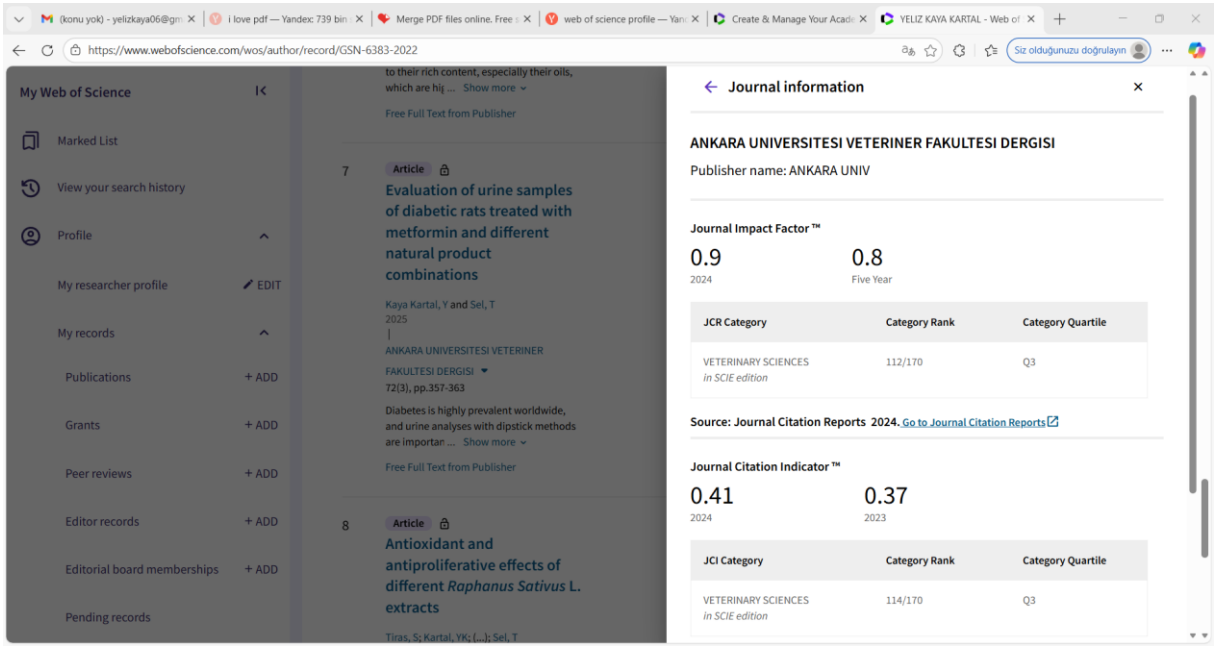

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Antioxidant content of *C. maxima and C. pepo* seeds and the cytotoxic effect on chronic lymphocytic leukemia cell lines

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ABSTRACT

Pumpkin seeds are essential for health due to their rich content, especially their oils, which are high in phenolic compounds. These compounds can be an alternative treatment for diabetes mellitus by reducing blood glucose levels. This study examined the seeds of *C. maxima* and *C. pepo,* which were grown in the Sakarya-Arifiye region and harvested in November 2022. DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging activity, total polyphenol, and flavonoid content of methanolic extracts were measured spectrophotometrically, with three separate extraction replicates for each sample. The total phenolic content was 0.31 ± 0.09 mg/g for *C. pepo* seeds and 0.19 ± 0.02 mg/g for *C. maxima* seeds. Total flavonoid content was 217.2 ± 20.9 µg/g in *C. pepo* and 162.9 ± 19.3 µg/g in *C. maxima*. Antioxidant activity levels demonstrated 12.7 ± 2.4% inhibition for *C. pepo* and 15.09 ± 0.4% for *C. maxima* in DPPH scavenging. In the MEC-1 (mutant p53 chronic B cell leukaemia) cell line, the IC50 value for *C. pepo* was 205 mg/ml, while for *C. maxima*, it indicated a proliferative effect. In the HG-3 (wild-type chronic B cell leukaemia) cell line, IC50 was 209 mg/ml for *C. pepo* and 940 mg/ml for *C. maxima*. These findings indicate that antioxidant, flavonoid, and phenolic content vary by species and growing conditions, influencing antiproliferative effects on cancer cells. Further studies on pumpkin seeds' effects on metabolic pathways in various diseases will be beneficial.

