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Research Article

Investigation of the Dual Role of *Scorzonera pygmaea*: Cytotoxic Activity and Antioxidant Potential

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Scorzonera pygmaea, an indigenous plant of western Anatolia, belongs to the *Scorzonera* genus, which includes various species recognized for their edible and medicinal properties. This study is aimed at investigating the phenolic composition of *S. pygmaea*; evaluating its antioxidant, cytotoxic, apoptotic, and genotoxic activities; and assessing the impact of *S. pygmaea* on the expression of cell cycle regulatory genes. HPLC-DAD analysis identified chlorogenic acid as the predominant phenolic compound, potentially linking plant to dietary benefits. The antioxidant potential, evaluated using DPPH radical scavenging and CUPRAC assays, yielded an IC_{50} value of $63.53 \pm 6.29\,\mu g/ml$ and a Trolox equivalent of 0.061 ± 0.013 g/g, respectively. To assess antigrowth activity, the *S. pygmaea* extract was tested against MCF-7 and MDA-MB-231 breast cancer cell lines, along with the nonmalignant MCF-10A cell line, using sulforhodamine B and ATP cell viability assays. The results exhibited highly consistent IC_{50} values of 104.63, 179.27, and 90.83 μg/ml, respectively. Notably, the *S. pygmaea* extract induced apoptosis in all cell lines, with MDA-MB-231 cells displaying a higher percentage of early apoptotic cells under fluorescence microscopy. The comet assay revealed that *S. pygmaea* extract induced DNA damage in all cell lines, with MCF-7 and MCF-10A cells showing an accumulation of DNA damage over time. Gene expression analysis revealed significant alterations in cell cycle regulators, with each cell line displaying distinct and time-dependent patterns after exposure to *S. pygmaea*. Collectively, these findings underscore the cytotoxic, apoptotic, and genotoxic activities of *S. pygmaea* while simultaneously emphasizing its antioxidant potential, especially given its high chlorogenic acid content. The dual role of *S. pygmaea*, evidenced by its cytotoxic and antioxidant properties, suggests the potential for further exploration of its utilization within the food industry as a possible dietary resource, while necessitating further studies to fully understand its implications.

1. Introduction

The genus *Scorzonera*, which comprises 180–190 species, is part of the Asteraceae family and is distributed in arid regions of Eurasia and Northern Africa [1]. Turkey has a high endemism rate for this genus, with 31 of its 52 endemic species [2]. Some *Scorzonera* species are used as edible plants, such as *Scorzonera hispanica*, commonly known as black salsify, which is grown for its roots and other parts that can be consumed [3]. In the 19th and 20th centuries, tender leaves and stems of *Scorzonera laciniata* were consumed during famines in Europe, particularly in Spain [4].

Scorzonera species have deep-rooted traditions in medicinal use across diverse geographical regions. In Turkey, they play a significant role in treating ailments, ranging from arteriosclerosis, rheumatism, and wounds to antidiabetic and antihypertensive conditions [5]. Similarly, in Mongolian folk remedies, they are recognized as antipyretics, diuretics, and agents for treating ulcers and stomach tumors, whereas in India's traditional medicine, they are primarily used for the treatment of jaundice [6, 7]. The versatility of *Scorzonera* species is further underscored in European, Chinese, and Tibetan traditions, where they have been employed to treat pulmonary diseases, reduce fever, and address inflammation, respectively [8]. Despite these regional variances in application, commonalities emerge, such as the treatment of wounds, infertility, lung edema, hypertension, gastric ulcers, and even cancer [8, 9]. Recent studies have expanded our

understanding of the pharmacological activities of these plants, including their cytotoxic, anti-inflammatory, analgesic, hepatoprotective, antimicrobial, wound healing, and antioxidant properties [8, 9]. Biochemical analyses attribute these medicinal properties to an array of phytochemicals present in *Scorzonera*, including sesquiterpenoids, triterpenoids, flavonoids, coumarinoids, and quinic acid derivatives, such as chlorogenic acid [8, 9]. Numerous *Scorzonera* species have been rigorously studied for their antioxidant activities, and several compounds within *Scorzonera* species have been identified for their anticancer properties [8, 10, 11]. For instance, scorzoaustriacin, a sesquiterpene lactone derived from *Scorzonera austriaca*, demonstrated an IC_{50} value of 11.3 μM against the K562 human erythroleukemia cell line [12]. Similarly, a quinic acid derivative isolated from *Scorzonera divaricata* was found to be cytotoxic to HepG2 hepatocellular carcinoma cells, with an IC_{50} value of 20.1 μM [13]. In addition, a study evaluated the aerial and subaerial parts of 27 *Scorzonera* taxa for cholinesterase inhibition, which is crucial for degenerative diseases, as well as antityrosinase activity. The latter is particularly relevant to the food industry, as inhibiting tyrosinase activity prevents browning [14].

Scorzonera pygmaea Sibth. & Sm. is a perennial herb with pale yellow flowers that grows 1.5 to 11 cm tall and is endemic to western Anatolia [15]. Previous studies have identified phenolic compounds in the aerial and subaerial parts of *S. pygmaea* and reported their antioxidant, antimicrobial, and anti-inflammatory activities [16, 17]. In a recent study, 29 terpenoids and fatty acids were profiled from *S. pygmaea*, and the extracts were evaluated for tyrosinase and urease inhibitory activity [18]. Despite various studies on *Scorzonera* species, there is a glaring gap in our understanding of the cytotoxic, apoptotic, and genotoxic effects of *S. pygmaea*. The relationship between the cytotoxic activities of the genus *Scorzonera* and its potential for causing DNA damage remains elusive. Venturing into this unexplored territory, this study seeks to unravel the phenolic profile of *S. pygmaea* and assess its antioxidant potential and its impact on cellular growth, apoptosis, and genotoxicity. To further deepen the scope, this study uniquely investigated the influence of the plant on the expression of key genes central to cell cycle regulation, an aspect yet to be explored in any *Scorzonera* species.

