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BIOACTIVE COMPOUNDS CONTENT AND ANTIOXIDANT POTENTIAL OF BEETROOT (*Beta vulgaris* L.) PEEL, POMACE, AND JUICE

SUMMARY

Scientific research in the 21st century has increasingly focused on bioactive compounds from medicinal plants and their antioxidant properties due to their potential benefits for human health. The aim of this study was to evaluate the antioxidant activity and determine the total content of bioactive compounds (phenols, flavonoids, tannins, and betalains) in peel and pomace extracts, as well as in freshly squeezed juice of *Beta vulgaris* L. Plant material was collected from two locations in Montenegro (Nikšić and Mojkovac) in September 2023.To compare extraction efficiency, two extraction methods (Soxhlet extraction and conventional maceration) and three solvents (water, methanol, and ethyl acetate) were used. Antioxidant activity was assessed using DPPH and FRAP assays. The highest total phenolic content (70.01 mg GAE/g extract) and total tannin content (49.27 mg TAE/g extract) were observed in the ethyl acetate peel extract obtained by the Soxhlet method from the Nikšić location. The highest total flavonoid content (88.31 mg QuE/g extract) was recorded in the ethyl acetate peel extract from Mojkovac. Higher concentrations of betacyanins and betaxanthins in peel and pomace were detected in samples from Nikšić. Freshly squeezed beetroot juice from this location exhibited the highest levels of all analyzed bioactive compounds and showed pronounced antioxidant activity (DPPH IC_{50}=24.99 μg/mL; FRAP=49.93 mmol Fe^{2+}/L). Among the extracts, the strongest DPPH scavenging activity was observed in the ethyl acetate peel extract from Mojkovac, while the highest FRAP value was recorded for the methanolic peel extract from Nikšić. These results indicate that *Beta vulgaris* L., particularly its peel and fresh juice, is a valuable source of bioactive compounds with strong antioxidant potential, influenced by extraction method, solvent type, and geographical origin.

Keywords: *Beta vulgaris* L., antioxidant activity, phenolics, flavonoids, tannins, betalains

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INTRODUCTION

For centuries, medicinal and aromatic plants have played an essential role in human life and culture worldwide. They have traditionally been used in pharmacy, cosmetics, and nutrition, and in recent years their application has expanded into food technology, particularly as natural sources of antioxidants (Stefanou *et al.*, 2014). Herbal-based medical systems represent some of the oldest forms of health care, dating back to the earliest stages of human civilization (Thirumalai *et al.*, 2019). A thorough understanding of plant biochemical composition is fundamental for elucidating their potential medicinal properties, which are primarily attributed to secondary metabolites. These compounds have historically been used to alleviate various ailments in traditional and folk medicine and are currently exploited in the development of pharmaceutical agents targeting a wide range of diseases, from migraines to cancer (Hussein *et al.*, 2019).

Plants of the genus Beta originate from North Africa, from where their distribution expanded across the Mediterranean region and subsequently to Europe, Asia, and the Americas (Ceclu and Nistor, 2020). Beetroot has been cultivated for several centuries in temperate coastal regions, initially for its leaves, which were consumed as salad greens, while the thin and fibrous roots were occasionally used for medicinal purposes. Systematic cultivation for root consumption began in the 16th century in Germany and Italy, leading to the development of beet varieties that are still cultivated today (Sentkowska and Pyrzyńska, 2023). Currently, the largest production of beetroot occurs in northern and central Europe, although it is also widely grown in North Africa and Asia, extending as far as India (Yashwant, 2015). Beetroot thrives in moderately warm climates and grows optimally in clayey, slightly acidic soils with a pH ranging from 5.5 to 6.2. Sowing is generally performed between March and June to avoid low temperatures, while optimal growth occurs at temperatures between 15 and 19 °C; lower temperatures are known to enhance the development of the characteristic dark red pigmentation (Giampaoli *et al.*,2021, Chhikara *et al.*, 2019). In recent decades, *Beta vulgaris* L. has attracted increasing scientific interest as a functional food with significant health-promoting properties. Although its medicinal use was documented as early as Roman times, systematic scientific investigations into its bioactive potential have intensified only recently. Beetroot is recognized as a rich source of bioactive compounds, many of which are associated with beneficial effects related to chronic inflammatory conditions (Clifford *et al.*, 2015).

Beetroot contains dietary fiber, essential minerals, and vitamins, including vitamins A, C, and those of the B complex, as well as betalains and phenolic compounds. Its high nutritional value is largely attributed to its considerable sucrose content. These bioactive constituents exert their effects through diverse mechanisms, including free radical scavenging, interaction with enzymatic active sites, and modulation of gene expression involved in intracellular defense systems against oxidative damage (Baião *et al.*, 2017). Betalains derived from beetroot

have been reported to reduce the risk of certain cancers, cardiovascular and cerebrovascular diseases, as well as liver and kidney damage (Chen *et al.*, 2021). Moreover, beetroot extracts exhibit a wide range of biological activities, such as antioxidant, anti-inflammatory, antihyperglycemic, and anticancer effects. However, the extraction techniques and solvents employed significantly influence the qualitative and quantitative composition of bioactive compounds and, consequently, their biological potential (Borjan *et al.*, 2022). Additionally, substantial variability in phytochemical content has been observed among different beetroot varieties, as well as a result of processing and storage conditions, including temperature and light exposure (Wruss *et al.*, 2015, Pavlović *et al.*, 2022).

