Scientia Pharmaceutica

ÖSTERREICHISCHE PHARMAZEUTISCHE GESELLSCHAFT

MDPI

Article

Characterization of Phytochemical Components of *Crocus sativus* Leaves: A New Attractive By-Product

Olha Mykhailenko [1], Liudas Ivanauskas [2], Ivan Bezruk [1], Lyudmila Sidorenko [1], Roman Lesyk [3,*] and Victoriya Georgiyants [1]

[1] Department of Pharmaceutical Chemistry, National University of Pharmacy, 4-Valentynivska st., 61168 Kharkiv, Ukraine; mykhailenko.farm@gmail.com (O.M.); vania.bezruk@gmail.com (I.B.); slv.ludmila16@gmail.com (L.S.); vgeor@nuph.edu.ua (V.G.)

[2] Department of Analytical and Toxicological Chemistry, Lithuanian University of Health Sciences, A. Mickevičiaus g. 9, LT 44307 Kaunas, Lithuania; liudasivn@gmail.com

[3] Department of Public Health, Dietetics and Lifestyle Disorders, Faculty of Medicine, University of Information Technology and Management in Rzeszow, Sucharskiego 2, 35-225 Rzeszow, Poland

* Correspondence: dr_r_lesyk@org.lviv.net; Tel.: +38-067-703-8010

Abstract: *Crocus sativus* L. is one of the world's most famous saffron production crops and its enormous by-products, such as leaves, are an excellent source of bioactive compounds with potential nutritional applications. The total phenolic content of *Crocus* leaves was 5.44 ± 0.01 mg GAE/g, and the total flavonoid content was 2.63 ± 0.05 mg RE/g, respectively. The main bioactive compounds in the leaves, such as polyphenols, flavonoids by HPLC and carboxylic acids, and amino acids, were also identified by GC-MS. HPLC analyses revealed mangiferin as a dominant constituent (1.26 ± 0.02 mg/g). *C. sativus* contains seven essential amino acids (ILE, LEU, LYS, MET, PHE, THR, TRP, VAL) in high concentration. Among them, isoleucine (7965 µg/g) was the dominant compound. In addition, the K and Ca concentrations in the leaves were significant ($p < 0.05$). The chemical composition revealed α-linolenic acid (22,490 µg/g) and linoelaidic acid (9880 µg/g) to be major constituents among all the acids found in the *Crocus* leaves. The extracts of *C. sativus* leaves showed the highest inhibitory activity for Gram-positive (*B. subtilis* and *S. aureus*) bacteria in the *in vitro* assay. The current results identify and underline the potential of natural products from *C. sativus* leaves that can add value to saffron production.

Keywords: *Crocus sativus* leaves; HPLC; UPLC-MS/MS; GC-MS; atomic emission spectroscopy; antibacterial assay; value-added byproducts

check for updates

Citation: Mykhailenko, O.; Ivanauskas, L.; Bezruk, I.; Sidorenko, L.; Lesyk, R.; Georgiyants, V. Characterization of Phytochemical Components of *Crocus sativus* Leaves: A New Attractive By-Product. *Sci. Pharm.* **2021**, *89*, 28. https://doi.org/10.3390/scipharm89020028

Academic Editor: Helen D. Skaltsa

Received: 15 May 2021
Accepted: 8 June 2021
Published: 15 June 2021

Publisher's Note: MDPI stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Copyright: © 2021 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https://creativecommons.org/licenses/by/4.0/).

1. Introduction

Recently, traditional herbal medicine has gained more attention from consumers, especially when the use of synthetic drugs does not provide results or drug side effects occur. Herbal medicine is still the most common alternative medicine that is based on natural products and is safe [1]. As a rule, the pharmacological activity of the plant extracts is not determined by a specific active ingredient but by the interaction of a group or a whole complex of components. Their content may vary depending on the type of plant, cultivation season, geographical location, method of drying, grinding, storage, etc. [2–4]. *Crocus sativus* L. is a famous plant from the *Iridaceae* family. From its stigmas, the most expensive spice in the world, "saffron", is obtained. *Crocus* is cultivated in different countries, such as Iran, India, Afghanistan, Italy, France, New Zealand, Spain, Portugal, Greece, Morocco, Turkey, and some parts of China [5,6]. Since saffron is widely cultivated and harvested in different parts of the world, planting methods can also vary based on climate, soil type, planting depth, and distance between corms. Recent studies [6–8] have shown that saffron has many pharmacological effects, such as anticonvulsant, antihypertensive, antitussive, antigenotoxic, anxiolytic, cytotoxic, antioxidant, antidepressant, anti-inflammatory, and

relaxant properties. Saffron also helps to improve memory and learning skills and to increase blood flow in the retina and choroid. These pharmacological activities occur because of several active compounds, such as crocin, picrocrocin, and safranal [9].

The production of only 1 kg of saffron generates a huge amount of residues, such as flower waste (350 kg), low-quality corms (several hundred), and *Crocus* leaves (1500 kg), which gradually dry out and are not used [5,10,11]. However, these plant raw materials also contain various biologically active compounds. Thus, they can be considered as useful byproducts for obtaining biologically active complexes or additional raw materials for the development of herbal drugs. It is known that plant leaves can exhibit pronounced antioxidant and antibacterial activities, especially due to the presence of phenolic compounds. Analysis of the literature has shown that study of the chemical composition of *C. sativus* leaves is very limited. Smolskaite et al. [12] analyzed 46 samples of *Crocus* leaves from Azerbaijan, India, New Zealand, Morocco, France, Turkey, Iran, Italy, and Spain by High-performance liquid chromatography with ultra-violet (UV) spectroscopy (HPLC-UV) and Liquid chromatography with mass spectrometry (LC-MS). Several flavonoids have been identified; among them, kaempferol-8-C-gluco-6,3-*O*-diglucoside and kaempferol-8-C-gluco-6-*O*-glucose were found for the first time. At the same time, the authors noted that different samples of *Crocus* leaves did not differ in chemical composition but differed in the concentration of each component. Jadouali et al. [13] only studied the total phenolic compounds and total flavonoid contents in the leaves and flowers of *C. sativus* from Morocco. Naringenin was found in *C. sativus* leaves using LC-MS [14]. A qualitative analysis of *C. sativus* leaves from Morocco showed that glycosylated derivatives of luteolin (luteolin-C-(O-caffeoyl-hexosyl)-*O*-hexoside) and glycosylated kaempferol (kaempferol 3,7-di-*O*-glucoside) were the major identified polyphenols in leaves [15]. A significant amount of polyphenols, the main glycosides being kaempferol, luteolin, and quercetin, have been determined in *C. sativus* leaves from Spain [2]. Thus, data from the literature on the chemical composition of *C. sativus* leaves are very scarce and contradictory.

Ukraine is a new country to the saffron market, and its climatic features affect the composition of secondary metabolites, which determine the pharmacological value of the plant. Therefore, the research objectives included analysis of the chemical composition of the phenolic compounds, carboxylic acids, amino acids, and mineral components of the leaves of Ukrainian *C. sativus* as well as the potential of antibacterial activity of plant extracts.

2. Materials and Methods

2.1. Plant Material

C. sativus leaves were collected from a plantation in the village Lyubimivka (Ukraine, Kherson region) in November 2020. Raw material was collected and identified by Dr. Mykhailenko, and the identification was verified by Dr. Gamulya (V.M. Karazin Kharkiv National University, Ukraine). A specimen was deposited at the Herbarium of V.M. Karazin Kharkiv National University, Ukraine (CWN, voucher specimen No. CWN0056541). Leaves were air dried at room temperature with suitable ventilation and then crushed to grit (1.0 mm size) for further extraction.

2.2. Chemicals

The acetonitrile and methanol were of HPLC grade and purchased from Roth GmbH (Karlsruhe, Germany). Reference compounds (chlorogenic acid, *trans*-cinnamic acid, mangiferin, kaempferol, ononin, irigenin) were purchased from ChromaDex (Santa Ana, CA, USA), Sigma-Aldrich (Saint Louis, MO, USA), HWI ANALYTIK GmbH, and Roth GmbH (Karlsruhe, Germany). All the other chemicals were of analytical grade.

2.3. Qualitative Analysis

Crushed *Crocus* leaves were extracted with 70% methanol (in a ratio of raw material to extractant of 1:5 w/v) and kept in an ultrasonic bath at room temperature (20 $\pm$ 2 °C) for

20 min. Then, the sample was filtered through microfilters into a 10 mL flask and diluted with methanol to the mark. Preliminary qualitative analysis of the phenolic compounds of the leaves was established as described by Morsy [16] and by TLC analysis [17]. The following methods were used to separate or identify substances on the TLC plates (silica gel 60 F_{254}, Merck, Germany) of *Crocus* samples: physical (individual color of a substance or fluorescence of a substance in UV light) and chemical (colored reactions of separated substances with imaging reagents). Phenolic compounds have a weak natural fluorescence in UV light at 365 nm and 254 nm wavelengths and must be enhanced during separation on chromatography plates. The phenolic compounds' fluorescence was enhanced by spraying the TLC plates with different complex agents (ammonium vapors; 2% alcoholic solution of aluminum chloride; 10% sodium/potassium hydroxide solution; 5% alcoholic solution of diazotized sulfanilic acid).

