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Spelt Grass Juice: Phytochemicals and Antiproliferative Activity

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Abstract: Spelt is gaining popularity due to its nutritional and ecological benefits, particularly in organic farming. Juice from young spelt grass is rich in potassium, phosphorus, manganese, and chromium, along with beneficial pigments and phenolic compounds, all of which support human health. This study examines the phytochemical composition (photopigments and phenolics) determined by visible spectrophotometry and liquid chromatography, as well as the antiproliferative effects of spelt grass juice extracts assessed by the MTT assay. It also explores their selectivity towards human malignant cells (lung A-549 and cervical HeLa) and their potential synergy with doxorubicin, an anticancer drug. Spelt grass juice extracts, particularly aqueous and methanol–water extracts, contained significant amounts of ferulic acid and its derivatives. The methanol–water extracts were similar to aqueous extracts in terms of total phenolics (3464–3601 μg/g DW), while the acetone–water extracts had a significantly lower content (around 2700 μg/g DW). The antiproliferative effect of spelt extracts was moderate, with the acetone–water extract showing the highest selectivity towards HeLa cells, likely due to its ability to extract both photopigments and phenolics. Co-treatment with doxorubicin enhanced the cytotoxic effects. These findings highlight the potential of *Triticum spelta* extracts to improve the efficacy of conventional chemotherapeutic agents.

Keywords: spelt grass juice; phenolic; pigment; malignant cell; antiproliferative activity

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1. Introduction

Cultivated since at least 5000 B.C., spelt (*Triticum aestivum* ssp. *spelta*) is most likely developed by spontaneously crossing older varieties of wheat originating in the Middle East. Nowadays, spelt has earned an increasingly important place in the production of healthy and safe food, especially due to the exceptional benefits of production (the tolerance to abiotic and biotic stresses—diseases, drought, and soil nutrient shortage) especially in the organic farming system [1]. In addition, the juice/extract obtained from young spelt grass has beneficial effects on human health as a rich source of some major (potassium and phosphorus) and trace (manganese and chromium) elements, pigments (especially chlorophyll and pheophytin) and phenolics [2,3]. The antioxidant potential of young spelt grass extract was positively correlated with the total phenolic content [2]. The application of antioxidants and anti-inflammatory agents helps to diminish the tumor-supportive environment, thereby reducing cancer progression [4]. Spelt is making a comeback in human diets lately, as it is claimed to be even healthier than commercial wheat. Spelt products are recommended for people with gastrointestinal issues, children, and the elderly [2]. While wheatgrass and its juice have been reported to possess antioxidant, antimutagenic, antimicrobial, antiallergenic, nephroprotective, anti-inflammatory, diuretic, and anticancer

properties [5–13], similar effects of spelt grass extracts remain insufficiently investigated. Spelt grass juice supplementation exerts beneficial effects on the pro-oxidant–antioxidant balance and demonstrates anti-inflammatory properties, as evidenced by the changes in hsCRP concentrations [14]. Several studies on malignant cells have demonstrated the antiproliferative potential of ethanol and ethyl acetate extracts of *Triticum spelta* against human liver (HepG2) and colorectal (Caco-2) cancer cells, as well as the methanolic extract of *Triticum aestivum* in HeLa cervical carcinoma cells [3,5]. Studies employing protein extracts from both ancient and contemporary wheat cultivars showed that immunological responses, as shown by epitope-specific T-cell responses, varied significantly by genotype [15].

It is well-known that the combination of extracts from natural products with antineoplastic agents can result in enhanced antitumor effects on cancer cell lines [13,16]. Polyphenols enhanced the efficacy of chemotherapeutic agents by modulating the resistance mechanisms in cancer cells, particularly those exhibiting a multidrug resistance phenotype. In vitro studies have demonstrated that combining polyphenols with chemotherapeutics can modulate the genes associated with proliferation, induce apoptosis, cause cell cycle arrest, and increase oxidative stress, thereby contributing to more effective tumor suppression [17]. Meanwhile, in vivo studies indicate that the integration of phytochemicals with conventional chemotherapeutic agents may modulate tumor microenvironment dynamics by suppressing angiogenesis, promoting tumor necrosis, and reducing vascular density, though additional clinical validation is required [16].

This study focuses on the phytochemical composition (photopigments and phenolic compounds) and the antiproliferative activity of aqueous, and methanol– and acetone–water extracts obtained from spelt grass juice, their selectivity towards malignant cells, and the effect of co-treatment with doxorubicin, an antineoplastic drug.

2. Materials and Methods

2.1. Production Process of Young Spelt Grass Juice

The juice from young spelt grass was prepared by a local manufacturer in Podgorica for the Montenegrin market. The production process consists of three key steps: (1) Seed selection and initial treatment—The seeds are stored at low temperatures and initially soaked in a 5% $NaHCO_3$ solution, followed by water. Over the next 36 h, the seeds are rinsed and drained every 8 h in the same manner. During this period, the seeds begin to sprout, and the sprouts are then placed in a thin, dense layer on a perforated plastic or stainless-steel tray, sprayed with a 0.1% H_2O_2 solution, and covered with an opaque film. (2) Crop maintenance—In the greenhouse (temperature 21 °C and humidity 60%, with constant ventilation and air circulation provided by fans), a combination of natural and artificial light is used, along with precise irrigation and continuous monitoring of the microclimate. Irrigation is stopped during the dark phase. (3) Harvesting and extraction—The grass is harvested between the 8th and 10th day after sprouting, once the nutritional content of the seed has been exhausted. Extraction is carried out using dual-cascade extractors to preserve the maximum concentration of nutrients and enzymes. In the first cascade, the raw material is crushed with special stainless-steel blades, and pressure extraction (squeezing) is performed in the subsequent cascade. The obtained juice is stored under deep-freezing conditions until analysis.

2.2. Sample Preparation

Considering that the foam produced during the process of squeezing spelt grass probably contains bioactive compounds, for the purposes of this study, the samples were prepared in two series: one with foam and one with the foam removed. They were dried in a lyophilizer (Alpha 1–4 LD, Martin Christ, Osterode am Harz, Germany) to preserve the

biological activity of juice. The average share of dry matter in the samples without foam was 6.28%, and, in those with foam, 6.61%.

