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ELLAGITANNINS IN FINNISH PLANT SPECIES — CHARACTERIZATION, DISTRIBUTION AND OXIDATIVE ACTIVITY

Johanna Moilanen

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ABSTRACT

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Ellagitannins are secondary metabolites that are produced by plants. Among other features, they are assumed to function as plants' defensive compounds against plant-eating herbivores. This thesis focuses on a theory, which suggests that the biological activity of ellagitannins is based on their tendency to oxidize at the highly alkaline gut conditions of insect herbivores (oxidative activity).

To study the biological activities of ellagitannins, a wide variety of structurally different ellagitannins were purified from different plant species by using liquid chromatographic techniques. The structures were characterized with the aid of spectrometric methods. Based on the acquired data, it was also possible to create a scheme, which enables the classification and even identification of ellagitannins from plant extracts without the need to isolate each compound for individual characterization.

The biological activities of ellagitannins were determined with methods that are based on the abilities of the compounds to scavenge radicals, chelate iron ions, and on their rate of oxidation at high pH. The results showed that ellagitannins possess oxidative activities both at high and neutral pH, and that their activities depend on structure.

The occurrence, distribution and content of ellagitannins in Finnish plant species were also studied. The specific ellagitannin profiles of the studied plant species were found to correlate well with their taxonomic classification.

Keywords: Ellagitannin, biological activity, taxonomy

TIIVISTELMÄ

TURUN YLIOPISTO
Kemian laitos/Matemaattis-luonnontieteellinen tiedekunta

MOILANEN, JOHANNA: Ellagitanniinit suomalaisissa kasveissa – Karakterisointi, esiintyminen ja oksidatiivinen aktiivisuus

Väitöskirja, 156 s.
Luonnonyhdisteiden kemia
Kesäkuu 2015

Ellagitanniinit ovat kasvien tuottamia sekundääriyhdisteitä, joiden oletetaan toimivan muun muassa kasvien puolustusyhdisteinä niitä ravintonaan käyttäviä kasvinsyöjiä vastaan. Tämän väitöskirjan pääpaino on teoriassa, jonka mukaan ellagitanniinien biologinen aktiivisuus perustuu niiden hapettumisherkkyyteen (oksidatiiviseen aktiivisuuteen) hyönteistoukkien ruoansulatuskanavan korkeassa pH:ssa.

Biologisen aktiivisuuden määrittämiseksi rakenteeltaan erilaisia ellagitanniineja puhdistettiin eri kasvilajeista nestekromatografisin menetelmin. Rakenteet karakterisoitiin spektrometrisin menetelmin. Saadun aineiston pohjalta luotiin myös kaavio, jonka avulla ellagitanniinien luokittelu ja jopa tunnistaminen on mahdollista tehdä suoraan kasviuutteesta, ilman että yksittäisiä yhdisteitä tarvitsee eristää ja puhdistaa tunnistamista varten.

Biologisen aktiivisuuden määrittämiseksi käytettiin menetelmiä, jotka perustuvat yhdisteiden radikaalinsieppauskykyyn, kykyyn kelatoida metalli-ioneja sekä hapettumisnopeuteen korkeassa pH:ssa. Tulokset osoittivat, että ellagitanniinit ovat oksidatiivisesti aktiivisia sekä korkeassa että neutraalissa pH:ssa, ja että niiden aktiivisuus riippuu rakenteesta.

Lisäksi työssä tutkittiin ellagitanniinien esiintymistä suomalaisissa kasveissa sekä sitä, kuinka paljon ja minkä tyyppisiä ellagitanniineja eri kasvilajit tuottavat. Kasvien ellagitanniinikoostumusten havaittiin korreloivan hyvin niiden taksonomisen luokittelun kanssa.

Asiasanat: Ellagitanniini, biologinen aktiivisuus, taksonomia

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PREFACE AND ACKNOWLEDGEMENTS

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Forssa, May 2015

Johanna Moila

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications and some unpublished results. The publications are referred to in the text by their Roman numerals.

I Moilanen, J., Sinkkonen, J., Salminen, J.-P., 2013. Characterization of bioactive plant ellagitannins by chromatographic, spectroscopic and mass spectrometric methods. *Chemoecology* 23: 165–179.

II Moilanen, J., Koskinen, P., Salminen, J.-P., 2015. Distribution and content of ellagitannins in Finnish plant species. *Phytochemistry* http://dx.doi.org/10.1016/j.phytochem.2015.03.002.

III Moilanen, J., Salminen, J.-P., 2008. Ecologically neglected tannins and their biologically relevant activity: chemical structures of plant ellagitannins reveal their in vitro oxidative activity at high pH. *Chemoecology* 18: 73–83.

IV Moilanen, J., Karonen, M., Tähtinen, P., Jacquet, R., Quideau, S., Salminen, J.-P. Biological activity of ellagitannins: effects as antioxidants, pro-oxidants and metal chelators. Manuscript submitted to *Chemoecology*.

ABBREVIATIONS

1D	one dimensional
2D	two dimensional
2-DR	2-deoxyribose
AA	ascorbic acid
Abs	absorbance
ACN	acetonitrile
CD	circular dichroism
$CDCl_3$	deuterated chloroform
CID	collision-induced dissociation
COLOC	correlation via long-range coupling
COSY	correlation spectroscopy
CPC	centrifugal partition chromatography
D_2O	deuterated water
DAD	diode array detector
DC	direct current
DCCC	droplet counter-current chromatography
DHA	dehydroascorbate
DHHDP	dehydrohexahydroxydiphenoyl
DNA	deoxyribonucleic acid
DPPH	2,2-diphenyl-1-picrylhydrazyl radical
DQF-COSY	double-quantum filtered correlation spectroscopy
dw	dry weight
ECI	efficiency of conversion of ingested food to biomass
EDTA	disodium ethylenediaminetetraacetic acid
ESI	electrospray ionization
ET	ellagitannin
EtOH	ethanol
FAB	fast atom bombardment
$FeCl_3$	iron chloride
fw	fresh weight
GC	gas chromatography

GSH	glutathione
GSSG	oxidized glutathione
GT	gallotannin
H_2O	water
H_2O_2	hydrogen peroxide
H_3PO_4	phosphoric acid
HCOOH	formic acid
HHDP	hexahydroxydiphenoyl
HILIC	hydrophilic interaction chromatography
HMBC	heteronuclear multiple bond correlation
$HO_2^{\bullet-}$	hydroperoxyl radical
HOCl	hypochlorous acid
HPLC	high-performance liquid chromatography
HSQC	heteronuclear single quantum coherence
HT	hydrolysable tannin
IC_{50}	inhibition concentration at 50 % inhibition point
KIO_3	potassium iodate
K_{ow}	water-octanol partition coefficient
LC	liquid chromatography
m/z	mass-to-charge ratio
MALDI	matrix-assisted laser desorption ionization
m-DOG	valoneoyl
Me_2CO	acetone
MeOH	methanol
m-GOD	sanguisorboyl
m-GOG	isodehydrodigalloyl
MS	mass spectrometry
MS/MS	tandem mass spectrometry
MS^n	multistage mass spectrometry
MW	molecular weight
N_2	nitrogen
$NaBH_4$	sodium borohydride
NaH_2PO_4	sodium dihydrogen phosphate
$NaNO_2$	sodium nitrite

NaOH	sodium hydroxide
NHTP	nonahydroxytriphenoyl
NMR	nuclear magnetic resonance
NOESY	nuclear Overhauser effect spectroscopy
NP	normal phase
O_2	oxygen
$O_2^{\bullet -}$	superoxide anion radical
O_3	ozone
$OH^{\bullet}$	hydroxyl radical
PA	proanthocyanidin
PDA	photodiode array detector
p-DOG	tergalloyl
PE	peritrophic envelope
PGG	pentagalloyl glucose
p-GOG	dehydrodigalloyl
PPC	protein precipitation capacity
ppm	parts per million
Q	quadrupole
QIT	quadrupole iontrap
RCR	relative consumption rate
RF	radio frequency
$RO^{\bullet}$	alcoxyl radical
$RO_2^{\bullet}$	peroxyl radical
ROS	radical oxygen species
RP	reversed phase
SEM	standard error of the mean
TBA	2-thiobarbituric acid
TCA	trichloroacetic acid
TMS	tetramethylsilane
TOCSY	total correlation spectroscopy
TOF	time-of-flight
TQ, QqQ	triple quadrupole
UHPLC	ultra-high-performance liquid chromatography
V, U	electric potential

1 INTRODUCTION

Plants metabolize hundreds of compounds in their cells. Some compounds, such as amino acids, sugars and proteins, are essential for the survival, growth and reproduction of plants and because of that are called primary compounds or primary metabolites. In addition to primary compounds, plants produce a wide variety of secondary compounds, i.e., metabolites that are not considered to be essential for the survival of the plant. These secondary compounds include, among many others, tannins, alkaloids, flavonoids, phenolic acids and terpenes (Crozier et al., 2006). Although these compounds do not have a role in the primary metabolism of a plant, they are believed to have protective functions, such as protection against ultraviolet radiation and plant-eating herbivores (Crozier et al., 2006).

Tannin is a common word that has been (and still is) used to name plant-based polyphenolic compounds. However, the original meaning of this word clearly refers to plant material that can turn hide into leather; the word tannin comes from the French word ‘tan’ that means powdered oak bark extract, which in turn has been derived from Celtic prefix ‘tann-‘ meaning oak (Haslam, 1988, 1989; Quideau et al., 2011). Nevertheless, the only “true” tannins can be divided into three subclasses: hydrolysable tannins, proanthocyanidins (condensed tannins) and phlorotannins.

Hydrolysable tannins (HTs) are esters of gallic acid and a polyol, which in most cases is D-glucose (Quideau and Feldman, 1996). HTs are divided into two subgroups, i.e., gallotannins and ellagitannins, based on their structures. In both cases the basic structure from which they are derived is pentagalloyl glucose (PGG; Fig. 1). Gallotannins (GTs) are formed when additional galloyl units are attached to PGG via *m*- or *p*-depside bonds (Fig. 1; Haslam, 1988). Ellagitannins (ETs) are formed when two or more neighboring galloyl groups of PGG are attached to each other by carbon-carbon (C–C) bonds, thus forming the characteristic feature of ETs, a hexahydroxydiphenoyl (HHDP) group (Fig. 1; Quideau and Feldman, 1996). Proanthocyanidins (PAs) are oligo- and polymers of flavan-3-ols (Fig. 1; Haslam, 1988). Phlorotannins are also oligo- and polymers, but their basic building unit is phloroglucinol (Fig. 1; Quideau et al.,

2011). Phlorotannins have only been detected in brown algae (Steinberg, 1995), while PAs and HTs are widely distributed in the plant kingdom.

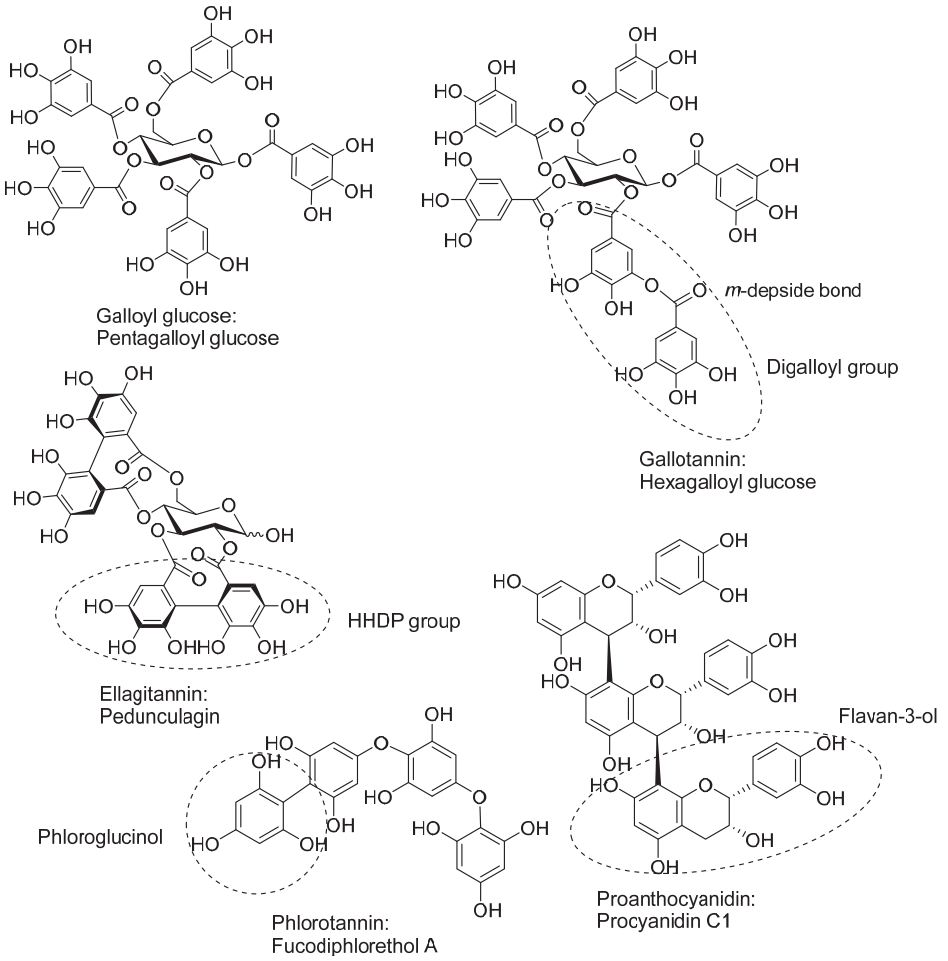


Figure 1. Example structures of tannin subgroups. Pentagalloyl glucose is a precursor molecule for gallotannins (hexagalloyl glucose) and ellagitannins (pedunculagin). Procyanidin C1 is an example of proanthocyanidins and fucodiphlorethol A is an example of phlorotannins. HHDP = hexahydroxydiphenoyl group.

Tannins are considered to function as anti-herbivore agents by two different mechanisms: by reducing the nutritive value of the plant by precipitating proteins in the guts of herbivores (Feeny, 1968, 1969; Haslam, 1989) and on the other hand by oxidative reactions that can cause oxidative stress to herbivores and damage cellular components and nutrients (Appel, 1993). In the early years of tannin research, the focus was set on the analysis of PAs, probably because of

their greater protein precipitation capacity (PPC; e.g., Feeny, 1968, 1969) which has been the most fundamental characteristic of tannins for decades, and because they have been said to be easier to analyze. Although ETs have also been studied at some level, the extent of their research has been far behind from that of PAs. The turning points for ET research have been the development of chromatographic separation and analysis techniques and the realization of their biological activity (Quideau and Feldman, 1996). Especially the developments in reversed phase (RP) column materials and detection techniques, particularly those concerning diode array detection (DAD) and mass spectrometry (MS), have had significant impact on ET research. These improvements in isolation and analysis techniques together with the advantages of using nuclear magnetic resonance (NMR) spectroscopy has enabled the characterization of approximately a thousand different ET structures (Quideau et al., 2011). As a result, the number of studies on the biological activity of ETs has increased significantly. However, the majority of these researches have mainly been focused on measuring the activity from the human health-promoting perspective (reviewed by e.g., Quideau et al., 2011; Serrano et al., 2009) and not so much on the activities ETs may have on herbivores.

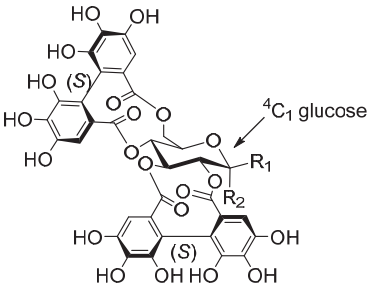
Although the literature on ET analysis, characterization, biological activity and their presence in a wide variety of different plant species has increased in the past few decades, the information is scattered and finding comprehensive data on, e.g., characterization of certain type of ET structures may be difficult. Thus, there is still room for improvements in that field and, more specifically, in the field of making the characterization easier without the necessity to isolate all the compounds in a plant sample for individual characterizations. This comes particularly useful in the case of screening studies, in which numerous samples need to be effectively and quickly analyzed. Therefore, being able to recognize the compound composition of a plant sample, one can easily deduce if that particular plant sample is suitable for large-scale isolation of some specific compound of interest. This is of importance in the case of biological activity measurements, which usually require relatively lot of purified compounds.

2 STRUCTURAL FEATURES OF ELLAGITANNINS

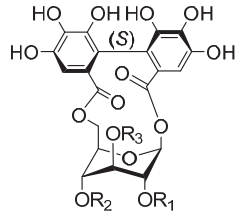
2.1 Monomeric ellagitannins

2.1.1 Glucopyranose-based ellagitannins

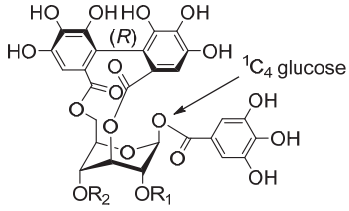
The precursor for the vast variety of different ET structures is PGG and the defining character of ETs is the HHDP group. Majority of the monomeric ETs have a D-glucose as the central polyol but ETs with e.g., a gluconic acid (Yoshida et al., 2009) core have also been found. D-glucose may adopt several conformations from which the chair ($^4C_1$) conformation is the most often found in ET structures, but compounds with the less energetically favored $^1C_4$ conformation also exist (Fig. 2). Additionally, the C-1 of the D-glucose core can have either α or β configuration (Fig. 2: potentillin and casuarictin, respectively). Already these features enable several possible ET structures to be produced by plants. Further, due to axial chirality of the HHDP group (Eliel et al., 1994), it may adopt two different configurations, namely *S* and *R* configurations. However, it has been noticed that in the case of $^4C_1$-glucose-based ETs where the HHDP group is attached to the 2,3- and/or 4,6-position(s) of the glucose core, the HHDP group is in *S* configuration (Okuda et al., 2009). On the other hand, in the case of $^1C_4$-glucose-based ETs where the HHDP group is at 2,4- and/or 3,6-position(s) the configuration of the HHDP goup is *R* (Khanbabaee and van Ree, 2001; Okuda et al., 2009; Quideau and Feldman, 1996). ETs which incorporate an HHDP group in 1,6-position are rare in nature, and so far the only known compounds are davidiin (Hatano et al., 1990a; Self et al., 1986), hellioscopinin A and carpinusin, in which the 1,6-HHDP is in *S* configuration (Fig. 2) (Quideau and Feldman, 1996; Yoshida et al., 2009).



Casuarictin R_1 = (*O*)-galloyl, R_2 = **H**
Potentillin R_1 = H, R_2 = (*O*)-galloyl

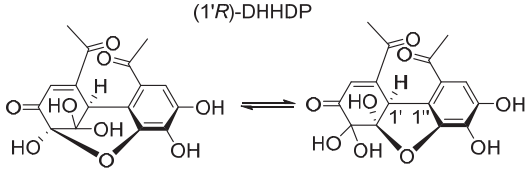


Davidiin R_1 = R_2 = R_3 = galloyl
Hellioscopinin A R_1~R_2 = (1''*S*)-DHHDP, R_3 = galloyl
Carpinusin R_1~R_2 = (1'*R*)-DHHDP, R_3 = galloyl

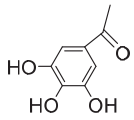


Geraniin R_1~R_2 = (1'*R*)-DHHDP
Chebulagic acid R_1~R_2 = chebuloyl

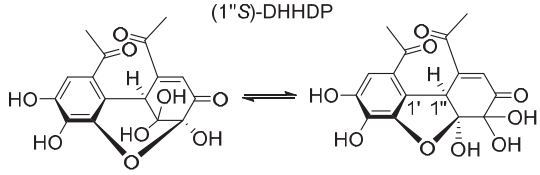
(1'*R*)-DHHDP



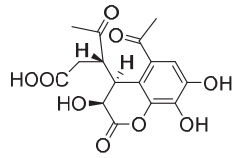
Galloyl

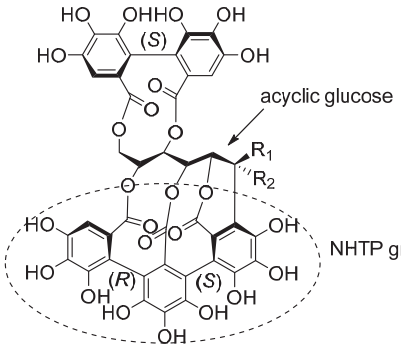


(1''*S*)-DHHDP



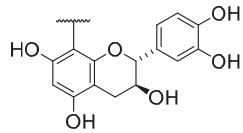
Chebuloyl



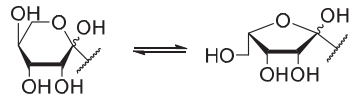


NHTP group

Catechin



Lyxose



Vescalagin R_1= OH, R_2 = **H**
Castalagin R_1= H, R_2 = OH
Acutissimin A R_1 = catechin, R_2 = **H**
Grandinin R_1 = lyxose, R_2 = **H**

Figure 2. Example structures of monomeric ellagitannins. DHHDP = dehydrohexahydroxydiphenoyl, NHTP = nonahydroxytriphenoyl.

