Concentration of Bioactive Compounds from Elderberry (Sambucus nigra L.) Juice by Nanofiltration Membranes

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Abstract

For the first time the chemical profile, physico-chemical parameters, inhibition of carbohydrate hydrolysing enzymes associated with type 2 diabetes, and radical scavenging properties of Sambucus nigra L. (elderberry) juice treated by nanofiltration (NF) were investigated. Three commercial NF membranes with different molecular weight cut-off (MWCO) (400 and 1000 Da) and polymeric material (composite fluoro-polymer and polyethersulphone) were tested. According to HPLC analyses, most part of bioactive compounds were retained by the NF membranes producing a retentate fraction of interest for the production of functional foods. The NP030 membrane, a polyethersulphone membrane with a MWCO of 400 Da, exhibited the highest rejection towards phenolic compounds when compared with the other selected membranes. Accordingly, the produced retentate fractions exhibited the highest radical scavenging activity.

Keywords: Sambucus nigra L. .Juice processing . Phenols . Radical scavenging activity . Type 2 diabetes . Nanofiltration

Introduction

Sambucus nigra L. (elderberry) is a widespread plant of the Caprifoliaceae family growing in Europe, North Africa, West Asia, and USA [1]. S. nigra berries or juices are used in the processing of jellies and jams, and in the preparation of pies and liqueurs. Berries have been utilised also as dietary supplements worldwide. All plant parts have been used in traditional medicine for their purgative, diuretic, haemostatic effects as well as for the prevention of diabetes, cardiovascular diseases, and cancer [2, 3]. The health benefits of S. nigra can be attributed to its characteristic chemical profile, which includes anthocyanins, flavonols, flavanones, and flavones as the main secondary metabolites [4]. Elderberry constituents demonstrated interesting antioxidant, anti-bacterial, anti-inflammatory, anti-viral, and anti-allergic properties in both in vitro and in vivo studies [5,6,7,8]. Several studies reported the beneficial role of anthocyanins-rich plants in diabetes due to their capacity to reduce hyperglycaemia-induced oxidative stress and stimulate insulin secretion [9]. A therapeutic approach to treat diabetes is to decrease post-prandial hyperglycaemia by the inhibition of carbohydrates-hydrolysing enzymes such as α-amylase and α-glucosidase [10].

Recently, the recovery of bioactive compounds from elderberry juice has gained a grown interest in the production of functional foods based on their positive contribution to the prevention of several diseases. Organic solvent extraction is the most common technique used to extract bioactive compounds but it is limited by harsh conditions such as high temperatures, which could lead to degradation of thermolable compounds, and the use of potentially toxic solvents for human consumption. Alternative green extraction techniques, such as ultrasound assisted extraction, and super critical extraction guarantee safer conditions and preservation of thermolable compounds but are characterized by high capital costs, which limit their widespread use. Pressure-driven membrane

processes, such as microfiltration (MF), ultrafiltration (UF), nanofiltration (NF) and reverse osmosis (RO), offer key advantages over conventional methodologies since the separation process does not involve phase changes or chemical agents; additionally, they can operate at room temperature with high efficiency, simple equipment and low energy consumption [11]. These processes are based on the principle of selective permeation of molecules through semipermeable membranes under a mechanical pressure as driving force. In particular, NF membranes are characterized by narrow pore sizes from 0.5 to 2 nm: they are able to retain lower particles when compared to MF and UF membranes, offering particular advantages for the fractionation of molecules of similar molecular weight (in the range 100–1000 Da) through the selection of membranes with suitable molecular weight cut-off (MWCO). At this purpose, NF has been widely used for the separation, fractionation and concentration of bioactive compounds from a large variety of products [12]. There are no reports in the literature about membrane fractionation of S. nigra juice. Therefore, following our previous studies [13, 14, 15, 16, 17, 18, 19], this work was aimed at evaluating the viability of NF in the separation and concentration of bioactive compounds from the juice in order to produce fractions to be used for the formulation of functional foods. At this purpose, the performance of three commercial polymeric membranes was compared in terms of productivity and rejection towards bioactive compounds of the juice. The antioxidant and hypoglycaemic properties of both permeate and retentate fractions were also analysed and compared to those of the untreated juice.

