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Chemical Profile, Antioxidant and Antimicrobial Activity of Red Grapefruit (*Citrus paradisi* Macf.) from Serbian Market

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Abstract The aim of this work is to give in one place as much information as possible on red grapefruit (*Citrus paradisi* Macf.) present on Serbian market. In order to provide chemical composition UHPLC-ESI-MS was employed for both polar and non-polar red grapefruit extracts obtained with solvent mixture consisting of hexane, acetone, and ethanol in volumes ratio 2:1:1, respectively and 0.05% (w/v) butylated hydroxytoluene. In addition, total content of lycopene, *β*-carotene, total polyphenols, and flavonoids content were determined by the means of UV-Vis spectrophotometry. The antioxidant activity of the samples was tested by applying four different methods: ABTS, DPPH, FRAP, and CUPRAC. In the chemical profile of the red grapefruit, 11 compounds of interests were identified by UHPLC method and they all were correlated to antioxidant and antimicrobial activity. The antimicrobial activity was tested against gram-positive (*Staphylococcus aureus*, *Enterococcus faecalis*, *Bacillus cereus*) and gram-negative (*Escherichia coli*, *Salmonella enteritidis*, *Enteroabacter aerogenes*, *Pseudomonas aeruginosa*) bacteria and yeast *Candida albicans*. The obtained results are as follows: lycopene, *β*-carotene, total polyphenols, and flavonoids content 34.2 mg/kg fruit weight, 18.6 mg/kg fruit weight, 298 mgGAE/kg fruit weight, 216.4 mgCE/kg 216.4, respectively; antioxidant activity by: ABTS 1.8 mmol of TE/kg of sample, DPPH 0.72 mmol of TE/kg of sample, FRAP 9.7 µmol of Fe^{2+} equivalents/kg of the sample, CUPRAC 4.66 mmol of TE/kg of sample; and they are the basis for explanation of antimicrobial activity obtained in this work. Results obtained in this study are recommending red grapefruit in everyday diet as valuable source of health beneficial bioactive compounds.

Keywords: *red grapefruit, lycopene, β-carotene, antimicrobial activity*

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1. Introduction

Citrus fruits are widely consumed around the world. Among them, grapefruit (*Citrus paradisi* Macf.) stands out with its distinctive appearance, flavor, and health benefits. It is a subtropical tree that belongs to the *Rutaceae* family and is known for its mild sour to semi sweet fruit [1]. It was proved that grapefruit juice has antiseptic potential including bone growth promotion and weight loss [2] while it's peel can be effectively used as biopesticides against mosquito larvae [3]. Certainly, grapefruit's positive influence on various biochemical processes is owed to it's content of important bioactive compounds, so called, phytochemicals.

Phytochemicals frequently found in fruits and vegetables are an excellent basis for a healthy diet. It is confirmed that phytochemicals may exhibit a potential for modulating human metabolism in a manner favourable for the prevention of chronic and degenerative diseases [4]. Their role in mediating antioxidant processes is a key factor in prevention of cell damage, reducing the risks of many diseases, boosting the immune system, balancing hormone levels, and notable antibacterial and antiviral response of the organism [5]. Phytochemicals cover wide classes of chemicals including polyphenols, carotenoids, vitamins, omega-3 fatty acids, organic acids, nucleosides and nucleotides, phytosterols, etc. Each of these classes of compounds give natural products unique nutritive and bioactive characteristics as a consequence of defined chemical composition. Bioactive compounds have specific

place in pharmacological and cosmetic industry in the form of different commercial products able to provide health and beauty care. The groving interest in this field is covered by novel studies based on fruits and vegetables analyses especially in terms of phytochemicals content and their impact on the healing or prevention of disease.

Carotenoids are a class of phytochemicals of yellow to red color which are often found in vegetables and fruits as an essential matter valuable to human diet. They have specific impact in mediating biochemical reactions which is crucial in elimination of the negative effects of free radicals. The most popular among carotenoids are lycopene and *β*-carotene. Lycopene is known as a strong antioxidant and antimicrobial agents which is found in higher quantities in tomatoes, pink grapefruit, apricots, red oranges, watermelon, and guava. Its ability to quench singlet oxygen and interact with free radicals is a proven effect which is crucial in reducing the risk of degenerative diseases such as cancer of the lungs, bladder, cervix, prostate, breast, and skin, atherosclerosis, and associated coronary artery disease [6-8]. *β*-Carotene is yellow-orange carotenoid which is found in many plants, fruits, and fungi. Extensive conjugation of double bonds in it's molecule is responsible for intense absorption of visible light which is important in antioxidation processes [9].

Flavonoids are a group of polyphenolic compounds often found in fruits and vegetables which cover flavonols, flavones, flavanones, catechins, anthocyanidins, and isoflavones. It is reported that specific flavonoids such as quercetin, naringenin, rutin, and chlorogenic acid can help against inflammation, cardiovascular diseases, and in cancer prevention [10].

One should be very careful, one up to two glasses of grapefruit juice acts by inhibiting presystemic drug metabolism via CYP3A isoforms in the small bowel and can change drug concentration [11]. This interaction is reported with more than 40 different drugs (Amiodarone, Atrovastatin, Buspirone, Carbamazepine, Dazepam, Felodipine, etc.) [12].

Grapefruits contain several phytochemicals including carotenoids, flavonoids (naringin), limonoids, organic acids, pectin, and folate [13-15]. Their content varies with the fruit variety, along with the way of it's cultivation, harvesting and storage. Considering the worldwide consumation of grapefruit and importance of compounds responsible for it's antioxidant and antimicrobial activity, there is a justified need to estimate content of these compounds. Hence, in this paper, concentrations of lycopene, *β*-carotene, total polyphenols, and flavonoids were determined and correlated to antimicrobial and antioxidant activity of grapefruit available in Serbia.

2. Material and Methods

2.1. Material

For this experimental research fresh red grapefruits bought from the local supermarket in Niš, Serbia was used. Country of their production is Turkey.

