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Accepted Version

Ropiak, H. M., Ramsay, A. and Mueller-Harvey, I. (2016) Condensed tannins in extracts from European medicinal plants and herbal products. Journal of Pharmaceutical and Biomedical Analysis, 121. pp. 225-231. ISSN 0731-7085 doi: https://doi.org/10.1016/j.jpba.2015.12.034 Available at http://centaur.reading.ac.uk/49645/

To link to this article DOI: http://dx.doi.org/10.1016/j.jpba.2015.12.034

Publisher: Elsevier

Condensed tannins in extracts from European medicinal plants and herbal products

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ABSTRACT

Medicinal plant materials are not usually analysed for condensed tannins (CT). Thirty commercially available European medicinal plants and herbal products were screened for CT and fourteen CT samples were analysed in detail. This is also the first comprehensive CT analysis of pine buds, walnut leaves and heather flowers and great water dock roots. Acetone/water extracts contained between 3.2 and 25.9 g CT/100 g of extract, had CT with mean degrees of polymerisation of 2.9 to 13.3, procyanidin/prodelphinidin ratios of 1.6/98.4 to 100/0 and *cis*/*trans* flavan-3-ol ratios of 17.7/82.3 to 97.3/2.7. The majority of samples contained procyanidins, four contained A-type linkages (blackthorn flowers, heather flowers, bilberry leaves and cowberry leaves) and one sample also had galloylated procyanidins (great water dock roots).

Keywords: Proanthocyanidins, Flavan-3-ols, Molar response factors, Thiolysis, Mean degree of polymerization

1. Introduction

Folk medicine in Europe uses plants against a wide range of illnesses [1, 2], as food or dietary supplements and as herbal products [3]. The most popular oral intake is via herbal infusion, decoction or as ethanol extracts [2]. Several beneficial actions of medicinal plants have been attributed to tannins [4, 5], and their traditional uses include treatments of diarrhoea, heavy metal poisoning [2] or mild peptic ulceration [5]. Condensed tannins (CT, Fig. 1) are also of interest for their antimicrobial, antiviral and antitumour effects; and for their health benefits in cases of cardiovascular, diabetes and inflammatory issues and effects on innate immune responses [6-8]. However, commercially available medicinal plants are not usually analysed for CT and the European Pharmacopeia recommends that all tannins be quantified simply as pyrogallol equivalents [9]; but this provides no accurate information on CT contents or composition. Detailed information on these well-known antioxidants [6] in medicinal plants could prove useful for research into their bioactivities, whether on their own or in combination with other plant compounds [2, 10] and may also contribute to the stability of active ingredients. Therefore, we first screened several medicinal plants and herbal products that are widely used in European folk medicine. A subset of extracts from materials with the highest CT contents was then analysed in detail for their flavan-3-ol compositions [11].

2. Materials and methods

2.1. Reagents

Hydrochloric acid (37%, AR grade), butan-1-ol, acetic acid glacial (AR grade), acetone (AR grade), acetonitrile (HPLC grade), dichloromethane (LR grade), hexane (GLC, pesticide residue grade) and methanol (HPLC grade) were obtained from ThermoFisher Scientific (Loughborough, UK); benzyl mercaptan (99%), catechin hydrate (≥98%), epicatechin (90%), epigallocatechin (95%), gallocatechin (≥97%), catechin gallate (≥98%), epicatechin gallate (≥98%), epigallocatechin gallate (≥95%), gallocatechin gallate (≥98%), quercetin (≥99%), isoquercitrin (≥90%), rutin hydrate (≥95%), naringin (≥95%), (±)-eriodictyol (≥90%) from Sigma-Aldrich (Poole, UK); naringenin (97%) from Alfa Aesar (Lancashire, UK); (±)-taxifolin (98%) from Apin Chemicals (Abingdon, UK); procyanidin A2 (PC A2, ≥99%) and naringenin-7-*O*-glucoside (≥99%) from Extrasynthese (Genay Cedex, France); afzelechin (96-98%) from Plantech UK (Reading, UK) and Sephadex LH-20 from GE Healthcare (Little Chalfont, UK).

2.2. Plant materials

Pruni spinosae flos, Callunae vulgaris flos, Crataegi inflorescentia, Tiliae inflorescentia, Betulae folium, Myrtilli folium, Vitis idaeae folium, Ribis nigri folium, Salicis cortex, Lupuli flos, Hydrolapathi radix (from Poland) and *Pini gemmae* (typically from Ukraine) were obtained from Flos (Mokrsko, Poland); *Juglandis folium* (collected in June to August 2012, Poland) from Kawon (Gostyń, Poland); white clover flowers (*Trifolium repens* L., collected in April 2012, Poland) from Zioła z Kurpi (Jednorożec, Poland); (see Table 1). Details of other samples are in Table A1 and Appendix A.1.1. Plant materials were purchased in 2012/2013 and ground to pass a 1 mm sieve (impeller SM1 cutting mill, Retsch, Haan, Germany). Leaves and stalks were removed from blackthorn flowers and only flowers were used.

2.3. Screening plant material for CT with HCl/butan-1-ol

Plant materials were screened for CT presence with HCl/butan-1-ol [12] (see Appendix A.1.2).

2.4. Preparation of plant extracts

Acetone/water was used to prepare the CT extracts [11] (see Appendix A.1.3).

2.5. CT derivatisation with benzyl mercaptan, HPLC and LC-MS analysis

CT in extracts were derivatised with benzyl mercaptan in triplicate [11]. Samples were analysed within 48 h by HPLC using gradient 1 [13]. However, heather flowers, bilberry and cowberry leaf extracts were analysed with gradient 2 (solvent A: 1% acetic acid/Milli-Q H_2O; solvent B: acetonitrile) as follows: 0-52 min, 0-36% B; 52-60 min, 36-50% B; 60-65 min, 50-100% B; 65-73 min, 100-0% B; 73-80 min, 0% B). Flavan-3-ols and their benzyl mercaptan (-BM) adducts were identified [14] and quantified [11] using peak areas at 280 nm and molar response factors relative to taxifolin: 0.30 for catechin and epicatechin; 0.06 for gallocatechin and epigallocatechin; 0.26 for catechin-BM and epicatechin-BM; 0.06 for gallocatechin-BM and epigallocatechin-BM [14-16]; 0.55 for PC A2, PC A-type trimers and their corresponding -BM adducts [17]; and 1.01 for epicatechin gallate and epicatechin gallate-BM [18] (Appendix A.1.4, A.1.5 and Table A.3). LC-MS was used to confirm the identity of terminal and extension units; MS spectra were recorded in the negative and positive ionisation scan mode and UV spectra at 280 nm [13] (Table A.3 provides information on $[M\text{-}H]^-$ ions of each detected compound).

2.6. Quantification of free flavan-3-ols

Extracts were also assayed for free flavan-3-ol monomers and their 3-*O*-galloylated derivatives as these interfere with the calculation of CT concentration and composition [19]. Extracts (4 mg) were dissolved in a mixture of methanol (2.05 ml), H_2O (2.5 ml) and the internal standard (taxifolin, 0.5 ml; 0.05 mg/ml in methanol), vortexed and centrifuged (5 min, 3000 rpm) prior to RP-HPLC or LC-MS analysis. Samples were analysed in duplicate within 24 h.