2. Materials and Methods

2.1. Plant Material Extraction. Aerial parts of *S. pygmaea* were collected from Uludag National Park at an altitude of 2200 m. The plant was authenticated by Professor Gurcan Guleryuz from the Biology Department of Bursa Uludag University. A voucher specimen was deposited in the university herbarium with the herbarium number BULU 45698. The collected plant specimens were first evaluated for any contamination or presence of foreign materials and then dried in the shade at room temperature. The dried specimens were subsequently ground to a fine powder. An extract was prepared from a 30 g aliquot of the powder using a Soxhlet apparatus with 150 ml methanol. The resulting crude extract was concentrated using a rotary vacuum evaporator, lyophilized with equipment from Christ, Osterode am Harz, Germany, and preserved at -80°C for later analyses.

2.2. Detection of Phenolic Compounds. For the analysis of phenolic content in *S. pygmaea*, a lyophilized extract was first prepared for investigation. It was dissolved in a 1 : 1 (*v*/*v*) mixture of ethanol and water at a concentration of 5 mg/ml. A sonicator was used to ensure that the solution was uniform. Following the preparation, the analysis of phenolic compounds was undertaken using a high-performance liquid chromatography (HPLC) system—an Agilent 1200 series (Waldbronn, Germany)—equipped with a diode array detector (DAD), an autosampler, a vacuum degasser, and a binary pump. The detailed procedure for this analysis has been described previously [19].

The antioxidant activity of *S. pygmaea* was determined using the DPPH radical scavenging [20] and cupric reducing antioxidant capacity (CUPRAC) method [21], utilizing the adapted protocols as described by Sarimahmut and Celikler [22]. For both assays, an initial stock solution was prepared from the *S. pygmaea* extract at a concentration of 1 mg/ml using a mixture of ethanol and water in a 1 : 1 (*v*/*v*) ratio. To guarantee the integrity of the antioxidant compounds and circumvent potential activity loss, all solutions were prepared immediately prior to conducting the assays. The solutions underwent a twofold serial dilution process within 96-well microplates, resulting in a concentration range of the extract solutions spanning from 1.95 to 1000 μg/ml. For the DPPH radical scavenging assay, the reagent was added to each well, ensuring a final concentration of 0.1 mM. Following the addition of reagents, microplates were incubated on orbital plate shaker set at 600 rpm, maintaining a temperature of 37°C. Absorbance values were subsequently recorded using a spectrophotometer (FlashScan S12, Germany). For the DPPH radical scavenging assay, absorbance was measured at 517 nm, while for the CUPRAC method, the measurement was taken at 450 nm.

2.3. Cell Culture. Cultures of MCF-7, MDA-MB-231, and MCF-10A were propagated as monolayers under conditions of 37°C with a 5% CO_2 atmosphere. The culture media for the MCF-7 and MDA-MB-231 human breast cancer cells consisted of RPMI 1640 medium, while the nonmalignant human breast cell line, MCF-10A, was grown in equal proportions of DMEM and Ham's F12 medium. Both media were supplemented with 5% fetal bovine serum, 1% penicillin G (100 U/ml), and streptomycin (100 μg/ml). Additionally, the DMEM and Ham's F12 medium were fortified with epidermal growth factor (20 ng/ml), cholera toxin B subunit (10 ng/ml), and insulin (0.12 IU/ml).

Regarding the cell culture experiments, the lyophilized extract of *S. pygmaea* was reintroduced to dimethyl sulfoxide, forming a stock solution at a concentration of 100 mg/ml. The necessary dilutions were then conducted using the respective cell culture medium.

2.4. Antigrowth Activity and Apoptosis Detection Using Fluorescent Microscopy. Inhibition of growth by the *S. pygmaea* extract was evaluated through sulforhodamine B and

ATP assays. The ATP assay was employed to more sensitively determine the inhibition of growth [23]. The propensity of the *S. pygmaea* extract to induce apoptosis was investigated through fluorescence microscopy using the nuclear-staining dyes propidium iodide (PI) and Hoechst 33342. Briefly, *S. pygmaea* extract was diluted in a series of twofold steps to create a concentration gradient ranging from 3.13 to 200 μg/ml in 96-well microplates. MCF-7, MDA-MB-231, and MCF-10A cells grown in the exponential phase were seeded at 5×10^3 cells per well and incubated for 48 h at 37°C. Evaluation of cell viability and detection of apoptosis were performed as described previously [22].

2.5. Genotoxic Effects of S. pygmaea Extract. The genotoxic effects of the *S. pygmaea* extract were evaluated using an alkaline comet assay, with slight modifications to the method described by Singh et al. [24]. MCF-7, MDA-MB-231, and MCF-10A cells (5×10^5 cells/well) were seeded in 6-well plates and treated with the IC_{50} dose of the *S. pygmaea* extract after a 24 h incubation period. Cells were subjected to two time points of treatment with the extract at 6 and 18 h. The slides were then prepared and placed in an electrophoresis tank filled with alkaline electrophoresis buffer. Electrophoresis was performed for 30 min at 25 V and a current of 300 mA. After the slides were neutralized and air-dried, they were stained with a 2 μg/ml solution of ethidium bromide and subsequently visualized at a magnification of ×200. The tail length, tail DNA percentage, and Olive tail moment parameters were analyzed using specific software for the comet assay (Kameram 21; Argenit, Istanbul).