All chemicals used in the present study were of analytical grade. Acetone was obtained from Fisher Scientific (Loughborough, United Kingdom). Hexane, butylated hydroxytoluene, sodium acetate, ethanol, glacial acetic acid, Folin & Ciocalteu'S phenol Reagent Pure (Fisher Chemical™, Germany), Na_2CO_3 (Merck, Germany), 5% $NaNO_2$ (Merck, Germany), $AlCl_3$ (Sigma Aldrich Chemical Co. St Louis, Mo, USA), NaOH (Sigma Aldrich Chemical Co. St Louis, Mo, USA), 2,2-diphenyl picrylhydrazyl (DPPH), and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) were purchased from Sigma-Aldrich (Steinheim, Germany).

The antimicrobial activity of the samples was evaluated against a collection of microorganisms in the laboratory, including Gram-positive bacteria such as *Staphylococcus aureus* (ATCC 6538), *Enterococcus faecalis* (ATCC 19433), and *Bacillus cereus* (ATCC 11778), as well as Gram-negative bacteria including *Escherichia coli* (ATCC 25922), *Salmonella enteritidis* (ATCC 13076), *Enterobacter aerogenes* (ATCC 13048), and *Pseudomonas aeruginosa* (ATCC 9027), and yeast *Candida albicans* (ATCC 24433). The bacterial analysis was performed using overnight cultures grown on Mueller-Hinton agar (Institute of Virology, Vaccines and Sera "Torlak", Belgrade, Serbia), while yeast was analyzed on Sabouraud dextrose agar (Institute of Virology, Vaccines and Sera "Torlak", Belgrade, Serbia).

2.2. Extraction Procedures

Representative fruits were washed with deionized water and seedless pulp was then sliced and homogenized by chopping. The method applied was same as in work by Miljkovic et al. [16]. Briefly, a known quantity of the grapefruit sample was added to a mixture consisting of organic solvents (hexane, acetone, and ethanol in volumes ratio 2:1:1, respectively) and 0.05% (w/v) butylated hydroxytoluene. The mixture was stoppered and mixed on an orbital shaker at 180 rpm for 15 minutes. The temperature during the mixing was kept at 5 °C in the water bath. Afterward, 7.5 ml of cold deionized water on every 10 grams of starting sample was added to the mixture and agitated for another 5 min. The suspension was transferred to a separation funnel to separate the upper (non-polar) from the lower (polar) phase for 10 minutes at room temperature.

Polar extract was obtained by homogenizing a precisely determined mass of the grapefruit sample and mixing it with three times the amount of ethanol (70% v/v). This mixture was magnetically stirred for 10 min at 5 °C in a water bath and subsequently centrifuged at 5000 rpm for 10 min. After decanting the supernatant, the remainder of the sample was re-extracted (as previously explained) with twice the volume of ethanol. The obtained supernatants were combined and made up to a known volume with 70% v/v ethanol. UHPLC analysis in negative and positive ion mode was employed to detect as much as possible of the compounds present in the grapefruit sample.

2.3. UV-Vis analysis

The non-polar phase was analyzed using UV-Vis spectroscopy to determine the content of lycopene, *β*-carotene, total polyphenols, and flavonoids, as well as the antioxidant activity. For this purpose, UV-1800 spectrophotometer (Shimadzu, Kyoto, Japan) was

employed. The concentrations of lycopene and β-carotene in the grapefruit sample were determined by measuring the absorbance of the non-polar layer in a 1 cm path length glass cuvette at 450 nm and 503 nm relative to a blank solvent mixture. The carotenoid content was obtained from the system of linear equations:

$$A_{450} = \varepsilon_{L450} C_L + \varepsilon_{\beta 450} C_\beta \quad (1)$$

$$A_{503} = \varepsilon_{L503} C_L + \varepsilon_{\beta 503} C_\beta \quad (2)$$

where A_{450}, A_{503}, ε_{L450}, ε_{L503}, are absorbances and molar absorbance coefficients at 450 nm and 503 nm, respectively. Concentrations of lycopene and β-carotene in the working solution, expressed in moles per liter, are designated by the symbols C_L and C_β, respectively. Content was calculated and expressed in milligrams per kilogram.

2.3.1. Total Polyphenols Content

The present study employed the Folin-Ciocalteu method, as described by Huang et al. [17], to determine the total polyphenol content in tomato and tomato juice samples. To do so, 0.4 ml of pre-prepared and degreased sample was combined with 0.5 ml of Folin-Ciocalteu reagent and 2 ml of 20% Na_2CO_3 water solution in a 10 ml volumetric flask. The flask was then filled with deionized water and thermostated for 2 hours at 20 °C, after which absorbance was measured at 760 nm using deionized water as the reference solution. The resulting calibration line was found to be linear for analyte concentrations ranging from 1 mg·l^{-1} to 9 mg·l^{-1}. The polyphenolic compound content in the samples was expressed as milligrams of gallic acid equivalents (GAE) per kilogram of the sample, as determined by the equation of the calibration line:

$$A = 0.10262 c_x + 0.05719 \left(r^2 = 0.999469\right) \quad (3)$$

where A represents absorbance, c_x is analyte concentration, and r^2 is dimensionless coefficient.

2.3.2. Total Flavonoids Content

The approach used to determine the total flavonoid content followed the procedure outlined by de Souza et al. [18]. A reaction mixture was created by combining 0.25 ml of the sample, 3 ml of deionized water, and 0.3 ml of 5% $NaNO_2$. After 5 minutes, 1.5 ml of $AlCl_3$ was added, followed by 2 ml of 1 mol·l^{-1} NaOH and deionized water to reach a total volume of 10 ml. The solution's absorbance was measured at 510 nm using deionized water as the reference solution in a UV/Vis spectrophotometer. The absorbance of the "blank" solution was also measured and subtracted from the 510 nm absorbance measurement. To construct a linear calibration plot in the concentration range of 5 mg·l^{-1} to 40 mg·l^{-1}, a series of working solutions was prepared from a (+)-catechin solution. Using the obtained equation of the calibration line:

$$A = 0.03612 c_x - 0.0091 \left(r^2 = 0.9993\right) \quad (4)$$

where A is absorbance, c_x is analyte concentration, and r^2 is a dimensionless coefficient, the total flavonoid content was computed and expressed as milligrams of catechin equivalents (CE) per kilogram of the sample.