For the efficient extraction of photopigments and phenolic compounds (components with biological activity), two extraction solvents were used for the preparation of extracts: acetone–water (Ac:H_2O 4:1) and methanol–water (MeOH:H_2O 4:1). The mass of the lyophilized juice (about 0.1 g) was weighed and 10 mL of extraction solvent was added. Extraction with Ac:H_2O for 2 h and with MeOH:H_2O for 3 h was performed in an ultrasonic bath with the addition of ice. The extracts were centrifuged and filtered through the appropriate membrane filters (0.20 μm). Samples of fresh juice obtained by squeezing of young spelt grass were also centrifuged and filtered in the same way as mentioned extracts, for purpose of obtaining the liquid phase with water-soluble components, i.e., aqueous extract of spelt grass juice. All prepared samples of extracts were deep-frozen until preparation for performing of analyses.

2.3. Chemicals

For determination of derivatives of hydroxybenzoic and hydroxycinnamic acids as phenolic compounds, the following standards were used: gallic acid, ferulic acid, *p*-coumaric acid, and caffeic acid from Merck KGaA (Darmstadt, Germany). Methanol from J.T. Baker (Phillipsburg, NJ, USA) and acetone from Lachner (Neratovice, Czech Republic) and ultrapure water produced by Milli-Q system (Millipore, Bedford, MA, USA) were used for preparation of extracts. The chemicals for the mobile phases were HPLC-MS-grade acetonitrile and formic acid from Sigma-Aldrich. Water for the mobile phase was double-distilled and purified with the Milli-Q system (Millipore, Bedford, MA, USA).

2.4. Determination of Photosynthetic Pigments in Extracts

The concentration of pigments (chlorophyll a, chlorophyll b, and carotenoids) in acetone–water and methanol–water extracts was determined by the spectrophotometric method according to Marković [18]. Absorbances (A662, A644, and A440 for acetone–water extract; A666, A653, and A470 methanol–water extract) of photosynthetic pigments were read on a Cary 100 UV–visible spectrophotometer (Varian Inc., Palo Alto, CA, USA). The amounts of chlorophyll a, chlorophyll b, and carotenoids (μg/mL) in acetone–water extract were calculated using the formula and molar absorption coefficients (Holm and Wettstein) [18]:

$$\text{Chlorophyll a} = 9.784 \cdot A662 - 0.990 \cdot A644, \quad (1)$$

$$\text{Chlorophyll b} = 21.426 \cdot A644 - 4.650 \cdot A662, \quad (2)$$

$$\text{Carotenoids} = 4.695 \cdot A440 - 0.268 \cdot (\text{Chl a content} + \text{Chl b content}). \quad (3)$$

The amounts of chlorophyll a, chlorophyll b, and carotenoids (μg/mL) in methanol–water extract were calculated using the formula and molar absorption coefficients according to Wellburn and Lichtenthaler [19]:

$$\text{Chlorophyll a} = 15.65 \cdot A666 - 7.34 \cdot A653, \quad (4)$$

$$\text{Chlorophyll b} = 27.05 \cdot A653 - 11.21 \cdot A666, \quad (5)$$

$$\text{Carotenoids} = 4.082 \cdot A470 - 0.012 \cdot \text{Chl a content} - 0.527 \cdot \text{Chl b content}, \quad (6)$$

Concentrations were converted to μg/g DW.

2.5. Determination of Individual Phenolic Compounds in Extracts Using HPLC-DAD-MS^n Analysis

Phenolic compounds were analyzed by an HPLC system Ultimate 3000 (Thermo Fisher Scientific, Waltham, MA, USA) equipped with a DAD detector set at 280 nm, 350 nm, and 530 nm as reported by Mikulic-Petkovsek et al. [20]. The elution solvents were aqueous 0.1% formic acid and 3% acetonitrile in double-distilled water (A) and 0.1% formic acid and 3% water in acetonitrile (B). For phenolic determination, a Gemini C18 (Phenomenex, Torrance, CA, USA) column operated at 25° C was used.

The phenolic compounds were identified by comparing their UV–Vis spectra and retention times with standards, and also confirmed using a mass spectrometer (LTQ XL Linear Ion Trap Mass Spectrometer, Thermo Fisher Scientific, Waltham, MA, USA) with an electrospray interface (ESI) operating in negative ion mode. Full-scan data-dependent MS^n scanning from m/z 115 to 1900 was performed. All mass spectrometer conditions were the same as reported by Mikulic-Petkovsek et al. [20].

Phenolic contents were expressed in mg/g DW.

2.6. Determination of the Antiproliferative Activity of Spelt Grass Juice Extracts

2.6.1. Growth and Culture of the Cell Lines

For the estimation of antiproliferative activity of various spelt grass juice extracts, one non-transformed human cell line, MRC-5 (a non-transformed (normal) fetal lung fibroblast, ECACC 84101801), and two human malignant transformed cell lines, A-549 (ECACC 86012804—lung cancer) and HeLa (ECACC 93021013—cervix cancer), were utilized. The cell lines were cultured and maintained in Dulbecco's Modified Eagle's Medium (DMEM, Merck KGaA, Darmstadt, Germany) supplemented with 10% Fetal Calf Serum (FCS), 100 Units/mL penicillin, and 100 µg/mL streptomycin, referred to as complete medium. The cells were grown in 25 cm^2 flasks at 37 °C in an atmosphere of 5% CO_2 and high humidity and were sub-cultured twice a week. A single-cell suspension was obtained using 0.1% trypsin with 0.04% EDTA.

2.6.2. MTT Assay with Extracts of Spelt Grass Juice

Cell growth was assessed using the MTT assay [21]. Cell lines were seeded into 96-well microtiter plates (Sarstedt, Newton, MA, USA) at varying densities: 5×10^3 cells per well for MRC-5 and A-549, and 4×10^3 cells per well for HeLa in a volume of 180 µL. The cells were pre-incubated in complete medium supplemented with 5% FCS at 37 °C for 24 h.