2.1.2 Ellagitannins with modified HHDP group

The oxidation of an HHDP group results in a dehydrohexahydroxydiphenoyl (DHHDP) group (Fig. 2). It has been proposed that the DHHDP group is formed from the HHDP group by dehydrogenation, which in turn results to a cyclohexenetrione structure that is stabilized by hydration of one of the ketone carbonyls (Luger et al., 1998; Okuda et al., 1976). In solution, the DHHDP group equilibrates between six- and five-membered rings, and that is why, e.g., the structure of geraniin is usually presented as in Fig. 2. Further oxidation of the DHHDP group leads to modified dehydroETs (Okuda et al., 2009). One typical example of these is the chebuloyl group (Fig. 2). There are still a variety of different oxidized substructures found, but as they are more rarely found they are not presented here.

2.1.3 C-*glucosidic ellagitannins*

Another special subgroup of ETs is the *C*-glucosidic ETs. These compounds have an acyclic glucose core (Fig. 2; Okuda et al., 2009). They are also the only ETs in which three galloyl groups can be joined together to form a nonahydroxytriphenoyl (NHTP) group (vescalagin in Fig. 2). *C*-glucosidic ETs can be divided into vescalagin-type and castalagin-type, depending on the configuration of the hydroxyl (OH) group at C-1. In vescalagin-type ETs, the configuration is β, and in castalagin-type ETs α. *C*-glucosidic ETs are prone to react with other compounds. FlavanoETs or complex tannins are formed when a *C*-glucosidic ET and a flavan-3-ol unit are attached to each other (e.g., acutissimin A in Fig. 2; Okuda et al., 2009). *C*-glucosidic ETs are also often found to be attached to an additional glycosyl unit, such as xylose and lyxose (e.g., grandinin in Fig. 2; Yoshida et al., 2009).

2.2 Oligomeric ellagitannins

The structural diversity of ETs is already versatile within the monomers, but the variety of different compounds becomes even wider when oligomeric ETs are taken into account. Until recently, the largest oligomeric ETs isolated and purified were pentamers (Yoshida et al., 2005), but with the aid of modern mass

spectrometric methods, even an undecameric ET has been characterized (Salminen et al., 2011). The oligomerization of monomeric ETs occurs usually either between two galloyl groups or between a galloyl group of the other monomer and an HHDP group of the other monomer via an ether (C–O–C) linkage (Okuda et al., 2009). *C*-glucosidic ETs may also oligomerize through carbon-carbon (C–C) bonds (Okuda et al., 2009).

2.2.1 Oligomers linked via dehydrodigalloyl groups

Oligomeric ETs with dehydrodigalloyl linkages are formed when two monomeric ETs are bound to each other via ether bonds between two galloyl groups. One galloyl group serves as an *O*-donating group, i.e., one of the OH groups of the galloyl group forms the ether linkage to the carbon atom of another galloyl group. Depending on the position of the ether bond, the group is called either a dehydrodigalloyl group (*m*-GOG) or an isodehydrodigalloyl group (*p*-GOG). The first oligomeric ET isolated was agrimoniin (Fig. 3; Okuda et al., 1982a), which possesses the *m*-GOG group.

2.2.2 Oligomers linked via valoneoyl and sanguisorboyl groups

These linking units are most frequently found from oligomeric structures (Okuda et al., 2009). In a valoneoyl linkage, the other monomer donates one of the OH groups in an HHDP group to the ether bond and the other monomer accepts this bond to its unsubstituted galloyl group carbon atom. This is also referred to as a DOG-linkage (Okuda et al., 2009). Again, there are different possibilities how the linkage can be formed: in the valoneoyl group, the bond is formed from the *m*-position of the HHDP group, as in rugosin D (Fig. 3; Hatano et al., 1990b; Okuda et al., 1982b). In a tergalloyl group, the ether bond is formed from the *p*-position of the HHDP group. A special modification of the valoneoyl group linking unit are macrocyclic ETs, in which two HHDP groups are coupled to two galloyl groups to form two valoneoyl groups as in oenothein B (Fig. 3; Hatano et al., 1989a). In a sanguisorboyl linkage, the OH-donating group is the galloyl group and the HHDP group accepts the bond. This is also referred to GOD-linkage and is found, e.g., from sanguiin H-6 (Fig. 3; Nonaka et al., 1982).

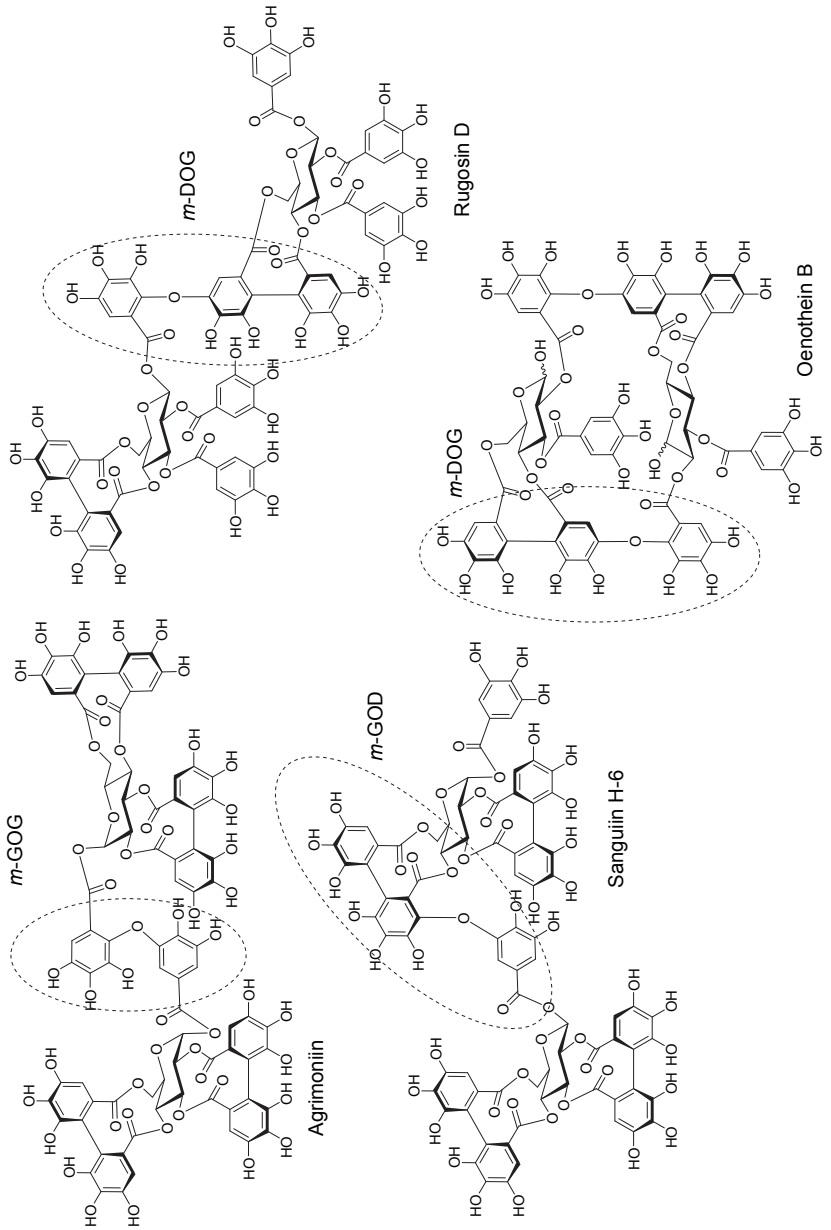


Figure 3. Structural examples of different linking units formed during the oligomerization of ellagitannins. *m*-GOG = dehydrodigalloyl linkage, *m*-DOG = valoneoyl linkage, *m*-GOD = sanguisorboyl linkage

2.2.3 C-glucosidic oligomers

Most of the reported *C*-glucosidic ET oligomers are formed via C–C bonds between the C-1 of the sugar moiety of the other monomer and the HHDP or galloyl group of the other monomer (Fig. 4 roburin A; Hervé du Penhoat et al., 1991). It is worth noting that in these oligomers, the C-1 participating to the C–C bond in most cases possesses the *β*-orientation, which means that this monomer is of vescalagin-type (Yoshida et al., 2009). However, there are some exceptions to this rule. These exceptions rise from the fact that the oligomerization can also take place at other parts of the structure. Examples of these kinds of ET structures are cocciferin D_2, which is composed of a *C*-glucosidic ET (castalagin) and a glucopyranose-based ET (casuarictin) (Ito et al., 2002) and salicarinins A–C, which are composed of two *C*-glucosidic ETs (vescalagin/castalagin and stachyurin/casuarinin pairs; Fig. 4) (Piwowarski and Kiss, 2013). In both of these cases, the oligomerization occurs via a valoneoyl (*m*-DOG) group.

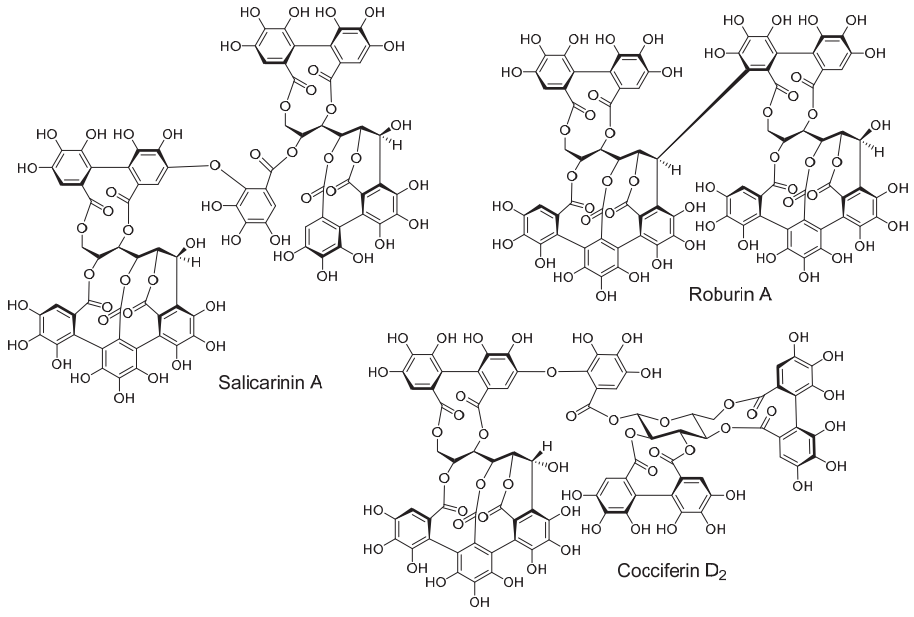


Figure 4. Structural examples of *C*-glucosidic ellagitannin oligomers.

3 ANALYSIS, PURIFICATION AND CHARACTERIZATION OF ELLAGITANNINS

In the early stages of ET research, the main methods for analysis and characterization were paper chromatography, NMR spectroscopy, different colorimetric methods (sodium nitrite, rhodanine and potassium iodate assays) and hydrolysis (e.g., Gupta et al., 1982; Haddock et al., 1982a). However, the resolution of paper chromatography is poor (Okuda et al., 1979; Scalbert et al., 1990), and the specificity and accuracy of the colorimetric methods are questionable (Waterman and Mole, 1994). The hydrolysis, on the other hand, is time-consuming and laborious as each hydrolysis product has to be purified and characterized separately. Later, especially the colorimetric methods and hydrolysis have been replaced by other methods, such as high-performance liquid chromatography (HPLC) coupled to a diode array detector (DAD) or different mass detectors. Still, NMR spectroscopy is the most widely used method for the characterization of purified compounds and with the aid of circular dichroism (CD) spectroscopy the absolute configuration of the compounds can be obtained.

3.1 Methods for analyzing ellagitannins

3.1.1 Colorimetric assays

The rhodanine assay (Inoue and Hagerman, 1988) is a specific method for the determination of gallic acid. Thus, this method is applicable for the detection of both types of hydrolysable tannins, i.e., GTs and ETs. In the reaction, esterified gallic acid groups are hydrolyzed to free gallic acid, which then reacts with rhodanine to give a gallic acid-rhodanine complex whose absorbance can be measured spectrophotometrically at 518 nm (Inoue and Hagerman, 1988). The shortcoming of the assay is that two separate determinations have to be performed: one that is made before the hydrolysis and second that is made after the hydrolysis. The first measurement will reveal if the sample contains free gallic acid and this result is subtracted from the final results after the hydrolysis

to obtain the amount of esterified gallic acid residues (Inoue and Hagerman, 1988). Another shortcoming is that the method does not distinguish GTs from ETs, since it only detects gallic acid. Thus, if plant extracts are analyzed, one has to use other methods to find out whether the samples contain ETs or not.

The potassium iodate (KIO_3) assay (Bate-Smith, 1977) detects both gallic acid and ellagic acid. However, the method is sensitive to timing and temperature, and in addition, to side reactions and formation of precipitates (Bate-Smith, 1977; Waterman and Mole, 1994). The method was later optimized by Hartzfeld et al. (2002) for the detection of gallic acid. A more specific assay for the determination of ETs is the sodium nitrite ($NaNO_2$) assay (Bate-Smith, 1972; Wilson and Hagerman, 1990). In this method, ETs are detected from a plant sample by hydrolyzing the sample in acidic conditions and by applying $NaNO_2$ solution to obtain blue-colored solution, which indicates the presence of ellagic acid (Bate-Smith, 1972). However, there are several problems concerning the analytical conditions. These include long hydrolysis time, the use of anoxic reaction conditions, the use of pyridine and the need to use absolutely clean glassware (Waterman and Mole, 1994; Wilson and Hagerman, 1990).

3.1.2 Chromatography

Before the development of HPLC, paper chromatography was used for analytical separations. The detection of ETs was achieved by spraying the paper with color reagents, such as a solution consisting of nitrous acid, ice-cold 10 % $NaNO_2$ and glacial acetic acid solution, which revealed ETs as rose-red spots that turned rapidly through green, brown and purple to indigo-blue (Gupta et al., 1982). However, as the liquid chromatographic equipment evolved, this method was quickly replaced.

HPLC can be regarded to be the most sensitive and comprehensive method to determine phenolic compounds from plant samples. Majority of ET analyses are nowadays performed with RP-HPLC. In the RP-HPLC, the column material is nonpolar and widely used materials include silica to which octadecyl (C_{18}) groups are bound. Elution is performed with relatively polar solutions, usually with water containing some acid [e.g., formic acid (HCOOH) or *o*-phosphoric acid (H_3PO_4)] and acetonitrile (ACN) or methanol (MeOH). The acid is added to

the eluent(s) for preventing ionization of compounds, and thus for improving the chromatographic separation. Various analysis conditions and column materials have been reviewed by, e.g., Arapitsas (2012).

The latest achievement in the development of LC technology is the ultra-high-performance liquid chromatography (UHPLC). The development of ultra-high pressure pumps and sub-2 μm particles in column materials have had a significant impact on the separation, speed and efficiency of LC analyses (Motilva et al., 2013). These improvements have enabled 5- to 10-fold faster analysis times, better resolution, higher sensitivity and reduced solvent consumption in comparison to HPLC (Motilva et al., 2013).

In addition, other kinds of column materials that are based on hydrophilic interactions (HILIC) have been developed. HILIC is referred to be a variant of normal phase (NP)-HPLC in which the mobile phase resembles that used in RP-HPLC (Buszewski and Noga, 2012). It is most applicable for those compounds, which were earlier analyzed by NP-HPLC.

3.2 Purification methods

3.2.1 Gel chromatography

Sephadex LH-20 (hydroxypropylated dextran) gel chromatography is a widely used method for fractionation of plant crude extracts and in pre-purification of ETs. The choice of used elution solutions varies depending on the target molecules under investigation. For ETs, usually aqueous alcoholic [ethanol (EtOH) or methanol (MeOH)] and/or aqueous solutions of acetone (Me_2CO) are the most frequently used ones (Kashiwada et al., 1992; Pfundstein et al., 2010; Salminen and Karonen, 2011; Scalbert et al., 1990). Since ETs are adsorbed relatively tightly on the gel, gradual decrease of the elution solvent polarity, e.g., from an alcoholic solution to an acetone solution, provides good separation of different ET structures. In a typical procedure the sample is first dissolved in water and applied to the column. The elution is started with water followed by aqueous methanol (Kashiwada et al., 1992) and finally with aqueous acetone solutions (Salminen and Karonen, 2011). Other gel materials, such as Toyopearl

HW-40 F (e.g., Fukuda et al., 2003; Okuda et al., 1986a, b; Piwowarski et al., 2014; Piwowarski and Kiss, 2013;) which is a vinyl polymer resin (Okuda et al., 1989), MCl CHP-20P and Fuji gels are used in addition to or instead of Sephadex LH-20 purification (e.g., Ishimaru et al., 1987; Kashiwada et al., 1992). The fractions obtained from the crude extract usually require one or more additional fractionations before the final purification by other methods, such as preparative RP-LC.

3.2.2 Preparative RP-LC

In most cases, the final purification of individual ETs is achieved by preparative RP-LC. The preparative RP-LC purification is often performed using aqueous methanol or aqueous acetonitrile solutions as elution solvents (e.g., Pfundstein et al., 2010; Piwowarski et al., 2014; Piwowarski and Kiss, 2013; Puech et al., 1999; Quideau et al., 2004). There are several features that affect the effectiveness of the purification step. For example, resolution is affected by the solvent into which the sample is dissolved. Water is the best choice, since organic solvents may cause peak multiplication that results from the autoassociation of polar compounds in organic solvents due to their limited solubility (Scalbert et al., 1990). In some specific cases, such as in ETs which contain the DHHDP group, alcoholic organic solvents (Hatano et al., 1988a) and acetone (Tanaka et al., 1992) may cause formation of adducts. These adducts affect the chromatography and naturally, the structures of ETs. When acetonitrile is used as the eluent, peak multiplication is not observed (Hatano et al., 1988a). Addition of an acid into the eluent solution is recommended to prevent ionization, but it should be removed to prevent hydrolysis of the purified ETs (Puech et al., 1999; Scalbert et al., 1990).

3.2.3 Droplet counter-current chromatography and centrifugal partition chromatography

Other fractionation methods include droplet counter-current chromatography (DCCC) and centrifugal partition chromatography (CPC). DCCC's advantage is that it does not contain a solid phase on which the ETs could adsorb. The

apparatus (Fig. 5) consists of a set of columns that are filled with the stationary phase. The columns are attached to each other by tubing. The sample is first dissolved in either of the phases or in a mixture of these and is then applied to a sample chamber (Hostettmann, 1980). The mobile phase is immiscible with the stationary phase, and it is pumped into the system as droplets. The sample molecules move with the mobile phase and are partitioned between the phases according to their partition coefficients. Finally, the partitioned compounds end up to the fraction collector (Hostettmann, 1980; Ogihara et al., 1976; Tanimura and Ito, 1974).

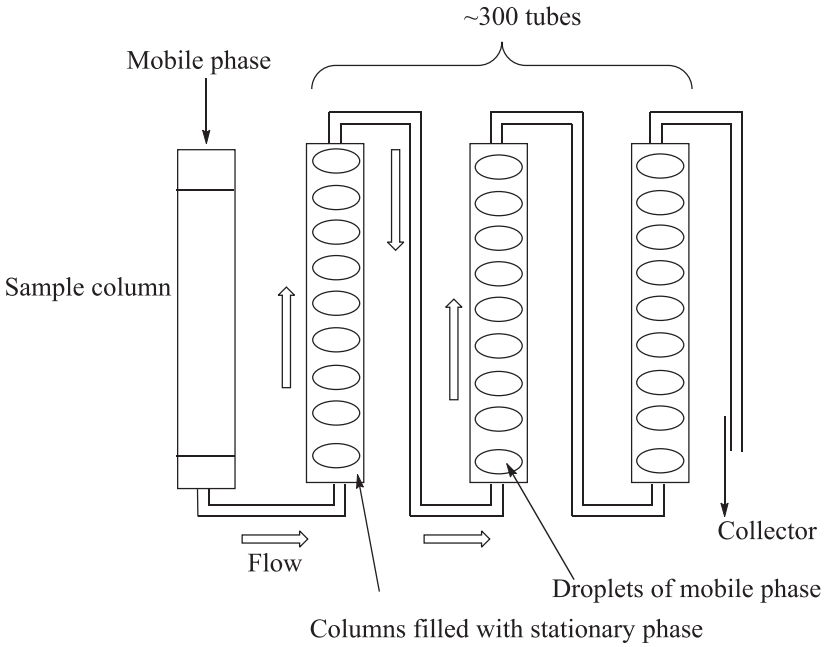


Figure 5. Schematic illustration of the operating principle of droplet counter-current chromatography in the ascending mode. The mobile phase is pumped throught the sample column and inserted into the first column from the bottom. A steady stream of droplets are formed. When the droplets reach the top of the column, they are delivered to the bottom of the next column, and the droplet formation begins again. Adapted from Hostettmann (1980).

CPC, also known as centrifugal counter-current chromatography, is also based on partition between two liquid phases (Hostettmann and Marston, 1990). In CPC, a centrifugal force is applied that allows faster separation when

compared to that of DCCP and in addition, a wider range of solvent systems are applicable because there is no need for droplet formation (Hostettmann and Marston, 1990). The instrument consists of a series of cartridges located at the circumference of a centrifuge rotor with their longitudinal axes parallel to the direction of the centrifugal force. The cartridges are connected to each other as in DCCC (Hostettmann and Marston, 1990). CPC is faster than DCCC (Okuda et al., 1986b) which results in lower consumption of solvents and evaporation times (Okuda et al., 1989). In addition to other ETs (Okuda et al., 1986a, b; Okuda et al., 1987), CPC has been found to be particularly efficient in the separation of oligomeric ETs from each other (Okuda et al., 1989). These methods have also been widely used in isolation of other secondary metabolites (reviewed by Marston and Hostettmann, 1994).

