

Effect of Nanofiltration on the Antioxidant Properties of *Crataegus monogyna* Curative Extracts*

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Abstract. Treatment of pathological conditions with medicinal and aromatic plants is an ancient approach of treatment and prevention. Basis of a properly conducted therapy is the effective component and precise dosage. To realise the therapeutic properties, in many cases separation and concentration of the active ingredients are essential steps. We chose *Crataegus monogyna* (“hawthorn”) as one of the most common species used in traditional herbalism, which is of considerable interest as a natural sedative and for treating cardiac ailments. The plant parts used medicinally are usually sprigs with both leaves and flowers, or alternatively the fruit. Both of them showed substantial content of antioxidant phytochemicals, although quantification of total phenolic/reducers content (TPC) and radical scavenging ability in parallel expressed significant prevalence of “leaves with flowers” extract vs. “fruits” one. Membrane concentration was shown an effective means for direct increase of bioactive components. A strong correlation between antiradical capacities of the extracts and their TPC was established.

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1 Introduction

Curative properties of the medicinal plants attract vast attention throughout the whole historic period, since the advent of mankind.

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Wide variety of approximately 4000 plant species in different communities and habitats can be met in Bulgaria, favoured by varied topography, geology and soils, specific microclimate conditions and millennia of human activity in the Balkans. About 770 species or 19% of all plants in the country are healing; most of them, about 760 species are wild. About 250 of them are used in large quantities for trading and processing. Others are still not subject to economic interest for the time being, but there is enough scientific data and practical evidence on their healing effectiveness [1-2].

Besides, Bulgaria is among the leaders in Europe on the export of unprocessed medicinal and aromatic plants. About 200 herbal products of 140 plant species are subject to export. About 13,000–15,000 tons of Bulgarian herbs are exported annually: to Germany 65%, Spain 10%, Italy 5%, France 5%, other countries 15%. Additionally, about 2000-3000 tons of medicinal and aromatic plants are processed or consumed in Bulgaria each year, with around 300,000 people employed in the collection and processing of those herbs [3-4].

A relatively broad range of medicinal plants and related products has been studied for antioxidant activity (AOA), in particular total polyphenolic content (TPC) and radical quenching activity (RQA), along with some local foods and beverages. Some examples are extracts of *Allium ursinum* L. [5], *Allium bulgaricum* L. [6-7], *Chrysanthemum balsamita* L. [8-9], Mulberry tree leaves [10], Mulberry fruit [11], *Alchemilla mollis* [12], *Pelargonium graveolens* [13], *Melissa officinalis* L. [14], a range of nine plants (*Clinopodium vulgare* L., *Matricaria chamomilla* L., *Melissa officinalis* L., *Thymus vulgaris* L., *Mentha piperita* L., *Sideritis scardica* L., *Origanum vulgare* L., *Salvia officinalis* L., *Cotinus coggygria* Scop.) [15]. AOA and TPC of 25 Bulgarian medicinal plants were studied in [16] (*Achillea millefolium*, *Arctium lappa*, *Betula pendula*, *Calendula officinalis*, *Cichorium intybus*, *Clinopodium vulgare*, *Crataegus monogyna*, *Glycyrrhiza glabra*, *Humulus lupulus*, *Hypericum perforatum*, *Laurus nobilis*, *Matricaria chamomilla*, *Melissa officinalis*, *Mentha piperita*, *Mentha spicata*, *Ocimum basilicum*, *Rubus idaeus*, *Salvia officinalis*, *Sideritis scardica*, *Taraxacum officinale*, *Thymus vulgaris*, *Tilia cordata*, *Tribulus terrestris*, *Trigonella foenum-graecum* and *Urtica dioica*). Antioxidant activity of Bulgarian culinary plants [17-18], fruits [19-21] and vegetables [22], were studied.

