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Fractionation of Biologically Active Compounds Extracted from Propolis by Nanofiltration

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Abstract

Fractionation of biologically active compounds (BAC) extracted from propolis was performed by nanofiltration. Direct nanofiltration with the feed solution was applied, as well as sequential filtration of each permeates; Duramem membranes with different molecular weight cut-off (MWCO), ranging from 300 to 900Da were used. During each filtration a constant flux was observed. When direct filtration of the raw extract was applied the measured flux was about 12% lower, but no fouling of the membrane was observed. The dependence of rejection on the MWCO of the membrane was obtained.

The free radical scavenging activity of the feed, permeates and retentates as well as the kinetics of inhibition of free radicals were studied by DPPH test. Fractions of different composition were obtained, characterized by the relative content of flavonoids in the total phenols (0.08 to 0.21). They have shown very different antioxidant activity, ranging from 19 to 98% and proportional to the flavonoids content.

Keywords: Propolis; Biologically active compounds; Nanofiltration.

Introduction

Propolis (Apis mellifera) is a resinous material that is collected by honeybees from buds, leaves, bark, and exudates of several trees and plants1. [2] Propolis is composed of 45% resins, 30% waxes and fatty acids, 10% essential oils, 5% pollens and 10% organic compounds and minerals. [3,4]. Wax and organic debris are removed during processing, usually by ethanolic extraction, and the propolis tincture (balsam) thus obtained, contains the bulk of propolis biologically active compounds (BAC). This natural product has a long history of use in traditional medicine dating back at least to 300 BC, because it possesses a broad spectrum of biological activities including antibacterial, antifungal, antiprotozoan, antiviral, immunomodulatory, anti-inflammatory and antitumor activity [5-9].

Moreover, in the last decades the interest of propolis is increased due to its high antioxidant activity (AA). Antioxidants play a very important role in the body defense system against reactive oxygen species (ROS). The ROS are the harmful byproducts generated during normal cell aerobic respiration. In addition, different environmental stress factors such as pollution, drought, temperature, excessive light intensities and nutritional limitation are able to increase the production of ROS. [6]. The AAs are associated with the phenolic constituents, especially flavonoids and phenolic acids [7]. Many protective effects, related to the antioxidant activity of propolis, including effects against doxorubicin-induced myocardiopathy [8], carbon tetrachloride-induced liver damage [9], galactosamine-induced hepatitis, and γ-irradiation [10] were demonstrated.

Propolis antioxidant, antibacterial and antifungal properties, combined with the fact that several of its constituents are present in food and/or food additives, specified as GRAS (Generally Recognised as Safe), make it an attractive candidate as a natural preservative in new food applications. This meets the demand for natural antioxidants and antimicrobials, fuelled by the increasing consumer awareness for natural, minimally processed foods with traditional preservatives absent or at very low concentrations. [11] As a result of this wide range of biological activities, propolis is extensively used in the food industry such as beverages, health foods, nutritional supplements and cosmetic applications, such as dental hygiene products, wound healing creams, antibacterial soaps, immune system booster supplements as well as anti-rheumatic preparations[12].

More than 300 BAC have been identified in propolis sample, which indicated propolis was potential for new drugs. However, due to the complexity and variety of active constituents in propolis depending on its geographical and botanical origins, it has not been easy to separate/purify the functional components from propolis, and only several active components against clinical pathogens have been identified [13]. In order to understand how chemical structure, molecular weight, etc. of BAC, particularly flavonoids and polyphenols, influence on AA, their separation by fractionation using cheap and easy methods is required.

Traditional approaches, including simple steam distillation and vacuum distillation, are adopted to yield fractions. Generally these methods require an increased temperature and high energy consumption. The first is inappropriate for heat-sensitive products. These methods may also result in a loss of compounds of low molecular weight, which can be removed together with the solvent during evaporation [14].

The concentration of BAC from propolis by nanofiltration was recently reported [18,15] as an advantageous alternative to above mentioned separation methods. [16] Dead-end [18] and cross-flow [19] nanofiltration was used for concentrating the extracts from propolis. In both articles ethanol and ethanol-water solvent was used and the concentration of extracted polyphenols and flavonoids was followed.

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The complex composition of the phenolic fraction (pinocembrin, pinobanksin, caffeic acid, p-coumaric acid, quercetin, chrysin etc. [17]) is characterized by molecular weights ranging from about 180 to 410. Duramem membrane (MWCO 200Da) showed practically complete rejection of the BAC, contained in the water-ethanolic extract (total phenols, flavones and flavonols, flavonones and dihydroflavonols). [18] Fractionation of BAC from propolis by nanofiltration is not reported.

