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Characterization of phytochemical profile and biological activity of *Prunus spinosa* L. flower extracts in the context of cardiovascular diseases

PhD dissertation based on a series of scientific publications

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Summary

The major goal of contemporary medicine is the development of more effective strategies for prevention and therapy of cardiovascular diseases (CVDs), one of the primary health problems in modern societies. The studies carried out in the last two decades have revealed that pathogenesis of CVDs is related to two closely intertwined processes of oxidative stress and inflammation. As a result, a large amount of research has focused on the exogenous antioxidants, in particular plant polyphenols, which may exert multidirectional effects on human body primarily by the regulation of redox homeostasis and inflammation processes in living cells. The activity of the polyphenolic compounds within the circulatory system has been confirmed by successful centuries-old traditional use of polyphenol-rich plant materials in the treatment of CVDs, as well as by the results of the clinical and epidemiological studies, which indicated the direct relationship between the polyphenol-rich diet and decreased incidence of CVDs. Thus, the search for novel source of polyphenols as well as phytochemical, pharmacological and toxicological investigations of traditional polyphenolic plant materials seem justified, as in further perspective they may lead to development of new medicinal plant preparations relevant for prophylaxis and treatment of CVDs.

In an attempt to follow this scientific trend, the presented dissertation focuses on blackthorn flowers (*Pruni spinosae flos*), a valued traditional polyphenolic plant remedy from Central and Eastern Europe, recommended among others in various circulatory disorders. Ethno- pharmacological sources document the use of the flowers as ingredients of compound herbal prescriptions applied, e.g. to treat numerous cardiac complaints, such as myocarditis, cardiac neurosis and atherosclerosis. The biological studies conducted in the 60s and 70s of the 20th century suggested that the flavonoid fraction of blackthorn flowers significantly reduces capillary permeability and shows anti-inflammatory effects in internal organs of experimental animals, normalizes the blood cholesterol and cholesterol/phospholipid ratio in atherogenic rabbits, and increases the amplitude of heart contractions in perfusion of isolated frog hearts. Thus, *P. spinosa* flowers seem to present promising therapeutic potential, in particular within the circulatory system; that however, due to the scarcity of data confirming their biological value, cannot be fully utilized. For wider recommendation of the plant material, further phytochemical, pharmacological and toxicological studies are required, as is the development of standardization methodology. Taking into account the above mentioned premises, the presented dissertation became the initial part of a wider project aiming at comprehensive assessment of *P. spinosa* flowers as a source of natural, bioactive polyphenols with potential influence on the circulatory system. The particular objectives of the dissertation were the detailed characterization of the phytochemical profile, development of quality control procedure in terms of polyphenolic compounds, as well as the preliminary evaluation of the antioxidant and anti-inflammatory activity.

The studies were carried out using dry extracts/fractions from blackthorn flowers, which are preparations recommended by modern phytotherapy due to their higher effectiveness in comparison to the unprocessed plant materials. The phytochemical part of the studies included comprehensive profiling of the extracts/fractions composition (qualitative and quantitative analyses), development of an adequate quality control methodology for the flower and extracts prepared thereof, and evaluation of the variation in the individual polyphenols contents in commercial plant materials. The qualitative UHPLC-PDA-ESI-MS^3 analysis showed huge complexity of the phenolic matrix and led to full or partial

identification of over 50 constituents (36 new for the flowers); among them, 37 were classified as flavonoids (mostly kaempferol and quercetin mono- and diglycosides), while the other as flavan-3-ols derivatives (catechins and A-type procyanidins), caffeic acid pseudodepsides and simple phenolic acids. The results of the quantitative studies showed that extracts are rich sources of phenolic compounds as the total phenolic content (TPC) in the methanol-water extract amounted to 206.1 mg GAE/g dw, whereas in the polyphenol-richest ethyl acetate (EAF) and diethyl ether fractions (DEF) – to 584.1 and 464.6 mg GAE/ g dw, respectively. Moreover, the spectroscopic and chromatographic quantification of the main groups of polyphenols (flavonoids, procyanidins, phenolic acids) revealed that flavonoids are the dominant phenolic components and the highest total flavonoid contents was observed for DEF (459.6 mg/g dw, counted as glycosides). In the next step, the HPLC-PDA-fingerprint method for determination of individual blackthorn flowers constituents was optimized and validated. The developed method allowed for efficient separation of the matrix peaks and simultaneous quantification of crucial analytes with the use of 30 calibration standards typical of the analyzed species. Moreover, the relative response factors (RRFs) were established, that would enable an absolute quantification of the abovementioned compounds using only 5 commercially available reference standards. The real sample analyses demonstrated the applicability of the developed procedure for quality control, as it was successfully employed for quantification of the phenolics both in commercial samples of *P. spinosa* flowers, as well as in extracts prepared thereof. Among the standards used in the LC-MS and HPLC-fingerprint studies were flavonoid diglycosides isolated from the leaves of *P. spinosa* by preparative HPLC-PDA method optimized by application of the response surface methodology (RSM).