2.6. qRT-PCR Analysis. Exponentially growing MCF-7, MDA-MB-231, and MCF-10A cells, cultivated in T25 flasks, were exposed to the *S. pygmaea* extract at IC_{50} for 6, 12, and 18 h. The cells were trypsinized at the end of treatment. Total RNA isolation (catalog # 12183018A; Life Technologies, CA, USA) and first-strand cDNA synthesis (catalog # E6300S; New England Biolabs, MA, USA) were performed according to the manufacturer's instructions. The A260/A280 ratios were determined using a microvolume UV-Vis spectrophotometer (NanoDrop™, Thermo Scientific) to detect RNA quality and purity. The synthesized cDNAs were analyzed using a Roche LightCycler 480 II instrument. Primers designed for specific gene regions were used (Table S1), and the LightCycler® 480 SYBR Green I Master kit (catalog # 04707516001, Roche, Basel, Switzerland) was employed for amplification. The PCR mix components and volumes, as well as the temperature and duration conditions, are provided in the Supplementary Material (Tables S2 and S3). For relative quantification, the obtained Ct values for the target genes were normalized to ACTB, and the expression values were calculated.

2.7. Statistical Analysis. All statistical analyses were performed using SPSS 28.0 statistical software for Windows. Results are expressed as mean ± standard deviation or standard error. One-way analysis of variance (ANOVA) was conducted following a normality test to determine significance, and intergroup differences were determined using Tukey's honest significant difference (HSD) or Tamhane's T2 post hoc test. A p value of less than 0.05 was considered statistically significant.

3. Results and Discussion

3.1. Phenolic Composition of S. pygmaea by HPLC-DAD. The phenolic compounds in the methanol extract of *S. pygmaea* were characterized by HPLC-DAD, and the results are summarized in Table 1 and Figure S1. Of the seven detected compounds, chlorogenic acid was the most abundant constituent in the aerial parts of *S. pygmaea*. The presence of chlorogenic acid, a recognized antioxidant with proven hepatoprotective, cardioprotective, antiobesity, and anti-inflammatory properties, is significant [25]. Moreover, its antimicrobial properties and ability to inhibit lipid oxidation highlight its potential application in food preservation [26].

Studies on phenolic compounds in the aerial and subaerial parts of the *Scorzonera* genus have identified chlorogenic acid in 20 species, making it one of the most prevalent phenolic constituents, although concentrations can vary notably between species [8]. Quercetin 3-β-D-glucoside and rutin are also commonly found in numerous *Scorzonera* species. Conversely, ferulic acid has been detected in *S. tomentosa*, *S. papposa*, and *S. semicana*; caffeic acid in *S. divaricata*, *S. hieraciifolia*, and *S. hispanica*; and *p*-coumaric acid in two species, while protocatechuic acid has so far only been identified in *S. pygmaea*, as per the available literature [8, 9]. The exclusive detection of protocatechuic acid in *S. pygmaea* suggests its potential value as a chemosystematics marker for this species.

Previous studies have highlighted the identification of dihydroisocoumarins, fatty acids, terpenoids, and phenolic acid derivatives, including scorzopygmaecoside and scorzonerol, in the aerial and subaerial parts of *S. pygmaea* [16, 17]. Notably, the latter two compounds were detected in this species for the first time, underscoring the rich chemical composition of the plant [17]. Although the current research and prior studies both identified protocatechuic acid as a consistent constituent, there were several differences in the phenolic profiles. Compounds such as thunberginol C, cudrabibenzyl A, scorzocreticin, scorzocreticoside I and II, scorzopygmaecoside, scorzonerol, and 3,5-di-O-caffeoylquinic acid, which were present in earlier research, were absent from this study. In contrast, phenolic compounds, such as caffeic acid, ferulic acid, *p*-coumaric acid, quercetin 3-β-D-glucoside, and rutin, were identified in the present study, which were not indicated in previous research. Potential reasons for these discrepancies might encompass variations in the plant parts studied; notably, one prior study focused on the subaerial sections. Other factors could include differences in the extraction methods, seasonal variations, plant age, geographical origins, and detection methodologies [16, 17].

3.2. Antioxidant Activity of S. pygmaea. The antioxidant activity of *S. pygmaea* was evaluated using the DPPH assay and Trolox equivalents (g TE/g sample); the results are shown in Table 2. The values are expressed as IC_{50} for the DPPH assay and as Trolox equivalents, which represent

Table 1: Phenolic content analysis of *S. pygmaea* using HPLC-DAD.

Compound	Amount (mg/100 g)
Caffeic acid	55.21 ± 0.45
Chlorogenic acid	1213.72 ± 0.58
Ferulic acid	53.38 ± 0.07
p-Coumaric acid	10.27 ± 0.17
Protocatechuic acid	0.62 ± 0.04
Quercetin 3-β-D-glucoside	31.16 ± 0.10
Rutin	18.69 ± 0.10

Table 2: Evaluation of the antioxidant potential of *S. pygmaea* extract via DPPH and CUPRAC assays.

Sample	DPPH scavenging IC_{50} (μg/ml)	CUPRAC g TE/g sample
S. pygmaea extract	63.53 ± 6.29	0.061 ± 0.013
Ascorbic acid	3.14 ± 0.27	0.738 ± 0.064
Trolox	4.00 ± 0.06	NA

CUPRAC: cupric reducing antioxidant capacity; DPPH: 2,2-diphenyl-1-picrylhydrazyl; NA: not available; TE: Trolox equivalent.

the amount of Trolox solution that has an equivalent antioxidant capacity to 1 g of the sample. Ascorbic acid was used as the reference for antioxidant capacity. These results indicate that *S. pygmaea* has significant antioxidant activity, which can be attributed to its high phenolic content. Both the methods yielded consistent results.

