https://doi.org/10.31925/farmacia.2024.6.4 **ORIGINAL ARTICLE**

EVALUATION OF ANTI-MELANOMA, ANTIBACTERIAL AND ANTIOXIDANT EFFECTS OF A PHYTOCHEMICALLY CHARACTERIZED WATER-SOLUBLE FRACTION FROM GREEN COFFEE SEEDS

GRAȚIANA RUSE [1], DIANA-SIMONA TCHIAKPE-ANTAL [1,8], ELENA-ALINA MOACĂ [2,8], ALEXANDRA-DENISA SEMENESCU [2,8], ANA-MARIA VLASE [3], DELIA MUNTEAN [4], FLORINA ARDELEAN [1,8], ALINA-ARABELA JOJIC [5,8], VOICU MIRELA [5,8], ADELINA CHEVEREȘAN [6]*, ALINA HEGHEȘ [7]

[1]*Discipline of Pharmaceutical Botany, Faculty of Pharmacy, "Victor Babeș" University of Medicine and Pharmacy Timișoara, 2 Eftimie Murgu Square, 300041, Timișoara, Romania*
[2]*Discipline of Toxicology, Drug industry, Management and Legislation, Faculty of Pharmacy, "Victor Babeș" University of Medicine and Pharmacy Timișoara, 2 Eftimie Murgu Square, 300041, Timișoara, Romania*
[3]*Discipline of Pharmaceutical Botany, Faculty of Pharmacy, "Iuliu Haţieganu" University of Medicine and Pharmacy, 8 Victor Babeș Street, Cluj-Napoca, Romania*
[4]*Discipline of Microbiology, Faculty of Medicine, "Victor Babeș" University of Medicine and Pharmacy Timișoara, 2 Eftimie Murgu Square, 300041, Timișoara, Romania*
[5]*Discipline of Pharmacology-Pharmacotherapy, Faculty of Pharmacy, "Victor Babeș" University of Medicine and Pharmacy Timișoara, 2 Eftimie Murgu Square, 300041, Timișoara, Romania*
[6]*Discipline of Pharmacology, Faculty of Medicine, "Victor Babeș" University of Medicine and Pharmacy Timișoara, 2 Eftimie Murgu Square, 300041, Timișoara, Romania*
[7]*Discipline of Pharmaceutical Technology, Faculty of Pharmacy, "Victor Babeș" University of Medicine and Pharmacy Timișoara, 2 Eftimie Murgu Square, 300041, Timișoara, Romania*
[8]*Research Centre for Pharmaco-Toxicological Evaluations, Faculty of Pharmacy, "Victor Babeș" University of Medicine and Pharmacy Timișoara, 2 Eftimie Murgu Square, 300041, Timișoara, Romania*

**corresponding author: cheveresan.adelina@umft.ro*

Manuscript received: August 2024

Abstract

The green seeds of *Coffea arabica* are appreciated for the promotion of health and wellness. The current research focuses on the phytochemicals and bioactivity of the water-soluble fraction prepared from green coffee seeds. The first steps were to characterize the extract with the aid of HPLC, to record the FT-IR spectrum and to evaluate the content in total polyphenols. Subsequently, the antioxidant and antibacterial effects were determined. The last part of the research concerns the effect of the extract on the melanoma cell line A375. The water-soluble fraction, rich in chlorogenic acid, displayed a modest antibacterial effect, stronger against the fungus *Candida parapsilosis* than against the tested bacteria. It reduced the viability of A375 cells in a concentration-dependent manner, decreased cell number and confluence, presented a negative impact on the cell membrane integrity, and induced apoptosis. These effects warrant further investigations into the potential of the water-soluble fraction in the nutraceutical and dermatologic fields.

Rezumat

Semințele netorefiate de *Coffea arabica* sunt apreciate pentru menținerea stării de sănătate generale. Prezenta cercetare se concentrează pe compoziția fitochimică și bioactivitatea fracției hidrosolubile din aceste semințe. Primii pași au fost reprezentați de caracterizarea extractului cu ajutorul HPLC, înregistrarea spectrului FT-IR și evaluarea conținutului în polifenoli totali. Ulterior, au fost determinate efectele antioxidante și antibacteriene. Ultima parte a cercetării se referă la efectul extractului asupra liniei celulare de melanom A375. Fracția solubilă în apă, bogată în acid clorogenic, a prezentat un efect antibacterian modest, mai intens contra ciupercii patogene *Candida parapsilosis* decât împotriva bacteriilor testate. Extractul testat a redus viabilitatea celulelor A375 într-o manieră dependentă de concentrație, a scăzut numărul de celule și confluența lor, a avut un impact negativ asupra integrității membranei celulare și a indus apoptoza.

Keywords: *Coffea arabica*, green seeds, A375 melanoma cell line, HPLC

Introduction

Green coffee seeds are gaining momentum in disease prevention and treatment due to their impressive multi-targeted potential [8]. One of the easiest ways to exploit the favourable effects of green coffee is under the form of aqueous extracts. These extracts contain

mainly derivatives of phenolic acids, with chlorogenic acid (5-O-caffeoylquinic acid) being the main constituent [34]. They exert numerous positive effects in cardio-metabolic diseases: they are able to reduce the blood pressure after oral ingestion, an effect that is linked to ferulic acid, a metabolite of 5-caffeoylquinic acid [39]. Water-based green coffee extracts improve metabolic parameters such as insulin resistance, glycaemia, serum leptin and the levels of liver triglycerides [10]. Added effects on body weight [35, 42] are of high importance in the management of metabolic syndrome. The protective effect in stroke was demonstrated in rats with experimentally induced bilateral carotid artery occlusion, with green coffee seeds extract being more effective than the extract obtained from roasted seeds [33]. In fact, the levels of chlorogenic acids decrease with roasting, whereas caffeine levels are higher in roasted seeds [7]. Inflammatory processes are core components of various pathologies and studies have shown that green coffee water-soluble bioactive compounds can effectively decrease cytokines and enzymes. The survival of neurons, neuroprotection, the improvement of the neurological function, antioxidant and anti-inflammatory effects in this cell type resulting in the restoration of neurochemistry are key findings in several studies [3, 33]. In rats with fructose-induced Alzheimer's disease, decaffeinated green coffee extract was shown to impede the accumulation of beta-amyloid plaques and to increase the level of acetylcholine [26].

