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Original research paper

ANTIOXIDANT AND ANTIMICROBIAL POTENTIAL OF BLACKBERRY POLYPHENOLIC EXTRACTS: INFLUENCE OF DIFFERENT EXTRACTION TECHNIQUES AND SOLVENTS

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Abstract: Blackberries (*Rubus* sp.) are considered a good source of bioactive compounds, especially polyphenols. The high antioxidant and antimicrobial activity associated with polyphenols offers the possibility of using blackberry extracts in various food applications. This study investigates the potential of blackberry extracts obtained with various solvents and extraction techniques as a source of natural antioxidants and antimicrobial compounds. Ethanol and two natural deep eutectic solvents (NADES) (N1 − choline chloride:glycerol (1:2) and N2 − choline chloride:lactic acid (1:4)) and three different extraction techniques (maceration, ultrasound-assisted extraction, and microwave-assisted extraction) were applied. The extracts were evaluated in terms of total monomeric anthocyanin content, polymeric color, total polyphenol content, and ferric reducing antioxidant power (FRAP). The antimicrobial potential against two Gram-positive and two Gram-negative bacteria, as well as one yeast, was assessed using agar diffusion and broth microdilution methods. The N2 solvent gave a better yield of bioactives than N1 and ethanol, while the microwave-assisted extraction had the most pronounced effect on the extracts' polyphenol content and color. All samples displayed significantly higher antimicrobial activity than the ethanolic extract, with the generally highest efficacy observed against Gram-positive bacteria. Overall, these preliminary results demonstrate the potential of acidic NADES for the extraction and application of blackberry polyphenols. Future steps should include extraction optimization and food application studies.

Key words: *blackberry, polyphenols, NADES, extraction techniques, antioxidants, antibacterial activity*

INTRODUCTION

Blackberries are small, dark purple to black fruits belonging to different species of the *Rubus* genus. They are grown across all continents except Antarctica, with a global market estimated at 1.61 billion USD in 2024 (Business Research Insights, 2025). Their sweet and tangy flavor, along with their high nutritional value, makes them a popular choice for preparing smoothies, jams, and desserts.

Apart from being abundant sources of fiber and vitamin C, blackberries are particularly rich in polyphenols, secondary plant metabolites characterized by great antioxidant and antimicrobial potential (Pandey & Rizvi,

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2009). The most prevalent blackberry polyphenols include phenolic acids (ellagic acid, gallic acid, caffeic acid, etc.), anthocyanins (mainly cyanidin-based glycosides), flavan-3-ols (catechin and epicatechin), flavonols (kaempferol, myricetin, rutin, etc.), and tannins (ellagitannins and proanthocyanidins) (Robinson, Bierwirth, Greenspan & Pegg, 2020; Tripathi et al., 2024).

Given the prevailing trends in the food industry favoring functional products and formulations free of artificial ingredients, blackberries and their derived extracts exhibit considerable promise as candidates for incorporation into value-added food systems. Blackberry polyphenols can be utilized as natural preservatives, protecting food products from oxidation and microbial spoilage, while anthocyanins, as natural pigments, can be used as healthy substitutes for artificial colorants (Babaoğlu, Unal, Dilek, Poçan & Karakaya, 2022; Vidana Gamage, Goh & Choo, 2024). The radical-scavenging potential of blackberry polyphenols has been shown to offer numerous health benefits, including the prevention of cardiovascular diseases, neurodegenerative diseases, obesity, and cancer (Kaume, Howard & Davareddy, 2011; Tatar, Bagheri, Varedi & Naghibalhossaini, 2019; Najjar, Knapp, Wanders & Feresin, 2022; de Mello et al., 2023; Gil-Martínez et al., 2023).

Various techniques have been employed to extract polyphenols from plant material. Traditional techniques include maceration, percolation, and Soxhlet extraction, whereas ultrasound-assisted extraction, microwave-assisted extraction, and supercritical fluid extraction are some of the more advanced techniques. Despite the higher investment costs, modern techniques are becoming more widespread due to higher efficiency and lower energy consumption (Sridhar et al., 2021). Regarding extraction solvents, natural deep eutectic solvents (NADES) have become a popular choice for the extraction of different bioactives due to their biodegradability, simple preparation, low cost, and versatility (Liu et al., 2018). By definition, they represent mixtures of pure compounds − a hydrogen bond acceptor (HBA) and a hydrogen bond donor (HBD) having a eutectic point lower than that of an ideal liquid mixture (Martins, Pinho & Coutinho, 2018). As the name implies, NADES components are naturally present in biological systems, including plant and animal cells, and typically include ammonium quarternary salts, e.g., choline chloride as HBA; organic acids and sugars play the role of HBD, while alcohol, amine, aldehyde, ketone, and carboxylic groups can act as both (Mišan et al., 2019). Furthermore, the composition of NADES can be adjusted based on their desired applications and may influence the properties of the obtained extracts (Cajnko, Vicente, Novak & Likozar, 2023).