DPPH assay is routinely practiced for assessment of free radical scavenging potential of an antioxidant molecule and considered as one of the standard and easy colorimetric methods for the evaluation of antioxidant properties of pure compounds. In these methods, expensive reagents or sophisticated instrumentation are not required,

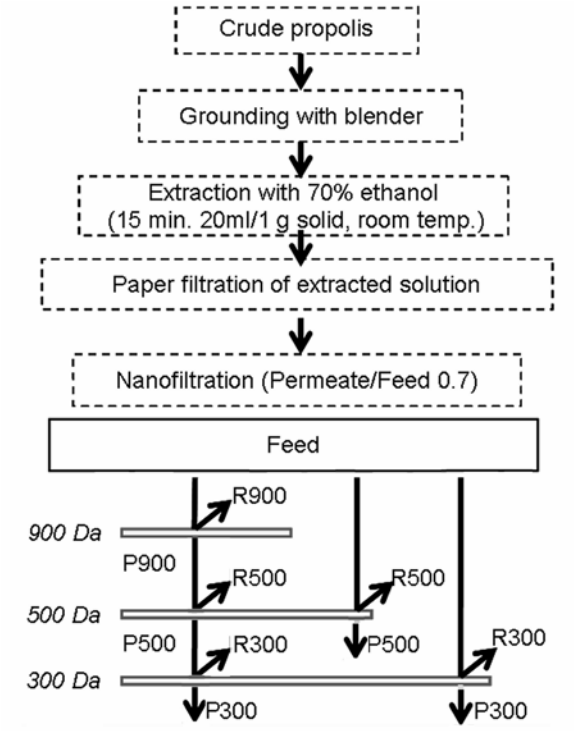

Figure 1: Schematic representation of the nanofiltration experiments.

Membrane	Feed, used for nanofiltration	Fraction	Antioxidant activity [%] of diluted fraction 1/20 (V/V)	Flavonoids [mg/ml]±sd*	Total Phenols [mg/ml]±sd*	Rejection of flavonoids [%]	Rejection of total phenols [%]	Degree of concentration of flavonoids [%]	Degree of concentration of total phenols [%]
		Extract	93	3.078±0.005	17.821±0.055				
Duramem™ 900 (MWCO 900 Da)	Extract	Retentate (R900)	94	4.468±0.003	23.356±0.068	34	31	145	131
		Permeate (P900)	76	2.030±0.003	12.308±0.009				
Duramem™ 500 (MWCO 500 Da)	Permeate from Duramem™ 900	Retentate (R500)	91	4.020±0.008	20.935±0.046	65	53	198	170
		Permeate (P500)	38	0.720±0.002	5.838±0.023				
	Extract	Retentate (R500)	97	7.180±0.013	33.994±0.023	77	68	233	191
		Permeate (P500)	39	0.709±0.001	5.762±0.011				
Duramem™ 300 (MWCO 300 Da)	Permeate from Duramem™ 500	Retentate (R300)	48	1.474±0.001	10.796±0.023	70	59	204	185
		Permeate (P300)	19	0.216±0.001	2.395±0.005				
	Extract	Retentate (R300)	98	8.034±0.025	37.636±0.046	93	87	261	210
		Permeate (P300)	19	0.207±0.001	2.402±0.023				

Table 1:

the reduction of DPPH radical by radical scavengers is evaluated spectrophotometrically by monitoring the decrease in absorbance at 517 nm, as the DPPH radical is decolorised from deep violet to pale yellow [18].

The object of the present investigation is the fractionation of biologically active compounds with different MW, extracted from propolis into ethanol-water solvent by nanofiltration, as well as determination of the antioxidant activity of the different fractions after nanofiltration.

Experimental

Propolis was provided by the Centre of Phytochemistry of the Institute of Organic Chemistry, BAS, (Bulgaria); Ethanol (99,9%) and Methanol (99,9%) were supplied by Valerus (Bulgaria); Aluminium chloride anhydrous, Potassium hydroxide (ISO), Sodium carbonate anhydrous (ISO), Sulfuric acid (96%), Folin-Ciocalteu's phenolic reagent and Methanol Lichrosolv (99,8%), were supplied by Merck; Galangin was supplied by Fluka and 2,2-Diphenyl-1-picrylhydrazyl (free radical DPPH, 95%) was supplied by Alfa Aesar.