This study aimed to evaluate the bioactive profile of *Beta vulgaris* L., focusing on phenols, flavonoids, tannins, and betalains in various extracts and freshly squeezed juices. In addition to qualitative and quantitative characterization, the antioxidant activity of these samples was assessed. Although beetroot has been widely recognized as a rich source of bioactive compounds, comparative data on different extraction methods and freshly squeezed juices remain limited. Therefore, this research seeks to clarify how extraction procedures influence the recovery of key phytochemicals and their antioxidant potential. Furthermore, the associations between different polyphenol classes and antioxidant capacity were explored using Pearson's correlation coefficient to better understand their relative contribution to oxidative stress mitigation. The findings aim to provide a clearer insight into the functional potential of beetroot-derived products and support their application in food and nutraceutical development.

MATERIAL AND METHODS

Chemicals and Instruments

All phenolic standards and other chemicals/reagents were obtained from Cayman Chemical (Michigan, USA), Acros Organics (Antwerp, Belgium), Sigma-Aldrich Chem (Steinheim, Germany), VWR International (Pennsylvania, USA), MP Biomedicals (California, USA), Alfa Aesar (Massachusetts, USA), Carl Roth (Karlsruhe, Germany), and Centrohem (Stara Pazova, Serbia). All reagents used in this study were of analytical grade. The instruments employed included a UV-Vis spectrophotometer (Cecil Aurius Series CE 2021, UK), a digital pH meter (Jenway 3505, UK), an incubator (LSW-33, Vims Elektrik, Serbia), and an analytical balance (Kern ABS 220-4, Philippines).

Plant Samples

The sample was collected at two locations in the municipality of Nikšić (Vidrovan, 42° 50' 21.3" N 18° 55' 36.3" E) and Mojkovac (Polja, 42° 57' 35.3" N 19° 32' 57.8" E) in September 2023. The beetroot sample was collected from arable fields/gardens from local households that grow beetroot only for household needs.

Preparing samples for extraction

The collected beets were washed, the petioles and leaves were separated from the roots. The beet root was peeled, cut into pieces and left to dry at room temperature. The juice was separated from the pomace with a juicer and immediately subjected to analysis. The pomace, which was additionally squeezed for faster and more efficient drying, was placed in the drying room together with the peel. Before the actual extraction, the peel (root periderm) and the pomace were additionally ground with an electric blender. The same procedure was carried out for both localities. For the analysis of the total content of phenols, flavonoids, and tannins, as well as for the determination of antioxidant activity through the FRAP test, the extracted juice was diluted 10 times. All analyses were performed in triplicate.

Soxhlet solid–liquid extraction

Peel (root periderm) and pomace from two localities were extracted using the Soxhlet solid-liquid extraction method. Two solvents were used for this type of extraction: methanol and ethyl acetate. 15 g of mechanically ground sample was packed in filter paper and then sealed. 300 ml of solvent was added to the distillation flask in contact with the heat source. After the boiling of the solvent, the extraction cycle is started, which was continuously repeated for the next 6 hours that the extraction lasted. Solvents were evaporated by air-drying at room temperature until a constant weight was achieved. The procedure was repeated for both solvents, for peel and pomace from two different sites, yielding 8 different samples. A total of eight final extracts were stored at 4°C in an airtight container until further use. Stock samples were made by dissolving 0.01g of each of the six obtained extracts in 10 ml of methanol. Their concentration was 1 mg/ml.

Maceration solid-liquid extraction

Three solvents were used for this type of extraction: distilled water, methanol and ethyl acetate.10 g of mechanically ground sample was extracted with 100 ml of solvent, then left to stand for 48 h at room temperature. After every 24 h, the samples were macerated. When the maceration procedure was finished, the macerate was filtered with filter paper, and the sample was steamed in a water bath. The procedure yielded 12 samples: peel and pomace from two localities extracted with three different solvents. A total of twelve final extracts were stored at 4°C in an airtight container until further use. Stock samples were made by dissolving 0.01 g of each of the six obtained extracts in 10 ml of methanol. Their concentration was 1 mg/ml.

Determination of total phenolics

The colometric Folin-Ciocalteu method (Singleton and Rossi, 1965). was used to determine the content of total phenols in different extracts and extracted juice. Reaxtion mixture were obtained with 1 ml of sample (1 mg/ml), 0.5 ml of

Folin-Ciocalteu solution and 2.5 ml of 7.5% Na2CO3. Three repetitions were performed for each sample. A blank sample was also measured, which differed in the preparation in the addition of 1 ml of distilled water instead of the sample. The prepared samples were stored in a dark room for 120 minutes, then their absorbance was measured at 740 nm. The gallic acid calibration curve was used to calculate the content of total phenols. TPC (Total phenolic content) was expressed in mg/g equivalent of gallic acid (mg/g GAE).

Determination of flavonoids

The colorimetric aluminum-chloride method was used to determine the content of total flavonoids in different extracts and extracted juice (Ardekani *at al.*, 2011). 1.2 ml of 2% AlCl3 solution and 1.2 ml of sample (1 mg/ml) are added to the test tube in order to obtain a reaction mixture. Three repetitions were performed for each sample. Incubation of the prepared mixture took place at room temperature for an hour, then the absorbance was read at 420 nm. The absorbance of the blank (extraction solvent - methanol) was also measured. The flavonoid content was calculated using the calibration curve for quercetin. TFC (Total flavonoid content) was expressed in mg/g quercetin equivalent (mg/g QuE).

