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Phytochemical Profiling, Antioxidant Activity, and Protective Effect against H_2O_2-Induced Oxidative Stress of *Carlina vulgaris* Extract

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Abstract: *Carlina vulgaris* is a little-understood plant with unexplored biological potential, and the papers regarding its chemical composition are scarce. In our study, for the first time, the phytochemical profile of the plant, focusing on polar metabolites, was established using modern chromatographic techniques including LC-HRMS-QTOF-CAD, UHPLC-PDA-MS. Phytochemical analysis revealed that the species is a rich source of polyphenolic components, with the most abundant being chlorogenic acid and C-glycosides of luteolin, including carlinoside, orientin, isoorientin, and C-glycosides of apigenin, schaftoside, isoschaftoside, and vitexin. Furthermore, we assessed the impact of the polyphenolic-rich fraction of *C. vulgaris* extracts on human skin fibroblasts using the MTT and NR assays. It was found that the extract was non-toxic and exhibited potent antioxidant activity in the cells subjected to induced oxidative stress. Additionally, it effectively protected the cells against H_2O_2-induced cytotoxicity. Our study contributes to the general trend of searching for new phytotherapeutics with potential applications in pharmacy and medicine. The results indicate that further exploration of *C. vulgaris* species is worthwhile, as they can serve as valuable plant material for cosmetic use.

Keywords: polyphenols; flavonoid C-glycosides; antioxidant; H_2O_2-induced stress; human skin fibroblast

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1. Introduction

The genus *Carlina*, belonging to the Asteraceae family, comprises over 30 species that can be found in Europe and Asia in their natural environment. Plants from the genus *Carlina* have been widely used in traditional medicine in many countries, including Spain, Italy, Hungary, Lithuania, Poland, and the Balkan countries, due to their high potential for medicinal purposes [1–8]. They have been applied to gastrointestinal dysfunctions such as gastritis and dyspepsia and as a cholagogic agent [2,3]. Furthermore, extracts from the herbs have been found to be useful in facilitating the healing of skin lesions, wounds, ulcerations, skin infections, rough skin, and swellings [1,9–11].

However, to date, most species from the genus are poorly understood in terms of both biological activity and phytochemistry. Currently, the most knowledge is available about *C. acaulis*, which is the subject of intensive research. Many scientific reports have confirmed its multidirectional biological activity, including antioxidant, antibacterial, antifungal, insecticidal, and anti-ulcer properties and cytotoxicity against a few types of cancer lines [12–21]. The plant has also been shown to be a rich source of valuable components including volatile compounds of essential oil, phenolic acids, and pentacyclic triterpenes [22–25]. These findings justify the traditional usage of *Carlina* species and demonstrate the enormous potential of the genus.

C. vulgaris L. is one of the less studied species from the *Carlina* genus. However, due to its taxonomic affinity with *C. acaulis*, it could potentially serve as another source of metabolites with high biological activity. It is a monocarpic perennial plant widespread in central and western Europe, from the southern regions of the Iberian and Apennine Peninsulas to southern Sweden. It primarily colonizes calcareous grasslands [26]. The plant produces a stiff, erect, top-branching stem that can reach a height of up to 80 cm. The leaves are lanceolate, serrated, and strongly spiny. The inflorescence consists of one or several baskets, measuring 3.5–5 cm in diameter (Figure 1).

Figure 1. An example of *C. vulgaris* growing in its natural habitat.

Despite a relatively detailed botanical description [27], there are limited available data on the phytochemistry of this plant. Strzemski et al. discovered pentacyclic triterpenes, including oleanolic and ursolic acid, lupeol, lupeol acetate, α and ß amyrins, and ß amyrin acetate in the inflorescences, green parts, and roots of *C. vulgaris*. [23]. Furthermore, both the aboveground parts and roots of *C. vulgaris* plants were found to be a rich source of chlorogenic acid, with the highest content observed in the leaves (approximately 3 mg/g) [28]. Polyacetylenes, with the most abundant carlina oxide (33.7%) and 13-methoxy carlina oxide (11.5%), were identified as the main components of the root essential oil from *C. vulgaris* [29].

The knowledge regarding the biological properties of *C. vulgaris* is also scarce. It has been found that the plant extracts display a free radical scavenging effect in ABTS and DPPH tests [28], and the essential oil shows antioxidant and antifungal activity against *Penicillium expansum* and *Aspergillus niger* [29]. Additionally, carlina oxide found in the oil exhibited antibacterial and antifungal activity against various strains of bacteria and fungi. It also demonstrated an antiparasitic effect against *Trypanosoma brucei* [30].

The gathered information indicates that it is worthwhile to investigate *C. vulgaris* in more detail. Therefore, the aim of our research was to address this issue and expand our understanding of this species. The main goal was to establish the phytochemical profile of the plant, focusing on polar metabolites, using modern chromatographic techniques including

liquid chromatography with a quadrupole-time-of-flight high-resolution mass spectrometer (LC-HRMS-QTOF), a charged aerosol detector (CAD), and ultra-performance liquid chromatography (UHPLC) coupled to photodiode array detection (PDA) and mass spectrometry (MS). Furthermore, the antioxidant potential and protective effect against H_2O_2-induced oxidative stress were investigated in human skin fibroblasts (HSF), which could provide an explanation for the traditional application of *Carlina* plants in skin disorders.

2. Results

2.1. Plant Material, Phytochemical Profiling and Quantification of the Components

A total of 1.2 kg of the aboveground parts of the plants was obtained from field cultivation of *C. vulgaris*, which yielded 312 g of lyophilized material. A total of 57 g of dried extract was obtained after exhaustive extraction of 300 g of freeze-dried samples with 70% methanol (the methanolic extract of *C. vulgaris*—ECV).

The phytochemical composition of the methanolic/water extract from the aboveground parts of *C. vulgaris* was determined using UHPLC-MS. The components were analyzed in negative and positive ionization modes and identified based on mass data (M-H), fragmentation patterns, and UV-Vis spectra (200–600 nm). Representative chromatograms are shown in Figure 2.