For evaluation of antiproliferative activity, extracts of spelt grass juice were dissolved in DMSO and 0.9% NaCl. The final concentration of DMSO was under 0.2%, while the concentration of aqueous extracts of spelt grass juice ranged between 633.6 and 6700 µg/mL, and the concentration of acetone–water and methanol–water extracts 100–1000 µg/mL. Additionally, the antineoplastic drug doxorubicin for co-treatment was used at a final concentration of 2 µM. Microplates were incubated at 37 °C for an additional 48 h. Color development was measured using a Multiscan Ascent (Labsystems; Helsinki, Finland) photometer at 540 nm, with 620 nm as the background. Results of antiproliferative activity were expressed as the mean ± SD of two independent experiments, each performed in quadruplicate. All values presented in the figures and text represent the percentage of cell growth relative to the control, according to the following formula:

$$\text{Cell growth}(\%) = (\text{At}/\text{Ac}) \times 100, \quad (7)$$

where At represents the absorbance of the test sample and Ac represents the absorbance of the control.

Based on concentration–cell growth curves, IC50 values (the concentration that inhibits cell growth by 50%) were determined using CalcuSyn Version 1.1 (Mike Hayball Copyright Biosoft, 1996, St. Louis, MO, USA). Using IC50 values obtained from a non-tumor cell line and the respective tumor cell line, non-tumor/tumor IC50 ratios (NT/T) were calculated for the extract. The NT/T ratio (or selectivity index) indicates the selectivity of the tested samples towards tumor cells compared to a healthy cell line.

2.7. Statistical Analysis

Data were processed using the IBM SPSS Statistics 23 (Chicago, IL, USA). Descriptive analysis and one-way ANOVA with post hoc multiple range test (Duncan's test, significance level 0.05) was carried out.

3. Results and Discussion

3.1. Photosynthetic Pigments in Extracts

Various types of plant pigments are found in the photosynthetic apparatus as crucial components. By absorbing light of different wavelengths and allowing photosynthetic organisms to adapt permanently or temporarily to different environments, chlorophyll plays an important role in light harvesting. Among their diverse functions in photosynthesis, including light capture, carotenoids are essential antioxidants that lessen photodamage and photoinhibition [22].

In green plants, as the two main chemical structures of chlorophyll are Chl a and Chl b, typically in a 3:1.1 ratio. Chlorophyll has proven to be an anticancer agent through multiple biological activities like antigenotoxicity, the trapping of mutagens, antioxidant activities, apoptosis, and immunomodulation [23]. Some limitations of its pure natural form are related to its poor stabilization under physiological conditions, and its poor solubility in aqueous solutions, which reduces its accumulation in cancer cells [24].

The lyophilized samples of spelt grass juice (extracted with acetone–water) without foam had an average content of pigments as follows: 6.39 mg chlorophyll a/g DW, 2.47 mg chlorophyll b/g DW, and 1.81 mg carotenoids/g DW. Obviously, there were no significant differences between them and ones with foam: 6.91 mg chlorophyll a/g DW, 2.69 mg chlorophyll b/g DW, and 1.95 mg carotenoids/g DW (Figure 1, statistics not displayed). Methanol is known to be less efficient than acetone in the extraction of pigments. Consequently, the values for the mentioned pigments in the methanol–water extracts were about 5, 3, and 20 times lower than in to the acetone–water extracts, respectively.

Jančić et al. [2] chromatographically determined the content of photopigments in spelt grass extracts using pure acetone. The concentrations varied significantly, such as chlorophyll a 2.4–7.08 mg/100 g and chlorophyll b 8.55–28.1 mg/100 g of fresh spelt grass extract. These authors assume that significant amounts of chlorophyll a are converted into pheophytin a during the lyophilization process, for which they measured concentrations of 3.72–8.81 mg/100 g. Further, the sum of average values for carotenoids (α-carotene, β-carotene, lutein, and zeaxanthin) ranged from 2.45 to 3.08 mg/100 g. Our results showed a much higher content of chlorophyll a (about 40 mg/100 g FW) and carotenoids (more than 10 mg/100 g FW), while the content of chlorophyll b (about 15 mg/100 g FW) is in the range of values found in the study of Jančić et al. [2].

