

Article

Exploring the Biological Potential of Mountain Germander Polyphenolic Extract on Cellular Model Macromolecules, Human Cell Lines, and Microbiome Representatives

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Abstract: In the context of revitalizing the use of traditional plant species as remarkable sources of bioactive compounds, the determination of their biological effects is of utmost importance. Among Lamiaceae species, *Teucrium montanum* (Mountain Germander) represents understudied Mediterranean plant species; it is rich in polyphenols, which are well-studied biologically active compounds for human disease prevention and the reduction of oxidative stress, i.e., phenolic acids, phenylethanoid glycosides, and flavonoids. For that purpose, the aim of this study was to investigate the antioxidant, cytotoxic, and genotoxic effects of Mountain Germander (MG) polyphenolic extract (0.025, 0.050, 0.150, and 0.500 mg extract mL⁻¹) on the hepatocellular (HepG2), tongue (CAL 27), gastric (AGS), and colorectal (Caco-2) continuous human cancer cell lines, as well as its bacteriostatic potential on representative members of human microbiota. In addition, the antioxidant potential of the MG polyphenolic extract was determined using bovine serum albumin and DNA plasmid as cellular model macromolecules. In vitro analysis revealed a significant cytotoxic effect of all MG extract concentrations on AGS and Caco-2 cell lines after prolonged treatment (24 h). In addition, treatment with 0.500 mg extract mL⁻¹ showed the most pronounced antioxidant effect under prolonged treatment (24 h) on CAL 27 and HepG2 cell lines. All of the applied MG extract concentrations seem to have a genoprotective effect on DNA plasmid. Furthermore, a significant inhibitory effect on *E. coli* was detected upon the treatment with 0.150 mg extract mL⁻¹, reducing the cell viability by 56%.

Keywords: biological activity; cancer cell lines; Mountain Germander; phenylethanoid glycosides



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1. Introduction

The selection and systematization of plant species and their preparations, based on experience and practices passed down from generation to generation, shows their important historical role in the treatment and prevention of various diseases [1]. In the course of biological evolution, various plant metabolites have evolved that are specialized to play a mediating role in biological communication between species, so that they possess biologically active effects similar to those of drugs, and have been recognized by humans through a long tradition of use [2]. In recent years, there has been a significant revitalization of the status of traditional plant species at the global level through investment in the improvement of national health regulations and pharmacovigilance systems for herbal medicines [3]. The COVID-19 pandemic has further empowered the use of traditional plant species to strengthen the immune system and prevent diseases, which has been particularly

reflected in the markets of China and India [4]. Clinical trials conducted in China have shown the high efficacy of six formulations from Traditional Chinese Medicine (TCM) practices in the treatment of mild and moderate COVID symptoms [5,6], which is due to the presence of polyphenolic compounds, specifically phenylethanoid glycosides, as inhibitors of SARS-CoV-2 protease [7]. With the establishment of the World Centre for Traditional Medicine within the World Health Organization in India (2022), the economic potential and health capacity of traditional medicine, including the use of traditional plant species, was officially recognized, with the main aim of investing in pharmacological research and the development of a sustainable and standardized production of herbal formulations [8,9].

In addition to the commercially available aromatic plant species, such as mint, oregano, basil, sage, rosemary, bay leaf, and lemon balm, etc., there is a large number of understudied plant species from the Lamiaceae family that have limited use [10]. *Teucrium* L. is a cosmopolitan genus with 300 identified plant species, mostly growing in the Mediterranean region [11]. Among *Teucrium* species, *Teucrium montanum* (Mountain Germander) represents traditional species often used in the form of herbal infusions and decoctions for the prevention and treatment of digestive and respiratory diseases [12], diabetes [13], and asthma [14], as well as for immune strengthening [15]. Although there are only 11 available ethnobotanical studies to date that mention its medicinal use, the expression “raises the dead to life” is often used in the local population to emphasize the importance of the Mountain Germander remedy [16,17]. So far, immunomodulatory [18], cytotoxic, and genotoxic effects [19] of MG polyphenolic extract responded on normal T cells and normal human lung fibroblast cell lines, as well as having antiproliferative [20,21], antimicrobial [22,23], genoprotective [24], neuroprotective [25,26], and antioxidant [23,26,27] activity on various human cancer cell lines (human colon cancer cells, human melanoma cells, human lymphoblast cells, human breast adenocarcinoma cells); additionally, cellular model macromolecules (plasmids, proteins, and lipids) and bacterial cultures were reported. The expressed biological effects in all reported studies were primarily correlated with the presence of phenolic acid, flavonoids, and phenylethanoid glycosides.

To date, there are numerous studies confirming their role in neutralizing reactive oxygen species (ROS), and thus indirectly affecting the activation of different signaling metabolic pathways, reducing oxidative damage of biological macromolecules, modulating gene expression, and, thereby, regulating redox status in the cells [28,29]. However, a pro-oxidant character of polyphenols was also reported in terms of the ability to generate ROS in cell systems and cell-free media [30]. Compared to the dual character of some of the most investigated phenolic compounds, e.g., phenolic acids, flavonoids, and *Camelia sinensis* extracts [31,32], phenylethanoid glycosides as relatively understudied phenolic compounds in terms of biological activity, shown to have a primarily protective role in suppressing the ROS formation [33], inhibiting the proliferation of cancer cells by downregulating the PI3K/AKT pathway [34], increasing the activity of endogenous antioxidant enzymes and inflammatory marker production [35], etc.

It is a common misconception among consumers that the consumption of herbal remedies as a natural source of phytochemicals does not pose a health risk. Aside from expected adverse effects as the consequence of the numerous factors, such as inherent toxic substances in plants, misidentification of plant species, inappropriate doses, interactions with other drugs, and the presence of potentially hazardous substances, etc., limited knowledge of the appropriate preparation of herbal remedies and safe doses are other drawbacks related to the consumption of medicinal herbs [36]. Hence, expanding the literature base for herbal remedies in the context of their potential in provoking or disabling the free radical formation, inflammatory processes, cell apoptosis, and changes in DNA structure, etc., represents an important step for minimizing the long-term and dose-dependent toxicity.

Since herbal preparations are traditionally consumed orally, cytotoxic, pro-oxidative, and genotoxic effects were investigated on the hepatocellular (HepG2), tongue (CAL 27), gastric (AGS), and colorectal (Caco-2) continuous human cancer cell lines, as well as the influence of oxidative potential on the cellular protein model and DNA plasmid.

Additionally, the influence of Mountain Germander extract on *L. plantarum*, *E. coli*, and *S. aureus* survival was determined. Results of this study could give an insight into the ability of Mountain Germander extract to influence cancer cell or bacterial strain survival, to act as an antioxidant and/or pro-oxidant, or to cause DNA damage.

2. Materials and Methods

2.1. Materials

2.1.1. Plant Material, Model Macromolecules, and Biological Test Systems

Mountain Germander (*T. montanum* L.) was purchased from the local supplier (Ljekovito bilje j.d.o.o, Zadar, Croatia). The plant was collected in August 2020 (municipality of Kistanje, Šibenik-Knin County, Croatia), with the voucher specimen (ID: 75518) deposited in the Herbarium Croaticum (Faculty of Science, Zagreb, Croatia). Sorted and sieved areal parts (flowers, stems, and leaves) of the plant (<450 µm) were used in all experiments.

Bovine serum albumin was obtained from Sigma-Aldrich (Steinheim, Germany). A double-stranded, covalently closed, circular form of PhiX174 RFI DNA was purchased from the Promega Corporation (Madison, WI, USA).

Continuous human cell lines, i.e., epithelial tongue carcinoma (CAL 27; ATCC CRL-2095), gastric adenocarcinoma (AGS, ATCC CRL-1739), epithelial colorectal adenocarcinoma (Caco-2; ATCC HTB-37), and hepatocellular carcinoma (HepG2; ATCC HB-8065), were grown in a monolayer in T-flasks (37 °C, controlled CO₂ atmosphere), in a complete RPMI culture medium (Capricorn Scientific GmbH, Ebsdorfergrund, Germany) with 10% fetal bovine serum (FBS), except for the AGS cell line, for which Ham's F-12 culture medium (Capricorn Scientific GmbH, Ebsdorfergrund, Germany) was used. Based on the number of cells determined using the Bürker-Türk chamber in the suspension after the trypsinization of cultivated cells, an initial cell concentration of 10⁵ cells mL⁻¹ was set for further experiments.

Bacterial strains, i.e., *Escherichia coli*, *Lactobacillus plantarum*, and *Staphylococcus aureus*, were stored at −80 °C with the addition of 10% glycerol, as the part of the Collection of Organisms of the Laboratory for Biology and Genetics of Microorganisms at the Faculty of Food and Biotechnology (University of Zagreb, Zagreb, Croatia). For the experiments, bacterial cultures were cultivated in the complete Luria–Bertani culture medium; a complete Man, Rogosa, and Sharpe broth culture medium; and a selective minimal culture medium with mannitol, respectively. The culture media mentioned were prepared according to the standard instructions.

2.1.2. Chemicals

All chemicals and reagents used in the experiments were of per analysis and HPLC grade. All of the used organic and inorganic salts, as well as the ascorbic acid, bromothymol blue, dimethyl sulfoxide, glycerol, ethyl acetate, glacial acetic acid, ethanol, hydrochloric acid, and hydrogen peroxide were obtained from Kemika (Zagreb, Croatia). Echinacoside (>98%) and verbascoside (>98%) were obtained from Biosynth s.r.o. (Bratislava, Slovakia). 2,4-dinitrophenylhydrazine (DNPH), dichlorofluorescein diacetate (DCF-DA), Neutral Red (3-amino-7-dimethylamino-2-methylfenasine hydrochloride), ethidium bromide, sodium dodecyl sulfate, sodium laurylsarcosinate, and D-mannitol were purchased from Sigma-Aldrich (St. Louis, MO, USA). Agar, bacto-tripton, MRS broth, and yeast extract were supplied from Biolife (Monza, Italy). Trypsin was supplied from Capricorn Scientific (Ebsdorfergrund, Germany). Thiamine hydrochloride was obtained from Koch-light Ltd. (Haverhill, UK). Guanidine hydrochloride and triton X-100 were purchased from Acros Organics (Boston, MA, USA). Formic acid was purchased from Carlo Erba (Emmendingen, Germany), while acetonitrile and trichloroacetic acid were supplied from Fisher Scientific (Waltham, MA, USA).

2.2. Methods

2.2.1. Preparation and Bioactive Characterization of Mountain Germander Extract

Aqueous extract was prepared according to the previously optimized extraction parameters (1 g: 100 mL, 100 °C, 30 min) using a heat-assisted extraction technique [37]. A concentrated extract under a vacuum evaporator (IKA RV8, Staufen, Germany) was then subjected to freeze drying (Alpha 1-2 LD plus freeze-dryer, Martin Christ, Osterode am Harz, Germany) in order to obtain a lyophilized extract, which was used for all further experiments. Firstly, the working concentrations of the freeze-dried extract were defined, i.e., 0.025 mg mL⁻¹, 0.05 mg mL⁻¹, 0.15 mg mL⁻¹, and 0.5 mg mL⁻¹, which correspond to half (0.5×), one (1×), three (3×), five (5×), and ten (10×) cups of an herbal decoction prepared for consumption, respectively.