Materials and Methods

Chemicals and Reagents

All HPLC grade solvents were obtained from Carlo Erba Reagents (Milan, Italy). Astragalin, cyanidin-3-O-sambubioside, cyanidin-3-O-glucoside, (+)-catechin, gallic acid, quercetin, protocatechic acid and p-coumaric acid were purchased by Extrasynthese (Genay, France). Acarbose was obtained from Serva (Heidelberg, Germany). Ascorbic acid, aluminium chloride hexahydrate, ferrous sulfate, sodium molybdate, sodium nitrite, sodium hydroxide, sodium carbonate, 2,2′-azino-

bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), 2,2-diphenyl-1picrylhydrazyl (DPPH), o-dianisidine, and peroxidase/glucose oxidase were obtained from Sigma-Aldrich (Milan, Italy).

Preparation of Raw Juice

S. nigra berries were collected in Southern Italy (Calabria) at the end of September 2015 near the river Crati (Tarsia, Cosenza, Italy). Samples were identified by Dr. NG Passalacqua, Natural History Museum of Calabria and Botanic Garden, University of Calabria. Berries were destemmed, washed and then pressed by using a manual press. The extracted juice was pre-filtered through a nylon cloth in order to remove solid residues and impurities, stored in a refrigerator cell at −17 °C and defrosted before use. The extraction procedure gave an average juice yield of 52.4% (w/w).

Membranes and Nanofiltration Set-Up

Membrane filtration tests were performed by using a high-pressure cross-flow filtration cell (model HP4750) supplied by Sterlitech Corporation (Washington, USA) with a feed volume capacity of 300 mL, a diameter of 5.1 cm, and an effective membrane area of 20.4 cm2. Experiments were performed at room temperature. Before juice treatment, each membrane was conditioned in pure water for 24 h, and then placed in the cell. Experiments were carried out by applying a N2 gas transmembrane pressure of 20 bar. Each membrane filtration test was conducted three times. The permeate flux (Jp) through each membrane, at a given pressure, was defined as the volume permeated per unit area and per unit time, according to Eq. (2):

$$\mathbf{Jp=VpA\cdot\Delta t}$$

where Vp (L) is the volume of permeate, A (m2) is the membrane area, and Δt (h) is the operation time. The average and relative standard deviation were calculated. Three different commercial flat-sheet membranes namely Etna 01PP (from Alfa Laval, Lund, Sweden), NP010 and NP030 (from Mycrodin-Nadir, Wiesbaden, Germany) were used for the treatment of the juice. Their typical characteristics according to the manufacturers' data sheet are reported in Table 1.

The rejection (R, %) of bioactive compounds was calculated by using the following Eq. (2):

$$R=(1-\frac{Cp}{Cf})\cdot 100$$

where Cf and Cp are the feed and permeate concentration, respectively.

Rejection values, expressed as average ± SD, are the means of three different experimental runs and data statistically significant were evaluated according to the ANOVA test followed by Bonferroni t-test ($p < 0.05$).

Physico-Chemical Parameters of Elderberry Juice

The total soluble solids were estimated by a digital refractometer PR-201α (Atago, Tokyo, Japan) and expressed as °Brix (sucrose percentage) at 20 °C. The pH of S. nigra juice was measured by a pH meter (Basic Model 20, Crison). Fresh-juice colour was measured using a Konica Minolta CM-700/600d spectrophotometer (Konica Minolta Sensing, Japan) at 25 °C. Data were expressed as L* (lightness/darkness in a range 0–100), a* (greenness/redness in a range between −60 and + 60) and b* (blueness/yellowness in a range between −60 and + 60).

High Performance Liquid Chromatography-Diode Array Detection (HPLC-DAD) Analyses

Analyses were carried out by using a Knauer (Asi Advanced Scientific Instruments, Berlin) system equipped with two pumps (Smartiline Pump 1000), a Rheodyne injection valve, a photodiode array detector uv/vis equipped with a semi-micro cell, and a Knauer RP C18 column (250 mm × 4.6 mm, 5 μm). The mobile phase was water/formic acid (solvent A: 99.9:0.1, v/v) and acetonitrile/formic acid (solvent B: 99.9:0.1, v/v) with the gradient profile of 0.01–20.00 min 5% B isocratic; 20.01–50 min, 5–40% B; 50.01–55 min, 40–95% B; 55.01–60 min 95% B isocratic. The flow rate was 1 mL/min. Peaks were monitored at 280 and 350 nm. Cyanidin-3-O-sambubioside, cyanidin-3-O-glucoside, quercetin, quercetin-3-O-rutinoside, astragalin, (+)-catechin and protocatechic acid were quantified. A calibration straight for each standard was obtained by analysing the standard solution diluted at

different concentrations. Identification and quantification were carried out based on recorded retention times in comparison with authentic standards. Analyses were performed in triplicate.

