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Plasma protein binding of polyphenols from maritime pine bark extract (USP)

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ABSTRACT

Maritime pine bark extract is monographed in the United States Pharmacopeia (USP) as a dietary supplement. As knowledge about active principles – protein interactions contribute to insights into pharmacokinetic and pharmacodynamic properties we elucidated the plasma protein binding of various constituents and metabolites of this extract. We chose high performance affinity chromatography for fast characterization of the analytes' binding extent to human serum albumin. For selected maritime pine bark compounds that were previously detected in human plasma samples we additionally employed ultrafiltration. The flavonoids catechin and taxifolin revealed highest plasma protein binding of close to 100%, followed by procyanidin B1 and the cinnamic acid derivates ferulic acid ($73.5 \pm 0.12\%$), caffeic acid ($66.0 \pm 0.23\%$) and p-cumaric acid ($65.4 \pm 4.84\%$). Lower protein binding was observed for the benzoic acid derivates vanillic acid ($56.3 \pm 1.16\%$), p-hydroxy benzoic acid ($35.3 \pm 10.9\%$), gallic acid ($31.6 \pm 0.56\%$) and protocatechuic acid ($20.7 \pm 0.09\%$). Lowest protein binding was measured for the maritime pine bark metabolites δ-(3,4-dihydroxy-phenyl)-γ-valerolactone ($34.9 \pm 1.28\%$) and δ-(3-methoxy-4-hydroxy-phenyl)-γ-valerolactone ($26.4 \pm 0.03\%$). For all but one compound the results of both methods revealed excellent correlation. Thus, we provide new data as a basis for a more comprehensive understanding of the bioactivity of a complex plant extract.

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1. Introduction

Maritime pine bark extract is monographed in the United States Pharmacopeia (USP) as a dietary supplement [1]. The USP provides the latest FDA-enforceable standards for quality, identity, strength, and purity of drug ingredients, dosage forms, medical devices, and also of various plant extracts. A standardized bark extract that conforms with the monograph of maritime pine bark extract is derived from *Pinus pinaster, Ait.* (Pycnogenol®, Horphag Research Ltd., UK). About 65–75% of this extract are procyanidins consisting of catechin and epicatechin moieties of varying chain lengths [2,3]. Other constituents are polyphenolic monomers, phenolic or cinnamic acids and their glycosides.

The procyanidine-rich maritime pine bark extract exhibited diverse pharmacological actions in human trials, e.g. cardiovascular, anti-diabetic and anti-inflammatory effects [2,3]. So far there is still limited information on which compound(s) of the complex extract are mainly responsible for the documented bioefficacy. Although oligomeric procyanidins cannot be absorbed due to their high molecular mass they have been shown to exhibit local effects in the gastrointestinal tract [4]. They potently inhibit α-glucosidase and might thus contribute to lowering post-prandial glucose elevation in diabetic patients after intake of Pycnogenol [5,6].

After oral intake of maritime pine bark extract free and conjugated ferulic acid and taxifolin were detected in urine of volunteers [7,8]. Both taxifolin and ferulic acid are genuine components of the extract (Fig. 1). Furthermore, two metabolites that were not originally present in the pine bark extract were identified as δ-(3,4-dihydroxy-phenyl)-γ-valerolactone (M1, Fig. 1) and δ-(3-methoxy-4-hydroxy-phenyl)-γ-valerolactone (M2) [7]. We recently demonstrated that these metabolites inhibit various matrix metalloproteinases (MMPs) and exhibit antioxidant activity [9]. In a pharmacokinetic study we analyzed which extract components were detectable in plasma samples of volunteers after single and multiple intake of Pycnogenol [10]. Besides taxifolin, ferulic acid and the metabolite M1 that were already detected in urine samples we found catechin and caffeic acid (Fig. 1) in plasma. Moreover, we described the time course of ten unknown compounds in plasma. Interestingly, the plasma samples obtained from volunteers after ingestion of Pycnogenol revealed significant efficacy in inhibition of NF-κB activation, MMP-9 secretion and inhibition of COX-1 and COX-2 activity [11,12].