The total phenolic content, total hydroxycinnamic acid content, as well as the results of the identification and quantification of polyphenols in analyzed MG extract, were determined by methods provided in our previous studies [38,39]. Nine phenylethanoid glycosides and three flavonoids were identified and quantified in total, as reported in Table 1.

Table 1. Bioactive characterization of Mountain Germander freeze-dried polyphenolic extract.

TPC (mg GAE g ⁻¹ *)			Total Hydroxycinnamic Acids (mg ECH g ⁻¹ *)					
352.24 ± 1.56			275.23 ± 0.97					
phenylethanoid glycosides (mg g ⁻¹ **)								
β-OH-forsythoside B	echinacoside	jionoside A	teupolioside	stachysoside A	poliumoside	verbascoside	forsythoside B	isoverbascoside
2.73 ± 0.03	75.37 ± 1.10	4.73 ± 0.10	22.19 ± 0.42	42.86 ± 0.83	29.27 ± 0.39	25.29 ± 0.46	9.03 ± 0.33	6.43 ± 0.24
flavonoids (mg g ⁻¹ **)								
vicenin-2			diosmin			acacetin-7-O-rutinoside		
1.86 ± 0.03			1.76 ± 0.10			1.19 ± 0.02		

* expressed in mg equivalents of gallic acid (GAE) or echinacoside (ECH) per g of lyophilized extract, ** expressed in mg per g of lyophilized extract.

2.2.2. Determination of Antioxidant/Pro-Oxidant Potential of Mountain Germander Extract on the Model Protein Macromolecule

The derivatization method using 2,4-dinitrophenylhydrazine (DNPH) was employed to examine the influence of polyphenolic extract on bovine serum protein oxidation [40]. The reaction mixture contained the following: 240 µL of BSA (1 mg mL⁻¹), FeCl₃ solution (60 µL), ascorbic acid (60 µL), and 25 mM H₂O₂ (24 µL), as well as the corresponding volumes of extract to obtain the previously defined extract concentrations, and demineralized water to reach a final volume of 600 µL, with an incubation time for 30 min at 37 °C. The negative control contained the same amount of water instead of the extract. After incubation, 2,4-dinitrophenylhydrazine (DNPH) was added (500 µL), and the mixture was incubated for 30 min at room temperature. In order to precipitate the carbonylated proteins, 500 µL of 10% trichloroacetic acid was added, with an incubation time of 15 min. After the centrifugation, the precipitate was washed with ethyl acetate (1 mL). Finally, the samples were resuspended in 500 µL of guanidine hydrochloride (6 M) and left overnight. The absorbance was measured at 370 nm, while the degree of protein oxidation was calculated in relation to the negative control (water), according to the Formula (1), which is as follows:

$$\text{rate of protein oxidation (\%)} = \frac{A_{370}(\text{sample})}{A_{370}(\text{control})} \times 100 \quad (1)$$

2.2.3. Determination of Genotoxic/Genoprotective Effect of Mountain Germander Extract on Model DNA Macromolecule

The genotoxic/genoprotective effect of MG extract on the model plasmid phi174 RF1 DNA was tested by electrophoresis in agarose gel, according to the modified protocol [41]. After preparing a 1% (w/w) agarose gel in a buffer solution of Tris base, acetic acid, and EDTA (TAE), and forming wells on the gel in an electrophoresis mold, the prepared reaction

mixture (30 μL) consisted of: an extract at a certain concentration, 0.2% (v/v) of hydrogen peroxide, plasmid (0.1 mg mL^{-1}) and TAE buffer. All samples are irradiated with UV radiation for 3 min. Simultaneously, a positive control consisting of plasmid and hydrogen peroxide is placed under a UV lamp and exposed to the radiation. In addition, three negative controls were prepared, as follows: (i) a sample containing only the plasmid, (ii) a plasmid and hydrogen peroxide without UV irradiation, and (iii) a plasmid irradiated with UV irradiation. After treatment, 1 μL of application buffer containing bromothymol blue and glycerol is added, allowing visual monitoring of the electrophoresis (1 h, 150 mA). At the end of the electrophoresis, the samples are stained with ethidium bromide solution ($20 \mu\text{g mL}^{-1}$) and visualized with a transilluminator. The gel images showing the intensity of the supercoiled (lower band) and relaxed (upper band) forms of the plasmid were processed using the GelAnalyzer 19.0 software program. The results of the antioxidant potential of the MG extract were expressed as the ratio of the area of the supercoiled and relaxed forms of the band, along with the positive and negative controls.

2.2.4. Determination of Antioxidant/Pro-Oxidative Effect of Mountain Germander Extract on Human Cell Lines

The DCFH-DA method (2',7'-dichlorofluorescein diacetate) was used to determine the reactive oxygen species (ROS) after treatment with the extract [42]. After treating the cells with the working concentrations of the extract (2 and 24 h), they are washed with PBS buffer, and 100 μL of DCFH-DA dye (0.05 mM in PBS) is added to the wells of the black microtiter plate. After treatment (15 min in a CO_2 incubator at 37°C), the fluorescence intensity is proportional to the amount of ROS, and it is measured at 485 nm (emission) and 530 nm (excitation). The induction of free radicals is given in relation to the negative control water (100%) and calculated using Formula (2), which is as follows:

$$\text{free radical induction (\%)} = \frac{\frac{\text{fluorescence (extract)}}{\% \text{ of survival}}}{\frac{\text{fluorescence (control)}}{100}} \quad (2)$$

2.2.5. Determination of Cytotoxic/Proliferative Effect of Mountain Germander Extract on Human Cell Lines

The Neutral Red method was used to analyze the cytotoxic or proliferative effect of the Mountain Germander extract [43]. Microtiter plates with 96 wells were inoculated with 100 μL of a specific cell suspension during 24 h cultivation. After removing the spent medium, cells were treated (100 μL) with previously dissolved Mountain Germander extract in the nutrient medium (0.025, 0.050, 0.150, and 0.500 mg mL^{-1}). The control contained 100 μL of the appropriate nutrient medium instead of the extract. After the treatment (2 and 24 h), the spent nutrient medium with the extract was removed, and the Neutral Red dye working solution (100 μL) was added to each well. After incubation in a CO_2 incubator (37°C , 3 h), the dye solution is removed from the wells, and the dye accumulated in the cells is extracted with a decolorizing solution, followed by a measurement of its absorbance at 540 nm. The intensity of the staining is proportional to the concentration of viable cells, and the percentage of surviving cells relative to the negative control (water) is calculated using the following Formula (3):

$$\text{survival rate (\%)} = \frac{A_{540} (\text{sample})}{A_{540} (\text{control})} \times 100 \quad (3)$$

2.2.6. Determination of Genotoxic/Genoprotective Effect of Mountain Germander Extract on Human Cell Lines

Oxidative damage of the genetic material of continuous human cell lines in the presence of MG extract was tested using a comet assay with some modifications [44]. First, CAL 27, HepG2, AGS, and Caco-2 cells (initial concentration: $10^5 \text{ cells mL}^{-1}$, incubated at 37°C under controlled atmosphere) were centrifuged from the spent culture medium,

and then treated with MG extract in defined concentrations (3 mL) for 2 and 24 h. Simultaneously, the negative control contained only the same amount of corresponding fresh culture medium. After treatment, the medium containing the extract was removed from the Petri dishes, and the cells were washed with PBS buffer, trypsinized, and transferred to Eppendorf tubes. After centrifugation ($5000 \times g$, 5 min), sediment with the cells is used for the rest of the experiment. After applying resuspended cells (10 μL) and 0.5% agarose (100 μL) to the polymerized agarose layer on a glass slide (1.5% normal melting point (NMP) agarose solution prepared by boiling in PBS buffer), the cells are lysed overnight at 4°C in buffer. Electrophoresis is then performed in an alkaline buffer (300 mA, 25 V, 20 min). For neutralization, the slides are washed three times with Tris-HCl buffer (pH = 7.5, 5 min interval). Immediately before analysis on the epifluorescence microscope, the slides are stained with ethidium bromide solution (10 mg mL^{-1}). There were 50 comets measured on each slide, while tail intensity and tail length parameters were used as indicators of genetic material damage.

2.2.7. Determination of Bacteriostatic/Proliferative Effect of Mountain Germander Extract on Representative Bacterial Strains of Human Microbiota

Previously prepared suspensions of tested bacterial strains in adequate nutrient media, i.e., *E. coli*, *S. aureus*, and *L. plantarum*, were used in the exponential growth phase. Then, a microtiter plate (96 wells) was inoculated with the appropriate bacterial suspension (100 μL). The bacterial suspension was treated with different MG extract concentrations (100 μL). The control was a bacterial suspension treated with 100 μL of the respective liquid nutrient medium instead of extract. After incubation (40 min, 37°C), microdilutions were made (bacterial + nutrient medium mixture: PBS buffer = 1:9). Then, each dilution (10 μL) was inoculated onto the appropriate solid nutrient and cultivated (24 h, 37°C). The results of the survival of bacteria after treatment with MG extract were calculated according to the following Formula (4):

$$\text{bacterial survival rate (\%)} = \frac{\frac{\text{CFU}}{\text{mL}} \text{ bacterial suspension}}{\frac{\text{CFU}}{\text{mL}} \text{ control}} \times 100 \quad (4)$$

2.2.8. Statistical Analysis

The results were statistically analyzed with Statistica software (version 10, TIBCO Software, Palo Alto, CA, USA), using a one-way analysis of variance with Tukey's post hoc test ($p < 0.05$), and a Mann-Whitney U-test. All measurements were performed in triplicate ($n = 3$).

3. Results and Discussion

3.1. Antioxidant/Pro-Oxidant Potential of Mountain Germander Extract on the Model Protein Macromolecule

Excessive production of reactive oxygen radicals due to increased activity of endogenous and exogenous sources ultimately leads to cumulative structural changes and oxidative damage to cellular macromolecules, i.e., the formation of reactive carbonyl groups that react with nucleophilic groups of proteins. In this study, results showed a positive correlation between an increased concentration of MG extract and protein oxidation ($p < 0.05$). Treatment with extract concentrations of 0.025 and 0.050 mg mL^{-1} resulted in lower protein carbonylation for approximately 40% and 24%, respectively, while higher MG extract concentrations (0.125 and 0.500 mg mL^{-1}) showed a pro-oxidant effect by increasing protein oxidation for 14%, in both cases (Figure 1).

The pro-oxidant effect of *T. arduini* methanol extract (0.01–0.25 mg mL^{-1}) on BSA protein was also reported in the study by Šamec et al. (2015) [45]. However, Bektašević et al. (2023) [26] reported a protective role of treatment with aqueous and methanolic MG extract (0.167 $\mu\text{g}/\text{mL}^{-1}$) after 1 h by inhibiting the protein carbonylation by 8.49%, in comparison to the BHT (11.12%). With prolonged treatment (24 h), pro-oxidative activity

was observed. It is well-known that polyphenols have a dual character, i.e., an antioxidant and a pro-oxidant role, which largely depends on the physiological conditions of the cell, their chemical structure (number of hydroxyl, acetyl groups, conjugated bonds, chelating ability, etc.), the presence of metal ions, bioavailability, etc. [30]. Many herbal extracts with high antioxidant activity, e.g., juniper (*Juniperus communis* L.), basil (*Ocimum basilicum* L.), and laurel (*Laurus nobilis* L.), also showed the ability to cause oxidative damage on BSA protein [46].