α-Amylase and α-Glucosidase Inhibitory Assay

The α-amylase inhibitory test was performed as previously described [20]. Concisely, α-amylase solution, starch solution, and colorimetric reagent were prepared. Control and samples (at concentrations in the range 0.01–1 mg/mL) were added to starch solution and left to react with the enzyme at room temperature for 5 min. The generation of maltose was quantified at 540 nm by the reduction of 3,5-dinitrosalicylic acid to 3-amino-5-nitrosalicylic acid. The α-glucosidase inhibition was measured as previously described [21]. Both control and samples (at concentrations in the range 0.01–1 mg/mL) were added to maltose solution and left to equilibrate at 37 °C. The reaction was started by adding the enzyme and left to incubate at 37 °C for 30 min. A perchloric acid solution was used to stop the reaction. The supernatant was collected and mixed with peroxidase/glucose oxidase and o-dianisidine and left to incubate for 30 min at 37 °C. The absorbance was measured at 500 nm. Acarbose was the positive control in both tests.

Radicals Scavenging Activity (ABTS and DPPH Assays)

ABTS test was used to investigate the anti-radical activity of S. nigra samples (concentrations range of 40–2.5 mg/mL) [22]. ABTS radical cation was produced by reacting 7 mM ABTS solution with 2.45 mM potassium persulphate and allowing the mixture to stand in the dark at 25 °C for 12 h before use. After addition of 25 μL of sample to 2 mL of diluted ABTS solution, absorbance at 734 nm was measured at 6 min. The antioxidant properties were assessed also by using DPPH assay [22]. A mixture of DPPH (0.25 mM in ethanol) and samples at different concentrations (5–1000 μg/mL) was prepared and left for 30 min at room temperature. The bleaching of DPPH was measured at 517 nm. The positive control was ascorbic acid.

Results and Discussion

Physico-Chemical Parameters and Chemical Profile of S. nigra Juice

S. nigra juice had a pH of 3.47 and showed a content of tartaric and citric acid of 1.04 and 19.86 g/L, respectively. The content of glucose and fructose was of 27.3 and 21.1 g/L, respectively (Table 2). The total soluble solids value was of 11.60 °Brix. Hunter colour values (L*, a* and b*) of the juice were measured. The brightness/whiteness colour coordinate L* was of 10.94; the redness (a*) and yellowness (b*) indicators were of 0.71 and 0.58, respectively (greenness/redness in a range between −60 and + 60). A chroma value (C*) of 0.92 was calculated.

By referring to the HPLC profile, cyanidin-3-O-sambubioside (300.7 mg/L), followed by cyanidin-3-O-glucoside (147.1 mg/L) and quercetin (142.9 mg/L), were the main constituents of untreated elderberry juice. These data are in agreement with those reported by Wu et al. [23] for the European elderberry variety. Other studies reported significant amounts of anthocyanins in elderberry juice [24,25,26]. In particular, cyanidin-3-O-sambubioside and cyanidin-3-O-glucoside represented over 83% of total anthocyanins in S. nigra juice [25]. In a previous work, Lee and Finn [27] indicated also quercetin-3-O-rutinoside as a major constituent of elderberry juice.

Water Permeability of Selected Membranes

Figure 1 shows the water permeability of selected membranes at 25 ± 1 °C. Among the selected membranes the ETNA 01PP showed the highest water permeability of about 15.5 L/m2 h. As expected, the NP010 membrane presented a higher water permeability when compared to that of NP030 being of the same material but with a higher molecular weight cut-off (1000 Da in comparison to 400 Da).

Evaluation of Permeate Flux

The performance of membrane separation processes is usually expressed in terms of two parameters: flux and rejection. Both parameters depend not only on the membrane properties but also on the

operational conditions [28]. The time evolution of the permeate flux measured in the treatment of the elderberry juice with the investigated membranes in selected operating conditions is illustrated in Fig. 2. A typical flux decay was observed due to concentration polarization and fouling phenomena until to reach a steady-state value. In particular, the Jp vs. time curve could be divided in two periods: an initial period characterized by a decrease of permeate flux, more significant for the Etna 01PP membrane; a second period in which the permeate flux reaches a steady-state value. Experimental results are in agreement with data observed for water permeability: the ETNA 01PP membrane exhibited the highest permeate flux with a steady-state value of about 6 L/m2 h, followed by the NP010 and NP030 membranes with steady-state values of 4 and 2 L/m2 h, respectively.