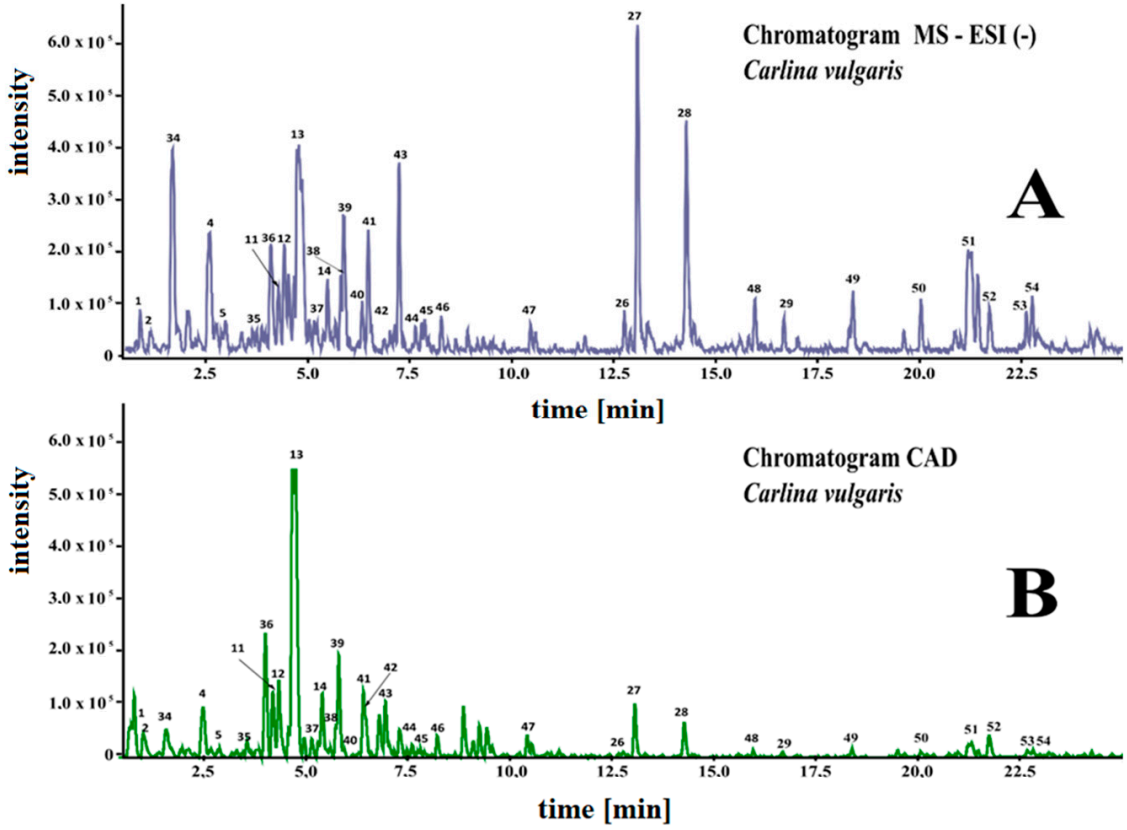


Figure 2. Chromatograms of extracts from *C. vulgaris* obtained using liquid chromatography with a quadrupole-time-of-flight high-resolution mass spectrometer (LC-HRMS-QTOF) and a charged aerosol detector (CAD). (**A**)—MS chromatogram (electrospray ionization—ESI); (**B**)—CAD chromatogram.

The results of qualitative analysis and quantification, expressed per gram of dried plant material, are shown in Table 1.

Table 1. Compounds found in the extract of aerial parts of *C. vulgaris*. Amount was expressed in milligrams per gram of dry weight (mg/g d.w).