With the present research we studied antioxidant properties of curative extracts of *Crataegus monogyna* (hawthorn), before and after concentration via nanofiltration. This technology gives an opportunity to perform the concentration using mild process conditions (as opposed to the traditional evaporation technology) which is crucial for the preservation of the antioxidant properties and potential healing effect of the obtained phytotherapeutic product.

2 Experimental Part

2.1 Materials

All chemical reagents used were of analytical grade quality: Folin & Ciocalteu's phenol reagent, gallic acid, anhydrous sodium carbonate from Sigma-Aldrich, Taufkirchen, Germany. Syringe filters of 0.45 μm (Valerus Ltd., Sofia, Bulgaria). Distilled water (water still GFL Typ 2004, Burgwedel, Germany) was used throughout the work. Hawthorn plant products (*Flores Crataegi cum floria* and *Fructus Crataegi*) dried leaves & flowers and dried fruits were purchased as local pharmacy commodities (Sofia, Bulgaria). They were stored at room temperature in dark until the time of processing.

2.2 Instruments

Following instrumentation was used throughout the work:

Membrane filtration. "Dead-end" filtration cell of type "METcell" (Membrane Extraction Technology Ltd., London, UK), fitted with 0.0054 m^2 nanofiltration membrane "Microdyn Nadir" NP 030 P (Microdyn-Nadir GmbH, Wiesbaden, Germany); operating pressure 40 bar (compressed nitrogen) under internal stirring 1000 min^{-1} .

Spectrophotometry. An S-22 UV/Vis type of spectrophotometer (Boeco, Germany) was used for determination of the maximal spectral absorption at wavelength of 760 nm. The determination of standard curves (Gallic acid equivalents; GAE) was done in accordance with [23].

Centrifugation. "Janetzki" T32a centrifuge (Berlin, Germany).

2.3 Preparation of the hawthorn extracts

All plant materials were deliberately processed following the traditional preparation procedures for hawthorn medical extracts, without mass normalisation. This approach helped to reveal the curative properties of the extracts based on their therapeutic antioxidant content.

For this purpose 10 g of dried fruits were added to 250 ml boiling distilled water. After 10 min incubation at 100°C it was left covered at room temperature for 20 minutes. The supernatant was collected and was used for further analysis.

The leaves & flowers extract was prepared by 1.25 g of dried leaves and flowers that were added to 250 ml boiling water at 100°C. Then sample was left covered at room temperature for 15 minutes. The supernatant was collected and was used for further analysis.

In order to remove hampering colloid admixtures centrifugation at 3000 min^{-1} for 60 minutes was applied as pretreatment, followed by filtration using "Boeco"

391 filter paper (2–3 μm porosity) and finally 0.45 μm syringe filters. Filtered this way extracts were eventually subjected to nanomembrane separation.

2.4 Nanomembrane filtration

60 ml of the hawthorn extracts were subjected to nanofiltration at temperature of 298.15 K. The membrane separation continued until 30 ml of permeate (P) were collected, with retentate (R) being the rest.

2.5 Total phenolic content (TPC) determination

A spectrophotometric assay of TPC was applied using a modified method of Folin-Ciocalteu method [24,25]. The assay was carried out according to the following procedure: corresponding amount of the phenolic/reductive hawthorn extract (0.050 to 1.000) was brought to a volume of 7 ml through dilution with distilled water. 0.5 ml of Folin–Ciocalteu’s reagent were subsequently added, and after thorough shaking 10 minutes of dead time was applied. Then 2.5 ml of 10% (w/w) aqueous sodium carbonate solution was added. The colourisation reaction developed in 20 min at room temperature. Afterwards the specific absorbance at 760 nm was finally measured with a UV-Vis spectrophotometer; an identical mixture without hawthorn extract was used as a blank sample. The total phenolic/reducing contents were rated and normalised as mg/kg gallic acid equivalents (GAE), through a calibration curve of gallic acid equivalents.