Modified polyimide flat sheet Duramem™ nanofiltration membranes with Molecular Weight Cut-Off (MWCO) from 200 to 900 Da were supplied by Evonik Membrane Extraction Technology Ltd, UK.

Propolis (cooled at 5°C) was ground before extraction. Extraction was carried out with 70% (v/v) EtOH-water solvent at room temperature and liquid to solid ratio (20 ml liquid/g solid) for 15 minutes.

UV-VIS analysis of BAC concentration in the extract and antioxidant activity was performed on a Hexiosy v 7.06 spectrophotometer. Every assay was carried out in triplicate.

Flavonoid substances (flavones and flavonols) were measured by spectrophotometric assay based on aluminum chloride complex formation. [19] Flavonoids content was estimated using a calibration curve of galangin, concentration range of 0.0052–0.052 mg/ml [18].

Total phenolic substances were measured by the Folin– Ciocalteu's method. [20] Total phenolics content was estimated using calibration curve of standard mixture pinocembrin–galangin 2:1, concentration range 0.025–0.3 mg/ml [18].

The free radical scavenging activity of the feed, permeates and retentates as well as the kinetics of inhibition of free radicals were studied by DPPH test. [20] The values of $[DPPH]_t$ at each reaction time were calculated according to the calibration curve, determined by least square method in the concentration range of 3.75 to 25 μg/ml): Abs= 2740 $[DPPH]_t$ – 0.0036 (R=0.999), where the concentration $[DPPH]_t$ is expressed in mg/ml. The radical scavenging activity (RSA) was calculated as [21]:

$$RSA = \frac{[DPPH]_0 - [DPPH]_{20}}{[DPPH]_0} \cdot 100\% \quad (1)$$

where $[DPPH]_0$ is the concentration of the DPPH· solution (without sample) at t=0 and $[DPPH]_{20}$ is the remained DPPH·concentration at t=20 min. Lower $[DPPH]_t$ in the reaction mixture indicates higher free radical scavenging activity.

Membrane stirred cell and set-up A 270 ml stirred cell, Evonic Membrane Extraction Technology Ltd, UK was used to conduct the dead-end nanofiltration experiments: effective membrane area was 54 cm^2; operating pressure was 10 bar; permeate to feed ratio was kept constant and equal to 0.7. The nanofiltration experiments were

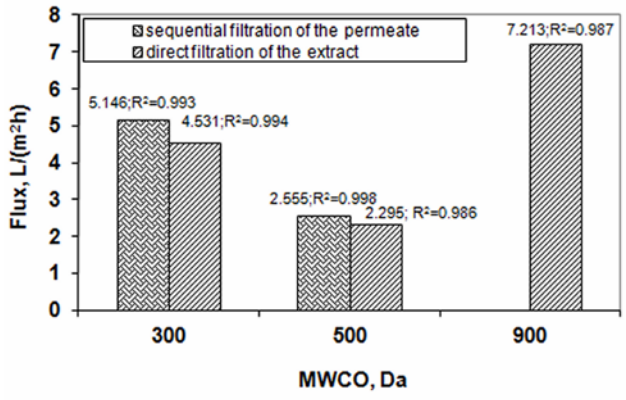

Figure 2: Flux vs MWCO of the membranes.

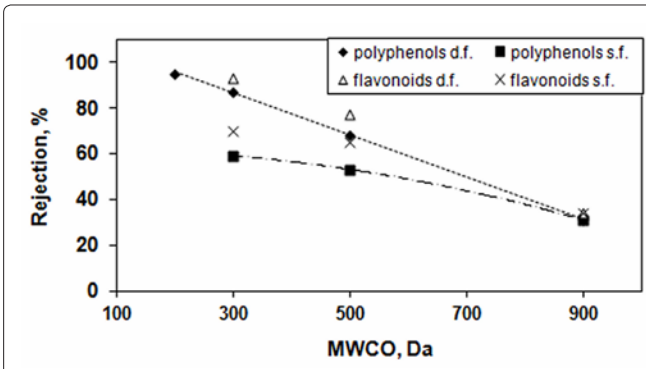

Figure 3: Rejection of BCA as a function of MWCO of the membrane (d.f. - direct filtration of raw extract; s.f. - sequential filtration of permeates).