Based on all the above, this study aimed to investigate the effects of different extraction techniques and solvents on the functional properties of blackberry polyphenolic extracts. The study involved the application of three extraction techniques: traditional maceration and two modern techniques − ultrasound-assisted extraction and microwave-assisted extraction, using 70% ethanol as a conventional solvent and two NADES composed of choline chloride with polyol/organic acid as hydrogen bond donors. The obtained extracts were assessed based on their anthocyanin content, total polyphenol content, polymeric color, as well as antioxidant and antimicrobial potential.

MATERIALS AND METHODS

Plant material

The blackberries used in this study were collected during August 2023 in the Belgrade area (44°53'25"N, 20°27'2"E). The plant material was homogenized, frozen (−80 °C, 48 h), and freeze-dried using an Alpha 1-4 LSCplus freeze dryer (Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany) according to the following regime: −20 ºC, 10 h, 0.1 mbar; −10 ºC, 5 h, 0.1 mbar; 0 ºC, 5 h, 0.1 mbar; 20 ºC, 5 h, 0.05 mbar. The freeze-dried blackberries were manually ground into a coarse powder and stored in a refrigerator at 4 °C until further use.

Solvent preparation

Two choline chloride-based NADES were used in this study to investigate how their different chemical natures influence the extraction efficiency of the NADES: choline chloride:glycerol (molar ratio of 1:2) − solvent N1, and choline chloride:lactic acid (molar ratio of 1:4) − solvent N2. The following solvents and their HBA:HBD ratios were chosen based on previously published studies, which demonstrated their stability and effec-

tiveness in extracting anthocyanins and other polyphenols from plant materials (Milošević et al., 2024; Pavlić et al., 2022). The water content of each compound was taken into account for the preparation. The solvents were prepared by mixing in a water bath placed under a magnetic stirrer hot plate at 80 °C until a homogeneous, transparent liquid was formed. The 70% ethanol used for the extraction was prepared from absolute ethanol.

Extraction

Three different techniques and the above-described solvents were used to extract polyphenols from blackberry powder: maceration (MAC), ultrasound-assisted extraction (UAE) and microwave-assisted extraction (MAE). MAC was performed on a magnetic stirrer at room temperature (22 °C) and 1500 rpm for 30 minutes (samples MAC1 and MAC2). UAE was conducted in an ultrasonic bath (EUP540A, Euinstruments, France) for 30 minutes at a frequency of 40 kHz and two different ultrasound powers (100% and 50%) (samples $UAE1_{100}$, $UAE2_{100}$, $UAE1_{50}$, and $UAE2_{50}$, respectively). MAE was carried out in a modified domestic microwave oven as described by Pavlić et al. (2020) for 30 minutes at two microwave irradiation powers (180 W and 600 W) (samples $MAE1_{180}$, $MAE2_{180}$, $MAE1_{600}$, and $MAE2_{600}$, respecttively).

All extractions were done by mixing 1 g of blackberry powder with 16 g of NADES and 4 g of distilled water. The water was added to reduce the solvents' viscosity, i.e., improve the yield of extracted bioactives. For the ethanol-based extraction, 1 g of blackberry powder was mixed with 20 mL of 70% ethanol, and maceration was performed under the same conditions as for the MAC samples (sample E). After extraction, all samples were filtered through filter paper (Whatman, No.1) and stored at 4 °C until further analysis.

Total monomeric anthocyanin content

The total monomeric anthocyanin content (TAC) of the extracts was determined by the pH differential method according to Lee et al. (2005) after 15 minutes of incubation. Distilled water was used as a blank. The results were calculated using the following formula and expressed in µg cyanidin-3-glucoside equivalents (CGE) per mL of extract (Eq. 1):

$$Total\ monomeric\ anthocyanins = \frac{A \times MW \times DF \times 10^3}{\varepsilon \times L} \quad (1)$$

where A = $(A_{520nm} - A_{700nm})pH1.0 - (A_{520nm} - A_{700nm})pH4.5$, MW and ε are the molecular weight and molar extinction coefficient of cyanidin-3-glucoside (449.2 g x mol^{-1} and 26900 L x mol^{-1} x cm^{-1}, respectively); DF is the dilution factor, 1000 is the conversion factor from g to mg, and L is the pathlength (cm).