Previous studies have determined the antioxidant activities of different *Scorzonera* species. According to Şahin et al. [16], *S. pygmaea* demonstrated the highest antioxidant capacity in the ethyl acetate fraction of the ethanol extract measured by DPPH and ABTS radical scavenging assays as 1.24 mg/ml EC_{50} value and 1.856 Trolox equivalents (as mM, determined at a 2.5 mg/ml fraction concentration), respectively. Antioxidant activity of *Scorzonera mollis* was recorded as 68.55 μg/ml EC_{50} value by DPPH radical scavenging assay and 241.66 μg/ml $A_{0.50}$ value by CUPRAC assay [27]. Nasseri et al. [28] reported that IC_{50} value of *Scorzonera paradoxa* leaf extract measured by DPPH radical scavenging assay was 18.81 mg/ml. Sezer Senol et al. [14] studied the DPPH radical scavenging activity of aerial parts and roots from 27 *Scorzonera* species at a concentration of 1000 μg/ml. Notably, the aerial part extracts of *S. pisidica* and *S. kotschyi* exhibited significant scavenging activities, with rates of 84.55% and 61.53%, respectively. The findings of this study are in parallel with some of the previous studies made so far, but variations are noted in different studies, which might conceivably originate from interspecies variation and methodological differences.

3.3. Antigrowth Activity of S. pygmaea. The antigrowth potential of *S. pygmaea*, as determined by SRB and ATP assays, is shown in Figure 1. The IC_{50} values derived from the SRB assay were 87.31, >200, and 78.21 μg/ml for MCF-7, MDA-MB-231, and MCF-10A cell lines, respectively. An ATP assay was performed to validate the results, yielding IC_{50} values of 104.63, 179.27, and 90.83 μg/ml for MCF-7, MDA-MB-231, and MCF-10A cell lines, respectively. In both assays, MCF-10A cells displayed the highest sensitivity to *S. pygmaea* extract, whereas MDA-MB-231 cells exhibited the highest resistance. The higher sensitivity of nonmalignant MCF-10A cells to *S. pygmaea* treatment implies a lack of selective toxicity towards cancer cells. The differences between the dose and response curves of MCF-7 and MCF-10A in the separate cell viability assays were minimal. However, the viability of MDA-MB-231 cells following treatment with 100 and 200 μg/ml *S. pygmaea* extract was considerably higher according to the SRB assay. This was presumably due to the treated cells depleting their ATP reserves, yet retaining a higher protein content, as inferred from the SRB assay's reflection of cellular protein amounts.

Recent years have witnessed an upsurge in research on the biological activities and anticancer potential of *Scorzonera* species. A significant proportion of extracts derived from *Scorzonera* species exhibit antiproliferative activity over a broad concentration range, with certain species demonstrating notable activity. Keser and Kak [29] evaluated the anticancer activity of the aerial parts of *Scorzonera semicana* and reported IC_{50} values of 7.82, 7.97, and 20.35 μg/ml against HCT-116, LNCaP, and MCF-7 cell lines, respectively. Furthermore, Deveci [27] noted that the methanol extract from the aerial parts of *S. mollis*, which was the most active among hexane and acetone extracts, demonstrated an IC_{50} value of 19.46 against DLD-1 colorectal adenocarcinoma cells. Extractions from the leaves and roots of *Scorzonera austriaca* were screened for cytotoxicity against MCF-7, A431, and HeLa cell lines. Notably, the chloroform root extract exhibited remarkable antigrowth activity, with IC_{50} values spanning from 4.71 to 6.42 μg/ml. In contrast, the most potent extract from the remaining samples achieved a mere 48.11% growth inhibition at a concentration of 10 mg/ml [30]. Other studies have reported weak antigrowth activities. Essential oils extracted from the aerial parts of *Scorzonera calyculata* had an IC_{50} value of 9.8 mg/ml after 24 h of treatment of the A549 non-small-cell lung carcinoma cell line [31]. Similarly, the whole plant methanol extract of *Scorzonera musilii* showed marginal cytotoxicity against HepG2 hepatocellular carcinoma cells, inhibiting cell viability by only 13.9% at 50 μg/ml [32]. Comparatively, the IC_{50} values of *S. pygmaea* did not lie at the extreme ends of this broad IC_{50} range. The discrepancies in the antigrowth activity of the extracts could be attributed to a multitude of critical variables, such as extraction methodologies, *Scorzonera* species, and targeted cancer cell lines. Although it remains challenging to attribute the antiproliferative effects of *S. pygmaea* extract to a particular phenolic molecule or group of molecules, it is noteworthy that chlorogenic acid, in previous studies, has exhibited anticancer activity against MCF-7 and MDA-MB-231 cells with IC_{50} values of 52.5 and 75.88 μM, respectively, when used in isolation. However, the concentration of chlorogenic acid used in these studies was substantially higher than its estimated presence in the *S. pygmaea*

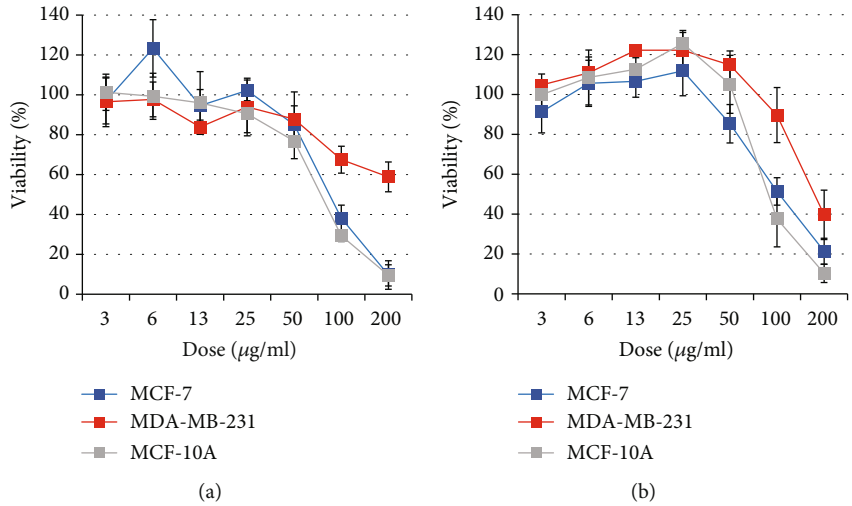

Figure 1: Viability (%) of MCF-7, MDA-MB-231, and MCF-10A cells following 48 h of *S. pygmaea* extract treatment determined by the SRB (a) and ATP (b) assays.

extract, implying that chlorogenic acid may not be the primary factor driving the growth inhibitory effects of *S. pygmaea* [33].