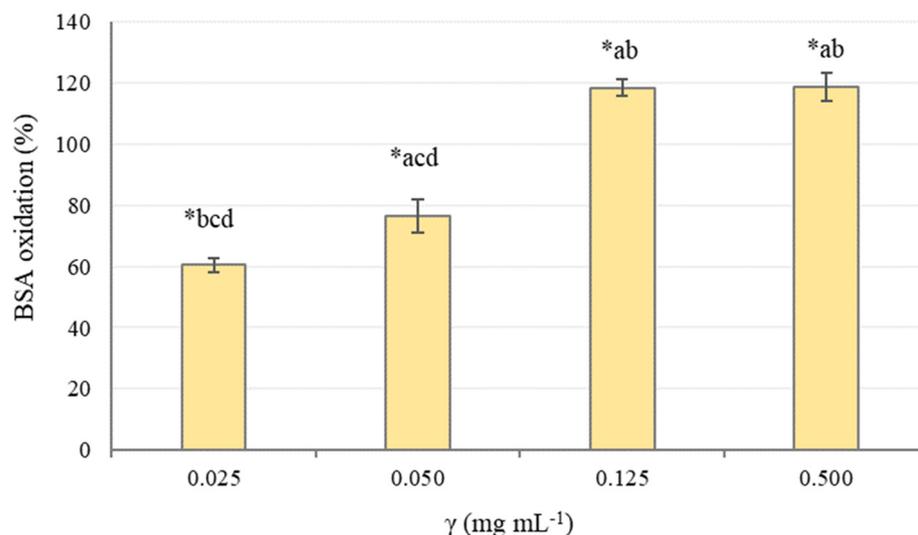


Figure 1. Protein oxidation rate of bovine serum albumin (BSA) protein treated with Mountain Germander extract concentrations (0.025–0.500 mg mL⁻¹). Statistical data processing was carried out by one-way analysis of variance with Tukey's post hoc test. * = statistically significantly different ($p < 0.05$) compared to the negative control (water); a = statistically significantly different ($p < 0.05$) compared to 0.025 mg mL⁻¹; b = statistically significantly different ($p < 0.05$) compared to 0.050 mg mL⁻¹; c = statistically significantly different ($p < 0.05$) compared to 0.150 mg mL⁻¹; d = statistically significantly different ($p < 0.05$) compared to 0.500 mg mL⁻¹.

3.2. Genotoxic/Genoprotective Effect of Mountain Germander Extract on Model DNA Macromolecule

The potential of MG extract to indirectly induce or suppress the oxidation of genetic material through the ROS formation was tested on the model plasmid phiX174 RF1 DNA. Figure 2 shows a gel image with detected bands of the relaxed (RCP) and supercoiled (SCCP) forms of plasmid after the treatment with the extract within a certain concentration range, and under H₂O₂ photolysis stimulated by UV radiation.

The processing of the obtained images in the GelAnalyzer program resulted in the calculated ratios of SCCP and RCP plasmid (Figure 3).

Due to the action of the hydroxyl radical, higher DNA damage will visually result in a more intense upper band of the relaxed form of DNA, or, in other words, higher content of DNA fragments as a result of the unwinding of the supercoiled DNA form. Considering the positive and negative control, a statistically significant ($p < 0.05$) genoprotective effect of the highest concentration of MG extract (0.500 mg mL⁻¹) on the model plasmid was observed. Although the concentration of 0.050 mg mL⁻¹ was not statistically significant compared to the positive control, it shows that the ratio between the supercoiled and the relaxed form increased by 64% compared to the treatment of the plasmid with a combination of UV radiation and H₂O₂ (PC), i.e., the pro-oxidant damage was significantly lower. It is important to observe that, although there is significantly ($p < 0.05$) higher plasmid damage when treated with concentrations of 0.025, 0.050, and 0.150 mg mL⁻¹ compared to the negative control, this effect generally decreases with an increasing concentration MG extract.

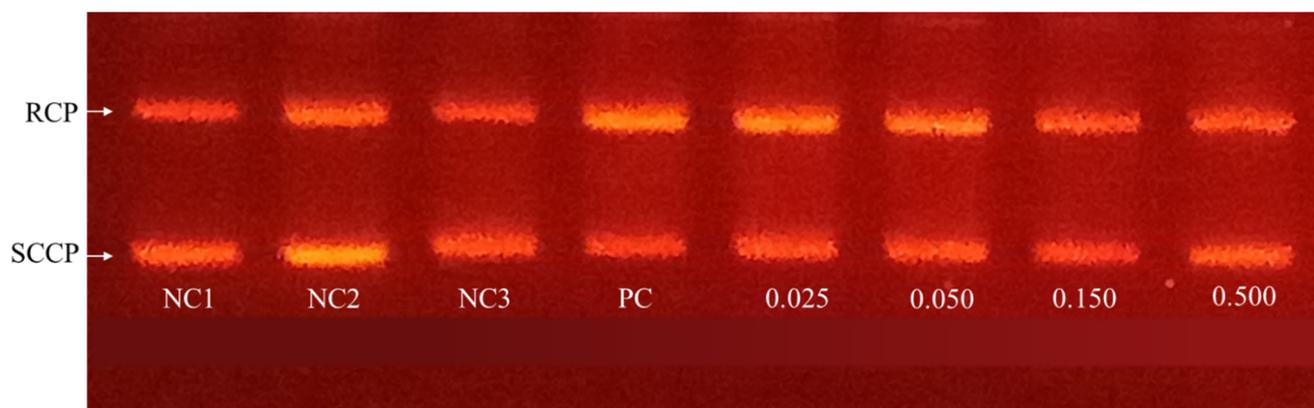


Figure 2. Visualization of oxidative damage of model DNA plasmid induced by UV-photolysis of H_2O_2 during treatment with MG extract concentrations ($0.025\text{--}0.500\text{ mg mL}^{-1}$). RCP: relaxed circular plasmid; SCCP: supercoiled circular plasmid; NC1: plasmid+buffer; NC2: H_2O_2 ; NC3: UV radiation; PC: plasmid + H_2O_2 + UV radiation; 0.025–0.500: extract concentration for plasmid treatment (mg mL^{-1}).

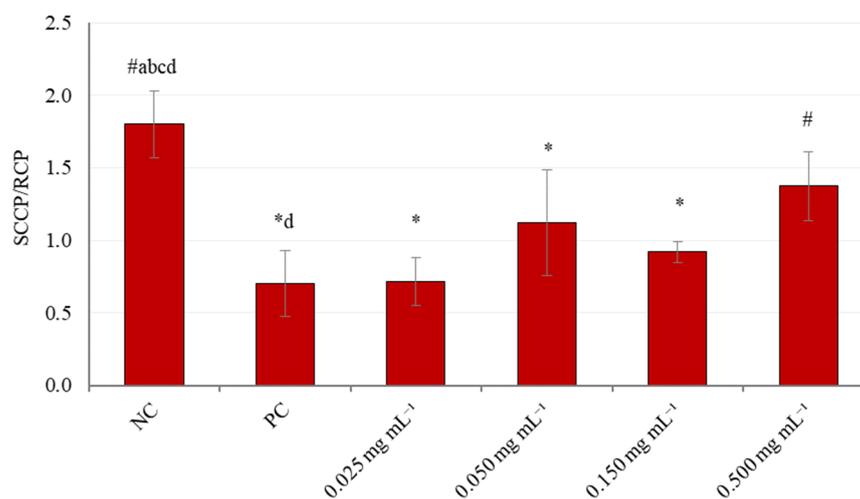


Figure 3. The effect of Mountain Germander extract concentrations ($0.025\text{--}0.500\text{ mg mL}^{-1}$) on DNA plasmid oxidative damage. SCCP/RCP = ratio of supercoiled and relaxed circular plasmid; NC = sum of all tested negative control; PC = positive control (plasmid + H_2O_2 + UV radiation). Statistical data processing was carried out by one-way analysis of variance with Tukey's post hoc test. # = statistically significantly different ($p < 0.05$) compared to the positive control; * = statistically significantly different ($p < 0.05$) compared to the negative control; a = statistically significantly different ($p < 0.05$) compared to 0.025 mg mL^{-1} ; b = statistically significantly different ($p < 0.05$) compared to 0.050 mg mL^{-1} ; c = statistically significantly different ($p < 0.05$) compared to 0.150 mg mL^{-1} ; d = statistically significantly different ($p < 0.05$) compared to 0.500 mg mL^{-1} .

Oalde et al. (2020) [19] also investigated the effect of aqueous, methanolic, and ethanolic extracts of MG at concentrations of $0.1, 0.5,$ and 1 mg mL^{-1} on the model plasmid pUC19, isolated from *E. coli* by applying a combined treatment of H_2O_2 and UV radiation to the supercoiled DNA to induce damage. The lowest percentage of the damaged or relaxed form was found in the aqueous extract ($<5\%$) compared to the alcoholic extract ($10\text{--}15\%$), with a significantly higher genoprotective effect in relation to the tested positive control. The genoprotective effect on the model plasmid may be partly attributed to the presence of phenylethanoid glycosides in the polyphenolic extract. Although the effects on preventing and/or slowing down the oxidation processes of genetic material have been insufficiently studied, the available studies indicate the valuable antioxidant potential of

phenylethanoid glycosides in inhibiting DNA damage. For example, Jang et al. (2020) [47] found a significant inhibition of oxidative damage to plasmid Φ X-174 RF I DNA induced by Fe^{2+} ions and hydroxyl radicals, during treatment with verbascoside that was isolated from the extract of the traditional Korean plant species *Abeliophyllum distichum* (Lamiaceae). In both cases, a dose-dependent effect was observed, with a reduction in damage from 7.09% at 0.32 μM verbascoside to 88.05% at 200 μM verbascoside in the case of divalent iron-induced damage, while in the case of hydroxyl radical treatment, damage was reduced by 47.14% at 0.32 μM verbascoside and 97.66% at 200 μM verbascoside. Zhang et al. (2017) [48] determined the protective effect of echinacoside on the reduction of UVB-induced DNA fragmentation on keratinocyte cells (HaCaT). They found a significant reduction of the DNA fragmentation in echinacoside-pretreated cells (25, 50, and 100 μM) before UVB radiation, with the highest applied echinacoside concentration as the most effective (DNA damage reduction for approximately 35%).

3.3. Cytotoxic/Proliferative Effect of Mountain Germander Extract on Human Cell Lines

One of the most important indicators of the biological activity of a target compound or a mixture of bioactive compounds is the property of a cytotoxic or proliferative effect on certain cell lines, which depends on the concentration applied and the duration of treatment. A statistically significant ($p < 0.05$) decrease in viable cells, i.e., a cytotoxic effect, was observed in Caco-2 and AGS after prolonged treatment (24 h) for all concentrations of MG extract (0.025, 0.050, 0.150, and 0.500 mg mL^{-1}) (Figure 4).