nr	RT (min)	M/Z	MS2	Ion Formula [M/Z-H]	Δppm	Identified	Amount (mg/g d.w)
1	0.8	191.055878	191, 135	$C_7H_{11}O_6$	1.2	quinic acid	0.23 ± 0.01
2	1.3	153.019395	153, 109	$C_7H_5O_4$	−0.4	di-hydroxybenzoic acid	0.09 ± 0.01
34	2.0	203.082571	203, 116	$C_{11}H_{11}N_2O_2$	0.2	L-tryptophan	
4	2.5	353.087769	353, 351, 191, 133	$C_{16}H_{17}O_9$	0.1	neochorogenic acid	1.02 ± 0.01
5	2.6	353.087764	353, 191	$C_{16}H_{17}O_9$	0.1	chlorogenic acid	6.90 ± 0.01
35	2.9	215.082690	215, 171, 142, 116	$C_{12}H_{11}N_2O_2$	−0.4	methyltryptophan	
36	4.0	579.136415	579, 489, 399, 369	$C_{26}H_{27}O_{15}$	−1.5	carlinoside	3.10 ± 0.01
11	4.4	563.141367	563, 473, 443, 383, 353	$C_{26}H_{27}O_{14}$	−1.3	schaftoside	1.59 ± 0.01
12	4.6	447.094105	447, 429, 357, 327, 297	$C_{21}H_{19}O_{11}$	−1.8	orientin	1.86 ± 0.02
13	4.7	563.141444	563, 503, 473, 443, 383, 353	$C_{26}H_{27}O_{14}$	−1.4	isoschaftoside	3.88 ± 0.01
37	5.1	303.051651	303, 285, 217, 125	$C_{15}H_{11}O_7$	−2.1	taxifolin	0.06 ± 0.01
14	5.2	563.141926	563, 503, 473, 443, 383, 353	$C_{26}H_{27}O_{14}$	−2.3	isoorientin	1.28 ± 0.01
38	5.4	609.147050	609, 300	$C_{27}H_{29}O_{16}$	−1.5	rutin	1.30 ± 0.01
39	5.6	431.099416	431, 341, 311, 283	$C_{21}H_{19}O_{10}$	−2.4	vitexin	4.02 ± 0.01
40	5.9	533.131166	533, 515, 473, 443, 383, 353	$C_{25}H_{25}O_{13}$	−2.1	apigenin di-C arabinoside	0.05 ± 0.01
41	6.3	385.115218	385, 207, 177, 129	$C_{17}H_{21}O_{10}$	−3.1	densifloside	4.98 ± 0.01
42	6.4	593.152523	593, 285	$C_{27}H_{29}O_{15}$	−2.2	nicotiflorin	0.99 ± 0.01
43	7.2	187.098392	187, 169, 125	$C_9H_{15}O_4$	−4.3	azelaic acid	
44	7.7	243.124940	243, 225, 199, 181, 163	$C_{12}H_{19}O_5$	−4.7	4-oxododecaneoic acid	
45	7.8	340.095398	340, 296, 257, 241, 210	$C_{17}H_{14}N_3O_5$	−4.4	unknown	
46	8.2	551.215501	551, 341, 329, 205	$C_{28}H_{31}N4O_8$	−1.4	unknown	
47	10.4	609.293709	609, 565, 463, 301, 113	$C_{31}H_{45}O_{12}$	−3.4	unknown	
26	12.7	227.128985	227, 183, 165	$C_{12}H_{19}O_4$	−0.5	traumatic acid	3.19 ± 0.01
27	13.0	327.217951	327, 211, 171	$C_{18}H_{31}O_5$	−0.8	9,10-dihydroxy-8-oxooctadec-12-enoic acid	0.07 ± 0.01
28	14.2	329.233675	329, 229, 211, 171	$C_{18}H_{33}O_5$	−1.0	pinellic acid	0.06 ± 0.01
48	15.9	307.191932	307, 235, 211, 185, 121	$C_{18}H_{27}O_4$	−1.5	linoleic acid derivat.	
29	16.6	311.187090	311, 293, 267	$C_{17}H_{27}O_5$	−2.2	octadecdienoic acid derivat.	
49	18.3	311.223767	311, 293, 211	$C_{18}H_{31}O_4$	−3.2	9(S)-HPODE	
50	20.0	313.239690	313, 295, 201	$C_{18}H_{33}O_4$	−4.0	9,10-DHOME	
51	21.1	293.213413	293, 275, 235, 183	$C_{18}H_{29}O_3$	−4.1	linoleic acid derivat.	
52	21.3	293.213318	293, 275, 223, 195	$C_{18}H_{29}O_3$	−3.7	linoleic acid derivat.	
53	22.5	295.228378	295, 277, 195	$C_{18}H_{31}O_3$	−1.7	linoleic acid derivat.	
54	22.7	295.228280	295, 277, 171	$C_{18}H_{31}O_3$	−1.4	linoleic acid derivat.	

Compounds were identified based on: Compound Crawler Bruker, Sirius 4.0.1. and confirmed by standards when available. RT—retention time, M/Z—mass to charge ratio.

A total of fifteen different phenolic compounds were identified in the *C. vulgaris* extract. Among the phenolic acids, chlorogenic acids, including 3-caffeoquinic and 5-caffeoquinic (neochlorogenic), were the most abundant, with contents of 6.90 and 1.02 mg/g of dry weight, respectively. This was followed by densifloside (4.98 mg/g) and a low amount of dihydroxybenzoic acid (0.09 mg/g). A total of 0.23 mg/g of quinic acid (cyclohexanecarboxylic acid) was also found in the aboveground part of *C. vulgaris*. Flavonoids were mainly represented by C-glycosides of luteolin and apigenin. Vitexin (apigenin-8-C-glucoside), isoschaftoside (apigenin-6-C-arabinoside-8-C-glucoside), and carlinoside (luteolin 6-C-glucoside-8-C-arabinoside) were detected at the highest amounts with mean contents of 4.0 mg/g, 3.9 mg/g, and 3.1 mg/g, respectively. Orientin (luteolin-8-C-glucoside), schaftoside (Apigenin-6-C-glucoside-8-C-arabinoside), and isoorientin (luteolin-6-C-glucoside) were present in amounts ranging from 1.3 mg/g to 1.9 mg/g. Additionally, a small quantity (0.05 mg/g) of apigenin di-C-arabinoside was identified. Rutin (quercetin-3-O-rutinoside,1.30 mg/g) and taxifolin (dihydroquercetin, 0.06 mg/g) were also found in the extract. Furthermore, several lipidic constituents including linoleic and octadecenoic acid

derivatives and two amino acids (L-tryptophan and methyltryptophan) were present in methanol/water extract from the plant.

2.2. Fractionation and Phytochemical Characterization of the Fractions

The methanolic extract was fractionated using solvents with different polarity to obtain a polyphenolic-rich fraction. The yields of extracts obtained from subsequent fractionation of ECV with hexane (HCV), ethyl acetate (EaCV), and n-butanol (BCV) were 1.55 g, 0.7 g, and 1.45 g, respectively. The remaining 53.3 g of solid residues were dissolved in water (H_2OCV). The UHPLC-MS and PDA chromatograms of the fractions are shown in the Figure 3, and the results of quantitative analysis of the components in the fractions, expressed per gram of dried extract, are summarized in Table 2.