3.3 Characterization methods

3.3.1 Analysis of hydrolysis and derivatization products

Hydrolysis products can be used to aid the structure characterization of an unknown ET. Hydrolysis in boiling water or in aqueous acid (also by tannase enzyme) yields different kinds of hydrolysis products depending on the structure of the ET. These include, e.g., gallic acid (from esterified galloyl groups), ellagic acid (from HHDP groups), valoneic acid dilactone (from valoneoyl group), brevifolincarboxylic acid (from DHHDP group) and other partially hydrolysed ET structures (e.g., Gupta et al., 1982; Haddock et al., 1982a, b; Haslam, 1989; Hatano et al., 1990b; Tanaka et al., 1990). The identification of hydrolysis products is achieved by purification of individual hydrolysis products with different chromatographic methods (e.g., Sephadex LH-20 or silica gel) and subsequent NMR measurements. Methylation, acetylation, methanolysis and reactions with bases have also been used in characterizations (e.g., Hatano et al., 1989b; Ishimaru et al., 1987; Okuda et al., 1976; Tanaka et al., 1990). Again, the products have been purified chromatographically and studied with NMR. Reactions with *o*-phenylenediamine that result in phenazine derivatives of corresponding ETs, have also been used to aid the characterization. However,

phenazine derivatives can be unstable and decompose before the characterization is performed (Tanaka et al., 1992).

3.3.2 UV/Vis spectrum and chromatographic behavior

By using DAD [also photodiode array detector (PDA)] for the detection of ETs in chromatographic analyses, a significant advantage over single wavelength detectors can be achieved, since it enables multiple wavelengths to be monitored at the same time (Waterman and Mole, 1994). As a result, the UV spectrum for each peak in the chromatogram can be obtained. It would be highly informative if the UV spectra were presented in the publications, but this is very seldom done. Instead, the UV data are generally reported by presenting the wavelength(s) at which the absorption maxima are observed (see Määttä-Riihinen et al., 2004; Salminen et al., 1999, 2011; Silva et al., 2000 for exceptions).

The retention order of ETs is also a very informative characteristic that could be more effectively used in the characterization of ETs, as has been realized in the case of other phenolic compounds, such as anthocyanidins, proanthocyanidins and flavonoids (e.g., Santos-Buelga and Williamson, 2003). Of course, it has to be kept in mind, that the retention times of compounds in LC analyses are affected by several factors, e.g., the equipment, the column material, the elution solvents and the flow rates used. Despite the features listed, there are general characteristics that are universal, regardless of the equipment used. Furthermore, the retention order of compounds remains usually unchanged between different LCs and columns.

Those glucopyranose-based ETs, which have a free -OH group at C-1, show two peaks in their chromatogram. This results from the α and β anomers that are usually present almost at 1:1 ratio (Hatano et al., 1988a). The presence of these kinds of ETs, e.g., pedunculagin (Fig. 1), can be verified by their reaction with sodium borohydride ($NaBH_4$) that reduces the hemiacetal group at C-1 and results as one peak in the chromatogram. Those ETs that are acylated, i.e., galloylated at C-1 are not affected by the treatment with $NaBH_4$ (Hatano et al., 1988a; Okuda et al., 1989).

The number of galloyl groups in the ET structures affects their retention: the more there are free galloyl groups the later the ET will elute as the hydrophobicity of the ET increases (Tanaka et al., 1997). Likewise it can be said that the more there are HHDP groups, the earlier the ET will elute as the hydrophobicity of the ET decreases (Tanaka et al., 1997). Thus, the elution order of ETs is correlated with their solubility in water that is expressed by the water-octanol partition coefficient (K_{ow}).

3.3.3 Mass spectrometry

Mass spectrometry (MS) is an important tool in the characterization of ETs. Since ETs are highly polar, thermally labile and have poor volatility, a gas chromatograph coupled to a mass spectrometer (GC-MS) is not practical in ET analysis. However, GC-MS has sometimes been used to aid the characterization of hydrolysis products of ETs after trimethylsilylation (Hatano et al., 1990c; Yoshida et al., 1991a). Instead, LC-MS is a very practical and convenient tool to characterize ETs. This has been enabled by the development of electrospray ionization (ESI) interface between the LC and the MS.

Ionization techniques

The principal mechanism by which the ions to be analyzed are produced in the ESI source is as follows: the eluent from the LC is introduced to the ESI source by a capillary, to which a voltage of 3–4 kV (positive or negative depending on the ionization mode used) is applied (El-Aneed et al., 2009; Westman-Brinkmalm and Brinkmalm, 2009a). At the end of the ion-source, i.e., the nozzle, an opposite charge relative to that of the needle is applied (Westman-Brinkmalm and Brinkmalm, 2009a). The electric field at the needle tip charges the surface of the flowing liquid and a so-called Taylor cone is formed (Fig. 6). The cone is enriched with positive or negative ions from which charged droplets are ejected by the electric field, dispersing it into a fine spray of charged droplets. At the same time, a heated gas flow [usually nitrogen (N_2)] is applied into the ion source that evaporates the solvent from the droplets and decreases their diameter (Westman-Brinkmalm and Brinkmalm, 2009a). As a result, the

charge density on the surface of the droplets increases until the charge repulsion and surface tension becomes so large that the droplets explode into smaller droplets. This repeats sequentially until the droplets are so small that ions can be desorbed from it into the ambient gas, from which they are directed to the mass analyzer (Fenn et al., 1989; Niessen and Tinke, 1995).

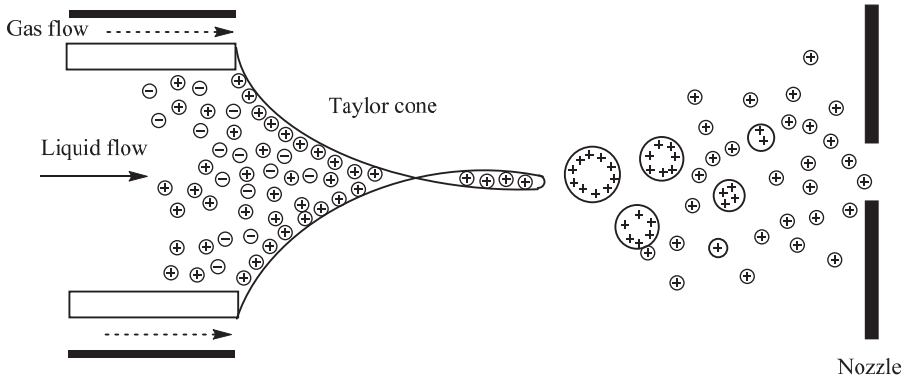


Figure 6. Schematic of an electrospray ionization source. Adapted from Westman-Brinkmalm and Brinkmalm (2009a).

The advantages of ESI are high droplet charge-to-mass ratios, which result in increased analytical sensitivity and extensive multiply charging of ions that enables the analysis of large molecular weight compounds, e.g., proteins (Fenn et al., 1989). In ET analysis, the multiple charging of ions in ESI and the availability of high resolution mass analyzers (e.g., time-of-flight (TOF) analyzer) have enabled the characterization of new ET oligomers (up to undecamers) that was not possible earlier (Karonen et al., 2010; Salminen et al., 2011).

Another widely used ionization technique is the matrix-assisted laser desorption ionization (MALDI), which is particularly useful in protein, DNA, lipid and glycoconjugate studies (El-Aneed et al., 2009). In MALDI, ions are desorbed from the solid phase, i.e., from the matrix. The dissolved sample is mixed with a suitable matrix material, which is then dried and results in the formation of crystallized matrix-analyte mixture. When a laser beam (usually N_2 laser at the wavelength of 337 nm) is directed to the matrix-analyte crystal, the matrix is excited and transfers its energy to the analyte molecules resulting in the

ionization and desorption of the analyte ions into the gas phase (El-Aneed et al., 2009). The difference between ESI and MALDI is that with MALDI mainly singly charged ions are formed (El-Aneed et al., 2009). The mass range of MALDI is dependent on the analyzer used, but proteins with masses up to 1 MDa have been succesfully analyzed with MALDI-TOF-MS (Westman-Brinkmalm and Brinkmalm, 2009a).

Before ESI, methods such as fast atom bombardment (FAB) were used to confirm the molecular weight of isolated ETs and to obtain the molecular formula (e.g., Hatano et al., 1990c; Ishimaru et al., 1987; Yoshida et al., 2000). In FAB, the sample is dissolved into a liquid matrix, e.g., glycerol. Then the sample is bombarded with energetic atoms, such as argon or xenon, with a kinetic energy of ~10 keV. The atom impact initiates a cascade of collisions between the impacting particles and atomic nuclei of the sample, resulting in the ejection of neutral molecules and ions (Westman-Brinkmalm and Brinkmalm, 2009a). In ET analysis, matrices such as glycerol (Self et al., 1986) and hexamethylphosphoric triamide-glycerol (1:1, v/v) (Isobe et al., 1989) have been used with a xenon beam.

Mass analyzers

After ionization, the ions formed need to be analyzed. Two most commonly used mass analyzers in ET analyses are the quadrupole (Q) and TOF analyzers. However, quadrupole ion trap (QIT) and Orbitrap analyzers are becoming more common as well (e.g., Grace et al., 2014; Granica et al., 2012, 2014; Hager et al., 2008; Regueiro et al., 2014).

The quadrupole mass analyzer is composed of four rods (Fig. 7). A direct current (DC) potential (U) is applied to two of the rods and an alternating radio-frequency (RF, ω) potential (V) to the other two. Opposite rods are connected to each other electrically as pairs, and these pairs have (at any given time) same potentials, but of opposite sign (Westman-Brinkmalm and Brinkmalm, 2009a). The ions are moving towards the quadrupole due to the electric field and to the direction of opposite charge (i.e., negatively charged ions towards positively charged rods and vice versa). However, the polarities of the rods are interchanged continuously, which prevents the ions from striking the rods. As a

result, only ions with an appropriate mass-to-charge (*m/z*) ratio will pass towards the detector. The remaining ions will collide with one of the rods. By changing the *V*, *U* and ω values, transmission of various ions towards the detector with different *m/z* values can be obtained (El-Aneed et al., 2009; Westman-Brinkmalm and Brinkmalm, 2009a). The advantages of quadrupole analyzers are low cost, relatively small size, robustness and ease of maintenance (El-Aneed et al., 2009). The drawbacks include limited mass range, typically up to 2000 Da (Westman-Brinkmalm and Brinkmalm, 2009a) and low resolving power (El-Aneed et al., 2009a).

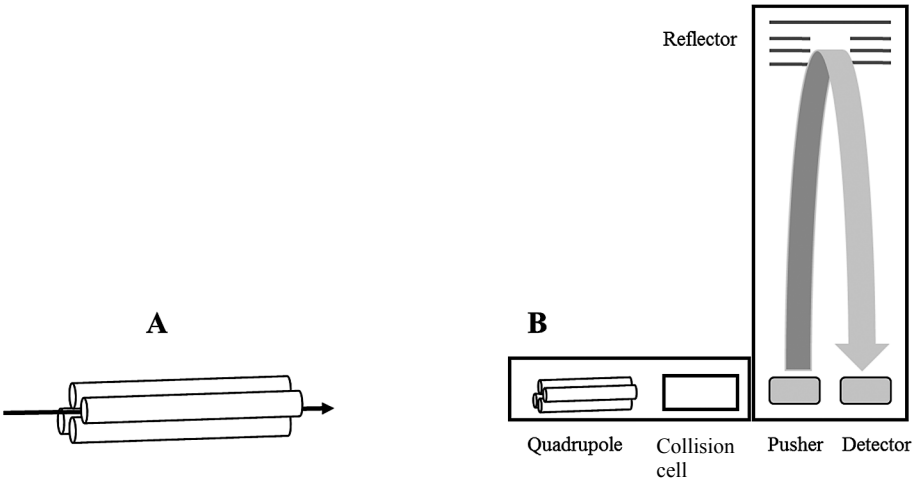


Figure 7. Representations of a quadrupole (A) and a quadrupole-time-of-flight (B) mass analyzers.

The other commonly used analyzer, TOF, is one of the simplest: it relies on the free flight of the ions in a tube before reaching the detector (Fig. 7). The main advantage of TOF is that all the ions formed are also detected. The separation of different ions is based on their mass: the lighter the ion, the more quickly it will reach the detector and the heavier the ion, the slower it will reach the detector. The resolving power of TOF can be enhanced with a reflector (an electrostatic ion mirror) that changes the path of the ions within the TOF. Those ions having higher kinetic energy will penetrate deeper into the mirror and hence ions will be gradually repelled, improving the resolution (El-Aneed et al., 2009). Another factor is the tube length: when reflector is used, the ions travel a longer

path, thus resulting in better resolution and more accurate mass measurements (El-Aneed et al., 2009). Two or more reflectors can also be arranged in series, which enhances the mass resolving power even more (Westman-Brinkmalm and Brinkmalm, 2009a). The advantages of TOF analyzers include unlimited mass range (in theory) and the speed of the analysis (Westman-Brinkmalm and Brinkmalm, 2009a). A major drawback of TOF analyzers is their low quantification capability (Westman-Brinkmalm and Brinkmalm, 2009a).

QIT is based on the same principle as a Q analyzer, but its geometry is different (Westman-Brinkmalm and Brinkmalm, 2009a): the quadrupoles are arranged so that they form a cylinder (can also be arranged linearly as in Q) into which the ions are trapped and stored. The mass spectrum is obtained by ejecting ions from the trap by changing the RF voltage. QIT is particularly useful in tandem mass spectrometry (MS/MS) and multistage mass spectrometry (MS^n), since ions with particular *m/z* can be stored inside the trap for consecutive fragmentations. Drawbacks include low mass accuracy and limited dynamic range due to which it is not good for quantification (Westman-Brinkmalm and Brinkmalm, 2009a). In Orbitrap analyzer, the ions are trapped around a central electrode by applying an electrostatic field between the inner and outer electrodes, as well as between the end caps (Fig. 8) (Westman-Brinkmalm and Brinkmalm, 2009a). The ions move and oscillate in stable trajectories around the central electrode (Westman-Brinkmalm and Brinkmalm, 2009a). The frequency of the harmonic axial oscillation can be measured by image current detection (Gross, 2011) and as a result a sine wave for each *m/z* value is obtained. The image current signal is then translated into frequency domain signal by Fourier transform (Gross, 2011). This enhances the resolution and the mass accuracy of Orbitrap when compared to those of QIT (Westman-Brinkmalm and Brinkmalm, 2009a).

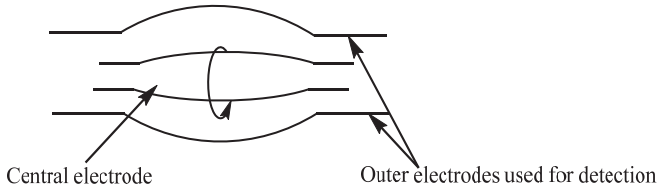


Figure 8. Schematic presentation of an Orbitrap analyzer.

By combining mass analyzers, e.g., a quadrupole with another quadrupole or with a TOF analyzer (Fig. 7), it is possible to do MS/MS. In these cases, the MS/MS method is called tandem-in-space, as the ions move from one part of the instrument to another. In the case of QIT, the method is called tandem-in-time, since the trapping, isolation, fragmentation and detection of selected ion(s) is performed in the same physical device (Westman-Brinkmalm and Brinkmalm, 2009b). Basically MS/MS relies on the selection of a specific ion, which is then subjected to dissociation and subsequent production of fragment or product ion(s) that are detected. When three quadrupoles are combined, it is called a triple quadrupole (QqQ or TQ) instrument. The second quadrupole (q), which is in fact usually a hexapole or an octapole refers to a collision cell, in which the fragmentation of the selected ion is achieved (Westman-Brinkmalm and Brinkmalm, 2009b). When MS/MS analysis is performed with a TQ instrument, the process is as follows: the selected ion is filtered at the first quadrupole (Q_1) from which it continues to the collision cell (q). A continuous stream of inert gas, e.g., helium, N_2, argon or xenon, is supplied to the collision cell and when the ions collide with the gas (as well as with each other), some of the kinetic energy of the gas will transfer to the ions, causing their fragmentation (the process is called collision-induced dissociation, CID). The fragments are then analyzed at the third quadrupole (Q_2). The basic principle of an MS/MS analysis in a quadrupole-time-of-flight (Q-TOF) spectrometer is the same as with TQ, the only difference being that the analysis of fragments is achieved with a TOF analyzer instead of the Q. Usually Q-TOF instruments are equipped with an additional quadrupole before the Q_1 as an ion focusing device to provide collisional cooling of the ions so that the quality of the ion beam gets improved. This quadrupole is referred to as q_0 (El-Aneed et al., 2009).

The advantages of Q-TOF instruments include ease of operation, higher resolution and higher mass accuracy when compared to TQ (Westman-Brinkmalm and Brinkmalm, 2009b). However, the TQ instruments are favored in quantitative studies, because they have better linear dynamic range and because the sensitivity for quantification of targeted compounds is better than with Q-TOF (El-Aneed et al., 2009; Westman-Brinkmalm and Brinkmalm, 2009b).

The mass spectra of ETs typically exhibit the molecular ion $[M–H]^-$ (in negative ionization mode) and some characteristic fragment ions. If the ET contains galloyl groups, typical fragment ions observed are those in which a galloyl group $[M–152]^-$ or a gallic acid group $[M–170]^-$ has been lost. Another typical fragment that is observed for ETs is the loss of an HHDP group. It is observed as a deprotonated ellagic acid $[M–H]^-$ at *m/z* 301, since the HHDP group forms a lactone spontaneously (Self et al., 1986). In the mass spectra of dimeric ETs, the molecular ion $[M–H]^-$ is usually obtained. In addition, the spectra are usually characterized by a doubly charged molecular ion $[M–2H]^{2-}$, which can be recognized from the isotopic peaks that are separated by 0.5 Da from each other (Self et al., 1986).

3.3.4 Nuclear magnetic resonance spectroscopy

The ultimate method for structure determination is NMR spectroscopy. As the name suggests, this technique relies on the magnetic resonances of nuclei that are brought about by applying electromagnetic radiation (radio frequency) in a form of pulses to a sample inside a powerful static magnetic field (Butler, 2008; Waterman and Mole, 1994). The advantage of NMR is that it provides constitutional, configurational and conformational information (Balci, 2005). The basic principle of operation is that some atomic nuclei are able to take up electromagnetic energy at characteristic frequencies to attain a higher resonance state. As this excitation relaxes back to the initial state, the emitted radiation is measured and transformed into a spectrum by Fourier transform (Butler, 2008; Waterman and Mole, 1994). The operating frequencies of NMR spectrometers can vary from 60 MHz to 1 GHz. Usually the frequencies of the equipment used in ET characterizations are in the range of 400–600 MHz.

Those isotopes, which are part of basic NMR measurements, are proton (^{1}H) and carbon (^{13}C) measurements. A ^{1}H measurement requires typically a limited amount of sample (0.5–1 mg), whereas the natural abundance of ^{13}C is only 1 %, and thus a ^{13}C measurement requires much more sample (e.g., a 10 mg sample relates only to 0.1 mg as far as the ^{13}C nuclei are considered). The measurements are usually performed using solution samples (although solid-state NMR experiments can also be performed). The solvents must be deuterated, i.e., the ^{1}H nuclei of the solvent have been replaced by ^{2}Hs (e.g., methanol-d_4, D_2O, acetone-d_6, $CDCl_3$), because the ^{1}Hs of the solvent resonate at similar frequencies as the ^{1}Hs in the molecules under investigation and thus, show signals in the same part of the spectrum. The chemical shift (δ) scale of the spectra are calibrated either with tetramethylsilane (TMS) or by ^{1}H signal(s) arising from the residual ^{1}H in the deuterated solvent so that $\delta_{TMS} = 0$ ppm (Waterman and Mole, 1994). Each δ unit represents one millionth (ppm) of the resonance frequency employed and thus is not dependent on the magnetic field used. This means that the δ values observed for the nuclei of a particular compound are comparable, and thus, the same regardless of the used field strength of the magnet. This in turn means that by using more powerful magnets some increase in sensitivity is achieved but more importantly, a considerable enhancement in resolution is gained (Waterman and Mole, 1994).

From basic 1D ^{1}H and ^{13}C NMR measurements, the chemical shifts of protons and carbons, respectively, are obtained. The area of the signals in the ^{1}H spectrum can be integrated to obtain the relative amount of different kind of hydrogens present in the molecule. It is also possible to determine the magnitude of coupling constants (J_{HH}) between different protons, which give information on the proximity and spatial position of hydrogens in relation to each other.