Though some basic information for understanding bioavailability and bioefficacy of the maritime pine bark extract is thus accessible, no data of plasma protein binding is available yet. The pharmacological activity of a compound depends on its pharmacokinetic as well as on its pharmacodynamic properties and plasma

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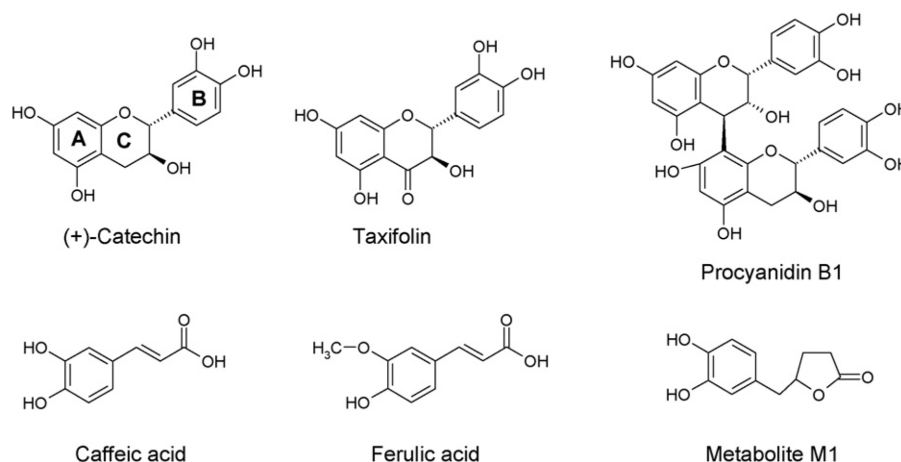

Fig. 1. Structural formulas of selected constituents and a metabolite of maritime pine bark extract.

protein binding might affect both [13]. Thus, knowledge about protein binding of constituents and metabolites of maritime pine bark extract is warranted to gain further insight into the bioactivity of a plant extract. Generally, rather sparse information about plasma protein binding of plant extract components is accessible and only recently the attention towards this issue increases [14–17].

For determination of plasma protein binding various methods have been employed among which equilibrium dialysis and ultracentrifugation play a prominent role [18]. The value of each method and the comparability of results is subject of incessant discussions [19–21]. For our experimental approach we chose high performance affinity chromatography (HPAC) for fast characterization of the analytes' binding extent to human serum albumin (HSA) [22]. For selected maritime pine bark compounds that were previously detected in human plasma samples [10] we additionally employed a modified ultrafiltration method [23] for comparison of results derived from HPAC.

2. Materials and methods

2.1. Chemicals and reagents

Acetaminophen, allopurinol, atenolol, salicylamide, salicylic acid, sotalol, triamterene, ferulic acid, (±)-taxifolin, (+)-catechin, caffeic acid, 3,4-dihydroxybenzoic acid, p-coumaric acid, gallic acid, p-hydroxybenzoic acid, vanillic acid and procyanidin B_1 were obtained from Sigma–Aldrich (St. Louis, MO, USA). The metabolites M1 (δ-(3,4-dihydroxy-phenyl)-γ-valerolactone) and M2 (δ-(3-methoxy-4-hydroxy-phenyl)-γ-valerolactone) were synthesized by Große Düweler [7]. Methanol, isopropanol (both HPLC grade) and polysorbat 80 were obtained from (Merck, Darmstadt).

2.2. Buffers and human plasma

The 20 mM potassium phosphate buffer (pH 7.0) consisted of 7.5 mM $NaH_2PO_4 \times 2\ H_2O$ and 12.2 mM Na_2HPO_4. Krebs–Ringer–HEPES buffer (pH 7.4) consisted of 118 mM NaCl, 4.84 mM KCl, 1.2 mM KH_2PO_4, 2.43 mM $MgSO_4$, 2.44 mM $CaCl_2 \times 2\ H_2O$ and 10 mM HEPES.

