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

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

32 Rosa Tundis<sup>1</sup>, Monica R. Loizzo<sup>1</sup>, Marco Bonesi<sup>1</sup>, Vincenzo Sicari<sup>2</sup>, Claudia Ursino<sup>3</sup>, Ilaria  
33 Manfredi<sup>3</sup>, Carmela Conidi<sup>3</sup>, Alberto Figoli<sup>3</sup> & Alfredo Cassano<sup>3</sup>

34

35 \* Monica R. Loizzo [monica\\_rosa.loizzo@unical.it](mailto:monica_rosa.loizzo@unical.it)

36 \* Alberto Figoli [a.figoli@itm.cnr.it](mailto:a.figoli@itm.cnr.it)

37 1 Department of Pharmacy, Health and Nutritional Sciences, University of Calabria, 87036 Rende  
38 (CS), Italy

39 2 Department of Agricultural Science, Mediterranean University of Reggio Calabria, Feo di Vito,  
40 89123 Reggio Calabria, Italy

41 3 Italian National Research Council (ITM-CNR), Institute on Membrane Technology, via Pietro  
42 Bucci, 17/C, 87036 Rende (CS), Italy

43

44 **Abstract**

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

55

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

58

## 59 **Introduction**

60 Sambucus nigra L. (elderberry) is a widespread plant of the Caprifoliaceae family growing in Europe,  
61 North Africa, West Asia, and USA [1]. S. nigra berries or juices are used in the processing of jellies  
62 and jams, and in the preparation of pies and liqueurs. Berries have been utilised also as dietary  
63 supplements worldwide. All plant parts have been used in traditional medicine for their purgative,  
64 diuretic, haemostatic effects as well as for the prevention of diabetes, cardiovascular diseases, and  
65 cancer [2, 3]. The health benefits of S. nigra can be attributed to its characteristic chemical profile,  
66 which includes anthocyanins, flavonols, flavanones, and flavones as the main secondary metabolites  
67 [4]. Elderberry constituents demonstrated interesting antioxidant, anti-bacterial, anti-inflammatory,  
68 anti-viral, and anti-allergic properties in both in vitro and in vivo studies [5,6,7,8]. Several studies  
69 reported the beneficial role of anthocyanins-rich plants in diabetes due to their capacity to reduce  
70 hyperglycaemia-induced oxidative stress and stimulate insulin secretion [9]. A therapeutic approach  
71 to treat diabetes is to decrease post-prandial hyperglycaemia by the inhibition of carbohydrates-  
72 hydrolysing enzymes such as  $\alpha$ -amylase and  $\alpha$ -glucosidase [10].

73

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

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

100

## 101 **Materials and Methods**

### 102 *Chemicals and Reagents*

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

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

110

### 111 **Preparation of Raw Juice**

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

118

### 119 **Membranes and Nanofiltration Set-Up**

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

$$128 \mathbf{J_p = V_p A \cdot \Delta t}$$

129 where  $V_p$  (L) is the volume of permeate,  $A$  (m<sup>2</sup>) is the membrane area, and  $\Delta t$  (h) is the operation  
130 time. The average and relative standard deviation were calculated. Three different commercial flat-  
131 sheet membranes namely Etna 01PP (from Alfa Laval, Lund, Sweden), NP010 and NP030 (from  
132 Mycrodin-Nadir, Wiesbaden, Germany) were used for the treatment of the juice. Their typical  
133 characteristics according to the manufacturers' data sheet are reported in Table 1.

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

$$135 R=(1-C_pC_f)\cdot 100$$

136 where  $C_f$  and  $C_p$  are the feed and permeate concentration, respectively.

137

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

141

### 142 **Physico-Chemical Parameters of Elderberry Juice**

143 The total soluble solids were estimated by a digital refractometer PR-201 $\alpha$  (Atago, Tokyo, Japan) and  
144 expressed as  $^{\circ}$ Brix (sucrose percentage) at 20  $^{\circ}$ C. The pH of *S. nigra* juice was measured by a pH  
145 meter (Basic Model 20, Crison). Fresh-juice colour was measured using a Konica Minolta CM-  
146 700/600d spectrophotometer (Konica Minolta Sensing, Japan) at 25  $^{\circ}$ C. Data were expressed as  $L^*$   
147 (lightness/darkness in a range 0–100),  $a^*$  (greenness/redness in a range between –60 and + 60) and  
148  $b^*$  (blueness/yellowness in a range between –60 and + 60).