A notable research direction of green coffee extracts is the investigation of their anticancer properties. Tests were performed on cell lines of colon cancer [28], oral squamous cell carcinoma [31], lung, oesophageal and urinary bladder human carcinoma cells [5] with encouraging results. To our knowledge, the potential against melanoma cells was not yet investigated. The increased incidence of melanoma is one of the current concerns in cancer research. Around 331,700 new cases of skin melanoma were diagnosed in 2022, being the 17th most common type of cancer worldwide [19]. Melanoma cases have increased in recent years in developed countries, predominantly in fair-skinned people and it is responsible for most skin cancer deaths. Risk factors such as exposure to UV radiation, photo-type (phototypes I and II are more likely to develop melanoma), moles, indoor tanning, immunosuppression, genetic factors and even obesity have been related to this malignancy [13, 36].

Melanoma is a malignant transformation of melanocytes with a high metastatic potential. Melanocytes are cells derived from the neural crest whose main function is the production of melanin, the pigment responsible for the colour of the skin, eyes and hair [24]. Several clinical melanoma subtypes have been described, namely superficial spreading melanoma, nodular melanoma, lentigo malign melanoma and acral lentiginous melanoma, the first two being the most common [32].

The new therapeutic options have determined a significant improvement in the prognosis and survival rate of melanoma patients. The recently introduced immune checkpoint inhibitors and *BRAF/MEK* tyrosine kinase inhibitors have reduced mortality [40]. Despite the recorded progress, finding new therapeutic strategies is of particular interest, considering the side effects of the treatment and the different mechanisms of drug resistance. Drug combinations and new molecules have been studied to address these limitations [15]. Considering that plants are the oldest sources of bioactive compounds, recent studies have also focused on the anticancer effects of plant compounds and plant extracts. Tumour shrinkage, regression in tumour angiogenesis as well as a decrease in metastatic potential have been reported in studies for plant extracts [4]. Epigallocatechin-3-gallate, quercetin, lycopene, apigenin, piperine or resveratrol are some of the plant derived compounds that have shown positive effects in studies on melanoma [22].

In the current research, the authors aim to contribute to the exploration of the preventative and therapeutic potential of green coffee, taking as a subject the water-soluble fraction of a crude ethanol extract. After a thorough physicochemical characterization using HPLC, FT-IR and Folin-Ciocâlteu assay, its anti-oxidative, antibacterial and anti-melanoma efficacy were explored.

Materials and Methods

Reagents and plant material

Ethanol was purchased from Thermo Fisher Scientific; petroleum ether, diethyl ether, ethyl acetate and *n*-butanol (of analytical grade) were obtained from Sigma-Aldrich. HPLC reagent were of gradient grade and obtained as well from Sigma-Aldrich. The plant material, represented by dried, unroasted (green) seeds of *Coffea arabica* was purchased from Ethiopia Sidamo.

Extraction protocol

For the obtainment of the extract, 300 g seeds were milled with an IKA A11 basic equipment. One litre ethanol 95% was added to the powder and the mixture was left to macerate for 24 h at 22 ± 2°C. After that, the mixture was sonicated for 30 min using an ultrasonic water bath (ELMA S120 Elmasonic from Elma Schmidbauer GmbH, Singen, Germany, operated with ultrasonic frequency 37 kHz, ultrasonic power 200 W), followed by a filtration procedure using the Whatman Grade 4 filter. The ethanol phase (Ca-EtOH) was obtained by concentrating the ethanolic extract using a rotary evaporator (Laborata 4000eco from Heidolph Instruments, GmbH & Co. KG, Schwabach, Germany), at 97 mbar and 35°C. The initial parameters set were water bath temperature at 35°C, 40 rpm, refrigerant water at 10°C and starting pressure at 300 mbar. The separation operation was repeated twice, and it used 2 x 1000 mL ethanol 95% (EtOH 95%) respectively. Thus, 29.20 g of crude extract were obtained, of which

4.20 g was preserved for further investigations and analysis, and the remaining 25.00 g, were partitioned with various solvents (petroleum ether, diethyl ether, ethyl acetate and *n*-butanol). To this end, the crude extract was suspended in 135 mL distilled water, stirred and transferred into a separation funnel. After this step, organic solvents of increasing polarity (petroleum ether, diethyl ether, ethyl acetate and n-butanol) were sequentially added over the *C. arabica* aqueous suspension. Each solvent was used in 4-fold repetitions of 200 mL each. Each organic phase was subsequently concentrated using a rotary evaporator (parameters are detailed in Figure 1 yielding the petroleum-ether, diethyl ether, ethyl acetate and n-butanol-soluble fractions of the crude extract, respectively. The remaining aqueous phase was concentrated at 20 mbar and 35°C, giving the extract tested in the current research (3.95 g). The yield of the water-soluble fraction (15.8%) was calculated by dividing the amount of this fraction through the amount of crude extract subjected to fractionation, followed by multiplication with hundred. All the extracts obtained were stored in a refrigerator at 4°C until further use.

