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Quantitative and Qualitative Determination of Polyphenolic Compounds in *Castanea sativa* Leaves and Evaluation of Their Biological Activities

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Abstract: The aim of the study was to evaluate the polyphenol profile of *Castanea sativa* leaf methanolic extract and further evaluate its biological activities in vitro. After purification with an RP-18 resin, the extract was assessed for its polyphenol profile by UPLC-PDA-MS/MS, as well as for the antioxidant potential (ABTS, CUPRAC, ChA, ROS scavenging methods), anticancer, antiobesity, antidiabetic and antimicrobial potential. Eighteen polyphenols were identified and the dominant compounds were chestatin followed by quercetin 3-*O*-glucoside. The total phenolic content of the extract showed a value of 1426.55 mg/100 g d.w. The obtained preparation showed the ability to scavenge $O_2^{\bullet -}$ (0.067 mg/mL) and $OH^{\bullet}$ (0.207 mg/mL) radicals and had a stronger anti-obesity than anti-diabetic effect. Additionally, this extract exhibited a strong anticancer activity against the Caco-2 line (153.54 µg/mL), with anti-migratory and anti-proliferative activity. In turn, among the tested strains, the highest activity was demonstrated against *Staphylococcus aureus*. Moreover, the effects demonstrated were strongly dependent on the content of polyphenols. In conclusion, *C. sativa* is a promising source of natural antioxidant, antibacterial, antiobesity, antidiabetic and chemopreventive compounds for food-pharma industry; however, further experimental studies are needed to validate its pharmacological properties.

Keywords: *Castanea sativa*; leaves; polyphenols; antioxidant; anticancer; antibacterial activities

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Citation: Żurek, N.; Pawłowska, A.M.; Pycia, K.; Potocki, L.; Kapusta, I.T. Quantitative and Qualitative Determination of Polyphenolic Compounds in *Castanea sativa* Leaves and Evaluation of Their Biological Activities. *Appl. Sci.* **2024**, *14*, 3859. https://doi.org/10.3390/app14093859

Academic Editors: Marta Mesías and Tiane Finimundy

Received: 20 March 2024
Revised: 29 April 2024
Accepted: 29 April 2024
Published: 30 April 2024

1. Introduction

Oxygen (O_2) is an essential factor for the life of humans, plants and animals. Particularly dangerous to human health are reactive and highly unstable oxygen molecules, i.e., free radicals (ROS), which are formed as a result of cellular metabolism [1–3]. Chemically, they are independent atoms or molecules with at least one unpaired electron in their valence shell. They demonstrate the ability to withdraw electrons from other molecules, striving for stability [2]. In a properly functioning body, they perform a number of important functions, such as stimulating the transport of glucose into cellular structures, controlling endothelial function and participating in the transmission of impulses to cells. However, their excessive production in the body under the influence of, among others, stress factors leads to the so-called oxidative stress, i.e., oxidation of important cellular structures. As a result, ROS generate cardiovascular diseases, atherosclerosis, eye diseases, including cataracts, chronic inflammation, neurodegenerative diseases such as Alzheimer's or Parkinson's disease, and numerous cancer diseases [1,4,5]. Reactive oxygen species can be neutralized using phenolic compounds found in various plants such as fruits, vegetables, herbs and others [1,6], because plants are a natural, easily accessible and cheap source of bioactive

substances. These compounds differ in chemical structure, properties and applications. Due to the content of biologically active health-promoting compounds, a significant part of plants was and still is used for preventive or therapeutic purposes as an ingredient of medicines. The use of medicinal plants to prevent or treat diseases is the oldest practice, dating back to folk medicine. According to estimates by the World Health Organization (WHO), approximately 70–95% of the population of developing countries use medicinal plants to combat diseases [7,8]. An example of a plant with valuable health properties is the edible chestnut *Castanea sativa* Mill. (*Fagaceae*). Botanically, the chestnut is a large deciduous tree most commonly growing in Southern Europe, the Mediterranean region and the Balkans. The useful part of the chestnut is the nut, called the chestnut, which is edible and therefore processed into various dishes [9,10]. In addition to chestnuts, by-products such as leaves or husks are also valued for their strong antioxidant properties because Cerulli et al. (2020) confirmed the presence of hydrolysable tannins, flavonoids and triterpenoids in chestnut shell extract [11]. The literature contains information that the leaf extract of the described plant is rich in a number of compounds with strong antioxidant properties, such as gallic acid, protocatechuic acid, 4-hydroxybenzoic acid, vanillic acid, rutin, quercetin and apigenin [1,12,13] and strong antimicrobial potential [7,14]. For this reason, decoctions or infusions of *C. sativa* leaves were used in regions of northern Portugal to treat cough in children, diarrhea, hypertension and even infertility. However, in addition, from the point of view of composing medicines or functional foods, knowledge about the antibacterial and anticancer properties of plant extracts, including *C. sativa* leaf methanolic extract, is also important [7]. It is also particularly important to expand knowledge about the cytotoxic effects of chestnut leaf extract on various cancer cell lines. Therefore, the aim of the study was to comprehensively evaluate the *C. sativa* leaf extract in terms of antioxidant potential, polyphenol profile, antimicrobial, anticancer activity and in vitro biological potential. This will allow the expansion of knowledge of the properties and acquisition of nutraceuticals from new plant sources.

2. Material and Methods

2.1. Plant Material

Castanea sativa leaves were collected in the Subcarpathian region in Poland. After delivery to the laboratory, the leaves were freeze-dried (ALPHA 1-2 LD plus Martin Christ Gefriertrocknungsanlagen GmbH, Osterode, Germany), ground into powder and stored in tightly closed glass jars at −20 °C until the extract was prepared.

2.2. Preparation of Extract

The *J. regia* leaf preparation was obtained using the solid phase extraction method in accordance with our previous reports [15,16]. Briefly, the ground material (5 g) was mixed with methanol (50%; 45 mL) and sonicated in an ultrasonic bath for 30 min at 30 °C (Sonic 10, Polsonic, Krakow, Poland). The suspension was centrifuged at 19,000× *g* for 10 min (Centrifuge 5430, Eppendorf, Hamburg, Germany), the supernatant was collected, and the residue resubmitted to the above extraction using methanol (70%; 45 mL). The resulting supernatants were combined and concentrated using a rotary evaporator at 40 °C (R-215 Rotavapor System, Buchi, Flawil, Switzerland). Concentrated samples were applied to the SPE column (LiChroprep RP-18; pore size 40–63 µm), previously conditioned with methanol and equilibrated with water. The polyphenol fraction was eluted with methanol. The methanolic polyphenol extract was evaporated in a vacuum and lyophilized.