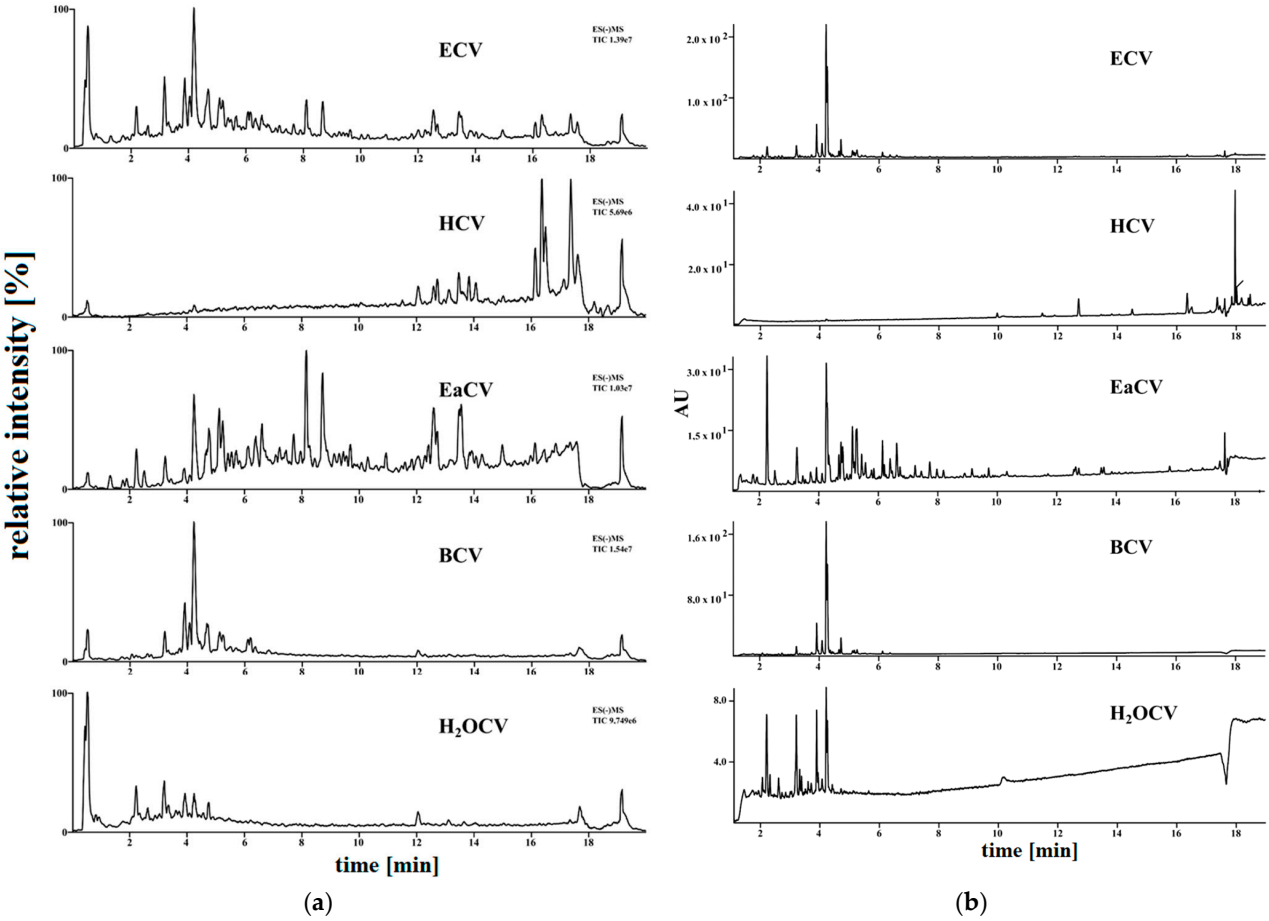


Figure 3. The chromatograms obtained from ultra-performance liquid chromatography with mass spectrometry and electrospray ionization UHPLC-ESI-MS(−) chromatograms (**a**) and the UHPLC with photodiode detector—PDA (254 nm) (**b**). Fractions obtained through liquid–liquid extraction from the extract of *C. vulgaris*. ECV—methanol extract, HCV—hexane fraction, EaCV—acetate fraction, BCV—butanol fraction, H_2OCV—water fraction.

The results of the chromatographic analysis showed that the ethyl acetate fraction (EaCV) contained the highest content of chlorogenic acids (181 mg/g of dried fraction), densifloside (113.73 mg/g), C-glycosides of apigenin (174.7 mg/g), followed by C-glycosides of luteolin (57.7 mg/g), and rutin (23.9 mg/g). The butanol fraction (BCV) contained the highest content of orientin and isoorientin (57.5 mg/g), a low amount of densifloside (7.9 mg/g), and dihydroxybenzoic acid (5.8 mg/g), as well as trace amounts of polyphenolic compounds. No phenolic constituents were identified in HCV, which is not surprising because they are polar compounds poorly dissolved in nonpolar solvents such as hexane.

Table 2. The compounds identified in individual fractions obtained from *C. vulgaris* methanol/water extract (mg/g ± SD of fraction).

Nr	Compounds	HCV	EACV	BCV	H_2OCV
1	quinic acid	ND	ND	ND	17.63 ± 0.11
2	dihydroxybenzoic acid	ND	ND	5.8 ± 0.12	1.1 ± 0.02
34	L-typtophan	ND	ND	ND	+
4	neochorogenic acid	ND	23.20 ± 0.12	0.09 ± 0.01	0.06 ± 0.01
5	chlorogenic acid	ND	157.69 ± 0.12	1.32 ± 0.18	0.12 ± 0.01
35	methyltryptophan	ND	ND	ND	+
36	carlinoside	ND	57.71 ± 0.23	1.92 ± 0.12	ND
11	schaftoside	ND	29.07 ± 0.02	1.01 ± 0.10	ND
12	orientin	ND	ND	35.03 ± 0.03	ND
13	isoschaftoside	ND	71.46 ± 0.02	1.90 ± 0.12	ND
37	taxifolin	ND	1.17 ± 0.11	0.42 ± 0.06	ND
14	isoorientin	ND	ND	22.45 ± 0.12	ND
38	rutin	ND	23.88 ± 0.11	1.65 ± 0.10	ND
39	vitexin	ND	74.18 ± 0.13	2.98 ± 0.12	ND
40	apigenin di-C arabinoside	ND	0.93 ± 0.02	0.02 ± 0.01	ND
41	densifloside	ND	113.73 ± 0.26	7.89 ± 0.12	ND
42	nicotiflorin	ND	18.17 ± 0.02	2.90 ± 0.13	ND
43	azelaic acid	ND	+	+	ND
44	4-oxododecaneoic acid	ND	+	+	ND
45	NZ	ND	+	+	ND
46	NZ	ND	ND	+	ND
47	NZ	ND	+	+	ND
26	traumatic acid	ND	43.04 ± 0.05	ND	ND
27	9,10-dihydroxy-8-oxooctadec-12-enoic acid	ND	29.60 ± 0.08	ND	ND
28	pinellic acid	ND	36.57 ± 0.12	ND	ND
48	linoleic acid derivat.	ND	+	ND	ND
29	octadecdienoic acid derivat.	ND	+	ND	ND
49	9(S)-HPODE	ND	+	ND	ND
50	9,10-DHOME	ND	+	ND	ND
51	linoleic acid derivat.	+	ND	ND	ND
52	linoleic acid derivat.	+	ND	ND	ND
53	linoleic acid derivat.	+	ND	ND	ND
54	linoleic acid derivat.	+	ND	ND	ND