Human plasma was obtained from the transfusion medicine of the University of Würzburg.

2.3. High performance affinity chromatography (HPAC)

HPAC was performed using a Waters HPLC (Milford, MA, USA) with a 1525 binary pump, a 717plus autosampler, a 2487 dual wavelength absorbance detector set at the detection wavelength of 280 nm. Data collection and integration were accomplished using Breeze™ software version 3.30. Analysis was performed on a CHIRAL-HSA column (50 mm × 3.0 mm I.D., 5 μm particle size; Chrom Tech Ltd., Congelton, Cheshire, UK). The column temperature was maintained at 37 °C. The mobile phase consisted of 95% potassium phosphate buffer (20 mM, pH 7.0) and 5% (v/v) isopropanol, the flow rate was 0.5 mL/min. A sample volume of 10 μL was injected. All sample compounds were dissolved in phosphate buffer (concentration 0.03 mg/mL). For every compound five replicates where analyzed.

Reference compounds with known plasma protein binding extents (acetaminophen, allopurinol, atenolol, salicylamide, salicylic acid, sotalol, triamterene) were subjected to HPAC to calculate the capacity factor (k') from the retention time using Eq. (1):

$$k' = \frac{t_R - t_M}{t_M} \quad (1)$$

t_R = retention time of the compound and t_M = retention time of the unretained compound (sotalol).

The known binding values of the reference compounds [24,25] were plotted against the measured protein binding values calculated according to Eq. (2):

$$\%\,\text{Protein binding} = \left(\frac{k'}{k'+1}\right) \times 100 \quad (2)$$

A calibration line was generated by linear regression and the % protein binding of the test compounds with unknown protein binding was calculated.

2.4. Ultrafiltration

For the ultrafiltration Microcon (YM-10; Millipore, Bedford, MA, USA) collection tubes and ultrafiltration devices with a MWCO of 10 kDa were used.

2.4.1. Pretreatment of the ultrafiltration devices

For reduction of nonspecific binding the ultrafiltration devices were pretreated using the method of Lee et al. [26] with some modifications. Briefly, all filters were loaded with 25 μL of a 5% (m/m) polysorbat 80 solution and incubated for 10 min at 25 °C. After centrifugation (Microfuge®, 22R Centrifuge, Beckman Coulter, CA, USA) at 3000 × g for 10 min 200 μL of Krebs–Ringer–HEPES buffer was added and centrifuged as described above.

2.4.2. Determination of nonspecific binding of the test compounds

All test compounds were dissolved in Krebs–Ringer–HEPES buffer (concentrations see Section 2.4.3) and 100 μL were applied to the pretreated ultrafiltration unit. After centrifugation at 14,000 × *g* for 30 min 10 μL of the filtrate were subjected to HPLC analysis. The nonspecific binding was expressed as the ratio of the compounds' concentration recovered after ultrafiltration (C_{post}) and compounds' initial concentration before filtration (C_{pre}). The nonspecific binding (NSB) was calculated according Eq. (3):

$$\text{NSB}\,[\%] = \frac{(C_{pre} - C_{post})}{C_{pre}} \times 100 \qquad (3)$$