3.4. Apoptotic Activity of S. pygmaea Extract. The apoptotic potential of *S. pygmaea* extract was evaluated in MCF-7, MDA-MB-231, and MCF-10A cells. Cellular changes were observed (Figure 2) following exposure to a range of 3.13-200 μg/ml of the extract for 48 h. Figure 2 illustrates examples of apoptotic cells characterized by pyknotic or fragmented nuclei, marked with arrows. Cells that did not contain PI were considered to be in the early apoptotic stage. Hoechst 33342 and PI positivity indicated late apoptosis and necrosis. The signs of apoptotic cell death, such as pyknotic and fragmented nuclei, were more evident at 100 and 200 μg/ml for all cell lines. The ratio of early to late apoptotic cells was significantly higher in the MCF-7 cell line at concentrations of 100 and 200 μg/ml *S. pygmaea* extract, with virtually all cells undergoing apoptosis at a dose of 200 μg/ml. At lower doses of 25-50 μg/ml, late apoptotic figures were observed in MCF-7 cells. MDA-MB-231 cells treated with *S. pygmaea* extract showed an increasing number of apoptotic cells, starting at concentrations as low as 12.5 μg/ml. Most MDA-MB-231 cells observed with 12.5-200 μg/ml *S. pygmaea* extract were in the early stage of apoptosis and had not yet lost cell membrane integrity, suggesting higher resistance to *S. pygmaea* treatment. Similarly, the nuclear morphology of MCF-10A cells was consistent with apoptotic cell death, but the number of cells in the same image field declined significantly at 100 and 200 μg/ml doses owing to extreme cytotoxicity. To date, a recent study examined the apoptotic effects of a *Scorzonera* species, which showed that the chloroform fraction of *Scorzonera hispanica* seeds promoted apoptosis by downregulating the expression of prosurvival proteins, including phosphorylated Akt, FAK, and ERK 1/2 in MCF-7 cells [34]. These findings suggest that the dominant mechanism of cell death is apoptosis rather than necrosis, and microscopic examination seems to be consistent with the antigrowth activity of *S. pygmaea*.

3.5. Genotoxic Effects of S. pygmaea Extract. The comet assay results, as displayed in Figure 3, underscored the genotoxic effects of the *S. pygmaea* extract. The extract was applied at a single IC_{50} dose for short-term (6 h) and long-term (18 h) incubation. The degree of DNA damage was determined by analyzing various comet parameters, including tail length, % tail DNA, and Olive tail moment. While tail length directly measures the movement of smaller, damaged DNA particles, the percentage of tail DNA approximates the overall quantity of damaged DNA. The Olive tail moment, computed as the product of tail length and the distance between the centers of mass of the comet head and tail, serves as a comprehensive indicator of the severity of DNA damage.

Significant increases in DNA damage compared with the untreated control group (0 h) were observed across all cell lines in a time-dependent manner ($p < 0.05$). In both MCF-7 and MCF-10A cell lines, DNA damage was delayed but became more substantial between 6 and 18 h of incubation with *S. pygmaea* extract. Meanwhile, for the MDA-MB-231 cell line, all comet parameters increased significantly after 6 h of treatment with *S. pygmaea* ($p < 0.05$), but no significant changes were observed after 18 h of incubation ($p > 0.05$).

Intriguingly, the *S. pygmaea* extract could inflict DNA damage in both malignant and nonmalignant cells. Despite the genotoxic effects noted in this study, *S. pygmaea* extract also contains chlorogenic acid, an antigenotoxic compound that suppresses reactive oxygen species [35]. Chlorogenic acid has been well documented for its cytoprotective effects, encompassing cancer chemopreventive properties and wound-healing abilities in diabetics [36, 37]. Notably, the wound-healing capabilities of several Anatolian *Scorzonera* species have been attributed to the presence of chlorogenic acid in *in vivo* studies [38]. Further research has highlighted that *Scorzonera tomentosa* extract demonstrates anti-inflammatory effects, largely attributed to its chlorogenic acid content, through the inhibition of NF-κB activation. Additionally, other constituents, such as dicaffeoyl-quinic acid derivatives