ND—not detected; +—detected.

2.3. Antioxidant Assay

In order to assess the antioxidant properties of the tested samples, two different tests, namely DPPH and FRAP, were used. The free radical scavenging capacity (DPPH assays) was expressed as Trolox equivalent. Ferric reducing antioxidant power (FRAP) was calculated as equivalent of ascorbic acid. The results for the DPPH and FRAP assays are shown in Table 3.

Table 3. The results of radical scavenging activity (DPPH) and ferric reducing antioxidant power (FRAP) obtained for hexane (HCV), ethyl acetate (EaCV), butanol (BCV), and water (H_2OCV) fractions from the methanolic extract of *Carlina vulgaris*. Values are means ± standard deviation (SD) of triplicate.

Fractions	Concentration (µg/mL)	Equivalent of Trolox Concentration (DPPH)	Equivalent of Ascorbic Acid Concentration (FRAP)
H_2OCV	25	5.062 ± 0.698	4.581 ± 0.223
	100	14.221 ± 0.451	17.207 ± 0.632
	200	22.322 ± 0.516	34.860 ± 0.258
BCV	25	3.330 ± 0.799	2.793 ± 0.428
	100	10.429 ± 0.296	11.397 ± 0.011
	200	20.458 ± 0.309	21.676 ± 0.223
EaCV	25	10.988 ± 0.420	10.056 ± 0.365
	100	29.581 ± 0.390	39.33 ± 0.447
	200	42.992 ± 0.160	74.413 ± 1.210
HCV	25	0.081 ± 0.011	0.894 ± 0.447
	100	0.773 ± 0.535	3.017 ± 0.223
	200	1.133 ± 0.579	4.804 ± 0.223

The term "equivalent of ascorbic acid/Trolox" means that the reducing/antioxidant power of the extract at a given concentration is equivalent to the reducing power of a given concentration of ascorbic acid/Trolox.

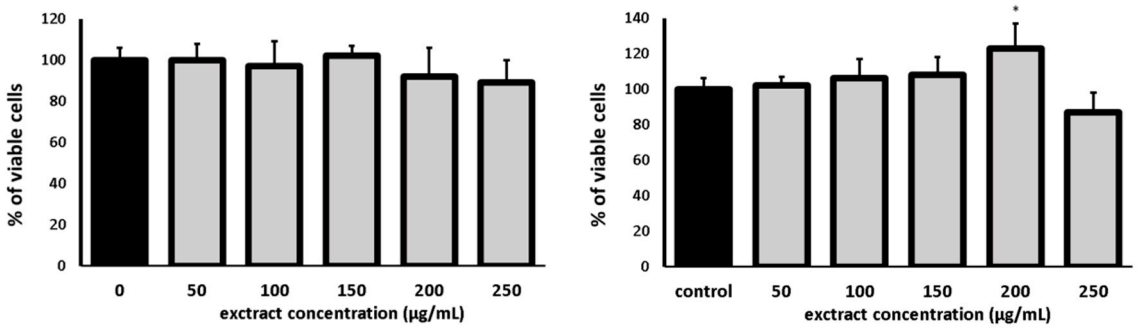
As can be seen, EaCV exhibited the highest free radical scavenging activity in the DPPH test and the highest ferric reducing ability. It was followed by BCV, H_2OCV, and HCV in terms of antioxidant potential. These findings were consistent with the results of quantitative analysis, as EaCV showed significantly higher content of polyphenolic compounds from phenolic acids and flavonoid classes.

2.4. Antioxidant Assay Using Human Cell Fibroblasts

Given that EaCV is the richest in polyphenolic compounds fraction with the highest antioxidant potential, it was selected for further antioxidant testing using HSF cells.

2.4.1. Cell Viability Assay

To determine the non-toxic concentration of EaCV extract, the cytotoxicity on fibroblast cells was evaluated using two complementary assays: the neutral red (NR) test and the MTT test (Figure 4). The NR test assesses the stability of cell membranes based on the uptake of the dye via active transport and its accumulation in lysosomes of viable cells. In turn, the MTT test demonstrates the impact on cellular metabolism, specifically the activity of NAD(P)H-dependent cellular oxidoreductases.



(**a**) (**b**)

Figure 4. Effect of the different concentrations of ethyl acetate fraction (EaCV), obtained from methanol/water extract of *Carlina vulgaris* on cell viability determined by the NR (**a**) and MTT (**b**) assay, expressed as a % of control (0.5% of DMSO in medium). The data are means (n = 3) ± SD. One-way ANOVA followed by Dunnett's post hoc test; the differences were considered significant at $p < 0.05$. * indicates statistically significant difference.

The NR assay demonstrated that the extract did not have a negative impact on cell viability and did not decrease, in a statistically significant manner, the number of living cells in the culture within the tested concentrations range. Furthermore, at a concentration of 200 µg/mL, it even slightly stimulated cell metabolism.