On the other hand, Stanković et al. (2011) [27] found a dose-dependent cytotoxic effect of the methanolic MG macerate in the concentration range of 0.050–1 mg mL^{-1} on HCT-116 colon cancer cells. Using the MTT assay, a significant cytotoxic effect ($p < 0.05$) of the extract was observed after 24 h of treatment at all concentrations. However, prolonged treatment (72 h) at the lowest applied concentration of the extract (0.050 mg mL^{-1}) resulted in the strong proliferative effect. When comparing results, a much longer exposure time, methanol as the extraction solvent, the cell line type, and the applied assay for cytotoxicity determination should be considered. Furthermore, in the work of Stanković et al. (2015) [20], human melanoma cells (Fem-x), human breast cancer cells (MDA-MB-361), chronic myeloid leukemia cells (K562), and cervical adenocarcinoma cells (HeLa) were treated with the same prepared MG extract. A pronounced dose- and cell line-dependent cytotoxic effect of the tested extract in the range of 0.0125–0.200 mg L^{-1} was observed. The highest concentration of the extract had the strongest antitumor effect on K562 and HeLa cells, reducing their viability by ~97% and ~85%, respectively, during 72 h at a concentration of 0.200 mg L^{-1} . In addition, it is important to mention the results of the treatment of human normal immunocompetent blood cells (PBMC) and proliferation-stimulated blood cells (PBMC-PHA) with phytohemagglutinin (conditions the coagulation of leukocytes) in the same work with the same concentration of MG extract, whereby the viability of the tested cells was reduced by 20% only for PBM-PHA, which indicates the selectivity of the polyphenol extract towards certain cell lines, and thus modulates the antitumor effect.

The cytotoxic effect of the MG extract on AGS, Caco-2, and HepG2 cells at the highest concentration and during the 24 h of exposure may be partly related to the presence of phenylethanoid glycosides, which account for about 22% of the freeze-dried MG extract used in this study, with echinacoside representing approximately 40% of the total. In the study by Ye et al. (2019) [49], a significant increase in antiproliferative activity by increasing the concentration of echinacoside (20 $\mu\text{g mL}^{-1}$ —9.57%; 50 $\mu\text{g mL}^{-1}$ —26.67%; 100 $\mu\text{g mL}^{-1}$ —37.20%) on HepG2 cells and myeloid cells was observed. The influence of structurally similar polyphenolic compounds, such as verbascoside, should also not be excluded. For example, Mulani et al. (2014) [50] reported a stronger cytotoxic effect of verbascoside (23–30%) compared to echinacoside (10–18%) and calkeolarioside A (13–18%) and B (5–15%). Additionally, Attia et al. (2018) [51] reported a significant cytotoxic effect of verbascoside in the treated range of 0.01–100 μM on Caco-2 (decreased cell viability by 70%) and HCT-116 (decreased cell viability by 80%) cells, while the combination of a verbascoside

dose of 0.1 μM and 10 μM fluorouracil as a standard chemotherapeutic agent resulted in a significant synergistic cytotoxic effect (Caco-2: 80%, HCT-116: 90%). Numerous studies also confirm the antiproliferative effect of other plant extracts that are rich in phenylethanoid glycosides. For example, Yuan et al. (2021) [52] found the concentration- (200–600 $\mu\text{g mL}^{-1}$) and time-dependent (24 and 48 h) cytotoxic effect of *Cistanche tubulosa* extract (28% echinacoside, 9.9% verbascoside, 34.8% of total polysaccharides) on liver cancer cells—BEL-7404 and HepG2—through the induction of the cell cycle arrest and apoptosis associated with the activation of the MAPK (mitogen-activated protein kinase) signaling pathway. The increased efficacy of the antitumor properties of the conventional chemostatic agent cisplatin, with a simultaneous reduction of side effects, as well as the independent cytotoxic role of the tested extract through a direct antiproliferative effect and an indirect improvement of the immune response, were also observed.

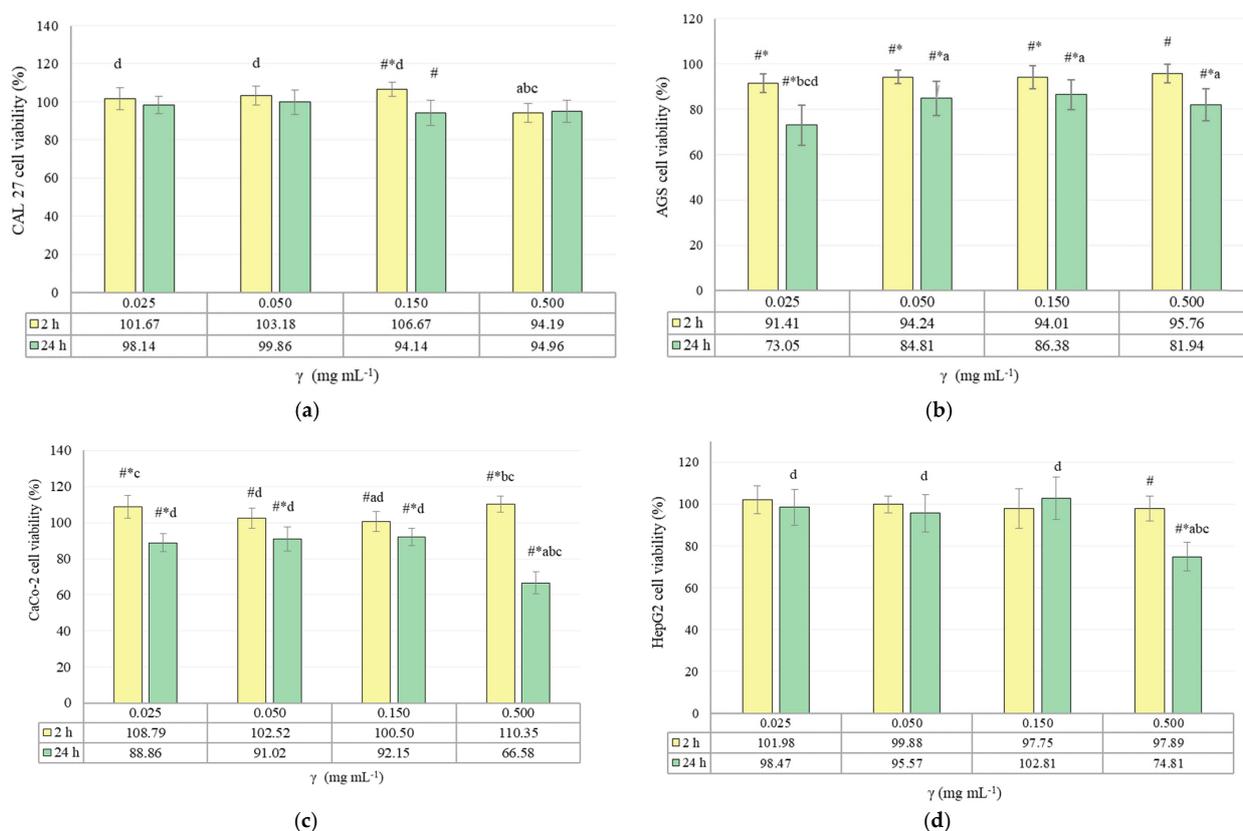


Figure 4. Cell viability of tested lines: (a) CAL 27; (b) AGS; (c) Caco-2; and (d) HepG2 after 2 and 24 h of treatment with Mountain Germander extract concentrations (0.025–0.500 mg mL^{-1}). One-way analysis of variance was carried out between the tested concentrations with Tukey’s post hoc test. The values marked with the sign # are statistically significantly ($p < 0.05$) different in regard to the treatment time for each extract concentration, and were obtained by performing the Mann–Whitney test ($p < 0.05$). * = statistically significantly different ($p < 0.05$) compared to negative control (water); a = statistically significantly different ($p < 0.05$) compared to 0.025 mg mL^{-1} ; b = statistically significantly different ($p < 0.05$) compared to 0.050 mg mL^{-1} ; c = statistically significantly different ($p < 0.05$) compared to 0.150 mg mL^{-1} ; d = statistically significantly different ($p < 0.05$) compared to 0.500 mg mL^{-1} .

3.4. Antioxidant/Pro-Oxidative Effect of Mountain Germander Extract on Human Cell Lines

To determine the correlation between the survival of cells treated with the extract and the decrease or increase in the proportion of highly reactive oxygen radicals (ROS) produced, an analysis of the antioxidant/pro-oxidant activity was also carried out. In addition to the fact that the induction of free radicals differed significantly depending

on the cell culture treated, a dose-dependent effect of the extract was generally observed (Figure 5).

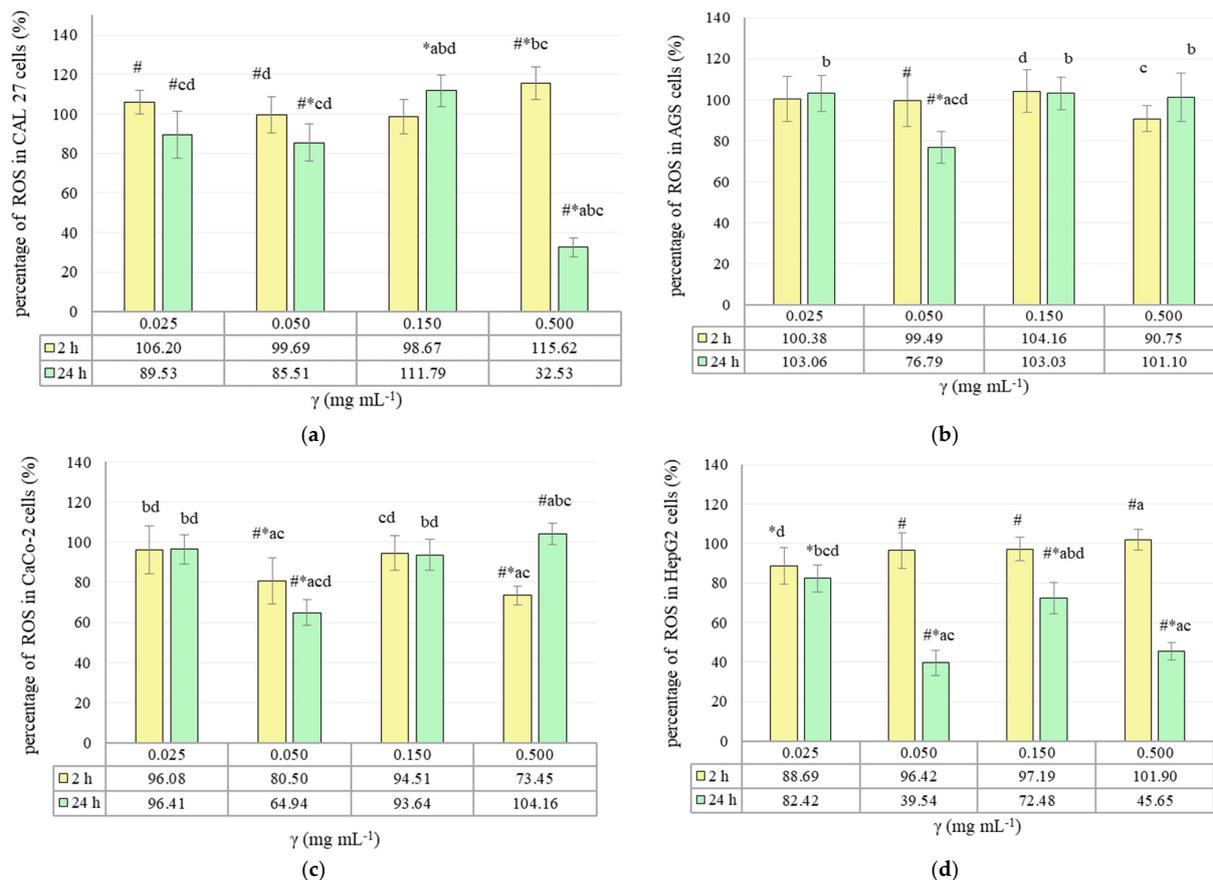


Figure 5. Induction of free radicals (ROS) on cell lines: (a) CAL 27; (b) AGS; (c) Caco-2; and (d) HepG2 after 2 and 24 h of treatment with Mountain Germander extract concentrations (0.025–0.500 mg mL⁻¹). One-way analysis of variance was carried out between the tested concentrations with Tukey's post hoc test. The values marked with the sign # are statistically significantly ($p < 0.05$) different in regard to the treatment time for each extract concentration, and were obtained by performing the Mann–Whitney test ($p < 0.05$). * = statistically significantly different ($p < 0.05$) compared to negative control (water); a = statistically significantly different ($p < 0.05$) compared to 0.025 mg mL⁻¹; b = statistically significantly different ($p < 0.05$) compared to 0.050 mg mL⁻¹; c = statistically significantly different ($p < 0.05$) compared to 0.150 mg mL⁻¹; d = statistically significantly different ($p < 0.05$) compared to 0.500 mg mL⁻¹.