2.3. Determination of Total Phenolic and Flavonoid Content

The total phenolic content (TPC) was evaluated using the method described by Gao et al. (2000) [17]. The plant extract was mixed with distilled water (2.0 mL), Folin–Ciocalteau reagent (0.2 mL) and 20% sodium carbonate (1.0 mL). After 1 h, the absorbance was measured at a wavelength of 765 nm using a UV–vis spectrometer (Type UV2900, Hitachi, Tokyo, Japan).

The total flavonoid content (TFC) was estimated by following the procedure developed by Chang et al. (2020) [18]. The extract was mixed with ethanol (1.5 mL), aluminum chloride (0.1 mL), distilled water (2.8 mL) and 1 M sodium acetate (0.1 mL). After 30 min, the absorbance was measured at 415 nm.

2.4. Determination of the Polyphenol Profile by UPLC-Q-TOF-MS

Polyphenolic compounds were identified and quantified using the Ultra-Performance Liquid Chromatography Array Detector (UPLC-Q-TOF-MS, Waters, Milford, MA, USA) according to the protocol described by Żurek et al. (2022) [19]. Briefly, the separation of individual phenols was performed at 50 °C using a UPLC BEH C18 column (100 mm × 2.1 mm, 1.7 µm, Waters, Warsaw, Poland), with an injection volume of 5 µL and an isocratic rate flow rate 0.35 mL/min. Solvent A (water) and Solvent B (40% acetonitrile in water, *v/v*) were used as the mobile phase. The following parameters were used for triple–quadrupole detection: gas flow con 100 L/h; voltage 30 V; capillary voltage 3.5 kV; source temperature 120 °C; desolvation temperature 350 °C and desolvation gas flow 800 L/h. All of the compounds were identified by comparison of their retention times, elution orders, ESI-MS spectrometric data, and photodiode array PDA/UV–vis with authentic reference standards, which were gallic acid, quercetin 3-*O*-glucuronide, quercetin 3-*O*-glucoside, kaempferol 3-*O*-glucoside, isorhamnetin 3-*O*-glucoside and/or the literature data. Results are expressed in mg/g dw.

2.5. Determination of Antioxidant Activity

The scavenging activity of leaf extracts on $ABTS^{\bullet+}$ radicals was determined according to the method of Re et al. (1999) [20]. The plant extracts were mixed with the $ABTS^{\bullet+}$ solution (0.03 mL) diluted with distillated water to an absorbance of 0.7. After 6 min, the absorbance was measured at 734 nm. The results were expressed as Trolox Equivalent (mmol TE/g dw).

The CUPRAC test was conducted by a spectrophotometric method described by Apak et al. (2006) [21]. The plant extract was mixed with 10 mM copper chloride (1.0 mL), 7.5 mM neocuproine (1.0 mL) and 1 M acetate buffer (1.0 mL). After 30 min, the absorbance was measured at 450 nm. The results were expressed as Trolox Equivalent (mmol TE/g dw).

The chelating ability of ferrous ions was assessed according to the method described by Żurek et al. (2022) [19]. The plant extract was mixed with 0.1 mM iron II sulfate (0.2 mL) and 0.25 mM ferrozine (0.4 mL). After 10 min, the absorbance was measured at 562 nm.

Superoxide radical scavenging activity was measured based on the method described by Robak and Gryglewski (1988) [22]. The plant extract was mixed with 150 µM NBT (1.0 mL), 468 µM NADH (1.0 mL) and 60 µM PMS (1.0 mL). After 5 min, the absorbance was measured at a wavelength of 560 nm.

Hydroxyl radical scavenging activity was evaluated by the method of Żurek et al. (2022) [19]. The plant extract was mixed with 0.2 mM 2-deoxyribose, 1.0 mM iron ammonium sulphate, 1.04 mM EDTA, 1.0 mM ascorbic acid, 0.1 M perhydrol, 2.8% trichloroacetic acid, 1% thiobarbituric acid. After being heated to 100 °C for 15 min and cooled to room temperature, the absorbance was measured at 532 nm.

The results of ferrous ion chelating capacity, superoxide activity and hydroxyl radical scavenging activity were calculated according to equation

$$I\ (\%) = [[A0 - A1]/A0] \times 100$$

where A0 is the absorbance of the control sample and A1 is the absorbance of the test sample. The IC_{50} values (mg/mL), calculated from the regression curve, indicate the extract concentration inducing 50% radical scavenging.

2.6. Antidiabetic and Antiobesity Activity

α-amylase inhibition activity was measured according to the method of Zurek et al. (2023) [23]. Porcine pancreatic α-amylase (220 U/mL) was dissolved in 100 mM phosphate

buffer (pH 5.6). To measure α-amylase inhibitory activity, a mixture of 0.25 mL of α-amylase solution and extract was first incubated in a water bath at 40 °C for 15 min. Then, 0.5 mL of 1% starch solution was added. After 10 min, the reaction was stopped by adding 1 mL of 3,5-dinitrosalicylic acid and heating for 10 min. The mixture was then made up to 10 mL with distilled water and the absorbance was measured at 415 nm against a blank.

For α-glucosidase inhibition activity measurement, 0.04 mL of α-glucosidase (1 U/mL) and 0.2 mL of sample were added to 0.9 mL of a 0.1 mol/L phosphate buffer at pH 6.8 [23]. After incubation at 37 °C for 5 min, 0.04 mL of 1% sucrose was added. The reactions were incubated at 37 °C for 50 min, stopped by adding 0.2 mL of 3,5-dinitrosalicylic acid (DNS) and heated for 10 min. Absorbances were tested at 540 nm.

Lipase activity was measured using 4-nitrophenyl acetate as a substrate [23]. The reaction mixture contained 0.44 mL of a 100 mM TRIS-HCL buffer, pH 7.6, 0.02 mL of the enzyme (10 µg/mL, pancreatin from porcine pancreas), 0.02 mL of the studied extract and 0.04 mL of the substrate (10 mmol/L 4-nitrophenyl acetate). The change in absorbance was measured at 410 nm.

The ability of the tested samples to inhibit α-amylase, α-glucosidase and lipase activity was calculated according to the following equation:

$$I\ (\%) = [[A0 - A1]/A0] \times 100$$

where A0 is the absorbance of the control sample and A1 is the absorbance of the test sample. The IC_{50} values (mg/mL), calculated from the regression curve, indicate the extract concentration inducing 50% radical scavenging.

2.7. Cell Culture

Seven human cell lines were selected for the study. Cell lines Caco-2, DLD-1 (colon adenocarcinoma), MCF-7 (breast adenocarcinoma) and CCD 841 CoN (human colon epithelial cells) were obtained from the Sigma-Aldrich company (ECACC, Steinheim, Germany). Then, SK-Mel-28 (melanoma), U87MG (glioblastoma) and U251MG (astrocytoma) cell lines were obtained from the collection of the Nencki Institute of Experimental Biology, PAS, Warsaw, Poland. Cell lines were cultured in DMEM or McCoy's medium supplemented with heat-inactivated fetal bovine serum and antibiotics (penicillin/streptomycin). Cultivation was carried out under conditions recommended for tissue culture, at 37 °C and a CO_2 atmosphere (5%) (CB170, Binder, Tuttlinen, Germany).