Usually the characterization of an unknown compound requires 2D NMR measurements in order to resolve the structure. Typical 2D measurements include: ^{1}H,^{1}H-homocorrelation spectroscopy [COSY or usually double-quantum filtered correlation spectroscopy (DQF-COSY)] experiment, which correlate the chemical shifts of ^{1}H nuclei that are *J*-coupled to each other; total correlation spectroscopy (TOCSY) experiment that shows correlations between hydrogens which belong to the same spin network; nuclear Overhauser effect spectroscopy (NOESY) experiment, which show correlations between nuclei

that are spatially close to each other. With ^{1}H-detected heteronuclear multiple bond correlation (HMBC) and ^{13}C-detected correlation via long-range coupling (COLOC) experiments the long-range ^{1}H-^{13}C connectivities can be obtained. With heteronuclear single quantum coherence (HSQC) experiment the single-bond ^{1}H-^{13}C connectivities are obtained (i.e., which hydrogens are attached to which carbons) (Bruker, 2003).

In the course of ET characterizations, NMR spectroscopy has played a central role and there is a vast amount of literature available (see for example, Haslam, 1989; Hatano et al., 1988b, c; Yoshida et al., 1984, 2000). For example, it has been observed that the types of the polyphenolic acyl units (e.g., galloyl, HHDP and DHHDP groups) and the position to which these groups are attached in the glucose core, affect characteristically the chemical shifts observed for the glucose carbon atoms (Hatano et al., 1988b, c; Yoshida et al., 1984) and can thus be used in the characterization of unknown ET structures.

The differences in the chemical shifts of α and β anomers can be used to distinguish these isomers from each other. For example, in the case of casuarictin (β-anomer) and potentillin (α-anomer) there are no significant differences observed for the chemical shifts of C-4 and C-6 of the glucose core (Table 1). However, for the rest of carbons (C-1–C-3 and C-5) an upfield shift is observed in the case of the α-anomer (Table 1; Yoshida et al., 1984). The effects of the formation of an HHDP group from two galloyl groups on the chemical shifts can be seen by comparing the differences of chemical shifts of pedunculagin-tellimagrandin I pairs (Table 2). Thus, when an HHDP groups is formed a downfield shift of the C-2 and C-3 signals are observed. This can be explained by increased rigidness of the structure, which results as constraints on the glucopyranose ring (Hatano et al., 1988b). In the case of those ETs which form anomeric mixtures, the effects on the changes in the chemical shifts are larger when compared to those ETs which are acylated at C-1 (compare the chemical shift changes of tellimagrandin I and pedunculagin to that of potentillin-casuarictin) (Hatano et al., 1988b). In addition, the acetylation of the C-1 affects characteristically to the chemical shifts of C-1 and C-5 of the glucose core. Thus, the acetylation of C-1 shifts the chemical shift of C-1 upfield and the chemical shift of C-5 downfield (note the chemical shift diffrences of α-

pedunculagin-potentillin, *β*-pedunculagin-casuarictin and β-tellimagrandin I-tellimagrandin II pairs in Table 2) (Hatano et al., 1988b).

Those ETs, which contain a DHHDP or a chebuloyl group in their structure, the glucose core adopts $^1C_4$ conformation. By comparing the chemical shifts of geraniin (contains a DHHDP group) and chebulagic acid (contains a chebuloyl group) to those of corilagin (contains two OH groups instead of the DHHDP or chebuloyl group) (Table 2), the effects of the substitution on the changes of chemical shifts can be seen. Thus, a significant downfield shift of the C-4 signals of geraniin and chebulagic acid are observed when compared to that of corilagin (Hatano et al., 1988c).

C-glucosidic ETs can also be distinguished from each other by the characteristic chemical shift changes. Thus, in the case of those ETs which have *β*-configuration at C-1 of the acyclic glucose core (e.g., stachyurin), a significant downfield shift of the C-2 signal (+4.3 ppm) and an upfield shift of the C-1 signal (-2.1 ppm) is observed, when compared to those of casuarinin (Table 1; Hatano et al., 1988c). Similar changes are also observed for vescalagin and castalagin (Table 1). In addition, the chemical shift of C-2 appears at the lowest field among the glucose carbon signals (Hatano et al., 1988c), and can be used to determine the configuration of C-1 (Hatano et al., 1988c; Okuda et al., 1989).

The characterization of monomeric ETs is quite easy, but in the case of oligomers the situation is more complicated. For example, the type of the linking unit between the monomers (e.g., dehydrodigalloyl, valoneoyl, sanguisorboyl etc.) cannot be straightforwardly determined, but is achieved by using long-range 2D correlation measurements, such as HMBC or ^1H-^{13}C COLOC (Yoshida et al., 2000). In addition, by comparing the NMR data (especially the ^{13}C data) of known monomeric structures to those obtained for the oligomeric ETs, the characterization process is made easier (Hatano et al., 1988c; Okuda et al., 1989; Yoshida et al., 1984).

Table 1. Chemical shifts for the glucose carbon atoms of selected monomeric ETs.

	α-anomer						*β*-anomer						
	C-1	**C-2**	**C-3**	**C-4**	**C-5**	**C-6**	**C-1**	**C-2**	**C-3**	**C-4**	**C-5**	**C-6**	**Reference**
$^4C_1$ ETs													
Casuarictin							92.4	76.0	77.3	69.3	73.5	63.1	Yoshida et al., 1984
Potentillin	90.7	74.1	76.0	69.1	71.0	63.2							Yoshida et al., 1984
Tellimagrandin II							93.8	71.8	73.3	70.8	73.1	63.1	Yoshida et al., 1984
$^4C_1$ ETs forming anomeric mixtures													
Pedunculagin	91.8	75.6	75.8	69.9	67.4	63.6	95.4	78.3	77.6	69.9	72.5	63.6	Hatano et al., 1988b
Tellimagrandin I	91.2	72.9	71.1	71.1	67.2	63.5	96.7	74.1	73.5	71.1	72.0	63.5	Hatano et al., 1988b
$^1C_4$ ETs													
Corilagin	94.2	68.8	70.4	62.2	75.5	64.3							Hatano et al., 1988c
Geraniin[a]	90.8	69.9	63.3	65.9	72.6	63.6							Hatano et al., 1988c
Geraniin[b]	91.8	70.4	62.3	66.8	73.1	63.8							Hatano et al., 1988c
Chebulagic acid	91.5	70.5	61.7	66.1	73.5	63.9							Hatano et al., 1988c
***C*-glucosidic ETs**													
Stachyurin							65.5	81.0	70.9	73.3	72.0	64.5	Hatano et al., 1988c
Casuarinin	67.6	76.7	69.8	74.2	71.2	64.6							Hatano et al., 1988c
Vescalagin							66.1	78.3	71.7	69.1	70.0	66.0	Yoshida et al., 2000
Castalagin	67.2	74.0	66.2	69.2	71.1	65.3							Yoshida et al., 2000

[a] Six-membered hemiacetal form, [b] Five-membered hemiacetal form

Table 2. Effects of different structural features on the chemical shift changes of the glucose carbon atoms.

	C-1	C-2	C-3	C-4	C-5	C-6	Reference
Pedunculagin-tellimagrandin I (α-α)	+0.6	+2.7	+4.7	-1.2	+0.2	+0.1	Hatano et al., 1988b
Pedunculagin-tellimagrandin I (β-β)	-1.3	+4.2	+4.1	-1.5	+0.5	+0.1	Hatano et al., 1988b
Tellimagrandin I (Δαβ)	-5.5	-1.2	-2.4	0	-4.8	0	Hatano et al., 1988b
Pedunculagin (Δαβ)	-3.6	-2.7	-1.8	+0.3	-5.1	0	Hatano et al., 1988b
α-pedunculagin-potentillin	-1.1	-1.5	+0.2	-0.8	+3.6	-0.4	Hatano et al., 1988b
β-pedunculagin-casuarictin	-3.0	-2.3	-0.3	-0.3	+1.0	-0.5	Hatano et al., 1988b
β-Tellimagrandin I-tellimagrandin II	-2.9	-2.3	-0.2	-0.3	+1.1	-0.4	Hatano et al., 1988b
Corilagin-geraniin (six-membered hemiacetal form)	-3.4	+1.1	-7.1	+3.7	-2.9	-0.7	Hatano et al., 1988c
Corilagin-geraniin (five-membered hemiacetal form)	-2.4	+1.6	-8.1	+4.6	-2.4	-0.5	Hatano et al., 1988c
Corilagin-chebulagic acid	-2.7	+1.7	-8.7	+3.9	-2.0	-0.4	Hatano et al., 1988c

3.3.5 Circular dichroism spectroscopy

Circular dichroism (CD) refers to the differential absorption of left and right circularly polarized radiation that is observed for chiral compounds (Wallace and Janes, 2009). Thus, a CD spectrum is a type of an absorption spectrum. For ETs, usually the CD spectra are measured in the UV-Vis range (which corresponds to the electronic transitions in the molecules), and thus, the specific method applied is electronic circular dichroism (ECD). In an ECD spectrum the molar ellipticity, [θ], as degrees cm^2 $dmol^{-1}$ is often given as a function of the wavelenghts used (Wallace and Janes, 2009). The absorptions observed may be either negative or positive in their signs. This depends on whether the right- or left-handed radiation is absorbed more efficiently by the molecule at a given wavelenght. These absorption maxima/minima are usually referred to as Cotton effects. In ETs, the chirality results especially from the HHDP group(s) (due to axial chirality; Eliel et al., 1994) and its modifications. There are several features that affect the ECD spectra of ETs, and these features can be used to aid the structure elucidation of unknown ETs.

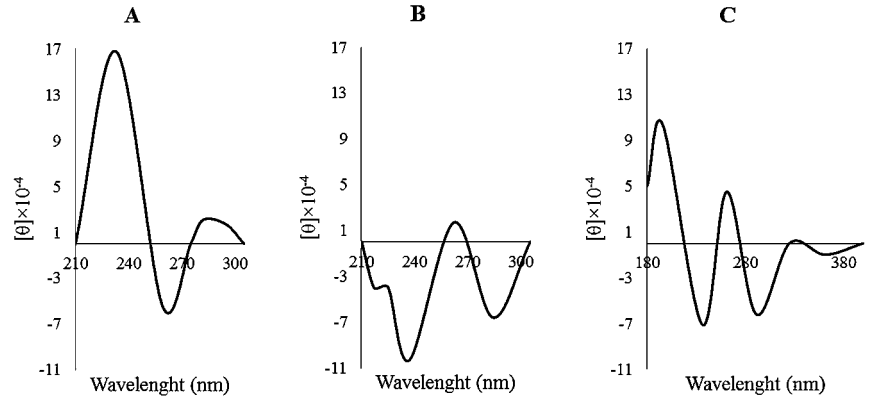


Figure 9. Electronic circular dichroism spectra of pedunculagin (A), corilagin (B) and geraniin (C). Modified from Okuda et al. (1982c, d).

In the ECD spectra of those ETs that have only one HHDP group (i.e., HHDP-glucoses) in their structure, two Cotton effects (at 235 and 265 nm) are observed in the spectrum. If the observed Cotton effect at ~265 nm is positive, the configuration is *R*, and if it is negative, the configuration is *S*. When the HHDP group is accompanied by one to three galloyl groups in the ET structure, three Cotton effects are observed in the ECD spectrum. These maxima are situated at ~235, ~265 and ~285 nm (Fig. 9A). Again, if a positive Cotton effect is observed at around 265 nm, the configuration is *R*, and if the effect is negative the configuration is *S* (at 235 nm, the observed Cotton effects are opposite at their signs in comparison to that at 265 nm) (Okuda et al., 1982c). However, it has been observed that the Cotton effects at 265 nm are affected by the interactions between galloyl and HHDP groups, and thus, the Cotton effect at 235 nm is considered to be more diagnostic in determining the absolute configuration of the HHDP group (Yoshida et al., 2000). If there is no Cotton effect detected at the 235 nm, the compound does not contain an HHDP group. The amplitude of the observed Cotton effect at 235 nm is also informative: if the ET contains two HHDP groups, the amplitude of the Cotton effect is about twofold compared to those ETs that have only one HHDP group (Okuda et al., 1982c; Yoshida et al., 2000). Similarly,

the configurations of linking units in the oligomeric ETs can be determined by ECD spectroscopy (Yoshida et al., 2000).

Chebuloyl and DHHDP groups are also chiral. Those compounds that have only a DHHDP group in their structure have three maxima in their ECD spectra: negative at 206 nm and two positive ones at 237 and 375 nm when the configuration is 1'*S*. The sign is opposite at 237 nm in the case of 1'*R* configuration. Other groups (galloyl and HHDP) in the structure affect the observed ECD spectra: first, two more maxima are observed (five in total; Fig. 9C) and second, the maximum at 230 nm region is affected by these groups. Thus, the configuration of the DHHDP group is best determined from the sign of the Cotton effect at the 200 nm region: positive Cotton effect means *R* configuration and negative *S* configuration (Okuda et al., 1982d).

Some *C*-glucosidic ETs have, in addition to the HHDP group, an NHTP group in their structure. This group is also chiral, and thus it contributes to the ECD spectra of such compounds. Earlier studies suggested that the configuration of the NHTP group is (*S*, *S*) (Nonaka et al., 1987), but when these structures were re-examined by both computational and experimental methods (Matsuo et al., 2015), it was found that the actual configuration is (*S*, *R*) (Fig. 2). Thus, all the structures of the *C*-glucosidic ETs presented in this thesis have been corrected according to Matsuo et al. (2015).

4 DISTRIBUTION OF ELLAGITANNINS IN THE PLANT KINGDOM

ETs can be found from leaves, roots, bark, seeds, fruits and berries of several plant taxa and they are often the principal component of those plants that have been or are used as herbal medicines (e.g., Clifford and Scalbert, 2000; Okuda, 2005; Serrano et al., 2009). Although ETs are often said to be widely distributed in the plant kingdom, their occurrence is well defined and restricted only to certain taxonomic branches. Thus, they are only found in Angiosperms (flowering plants) that are dicots and more specifically, they are found in rosids but not in asterids (Gardner, 1977; Mole, 1993; Okuda et al., 2000). It has also been suggested that it is more likely to find tannins from the apparent plants (such as trees) than from herbaceous plants (Mole, 1993; Sporne, 1975), although divergent opinions exist (Okuda et al., 1992, 1993). Furthermore, tannins are more likely to be found from the early developed and primitive species than from the more recently developed species (Mole, 1993; Sporne, 1975).

The generalizations mentioned above would not have been possible without comprehensive screening studies. These kinds of chemotaxonomic researches, i.e., the classification of plants based on natural products, have been conducted from the early stages of tannin and ET research (e.g., Gardner, 1977 and references therein; Haddock et al., 1982c). Even though the analysis methods were not as sophisticated as they are today, the results of these studies are still valid, but have been fine-tuned.

The taxonomic distribution of ETs has been recognized early on and, e.g., Haddock et al. (1982c) concluded that the occurrence of dehydroETs can be used as a chemotaxonomic marker for Aceraceae (Sapindales) and Geraniaceae (Geraniales). More detailed results were obtained by Okuda et al. (1992) in a study, in which species belonging to family Rosaceae were studied. In this study, several monomeric and oligomeric ETs were used as markers. It was found that the subfamily Rosoideae was the only

one containing ETs, while the other subfamilies (Spiraeoideae, Amygdaloideae and Maloideae) did not contain ETs. The results also showed that several monomeric ETs are present in the ET-containing species, and thus are not suitable to be used as chemotaxonomic markers. Oligomeric ETs, on the other hand, were found to correlate with tribes and genera. For example, it was found that sanguiins H-6 and H-11 were only found in *Sanguisorba* and *Rubus* species, agrimoniin was limited to *Agrimonia* species and to the tribe Potentilleae (includes genera *Fragaria*, *Potentilla* and *Duchesnea*) and gemin A was limited to *Geum* species. Later Okuda et al. (1993, 2000) extended these results by combining the structural data of ETs with the Cronquist's classification for flowering plants (Cronquist, 1988; Fig. 6).

A
Rosales
Fabales
Proteales
Podostemales
Haloragales
Myrtales
Rhizophorales
Cornales
Santalales
Rafflesiales
Celastrales
Euphorbiales
Rhamnales
Linales
Polygalales
Sapindales
Geraniales
Apiales
No ETs
Dilleniales
Theales
Malvales
Lecythidales
Nepenthales
Violales
Salicales
Capparales
Batales
Ericales
Diapensiales
Ebenales
Primulales
Trochodendrales
Hamamelidales
Daphniphyllales
Didymelales
Eucommiales
Urticales
Leitneriales
Juglandales
Myricales
Fagales
Casuarinales
Caryophyllales
Polygonales
Plumbaginales
Magnoliales
Laurales
Piperales
Aristolochiales
Illiciales
Nymphaeales
Ranunculales
Papaverales
Magnoliidae
Hamamelidae
Caryophyllidae
Dilleniidae
Rosidae
Asteridae
Magnoliopsida

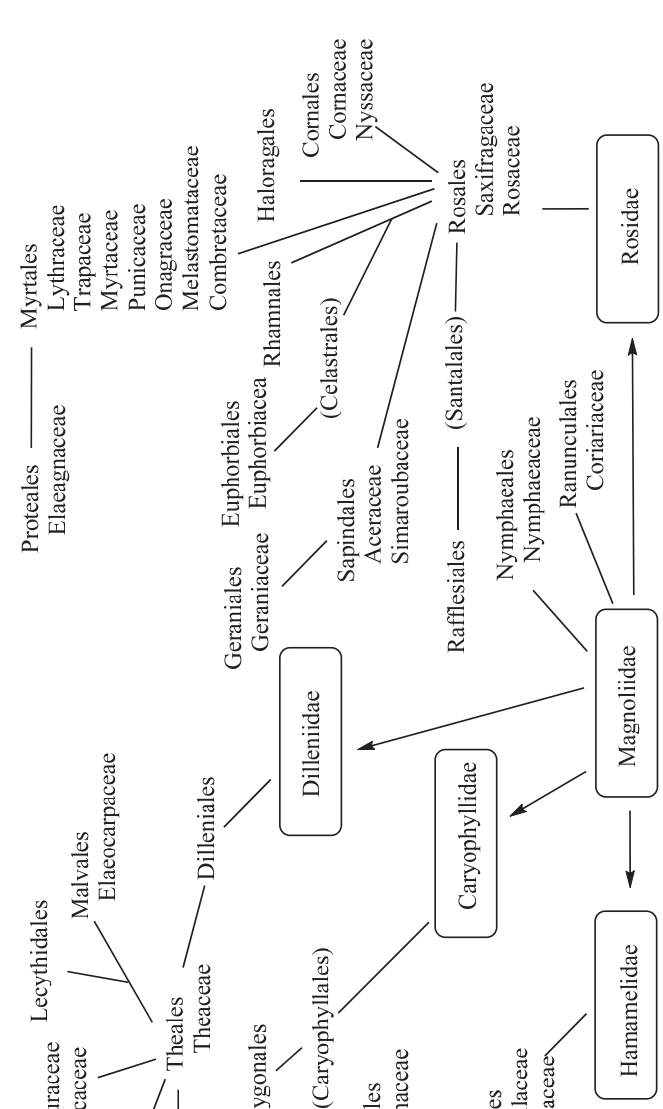


Figure 6. Classification of Magnoliopsida according to Cronquist (1988). In (A), subclasses and orders are presented. Those orders from which ellagitannins have been found are in bold. In (B), the evolutionary route of orders (according to Cronquist, 1988) that produce ellagitannins is presented. Those orders which have not been found to contain ellagitannins, but are shown for evolutionary purposes are in parenthesis. Modified from Okuda et al. (1993).

It was concluded that oxidized tannins, i.e., dehydroETs such as geraniin (classified as type III ETs in Okuda et al., 2000) and transformed dehydroETs such as chebulagic acid (classified as type IV ETs in Okuda et al., 2000) were frequently found in the Rosidae, but only in some species of the Dilleniidae and Hamamelidae (Fig. 6). These types of ETs were neither found from the earliest subclasses of Dicotyledonae namely Caryophyllidae and Magnoliidae (Fig. 6). The earliest order of Rosidae, Rosales, produce mostly gallotannins such as PGG (classified as type I in Okuda et al., 2000) and basic glucopyranose-based ETs such as pedunculagin (classified as type II ETs in Okuda et al., 2000). Oxidative transformations to types III and IV progress from Rosales to Sapindales to Geraniales without the production of oligomeric ETs (Fig. 6). Oligomeric ETs together with type II and II+ (e.g., *C*-glucosidic ETs such as casuariin) ETs occur in Rhamnales, Euphorbiales, Cornales, Myrtales and Proteales. In Hamamelidae the oxidative progression of ETs is not clear, but the occurrence of *C*-glucosidic ETs in many genera of Fagales is of note.

The Cronquist's classification is an example of a morphology-based classification, i.e., plants are classified based on similarities found in their phenotypic features. Nowadays, the taxonomists aim to ground classifications on genetic data, and a new classification for the Angiosperms has been proposed (APG, 1998, 2003, 2009). Since this is relatively new classification system, there is no literature available where the chemotaxonomy of ETs has been studied with respect to this classification.

Although a wide variety of different plant species, belonging to a variety of genera, families and orders have been studied worldwide, the research of Finnish plant species is limited (Table 3). Some of these studies have primarily focused on the quantification of ellagic acid content in the samples after hydrolysis (Koponen et al., 2007) or on the total ET content without further characterization of individual ETs (Kähkönen et al., 2001). In recent years, however, the situation seems to have changed,

and more detailed studies on the ET composition of Finnish plant species have been published (e.g., Hanhineva et al., 2008; Kähkönen et al., 2012; Tuominen et al., 2013). The available data about Finnish ET-containing plant species is listed in Table 3.