Polymeric color index and browning index

The polymeric color and browning indexes of the extracts were determined according to the method established by Giusti & Wrolstad (2001). For all samples, 2.8 mL of the sample was appropriately diluted with distilled water and mixed with 0.2 mL of 0.025 M potassium metabisulfite solution in one cuvette and in the second cuvette, the same amount of sample was mixed with 0.2 mL of distilled water.

The samples were incubated for 15 minutes at room temperature, followed by an absorbance reading at 420 nm, 520 nm, and 700 nm against a distilled water blank on a HALO DB-20S UV-Vis spectrophotometer (Dynamica Scientific Ltd., Livingston, UK). The polymeric color index was calculated based on the polymeric color and color density using Eq. 2:

$$Polymeric\ color\ index\ (\%) = \frac{Polymeric\ color}{Color\ density} \times 100 \quad (2)$$

Polymeric color was calculated using the absorbance values for the sample with added potassium metabisulfite (Eq. 3):

$$Polymeric\ color\ = [(A_{420nm} - A_{700nm}) + (A_{520nm} - A_{700nm})] \times dilution\ factor \quad (3)$$

Color density, on the other hand, was determined based on the absorbances of the samples with added distilled water (Eq. 4):

$$Color\ density = [(A_{420nm} - A_{700nm}) + (A_{520nm} - A_{700nm})] \times dilution\ factor \quad (4)$$

Browning index was also calculated using the absorbances of the water-diluted samples (Eq. 5):

$$Browning\ index = \frac{A_{420nm}}{A_{520nm}} \quad (5)$$

Total polyphenol content

The total phenolic content (TPC) of the obtained extracts was determined by the Folin–Ciocalteu method according to Terpinc, Čeh, Ulrih & Abramovič (2012). The absorbance was read at 765 nm on a HALO DB-20S UV-Vis spectrophotometer (Dynamica Scientific Ltd., Livingston, UK) with distilled water used as a blank. Total phenolic contents were determined from the gallic acid calibration curve (R^2 = 0.9989), and the results were expressed in μg gallic acid equivalents (GAE) per mL of extract.

Ferric reducing antioxidant power

The ferric reducing antioxidant power (FRAP) of the extracts was determined according to the method established by Benzie & Strain (1996). After incubation (37 °C, 40 min), the absorbance reading was done at 593 nm on a HALO DB-20S UV-Vis spectrophotometer (Dynamica Scientific Ltd., Livingston, UK) against a distilled water blank. The values were determined based on a Trolox calibration curve (R^2 = 0.9991), and the results were expressed in μmol Trolox equivalents (TE) per mL of extract.

Microbial culture preparation

Antimicrobial activity of the extracts was tested against two Gram-positive strains (*Staphylococcus aureus* ATCC 25923 and *Listeria monocytogenes* ATCC 19111), two Gram-negative strains (*Escherichia coli* ATCC 25922 and *Salmonella enterica* subsp. *enterica* serovar Enteritidis ATCC 13076), and one yeast strain (*Candida albicans* ATCC 10231), using the agar diffusion method and broth microdilution method. The fresh inocula of *S. aureus*, *L. monocytogenes*, and *E. coli* were prepared in tryptone soy broth, *S.* Enteritidis was grown in Mueller–Hinton broth, whereas *C. albicans* was prepared in malt extract broth.

Antimicrobial activity − agar diffusion method

The overnight microbial cultures were evenly spread on their respective agar surface using a sterile swab. Sterile diameter discs (⌀ 6 mm) were placed on the inoculated dishes under sterile conditions, and each was impregnated with 10 μL of undiluted extract. Discs with pure NADES were also prepared in parallel with the extracts, and the antibiotic discs served as positive controls (penicillin for Gram-positive bacteria, chloramphenicol for Gram-positive and Gram-negative bacteria, and nystatin for the yeast strain). The inoculated dishes were incubated at 37 °C for bacterial strains or 30 °C for the yeast strain for 24 hours. After incubation, the inhibition zones were measured to evaluate the microbial growth.