149

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

151 Analyses were carried out by using a Knauer (Asi Advanced Scientific Instruments, Berlin) system  
152 equipped with two pumps (Smartiline Pump 1000), a Rheodyne injection valve, a photodiode array  
153 detector uv/vis equipped with a semi-micro cell, and a Knauer RP C18 column (250 mm  $\times$  4.6 mm,  
154 5  $\mu$ m). The mobile phase was water/formic acid (solvent A: 99.9:0.1, v/v) and acetonitrile/formic acid  
155 (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,  
156 5–40% B; 50.01–55 min, 40–95% B; 55.01–60 min 95% B isocratic. The flow rate was 1 mL/min.  
157 Peaks were monitored at 280 and 350 nm. Cyanidin-3-O-sambubioside, cyanidin-3-O-glucoside,  
158 quercetin, quercetin-3-O-rutinoside, astragalín, (+)-catechin and protocatechic acid were quantified.  
159 A calibration straight for each standard was obtained by analysing the standard solution diluted at

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

162

### 163 **$\alpha$ -Amylase and $\alpha$ -Glucosidase Inhibitory Assay**

164 The  $\alpha$ -amylase inhibitory test was performed as previously described [20]. Concisely,  $\alpha$ -amylase  
165 solution, starch solution, and colorimetric reagent were prepared. Control and samples (at  
166 concentrations in the range 0.01–1 mg/mL) were added to starch solution and left to react with the  
167 enzyme at room temperature for 5 min. The generation of maltose was quantified at 540 nm by the  
168 reduction of 3,5-dinitrosalicylic acid to 3-amino-5-nitrosalicylic acid. The  $\alpha$ -glucosidase inhibition  
169 was measured as previously described [21]. Both control and samples (at concentrations in the range  
170 0.01–1 mg/mL) were added to maltose solution and left to equilibrate at 37 °C. The reaction was  
171 started by adding the enzyme and left to incubate at 37 °C for 30 min. A perchloric acid solution was  
172 used to stop the reaction. The supernatant was collected and mixed with peroxidase/glucose oxidase  
173 and o-dianisidine and left to incubate for 30 min at 37 °C. The absorbance was measured at 500 nm.  
174 Acarbose was the positive control in both tests.

175

### 176 **Radicals Scavenging Activity (ABTS and DPPH Assays)**

177 ABTS test was used to investigate the anti-radical activity of *S. nigra* samples (concentrations range  
178 of 40–2.5 mg/mL) [22]. ABTS radical cation was produced by reacting 7 mM ABTS solution with  
179 2.45 mM potassium persulphate and allowing the mixture to stand in the dark at 25 °C for 12 h before  
180 use. After addition of 25  $\mu$ L of sample to 2 mL of diluted ABTS solution, absorbance at 734 nm was  
181 measured at 6 min. The antioxidant properties were assessed also by using DPPH assay [22]. A  
182 mixture of DPPH (0.25 mM in ethanol) and samples at different concentrations (5–1000  $\mu$ g/mL) was  
183 prepared and left for 30 min at room temperature. The bleaching of DPPH was measured at 517 nm.  
184 The positive control was ascorbic acid.

185

## 186 **Results and Discussion**

### 187 *Physico-Chemical Parameters and Chemical Profile of S. nigra Juice*

188 *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,  
189 respectively. The content of glucose and fructose was of 27.3 and 21.1 g/L, respectively (Table 2).  
190 The total soluble solids value was of 11.60 °Brix. Hunter colour values (L\*, a\* and b\*) of the juice  
191 were measured. The brightness/whiteness colour coordinate L\* was of 10.94; the redness (a\*) and  
192 yellowness (b\*) indicators were of 0.71 and 0.58, respectively (greenness/redness in a range between  
193 -60 and + 60). A chroma value (C\*) of 0.92 was calculated.

194 By referring to the HPLC profile, cyanidin-3-O-sambubioside (300.7 mg/L), followed by cyanidin-  
195 3-O-glucoside (147.1 mg/L) and quercetin (142.9 mg/L), were the main constituents of untreated  
196 elderberry juice. These data are in agreement with those reported by Wu et al. [23] for the European  
197 elderberry variety. Other studies reported significant amounts of anthocyanins in elderberry juice  
198 [24,25,26]. In particular, cyanidin-3-O-sambubioside and cyanidin-3-O-glucoside represented over  
199 83% of total anthocyanins in *S. nigra* juice [25]. In a previous work, Lee and Finn [27] indicated also  
200 quercetin-3-O-rutinoside as a major constituent of elderberry juice.

