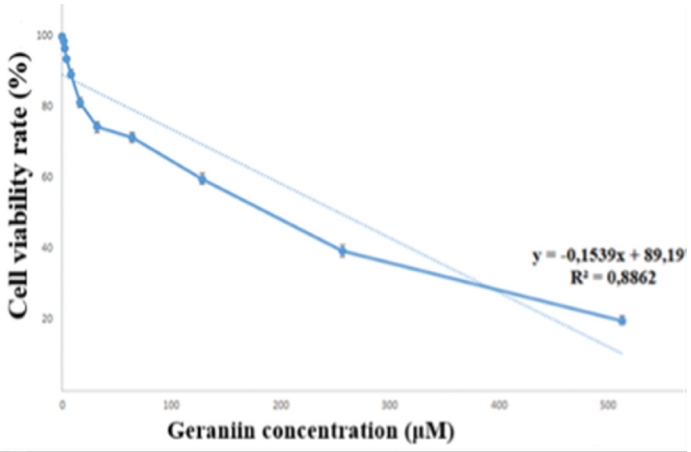

Figure 1. Effect of Geraniin on cell viability rates

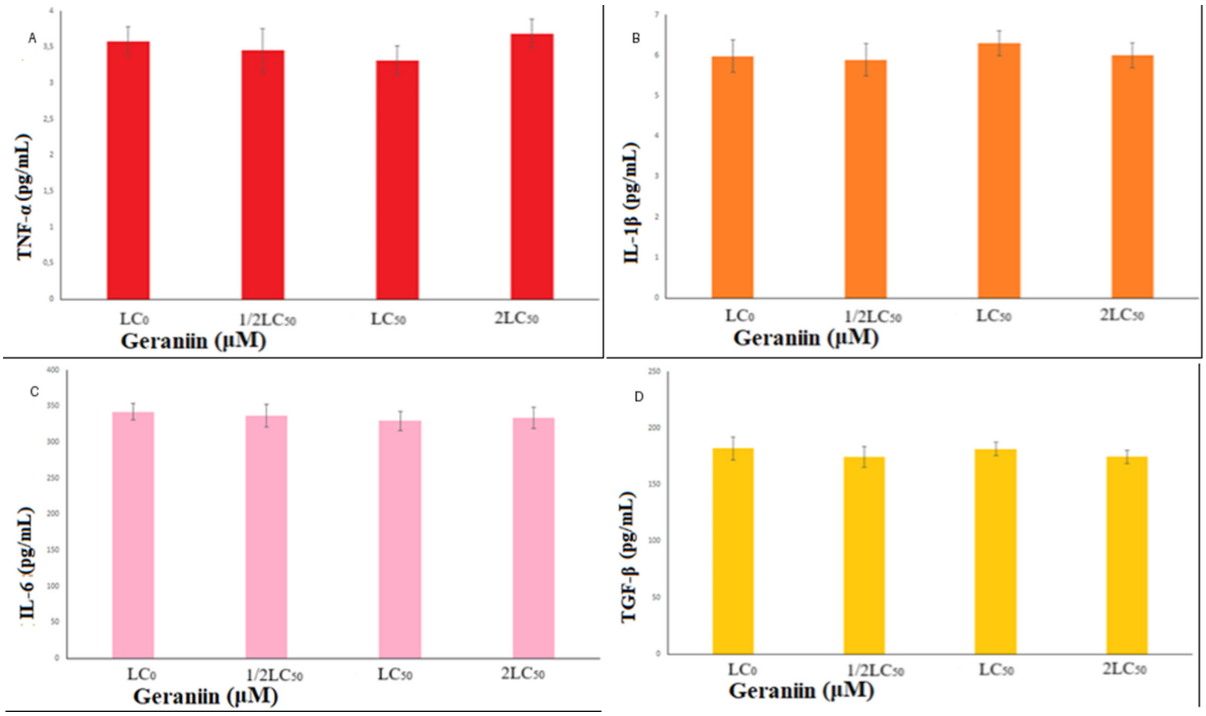

Figure 2. Effect of Geraniin on proinflammatory cytokines. Panels: (A) TNF-α; (B) IL-1β; (C) IL-6; (D); TGF-β levels