Antimicrobial activity − broth microdilution method

The antimicrobial activity of the extracts against the abovementioned strains was also evaluated by determining the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) using the BioLiteTM 96-well microtiter plates (Thermo Fisher Scientific Inc., Rochester, NY, USA). The concentration of the overnight bacterial cultures was adjusted to an optical density equivalent of 10^5–10^6 CFU/mL using the DEN-1 McFarland densitometer (Biosan, Riga, Latvia), and 150 μL of resazurin, a color indicator of microbial growth, was added into 10 mL of each suspension. Two-fold serial dilutions were prepared (extract concentrations: 50–0.006% v/v), and 50 μL of bacterial suspension was transferred into wells containing 50 μL of extract dilutions. The wells containing broths and bacterial suspensions with resazurin served as negative and positive controls, respectively. The microtiter plates were incubated at 37 °C (bacterial strains) or 30 °C (yeast strain) for 24 hours. The highest dilution (% v/v) of the sample that preceded the change in color of the indicator from violet to pink was determined as MIC. The contents of the wells with concentrations greater than or equal to the MIC were plated onto their respective agar and incubated for 24 hours at the corresponding temperature to determine MBC. The lowest concentration of the extract, which resulted in no visible growth on the agar surface, was defined as MBC.

Statistical analysis

Statistical analysis was performed using the GraphPad Prism 9.0 software (GraphPad Software, San Diego, CA, USA). Analysis of variance (ANOVA) and Tukey's test for pair comparison were carried out using a significance level of 95% confidence ($p \leq 0.05$). All

analyses were performed in triplicates and the results are presented as means ± standard deviation.

RESULTS AND DISCUSSION

Total monomeric anthocyanin content

The total monomeric anthocyanin content (TAC) of blackberry extracts was greatly influenced by the used solvent (Fig. 1). The TAC values were similar to those reported in the literature for blackberries and other berry fruits, indicating good yields (Kopjar, Bilić & Piližota, 2011; Le et al., 2019). Out of all samples, the sample $UAE2_{50}$ had the highest TAC (312.27 ± 13.67 μg CGE/mL) but did not differ significantly ($p > 0.05$) from MAC2.

The use of choline chloride:lactic acid NADES generally yielded higher anthocyanin concentrations than choline chloride:glycerol, most probably due to its low pH. In an acidic environment, anthocyanins exist in their most stable form, known as flavilium cation, resulting in facilitated extraction and lower degradation rates (Bridgers, Chinn & Truong, 2010; Yu, Shiau, Pan & Yang, 2024).

Compared to ethanol, only the abovementioned $UAE2_{50}$ sample had a significantly higher ($p \leq 0.05$) TAC. In terms of the employed extraction technique, although there was no significant difference between the MAC and UAE_{50} samples, a similar trend could be observed for both NADES, with the UAE at 50% power showing the highest efficiency. On the other hand, using microwaves had a negative and the most pronounced effect. Intensive heating during MAE over a long period may result in anthocyanin degradation and the formation of brown (polymer) products, which in this case most probably made anthocyanin quantification impossible (Zhang, Deng, Zheng, Liu & Zhang, 2020).

Polymeric color index and browning index

As mentioned above, heat processing can cause anthocyanin degradation and polymerrization, resulting in the loss of red color and the formation of brown-colored products (Jiang et al., 2019). Therefore, the polymeric color index and browning index were determined to estimate the degradation rate of anthocyanins and potentially explain the loss of monomeric anthocyanins noted in the previous section. The results presented in Table 1 indicate that the microwave-assisted extraction, particularly at higher power (600 W), caused the most significant changes in anthocyanin extracts, resulting in 3−5 times higher PCI and BI compared to maceration and ultrasound.

The PCIs of over 80% and BIs above 1.0 indicate a loss of almost total anthocyanin content (Zhang et al., 2020). The browning was the least pronounced in N2 samples and ethanol, UAE2 samples in particular, which could be attributed to the abovementioned protective effects of acid-based NADES on anthocyanins. The 50% ultrasound power led to the lowest losses of anthocyanins, especially in the case of N2 extract.

Total polyphenol content

Fig. 2 shows the total phenolic contents (TPC) of the extracts, with the highest and lowest values observed in $MAE2_{600}$ (649.28 ± 38.26 μg GAE/mL) and $UAE1_{50}$ (17.07 ± 0.73 μg GAE/mL), respectively. As with anthocyanins, the N2 solvent generally resulted in a higher recovery of polyphenolic compounds compared to the N1 solvent.