Determination of tannins

The total content of tannins in different extracts and the squeezed juice was determined according to the modified method according to Price and Butler, 1977. 500 μl of sample (1mg/ml), 0.5 ml of 0.1 M FeCl3, 0.5 ml of 0.008 M K3Fe(CN)6 and 8 ml of distilled water were used to obtain a reaction mixture. Three repetitions were performed for each sample. A blank sample was measured, which differed in the preparation only in the addition of 500 μl of distilled water instead of the sample. The samples were left for 10 minutes in room temperature, after which the absorbance was measured at a wavelength of 720 nm. The tannin content was calculated according to the calibration curve of the tannic acid standard. TTC (Total tannin content) was expressed in mg/g tannic acid equivalent (mg/g TAE).

Determination of betalains

The method is based on spectrophotometric measurement of absorbance at different wavelengths for betacians and betaxanthins (Shakir and Simone, 2024). 0.1 g of mechanically ground sample was dissolved in 10 ml of distilled water. The mixture was stirred for one minute, and then the contents were centrifuged for 10 minutes at 15,000 g. All four samples (peel and pomace from two locations) were made in three repetitions. After centrifugation, the supernatant was separated and subjected to spectrometric analyses. The absorbance was measured at two different wavelengths: 480nm for betaxanthines and 538nm for betacyanins. The content of total betalains was calculated using a mathematical equation.

The betalain content in the squeezed beetroot juice was determined by a spectrophotometric method, measuring content betanin and vulgaxanthin. 15 ml of phosphate buffer pH 6.5, 0.15 ml of squeezed juice sample were used to obtain a reaction mixture. Absorbance of the solution was determined at three different wavelengths: 600, 476, 538. Both juice samples were made in three repetitions. Phosphate buffer was measured on a spectrophotometer as a blank. The content of total betalains was calculated using a mathematical equation

DPPH radical-scavenging activity

The DPPH test was used to evaluate the free radical scavenging activity of various extracts as well as beetroot juice (Gülçin and Alwasel, 2023; Baliyan *et al.*, 2022). For the analysis of the antioxidant activity, serial dilutions of the extracts were made at concentrations of 1, 0.5, 0.25, 0.125, 0.0625, 0.03125, 0.015, 0.0075 mg/ml. Three repetitions were made for each dilution, 24 test tubes for each sample. In each test tube, 0.1mM DPPH solution and 3 ml of juice/extract of a certain concentration were prepared. All test tubes were incubated in a darkened room for a period of thirty minutes, and then measured at a wavelength of 517 nm. A blank sample consisting of 3 ml of methanol, a control prepared with 3 ml of methanol, and 1 ml of DPPH were also measured.

The percent DPPH inhibition (percentage of antioxidant activity - %AA) was calculated by the equation: %AA=(A0–A1)/A0x100, where A0 was the absorbance of the control, and A1 is the absorbance of the sample. Antioxidant activity was expressed as the IC_{50} value, which represents the concentration of the extract or reference compound required to inhibit 50% of DPPH radicals. IC_{50} values were determined by plotting the percentage of DPPH inhibition against the sample concentration, followed by interpolation using nonlinear regression.

FRAP assay

The FRAP test was used to determine the total antioxidant activity of various extracts of the bark and roots, as well as of the squeezed beetroot juice (Benzie and Devaki, 2017). 100µl of sample, 3 ml of TPTZ reagent and 1 ml of distilled water were added to the test tube to make the reaction mixture. Each one of the samples had three replicates. The reaction mixture was incubated in a thermostat (37ºC) for 15 minutes. After the test tubes were removed from the thermostat, they stood for five minutes (room temperature), after which the absorbance value was read at a wavelength of 595 nm. A blank sample was also measured, with the addition of 100µl of distilled water instead of the sample. The value of the antioxidant potential is expressed as the equivalent of mmol Fe2+ in 1 ml of the sample.

Statistical analysis

All experimental data were processed using the statistical software package Statistica 10.0. The relationships between the contents of total phenolics, flavonoids, and tannins in the plant extracts and their antioxidant activities,

determined by the DPPH and FRAP assays, were evaluated using Pearson's correlation analysis ($p<0.05$). The strength of the correlation coefficient (r) was interpreted as follows: $|r|<0.3$, negligible or no correlation; $0.3\leq|r|<0.5$, weak correlation; $0.5\leq|r|<0.7$, moderate correlation; $0.7\leq|r|<0.9$, strong correlation; and $0.9\leq|r|\leq1.0$, very strong correlation.

Differences among experimental groups were analyzed using one-way analysis of variance (ANOVA). When statistically significant differences were detected, Tukey's honestly significant difference (HSD) post hoc test was applied to identify differences between individual mean values. A p-value <0.05 was considered indicative of statistical significance. Furthermore, principal component analysis (PCA) was conducted to characterize the distribution of bioactive compounds in the tested *Beta vulgaris* L. extracts in relation to the extraction solvent used.

RESULTS AND DISCUSSION

Extraction Yields

Comparing the two extraction methods, a significantly higher yield (34.2%) was obtained by the Soxhlet method using methanol as a solvent, while the ethyl acetate extract had the lowest yield in both extraction methods (0.17%). Overall, the yield of the pomace extracts was significantly higher compared to the peel.

Bioactive Compounds Content of Beetroot Peel, Pomace, and Juice

The content of bioactive compounds in different peel and pomace extracts and in squeezed juice of *Beta vulgaris* L. is presented in Tables 1, 2, and 3.