Introduction

Pumpkin is an important vegetable from the *Cucurbitaceae* family (10). *Cucurbita maxima*, *C. pepo*, and *C. moschata* are common species harvested worldwide (9). Pumpkin has also been traditionally cultivated in many countries as a pharmaceutical product (20). In recent years, it has gained popularity in the healthcare industry due to its therapeutic effects (11).

Pumpkin seeds are rich in nutrients, just like other seeds (7). The seeds attract attention due to their biological benefits and contain fibre (20), carbohydrates, protein (18), lipids, essential omega fatty acids (omega-3, 6, and 9) (8), vitamins, provitamins, squalene, tocopherols (vitamin E), cucurbitacin, carotenoids, phytosterols, and phenolic content and their derivatives (7, 21, 22). Furthermore, pumpkin seeds are a source of minerals such as magnesium, phosphorus, potassium, manganese, zinc, iron, sodium, and calcium (6). Although pumpkin seeds are a popular snack in many countries and can be added to meals as a protein supplement, the consumption of plants rich in phenolic compounds has been reported to aid in recovering from oxidative stress (22, 32). This may be attributed to the presence of natural antioxidant agents (1). The consumption of antioxidant agents is associated with reducing the risk of Alzheimer's disease (15).

The popularity of pumpkin in traditional medicine has attracted increasing attention from researchers due to its nutritional profile (25). Pumpkin and its extracts can prevent prostate cancer and urinary problems (22). They are also beneficial for diabetes mellitus (2), cardiovascular diseases (13), and hypertension (24). In addition to these benefits, pumpkin has been proven to have anthelmintic (4), antibacterial (21), and antitumour effects (22). Several studies have also shown that pumpkin seed oil can slow the progression of hypertension and arthritis (32).

This study aimed to analyse the antioxidant content of pumpkin seeds, a natural product known for its numerous benefits to human and animal health. Within this framework, the seeds of two different types of pumpkin (*C. maxima* and *C. pepo*) were comparatively investigated, and their cytotoxic effects on the HG-3 and MEC-1 chronic lymphocytic leukaemia (CLL) cell lines were examined.

Materials and Methods

C. maxima and *C. pepo* harvested in the Sakarya-Arifiye region in November 2022 were used in this study. The seeds of *C. maxima* and *C. pepo* can be seen in Figure 1. The extraction of the pumpkin seeds was performed according to the method described by Scalzo et al. (2005) (26). Pumpkin seeds were crushed using a mortar and pestle. Four grams of seeds were weighed, and 8 ml of 80% methanol solution was added to each sample. The mixture was kept in an ultrasonic water bath for 15 minutes. After 15 minutes, they were shaken on a shaker for 2 hours. Following shaking, the tubes were centrifuged at 9000 rpm for 10 minutes. The supernatants were collected and stored in the refrigerator until analysis.

Determination of Total Polyphenol Content: In this analysis, the method developed by Ullah et al. (2014) (30) was used. In this method, gallic acid (GA) was employed as the standard phenolic substance, and 6 different concentrations of GA solution were prepared to create a standard curve. A volume of 500 µl distilled water and 100 µl Folin-Ciocalteu reagent were added to 100 µl of the extraction sample and shaken on a shaker in the dark for 6 minutes. After shaking, 1 ml of Na_2CO_3 and 500 µl of distilled water were added and incubated in the dark for 90 minutes. After the 90-minutes incubation, the blue-coloured solution was measured at 760 nm using a spectrophotometer. The intensity of the blue colour is directly proportional to the amount of phenolic substance.

Determination of Flavonoid Content: Flavonoid content analysis was performed by modifying the method developed by Boateng et al. (2008) (6). For the standard curve, five different concentrations of routine solutions were prepared. A volume of 2 ml distilled water and 150 µl $NaNO_2$ were added to 0.5 ml of the extraction sample and shaken for 5 minutes. After 5 minutes, 150 µl of $AlCl_3$ was added and shaken again for 5 minutes. At the end of the incubation period, 1 ml of NaOH was added, and the intensity of the orange-coloured solution formed was measured at 510 nm using a spectrophotometer.

Determination of Antioxidant Activity: The method of Ullah et al. (2014) (30) was modified and applied to determine antioxidant activity. The antioxidant levels of the extracts were analysed by examining the scavenging effect of DPPH (31). Five different concentrations of GA solution were prepared to create a standard curve. A 3 ml DPPH solution was added to 50 µl of the extract and incubated in the dark for 15 minutes. After 15 minutes, a measurement was taken at 515 nm using a spectrophotometer.



Figure 1. *C. maxima* (a) and *C. pepo* seeds (b).