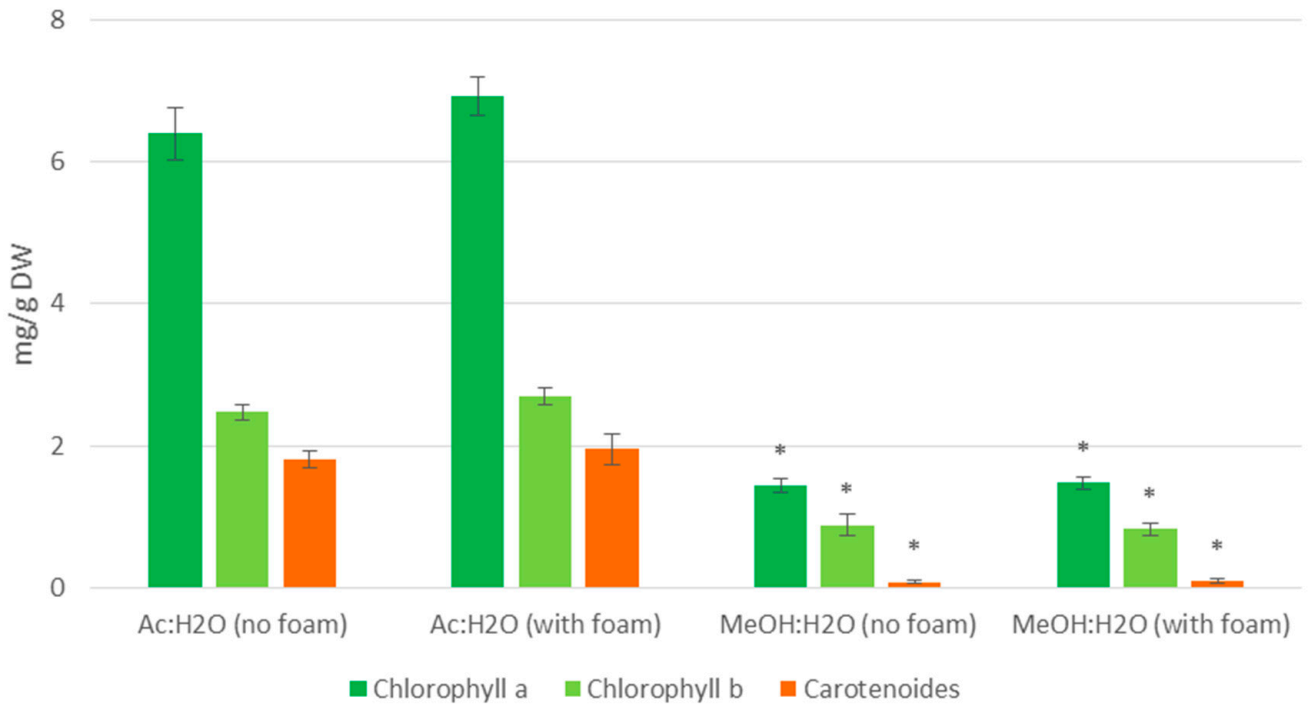


Figure 1. Photopigment content in extracts of lyophilized spelt grass juice with and without foam (Ac:H_2O no foam, Ac:H_2O with foam, MeOH:H_2O no foam, and MeOH:H_2O with foam), obtained using solvent–water (4:1) extraction. Statistically significant differences between pigment means, indicated by * above the bars, were determined using one-way ANOVA and Duncan's test at $p < 0.05$.

Skoczilas et al. [8] measured the content of total chlorophyll pigments in wheatgrass juice 481 mg/L for the summer and 270 mg/L for the winter period with a chlorophyll a/chlorophyll b ratio of 1:0.34 and 1:0.27, respectively. For carotenoids, the concentrations were 94.3 mg/L in summer and 59.4 mg/L in winter. In our experiment with young spelt grass grown under controlled conditions, the total chlorophyll content was about 550 mg/L with a chlorophyll-a-to-b ratio of 1:0.38, and the carotenoid content was about 110 mg/L, which could be considered quite similar to their results for summer wheatgrass juice. It is important to note that Skoczilas et al. [8] also used 80% acetone for the extraction, and a spectrophotometric method to determine the pigment concentration.

3.2. Phenolic Composition of Extracts

Phenolic compounds are strong natural antioxidants that are very important for the plant's defense system. Their content is influenced by the growing conditions, the degree of ripeness, and genetic variations. Table 1 shows the concentrations of phenolic compounds analyzed in spelt grass juice extracts. In general, almost no differences in phenolic composition were found within the same type of extracts (with or without foam).

Among the phenolics analyzed, the aqueous extracts of spelt grass juice showed the highest concentration of ferulic acid (more than 1100 µg/g DW) and its derivatives. The proportion of this hydroxycinnamic acid, not including its derivatives, amounted to one-third of the total phenolics analyzed. Protocatechuic acid, as the dominant hydroxybenzoic acid in the aqueous extract of spelt grass juice, was found in a concentration of about 150 µg/g DW.

Table 1. The content (μg/g DM) of phenolic compounds (mean ± SD) [1] in spelt grass juice extracts.

Phenolic Compound	Standard Compound	Phenolic Group	Spelt Grass Juice Extract					
			AQUEOUS (No Foam)	AQUEOUS (with Foam)	Ac:H_2O (No Foam)	Ac:H_2O (with Foam)	MeOH:H_2O (No Foam)	MeOH:H_2O (with Foam)
Protocatechuic acid	Gallic acid	HBA	151.93 ± 8.16 a	150.16 ± 9.80 a	12.50 ± 7.14 d	16.87 ± 6.88 d	57.07 ± 1.55 b	40.51 ± 2.36 c
Syringic acid	Gallic acid	HBA	92.23 ± 4.95 a	91.15 ± 5.95 a	7.59 ± 4.33 d	10.24 ± 4.18 d	34.64 ± 0.94 b	24.59 ± 1.43 c
Salicylic acid	Gallic acid	HBA	22.04 ± 1.18 a	21.78 ± 1.42 a	1.81 ± 1.04 d	2.45 ± 1.00 d	8.28 ± 0.22 b	5.88 ± 0.34 c
Ferulic acid derivative 1	Ferulic acid	HCA	204.18 ± 14.74 a	201.02 ± 4.45 a	98.14 ± 8.83 c	89.52 ± 14.24 c	128.85 ± 3.40 b	121.29 ± 1.95 b
Ferulic acid derivative 2	Ferulic acid	HCA	52.96 ± 3.72 c	48.92 ± 5.42 c	140.28 ± 7.64 a	137.88 ± 20.26 a	116.18 ± 2.79 b	110.01 ± 3.76 b
p-coumaric acid	*p*-coumaric acid	HCA	1.43 ± 0.11 a	1.44 ± 0.03 a	0.64 ± 0.03 b	0.68 ± 0.14 b	0.73 ± 0.01 b	0.73 ± 0.01 b
Vanillic acid	Gallic acid	HBA	0.66 ± 0.05 a	0.67 ± 0.02 a	0.30 ± 0.02 b	0.31 ± 0.06 b	0.34 ± 0.00 b	0.34 ± 0.01 b
Ferulic acid derivative 3	Ferulic acid	HCA	200.41 ± 15.57 a	202.24 ± 4.77 a	89.69 ± 4.62 b	95.03 ± 19.56 b	102.54 ± 1.47 b	102.27 ± 1.68 b
o-coumaric acid	*p*-coumaric acid	HCA	9.35 ± 2.11 c	7.62 ± 1.66 c	618.36 ± 20.19 a	595.05 ± 31.20 a	297.69 ± 5.17 b	277.02 ± 9.86 b
p-hydroxybenzoic acid	Gallic acid	HBA	71.78 ± 2.69 a	68.47 ± 4.07 a	40.78 ± 5.26 d	40.85 ± 4.32 d	61.39 ± 1.36 b	56.77 ± 0.66 c
Ferulic acid	Ferulic acid	HCA	1177.37 ± 44.12 a	1122.94 ± 66.78 a	668.80 ± 86.22 d	669.93 ± 70.86 d	1006.91 ± 22.38 b	931.04 ± 10.81 c
3-feruloylquinic acid	Ferulic acid	HCA	87.42 ± 6.20 a	100.78 ± 7.09 a	65.34 ± 7.06 b	66.61 ± 15.93 b	97.06 ± 12.06 a	92.82 ± 3.29 a
Unknown 385	Ferulic acid	HCA	195.24 ± 11.32	181.07 ± 6.58	200.27 ± 40.41	182.49 ± 50.41	204.08 ± 8.46	190.72 ± 12.21
Caffeic acid	Ferulic acid	HCA	59.95 ± 7.99 c	51.31 ± 2.14 cd	92.55 ± 6.83 b	114.99 ± 16.61 a	48.79 ± 1.38 cd	42.86 ± 5.71 d
Unknown 579	Ferulic acid	HCA	7.86 ± 1.70 c	8.05 ± 2.30 c	37.80 ± 1.95 ab	27.08 ± 16.80 b	50.44 ± 3.74 a	47.07 ± 5.76 a
Unknown 529	Ferulic acid	HCA	165.95 ± 8.93 a	152.74 ± 10.95 a	75.34 ± 3.06 b	64.73 ± 12.75 b	166.74 ± 11.54 a	161.41 ± 5.23 a