2.4.3. Determination of human plasma protein binding of the test compounds

For plasma protein binding determination caffeic acid (1.3 μM), (+)-catechin (2.0 μM), ferulic acid (80.0 μM), M1 (6.0 μM), procyanidin B_1 (10.0 μM) and (±)-taxifolin (6.0 μM) were incubated with human plasma for 30 min at 37 °C to allow equilibration. The different concentrations of the test compounds were chosen based on the consideration to recover concentrations above the lower limit of detection even if extensive protein binding occurred. The ultrafiltration procedure was performed according to Taylor and Harker [23]. Briefly, aliquots of 150 μL compound-spiked plasma were pipetted into the ultrafiltration unit. In parallel, aliquots of 150 μL blank plasma where applied on another filter tube. Each experiment consisted of two tubes. The devices where centrifuged at 25 °C for 30 min at 14,000 × *g*. The compounds' fraction bound to plasma proteins was retained in the upper part of the filtration unit and the free fraction in the filtrate. In the second device only a separation of proteins and plasma water occurred. The membrane filter were inverted and placed on the other tube. By centrifugation at 3000 × *g* for 10 min at 25 °C the compound was transferred from the filter membrane into the filtrate collection reservoir. As result the protein bound fraction was recovered in the filtrate of the respective other ultrafiltration device. Thus, each experiment provided information about both, the free as well as the protein bound fraction of each compound.

2.4.4. Sample preparation and analysis

After centrifugation 100 μL plasma was mixed with 130 μL methanol and 40 μL 0.5 M HCl. The samples were centrifuged at 4 °C for 30 min at 14,000 × *g*. 20 μL of the supernatant was subjected to HPLC analysis (HPLC system see Section 2.3) on a Zorbax SB C8 column (150 mm × 4.6 mm I.D., 5 μm particle size, Agilent Technologies, Palo Alto, CA, USA). (+)-Catechin, caffeic acid, M1 and (±)-taxifolin were analyzed by electrochemical detection using the internal standard p-coumaric acid (6 μM). The electrochemical detector CLC 100 (Chromsystems, Munich, Germany) was set at an oxidation voltage of 0.5 V. Ferulic acid was analyzed by UV detection (280 nm); this detector was connected to the control system by a satellite interface (Waters). The flow rate was 1 mL/min, the injection volume 20 μL. Isocratic elution was performed using 88% aqueous phase (containing 0.6 mM 1-octanesulfonic acid sodium salt, 0.27 mM ethylenediaminetetraacetic acid disodium salt, 0.04 M triethylamine; pH 2.95 adjusted with phosphoric acid) and 12% acetonitrile (ACN, HPLC gradient quality, Fisher Scientific, Schwerte, Germany). The method was validated according ICH guidelines. The method fulfilled the quality criteria for linearity, selectivity and intra- and inter-day precision.

2.4.5. Result calculation

Protein binding was calculated by using Eq. (4):

$$\%\,\text{Protein binding} = (1 - f) \times 100 \qquad (4)$$

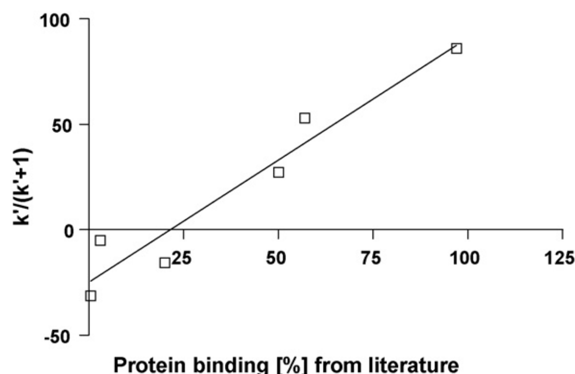

Fig. 2. Calibration curve for determination of protein binding using high performance affinity chromatography. Published protein binding values of various compounds [24,25] were plotted against measured retention times on the HSA column. Linear regression revealed a coefficient of correlation of $r = 0.964$ ($p < 0.01$). Each data point represents the mean and standard deviation of five replicates.

The free, non-protein bound fraction f was determined by Eq. (5):

$$f = \frac{F_f}{[(1 - \text{NSB}) \times F_E]} \qquad (5)$$

F_f was defined as the area ratio of the compound after ultrafiltration and the internal standard, NSB is the nonspecific binding (see Section 2.4.2), F_E the area ratio of the compound before ultrafiltration and the internal standard.