2.4. UHPLC-ESI-MS Analysis

Qualitative analysis of both polar and non-polar extracts was conducted using ultra-high performance liquid chromatography coupled with electrospray ionization and mass spectrometry (UHPLC-ESI-MS). The UHPLC-ESI-MS analysis was performed using a Hypersil gold C18 column (50 mm × 2.1 mm, 1.9 µm) at 25 °C, employing a Dionex Ultimate 3000 UHPLC+ system equipped with a diode array detector and an LCQ Fleet Ion Trap Mass Spectrometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA). For this analysis, the method described elsewhere was utilized [19]. For the non-polar extract, an isocratic method was employed using a mixture of methanol and acetonitrile (1:1) as eluents at a flow rate of 0.25 ml/min. The volume of sample injection was set at 4 µl.

The polar extract was analyzed using a mobile phase flow rate of 0.250 ml/min and a sample volume of 8 µl. The mass spectrometric analysis was conducted using a 3D-ion trap with electrospray ionization (ESI) in positive ion mode for non-polar extracts and in both negative and positive ion modes for polar extracts. Full-range acquisition of *m/z* 100-700 was performed for the mass spectra. Tandem mass spectrometry analysis was carried out using a data-dependent scan with collision-induced dissociation of detected molecular ion peaks ($[M-H]^-$ /$[M+H]^+$) at 30 eV for both ionization modes. The capillary temperature was set at 350 °C, and the nitrogen sheath and auxiliary gas flow were 32 and 8 arbitrary units, respectively. Xcalibur software (version 2.1, Thermo Fisher Scientific) was used for instrument control, data acquisition, and data analysis.

The qualitative analysis of the identified compounds relied on comparing their retention times and MS spectra with the corresponding molecular ion peaks, as well as examining the characteristic ion fragmentation of selected peaks (MS/MS) from corresponding UHPLC chromatograms, in addition to comparing them with the mass spectral database accessible online (MassBank, MassBank consortium) and relevant literature. Reference standards were used for the full identification of some compounds, such as citric acid, chlorogenic acid, and rutin dihydrate.

The eluents used in the mobile phase were methanol, acetone, and water (LC-MS purity) from Thermo Fisher Scientific, along with HPLC-grade formic acid purchased from Carlo Erba (Emmendingen, Germany). Reference HPLC standards, such as citric acid, chlorogenic acid, and rutin dihydrate, were purchased from Merck (Darmstadt, Germany).

2.5. Antioxidant Activity

2.5.1. ABTS Method

In 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) method [20], the decolorization of a blue-green ABTS radical cation produced through chemical or

enzymatic oxidation of ABTS solution is used as a main principle. To perform the method, 0.1 ml of tomato sample was mixed with 2 ml of ABTS radical cation working solution, then kept in the dark for 6 min, after which, the absorbance of the solution was measured at 734 nm with methanol as the reference solution. The absorbances of the standard solutions were subtracted from the blank absorbance. Calculations were made by the equation 5:

$$\Delta A = A_0 - As = f(c_x) \quad (5)$$

where A_0 is the absorbance of the blank, and A_s is the mean value of three samples of the standard solutions which have given concentrations. The calibration curve is linear in the range of concentrations from 0.5 µmol·l^{-1} to 2 µmol·l^{-1} and can be expressed as:

$$\Delta A = 0.0316c_x + 0.068 \left(r^2 = 0.9998\right) \quad (6)$$

where c_x is ABTS radical cation concentration expressed in micromole per litre, and r^2 is the coefficient of determination. Based on the obtained equation, the antioxidant activity was calculated and expressed as milimoles of Trolox equivalents (TE) per kilogram of the sample.

2.5.2. DPPH Method

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) method [21] employs a similar mechanism to the ABTS method. A purple solution of DPPH radicals reacts with antioxidants present in the sample, resulting in the reduction of these radicals to a yellow DPPH form and a subsequent decrease in absorbance at 515 nm. To perform the assay, 0.1 ml of the sample was mixed with 5 ml of the DPPH radical working solution in a volumetric flask, and the volume was made up to 10 ml with methanol. The resulting solution was left to stand at 20 °C for 6 min, and the absorbance was measured at 734 nm relative to methanol as the reference solution. A series of standard solutions was prepared by adding 5 ml of DPPH to a certain volume of Trolox and making up to 10 ml with methanol. The absorbance of the resulting solutions was measured after 30 min and the mean of three measurements was taken. The absorbance of the blank was subtracted from the absorbance of the standard solutions. The calibration plot is linear in the concentration range from 0.5 µmol·l^{-1} to 5 µmol·l^{-1} and can be expressed as follows:

$$\Delta A = 0.02449c_x - 0.00913 \left(r^2 = 0.9988\right) \quad (7)$$

where c_x is DPPH radical concentration expressed in micromoles per litre and r^2 is the coefficient of determination. Using this equation, the antioxidant activity of the sample was calculated and expressed in millimoles of TE per kilogram.