2.8. Cell Viability Assay

Viability of all cell lines was assessed by MTS assay as previously reported [24,25]. Briefly, 200 µL of culture medium (8.0×10^3 cells/well) was incubated in 96 microplates for 24 h. After this time, the medium was changed to the test extract (10–750 µg/mL) and incubated for 48 h. The MTS assay was then performed and the absorbance at 490 nm was measured after 2 h (SmartReader 96 Microplate Absorbance Reader, Accuris Instruments, Edison, NJ, USA). Cell viability was calculated according to the following equation:

$$I\ (\%) = [Ac - As]/[Ac - Ab] \times 100$$

where Ac is the absorbance of the well containing the sample and the cell culture, As is the absorbance of the well containing the cell culture and Ab is the absorbance of the well containing the medium without cells. Calculated from the regression curve, the IC_{50} values (µg/mL) indicate the extract concentration inducing 50% inhibition of cell viability.

2.9. Cell Scratch Assay

Caco-2 cells were plated in a 12-well microplate at a concentration of 2.0×10^4 cells/well and grown to 90% confluency. A wound was then made with a sterile pipette tip and the plate was washed twice with PBS. Cells were treated with non-cytotoxic concentrations of *C. sativa* leaf methanolic extract. Images were taken 0, 12, 24 and 48 h after scratching using

an inverted light microscope (OX.2453-PLF Oxion Inverso, Euromex, Mataró, Spain). The results, compiled using ImageJ 1.54i software, are expressed as the average % wound area closure, calculated according to the following equation:

$$\text{Wound area closure (\%)} = [A0 - A1]/A0 \times 100$$

where A0 is the area of the scratch surface at time point Oh and A1 is the area of the scratch surface at the tested time points.

2.10. Colony Formation Assay

Caco-2 cells were plated at a concentration of 1.0×10^3 cells/dish. After 16 h of incubation, cells were treated for 24 h with extract at a concentration of 10–750 μg/mL. Untreated cells were used as control. After this time, the extracts were exchanged with fresh medium and the cells were cultured for 14 days; the medium was changed every 3 days. The formed colonies were fixed with ethanol, stained with 3% crystal blue and counted with an inverted light microscope. Results are expressed as % colony formation relative to control.

2.11. Bacterial Strains and Antimicrobial Potential

To obtain the water extract, 100 mg of freeze-dried *C. sativa* leaves were added to sterile ultra-pure water to a final concentration of 100 mg/mL. The suspension was boiled at 100 °C for 30 min. In the next step, the suspension was centrifuged at 12,000 RPM for 10 min and then the supernatant was transferred to a new eppendorf tube. The supernatant was collected and stored until use at −20 °C. To obtain the ethanolic extract, 100 mg of freeze-dried *C. sativa* leaves were added to 80% ethanol to a final concentration of 100 mg/mL. The samples were then incubated at 37 °C for 24 h with shaking (1000 RPM). They were then centrifuged at 12,000 RPM, RT, for 10 min. The supernatants were collected and stored until use at −20 °C.

The antimicrobial potency of *C. sativa* leaf extracts was evaluated on the following microorganisms: four strains of Gram-negative (*Escherichia coli* PCM 2209, *Klebsiella pneumoniae* PCM 1, *Pseudomonas aeruginosa* DSM 19880, Serratia marcescens PCM 501) and three strains of Gram-positive (*Staphylococcus aureus* PCM 458, *Listeria monocytogenes* PCM 2191 and *Enterococcus faecalis*). Additionally, the antifungal activity of the tested extracts was checked using the *Candida albicans* ATCC 14053 strain. Clinical strains *Enterococcus faecalis* were received from the collections of microorganisms of the Frederic Chopin Provincial Specialist Hospital in Rzeszów, Poland which were obtained during routine diagnostic cultures. The microorganisms were cultured on Nutrient Agar NA (meat extract—10 g/L, peptone—10 g/L, sodium chloride—5 g/L, agar—20 g/L, pH—7.0) and on YPD medium (yeast extract—10 g/L, peptone—20 g/L, glucose—20 g/L, pH—7.2). The water and ethanolic extracts were used to prepare dilutions, i.e., 1:10, 1:100 with which the tested microorganism species were treated. Antibacterial and growth inhibitory properties of *C. sativa* leaf extracts against selected microorganisms were tested using a spot-on-lawn method. Briefly, microorganism assay plates were prepared whereby the tested bacteria and yeast (8-log CFU/mL) were seeded. Then, 5 μL of each *C. sativa* leaf extract was surface-spotted onto the indicator lawn. In the case of ethanol extracts, positive controls of appropriately diluted ethanol, i.e., 80%, 8%, 0.8% were also surface-spotted onto the indicator lawn. Plates were allowed to incubate overnight. Growth inhibition was evaluated after incubation (24 h at 37 °C—bacteria and 29 °C—yeast) by observations of the zone of inhibition around the spots with the test organism and were recorded using a Canon EOS 600D camera (Canon, Warsaw, Poland).

2.12. Statistical Analysis

Test results are expressed as the mean of three independent tests ± standard error (SD). Differences between results were assessed by statistical analysis including Duncan's test,

Student's *t*-test and Pearson's correlation ($p < 0.05$) using Statistica 13.3 software (StatSoft, Krakow, Poland).

3. Results and Discussion

3.1. Evaluation of the Total Phenolic and Flavonoid Contents

C. sativa leaf extract was calorimetrically assessed for total phenolic (TPC) and flavonoid (TFC) contents. The results obtained are presented in Table 1.

Table 1. Total phenolic (TPC) and flavonoid (TFC) content in *C. sativa* leaf methanolic extract.

	TPC mg GAE/g	TFC mg QE/g
C. sativa leaf methanolic extract	458.22 ± 1.02	24.55 ± 0.08

Values are expressed as mean ± SD.