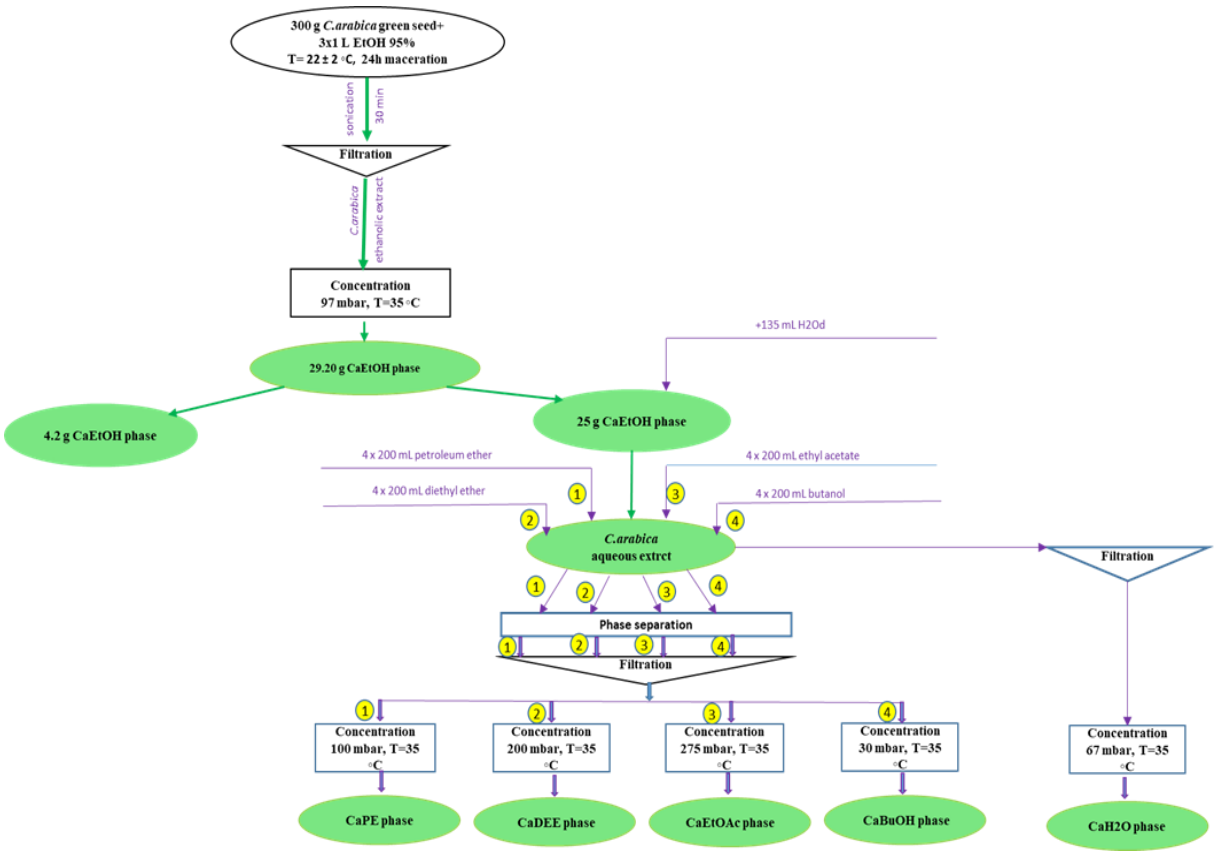

Figure 1.
The schematic representation of the extraction protocol

Phytochemical Analysis of Plant Extract by HPLC

The phytochemical composition of the extract was assessed using liquid chromatography tandem mass spectrometry (LC-MS/MS) through two previously validated analytical methods [2, 41, 43]. The employed equipment consisted in the Agilent Technologies 1100 HPLC Series system (Agilent, Santa Clara, CA, USA), which featured an autosampler, column thermostat, binary gradient pump, degasser and UV detector. This setup was coupled with an Agilent Ion Trap 1100 SL mass spectrometer (LC/MSD Ion Trap VL) [20].

Chromatographic separation was achieved on a reverse-phase Zorbax SB-C18 analytical column (100 mm x 3.0 mm i.d., 3.5 µm particle size, Agilent Technologies). The mobile phase consisted of methanol and 0.1% acetic acid (v/v) in a binary gradient. The elution began with a linear gradient from 5% methanol, rising to 42% over 35 minutes, followed by isocratic elution at 42% methanol for 3 minutes, and then re-equilibrating with 5% methanol for the next 7 minutes [6]. The column temperature was set at 48°C, with a flow rate of 1 mL/min and an injection volume of 5 µL. Detection was performed using both UV and MS modes, with the UV detector set at 330 nm for poly-phenolic acids up to 17 minutes and at 370 nm for flavonoids and their aglycones up to 38 minutes. The MS operated in electrospray ionization (ESI) negative mode, with a capillary voltage of 3000 V, a nebulizer pressure of 60 psi (nitrogen) and a gas flow rate of 12 L/min at 360°C [18, 38].

The identification of each bioactive compound involved comparing MS spectra/traces with library standards, followed by quantification using UV detection, considering calibration curves of corresponding analytical standards. The results were processed using DataAnalysis (v5.3) and ChemStation (vB01.03) software from Agilent, with quantifications expressed as micrograms *per* mL of plant extract.

Fourier-Transformed Infrared Spectroscopy (FT-IR)

The functional groups of the phytochemicals contained in the water-soluble fraction of *Coffea arabica* seeds were investigated through the qualitative method FTIR spectroscopy, using the Prestige-21 spectrometer (Shimadzu, Duisburg, Germany). The extract was mixed with KBr until a pellet was obtained and further analysed at room temperature (22 ± 2°C) in the spectral region from 4000 cm^{-1} to 400 cm^{-1}, with a resolution of 4 cm^{-1}. The spectra were interpreted based on the match between the recorded absorption bands of the aqueous extract of *Coffea arabica* green seeds at a specific wavenumber and the absorption band frequencies contained in the electronic library [11].

Total polyphenol content (TPC)

The total phenolic content was determined using the Folin-Ciocâlteu method, slightly modified [14]. Briefly, the extract was treated with a mixture of acetone, methanol, distilled water and acetic acid by (40 mL acetone + 40 mL methanol + 20 mL distilled water + 0.1 mL acetic acid), heated at 60°C for 1 hour and cooled to room temperature. Determinations were made in triplicate with Folin-Ciocâlteu and 7.5% Na_2CO_3 solution. Absorbance was read at 726 nm using an Agilent BioTek Synergy H1 Hybrid Multi-Mode Reader. Total polyphenol content was expressed as chlorogenic acid equivalents in mg/g of extract.