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Performing Cell Viability Tests: After the methanol content of *C. maxima* and *C. pepo* seed extracts was evaporated, the extracts were dissolved in DMSO and reconstituted with the medium. The cytotoxic effects of pumpkin seed extracts on MEC-1 and HG-3 CLL cell lines were determined using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay method (Meral, 2018) (17), and IC50 values were calculated. The dehydrogenase enzyme in the mitochondria of viable cancer cells or those in early apoptosis breaks down the tetrazolium ring in MTT, resulting in the formation of coloured crystals called formazan. This reaction does not occur in dead cells. Thus, alive and dead cells can be distinguished using a spectrophotometer (14). Studies have demonstrated the effects of pumpkin seeds on breast, lung, colorectal, gastric, prostate, thyroid, and hepatic cancer cell lines (5, 16, 31). Since there is limited literature on its effects on CLL cells, it was chosen for this study.

Statistical Analysis: Statistical analysis was performed using the SPSS 21.0 software. Descriptive statistics were calculated for the data and reported as the "Arithmetic Mean ± Standard Error".

Results

Total phenolic content (Figure 2) was found to be 0.31 ± 0.09 mg/g Gallic Acid Equivalent (GAE) in *C. pepo* seeds and 0.19 ± 0.02 mg/g GAE in *C. maxima* seeds. *C. pepo* seeds contained more phenolic content than *C. maxima* seeds. However, the difference in total phenolic contents between the two types of pumpkin seeds was statistically insignificant (P>0.05).

Total flavonoid content (Figure 3) was found to be 217.2 ± 20.9 µg/g RE in *C. pepo* seeds and 162.9 ± 19.3 µg/g RE in *C. maxima* seeds. As with the total phenolic content analysis, *C. pepo* seeds were found to have higher flavonoid content than *C. maxima* seeds. However, the difference in total flavonoid content between the two types of pumpkin seeds was statistically insignificant (P>0.05).

Antioxidant activity (Figure 4) was found to be 12.7 ± 2.4 % inhibition in *C. pepo* seeds and 15.09 ± 0.4 % inhibition in *C. maxima* seeds. The antioxidant activity analysis revealed that C. *maxima* seeds had a higher percentage inhibition value than *C. pepo* seeds, in contrast to the phenolic and flavonoid content. However, the antioxidant activity values were statistically insignificant between the two types of pumpkin seeds (P>0.05).

The percentage viability rates of MEC-1 cells after 24 hours of incubation with *C. pepo* seed and *C. maxima* seed extracts were calculated and compared to the control group (Figure 5).

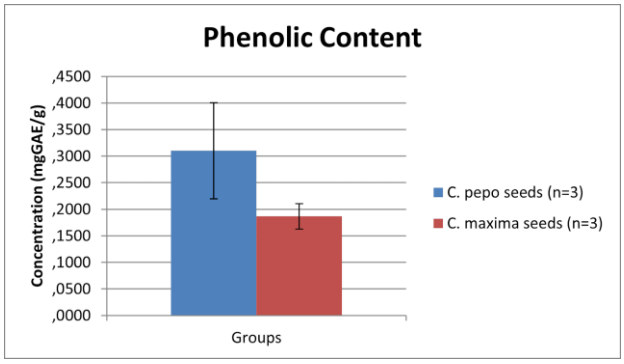


Figure 2. Phenolic content of *C. pepo* and *C. maxima* seeds (mg/g).

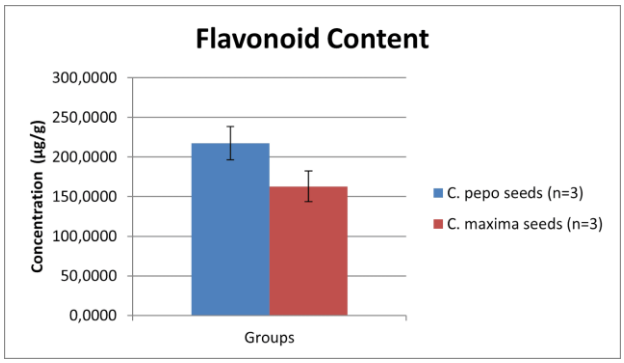


Figure 3. Flavonoid content of *C. pepo* and *C. maxima* seeds (µg/g).