2.4. Determination of Total Phenolic and Flavonoid Contents

The total phenolic and flavonoid content of the ethanol extract of *Crocus* leaves was estimated using the methods of the State Pharmacopoeia of Ukraine 2.0 [18] by absorption spectrophotometry on an Evolution TM 60S UV–visible light spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) with slight modifications. Extract solutions (1 mL of the concentration of 2 mg/mL) were mixed with ethanol/water 70/30 (*v*/*v*) (by volume 25 mL) and the solutions were incubated for 5 min at room temperature. The determination was at the wavelength 270 nm and the reference solution was 70% ethanol. The amount of total phenols was determined based on a mg of gallic acid equivalent per gram of sample (mg GAE/g). The total flavonoid content was assessed based on the aluminum trichloride method [19] at the wavelength 410 nm. Extract solutions (0.5 mL of the concentration of 2 mg/mL) were mixed with 1 mL of $AlCl_3$ ethanolic (3%). The absorbance of the extracts was measured at 410 nm after a 40 min incubation (room temperature, in the dark). The total content of flavonoids was expressed as mg of rutin equivalents per gram of sample (mg RE/g), using a rutin calibration curve.

2.5. Sample Extraction

A measurement of 0.1 g of the dried *Crocus* leaves was extracted with 10 mL of methanol in an ultrasonic bath at room temperature (20 ± 2 °C) for 30 min. The solutions were filtered through a membrane filter (0.45 μm) prior to use. An aliquot of 10 μL was injected into the HPLC system for analysis.

2.6. Standard Preparation

A standard stock solution of the phenolic reference compounds at a concentration of 1.0 mg/mL for each compound was prepared by dissolving the substance in methanol. After preparation of standard stock solutions, all of them were mixed and dissolved with methanol to obtain working concentrated solutions. All samples were kept at 4 °C before use.

2.7. HPLC Conditions

For separation, the Shimadzu Nexera X2 LC-30AD HPLC chromatographic system (Shimadzu, Japan) composed of a quaternary pump, an on-line degasser, and a column temperature controller; the SIL-30AC autosampler (Shimadzu, Japan); the CTO-20AC thermostat (Shimadzu, Japan); and the SPD-M20A diode array detector (DAD) were used. Chromatographic separation of phenolic compounds was carried out using an ACE C18 column (250 mm × 4.6 mm, 5.0 μm; Bellefonte, PA, USA) with the column thermostat at 25 °C (±1 °C) as described previously [20]. Briefly, the mobile phase consisted of 0.1% acetic acid in water (solvent A) and acetonitrile (solvent B). The flow rate was maintained at 1 mL/min. The gradient elution was performed as follows: 0 min—95% A and 5% B, 7 min—95% A and 5% B, 67 min—0% A and 100% B, 69 min—95% A and 5% B, and 75 min—95% A and 5% B. The individual phenolic compounds were identified using MS spectra and

UV spectra and verified by reference substances. The analyses were performed in duplicate. Validation of the HPLC method was performed according to the ICH guidelines [21] by the following parameters: specificity, linearity, precision, LOD, and LOQ.

2.8. UPLC-MS/MS Analysis of Components

Separation of the samples' components was carried out with the ACQUITY H-class UPLC system (Waters, Milford, MA, USA) equipped with ACQUITY UPLC BEH C18 (50 × 2.1 mm, particle size 1.7 µm) (Merck Millipore, Darmstadt, Germany). Gradient elution was performed with 0.1% formic acid water solution (solvent A) and acetonitrile (solvent B) with the flow rate at 0.5 mL/min. The following proportions of the solvent system were applied using a linear gradient profile B: initial 5%, 3 min 30%, 7 min 50%, 7 to 8 min 95%, and 15 to 16 min 5%. A Xevo TQD triple quadrupole mass spectrometer detector (Waters) was used to obtain MS/MS data. Positive electrospray ionization was applied with the following settings: capillary voltage was 1.5 kV, source temperature was 150 °C, desolvation temperature was 350 °C, with a desolvation gas flow of 650 L/h and cone gas flow of 25 L/h. Collision energy and cone voltage were optimized for each compound separately. Collision energy varied in the range from 6 eV to 20 eV and cone voltage was selected from 8 V to 38 V.

2.9. Mineral Composition

The graphite furnace atomic absorption spectroscopy (GF-AAS) method with photographic registration on the DFS-8 device was used to determine the qualitative and quantitative content of macro- and microelements in *Crocus* leaves. The method is based on the evaporation of samples from craters of graphite electrodes and the excitation of spectra in the arc and registration of the obtained spectra on IBS-28 photographic plates. Measuring conditions: amperage arc—16 A, ignition phase—60 °C, ignition pulse frequency—100 bps, analytical gap—2 mm, gap width—0.015 mm, and exposure—60 s. The spectra were photographed in the 240–350 nm region. A set of standard LNG-24 samples (GSO 2820-83) was used in the determination.

2.10. Amino Acids and Carboxylic Acids Assays

The methanol extracts (prepared as indicated above) were evaporated under nitrogen to dry the residue. It was dissolved in a 200 µL mixture of acetonitrile and MTBSTFA (1:1), then heated in a glycerol bath and subjected to amino acid gas chromatography analysis. The gas chromatography method has been previously described by Mykhailenko et al. [22] and Jarukas et al. [23]. Briefly, the SHIMADZU GC-MS-QP2010 system and Rxi-5 ms (Restek Corporation, Bellefonte, PA, USA) capillary column (30 m, 0.25 mm outer diameter, 0.25 µm) were applied. The MS was operated in positive mode (electron energy 70 eV). The full-scan acquisition was performed with the mass detection range set to 35–500 m/z to determine the retention times of analytes, optimize the oven temperature gradient, and observe characteristic mass fragments for each compound. Amino acids and carboxylic acids were identified by comparison with the database (NIST14, NIST14s, WR10, WR10R) mass spectra of compounds, analyzing ions characteristic of mass spectra and retention times of standards. 4-Aminobutyric acid was quantified in glutamic acid equivalents. The content of the carboxylic acids was calculated and expressed in terms of the lactic acid equivalent; the contents of 2-aminobutyric acid, γ-aminobutyric acid, and L-aspartic acid were re-calculated in glutamic acid; the content of β-alanine was calculated via L-alanine; and L-tryptophan was calculated via L-proline.

2.11. Extraction Procedure of C. sativus Leaves for Bioassay

C. sativus leaves were dried and ground, and the powder was extracted with distilled water in a water bath at 100 °C (100 g, 1 L, 60 min × 3) or 70% ethanol at room temperature (100 g, 1 L, 60 min × 3). The extracts were concentrated to dryness.

2.12. Antibacterial Activity Assays

The agar well diffusion method and serial dilution technique were used to screen the antibacterial and antifungal activities of water and ethanolic *C. sativus* leaf extracts [24]. According to the WHO recommendations [25], the following test strains were used: *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922, *Proteus vulgaris* NCTC 4636, *Pseudomonas aeruginosa* ATCC *27853, Bacillus subtilis* ATCC *6633*, and *Candida albicans 885/653* ATCC. Detailed information concerning the method conditions has been previously provided [26]. The concentration of extracts (1% *w*/*v*) was selected based on our pre-experiments and the previous literature. An alcohol solution of chlorophyllipt ("Chlorophyllipt ethanolic 1%solution" 100 mL, "DP", "OZ GNTSLSHOOKUkrmedprom", Pilot Plant Medicines, Kharkiv, Ukraine) at a dose of 10 mg/mL was employed as a positive control. Antibacterial activity was evaluated by measuring the diameter of the inhibition zone (including the wells diameter) appearing after the incubation period. The assay was repeated thrice. Antibacterial activity was expressed as the mean zone of inhibition diameters (mm) produced by the *Crocus* leaf extract. The method of serial dilutions was used for the examination of the antibacterial properties of selected extracts. The principle of the method consists in the determination of the minimal inhibitory concentration (MIC) that characterizes the bacteriostatic properties of the objects of the examination. The concentration of substances was 1000 μg/mL; 500 μg/mL; 250 μg/mL; 125 μg/mL; and 62.5 μg/mL. The MIC was considered as the lowest concentration which inhibited the growth of the respective microorganisms. All assays were performed in triplicate.

2.13. Data Analysis

All data processing was carried out using the LabSolutions Analysis Data System (Shimadzu Corporation, Kyoto, Japan). Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test with the software package Prism v.5.04 (GraphPad Software Inc., La Jolla, CA, USA). The value of $p < 0.05$ was taken as a level of significance.

3. Results and Discussion

3.1. Preliminary Phytochemical Screening

The phytochemical study of the methanol extract of *Crocus* leaves revealed the presence of various bioactive compounds (Table 1), including polyphenols, tannins, flavonoids, xanthones, hydroxycinnamic acids, hydrolysable tannins, triterpenoids, saponins, aminoacids, and proteins. Alkaloids and coumarins were not found in this raw material.

Table 1. Qualitative analysis of biological active compounds in *C. sativus* leaves.