The TPC in *C. sativa* leaf methanolic extract was 458.22 mg GAE/g, and TFC was 24.55 mg QE/g. The obtained TPC and TFC were much higher compared to previously published studies. In the reports of Cerulli et al. (2021), Vella et al. (2017), Barreira (2007), Mujić et al. (2011), Zivković et al. (2010) and Almeida et al. (2010), the TPC in *C. sativa* leaves extracts ranged from 14.0 to 298.96 mg GAE/g, while the TFC, expressed so far in units other than those in this work, ranged from 3.2 to 23.4 mg CE/g [11,26–30]. This significant difference in the TPC between our own results and the cited reports may result from different methods of performing the assay, as well as from the fact that the preparation obtained as a result of fractionation on the RP-18 resin was cleaned of ballast compounds, thus enriched in phenolic compounds and flavonoids. This fact is also confirmed by the article published by Formato et al. (2022), whose analyzes assessed the macerate obtained from *C. sativa* leaves, the fraction from liquid–liquid extraction and the preparation obtained from the Amberlite XAD-4 absorption resin [31]. The highest TPC and TFC was also found for the preparation obtained from the deposit (872.4 mg GAE/g and 83.3 mg QE/g, respectively). The obtained value was much higher than that of the other extracts assessed, as well as the result obtained in this study, which may indicate that polymer resins with larger particle sizes than silica resins are more advantageous for the recovery of polyphenolic compounds from *C. sativa* leaves.

With respect to the remaining morphological parts of the *C. sativa* tree, the TPC in the tested leaf extract was 1.5 times higher compared to that of flowers [28], 122.8 times higher compared to that of fruits [27], 16.2 times higher than that of bur [32], 2.2 times higher for the shell [33], including 1.04 and 1.11 times lower than those estimated for the inner and outer shells [27], respectively. In turn, the TFC in other morphological parts of the *C. sativa* tree was expressed in different units in previous reports, hence these values cannot be compared.

3.2. Quantification of the Polyphenols with UPLC-Q-TOF-MS

The phenolic profile of chestnut leaves was carried out by means of UPLC-Q-TOF-MS analyses. All of the compounds were identified by comparison of their retention times, elution orders, ESI-MS spectrometric data, and photodiode array PDA/UV–vis with authentic reference standards and/or the literature data. The chromatographic, spectroscopic and spectrometric data, as well as the quantitative amounts of individual compounds are shown in Table 2.

In total, 18 phenolic compounds were putatively identified. The main class (six compounds) consisted of hydrolysable tannins. Hydrolysable tannins are polymers containing a center core of glucose esterified with gallic or ellagic acids (gallo- and ellagitannins, respectively). They exhibit anti-cancer, anti-angiogenic, antioxidant, anti-inflammatory and anti-ulcerative properties [34]. Compounds **2**, **4**, **5**, and **10** were characterized as ellagitannins, the isomers of galloyl-bis-HHDP-glucose. They all displayed a dominant precursor [M-H]- ion at *m/z* 935 and ion at *m/z* 467 corresponding to doubly charged molecular

ion $[M\text{-}2H]^{2-}$. Component **3** showed [M-H]- at *m/z* 953 and the product ion at *m/z* 785, which was received by the loss of 168 Da. It also produced a fragmentation ion at *m/z* 301 coinciding to ellagic acid and at the same time being the most significant diagnostic ion of ellagitannins [35]. Therefore, this component was assigned to galloyl-chebuloyl-HHDP-glucose (chebulagic acid). In turn, Compound **9** was identified as galloyl glucose derivative—trigalloyl-glucose. It displayed parent ion [M-H]- at *m/z* 635 with a typical for gallotannins fragmentation pathway with the loss of gallic acid moiety (170 Da).

Table 2. Individual phenolic compounds identified by UPLC-PDA-MS/MS in *C. sativa* leaf extract.

	Compound	Rt	λ_{max}	[M-H] *m/z*		Concentration mg/100 g d.w.
	HYDROLYSABLE TANNINS					
	Ellagitannis					
2	Galloyl-bis-HHDP-glucose I	2.78	276	935	637, 467	138.28 ± 9.98
3	Galloyl-chebuloyl-HHDP-glucose (chebulagic acid)	2.88	276	953	785, 301	77.41 ± 7.04
4	Galloyl-bis-HHDP-glucose II	2.92	276	935	637, 467	132.47 ± 7.08
5	Galloyl-bis-HHDP-glucose III	2.99	276	935	767, 467	59.29 ± 2.40
10	Galloyl-bis-HHDP-glucose IV	3.73	276	935	637, 467	29.48 ± 1.12
	Gallotannins					
9	Trigalloyl-glucose	3.68	269	635	465	13.51 ± 0.19
	FLAVONOIDS					
14	Quercetin 3-*O*-glucuronide	4.65	255, 353	477	301	137.73 ± 4.19
15	Quercetin 3-*O*-glucoside	4.74	255, 353	463	301	184.30 ± 4.02
17	Kaempferol 3-*O*-glucoside	5.30	264, 347	447	285	61.60 ± 1.39
18	Isorhamnetin 3-*O*-glucoside	5.49	253, 352	477	315	22.33 ± 0.64
	PHENOL GLUCOSIDE DERIVATIVES					
6	Digalloyl phenol glucoside (chesnatin)	3.17	272	637	467, 305	90.38 ± 2.68
8	Digalloyl phenol glucoside (isochesnatin)	3.59	271	637	467	42.18 ± 1.43
11	Galloyl phenol glucoside (cretanin)	4.01	274	469	169	15.04 ± 1.16
13	Galloyl phenol glucoside dimer (chestanin)	4.37	274	937	467, 301	266.81 ± 8.36
16	Galloyl phenol glucoside dimer (isochestanin)	5.04	269	937	637, 467	39.54 ± 1.29
	PHENOLIC ACID DERIVATIVES					
1	Gallic acid	2.35	270	169	125	81.52 ± 0.99
7	Methyl-gallate	3.53	269	183	168	19.07 ± 0.17
12	Ellagic acid pentoside	4.12	274	433	301	15.59 ± 2.49
	TOTAL					1426.55 ± 54.99

The second most abundant group of compounds were phenol glucoside derivatives. Components **6** and **8** both exhibited pseudomolecular ions [M-H]- at *m/z* 637 with fragmentation [M-H-170-162]- at *m/z* 467 and 305 attributable to the loss of galloyl and glucose units, respectively. They were identified as chesnatin and isochesnatin. Peaks 13 and 16 both showed precursor ion [M-H]- at *m/z* 937 that produced fragment ion [M-H-470]- at *m/z* 467 due to the loss of the glucose, the 3,4,5-trihydroxybenzyl alcohol and the galloyl moieties; thus, they were tentatively characterized as chestanin and isochestanin. Compound **11** showed [M-H]- at 469 and the daughter ion [M-H-300]- at *m/z* 169, indicating the simultaneous loss of the 3,4,5-trihydroxybenzyl alcohol unit and the glucose molecule.

Further, five constituents were identified as flavonol glycosides, namely quercetin 3-*O*-glucuronide (**14**), quercetin 3-*O*-glucoside (**15**), kaempferol 3-*O*-glucoside (**17**) and isorhamnetin 3-*O*-glucoside (**18**). Their full mass spectra showed deprotonated molecules [M-H]- at *m/z* 477, 463, 447 and 417, respectively. The fragmentation pathway displayed common diagnostic product ions at *m/z* 301 (**14** and **15**), 285 (**17**) and 315 (**18**), confirming the presence of three different aglycones, corresponding to quercetin, kaempferol and isorhamnetin, respectively, and, at the same time, the neutral loss of the sugar unit, which was represented by glucose (162 Da) and glucuronic moiety (176 Da).

