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Screening of Natural Deep Eutectic Solvents for the Recovery of Valuable Phenolics From Waste of Shalgam Juice Process

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ABSTRACT

Shalgam juice is one of the most popular non-alcoholic beverages in Türkiye and also in many countries. The high amount of production, regarding the high amount of consumption leads to an inevitable amount of solid waste. This amount reaches to almost 12 million kg of fermented black carrot annually. The accumulation of this waste causes fatal environmental pollution. The waste of shalgam juice process, fermented black carrot, retains significant amount of valuable components, such as phenolics, flavonoids, and anthocyanins. In this study new generation green solvents, Natural Deep Eutectic Solvents (NADESs) were screened for the extraction of valuable phenolics from fermented black carrot using ultrasound assisted extraction. The NADESs used were selected from four different groups such as acidic, sugar-based, choline chloride-sugar based and polyol-based. According to the results, the members of polyol and choline chloride sugar NADESs showed up. Choline chloride-glycerol (polyol group), extracted the highest total phenol amount (16.04 mg/g) and also provided the highest antioxidant activity (81.77%). On the other hand, NADESs belonging to choline chloride-sugar group were effective for the extraction of flavonoids and monomeric anthocyanins. Namely, choline chloride-fructose-water extract contained 21.45 mg/g of total flavonoids, while choline chloride-sucrose-water extract contained 1680.51 mg/kg of total monomeric anthocyanins. The performances of NADESs tested were found to be higher than that of water and ethanol showing the high yield recovery of valuable phenolics with NADESs. The results exhibited the significance of the components inside the waste. The remaining valuable content could be easily and efficiently extracted using NADESs and these extracts –as a mixture or after purification- can furtherly be used for different purposes in different fields, such as cosmetics, antioxidant preparations, etc, using a zero-cost waste as the input of the processes.

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Introduction

Shalgam juice is one of the most popular non-alcoholic beverages in Türkiye. The bitter-sour taste matches perfectly with the traditional local food therefore, it is preferred together with almost every meal (Canbaş and Fenercioğlu, 1984). It is a savory beverage and helps with the digestion system. Since ancient times people have prepared this beverage by their own fermentation methods, especially in Adana, Mersin and Hatay –in the south part of Türkiye (Canbaş and Fenercioğlu, 1984). Nowadays the consumption of shalgam juice is widespread in Türkiye and it is commercially produced at high amounts and exported to more than 30 countries (*Dünya şalgam suyunu keşfetti*, 2020). The production of shalgam juice in Türkiye was reported as 60 million liters per year in 2020 and increased to 70 million liters per year in 2022 which is quite high (*Dünya şalgam suyunu keşfetti*, 2020; Ekinci, 2022). This high amount of production leads to an inevitable amount of solid waste that corresponds to almost 20% of the juice produced (Tanrıseven et al., 2018) that is about 12 million kg of fermented black carrot annually. The accumulation of this waste causes environmental pollution in terms of stench and the physical presence of the waste.

Due to the increasing consciousness about the environmental pollution researchers struggle for the evaluation of the wastes which cannot be prevented. In this aspect, some types of the waste, especially in the food industry have been investigated in order to be reevaluated for their potential to be a zero-cost waste as an input of many different processes in other fields (Ahmad and Danish, 2018; Cheok et al., 2018).

Fresh black carrot is a well-known source of various types of phenolics such as anthocyanins, carotenoids, flavonoids, antioxidants, vitamins, and etc. (Khandare et al., 2011; Akhtar et al., 2017). In the production process of shalgam juice, black carrots are fermented in the tanks in the form of chopped pieces in 2-3 cm in length. The thick form of the pieces lets the vegetable to retain various beneficial components inside during fermentation process.

In the literature there are many studies that reported the evaluation of bioactive compounds from fresh black carrot (Türker and Erdoğdu, 2006; Ersus and Yurdagel, 2007; Gizir et al., 2008; Gras et al., 2015; Guldiken et al., 2016) but there are only a few studies on the extraction of the waste of black carrot from processing plants (Ağçam and Akyıldız, 2015; Agcam et al., 2021). Most of these studies use conventional extraction processes that require the utilization of abundant organic solvents. One of these studies used acidified methanol, ethanol and water for the extraction of anthocyanins from black carrot waste and reported high amount of total monomeric anthocyanins (Ağçam and Akyıldız, 2015). They also reported that the acidity of these solvents was effective on the recovery. Despite the high yields provided by organic solvents, the harms of these processes to the environment are inevitable. On the other hand, organic solvent free extraction was also performed, however, the system used required high pressure, that resulted in high cost (Agcam et al., 2021).