2.7. Calculation of CT composition

The mDP-values of B-type CT and galloylated B-type CT [14, 20], molar percentages of galloylated flavan-3-ols [20], procyanidin/prodelphinidin (PC/PD) ratios and *cis*/*trans* flavan-3-ol ratios [14] were calculated as previously reported (see Appendix A.2 for equations to calculate CT composition); however, A-type units were not included in the calculations of *cis*/*trans* ratios. Flavan-3-ols in terminal and extension units [21] are reported as relative molar percentages.

The mDP-values of CT that had both B-type and A-type linkages were calculated according to Equation 1, which is derived from a published formula for A-type dimers [7, 22, 23] and refers to molar ratios of terminal and extension flavan-3-ol units:

$$\underset{\substack{\text{(CT with B-type}\\ \text{and A-type linkage)}}}{\text{mDP}} = \frac{\sum(\text{B-type TU}) + \sum(\text{B-type EU}) + \sum(\text{n} \times \text{A-type TU}) + \sum(\text{n} \times \text{A-type EU})}{\sum(\text{B-type TU}) + (\text{A-type TU})}$$

Equation 1

where: TU – terminal unit; EU – extension unit; n = 2 or 3 and is the degree of polymerisation of terminal or extension units. The percentage of A-type linkages was calculated according to Equation 2 [23] and takes A-type trimers into account:

$$\%\text{A} - \text{type linkage} = \frac{\sum(\text{A} - \text{type TU}) + \sum(\text{A} - \text{type EU})}{\sum(\text{A} - \text{type TU}) + \sum(\text{B} - \text{type EU}) + \sum(\text{ n } \times \text{ A} - \text{type EU })}$$

Equation 2

3. Results and discussion

Commercially available medicinal plants, and for that matter also other plants, are rarely analysed for CT contents or compositions, but these compounds are of interest as they have been implicated in numerous health effects [6-8]. Such information could be useful when searching for CT bioactivities or combination effects with other compounds that might be linked to their traditional uses. The main uses in traditional medicine of the samples investigated here are for treating minor urinary tract infections, feverish colds or mild rheumatism (Table 1).

3.1. Analysis of CT

Initial screening with HCl/butan-1-ol revealed the presence of CT in 20 of the 30 plant materials (Table A.2). Samples with >3 g CT/100 g extract were selected for further analysis (see Appendix A.3).

3.1.1. Discussion of response factors for quantifying terminal and extension units in CT

Response factors relative to taxifolin at 280 nm are widely used for CT analysis after depolymerisation with benzyl mercaptan [14-16, 24]. However, the literature contains several different response factors for flavan-3-ol terminal and extension units [15, 16] and these can be affected to some extent by analysis conditions [25]. Some authors have also quantified extension units with response factor ratios against the epicatechin-BM adduct [26] by using the molar response factors reported previously [27]. Others have reported that catechin and epicatechin had the same molar response factors as their corresponding -BM adducts [28] or had relatively similar factors as in the case of epigallocatechin and its -BM adduct [27]. The relative molar responses of flavan-3-ols against taxifolin were, therefore, checked and found to be close to previous reports [14, 15, 24] (see Materials and Methods 2.5).

Although mass responses of B-type dimers and trimers relative to epicatechin were similar, i.e. 0.96 and 1.04 [29], no information exists on the relative mass or molar response factors of A-type dimers and trimers against taxifolin. Two reports stated that the molar absorptivity of the epicatechin dimer (PC A2) was not equal to two times the molar absorptivity of epicatechin at 280 nm [30, 31]. Indeed, we found a mass response factor of 0.29 and a molar response factor of 0.55 for the PC A2 dimer against taxifolin, and used the molar response in this study. This is in line with other work, where the relative molar response was twice that of the corrected relative mass response of PC A2 against epicatechin [30]. Finally, the same relative molar response factors were used for the A-type dimer- and trimer-BM adducts in line with a previous report on A-type dimers and their BM adducts [23].

3.1.3. Characterisation of B-type CT

Overall, CT contents ranged from 3.2 to 20.2 g CT/100 g extract, mDP-values from 4.2 to 13.3, PC/PD ratios from 1.6/98.4 to 100/0 and *cis*/*trans* flavan-3-ol ratios from 17.7/82.3 to 97.3/2.7 (Table 2). Interestingly, most samples contained only B-type PC, i.e. extracts from hawthorn flowers, hop strobiles, *Tilia* flowers, willow bark and walnut leaves. Only the extract from great water dock roots had CT with galloylated flavan-3-ol subunits. White clover flowers had the highest percentage of PD (98.4%). *Cis*-flavan-3-ols accounted for >90% of the CT subunits in extracts from *Tilia* flowers, great water dock roots and hawthorn flowers; and blackcurrant leaves had the highest percentage of *trans*-flavan-3-ols (82.3%).

This is the first report of the CT flavan-3-ol composition from pine buds and walnut leaf extracts: pine bud CT had an mDP of 7.8, a PC/PD ratio of 61.5/38.5 and a *cis*/*trans* flavan-3-ol ratio of 70.7/29.3 (Table 2); walnut leaf CT had an mDP of 5.1, consisted only of PC with a *cis*/*trans* flavan-3-ol ratio of 62.6/37.4. In the following paragraphs, we compare our results from medicinal and herbal products with literature data.

White clover flowers contained CT with the highest mDP-value (13.3), the highest PD percentage (98.4%) and a moderate *cis*/*trans* ratio (61.1/38.9; Table 2). This closely resembles previous results where PD percentage was 99%, *cis*/*trans* ratio of 66/34; however, the mDP of 4.4 was much lower [13]. Epigallocatechin was the main extension unit and gallocatechin was the only terminal unit (Table 3). The blackcurrant leaf extract also contained CT that consisted mostly of PD (95.3%), had an mDP of 6.0 and a *cis/trans* ratio of 17.7/82.3 (Table 2). Whilst the mDP-value and PD percentage were similar to previous report (5.4 and 94.2, respectively), the *cis*/*trans* ratio differed noticeably (9.1/90.9) [13]. The birch leaf extract had mixed CT with, a PC/PD ratio of 58.9/41.1, an mDP of 4.2 and a *cis*/*trans* ratio of 62.9/37.1. These CT contained catechin as the main terminal unit and epicatechin as the main extension unit.

The hawthorn flower extract had only PC with an mDP of 4.8 and a *cis*/*trans* ratio of 97.3/2.7 (Table 2). Epicatechin was found in extension units and both catechin and epicatechin in terminal units (Table 3). Willow bark extract is one of the most studied medicinal plant preparations due to its anti-inflammatory and pain relieving effects [10]. In agreement with others, these CT consisted of pure PC [32] and with a low mDP-value (4.6). Catechin was mainly in the terminal position [32] and the *cis*/*trans* ratio was 68.7/31.3. Hop strobiles had pure PC in agreement with the literature [32], although LC-MS analysis detected traces of gallocatechin and epigallocatechin in extension units (Table 3). These CT had an mDP of 5.1 and a *cis*/*trans* ratio of 64.0/36.0 (Table 2). The *Tilia* flower extract also had pure PC with an mDP of 5.8, which concurs with a report that described PC oligomers up to pentamers [33]. These CT had a very high proportion of *cis*-flavan-3-ols (90.6%).

