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In vitro intestinal transport of oligomeric procyanidins (DP 2 to 4) across monolayers of Caco-2 cells

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ABSTRACT

Extracts from hawthorn leafs and flowers (*Crataegus* sp., Rosaceae) are widely used as a rational based phytomedicine for declining cardiac performance. According to present literature C-glycosylated flavones and oligomeric procyanidins are considered to be the active ingredients, despite the fact that no systematic data are available on systemic bioavailability of proanthocyanidins after oral intake. The present study aims to review the actual state of literature in this field and to investigate the intestinal absorption mechanisms of defined hawthorn PAs with different degrees of polymerization by validated *in vitro* Caco-2 monolayer permeation system. Hawthorn OPCs with DP 2 to 6 were isolated as defined clusters. Procyanidin B2 and the procyanidin clusters DP 4, 5 and 6 had very low P_{app} values between 0.6 and 6×10^{-7} cm/s for apical to basolateral permeation. The higher the molecular weight the lower permeation coefficients were calculated. The observed low-level transport was mainly due to passive paracellular permeation. Additionally cellular uptake of OPCs by transcellular permeation was possible; on the other side procyanidins were shown to be p-glycoprotein substrates, which leads to subsequent excretion of PAs by the efflux pump to the apical side. Mixtures of the different OPCs did not have an increased permeation. Transport experiments of complex OPC mixtures together with hawthorn flavonoids did not indicate any improved permeation or synergistic effects. In principle this raises the question if systemic pharmacological activities of hawthorn extracts, can really be attributed to OPCs with very low systemic bioavailability.

1. Introduction

Hawthorn leafs and flowers consist of the dried flower bearing branches of *Crataegus monogyna* Jacq. Emend. Lindm., *Crataegus laevigata* (Poiret) and, more rarely, *Crataegus pentagyna* Waldst. et Kit. Ex Willd., *Crataegus nigra* Waldst. et Kit. and *Crataegus azarolus* L. Pharmaceutical preparations of hawthorn are considered as a rational based phytomedicine for declining cardiac performance corresponding to functional capacity class I and II, as defined by the New York Heart Association (NYHA) [1]. Also a recent metastudy indicates a significant benefit in symptom control and physiologic outcome from hawthorn extract as an adjunctive treatment for chronic heart failure [2]. Main constituents are flavonoids (up to 2%), such as vitexin, vitexin-2″-rhamnoside, hyperoside, rutin, approximately 3% of oligo- and polymeric B-type procyanidins [3–6], oligomeric procyanidin hexosides [7], oligomeric chinchonains [7], triterpens, phenolic acids, amines, xanthines and polysaccharides [8]. From the functional point of view flavon-C-glycosides and oligomeric procyanidins (OPCs) are considered to be the main active compounds [1]. Despite the fact that OPCs are claimed to be part of the active ingredients data published on cardiac activity of hawthorn did not use pure OPCs but multi-compound extracts, standardized on procyanidin content [9–11]. For that reason intensified work is obviously needed for more detailed pharmacological data on the influence

Abbreviations: DP, degree of polymerisation;GUT, gastro intestinal tract; HBSS, Hanks balanced buffer system;HBSS+, Hank's balanced buffer plus ascorbic acid;OPC, oligomeric procyanidins;PC, procyanidin;pgp, p-glycoprotein;TEER, transepithelial electrical resistance

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of isolated OPCs on e.g. cardiac contractility or myocardial circulation. On the other side the B-type OPCs from hawthorn are a complex heterologous series from DP 2 to 13 and accompanied by strong polymeric fraction. For detailed structure-activity studies an efficient methodology for the purification of oligomeric procyanidins with defined DP has been established in order to obtain sufficient amounts of test compounds [12]. On the other side prior to a potential functional testing the question on systemic bioavailability of the test compounds after oral uptake has to be investigated. What is known until now on kinetic properties of proanthocyanidins after oral intake?

While the bioavailability of monomeric flavan-3ols is well investigated [13–15] data for oligomeric proanthocyanidins are limited. According [16] saliva has no influence on PAs from *Camellia sinensis*, while [17] described degalloylation of epigallocatechin-3-O-gallate to occur after contact with human saliva. Ungalloylated oligomeric PAs (DP 2 to 6) are stable in human saliva and are not degraded [18]. Stability of PAs during stomach passage was shown by [19] under *in vivo* conditions, a finding which was confirmed in another study [20]. For that it can be concluded that PAs reach ileum after oral intake.