201

### 202 **Water Permeability of Selected Membranes**

203 Figure 1 shows the water permeability of selected membranes at  $25 \pm 1$  °C. Among the selected  
204 membranes the ETNA 01PP showed the highest water permeability of about 15.5 L/m<sup>2</sup> h. As  
205 expected, the NP010 membrane presented a higher water permeability when compared to that of  
206 NP030 being of the same material but with a higher molecular weight cut-off (1000 Da in comparison  
207 to 400 Da).

208

### 209 **Evaluation of Permeate Flux**

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

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

221

## 222 **Characterization of Nanofiltered Juice**

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

226

227 Table 3 shows the composition of the juice and permeate samples in terms of bioactive compounds  
228 determined according to HPLC measurements. All selected membranes showed high rejections  
229 towards anthocyanins (cyanidin-3-O-sambubioside and cyanidin-3-O-glucoside), quercetin-3-O-  
230 rutinoside and astragalin (higher than 75%) and lower rejections towards protocatechic acid and  
231 catechin (between 25 and 42%). The NP030 membrane, with the lowest MWCO, showed higher  
232 rejections for all bioactive compounds in comparison to the other two NF membranes (Fig. 3). For  
233 this membrane, anthocyanins and quercetin-3-O-rutinoside were completely rejected. NF membranes  
234 with MWCO of 1000 Da exhibited a similar selectivity towards investigated compounds indicating  
235 that rejections values were not affected by the polymeric nature of the membranes. According to these  
236 results, the selected membranes displayed a good separation efficiency of sugars and organic acids  
237 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 IC<sub>50</sub> value of 0.3 mg/mL, followed by  
249 retentates of ETNA01PP and NP010 membranes, with IC<sub>50</sub> values of 0.5 mg/mL. The same trend  
250 was observed in ABTS test: the retentate of NP030 membrane showed an IC<sub>50</sub> value of 0.5 mg/mL,  
251 followed by retentates of ETNA01PP and NP010 membranes with IC<sub>50</sub> values of 0.7 and 0.8 mg/mL,  
252 respectively

253 The potential hypoglycaemic properties were analysed by means of the inhibition of  $\alpha$ -amylase and  
254  $\alpha$ -glucosidase assays (Table 4). The most promising activity was found against  $\alpha$ -glucosidase. In  
255 particular, the permeate of NP010 membrane was the most active with an IC<sub>50</sub> value of 0.21 mg/mL,  
256 followed by NP030 permeate (IC<sub>50</sub> of 0.3 mg/mL). Both permeates were more active than the  
257 untreated juice (IC<sub>50</sub> of 0.4 mg/mL) and retentates (IC<sub>50</sub> of 0.7 and 0.6 mg/mL, respectively). The  
258 best  $\alpha$ -amilase inhibitory activity was observed for the NP030 permeate with an IC<sub>50</sub> value of 0.5  
259 mg/mL. A variety of phenolic compounds, identified in elderberry, are able to inhibit the activity of  
260  $\alpha$ -amylase and  $\alpha$ -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  $\alpha$ -amylase and  $\alpha$ -glucosidase evidenced that the presence of  
263 glucose moiety at the 3-O-position of cyanidin increases the potency of pancreatic  $\alpha$ -amylase

264 inhibition with IC<sub>50</sub> values of 0.4 and 0.3 μM, respectively. Moreover, when cyanidin or cyanidin-  
265 3-O-glucoside at concentration of 1 μM were added to acarbose (3.12 μM) a synergistic effect against  
266 α-amylase activity was observed. A good activity was observed also for quercetin with IC<sub>50</sub> values  
267 of 0.5 mM and 8 μM for α-amylase and α-glucosidase, respectively [31, 32]. These compounds are  
268 the most representative of our samples.