A statistically significant ($p < 0.05$) pro-oxidant effect on CAL 27 after a shorter treatment (2 h) was observed only at 0.500 mg mL⁻¹, while after 24 h, the opposite effect occurred, i.e., a reduction in ROS formation by 72% ($p < 0.05$). The antioxidant activity of the extract in the treatment of AGS cells is only visible after 24 h, at 0.050 mg mL⁻¹. In Caco-2 cells, a statistically ($p < 0.05$) significant antioxidant effect of MG extract at 0.050 mg mL⁻¹ was observed after 2 h of treatment, which persisted even after prolonged exposure (24 h), while the treatment with 0.500 mg mL⁻¹ showed statistically significant ($p < 0.05$) antioxidant activity only after 2 h. On the contrary, after 24 h, a statistically significant ($p < 0.05$) pro-oxidant effect of a concentration of 0.500 mg mL⁻¹ was observed. In HepG2 cells, a statistically significant ($p < 0.05$) antioxidant activity was observed, with a longer incubation of the cells (24 h) compared to a shorter exposure to the extract, with extract concentrations of 0.050 and 0.500 mg mL⁻¹ leading to the most pronounced effect, i.e., a reduction in the generation of ROS by approximately 60% and 54%, respectively. Based on the results obtained, a statistically significant ($p < 0.05$) trend between the antioxidant effect of the extract

and prolonged time exposure (24 h) in CAL 27 and HepG2 can be noticed. Furthermore, no statistically significant ($p > 0.05$) dependence of ROS generation on the reduction of cell viability was observed in the tested cell lines compared to the control. On the other hand, it should be noted that a statistically significant ($p < 0.05$) reduction in reactive radical induction was observed in HepG2 cells at all concentrations, and partially in CAL 27 and Caco-2, while the percentage of cell survival (%) remained relatively unchanged. A similar trend was observed in the study by Stanković et al. (2011) [27], in which the correlation between the increased formation of superoxide ($O_2^{\cdot-}$) and nitrite ($NO_2^{\cdot-}$) radicals and the decrease in proliferation of HCT-116 colon tumor cells in the presence of a methanolic extract of MG extract ($0.050^{-1} \text{ mg mL}^{-1}$) was investigated. The results did not indicate a statistically significant increase in generated radicals, while an increase in viable cells was observed at lower concentrations during the same treatment (72 h), which implies that the proliferative effect is independent of the ROS presence. On the other hand, Živanović et al. (2016) [53] investigated the migration potential of MG extract ($1\text{--}100 \mu\text{g mL}^{-1}$) on human colon cancer cell lines, SW-480 and breast cancer MDA-MB-231, depending on the change in redox status measured by the concentrations of reduced glutathione (GSH), superoxide, and nitrite radicals after 24 and 72 h of treatment. Generally, the migration potential is the first step in cancer metastasis. By penetrating into the surrounding tissue and bloodstream, it creates the conditions for the formation of secondary tumors. The results showed that only the lowest concentration of the extract ($1 \mu\text{g mL}^{-1}$ for SW-480; $10 \mu\text{g mL}^{-1}$ for MDA-MB-231) reduced the migration potential, while a further increase in concentration had the opposite effect. Additionally, no correlation was found between the reduction in migration potential and the pro-oxidant effect of the extract due to the increased formation of the tested radicals, indicating a possible modulation of tumor cell migration by mechanisms independent of the redox status. It is known that polyphenols have the ability to have a very selective pro-oxidant effect on cancer cells by inducing the formation of free radicals, leading to a toxic effect and cell death, while not damaging normal cells [30]. On the other hand, their antioxidant role is based on the elimination and reduction of free radical formation, which is the main role in the regulation of oxidative stress and the prevention of cancer. The lack of a positive correlation between the ROS induction and the cytotoxic effect during treatment with MG extract can be partly explained by the extract's pronounced antioxidant properties after 24 h of treatment, such as the ability to chelate metals in a cancer environment, activate the defense system, and regenerate cells [27]. In this context, it can be partially concluded that the polyphenolic compounds of MG extract play a role in the prevention of pathogenesis, which can be used for chemopreventive purposes [54]. In contrast to some phenolic acids (gallic acid, ellagic acid) and flavonoids (epigallocatechin gallate, rutin, myristic acid, resveratrol), for which both antioxidant and pro-oxidant roles have been demonstrated, the available research on various phenylethanoid glycosides, such as echinacoside and salidroside, points to their preventive, antioxidant role by reducing lipid peroxidation, improving mitochondrial functions, inhibiting oxidative stress, improving intercellular communication, regulating the MAPK signaling pathway (mitogen-activated protein kinase/extracellular signal-regulated kinase), as one of the fundamental cascade reactions in the proliferation, differentiation, survival, and apoptosis of inflammatory cells and others [55,56].

3.5. Genotoxic/Genoprotective Effect of Mountain Germander Extract on Human Cell Lines

The induction of oxidative DNA damage of the tested human cell with MG extract was evaluated by the following parameters of the alkaline comet assay: tail length and intensity. In CAL 27 cells, no statistically significant ($p > 0.05$) effect on genetic material was observed for any of the tested extract concentrations (Figure 6).

In other cell lines, concentrations of 0.050 and 0.500 mg mL^{-1} were found to be statistically significant ($p < 0.05$) in exhibiting genoprotective or genotoxic effects. In AGS cells, a statistically significant ($p < 0.05$) genoprotective effect of tested polyphenolic extract was found at concentrations of 0.025 and 0.050 mg mL^{-1} when considering the reduction

in the size of oxidation damage (tail length) after a shorter treatment time, which was prolonged with a 24 h treatment. Treatment with the extract showed a genotoxic effect on Caco-2 cells, considering that the size of the measured DNA fragments (tail length) increased statistically significantly ($p < 0.05$) after 2 h at a concentration of 0.050 mg mL^{-1} compared to the control. In HepG2, a statistically significant ($p < 0.05$) genotoxic effect of the extract was observed during a shorter exposure time (2 h) by analyzing the tail length results, where the concentrations of 0.050 and 0.500 mg mL^{-1} again stood out. Based on the measured intensity of the tail, an increase in the amount of oxidative damage to DNA can be observed after prolonged treatment (24 h) at concentrations of 0.050 and 0.150 mg mL^{-1} . The oxidative effect of treatment with aqueous, ethanolic, and methanolic extracts from selected plant species of the genera *Tyhmus*, *Origanum*, and *Teucrium* on the genetic material of normal human lung fibroblasts (MRC-5) was investigated in the study by Oalđe et al. (2020) [19]. The following concentrations were used— 0.025 , 0.05 , and 0.1 mg mL^{-1} —and the results of the comet assay were compared to the negative (water, ethanol, or methanol) and positive controls (H_2O_2). According to the tail intensity results for all prepared extracts, a decrease in the percentage of DNA fragments generated was observed, which is as follows: 0.025 mg mL^{-1} for the methanol extract, 0.05 mg mL^{-1} for the ethanol extract, and 0.05 mg mL^{-1} for the aqueous extract. Furthermore, the results were positively correlated with certain proportions of phenolic acids and flavonoids, with rosmarinic acid, i.e., luteolin-7-*O*-glucoside, dominating. In a more recent study by Tureyen et al. (2023) [57], ovarian cancer cells (A2780) were found to have a significant increase in tail intensity when treated with tubuloside A, one of the major phenylethanoid glycosides from the traditional Chinese plant *Cistanche tubulosa*, at a concentration of $100 \text{ }\mu\text{M}$ ($p < 0.05$), compared to the standard chemotherapeutic agent ($100 \text{ }\mu\text{M}$ 5-fluorouracil), with the confirmed induction of apoptosis of cancer cells through activation of the transcription factor p53 and the cascade of caspase enzyme reactions for programmed cell death.