3. Results

3.1. Protein binding determined by high performance affinity chromatography (HPAC)

Reference compounds with protein binding values of 0.5% (allopurinol) to 97% (salicylic acid) were subjected to high performance affinity chromatography (HPAC). Published protein binding data were plotted against measured binding values on the HSA column (Fig. 2). Linear regression revealed a good coefficient of correlation of $r = 0.964$ ($p < 0.01$). Eight polyphenols that were previously detected in maritime pine bark extract [2] as well as two metabolites known to be formed in humans after intake of the extract [7] were subsequently analyzed by HPAC. Each measurement consisted of five replicates and results were expressed as mean and standard deviation. Two of the analyzed compounds, the flavonoids catechin and taxifolin, were so strongly bound to the affinity column that they were not eluted with the described chromatographic conditions. Only a higher proportion of isopropanol in the mobile phase detached these compounds from the affinity column. Thus, a protein binding of 100% was assumed for these substances (Table 1). Among the other constituents or metabolites of maritime pine bark extract procyanidin B1 revealed the highest binding (91.8 ± 0.94%), followed by the cinnamic acid derivates ferulic acid (73.5 ± 0.12%), caffeic acid (66.0 ± 0.23%) and p-cumaric acid (65.4 ± 4.84%). Lower protein binding was observed for the benzoic acid derivates vanillic acid (56.3 ± 1.16%), p-hydroxy benzoic acid (35.3 ± 10.9%), gallic acid (31.6 ± 0.56%) and protocatechuic acid (20.7 ± 0.09%). Lowest protein binding was measured for the maritime pine bark metabolites δ-(3,4-dihydroxy-phenyl)-γ-valerolactone (34.9 ± 1.28%) and δ-(3-methoxy-4-hydroxy-phenyl)-γ-valerolactone (26.4 ± 0.03%).

Table 1
Protein binding of various constituents and metabolites of maritime pine bark extract determined in the present study with two different methods. Five replicates of every experiment were performed. The results are displayed as mean with standard deviation. For comparison, protein binding results of other studies are given. Methods: high performance affinity chromatography (HPAC), ultrafiltration (UF), equilibrium dialysis (ED), capillary electrophoresis (CE).

	Present study HPAC	Present study UF	Other studies HPAC	Other studies UF	Other studies ED	Other studies CE
	% Binding to human serum albumin (HSA) or human plasma proteins					
Caffeic acid	66.0 ± 0.23	79.1 ± 2.52	81.8 [29]	18.9 [16]	61–95 [30]	
(+)-Catechin	100	95.9 ± 1.47	42[a] [14]			90 ± 3 [15]
Ferulic acid	73.5 ± 0.12	78.4 ± 4.00			36.7 [17]	
Taxifolin	100	95.4 ± 1.13		>90 [31]		
Procyanidin B1	81.5 ± 1.09	35.3 ± 3.65				
δ-(3,4-Dihydroxy-phenyl)-γ-valerolactone (M1)	34.9 ± 1.28	58.8 ± 19.4				
δ-(3-Methoxy-4-hydroxy-phenyl)-γ-valerolactone (M2)	26.4 ± 0.03					
p-Coumaric acid	65.4 ± 4.84					
Gallic acid	31.6 ± 0.56		48[a] [14]			
p-Hydroxy benzoic acid	35.3 ± 10.9					
Protocatechuic acid	20.7 ± 0.09					
Vanillic acid	56.3 ± 1.16					

[a] Calculated from affinity constants according to the method described by Valko et al. [37].

Table 2
Nonspecific binding of selected constituents and a metabolite (M1, δ-(3,4-dihydroxy-phenyl)-γ-valerolactone) of maritime pine bark extract to pretreated ultrafiltration membranes. For comparison, the respective log P, numbers of H-bond donors/acceptors and topological polar surface area (TPSA) is given according PubChem database information (http://pubchem.ncbi.nlm.nih.gov/). Nonspecific binding data represent the mean and mean deviation of the mean of triplicate experiments.