Group of BACs	Test	Reactions
Xanthones	TLC: *n*-butanol–acetic acid–water (4:1:2); 15% acetic acid	++
Flavonoids	$AlCl_3$ test, Ammonia test, Shinoda; cyanidine reaction by Bryant. TLC: *n*-butanol–acetic acid–water (4:1:2)	+++
Coumarins	Lactone reaction; formation of azo dye	-
Hydroxycinnamic acids	TLC: *n*-butanol–acetic acid–water (4:1:2); 15% acetic acid	+++
Tannins	Ferric chloride; gelatin solution; quinine hydrochloride solution	+++
Alkaloids	Dragendorff, Mayer'test	-
Saponins	Foam test, Lieberman-Bourchard	++
Amino acids	0.25% Ninhydrin	+++
Proteins	Xantoproteica	++
Triterpenoids	Salkowski	++
Polysaccarides	Molisch reaction	++

Note: evaluation of reactions: "-"—any reaction; "+"—weak reaction; "++"—strong reaction, but with some deficiencies in coloration; "+++"—best reaction.

Quantitative phytochemical analysis showed that *C. sativus* leaves contained the largest amount of biologically active compounds, primarily phenolic and flavonoid compounds.

Previously, Baba et al. [14] reported that the total phenolic content of ethanolic extracts of *C. sativus* leaves from Jammu and Kashmir (India) was 5.62 mg/g, using the Folin–Ciocalteu reagent method, and that the total flavonoid content was 1.61 ± 0.12 mg RE/g. According to Jadouali et al. [13], the total phenolic content for *C. sativus* leaves from Morocco was 81.69 ± 1.58 mg GAE/mg of powder crude extract, and the total flavonoids content was 50.64 ± 1.63 mg of CE/g of dry plant material. In another investigation [15] of saffron from Morocco, the total phenolic and flavonoid content of the methanolic *C. sativus* leaf extracts was 94 ± 17 mg of GA eq/g and 27 ± 4 mg of quercetin eq/g, respectively. This data is practically correlated with previous studies and is probably due to a single area of plant cultivation. In Spain, in *C. sativus* leaves, the total polyphenol content was 1.5 g GAE/100 g [11]. Thus, it can be seen that the place of cultivation and climatic factors [27–29] have a great influence on the composition of phenolic compounds in *Crocus* leaves.

The current phytochemical analysis revealed the high total phenolic content of *C. sativus* leaves from Ukraine, corresponding to 5.44 ± 0.01 mg GAE/g DW and a flavonoid content of 2.63 ± 0.05 mg RE/g DW. Phenolic compounds play a key role in scavenging free radicals, inhibiting hydrolytic and oxidative enzymes, and exhibiting anti-inflammatory effects [30]. Therefore, it is possible to expect a pronounced antioxidant effect for *Crocus* leaves from Ukraine. Therefore, a deeper study of the chemical profile of *Crocus* leaves was carried out.

3.2. Identification and Content of Phenolic Compounds by HPLC

The most common constituents in the leaves of the Iridaceae plant family are flavone C-glycosides, flavanols, and isoflavones derivatives [7,20,31]. The most studied are leaves and rhizomes of *Iris* spp. In plant derivatives of 7-methyl ester and 7,4-dimethyl ester of apigenin, derivatives of 6,7-methylenedioxyflavans, as well as various isoflavonoids and xanthones, have been found. Derivatives of myricetin, kaempferol, and quercetin were found in the leaves of different *Crocus* species [6]. However, data on the composition of flavonoids in *Crocuses* are very limited.

Compounds identification in *Crocus* leaves extract was based on their coelution with reference compounds and on the UV/MS spectroscopic data according to the HPLC-DAD method. For the identification of phenolic compounds, a more selective and sensitive UPLC method for plants using the negative ionization mode was selected [32]. Six phenolic compounds were detected in the studied *Crocus* leaves for the first time (Figure 1). The quantification of the phenolic compounds using the available standards was carried out (Table 2). All identified compounds were of a minor amount, among them two hydroxycinnamic acid derivatives, i.e., *trans*-cinnamic acid (0.15 ± 0.002 mg/g) and chlorogenic acid (0.31 ± 0.004 mg/g); flavonoid kaempferol (0.032 ± 0.001 mg/g); and two isoflavones—ononin (0.04 ± 0.001 mg/g) and irigenin (0.002 ± 0.001 mg/g)—were determined. Additionally, xanthone mangiferin was found in *C. sativus* leaves for the first time, and its content was the highest (1.26 ± 0.02 mg/g) among all identified compounds. As a result, *trans*-cinnamic acid, chlorogenic acid, ononin, irigenin, and mangiferin were identified first in *C. sativus* leaves from Ukraine.

The HPLC method used was validated for such parameters as linearity range, LOD, LOQ, accuracy, precision, repeatability, and specificity for each analyte. These are presented in Tables 3 and 4. A regression equation and correlation coefficient ranging from 0.9999 to 1.000 revealed a good linearity response within the tested ranges. The series of calibration solutions were prepared and separated under the optimal conditions as described above. The LOD and LOQ values (Table 3) indicate that the proposed method demonstrates good sensitivity for quantifying seven phenolic compounds in *Crocus* leaves. The repeatability was observed in the range of 0.24–1.05%, which was satisfactory and demonstrated the good repeatability of the proposed method. The determination of the main compounds in the test solutions was carried out by comparing the retention times of peaks and UV spectrum obtained from the chromatogram of the standard solution (Table 2). All results showed the repeatability, accuracy, high sensitivity, and good linearity of the method.

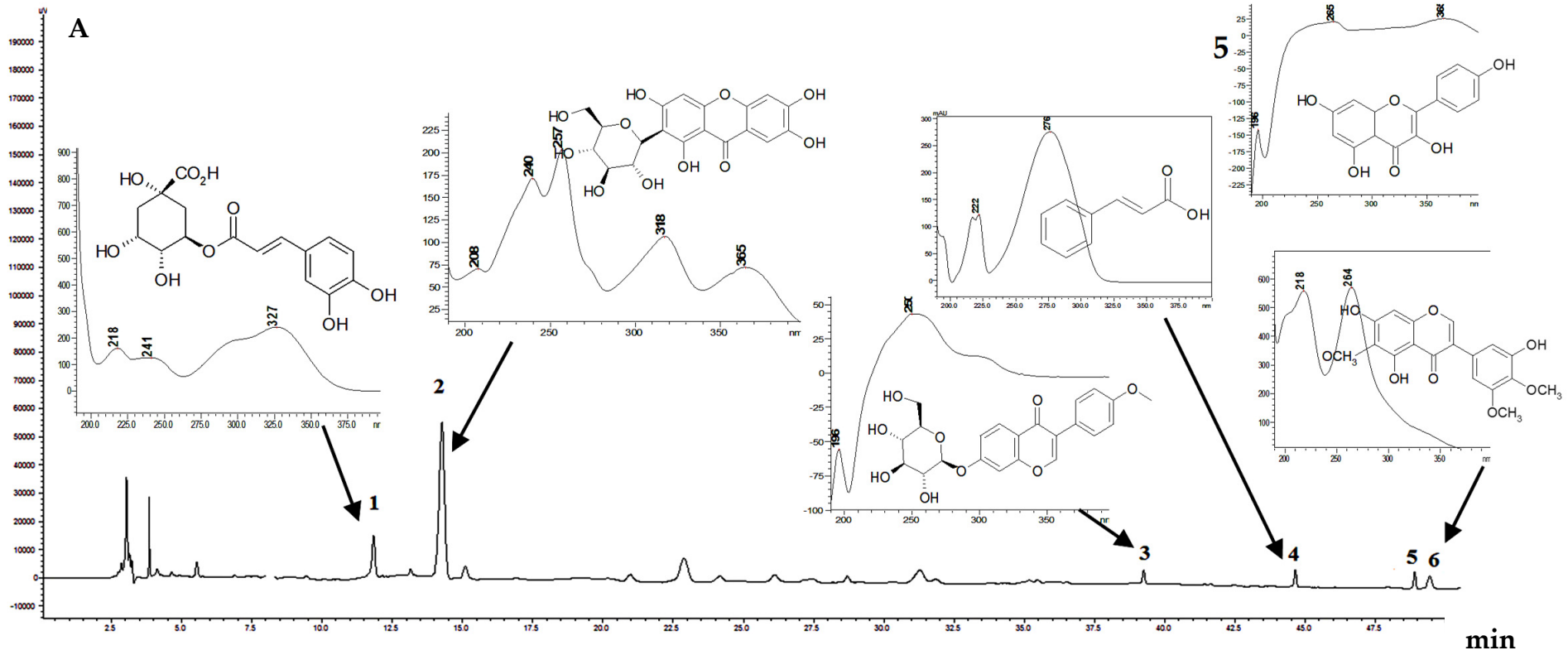

Figure 1. HPLC-DAD chromatogram of the methanolic extract of *C. sativus* leaves: chlorogenic acid (**1**), mangiferin (**2**), ononin (**3**), *trans*-cinnamic acid (**4**), kaempferol (**5**), and irigenin (**6**).

Table 2. HPLC identification of the major constituents of *C. sativus* leaf extracts: chromatographic, UV, mass spectroscopic data of identified compounds, and their content (mg/g).