Of particular interest was the great water dock root (*Rumex hydrolapathum*) extract as it had the highest CT content (25.9 g/100 g extract), a high percentage of *cis*-flavan-3-ols (94.7%) and PC that contained 52% of the flavan-3-ols as gallate esters (Table 2). The chromatographic profile (Fig. A.2-A) was similar to CT from *R. obtusifolius* leaves [24]. *R. hydrolapathum* roots and *R. obtusifolius* leaves contained only PC with similar mDP-values (6.2 vs. 4.3) [24]. However, the extent of galloylation was much higher in *R. hydrolapathum* roots (52 vs. 8%) [24].

3.1.4. Characterisation of A-type CT in extracts

A-type linkages were only found amongst PC from blackthorn flowers, cowberry leaves, bilberry leaves, heather flowers and accounted for 25.3, 17.1, 7.3, and 6.7% of the CT, respectively (Table 2; Fig. A.2-B, C, D and E). A-type linkages are usually released by thiolysis as A-type dimers from terminal units or as A-type dimer-BM adducts from extension units [19, 28]. Although one study also reported the release of an A-type trimer from extension units [34]. The PC from blackthorn flower extract had a particularly low mDP (2.9) and a moderate *cis*/*trans* ratio (67.0/33.0), but had the highest percentage of A-type linkages (25.3%) which were present in terminal units (Table 2 and 3). Cowberry leaf extract had PC with an mDP of 6.5, a *cis*/*trans* ratio of 68.5/31.5 and 17.1% of the flavan-3-ols had A-type linkages (Table 2). The presence of pure PC agrees with previous report [35]. A-type dimers occurred in terminal units and both A-type dimers were released from extension units (Table 3); somewhat unusually, A-type trimers also came from extension units. Bilberry leaf extract had PC with an mDP of 6.6, a *cis*/*trans* ratio of 92.2/7.8 and a low percentage of A-type linkages (7.3%, Table 2). A-type trimers were detected as terminal units and both A-type dimers and trimers as extension units.

However, no gallocatechin and epigallocatechin were detected in our sample and this suggests that the percentages of PD can vary in bilberry leaves [35].

The flavan-3-ol composition of CT from heather flower extract is reported here for the first time. These PC had an mDP of 7.2 and a *cis*/*trans* ratio of 87.5/12.5; and 6.7% of flavan-3-ols were present in A-type linkages. A-type trimers were detected as terminal units and A-type dimers as extension units.

3.2. Other flavonoids

Whilst this work focussed on CT we also provide information on a few flavonoids (rutin, quercetin and quercetin-hexoside) in the Appendix (Table A.3 and Fig. A.1 and A.2). Of particular interest here is an unusual observation in the thiolysed willow bark sample. This solution contained a major peak at 44.6 min that yielded an $[M-H]^-$ ion at *m/z* of 271 (Fig. A.1-I) and a UV/VIS spectrum and retention time that matched authentic naringenin. However, this compound was not present in the original extract, which had contained two distinctive peaks at 32.4 and 32.9 min that gave rise to $[M-H]^-$ ions at *m/z* 433, $[M+H]^+$ ions at *m/z* 435 and cleavage products of *m/z* 273 (Table A.3). Both peaks were reduced to two minor peaks after thiolysis (Fig. A.1-J), which probably suggests that the naringenin peak was generated during thiolysis. However, authentic naringenin-7-*O*-glucoside (with a retention time of 34.5 min) was not cleaved during thiolysis. Given that naringenin, naringenin-7-*O*-glucoside and (±)-naringenin-5-*O*-glucoside (as two peaks) were previously detected in willow bark [10], we propose that, surprisingly, (±)-naringenin-5-*O*-glucoside was degraded under the relatively mild conditions of this thiolysis reaction.

4. Conclusions

Condensed tannins were characterised in acetone/water extracts from fourteen widely used medicinal plants and herbal products. Ten samples had pure procyanidins, two had almost pure prodelphinidins, and another two had CT as mixtures of procyanidins and prodelphinidins. Four extracts also contained A-type procyanidins and one extract had CT with 52% of the flavan-3-ol units as galloylated derivatives. To our knowledge, this is the first detailed analysis of CT in extracts from pine buds, walnut leaves, great water dock roots and heather flowers. Tannins occur in >80 dicotyledonous plant families [36]; however, information on tannin composition is generally hard to find, as their analysis is not trivial [31]. In contrast to food databases [37], European medicinal plants are not generally screened for CT contents or compositions [9] despite the fact that information on CT in medicinal plants would present opportunities for studying their health effects and could add useful information to a medicinal plant database. Such a database could provide a valuable tool for research into CT bioactivities or on their additive or synergistic effects with other compounds. In addition, it could contribute to the standardisation and quality control of herbal products.

Acknowledgements

This study was supported by an EU Marie Curie Initial Training Network (PITN-GA-2011-289377, "LegumePlus"). We thank Ms T.D. Ropiak for help in obtaining plant material Prof E.M. Williamson and Dr P. Lachmann for constructive discussions, Mr R.H. Brown, Mr C. Drake, C. Fryganas, Ms C. Klongsiriwet and Ms P.H. Majid for help in the laboratory.

Appendix A. Supplementary data

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Table 1 Traditional uses of medicinal plant and herbal product samples investigated in this study.

English vernacular name and part used	Latin name of herbal substance	Botanical name(s)	Examples of pharmacological activities/indications (in single form for traditional uses)	Manufacturers' directions of use in form of infusion or decoction
Bilberry leaves (red)	*Myrtilli folium* [b*, c]	*Vaccinium myrtillus* L. [b, d]	-	Relaxing and soothing (bath) [g]
Birch leaves	*Betulae folium* [b, c]	*Betula pendula* Roth and/or *Betula pubescens* Ehrh. [a, b] (*Betula* spp.[d])	Rheumatic complains, urinary illness (irrigation) [1]	Mild urinary tract infections [e]
Blackcurrant leaves	*Ribis nigri folium* [b, c]	*Ribes nigrum* L. [a, b]	Diuretic, inflammatory disorders such as rheumatic conditions, spasmodic cough, colic, diarrhoea and topically to aid wounds [3]	Mild anti-rheumatic [e]
Blackthorn flowers	*Pruni spinosae flos* [c]	*Prunus spinosa* L.	-	General health benefits [f]
Cowberry leaves	*Vitis idaeae folium* [c]	*Vaccinium vitis-idaea* L.	-	Diuretic for mild urinary tract inflammation, renal calculus [e]
Great water dock roots	*Hydrolapathi radix* [c]	*Rumex hydrolapathum* Huds.	-	Relaxing and soothing (bath) [g]
Hawthorn flowers	*Crataegi inflorescentia* [c]	*Crataegus laevigata* Poir. [a] (leaves with flowers of *Crataegus* spp. [d])	Reduction of cardiac performance (leaves with flowers – more recent use) [3]	Fatigue, initial mild cardiac failure where medication is not required [e]
Heather flowers	*Callunae vulgaris flos* [c]	*Calluna vulgaris* (L.) Hull. (leaves with flowers from inflorescence) [d]	Cystitis, urinary infections, rheumatism (leaves with flowers from inflorescence) [1]	Dietary supplement [f]
Hop strobiles	*Lupuli flos* [b, c]	*Humulus lupulus* L. [a, b, d]	Sedative, hypnotic, bactericidal (topically) [38] insomnia, excitability [1] neuralgia, priapism, mucous colitis, crural ulcers, restlessness (due to nervous tension headache and/or indigestion) [3]	Insomnia, nervous tension, calming [e]
Lime tree (*Tilia*) flowers	*Tiliae inflorescentia* [c] (*Tiliae flos* [b])	*Tilia cordata* Miller, *Tilia platyphyllos* Scop., *Tilia x vulgaris* Heyne or their mixtures [a] (*Tilia* spp. [d])	Sedative, antihypertensive [38], migraine, feverish cold [1], hysteria, arteriosclerotic hypertension, arterial pressure (due to arteriosclerosis and nervous tension) [3]	Feverish cold, diaphoretic [e]