2.4.2. Protective Effect of Extract on H_2O_2-Induced Cytotoxicity

The protective effect of the extract against H_2O_2-induced cytotoxicity was investigated by assessing cell viability and cell metabolism. The cells were pretreated with increasing concentrations of the extract and then exposed to H_2O_2. The results are presented in Figure 5.

It was observed that H_2O_2 treatment significantly reduced the percentage of viable cells and suppressed the enzymatic activity of the cells compared to the control. However, when the extract was added 30 min prior to H_2O_2 exposure, at a concentration of 200 µg/mL, it protected the cells from these negative effects.

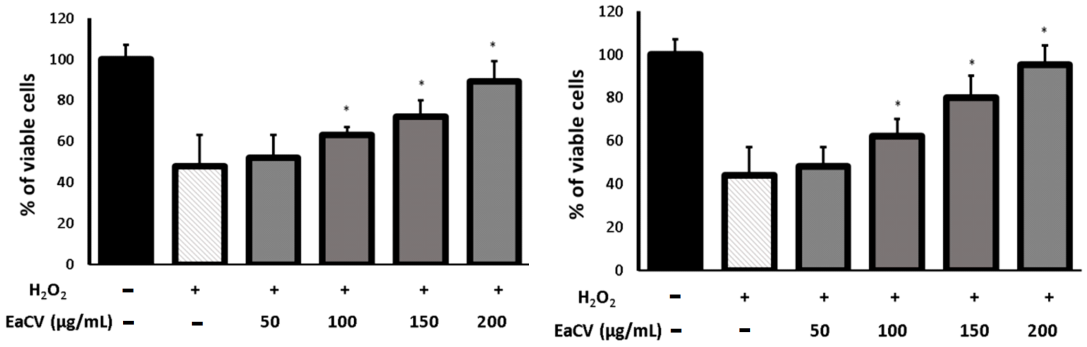


Figure 5. Effect of different concentrations of ethyl acetate fraction (EaCV), obtained from the methanol/water extract of *Carlina vulgaris*, on H_2O_2-treated cells evaluated in terms of (**a**) cell viability (NR) and (**b**) cellular metabolism (MTT). Cells were pretreated with extract at different concentrations prior to the H_2O_2 exposure. The results are expressed as a percentage of the control (0.5% DMSO). The data are means ± SD (n = 3). * indicates a statistically significant difference ($p < 0.05$) versus H_2O_2-treated cells assessed using one-way ANOVA followed by Dunnett's multiple comparison post hoc test.

2.4.3. Antioxidant Activity of the Extract in H_2O_2-Induced Oxidative Stress

The assay aimed to find out whether the protective effect of the ethyl acetate fraction (EaCV) on human skin fibroblasts exposed on H_2O_2 induced oxidative stress is a result of its impact on oxidative imbalance. To evaluate the protective action of EaCV against disturbance in the oxidative balance, the influence on the intracellular production of reactive oxygen species was determined using the H_2DCFDA test. The results are shown in Figure 6.

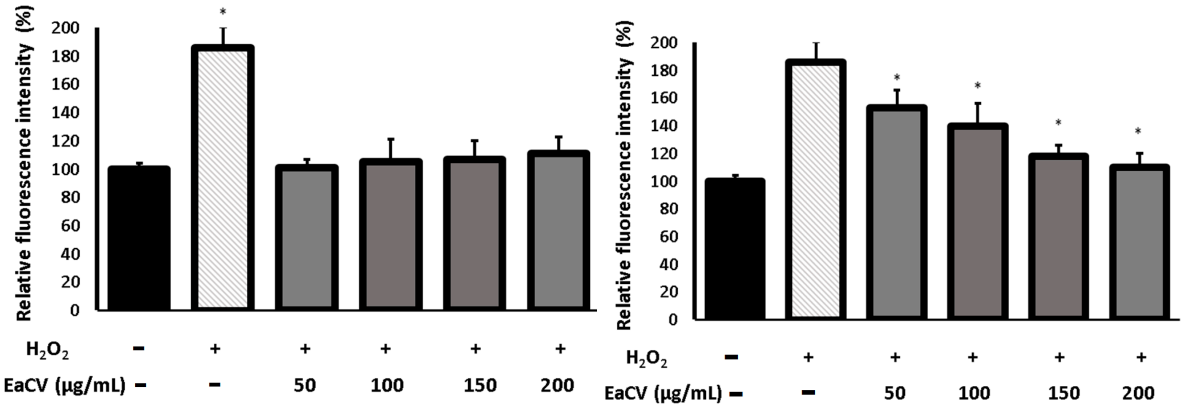


Figure 6. Relative fluorescence of 2′,7′-dichlorodihydrofluorescein (DCF) in human skin fibroblast cells calculated as a percentage in comparison with untreated control cells. (**a**)—the cells were treated with H_2O_2 or different concentrations of ethyl acetate fraction (EaCV), obtained from the methanol/water extract of *Carlina vulgaris*. * indicates a statistically significant difference ($p < 0.05$) versus untreated controls. (**b**)—the cells were pretreated with EaCV prior to the H_2O_2 exposure. * indicates a statistically significant difference ($p < 0.05$) versus the H_2O_2-treated cells. The data are means ± SD (n = 3). One-way ANOVA followed by Dunnett's multiple comparison post hoc test.

As can be seen, H_2O_2 strongly induced oxidative stress and the level of ROS significantly increased (up to 184%) compared to the control. In turn, the EaCV extract did not affect the oxidative balance in a statistically significant manner (Figure 6a). Pretreatment with the EaCV suppressed ROS production in H_2O_2-stimulated cells in a concentration-dependent manner.

3. Discussion

Carlina vulgaris is a little-understood plant with unexplored biological potential, and papers regarding its chemical composition are scarce. To date, only a few triterpenic compounds and chlorogenic acid have been identified in the aboveground parts and roots of *C. vulgaris* [23,28]. In turn, analysis of the root essential oil revealed the presence of thirteen volatile components, with the prevalence of polyacetylenes, carlina oxide, and its 13-methoxy derivative [29].