Table 1. Total phenolic (TPC), flavonoid (TFC), tannin (TTC) and in peel extracts (PL) and pomace extracts (PC) of *Beta vulgaris* L obtained with solid-liquid Soxhlet method, as well as squeezed juice (SJ); values represent mean + SD of three measurements (n=3)

Sample/solvent	Method/location	TPC mgGAE[1]/g dw	TFC mg QE[2]/g dw	TTC mg TAE[3]/g dw
PL/ethyl acetate	S/Nikšić	70.01 ± 0.78^{c}	88.318±1.33^{c}	49.27± 1.77^{c}
PL/ethyl acetate	S/Mojkovac	64.34 ± 1.27^{c}	73.20± 0.45^{c}	28.60± 1.32^{d}
PL/methanol	S/Nikšić	63.91 ± 0.89^{c}	n.d[4]	38.46± 0.78^{cd}
PL/methanol	S/Mojkovac	65.35 ± 1.23^{c}	n.d[4]	26.58± 0.24^{d}
PC/ethylacetate	S/Nikšić	50.43 ± 0.32^{d}	32.61± 1.76^{d}	16.26±0.68^{e}
PC/ethylacetate	S/Mojkovac	41.191 ± 2.21^{e}	n.d[4]	8.634±0.56^{ef}
PC/methanol	S/Nikšić	46.451±0.67^{d}	n.d[4]	24.09±0.75^{d}
PC/methanol	S/Mojkovac	40.53 ±1.44^{e}	2.03±1.11^{e}	12.914±1.28^{e}
Squeezed juice (SJ)	Nikšić	722.6 ±1.12^{a}	698.44±0.97^{a}	601.6± 0.56^{a}
Squeezed juice (SJ)	Mojkovac	554.37 ±0.76^{b}	293.27±0.86^{b}	336.84± 1.16^{b}

**GAE–gallic acid equivalent; 2QE–quercetin equivalent; 3TAE–tannic acid equivalent; 4n.d.–not detected with the standard concentration range. Different superscript letters within each column indicate statistically significant differences (Tukey HSD test, p < 0.05). The contents of phenols, flavonoids and tannins for the juice samples are expressed per liter (mg/L)*

Table 2. Total phenolic (TPC), flavonoid (TFC), tannin (TTC) and in peel extracts (PL) and pomace extracts (PC) of *Beta vulgaris* L. obtained with solid-liquid maceration method; values represent mean + SD of three measurements (n=3)

Sample/solvent	Method/location	TPC mgGAE[1]/g dw	TFC mg QE[2]/g dw	TTC mg TAE[3]/g dw
PL/ethyl acetate	M/Nikšić	69.70 ± 2.41[a]	85.71±5.34[a]	28.71± 1.76
PL/ethyl acetate	M/Mojkovac	60.70 ±1.56[a]	60.09± 3.21[b]	16.56±2.34
PL/methanol	M/Nikšić	59.42 ±1.12[a]	n.d[4]	24.53± 3.44
PL/methanol	M/Mojkovac	61.82 ± 2.78 [a]	n.d[4]	21.57±1.56
PL/water	M/Nikšić	52.12 ±3.56[b]	n.d[4]	25.72±0.95
PL/water	M/Mojkovac	48.08 ±0.78[b]	n.d[4]	24.29±0.78
PC/ethylacetate	M/Nikšić	50.02 ± 4.56[b]	26.20± 0.98[c]	7.34±1.67
PC/ethylacetate	M/Mojkovac	36.378 ± 1.23[c]	29.04±1.32[c]	8.53±1.66
PC/methanol	M/Nikšić	40.04±0.78b[c]	n.d[4]	9.03±2.34
PC/methanol	M/Mojkovac	35.893 ±2.15[c]	n.d[4]	9.96±0.68
PC/water	M/Nikšić	34.846 ±2.23[c]	n.d[4]	13.01±0.88
PC/water	M/Mojkovac	27.974 ±1.65c[d]	n.d[4]	8.53± 1.45

**GAE–gallic acid equivalent; 2QE–quercetin equivalent; 3TAE–tannic acid equivalent; n.d.–not detected with the standard concentration range. Different superscript letters within each column indicate statistically significant differences (Tukey HSD test, p<0.05)*

Table 3. Betalain content of peel (PL), pomace (PC) and squeezed juice (SJ) of *Beta vulgaris* L. at two different locations; values represent mean + SD of three measurements (n=3)

Sample/location	Betacyanins (mg/l) for extracts (mg/ml) for SJ	Betaxanthin (mg/l) for extracts (mg/ml) for SJ
PL/Nikšić	206.25 ±6.3 [a]	132.82± 0.98[a]
PL/Mojkovac	181.5 ± 4.22[b]	111.65± 1.24[b]
PC/Nikšić	143 ± 2.39[c]	90.47±2.22[c]
PC/ Mojkovac	132± 2.67[c]	90.47± 1.76[c]
SJ/Nikšić	185.62±1.98[b]	94.5± 1.89[c]
SJ/Mojkovac	71.69±3.87[d]	30.8±3.14[d]

**Different superscript letters within each column indicate statistically significant differences (Tukey HSD test, p < 0.05)*

The total phenolic content (TPC) in the extracts ranged from 27.97 to 70.01 mg GAE/g dw. The highest TPC was found in the ethyl acetate peel extract from the Nikšić locality, while the lowest value was recorded in the aqueous pomace extract from the Mojkovac site. Regardless of the extraction method, the most pronounced differences in TPC were observed between aqueous and ethyl acetate extracts of both peel and pomace at both localities. Analysis of the results indicates that ethyl acetate and methanol were more efficient solvents for the extraction of total phenols compared to water, which proved to be the least

effective solvent. Slight variations in TPC were observed depending on the extraction method, with Soxhlet extraction yielding marginally higher values than maceration. In general, except for peel extracts, TPC values were slightly higher in samples from the Nikšić locality. At both localties, the TPC was significantly higher in peel extracts compared to pomace, regardless of the solvent and extraction method used.The total phenolic content in juice samples ranged from 554.37 to 722.60 mg GAE/L. Beetroot juice from the Nikšić locality exhibited higher TPC values than juice samples from Mojkovac.