Pine buds	*Pini gemmae* [c]	*Pinus* spp.	-	Illness of upper respiratory tract, mucolytic agent [e]
Walnut leaves	*Juglandis folium* [b, c]	*Juglans regia* L. [b, d]	Mild inflammation of skin, excessive perspiration of hand/feet (external use) [1]	Mild inflammation of skin, excessive perspiration of hand/feet (external) [e]
White clover flowers	*Trifolii albi flos*	*Trifolium repens* L.	-	Relaxing and soothing (bath) [g]
Willow bark	*Salicis cortex* [b, c]	*Salix* spp. (various including *S. purpurea* L.; *S. daphnoides* Vill.; *S. fragilis* L.) [a, b]	Anti-inflammatory, anti-rheumatic [38] muscular and arthrodial rheumatism with pain and inflammation, gouty and rheumatoid arthritis, systemic connective tissue disorders with inflammation, influenza, respiratory catarrh, ankylosing spondylitis [3]	Feverish illness, mild anti-rheumatic [e]

Note: [a] – [9], [b] – [39], [b*]– no final opinion [39], [c] – as described by manufacturer, [d] – [1]; sold by manufacturer as either: [e] – medicinal product, [f] – dietary supplement or [g] – herbal product.

Table 2 Condensed tannin (CT) contents, mean degree of polymerisation (mDP), procyanidin/prodelphinidin (PC/PD) and *cis*-/*trans*-flavan-3-ol ratios, percentages of galloylation and A-type linkages in aqueous acetone extracts of medicinal plants or herbal products.

Extract derived from	CT (g/100 g extract)	mDP	PC / PD	*cis* / *trans*	% galloylation
Great water dock roots	25.9 ± 0.7	6.2 ± 0.1	100.0 / 0.0 ± 0.1	94.7 / 5.3 ± 0.1	52.0 ± 0.1
					% A-type bond
Cowberry leaves	16.6 ± 0.4	6.5 ± 0.0	100.0 / 0.0 ± 0.0	68.5 / 31.5 ± 0.1	17.1 ± 0.0
Heather flowers	19.3 ± 0.4	7.2 ± 0.1	100.0 / 0.0 ± 0.1	87.5 / 12.5 ± 0.1	6.7 ± 0.1
Bilberry leaves	12.2 ± 0.5	6.6 ± 0.0	100.0 / 0.0 ± 0.0	92.2 / 7.8 ± 0.0	7.3 ± 0.0
Blackthorn flowers	4.0 ± 0.2	2.9 ± 0.1	100.0 / 0.0 ± 0.1	67.0 / 33.0 ± 0.2	25.3 ± 0.1
Hawthorn flowers	11.5 ± 0.4	4.8 ± 0.0	100.0 / 0.0 ± 0.0	97.3 / 2.7 ± 0.0	
Hop strobiles [a]	3.2 ± 0.1	5.1 ± 0.1	100.0 / 0.0 ± 0.1	64.0 / 36.0 ± 0.1	
Tilia flowers	19.5 ± 0.4	5.8 ± 0.1	100.0 / 0.0 ± 0.0	90.6 / 9.4 ± 0.0	
White clover flowers [a]	13.1[b] ± 0.6	13.3 ± 0.1	1.6 / 98.4 ± 0.1	61.1 / 38.9 ± 0.1	
Pine buds	4.8 ± 0.3	7.8 ± 0.1	61.5 / 38.5 ± 0.0	70.7 / 29.3 ± 0.0	
Birch leaves [a]	4.8 ± 0.1	4.2 ± 0.1	58.9 / 41.1 ± 0.0	62.9 / 37.1 ± 0.0	
Blackcurrant leaves [a]	20.2[b] ± 0.7	6.0 ± 0.1	4.7 / 95.3 ± 0.0	17.7 / 82.3 ± 0.0	
Willow bark	14.6 ± 0.7	4.6 ± 0.1	100.0 / 0.0 ± 0.1	68.7 / 31.3 ± 0.1	
Walnut leaves	5.9 ± 0.3	5.1 ± 0.1	100.0 / 0.0 ± 0.1	62.6 / 37.4 ± 0.1	

Note: [a] – no free flavan-3-ols detected, [b] – previously reported [11].

Table 3 Composition of condensed tannins in terms of flavan-3-ols in terminal and extension units (as molar percentages).

Extract derived from	Flavan-3-ols (%)													
	GC	EGC	C	EC	GC -BM	EGC -BM	C -BM	EC -BM	ECg	ECg -BM				
Great water dock roots	0.0	0.0	5.3	2.4	0.0	0.0	0.0	40.3	8.5	43.5				
±	0.0	0.0	0.1	0.1	0.0	0.0	0.0	0.1	0.1	0.1				
									PC A-type trimer I	PC A-type trimer II	PC A-type dimer I	PC A-type dimer II	PC A-type trimer -BM	PC A-type dimer -BM
Cowberry leaves	0.0	0.0	9.0	2.6	0.0	0.0	16.8	53.7	0.0	1.5	5.8	0.4	5.8	4.4
±	0.0	0.0	0.1	0.1	0.0	0.0	0.2	0.1	0.0	0.1	0.0	0.1	0.0	0.1
Heather flowers	0.0	0.0	8.8	4.2	0.0	*	2.9	78.0	1.9	0.5	0.0	0.0	0.0	3.7
±	0.0	0.0	0.1	0.1	0.0	0.0	0.1	0.1	0.1	0.1	0.0	0.0	0.0	0.1
Bilberry leaves	0.0	0.0	2.1	13.3	0.0	0.0	5.3	72.7	0.0	1.3	0.0	0.0	2.5	2.9
±	0.0	0.0	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.2	0.0	0.0	0.0	0.0
Blackthorn flowers	0.0	0.0	18.6	3.6	0.0	0.0	7.9	50.2	0.0	0.0	15.9	3.9	0.0	0.0
±	0.0	0.0	0.2	0.2	0.0	0.0	0.1	0.2	0.0	0.0	0.1	0.3	0.0	0.0
Hawthorn flowers	0.0	0.0	2.7	18.0	0.0	0.0	*	79.3						
±	0.0	0.0	0.1	0.0	0.0	0.0	0.0	0.0						
Hop strobiles [a]	0.0	0.0	16.6	3.0	*	*	19.4	61.0						
±	0.0	0.0	0.1	0.1	0.0	0.0	0.1	0.1						
Tilia flowers	0.0	0.0	4.2	13.1	0.0	0.0	5.2	77.5						
±	0.0	0.0	0.1	0.1	0.0	0.0	0.1	0.0						
White clover flowers [a]	7.5	*	0.0	0.0	30.8	60.1	0.0	1.0						
±	0.1	0.0	0.0	0.0	0.1	0.1	0.0	0.1						
Pine buds	*	0.0	12.8	0.0	*	38.5	16.5	32.3						
±	0.0	0.0	0.1	0.0	0.0	0.0	0.1	0.0						