Peak No	t_R (min)	UV λ_{max} (nm)	Mol. Formula	Mol. Weight, g/mol	Retention Time, min (UPLC-MS)	$[M-H]^-$ (*m*/*z*)	Fragment Ions (−)	Compound	Content, mg/g
1	11.66	218, 241, 327	$C_{16}H_8O_9$	354.31	3.69	353	191, 179, 135	Chlorogenic acid	0.31 ± 0.01
2	14.18	240, 318, 257, 365	$C_{19}H_{18}O_{11}$	422.30	4.21	421	403, 331, 301, 259, 271	Mangiferin	1.26 ± 0.02
3	39.11	250	$C_{22}H_{22}O_9$	430.40	-	-	-	Ononin	0.04 ± 0.00
4	44.76	322, 276	$C_9H_8O_2$	148.16	6.80	147	103	*trans*-Cinnamic acid	0.15 ± 0.00
5	48.99	265, 365	$C_{15}H_{10}O_6$	286.24	7.41	285	239, 187	Kaempferol	0.03 ± 0.00
6	49.78	269	$C_{18}H_{16}O_8$	360.30	7.58	359	344, 329, 314, 286, 258	Irigenin	0.002 ± 0.00

Values of mean ± standard deviation are reported. Statistical comparisons were performed using ANOVA test ($p < 0.05$).

Table 3. Calibration curves, limit of detection (LOD), and limit of quantification (LOQ) data of 6 phenolic reference compounds.

Peak No	Compound	Calibration Curve [a]	Correlation Coefficient r^2 (n = 6)	Linear Range (μg/mL)	RSD (%)	LOD [b] (ng/mL)	LOQ [c] (ng/mL)
1	Chlorogenic acid	y = 29930.2x − 538.361	0.9999	0.36–46	1.29	20	70
2	Mangiferin	y = 29263.5x + 13863.9	0.9997	0.28–145.00	1.32	310	940
3	Ononin	y = 37456.2x + 5132.42	0.9998	0.21–26.24	1.29	49	150
4	*trans*-Cinnamic acid	y = 78502.3x + 0	1.0000	0.30–38.75	0.12	30	90
5	Kaempherol	y = 29888.8x + 1814.27	0.9999	0.14–18.32	0.90	37	110
6	Irigenin	y = 81832.6x + 137668	0.9994	0.54–277.00	0.64	50	160

[a] Compound concentration (mg/mL); y, peak area; [b] LOD, limit of detection (S/N = 3); [c] LOQ, limit of quantification (S/N = 10).

Table 4. Precision and stability of 6 quantified compounds.

Peak No.	Compound	Concentration (μg/mL)	Precision				Repeatability	
			Intra-Day (n = 3)		Inter-Day (n = 3)		Recovery (%)	RSD (%)
			RSD (%)	Accuracy (%)	RSD (%)	Accuracy (%)		
1	Chlorogenic acid	5.75	1.31	101.12	0.38	98.40	100.69	0.86
		23	0.42	99.08	0.73	99.43	99.58	1.05
		46	0.96	100.27	0.48	98.24	101.91	0.97
2	Mangiferin	9.06	0.33	100.46	0.29	100.41	100.29	0.25
		36.25	0.24	99.66	0.32	100.45	100.03	0.39
		145	0.22	100.32	1.10	98.45	99.58	0.99
3	Ononin	3.28	0.81	100.12	0.32	98.39	100.56	0.79
		13.12	0.69	100.44	0.75	99.08	100.72	0.93
		26.24	0.84	99.50	0.82	100.00	99.75	0.75
4	*trans*-Cinnamic acid	4.77	0.68	100.41	0.62	100.09	99.41	0.29
		21.01	1.09	99.55	0.80	100.97	99.55	0.31
		38.795	1.10	98.99	0.47	101.53	99.99	0.56
5	Kaempherol	2.29	0.48	100.69	0.44	99.81	100.35	0.49
		9.16	0.77	100.97	1.01	99.54	100.49	0.68
		18.32	1.14	99.66	1.12	100.84	99.83	0.34
6	Irigenin	17.31	1.08	101.54	1.29	101.84	101.12	0.98
		69.25	0.80	101.14	1.16	101.65	100.93	0.84
		277	0.33	99.53	0.20	99.71	99.74	0.24

3.3. UPLC-MS/MS Analysis of Phenolic Compounds

To confirm the identified compounds in *Crocus* leaves, further UPLC-MS/MS analysis of these components was performed in negative ion mode. Their retention times (t_R), calculated molecular weights, and MS/MS data are shown in Table 2, respectively. All compounds were unambiguously identified with the reference standards comparison. Peak **1** ($[M^-H]^-$ at m/z 353) was assigned to monocaffeoyquinic acids. The MS spectrum of peak **1** was characterized by the loss of one moiety of caffeic acid, due to the ester bond, which readily dissociated and was identified as 3-*O*-caffeylquinic acid or chlorogenic acid in comparison with the reference standards. In the MS spectrum, peak **2** produced the $[M^-H]^-$ ion at m/z 421. The MS^2 spectrum gave the predominant fragment ions at m/z 331 and 301 owing to the neutral loss of $C_3H_6O_3$ and $C_4H_8O_4$, respectively. The low-intensity product ion at m/z 259 appeared in the MS^2 spectrum due to the loss of the glucose residue from the $[M^-H]^-$ ion. The MS^3 spectrum of the precursor ion at m/z 301 gave the product ion at m/z 273, 271, and 257 resulting from the loss of CO, CH_2O, and CO_2, respectively. The RDA fragmentation reaction was also observed in the MS/MS spectrum and corresponded with the literature data [33]. Peak **4** produced the $[M^-H]^-$ ion at m/z 147 at 44.76 min and was identified as *trans*-cinnamic acid. Peaks **5** produced the $[M^-H]^-$ ion at m/z 285 and were identified as kaempferol (calculated for $C_{15}H_{10}O_6$, m/z 286). They were further confirmed by comparing the retention time and the MS/MS spectra with the corresponding standards. In the MS spectrum, peak **6** produced the $[M^-H]^-$ ion at m/z 459 and lost three methyl groups, showing fragments at m/z 344 and m/z 329. The isoflavonoid ononin was identified in the positive ionization mode according to the retro-Diels–Adel reaction (RDA) [33]. Since we were able to identify almost all substances in the negative ion mode, we did not carry out a separate determination for ononin. According to Luo et al. [33], the excimer ion peak $[M^+H]^+$ of ononin has a m/z of 431. However, we did not perform such a definition.

3.4. GF-AAS Analysis of Mineral Composition

The literature data of macro- and microelement analysis in *C. sativus* leaves are limited. It is known that Jadouali et al. [13] only established the calcium, potassium, sodium, nitrogen, iron, and zinc elements, as well as a high concentration of iron (985 mg/kg), in *C. sativus* leaves from Morocco.

As a result of the element profile of *Crocus* leaves from Ukraine, the presence of 15 minerals has been determined (Table 5). Among the identified elements were three macroelements, five mezoelements, and seven ultramicroelements. It is interesting to note the presence of a significant amount of essential and conditionally essential elements, such as calcium (Ca), iron (Fe), manganese (Mn), zinc (Zn), silica (Si), phosphorus (P), and nickel (Ni).

Table 5. Mineral profile of *C. sativus* leaves.

Mineral	Content, mg/100 g	Mineral	Content, mg/100 g
Fe	60.02 ± 1.05	Mg	6.73 ± 0.12
Si	395.11 ± 9.95	Pb	0.03 ± 0.01
Al	73.21 ± 1.28	Mo	0.06 ± 0.01
Mn	8.04 ± 1.14	P	130.04 ± 2.29
K	1525.10 ± 26.81	Ni	0.06 ± 0.01
Ca	550.12 ± 9.67	Cu	0.30 ± 0.01
Zn	7.30 ± 0.13	Na	75.02 ± 1.32
Sr	2.41 ± 0.04		

Co < 0.03; Cd < 0.01; As < 0.01; Hg < 0.01. Values of mean ± standard deviation are reported. Statistical comparisons were performed using ANOVA test ($p < 0.05$).

The results of the mineral content of *C. sativus* leaves from Ukraine are presented in Table 5. In leaves, potassium and calcium were found to be the most abundant minerals with a value of 1525 mg/100 g and 550 mg/100 g, respectively. The high content of potassium in *Crocus* leaves is due to the physiological function of potassium in the process of transport in plants: first and foremost, with the participation of a highly osmotic active substance in the induction of corms pressure and transport of assimilates in the leaf phloem [34]. Calcium accumulates at the deposit places in the cell (vacuole, endoplasmic reticulum), is gradually released, and performs its physiological function [35]. The plant has the highest calcium content in the photosynthetically active leaves vacuole in the form of oxalate or other hardly soluble salts. Calcium passes slowly through the xylem, so its content is lower than potassium. A significant decrease in the content of magnesium (6.73 mg/100 g) in *Crocus* leaves is directly related to the content of potassium in the raw material and is associated with the mechanisms of absorption of these nutrients from the soil [36,37]. It should be noted that the crop *C. sativus* is cultivated without any nitrates, pesticides, or additional growth stimulants [29,38]. Nitrogen, phosphorus, and potassium fertilizers are applied only in April after harvesting *Crocus* corms from the field. The current study showed that *C. sativus* leaves have a pronounced mineral composition without additional fertilizers.

3.5. GC-MS Analysis of Amino Acids and Short-Chained and Long-Chain Carboxylic Acids

The amino acid composition was studied only for *C. sativus* stigma from Spain, Italy, Greece, and Iran, and high levels of alanine, proline, and aspartic acid were found [39]. Fatty acids, such as myristic, stearic, palmitic, palmitoleic, oleic, linoleic, linolenic, and arachidonic acids, have also been found in *C. sativus* stigmas [40,41].