Table 1.
Polymeric color index and brown index of blackberry-based extracts

Sample	Polymeric color index (PCI, %)	Browning index (BI)
MAC1	30.35 ± 0.00^{a}	0.732 ± 0.000^{a}
$UAE1_{50}$	17.99 ± 1.18^{be}	0.592 ± 0.004^{b}
$UAE1_{100}$	30.24 ± 0.25^{a}	0.703 ± 0.003^{a}
$MAE1_{180}$	88.56 ± 0.10^{c}	1.762 ± 0.016^{c}
$MAE1_{600}$	90.10 ± 2.62^{c}	2.072 ± 0.040^{d}
MAC2	19.90 ± 0.49^{b}	0.487 ± 0.005^{e}
$UAE2_{50}$	11.42 ± 0.52^{d}	0.396 ± 0.004^{f}
$UAE2_{100}$	15.23 ± 0.19^{e}	0.432 ± 0.004^{f}
$MAE2_{180}$	83.35 ± 2.47^{f}	1.750 ± 0.010^{c}
$MAE2_{600}$	88.40 ± 1.96^{c}	1.768 ± 0.011^{c}
E	21.51 ± 0.81^{b}	0.541 ± 0.010^{g}

Values represent the mean ± SD of triplicate tests. Values with different superscript letters within the same column are significantly different ($p \leq 0.05$)

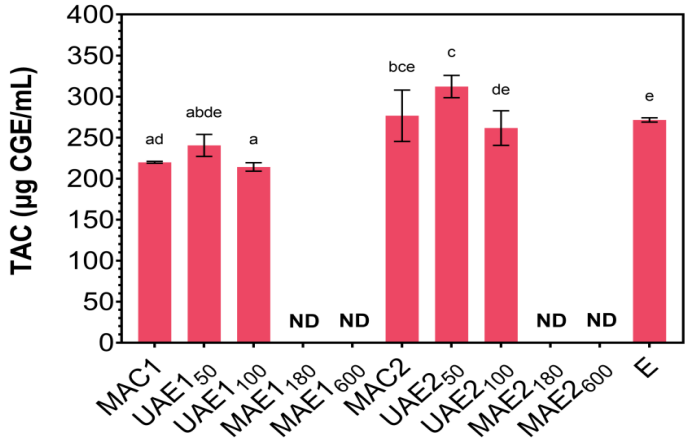

Figure 1. Total monomeric anthocyanin contents (TAC) of the obtained extracts. Different letters indicate significant differences ($p \leq 0.05$) between the values for each extract. MAC − maceration, UAE − ultrasound-assisted extraction (50% and 100% power), MAE − microwave-assisted extraction (180 W and 600 W power). Codes with number 1 and 2 refer to solvents N1 and N2, respectively.

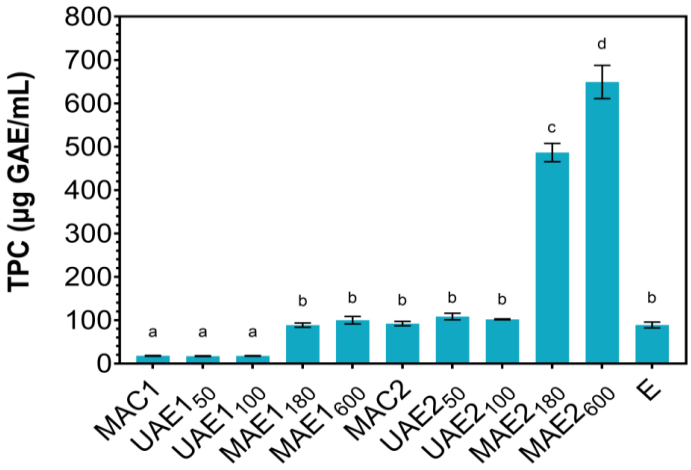

Figure 2. Total phenolic contents of the blackberry extracts. Different letters indicate significant differences ($p \leq 0.05$) between the values for each extract.

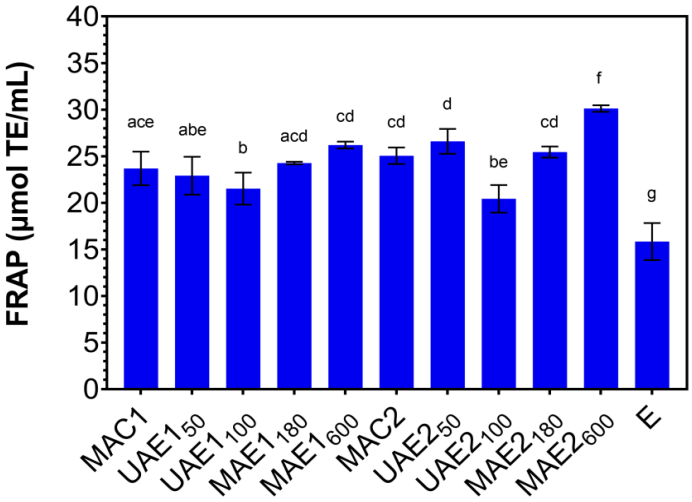

Figure 3. Ferric Reducing Antioxidant Power (FRAP) of the blackberry extracts. Different letters indicate significant differences ($p \leq 0.05$) between the values for each extract.