Additionally, Component 1 was found to be gallic acid, Compound **3** was methyl-gallate and Constituent 12 was ellagic acid pentoside. Gallic acid (**1**) fragmentation produced the [M-H]- ion at *m/z* 169 and the MS/MS ion spectrum [M-H-44]- at *m/z* 125, suggesting the loss of carbon dioxide. Methyl-gallate (**3**) was characterized by the diag-

nostic mass ion of *m/z* 183 and its mass fragments [M-H-15]- of *m/z* 168 after the loss of the methyl group. Instead, ellagic acid pentoside (**12**) showed [M-H]- at *m/z* 433 and a fragmentation ion [M-H-132]- at 301 arising from the loss of the pentosyl unit. All of the compounds were previously characterized in *C. sativa* leaf extracts [10,11,36–38].

The total amount of flavonoids of *C. sativa* leaves was 1426.55 µg/10 mg of dried plant material. Chestanin was the most abundant component with 18.70%, followed by quercetin 3-*O*-glucoside and galloyl-bis-HHDP-glucose isomer I with 12.92% and 9.69%, respectively. Our results are in agreement with previous reports on the chestnut leaves, where the major constituents were ellagitanins, phenol glucoside and quercetin derivatives [36,37].

3.3. Antioxidant Activity

The antioxidant activity of *C. sativa* leaf methanolic extracts was assessed using five in vitro methods, such as scavenging activity $ABTS^{\bullet+}$ radicals (ABTS), the ability to copper ion reduction (CUPRAC), iron ion chelation (ChA) and scavenging reactive oxygen species (ROS), namely superoxide radicals ($O_2^{\bullet-}$) and hydroxyl radicals ($OH^{\bullet}$). The obtained results are presented in Table 3.

Table 3. Antioxidant activities of *C. sativa* leaf methanolic extract.

	Antioxidant Activities				
	ABTS	CUPRAC	ChA	$O_2^{\bullet-}$	OH^{-}
	mmol TE/g		IC_{50} (mg/mL)		
C. sativa leaf methanolic extract	11.52 ± 0.16	1.96 ± 0.01	0.31 ± 0.08	0.067 ± 0.09	0.207 ± 0.02

Values are expressed as mean ± SD.

In the assessment of the antioxidant potential using the ABTS and CUPRAC methods for *C. sativa* leaf methanolic extract, the obtained values were 11.52 and 1.96 mmol TE/g, respectively. In turn, for the remaining three methods, chelating iron ions and scavenging $O_2^{\bullet-}$ and $OH^{\bullet}$ radicals, the activity was 0.310, 0.067 and 0.207 mg/mL, respectively.

There are very few reports on the antioxidant potential of *C. sativa* leaves. To date, the analysis of ABTS activity expressed in Trolox equivalents has only been performed by Silva et al. (2020), where the obtained value was lower than in this work (5.81 mmol TE/g) [37]. Higher activity was demonstrated by Calliste et al. (2005) and Almeida et al. (2008) in the analysis of $O_2^{\bullet-}$ radical scavenging (0.0016–0.014 mg/mL) and $OH^{\bullet}$ radical scavenging (0.160–0.216 mg/mL) [1,39]. No reports have been published yet for the remaining two methods—CUPRAC and ChA.

With respect to the remaining morphological parts of the *C. sativa* tree, the ABTS activity of the tested leaves was 3.2 times higher than the bur activity [40], 2.4 times higher than the shell activity [41], including 3.3 and 57.6 times higher than the inner and outer shell activity, respectively [37]. On the other hand, the $O_2^{\bullet-}$ radical scavenging capacity was 5.1 times lower than the activity of the shell extract [42]. The indicated differences in the measurement of antioxidant activity compared to the cited works assessing the activity of leaves and other morphological parts of the *C. sativa* tree may result from the date of harvesting the raw material, location and extraction method. Reinoso et al. (2012), in the analysis of extraction conditions using the Box–Wilson experimental design (CCC), showed that the highest antioxidant activity of the leaf extract is provided by the use of ethanol as a solvent while maintaining the extraction time and temperature of >90 min and 50 °C, respectively [12]. In turn, Calliste et al. (2005), in the analysis of *C. sativa* leaves, proved that the radical scavenging activity in the DPPH method increases with the TPC, and the ability to scavenge $O_2^{\bullet-}$ and $OH^{\bullet}$ radicals depends on the ratio of tannins to other phenolic compounds [1]. Our own analyzes showed a strong correlation between the CUPRAC method and the sum of phenol compounds ($r = 0.999$, $p < 0.05$), the ABTS method and TPC ($r = 0.994$, $p < 0.05$).

3.4. Antidiabetic and Antiobesity Activity

Pancreatic α-amylase and α-glucosidase are enzymes that hydrolyze bonds in complex carbohydrate molecules, breaking them down into simple sugars, compounds suitable for absorption. In turn, pancreatic lipase participates in the breakdown of lipid bonds into free fatty acids and monoacylglycerols. Inhibiting the activity of these enzymes may play an important role in the strategy of preventing and/or treating type II diabetes and obesity. Hence, the search for new sources of inhibitors among materials of plant origin is important in contemporary phytotherapy. The ability of *C. sativa* leaf extract to inhibit the activity of α-amylase, α-glucosidase and lipase is shown in Table 4.

Table 4. Anti-diabetic and -obesity activities of *C. sativa* leaf methanolic extract.

	Anti-Diabetic and Anti-Obesity Activities		
	α-amylase	α-glucosidase	lipase
		IC_{50} (mg/mL)	
C. sativa leaf methanolic extract	1.62 ± 0.24	2.20 ± 0.22	0.72 ± 0.04

Values are expressed as mean ± SD.