Table 3. Finnish plant species which have been studied for their ellagitannin content and/or composition.

Species		ET content or individual ETs characterized	Reference
Rubus chamaemorus	cloudberry, berries	1090–1630 mg/100 g dw[a]	Kähkönen et al., 2001
		311.8 mg/100 g fw[a]	Koponen et al., 2007
		Phyllanthusiin G, trisgalloyl-HHDP-glucose isomers, pedunculagin/casuariin, casuarictin/ potentillin, sanguiins H-10, H-6, H-2, lambertianin C	Kähkönen et al., 2012
		Galloyl-bis-HHDP-glucose	Määttä-Riihinen et al., 2004
Rubus idaeus	raspberry, berries	1692–1754 mg/100g dw[a]	Kähkönen et al., 2001
		260.0–326.2 mg/100 g fw[a]	Koponen et al., 2007
		Pedunculagin, casuarictin/potentillin, sanguiin H-6 and H-10 isomers, lambertianin C	Kähkönen et al., 2012
		Galloyl-bis-HHDP-glucose	Määttä-Riihinen et al., 2004
Rubus arcticus	arctic bramble, berries	Galloyl-bis-HHDP-glucose	Määttä-Riihinen et al., 2004
Fragaria ananassa	strawberry, berries	81–184 mg/100 g dw[a]	Kähkönen et al., 2001
		67.6–83.2 mg/100 g fw[a]	Koponen et al., 2007
	flowers	Casuarictin, potentillin, agrimoniin, HHDP-glucose, bis-HHDP-glucoses, galloyl-HHDP-glucoses, digalloyl-HHDP-glucose, tris-galloyl-HHDP-glucose	Hanhineva et al., 2008
	leaves	Pedunculagin, galloyl-HHDP-glucoses, tellimagrandin I, agrimoniin, other dimeric and trimeric ETs	Kårlund et al., 2014
	berries	Galloyl-bis-HHDP-glucose	Määttä-Riihinen et al., 2004
Rosa rugosa	hip	107.5 mg/100 g fw[a]	Koponen et al., 2007
		Pedunculagin, tellimagrandin II	Salminen et al., 2005
Hippophae	sea buckthorn,	1.0 mg/100 g fw[a]	Koponen et al., 2007

Species		ET content or individual ETs characterized	Reference
rhamnoides	berries		
Lythrum salicaria	purple loosestrife, herb	Vescalagin, pedunculagin	Rauha, 2001
Betula pubescens spp. *pubescens*	white birch, leaves	Pedunculagin, pedunculagin derivative, tellimagrandins I and II, casuarictin, potentillin, isostrictinin, 1,2,3-trigalloyl-4,6-HHDP-α-glucose, 2,3-HHDP-glucose	Salminen et al., 2001, 2002
Betula pubescens spp. *czerepanovii*	mountain birch, leaves	Pedunculagin, pedunculagin derivatives, casuarictin Pedunculagin, pedunculagin derivative, tellimagrandins I and II, casuarictin, potentillin, isostrictinin, 1,2,3-trigalloyl-4,6-HHDP-α-glucose, 2,3-HHDP-glucose	Haviola et al., 2012 Salminen et al., 2002
Betula pendula	silver birch, leaves	Pedunculagin, tellimagrandins I and II, casuarictin, potentillin, isostrictinin, 1,2,3-trigalloyl-4,6-HHDP-α-glucose, 2,3-HHDP-glucose	Salminen et al., 2002
Betula nana	dwarf birch, leaves	Pedunculagin, tellimagrandins I and II, casuarictin, potentillin, isostrictinin, 1,2,3-trigalloyl-4,6-HHDP-α-glucose, 2,3-HHDP-glucose	Salminen et al., 2002
Quercus robur	English oak, leaves	Tellimagrandin II, casuarictin, pedunculagin, stachyurin/casuarinin, vescalagin, castalagin, castavaloninic acid, cocciferin D_2	Salminen et al., 2004
Geranium sylvaticum	wood cranesbill, different parts	Geraniin, ascorgeraniin, euphorbin, carpinusin, geraniinic acid derivatives, chebulagic acid-type ET, repandusidic acid-type ETs,1,2-digalloyl-4,6-HHDP-glucose, galloyl-HHDP-glucose, digalloyl-HHDP-glucose, trigalloyl-HHDP-glucose, trigalloyl-DHHDP-glucose, digalloyl-HHDP-DHHDP-glucose	Tuominen et al., 2013

[a] different cultivars, sampling locations and years. Minimum and maximum contents are shown. Abbreviations: dw = dry weight, fw = fresh weight

5 BIOLOGICAL ACTIVITY OF ELLAGITANNINS FROM THE POINT OF VIEW OF AN INSECT HERBIVORE

As was mentioned in the Introduction, there are two different theories by which tannins are suggested to function as anti-herbivore agents, i.e., by protein precipitation and by oxidative activation. The effect of the protein precipitation theory was emphasized by Feeny's findings in the late 60's and early 70's when he concluded that tannins were responsible for the negative effects on the growth and development of winter moth (*Operophtera brumata*, Lepidoptera: Geometridae) larvae (Feeny, 1968) probably caused by complexation of oak leaf tannins with proteins (Feeny, 1969). Feeny showed that PAs were more effective protein precipitants than were HTs (Feeny, 1969) and after that, the protein precipitation capacity (PPC) of various tannins in various conditions and with various methods and proteins have been studied (e.g., Asquith and Butler, 1986; Hagerman and Butler, 1981; Hofmann et al., 2006; Kilkowski and Gross, 1999). Some of these studies included also ETs (e.g., Kilkowski and Gross, 1999; Hofmann et al., 2006), but since their PPC was significantly lower than that of PAs, galloyl glucoses and GTs, they were not considered to be biologically relevant compounds.

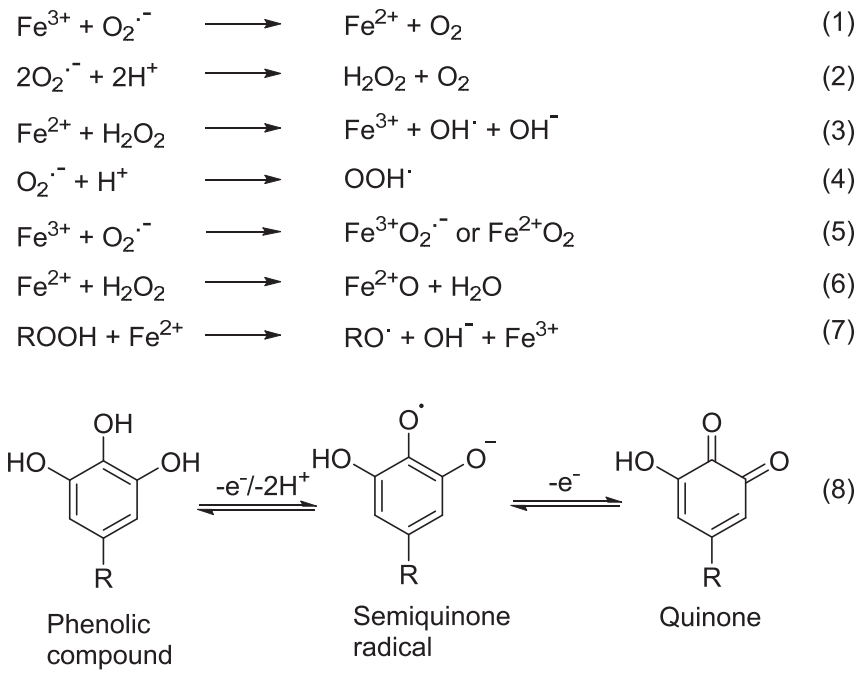
On the other hand, the results of Feeny were challenged by results obtained from feeding experiments done with grasshopper (Orthoptera: Acrididae) (Bernays, 1978; Bernays and Chamberlain, 1980) and swallowtail (Lepidoptera: Papilionidae) caterpillars (Berenbaum, 1983). In Bernays' study (1978), PAs were not found to have effects on the survival, growth, consumption or digestion of the larvae. Similar results were obtained when tannic acid (a mixture of GGs and GTs) was added to the food (Bernays, 1978; Bernays and Chamberlain, 1980), except for one grasshopper species (*Locusta migratoria*) that was severely affected by the tannic acid-containing food (Bernays, 1978). The effects of HT-containing food were explained by the passage of tannic acid through the peritrophic envelope (PE) which resulted as lesions in midgut and caeca (Bernays, 1978). Likewise, in Berenbaum's study, no effects were observed and it was concluded that tannins do not act as digestibility-reducing substances, but

there must be some other mechanism for their activity (Berenbaum, 1983; Bernays, 1978).

It was suggested that pH conditions together with redox conditions in the guts of different types of herbivores could explain these differences. Soon it was proposed that the activity of tannins cannot be based on PPC (at least in the cases of all herbivores), because for example lepidopteran larvae have basic gut conditions that prevent precipitation processes (Appel, 1993) and there are surfactants in the gut fluids that inhibit protein precipitation (Martin and Martin, 1984). Instead, it was suggested that the biological activity resulted from oxidation and reactions related to that, such as the generation of reactive oxygen species (ROS) (Appel, 1993).

5.1 Oxidative activity of tannins in herbivores

Oxidation is a reaction that is involved in the normal metabolism of cells. In these oxidative reactions several radicals are formed which are involved in enzymatic reactions, mitochondrial electron transport, signal transduction, activation of nuclear transcription factors and gene expression (Bayir, 2005). The products of oxidative reactions are called reactive oxygen species (ROS) and include both oxygen-based radicals and non-radical oxidizing agents. Radicals include hydroxyl ($OH^{\bullet}$), superoxide ($O_2^{\bullet -}$), peroxyl ($RO_2^{\bullet}$), hydroperoxyl ($HO_2^{\bullet}$; Bayir, 2005) and alcoxyl ($RO^{\bullet}$) radicals (Barbehenn and Stannard, 2004). Non-radical oxidizing agents include hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl) and ozone (O_3), which all can be converted to radicals (Bayir, 2005). The formation of these various radicals are presented and discussed in Scheme 1. From these reactions those involving hydrogen peroxide and peroxides in general, can be regarded to be the most important ones with respect to plant-eating insect herbivores. This is because $OH^{\bullet}$ and $O_2^{\bullet -}$ radicals should be scavenged in the gut lumen by, e.g., the peritrophic envelope (Barbehenn, 2001; Section 5.2). In addition, peroxides are stable enough to be able to oxidize other luminal components and diffuse into midgut epithelial cells where they can decompose to ROS (Barbehenn, 2001).



Scheme 1. Reactions related to the formation of reactive oxygen species (ROS). Catalytic amounts of free ferric ion (Fe^{3+}) may react with superoxide radical ($O_2^{\bullet -}$) to generate the ferrous iron (Fe^{2+}) and molecular oxygen (Reaction 1). If hydrogen peroxide (H_2O_2) is generated within the system by the chemical dismutation of superoxide radical (Reaction 2), the extremely reactive hydroxyl radical ($OH^{\bullet}$) may be formed (Reaction 3; Fenton reaction). Other ROS may be formed, such as the peroxyl radical, the perferryl ion, the ferryl ion or alcoxyl radical (Reactions 4, 5, 6 and 7 respectively). At the high pH of the digestive tract of insect larvae, the autoxidation of (poly)phenolic compounds produces semiquinone radicals and quinones (Reaction 8). Adapted from Summers and Felton (1994), Barbehenn and Stannard (2004) and Barbehenn and Constabel (2011).

Organisms use antioxidants to overcome the destructive consequences of oxidation. These antioxidants include, e.g., ascorbic acid and glutathione (Summers and Felton, 1994). However, if the protective effect of antioxidants are overwhelmed by the production of ROS, oxidation-related damages will occur and this phenomenon is called oxidative stress. This can result in protein oxidation and lipid peroxidation, which in turn may affect the growth and development of larvae, reduce reproductive potential and decrease adult longevity (Summers and Felton, 1994).

In many lepidopteran larvae digestive tracts the oxygen levels are low (Johnson and Barbehenn, 2000) and the pH is high (Berenbaum, 1980; Dow,

1984; Gringorten et al., 1993). Despite the low oxygen levels, the overall redox conditions in the midgut (that is the place of food assimilation) may still be oxidizing (Appel and Martin, 1990), thus enabling the oxidation of phenolic compounds. It has also been observed that the amount of iron ions in the midgut is high enough (40–70 μM; Barbehenn et al., 2005a) to enhance the production of ROS through Fenton-type reactions (Barbehenn et al., 2005a; Scheme 1). Moreover, the midgut redox and pH conditions have been shown to be affected by the plant material that herbivores consume (Appel and Maines, 1995; Johnson and Felton, 1996; Schultz and Lechowicz, 1986). Also, the content and quality of phenolic compounds in the plant material affects significantly the semiquinone radical levels they produce in the guts of herbivores, and thus the level of oxidative stress (Barbehenn et al., 2005b, 2006a, 2008a, b). For example, it has been shown that sugar maple leaves, which have low ascorbate:phenolic ratio produce higher levels of semiquinone radicals in the midguts of two caterpillar species than red oak leaves do, in which the ascorbate:phenolic ratio is higher (Barbehenn et al., 2005b). In addition, semiquinone radical levels together with markers that indicate oxidative stress (protein carbonyls (oxidized proteins), peroxides, dehydroascorbate (oxidized):total ascorbate ratio and glutathione disulphide (oxidized): total glutathione ratio) are higher in tannin-sensitive species (*Malacosoma disstria*) than in tannin-tolerant species (*Orgyia leucostigma*) (Barbehenn et al., 2005b, 2008b). Moreover, Barbehenn et al. (2003, 2009a) have shown that HTs increase the level of radical production in the digestive tracts of several larvae species. Furthermore, it has been shown that the ETs produce the highest amounts of semiquinone radicals, followed by galloyl glucoses. On the other hand, it was found that PAs do not produce radicals at all (Barbehenn et al., 2006b). Previously, Barbehenn and Martin (1994) have found that oxidizing gut conditions do not show oxidation of polyphenols in tannin-tolerant larvae and similar results (browning of midgut material was more pronounced in the most reducing midguts) have been obtained by Johnson and Felton (1996). This paradoxical relationship has been suggested to reflect the differences in the concentrations of oxidizable compounds in different host foliage or to indicate that the negative redox potentials may be a product of extensive oxidation, and not a larvae's way to prevent oxidation (Johnson and Felton, 1996). Thus it is

possible, despite the reductive conditions that herbivores typically have in their midguts that the food material they ingest still autoxidizes in the highly alkaline gut conditions (Appel, 1993). It has also been suggested that even though some parts of the digestive tracts of herbivores might be anoxic, oxygen is always present close to the epithelium which is the prime target of oxidative stress, thus enabling oxidative reactions (Gross et al., 2008).

5.2 Antioxidative mechanisms of herbivores

Although there is evidence that ETs and other polyphenols have oxidative effects in the guts of lepidopteran herbivores (e.g., Barbehenn et al., 2003, 2005b, 2009a), the herbivores have antioxidative mechanisms to diminish the damage. One of these is the PE (also called a peritrophic membrane) that insects secrete around the ingested food in their midguts. It is composed of a meshwork of chitin which is surrounded by a gel-like matrix of glycoproteins, proteins and proteoglycans (Barbehenn and Stannard, 2004). PE is assumed to protect the midgut epithelium from abrasive food, invasive pathogens and plant toxins, but it may also function as an iron and radical-scavenging antioxidant (Barbehenn and Stannard, 2004; Summers and Felton, 1996). There are four different mechanisms by which the PE is proposed to function: adsorption, ultrafiltration, polyanion exclusion and as an antioxidant (Barbehenn, 2001; Summers and Felton, 1996).

The adsorption of tannins on PE has been evidenced only in the case of grasshoppers (Bernays, 1978). Barbehenn and Martin (1992) studied one herbivorous insect, *Orgyia leucostigma,* but found no evidence for the adsorption of tannic acid on the PE. The second mechanism, ultrafiltration, has not either gained support, because of the fact that for the filtration to occur, the molecular weights of ingested compounds should be high or the diameter of the compounds should be large (Barbehenn, 2001). However, this mechanism might have some significance, since it has been shown that tannic acid forms aggregates and colloidal structures in the gut of *Manduca sexta* larvae (Barbehenn and Martin, 1998). Polyanion exclusion might seem plausible mechanism for the PE to function. Proteoglycans that are found in the PE possess negatively charged sites. This, together with the fact that polyphenolic

compounds ionize at the highly alkaline gut conditions of insect herbivores to negatively charged phenolate ions, could explain the mode of action. However, the results obtained have not gained support for this mechanism either (Barbehenn and Martin, 1997). The fourth mechanism suggesting that the PE functions as an antioxidant that can scavenge hydroxyl radicals and inhibit the formation of hydroperoxides from hydroxyl radical generating systems could be an important one for several reasons (Summers and Felton, 1996): first, the midgut epithelium is sensitive to oxidative damage by dietary pro-oxidants and thus needs to be protected; second, by acting as an antioxidant, the radical scavenging capacity of the PE would prevent the depletion of antioxidant vitamins (e.g., ascorbic acid) and in additon, it would also prevent damage to amino acids and small peptides as well as to the transporters that are responsible for their uptake; third, as an antioxidant, the PE might also interfere the generation of pro-oxidants, e.g., by chelating metal ions (Summers and Felton, 1996). The role of PE as a sacrificial antioxidant has been studied by Barbehenn and Stannard (2004). In this feeding experiment, *Malacosoma disstria* (tannin-sensitive species) larvae were fed with diets containing elevated iron concentrations and a diet with elevated iron content and tannic acid as a source of free radicals. In both of these cases, the PE showed properties of sacrificial antioxidant: elevated iron content in the diet resulted in elevated iron levels in the PE and the PE protected proteins of the midgut epithelium by directing the oxidative damages to it, which was detected as elevated levels of protein carbonyls in the PE (Barbehenn and Stannard, 2004).

Another antioxidative process that is important for insect larvae is the ascorbate-recycling system (Barbehenn et al., 2001). Ascorbic acid (or ascorbate) is a well-known antioxidant both in humans and in insects. Its antioxidant power is further enhanced by the presence of glutathione. The complete redox-cycle of these two antioxidants is presented by the following reactions (9 and 10).

$$AA + H_2O_2 \rightarrow DHA + 2H_2O \quad (9)$$

$$DHA + 2GSH \rightarrow AA + GSSG \quad (10)$$

At the first stage (Reaction 9), hydrogen peroxide (H_2O_2) reacts with ascorbic acid (AA) in the presence of ascorbate peroxidase to form dehydroascorbate (DHA) and water (H_2O). In the second step (Reaction 10), glutathione (GSH)

converts DHA back to AA in the presence of dehydroascorbate reductase. In the process, glutathione itself is oxidized to GSSG.

To study the effectiveness of the ascorbate-recycling system, Barbehenn et al. (2001) used *O. leucostigma* and *M. disstria* larvae. With both larvae, elevated peroxide concentrations were observed when the larvae fed on a diet containing tannic acid, but no added ascorbate. When ascorbate was added to the diet together with tannic acid, the peroxide levels of *O. leucostigma* were reduced, but were not changed in the case of *M. disstria*; high levels of peroxides were still observed regardless of the addition of the ascorbate. The same phenomenon was observed with *Helicoverpa zea* (Noctuidae) larvae when they were fed with a diet containing caffeic or chlorogenic acid and ascorbate (Summers and Felton, 1994). It was concluded that the tannin-tolerant *O. leucostigma* has more efficient antioxidant system compared to that of the tannin-sensitive *M. disstria*, since the levels of antioxidative enzymes were similar in both species (Barbehenn et al., 2001).

Another way of avoiding the harmful effects of ingested HTs is by compensatory feeding (Barbehenn et al., 2009a; Haukioja et al., 2002; Kause et al., 1999; Lempa et al., 2000). For example, it has been studied that when larvae fed on leaves painted with HTs (compounds used were PGG and pedunculagin), the semiquinone radicals formed in the midgut were significantly increased. However, no effects on larval performance were detected. This was explained by compensatory feeding, i.e., the larvae eat more to compensate the harmful effects of oxidative reactions, that reduce the quality of the food (Barbehenn et al., 2009a).

In a another study, birch leaves of different ages and with varying amounts of GTs and PAs were fed to *Epirrita autumnata* larvae (Kause et al., 1999). In this study, it was found that when larvae were fed with young leaves with high GT content, the efficiency of conversion of ingested food into larval biomass (ECI) was negatively affected by the high GT content. However, this was compensated by the larvae by significant increase in the relative consumption rates (RCR) of leaves, thus indicating compensatory feeding (Kause et al., 1999). On the other hand, when the larvae were fed with mature leaves containing high concentration of PAs, the effects on ECI and RCR were not significant and thus, the larvae did not display compensatory feeding in the case of mature leaves (Kause et al., 1999).