Table 1. *Cont.*

Phenolic Compound	Standard Compound	Phenolic Group	Spelt Grass Juice Extract					
			AQUEOUS (No Foam)	AQUEOUS (with Foam)	Ac:H_2O (No Foam)	Ac:H_2O (with Foam)	MeOH:H_2O (No Foam)	MeOH:H_2O (with Foam)
Unknown 563	Ferulic acid	HCA	741.56 ± 38.55 b	783.23 ± 29.76 b	357.67 ± 12.69 c	396.78 ± 7.59 c	865.11 ± 22.77 a	902.55 ± 19.12 a
Unknown 371	Ferulic acid	HCA	134.93 ± 7.01 b	142.52 ± 5.42 b	65.08 ± 2.31 c	72.20 ± 1.38 c	157.41 ± 4.14 a	164.23 ± 3.48 a
4-feruloylquinic acid	Ferulic acid	HCA	3.70 ± 0.19 b	3.90 ± 0.15 b	1.78 ± 0.06 c	1.98 ± 0.04 c	4.31 ± 0.11 a	4.50 ± 0.10 a
Unknown 368	Ferulic acid	HCA	18.55 ± 1.72 c	20.13 ± 3.41 c	25.95 ± 0.55 b	29.34 ± 1.31 b	56.51 ± 1.80 a	59.95 ± 3.62 a
Unknown 469	Ferulic acid	HB	76.63 ± 2.41 b	80.43 ± 1.58 a	36.90 ± 2.94 e	34.32 ± 0.75 e	64.36 ± 1.53 c	60.16 ± 0.65 d
Sinapic acid	Caffeic acid	HB	23.29 ± 3.44 c	25.05 ± 3.54 c	47.00 ± 3.60 b	50.46 ± 3.33 b	71.20 ± 4.19 a	67.49 ± 18.45 a
Sum of total analyzed phenolics			3499 ± 177 a	3466 ± 166 a	2685 ± 182 b	2700 ± 263 b	3601 ± 83 a	3464 ± 80 a

[1] The significant differences between the means (labeled with different letters in rows) were determined with one-way ANOVA and Duncan's test at $p < 0.05$.

Eissa et al. [10] found that sinapic (27.98 μg/mL), protocatechuic (22.34 μg/mL), caffeic (12.04 μg/mL), and rosmarinic (11.32 μg/mL) acids were present in the highest concentrations in fresh wheatgrass juice. Differently from these authors, Hebbani et al. [9] identified pyrogallol, syringic acid, vanillic acid, and ferulic acid as the dominant phenolics. Skoczylas et al. [8] analyzed the phenolic profile of frozen wheatgrass juice, with catechin (341.1 μg/mL) and ferulic acid (92.8 μg/mL) being the dominant phenolics. Compared to our results (converted in same units: sinapic acid—1.59 μg/mL, protocatechuic acid—9.93 μg/mL, caffeic acid—3.65 μg/mL, syringic acid—6.03 μg/mL, vanillic acid—0.04 μg/mL, and ferulic acid—75.56 μg/mL), wheatgrass juice is considerably richer in these compounds.

Previous studies on the phenolic profile of wheat and spelt grains have shown that the phenolic acids are strongly bound to the matrix and are effectively released during hydrolysis. The most common phenolic acids found in whole grains were as follows: ferulic acid (as predominant), vanillic acid, caffeic acid, syringic acid, and *p*-coumaric acid [25,26]. Gawlik-Dziki et al. [26] found a significantly different content of ferulic acids in grains of spelt cultivars, with the highest value around 700 μg/g DW. Overall, phenolic acids protect wheat grains by providing both physical and chemical barriers through carbohydrate cross-linking, antioxidant activity to combat destructive radicals, and astringency to prevent consumption by insects and animals. Baranski et al. [27] investigated older wheat species and found that the content of almost all free and bound phenolic acids was significantly higher in the einkorn (*Triticum monococcum* L.) than in emmer (*Triticum dicoccon* Schrank) and spelt (*Triticum spelta* L.) wheat species. In whole spelt grain, the highest concentration of protocatechuic acid (in free phenolic fraction) was almost 90 μg/g DW [26].