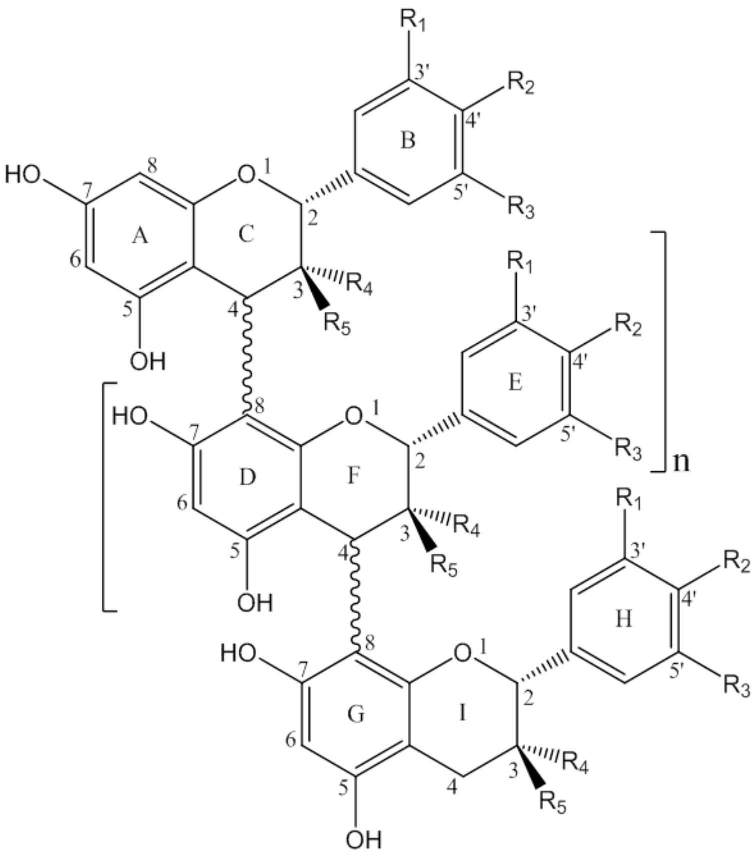
Birch leaves [a]		9.0	0.0	14.9	0.0	6.5	25.6	6.7	37.2
	±	0.1	0.0	0.1	0.0	0.2	0.0	0.1	0.0
Blackcurrant leaves [a]		12.7	2.2	1.6	0.0	66.2	14.2	1.9	1.2
	±	0.1	0.1	0.1	0.0	0.1	0.1	0.0	0.1
Willow bark		0.0	0.0	21.7	*	0.0	0.0	9.6	68.7
	±	0.0	0.0	0.1	0.0	0.0	0.0	0.2	0.1
Walnut leaves		0.0	0.0	19.7	0.0	0.0	0.0	17.7	62.6
	±	0.0	0.0	0.1	0.0	0.0	0.0	0.1	0.1

Note: [a] – no free flavan-3-ols detected; * – trace amounts detected; GC – gallocatechin, EGC – epigallocatechin, C – catechin, EC – epicatechin, GC-BM – gallocatechin benzyl mercaptan adduct, EGC-BM – epigallocatechin benzyl mercaptan adduct, C-BM – catechin benzyl mercaptan adduct, EC-BM – epicatechin benzyl mercaptan adduct, ECg – epicatechin gallate, ECg-BM – epicatechin gallate benzyl mercaptan adduct, PC – procyanidins.

Figure Caption

Fig. 1. Example of B-type condensed tannins.

Figure 1



Flavan-3-ol subunits	R_1	R_2	R_3	R_4	R_5
Catechin	OH	OH	H	H	OH
Epicatechin	OH	OH	H	OH	H
Gallocatechin	OH	OH	OH	H	OH
Epigallocatechin	OH	OH	OH	OH	H

Appendix A.

A.1. Material and methods

A.1.1 Other plant materials

Plant material of *Robiniae flos, Plantaginis maioris folium, Morus alba* L. *folium, Salviae folium, Menthae piperitae folium, Crataegi fructus, Pruni spinosae fructus, Sambuci fructus, Urticae radix, Frangulae cortex Calami rhizoma* (country of origin: Poland) and *Myrtilli fructus* (typical country of origin: Poland and Albania) was obtained from Flos (Mokrsko, Poland; GMP and GLP standard compliant); *Juniperi fructus* (collected in November to December 2012, Poland), *Aroniae fructus* (collected in August to September 2012, Poland) from Kawon (Gostyń, Poland; ISO 9001:2000 and HACCP compliant); *Linum usitatissimum* L. (golden linseeds variety, collected in July to August 2011 and/or 2012 from various European countries, de-fatted and milled in Poland) and *Chamomillae anthodium* (collected in June to July 2011 and/or 2012, Poland) from Herbapol (Lublin, Poland; GMP, BRC Global Standard Food and ECO compliant). Roots and fruits were ground first to pass a 5 mm and then a 1 mm sieve. Plant materials were stored in the dark at room temperature.

A.1.2 Screening of plant materials for CT with HCl/butan-1-ol

Plant materials were first screened for CT presence with HCl/butan-1-ol in duplicate [12]. HCl/butan-1-ol (5 ml, 5:95 v/v) was added to the plant material (50 mg) in a 10 ml test tube and heated at 95 °C for 60 min. Corresponding blanks were kept at room temperature to check for the presence of flavan-4-ols or flavan-3,4-diols [40]. Plant materials that gave dark red colour were used for preparing CT extracts (see Table A.2).

A.1.3 Preparation of plant extracts

Acetone/water was used to prepare the CT extracts [11]. Plant material (20 g) was extracted once with 70% acetone/H_2O (250 ml) by stirring for 60 min and filtered under vacuum.

Chlorophyll and lipids were removed with dichloromethane (125 ml). Solvents were removed on a rotary evaporator at 35 °C and the aqueous phase was centrifuged for 3 min at 4500 rpm (Jouan CR3i Multifunction Centrifuge, Thermo Electron Corporation, Basingstoke, UK). Extracts were freeze-dried and stored at -20 °C (see Table A.2 for extract yields). Deionised water was purified in an Option 3 water purifier (ELGA Process Water, Marlow, UK).