Increasing consciousness on the environmental and health issues directed researchers to the utilization of green processes. One of the most popular green strategies involves the replacement of organic solvents with green solvents. A significant class of green solvents are Deep Eutectic Solvents (DESs). They are regarded as new generation green solvents and they have been investigated to replace organic solvents in many fields (Tang et al., 2015; Li and Row, 2016; Cunha and Fernandes, 2018; Ünlü, 2021; Deniz et al., 2023). Besides their advantages such as easy preparation, low volatility, non-toxicity, high solvation capacity; the recent research conducted on the synthesis of different DESs resulted in a new sub-class that are called Natural Deep Eutectic Solvents (NADESs) (Dai et al., 2013b; Paiva et al., 2014; Vanda et al., 2018). The additional advantage of these new solvents were declared as the natural structure of the constituents. Their natural substance origin makes them attractive for researchers especially in the various extraction processes (Tang et al., 2015; Cvjetko Bubalo et al., 2016; Li and Row, 2016; Peng et al., 2016; Ruesgas-Ramón et al., 2017; Cunha and Fernandes, 2018; Meng et al., 2018; Ünlü, 2021; Deniz et al., 2023).

Among the non-conventional procedures, ultrasound assisted extraction takes attention as a simple, cheap and effective process (Huang et al., 2009). During exposure to ultrasound waves, cavitation is increased and effective mass transfer is achieved (Knorr et al., 2002). Additionally, ultrasound-assisted extraction was reported to be safe for heat sensitive components (Tiwari, 2015).

In this study, we aimed to reveal the potential of fermented black carrot that is the waste of shalgam juice process in terms of valuable ingredients that remains inside even after fermentation. In accordance with the aim to evaluate the waste for a sustainable process, toxic organic solvent utilization was avoided in this study. Instead, eleven different NADESs were used as green, natural and effective extraction solvents. Ultrasound waves were utilized in order to achieve high yield extractions at mild conditions. We evaluated the presence of phenolics in terms of total phenol amount, total flavonoid amount, total monomeric anthocyanin amount and presented antioxidant activities of the extracts. To our knowledge this is the first study to evaluate the waste of shalgam juice plant using NADESs as green solvents.

Materials and Methods

Preparation of Shalgam Juice Waste

Fermented black carrot was obtained from Yeni Kavaklıdere Company (Ankara, Turkey) with no cost. Solid waste was ground by blender (Braun MQ9078X) and lyophilized at 0.04 mbar and -50 °C (Hetosicc,CD 52-1, Heto Lab). The particles were then separated using molecular sieves (Endecotts, Octagon 200, England) and 425 µm-1 mm sized particles were used for the extraction processes.

Preparation of NADESs

Required amount of the components (Table 1) were weighed in a screw-capped bottle and heated (50, 60 or 80°C) for 2h, till clear liquid was formed. Choline chloride was dried under vacuum over silica gel in a desiccator prior to use. The NADESs that were prepared, their abbreviations and synthesis temperatures were given in Table 1.

Extraction of Shalgam Juice Process Waste

For the extractions, a modified method for anthocyanin extraction was used (Bubalo et al). 12 ml of total volume was added on 0.8 g of grounded lyophilized waste. NADESs were used as a mixture with water at 50% (v/v), to increase the polarity and also decrease the viscosity in order to provide easy handling (Altundağ et al., 2021; Ünlü, 2021; Deniz et al., 2023). Experiments was conducted using an ultrasonic water bath with a plug-in temperature controller, at 50 °C for 30 min (Elma S30H, Singer, Germany) with a frequency of 37 kHz and a power of 140W. After extraction, the extract was filtered through a 0.45 µm syringe filter and stored at -30 °C in the dark.

Total Phenol Amount

Total phenolic amounts of the samples were determined by the Folin-Ciocalteu method. 20 µL of extracts were mixed with 1580 µL of distilled water and 100 µL Folin-Ciocalteu reagent was added. After 1 min of incubation, 300 µL of 20% Na_2CO_3 was added and vortexed. The mixture was incubated at room temperature in the dark for 2h. At the end of the incubation period the absorbance was read at 750 nm. The results are given as gallic acid equivalent (Arnous et al., 2001).