	Nonspecific binding (%)	Log P values	H-bond donors	H-bond acceptors	TPSA
Caffeic acid	29.7 ± 0.11	1.20	3	4	77.8
Catechin	45.8 ± 0.09	1.80	4	5	90.2
Ferulic acid	45.2 ± 0.09	1.58	2	4	66.8
M1	25.6 ± 0.06	1.50	2	4	66.8
Procyanidin B1	8.5 ± 0.05	2.40	10	12	221
Taxifolin	11.7 ± 0.10	1.50	5	7	128

3.2. Protein binding determined by ultrafiltration

Constituents and one metabolite of maritime pine bark extract that were previously detected in human plasma samples [10] and procyanidin B1 were subjected to ultrafiltration. Though pretreatment of the filter membranes with polysorbat 80 solution clearly reduced nonspecific binding, residual nonspecific binding was still high for some of the compounds (Table 2). Highest nonspecific binding was observed for catechin (45.8 ± 0.09%) ferulic acid (45.2 ± 0.09%) while procyanidin B1 (8.5 ± 0.05%) revealed the lowest nonspecific adsorption to the ultrafiltration unit. There was no apparent correlation of nonspecific binding with the lipophilicity of the compound expressed as log P value. In contrast, a weak correlation of nonspecific binding and the compounds' topical polar surface area (TPSA) was apparent after logarithmic regression ($r = 0.8529$; $p < 0.05$).

The plasma protein binding, corrected for nonspecific binding, was highest for the flavonoids catechin (95.9 ± 1.47%) and taxifolin (95.4 ± 1.13%) (Table 1). Five replicates of every experiment were performed. Lower protein binding was measured for the cinnamic acid derivates caffeic acid (79.1 ± 2.52%), ferulic acid (78.4 ± 4.0%) and for the maritime pine bark metabolite δ-(3,4-dihydroxy-phenyl)-γ-valerolactone (58.8 ± 19.4%). Lowest protein binding was determined for procyanidin B1 (35.3 ± 3.65%). There was no apparent correlation of the plasma protein binding extent with the compounds' previously analyzed nonspecific binding to the ultrafiltration units.

3.3. Comparison of methods for determination of protein binding

Compounds that were previously detected in human plasma samples after ingestion of maritime pine bark extract [10] and procyanidin B1 were analyzed with both methods, HPAC and ultrafiltration. This allows a direct comparison of the obtained results (Fig. 3). An excellent and statistically significant correlation ($r = 0.9933$; $p < 0.01$) of binding data obtained with HPAC and ultrafiltration was found for caffeic acid, (+)-catechin, ferulic acid, taxifolin and the metabolite M1 (δ-(3,4-dihydroxy-phenyl)-γ-valerolactone). The protein binding results were highly comparable for catechin, ferulic acid and taxifolin and well comparable for caffeic acid. The metabolite M1 exhibited higher protein binding when

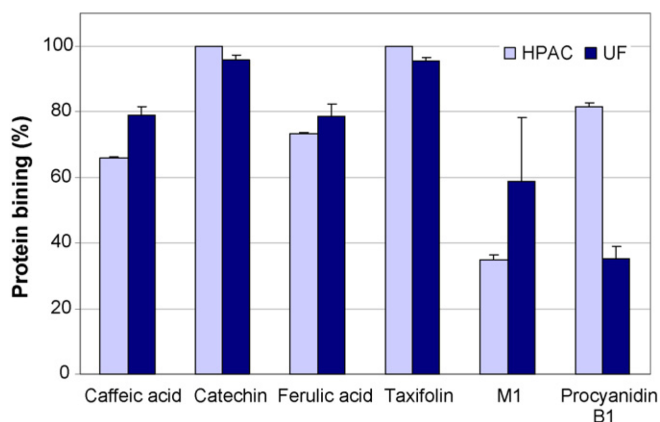

Fig. 3. Comparison of methods for determination of protein binding. Compounds that were previously detected in human plasma samples after ingestion of maritime pine bark extract [10] and procyanidin B1 were analyzed with both high performance affinity chromatography (HPAC) and ultrafiltration (UF). Binding data obtained with both methods revealed an excellent correlation ($r = 0.9933$; $p < 0.01$) for caffeic acid, (+)-catechin, ferulic acid, taxifolin and the metabolite M1 (δ-(3,4-dihydroxy-phenyl)-γ-valerolactone) while procyanidin B1 showed clearly deviating results.