Concerning intestinal absorption of OPCs the following studies have been reported: Using a rat perfusion system the degradation of procyanidin B2 and B5 to epicatechin was observed, which again was identified as absorbed monomer [18]. Also [21] observed a significant decrease of degree of polymerisation of OPCs after feeding rats with ^{14}C-labeled proanthocyanidins. [22] identified procyanidin B2 after feeding rats (very high concentrations of 50 mg/kg body weight) with this compound after 30 min at 0.5 μM in the plasma. [23] reported the occurrence of di- and trimeric proanthocyanidins, monomeric flavan-3-ols and methylated metabolites after feeding of grape seed extract (300 mg/kg, 2× per day) to rats. Application of cocoa, containing 323 mg flavan-3-ols and 256 mg dimeric PC, to humans led to the identification of low amounts of procyanidin B2 in the serum at 16 nmol/L level after 30 min and 41 nmol/L after 2 h [24]. [25] identified procyanidin B2 in human urine (0.2 nmol/mg Kreatinin) after oral intake of 49 g cocoa. In contrast to the investigations of [24,25] the presence of procyanidin B3 and C2 could not be proven after the feeding of rats [26]. Again another group [27] identified oligomeric PCs in rat plasma after the feeding of apple seed PC-extract and showed that polymeric PC in the extract positively influenced the intestinal absorption.

In vitro absorption studies in Caco-2 cell culture system indicated low permeability coefficients (0.9 to 2.0×10^{-6} cm/s) for catechin, di- and trimeric PAs, while higher oligomers had very low coefficients [28]. These data indicate low absorption of dimers and trimers, while higher oligomers are very limited concerning permeation across Caco-2 cells.

For that it is assumed that the largest part of PAs after oral intake is transferred to the colon, were extensive degradation to phenyl acetic acid or phenyl propionic acid takes place [26,29]. Detailed structure elucidation of catabolites from A-type procyanidins, procyanidin A2 and cinnamtannin B1, after incubation with a pig cecum model to mimic the degradation caused by the microbiota was reported recently by [30] indicating degradation of 80% of PC A2 and 40% degradation of the trimer cinnamtannin B1 to hydroxylated phenolic compounds. Strong metabolic degradation by cecal microbiota was additionally shown for procyanidins in pigs [31].

So far, the data indicate a very limited GUT absorption of OPCs, but available data are not systematic and in part contradictory, presumably in many cases because of a lack of defined OPC reference material. In the present study pure OPC clusters (DP2 to DP4) with defined molecular weight were isolated on a large scale and investigated concerning its transport and cellular uptake across the Caco-2 *in vitro* model of the gastrointestinal mucosa.

2. Experimental

2.1. Materials

Herbal material *Crataegi folium cum flore* (Ch.-B.: 52467097) conforming to specification of Ph. Eur. 6 was obtained from Caesar & Loretz, Germany. A reference sample (voucher sample IPBP 240) is stored at the Institute of Pharmaceutical Biology and Phytochemistry.

DMEM, NEAA, trypsin, penicillin/streptomycin and HBSS were purchased from PAA Laboratories, Austria. FBS was obtained from Hyclone, UK, glucose D-[^{14}C(U)] (300mCi/mmol) from American Radiolabeled Chemical, USA. $EDTANa_2$, HEPES buffer grade, Triton®X-100 were from Applichem, Germany. D,L-propranolol HCl was obtained from Acros Organics, USA and (±)-verapamil HCl from Sigma-Aldrich, USA. Ascorbic acid was purchased from Merck, Germany.

2.2. Isolation of OPC + flavonoid-enriched extract M, OPC-enriched subfraction c, procyanidin B2, C1, and procyanidin clusters with DP 4 to 6

Isolation and characterization of OPC clusters were performed as described recently [12,40,41]. In principle the isolation protocol exhaustively extracted herbal material *Crataegi folium cum flore* (5.15 kg) with acetone/water (7:3 v/v). The extract was evaporated *in vacuo*, filtered to remove precipitated chlorophyll, concentrated and defatted with petroleum benzine. Successive extraction of the aqueous phase with EtOAc yielded an EtOAc-extract (126 g, corresponding to 2.6%) and a water extract (859 g, corresponding to 16.7%, related to the starting material).

Procyanidin B2 and C1 were isolated from the EtOAc extract, which was resuspended in ethanol, centrifuged (2.833×g) and the resulting supernatant, (corresponding to 85 g dry mass) was applied to Sephadex® LH-20 (675×55 mm, mobile phase ethanol 96% 13 L→methanol 9 L→acetone/water (7:3) 3,9 L, flow 1.5→2 mL/min, fraction size 15 mL). All fractions eluated were investigated by TLC and fractions with comparable compositions were collected.

Subfraction E4 (elution from 5.8 to 8.4 L, yield 1.83 g) and E6 (elution from 9.7 to 11.2 L, yield 0.81 g) were subjected to preparative HPLC on diol stationary phase (Uptisphere®120 A bonding OH, 6 μm, 21.2×250 mm, Interchim, France) using acetonitrile, methanol:water gradient for isolation of procyanidins B2 (yield 240 mg) from E4 and procyanidin C1 (yield 113 mg) from E6.

The water extract was divided into a MeOH-soluble extract (extract M) and a MeOH-insoluble fraction. An aliquote of extract M (5.47 g) was further fractionated by MPLC on RP18