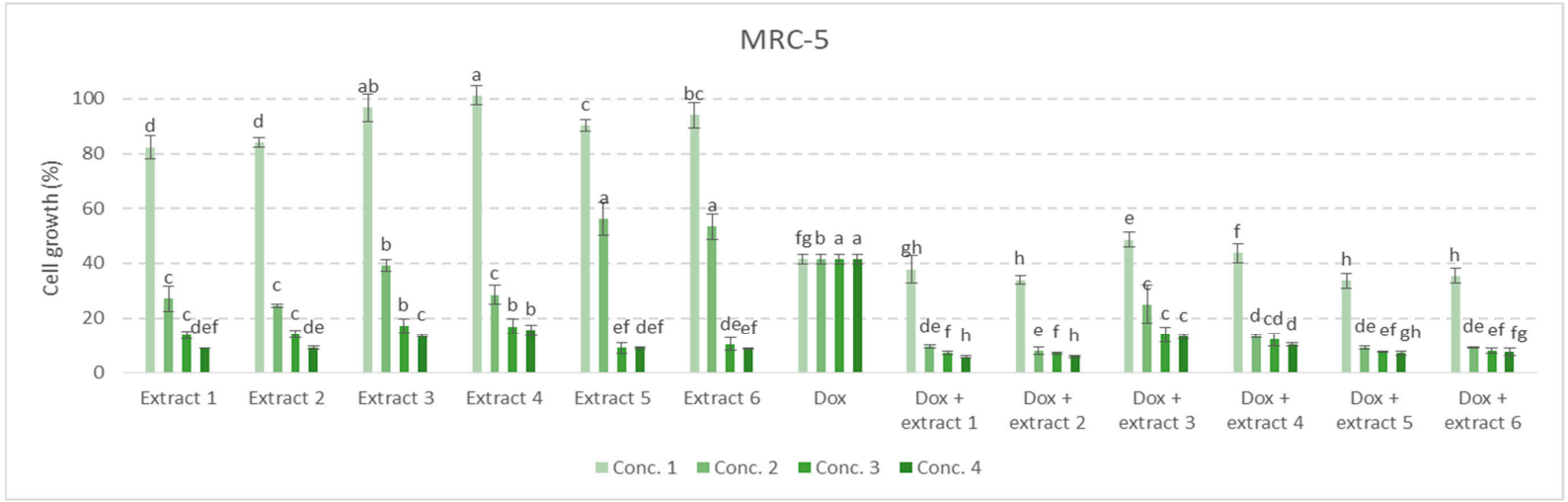
The phenolic compounds were analyzed in both acetone–water and methanol–water extracts, and the methanol–water extract was similar to the aqueous extract of spelt juice regarding total phenolics with values in the range of 3464–3601 μg/g DW. Considering the individual phenolic compounds, the concentration of 14 compounds was lower and that of 8 compounds higher compared to the aqueous extract of spelt grass juice. The difference in the content of *o*-coumaric acid was especially high. The concentration in the methanol–water extract was about 30 times higher than in the aqueous extract. This is to be expected, due to the low solubility of *o*-coumaric acid in water. In the acetone extract, the content of total phenolics (around 2700 μg/g DW) was significantly lower compared to the other extracts, as was the case for almost all compounds except *o*-coumaric acid and the ferulic acid derivative. The content of *o*-coumaric acid was even 60 times higher compared to aqueous extract of spelt grass juice.

3.3. Antiproliferative Activity of Extracts

The antiproliferative effects of six whole extracts derived from spelt grass juice were evaluated on three distinct cell lines. This study aimed to assess the influence of the extract type and solvent choice on their antiproliferative properties as well as the selectivity of the extracts towards two malignant cell lines, and their potential to enhance the efficacy of doxorubicin in co-treatment.

All tested spelt grass juice extracts, irrespective of the presence of foam, exhibited a concentration-dependent antiproliferative effect (Figure 2). Co-treatment with doxorubicin led to an enhanced inhibitory response across all examined cell lines. The presence of foam did not induce significant changes in the observed effects.

(**a**)



(**b**)

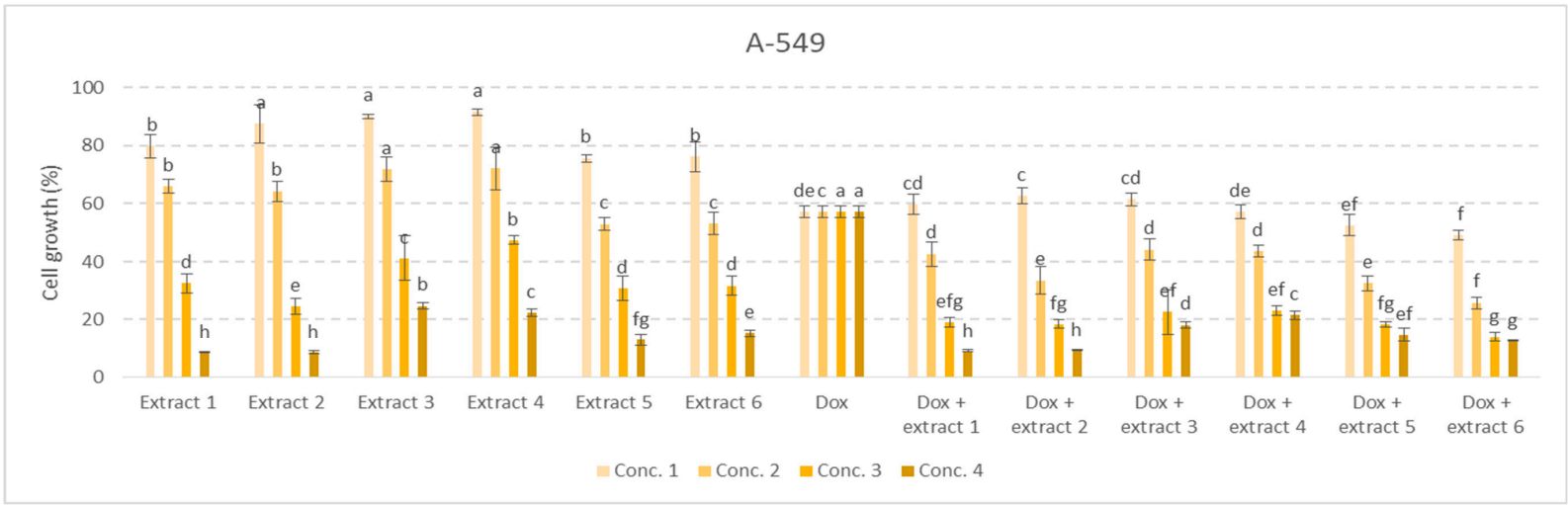


Figure 2. *Cont*.

(**c**)