The maceration and ultrasound-assisted extraction gave results similar to ethanol ($p > 0.05$); however, for both solvents, the MAE resulted in 4−6 times higher TPC compared to the other two techniques. As explained by Zhang et al. (2020) and Wang et al. (2024), anthocyanin degradation products include various phenolic acids and aldehydes which all react with the Folin–Ciocalteu reagent.

Furthermore, the present and newly formed amino compounds and sugars, stimulated by the high temperatures, could have undergone Maillard reactions or caramelization, with their products also giving positive reactions (Žilić, Kocadağlı, Vančetović & Gökmen, 2016).

Ferric reducing antioxidant power

The results of the FRAP assay (Fig. 3) were generally more consistent among the samples. Ethanolic extract showed the lowest antioxidant activity (16.76 ± 1.20 μmol TE/mL), while the highest value was observed for $MAE2_{600}$ (30.12 ± 0.35 μmol TE/mL). The N1 and N2 extracts produced using the same techniques did not differ significantly ($p > 0.05$) except for UAE_{50} and MAE_{600} samples. The effect of extraction techniques was more prominent among N2 extracts, with the lower ultrasound power and higher microwave power resulting in higher ferric-reducing power. In a study by Shang et al. (2020), an increase in microwave power also resulted in higher TPC and antioxidant activity of sweet tea extracts, with the microwave power of 600 W chosen as the optimal.

Antimicrobial activity − agar diffusion method

The results of the agar diffusion method are given in Table 2. The N1- and ethanol-based extracts and pure solvents did not inhibit bacterial growth; however, the addition of N2 samples resulted in inhibition zones for all microorganisms except *C. albicans*.

The highest activity was observed against *L. monocytogenes*, particularly for $UAE2_{50}$ and MAE samples ($p > 0.05$). According to the literature, blackberry juices and extracts exhibit activity against both Gram-positive and Gram-negative bacteria, including the foodborne pathogens investigated in this study, whereas yeast strains tend to be more resistant (Yang, Hewes, Salaheen, Federman & Biswas, 2014; Četojević-Simin et al., 2017).

The antibacterial activity of the extracts can also be attributed to the low pH of the solvent, which is known to inhibit bacterial growth; the results of the pure N2 also confirm this (Trusheva et al., 2024).

Table 2.
Antimicrobial activity by the agar diffusion method

Sample	Zones of inhibition (mm)				
	S. aureus	***L. monocytogenes***	***S.* Enteritidis**	***E. coli***	***C. albicans***
MAC1	ND	ND	ND	ND	ND
$UAE1_{50}$	ND	ND	ND	ND	ND
$UAE1_{100}$	ND	ND	ND	ND	ND
$MAE1_{180}$	ND	ND	ND	ND	ND
$MAE1_{600}$	7.5 ± 0.71^{a}	ND	ND	ND	ND
N1	ND	ND	ND	ND	ND
MAC2	13.67 ± 0.58^{b}	25.00 ± 1.00^{a}	14.00 ± 1.73^{ad}	11.00 ± 1.00^{ac}	ND
$UAE2_{50}$	15.00 ± 2.00^{bc}	29.00 ± 1.00^{b}	14.00 ± 1.00^{ad}	13.00 ± 1.00^{ab}	ND
$UAE2_{100}$	15.33 ± 1.15^{bc}	25.00 ± 1.00^{a}	15.00 ± 0.00^{abd}	12.33 ± 1.53^{ab}	ND
$MAE2_{180}$	15.33 ± 0.58^{bc}	27.00 ± 1.00^{ab}	15.00 ± 0.00^{abd}	14.00 ± 0.00^{b}	ND
$MAE2_{600}$	17.00 ± 1.00^{cd}	28.00 ± 1.00^{ab}	16.33 ± 0.58^{b}	13.67 ± 0.58^{b}	ND
N2	18.76 ± 1.53^{d}	30.33 ± 0.58^{b}	19.00 ± 0.00^{ce}	13.33 ± 1.15^{ab}	ND
E	ND	ND	ND	ND	ND
E solvent	ND	ND	ND	ND	ND
Antibiotic/ Antimycotic*	37.00 ± 0.00^{e} / 20.00 ± 0.00^{d}	37.00 ± 1.73^{c} / 30.50 ± 2.50^{b}	13.00 ± 0.00^{d} / 18.00 ± 0.00^{e}	9.00 ± 0.00^{c} / 24.00 ± 0.00^{d}	27.67 ± 0.58