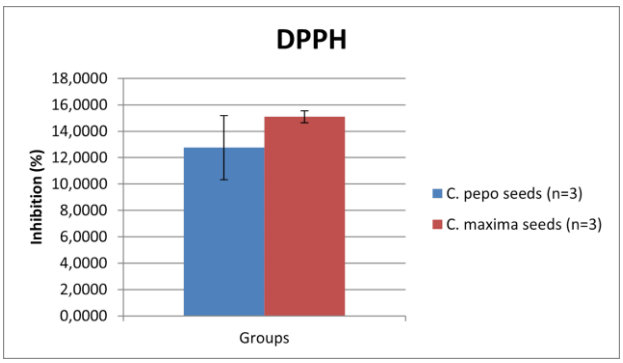


Figure 4. DPPH radical scavenging activity of *C. pepo* and *C. maxima* seeds (% inhibition).

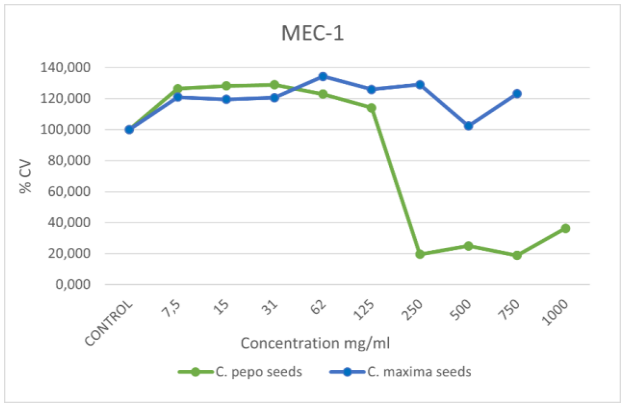


Figure 5. MTT results of pumpkin seeds in MEC-1 cell line.

The analysis reveals that for *C. pepo*, dilutions of 7.5, 15, 31, and 62 mg/ml exhibit proliferative effects on the cells, whereas concentrations of 62, 125, and 250 mg/ml demonstrate antiproliferative effects. In contrast, *C. maxima* generally shows a proliferative effect. The IC50 value of *C. pepo* in the MEC-1 cell line was calculated to be 205 mg/ml, as illustrated in the graphic. However, since no antiproliferative effect was observed in *C. maxima* extracts, the IC50 value could not be determined.

When statistical calculations were made according to the viability test results, statistical significance was found between the control group and all dilutions except *C. pepo* 1000 mg/ml and *C. maxima* 500, and 125 mg/ml dilutions in MEC-1 cells ($P<0.05$). The statistical significance between *C. pepo* 750, 500, and 250 mg/ml dilutions and the control group is due to decreased cell viability. The statistical significance between the other dilutions and the control group is due to increase in cell viability.

The % viability rates of HG-3 cells after 24 hours of incubation with *C. pepo* seed and *C. maxima* seed extracts were calculated compared to the control group (Figure 6).

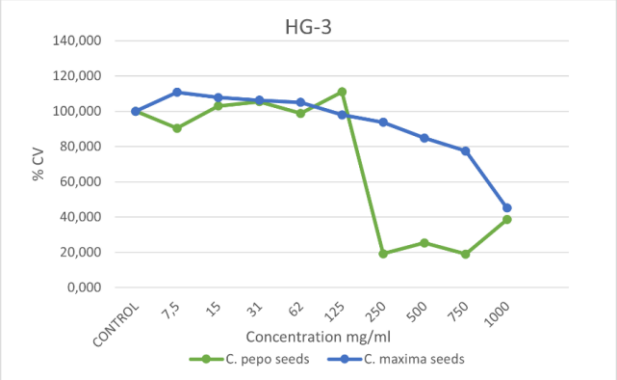


Figure 6. MTT results of pumpkin seeds in HG-3 cell line.

When the graphic is analysed, it is observed that *C. pepo* has an antiproliferative effect in the range of 125 and 250 mg/ml, while for other dilutions of *C. pepo*, there is generally a proliferative effect. For *C. maxima*, a proliferative effect is observed at dilutions from 7.5 to 125 mg/ml and an antiproliferative effect at concentrations of 250, 500, 750, and 1000 mg/ml. Again, from the graphic, the IC50 value of *C. pepo* was calculated as 209 mg/ml and *C. maxima* as 940 mg/ml in HG-3 cells.

When statistical calculations were made according to the viability test results in HG-3 cells, statistical significance was found only between *C. pepo* 1000, 750, 500, and 250 mg/ml, and *C. maxima* 1000 mg/ml dilutions with the control group ($P<0.05$). Here, statistical significance is due to the decrease in cell viability. There is no statistical significance related to the increase in cell viability.