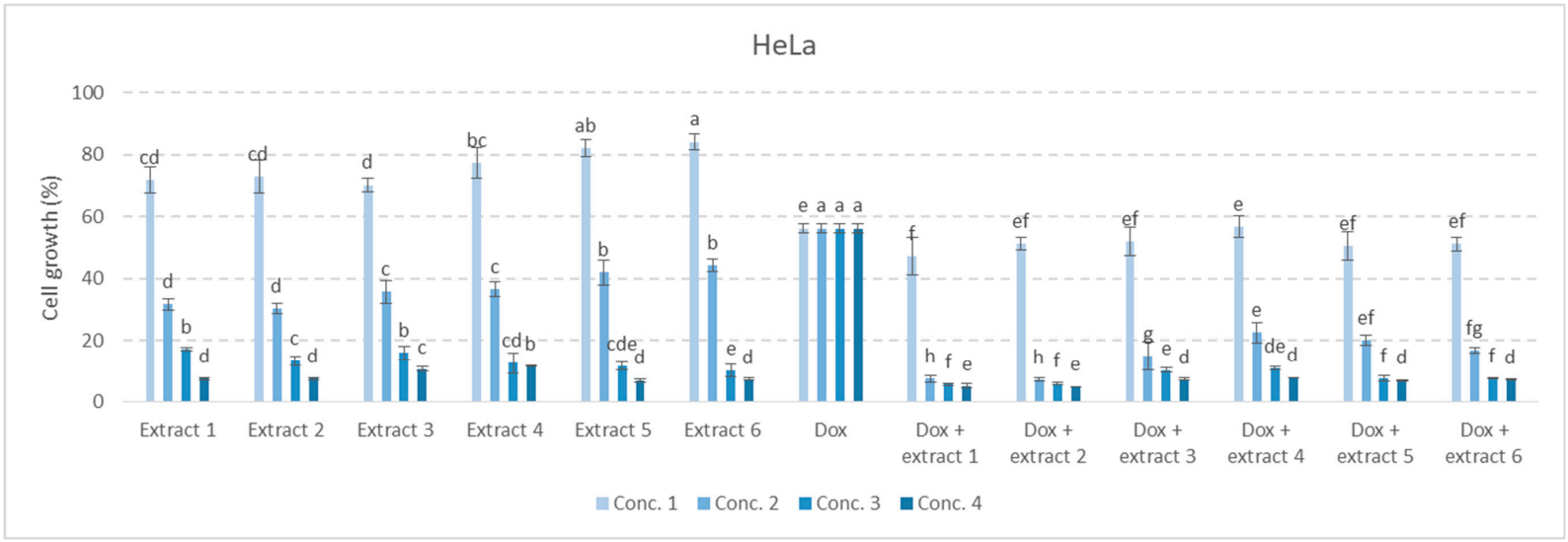


Figure 2. Antiproliferative effect of spelt grass juice extracts on cell growth after 48 h incubation at different concentrations, individually and in combination with 2 µm doxorubicin. Cell lines tested: (**a**) MRC-5—non-transformed cells; (**b**) A-549 lung cancer cells; and (**c**) HeLa—cervix cancer cells. Extracts: Extract 1—Aqueous extract of spelt grass juice without foam; Extract 2—Aqueous extract of spelt grass juice with foam; Extract 3—Ac:H_2O (4:1) extract of lyophilized spelt grass juice without foam; Extract 4—Ac:H_2O (4:1) extract of lyophilized spelt grass juice with foam; Extract 5—MeOH:H_2O (4:1) extract of lyophilized spelt grass juice without foam; and Extract 6—MeOH:H_2O (4:1) extract of lyophilized spelt grass juice with foam, and their combination with Dox (doxorubicin). Concentrations tested: Conc. 1—633.6 µg/mL (Extract 1), 663.3 µg/mL (Extract 2), and 100 µg/mL (Extracts 3–6); Conc. 2—1920 µg/mL (Extract 1), 2010 µg/mL (Extract 2), and 300 µg/mL (Extracts 3–6); Conc. 3—3200 µg/mL (Extract 1), 3350 µg/mL (Extract 2), and 500 µg/mL (Extracts 3–6); and Conc. 4—6400 µg/mL (Extract 1), 6700 µg/mL (Extract 2), and 1000 µg/mL (Extracts 3–6). All results are expressed as percentages of cell growth relative to the untreated control groups. Statistically significant differences between means at each concentration, indicated by different letters above bars, were determined using one-way ANOVA and Duncan's test at $p < 0.05$.

Solvent selection, determined by variations in polarity and solubility, significantly influenced the extraction efficiency and the phytochemical composition of the obtained extracts. Among the tested extracts, the methanol–water fraction demonstrated the most potent inhibitory effect on MRC-5 normal fibroblasts and A-549 lung cancer cells, whereas the acetone–water extract exhibited the highest antiproliferative activity against HeLa cervical carcinoma cells, likely due to its ability to extract both polar and non-polar bioactive compound. In contrast, the aqueous extract consistently showed the weakest inhibitory effects across all tested cell lines (Table 2). These findings underscore the importance of solvent selection in the extraction process, as it significantly influences the phytochemical composition and, consequently, the biological activity of the extracts.

Table 2. IC50 values for various spelt grass juice extract (μg/mL) and their selectivity towards tumor cells (NT/T).

Spelt Grass Juice Extract		IC50			NT/T	
		MRC-5	**A-549**	**HeLa**	**A-549**	**HeLa**
Extract 1	Aqueous (no foam)	1319.30	1887.50	1162.10	0.70	1.14
Extract 2	Aqueous (with foam)	1405.90	2060.12	1184.97	0.68	1.19
Extract 3	Ac:H_2O (no foam)	331.07	530.68	182.05	0.62	1.82
Extract 4	Ac:H_2O (with foam)	416.84	529.20	203.07	0.79	2.05
Extract 5	MeOH:H_2O (no foam)	271.96	453.22	221.82	0.60	1.23
Extract 6	MeOH:H_2O (with foam)	293.13	411.13	228.89	0.71	1.28

Regarding the IC50 values for all types of extracts, cervical cancer cells were more susceptible compared to lung cancer cells. The higher efficacy towards cervical cancer cells was observed for the acetone–water and methanol–water extracts compared to aqueous ones. Our results align with previous studies [5,17] that demonstrated the antiproliferative properties of *Triticum aestivum* extracts on the HeLa cell line. Patel et al. [5] showed that the methanolic leaf extract of *Triticum aestivum* exhibited moderate anticancer activity with an IC50 value of 156 μg/mL against HeLa cervical carcinoma cells. The apoptosis induced by the *Triticum aestivum* extract was correlated with high free radical scavenging activity, supporting the role of antioxidants in reducing cancer cell viability [12]. Namely, as stated by these authors, an analysis of wheatgrass extract revealed the presence of biologically active compounds containing hydroxyl groups and double bonds, which help stabilize free radicals and, thus, exhibit antioxidant properties. Reactive oxygen species, or free radicals, are converted into hydrogen peroxide and an oxygen molecule by the antioxidant enzymes superoxide dismutase and cytochrome oxidase. This transformation leads to the destruction of cancer cells. Furthermore, Jančić et al. [2] demonstrated the antioxidant activity of spelt grass juice samples obtained from the same geographic region and produced by the same local manufacturer in Montenegro as the samples analyzed in our study. Their findings, based on FRAP, DPPH, and ABTS assays, revealed a positive correlation between antioxidant activity and the phenolic and flavonoid content of the juice. The antioxidant-dependent antiproliferative properties of *Triticum spelta* could be significantly attributed to the presence of phenolic compounds and carotenoids, as well as their synergistic interactions [3,28]. Numerous studies have highlighted the anticancer potential of ferulic acid, which is predominant in spelt grass juice extracts [29]. Phenolic acids, including *p*-coumaric acid, ferulic acid, and sinapic acid, have been shown to induce the dose-dependent inhibition of proliferation, concentration-dependent apoptosis, and HDAC inhibition in HeLa cells, with *p*-coumaric and ferulic acid specifically causing cell