The total flavonoid content (TFC) in the extracts ranged from 26.20 to 88.32 mg QuE/g dw. Detectable flavonoid levels were observed exclusively in ethyl acetate extracts, regardless of the extraction method or locality. The highest TFC values were obtained in peel extracts prepared by Soxhlet extraction, while the lowest values were recorded in pomace extracts obtained by maceration. As observed for total phenols, flavonoid content was significantly higher in peel than in pomace samples, irrespective of the extraction method and locality. Overall, only minor variations in TFC were noted between localities and extraction methods. In juice samples, TFC values varied considerably, ranging from 293.27 mg QuE/L in Mojkovac to 698.44 mg QuE/L in Nikšić.

The total tannin content (TTC) in beetroot extracts ranged from 7.34 mg TAE/g dw, recorded in pomace extracts obtained by maceration with ethyl acetate, to 49.28 mg TAE/g dw in ethyl acetate peel extracts prepared by Soxhlet extraction. The efficiency of solvent extraction depended on both the extraction method and the plant part. Under Soxhlet extraction conditions, ethyl acetate showed the highest efficiency for tannin extraction from peel, whereas methanol was more effective for pomace samples. In contrast, no consistent solvent efficiency pattern was observed for maceration. Regardless of the extraction method, solvent, or locality, TTC values were significantly higher in peel than in pomace. Soxhlet extraction generally resulted in higher tannin yields compared to maceration, and samples from the Nikšić locality exhibited higher tannin contents, particularly in ethyl acetate extracts. The tannin content in juice samples was 336.84 mg TAE/L for Mojkovac and 601.60 mg TAE/L for Nikšić.

Betalain content ranged from 30.80 to 132.83 mg/L for betaxanthins and from 71.70 to 206.25mg/L for betacyanins. In all analyzed samples (peel, pomace, and juice), betacyanin content was higher than betaxanthin content. The peel samples from both localities contained higher levels of betalains compared to pomace samples. Only minor differences in betalain content were observed between the two localities.

Antioxidant Potential of Beetroot (*Beta vulgaris* L.) Peel, Pomace, and Juice

The antioxidant potential of peel and pomace extracts and squeezed juice of *Beta vulgaris* L., determined by the DPPH assay and expressed as IC_{50} values, is presented in Tables 4 and 5.

Table 4. Antioxidant activity of peel extracts (PL) and pomace extracts (PC) of *Beta vulgaris* L. obtained with solid-liquid Soxhlet method, as well as squeezed juice (SJ); values represent mean + SD of three measurements (n=3)

Sample/solvent	Method/location	DPPH IC50 (µg/ml)	FRAP (mmol Fe^{2+}/g dw)
PL/ethyl acetate	S/Nikšić	84.64 ± 1.55^{d}	19.91±0.88^{c}
PL/ethyl acetate	S/Mojkovac	52.98 ± 0.67^{e}	17.04± 0.76^{d}
PL/methanol	S/Nikšić	99.01 ± 1.73^{d}	21.37±1.22^{c}
PL/methanol	S/Mojkovac	81.08 ± 2.79^{d}	21.03±3.98^{c}
PC/ethylacetate	S/Nikšić	373.89 ± 3.45^{b}	12.87± 4.56^{e}
PC/ethylacetate	S/Mojkovac	923.46 ± 2.28**a**	4.22±1.57^{f}
PC/methanol	S/Nikšić	317.15±1.75^{b}	9.66±1.29^{ef}
PC/methanol	S/Mojkovac	332.32 ±1.66^{b}	10.26±2.33^{e}
Squeezed juice (SJ)	Nikšić	24.99 ±2.99^{f}	398.6±6.78^{a}
Squeezed juice (SJ)	Mojkovac	127.27 ±1.43^{c}	227.8±7.89^{b}

**Different superscript letters within each column indicate statistically significant differences (Tukey HSD test, p <0.05). For DPPH IC50, lower values indicate stronger antioxidant activity; for FRAP, higher values indicate stronger antioxidant activity. The FRAP value for juice is expressed per liter (mmol Fe^{2+}/L)*

Table 5. Antioxidant activity of peel extracts (PL) and pomace extracts (PC) of *Beta vulgaris* L obtained with solid-liquid maceration method; values represent mean + SD of three measurements (n=3)

Sample/solvent	Method/location	DPPH IC50 (µg/ml)	FRAP (mmol Fe2+/g dw)
PL/ethyl acetate	M/Nikšić	136.04 ± 5.7^{e}	12.31±2.35^{b}
PL/ethyl acetate	M/Mojkovac	331.7 ± 8.97 ^{cd}	6.91±1.22 ^{c}
PL/methanol	M/Nikšić	114.95 ± 3.87^{e}	13.18±2.09^{a}
PL/methanol	M/Mojkovac	131.98 ±3.56^{e}	14.4±1.89^{a}
PL/water	M/Nikšić	85.10±2.38^{f}	14.14±1.56^{a}
PL/water	M/Mojkovac	113.17±1.77^{e}	13.28±2.34^{a}
PC/ethylacetate	M/Nikšić	5298.82 ±7.67^{a}	4.11± 1.46^{d}
PC/ethylacetate	M/Mojkovac	2198.63 ±8.84^{b}	3.55±2.35^{ed}
PC/methanol	M/Nikšić	635.07±4.67^{c}	4.15±0.78^{d}
PC/methanol	M/Mojkovac	512.76 ±4.45^{c}	4.74±0.56^{d}
PC/water	M/Nikšić	563.8 ±3.45^{c}	7.24±2.78^{c}
PC/water	M/Mojkovac	1813.34 ±3.34^{b}	5.12±0.97^{cd}