269

## 270 **Conclusions**

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

281

## 282 **Abbreviations**

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

284 DPPH: 2,2-Diphenyl-1-picrylhydrazyl

285 IC<sub>50</sub> : Half maximal inhibitory concentration

286 MWCO: Molecular weight cut-off

287 NF: Nanofiltration

288

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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

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**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

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**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
Astragalín	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

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

S. nigra	ABTS test	DPPH test	$\alpha$ -Amylase inhibitory test	$\alpha$ -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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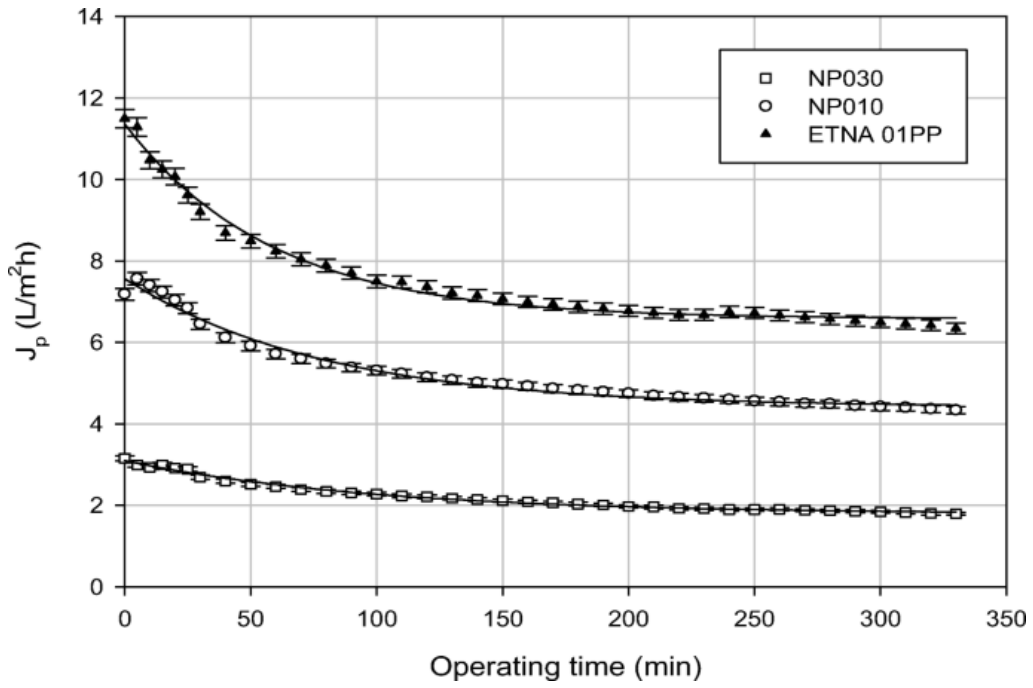