Linoleic acid, linolenic acid, and palmitic acid were the predominant fatty acids of *C. sativus* flowers [42]. Lauric acid and hexadecanoic acid have been found in *C. sativus* tepals, stamens, and stigmas [43]. Ursolic, oleanolic, palmitic, palmitoleic, oleic, linolenic, and linoleic acids were found in *C. sativus* corms [44], as well as amino acids (aspartic acid, glutamic acid, cysteine, serine, glycine, proline, phenylalanyl, leucine, valine, and

methionine) [45]. No published data on the amino acids and fatty acids composition of *C. sativus* leaves has been found.

For the investigation, a method for the simultaneous study of fatty acids, short-chained carboxylic acids, and amino acids in *C. sativus* leaves from Ukraine was used. As a result, 44 metabolites were found. This separation was achieved by the derivatization method. The derivatization procedure is highly dependent on the solvent used. Therefore, in our previous work [22,23], we developed the GC-MS method for organic acid investigation. Different times, temperatures and the effect of the reagent amount on the derivatization process were tested. The optimal conditions for the complete derivatization of most amino acids and organic acids were ultrasonic extraction for 10 min, followed by derivatization with acetonitrile and N-Methyl-N-tert-butyldimethylsilyltrifluoroacetamide MTBSTFA (1:1) for 4 h at 70 °C.

All compounds were identified by comparing the retention times of selected acids in specific MS chromatograms. The content of identified carboxylic acids and amino acids is presented in Table 6.

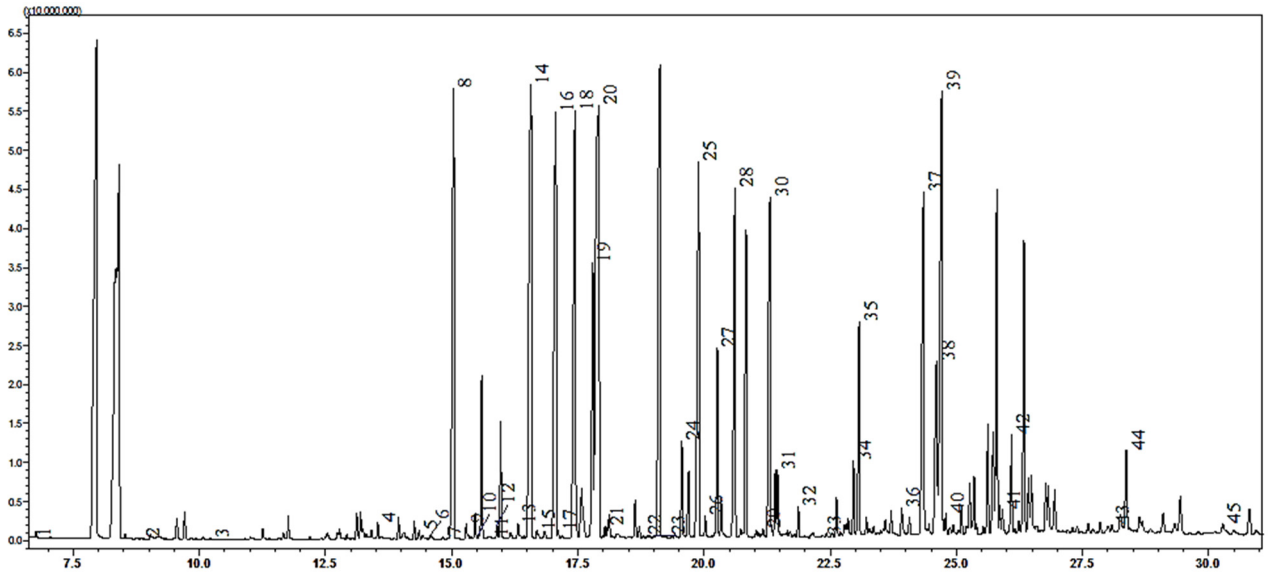

Figure 2. GC-MS chromatograms of the simultaneous study of fatty acids, short-chained carboxylic acids, and amino acids in *C. sativus* leaves from Ukraine.

Table 6. The content of carboxylic acids and amino acids in *C. sativus* leaves, μg/g.

Peak No. *	t_R (min)	Compound	Chemical Formula	Molar Mass, g·mol^{-1}	Mass Peaks	Content
Carboxylic acids						
1	6.71	Butyric Acid	$C_4H_8O_2$	88.11	235	11.97 ± 0.21
2	8.85	Crotonic acid	$C_4H_6O_2$	86.09	307	5.10 ± 0.09
3	10.23	Formic acid	CH_2O_2	46.03	254	4.03 ± 0.07
4	13.54	3,3-Dimethylacrylic acid	$C_5H_8O_2$	100.12	308	187.72 ± 0.30
5	14.36	Lactic acid	$C_3H_6O_3$	90.08	271	98.93 ± 1.74
6	14.59	Glycolic acid	$C_2H_4O_3$	76.05	285	51.90 ± 0.91
7	14.83	Phenylacetic acid	$C_8H_8O_2$	136.15	282	33.83 ± 0.60
10	15.54	3-Hydroxypropionic acid	$C_3H_6O_3$	90.08	252	21.85 ± 0.38
11	15.77	Malonic acid	$C_3H_4O_4$	104.06	245	29.23 ± 0.51
15	16.69	4-Hydroxybutanoic acid	$C_4H_8O_3$	104.10	310	162.36 ± 2.85
21	18.04	Fumaric acid	$C_4H_4O_4$	116.07	280	195.59 ± 3.44
22	18.80	Glutaric acid	$C_5H_8O_4$	132.11	320	51.19 ± 0.90
29	21.17	Myristic acid	$C_{14}H_{28}O_2$	228.37	274	304.08 ± 5.35
31	21.46	Malic acid	$C_4H_6O_5$	134.09	324	925.24 ± 16.27
35	23.07	Palmitic Acid	$C_{16}H_{32}O_2$	256.42	402	6997.26 ± 123.03
38	24.60	Linoelaidic acid	$C_{18}H_{32}O_2$	280.40	434	9880.26 ± 173.72

Table 6. *Cont.*

Peak No. *	t_R (min)	Compound	Chemical Formula	Molar Mass, g·mol^{-1}	Mass Peaks	Content
			Carboxylic acids			
39	24.71	α-Linolenic acid	$C_{18}H_{30}O_2$	278.40	441	22,490.08 ± 395.43
40	24.79	Stearic acid	$C_{18}H_{36}O_2$	284.50	336	974.33 ± 17.13
41	25.91	Citric acid	$C_6H_8O_7$	192.12	376	834.86 ± 14.68
43	28.08	Behenic acid	$C_{22}H_{44}O_2$	340.60	346	916.91 ± 16.12
45	30.28	Lignoceric acid	$C_{24}H_{48}O_2$	368.60	334	1611.20 ± 28.33
			Amino acids			
16	17.07	L-Leucine	$C_6H_{13}NO_2$	131.17	397	6955.65 ± 122.30
17	17.11	L-Serine	$C_3H_7NO_3$	105.09	291	35.87 ± 0.63
18	17.44	Isoleucine	$C_6H_{13}NO_2$	131.17	400	7965.70 ± 140.06
19	17.80	γ-Aminobutyric acid (recalculated to Glutamic acid)	$C_4H_9NO_2$	103.12	366	2809.52 ± 49.40
20	17.92	L-Proline	$C_5H_9NO_2$	115.13	370	1725.11 ± 30.33
23	19.01	L-Aspartic acid (recalculated to Glutamic acid)	$C_4H_7NO_4$	133.10	242	38.54 ± 0.68
25	19.90	L-Pyroglutamic acid	$C_5H_7NO_3$	129.11	439	6233.15 ± 109.60
26	20.03	L-Methionine	$C_5H_{11}NO_2S$	149.21	344	398.40 ± 7.00
27	20.26	L-Serine	$C_3H_7NO_3$	105.09	414	920.59 ± 16.19
28	20.61	L-Threonine	$C_4H_9NO_3$	119.12	443	2701.71 ± 47.50
30	21.30	L-Phenylalanine	$C_9H_{11}NO_2$	165.19	432	6032.94 ± 106.07
32	21.87	L-Aspartic acid (recalculated to Glutamic acid)	$C_4H_7NO_4$	133.10	399	350.57 ± 6.16
33	22.36	L-Cysteine	$C_3H_7NO_2S$	121.16	293	38.71 ± 0.68
34	22.96	L-Glutamic acid	$C_5H_9NO_4$	147.13	417	1000.94 ± 17.60
36	23.91	L-Lysine	$C_6H_{14}N_2O_2$	146.19	424	866.09 ± 15.23
37	24.35	L-Glutamine	$C_5H_{10}N_2O_3$	146.14	448	7374.10 ± 129.66
42	26.09	L-Tyrosine	$C_9H_{11}NO_3$	181.19	450	837.27 ± 14.72
44	28.37	L-Tryptophan (recalculated to Proline)	$C_{11}H_{12}N_2O_2$	204.22	430	1186.92 ± 20.87
			Phosphorus Compound			
24	19.56	*Tris*-Phosphoric acid	$C_{24}H_{51}O_4P$	434.6	417	1305.02 ± 22.05
			Total short-chained carboxylic acids			2613.80
			Total long-chain carboxylic acids			63,544.28
			Total amount of aminoacids			43,174.12
			Other compounds			1305.02

*—The numbering of compounds in Table 6 and Figure 2 are identical. Values of mean ± standard deviation are reported. Statistical comparisons were performed using ANOVA test ($p < 0.05$).