Total Flavonoid Amount

Total flavonoid amounts of extracts were measured as reported by Choi et al. (2011). 100 µL of extract, 1000 µL of diethylene glycol and 100 µL of NaOH were mixed in a test tube and incubated at 37 °C for 60 min. The absorbance was measured at 420 nm. The concentrations of flavonoids were presented in quercetin equivalent (Choi and Kim, 2011).

Total Monomeric Anthocyanin Amount

Total monomeric anthocyanin amount was determined using pH differential method (Giusti and Wrolstad, 2001). Samples were diluted with 0.025 M potassium chloride buffer (pH=1) and with 0.4 M sodium acetate buffer (pH=4.5). After equilibration for 15 min, absorbances at λ_{max} (527 nm) and 700 nm were measured. Total monomeric anthocyanin concentration (TMAP) was calculated as cyanidin-3-glucoside equivalents by following equation in mg/L; then corresponding amount (mg/kg) was calculated with the use of solid-liquid ratio.

$$\text{TMAP}\left(\frac{\text{mg}}{\text{liter}}\right)=\frac{A\times MW\times DF}{\varepsilon\times 1}\times 100$$

Where;
A= $(A_{527nm}-A_{700nm})_{pH1}-(A_{527nm}-A_{700nm})_{pH4.5}$
MW (molecular weight)= 449.2 g/mol for cyanidin-3-glucoside
DF= Dilution factor
l= path length in cm
ε= 26900 molar extinction coefficient in $Lxmol^{-1}\times cm^{-1}$ for cyanidin-3-glucoside
1000= Factor for conversion from g to mg.

Antioxidant activity

Antioxidant activities were measured using DPPH radical scavenging method (Fernando and Soysa, 2015). 60 µL extract was mixed with 1140 µL of 100 µM DPPH (in ethanol). The mixture was kept at room temperature in the dark for 30 min. The absorbance at 517 nm was recorded (A_{sample}). As control, the same procedure was carried out without sample ($A_{control}$). The percentage of DPPH scavenging effect (inhibition %) was calculated by following equation.

$$\text{Inhibition}(\%)=\frac{A_{control}-A_{sample}}{A_{control}}\times 100$$

Statistical analysis

All extractions and analysis were performed in triplicates and average values were presented. The results were analyzed by one-way analysis of variance (ANOVA) and t-test and $P<0.05$ were considered significantly different using GraphPad Prism Version 10.0.2 (GraphPad Software, Inc, La Jolla, CA, USA).

Results

NADESs used in this study were selected based on our previous studies such as extraction of phenolics from olive leaves (Ünlü, 2021) or apple pomace (Deniz et al., 2023). The broad range of NADESs let us the to screen the relevant types on a general basis. Regarding this, eleven green solvents from four different groups, as acidic, sugar based, choline chloride-sugar based and polyalcohol based NADESs were tested using one-pot-at-a-time method (Table 1).

Total Phenolic Amount

Figure 1 shows total phenolic amounts obtained using eleven different NADESs and also reference extractions performed using water and EtOH. According to the results the highest phenolic amount was achieved using CG (1:2) as 16.04 ± 0.87 mg/g, followed by CFW (5:2:5) as 14.23 ± 0.42 mg/g. On the other hand, total phenolic amounts obtained for GFW (1:1:11), CLa (1:9), GSW (1:1:11), CEG (1:2) were found to be around 10 mg/g (differences were not significant; p>0.05). Regarding different groups of NADESs, CG (1:2) was found to be the best in polyol group, CLa (1:9) in acidic group, GFW in sugar group and CFW (5:2:5) in choline-chloride sugar group. On the other hand, it was found that NADESs could extract higher amount of phenolics than water (6.29 ± 0.34 mg/g) and EtOH (2.50 ± 0.66 mg/g).