Discussion and Conclusion

C. pepo seeds were richer in total phenolic and flavonoid compounds than *C. maxima*, while their antioxidant activity was higher. Conversely, *C. maxima* exhibited higher inhibition activity than *C. pepo*. In the cell culture study, the antiproliferative effect of *C. pepo* seeds were more pronounced than that of *C. maxima*.

Peiretti et al. (2017) found 4.2 mg GAE/100g of total phenolic content in the methanolic extract of *C. pepo*. In this study, the total phenolic content was found to be higher than the result reported by Peiretti et al. (2017). Although the pumpkins are the same species, the differing results may be attributed to the regions where the pumpkins are grown (21).

According to the studies of Tuberosa et al. (2007) and Siger et al. (2008), the total phenolic content in pumpkin seeds was found to range between 0.28 and 2.46 mg GAE/100 g. In this study, the total phenolic content was higher (27, 29).

Xanthopoulou et al. (2009) investigated four different types of pumpkin seeds with two different extraction methods. They found total phenolic content to range between 42.49 and 64.07 µmol gallic acid/g. In this study, the total phenolic content was found to be higher than the results reported by Xanthopoulou et al. (2009). This difference may be attributed to the pumpkin species and different extraction methods used (32).

Nyam et al. (2013) reported the total phenolic content in roasted pumpkin seeds as 22.92 ± 0.61 mg GAE/100g and the DPPH antioxidant activity as 36.9 ± 1.76 %. In this study, the DPPH antioxidant activity and *C. maxima*'s total phenolic content were found to be lower than the results reported by Nyam et al. (2013). This difference is thought to be due to the pumpkin species and the roasting process (20).

Hussain et al. (2021) studied the methanolic extraction of *C. maxima* seed powder. They found the total phenolic content to be 224.61 ± 1.60 mg GAE / 100g and flavonoid content to be 139.37 ± 1.37 mg Catechin Equivalent (CE) / 100g. The results reported by Hussain et al. (2021) are higher than those of this study. This difference may be attributed to the different environmental conditions where the pumpkins are grown and the process of powdering the seeds (9).

Indrianingsih et al. (2019) investigated the ethanolic extraction of C. *moschata* and *C. maxima* seeds. They found *C. maxima*'s DPPH antioxidant activity to be 17.99 ± 0.8 %. In this study, the DPPH antioxidant activity was found to be lower than that reported by Indrianingsih et al. (2019). Although the pumpkins are the same species, the differing results may be due to the different regions where the pumpkins are grown, and the different extraction methods used (11).

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Peng et al. (2021) investigated the methanolic extraction of roasted *C. pepo* seeds in terms of total phenolic, total flavonoid content, and DPPH antioxidant activity. They found the total phenolic content to range from 2.44 to 3.82 mg GAE/g, the total flavonoid content to range from 1.56 to 2.81 mg Routine Equivalent (RE), and DPPH antioxidant activity to range from 0.31 to 0.47 µmol Trolox Equivalent (TE)/g. They also reported that seeds roasted at higher temperatures contained higher phenolic and flavonoid content (22).

Nawirska-Olszanska et al. (2013) found the total phenolic content to range from 35.4 to 65.7 mg GAE/100 g for *C. maxima* and from 34.3 to 113 mg GAE/100 g for *C. pepo* after 24 hours of ethanolic extraction of pumpkin seeds. The reason for this may be due to the differences in extraction methods, as well as the differences in the regions where the pumpkins are grown (19).

Akomolafe et al. (2015) studied the methanolic extraction of *C. pepo* seeds. They found the total phenolic content to be 32.9 mg GAE/g, the total flavonoid content to be 21.5 mg Quercetin Equivalent (QE)/g, and DPPH antioxidant activity for a 50 µg/ml concentration to be nearly 30%. The results of this study are lower than those of Peng et al. (2021) and Akomofale et al. (2015). This may be due to the different regions where the pumpkins were grown (1).

Quesada-Granados et al. (2023) analysed the total phenolic and total flavonoid content of pumpkin seeds. They found the total phenolic content to be 1230.0 ± 8.89 mg Rutin Hydrate/100g and the total flavonoid content to be 1100.0 ± 9.54 mg Quercetin/100g. The results of this were much lower compared to those of Quesada-Granados et al. (2023). The main reason for this may be the difference in extraction methods (23).