Antioxidant capacity

The water-soluble fraction of *Coffea arabica* green seeds was investigated in terms of antioxidant capacity using DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical-scavenging assay [37]. Six different concentrations (1 mg/mL, 0.8 mg/mL, 0.5 mg/mL, 0.3 mg/mL and 0.1 mg/mL) of this extract were prepared and tested. First, a 0.1 mM DPPH ethanolic solution was prepared and stored at 4°C until further use. As a standard, the ethanolic solution of ascorbic acid (1 mM in 95% EtOH) was used. The analysis consisted of measuring a volume of 0.3 mL of each test sample, mixed with 2.7 mL of 0.1 mM DPPH ethanolic solution, and the entire mixture was analysed spectrophotometrically at a wavelength of 517 nm in a quartz test cuvette (10 × 10 mm). By using the UviLine 9400 Spectrophotometer from SI Analytics (Mainz, Germany), were read in a continuous mode the absorbance, for 20 min. The antioxidant capacity (AOC%) was determined by using the following equation:

$$AOA\ [\%] = \left(\frac{A_{free\ radical} - A_{aqueous\ extract}}{A_{free\ radical}}\right) \times 100.$$

Assessment of antimicrobial effects

The antimicrobial activities of the water-soluble fraction were tested by Disk Diffusion Method (DDM) on known Gram-positive bacterial strains (*Staphylococcus aureus* ATCC 25923, *Streptococcus pyogenes* ATCC 19615), two Gram-negative bacterial strains (*Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853) and the fungus *Candida parapsilosis* ATCC 22019. All strains of microorganisms were purchased from ThermoScientific (Waltham, MA, USA). All bacterial strains were isolated on Columbia agar supplemented with 5% sheep blood, while for *Candida parapsilosis* Sabouraud agar with chloramphenicol (Oxoid, Wesel, Germany) was used. The microbial suspensions were produced in 0.85% NaCl (bioMérieux, Marcy-l'Étoile, France) at a concentration of 0.5 McFarland (approximately 1 - 2 x 108 colony-forming units/mL). The positive control was represented by disks with gentamicin for the antimicrobial tests (10 µg/disk for all bacteria except for streptococci where 120 µg were used) and fluconazole (25 µg) (from BioMaxima, Lublin, Poland) for the antifungal effect. Negative control was achieved by a blank disk impregnated with ethanol:water = 1:1. Antimicrobial activity was evaluated according to the recommendations of the EUCAST (European Committee on Antimicrobial Susceptibility Testing) [6] and the Clinical Laboratory and Standards Institute (CLSI) [12].

Cell-based assays

Reagents

A series of reagents were used to perform the *in vitro* analyses, including high glucose Dulbecco's Modified Eagle's Medium (DMEM) and foetal bovine serum (FBS) acquired from PAN-Biotech GmbH (Aidenbach, Germany). Penicillin/streptomycin mixture (Pen/Strep 10,000 IU/mL), trypsin-EDTA solution, phosphate-buffered saline (PBS) and dimethyl sulfoxide (DMSO) were purchased from Sigma Aldrich (Steinheim, Germany). The MTT (3-(4,5-dimethylthiazol2-yl)-2,5-diphenyltetrazolium bromide) kit, the lactate dehydrogenase (LDH) kit and the Hoechst 33342 dye were procured from ThermoFisher Scientific (Waltham, MA, USA).

Cell culture conditions and treatment protocol

The A375 cell line was purchased from the American Type Culture Collection (Manassas, VA, USA) as a frozen vial. Human melanoma cells were grown in standard conditions (5% CO_2 and 37°C) and cultured in a specific DMEM medium supplemented with 10% FBS and 1% Pen/Strep.

The water-soluble fraction was dissolved in 0.5% DMSO to obtain a 1 mg/mL stock solution. Next, the test samples were made by diluting the stock solution in six different concentrations (10, 25, 50, 75, 100 and 150 µg/mL).

Cellular viability

The impact of tested extract on cell viability was analysed using the MTT method. After stimulating

A375 cells for 24 hours with different concentrations of extract (10, 25, 50, 75, 100 and 150 µg/mL), 100 µL of fresh medium and 10 µL of reagent 1 MTT were added to the 96 wells, after which it was placed in the incubator for 3 hours. After the 3 hours, reagent 2 MTT was added for 30 minutes at room temperature. Absorbances were read at 570 nm using the Cytation 5 device.

Assessment of cell confluence and cell number

To determine the effect of water-soluble fraction on the confluence and number of A375 human melanoma cells after 24 hours of application, images were taken using the automatic Lionheart FX microscope at 4× objective (PL FL). Images were interpreted using the Cell Analysis Tool provided by the Gen5™ Microplate Data Collection and Analysis Software (Version 3.14) from BioTek Instruments Inc. (Winooski, VT, USA).

Cell morphology assay

The changes at the morphological level of A375 cells produced by the water-soluble fraction (10, 25, 50, 75, 100 and 150 µg/mL) were evaluated by making representative images of untreated and treated cells, with the microscope automatic Lionheart FX, under bright field illumination at magnification 20×.

Lactate dehydrogenase (LDH) leakage (Cytotoxic activity)

The cytotoxic activity of the water-soluble fraction (10, 25, 50, 75, 100 and 150 µg/mL) on cell membranes was determined by measuring the release of cytosolic LDH in the culture medium after 24 hours of treatment. Initially, 50 µL of medium with released LDH is transferred to a new plate with 96 wells over which 50 µL of reaction mixture is added, after which it is incubated for 30 minutes. Finally, 50 µL of stop solution was added to the wells, and the absorbances were read at two wavelengths: 490 nm and 680 nm using Cytation 5.

Hoechst 33342 nuclear staining

To highlight the changes induced by the water-soluble fraction on the shape of the nuclei of human melanoma cells, staining with Hoechst 33342 was performed. After the 24-hour treatment, the culture medium was removed from the plates (12 wells), and the Hoechst solution (1:2000 dilution in PBS) was added and left in the dark at room temperature for 5 - 7 minutes. After this period, the staining solution was removed and the cells were washed with PBS (2x). Image processing was performed using the Lionheart FX automated microscope and Gen5™ Microplate Data Collection and Analysis Software (Version 3.14) from BioTek Instruments Inc. (Winooski, VT, USA).

Statistical analysis

In vitro results were analysed using the GraphPad Prism program (GraphPad Software, software version 9.4.0 for Windows, San Diego, CA, USA). The data were indicated as means ± standard deviation, and the differences between the means were compared by one-way ANOVA and Dunnett's multiple comparison post-test (GraphPad Prism v. 9.4.0 Software, San Diego, CA, USA). Statistically significant differences between the results were highlighted with * (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$).

Results and Discussion

Green *Coffea arabica* seeds have garnered significant attention recently for their potential therapeutic applications. This interest is largely attributed to their rich phytochemical profile. The current research investigated only the water-soluble fraction contained in green coffee seeds. This fraction was prepared through the depletion of a crude extract with organic solvents of increasing polarity, as depicted in Figure 1. The first objective was to perform phytochemical analysis of the water-soluble fraction, using HPLC-MS, FT-IR and the spectrophotometric evaluation of total polyphenols. The HPLC chromatogram revealed the presence of five peaks, of which the identity of chlorogenic acid and 4-O-caffeoylquinic acid could be confirmed in comparison with references.