Characterization of Nanofiltered Juice

The analytical determination of organic acids (citric and tartaric acid) and sugars (glucose and fructose) in the feed and filtered juice samples indicated that these compounds are recovered in the permeate stream of the process (Table 2), as expected based on MWCO of selected membranes.

Table 3 shows the composition of the juice and permeate samples in terms of bioactive compounds determined according to HPLC measurements. All selected membranes showed high rejections towards anthocyanins (cyanidin-3-O-sambubioside and cyanidin-3-O-glucoside), quercetin-3-O-rutinoside and astragalin (higher than 75%) and lower rejections towards protocatechic acid and catechin (between 25 and 42%). The NP030 membrane, with the lowest MWCO, showed higher rejections for all bioactive compounds in comparison to the other two NF membranes (Fig. 3). For this membrane, anthocyanins and quercetin-3-O-rutinoside were completely rejected. NF membranes with MWCO of 1000 Da exhibited a similar selectivity towards investigated compounds indicating that rejections values were not affected by the polymeric nature of the membranes. According to these results, the selected membranes displayed a good separation efficiency of sugars and organic acids from phenolic compounds. High separation factors between sugars and phenolic compounds were

238 also observed by Conidi et al. [29] in the treatment of clarified pomegranate juice with thin-film
239 composite membranes having MWCO of 1000 and 2000 Da.
240
241 **In vitro Antioxidant and Hypoglycaemic Activity**
242 Negative impact of radicals on humans and animals is responsible for growing research studies in
243 antioxidant properties of compounds that could protect living organisms from the damaging influence
244 of these reactive species. Herein, the elderberry untreated juice as well as retentate and permeate
245 samples obtained with selected NF membranes were tested for their antioxidant potential by using
246 ABTS and DPPH assays. Retentate fractions showed a higher radical scavenging potential in
247 comparison to the untreated juice and permeate fractions (Table 4). In DPPH test, the retentate
248 obtained by membrane NP030 was the most active with an IC50 value of 0.3 mg/mL, followed by
249 retentates of ETNA01PP and NP010 membranes, with IC50 values of 0.5 mg/mL. The same trend
250 was observed in ABTS test: the retentate of NP030 membrane showed an IC50 value of 0.5 mg/mL,
251 followed by retentates of ETNA01PP and NP010 membranes with IC50 values of 0.7 and 0.8 mg/mL,
252 respectively
253 The potential hypoglycaemic properties were analysed by means of the inhibition of α-amylase and
254 α-glucosidase assays (Table 4). The most promising activity was found against α-glucosidase. In
255 particular, the permeate of NP010 membrane was the most active with an IC50 value of 0.21 mg/mL,
256 followed by NP030 permeate (IC50 of 0.3 mg/mL). Both permeates were more active than the
257 untreated juice (IC50 of 0.4 mg/mL) and retentates (IC50 of 0.7 and 0.6 mg/mL, respectively). The
258 best α-amilase inhibitory activity was observed for the NP030 permeate with an IC50 value of 0.5
259 mg/mL. A variety of phenolic compounds, identified in elderberry, are able to inhibit the activity of
260 α-amylase and α-glucosidase [30, 31]. Among them, cyanidin and its glycosides are considered
261 promising candidates for the use in the prevention of type 2 diabetes. Structure-activity relationship
262 of cyanidin and its glycosides against α-amylase and α-glucosidase evidenced that the presence of
263 glucose moiety at the 3-O-position of cyanidin increases the potency of pancreatic α-amylase

inhibition with IC50 values of 0.4 and 0.3 μM, respectively. Moreover, when cyanidin or cyanidin-3-O-glucoside at concentration of 1 μM were added to acarbose (3.12 μM) a synergistic effect against α-amylase activity was observed. A good activity was observed also for quercetin with IC50 values of 0.5 mM and 8 μM for α-amylase and α-glucosidase, respectively [31, 32]. These compounds are the most representative of our samples.