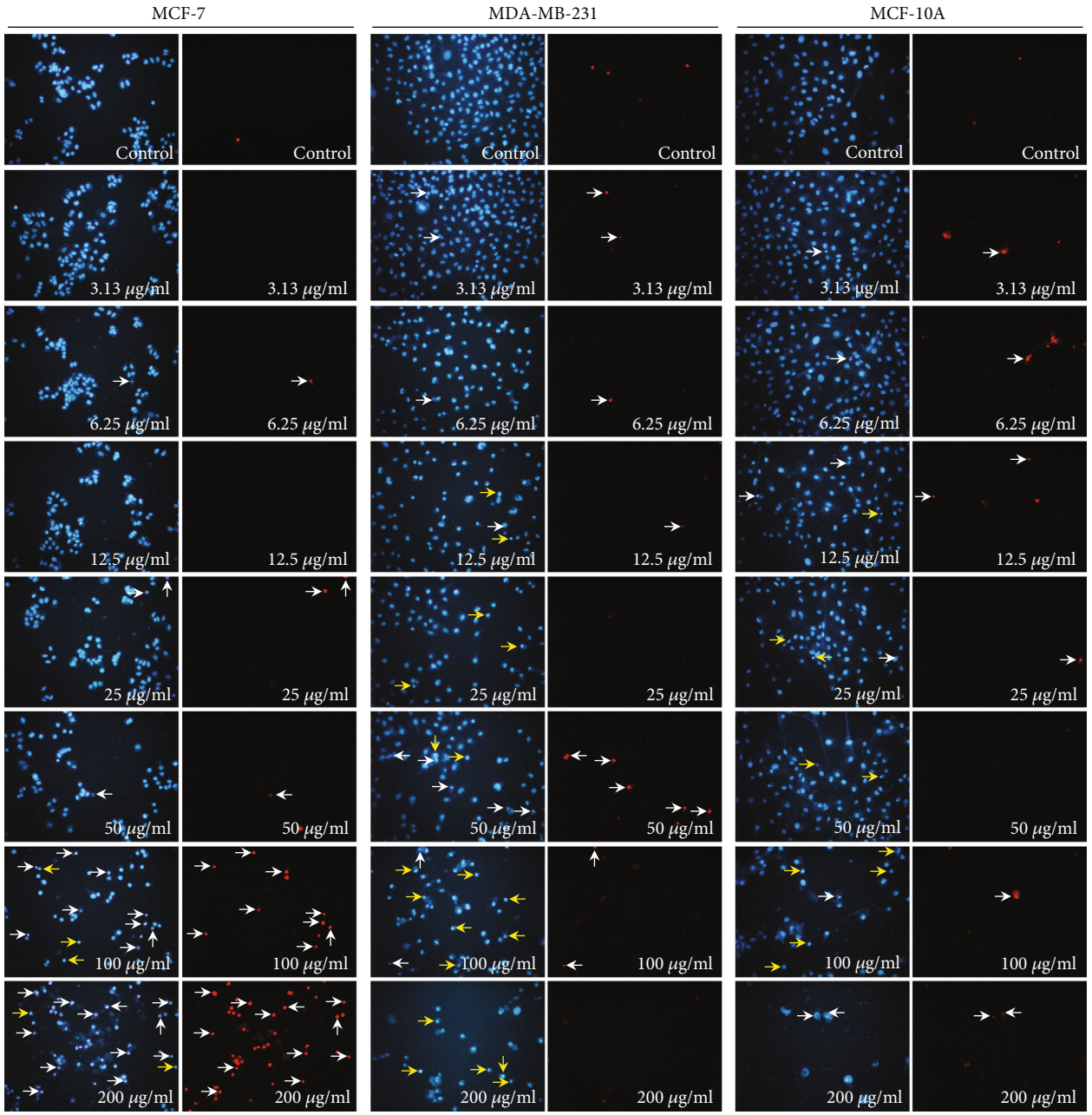

Figure 2: Fluorescence microscopy images illustrating the nuclei of MCF-7, MDA-MB-231, and MCF-10A cells treated with *S. pygmaea* extract for 48 h (×100). Cells in the early stage of apoptosis are denoted by yellow arrows, while secondary necrotic cells are marked with white arrows.

and specific flavonoids like quercetin-3-β-D-glucoside and hyperoside, contribute to this activity [39]. In addition, chlorogenic acid has been found to mitigate isoproterenol-induced DNA damage in vascular smooth muscle cells without causing cytotoxicity [35]. It is important to highlight that this is the first study to present data on DNA damage caused by a *Scorzonera* species, making it a pioneering investigation in this context. This revelation, that a member of the *Scorzonera* genus can be genotoxic, provides valuable insights that may shed light on the biological activities of other *Scorzonera* species. Thus, further studies are warranted to thoroughly understand the genotoxic potential of *S. pygmaea* extract and to explore its potential health benefits, particularly its applications in the food industry.

3.6. Gene Expression Analysis. Understanding the dynamic regulation of gene expression during the cell cycle is crucial for understanding the intricate mechanisms that govern cell cycle progression. In this context, several key regulators have been identified, including *CDKN1A*, which encodes the p21 protein and is known for its role in cell cycle control. DNA damage triggers the upregulation of p21 through the activation of p53, leading to the inhibition of cyclin-dependent kinases such as CDK1 [40]. Furthermore, the G2 DNA