For each compound, a limit of quantification and detection was imposed (0.1 µg/mL), calculated as the minimum concentration capable of producing a reproducible peak with a signal-to-noise ratio > 3. Analyses of the water-soluble fraction from green *Coffea arabica* seeds show that the major compound is chlorogenic acid (5-*O*-caffeoylquinic acid), followed by 4-*O*-caffeoylquinic acid (Table I). The prominent occurrence of various esters of caffeic acid with quinic acid are in accordance with the scientific literature [17].

Table I

Identification and quantification of compounds present in the water-soluble fraction from green *C. arabica* seeds by HPLC-MS

No.	Compound	Concentration in the fraction (µg/mL)
1	Chlorogenic acid	30.096 ± 0.903
2	4-*O*-caffeoylquinic acid	6.171 ± 0.864

< LOQ - below the limit of quantification of the analytical method

The analysis of the extract with the aid of FT-IR was able to point out the main functional groups of the metabolites present in this fraction. Details of the peak values as well as the functional groups recorded in the FT-IR spectrum are presented in Table II.

Table II

The peak values and functional groups recorded in the spectrum of the water-soluble fraction from green *Coffea arabica* seeds

Bond	Functional groups	Wavenumber (cm^{-1})
OH stretch/H bonded	alcohol	3446.79
OH stretching	carboxylic acid	3078.39
C-H stretching	alkane	2935.66
C=O stretch	α,β unsaturated esters/carboxylic acids	1716.65
C=O stretching	conjugated acid/conjugated aldehyde	1699.29
C=C stretching	conjugated alkene (mono-, disubstituted-cis)	1647.21
C-H bending/C=C stretch	alkane/aromatic compounds	1471.69
C-O stretch	acids/aromatic esters	1261.45
C-O stretching	tertiary alcohol/esters	1174.65
C-O stretching	secondary alcohol/aliphatic ether	1091.71
C-O stretch	ether	1028.06
C-H bend (meta)/C=C bending	aromatics/alkene	887.26
C=C bending (trisubstituted)	alkene	800.46

The most intense absorption bands are recorded at 3446.79 cm^{-1} (O-H stretching vibration functional groups from alcohols); at 2935.66 cm^{-1} (C-H stretching vibration functional groups from alkanes); 1716.65 cm^{-1} – attributed to the C=O stretching vibration of α,β unsaturated esters or carboxylic acids functional groups. The last intense absorption band recorded in the *C. arabica* aqueous extract spectrum is located at 1699.29 cm^{-1} - attributed to the C=O stretching vibration functional groups, present either in conjugated acid or in conjugated aldehyde.

Some medium absorption bands are recorded on the *C. arabica* aqueous extract spectrum, as follows: the band located at 1471.69 cm^{-1} could be attributed either to the C-H bending vibration functional group from alkane, or to the C=C stretching vibration functional group from aromatic compounds. At 1261.45 cm^{-1} could be recorded the presence of C-O stretching vibration functional groups from acids or aromatic esters present in the aqueous extract of *C. arabica*. Another C-O stretching vibration functional groups are recorded on the spectrum at 1174.65 cm^{-1}, and these groups could be assigned to the tertiary alcohols or the esters from aqueous extract; as well as recorded at 1028.06 cm^{-1} wavenumber, and this group could be attributed to the ethers present in the aqueous extract of *C. arabica*. At 887.26 cm^{-1} and 800.46 cm^{-1} wavenumbers recorded the C=C bending vibration functional groups from alkenes, but, at the same time, the absorption band recorded at 887.26 cm^{-1} could be assigned also to C-H bending vibration functional groups (meta position) from aromatic compounds. The rest of the absorption bands recorded on the *C. arabica* aqueous spectrum are of weak intensity, located at 3078.39 cm^{-1} (assigned to O-H stretching vibration functional groups from carboxylic acids); 1647.21 cm^{-1} (attributed to the C=C stretching vibration functional groups from conjugated alkene (mono-, or disubstituted in cis position)); and 1091.71 cm^{-1} (attributed to the C-O stretching vibration functional groups from secondary alcohol or aliphatic ethers).

The bands measured by FT-IR concur with the functional groups present in chlorogenic acid and its isomers, identified previously through HPLC-UV and HPLC-MS.

With the aid of the Folin-Ciocâlteu assay, it could be established that the water-soluble fraction of green coffee seeds had a total phenolic content of 253.25 mg chlorogenic acid equivalents (CAE)/g of extract. The results suggest that the water-soluble fraction contains a significant amount of health-promoting phenolic compounds, which will reflect on biological activities like antioxidant effects, and is also relevant from the viewpoint of daily coffee consumption as a beverage.

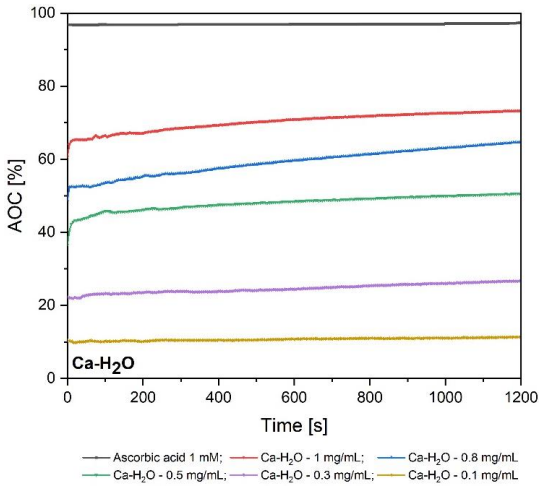

Figure 2.

The time-dependent antioxidant capacity (AOC) of the water-soluble fraction from green *Coffea arabica* seeds

The antioxidative capacity of the extract was subsequently established through the DPPH free radicals test. Evaluations were performed at five different

concentrations, and compared with ascorbic acid. The time-dependency of the radical-scavenging effect was recorded over a 20 minutes period and is depicted in Figure 2.