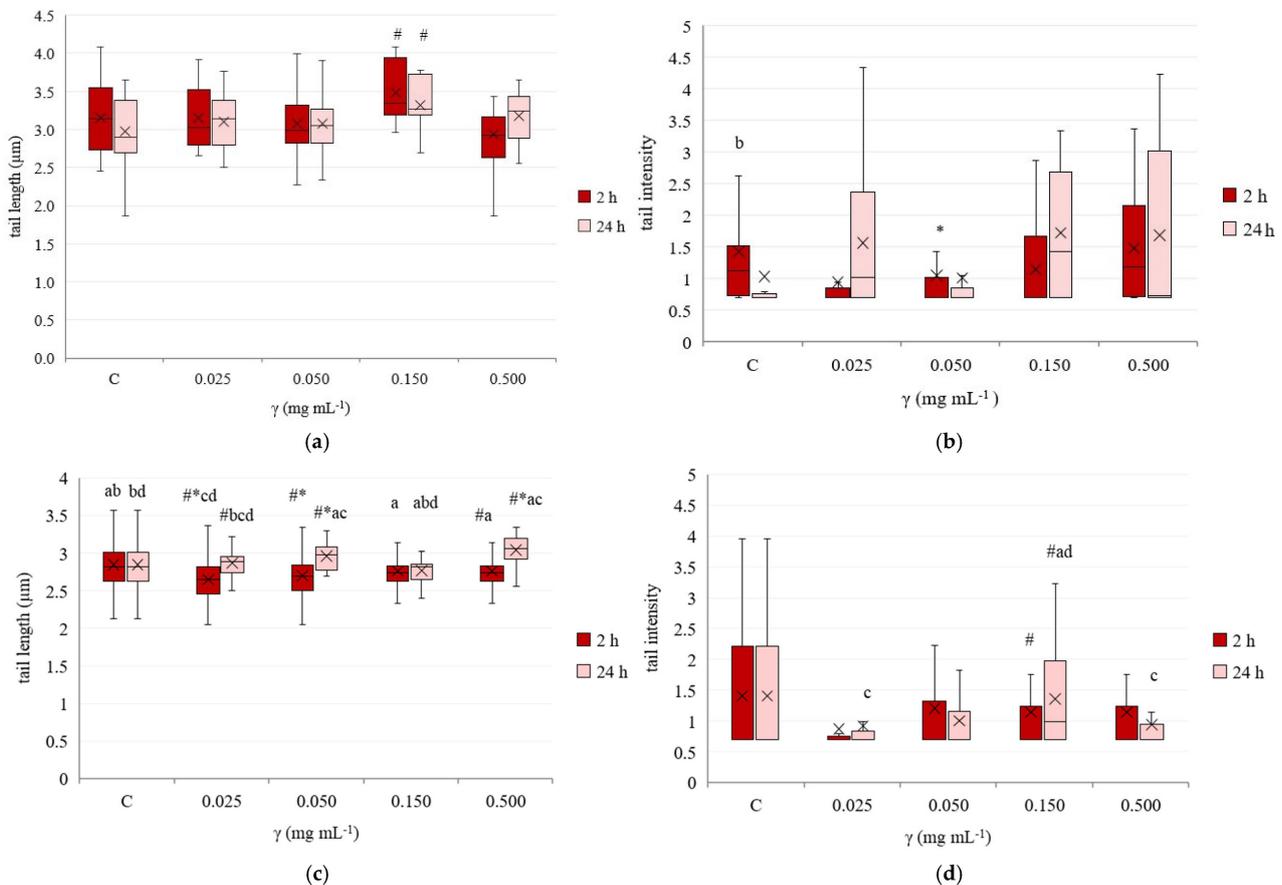


Figure 6. Cont.

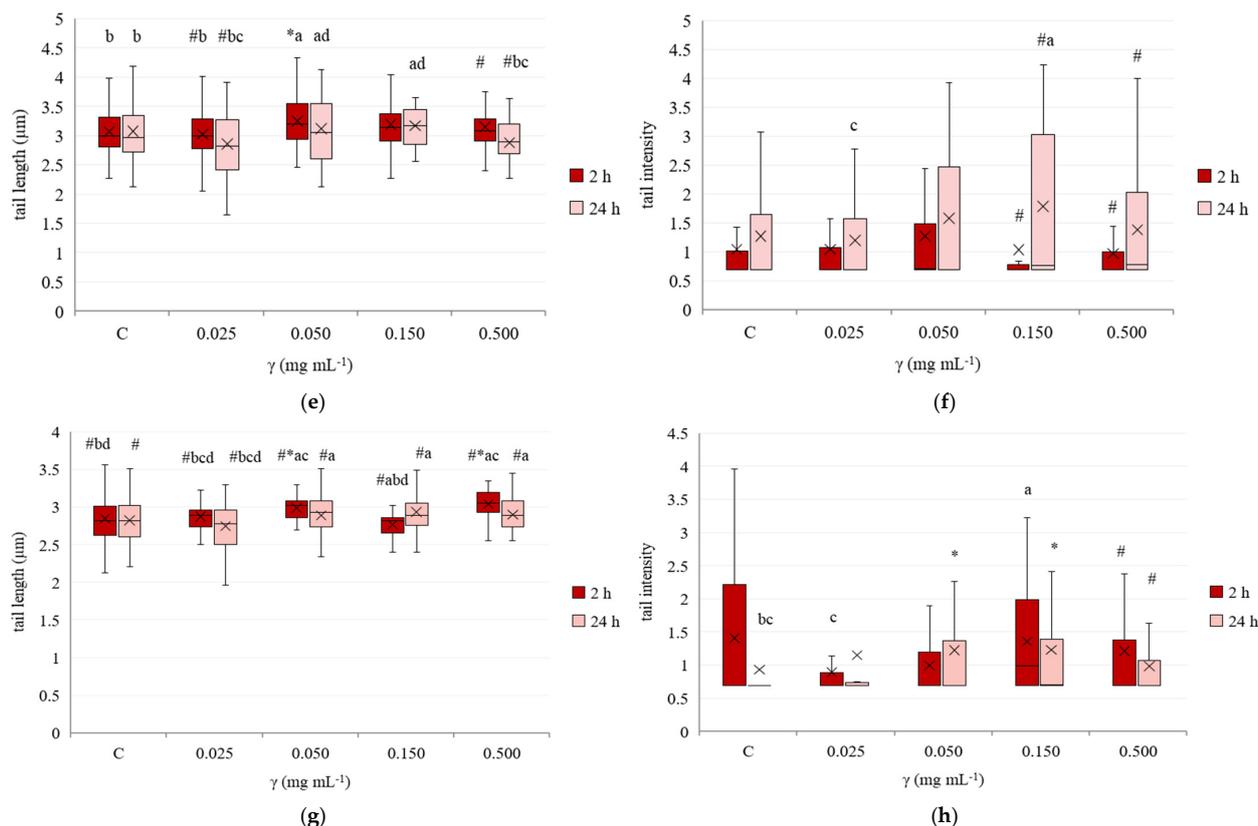


Figure 6. The effect of Mountain Germander extract concentrations (0.025–0.500 mg mL⁻¹) on DNA tail length and tail intensity of the cell lines: (a,b) CAL 27; (c,d) AGS; (e,f) Caco-2; and (g,h) HepG2 cells after 2 and 24 h of treatment. One-way analysis of variance was carried out between the tested concentrations with Tukey's post hoc test. The values marked with the sign # are statistically significantly ($p < 0.05$) different in regard to the treatment time for each extract concentration, and were obtained by performing the Mann–Whitney test ($p < 0.05$). * = statistically significantly different ($p < 0.05$) compared to negative control (culture medium); a = statistically significantly different ($p < 0.05$) compared to 0.025 mg mL⁻¹; b = significantly different ($p < 0.05$) compared to 0.050 mg mL⁻¹; c = statistically significantly different ($p < 0.05$) compared to 0.150 mg mL⁻¹; d = statistically significantly different ($p < 0.05$) compared to 0.500 mg mL⁻¹.

3.6. Bacteriostatic/Proliferative Effect of Mountain Germander Extract on Representative Bacterial Strains of Human Microbiota

To gain insight into the potential of MG extract as a bacteriostatic agent, the percentage of survival of bacterial cultures of *E. coli*, *S. aureus*, and *L. plantarum* was determined when they were exposed to the extract for 60 min. A statistically significant ($p < 0.05$) effect of all applied extract concentrations on reducing the viability of *E. coli* cells compared to the control was found, with the concentration of 0.150 mg mL⁻¹ showing the strongest effect, inhibiting its growth by 56% ($p < 0.05$) (Figure 7).

Although a statistically significant ($p < 0.05$) inhibitory effect of MG extract on the growth of *S. aureus* cells was observed at concentrations of 0.025, 0.150, and 0.500 mg mL⁻¹ ($p < 0.05$), it was relatively small (inhibition of growth by only 10–20%). In *L. plantarum*, a statistically significant decrease ($p < 0.05$) in bacterial viability was observed at higher concentrations (0.150 and 0.500 mg mL⁻¹). The observed inhibitory potential of *E. coli* during treatment with MG extract certainly represents a result of the greatest interest for further research, given the known facts related to the high antimicrobial resistance of this Gram-negative bacterium, which is significantly manifested in the ability of *E. coli* to participate in the transfer of its genes, but also in the uptake and accumulation of resistant genes from other microorganisms in the living environment [58]. In addition, the high resistance

of *S. aureus* also poses a challenge in the search for alternative antibacterial agents, as it has a thick peptidoglycan layer that is resistant to damage [59]. Previous studies have also confirmed the antimicrobial activity of methanolic polyphenolic extracts from other Lamiaceae species, such as pennyroyal (*Mentha pulgeum*), horehound (*Marrubium vulgare*), southern bugle (*Ajuga iva*), and felty germander (*T. polium*), compared to gallic acid and tannic acid as standards. A positive correlation was found between the enlargement of the inhibition zone and a certain MIC concentration for *E. coli* and *S. aureus*, depending on certain proportions of the total TPC, tannins, the total flavonoids, and antioxidant capacity [60]. In addition, Jerusalem artichoke sage (*Phlomis fruticose* L.) extract with a qualitative profile characterized in detail by HR MS-MS, in which the presence of phenylethanoid glycosides (nine compounds), flavonoids (sixteen compounds), and phenolic acids (fifteen compounds) was determined, showed moderate bacteriostatic activity against *S. aureus* and *E. coli*, but also a bactericidal effect against the methicillin-resistant strain of *S. aureus*, in contrast to the commercial antibiotic ampicillin, which did not even show an inhibitory effect [61]. On the other hand, in the same work, using the agar diffusion method, a greater antimicrobial activity of the metabolites of forsythoside A, more precisely hydroxytyrosol and dihydrocaffeic acid, against *S. aureus* was found than the tested component itself, which could partly indicate the relatively low bacteriostatic effect of the MG extract that is rich in phenylethanoid glycosides. However, recent findings related to the antimicrobial activity of these compounds indicate, for example, the synergistic effect of verbascoside with the commercial antibiotics vancomycin and ceftazidime in inhibiting certain strains of *S. aureus* and *P. aeruginosa* [62], or the inhibitory effect of echinacoside in combination with vancomycin on *S. aureus* activity [63].

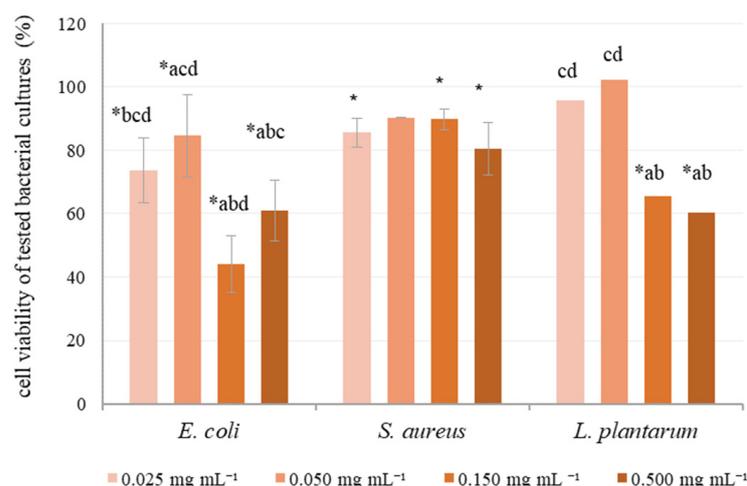


Figure 7. Cell viability of tested bacterial strains treated with Mountain Germander extract concentrations (0.025–0.500 mg mL⁻¹). One-way analysis of variance was carried out with Tukey’s post hoc test. * = statistically significantly different ($p < 0.05$) compared to negative control (PBS buffer); a = statistically significantly different ($p < 0.05$) compared to 0.025 mg mL⁻¹; b = significantly different ($p < 0.05$) compared to 0.050 mg mL⁻¹; c = statistically significantly different ($p < 0.05$) compared to 0.150 mg mL⁻¹; d = statistically significantly different ($p < 0.05$) compared to 0.500 mg mL⁻¹.