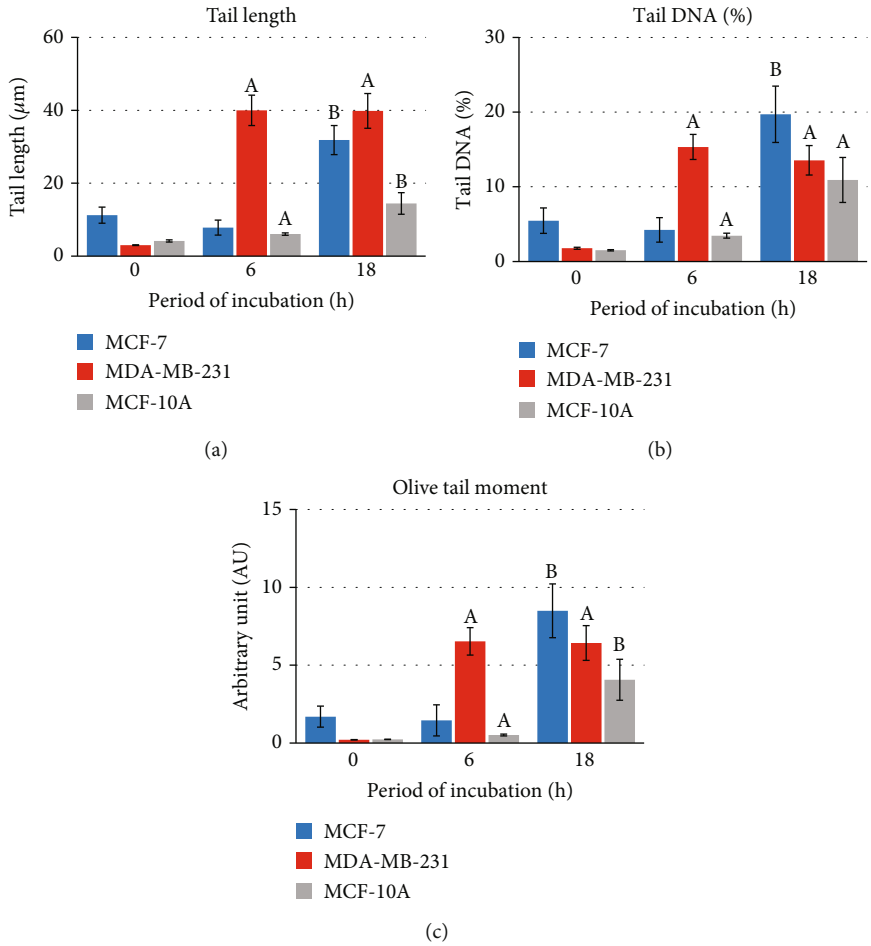

FIGURE 3: DNA damaging effects of *S. pygmaea* extract at IC_{50} dose for 6 and 18 h on MCF-7, MDA-MB-231, and MCF-10A cell lines are shown by comet assay parameters: (a) tail length, (b) % tail DNA, and (c) Olive tail moment. A: $p < 0.05$, compared to the control group. B: $p < 0.05$, compared to both the control and 6 h incubation groups.

damage checkpoint involves activation of Wee1 (*WEE1*) and inactivation of Cdc25 isomers (*CDC25A* and *CDC25C*) by protein kinases [41]. This orchestrated modulation of p21, Wee1, and Cdc25 isomers contributes to the phosphorylation and subsequent inactivation of CDK1, resulting in cell cycle arrest at the G2/M transition. Notably, the progression of the cell cycle is mediated by cyclins A2 (*CCNA2*) and B1 (*CCNB1*), which bind to CDK1 and facilitate cell cycle progression. By unraveling the intricacies of gene expression changes associated with cell cycle regulation, one can gain valuable insights into the fundamental processes that govern cell proliferation and division [42].

The results of gene expression analyses are shown in Figure 4. There were profound alterations in the gene expression patterns in both cell type- and time-dependent manners following *S. pygmaea* treatment. MCF10-A cells had elevated *TP53*, *CCNA2*, *CCNB1*, *CDC25A*, and *CDC25C* levels and lowered levels of *CDKN1A* and *WEE1*, which were not strictly time dependent. In the MDA-MB-231 cell line, there was a time-dependent increase in *CDKN1A* and *WEE1* expression with no outstanding changes in the positive regulators of cell cycle progression, suggesting an attempt to initiate cell cycle arrest and DNA damage repair following *S. pygmaea* treatment. In MCF-7 cells, increased expression of the cell cycle-promoting genes *CCNA2*, *CCNB1*, *CDK1*, *CDC25A*, and *CDC25C*, accompanied by decreased expression of the negative regulators of cell cycle progression, *WEE1* and *CDKN1A*, indicates an attempt by the cells to progress through the cell cycle phases. However, the absence of changes in the expression of cell cycle-promoting genes at 18 h suggests stabilization of the cellular response. The observed gene expression pattern in MCF-7 cells contributes to cell cycle progression and DNA replication and promotes the accumulation of DNA damage over time. In the canonical DNA damage response pathway, p21, encoded by *CDKN1A*, is activated to regulate the cell cycle, thereby protecting cells from apoptotic death. Yet, the selective transcriptional repression of *CDKN1A* observed in *S. pygmaea*-treated MCF-7 cells may induce a shift in the cellular response from cell cycle arrest to apoptosis [43]. Such repression has been attributed mainly to negative regulators that either hinder the transcriptional activity of positive regulators or block their binding to the promoter region [44].

Collectively, the relationship between gene expression patterns and DNA damage accumulation over time showed