2.5.3. FRAP Method

In the Ferric Ion Reducing Antioxidant Power (FRAP) method [22], the principle for determining the antioxidant activity of a sample is based on the disruption of the O-Phenanthroline-Fe^{2+} complex in the presence of chelating agents. To perform the assay, 20 µl of the test solution was diluted with 0.38 ml of deionized water, and 3 ml of FRAP reagent (a mixture of acetate buffer, 2,4,6-Tripyridyl-S-triazine (TPTZ), and $FeCl_3$ in a ratio of 10:1:1 v/v/v) was added. The mixture was thermostated for 5 min at 37 ºC, and the absorbance was measured at 595 nm relative to the blank. The blank consisted of the solvent only with no sample. A calibration plot was constructed using a series of standard solutions of $FeSO_4 \times 7H_2O$. The calibration curve was linear in the concentration range from 1.39 µmol·l^{-1} to 13.9 µmol·l^{-1}. Based on the obtained equation of the line, the antioxidant (FRAP) activity was calculated and expressed as micromoles of Fe^{2+} equivalent per kilogram of the sample. The calibration line is described by the equation:

$$\Delta A = 0.077c_x - 0.0286 \left(r^2 = 0.9999\right) \quad (8)$$

where c_x is concentration of Fe^{2+} ions expressed in milimoles per litre.

2.5.4. CUPRAC Method

The CUPRAC method, developed by Apak et al. [23], is founded on the principle of copper(I)-neocuproine complex formation, which exhibits a maximal absorbance at 450 nm. In our study, a sequence of standardized Trolox solutions was utilized to establish a calibration curve. The calibration line demonstrates a linear correlation within the concentration range of 3 µmol·l^{-1} to 18 µmol·l^{-1}. Subsequently, using the equation of the line, the antioxidant (CUPRAC) activity was calculated and expressed as micromoles of Trolox equivalents (TE) per kilogram of the sample. The calibration line is represented by the following equation:

$$\Delta A = 0.0606c_x - 0.0449 \left(r^2 = 0.9993\right) \quad (9)$$

where c_x is concentration of Trolox expressed in milimoles per litre.

2.6. Antimicrobial Activity

The antimicrobial activity of the extracts was determined using the microdilution method with slight modifications. Suspensions of microorganisms with a density of $1 \cdot 10^8$ CFU·ml^{-1} corresponding to 0.5 McFarland turbidity were prepared using overnight cultures of selected strains. Two types of sample solutions were made from the grapefruit extracts. The first extract was obtained using a mixture of solvents (250 ml of hexane, 125 ml of acetone, 62.5 ml of ethanol, and 62.5 ml of 50 mg·l^{-1} butylated hydroxytoluene) and dissolved in 100% dimethyl sulfoxide (DMSO), while the second extract was prepared with 70% ethanol. Samples were inoculated into a microtiter plate with 96 wells containing Mueller-Hinton broth (Institute of Virology, Vaccines and Sera "Torlak") and a series of double dilutions were prepared. The final concentration of microorganisms in each well was $1 \cdot 10^6$ CFU·ml^{-1}, and all microorganisms were cultivated at 37 °C for 18 hours according to the recommended Clinical and Laboratory Standards Institute procedure [24].

The minimum inhibitory concentration (MIC), on which there is no visible growth of microorganisms, was determined using an aqueous solution of triphenyltetrazolium chloride

(0.5%). The minimum microbicidal concentration (MMC) is defined as the sample concentration at which 99.9% of microorganism cells were killed. It was determined by transferring the contents of wells with no visible growth to Petri dishes and counting the colony forming units after incubation. All tests were performed in triplicate, and the results were analyzed using ANOVA with a 95% confidence level ($p \leq 0.05$). The testing time for *C. albicans* was sufficient, as the test is not meant to determine the exact number of cells, but rather to observe inhibitory or microbicidal activity.

3. Results and Discussion

3.1. Identification of Compounds by Chromatographic Analysis

Chromatogram of the non-polar extract of the red grapefruit sample is given in Figure 1. Two notable peaks for carotenoids lycopene and *β*-carotene are noticed. These two compounds have the same molecular mass, with same corresponding molecular ion peaks that are detected at the same *m/z* numbers (positive ESI-MS $[M+H]^+$ at *m/z* 537, Table 1) with similar MS/MS spectra. The assignation of the peaks is defined by the position of the peaks in chromatogram with first to elute lycopene and then *β*-carotene at C-18 column [25]. UHPLC chromatogram for the polar extract with MS detection in negative mode is given in Figure 2.

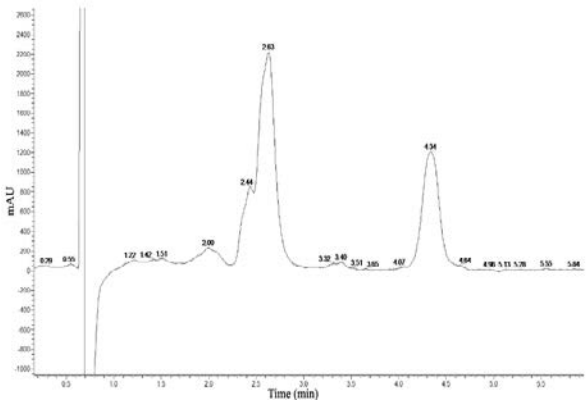

Figure 1. UHPLC-DAD chromatograms at the wavelength of 470 nm for the non-polar extract of grapefruit sample

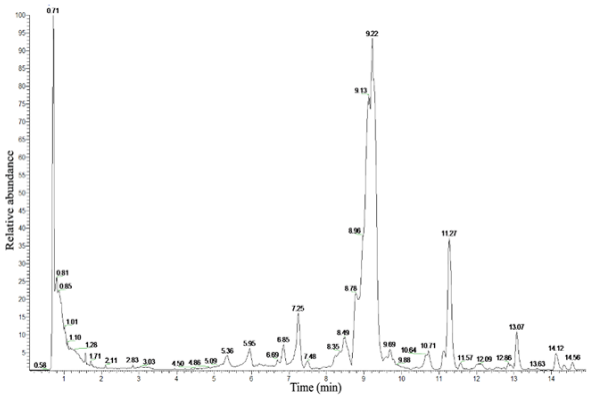

Figure 2. UHPLC chromatograms for the polar extract of grapefruit sample with MS detection in negative mode (ranged by the base peak)