**Different superscript letters within each column indicate statistically significant differences (Tukey HSD test, p <0.05). For DPPH IC50, lower values indicate stronger antioxidant activity; for FRAP, higher values indicate stronger antioxidant activity*

The IC_{50} values ranged from 81.08 µg/mL for the methanolic peel extract obtained by Soxhlet extraction from the Mojkovac locality to 5298.82 µg/mL for the ethyl acetate pomace extract obtained by maceration from Nikšić. In general, peel and pomace extracts obtained by the Soxhlet method exhibited higher antioxidant potential compared to those obtained by maceration, particularly ethyl

acetate and methanolic peel extracts, with only minor variations between localities. An exception was the aqueous peel extract from the Nikšić locality, which also demonstrated high antioxidant activity. Differences in antioxidant potential between the two localities were generally insignificant. Notably, peel extracts exhibited significantly higher antioxidant potential than pomace extracts. Analysis of the IC_{50} values of juice samples revealed a clear difference between the two localities, with juice from Nikšić showing stronger antioxidant activity (IC_{50} = 24.99 µg/mL) compared to juice from Mojkovac (IC_{50}=127.27 µg/mL).

Antioxidant potential measured by the FRAP assay showed the lowest value in the ethyl acetate pomace extract obtained by maceration from Mojkovac (3.55 mmol Fe^{2+}/g) and the highest value in the methanolic peel extract obtained by Soxhlet extraction from the same locality (21.37 mmol Fe^{2+}/g). Extracts obtained by the Soxhlet method generally exhibited higher FRAP values regardless of locality. Juice samples from both localities showed particularly high FRAP values, with 398.6 mmol Fe^{2+}/L for Nikšić and 227.8 mmol Fe^{2+}/L for Mojkovac.

DISCUSSION

Total Phenolic content

The total phenolic content (TPC) of beetroot extracts varied considerably depending on the plant part, solvent, and extraction method. In this study, TPC values ranged from 27.97 to 70.01 mg GA/g dry weight, with ethyl acetate extracts of the peel yielding the highest concentrations. These results are lower than previously reported ranges (136–1026 mg GA/g, Guamán-Balcáza *et al.* 2024), but are consistent with the trend observed in other studies showing that solvent polarity and extraction methodology significantly influence phenolic recovery (Čanadanović-Brunet, 2011, Arjeh *et al.*, 2022, Vaitkevičienė et al. 2022., Abadi *et al*. 2024). Specifically, water was generally less efficient for phenolic extraction, whereas ethyl acetate and methanol proved more effective. Soxhlet extraction also yielded slightly higher TPC values compared to maceration, corroborating earlier findings (Borjan *et al*., 2022). Across studies, peel extracts consistently contain higher phenolic levels than pomace extracts. Interestingly, the TPC of freshly squeezed juice in this study (up to 722.6 mg GA/L) exceeded values reported in earlier studies (11.58–98.08 mg GA/L, Abdo *et al.*, 2020, Olumese and Oboh. 2016), highlighting the efficiency of the extraction procedure and the influence of geographical origin.

The elevated total phenolic content (TPC) in beetroot peel can be attributed to its protective role against environmental stress. Phenolic compounds accumulate in the peel to provide defense against UV radiation, pathogens, and oxidative stress, which are more intense at the surface tissues (Duthie *et al.*, 2000). Additionally, secondary metabolites such as phenols are biosynthesized at higher levels in outer tissues to enhance antioxidant capacity and structural defense, whereas inner tissues, including the pomace, have lower phenolic

concentrations (Leong and Shui, 2002; Pérez-Gregorio *et al.*, 2010; De Vit *et al*,. 2020; Bhat *et al.*, 2025).

Total Flavonoid content

The flavonoid content in whole beetroot extracts has been reported as 38.5 mg QuE/g dry matter, (Nugraha *et al.* 2022) which is comparable to the pomace extracts obtained in the present study. Peel extracts, however, exhibited approximately twice this flavonoid content. Significantly lower flavonoid levels (1.54–8.35 mg QuE/g dry matter) have been reported (Hikmawanti *et al.*, 2021, El-Beltagi *et al.*, 2018) in various whole beetroot extracts, indicating that the flavonoid content observed in this study is considerably higher. Comparison of the two extraction methods, Soxhlet and maceration, revealed no significant differences in flavonoid yield (Dianursanti *et al.*, 2020), although other studies suggest that Soxhlet extraction may provide higher flavonoid recovery Factors such as an increased number of extraction cycles and optimal extraction temperature can further enhance flavonoid recovery using this method (Wardhani *et al.*, 2023). Furthermore, the flavonoid content in the juice samples (83.34±4.47 mg QuE/L) was substantially higher than values previously reported in the literature (Olumese and Oboh, 2016), indicating the high efficiency of the applied extraction procedure.

Total Tanin content

The tannin content in various beetroot extracts obtained in this study ranged from 7.34 mg TAE/g of dry matter, recorded in the pomace extract prepared with ethyl acetate by maceration, to 49.278 mg TAE/g of dry matter in the ethyl acetate peel extract obtained by Soxhlet extraction. The efficiency of tannin extraction was clearly dependent on both the method and the plant part analyzed. Soxhlet extraction with ethyl acetate showed the highest tannin recovery from peel at both collection sites, whereas methanol was more efficient for pomace extracts. In contrast, maceration did not show a consistent pattern in solvent efficiency; at the Mojkovac site, water provided the highest tannin content in peel extracts, while methanol was more effective for pomace. Overall, tannin content was consistently higher in peel than in pomace, independent of extraction method, solvent, or site, highlighting the differential accumulation of tannins in protective plant tissues.