A.1.4 Standards

Standards were injected in methanol for HPLC analysis: (+)-catechin hydrate, (-)-epicatechin, (-)-gallocatechin and (-)-epigallocatechin and (±)-taxifolin at 0.017 mg/ml; (-)-catechin gallate, (-)-epicatechin gallate, (-)-epigallocatechin gallate, (-)-gallocatechin gallate, PC A2 (+)-afzelechin and (±)-taxifolin at 0.013 mg/ml; (±)-eriodictyol, isoquercitrin, naringenin, naringin, naringenin-7-*O*-glucoside, quercetin and rutin at 0.1 mg/ml. Flavonoid standards were injected in methanol/water (80:20 v/v) for LC-MS analysis. Ultrapure water was purified in a Milli-Q Plus system (Millipore, Watford, UK).

Catechin hydrate, epicatechin, gallocatechin and epigallocatechin; catechin gallate, epicatechin gallate, epigallocatechin gallate, gallocatechin gallate, PC A2 and taxifolin were injected at above concentrations for molar response factor evaluation against taxifolin individually or in mixtures Corresponding -BM adducts of these compounds were assumed to have the same molar response factor. PC A-type trimers and their -BM adducts were assumed to have the same molar response factor as PC A2. See Materials and Methods 2.5 and Result and Discussion 3.1.1.

A.1.5. CT quantification

Due to interfering peaks, quantification was done with taxifolin as an external standard [11], which was prepared as thiolysis blank. Taxifolin was dissolved in the ‘thiolysis’ reagent, which was identical to the thiolysis solution used for samples, but it did not contain the sample and benzyl mercaptan was replaced by methanol.

A.2 Calculations of CT composition

The following equations were used for the calculation of:

a) mDP-values of B-type CT and galloylated B-type CT [14, 20]:

$$\mathrm{mDP} = \frac{\sum \mathrm{TU\ (mol)} + \sum \mathrm{EU(mol)}}{\sum \mathrm{TU\ (mol)}}$$

Equation A.2.1

where: TU – terminal flavan-3-ol units; EU – extension flavan-3-ol units;

b) molar percentages of galloylated flavan-3-ols [20] (i.e. % galloylation):

$$\%\ \mathrm{galloylation} = \frac{\sum \mathrm{galloylated\ TU} + \sum \mathrm{galloylated\ EU}}{\sum \mathrm{all\ types\ TU} + \sum \mathrm{all\ types\ EU}} \times 100$$

Equation A.2.2

c) procyanidin/prodelphinidin (PC/PD) ratios [14]:

$$\mathrm{PC}\ /\ \mathrm{PD} = \frac{(\sum \mathrm{C\ units} + \sum \mathrm{EC\ units}) \times 100}{\sum \mathrm{all\ units}} \Big/ \frac{(\sum \mathrm{GC\ units} + \sum \mathrm{EGC\ units}) \times 100}{\sum \mathrm{all\ units}}$$

Equation A.2.3

where: C – catechin, EC – epicatechin, GC – gallocatechin, EGC – epigallocatechin, all units – TU + EU;

d) *cis*/*trans* flavan-3-ol ratios [14], where A-type units were not included:

$$cis\ /\ trans = \frac{(\sum \mathrm{EC\ units} + \sum \mathrm{EGC\ units}) \times 100}{\sum \mathrm{all\ units}} \Big/ \frac{(\sum \mathrm{C\ units} + \sum \mathrm{GC\ units}) \times 100}{\sum \mathrm{all\ units}}$$

Equation A.2.4.

A.3. Analysis of CT

Extract yields ranged from 9 to 70 g/100 g plant dry weight (Table A.2); however, fruit sample extracts had particularly low contents (<3 g CT/100 g extract, data not shown).

No free flavan-3-ols were detected in hawthorn and white clover flowers, hop strobiles, birch and blackcurrant leaves (CT data without correction for free flavan-3-ol monomers are reported for comparison purposes and resulted in minor differences; Table 2 and 3 vs. Table A.5 and A.6).

Table A.1 List of other medicinal plants and herbal products screened for condensed tannins.

Example of English vernacular name and part of plant used	Latin name of herbal substance	Botanical name of plant(s)
Bilberry fruits	*Myrtilli fructus* [b, c]	*Vaccinium myrtillus* L. [a, b, d]
Black locust flowers	*Robiniae flos* [c]	*Robinia pseudoacacia* L.
Blackthorn fruits	*Pruni spinosae fructus* [c]	*Prunus spinosa* L.
Broadleaf plantain leaves	*Plantaginis maioris folium* [c]	*Plantago maior* L.
Calamus rhizome	*Calami rhizoma* [c]	*Acorus calamus* L.
Chamomile flowers	*Chamomillae anthodium* [c]	*Matricaria chamomilla* L.
Chokeberries fruits	*Aroniae fructus* [c]	*Aronia* Medik.
Elderberry fruits	*Sambuci fructus* [b*, c]	*Sambucus nigra* L.[b*]
Frangula bark	*Frangulae cortex* [b, c]	*Rhamnus frangula* L. [a, b, d] (*Frangula alnus* Miller) [a]
Hawthorn fruits	*Crataegi fructus* [c]	*Crataegus monogyna* Jacq. (Lindm.); *Crataegus laevigata* (Poir.) D.C. or other European [a] *Carataegus* spp. [a, d]
Juniper fruits/berry	*Juniperi fructus* [c] (*Juniperi pseudo-fructus* [b])	*Juniperus communis* L. [a, b]
Linseed seeds	*Lini semen* [b]	*Linum usitatissimum* L. [a, b, d]
Nettle roots	*Urticae radix* [b, c]	*Urtica dioica* L. [d]; *Urtica urens* L. [b]
Peppermint leaves	*Menthae piperitae folium* [b, c]	*Mentha x piperita* L. [a, b, d]
Sage leaves	*Salviae folium* [c] (*Salviae officinalis folium* [b])	*Salvia officinalis* L. [a, b, d]
White mulberry leaves	*Morus alba L. folium* [c]	*Morus alba* L.

Note: [a] – [9], [b] – [39], [b*]– no final opinion [39], [c] – as described by manufacturer, [d] – [1].

Table A.2 Results of HCl/butanol screening of plant materials and yields (g extract/100 g dry weight of plant) of extracts.

Plant material	HCl/butanol		Yield	Plant material	HCl/butanol		Yield
	sample	control	(%)		sample	control	(%)
Great water dock roots	+	-	26.5	Blackthorn fruits	+	-	29.0
Blackthorn flowers	+	-	24.5	Bilberry fruits	+	+	63.0
Hawthorn flowers	+	-	23.5	Heather flowers	+	+	9.0
Willow bark	+	-	20.5	Chokeberry fruits	+	+	46.0
Hop strobiles	+	-	18.0	Hawthorn fruits	+/-	-	65.0
Tilia flowers	+	-	21.5	Juniper fruits/berry	-	-	
White clover flowers	+	-	17.0	Black locust flowers	-	-	
Pine buds	+	-	15.0	Frangula bark	-	-	
Walnut leaves	+	-	19.0	Broadleaf plantain leaves	-	-	
Bilberry leaves	+	-	9.5	Linseed seeds	-	-	
Birch leaves	+	-	24.0	Sage leaves	-	-	
Blackcurrant leaves	+	-	17.5	Chamomile flowers	-	-	
Cowberry leaves	+	-	36.0	Peppermint leaves	-	-	
Elderberry fruits	+	-	44.0	White mulberry leaves	-	-	
Calamus rhizome	+	-	70.0	Nettle roots	-	-	

Legend: + positive (colour change to red), - negative (no colour change to red).