The bioactivity studies, conducted alongside the phytochemical investigations, included an *in vitro* assessment of the antioxidant and anti-inflammatory activity in complementary chemical and biological models. The simple chemical *in vitro* tests (DPPH, FRAP, TBARS) revealed significant and phenolic-dependent antioxidant effects of the blackthorn flowers extracts/fractions and indicated that scavenging of free radicals, reduction of metal ions and inhibition of lipid peroxidation might be among potential mechanisms of the antioxidant activity of the analytes. These analyses encouraged further studies in the biological model of human plasma, in which it was demonstrated that the extracts/fractions at *in vivo*-relevant levels available for polyphenols after oral administration (1–5 μg/mL) effectively protected components of human plasma against damage induced by $ONOO^-$, one of the most powerful secondary oxidative/nitrative species, generated among others in the circulatory system during acute and chronic inflammation, as well as in ischemia and reperfusion. Not only did the analyzed extracts/fractions effectively reduce the levels of oxidative stress biomarkers, including 3-NT (important marker of protein nitration, considered as predictor of cardiovascular incidents), FOX-1 and TBARS (lipid peroxidation markers), but they also enhanced the total antioxidant status (NEAC) of the $ONOO^-$-treated plasma. Taking into account that the potential sources of oxidative stress in the circulatory system *in vivo* include, besides $ONOO^-$, its direct precursors, i.e. $O_2^{\bullet-}$ and $NO^{\bullet}$, as well as $HO^{\bullet}$, H_2O_2 and HClO generated by the immune system cells (neutrophils and monocytes) during the inflammation, the next step involved the assessment of the antioxidant activity of the extracts/fractions towards multiple ROS/RNS ($O_2^{\bullet-}$, $HO^{\bullet}$, H_2O_2, $NO^{\bullet}$, $ONOO^-$, HClO). To identify the compounds that are crucial for the activity of the extracts and for more thorough evaluation of expected *in vivo* effects, the analyses included also selected polyphenolic compounds (considered as model constituents of the blackthorn flowers) and their main metabolites *in vivo*. All extracts exhibited

significant, concentration- and phenolic-dependent scavenging potential towards the tested oxidants, and the highest responses were observed for $O_2^{\bullet -}$, $HO^{\bullet}$, $ONOO^-$, and HClO. Importantly, the scavenging capacity of the most active fractions DEF and EAF was comparable or superior to the activity of positive standards, including ascorbic acid. The most active model polyphenols were quercetin, avicularin, procyanidin A2 and chlorogenic acid, which suggested decisive impact of these compounds on the activity of the blackthorn flowers and indicated them as valuable activity markers for standardization study. Among the metabolites, dihydrocaffeic acid and 2-(3',4'-dihydroxyphenyl)acetic acid were particularly active towards the investigated oxidants, in some tests exhibiting even stronger activity than their parent compounds. These results confirmed that phenolic metabolites deserve more attention during the assessment of the bioactivity of plant derived products. The anti-inflammatory activity study proved that the extracts/fractions might inhibit the activity of pro-inflammatory enzymes: lipoxygenase and hyaluronidase. The efficacies of the most active fractions DEF and EAF were between those of rutin (a flavonoid with proved vasoprotective activity) and indomethacin (a nonsteroidal anti-inflammatory drug). The juxtaposition of the activity parameters for extracts and model compounds suggested that all of the main groups of blackthorn phenolics (flavonols, chlorogenic acids, and procyanidins) might be responsible for this effect, with possible synergistic action. Furthermore, the preliminary assessment of the cellular safety with the use of human peripheral blood mononuclear cells (PBMCs) revealed that the blackthorn flowers extracts/fractions at the physiological concentration did not affect the viability of the cells, and might be regarded as safe.

The results of the studies described in the present dissertation demonstrated that *P. spinosa* flowers are a rich source of natural polyphenols with antioxidant and anti-inflammatory activity, and possess a significant potential in the prevention or adjunctive therapy of oxidative stress/inflammation-related diseases, including CVDs, partly confirming their traditional application. The blackthorn flowers, used so far only in the traditional medicine, seem therefore to be a promising material for wider investigations, which should include pharmacological studies focused on the protective effects within the circulatory system. A more thorough assessment of the plant material in this context may lead in further perspective to their introduction to the official medicine.