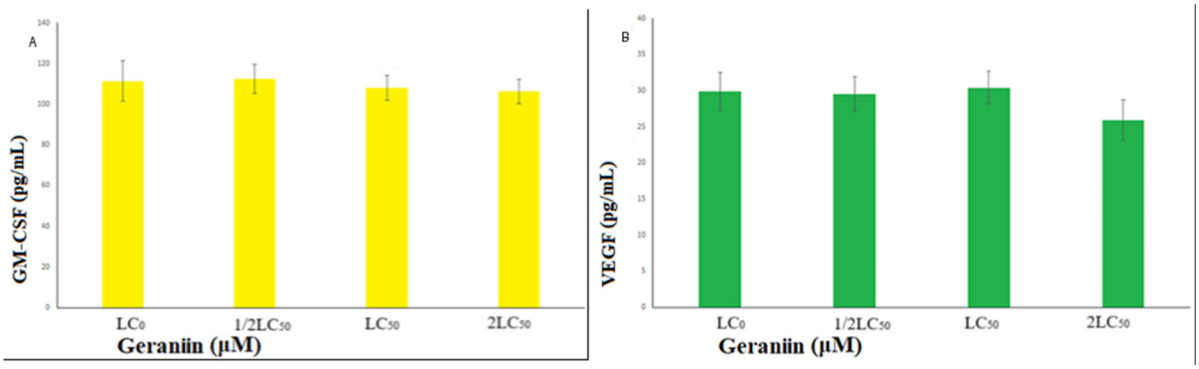

Figure 3. Effect of Geraniin on growth factors. Panels: (A) GM-CSF; (B) VEGF levels

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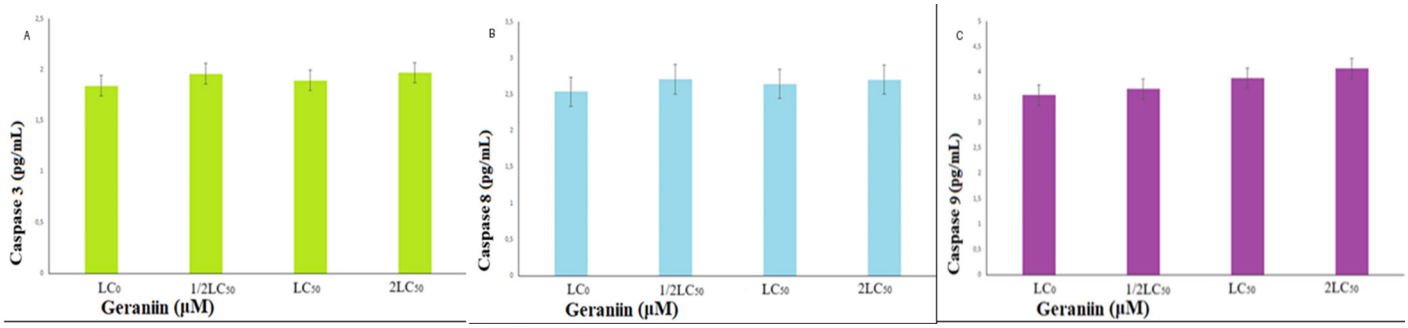

Figure 4. Effect of Geraniin on caspases. Panels: (A) Caspase-3; (B) Caspase-8; (C) Caspase-9

Discussion

In this study, the anti-proliferative, anti-inflammatory, anti-metastatic and apoptosis-related effects of Geraniin on laryngeal cancer Hep-2 cell line were investigated. As a result, it was determined that Geraniin had a suppressive effect on cell proliferation; however, no significant effect was observed on inflammatory cytokines (TNF-α, IL-1β, IL-6, TGF-β, GM-CSF), metastasis marker (VEGF) and apoptosis indicators (Caspase 3, 8, and 9). These results revealed that Geraniin limited anti-inflammatory, anti-metastatic and apoptosis-inducing effects in Hep-2 cell line. However, this study is important in terms of providing the first findings in the literature on the anti-proliferative effects of Geraniin on laryngeal cancer Hep-2 cells.