The methanolic extract showed higher activity against the obesity-promoting enzyme than against antidiabetic enzymes. The obtained values were 0.72 mg/mL (for pancreatic lipase) and 1.62 and 2.20 mg/mL (for pancreatic α-amylase and intestinal α-glucosidase, respectively). So far, only the antiobesity and antidiabetic activity of *C. sativa* shell has been assessed. Pinto et al. (2021) found comparable activity against lipase (IC_{50} 0.457 mg/mL) and overall weak activity against α-amylase (15.22% at 1 mg/mL) [42]. This means that *C. sativa* extracts have higher anti-obesity than anti-diabetic activity. Nevertheless, the reports of Mujić et al. (2011) showed a beneficial effect of *C. sativa* leaf extracts on rat pancreatic β cells, reducing the risk of cell death as a result of oxidative stress, thus preventing the development of diabetes. Importantly, this effect was attributed to the total polyphenol content, not to individual compounds isolated from the extract matrix [28]. Our own analyzes in the assessment of the Pearson coefficient showed a positive correlation between the sum of phenolic compounds and α-amylase ($r = 0.918$, $p < 0.05$), α-glucosidase ($r = 0.958$, $p < 0.05$) and pancreatic lipase ($r = 0.983$, $p < 0.05$). Also, Pinto et al. (2021) showed a strong correlation between TPC and α-amylase activity and a negative one between TPC and pancreatic lipase [42], which may indicate that *C. sativa* polyphenols have a significant impact on inhibiting the activity of digestive enzymes. Nevertheless, these are the first reports on the inhibitory effect of *C. sativa* leaves on digestive enzymes. Further research should be conducted to elucidate these relationships in detail.

3.5. Cell Viability

To assess cell viability after treatment with *C. satica* extract, the MTS test was performed. Seven human cell lines were selected for evaluation, such as Caco-2 and DLD-1 (colon adenocarcinoma), MCF-7 (breast adenocarcinoma), SK-Mel-28 (melanoma), U87MG (glioblastoma), U251MG cell lines (astrocytoma) and CCD 841 CoN (colon epithelial cells). The obtained results are presented in Figure 1A.

Among the tested cancer lines, the highest cytotoxic activity was demonstrated against Caco-2 cells (adenocarcinoma colon). The obtained IC_{50} value was 153.54 μg/mL. Cytotoxic activity against other tumor lines was in the following order: DLD-1 (166.31 μg/mL) > MCF-7 (184.86 μg/mL) > U87MG (210.67 μg/mL) > U251MG (268.49 μg/mL) > SK-Mel-2 (625.94 μg/mL). At the same time, the effect of the extract on the viability of human colonic epithelial cells (CCD 841 CoN) was assessed. The obtained IC_{50} value was 309.88 μg/mL. For the *C. sativa* leaf methanolic extract, this is a result above the bioactive effect against the five analyzed cancer lines. Only for the SK-Mel-28 cell line was higher cytotoxic activity observed.

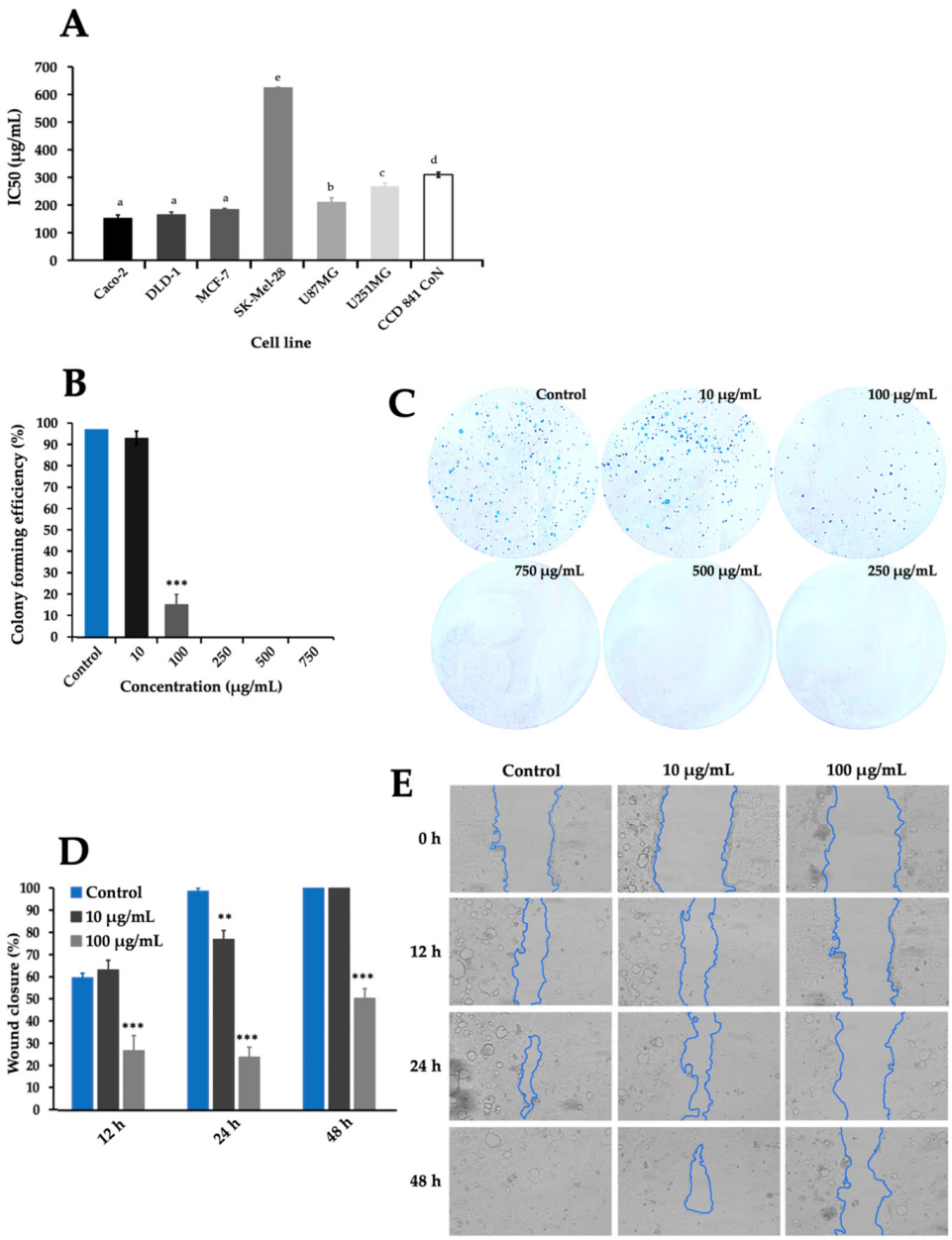


Figure 1. Test results for anticancer analysis. (**A**) Viability IC_{50} values of seven cell lines after 48 h of treatment with *C. sativa* leaf methanolic extract. (**B**) Graph showing the decrease in colony formation of colorectal adenocarcinoma (Caco-2) cells after 12 days of treatment with *C. sativa* leaf methanolic extract. Untreated cells were selected as a control. (**C**) Representative photos of stained Caco-2 cell colonies. (**D**) Graph showing the percentage of wound closure after 12, 24 and 48 h of treatment with *C. sativa* leaf methanolic extracts. A value of 100% means complete closure of the wound. (**E**) Representative images of cell migration after treatment with three noncytotoxic concentrations (values below IC_{50}). Cells were analyzed at four time points (0, 12, 24, 48 h). Images were taken under an inverted microscope at 20× magnification. All experiments were performed at least in triplicate $n \geq 3$. Data are expressed as mean ± SD. Significant differences (** p <0.01, *** p <0.001) were assessed using Student's *t*-test and Duncan's test (a–e).