Comparing these results with literature data, tannin levels in our pomace extracts were generally higher than those reported in whole beetroot extracts (6.055 mg TAE/g and 5.13 mg TAE/g; Odoh *et al.*, 2012; El-Beltagi *et al.*, 2018), while water extracts in previous studies often did not yield detectable tannin levels (Omogbai and Omoregie, 2016), in contrast to our measurable values. Methanolic extracts obtained by ultrasonic extraction also confirmed higher tannin content in peel 4.3 mg GA/g compared to pomace 3.7 mg GA/g (Arjeh *et al.*, 2022). While Manea (2013) reported extremely high tannin concentrations in juice (3059 g/L), the maximum tannin content observed in our study reached 601.5 mg TAE/g dw, suggesting notable differences that may be attributed to variations in plant material, extraction procedures, or analytical methods.

The relatively low tannin content observed in our extracts can be explained by several factors. Beetroot (*Beta vulgaris* L.) naturally contains comparatively low amounts of tannins relative to other phenolic compounds, such as flavonoids and betalains, which dominate its phytochemical profile, with betalains constituting the major class of phenolic-related constituents in red beetroot. (Mirmiran *et al.*, 2019).

Additionally, the distribution of specific phenolic classes in beetroot is influenced by biosynthetic pathways that prioritize betalain production a tyrosine derived pigment class that dominates the phenolic profile in *Beta vulgaris* L. over other phenolic secondary metabolites such as tannins, leading to comparatively limited tannin accumulation in this species. The betalain biosynthetic pathway has been extensively characterized as a tyrosine derived metabolic process responsible for high betalain content in beetroot tissues, which may reduce flux toward other phenolic pathways including tannin formation. (Khan and Giridhar, 2015).

Betalain Content

The obtained betalain content ranged from 30.8 to 132.825 mg/L for betaxanthins and from 71.696 to 206.25 mg/L for betacyanins, depending on the drying method and solvent used. These results are consistent with the study by Shakir and Simone (2024), highlighting that the method of drying plant material significantly affects betalain stability and content. Zin *et al.*, (2021) reported lower betacyanin (165.24 mg/L) and betaxanthin (97.56 mg/L) levels in peel extracts, whereas Aykın-Dinçer *et al*., (2021) and Jahan *et al.*, (2021) observed higher betalain concentrations in pomace (300.82 and 364.93mg/L for betacyanins; 261.27 and 249.40 mg/L for betaxanthins) compared to our results.

Freshly squeezed beetroot juice exhibited maximum values of betacyanin (119.53 mg betanin/100 mL) and betaxanthin (48.27 mg vulgasanthin I/100 mL) in the study by Fernández-López *et al*. (2023), which is consistent with the juice obtained from the Mojkovac locality in the present study. In all samples, betalain content was consistently higher in the peel than in the pomace, as reported by Sokolova (2022), in agreement with our findings. This pattern aligns with previous studies showing that betalains are predominantly localized in protective tissues such as the peel, where they contribute to antioxidant defense and color pigmentation (Kujala *et al.*, 2000; Mirmiran *et al.*, 2019).

Environmental factors, particularly temperature, influence betalain biosynthesis, with higher temperatures generally increasing betalain content (Sokolova *et al.*, 2024). This effect may partly explain the observed differences between the Mojkovac and Nikšić localities. Moreover, betacyanins are typically present at higher concentrations than betaxanthins in beetroot, reflecting their dominance in the plant's phenolic profile and metabolic prioritization over other phenolic compounds (Stintzing and Carle, 2004; Clifford *et al.*, 2017).

Antioxidant activity

The antioxidant potential of beetroot extracts was evaluated using DPPH and FRAP assays, revealing notable differences depending on solvent, plant part, and extraction method. In previous studies, IC_{50} values for methanol, water, and

ethyl acetate extracts of whole beetroot ranged from 34.9 to 88.6 μg/mL (Omer *et al.*, 2022), with ethyl acetate generally showing the lowest antioxidant activity. In contrast, in the present study, the ethyl acetate peel extract obtained by Soxhlet extraction exhibited the lowest IC_{50} value (52.98 μg/mL), indicating the highest antioxidant potential. These results emphasize the significant influence of extraction methodology on the recovery of bioactive compounds. Methanolic extracts consistently showed higher antioxidant activity than other solvents, in agreement with Saani and Lawrence (2017) and John *et al.* (2017). Similarly, Arjeh *et al.*, (2022) observed higher IC_{50} values in pomace compared to peel, consistent with our findings, where peel extracts exhibited stronger antioxidant activity than pomace. Overall, Soxhlet extraction proved more effective than maceration for antioxidant recovery.

In freshly squeezed juice samples, antioxidant activity measured by DPPH and FRAP was higher than previously reported values (IC_{50}=16.25 μg/mL, Khider *et al.*, 2019; FRAP=8.354 mmol/L, Wootton-Beard and Ryan, 2011), with juices from the Nikšić locality showing 51.54% inhibition. This activity also exceeded that reported for carrot juice in comparative studies (Arora *et al.*, 2019). The enhanced activity corresponds to the higher betacyanin content in juices from both Nikšić and Mojkovac, supporting the role of betacyanins as key contributors to antioxidant potential (Czapski *et al.*, 2009). Overall, trends observed in DPPH and FRAP assays were consistent across most samples, although minor variations between localities reflected differences in secondary metabolite composition.