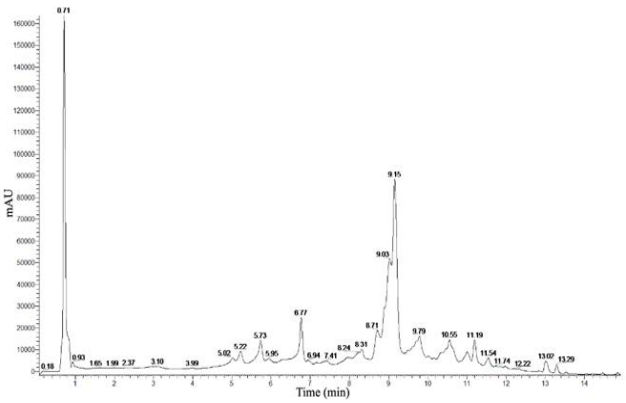

Figure 3. UHPLC chromatograms for the polar extract of the grapefruit sample with DAD detection at 350 nm

From the DAD signal at 300 nm, typical UHPLC-DAD chromatogram was recorded (Figure 3). The full list of all detected and identified compounds recorded at 300 nm with DAD signal by UHPLC-DAD along with their retention times and characteristic molecular and fragmentic ions is given in Table 1. Total of 16 compounds from two man classes (phenolic acids and flavonoids) were detected in the polar extract with 11 identifyed. The class of phenolic acids and their derivatives includes the following compounds: caffeic acid, caffeic acid hexosides (tentatively galactoside and glucoside), chlorogenic acid and 4-*O*-caffeoyl-quinic acid, and caffeic acid unknown derivative. Quinic and citric along with lycopene or *β*-carotene are present. It is the most probably that this pick of the compound No. 13 (Table 1) comes from lycopene [26]. In the ESI-MS/MS spectrum of the compound No. 13 (Table 1) assigned to lycopene with molecular ion peak $[M+H]^+$ detected at *m/z* 537, abundant ion was detected at *m/z* 457, which is to be in agreement to loss of 80 units - $[M+H–C_6H_8]^+$ [27].

Identification of quinic acid in spectrum was indicated by additional ions at *m/z* 85 and 127 [29]. For it's identification a reference standard solution was used. Caffeic acid was identified due to presence of an ion with *m/z* 179 [30]. The 4-*O*-caffeoyl-quinic acid derivatives were identified from the molecular ion peak [M-H]- at *m/z* 353 with characteristic peaks at *m/z* 191 (base peak) and 173 (base peak) [31]. With *m/z* 597 and same retention time phloretin-*C*-diglycoside was identified tentatively [32]. For identification of rutin it's reference standard solution was used. The fragment ions at *m/z* 271 and *m/z* 151 were leading to the identification of naringenin [32,33].

In similar study, Xu et al. [34] were also determining chemical composition of *Citrus paradisi* fruit, although they were using peel and flash portion for extraction with 80% methanol. Along the other components, HPLC-PDA with C-18 column was employed to identify caffeic and chlorogenic acid which is the same finding as in our study. Phenolic acids content decreases with fruit maturity and it is higher in peel than in flash [34]. By employing the similar analytical approach, Xu et al. [35] (other author with same surname) identified lycopene and beta carotene in different grapefruit cultivars, as Rouseff et al. did [36]. Furthermore, rutin, naringenin and quercetin were also identified by Sicari et al. [37]. Kelebek was determining composition of red grapefruit juices from 4 grapefruit varieties grown in Turkey (Rio Red, Star Ruby, Ruby Red

and Handerson) by using HPLC method with Aminex HPX-87H column. He identified 3 sugars (glucose, fructose and sucrose) and 15 phenolic compounds of which only 3 are mutual with results obtained in our study (caffeic acid, chlorogenic acid, naringenin). The reason for such a mismatch in results comparing to ours could be difference in sampling material as he analyzed juice instead of extract and different column for HPLC analysis and experimental conditions were applied [38].

Table 1. List of detected compounds by UHPLC-DAD-MS/MS analysis

Peak	t_R [min]	λ_{max} [nm]	Molecular ion $[M-H]^-$ *m/z*	MS/MS fragment ions *m/z*	Assignment
1	0.75	-	191	173, 127, 111, 85 (100%)	Quinic acid [13]
2	0.92	-	191	173, 111 (100%)	Citric acid (standard)
3	1.50	-	179	161, 143, 131, 119, 113, 101, 89 (100%), 71	Caffeic acid [17]
4	5.14	326 315sh	341	281, 251, 221, 179 (100%), 135	Caffeic acid hexoside *tent.* glucoside
5	5.30	325 303sh	353	191 (100%), 179, 173	Chlorogenic acid (standard)
6	5.76	325 300sh	353	191, 179, 173 (100%), 135	4-*O*-caffeoyl-quinic acid
7	7.04	291 305sh	427	381 (100%), 249, 161	not identified
8	8.18	354 262	471	425 (100%), 263	not identified flavonoide
9	8.99	351 289	597	487, 387, 357 (100%)	Phloretin-*C*-diglycoside [18]
10	8.99	355 258	609	301 (100%) 299,271	Rutin (quercetin-3-*O*-rutinoside) (standard)
11	9.26	321 293	579	533, 459 (100%), 357, 313, 271, 235	not identified
12	9.70	-	609	489,301(100%)	Quercetin-hexoside-rhamnoside (*tent.*)
13	10.90	-	537[#]	457 (100%)	Lycopene or *β*-carotene [28]
14	11.40	291	271	227, 177, 151 (100%), 107, 93	Naringenin [13, 14]
15	13.47	343	386[#]	201 (100%)	not identified
16	14.25	358	274[#]	256 (100%), 230, 106, 102, 88	not identified

[#]Electrospray Ionisation Mass Spectrometry (ESI-MS) data are corresponding to positive mode, ($[M+H]^+$),sh– shoulder
Presence in sample: (–) – compound is not present, (+) – compound is present, (±) – compound is present but less than in other samples.
tent.– tentatively.
Values in the brackets in the column of MS/MS fragment ions mean ion abundance of the base ion peak in the corresponding MS/MS spectrum of the compound.