The aqueous fraction provides a concentration-dependent antioxidant effect. The first two concentrations tested (1 mg/mL and 0.8 mg/mL) reacted with the DPPH free radicals throughout the entire analysis period, without the kinetics of the reaction reaching equilibrium. At 0.5 mg/mL extract concentration, the polyphenols present in the sample consumed the DPPH free radicals after 300 seconds, and then the equilibrium of the reaction was set. As the concentration of the aqueous extract decreases, *i.e.* as the amount of polyphenols in the sample decreases, the equilibrium of the reaction is reached faster (in the first 200 seconds in the case of 0.3 mg/mL aqueous extract concentration and the first 100 seconds in the case of 0.1 mg/mL aqueous extract concentration), the final colour of the solution being purple.

The antioxidant capacity of the tested fraction, compared with the antioxidant capacity of ascorbic acid (1 mM, dissolved in ethanol) is shown in Table III. The antioxidant capacity values, expressed in percent, obtained for all five concentrations tested of *C. arabica* aqueous extract represent an average of three measurements ± standard deviation (SD).

Table III

The initial and final AOC values (%) of the water-soluble fraction from green *Coffea arabica* seeds at five concentrations tested as compared with ascorbic acid ethanolic solution of 1 mM (standard)

Sample tested	Concentration [mg/mL]	Initial AOC ± SD [%] Time 0 seconds	Final AOC ± SD [%] After 1200 seconds
Ca-H_2O	1	63.24 ± 2.17	73.17 ± 0.02
	0.8	51.21 ± 2.09	64.66 ± 0.04
	0.5	39.73 ± 2.85	50.59 ± 0.02
	0.3	22.12 ± 0.16	26.66 ± 0.04
	0.1	10.25 ± 0.15	11.30 ± 0.04
Ascorbic acid	1 mM	96.83 ± 0.008	97.36 ± 0.04

The results of our study suggest that the water-soluble fraction of green *Coffea arabica* seeds has a modest, but concentration-dependent antioxidant capacity. The antioxidant property of *Coffea arabica* extracts has been extensively studied, revealing the capacity to scavenge free radicals and reduce oxidative stress. It is primarily attributed to the high total phenolic content, which acts as a reducing agent and hydrogen donor, thereby mitigating oxidative damage [29]. A comparative study on the antioxidant activities of green and roasted *Coffea arabica* extracts showed that both forms possess significant antioxidant properties, although the roasting process may lead to a reduction in certain phenolic compounds. In their study, Gligor and co-workers measured an antioxidant capacity of 9.160 mg/mL Trolox equivalent in green seeds of *Coffea arabica* [21]. Also, the study conducted by Masek and co-workers on the antioxidant activity of aqueous extract from green *Coffea arabica* seeds shows that it intensifies over time at a concentration of 4 mg/mL, where free radical inhibition increases from 69.6% to 81.6% in 30 minutes. This progress suggests a prolonged antioxidant effect, providing sustained protection against oxidative stress with long-term benefits [25]. Another study demonstrated that roasting coffee seeds leads to a 68.8% increase in antioxidant activity compared to green seeds. This enhancement is due to melanoidins, antioxidant compounds formed by Maillard reactions during roasting. These results indicate that thermal processing can modify the biochemical profile of coffee, thus increasing the antioxidant potential of the final product [1].

The health benefits associated with *Coffea arabica* extracts extend beyond their antioxidant properties. Research has indicated that these extracts can exhibit antimicrobial activity against various pathogens [23]. Concerning the antimicrobial activity of the extract prepared in the current study, our results showed a relatively modest activity against the tested microorganisms, in comparison to the positive control gentamicin. The effect varied depending on the type of pathogen (Table IV). The tested fraction showed moderate activity against *Staphylococcus aureus* and *Streptococcus pyogenes* suggesting that water-soluble compounds from green coffee may have a limited efficacy against Gram-positive bacteria commonly involved in skin infections. Antimicrobial activity was lowest against *Pseudomonas aeruginosa*. This can be explained by the structure of the cell wall of Gram-negative bacteria, which forms an effective barrier, reducing the access to antimicrobial compounds. In the case of *Candida parapsilosis*, the aqueous extract had a mild activity, with a zone of inhibition of about 11 mm, significantly lower than that of the antifungal positive control fluconazole. Altogether, these results suggest a modest antimicrobial effect of the tested extract, with the fungus *Candida parapsilosis* being the most sensitive. With regard to the effects of water-soluble extracts of green coffee seeds on bacteria, previous studies have shown their capacity to modulate the gut microbiota [10], and to reduce the bacterial charge in the oral cavity.

Table IV

The inhibition zone established through the disk diffusion method (DDM), after application of the water-soluble fraction from green coffee seeds

Microbial strains	DDM inhibition zone (mm) achieved by the tested fraction	DDM inhibition zone (mm) achieved by the positive control
Streptococcus pyogenes (Gram+)	**8**	**20***
Staphylococcus aureus (Gram+)	**8**	**20****
Escherichia coli (Gram–)	**9**	**20****
Pseudomonas aeruginosa (Gram–)	**7**	**19****
Candida parapsilosis (fungus)	**11**	**23*****

* positive control gentamicin (120 μg/disk), * positive control gentamicin (10 μg/disk), ** positive control fluconazole (25 µg/disk)

An important objective of our research was the evaluation of the water-soluble fraction against A375 human melanoma cells (A375 cell line). To evaluate the activity of the extract on the viability of these cells, MTT colorimetric analysis was performed as a first step. After 24 hours of stimulation with the samples containing six different concentrations, a decrease in cell viability was observed as the concentration increased, reaching a viability of 66% at 150 µg/mL. At the lowest concentration, a slight, but statistically insignificant cell proliferation is observed (Figure 3).

Next, the action of the water-soluble fraction from green coffee seeds on the confluence and the number of A375 cells was determined. Figure 4 shows that the decreasing values of the number of cells and the confluence are directly proportional to the increase in the applied dose. Thus, at 100 µg/mL values of ≈ 83% and 81% were observed, respectively.