Table A.3. Typical HPLC retention times and *m/z* values of flavan-3-ols and selected flavonoids.

No	Compound	Retention times (min) gradient 1	Retention times (min) gradient 2	*m/z* Molecular ion [M-H]$^-$	*m/z* Typical other fragments
1	Gallocatechin	20.9	19.4	305	340
2	Epigallocatechin	24.5	25.2	305	340
3	Catechin	26.2	26.8	289	325
4	Epicatechin	28.6	31.1	289	325
5	Taxifolin	34.2	38.8	303	
6	3,4-*trans*-gallocatechin-BM	40.6	50.7	427	303
7	3,4-*cis*-gallocatechin-BM	40.9	51.8	427	303
8	3,4-*trans*-epigallocatechin-BM	41.3	53.8	427	303
9	3,4-*trans*-catechin-BM	43.4	57.6	411	447, 287
10	3,4-*cis*-catechin-BM	43.9	58.2	411	447, 287
11	3,4-*trans*-epicatechin-BM	44.4	58.8	411	447, 287
12	Benzyl mercaptan and unidentified compounds	48.5	47.8	-	-
13	Epicatechin gallate	33.2	39.6	441	477
14	Epicatechin-BM gallate	43.2 and 45.3	61.5 and 62.8	563	599
15	PC A-type trimer	28.2 and 31.5	29.1 and 32.9	863	497, 325, 289, 141
16	PC A-type dimer	31.7 and 33.2	37.5 and 40.1	575	615, 633, 594, 319, 275, 141
17	PC A-type trimer-BM	42.2	56.1	986	862, 510, 430, 301, 141, 113
18	PC A-type dimer-BM	45.3	63.1	697	733, 573, 437, 141, 113
a	Rutin	31.7	38.5	609	321
b	Quercetin-hexoside [a, b]	32.9	40.1	463	
c	Quercetin [a]	39.6	51.9	301	
d	Possibly naringenin-*O*-hexoside [c]	32.3 and 32.9	38.4 and 39.4	433	593

e	Naringenin	44.9	60.3	271

Note: -BM – benzyl mercaptan adduct, PC – procyanidins; previously reported: [a] – quercetin-3-*O*-glucoside, quercetin-3-*O*-*β*-galactoside and quercetin were detected in bilberry leaves [35]; [b] – quercetin-3-*O*-glucoside in *Tilia* flowers [33]; [c] – in line with [10].

Table A.4 Data of B-type condensed tannins and galloylated procyanidins in extracts that were not corrected for free flavan-3-ols.*

Extract derived from	CT (g/100 g)	mDP	PC / PD	*cis* / *trans*	% galloylation
Great water dock roots	27.3 ± 0.7	4.9 ± 0.1	100.0 / 0.0 ± 0.1	92.1 / 7.9 ± 0.1	51.5 ± 0.1
					% A-type bond
Cowberry leaves	17.9 ± 0.4	4.5 ± 0.0	100.0 / 0.0 ± 0.0	60.7 / 39.3 ± 0.1	17.1 ± 0.0
Heather flowers	19.5 ± 0.4	6.8 ± 0.1	100.0 / 0.0 ± 0.1	86.6 / 13.4 ± 0.1	6.7 ± 0.1
Bilberry leaves	12.4 ± 0.5	6.1 ± 0.0	100.0 / 0.0 ± 0.0	92.3 / 7.7 ± 0.0	7.3 ± 0.0
Blackthorn flowers	4.3 ± 0.2	2.5 ± 0.1	100.0 / 0.0 ± 0.1	62.9 / 37.1 ± 0.1	25.3 ± 0.1
Hawthorn flowers	11.9 ± 0.4	4.2 ± 0.0	100.0 / 0.0 ± 0.0	97.4 / 2.6 ± 0.0	
Tilia flowers	21.0 [a] ± 0.4	5.1 ± 0.0	100.0 / 0.0 ± 0.0	90.8 / 9.2 ± 0.0	
Pine buds	4.8 ± 0.3	7.0 ± 0.1	62.2 / 37.9 ± 0.0	69.6 / 30.4 ± 0.0	
Willow bark	15.4 ± 0.7	3.8 ± 0.1	100.0 / 0.0 ± 0.1	64.8 / 35.2 ± 0.1	
Walnut leaves	6.2 ± 0.3	4.2 ± 0.1	100.0 / 0.0 ± 0.1	59.5 / 40.5 ± 0.1	

Note: * – for comparison purposes only, CT – condensed tannin, mDP – mean degree of polymerisation, PC/PD – procyanidin/prodelphinidin ratios, *cis*/*trans* – *cis*-/*trans*-flavan-3-ol ratios, [a] – previously reported [11].

Table A.5 Composition of flavan-3-ol in extracts as molar percentages that were not corrected for free flavan-3-ols. [a]

Extract derived from		Flavan-3-ols (%)													
		GC	EGC	C	EC	GC -BM	EGC -BM	C -BM	EC -BM	ECg	ECg -BM				
Great water dock roots		0.0	0.0	7.9	2.3	0.0	0.0	0.0	38.3	10.2	41.3				
	±	0.0	0.0	0.1	0.1	0.0	0.0	0.0	0.1	0.1	0.1				
										PC A-type trimer I	PC A-type trimer II	PC A-type dimer I	PC A-type dimer II	PC A-type trimer -BM	PC A-type dimer -BM
Cowberry leaves		0.0	0.0	17.7	2.3	0.0	0.0	15.2	48.6	0.0	1.4	5.3	0.4	5.2	3.9
	±	0.0	0.0	0.0	0.1	0.0	0.0	0.2	0.1	0.0	0.0	0.0	0.1	0.0	0.1
Heather flowers		0.0	0.0	9.7	4.2	0.0	*	2.9	77.2	1.9	0.4	0.0	0.0	0.0	3.6
	±	0.0	0.0	0.1	0.1	0.0	0.0	0.1	0.1	0.1	0.1	0.0	0.0	0.0	0.1
Bilberry leaves		0.0	0.0	2.0	14.8	0.0	0.0	5.2	71.4	1.3	0.0	0.0	0.0	2.4	2.9
	±	0.0	0.0	0.2	0.0	0.0	0.0	0.0	0.0	0.2	0.0	0.0	0.0	0.0	0.0
Blackthorn flowers		0.0	0.0	23.4	6.6	0.0	0.0	7.1	45.1	0.0	0.0	14.3	3.5	0.0	0.0
	±	0.0	0.0	0.1	0.1	0.0	0.0	0.1	0.1	0.0	0.0	0.1	0.1	0.0	0.0
Hawthorn flowers		0.0	0.0	2.6	21.0	0.0	0.0	*	76.4						
	±	0.0	0.0	0.1	0.0	0.0	0.0	0.0	0.0						
Tilia flowers		0.0	0.0	4.1	15.6	0.0	0.0	5.1	75.2						
	±	0.0	0.0	0.1	0.1	0.0	0.0	0.1	0.0						
Pine buds		*	0.0	14.2	0.0	*	37.8	16.2	31.7						
	±	0.0	0.0	0.1	0.0	0.0	0.0	0.1	0.0						
Willow bark		0.0	0.0	26.1	*	0.0	0.0	9.1	64.8						
	±	0.0	0.0	0.1	0.0	0.0	0.0	0.2	0.1						
Walnut leaves		0.0	0.0	23.7	0.0	0.0	0.0	16.8	59.5						
	±	0.0	0.0	0.1	0.0	0.0	0.0	0.1	0.1						