This is the first report on the cytotoxicity of *C. sativa* leaves. So far, there have only been reports of the anticancer effect of the *C. sativa* shell extract, for which Pinto et al. (2021) in the analysis of the cytotoxic effect on Caco-2 cells obtained a result 5.0 times weaker [42]. However, Jung et al. (2015), for the MCF-7 line, showed an effect that was 3.5 times weaker [43]. In turn, Cacciola et al. (2019) and Sorice et al. (2016) did not demonstrate any

cytotoxic effect of *C. sativa* shell extract against the same cancer line [44,45]. On the other hand, the strongest anticancer effect of *C. sativa* shell was demonstrated against HepG2 cells (hepatocellular carcinoma cells) (137.0 µg/mL), which, according to the authors, was related to the induction of depolarization of the cell mitochondrial membrane and the modification of cell cycle phases [43,45].

3.6. Wound Scratch

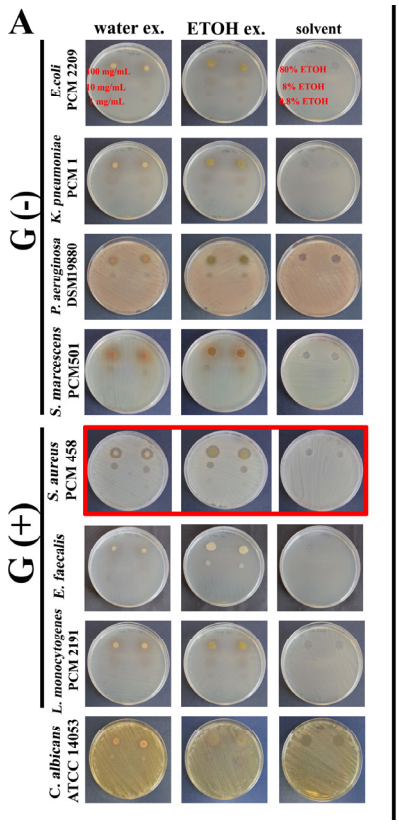
Migration is a characteristic feature of cancer cells promoting their aggressiveness and the formation of metastases. Counteracting metastases, which are responsible for 90% of all deaths, is one of the main goals of cancer treatment. To evaluate the effect of *C. sativa* leaf methanolic extract on cell migration, a wound healing assay was performed. The Caco-2 cell line was selected for testing, as it was characterized by the highest sensitivity to the tested extract. The test was carried out until the wound was completely closed in the control sample. Cells were treated with the extract at a concentration below the IC_{50} value. As shown in Figure 1D,E, the *C. sativa* leaf methanolic extract significantly inhibited Caco-2 cell migration compared to the control. Administration of the extract at a concentration of 10 µg/mL after 12 h of culture resulted in inhibition of cell migration by 63.00%. After extending the culture time to 24 and 48 h, the wound was closed in 76.74 and 99.95%, respectively. Complete wound closure occurred after 48 h of control cell culture. The strongest anti-migration effect was observed after treating cells with 100 µg/mL *C. sativa*. After 12, 24 and 48 h of incubation, 26.76, 23.84 and 50.26% of the scratch surface were covered by migrating cells, respectively. Previously unpublished results indicated that *C. sativa* leaves are a strong inhibitor of Caco-2 cell migration.

3.7. Clonogenic Test

Cancer cells have the ability to self-regenerate and endlessly produce offspring. For these reasons, resistance to chemotherapy, the formation of secondary tumors and cancer recurrence constitute serious clinical problems in effective oncological therapies [46]. In order to assess the ability of a single cell to form a colony, a clonogenic test was performed. Cells were treated with *C. sativa* extracts at five concentrations (10–750 µg/mL) for 12 days. Untreated cells were used as a control. As shown in Figure 1B,C, the tested extract significantly inhibited the ability of cells to form cell colonies in a dose-dependent manner. After incubation of cells with the extract at a concentration of >250 µg/mL, colony formation was completely inhibited, confirming the effectiveness of inhibiting cell proliferation assessed in the MTS assay. The percentage of cell colony formation formed after treatment with the extract at a concentration of 10 µg/mL was 95.84%, and the concentration of 100 µg/mL significantly suppressed the clonogenicity of cells to a value of 15.86%. The obtained results indicate that *C. sativa* leaf methanolic extract effectively inhibits the long-term ability of Caco-2 cells to proliferate.

3.8. Antimicrobial Activity

Aqueous and ethanolic extracts of *C. sativa* leaves at concentrations of 100 mg/mL and 10 mg/mL showed bactericidal activity against the Gram-positive bacterium *Staphylococcus aureus*. However, no antimicrobial effect was observed against other tested bacteria, including both Gram-negative (*Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Serratia marcescens*) and Gram-positive (*Enterococcus faecalis* and *Listeria monocytogenes*) strains. Additionally, the tested extracts did not exhibit antifungal activity against the *Candida albicans* yeast. For both aqueous and ethanolic extracts, the use of a concentration of 1 mg/mL did not show antimicrobial effects (Figure 2A,B).



B

Microorganisms	Zone of Inhibition (mm)			
	Concentrations (mg/mL)			
	water ex.		ethanol ex.	
	100	10	100	10
Escherichia coli PCM 2209	0 ^{A}	0 ^{A}	0 ^{A}	0 ^{A}
Klebsiella pneumoniae PCM1	0 ^{A}	0 ^{A}	0 ^{A}	0 ^{A}
Pseudomonas aeruginosa DSM 19880	0 ^{A}	0 ^{A}	0 ^{A}	0 ^{A}
Serratia marcescens PCM 501	0 ^{A}	0 ^{A}	0 ^{A}	0 ^{A}
Staphylococcus aureus PCM 458	11.5 ± 0.7 ^{B}	5.75 ± 0.4 ^{B}	8.5 ± 0.7 ^{B}	6.5 ± 0.7 ^{B}
Enterococcus faecalis	0 ^{A}	0 ^{A}	0 ^{A}	0 ^{A}
Listeria monocytogenes PCM 2191	0 ^{A}	0 ^{A}	0 ^{A}	0 ^{A}
Candida albicans ATCC 14053	0 ^{A}	0 ^{A}	0 ^{A}	0 ^{A}

Figure 2. Test results for antimicrobial analysis. (**A**) Water and ethanolic extracts of *C. sativa* leaf-mediated changes in microbial growth. The tested microorganisms were treated with surface spots of *C. sativa* leaf extracts at 100 mg/mL, 10 mg/mL and 1 mg/mL on indicator lawn media of NA agar (bacteria) and YPD agar (*Candida*). Representative micrographs of bacterial and yeast culture plates. (**B**) Table with diameters of bacterial growth inhibition (halos) induced by aqueous and ethanolic extracts of *C. sativa* leaves at concentrations of 10 and 100 mg/mL. The values are expressed as means ± SD of three biological replicates. Letters indicate statistically significant differences (capital letter < 0.001) among samples according to ANOVA and HSD Tukey's test.