The diameters of the inhibition zones include the zones under the disks. Values represent the mean ± SD of triplicate tests. Values within the same column with different superscripts are significantly different ($p \leq 0.05$). ND − not detected.
** Positive controls used for bacterial strains (penicillin and chloramphenicol) are presented in the first and second row, respectively. Nystatin was used as a positive control for the yeast strain*

The antibacterial effects of polyphenols and organic acids are thought to involve multiple mechanisms, including increased permeability and disruption of the cell membrane that cause leakage of intracellular content, acidification of the cytoplasm, inhibition of metabolic enzymes, denaturation of structural proteins and DNA, all of which ultimately result in cell death (Burdulis et al., 2009; Mani-López, García & López-Malo, 2011).

Antimicrobial activity − broth microdilution method

The results of the broth microdilution test for antibacterial activity against different bacterial strains and a yeast strain are shown in Table 3.

The minimal inhibitory and bactericidal concentrations (MIC and MBC, respectively) are presented as dilution percent of the samples, with lower values indicating higher antimicrobial activity.

Overall, extracts obtained using NADES with lactic acid showed the highest antimicrobial activity, especially against Gram-negative bacteria (0.01−1.56% v/v for *S.* Enteritidis and 0.006−0.78% v/v for *E. coli*). Similar to the disk diffusion method, this can be attributed to the acidity of the solvent.

On the other hand, the microdilution method also detected antibacterial activity of N1- and E-based extracts. The discrepancy between the two methods has already been reported in the literature and in this case could be due to the limited diffusion of extracts, particularly viscous NADES and high-molecular polyphenols, through the agar surface, which was not the case in a liquid medium (Hossain, Lim, Hammer, Hettiarachchi & Locher, 2022; Tan & Lim, 2015; Zheng, Tan, Yang & Liu, 1996). The highest activity was observed against Gram-positive bacteria, whereas *C. albicans* exhibited the highest resistance.

The influence of the extraction technique was only apparent for glycerol-based samples, with MAE samples being the most effective, which is likely due to the antimicrobial properties of different heat-induced degradation products (Song, Yang, Wei & Ruan, 2016).

Nevertheless, the fact that the MIC and MBC of extracts were generally lower than their respective solvents indicates the antimicrobial activity of extracted polyphenols and, potentially, synergistic effects.

Improved antimicrobial activity compared to the pure solvents is consistent with the findings of Trusheva et al. (2024) for NADES-based propolis extracts that exhibited significantly greater efficacy than the solvents alone, emphasizing the contribution of extracted bioactive compounds.

Table 3.
Antimicrobial activity by the broth microdilution method

	Concentration of diluted extract (% v/v)*									
Microorganism	***S. aureus***		***L. monocytogenes***		***S.* Enteritidis**		***E. coli***		***C. albicans***	
	MIC	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**
MAC1	1.56	50	12.5	12.5	25	50	12.5	25	25	50
$UAE1_{50}$	6.25	ND	12.5	12.5	25	50	12.5	25	25	50
$UAE1_{100}$	3.13	ND	12.5	12.5	25	50	12.5	25	25	50
$MAE1_{180}$	1.56	50	6.25	6.25	25	50	25	25	25	50
$MAE1_{600}$	1.56	25	3.13	3.13	25	50	25	25	12.5	50
N1	25	50	12.5	ND	12.5	25	25	25	25	25
MAC2	0.01	0.78	0.39	0.39	0.01	0.05	0.006	0.78	0.78	12.5
$UAE2_{50}$	0.01	1.56	0.39	0.39	0.01	0.02	0.01	0.01	0.78	12.5
$UAE2_{100}$	0.01	1.56	0.39	0.39	0.01	0.05	0.006	0.01	0.78	12.5
$MAE2_{180}$	0.01	0.10	0.39	0.39	0.01	0.01	0.01	0.78	0.78	12.5
$MAE2_{600}$	0.01	0.78	0.78	0.78	0.01	0.02	0.01	0.02	0.78	12.5
N2	0.78	0.78	0.39	0.78	0.39	1.56	0.78	0.78	6.25	6.25
E	1.56	50	6.25	25	25	50	25	50	50	50
E solvent	25	ND	50	50	50	ND	50	50	50	50