6 AIMS OF THE STUDY

This thesis focused on the study of ETs. On one part, the aims were to enhance their characterization from complex plant samples and to study their ocurrence in Finnish plant species, since these kinds of studies are limited. Another aspect was to examine their biological activity concentrating on the 'oxidation theory', i.e., could ETs function as plants' defensive compounds against plant-eating insect herbivores via oxidative reactions. To do this, and to obtain information about the structure-activity relationships of ETs, both *in vitro* and *in vivo* methods were used. Thus, the main aims can be summarized by posing the following questions:

1) How ETs can be effectively and quickly identified/characterized from plant crude extracts without the need for isolation and characterization of individual ETs? Are there useful characteristics and features in ETs' structures which can be used to aid the characterization by the data obtained from chromatographic, spectroscopic and mass spectrometric analyzes? (**I**)
2) Which Finnish plant species contain ETs? Which plants are rich in ETs and what kind of ETs these species produce? (**II**)
3) How different ET structures differ in their reactivity (i.e., activity) in different biological environments? (**III**, **IV**)

7 MATERIALS AND METHODS

7.1 Plant material

All plant materials used (Table 4) were collected from natural populations growing around Turku area, south-west Finland and from the Botanical Garden of the University of Turku. Several plants of a particular species were collected to obtain a pooled and representative sample; usually the amount of the collected plant material was high enough to obtain 50 g sample of the dried material to be extracted (Section 7.2). The plant species and parts to be analysed were selected based on availability and abundance. Thus, in some cases, e.g., alders and birches, leaves were collected but bark was not, because the trees could not be cut down. The sampling time also affected the decision which parts were collected. For large scale purification of individual ETs (**I, III and IV**) additional plant material was collected when needed. *Terminalia chebula* seeds (**I**) were from previous research projects (Saleem et al., 2001, 2002).

All plant materials were air-dried, ground into fine powder and stored in a freezer (–20 °C) until the time of extraction. Althought freeze-drying is the preferred choice for drying plant materials (Salminen, 2003), air-drying was used because of the substantial amount of plant material. However, this should not alter the chemical composition of the samples significantly (Keinänen and Julkunen-Tiitto, 1996; Salminen, 2003). Moreover, the grounding was performed as soon as the material was dry, and then stored in a freezer (–20 °C). After extraction and freeze-drying (Section 7.2), the crude extracts were stored in a freezer until the time of analysis (Section 7.6.1). All crude extracts and fractions were analyzed as soon as possible.

Table 4. Plant species used in this thesis work. Shown are plant families, species (scientific name and common name) and the plant part(s) that were used. The column 'Study' refers to publication(s) in which the particular plant species was used.

Plant family and species		Plant part(s)	Study
Betulaceae		Leaf	II
Alnus incana	Grey alder	Leaf	II
Alnus glutinosa	Black alder	Leaf	II
Betula pubescens	White birch	Leaf	II
Betula pendula	Silver birch	Leaf	II
Betula nana	Dwarf birch	Leaf	II
Corylus avellana	Common hazel	Leaf	II
Elaeagnaceae			
Hippophaë rhamnoides	Sea buckthorn	Leaf	I–III
Fagaceae			
Quercus robur	English oak	Leaf, seed, bark	I–III
Geraniaceae			
Geranium pratense	Meadow cranesbill	Leaf	II
Geranium sylvaticum	Wood cranesbill	Leaf	I–IV
Lythraceae			
Lythrum salicaria	Purple loosestrife	Leaf and flower (combined)	I–III
Myricaceae			
Myrica gale	Bog myrtle	Leaf	II
Onagraceae			
Epilobium hirsutum	Hairy willowherb	Leaf and flower (combined)	II
Epilobium montanum	Broad-leaved willowherb	Leaf	II
Epilobium angustifolium	Rosebay willowherb	Flower	I–III
Rosaceae			
Alchemilla	Lady's mantle	Leaf, root	II
Potentilla anserina	Silverweed	Leaf	II
Filipendula ulmaria	Meadowsweet	Inflorescence	I–III
Fragaria vesca	Wild strawberry	Leaf and flower (combined)	II
Geum urbanum	Wood avens	Leaf, seed	I–III
Geum rivale	Water avens	Leaf	II
Potentilla erecta	Tormentil	Root	I–IV
Comarum palustre	Marsh cinquefoil	Leaf	II
Rosa dumalis	Whitis-stemmed briar	Petals	II
Rosa mollis	Soft downy rose	Leaf	II
Rosa spinosissima	Burnet rose	Leaf	II
Rosa rugosa	Rugosa rose	Leaf	II
Rosa spp.		Hip	II
Rubus chamaemorus	Cloudberry	Leaf	II
Rubus idaeus	Raspberry	Leaf	I–III
Rubus saxatilis	Stone bramble	Leaf	II
Apiaceae			
Anthriscus sylvestris	Cow parsley	Inflorescence	II

Plant family and species		Plant part(s)	Study
Apocynaceae			
Vincetoxicum hirundinaria	Swallow-wort	Leaf	II
Araceae			
Calla palustris	Bog arum	Leaf	II
Asparagaceae			
Convallaria majalis	Lily of the valley	Leaf	II
Asteraceae			
Solidago virgaurea	Goldenrod	Leaf	II
Taraxacum spp.	Dandelion	Leaf, flower	II
Tussilago farfara	Coltsfoot	Leaf	II
Brassicaceae			
Alliaria petiolata	Garlic mustard	Leaf and flower (combined)	II
Bunias orientalis	Warty cabbage	Flower	II
Thlaspi arvense	Field pennycress	Leaf and silicles (combined)	II
Campanulaceae			
Campanula patula	Spreading bellflower	Flower	II
Campanula rapunculoides	Creeping bellflower	Flower	II
Caprifoliaceae			
Lonicera xylosteum	Fly honeysuckle	Leaf	II
Caryophyllaceae			
Saponaria officinalis	Soapwort	Leaf, flower	
Silene dioica	Red campion	Leaf	II
Stellaria sp.	Stitchwort	Leaf and flower (combined)	II
Ericaceae			
Rhododendron tomentosum	Labrador tea	Leaf	II
Vaccinium myrtillus	Blueberry	Leaf	II
Vaccinium uliginosum	Bog bilberry	Leaf	II
Fabaceae			
Caragana arborescens	Siberian peashrub	Leaf and flower (combined)	II
Lathyrus pratensis	Meadow vetching	Flower	II
Lupinus polyphyllus	Garden lupin	Leaf, flower	II
Vicia cracca	Tufted vetch	Leaf, flower	II
Grossulariaceae			
Ribes alpinum	Mountain currant	Leaf	II
Ribes uva-crispa	Gooseberry	Leaf	II
Malvaceae			
Tilia cordata	Small-leaved lime	Leaf	II
Oleaceae			
Fraxinus excelsior	Ash	Leaf	II
Orobanchaceae			
Melanpyrum sp	Cow-wheat	Leaf	II
Papaveraceae			

Plant family and species		Plant part(s)	Study
Chelidonium majus	Greater celandine	Leaf and flower (combined)	II
Pinaceae			
Picea abies	Norway spruce	Needle	II
Pinus sylvestris	Scots pine	Bark	II
Primulaceae			
Lysimachia vulgaris	Yellow loosestrife	Leaf, flower	II
Ranunculaceae			
Caltha palustris	Marsh marigold	Leaf	II
Ranunculus acris	Meadow buttercup	Flower	II
Rhamnaceae			
Frangula alnus	Buckthorn	Leaf	II
Rosaceae			
Amelanchier alnifolia	Saskatoon	Leaf	II
Cotoneaster lucidus	Cotoneaster	Leaf	II
Malus sp.	Apple	Leaf	II
Prunus cerasus	Cherry	Leaf	II
Prunus padus	Bird cherry	Leaf	II
Sorbus aucuparia	Rowan	Leaf	II
Sorbus hybrid	Finnish whitebeam	Leaf	II
Sorbus intermedia	Swedish whitebeam	Leaf	II
Salicaceae			
Populus tremula	Aspen	Leaf	II
Salix caprea	Goat willow	Leaf	II
Salix phylicifolia	Tea-leaved willow	Leaf	II
Sapindaceae			
Acer platanoides	Norway maple	Leaf, seed, flight wing	II
Acer rubrum	Red maple	Leaf	II
Aesculus hippocastanum	Horse-chestnut	Leaf	II
Solanaceae			
Solanum tuberosum	Potato	Leaf	II
Ulmaceae			
Ulmus glabra	Wych elm	Leaf	II
Urticaceae			
Urtica dioica	Stinging nettle	Leaf	II
Combretaceae			
Terminalia chebula	Myrobalan	Seed	I

7.2 Extraction of plant materials

All plant materials were extracted with aqueous acetone (Me_2CO:H_2O, 70:30, v/v) containing 0.1 % (m/v) ascorbic acid to prevent the oxidation of compounds. This solvent was selected because it had been found to be the best solvent for the extraction of hydrolysable tannins (Salminen, 2003). The extraction procedure was as follows: 50 g of finely ground plant material was extracted with 900 ml of aqueous acetone in a planary shaker at room

temperature usually overnight, but at least for eight hours per solvent batch. The extraction was repeated three times. These conditions were used to ensure that the extraction is efficient enough for quantitive determinations. Despite the rather long extraction time, no significant decomposition of ETs was observed (based on quantitative determination of ellagic acid; unpublished results). Before a new batch of extraction solvent was applied, the solvent was separated from the plant material by vacuum filtration with a Büchner funnel, the plant material was put back into the extraction vessel and a new batch of solvent was added. All three solvent batches were combined and acetone was evaporated with a rotary evaporator (<40 °C). The remaining aqueous phase was filtered to remove all the non-water-soluble material (chlorophyll, waxes, etc.) and freeze-dried.

7.3 Fractionation of the crude extracts

Two different fractionation procedures were used. In both cases, a Sephadex LH-20 column (400 × 40 mm, Pharmacia, Uppsala, Sweden) was used and 10 g of the crude extract was dissolved in 20 ml of water. The solution was applied onto the Sephadex LH-20 material, soaked into the material and eluted with different solvents. The crude extracts used in **II** were fractionated into five subfractions (Table 5). The crude extracts used in studies **I**, **III** and **IV** were fractionated with seven eluents (Table 5), but more than one fraction per eluent was collected if it was possible to obtain fractions with almost pure ETs (the elution was monitored by HPLC, Section 7.6.1). The fraction volume was 500 ml per eluent, except for the Fraction 1 that was collected in five 100 ml subfractions. These were discarded after HPLC analyses (Section 7.6.1) if they did not contain compounds of interest. To ensure that all compounds had eluted from the column before a new extract was applied, the column was washed with aqueous acetone (Me_2CO:H_2O, 80:20 v/v) solution (Fraction 5b or 7b) and combined with Fraction 5a or 7a. All fractions were analyzed with HPLC-DAD as described in Section 7.6.1, concentrated into aqueous phase with a rotary evaporator and freeze-dried.

Table 5. Fractionation procedures used for crude extracts in **II** (upper part of the table) and in **I**, **III** and **IV** (lower part of the table).

Fraction	H_2O	MeOH	Me_2CO	Compound group
1	100	–	–	sugars and non-phenolic compounds
2	70	30	–	phenolic acids
3	25	75	–	flavonoid glycosides
4	30	55	15	flavonoid glycosides
5a	30	–	70	hydrolysable tannins and
5b	20	–	80	proanthocyanidins
1	100	–	–	
2	70	30	–	monomeric acyclic ETs
3	50	50	–	monomeric acyclic and glucopyranose-based ETs
4	90	–	10	monomeric acyclic and glucopyranose-based ETs
5	70	–	30	monomeric glucopyranose-based and oligomeric ETs
6	50	–	50	oligomeric ETs
7a	30	–	70	oligomeric ETs
7b	20	–	80	

7.4 Purification of ellagitannins

Final purification of individual ETs was achieved with preparative and semi-preparative HPLC. The whole fraction (or max. 600 mg of the fraction or semi-pure compound) was dissolved in approximately 2 ml of water and filtered through 0.45 μm PTFE filter. Two different HPLC systems were used. In **I**, the preparative HPLC system consisted of a LiChroprep RP-18 (440 × 37 mm, 40–63 μm, Merck, Darmstadt, Germany) column and a Merck Hitachi 6200A pump (Merck-Hitachi, Tokyo, Japan). The eluent consisted of H_2O and MeOH and a linear gradient from 5:95 MeOH:H_2O to 80:20 MeOH:H_2O. The elution lasted for two hours with the flow rate of 5 ml min^{-1}. Fraction volume was 50 ml. All fractions were analyzed with HPLC-DAD (Section 7.6.1), pure fractions were combined, concentrated into aqueous phase and freeze-dried.

The semi-preparative HPLC system used in **III** and **IV** consisted of Waters Corporation (Milford, USA) HPLC, 2998 photodiode array detector, 600 controller, Delta 600 pump, programmable fraction collector and Gemini RP-18 (150 × 21.2 mm, 10 μm, Phenomenex) column. The eluent system consisted of 0.1 % formic acid (HCOOH) in water and acetonitrile (ACN) at the flow rate of

8 ml min^{-1}. Different linear gradient elutions were used depending on the retention time of a compound obtained from HPLC chromatograms (Section 7.6.1). In general, the elution begun with the same solvent composition as the compound eluted from the analytical HPLC. The elution gradient was increased to 80:20 ACN:H_2O (v/v) within two hours. The fraction collector was programmed to automatically collect fractions based on the retention time, the intensity of the peak and the volume of the collection vessel (2 or 10 ml). Based on the chromatograms obtained from the elution, the most promising fractions were analyzed with HPLC-DAD (Section 7.6.1). Fractions containing pure compounds were combined, concentrated into aqueous phase and freeze-dried.

7.5 Derivatization of ellagitannins

In order to obtain a wider selection of the structural features that may affect the activity of ETs in **III**, derivatives of vescalagin, stachyurin, hippophaenin B and a dimer from *Lythrum salicaria* were synthesized.

The methyl derivatives were obtained by refluxing ETs in MeOH for 2.5–48 h, depending on the efficiency of methylation. Methylation efficiency was monitored with HPLC-DAD (Section 7.6.1). The catechin derivatives of ETs were obtained by refluxing ETs with commercially available catechin for 24 h in 0.05 M *o*-phosphoric acid (H_3PO_4). HPLC-DAD was used to monitor the reaction (Section 7.6.1). In both cases, the derivatives were purified with preparative HPLC as described in Chapter 7.4.

7.6 Analysis and characterization

7.6.1 HPLC-DAD

Prior to analysis, all samples were filtered with a 0.45 μm PTFE filter. The analytical HPLC system used in all analyses consisted of a Merck Hitachi (Merck- Hitachi, Tokyo, Japan) liquid chromatography equipped with an interface module (D-7000), a diode array detector (L-7455), an autosampler (L-7200), a pump (L-7100), and a LiChroCART Superspher 100 RP-18 (75 × 4 mm, 4 μm, Merck, Darmstadt, Germany) column. The binary mobile phase

consisted of ACN (A) and 0.05 M *o*-H_3PO_4 (B). The elution profile was: 0–3 min, 2 % A in B (isocratic); 3–25 min, 2–30 % A in B (linear gradient); 25–30 min, 30–70 % A in B (linear gradient); 30–34 min 70 % A in B (isocratic; column wash); 34–36 min 70–2 % A in B (linear gradient); 36–50 min 2 % A in B (isocratic; column re-equilibration). Injection volume was 10 or 20 µl, flow rate was 1 ml min^{-1}, the detection wavelength was 280 nm, and the UV spectra were recorded between 195 and 450 nm.

Samples used in quantitations (**II**) were prepared by dissolving 10 mg of the crude extract (or fraction) in 1 ml of water. Gallic acid was used as an external standard. Four concentrations and three replicate injections per standard concentration were done. The standards were analyzed after every tenth sample. The calibration curve obtained was then used to quantitate those ten plant samples that followed the standards. The amount of total phenolic compounds (mg g^{-1}) in the crude extracts was calculated based on the total peak area of the chromatogram recorded at 280 nm.

7.6.2 HPLC-DAD-ESI-QTOF-MS

Samples for MS analyses were prepared by dissolving 3 mg of the crude extract in 1 ml of water. Samples were filtered through a 0.2 µm PTFE filter prior to analysis. The mass spectra were recorded with a Bruker Daltonics MicrOTOF-Q mass spectrometer (Bremen, Germany) with an ESI source. The mass spectrometer was controlled by Compass micrOTOF control software (Bruker Daltonics). Negative ion mode ESI conditions were: capillary voltage 4000 V with the end plate offset at –500 V, drying gas (N_2) temperature 200 ˚C with the flow rate of 8 l min^{-1}, nebulizer gas (N_2) pressure 1.6 bar, and mass range for data acquisition 100–3000 Da. The mass spectra were calibrated with 5 mM sodium formate solution injected at the end of each LC-MS run. Data obtained were handled by Compass DataAnalysis software (version 4.0, Bruker Daltonics).

The HPLC was controlled by Hystar software (version 3.2, Bruker BioSpin, Rheinstetten, Germany) and consisted of an Agilent Technologies (Waldbronn, Germany) 1200 Series pump, an autosampler, a diode array detector and an XBridge Phenyl (2.1×100 mm, 3.5 µm, Waters Corporation, Milford, USA)

column. The binary mobile phase consisted of ACN (A) and 0.1 % HCOOH (B) at the flow rate of 0.3 ml min^{-1}. The elution profile was: 0–1 min, 0 % A; 1–30 min, 0–30 % A in B (linear gradient); 30–32 min, 30–80 % A in B (linear gradient); 32–42 min 80 % A in B (isocratic; column wash); 42–44 min 80–2 % A in B (linear gradient); 44–60 min 0 % A (isocratic; column re-equilibration). Injection volume was 5 µl and chromatograms were recorded at 280 nm. The content of different ET subgroups in **II** were calculated from the chromatograms recorded at 280 nm and were expressed as percentage of the total peak area of the chromatogram. Characterization of individual ETs and compound groups were based on characteristic UV spectra as well as on mass spectra (**I**).

7.6.3 NMR

Structures of some of the ETs used in **I**, **III** and **IV** were confirmed by NMR. The NMR experiments were performed with a Bruker Avance 500 spectrometer operating at 500.13 MHz for ^{1}H and 125.77 MHz for ^{13}C. Spectra were recorded at 25 °C and methanol-d_4 was used as a solvent. The measurements included ^{1}H and ^{13}C spectra, and several 2D spectra, such as DQF-COSY, NOESY, ^{1}H–^{13}C HSQC and ^{1}H–^{13}C HMBC spectra.

7.7 Methods to study the activity of ellagitannins

7.7.1 pH 10 assay

The oxidative activities of the ETs in **III** and **IV** were measured with a 96-well plate reader (Multiskan Ascent, Thermo Electron Corporation) with our modification of the method reported by Barbehenn et al. (2006a, b). Ten mg of the purified and freeze-dried ET was dissolved in aqueous ethanol (EtOH:H_2O, 70:30 v/v) to prepare a 2.0 mM stock solution. Four dilutions (1.5 mM, 1.0mM, 0.50 mM and 0.25 mM) of the stock solution were prepared with EtOH:H_2O (70:30 v/v). One 12-well line of a 96-well plate was used in a single kinetic measurement. 3 × 30 µl of each of the four ET dilutions were pipetted into the 12 wells. 270 µl of a pH 10 carbonate buffer (50 mM, J.T. Baker, Deventer, Holland) solution was added into each well with a 12-line pipette; ET

concentrations in the reaction mixtures were thus 0.15 mM, 0.10 mM, 0.050 mM and 0.025 mM. Kinetic measurements were started immediately after the buffer addition; the delay was 1–2 s. The increase of absorbance at 415 nm (due to formation of quinones and other oxidation products) was monitored for each well for a period of 8 min in 8 s intervals. The results of the kinetic measurements were plotted as absorbance vs. time. From each plot, a maximum rate of oxidation (abs s^{-1}; maximum value for the tangential slope) was automatically calculated with the aid of the Multiskan Ascent software; in each case, the maximum rate was obtained during the first 100 s of oxidation. The three replicate samples within each ET concentration were used to calculate a mean value for the maximum rate of oxidation (abs s^{-1}) of a certain ET concentration (0.15 mM, 0.10 mM, 0.050 mM and 0.025 mM). These four mean values of maximum rates of oxidation were plotted against ET concentration. This type of a plot produced a straight line; only lines with correlation coefficients higher than 0.99 (as calculated by using Microsoft Excel) were accepted. The slope of the straight line gave the value of the maximum rate of oxidation as a function of concentration (abs s^{-1} mM^{-1}) for each of the tested ET. To maximise the reliability of the final results, each of the ETs were tested three to five times by separate 12-well measurements, i.e., each of the four ET concentrations were tested at least nine times (three replicate samples in three separate measurements). The final value of oxidative activity, i.e., the maximum rate of oxidation as a function of concentration (abs s^{-1} mM^{-1}) was reported as the mean value of these three to five measurements ± standard error of the mean (SEM).

7.7.2 2,2-diphenyl-1-picrylhydrazyl radical assay

The antioxidant activity of the purified ETs (measured for all those ETs in **III**; unpublished data) was measured with the DPPH (2,2-diphenyl-1-picrylhydrazyl) radical assay using the 96-well plate reader (Multiskan Ascent, Thermo Electron Corporation). Six dilutions (0.5, 0.25, 0.2, 0.1, 0.05 and 0.02 mM) were prepared from the 2 mM stock solution in EtOH:H_2O (70:30 v/v). The 0.25 mM DPPH solution was prepared in EtOH. For the measurements, 20 µl of each ET dilution was pipetted into four wells of the plate and 280 µl 0.25 mM DPPH solution was

added. In each set of measurements, positive and negative controls were included. The positive control sample, i.e., compound that produced 100 % inhibition of the radical, was 1 mM PGG in EtOH:H_2O (70:30 v/v). The negative control sample, i.e., compound that produced 0 % inhibition was EtOH:H_2O (70:30 v/v). The absorbance was monitored at 520 nm for 90 min. In this time, the reaction had completed, i.e., the absorbance of the sample did not change anymore. The absorbance was measured every ten minutes and the plate was shaken before reading the absorbance. The inhibition activity (%) for each dilution was calculated from the last absorbance readings by using the equation 1.

$$\text{Inhibition (\%)} = 1 - \frac{\text{Abs(sample)} - \text{Abs(pos.control)}}{\text{Abs(neg.control)} - \text{Abs(pos.control)}} \quad (1)$$

These calculated inhibition activities were plotted against concentration and the inhibition concentration at 50 % inhibition point (IC_{50}-value) was determined from the curves. Three independent replicates were measured for each ET.