analyzed by ultrafiltration compared to HPAC. The only outlier that did not show a good correlation of protein binding results obtained with the two methods was procyanidin B1. This dimer of a catechin and epicatechin unit was found to have more than twice as high protein binding when analyzed by HPAC compared to ultrafiltration.

4. Discussion

Though plant extracts are increasingly used as phytotherapeutics or dietary supplements information on human pharmacokinetics, bioefficacy and safety are still fragmentary. In the current study we investigated the plasma protein binding of various constituents and metabolites of maritime pine bark extract (USP quality) employing two different methods.

Determination of protein binding using high performance affinity chromatography (HPAC) permits rapid assays that allow conclusions about the test compounds' affinities to human serum albumin (HSA). This method requires a calibration using reference drugs with known protein binding values. The validity of obtained results is high when chemically similar molecules are used as reference and test compounds [27]. We therefore chose reference drugs with aromatic structure and an acidic functional group. The test system revealed excellent precision with low standard deviations of repeated test runs. The accuracy of the experimental approach appeared to be optimal for protein binding rates between 20% and 80%. In the course of method development we aimed at chromatographic conditions most closely related to the physiological situation. However, a mobile phase composition with a less than 5% addition of isopropanol to phosphate buffer resulted in insufficient linearity of the calibration curve. Likewise, a buffer of pH of 7.4 was not recommended in view of HSA column stability. Thus, a buffer of pH 7.0 containing 5% isopropanol was used as mobile phase for the assays. An alternative procedure for concluding on protein binding under strictly aqueous conditions has been described by Ashton et al. [28]. They suggested measuring protein binding employing multiple isopropanol concentrations and subsequently extrapolate to binding under exclusively aqueous conditions. However, this approach did not work for our compounds since no relationship between increasing percentage of organic solvent and analyte retention was seen.

With HPAC we determined the protein binding of eleven constituents and metabolites of maritime pine bark extract. So far no binding data were available for p-cumaric acid, vanillic acid, protocatechuic acid, procyanidin B1 and the procyanidin metabolites M1 (δ-(3,4-dihydroxy-phenyl)-γ-valerolactone) and M2 (δ-(3-methoxy-4-hydroxy-phenyl)-γ-valerolactone). In contrast, some binding data were already published for the other polyphenols. These were obtained with different methods; besides HPAC and ultrafiltration equilibrium dialysis and capillary electrophoresis were employed. Most data was available for caffeic acid [16,29,30] and all except one protein binding value [16] were consistent with our own results. Thus, the protein binding of caffeic acid appears to be around 70–80%. Likewise, the high protein binding of close to 100% we found for catechin and taxifolin was also reported by others [15,31]. Generally, binding data derived from literature reflects a certain variability of results obtained with different methodological approaches. Besides the already mentioned methods fluorescence quenching has been frequently used for determination of protein binding of polyphenols as well [32–35]. The results of this experimental approach are typically expressed protein affinity constants. The comparability of affinity constants with percentage of protein binding can be difficult, especially when protein binding is high [36]. Thus, in only two cases we converted affinity data obtained with HPAC [14] into percent protein binding using the method described by Valko et al. [37]. This provides an approximation of the extent of plasma protein binding.