Total Flavonoid Amount

The changes in total flavonoid amounts with different NADESs were presented in Figure 2. The highest flavonoid amount was obtained as 21. 45 ± 0.10 mg/g using CFW (5:2:5) which was almost 20% higher than the closest value obtained with GFW (1:1:11) (17.38 ± 0.70 mg/g). NADESs that provided the highest flavonoid amounts from each group were; CFW (5:2:5), GFW (1:1:11), CLa (1:9), CEG (1:2), from choline chloride sugar, sugar, acidic and polyol groups, respectively. Interestingly, three members from the sugar group of NADESs-except for GFSW (1:1:1:11) - lined up as second, third and fourth to extract the flavonoids. Among the acidic group, molar ratio of 1:9 showed up, however, no correlation could be detected between the extracted flavonoid amount and lactic acid molar ratio in NADES. On the other hand, water and EtOH were not effective on the extraction of flavonoids.

Total Monomeric Anthocyanin Amount

When total monomeric anthocyanin amounts are evaluated, CSW (4:1:4) stood out as shown in Figure 3, providing the highest monomeric anthocyanin amount as 1680.51 ± 22.21 mg/kg. Monomeric anthocyanins could be extracted at higher amounts using polyol and sugar containing NADESs whereas acidic NADESs were not effective in this aspect. However, total monomeric anthocyanin amount decreased with increasing amount of lactic acid in NADESs. On the other hand, water was found to extract anthocyanins at the sixth order, whereas EtOH was at the last order.

Table 1. List of NADESs prepared

NADES group	Name	Abbreviation	T	Reference
Acidic	Choline chloride-lactic acid	CLa (1:1)	60 °C	(Jablonský et al., 2018)
		CLa (1:6)	60 °C	(Jablonský et al., 2018)
		CLa (1:9)	60 °C	(Jablonský et al., 2018)
Sugar based	Glucose-fructose-sucrose-water	GFSW (1:1:1:11)	50 °C	(Mohammadpour et al., 2018)
	Glucose-fructose-water	GFW (1:1:11)	50 °C	(Mohammadpour et al., 2018)
	Glucose-sucrose-water	GSW (1:1:11)	50 °C	(Mohammadpour et al., 2018)
	Fructose-sucrose-water	FSW (1:1:11)	50 °C	(Mohammadpour et al., 2018)
Choline chloride-sugar based	Choline chloride-fructose-water	CFW (5:2:5)	80 °C	(Elgharbawy, 2018)
	Choline chloride-sucrose-water	CSW (4:1:4)	80 °C	(Elgharbawy, 2018)
Polyol-based	Choline chloride-ethylene glycol	CEG (1:2)	80 °C	(Zhang et al., 2012)
	Choline chloride- glycerol	CG (1:2)	80 °C	(Dai et al., 2013a)

T: Temperature

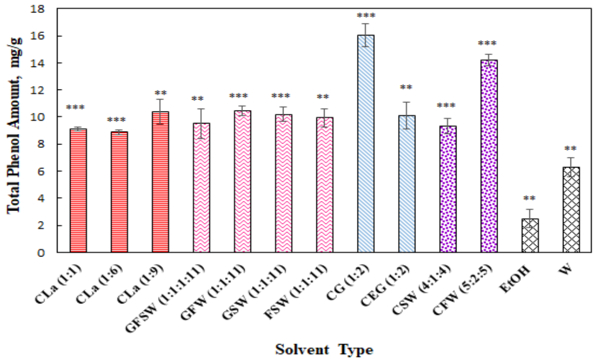

Figure 1. The effect of solvent types on total phenol amount ($p \leq 0.001$: ***, $p \leq 0.01$: **)

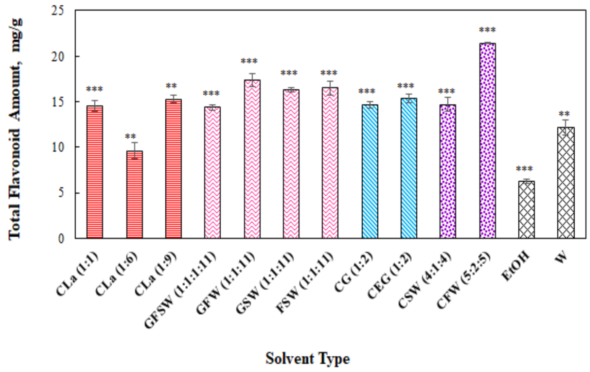

Figure 2. The effect of solvent types on total flavonoid amount ($p \leq 0.001$: ***, $p \leq 0.01$: **)