7.7.3 Deoxyribose assay

The radical scavenging activity, metal chelating ability and pro-oxidant activity of purified ETs were determined with the deoxyribose assay (**IV**; Aruoma, 1994; Soberon et al., 2009). The assay of Soberon et al. (2009) was slightly modified. The radical scavenging activity was measured as follows: 1240 µl of 20 mM sodium dihydrogen phosphate (NaH_2PO_4) buffer (pH 7.4), 160 µl of 0.1 mM 2-deoxyribose (2-DR), 40 µl of 4.16 mM disodium ethylenediaminetetraacetic acid (EDTA), 40 µl of 4 mM iron (III) chloride ($FeCl_3$), 160 µl of different sample dilutions [four dilutions (1 mM, 2 mM, 3 mM and 5 mM with three replicates)], 40 µl of 4 mM ascorbic acid (AA) and 40 µl of 40 mM hydrogen peroxide (H_2O_2) were pipetted into Eppendorf tubes in the indicated order. In blank samples, no 2-DR or sample solutions were included, instead they were replaced with buffer. In control samples, 2-DR solution was included, but sample solution was replaced with buffer.

Fresh EDTA, $FeCl_3$ (from stock solution of 0.1 M), AA, H_2O_2, 2-thiobarbituric acid (TBA) and trichloroacetic acid (TCA) (from stock solution of 100 % TCA) solutions were prepared daily. EDTA and $FeCl_3$ were not pre-mixed nor left to stand before adding of the rest of the reagents (Halliwell and Gutteridge, 1981).

The tubes were incubated in a 37 °C water bath for 2 hours, after which each sample was divided into two 300 µl subsample. After that the color reagents, 300 µl of 1 % TBA dissolved in 50 mM natrium hydroxide (NaOH) and 300 µl of 2.8 % TCA, were pipetted into the tubes in the indicated order and the tubes were incubated in an 80 °C water bath for 30 minutes. The tubes were then allowed to cool at room temperature for 30 minutes before the absorbances were measured. From the cooled tubes, two 250 µl aliquots were pipetted to a 96-well plate, and the absorbances of samples, controls and blanks were measured with 96-well plate reader (Multiskan Ascent, Thermo Electron Corporation) at 520 nm. The average absorbance of each dilution was calculated, the absorbance of the blank sample was subtracted and the inhibition activity (%) was calculated by the equation 2.

$$\text{Inhibition (\%)} = \frac{[\text{Abs(control)} - \text{Abs(sample)}]}{\text{Abs(control)}} \cdot 100 \qquad (2)$$

The metal chelating ability measurements were determined by omitting EDTA from the reaction mixture and by replacing it with buffer solution and using sample concentrations of 0.1 mM, 0.25 mM, 0.5 mM and 1 mM. The pro-oxidant activity measurements were determined by omitting AA from the reaction mixture and by replacing it with the buffer solution. Same sample concentrations were used as in the radical scavenging assay.

7.7.4 Feeding experiment with **Amphipyrea pyramidea** *larvae*

Feeding experiments with purified ETs and copper underwing (*Amphipyra pyramidea*) larvae were performed in spring/summer 2011. The copper underwing larvae were reared from eggs; in the experiment fourth instar larvae

were used. For every ET-treatment 16 larvae from four clutches were randomly selected. The larvae were placed in plastic containers into which the ET-painted leaf of bird cherry (*Prunus padus*) was placed. Six different ETs (vescalagin, vescavaloninic acid, chebulagic acid, chebulanin, punicalagin and cocciferin D_2) were painted on the leaves at the concentration of 25 mg g^{-1} leaf dw. ETs were dissolved in aqueous acetone (Me_2CO:H_2O, 70:30 v/v) solution, the solution was painted on each leaf with a brush and acetone was allowed to evaporate before the leaf was put into the container with the larva. Aqueous acetone (70:30 v/v) solution was painted on the control leaves. The larvae were weighted before the experiment and at the end of the experiment (72 h). A fresh leaf was provided daily, the uneaten remainders of the leaves were removed from the containers and the frass was collected. Remainders of the leaves and frass were freeze-dried and weighted. The larvae were kept at ambient outdoor conditions in a shed at the Botanical garden of the University of Turku at Ruissalo. After the 72 h experiment, the larvae were allowed to pupate in their containers (moss was put to the containers to prevent drying of the larvae and to offer a more natural environment for the pupation) and the pupa were weighted 14 days after pupation.

To identify the possible effects of the ETs on the different components of larval performance, four nutritional indices were used (Roslin and Salminen, 2008). These indices were:

1) Amount of food ingested (mg dw)
2) Digestability (absorption effiency):

$$\frac{\text{food ingested (mg dw)} - \text{frass excreted (mg dw)}}{\text{food ingested (mg dw)}}$$

3) Growth efficiency (efficiency of conversion of ingested food to body substance)

$$\frac{\text{biomass increment (mg fw)}}{\text{food ingested (mg dw)}}$$

4) Metabolic efficiency (efficiency of conversion of digested food to body substance)

$$\frac{\text{biomass increment (mg fw)}}{\text{food ingested (mg dw)} - \text{frass excreted (mg dw)}}$$

To examine the impact of treatment on different metrics of larval performance, a generalized linear mixed effects model was used. Each response metric was modeled separately as a function of treatment (a categorical fixed effect with seven levels) and the weight of the larva at the start of the experiment (a continuous variable). To account for potential correlations among larvae from the same clutch, a repeated-measures model with clutch treated as the subject was used. Relevant degrees of freedom were estimated by a Kenward-Roger approximation. The model was fitted in SAS System 9.2 for Windows (SAS Institute Inc., Cary, NC, USA).

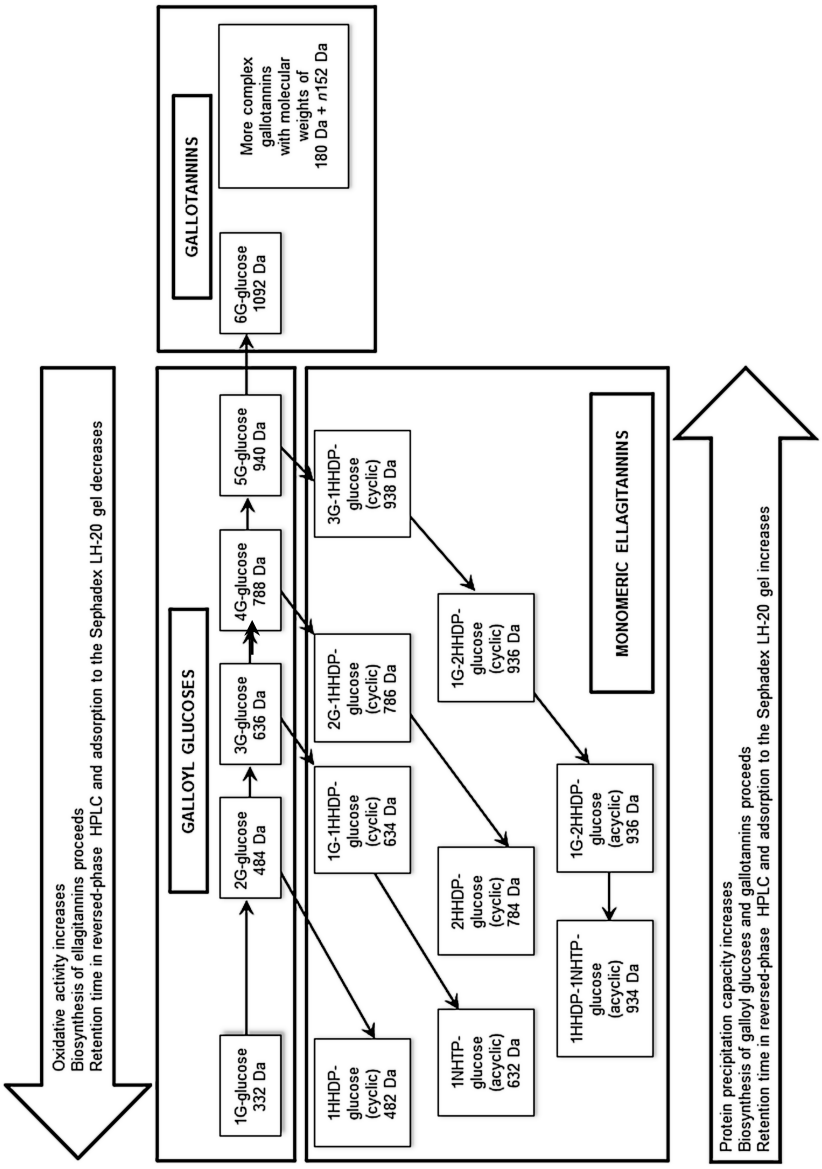
8 RESULTS AND DISCUSSION

8.1 Characterization of ellagitannins from plant samples

Nowadays the number of characterized ET structures exceeds a thousand (Quideau et al., 2011). This means that a lot of time has been used to extract, isolate, purify and characterize the structures of the isolated ETs. Thus, there is a vast literature available on different spectroscopic and spectrometric features for individual ET structures to aid phytochemists to identify their ETs. However, there is only little literature available that concerns thoroughly the characterization of ETs from different analytical steps, starting from the first chromatographic analysis of the crude extract in comparison to, e.g., flavonoids for which there is comprehensive data available (see e.g., Santos-Buelga et al., 2003). Moreover, there is a very limited selection of commercially available ETs, and thus reference compounds are not as readily available as they are for e.g., the above-mentioned flavonoids.

Fast compound classification and characterization is particularly useful in the case of screening studies, where there is a number of samples to be analyzed and the compounds of interest should be quickly recognized. Thus the aim of **I** was to overcome some of the characterization-related difficulties by providing a thorough representation on how ETs having different substructures can be readily and effectively characterized from complex plant samples.

Nevertheless, the data obtained by chromatographic and spectrometric methods is dependent on several factors, such as, the instrument, the column, the flow rate, the elution solvents and the ionization parameters used. Thus direct comparisons of, e.g., retention times between different studies is not meaningful. In the case of UV spectra, the data is in many cases published only as numerical data in a table which makes the application of the information inconvenient. Thus, in **I**, a characterization scheme (Scheme 2) was developed: it represents the regularities between different ET structures, their retention behavior in the RP-LC, mass spectrometry and even their biological activities.



Scheme 2. A simplified scheme showing how galloyl glucoses, gallotannins and ellagitannins are linked to each other in respect to their molecular weight, oxidative activity, protein precipitation capacity, HPLC retention order and affinity to Sephadex LH-20 gel. G = galloyl, HHDP = hexahydroxydihenoyl, NHTP = nonahydroxytriphenoyl

8.1.1 Structural information obtained from the retention order

The main feature that affects the retention of ETs is the ratio between galloyl groups and HHDP (or other HHDP derived) groups together with the configuration of the glucose core. As is seen from Scheme 2, the more there are galloyl groups in the ET structure, the later it will elute from the RP-HPLC column, and also from the Sephadex LH-20 column. Likewise, when the number of the HHDP groups in relation to the galloyl groups increases, the retention of ETs in the column decreases. With acyclic ETs, the adsorption to the column material decreases even more. Thus, *C*-glucosidic ETs elute earlier than their glucopyranose-based counterparts (Scheme 2, Table 1 in **I**). Furthermore, there are a couple of additional features of ETs, which should be kept in mind when interpreting the chromatographic data. Both glucopyranose-based and *C*-glucosidic ETs have two possible isomers with respect to the C-1 of their glucose core. If the C-1 of the glucopyranose-based ETs is unsubstituted, i.e., there is a OH group at C-1, two peaks with a nearly 1:1 ratio is usually seen in the chromatogram, namely α- and β- anomers. The presence of these kinds of ETs can be confirmed by the reaction with sodiumborohydride ($NaBH_4$) (Section 3.3.2; Hatano et al., 1988a). In addition, those ETs whose configuration at C-1 is β elute earlier than the isomers with α configuration at the C-1 (Table 1 in **I**; Salminen et al., 2001). Thus, e.g., vescalagin-type *C*-glucosidic ETs elute earlier than castalagin-type *C*-glucosidic ETs.

8.1.2 Structural information obtained from the UV spectra

The characterization of ETs is enhanced significantly, if the LC is equipped with a diode array detector, since then the UV spectrum can be obtained for each peak in the chromatogram. Based on the UV spectra, ETs can be distinguished from each other (Fig. 10) and from the other polyphenolic compounds present in the sample (Fig. 4 in **I**). Thus, ETs can be classified as belonging to either glucopyranose-based ETs or *C*-glucosidic ETs. The distinction between dehydroETs and chebuloyl-containing ETs may be difficult if only their UV spectra are considered (compare the spectra of geraniin and chebulagic acid to

that of tellimagrandin I in Fig. 10), but their characterization is simpler if also mass spectrometry can be utilized (Section 8.1.3).

The same features which affect the retention behaviour of ETs in RP-LC analysis, affect also their UV spectra. The UV spectra of glucopyranose-based ETs resemble those of gallic acid and PGG, in which there are two clear maxima at 220 and 280 nm region and a valley at 240 nm region (PGG in Fig. 10). As the number of HHDP groups increases with respect to the number galloyl groups in the ET structure, the valley between the two maxima gradually disappears (e.g., compare the spectra of tellimagrandin I, casuarictin and pedunculagin to each other in Fig. 10). With acyclic glucose-based ETs, as in the case of *C*-glucosidic ETs, the shape of the UV spectrum changes also characteristically: there is neither maximum observed at the 280 nm region nor there is a valley observed at the 240 nm region (vescalagin in Fig. 10). If a *C*-glucosidic ET contains an HHDP group and a galloyl group instead of an NHTP group, a plateu can be seen in the 260 nm region (e.g., stachyurin in Fig. 10), which is clearly separable from the valley present in the spectra of glucopyranose-based ETs.

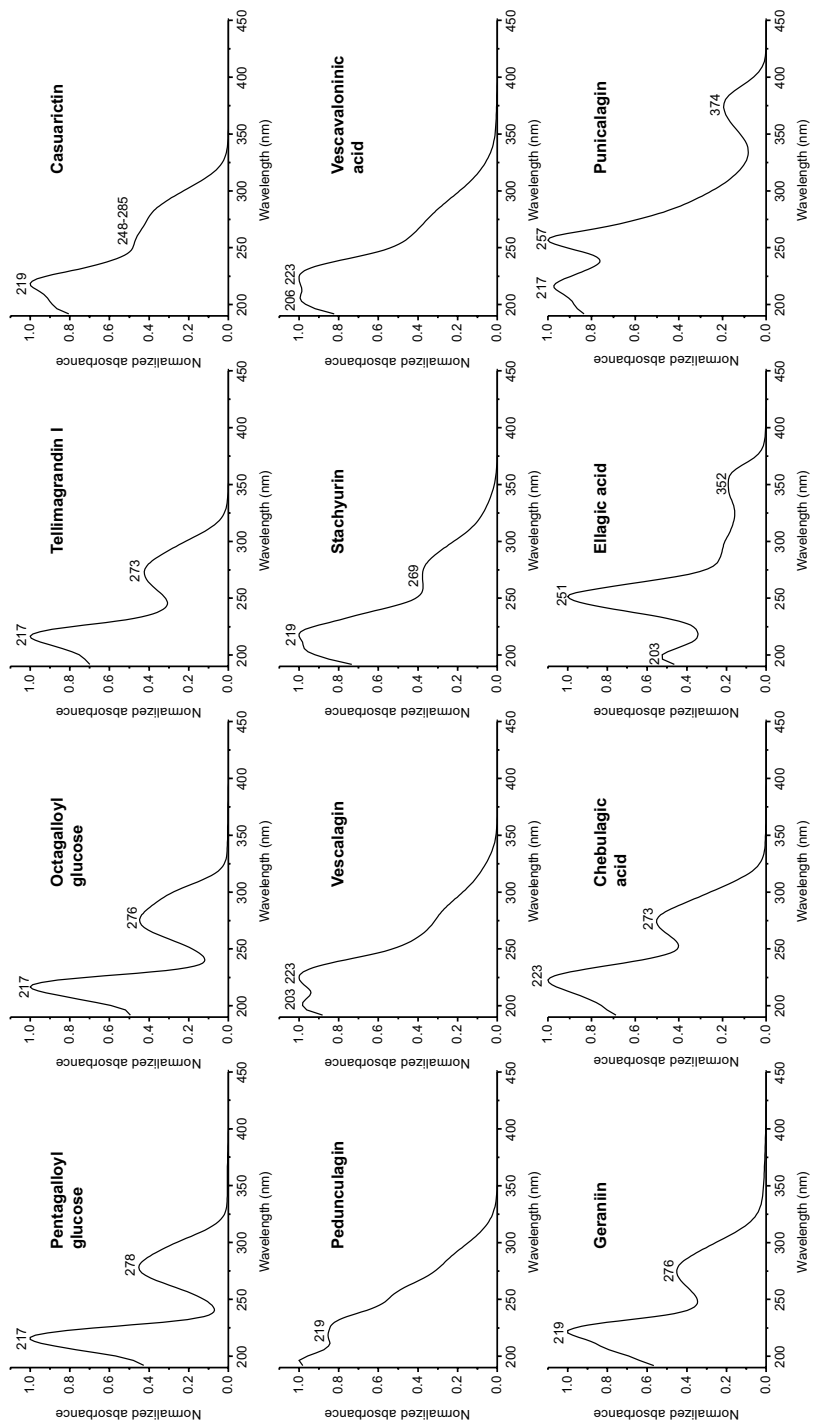


Figure 10. UV spectra of representative compounds belonging to hydrolysable tannin subclasses: galloyl glucoses (pentagalloyl glucose), gallotannins (octagalloyl glucose) and ellagitannins (rest of the spectra).

8.1.3 Structural information obtained from the mass spectra

The mass spectrometric analysis is an important tool in the characterization of ETs. The determination of the molecular weight (MW) is of importance, but additional information of the functional groups in the ET structure may also be obtained from the fragmentation data. Furthermore, if it is possible to combine the information from the chromatographic analysis and the UV spectra with the additional information obtained from the mass spectra, it may be possible to fully characterize ETs from the sample.

In the mass spectra of monomeric ETs, the most abundant peak represents usually the molecular ion $[M–H]^-$ of an ET. When the MW increases, as in the case of oligomeric ETs, the multiply charged ion $[M–2H]^{2-}$ may be the dominant one. These ions are distinguished from the singly charged ones by the isotopic distribution of the signals: if the isotopic signals differ by 1.0 Da the ion is singly charged, and if the signals differ by 0.5 Da, the ion is doubly charged (Fig. 6 in **I**).

Additional information about different structural details can be obtained from the fragment ions. For example, it is common that in the spectra of those ETs, which contain galloyl groups, an ion at *m/z* 169 is present, which corresponds to a deprotonated gallic acid. Likewise, the presence of an HHDP group is typically observed at *m/z* 301 as a deprotonated ellagic acid. In addition, information about substitution patterns can be obtained: for example, if an additional galloyl group is attached to an ET by an ether bond (C–O–C) as in the valoneoyl group of vescavaloninic acid (Fig. 2 in **I**), a fragment ion corresponding to the loss of a carboxylic acid (–COOH) group from the molecular ion (or from the doubly charged molecular ion) is seen, i.e., $[M–COOH]^-$ and/or $[M–H–COOH]^{2-}$. For those ETs which contain a DHHDP group, the loss of water $[M–H–H_2O]^-$ or $[M–2H–H_2O]^{2-}$ is typical. The loss of water is also characteristic for those *C*-glucosidic ETs, which have β configuration at their C-1, i.e., vescalagin-type ETs. From castalagin-type ETs (α configuration at C-1) the fragmentation of water is not observed. This has been explained to result from steric and intramolecular stabilization effects (Quideau et al., 2003, 2004, 2005; Yoshida et al., 1991b), i.e., in castalagin-type ETs, the OH group at C-1 forms a hydrogen bond to one of the OH groups of the NHTP group, which stabilizes the structure

of the castalagin-type ETs in comparison to the vescalagin-type ETs (Quideau et al., 2004, 2005).