The relationship between inflammation and cancer has been described previously [19]. Studies have also revealed various beneficial effects of Geraniin such as suppression of cell proliferation and inflammation, promotion of autophagy, reduction of oxidative stress and atherosclerosis, and protective effects against ischemia [4-6]. In addition, previous studies collectively demonstrate the potential of Geraniin in combating various types of cancer. Findings have shown Geraniin's pro-apoptotic activity, anti-inflammatory effect, and regulation of cancer signaling pathways. Geraniin's experimental therapeutic properties strongly present it as a potential anticancer agent in the future [20]. Although these promising effects, given that they are generally experimental results, further studies including both in vitro and in vivo methods are needed to reveal the roles of Geraniin in human health. In addition, the fact that the various beneficial roles of Geraniin [5,6] are opposed to the pathophysiological mechanisms of laryngeal cancer [2] provides a sensible and scientific basis to investigate the potential anti-cancer activity of Geraniin on the laryngeal cancer Hep-2 cell line.

In contrast to our expectations, the findings of this study suggest that Geraniin may not exhibit significant anti-cancer effects on the laryngeal cancer Hep-2 cell line. We found that Geraniin did not induce apoptosis, which is known to have anticancer activity, on the laryngeal cancer Hep-2 cell line. In fact, Geraniin has also been shown to have anti-apoptotic effects rather than inducing apoptosis in spinal cord and heart tissue [11,12]. On the other hand, the present study offers new insights into the topic and provides preliminary data on the potential anti-proliferative effects of Geraniin in Hep-2 cells.

Although numerous compounds have been studied for their effects on the laryngeal cancer Hep-2 cell line [9,10], no prior research has specifically examined the impact of Geraniin on this cell line. As a result, there is a lack of directly comparable findings in the existing literature. Nonetheless, earlier studies have demonstrated that Geraniin exhibits promising activity on various cancer-related pathophysiological mechanisms through experimental models, including in vitro and cell line-based approaches [4-6]. In this context, even though our study did not yield statistically significant outcomes, Geraniin remains a noteworthy compound warranting further investigation for its potential anti-cancer properties.

This study has both strengths and weaknesses. Its greatest strength relates to its specificity as it is the first assessment of the anti-cancer activity of Geraniin in the Hep-2 laryngeal cancer cell line. Its greatest strength lies in its originality, as it serves as a preliminary evaluation of the subject. Furthermore, this study examined not only one effect of Geraniin but multiple effects, such as anti-proliferative, anti-inflammatory, anti-metastatic and pro-apoptotic activity. On the other hand, the study also has some weaknesses. Its results did not reach significance levels and the discussion remained superficial due to the presence of limited relevant literature. In addition, the in vitro nature of the Hep-2 cell line suggests that the findings may not be directly transferable to in vivo human applications.

Conclusion

This study suggested that Geraniin may exert an inhibitory effect on cell proliferation in the laryngeal cancer Hep-2 cell line. However, the anti-inflammatory, anti-metastatic and apoptotic effects of Geraniin were limited. This may be due to the duration of exposure of cells to Geraniin. As this is the first study on this topic in the literature, further research is needed to confirm the findings and further clarify the effects.

Ethical Approval

Approved by the Van Yüzüncü Yıl University (Decision No: E-27552122-604.01.02-162820, Date: 03 February 2022).

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Patient Consent
Not applicable. This study did not involve human participants or patient data.

Conflict of Interest
The authors declare no conflict of interest.

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