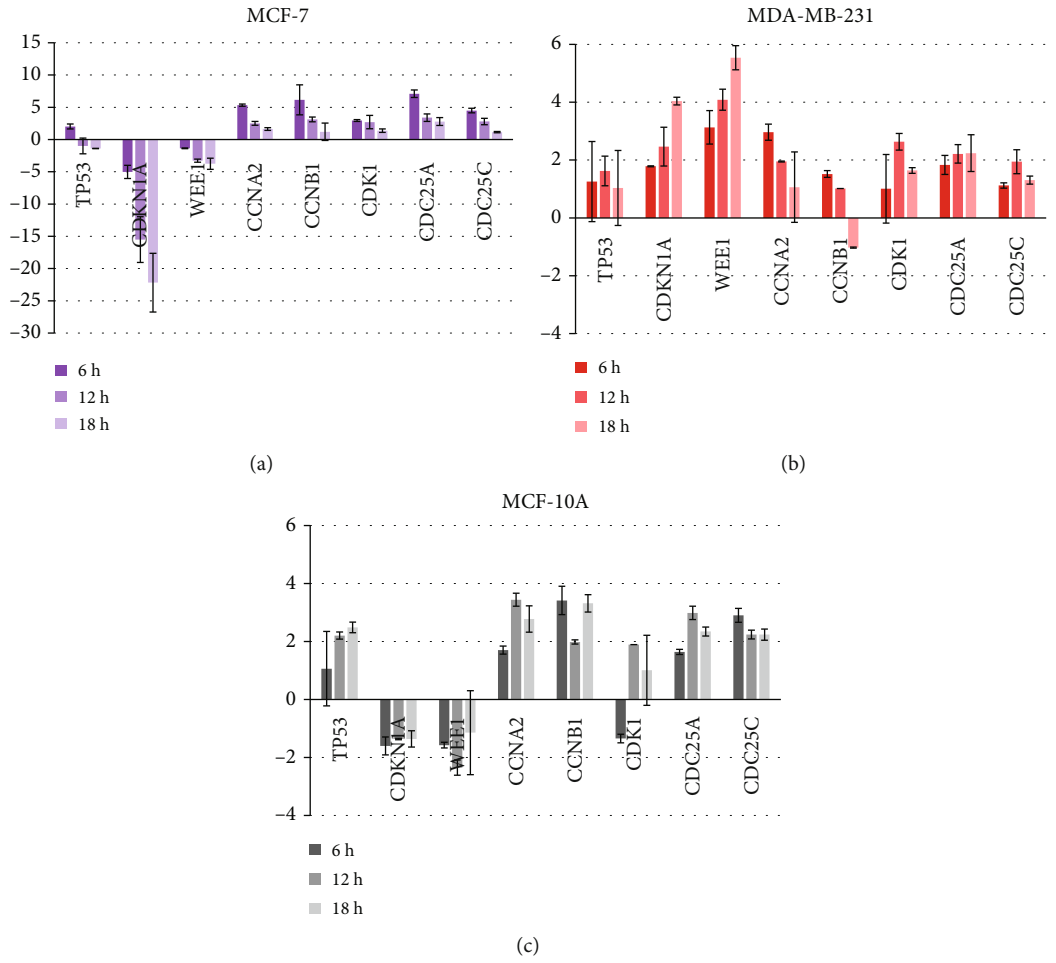

Figure 4: Fold changes in the expression of multiple genes following *S. pygmaea* extract treatment at IC_{50} doses for 6, 12, and 18 h in MCF-7 (a), MDA-MB-231 (b), and MCF-10A (c) cell lines.

similarities in MCF-7 and MCF-10A cells, in which MCF-7 cells displayed a heightened response in the expression of selected genes and consequently incurred more severe DNA damage over time than the MCF-10 cell line. One difference between the two cell lines is the contrasting trends in *TP53* expression, suggesting activation of the DNA damage response pathway in MCF-10A cells, which supports the notion that nonmalignant cells have a more intact and robust DNA damage response mechanism. In line with these findings, a prior study with MCF-10A cells showed that exposure to irradiation therapy, potent enough to induce reversible DNA damage, led to an increased expression of *CDC25C* and other positive cell cycle regulators [45]. On the other hand, due to the inherent dysregulated patterns of response to external stimuli often seen in malignant cells, MCF-7 cells exhibit compromised DNA damage responses [46]. MDA-MB-231 cells showed significantly higher levels of DNA damage at both 6 and 18 h without a significant difference between the two time points. Despite the absence of functional *TP53* in these cells [47], upregulation of *CDKN1A* and *WEE1* expression was observed, indicating the involvement of non-p53-dependent pathways in driving DNA damage-induced cell death in this cell line [48]. The distinct gene expression patterns observed in MDA-MB-231 cells following treatment with *S. pygmaea* may underlie their increased resistance to the extract. This resistance was manifested by higher cell viability percentages at higher doses and the absence of a significant increase in DNA damage over time. In fact, RNAi screening has identified that inhibiting *WEE1* is one of the primary strategies to increase the sensitivity of MDA-MB-231 cells to the chemotherapy drug cisplatin. Without this inhibition, cisplatin tends to shut down DNA replication and attenuate cytotoxic effects, especially when *WEE1* is highly expressed following treatment [49]. These findings underscore the important role of the distinct gene expression patterns induced by *S. pygmaea* treatment in determining the cellular response and potentially contributing to the relative resistance to the extract.

In conclusion, this study provides novel insights into the cytotoxic, apoptotic, and genotoxic activities of *S. pygmaea* extract. Our findings revealed that *S. pygmaea* extract induces apoptotic cell death in both malignant and nonmalignant cell lines, thereby suggesting its potential application in the development of nutraceuticals for cancer prevention. Furthermore, the extract elicited DNA damage, the extent of which varied depending on the duration of exposure

and the cell type, as determined by the comet assay. These differential responses across cell lines, driven by changes in the expression of cell cycle regulators, highlight the intricacies of the interactions between *S. pygmaea* constituents and biological systems. A promising area of future research is the exploration of the potential of *S. pygmaea* as a rich source of antioxidants, particularly chlorogenic acid, which is known for its substantial dietary benefits. Subsequent studies are required to fully understand the balance between the beneficial antioxidant properties and the potential genotoxic effects of *S. pygmaea*. The findings of this study underscore the potential utility of *S. pygmaea* in the food industry, contributing significantly to human health. These results serve as a compelling premise for further exploration of the properties and applications *S. pygmaea*.

Data Availability

The experimental data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The author declares that there are no conflicts of interest regarding the publication of this article.

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Supplementary Materials

Figure S1: HPLC chromatogram of phenolic compounds for S. pygmaea extract at 320 nm with their retention times. AME: 1, protocatechuic acid (11.8 min); 2, chlorogenic acid (15.7 min); 3, caffeic acid (20.1 min); 4, rutin (22.0 min); 5, quercetin 3-β-D-glucoside (22.9 min); 6, p-coumaric acid (23.4 min); 7, ferulic acid (24.3 min). Table S1: primer sequences for selected genes. The table provides the primer sequences, lengths, melting temperatures (Tm), and GC content (%) used in the qRT-PCR analysis. Table S2: PCR reaction mix components and volume. Table S3: PCR temperature and duration conditions. *(Supplementary Materials)*

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