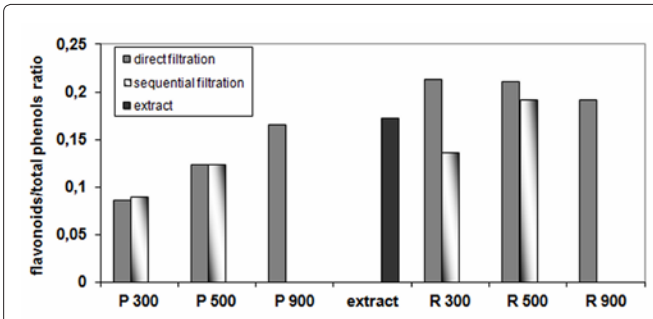

Figure 4: Flavonoids/polyphenols ratio in the fractions in relation to the MWCO.

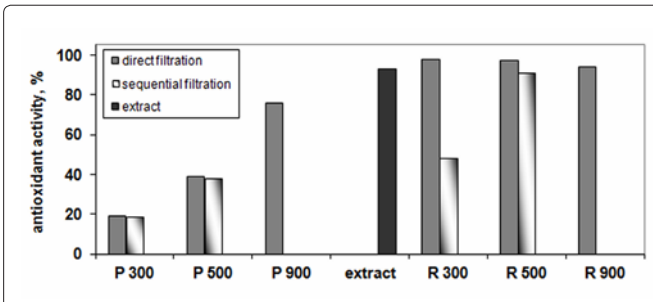

Figure 5: Antioxidant activity of the fractions in relation to the MWCO.

conducted at room temperature.

The rejection (R) was calculated using Eq.(2) where C_f, and C_p are the feed and permeate concentrations of the respective biologically active compounds.

$$R = \frac{C_f - C_p}{C_f} \cdot 100\% \quad (2)$$

The degree of concentration was calculated from the ratio $\frac{C_r}{C_f}$, as measured from the experiment.

Results and Discussion

The chemical composition of BAC in the feed, obtained as an average from 4 parallel runs by extracting at liquid/solid ratio 20 ml/g was: total phenolic compounds 385±4.92; total flavonoids 70±1.13 mg/g propolis. The separation of the obtained BAC was done by nanofiltration with Duramem membranes with different MWCO (from 300 to 900Da) and characterized by the flavonoids/polyphenols ratio in the different fractions. Direct nanofiltration of the extract was applied, as well as sequential filtration of each permeates, as shown in (Figure 1).

Permeate flux was calculated from the linear plot of the cumulative volume versus time. During each filtration a constant flux was observed. The measured values together with the coefficients of linear regression are shown in (Figure 2). As can be seen, the permeate flux was lower in the case of direct extract filtration, probably due to the higher feed concentration. No fouling of the membranes was observed. Details of the nanofiltration are given in Table 1.

The dependence of the experimental rejections on the MWCO of the membrane is illustrated in (Figure 3). Higher values for flavonoids rejection in comparison to polyphenols are observed. The difference in the rejection - MWCO dependence for the two sets of experiments is due to the different range of feed concentrations. In the case of constant feed concentration (direct filtration of the extract) a nearly linear relation is observed. When extended to include the previously reported rejection value for Duramem 200 [1], the agreement is also very good. By sequential filtration of each permeates rejections are obtained for decreasing (more than 3 times) feed concentrations (see Table 1), which considerably affect the observed dependence rejection-MWCO.

The membrane separation was characterized by the relative content of flavonoids in total phenols in the respective fraction. Illustration is given in Figure 4, where the flavonoids/polyphenols ratio is presented in function of the MWCO. As can be seen, fractions with different content of flavonoids are obtained, ranging from 0.08 to 0.22. Compared to the extract, the content on flavonoids is enriched in the retentates, and decreased in the permeates. By sequential filtration retentates with considerably different content of flavonoids are obtained (see R300 and R500). The different composition of the respective fractions appears also in their antioxidant activities, illustrated in (Figure 5). The relation between the flavonoids content and the antioxidant activity is obvious, when both (Figure 4) and (Figure 5) are compared. The measured different antioxidant activities (between 19 and 98%) are proportional to the flavonoids content of the fraction.

Conclusions

The fractionation of the BAC from propolis by nanofiltration gives rejections for total polyphenols and flavonoids, ranging from over 30% (with 900Da) up to 94% (with 300Da membrane). Constant flux and no fouling of the membranes are observed. Fractions of different composition are obtained, characterised by the relative content of flavonoids in the total phenols (0.08 to 0.21). They have shown different antioxidant activity, ranging from 19 to 98% and proportional to the flavonoids content. Based on obtained results nanofiltration processes in authors opinion could be consider as a new, cheap, easy method for new drugs separation from extracts obtained from natural products.

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