4. Conclusions

According to this study, the polyphenolic MG extract showed a dose-dependent dual effect on tested cellular macromolecules, i.e., it had a pro-oxidant effect by increasing the rate of BSA carbonyl protein formation at 0.125 and 0.500 mg mL⁻¹ of the extract and, on the contrary, acted as an efficient genoprotective agent on DNA plasmid at the highest tested concentration of MG extract (0.500 mg mL⁻¹). The most pronounced cytotoxic effect of the polyphenolic MG extract was observed on AGS, Caco-2, and HepG2 cells after 24 h of exposure at 0.500 mg mL⁻¹, while the genotoxic effect was significantly dependent on the concentration and exposure time for each cell line tested. However, the

lack of correlation between the percentage of induced ROS and reduced cell viability after treatment could indicate a possible antiproliferative effect of the polyphenolic MG extract, independent of ROS formation, as well as its dominant preventive and antioxidant role after prolonged treatment (24 h). In terms of the cell viability of the tested bacterial strains, the MG extract (0.150 and 0.500 mg mL⁻¹) showed remarkable bacteriostatic potential on *E. coli*, with the inhibition of the bacterial strain reducing viability by 56%. The present study expands the limited knowledge of Mountain Germander as an understudied medicinal plant species in terms of its biological activity at the gastrointestinal level as a primary route of administration, and provides the basis for further studies to discover the mechanisms of the demonstrated pro-oxidant, cytotoxic, and bacteriostatic activity.

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References

1. Aboelsoud, N.H. Herbal medicine in ancient Egypt. *J. Med. Plants Res.* **2010**, *4*, 82–86.
2. Li, F.S.; Weng, J.K. Demystifying traditional herbal medicine with modern approach. *Nat. Plants* **2017**, *3*, 17109. [[CrossRef](#)] [[PubMed](#)]
3. WHO. *WHO Global Report on Traditional and Complementary Medicine 2019*; World Health Organization: Geneva, Switzerland, 2019. Available online: <https://iris.who.int/handle/10665/312342> (accessed on 1 May 2024).
4. Zhang, D.; Zhang, B.; Lv, J.T.; Sa, R.N.; Zhang, X.M.; Lin, Z.J. The clinical benefits of Chinese patent medicines against COVID-19 based on current evidence. *Pharmacol. Res.* **2020**, *157*, 104882. [[CrossRef](#)] [[PubMed](#)]
5. NATCM. *Diagnosis and Treatment Protocol for Novel Coronavirus Pneumonia*; Trial Version 7; NATCM—National Administration of Traditional Chinese Medicine: Beijing, China, 2020.
6. Si, X.; Ma, X.; Wang, Y.; Li, Y.; Liu, L.; Yang, Y.; Guo, Z.; Liang, Y.; Pan, G. Efficacy and safety of Jinhua Qinggan granules in the treatment of coronavirus disease 2019 (COVID-19): A systematic review and meta-analysis. *Medicine* **2023**, *102*, e33545. [[CrossRef](#)] [[PubMed](#)]
7. Cheohen, C.F.D.A.R.; Esteves, M.E.A.; da Fonseca, T.S.; Leal, C.M.; Assis, F.D.L.F.; Campos, M.F.; Rebelo, R.S.; Allonso, D.; Leitão, G.G.; da Silva, M.L.; et al. In silico screening of phenylethanoid glycosides, a class of pharmacologically active compounds as natural inhibitors of SARS-CoV-2 proteases. *Comput. Struct. Biotechnol. J.* **2023**, *21*, 1461–1472. [[CrossRef](#)]
8. WHO. *WHO Establishes the Global Centre for Traditional Medicine in India*; World Health Organization: Geneva, Switzerland, 2022. Available online: <https://www.who.int/news/item/25-03-2022-who-establishes-the-global-centre-for-traditional-medicine-in-india> (accessed on 1 May 2024).
9. WHO TEAM. *WHO Traditional Medicine Global Summit 2023 Meeting Report: Gujarat Declaration*; WHO Global Centre for Traditional Medicine: Geneva, Switzerland, 2023. Available online: <https://www.who.int/publications/m/item/who-traditional-medicine-summit-2023-meeting-report--gujarat-declaration> (accessed on 1 May 2024).
10. Michel, J.; Abd Rani, N.Z.; Husain, K. A review on the potential use of medicinal plants from *Asteraceae* and *Lamiaceae* plant family in cardiovascular diseases. *Front. Pharmacol.* **2020**, *11*, 852. [[CrossRef](#)]
11. Tutin, T.G.; Heywood, V.H.; Burges, N.A.; Moore, D.M.; Valentine, D.H.; Walters, S.M.; Webb, D.A. *Flora Europaea III*; Cambridge University Press: Cambridge, UK, 1972.

12. Fatiha, B.A.; Ouafae, B.; Souad, S.; Fatima, E.H.; Jamila, D.; Allal, D.; Lahcen, Z. Ethnobotany study of medicinal plants used in the treatment of respiratory diseases in the middle region of Oum Rbai. *Int. J. Environ. Agric. Biotechnol.* **2017**, *2*, 1460–1468. [[CrossRef](#)]
13. Ferrier, J.; Šačiragić, L.; Chen, E.C.H.; Trakić, S.; Saleem, A.; Alikadić, E.; Cuerrier, A.; Balick, M.J.; Arnason, J.T.; Redžić, S. Ways the Lukomir highlanders of Bosnia and Herzegovina treat diabetes. In *Ethnobotany and Biocultural Diversities in the Balkans*; Pieroni, A., Quave, C.L., Eds.; Springer Publishing: New York, NY, USA, 2014; pp. 13–27, ISBN 978-1-4939-1492-0.
14. Šarić-Kundalić, B.; Fritz, E.; Dobeš, C.; Saukel, J. Traditional medicine in the pristine village of Prokoško lake on Vranica Mountain, Bosnia and Herzegovina. *Sci. Pharm.* **2010**, *78*, 275–290. [[CrossRef](#)]
15. Zlatković, B.; Bogosavljević, S.; Radivojević, A.; Pavlović, M. Traditional use of the native medicinal plant resource of Mt. Rtanj (Eastern Serbia): Ethnobotanical evaluation and comparison. *J. Ethnopharmacol.* **2014**, *151*, 704–713. [[CrossRef](#)]
16. Jarić, S.; Mitrović, M.; Pavlović, P. Ethnobotanical featured of *Teucrium* species. In *Teucrium Species: Biology and Applications*; Stanković, M., Ed.; Springer Nature: Cham, Switzerland; Berlin/Heidelberg, Germany, 2020; pp. 111–142, ISBN 978-3-030-52159-2.
17. Redžić, S. Wild medicinal plants and their usage in traditional human therapy (Southern Bosnia and Herzegovina, W. Balkan). *J. Med. Plant. Res.* **2010**, *4*, 1003–1027.
18. Li, J.J.; Ma, S.; Wang, Y.; Wang, M.; Li, M.; Gao, C.; Zhang, L.; Li, Y.; Liu, Y.; Stevanović, Z.D.; et al. *Teucrium montanum* extract drives effector and memory differentiation of CD8+ T cells. *Biomed. Res. Ther.* **2023**, *10*, 6023–6034. [[CrossRef](#)]
19. Oalđe, M.M.; Kolarević, S.M.; Živković, J.C.; Vuković-Gačić, B.S.; Marić, J.M.J.; Kolarević, M.J.K.; Đorđević, J.; Matin, P.D.; Šavikin, K.; Vuković-Gačić, B.; et al. The impact of different extracts of six *Lamiaceae* species on deleterious effects of oxidative stress assessed in acellular, prokaryotic and eukaryotic models in vitro. *Saudi Pharm. J.* **2020**, *28*, 1592–1604. [[CrossRef](#)] [[PubMed](#)]
20. Stanković, M.S.; Mitrović, T.L.; Matić, I.Z.; Topuzović, M.D.; Stamenković, S.M. New values of *Teucrium* species: In vitro study of cytotoxic activities of secondary metabolites. *Not. Bot. Horti Agrobot.* **2015**, *43*, 41–46. [[CrossRef](#)]
21. Vujanović, M.; Zengin, G.; Durović, S.; Mašković, P.; Cvetanović, A.; Radojković, M. Biological activity of extracts of traditional wild medicinal plants from the Balkan Peninsula. *S. Afr. J. Bot.* **2019**, *120*, 213–218. [[CrossRef](#)]
22. Stanković, M.; Stefanović, O.; Čomić, L.; Topuzović, M.; Radojević, I.; Solujić, S. Antimicrobial activity, total phenolic content and flavonoid concentrations of *Teucrium* species. *Open Life Sci.* **2012**, *7*, 664–671. [[CrossRef](#)]
23. Sailović, P.; Odžaković, B.; Bodroža, D.; Vulić, J.; Čanadanović-Brunet, J.; Zvezdanović, J.; Danilović, B. Polyphenolic composition and antimicrobial, antioxidant, anti-inflammatory, and antihyperglycemic activity of different extracts of *Teucrium montanum* from Ozren Mountain. *Antibiotics* **2024**, *13*, 358. [[CrossRef](#)]
24. Milošević-Djordjević, O.; Stošić, I.; Stanković, M.; Grujičić, D. Comparative study of genotoxicity and antimutagenicity of methanolic extracts from *Teucrium chamaedrys* and *Teucrium montanum* in human lymphocytes using micronucleus assay. *Cytotechnology* **2013**, *65*, 863–869. [[CrossRef](#)]
25. Vladimir-Knežević, S.; Blažeković, B.; Kindl, M.; Vladić, J.; Lower-Nedza, A.D.; Brantner, A.H. Acetylcholinesterase inhibitory, antioxidant and phytochemical properties of selected medicinal plants of the *Lamiaceae* family. *Molecules* **2014**, *19*, 767–782. [[CrossRef](#)]
26. Bektašević, M.; Jurin, M.; Roje, M.; Politeo, O. Phytochemical Profile, Antioxidant activity and cholinesterase inhibition potential of essential oil and extracts of *Teucrium montanum* from Bosnia and Herzegovina. *Separations* **2023**, *10*, 421. [[CrossRef](#)]
27. Stanković, M.; Ćurčić, M.; Žižić, J.; Topuzović, M.; Solujić, S.; Marković, S. *Teucrium* plant species as natural sources of novel anticancer compounds: Antiproliferative, proapoptotic and antioxidant properties. *Int. J. Mol. Sci.* **2011**, *12*, 4190–4205. [[CrossRef](#)]
28. Nair, S.; Li, W.; Kong, A.N.T. Natural dietary anti-cancer chemopreventive compounds: Redox-mediated differential signaling mechanisms in cytoprotection of normal cells versus cytotoxicity in tumor cells. *Acta Pharmacol. Sin.* **2007**, *28*, 459–472. [[CrossRef](#)] [[PubMed](#)]
29. Pelicano, H.; Carney, D.; Huang, P. ROS stress in cancer cells and therapeutic implications. *Drug Resist. Update* **2004**, *7*, 97–110. [[CrossRef](#)] [[PubMed](#)]
30. Babich, H.; Schuck, A.G.; Weisburg, J.H.; Zuckerbraun, H.L. Research strategies in the study of the pro-oxidant nature of polyphenol nutraceuticals. *J. Toxicol.* **2011**, *2011*, 467305. [[CrossRef](#)] [[PubMed](#)]
31. Weisburg, J.H.; Weissman, D.B.; Sedaghat, T.; Babich, H. In vitro cytotoxicity of epigallocatechin gallate and tea extracts to cancerous and normal cells from the human oral cavity. *Basic Clin. Pharmacol. Toxicol.* **2004**, *95*, 191–200. [[CrossRef](#)] [[PubMed](#)]
32. Fukumoto, L.R.; Mazza, G. Assessing antioxidant and prooxidant activities of phenolic compounds. *J. Agric. Food Chem.* **2000**, *48*, 3597–3604. [[CrossRef](#)]
33. Zhang, D.; Li, H.; Wang, J.B. Echinacoside inhibits amyloid fibrillization of HEWL and protects against A β -induced neurotoxicity. *Int. J. Biol. Macromol.* **2015**, *72*, 243–253. [[CrossRef](#)]
34. Liu, J.; Tang, N.; Liu, N.; Lei, P.; Wang, F. Echinacoside inhibits the proliferation, migration, invasion and angiogenesis of ovarian cancer cells through PI3K/AKT pathway. *J. Mol. Histol.* **2022**, *53*, 493–502. [[CrossRef](#)]
35. Khorashadizadeh, N.; Neamati, A.; Moshiri, M.; Etemad, L. Verbascoside inhibits paraquat-induced pulmonary toxicity via modulating oxidative stress, inflammation, apoptosis and DNA damage in A549 cell. *Drug Chem. Toxicol.* **2022**, *45*, 2212–2220. [[CrossRef](#)]
36. WHO. *WHO Guidelines on Safety Monitoring of Herbal Medicines in Pharmacovigilance Systems*; World Health Organization: Geneva, Switzerland, 2004. Available online: <https://www.who.int/publications/i/item/9241592214> (accessed on 26 August 2024).