Conclusions

The radical scavenging activity and the carbohydrate hydrolysing enzymes inhibitory properties of elderberry juice treated by NF were investigated in relation to the phytochemical content. For this purpose, fractions obtained with three commercial NF membranes were characterized and compared with the untreated juice. All selected membranes showed high rejections towards anthocyanins, quercetin-3-O-rutinoside and astragalin and lower rejections towards protocatechuic acid and catechin. Retentate fractions exhibited a higher antioxidant activity in comparison to the untreated juice and permeate fractions with the NP030 membrane, characterized by the lowest MWCO, the most promising one. Permeate samples from the NP030 membrane exhibited also a higher hypoglycaemic activity in comparison to that of the untreated juice, as demonstrated by α-amylase and α-glucosidase inhibitory assays.

Abbreviations

ABTS: 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)

DPPH: 2,2-Diphenyl-1-picrylhydrazyl

IC50 : Half maximal inhibitory concentration

MWCO: Molecular weight cut-off

NF: Nanofiltration

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Table 1 Characteristics of selected flat-sheet membranes for the treatment of S. nigra juice

Membrane type	Etna 01 PP	NP010	NP030
Manufacturer	Alfa Laval	Mycrodin-Nadir	Mycrodin-Nadir
Membrane material	Composite fluoropolymer	Polyethersulphone	Polyethersulphone
Molecular weight cut-off (Da)	1000	1000	400
pH operating range	1–11	0–14	0–14
Max. operating temperature (°C)	60	95	70

Table 2 Analytical determination of sugars and organic acids in filtered samples

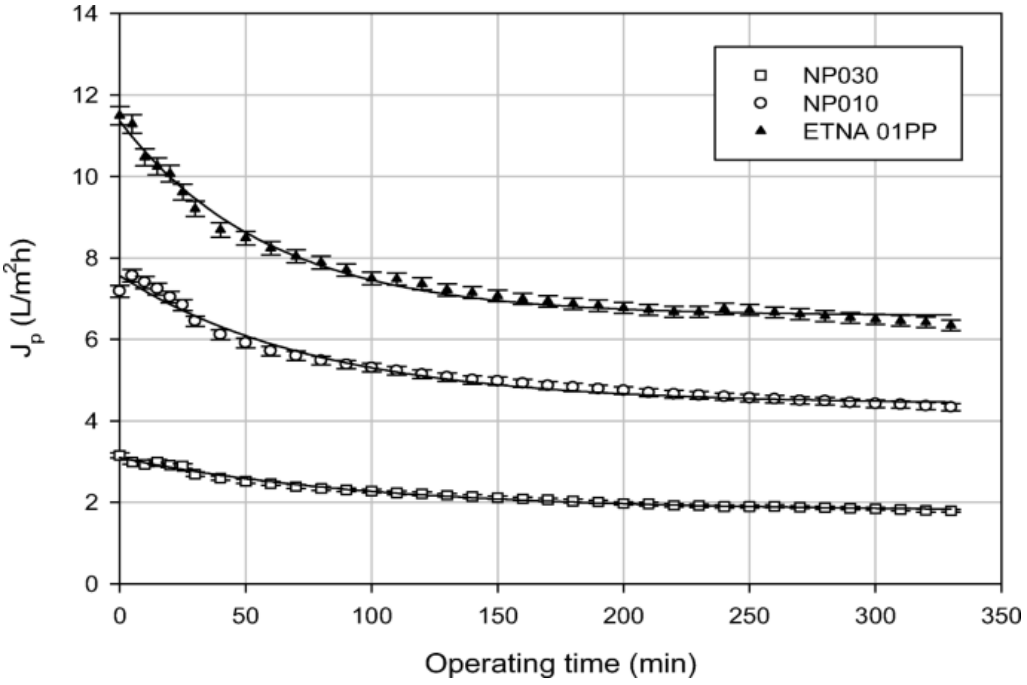
Compounds (g/L)	Feed	Permeate		387
		NP010	NP030	ETNA01PP
Tartaric acid	1.1 ± 0.1	2.1 ± 2.2	1.8 ± 0.6	2.3 ± 9.3
Citric acid	19.9 ± 1.1	19.1 ± 1.8	18.3 ± 1.1	19.7 ± 2.5
Glucose	27.3 ± 2.1	26.6 ± 2.3	25.1 ± 2.1	27.1 ± 2.8
Fructose	21.1 ± 2.0	20.1 ± 1.9	19.8 ± 2.0	20.9 ± 2.6