In the current study, we have determined the presence and quantity of 22 amino acids, 14 short-chained carboxylic acids, 7 fatty acids, and 1 phosphorus acid (*tris*-phosphoric acid) in *C. sativus* leaves by the GC-MS method. According to the results, 45 different acids are present in *Crocus* leaves. These are the first complete results of the composition of carboxylic and amino acids in *Crocus* leaves. Furthermore, this method was used to quantify the acids content in the raw material (Table 6, Figure 2).

L-Glutamine, Isoleucine, L-alanine, L-leucine, L-pyroglutamic, and L-phenylalanine acids were present in large amounts—7374 μg/g, 7965 μg/g, 6744 μg/g, 6955 μg/g, 6233 μg/g, and 6032 μg/g, respectively. High amounts of L-glutamine may be due to the glutamate synthase cycle [46], where L-glutamine is formed from simple compounds, such as ammonia, and only then are other amino acids, such as arginine, tryptophan, histidine, or asparagine, formed [47]. The seven essential amino acids in leaves that were present in high concentration included isoleucine (7965 μg/g), L-leucine (6955 μg/g),

L-lysine (866 μg/g), L-methionine (398 μg/g), L-phenylalanine (6032 μg/g), L-threonine (2701 μg/g), L-tryptophan (1186 μg/g), and L-valine (6151 μg/g). As for the total amino acids content in *C. sativus* leaves, it exceeds 43,174 μg/g and shows that this raw material can be an excellent and underestimated source of proteinogenic amino acids [48], especially glutamine, phenylalanine, isoleucine, alanine, and leucine.

For the composition of amino acids, a high content of isoleucine, L-leucine, L-phenylalanine, L-pyroglutamic acid, L-glutamine, L-alanine, and L-tryptophan was observed along with a low content of L-glycine, which indicates the plant's response to cold stress [49,50] as well as consistent with previously published data for this genus [51].

Among the organic acids of the tricarboxylic acid cycle (TCA cycle) in *C. sativus* leaves were found citric acid (834 μg/g), fumaric acid (195 μg/g), and malic acid (925 μg/g), which had the highest content of all short-chain carboxylic acids. In a TCA cycle, citric acid and malic acid are generally the most accumulated organic acids in plants [52]; at the same time, *Crocus* leaves have an increased content of malic acid during acclimatization to the low temperature [53,54] of Ukraine in the postharvest period. In addition, the path analysis of the effects of different acids on malic acid content revealed that citric acid has a maximum direct effect on malic acid [55]. Furthermore, 3,3-dimethylacrylic acid, 4-hydroxybutanoic acid, and lactic acid were found in amounts of 187 μg/g, 162 μg/g, and 98 μg/g, respectively. The content of other organic acids found did not exceed 50 μg/g. Additionally, polyphenols and organic acids may be responsible for the antioxidant activity of the plant extracts.

The analysis of fatty acids of *C. sativus* leaves showed that seven acids (myristic acid, palmitic acid, linoelaidic acid, α-linolenic acid, stearic acid, behenic acid, and lignoceric acid) were detected. The total long-chain carboxylic acids content was 63,544 μg/g of the total acids yield and is the largest part of all identified acids in the *Crocus* leaves. The dominant saturated acid was palmitic (C16:0) in *Crocus* leaves, with a content of 6997 μg/g, followed by lignocerinic acid ($C_{24:0}$), with a content of 1611 μg/g. The content of unsaturated fatty acids was two times higher than that of saturated acids.

The GC-MS analyses revealed the essential fatty acids α-linolenic acid (ω3) (22,490 μg/g) and linoelaidic acid (ω6) (9880 μg/g) as major constituents among the acids that were found in *Crocus* leaves for the first time. The biosynthesis of dienoic and trienoic acids 18:2ω6 and 18:3ω3 plays a defining role in the adaptation of plants to hypothermia. These polyunsaturated fatty acids regulate the "fluidity" of cell membranes in a wide temperature range and influence the resistance of plants to difficult environments. In stressful environments (low temperature, freezing, salinity, drought), the amount of unsaturated fatty acids is much higher than the saturated acids content in the plant organs [56,57]. The high content of linolenic acid in *Crocus* leaves is probably due precisely to the plant's adaptation to the low temperatures in Ukraine (December +3/−5 °C) during the harvested period.

The presence of *tris*-phosphoric acid (1305 μg/g) in *Crocus* leaves, according to Hu et al. [58], also signals the plant's response to temperature stress, which is expressed in shifts in the metabolite profile. *Tris*-phosphoric acid could supply adequate P secondary compounds for cell energy metabolism in stress conditions. In this case, an increase of organic acids (malic acid, citric acid, *tris*-phosphoric acid), amino acids (isoleucine, pyroglutamic acid, phenylalanine, alanine, glutamine), and unsaturated fatty acids (linoelaidic acid, linoleic acid) for this plant was established. Some of the phenolic compounds and organic acids might be responsible for the high antioxidant activities, antimicrobial activity, blood glucose control, and regulation of lipid abnormalities [59,60]. Thus, the leaves of *C. sativus* from Ukraine need to be further explored.

3.6. Antibacterial Activity

The results showed that ethanolic and water extracts of *C. sativus* leaves effectively suppressed the growth of the test strains of microorganisms with variable potency. As indicated in Table 7, the ethanolic and water leaf extracts had the maximum zone of inhibition against *B. subtilis* at 20.30 ± 0.36 mm and 18.00 ± 0.32 mm, respectively. Both

extracts showed almost the same inhibitory effect against four pathogenic strains (*E. coli*, *P. vulgaris*, *P. aeruginosa*, *C. albicans*). Concerning *S. aureus*, only ethanolic *C. sativus* leaves extract showed potential activity, with an inhibition zone of 18.00 ± 0.32 mm. It has been reported previously that the methanolic extract of *C. sativus* leaves from Morocco [13] did not show antibacterial activity against *S. aureus*, *B. subtilis*, *P. aeruginosa*, and *P. mirabilis*; however, it had antibacterial activity against *Listeria* spp. Our data confirmed that the leaf extracts of *C. sativus* from Ukraine possessed significant ($p < 0.05$) antibacterial activities against Gram-positive bacteria.

Table 7. Antimicrobial activity and minimum inhibitory concentration (MIC) of *C. sativus* leaf extracts.

Test Strains	Water Extract		Ethanolic Extract		*Chlorophyllipt*
	Zone of Inhibition (mm)	MIC Values (μg/mL)	Zone of Inhibition (mm)	MIC Values (μg/mL)	
S. aureus 25,923 ATCC	16.55 ± 0.30	125	18.00 ± 0.32	125	22.50 ± 0.40
E. coli 25,922 ATCC	16.00 ± 0.28	250	16.70 ± 0.30	250	12.50 ± 0.22
P. vulgaris 4636 NCTC	16.50 ± 0.30	500	17.00 ± 0.30	250	growth
P. aeruginosa 27,853 ATCC	15.40 ± 0.27	500	15.60 ± 0.27	250	growth
B. subtilis 6633 ATCC	18.00 ± 0.32	125	20.30 ± 0.36	125	growth
C. albicans 885/653 ATCC	15.20 ± 0.27	250	15.70 ± 0.28	125	growth

The difference is relatively accurate to control, ($p \leq 0.05$).

The data of the serial dilutions method showed that the lowest MIC values (125 μg/mL) were demonstrated by both leaf extracts against *S. aureus* and *B. subtilis*. In general, the ethanolic extract had lower MIC values than the water extract, due to the chemical composition of the extracts. In addition, this is consistent with the literature data that ethanolic extracts have a higher antimicrobial activity than aqueous extracts [61,62]. In further studies, it is necessary to select an extractant for the maximum yield of phenolic compounds, amino acids, fatty acids, and terpenes in crude extracts of *C. sativus* leaves for pharmacological assessment. The established antibacterial activity of *Crocus* leaves may be due to the presence of various phenolic compounds and other classes of plant compounds. Therefore, despite the well-known medicinal properties of *C. sativus* (saffron) stigma, our research suggests that *Crocus* leaves may also be used as a valuable plant source of naturally occurring bioactive molecules to develop new functional food and pharmaceutical ingredients, with the potential not only to promote human health but also to improve the biovalorisation of saffron.

4. Conclusions

The results obtained show that *C. sativus* leaves as a saffron by-product are a good source of phenolic compounds; they contain sufficient amounts of macro- and microelements, essential amino acids, different organic acids, and fatty acids. In addition, the aerial part of *Crocus* adapts to the weather conditions of Ukraine due to the increased content of polyunsaturated fatty acids and malic acid. The results of antimicrobial analyses showed that *Crocus* leaf extracts have significantly higher bactericidal activities than fungicidal ones ($p < 0.05$). This is the first complete study of the chemical composition of Ukrainian *C. sativus* leaves. Saffron obtained from *C. sativus* in Ukraine has shown good results as a food spice and, according to ISO 3632, *Crocus* stigma correspond to the I quality category [29]. Thus, the cultivation of this plant is in demand, and there is a wide raw material base of *Crocus* leaves for the pharmaceutical industry. The presented study shows the prospects for the integrated use of *Crocus* raw materials and excludes the formation of production waste.

Author Contributions: Conceptualization and methodology, O.M., R.L., and V.G.; investigation, O.M., I.B., L.S., and L.I.; writing—original draft preparation, O.M.; writing—review and editing, R.L., L.I., and V.G. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: UPLC-MS and mass spectra data of compounds will be supplied upon request.

Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

HPLC-UV	High-performance liquid chromatography with ultraviolet detection
HPLC-MS	High-performance liquid chromatography mass spectrometry
GF-AAS	Graphite furnace atomic absorption spectroscopy
TLC	Thin-layer chromatography
GAE/g	gallic acid equivalent
RE/g	rutin equivalent
DW	dry weight
MTBSTFA	N-Methyl-N-tert-butyldimethylsilyltrifluoroacetamide

References

1. Salmerón-Manzano, E.; Garrido-Cardenas, J.A.; Manzano-Agugliaro, F. Worldwide Research Trends on Medicinal Plants. *Int. J. Environ. Res. Public Health* **2020**, *17*, 3376. [CrossRef]
2. Sofowora, A.; Ogunbodede, E.; Onayade, A. The role and place of medicinal plants in the strategies for disease prevention. *Afr. J. Tradit. Complement. Altern. Med.* **2013**, *10*, 210–229. [CrossRef]
3. Zagayko, A.L.; Kolisnyk, T.Y.; Chumak, O.I.; Ruban, O.A.; Koshovyi, O.M. Evaluation of anti-obesity and lipid-lowering properties of *Vaccinium myrtillus* leaves powder extract in a hamster model. *J. Basic Clin. Physiol. Pharmacol.* **2018**, *29*, 697–703. [CrossRef]
4. Mykhailenko, O.; Gudžinskas, Z.; Kovalyov, V.; Desenko, V.; Ivanauskas, L.; Bezruk, I.; Georgiyants, V. Effect of ecological factors on the accumulation of phenolic compounds in *Iris* species from Latvia, Lithuania and Ukraine. *Phytochem. Anal.* **2020**, *31*, 1–19. [CrossRef] [PubMed]
5. Fernández, J.A.; Santana, O.; Guardiola, J.L.; Molina, R.V.; Heslop-Harrison, P.; De-Los-Mozos-Pascual, M. The world saffron and *Crocus* collection: Strategies for establishment, management, characterisation and utilisation. *Genet. Resour. Crop Evol.* **2011**, *58*, 125–137. [CrossRef]
6. Mykhailenko, O.; Kovalyov, V.; Goryacha, O.; Ivanauskas, L.; Georgiyants, V. Biologically active compounds and pharmacological activities of species of the genus *Crocus*: A review. *Phytochemistry* **2019**, *162*, 56–89. [CrossRef] [PubMed]
7. Maggi, M.A.; Bisti, S.; Picco, C. Saffron: Chemical composition and neuroprotective activity. *Molecules* **2020**, *25*, 5618. [CrossRef]
8. Almodóvar, P.; Briskey, D.; Rao, A.; Prodanov, M.; Inarejos-García, A.M. Bioaccessibility and pharmacokinetics of a commercial saffron (*Crocus sativus* L.) extract. *Evid-Based Compl. Alt.* **2020**, *2020*, 1575730. [CrossRef]
9. Lambrianidou, A.; Koutsougianni, F.; Papapostolou, I.; Dimas, K. Recent advances on the anticancer properties of saffron (*Crocus sativus* L.) and its major constituents. *Molecules* **2020**, *26*, 86. [CrossRef] [PubMed]
10. Moratalla-López, N.; Bagur, M.J.; Lorenzo, C.; Salinas, M.E.M.R.; Alonso, G.L. Bioactivity and bioavailability of the major metabolites of *Crocus sativus* L. flower. *Molecules* **2019**, *24*, 2827. [CrossRef]
11. Sánchez-Vioque, R.; Santana-Méridas, O.; Polissiou, M.; Vioque, J.; Astraka, K.; Alaiz, M.; Herraiz-Peñalver, D.; Tarantilis, P.A.; Girón-Calle, J. Polyphenol composition and in vitro antiproliferative eect of corm, tepal and leaf from *Crocus sativus* L. on human colon adenarcinoma cells (Caco-2). *J. Funct. Foods* **2016**, *24*, 18–25. [CrossRef]
12. Smolskaite, L.; Talou, T.; Fabre, N.; Venskutonis, P.R. Valorization of saffron industry by-products: Bioactive compounds from leaves. In *Innovations for Food Science and Production, Proceedings of the 6th Baltic Conference on Food Science and Technology FOODBALT-2011, Jelgava, Latvia, 5–6 May 2011*; Straumite, E., Ed.; Faculty of Food Technology: Riga, Latvia, 2011; pp. 67–72.
13. Jadouali, S.M.; Atifi, H.; Bouzoubaa, Z.; Majourhat, K.; Gharby, S.; Achemchem, F.; Elmoslih, A.; Laknifli, A.; Mamouni, R. Chemical characterization, antioxidant and antibacterial activity of Moroccan *Crocus sativus* L. petals and leaves. *J. Mater. Environ. Sci.* **2018**, *9*, 113–118. [CrossRef]
14. Baba, S.A.; Malik, A.H.; Wani, Z.A.; Mohiuddin, T.; Shah, Z.; Abbas, N.; Ashraf, N. Phytochemical analysis and antioxidant activity of different tissue types of *Crocus sativus* and oxidative stress alleviating potential of saffron extract in plants, bacteria, and yeast. *S. Afr. J. Botany* **2015**, *99*, 80–87. [CrossRef]
15. Lahmass, I.; Lamkami, T.; Delporte, C.; Sikdar, S.; Antwerpen, P.V.; Saalaoui, E.; Megalizzi, V. The waste of saffron crop, a cheap source of bioactive compounds. *J. Funct. Foods* **2017**, *35*, 341–351. [CrossRef]
16. Morsy, N. Phytochemical analysis of biologically active constituents of medicinal plants. *Main Group Chem.* **2014**, *13*, 7–21. [CrossRef]