37. Mandura Jarić, A.; Šeremet, D.; Vojvodić Cebin, A.; Jokić, S.; Komes, D. The multiple-response modeling of heat-assisted, microwave-assisted and subcritical water extraction on selected phenolics from traditional plant species *Teucrium montanum*. *Prep. Biochem. Biotechnol.* **2022**, *52*, 809–822. [[CrossRef](#)]
38. Mandura Jarić, A.; Čikoš, A.; Pocrnić, M.; Aladić, K.; Jokić, S.; Šeremet, D.; Vojvodić Cebin, A.; Komes, D. *Teucrium montanum* L.—Unrecognized Source of Phenylethanoid Glycosides: Green Extraction Approach and Elucidation of Phenolic Compounds via NMR and UHPLC-HR MS/MS. *Antioxidants* **2023**, *12*, 1903. [[CrossRef](#)]
39. Mandura Jarić, A.; Haramustek, L.; Nižić Nodilo, L.; Vrsaljko, D.; Petrović, P.; Kuzmić, S.; Jozinović, A.; Aladić, K.; Jokić, S.; Šeremet, D.; et al. A Novel Approach to Serving Plant-Based Confectionery—The Employment of Spray Drying in the Production of Carboxymethyl Cellulose-Based Delivery Systems Enriched with *Teucrium montanum* L. Extract. *Foods* **2024**, *13*, 372. [[CrossRef](#)] [[PubMed](#)]
40. Levine, R.L.; Garland, D.; Oliver, C.N.; Amici, A.; Climent, I.; Lenz, A.G.; Ahn, B.W.; Shaltiel, S.; Stadtman, E.R. Determination of carbonyl content in oxidatively modified proteins. *Methods Enzymol.* **1990**, *186*, 464–478. [[PubMed](#)]
41. Keum, Y.S.; Park, K.K.; Lee, J.M.; Chun, K.S.; Park, J.H.; Lee, S.K.; Kwon, H.; Surh, Y.J. Antioxidant and anti-tumor promoting activities of the methanol extract of heat-processed ginseng. *Cancer Lett.* **2000**, *150*, 41–48. [[CrossRef](#)] [[PubMed](#)]
42. Silveira, L.R.; Pereira-Da-Silva, L.; Juel, C.; Hellsten, Y. Formation of hydrogen peroxide and nitric oxide in rat skeletal muscle cells during contractions. *Free Radic. Biol. Med.* **2003**, *35*, 455–464. [[CrossRef](#)] [[PubMed](#)]
43. Repetto, G.; del Peso, A.; Zurita, J.L. Neutral red uptake assay for the estimation of cell viability/cytotoxicity. *Nat. Protoc.* **2008**, *3*, 1125–1131. [[CrossRef](#)]
44. Singh, N.P.; McCoy, M.T.; Tice, R.R.; Schneider, E.L. A simple technique for quantitation of low levels of DNA damage in individual cells. *Exp. Cell Res.* **1988**, *175*, 184–191. [[CrossRef](#)]
45. Šamec, D.; Durgo, K.; Grúz, J.; Kremer, D.; Kosalec, I.; Piljac-Žegarac, J.; Salopek-Sondi, B. Genetic and phytochemical variability of six *Teucrium arduini* L. populations and their antioxidant/prooxidant behaviour examined by biochemical, macromolecule- and cell-based approaches. *Food Chem.* **2015**, *186*, 298–305. [[CrossRef](#)]
46. Dorman, H.J.; Hiltunen, R. Antioxidant and pro-oxidant in vitro evaluation of water-soluble food-related botanical extracts. *Food Chem.* **2011**, *129*, 1612–1618. [[CrossRef](#)]
47. Jang, T.W.; Choi, J.S.; Park, J.H. Protective and inhibitory effects of acteoside from *Abeliophyllum distichum* Nakai against oxidative DNA damage. *Mol. Med. Rep.* **2020**, *22*, 2076–2084. [[CrossRef](#)]
48. Zhang, D.; Lu, C.; Yu, Z.; Wang, X.; Yan, L.; Zhang, J.; Li, H.; Wang, J.; Wen, A. Echinacoside alleviates UVB irradiation-mediated skin damage via inhibition of oxidative stress, DNA damage, and apoptosis. *Oxidative Med. Cell. Longev.* **2017**, *2017*, 6851464. [[CrossRef](#)]
49. Ye, Y.; Song, Y.; Zhuang, J.; Wang, G.; Ni, J.; Xia, W. Anticancer effects of echinacoside in hepatocellular carcinoma mouse model and HepG2 cells. *J. Cell Physiol.* **2019**, *234*, 1880–1888. [[CrossRef](#)] [[PubMed](#)]
50. Mulani, S.K.; Guh, J.H.; Mong, K.K.T. A general synthetic strategy and the anti-proliferation properties on prostate cancer cell lines for natural phenylethanoid glycosides. *Org. Biomol. Chem.* **2014**, *12*, 2926–2937. [[CrossRef](#)] [[PubMed](#)]
51. Attia, Y.M.; El-Kersh, D.M.; Wagdy, H.A.; Elmazar, M.M. Verbascoside: Identification, quantification, and potential sensitization of colorectal cancer cells to 5-FU by targeting PI3K/AKT pathway. *Sci. Rep.* **2018**, *8*, 16939. [[CrossRef](#)] [[PubMed](#)]
52. Yuan, P.; Fu, C.; Yang, Y.; Adila, A.; Zhou, F.; Wei, X.; Wang, W.; Lv, J.; Li, Y.; Xia, L.; et al. *Cistanche tubulosa* phenylethanoid glycosides induce apoptosis of hepatocellular carcinoma cells by mitochondria-dependent and MAPK pathways and enhance antitumor effect through combination with cisplatin. *Integr. Cancer Ther.* **2021**, *20*, 15347354211013085. [[CrossRef](#)] [[PubMed](#)]
53. Živanović, M.N.; Stojanović, A.Z.; Cvetković, D.M.; Milutinović, M.G.; Stanković, M.S.; Marković, S.D. Effects of *Teucrium* spp.: Extracts on migratory potential and redox status of human colon SW-480 and breast MDA-MB-231 cancer cells. *Kragujevac J. Sci.* **2016**, *38*, 161–172. [[CrossRef](#)]
54. Cardinali, A.; Pati, S.; Minervini, F.; D’Antuono, I.; Linsalata, V.; Lattanzio, V. Verbascoside, isoverbascoside, and their derivatives recovered from olive mill wastewater as possible food antioxidants. *J. Agric. Food Chem.* **2012**, *60*, 1822–1829. [[CrossRef](#)]
55. Bian, P.; Liu, C.; Hu, W.; Ding, Y.; Qiu, S.; Li, L. Echinacoside suppresses the progression of breast cancer by downregulating the expression of miR-4306 and miR-4508. *Integr. Cancer Ther.* **2021**, *20*, 15347354211062639. [[CrossRef](#)]
56. Wang, X.; Zhang, Z.; Cao, X. Salidroside inhibited the proliferation of gastric cancer cells through up-regulating tumor suppressor miR-1343-3p and down-regulating MAP3K6/MMP24 signal molecules. *Cancer Biol. Ther.* **2024**, *25*, 2322206. [[CrossRef](#)]
57. Tureyen, A.; Navruz, F.Z.; Gunay, S.; Erden, Y.; Ince, S. Tubuloside A induces DNA damage and apoptosis in human ovarian cancer A2780 Cells. *Eur. J. Ther.* **2023**, *29*, 900–906. [[CrossRef](#)]
58. Poirel, L.; Madec, J.Y.; Lupo, A.; Schink, A.K.; Kieffer, N.; Nordmann, P.; Schwarz, S. Antimicrobial resistance in *Escherichia coli*. *Microbiol. Spectr.* **2018**, *6*, 10–1128. [[CrossRef](#)]
59. Silhavy, T.J.; Kahne, D.; Walker, S. The bacterial cell envelope. *CSH Perspect. Biol.* **2010**, *2*, a000414. [[CrossRef](#)] [[PubMed](#)]
60. Khaled-Khodja, N.; Boulekbache-Makhlouf, L.; Madani, K. Phytochemical screening of antioxidant and antibacterial activities of methanolic extracts of some Lamiaceae. *Ind. Crops Prod.* **2014**, *61*, 41–48. [[CrossRef](#)]
61. Stojković, D.; Gašić, U.; Drakulić, D.; Zengin, G.; Stevanović, M.; Rajčević, N.; Soković, M. Chemical profiling, antimicrobial, anti-enzymatic, and cytotoxic properties of *Phlomis fruticosa* L. *J. Pharmaceut. Biomed.* **2021**, *195*, 113884. [[CrossRef](#)] [[PubMed](#)]

62. Shi, C.; Ma, Y.; Tian, L.; Li, J.; Qiao, G.; Liu, C.; Cao, W.; Liang, C. Verbascoside: An efficient and safe natural antibacterial adjuvant for preventing bacterial contamination of fresh meat. *Molecules* **2022**, *27*, 4943. [[CrossRef](#)]
63. Jiang, T.; Yuan, D.; Wang, R.; Zhao, C.; Xu, Y.; Liu, Y.; Song, W.; Su, X.; Wang, B. Echinacoside, a promising sortase A inhibitor, combined with vancomycin against murine models of MRSA-induced pneumonia. *Med. Microbiol. Immun.* **2023**, *212*, 421–435. [[CrossRef](#)]

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