Table 3 HPLC profile of permeate samples from NF processing of elderberry juice

Compounds (mg/L)	Feed	Permeate		
		NP010	NP030	ETNA01PP
Cyanidin-3-O-sambubioside	300.7 ± 2.6	24.9 ± 0.8	ND	35.5 ± 0.8
Cyanidin-3-O-glucoside	147.1 ± 1.4	9.8 ± 0.9	ND	30.9 ± 0.7
Quercetin-3-O-rutinoside	18.2 ± 0.4	1.2 ± 0.2	ND	2.1 ± 0.1
Quercetin	142.9 ± 2.5	61.3 ± 1.5	47.3 ± 2.1	81.1 ± 2.7
Astragalin	6.47 ± 0.2	1.0 ± 0.1	0.5 ± 0.2	1.5 ± 0.2
(+)-Catechin	19.3 ± 0.9	12.0 ± 1.1	11.1 ± 0.3	14.6 ± 0.8
Protocatechic acid	38.0 ± 1.0	24.7 ± 1.4	21.4 ± 0.6	28.6 ± 0.9

Table 4. Antioxidant properties and inhibition of carbohydrates-hydrolysing enzymes of elderberry juice

S. nigra	ABTS test	DPPH test	α-Amylase inhibitory test	α-Glucosidase inhibitory test
Untreated juice	> 0.5	2.9 ± 0.8	0.6 ± 0.8	0.4 ± 0.8
R-ETNA01PP	0.7 ± 0.06	0.5 ± 0.06	NA	0.6 ± 0.7
R-NP010	0.8 ± 0.08	0.5 ± 0.05	0.9 ± 0.8	0.7 ± 0.9
R-NP030	0.5 ± 0.09	0.3 ± 0.02	NA	0.6 ± 0.8
P-ETNA01PP	> 0.5	> 1	0.8 ± 0.8	0.8 ± 0.5
P-NP010	> 0.5	> 1	NA	0.2 ± 0.2
P-NP030	> 0.5	> 1	0.5 ± 0.8	0.3 ± 0.3



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411 **Fig. 1.** Water permeability of selected membranes at 25 ± 1 °C
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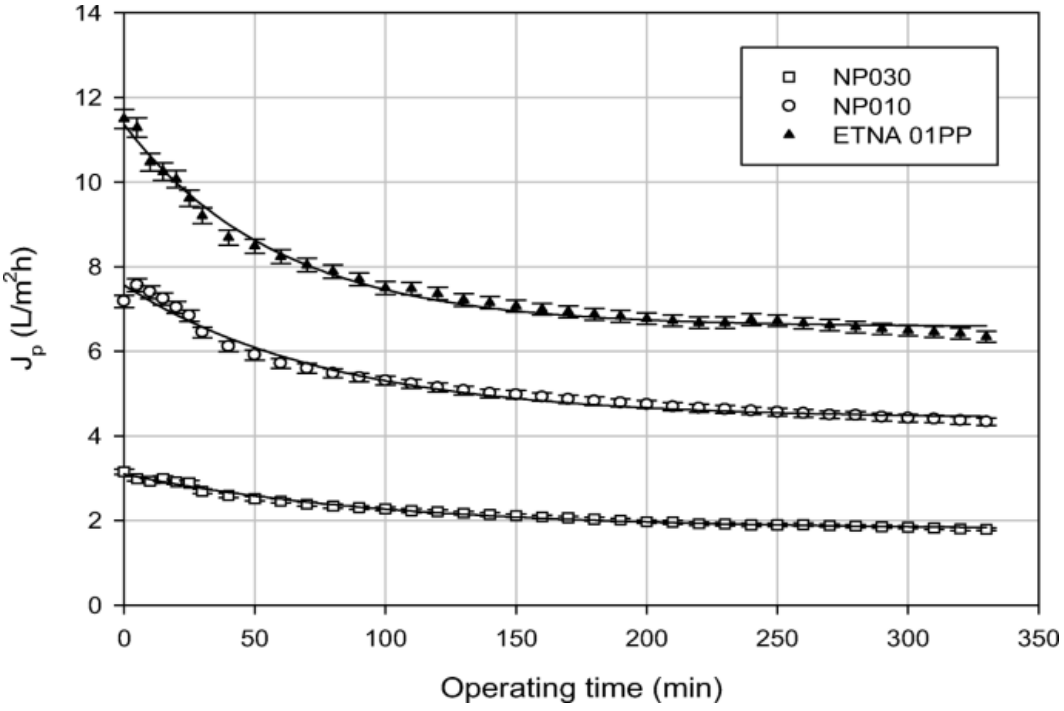