2.6 Radical scavenging assays

ABTS method: The total antioxidant capacity in the ABTS model system was determined by the method proposed by Re et al. [26]. The ABTS assay utilizes free mono-cation radical of 2’2-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid), generated by the oxidation of ABTS with potassium persulfate. The working solution was obtained by diluting the stock solution of the ABTS radical cation with buffer solution $\text{K}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$, pH 7.4, to produce a final solution with absorbance 0.700 at 734 nm. The samples reacted with 2 ml of $\text{ABTS}^{+\bullet}$ solution for 1 hour at room temperature. The discoloration of the pre-generated $\text{ABTS}^{+\bullet}$ radical was measured at 734 nm and the chemical response was compared with the blank one obtained under identical experimental conditions.

DPPH method: Analytical procedure was performed according to Goupy et al [27]. For this experimental procedure a working purple-coloured solution of the DPPH radical in ethanol was prepared, with absorbance of 1.000 at 517 nm. 2 ml of this working solution of the radical reacted with a certain volume of the studied extract fractions for 1 h at room temperature. After the incubation time, the decrease in the absorbance reading of the samples was measured at 517 nm.

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Results were presented as V-50 in μl : the added volume of the extract that provides 50% value of radicals in each of the systems. The bigger is the V-50 the smaller is antioxidant activity of the extract.

3 Results and Discussion

The aims of this research were to evaluate the nanofiltration performance for concentration of total polyphenols, reducers and other antioxidants in water extracts of hawthorn (leaves & flowers and separately fruits), and to establish the corresponding contents via TPC and radical scavenging assays.

3.1 Total phenolic content

Splitting 60 ml of feed extract into 30 ml concentrate and 30 ml permeate via nanofiltration enhanced the TPC in the concentrate 1.8 times for “leaves & flowers”, and 1.7 times for the “fruit” extracts; the reduction of the phenolic compounds in the permeates was evaluated correspondingly 36 and 11 times (Figure 1, Table 1). These results prove the process efficiency in direct enhancement of the curative concentrations.



Figure 1. (from left) Hawthorn extract leaves & flowers (permeate, concentrate, feed); hawthorn extract fruit (feed, concentrate, permeate).

3.2 Total antioxidant activity (TAOA)

The results concerning the AOA activity evaluation by the ABTS and DPPH model systems are presented in Figure 2. The experimental data from both assays show that the therapeutic extract prepared from leaves and flowers exhibits markedly better antioxidant properties, compared to the one derived from fruits; especially notable when taking into account concentrations' ratio of the extracts.

Table 1. Analytical results of the assays

Assay type	Units	Feed		Permeate		Retentate	
		Fr	L&F	Fr	L&F	Fr	L&F
ABTS	V50 in μl	17	2.5	180	130	10	2
DPPH	V50 in μl	60	7	570	260	35	5
TPC	GAE in mg/kg	210	1391	19	39	349	2486

Fr: fruits; L&F: leaves and flowers

Similar results with higher concentration of components possessing antioxidant activity including polyphenols in the leaves and flowers extract was established also for *Crataegus oxyacantha* by [28]. Given the fact that extracts from *Crataegus monogyna* have proven their effectiveness in medical treatment mainly in diseases which pathophysiological mechanism include free radical processes, better antioxidant properties could be well associated with stronger healing effect.

It is evident from the values listed in Figure 2 and Table 1 that the retentate has twice better TAOA, compared with the initial extract, which can be seen from