Correlation Between Bioactive Compounds and Antioxidant Activity

A very high positive correlation was observed between total phenolic content (TPC) and antioxidant potential (FRAP assay) in the methanolic peel extract ($r=0.97$, $p<0.05$) (Table 6).

Table 6. Pearson's correlation coefficients ($p<0.05$) between total phenols, flavonoids, tannins, and antioxidant capacity in methanol and ethyl acetate peel and pomace extracts of *Beta vulgaris* L. from Mojkovac, prepared by maceration

PEEL (PL)	TPC-EA	TPC-M	TPC-W	TFC-EA	TFC-M	TFC-W	TTC-EA	TTC-M	TTC-W
DPPH-EA	-0.29			0.53			0.26		
DPPH-M		-0.41			-0.21			-0.62	
DPPH-W			-0.07			-0.18			-0.11
FRAP-EA	-0.20			-0.34					
FRAP-M		**0.97***			-0.56			**0.96***	
FRAP-W			-0.61			-0.45			0.52

POMACE (PC)	TPC-EA	TPC-M	TPC-W	TFC-EA	TFC-M	TFC-W	TTC-EA	TTC-M	TTC-W
DPPH-EA	-0.31			-0,78			-0.63		
DPPH-M		-0.29			-0.98			0.73	
DPPH-W			0.97			-0.79			-0.79
FRAP-EA	0.45			**0.96***			**0.99***		
FRAP-M		0.14			0.65			0.62	
FRAP-W			0.34			-0.88			0.76

**Bold values represent strong correlations ($p < 0.05$)*

It is noteworthy that most extracts showed positive correlations when antioxidant activity was assessed using the FRAP method, whereas the DPPH assay predominantly revealed negative correlations. This observation is consistent with a previous study by Olumese and Oboh (2016), which reported a significant positive correlation (r=0.98) between flavonoid content and FRAP-measured antioxidant activity in various extracts of peeled beetroot and juice. However, in the same study, a relatively low correlation between total phenolic content and FRAP activity (r=0.36) was observed.

Furthermore, a strong positive correlation between betacyanin content and DPPH-determined antioxidant activity of whole beetroot (r=0.80) was reported by Sokolova *et al.*, (2024). This trend aligns with the present study, where a significant positive correlation was observed between betacyanin content and FRAP-measured antioxidant activity in pomace extracts (r=0.81, $p<0.05$) Additionally, beetroot juice showed significant correlations between FRAP-measured antioxidant potential and the contents of both total phenols and betacyanins, as reported by Wruss *et al.*, (2015). It is interesting to note that at the Nikšić locality, no statistically significant correlations were observed between the contents of the analyzed components and the values of antioxidant potential, regardless of the type of sample or the method used. The absence of statistically significant correlations at the Nikšić site may be due to the complex interplay of multiple bioactive metabolites beyond the measured components, environmental influences on phytochemical biosynthesis, and variability in how different antioxidant assays reflect the activities of specific compounds. Previous studies have noted that the overall antioxidant response of plant extracts can be affected by synergistic or antagonistic interactions among constituents, as well as by external ecological and methodological factors, which can obscure simple linear correlations between individual compounds and antioxidant outcomes. (Jacobo Velázquez and Cisneros Zevallos, 2009., Villegas-Aguilar *et al.*, 2024).

Principal component analysis (PCA)

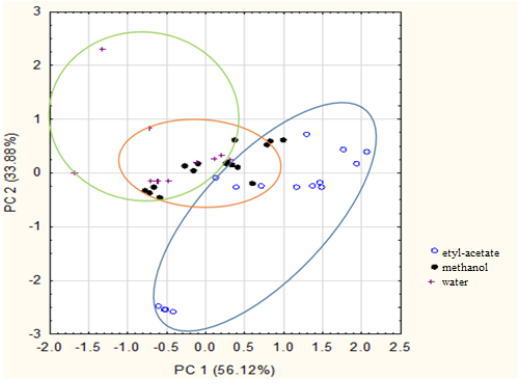

Figure 1. Principal Component Analysis (PCA) of total phenolic, flavonoid and tannin content in relation to type of solvent in peel and pomace samples of *Beta vulgaris* L. Projection of the samples on the first two principal components (PC1: 56.12%, PC2: 33.88%)

By analyzing the obtained data, it can be observed that the first two principal components account for approximately 90% of the variability in the content of phenols, flavonoids, and tannins (PC1: 56.12%, PC2: 33.28%) in relation to the type of solvent. The PCA results indicate that ethyl acetate extracts form a relatively distinct group, as evidenced by the ellipse in Figure 1, which slightly overlaps with the other solvent groups, highlighting differences in the composition of bioactive compounds compared to the remaining solvents.

CONCLUSIONS

The results of this study indicate that the content of secondary metabolites and the antioxidant potential of *Beta vulgaris* L. are significantly influenced by the extraction method, the solvent used, and the ecological conditions of the collection site. Soxhlet extraction, particularly with ethyl acetate, proved to be more effective in isolating phenolic compounds, flavonoids, tannins, and betalains compared to maceration. The peel generally contained higher amounts of bioactive components than the pomace, while freshly squeezed juices, especially from the Nikšić site, exhibited the highest antioxidant potential, which can be attributed to the absence of thermal degradation.

Comparative analysis of the DPPH and FRAP assays suggests that the FRAP method may provide a more reliable assessment of antioxidant activity. Although the collection site did not show a consistent effect on peel and pomace extracts, environmental factors clearly contribute to the variability of chemical composition. These findings contribute to a better understanding of the biochemical profile of *Beta vulgaris* L. and highlight the need for further research involving different varieties, additional extraction techniques, and other plant parts with potential bioactive properties.

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