3.2. Total Lycopene and *β*-carotene Content

The results for lycopene and *β*-carotene content in *Citrus paradisi* fruit extract are shown in Table 2. According to available literature results, there is a variation in lycopene and *β*-carotene content of *Citrus paradisi* fruit among the results reported by different authors. For the sake of the most adequate comparison, the paper by Rouseff et al. [36] presents the best agreement with the conditions we applied in terms of analytical method and plant material used, extraction procedure performed and the way of the results expression. In addition, published results by other authors are also discussed.

Table 2. Total lycopene and *β*-carotene content

Compound	Grapefruit sample	
	Content [$mg \cdot kg^{-1}$]	RSD [%]
Lycopene	34.2 ± 0.6	1.8
β-carotene	18.6 ±1.6	4.8
Lycopene/ *β*-carotene ratio	1.84	

Content is expressed per kilogram of fruit weight.
RSD – relative standard deviation.

Briefly, Rouseff et al. [36] performed extraction of flash part of *Citrus paradisi* fruit with the same solvents mixture (hexane:acetone:ethanol 2:1:1) and results are expressed in same way as presented in our study. Precisely, in different cultivars lycopene and *β*-carotene content was calculated to be (mg/kg of fruit weight): 2.9 and 4.2 in "Ruby red Int", 1.6 and 4.2 in "Ruby red IR", 7.9 and 8.6 in "Flame", 20.6 and 7.0 in "Ray Ruby", 33.3 and 9.6 in "Star Ruby" [36]. There is a difference which can be related to different cultivars, climate and growing conditions (interior central Florida and the Indian River) [39]. As it can be seen, results obtained in this study (34.2 and 18.6) are most similar with results for "Star Ruby" (33.3 and 9.6) and by them red grapefruit from supermarket in Serbia is in a class of highly pigmented red grapefruits.

Vanamala et al. were determining lycopene and *β*-carotene content extracted with solvent mixture acetone:hexane:double distilled water in ratio 1:1:1 from pulp of *Citrus paradisi* cultivar "Rio Red" grown in Texas. These two compounds of interest were found to be present in amount of ~150 mg/kg of fruit weight and ~43 mg/kg of fruit weight, respectively. They concluded that postharvest period, storage, and freeze drying have significant effects on the content of bioactive components [40].

Patil et al. analyzed early and late harvested "Rio Red" grapefruit fruit. They found that lycopene and *β*-carotene are present in amounts of: ~22 and ~4 mg/kg of fruit weight in early and ~18 and ~2.5 mg/kg of fruit weight in late harvested samples, respectively [41].

Red grapefruits ("Ruby Red" and "Star Ruby") from supermarket in U.K. were analyzed for lycopene and *β*-carotene content by Xu et al. [35] with solvent mixture consisting of chloroform/methanol/50mM Tris buffer, pH 7.5,containing 1M NaCl (2:1:1,v/v/v). Expressed as mg/kg of dry weight of sample, they extracted: lycopene 0.31 and 42.32 from peel and 0.16 and 159.35 from juice vesicle and *β*-carotene 0.84 and 13.97 from peel and 2.93

and 43.11 from juice vesicles of "Ruby Red" and "Star Ruby", respectively. It is interesting to note that these two carotenoids were absent in extract samples of ordinary white grapefruit [35]. Similar to this, Xu et al. [34] (other author with same surname) calculated lycopene and *β*-carotene concentration to be 46.77 and 0 in peel and 283.57 and 93.03 in flash of "Star Ruby" red grapefruit cultivar [34].

Color parameters of red grapefruits were described in details by Lee. The ratio of these two main carotenoid pigments is a function of maturity, it is related to red appearance in the flesh of grapefruits so that high ratio contributed to attractive red and red decreases as the season progresses. Lycopene to *β*-carotene ratios present in amount of (mg/kg) of juice samples from red grapefruit cultivars grown in Florida were: 3.82 and 1.6 in "Marsh Red", 1.51 and 1.19 in "Ruby Red", 9.55 and 3.26 in "Flame", 13.62 and 2.76 in "Star Ruby", 7.84 and 1.39 in "Ray Ruby", 11.27 and 1.8 in "Rio Red", respectively [42].

3.3. Total Polyphenols and Flavonoids Content

The results for total polyphenols and flavonoids content are shown in Table 3. The content of phenolic compounds is related to antioxidant activity and thereby health benefits [25]. The calculation of total polyphenols and total flavonoids content in *Citrus paradisi* fruit stands right in the context of health potential assessment. Flavonoids, the most present components in the total phenolic content, is the class of compounds that contribute to: antiatherosclerotic effect, anti-thrombogenic effect, anti-inflammatory effect, anti-oxidative effect, anti-tumor effect, anti-osteoporotic and antiviral effect [43-45].

Table 3. Total polyphenols and flavonoids content as determined by UV/Vis spectrophotometry

Analysis	Grapefruit sample	
	Content [mg·kg^{-1}]	RSD [%]
Total polyphenols	298.0 ± 7	2.3
Total flavonoids	216.4 ±15	7.0

Content of total polyphenols is expressed as milligrams of gallic acid equivalents (GAE) per kilogram fruit weight.
Content of total flavonoids is expressed as milligrams of catechin equivalents (CE) per kilogram of fruit weight.
RSD – relative standard deviation.

Considering the manner of results expression, the results obtained in this study are comparable with the ones obtained Mahdavi et al. who were determining polyphenols content in red grapefruits fresh and commercial juices from Tabriz-Iran market. They found that content of total polyphenols in fresh juice was 494 mg GAE/L and 418.1 mg GAE/L. These compounds are even more active in presence of vitamin C and saccharides which presence is also confirmed [46], because of enhanced assimilation [47]. The content of flavonoids in peeled red grapefruit is found to be 216.6 mg/kg of fruit weight according to Gorinstein et al. [48].