17. Mykchailenko, O.O.; Kovalyov, V.M. Phenolic compounds of the genus *Iris* plants (*Iridaceae*). *Ceska. Slov. Farm.* **2016**, *65*, 70–77.
18. SO Ukrainian Scientific Pharmacopoeial Center of Drugs Quality. *State Pharmacopoeia of Ukraine*, 2nd ed.; SO Ukrainian Scientific Pharmacopoeial Center of Drugs Quality: Kharkiv, Ukraine, 2015; Volume 3. (In Ukrainian)
19. Ilina, T.; Kashpur, N.; Granica, S.; Bazylko, A.; Shinkovenko, I.; Kovalyova, A.; Goryacha, O.; Koshovyi, O. Phytochemical profiles and in vitro immunomodulatory activity of ethanolic extracts from *Galium aparine* L. *Plants* **2019**, *8*, 541. [CrossRef]
20. Mykhailenko, O.; Korinek, M.; Ivanauskas, L.; Bezruk, I.; Myhal, A.; Petrikaitė, V.; El-Shazly, M.; Yen, C.-H.; Chen, B.-H.; Georgiyants, V.; et al. Qualitative and quantitative analysis of Ukrainian *Iris* species: A fresh look on their content and biological activities. *Molecules* **2020**, *25*, 4588. [CrossRef] [PubMed]
21. European Medicines Agency. *Validation of Analytical Procedures: Text and Methodology*; Q2 (R1); International Conference on Harmonization Secretariat: Geneva, Switzerland, 2005; p. 17.
22. Mykhailenko, O.; Ivanauskas, L.; Bezruk, I.; Lesyk, R.; Georgiyants, V. Comparative investigation of amino acids content in the dry extracts of *Juno bucharica*, *Gladiolus hybrid* zefir, *Iris hungarica*, *Iris variegata* and *Crocus sativus* raw materials of Ukrainian flora. *Sci. Pharm.* **2020**, *88*, 8. [CrossRef]
23. Jarukas, L.; Mykhailenko, O.; Baranauskaite, J.; Marksa, M.; Ivanauskas, L. Investigation of organic acids in saffron stigmas (*Crocus sativus* L.) extract by derivatization method and determination by GC/MS. *Molecules* **2020**, *25*, 3427. [CrossRef] [PubMed]
24. Clinical and Laboratory Standards Institute. *Performance Standards for Antimicrobial Susceptibility Testing*; Twenty-Second Informational Supplements; Document M100-S22; CLSI: Wayne, PA, USA, 2012; Volume 32, p. 188.
25. Coyle, M.B. *Manual of Antimicrobial Susceptibility Testing*; American Society for Microbiology: Washington, DC, USA, 2005; p. 236.
26. Kovalev, V.M.; Mykhailenko, O.O.; Krechun, A.V.; Osolodchenko, T.P. Antimicrobial activity of extracts of *Iris hungarica* and *Iris sibirica*. *Ann. Mechnikov. Inst.* **2017**, *2*, 57–64. [CrossRef]
27. Yuan, Y.; Tang, X.; Jia, Z.; Li, C.; Ma, J.; Zhang, J. The effects of ecological factors on the main medicinal components of *Dendrobium officinale* under Different Cultivation Modes. *Forests* **2020**, *11*, 94. [CrossRef]
28. Liu, W.; Yin, D.; Li, N.; Hou, X.; Wang, D.; Li, D.; Liu, J. Influence of environmental factors on the active substance production and antioxidant activity in *Potentilla fruticosa* L. and its quality assessment. *Sci. Rep.* **2016**, *6*, 28591. [CrossRef]
29. Mykhailenko, O.; Desenko, V.; Ivanauskas, L.; Georgiyants, V. Standard operating procedure of Ukrainian saffron cultivation according with Good Agricultural and Collection Practices to assure quality and traceability. *Ind. Crops Prod.* **2020**, *151*, 112376–112387. [CrossRef]
30. Frankel, E.N. Nutritional Benefits of Flavonoids. In *Food Factors for Cancer Prevention*; Ohigashi, H., Osawa, T., Terao, J., Watanabe, S., Yoshikawa, T., Eds.; Springer: Tokyo, Japan, 1997; pp. 613–616. [CrossRef]
31. Singab, A.N.B.; Ayoub, I.M.; El-Shazly, M.; Korinek, M.; Wu, T.-Y.; Cheng, Y.-B.; Chang, F.-R.; Wu, Y.-C. Shedding the light on *Iridaceae*: Ethnobotany, phytochemistry and biological activity. *Ind. Crops Prod.* **2016**, *92*, 308–335. [CrossRef]
32. Lim, T.K. *Edible Medicinal and Non Medicinal Plants*; Springer: Dordrecht, The Netherlands, 2014; Volume 8, pp. 77–136.
33. U.S. Department of Agriculture, Agricultural Research Service (USDA). USDA National Nutrient Database for Standard Reference, Release 26. Nutrient Data Laboratory Home Page. 2013. Available online: http://www.ars.usda.gov/ba/bhnrc/ndl (accessed on 9 May 2021).
34. Fabre, N.; Rustan, I.; de Hoffmann, E.; Quetin-Leclercq, J. Determination of flavone, flavonol, and flavanone aglycones by negative ion liquid chromatography electrospray ion trap mass spectrometry. *J. Am. Soc. Mass Spectrom.* **2001**, *12*, 707–715. [CrossRef]
35. Luo, L.; Gao, W.; Zhang, Y.; Liu, C.; Wang, G.; Wu, H.; Gao, W. Integrated phytochemical analysis based on UPLC-MS and network pharmacology approaches to explore the quality control markers for the quality assessment of *Trifolium pratense* L. *Molecules* **2020**, *25*, 3787. [CrossRef] [PubMed]
36. Taiz, L.; Zeiger, E. *Plant Physiology*, 2nd ed.; Sinauer Association Inc. Publ.: Sunderland, MA, USA, 1998. [CrossRef]
37. Tůma, J.; Skalický, M.; Tůmová, L.; Bláhová, P.; Rosůlková, M. Potassium, magnesium and calcium content in individual parts of *Phaseolus vulgaris* L. plant as related to potassium and magnesium nutrition. *Plant Soil Environ.* **2004**, *50*, 18–26. [CrossRef]
38. Marschner, H. *Mineral Nutrition of Higher Plants*, 2nd ed.; Academic Press Inc.: London, UK, 1997; 889p.
39. Tůma, J.; Matula, J. The effect of gradated nitrogen application rates on the yield, nutritive value, and mineral composition of orchard-grass herbage. *Rostl. Výr.* **1994**, *40*, 241–252.
40. Gresta, F.; Avola, G.; Lombardo, G.M.; Siracusa, L.; Ruberto, G. Analysis of flowering, stigmas yield and qualitative traits of saffron (*Crocus sativus* L.) as affected by environmental conditions. *Sci. Hort.* **2009**, *119*, 320–324. [CrossRef]
41. Del Campo, C.P.; Garde-Cerdan, T.; Sanchez, A.M.; Maggi, L.; Carmona, M.; Alonso, G.L. Determination of free amino acids and ammonium ion in saffron (*Crocus sativus* L.) from different geographical origins. *Food Chem.* **2009**, *114*, 1542–1548. [CrossRef]
42. Feizy, J.; Reyhani, N. Gas chromatographic determination of phytosterols and fatty acids profile in saffron petals. *Can. Chem. Trans.* **2016**, *4*, 389–397. [CrossRef]
43. Zheng, C.J.; Li, L.; Ma, W.H.; Han, T.; Qin, L.P. Chemical constituents and bioactivities of the liposoluble fraction from different medicinal parts of *Crocus sativus*. *Pharm. Biol.* **2011**, *49*, 756–763. [CrossRef]
44. Masuda, A.; Mori, K.; Miyazawa, M. Comparative analysis of volatile compounds from corms of *Crocus sativus* and *C. vernus*. *Chem. Nat. Com.* **2012**, *48*, 319–321. [CrossRef]
45. Bagri, J.; Yadav, A.; Anwar, K.; Dkhar, J.; Singla-Pareek, S.L.; Pareek, A. Metabolic shift in sugars and amino acids regulates sprouting in Saffron corm. *Sci. Rep.* **2017**, *7*, 11904–11914. [CrossRef]

46. Walker, M.C.; van der Donk, W.A. The many roles of glutamate in metabolism. *J. Ind. Microbiol. Biotechnol.* **2016**, *43*, 419–430. [CrossRef]
47. Keller, B.; Keller, E.; Salcher, O.; Lingens, F. Arogenate (Pretyrosine) pathway of tyrosine and phenylalanine biosynthesis in *Pseudomonas aureofaciens* ATCC 15926. *Microbiology* **1982**, *128*, 1199–1202. [CrossRef] [PubMed]
48. Ayon, N.J. Features, roles and chiral analyses of proteinogenic amino acids. *AIMS Mol. Sci.* **2020**, *7*, 229–268. [CrossRef]
49. Taïbi, K.; Del Campo, A.D.; Vilagrosa, A.; Bellés, J.M.; López-Gresa, M.P.; López-Nicolás, J.M.; Mulet, J.M. Distinctive physiological and molecular responses to cold stress among cold-tolerant and cold-sensitive *Pinus halepensis* seed sources. *BMC Plant Biol.* **2018**, *18*, 236–247. [CrossRef] [PubMed]
50. Chen, T.H.H.; Uemura, M.; Fujikawa, S. *Cold Hardiness in Plants: Molecular Genetics, Cell Biology, and Physiology*; CABI Publishing: Wallingford, UK, 2006.
51. Pastor-Férriz, T.; De-los-Mozos-Pascual, M.; Renau-Morata, B.; Nebauer, S.G.; Sanchis, E.; Busconi, M.; Fernández, J.A.; Kamenetsky, R.; Molina, R.V. Ongoing evolution in the genus *Crocus*: Diversity of flowering strategies on the way to hysteranthy. *Plants* **2021**, *10*, 477. [CrossRef]
52. Igamberdiev, A.U.; Eprintsev, A.T. Organic acids: The pools of fixed carbon involved in redox regulation and energy balance in higher plants. *Front. Plant Sci.* **2016**, *7*, 1042. [CrossRef]
53. Crecelius, F.; Streb, P.; Feierabend, J. Malate metabolism and reactions of oxido reduction in cold-hardened winter rye (*Secale cereale* L.) leaves. *J. Exp. Bot.* **2003**, *54*, 1075–1083. [CrossRef]
54. Streb, P.; Josse, E.; Gallouet, E.; Baptist, F.; Kuntz, M.; Cornic, G. Evidence for alternative electron sinks to photosynthetic carbon assimilation in the high mountain plant species *Ranunculus glacialis*. *Plant Cell Environ.* **2005**, *28*, 1123–1135. [CrossRef]
55. Kumar, V.; Sharma, A.; Bhardwaj, R.; Thukral, A.K. Analysis of organic acids of tricarboxylic acid cycle in plants using GC-MS, and system modeling. *J. Anal. Sci. Technol.* **2017**, *8*, 20–29. [CrossRef]
56. Upchurch, R.G. Fatty acid unsaturation, mobilization, and regulation in the response of plants to stress. *Biotechnol. Lett.* **2008**, *30*, 967–977. [CrossRef]
57. Mykhailenko, O.; Kovalyov, V.; Kovalyov, S.; Toryanik, E.; Osolodchenko, T.; Buidin, Y. Fatty acid composition of lipids of *Iris sibirica*. *Ceska Slov. Farm.* **2017**, *66*, 220–227.
58. Hu, T.; Liu, S.Q.; Amombo, E.; Fu, J.M. Stress memory induced rearrangements of HSP transcription, photosystem II photochemistry and metabolism of tall fescue (*Festuca arundinacea* Schreb.) in response to high-temperature stress. *Front. Plant Sci.* **2015**, *16*, 403. [CrossRef]
59. Liu, Q.; Tang, G.Y.; Zhao, C.N.; Feng, X.L.; Xu, X.Y.; Cao, S.Y.; Meng, X.; Li, S.; Gan, R.Y.; Li, H.B. Comparison of antioxidant activities of different grape varieties. *Molecules* **2018**, *23*, 2432. [CrossRef]
60. Liu, Q.; Tang, G.Y.; Zhao, C.N.; Gan, R.Y.; Li, H.B. Antioxidant activities, phenolic profiles, and organic acid contents of fruit vinegars. *Antioxidants* **2019**, *8*, 78. [CrossRef]
61. Al-Hashimi, A.G. Antioxidant and antibacterial activities of *Hibiscus sabdariffa* L. extracts. *Afr. J. Food Sci.* **2012**, *6*, 506–511. [CrossRef]
62. Xu, D.H.; Huang, Y.S.; Jiang, D.Q.; Yuan, K. The essential oils chemical compositions and antimicrobial, antioxidant activities and toxicity of three *Hyptis* species. *Pharm. Biol.* **2013**, *51*, 1125–1130. [CrossRef]