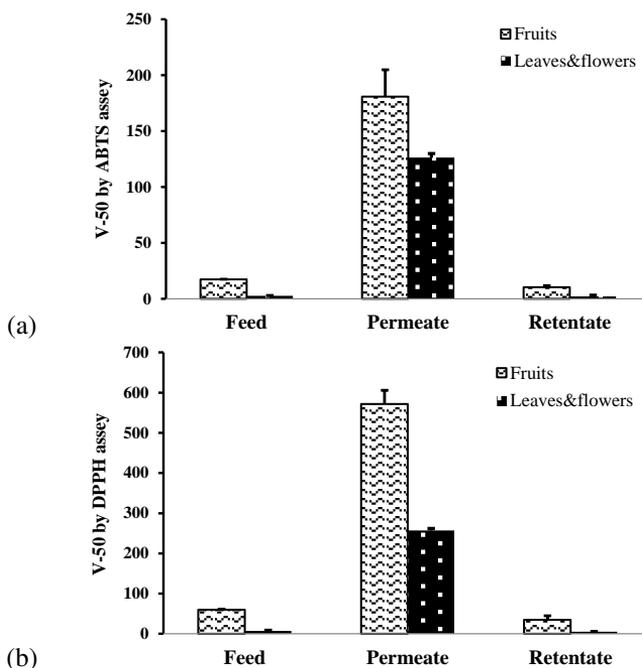


Figure 2. Antioxidant activity of the extracts before and after nanofiltration, determined using the ABTS (a) and DPPH (b) assay.

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the smaller V-50 values. We can conclude that the procedure of nanofiltration successfully concentrated the antioxidants in the extracts, which is visible for the TPC results too (Table 1). In total, this research proved considerably higher antioxidant activity of “leaves & flowers” extract versus the “fruits” ones.

3.3 Correlation between total phenolic content (TPC) and antioxidant activity (AOA)

The ratio between the above mentioned AOA of leaves and flowers extract versus the fruits extract for each assay is presented in Figure 3. This comparison gives us opportunity to make conclusion about the influence of the nanofiltration procedure on the total polyphenolic content and the other components contributing to the TAOA in the investigated model systems.

The data reveal correlation between the TPC and the measured TAOA in the ABTS system for the feed extracts. After the nanofiltration procedure a better correlation between the TPC of permeate and retentate and the TAOA is found with the results obtained using the DPPH assay. Despite this small difference for the feed extracts, our research did not confirm the conclusions of [16] about discrepancy between the antiradical capacity of the hawthorn extracts and their total polyphenolic content. On the contrary, we observe significant correlation of these two values.

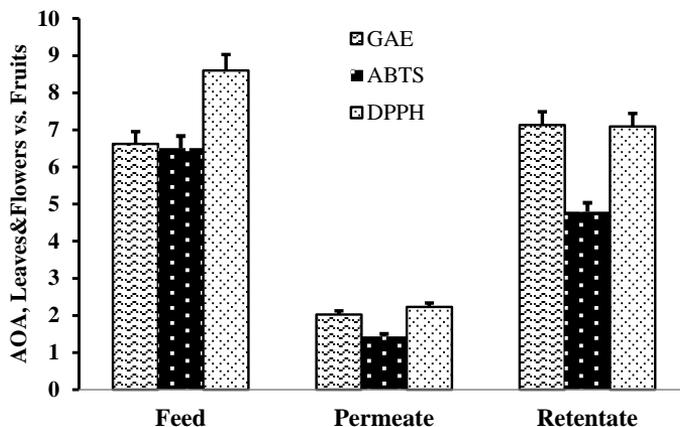


Figure 3. Ratio between the established analytical values for leaves & flowers and fruits for each of the used assays: TPC (as GAE), ABTS and DPPH (Table 1). The content of bioactive components is higher for L&F in each of the cases.

4 Conclusions

As an output of this work nanofiltration was identified as an effective method for “cold” concentration of the antioxidant content of hawthorn extracts. “Leaves & flowers” extract can be preferably used for curative purposes because of its considerably higher bioactive content. This extract showed 6.6-8.6 times higher content of polyphenols and reducing compounds in the feed extracts vs. the “fruits” extracts according to the different methods for antioxidant activity evaluation, and over 7 times in the retentates (concentrates). The ratio for the permeate solution is barely 2.0–2.2 times in favour of “leaves & flowers”, which evidences for an effective membrane rejection of the main bioactive components from the *Crataegus monogyna* extracts. The variations between the results from DPPH and ABTS assays on the antioxidant activity are associated with their different target components.

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