Sicari et al. were also analyzing red grapefruit juice grown in Italy. They calculated that in two red grapefruits cultivars total polyphenol and total flavonoids content is: 153.08 mg/L and 390.21 mg/L in "Marsh" while in "Star Ruby" 167.22 mg/L and 310.14 mg/L, respectively, with naringing as the one of major flavonoids [37].

Jayaprakasha et al. [49] were determining phenolics content of peeled "Rio Red" grapefruit cultivar grown in Texas as a function of different solvents. More precisely, expressed as catechin equivalents (CE) they achieved: 0 mg CE/kg with hexane, 2910 mg CE/kg with EtOAc, 200 mg CE/kg with acetone, 540 mg CE/kg with methanol, 860 mg CE/kg with methanol:water (80:20) as extraction solvents [49].

3.4. Antioxidant Activity

The ability to delay an oxidation process is attributed to antioxidant activity [50]. There are numerous assays available for measuring antioxidant activity but each of them has limitations. However, the most popular ones are DPPH and ABTS, with a reminder that dihydrohalcones and flavanones rich extracts do not react with DPPH radical and that structure-activity relationship is not clear for ABTS. In the end it comes down to difficulty to compare results obtained by different methods because they can be expressed in different ways e.g.: half maximal effective concentration (EC_{50}), time to reach the EC_{50} (T_{EC50}), antiradical efficiency, antiradical power (ARP), stoichiometry, kinetic behavior and rate constant, radical scavenging activity, or trolox equivalent antioxidant capacity (TEAC) [51].

Each of the mentioned assays for measuring antioxidant activity is based on different mechanisms or interactions with free radicals. With a purpose to give more detailed profile of *Citrus paradisi* fruit from Serbian market the antioxidant activity was tested by using four different methods based on electron transfer: ABTS, DPPH, FRAP, and CUPRAC. Comparing these methods, CUPRAC provides advantages such as: applicability to both hydrophilic and lipophilic antioxidants (different from DPPH), completion of the redox reactions for most common flavonoids (different FRAP), selective oxidation of antioxidant compounds without affecting sugars and citric acid and the capability to assay –SH bearing antioxidants (different from FRAP) [52]. The obtained results are shown in Table 4.

Table 4. Antioxidant activity of the analyzed grapefruit sample

Method	Grapefruit sample	
	Value	RSD [%]
ABTS	1.80 ± 0.03	1.7
DPPH	0.72 ± 0.01	1.4
FRAP	9.7 ± 0.3	3.1
CUPRAC	4.66 ± 0.04	0.9

ABTS – antioxidant activity determined by 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) method (expressed as millimoles of Trolox equivalents per kilogram of the sample), DPPH – antioxidant activity determined by 2,2-diphenyl-1-picrylhydrazyl method (expressed as millimoles of Trolox equivalents per kilogram of the sample), FRAP – antioxidant activity determined by ferric reducing antioxidant power method (expressed as micromoles of Fe^{2+} equivalents per kilogram of the sample), CUPRAC – antioxidant activity determined by cupric ion reducing antioxidant capacity method (expressed as millimoles of Trolox equivalents per kilogram of the sample).
RSD – relative standard deviation.

The Pearson correlation coefficient (Table 5) demonstrates a good agreement between the antioxidant assays and the content of total polyphenols and flavonoids.

Table 5. Pearson's correlation coefficient

	Total polyphenols	Total flavonoids	ABTS	DPPH	FRAP	CUPRAC
Total polyphenols	1.0000					
Total flavonoids	**0.9940**	1.0000				
ABTS	**0.9901**	**0.9995**	1.0000			
DPPH	**0.9980**	**0.9849**	**0.9791**	1.0000		
FRAP	**0.9679**	0.9344	0.9230	**0.9820**	1.0000	
CUPRAC	**0.9490**	0.9087	0.8953	**0.9672**	**0.9978**	1.0000

The ABTS and DPPH tests employ ABTS and DPPH radicals, which are reduced in the presence of antioxidants, leading to a color change, and enabling the assessment of antioxidant activity. The Pearson coefficient indicates the strongest correlation between these two tests (p = 0.9791). On the other hand, FRAP and CUPRAC tests involve the reduction of Fe^{2+} and Cu^{1+} ions. Since these two tests share similar mechanisms but differ from the previously mentioned tests, the Pearson coefficient confirms a high consistency in the results obtained from these tests (p = 0.9978). The total polyphenol content shows a good correlation with all applied antioxidant tests, indicating their significant contribution to the results of these tests. Conversely, total flavonoids exhibit a strong correlation only with the ABTS and DPPH methods. These are chemical tests used to evaluate antioxidant activity through different reactions and indicators, while the Pearson coefficient is utilized to assess how results from various tests are interrelated or aligned.

It is evident that the values vary depending on the applied method. That was also observed by Gorinstein et al. who tested antioxidant activity of peeled blond and red grapefruit. They obtained the following results: ORAC (oxygen radical absorbance capacity) ~0.6 mmolTE/kg and ~1.60 molTE/kg; by bleaching with β-carotene ~0.4 mmolTE/kg and ~0.55 mmolTE/kg; DPPH ~60 mmolTE/kg and ~160 mmolTE/kg, for blond and red grapefruit respectively. Results favor red grapefruit because of phenolic compounds such as anthocyanins and flavonoids that act as antioxidants [48].

It is interesting to compare results for antioxidant activity of "Marsh" and "Star Ruby" cultivars grapefruit juice. Concretely, determined by: ABTS method it is ~2.0 molTE/kg and ~1.3 molTE/kg; DPPH method it is ~1.0 molTE/kg and ~0.8 molTE/kg, respectively [37].