Kulaitine et al. (2017) found statistically significant differences in DPPH activities from the methanolic extraction of three different pumpkin seeds, with antioxidant activity increasing in proportion to total phenolic content. In this study, no statistically significant difference was found between antioxidant activities. Additionally, no relationship was found between antioxidant activity and total phenolic content (12).

Medjakovic et al. (2016) investigated the hydroethanolic extraction of *C. pepo* in inhibiting the growth of cancer cells, independent of steroid hormone receptors. In the context of the study, different cancer cell lines were investigated, including DU-145 (prostate), LNCaP (prostate), BPH-1 (benign prostate), CaCo-2 (colorectal), and MCF-7 (breast). As a result of the study, it was observed that *C. pepo* extracts inhibited growth in all cells except BPH-1 cells, where it increased cell growth by 40-50% (16).

Azari et al. (2018) reported the cytotoxic effects of ethanolic extracts of unshelled pumpkin seeds (*C. pepo subsp. pepo var.* Styriaka) on papillary thyroid carcinoma (PTC) cells. At a concentration of 1 mg/ml, they found no significant difference in cell viability after 24, 48, and 72 hours of incubation. However, at a concentration of 200 µg/ml the number of dead cells increased, and cell viability was found to be 15%. At 800 µg/ml, cell viability increased to 30% (3).

Bahadori et al. (2020) investigated the antiproliferative and apoptotic effects of pumpkin extract on PTC cells. The ethanolic extract prepared by the Soxhlet method demonstrated a limited effect at low concentrations (5-15% inhibition), while 30% inhibition was observed at 800 µg/ml and 60% inhibition at 1600 µg/ml. Higher concentrations (2400-6400 µg/ml) resulted in 70-90% inhibition, and statistically significant results were obtained (5).

Vinayashree et al. (2024) investigated the cytotoxic effect of pumpkin seed proteins on HepG2 and MCF-7 cell lines. They found no significant change in the HepG2 cell line at low concentrations after 48 and 72 hours. As the concentration increased (>50 µg), a decrease in cell viability was observed. They observed that there was no cytotoxic effect on the MCF-7 cell line (31).

In cell culture studies, pumpkin seeds have been reported to have an antiproliferative effect on cancer cells, as observed in this study.

Pumpkin seeds are a natural product widely used across the world. They have many positive effects in terms of both nutrition and disease prevention. The content properties of the seeds depend on factors such as the type of pumpkin, its genetics, the region where it is grown, the climate, soil type, and many other environmental factors, which are too numerous to be counted.

A previous study reported that the dry matter content and composition of radishes vary considerably depending on several factors, including growing conditions, climatic conditions, ripening stage, harvest time, and genotype (28).

Despite these variations, researchers in different countries have investigated pumpkin seeds and their effect on diseases. Among these diseases are various types of cancer, one of the major health challenges of today.

In this study, the methanolic extraction method was preferred for better analysis of total phenolic and flavonoid contents. The analyses indicated that *C. pepo* seeds contained higher phenolic and flavonoid contents than *C. maxima* seeds. However, in terms of antioxidant activity, *C. maxima* seeds exhibited higher antioxidant activity than *C. pepo* seeds. Although *C. pepo* seeds were richer in phenolic and flavonoid contents, their antioxidant activity was lower than *C. maxima* seeds, suggesting that antioxidant activity may be due to other compounds in the seed, such as minerals. In this respect, conducting

extensive content analyses of pumpkin seeds would be beneficial.

Cell culture studies demonstrated that the antiproliferative effect of *C. pepo* seeds were more effective than that of *C. maxima* seeds in both MEC-1 and HG-3 cells. In fact, the IC50 value could not be calculated since *C. maxima* seeds could not exhibit an antiproliferative effect in MEC-1 cells. This may be due to resistance caused by the p-53 gene mutation in MEC-1 cells. Although this study has demonstrated the effects of two different pumpkin seed types on MEC-1 and HG-3 cell lines, further detailed investigations and studies are needed.

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This study has not received the support of any organisation.

Ethical Statement

This study does not present any ethical concerns.

Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

TE, YKK, MP and TS conceived and planned the experiments. TE and YKK carried out the experiments. TE and YKK contributed to sample preparation. TE, YKK, MP and TS contributed to the interpretation of the results. TE took the lead in writing the manuscript. All authors provided critical feedback and helped shape the research, analysis and manuscript.

Data Availability Statement

The data supporting this study's findings are available from the corresponding author upon reasonable request.

Animal Welfare

The authors confirm that they have adhered to ARRIVE Guidelines to protect animals used for scientific purposes.

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