Our study shows that the aboveground parts of *C. vulgaris* are a rich source of polyphenolic components from the phenolic acids and flavonoids classes, with the most abundant being flavonoid C-glycosides. In contrast to the most widespread O-glycosides, in which the sugar is linked to the aglycone via an oxygen atom, the characteristic feature of C-glycosides is the attachment of sugar moieties directly to the flavonoid backbone through C-C covalent bonds. This makes their structure more stable than O-glycosides, and therefore, C-glycosides differ in pharmacokinetics and biological activities [31–33].

C-glycosides are relatively less studied flavonoid derivatives; however, recent literature data have shown that they possess many beneficial effects and health-promoting properties, including antioxidant, antibacterial, antiviral, anti-diabetic, anti-inflammatory, neuroprotective, and antihypertensive potential [31,34–38]. Our investigation for the first time reveals that *C. vulgaris* contains a high amount of C-glycosides of luteolin, including carlinoside, orientin, isoorientin, and C-glycosides of apigenin, schaftoside, isoschaftoside, and vitexin. Some of these C-glycosides, namely orientin, homoorientin, vitexin, and isoschaftoside, were previously detected in another *Carlina* species—*C. acaulis* [39,40]. However, quantitative data regarding their amounts were lacking.

Fractionation of the methanolic extract with ethyl acetate yielded a polyphenolic-rich fraction (EaCV) which showed a protective effect against H_2O_2-induced oxidative stress 253 and prevented H_2O_2-induced cytotoxicity. It can be supposed that the effect was related with polyphenolic compounds which are known for their potent antioxidant properties and beneficial effects on cell metabolism [41,42]. For example, it has been evidenced that chlorogenic acid (CA), an abundant component of *C. vulgaris*, stimulates the proliferation of skin fibroblasts and keratinocytes, and it contributes to the production of matrix proteins. It also exhibits a strong protective effect against oxidative stress [43–46]. Free radical scavenging effects and protective activities against reactive oxygen species (ROS) have also been found for many flavonoid C-glycosides in both in vitro and in vivo assays. It has been reported that vitexin acts as an effective radical scavenger and protects against lipid peroxidation and other oxidative damages in various oxidative stress-related diseases [47]. Additionally, orientin and vitexin have been shown to improve the endogenous antioxidant status in the organism and increase levels of superoxide dismutase, catalase, and glutathione peroxidase in the serum [48]. Furthermore, it has been demonstrated that isoschaftoside, isovitexin, vitexin, and orientin protect against lipopolysaccharide-stimulated inflammation [37,49].

Plant products with high antioxidant activity are strongly desirable components of skin care products. It is well known that oxidative stress plays a significant role in skin aging and favors the development of skin-related disorders. Excessive exposure to reactive oxygen species disturbs cellular redox balance and consequently leads to the damage and dysfunction of the cells. ROS may modify the micro-environment of the skin through modulation of the extracellular matrix and affect the matrix metalloproteinases responsible for tissue remodeling, which leads to structural and functional alterations in skin, including collagen fragmentation and disorganization of collagen fibers. Furthermore, ROS-related cell damage increases the level of pro-inflammatory cytokines and promotes a chronic inflammatory state [50–52]. All these processes accelerate skin aging.

Our study has revealed that *C. vulgaris* is rich in polyphenolic compounds with health-promoting properties. Therefore, it is worthwhile to continue investigating this plant and exploring other potential courses of action.

4. Materials and Methods

4.1. Reagents and Standards

Analytical standards of phenolic compounds and reagents, including 2,2-diphenyl-1-picrylhydrazyl (DPPH), o-phenanthroline and ferric chloride (FRAP reagent), were purchased in Fluka (Sigma-Aldrich Co., St. Louis, MO, USA).

LC-MS-grade methanol, acetonitrile, and formic acid were purchased from Merck KGaA (Darmstadt, Germany). The other solvents were analytical-grade (Merck).

Water was deionized and purified using Ultrapure Millipore Direct-Q® 3UV-R (Merck, KGaA, Darmstadt, Germany).

4.2. Plant Material

The seedlings of *Carlina vulgaris* plants were obtained from seeds collected from plants growing in the UMCS Botanical Garden in Lublin (voucher specimen no. 9/2009S). The cultivation lasted for 2 years (2018–2019) in the field at the Botanical Garden [53]. No fertilization was used. The plants were collected in August 2019 (during the flowering phase). The plant material was rinsed with running water, dried, freeze-dried and stored at a temperature of −20 °C until further processing.

4.3. Extraction and Fractionation

The aboveground parts of the plants were ground, and 300 g of material was exhaustively extracted with methanol and 70% methanol (3 × 5 L and 3 × 1.5 L for 15 min each) using an ultrasonic bath. The extracts (ECV) were combined, centrifuged at 8000 rpm, filtered, concentrated using a vacuum evaporator, frozen, and subjected to freeze-drying.

The freeze-dried ECV was suspended in 300 mL of MeOH and subjected to liquid–liquid extractions with n-hexane (5 × 100 mL) followed by ethyl acetate (5 × 100 mL). The residue was evaporated to dryness and suspended in water, then extracted with n-butanol (5 × 100 mL). The residue after extraction represented the H_2OCV fraction. The fractions were concentrated using a vacuum evaporator, frozen, and freeze-dried.

4.4. Chromatographic Analysis (UHPLC–HR/QTOF/MS–CAD–PDA)

The lyophilized samples were reconstituted in 50% MeOH in Milli-Q water with the addition of 5% dimethyl sulfoxide (DMSO), centrifuged, and filtered.