In the present study, compounds that were previously detected in human plasma samples after ingestion of maritime pine bark extract [10] and procyanidin B1 were additionally analyzed by ultrafiltration. This allows a direct comparison with the results acquired by HPAC. Protein binding values obtained with HPAC and ultrafiltration revealed an excellent and statistically significant correlation ($r = 0.9933$; $p < 0.01$) for all compounds except for procyanidin B1. Ferulic acid, caffeic acid and the metabolite M1 (δ-(3,4-dihydroxy-phenyl)-γ-valerolactone) showed a clear tendency towards higher binding when analyzed by ultrafiltration whereas catechin and ferulic acid displayed almost identical high binding in both experimental approaches. While HPAC reveals information about compounds' affinity to human serum albumin all plasma proteins are present in ultrafiltration. Thus, a higher protein binding observed with ultrafiltration compared to HPAC might be attributed to additional binding to plasma proteins such as α_1-acid glycoprotein or lipoproteins.

The low protein binding of procyanidin B1 seen with ultrafiltration is quiet surprising and unexpected. This dimer consists of a catechin and epicatechin unit and since the catechin monomer already exhibited high protein binding, it is not clear why the binding of the dimer was so low using the ultrafiltration approach. Though various procyanidins are known to be instable in buffers within acidic and alkaline pH ranges [38] the present experiments were conducted in human plasma in which procyanidins show reasonably stability at physiological pH [39]. Likewise, we observed no additional peaks indicating procyanidin instability in the HPLC chromatograms suggesting stability of procyanidin B1 under the chosen assay conditions.

Ultrafiltration membranes were pretreated with polysorbat 80 to reduce nonspecific binding of the tested compounds. Using this approach Lee et al. successfully diminished the nonspecific binding rate of propanolol from about 85% to 65% [26]. Despite membrane pretreatment and utilization of a modified ultrafiltration method described by Taylor and Harker [23] the residual nonspecific binding of the polyphenols was still considerable high. This observation calls for caution in performance and interpretation of experiments with polyphenols. We found a weak correlation of nonspecific binding and the compounds' topical polar surface area (TPSA) after logarithmic regression ($r = 0.8529$; $p < 0.05$) though the number of analyzed substances is probably too low to derive a well-supported conclusion.

A relationship between a compound's chemical structure and human serum albumin binding affinity has been elucidated for quercetin [40] and suggested an important role of the free 3′ hydroxyl group in the B-ring for protein adsorption. Also, it has been shown that the catechol structure with its vicinal diols in the B-ring generally enhanced the protein binding [41]. Recently it has been found that an additional insertation of a hydroxyl group in 5′ position of the B-ring contributed to the protein affinity as well. Interestingly, a triphenol moiety alone was not sufficient for pronounced protein binding, only the combination with the flavan-3-ol structure resulted in high protein adsorption [14]. This is consistent with our own observations. Both catechin and taxifolin carry a catechol structure in the B-ring and displayed high protein affinity while gallic acid with its triphenol moiety revealed significantly lower protein binding.

Plasma protein binding can influence both the pharmacokinetics as well as the pharmacodynamics of a compound [13]. The clinical significance of the protein binding has to be elucidated individually for the molecules of interest. So far it has been shown for polyphenols that the protein bound fraction is protected against degradation [40]. In return, the protein binding partner of the polyphenol is protected against peroxidation by the polyphenol.

This has been demonstrated for lipid peroxidation in LDL particles and for oxidative degradation of serum albumin [40,42].

To summarize, in the present study we analyzed protein binding data for various constituents and metabolites of maritime pine bark extract which is monographed in the USP. We compared and critically discussed two different methods for protein binding evaluation, the fast HPAC and ultrafiltration which provides conditions closer to physiology. For all but one compound the methods revealed excellent correlation of the obtained results. So far available binding data from literature were consistent with our findings. Beyond that we present for the first time protein binding data for seven polyphenolic compounds from the pine bark extract, of which some had previously been also detected in the plasma of volunteers after oral intake of the extract [10]. Thus, we provide new data as a basis more comprehensive understanding the pharmacokinetics and pharmacodynamics of a complex plant extract.

Conflict of interest

M.K. declares no conflict of interests.

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