Our findings corroborate previous studies indicating that *C. sativa* leaves possess antimicrobial activity against *Staphylococcus aureus* [14,47]. The results of microbiological analyses conducted by Kędzia et al. (2020) demonstrate a comparable inhibitory effect on *Staphylococcus* growth. Tenfold dilutions of a 45% aqueous extract of *C. sativa* produced diameter zones of inhibition of *Staphylococcus aureus* cultures in the range of 6 mm (20×–320× dilutions) to 13 mm (10-fold dilutions) [48]. Similarly, our analysis showed that aqueous extracts at concentrations of 10 and 100 mg/mL produced inhibition zones of 5.5–6 mm and 11–12 mm, respectively. For alcoholic extracts, the inhibition zones ranged from 7 to 10 mm and from 6 to 7 mm at concentrations of 10 and 100 mg/mL, respectively. In particular, other studies have shown a wider spectrum of susceptible microbial species compared to our work on the efficacy of the extract. In addition to inhibition of growth in *S. aureus*, a previous study demonstrated inhibitory effects against *Enterococcus faecalis* and Gram-negative bacteria, namely *Escherichia coli* and *Klebsiella pneumoniae* [48]. Similarly, another study reported that the soluble fraction of ethyl acetate from an aqueous leaf extract (pH 3.0) of *C. sativa* exhibited inhibitory activity against six bacterial strains in addition to *Staphylococcus*, with MICs ranging between 64 and 256 µg/mL. *E. aerogenes* and *S. aureus* were the most sensitive bacteria (MIC = 62.5 µg/mL), while *Proteus vulgaris*, *Pseudomonas aeruginosa* and *Enterococcus cloacae* showed MIC values of 125 µg/mL. The least sensitive bacteria were *E. coli* and *K. pneumoniae* (MIC = 250 µg/mL) [14], confirming our analysis. The differences in the growth inhibition (halos) of the analyzed bacterial species in different

research papers are likely due to the different methods of extract application to the culture medium. In this study, extracts were applied using the spot method, unlike the agar well diffusion method, where 0.05 mL of the extract was introduced into a well in agar [47]. The variability in antimicrobial activity of different *C. sativa* extracts is probably due to both the extraction solvent and the plant part used to produce them. This suggests that some active antimicrobial substances contained in the biomass dissolve to varying degrees in the applied solvents. The choice of solvent may depend on the desired metabolites to be extracted from the biomass of *C. sativa*, as well as on the target microorganisms. Literature studies have well documented that different organs of plants produce many different secondary metabolites with different biological functions [29,47,49]. Therefore, differences in the extract contents may determine the strong or mild antimicrobial and antifungal activity. This is supported by other studies showing that the choice of methanol as a solvent increases antifungal activity against *C. albicans* and *C. parapsilosis*, achieving MIC values of 31.2 μg/mL [47], in contrast to the aqueous and ethanol extracts used in our analyses. Furthermore, Avşar et al. (2016) demonstrated that a methanol extract derived from chestnut pollen grains exhibited mild antifungal activity against *C. albicans* and *C. parapsilosis* [50].

Another interesting thread suggesting that *C. sativa* leaves exhibit antimicrobial activity against *Staphylococcus aureus* is a study by Quave et al. (2015). This research provided evidence that extracts from *C. sativa* leaves can block virulence and pathogenesis in *Staphylococcus aureus* by altering quorum sensing [38]. Quorum sensing is a critical mechanism by which the bacterium regulates its virulence and its ability to form biofilms [51]. The active substances in the extracts effectively prevented bacteria from communicating with each other and coordinating the reduction in virulence factors. Additionally, researchers found that the extracts of *C. sativa* are not cytotoxic to human skin cells and do not inhibit the growth of normal skin microflora [38].

In conclusion, this study provides evidence that aqueous and alcoholic extracts of *C. sativa* leaves can serve as a potential source of anti-staphylococcus agents to support alternative treatment options for infections caused by multidrug-resistant (MDR) bacteria.

4. Conclusions

Comprehensive research on the polyphenol profile, antioxidant and antimicrobial activity, as well as in vitro biological activity research proved that *C. sativa* leaf methanolic extract is a valuable plant material with documented health-promoting potential. Eighteen compounds belonging to the hydrolysable tannins, flavonoids, phenol glucoside derivatives and phenolic acid derivatives group were identified in the polyphenol profile. Moreover, it was shown that the total content of polyphenols and flavonoids in the leaf extract was often many times higher compared to the content of these compounds in other anatomical parts of the chestnut tree, such as flowers, bark or shell. For the first time, it was shown that the extract from the tested leaves had an inhibitory effect on the activity of the main digestive enzymes. This discovery, however, requires further research to explain the mechanisms of action of bioactive substances, but is crucial in scientific work on ways to prevent and treat overweight and obesity. In addition, the studies showed that among the analyzed cancer lines, the highest cytotoxic activity was demonstrated against Caco-2 cells, i.e., colon adenocarcinoma. In turn, the results of tests on the antimicrobial potential confirmed that water and ethanolic extracts from the tested leaves had a bactericidal effect against the Gram-positive bacterium *Staphylococcus aureus*, but did not show any activity against Gram-negative bacteria or fungi. The scientific evidence obtained proves that more attention should be paid to the potential application possibilities of leaf extract as a possible ingredient of medicines, biocosmetics or even as an enriching agent for functional or designed foods.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/app14093859/s1, Table S1: Correlation between all parameters assessed for the *C. sativa* leaf methanolic extract; Figure S1: UPLC chromatogram of *C. sativa* leaf methanolic extracts.

Author Contributions: Conceptualization, N.Ż. and I.T.K.; methodology, N.Ż., L.P. and I.T.K.; software, N.Ż. and I.T.K.; validation, N.Ż. and I.T.K.; formal analysis, N.Ż. and I.T.K.; investigation, N.Ż., A.M.P., L.P. and I.T.K.; resources, N.Ż. and I.T.K.; data curation, N.Ż., A.M.P., L.P. and I.T.K.; writing—original draft preparation, N.Ż., A.M.P., K.P. and L.P.; writing—review and editing, N.Ż., A.M.P., K.P. and I.T.K.; visualization, N.Ż.; supervision, I.T.K.; project administration, N.Ż. and I.T.K.; funding acquisition, I.T.K. 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: Data are contained within the article and Supplementary Material.

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

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