cycle arrest at the S-phase [30]. Additionally, carotenoids, predominantly α- and β-carotene, contribute to the strong antioxidant capacity of *Triticum aestivum* by scavenging free radicals and inhibiting reactive oxygen species (ROS), potentially enhancing its antiproliferative effects on cancer cells through synergistic interactions with other bioactive compounds [28]. Given the previously mentioned results, our research further confirms the relationship between the presence of antioxidant compounds and antiproliferative effects.

The NT/T ratio indicates greater drug efficacy against tumor cells than toxicity to normal cells when exceeding 1.0 [31]. In our experiment, all six extracts showed selectivity toward HeLa cells, particularly acetone–water extracts, but not A-549 cells. The presence of photopigments such as chlorophyll and carotenoids (Figure 1) may contribute to this effect, as chlorophyll induces phase 2 detoxification enzymes and enhances oxygen delivery, which can be detrimental to oxygen-sensitive cancer cells [7]. Carotenoids, through their antioxidant properties, may further suppress tumor growth or trigger apoptosis [32]. Additionally, HeLa cells exhibit a high expression of proliferation-associated receptors, potentially increasing their susceptibility to the proapoptotic effects of carotenoids and phenolics, with *p*-coumaric and ferulic acid inducing S-phase arrest [30], further supporting the selective effect of the extract.

Beyond their general cytotoxic effects, natural bioactive compounds can modulate key signaling pathways and epigenetic mechanisms in cervical cancer cells, potentially explaining the selectivity of spelt extracts. Carotenoids like fucoxanthin and polyphenols such as epigallocatechin gallate (EGCG) have been shown to inhibit cervical cancer cell proliferation by downregulating oncogenic genes, promoting apoptosis, and inducing cell cycle arrest [33,34]. The PI3K/Akt/mTOR, MAPK/ERK, and NF-κB pathways, critical for tumor survival and proliferation, are known targets of dietary phytochemicals in HeLa cells. EGCG, for instance, inhibits PI3K/Akt signaling by downregulating PIK3CA and PIK3CB while upregulating PTEN, suppresses MAPK-mediated proliferation, and reduces NF-κB-driven inflammation by lowering IL1A, IL2, and IL6 expression [34]. Given that epigenetic changes are reversible, targeting them with bioactive compounds represents a promising therapeutic approach. The selective antiproliferative effects of spelt extracts on HeLa cells may be linked to their ability to modulate these oncogenic pathways, warranting further studies to elucidate their molecular mechanisms and potential therapeutic applications.

It is well-established that combining natural product extracts with antineoplastic drugs can enhance antitumor effects in cancer cell lines [13,16]. In our study, all tested *Triticum spelta* extracts in co-treatment with doxorubicin enhanced antiproliferative effects on HeLa and A549 cancer cells, aligning with the previously reported findings. While polyphenols contribute to these effects through direct antiproliferative and pro-apoptotic mechanisms, photopigments, including chlorophyll and carotenoids, may also play a crucial role. Chlorophyll has been shown to enhance chemotherapy efficacy by modulating oxidative stress, inducing phase 2 detoxification enzymes, and increasing cellular oxygenation, which may sensitize tumor cells to treatment [7,35]. Similarly, carotenoids exhibit antioxidant properties that can influence apoptotic pathways and enhance chemotherapy-induced cytotoxicity [32,36]. The interaction between these bioactive compounds and doxorubicin may contribute to improved tumor suppression by influencing cell cycle regulation, apoptosis, and the oxidative stress balance. Additionally, in vivo studies suggest that combining phytochemicals with chemotherapeutics can reduce angiogenesis, increase tumor necrosis, and lower blood vessel density, although further clinical validation is needed [16].

Our findings demonstrate that specific *Triticum spelta* extracts exert selective antiproliferative effects on cancer cell lines, suggesting their potential application in the formulation of functional foods or nutraceutical supplements with antitumor properties. Given that spelt is already a part of the human diet, its additional potential lies in the development

of enriched food products that could provide protective effects against malignancies. Future studies aimed at elucidating the molecular mechanisms activated by *Triticum spelta* extracts, as well as the characterization of their bioactive components, would enable their isolation and evaluation in clinical trials. By doing so, these findings could contribute to the development of dietary strategies for cancer risk reduction and establish a foundation for future therapeutic or preventive applications.

4. Conclusions

Based on the determination method, the total chlorophyll content, chlorophyll-a-to-b ratio, and total carotenoids in spelt grass juice were consistent with the literature data for summer wheatgrass juice. The aqueous extracts of spelt grass juice had the highest concentration of ferulic acid and its derivatives, with ferulic acid accounting for a third of the total phenolics. The methanol–water extracts were similar to the aqueous extracts of spelt juice regarding total phenolics, with its content being significantly lower in the acetone–water extracts.

The antiproliferative effect of *Triticum spelta* extracts was moderate, with solvent selection playing a crucial role in determining their efficacy. The acetone–water extract exhibited the highest selectivity toward HeLa cells, likely due to its ability to extract both phenolics and carotenoids. Co-treatment with doxorubicin further enhanced the cytotoxic effects, suggesting a synergistic interaction between phenolics, photopigments, and the chemotherapeutic agent. The presence of foam in spelt grass juice did not significantly influence the antiproliferative activity, likely due to its relatively low quantity.

These findings highlight the importance of the phytochemical composition in modulating antiproliferative activity and suggest the potential of *Triticum spelta* extracts for further exploration in cancer therapy.

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