** The results are presented as Minimum Inhibitory Concentrations (MIC) and Minimum Bactericidal Concentrations (MBC).*
ND − not detected

CONCLUSIONS

The results of this study indicate that the choice of solvent and extraction technique significantly influenced the properties of the blackberry polyphenolic extracts. NADES composed of choline chloride and lactic acid showed the most effective results due to their acidic nature, enhancing anthocyanin extraction and the antimicrobial activity of the extracts, particularly against Gram-positive pathogens. In terms of the extraction techniques, microwave-assisted extraction, especially at higher powers, had the most pronounced impact, yielding the highest total polyphenol content and antioxidant activity. However, the results suggest that high temperatures substantially altered the extract composition by causing anthocyanin degradation, which highlights the need for further investigation. For preserving the color and sensory properties of the extract, ultrasound-assisted extraction at moderate intensities appears to be the most suitable approach.

Anthocyanin extracts obtained using both conventional solvents and NADES offer a wide range of applications in the food industry, both as food additives and components for active packaging. Their antioxidant and antimicrobial properties may contribute to products' stability and color. Future steps should include optimization of the extraction parameters and food contact studies to further investigate the potential of the obtained extracts for food applications.

AUTHOR CONTRIBUTIONS

Conceptualization, A.D.C.K., A.B.T. and V.A.N.; Methodology, A.D.C.K., A.B.T., A.S.S., S.D.B. and M.M.M.; Investigation, formal analysis, validation, writing-original draft preparation, A.B.T., A.D.C.K., A.S.S. and S.D.B.; Writing-review and editing, S.M.L., A.S.S., A.D.C.K., A.S.S., M.M.M. and V.A.N.; Supervision, S.M.L. and V.A.N.

DATA AVAILABILITY STATEMENT

Data contained within the article.

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CONFLICT OF INTEREST

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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ANTIOKSIDATIVNI I ANTIMIKROBNI POTENCIJAL POLIFENOLNIH EKSTRAKATA KUPINE: UTICAJ RAZLIČITIH TEHNIKA EKSTRAKCIJE I RASTVARAČA

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Sažetak: Kupine (*Rubus* sp.) se smatraju dobrim izvorom bioaktivnih jedinjenja, naročito polifenola. Visoka antioksidativna i antimikrobna aktivnost povezana sa polifenolima pruža različite mogućnosti primene ekstrakata kupine u prehrambenoj industriji. Ova studija ispituje potencijal ekstrakata kupine dobijenih pomoću različitih rastvarača i tehnika ekstrakcije kao izvora prirodnih antioksidanasa i antimikrobnih komponenti. Primenjeni su etanol i dva prirodna duboko eutektička rastvarača (NADES) (N1 − holin-hlorid:glicerin (1:2) i N2 − holin-hlorid:mlečna kiselina (1:4)) kao i tri različite tehnike ekstrakcije (maceracija, ekstrakcija uz primenu ultrazvuka i ekstrakcija uz primenu mikrotalasa). Ekstrakti su analizirani u pogledu ukupnog sadržaja monomernih antocijana, polimerne boje, ukupnog sadržaja polifenola i FRAP antioksidativne aktivnosti (Ferric Reducing Antioxidant Power). Antimikrobni potencijal protiv dve Gram-pozitivne bakterije, dve Gram-negativne bakterije i jedne vrste kvasca procenjen je primenom agar-difuzione i mikrodilucione metode. Rastvarač N2 dao je bolji prinos bioaktivnih jedinjenja u poređenju sa rastvaračem N1 i etanolom, dok je ekstrakcija mikrotalasima imala najveći uticaj na sadržaj polifenola i boju ekstrakata. Svi uzorci su pokazali značajno jaču antimikrobnu aktivnost u poređenju sa etanolnim ekstraktom, pri čemu je generalno najveća efikasnost zabeležena protiv Gram-pozitivnih bakterija. Ovi preliminarni rezultati ukazuju na potencijal kiselih NADES-a za ekstrakciju i primenu polifenola iz kupine. Budući koraci istraživanja trebalo bi da uključe optimizaciju procesa ekstrakcije i ispitivanja vezana za primenu u hrani.

Ključne reči: *kupina, polifenoli, NADES, ekstrakcione tehnike, antioksidansi, antibakterijska aktivnost*

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