The chromatographic analyses of the extracts were performed using an ultra-high-performance liquid chromatography (UHPLC) system coupled with a charged aerosol detector (CAD) and a high-resolution/quadrupole-time-of-flight mass spectrometer (HR/QTOF/MS Impact II) employing electrospray ionization (ESI). The chromatographic separation was conducted on a BEH C18 column (2.1 × 150 mm, 1.7 µm; Waters) at a temperature of 40 °C. A linear gradient elution was applied with a flow rate of 0.5 mL/min, using solvent B (acetonitrile-0.1% formic acid (FA)) in solvent A (H_2O-0.1% FA), ranging from 2% B to 80% B, over 30 min. UV spectra of the compounds were recorded in the range of 190–750 nm with a resolution of 3.6 nm. MS spectra were acquired in negative modes, scanning in the range of 80–2000 m/z. Nitrogen gas was used as the cone and spraying gas, with flow rates of 800 L/h and 100 L/h, respectively. The capillary voltage was set at −2.8 kV for negative mode. The cone voltage was −25 V and 45 V, respectively, the source temperature was 140 °C, and the spraying gas temperature was 350 °C. The data acquisition and processing were performed using Waters Mass Lynx software. Quantitative analyses were performed using an ultra-performance liquid chromatography system UPLC-PDA-ESI-MS (ACQUITY, Waters) coupled with a PDA detector and a triple quadrupole mass spectrometer (ACQUITY TQD, Waters). The chromatographic conditions are described above.

4.5. Antioxidant Activity

4.5.1. DPPH Radical Scavenging Assay

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay was carried out according to procedure published previously [54]. EsCV was dissolved, diluted, and mixed with a 4 mM

methanolic DPPH solution. Absorbance was measured at the wavelength λ = 517 nm using a UV-VIS Filter Max 5 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Water with a DPPH solution was used as a control.

4.5.2. Ferric Ion Reducing Antioxidant Power (FRAP Assay)

The ferric-reducing activities of the samples were determined according to the method described by Sowa et al. [55] with some modifications. The sample (15 µL) was mixed with fresh FRAP reagent (350 µL). The 300 mM acetate buffer pH 3.6, 10 mM TPTZ in 40 mM HCl, and 20 mM $FeCl_3 \cdot 6H2O$ were mixed according to 10:1:1 as FRAP reagent and reacted in the dark for 5 min. The absorbance was measured at λ = 593 nm.

4.6. Cell Culture and Experimental Design

Human skin fibroblast cells line (ATCC® CRL-2522™) were from the American Type Culture Collection (Manassas, VA, USA). The cells were grown in Dulbecco's modification of Eagle's medium (DMEM, Biological Industries, Cromwell, CO, USA) supplemented with sodium pyruvate, L-glutamine, 10% fetal bovine serum (Gibco, Waltham, MA, USA), glucose (4.5 g/L), and 1% antibiotics (100 U/mL of penicillin and 1000 of µg/mL streptomycin, Gibco). Cultured cells were kept at 37 °C in a humidified atmosphere of 95% air and 5% carbon dioxide. When the cells obtained the required confluence, the medium was removed, and the cells were rinsed twice with sterile phosphate-buffered saline (PBS, Gibco, Waltham, MA, USA). The confluent layer was trypsinized (0.25% Trypsin/EDTA, Gibco) and placed in fresh medium.

For cytotoxicity assay, the cells (1×10^5 cells/mL) were plated in 96-well flat-bottom plates, incubated for 24 h at 37 °C, and then treated with the EaCV extract for 24 h.

For antioxidant assay, the cells (1×10^5 cells/mL) were seeded on the well bottom in 96-well plates. After 24 h of incubation at 37 °C, the cells were pretreated with the EaCV extract and, after 30 min, H_2O_2 (250 µM) was added to the medium to induce oxidative stress [56]. Stock solution of the EaCV extract was prepared using DMSO/culture medium (1:1) and appropriately diluted. The final concentration of DMSO did not exceed 0.5%. Cells treated with 0.5% of DMSO in culture medium was taken as control.

All experiments were performed in triplicates for each extract concentration and presented as a percentage of the control (100%).

4.7. Cell Viability Assay

4.7.1. MTT Assay

After 24 h of incubation with the EaCV extract, a 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide solution (MTT) at a concentration of 5 mg/mL (Sigma) was added to the cells (25 µL/well), followed by further incubation for 3 h. The insoluble formazan crystals were solubilized overnight in a mixture of 10% sodium dodecyl sulfate (SDS) in 0.01 M HCl. Absorbance was measured at 570 nm wavelength using an E-max Microplate Reader (Molecular Devices Corporation, Menlo Park, CA, USA).

4.7.2. Neutral Red Uptake Assay

After 24 h incubation of the cells with the EaCV extract, the solution was removed from the wells, and the cells were 2 h incubated with a solution of a neutral red dye (40 µg/mL) at 37 °C. The cells were washed with phosphate-buffered saline (PBS), PBS was removed, and 150 µL of decolorizing buffer was added. The plates were shaken for 10 min, and the optical density (OD) of the eluted dye was measured at 540 nm using an E-max Microplate Reader (Molecular Devices Corporation, Menlo Park, CA, USA). The results are presented as the percentage of the amount of dye retained compared to the control cells (100%)

4.8. Analysis of Intracellular Reactive Oxygen Species

The generation of intracellular reactive oxygen species in the human fibroblasts was performed as previously described [57]. After 24 h of incubation, the medium was removed

and replaced with 10 µM H_2DCFDA (Sigma Aldrich), and the cells were incubated for 45 min at 37 °C. The fluorescence was measured after 90 min using a FilterMax F5 microplate reader (Thermo Fisher Scientific) at a maximum excitation of 485 nm and emission spectra of 530 nm.

4.9. Statistical Analysis

All analyses were carried out in triplicate. The results were analyzed using Statistic ver. 13.3 software. One-way ANOVA followed by Dunnett's post hoc test was used. The values were expressed as the mean ± standard deviation (SD). The differences were considered significant at a *p*-value of $\leq$0.05.

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