Fig. 1. Water permeability of selected membranes at 25 ± 1 °C

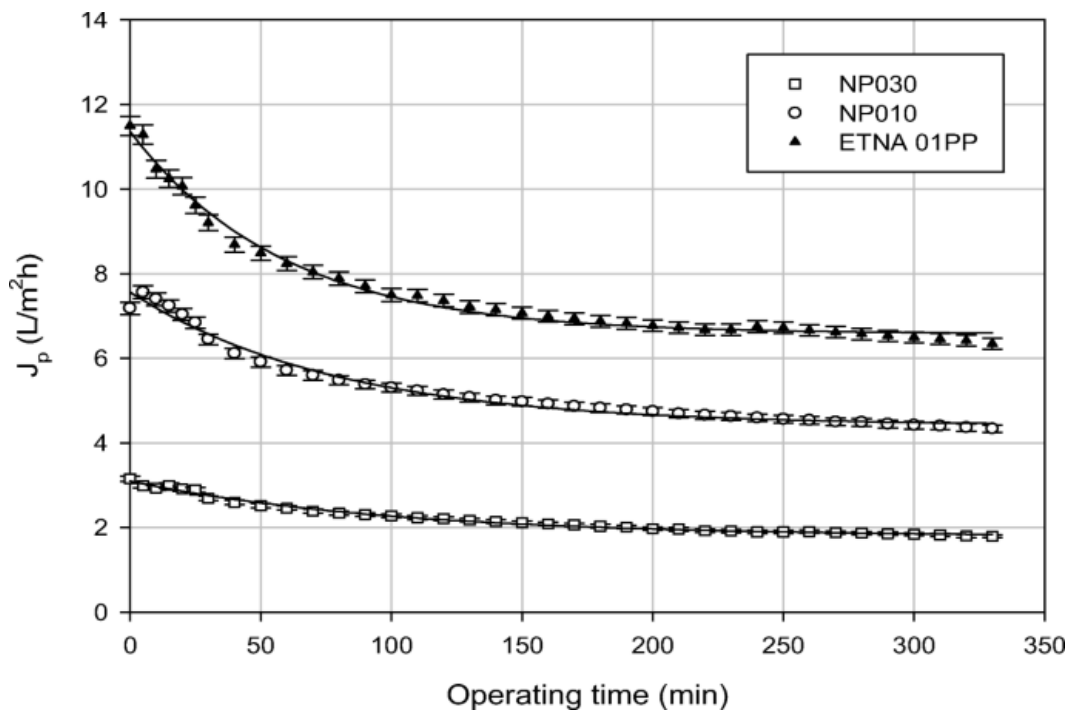
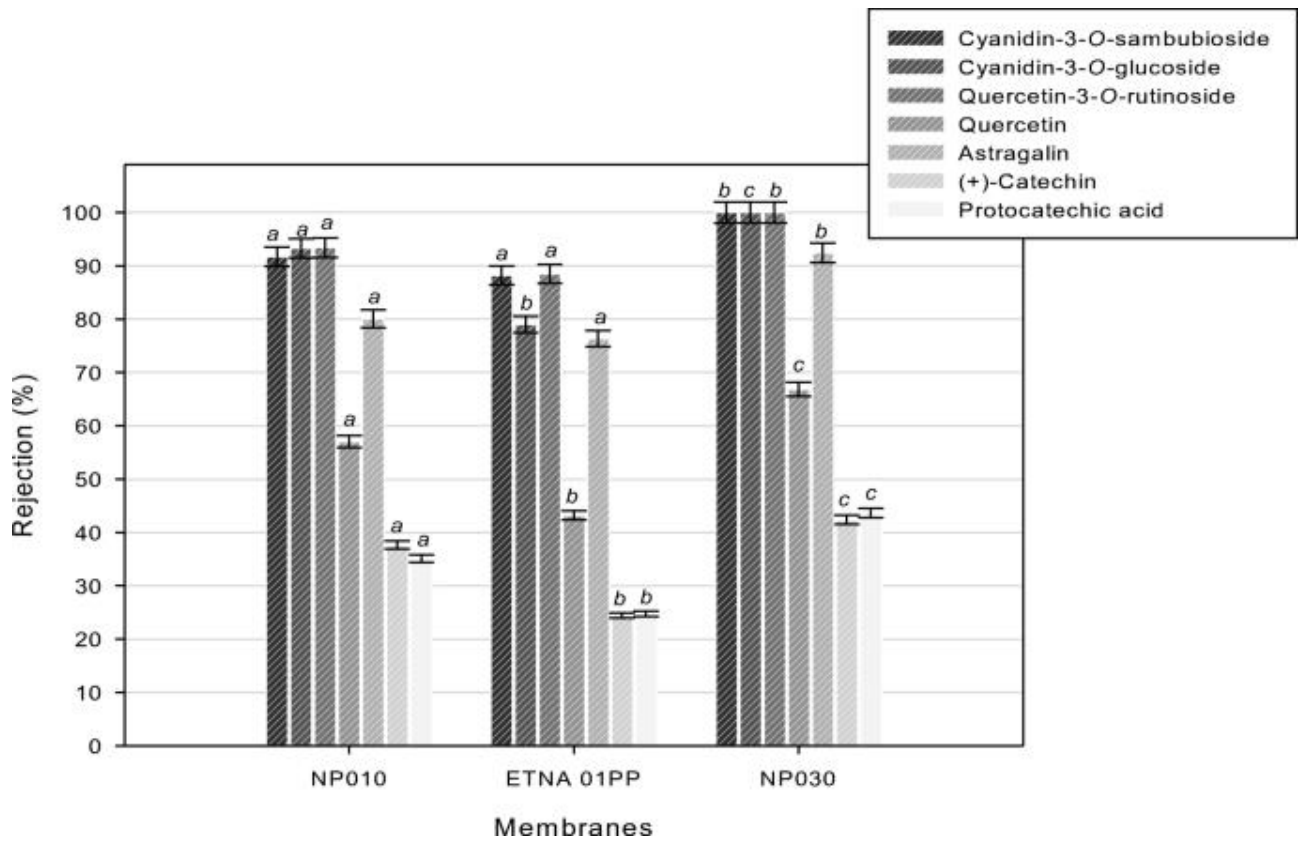


Fig. 2. Treatment of *S. nigra* juice with selected membranes. Permeate flux as a function of operating time

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