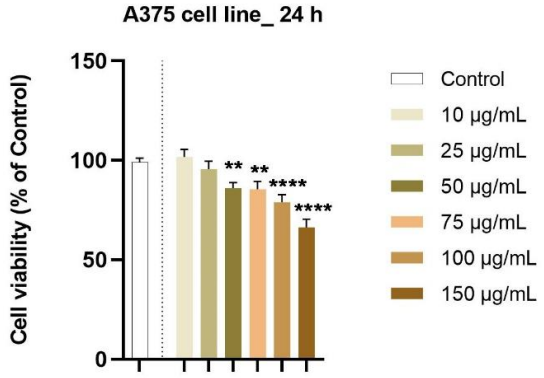

Figure 3.

In vitro cell viability assay of the tested extract (10, 25, 50, 75, 100 and 150 µg/mL) in A375 cells after 24 h treatment. A one-way ANOVA test followed by Dunnett's multiple comparisons post-test assessed the statistical differences between the control and the treated group (** $p < 0.01$; **** $p < 0.0001$)

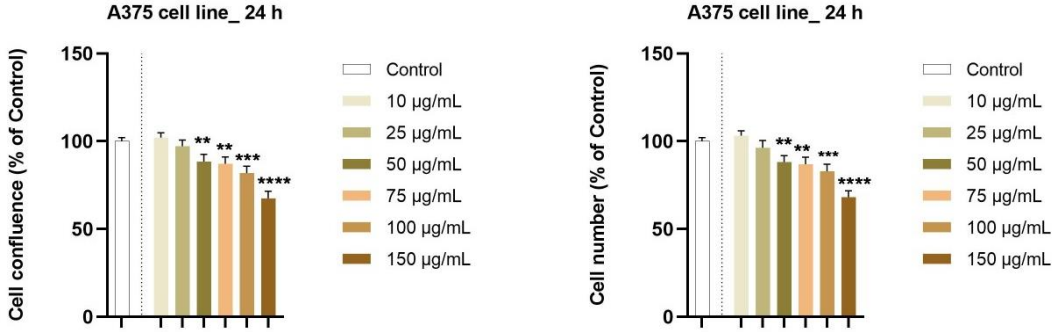

Figure 4.

Graphical representation of confluence and cell number in A375 cells at 24 h after treatment with the water-soluble fraction from green coffee seeds (10, 25, 50, 75, 100 and 150 µg/mL). A one-way ANOVA test followed by Dunnett's multiple comparisons post-test assessed the statistical differences between the control and the treated group (** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$)

The cytotoxic action of the water-soluble fraction from green coffee seeds on the cell membrane was quantified by measuring the release of LDH in the medium. The obtained results show a concordance with the data on cell viability. Thus, at the dose of 10 µg/mL, a value of ≈ 3% is noted, which increases up to 31% at the concentration of 150 µg/mL (Figure 5).

To complement the data, the effect of the extract on the morphology of A375 cells was explored. After treating the cells for 24 hours, changes in the cell shape were exposed as the dose increased, as can be seen in Figure 6. Thus, a shrinking of the cells is highlighted, a deformation of them with the appearance of round cells, signs observed especially from the concentration of 75 µg/mL.

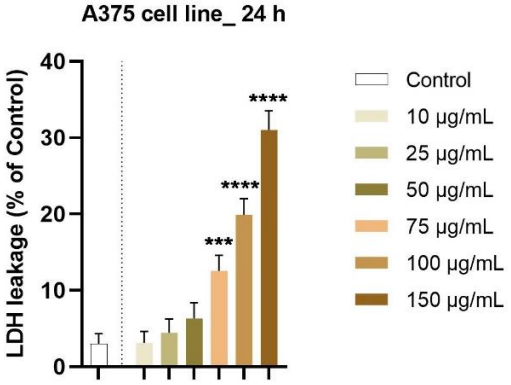

Figure 5.

Graphical illustration of LDH leakage in A375 cells at 24 h after treatment with the water-soluble fraction from green coffee seeds (10, 25, 50, 75, 100 and 150 µg/mL). A one-way ANOVA test followed by Dunnett's multiple comparisons post-test assessed the statistical differences between the control and the treated group (*** $p < 0.001$; **** $p < 0.0001$)

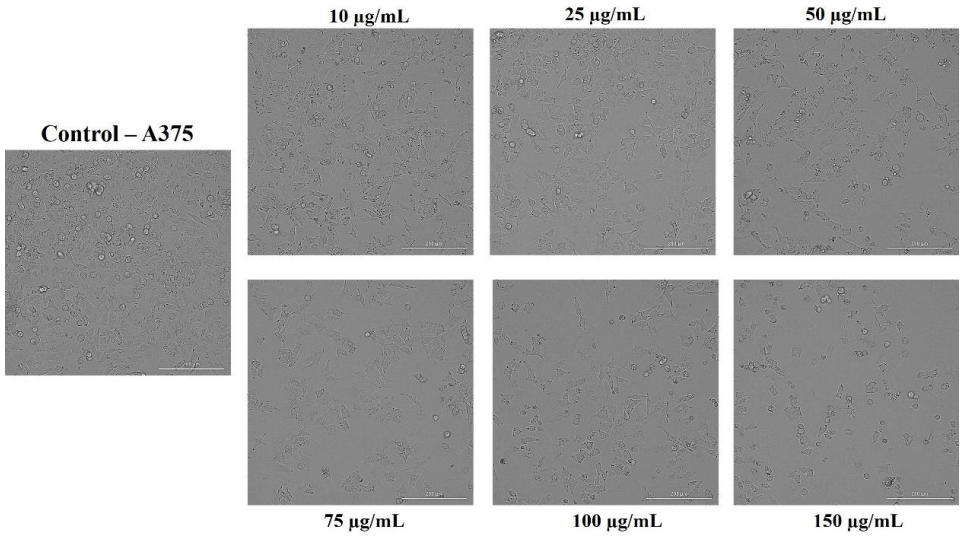

Figure 6.