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416 **Fig. 2.** Treatment of S. nigra juice with selected membranes. Permeate flux as a function of operating **time**
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418

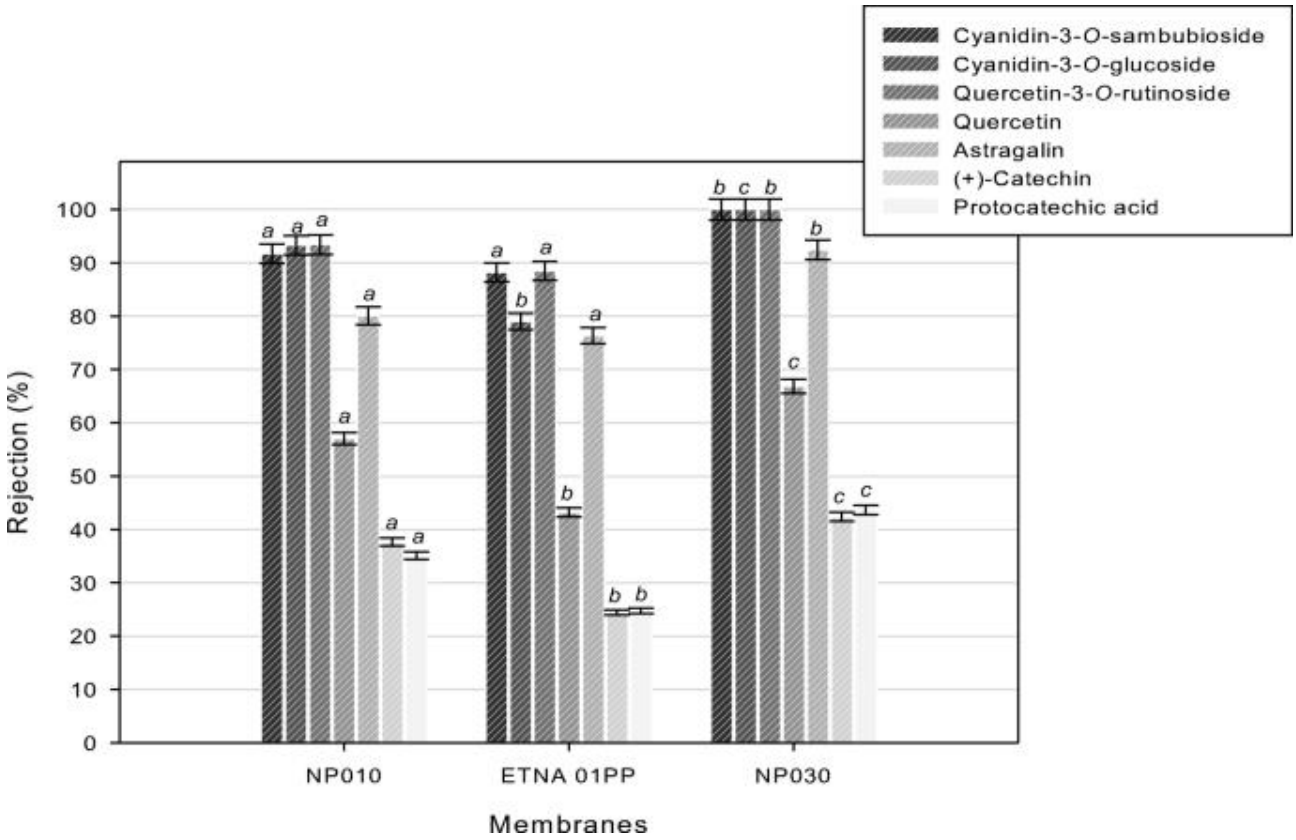

Fig. 3. Rejection of NF membranes towards bioactive compounds of S. nigra juice. Data are expressed as mean ± standard deviation (SD) (n = 3). For each specific compound, values for different membranes with different letters are significantly different at $p < 0.05$