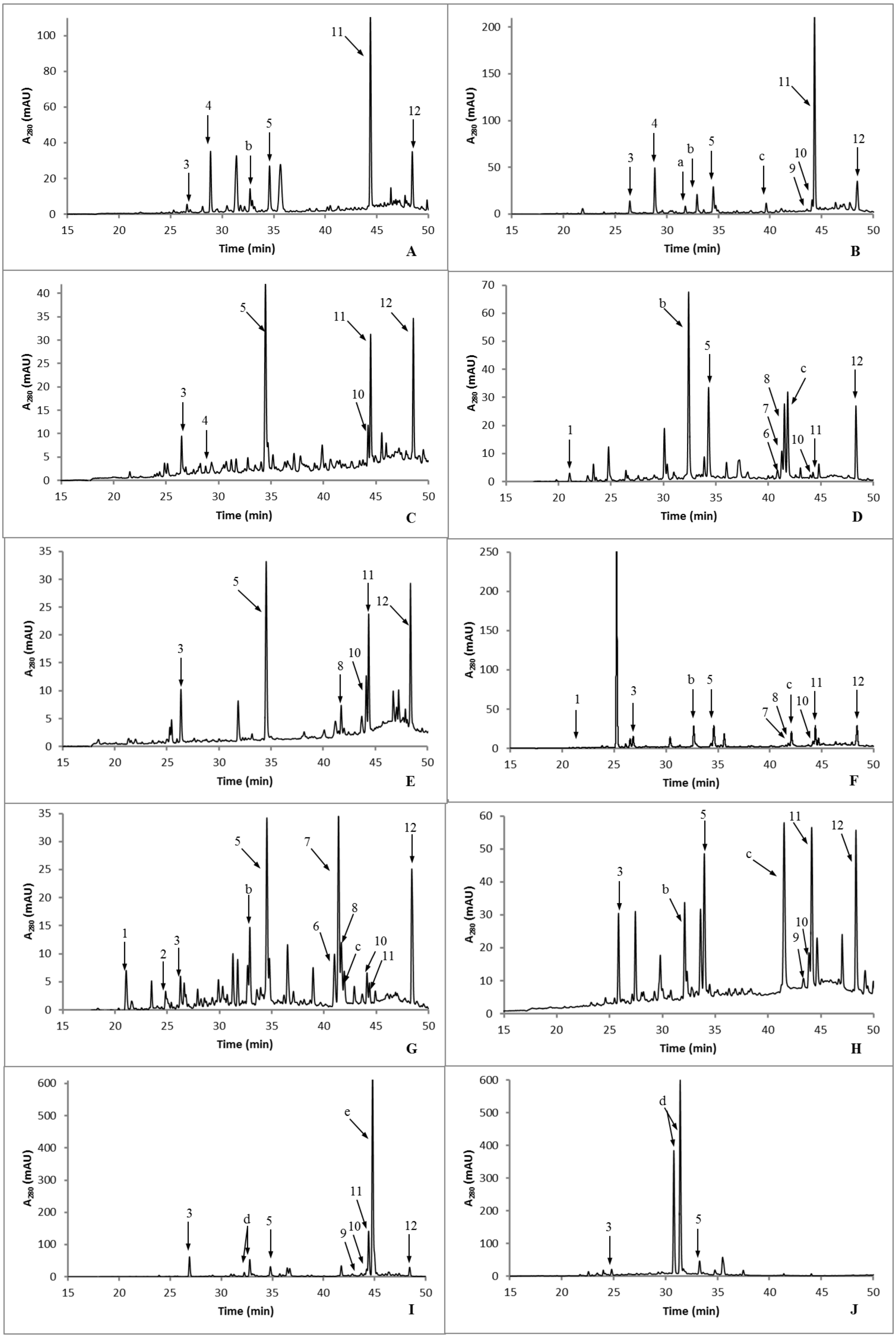
Note: [a] – for comparison purposes only, * – trace amounts detected; GC – gallocatechin, EGC – epigallocatechin, C – catechin, EC – epicatechin, GC-BM – gallocatechin benzyl mercaptan adduct, EGC-BM – epigallocatechin benzyl mercaptan adduct, C-BM – catechin benzyl mercaptan adduct, EC-BM – epicatechin benzyl mercaptan adduct, ECg – epicatechin gallate, ECg-BM – epicatechin gallate benzyl mercaptan adduct, PC – procyanidins.

Figure Captions

Fig. A.1. Examples of RP-HPLC chromatograms after thiolysis of extracts: A – hawthorn flowers, B – *Tilia* flowers, C – hop strobiles, D – white clover flowers, E – pine buds, F – birch leaves, G – blackcurrant leaves, H – walnut leaves, I – willow bark, J – willow bark (not thiolysed, shown for comparison); where: 1 – gallocatechin, 2 – epigallocatechin, 3 – catechin, 4 – epicatechin, 5 – taxifolin (internal standard), 6 – gallocatechin-BM (*trans*), 7 – gallocatechin-BM (*cis*), 8 – epigallocatechin-BM, 9 – catechin-BM (*trans*), 10 – catechin-BM (*cis*), 11 – epicatechin-BM, 12 – benzyl mercaptan and unidentified compounds; a – rutin, b – quercetin-*O*-hexoside, c – quercetin, d – possibly naringenin-*O*-hexoside.

Fig. A.2. Examples of RP-HPLC chromatograms after thiolysis of extracts: A – great water dock roots, B – cowberry leaves, C – heather flowers, D – bilberry leaves, E – blackthorn flowers; where: 1 – gallocatechin, 2 – epigallocatechin, 3 – catechin, 4 – epicatechin, 5 – taxifolin (internal standard), 6 – gallocatechin-BM (*trans*), 7 – gallocatechin-BM (*cis*), 8 – epigallocatechin-BM, 9 – catechin-BM (*trans*), 10 – catechin-BM (*cis*), 11 – epicatechin-BM, 12 – benzyl mercaptan and unidentified compounds, 13 – epicatechin gallate, 14 – epicatechin-BM gallate, 15 – procyanidin A-type trimer, 16 – procyanidin A-type dimer, 17 – procyanidin A-type trimer-BM, 18 – procyanidin A-type dimer-BM; a – rutin, b – quercetin-*O*-hexoside, c – quercetin.

Figure A.1


A280 (mAU)
Time (min)
A
3
4
b
5
11
12
B
3
4
a
b
5
c
9
10
11
12
C
3
4
5
10
11
12
D
1
b
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6
7
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c
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E
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G
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c
10
11
12
H
3
b
5
c
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10
11
12
I
3
d
5
9
10
11
e
12
J
3
d
5

Figure A.2