It should be kept in mind that ABTS and DPPH are two methods based on the same property that can rank grapefruit extracts differently because of the different solvents used [53]. Nevertheless, phenolic compounds including anthocyanins, flavonoids, and ascorbic acid are known to be responsible for antioxidant activities in fruits, and fruits with higher phenolic contents generally show stronger antioxidant activities. Bioactive compounds found in red grapefruit extracts: rutin, naringening [54], lycopene and β-carotene are responsible for antioxidant activity [55], but not quercetin and chlorogenic acid [53].

It is worth mentioning that Kelebek [38] determined antioxidant activity of juice from 4 grapefruit varieties grown in Turkey (Rio Red, Star Ruby, Ruby Red and Handerson) by DPPH method. According to them juice obtained from Star Ruby was strongest antioxidant followed by Handerson, Rio Red and Ruby Red [38]. Unfortunately, because of the different expression of the results they cannot be compared with results obtained in this study.

3.5. Antimicrobial Activity

The study of antimicrobial activity is of great importance in the field of nutrition quality, since it contributes to the profile of the grapefruit and allows addition of lower amounts of synthetic preservatives which means enhancing the food safety and shelf life of products [56,57]. The results for antimicrobial activity of red grapefruit extracts are shown in Table 6.

Table 6. Antimicrobial activity of the analyzed grapefruit sample

	Grapefruit sample	
Microbial strain	MIC [mg·ml^{-1}]	MMC [mg·ml^{-1}]
Gram (+) bacteria		
Staphylococcus aureus	4.0	0
Enterococcus faecalis	4.0	0
Bacillus cereus	4.0	2.0
Gram (-) bacteria		
Escherichia coli	4.0	0
Salmonella enteritidis	4.0	0
Enteroabacter aerogenes	4.0	0
Pseudomonas aeruginosa	2.0	0
Yeast		
Yeast *Candida albicans*	2.0	0

MIC – The minimum inhibitory concentration, MMC – The minimum microbicidal concentration.

The results for antimicrobial activity of grapefruit obtained by Cvetić & Vladimir-Knežević are the most suitable for comparison to results obtained in the present study. A comparison of activity against the strains used in both experiments is: against bacterial strain *Staphilococcus aureus* they needed 82.5 µg/mL instead of 4 mg/mL; against *Bacillus cereus* 82.5 µg/mL instead of 4 mg/mL; against *Escherichia coli* 41.3 µg/mL instead of 4 mg/mL; against *Salmonella enteritidis* 20.6 µg/mL instead of 4 mg/mL; against *Pseudomonas aeruginosa* 82.5 µg/mL instead of 2 mg/mL; against *Candida albicans* 82.5 µg/mL instead of 2 mg/mL. It can be concluded that they achieved MIC with lower concentrations of extract. One of the explanation could be based on the fact that juiceless pulp and seeds powdered plant as extraction material were used in contrast to full fruit as in this experimental research. However, both extracts are less effective than some commercially available extracts of grapefruit [58].

For determination of antimicrobial activity in this experimental work extract of red grapefruit in full was used as suggested by Liu. That is because antimicrobial effect of each phytochemical in extract by synergistic effect contributes to the complete antimicrobial effect of extract itself [59].

Quinic acid appears to be antimicrobial agent worth noting against this bacterial strain [60]. Citric acid has antimicrobial activity against *Staphylococcus aureus* [53], *Klebsiella aerogenes* [61] and *Escherichia coli* [53,61,62]. Caffeic acid's antibacterial activity as MIC against *S. aureus 6538*, strain used in this experimental research, was found to be 256 µg/ml [63] and 625 µg/ml [64]. The cognition that it augments the effects of antibiotics is of importance [63]. Also chlorogenic acid has shown noteworthy antimicrobial activity against *S. aureus* in experimental research [65]. Rutin's antimicrobial activity as MIC was found to be against: *S. aureus* 16 mg/mL; *Enterococcus faecalis* 8 mg/mL; *E. coli* 16 mg/mL; *P. aeruginosa* 16 mg/mL; *C. albicans* 16 mg/mL [66]. Lycopene's antimicrobial activity has been shown against *Staphylococcus aureus*, *E.coli*, *P. aeruginosa*, *C.albicans* [67]. Naringenin's antimicrobial activity affects both Gram-positive and Gram-negative bacterial strains [68]. Concretely and accurately, against Gram-positive strains of *Bacillus cereus* ATCC 27348 and *Staphylococcus aureus* ATCC 12598 naringenin was efficient in concentrations of MIC 300 and 100 µg/ml respectively; against Gram-negative strains of *E. coli* and *Pseudomonas aeruginosa* ATCC 10145 MIC was reached with 550 and 500 µg/ml, respectively [68]; against MRSA it is 125µg/ml [61].

It is not out of question to mention that essential oil of red grapefruit also showed antimicrobial activity against bacterial strains that were used in this work: *Staphylococcus aureus*, *Enterococcus faecalis*, *Bacillus cereus* and *Escherichia Coli* [69,70].

4. Conclusion

Analysis of red grapefruit (*Citrus paradisi*) carried out by UHPLC provides helpful evidence for explanation of differences between different cultivars of this plant species grown in different geographical regions. Results for lycopene and *β*-carotene obtained in this work are in the range of the values determined by other authors. The same is observed for total polyphenols and flavonoids content. Four assays used for estimating the antioxidant activity of analyzed red grapefruit extract provided a results notable for assessment of health benefits. The antimicrobial activity of extracts used in this research is lower than of the other authors. However, there are objective reasons for that kind of results (different solvents used for extraction, not the same plant material) that contribute to completely microbial profile of *Citrus paradisi* fruit extract. Microbial activity was tested and more complete antimicrobial profile of this plant species is fulfilled. Results obtained in this work are recommending red grapefruit in everyday diet, except when taking drugs when it should be avoided, as valuable source of health beneficial bioactive compounds.

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