The morphological aspect of A375 cells after 24 h of treatment with the water-soluble fraction from green coffee seeds. The scale bars represent 200 µm

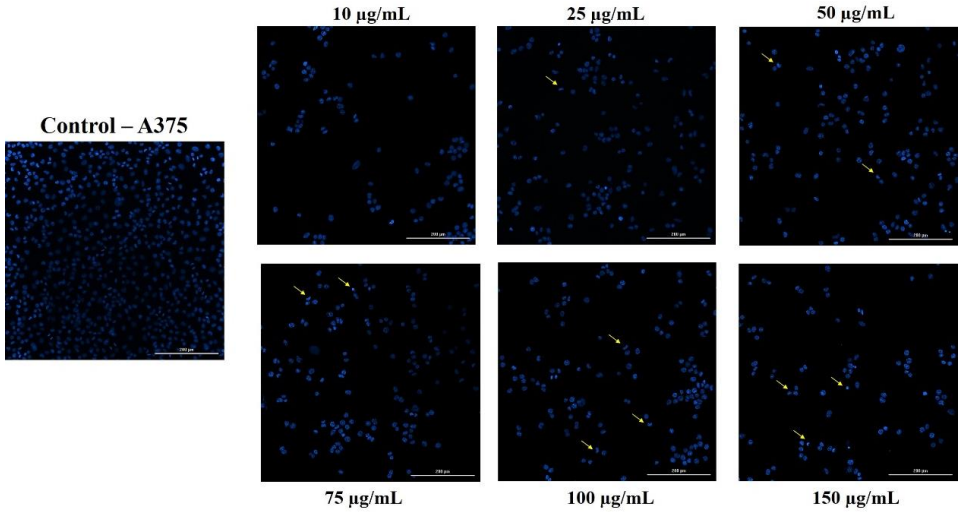

Figure 7.

Nuclear changes after 24 h of treatment with the water-soluble fraction from green coffee seeds (10, 25, 50, 75, 100 and 150 µg/mL). The yellow arrows underlined the apoptosis signs. The scale bars indicate 200 µm

Finally, the impact on nuclear morphology was determined by Hoechst 33342 staining. After applying the samples, slight signs of apoptosis were observed at the concentrations of 50 and 75 µg/mL, respectively. While at the highest concentrations, the changes were more obvious, a decrease in the size of the nuclei was observed, with the presence of apoptotic bodies (Figure 7).

Our *in vitro* results showed the water-soluble fraction from the green coffee seeds exerts a modest, but concentration-dependent cytotoxic effect on human melanoma A375 cells. As the concentration of the extract increases, cell viability decreases, reaching 66% at a concentration of 150 µg/mL. This effect is accompanied by a reduction in the cell number and confluency, as well as increased lactate dehydrogenase (LDH) release, indicating cell membrane damage. Obvious morphological changes such as cell shrinkage and rounding were also observed, especially at higher concentrations. Analysis of nuclear morphology revealed clear signs of apoptosis, including the presence of apoptotic bodies and a reduction in the size of the nuclei. Our data are in line with other studies that have indicated that aqueous extracts from coffee exhibit low toxicity to normal cells while targeting cancer cells. In this context, El-Garawani *et al.* revealed that the combination of green and roasted coffee extracts with Vitamin C significantly enhanced cell death in MCF-7 breast cancer cells, highlighting the potential of these extracts as adjunctive therapies in cancer management. This combination not only increased the cytotoxic effects on cancer cells but also demonstrated selective toxicity towards malignant cells compared to normal lymphocytes, suggesting a promising window for *Coffea arabica* extracts in oncology [16]. Chlorogenic acid, a prominent polyphenol present in green coffee seeds, was investigated for its anticarcinogenic potential through several cellular and molecular mechanisms that contribute to tumour growth inhibition. According to a study by Ombra *et al*., chlorogenic acid has a notable antioxidant effect, reducing levels of reactive oxygen species (ROS) and oxidative stress, thereby helping to protect the integrity of DNA and cellular proteins against oxidative damage [27]. The study demonstrated that chlorogenic acid exerts the anti-proliferative effects by inducing cell cycle arrest, limiting the uncontrolled replication of tumour cells. In addition, chlorogenic acid promotes apoptosis in cancer cells, facilitating their elimination and reducing tumour progression. Through its anti-inflammatory effects, chlorogenic acid helps to reduce chronic inflammation, a known risk factor for cancer. Also, by modulating metabolic pathways, including glucose and lipid metabolism, chlorogenic acid affects metabolic processes involved in tumour growth, indicating its adjuvant role in preventing cancer risk and progression [27]. In another recent study, chlorogenic acid was shown to inhibit the proliferation of cancer cells, including melanoma, under hypoxic conditions, which limits their ability to multiply and survive in unfavourable environments. Chlorogenic acid was found to interact with the ACAT1 (acetyl-CoA acetyltransferase 1) protein, influencing metabolic pathways essential for tumour cell survival. The study mentions the clinical relevance of chlorogenic acid, as it has been investigated in clinical trials for solid tumours, highlighting its viability as an oncological therapeutic agent. Another study indicates that chlorogenic acid may inhibit the migration and invasion of cancer cells, thereby preventing metastasis and limiting tumour dissemination. An important mechanism by which chlorogenic acid acts is the modulation of signalling pathways involved in cell survival, such as the PI3K/AKT pathway, having a direct effect on cell growth control and apoptosis. These properties support the promising potential of chlorogenic acid as a chemo-preventive agent in anticancer treatments [30], an important aspect for the current research as the water-soluble fraction from green coffee is a major source of chlorogenic acid. However, taking into account the content of 30 µg/mL chlorogenic acid in our extract, it can by hypothesized that the observed effect against melanoma is due to a synergistic effect of this major compound with other phytochemicals that are also present. Their identification and a better understanding of the combined mechanism of action warrants further, meaningful studies.

Conclusions

The present research investigated the water-soluble fraction of *Coffea arabica* seeds, establishing that it has a high content in total polyphenols, its main constituent being is chlorogenic acid. This extract showed concentration-dependant antioxidative effects in the DPPH assay, having a prolonged radical-quenching effect as recorded over a twenty minutes timespan. The antimicrobial and anti-melanoma effects observed in our study, albeit modest, testify for an essentially protective effect of the water-soluble compounds present in green coffee, in agreement with its nutritionally (as opposed to pharmacologically) relevant properties.

Acknowledgement

We would like to acknowledge the "Victor Babeș" University of Medicine and Pharmacy Timișoara for their support in covering the costs of publication for this research paper. The authors also thank Prof. Filomena Conforti from the University of Calabria, Italy, for the collaboration in the analysis of total polyphenols and acknowledge the support given through project 2023-1-RO01-KA220-HED-000164767.

Conflict of interest

The authors declare no conflict of interest.

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