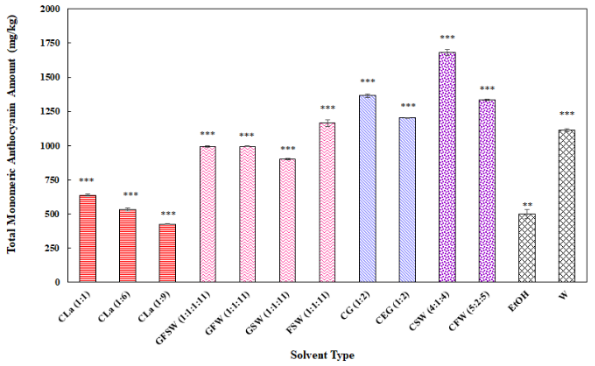

Figure 3. The effect of solvent types on total monomeric anthocyanin ($p \leq 0.001$: ***, $p \leq 0.01$: **)

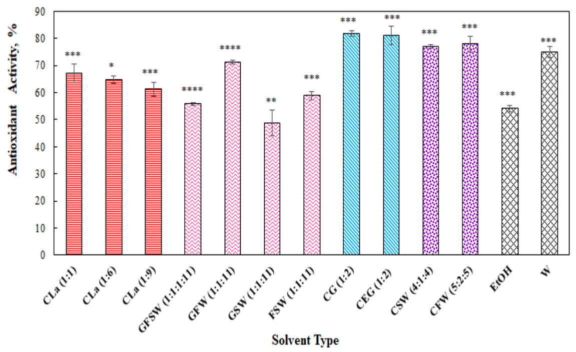

Figure 4. Antioxidant activities of the extracts ($p \leq 0.001$: ***, $p \leq 0.01$: **, $p \leq 0.05$: *)

Antioxidant Activity

Antioxidant activities of the extracts were investigated using DPPH as the radical. Control absorbance value to be used in Equation (1) was measured using NADESs without the extracts to eliminate the potential interference of the solvents with the procedure. The range of the antioxidant activities was found to change in the range of 81.77-48.63% (Figure 4). According to the results, the highest two values were very close to each other as 81.77± 1.01% and 81.22 ± 0.99% provided by CG (1:2) and CEG (1:2)), respectively. The extracts generally showed high antioxidant activities. Interestingly, antioxidant activity values decreased with increasing amount of lactic acid in NADESs, similar to monomeric anthocyanins.

Discussion

In this study, waste of shalgam juice was extracted using eleven different NADESs mostly selected from of our previous research (Ünlü, 2021; Deniz et al., 2023). Choline chloride sugar and sugar containing NADESs such as CFW (5:2:5), GFW (1:1:11) and CSW (4:1.4) were found to be prominent solvents. When the results are compared with the literature, it was reported that total phenol and flavonoid of olive leaves were extracted the most by choline-chloride sugar containing NADES; CFW (5:2.5) (Ünlü, 2021). On the other hand, GSW (1:1:11) as a sugar group NADES was declared to show up as the green solvent to extract phenolics from apple pomace (Deniz et al., 2023). Hence, similar findings of the extractions from different wastes revealed that sugar/choline chloride sugar group NADESs may be regarded as effective green solvents for phenolic components.

On the other hand, polyol group NADES; CG (1:2) extracted the highest amount of total phenols from the waste; fermented black carrot and also provided the highest antioxidant activity. When compared to the other member of polyol group that is CG (1:2); CEG (1:2) has extra hydroxyl group in the structure which might have positive effect on the efficiency of the extraction.

To observe the effect of the lactic acid content of NADESs, three different molar ratios of choline chloride-lactic acid NADESs were prepared. Total flavonoid amount and antioxidant activity results were inversely proportional to the increase in lactic acid amount of NADESs while total phenol and flavonoid amounts could not be correlated with the amount of lactic acid in NADES. On the contrary, the highest lactic acid containing NADES extracts contained the highest phenol and flavonoid amounts, while the lowest lactic acid containing NADES extracts were found to have the highest total monomeric anthocyanin and antioxidant activity. In contrast, apple pomace flavonoids were reported to be proportional to lactic acid content of NADES (Deniz et al., 2023).

Apart from NADESs, water and EtOH were also used to highlight their performances as commonly used solvents. Contrary to expectations, water was found to extract higher amounts of phenolics than ethanol as shown in Figs 1-3. This may be due to one of the ingredients of shalgam juice process; NaCl, that might have facilitated the extraction of phenolics from the waste. In the literature, the increase in the NaCl concentration in water was reported to increase the solubility of some phenolic compounds

(Noubigh et al., 2007). On the contrary, NaCl content of shalgam juice might have caused a negative effect on the extraction of phenolics by ethanol, since NaCl was reported to have quite low solubility in ethanol (Pinho and Macedo, 2005).

The results of the total phenolic amount could only be compared with Agcam et al. (2021) as this was the only study to present the phenolic amounts from the waste of black carrot pomace. The authors used a high pressure treatment system for the pomace-water mixture and also propylene glycol to transfer the pressure. They reported 350.93 mg/L of total phenolic amount and 107.0 mg/L of total monomeric anthocyanin at around 300 MPa and 60-80 °C (Agcam et al., 2021). Considering the discrepancy of the extraction methods one-to-one comparison would be inadequate, however, comparison of the highest total phenol and flavonoid amounts together with the cost of the systems used, one can conclude the present method as more advantageous, since almost three-fold higher total phenolic amount (CG (1:2) - 1067 mg/L, (16 mg/g)) could be obtained with a lower cost procedure. Total monomeric anthocyanins were compared to a study that presented vortex assisted acidified organic solvent extraction (Ağçam and Akyıldız, 2015). The range of total monomeric anthocyanins were reported as 656.2 - 1191.9 mg/kg. Compared to our results one can conclude that ultrasound assisted NADES extraction yielded almost 1.5-fold higher amount of total monomeric anthocyanins (1680.51 ± 22.21 mg/kg).

To highlight the amount of the valuable components retained inside the fermented black carrot as the waste of shalgam juice, examples of the extractions from fresh black carrot are presented below.

Jabbar et al. (2015) optimized the extraction of fresh black carrot using ultrasonic waves and organic solvents (Jabbar et al., 2015). They reported 316.74 µg/g phenolic amount at the optimized conditions (17 min, 34 °C, %48 EtOH) which is quite low than the results obtained in this study (16000 µg/g). On the other hand, Kumar et al. (2019) used microwave assisted extraction with EtOH and reported considerably higher phenolic amount as 264.9 ± 10.02 mg GAE/100 mL which is almost two-fold of the waste of shalgam juice (Kumar et al., 2019). Türker and Doğan (2021) used five different DESs to extract monomeric anthocyanins from fresh black carrot by ultrasound assisted extraction and the highest amount was obtained using choline chloride-citric acid (1:1) DES as around 6 mg/g (Aslan Türker and Doğan, 2021). This value is almost three-fold higher than the waste of shalgam juice. Hence we can conclude that roughly, one third to half of the phenolics in the fresh carrot remain inside the waste.

Conclusion

The waste; fermented black carrot was obtained from shalgam juice plant with no cost. After some preliminary steps to protect the waste from spoiling ultrasound-assisted NADES extraction was performed to recover valuable components. This study reveals the first time screening of NADESs in the extraction of valuable components from shalgam juice waste. With this aim, total phenol, flavonoid, monomeric anthocyanin amounts, as well as antioxidant activities were presented. Though the best solvent for each parameter varied, a general conclusion could be exhibited as, among the different groups tested, sugar / choline chloride sugar containing NADESs and also a member of polyol group NADES, that is CG(1:2) came forward. On the bright side, when compared to different extraction strategies, the green route presented in this study provided higher amounts of phenolics. The advantages of the presented procedure are; lower cost strategy than a high pressure process and a greener alternative to acidified organic solvents, besides, the procedure is effective and mild. So that the presented procedure has the potential to be an opt for the extraction of shalgam juice waste. On the other hand, flavonoid amounts and antioxidant activities were brought in as additional data to the literature. Additionally, hidden and/or neglected potential of the waste of shalgam juice process was revealed.

High-value-added residual components inside the waste are significant candidates as a potential to be used in different fields of industry both as a mixture or pure substance, if purification is performed. Further studies should be performed in order to optimize the extraction and detailed analysis can be performed to analyze the composition of phenolics, flavonoids and anthocyanins.

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The Declaration of Conflict of Interest

The authors declare that there are no competing financial and non-financial interest.

The Declaration of Ethics Committee Approval

This study does not require ethics committee permission or any special permission.

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