The interpretation of the mass spectra of monomeric ETs is quite straightforward and the building blocks of an ET can, at least to some extent, be resolved by the aid of Scheme 2 and the fragmentation patterns given above. It is also simple to determine the MWs of oligomeric ETs. However, it is also possible to further evaluate the building blocks of oligomeric ETs. To do this, it is worth remembering that the oligomeric ETs are comprised of those monomeric ETs that are present in the sample. Thus, if it is known which type of monomeric ETs a plant species produce, then it is likely that the oligomeric ETs are constructuted from these monomers. Secondly, the linkage between the monomers is formed either via C–O–C or via C–C bonds between the monomers. In the former case, the other monomer functions as an *O*-donor, i.e., its OH group is etherified to an aromatic carbon atom of the other monomer (functions as an *O*-acceptor) (Okuda et al., 2009). Thus, in the process, a total of 2 Da is lost. The latter coupling is only possible for *C*-glucosidic ETs and flavanoETs (Okuda et al., 2009). This coupling takes place between the C-1 of the other monomer and an aromatic carbon atom of the other monomer. In this linkage, a total of 18 Da is lost (cleavage of water). Thus, different variations of the theoretical oligomer masses can be calculated by the following equations (3–4):

MW of an oligomeric C–O–C linked ET =
(MWs of the n monomers) – 2(n–1) (3)

MW of an oligomeric C–C linked ET =
(MWs of the n monomers) – 18 (n–1) (4)

where n stands for the number of the monomers

However, as for every generalization, exceptions do exist. Examples of these exceptions are the macrocyclic oligomers (e.g., oenothein B) and dimers that consist of monomers that belong to different subgroups (e.g., cocciferin D_2). In macrocyclic ETs, there is an additional C–O–C bond that decreases the MW of the oligomer by another 2 Da. However, if one wants to use Equation 1 to

estimate the MWs of these types of oligomers, it is worth remembering that the double C–O–C coupling typically occurs only once in the structure (Karonen et al., 2010). Thus, this additional feature allows the decrease of the MW derived from Equation 1 only by 2 Da.

8.2 Distribution and content of ellagitannins in Finnish flora

As was shown in Table 3 (Chapter 4), the ET content of Finnish plant species have not been widely studied. Thus, in **II** a wide variety of different plant species were screened for their ET composition and content. The information obtained from the study **II** has been used in **III** and **IV**, when ET-containing plant species were needed for large scale ET isolation and purification purposes.

In total, 82 plant species were studied. These species represented 34 different plant families. In addition, different parts of plants were studied in some cases. The studied plant species and parts are listed in Table 4 (Section 7.1). From the 82 species, 30 contained ETs. These species were taken into further examination, and the total ET content of the crude extract was quantitated with HPLC-DAD. In addition, the crude extracts were analyzed with HPLC-DAD-ESI-QTOF-MS to obtain detailed information about the ET composition of individual plant species. The ETs found were characterized based on the characterization patterns presented in **I** and were divided into four subgroups (glucopyranose-based ETs, *C*-glucosidic ETs, dehydroETs and oligomeric ETs), whose contents were also determined. Individual ETs were identified based on **I** and tentative characterizations were based on literature. Taxonomic distribution of ETs in the studied plant species was examined with the aid of phylogenetic trees, and the results were reflected to the APG III (2009) classification.

The plant species containing ETs, studied plant parts, the total content of ETs in the crude extract and individual ETs characterized from the plant samples are presented in Table 1 (**II**). The phylogenetic relationships between ET-containing species and the content of different ET subgroups are presented in Figure 11.

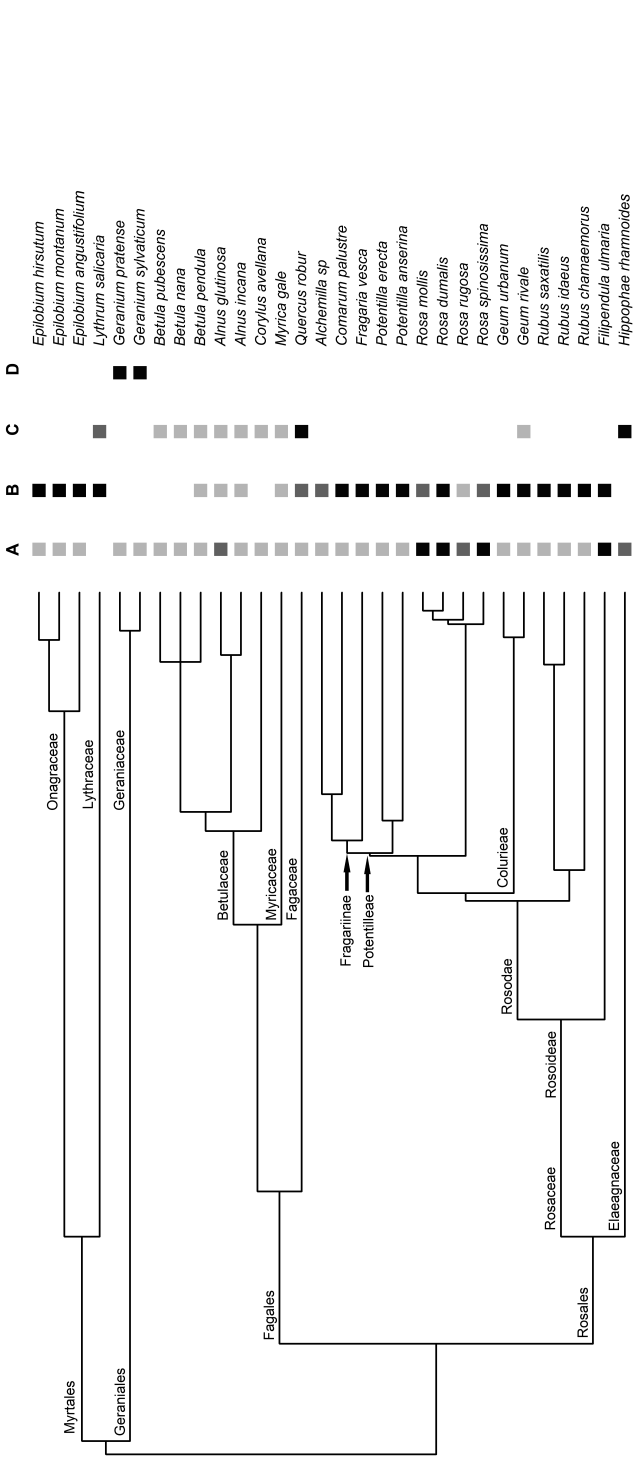


Figure 11. Phylogenetic tree representing those species which contained ellagitannins. The letters A–D denote different ET subgroups: A = glucopyranose-based ETs, B = oligomeric ETs, C = *C*-glucosidic ETs, D = dehydroETs. Content of ET subgroups: ■ <1–10%, ■ 11–29%, ■ 30–50%, ■ >50%. Classification of Rosaceae is according to Potter et al. (2007).

The Cronquist's classification is presented in Fig. 6 (Chapter 4) and the APG classification in Fig. 11. There are three major differences between these classifications. First, in the Cronquist's classification the subclass Hamamelidae includes orders Fagales (Fagaceae and Betulaceae) and Myricales (Myricaceae). In the APG classification the subclass Hamamelidae no longer exists, and the order Myricales is combined with Fagales. Thus, in the APG classification Fagales includes families Fagaceae, Betulacea and Myricaceae. Second, in the Cronquist's classification the subclass Rosidae includes order Proteales into which the family Elaeagnaceae is placed. In the APG classification, Elaeagnaceae is placed into order Rosales. Third, in the APG classification there is no ranking to subclasses as is in the Cronquist's classification, but the orders are placed under two unranked and informally named subgroups Malvids and Fabids. Thus, Malvids includes orders Geraniales and Myrtales and Fabids includes orders Rosales and Fagales (Fig. 11).

The change that family Myricaceae is placed under Fagales made by the APG is supported by the results of **II**: the ET profiles and contents of ETs in all of the studied plant species (except that of Fagaceae) are similar (Table 1 in **II**). They all contain low amounts of ETs, and the ETs found are both glucopyranose-based and *C*-glucosidic ETs. The content of ETs in *Quercus robur* (Fagaceae) is substantially higher than in those species that belong to the families Betulaceae (*Alnus* species, *Betula* species and *Corylus avellana*) and Myricaceae (*Myriga gale*). Also the ET profile of *Q*. *robur* is different: the species is characterized by having a high content of *C*-glucosidic ETs and especially by producing a dimeric ET cocciferin D_2, which is not detected in any other plant species studied (Fig. 11; Table 1 in **II**).

The family Elaeagnaceae has been a dilemma to taxomists, and it has been classified into several different orders (Hyvönen, 1996). In the Cronquist's classification it is placed under Proteales, which is suggested to be evolved from Myrtales (Fig. 6; Okuda et al., 2000). In the APG classification, Elaeagnaceae is placed under Rosales (Fig. 11). However,

as the ET composition in Figure 11 and in Table 1 (**II**) points out, it does not seem to fit in there either. The other family of Rosales is Rosaceae and all the other species and genera studied from Rosaceae have clearly different ET compositions compared to that of Elaeagnaceae (Fig. 11; Table 1 in **II**). At this point one could argue that only one species (*Hippophae rhamnoides*) from Elaeagnaceae was studied. However, the genus *Hippophae* is very small with only five species (Hyvönen, 1996). The whole family is also small; in addition to *Hippophae*, it includes only two other genera, *Shepherdia* with three species and *Elaeagnus* with 20–50 species [in comparison, Rosaceae includes 95 genera and ca. 2800 species (Encyclopedia of Life)]. *Shepherdia* and *Elaeagnus* species do not naturally grow in Finland, but have previously been studied by Ito (1999). All these genera are characterized by producing *C*-glucosidic ETs and their derivatives. Same types of *C*-glucosidic ETs have been found from species belonging to families Lythraceae and Fagaceae (Ito, 1999). Thus, in this case it would seem more appropriate to place Elaeagnaceae to Myrtales, as was suggested by Cronquist. Overall, the order Myrtales is comprised of families whose ET composition is exceptional: species belonging to Onagraceae (*Epilobium* species) are characterized by producing macrocyclic oligomeric ETs (e.g., oenothein A and B) and *Lythrum salicaria* (Lythraceae) by producing oligomeric ETs that are composed of *C*-glucosidic ETs (Table 1 in **II**).

In addition, Myrtales and Geraniales are classified in the APG system to Malvids. Geraniales includes the family Geraniaceae, which is also exceptional in its ET composition: *Geranium* species are characterized by producing dehydroETs and their derivatives. Thus, if Elaeagnaceae is included in Myrtales, this branch of Malvids would be composed of families which have special ET composition. Furthermore, in the phylogenetic tree of APG (APG III, 2009), Myrtales and Geraniales form their own well-separated branch, which indicates they are not closely related to other orders belonging to Malvids.

In general, the APG classification matches well with the ET data obtained in **II**. The only drawback of the APG classification is that it is focused only on the order and family level. In the case of Rosaceae, this level of classification was considered to be too wide: the family included 26 species, with several genera, which in turn had different ET profiles. Thus, the classification has been deepened into subfamily, supertribe, tribe and subtribe level according to Potter et al. (2007) (Fig. 11). Overall, this classification is in good accordance with the ET data, but if only the chemical data is considered, some modifications to this could be done. These modifications are illustrated in Scheme 1 (**II**), in which all the genera from Rosaceae are divided into groups based on the type of oligomeric ETs they produce.

Thus, in comparison with the classification of Potter et al. (2007; Fig. 11) the following changes could be made: 1) *Filipendula* could be placed to supertribe Rosodae as it has similar ET composition as *Rosa* species. 2) *Rubus* and *Rosa* species could be classified under different tribes because of the differences in their ET composition. 3) *Comarum*, *Fragaria* and *Alchemilla* species could be classified into the same tribe with *Potentilla* since they have the same main ET (agrimoniin). However, *Geum* species should be kept in their own tribe although they are placed into the same group with *Alchemilla*, *Potentilla*, *Comarum* and *Fragaria* species (Scheme 1 in **II**). This is because in *Geum* species the main ET is gemin A, not agrimoniin.

8.3 Biological activity of ellagitannins

Three different assays were used to measure the *in vitro* activity of ETs. There are two main reasons for this. First, the pH 10 assay was used to assess the propensity of ETs to oxidize at high pH (**III**), i.e., in conditions that many lepidopteran larvae have in their digestive tract. Second, since not all herbivorous insect larvae have alkaline gut conditions, it was of interest to study how ETs would react in different pH conditions. Thus, the deoxyribose assay (**IV**), in which the reaction conditions are buffered

to pH 7.4, was used. Furthermore, the advantage of the deoxyribose assay is that it can be modified so that it can be used to evaluate both the antioxidant activity and pro-oxidant activity of ETs. The antioxidant activity can be measured as non-site-specific radical scavenging activity by measuring the hydroxyl radical scavenging activity. Site-specific scavenging activity can be measured by the ability to chelate iron (Fe) ions. The pro-oxidant activity is measured as the ability to reduce Fe^{3+} ions. The activities of ETs in **III** were also measured with the DPPH assay, which is a widely used method for the determination of antioxidant capacity. For the activity measurements in **III** and **IV**, a wide variety of structurally different ETs were purified (Table 6).

Table 6. Ellagitannins used in the activity measurements in **III** and **IV**. Oxidative activity (Ox. act.) was measured with the pH 10 assay, antioxidative activity (Antiox. act.) with the DPPH assay and hydroxyl radical scavenging activity (Antiox. act.), pro-oxidant activity (Pro-ox. act.) and metal ion chelating ability (Metal ion chelation) with the deoxyribose (2-DR) assay. Note, that the negative values of inhibition (2-DR) indicate pro-oxidant activity.

	Ellagitannin	Plant source	Purity (%)[a]	Ox. act. (10^{-3} abs s^{-1} mM^{-1} ± SEM)	Antiox. act. IC_{50} (μM) (DPPH)	Antiox. act. (%) (2-DR)[b]	Pro-ox. act. (%) (2-DR)[b]	Metal ion chelation (%) (2-DR)[c]
1	Agrimoniin	*P. erecta*	98.1	4.0 ± 0.2	5.2 ± 1.6	55.5 ± 0.3	46.1 ± 1.4	66.9 ± 0.2
2	Carpinusin	*G. sylvaticum*	99.3	1.1 ± 0.1	nd	4.4 ± 0.5	–60.5 ± 0.1	24.3 ± 4.4
3	Castalagin	*L. salicaria*	98.8	13.8 ± 0.3	9.3 ± 1.0	–9.9 ± 4.7	–70.6 ± 1.1	28.6 ± 1.0
4	Castavaloninic acid	*Q. gambelii/robur*	99.4	22.6 ± 0.2	6.5 ± 0.8	–49.9. ± 2.3	–77.4 ± 2.7	20.8 ± 3.2
5	Casuarictin	*H. rhamnoides*	95.3	3.4 ± 0.2	10.0 ± 0.7	nd	nd	nd
6	Casuarinin	*H. rhamnoides*	99.0	8.4 ± 0.1	8.1 ± 0.9	nd	nd	nd
7	Catechin deriv. of 17		93.3	18.9 ± 0.1	7.5 ± 0.2	nd	nd	nd
8	Catechin deriv. of 31		96.3	4.2 ± 0.1	9.5 ± 0.8	nd	nd	nd
9	Catechin deriv. of 34		92.0	7.3 ± 0.2	9.5 ± 1.8	nd	nd	nd
10	Chebulagic acid	*T. chebula*		0.6 ± 0.3	nd	22.9 ± 4.3	–17.9 ± 0.5	20.3 ± 4.2
11	Chebulanin	*T. chebula*	95.0	0.8 ± 0.1	nd	26.4 ± 4.9	–36.7 ± 0.5	3.8 ± 1.7
12	Cocciferin D_2	*Q. robur*	93.8	12.7 ± 0.1	4.8 ± 1.0	nd	nd	nd
13	Elaeagnatin A	*H. rhamnoides*	96.3	21.0 ± 0.4	9.9 ± 1.7	nd	nd	nd
14	Gemin A	*G. rivale*	97.4	3.3 ± 0.1	4.8 ± 1.8	50.9 ± 0.2	46.8 ± 0.5	55.4 ± 1.8
15	Geraniin	*G. sylvaticum*	82.4	1.5 ± 0.2	nd	8.1 ± 2.6	–11.7 ± 2.3	21.7 ± 4.9
16	Grandinin	*Q. robur*	100.0	8.1 ± 0.3	nd	–40.8 ± 1.8	–136.6 ± 5.6	31.5 ± 0.3
17	Hippophaenin B	*H. rhamnoides*	95.7	16.7 ± 0.1	9.9 ± 1.8	nd	nd	nd
18	Isostrictinin	*H. rhamnoides*	98.7	4.9 ± 0.2	12.8 ± 1.5	nd	nd	nd

	Ellagitannin	Plant source	Purity (%)[a]	Ox. act. (10^{-3} abs s^{-1} mM^{-1} ± SEM)	Antiox. act. IC_{50} (μM) (DPPH)	Antiox. act. (%) (2-DR)[b]	Pro-ox. act. (%) (2-DR)[b]	Metal ion chelation (%) (2-DR)[c]
19	Lambertianin C	*R. idaeus*	85.9	7.1 ± 0.3	3.4 ± 0.8	nd	nd	nd
20	Methyl deriv. of 17		92.5	15.9 ± 0.1	9.9 ± 1.3	nd	nd	nd
21	Methyl deriv. of 28		92.6	11.0 ± 0.2	5.8 ± 0.8	nd	nd	nd
22	Methyl deriv. of 31		99.0	4.3 ± 0.1	9.5 ± 1.9	nd	nd	nd
23	Methyl deriv. of 34		99.2	8.8 ± 0.2	10.0 ± 2.0	nd	nd	nd
24	Oenothein B	*E. angustifolium*	94.9	5.7 ± 0.3	8.3 ± 1.4	nd	nd	nd
25	Pedunculagin	*H. rhamnoides*	97.8	5.0 ± 0.2	11.2 ± 1.2	nd	nd	nd
26	Pterocarinin A	*H. rhamnoides*	91.9	8.1 ± 0.2	9.7 ± 1.9	nd	nd	nd
27	Punicalagin	*T. chebula*	98.8	1.4 ± 0.3	nd	22.1 ± 3.5	–21.2 ± 1.1	34.2 ± 3.5
28	*C*-glucosidic dimeric ET	*L. salicaria*	94.6	11.9 ± 0.5	nd	nd	nd	nd
29	Roburin E	*Q. robur*	75.1	11.2 ± 0.1	nd	–44.9 ± 0.6	nr	38.0 ± 6.4
30	Sanguiin H-6	*R. idaeus*	97.7	6.4 ± 0.1	5.3 ± 1.8	nd	nd	nd
31	Stachyurin	*H. rhamnoides*	98.8	5.4 ± 0.1	8.6 ± 1.0	nd	nd	nd
32	Tellimagrandin I	*F. ulmaria*	85.2	3.3 ±0.2	11.4 ± 2.3	–7.9 ± 1.4	–44.4 ± 3.0	14.8 ± 1.9
33	Tellimagrandin II	*F. ulmaria*	99.2	1.5 ± 0.2	nd	14.5 ± 1.4	7.9 ± 1.0	27.0 ± 0.8
34	Vescalagin	*L. salicaria*	98.0	10.2 ± 0.5	9.5 ± 2.5	nd	nd	nd
35	Vescavaloninic acid	*Q. gambelii/robur*	96.8	20.8 ± 0.5	8.9 ± 2.0	nd	nd	nd

[a] HPLC purity at 280 nm, [b] Results reported at the concentration of 1 mM, [c] Results reported at the concentration of 0.1 mM, SEM = standard error of the mean, nr = no result, nd = not determined

8.3.1 Oxidative activity with pH 10 assay

Previously Barbehenn et al. (2006a, b) have successfully shown that the oxidation of ETs occurs at high pH. In that study, a simple spectrophotometric assay was introduced to evaluate the propensity of tannins to oxidize: tannins are oxidized at high pH and the formation of brown oxidation products (quinones and polymeric pigments) is monitored spectrophotometrically at 415 nm (Barbehenn et al., 2006a, b). We slightly modified this assay. First, we changed the starting time for the measurements. In the original assay the measurements were started 15 sec after the addition of the buffer. In this time period, the oxidation process has proceeded for a quite long time (Fig. 12). Therefore, in our method the measurements were started immediately after the addition of the buffer; the delay was in the range of 1 to 2 sec. Second, our measuring time was longer (8 min) than in the original method (3 min). Third, we used the oxidation data from the first 100 secs to calculate the maximum rate of oxidation; in the original method, the maximum rate was calculated from the data collected during the first 60 secs.

The oxidation curves obtained from the data varied in their shapes depending on the ET (Fig. 12) indicating their varying propensities to oxidize. The differences in the activities between the most inactive (chebulagic acid, Table 6; **IV**) and the most active (castavaloninic acid, Table 6; **III**) ETs were almost 40-fold. By comparing structurally closely related ETs (e.g., vescalagin and stachyurin), it is possible to deduce, which type of structural features affect the activity. Additionally, it is possible to draw conclusions on the direction and magnitude of these effects caused by different structural features, i.e., whether they are positive or negative, large or small. Based on these comparisons (**III)**, it was concluded that the structural features affected the oxidative activity of ETs in the following descending order: valoneoyl group with a free carboxylic acid (–COOH) group > acyclic glucose core with α-configuration at C-1 > glycosyl substituent (xylose/lyxose) > NHTP group > bound valoneoyl group ≈ sanguisorboyl group > HHDP group ≈ acyclic glucose with *β*-configuration at C-1. In addition, an equation that can be used to estimate the oxidative activity of

other ET structures was developed (**III**) and a good correlation between the measured and calculated activity was obtained ($r \approx 0.97$; **III**).

It is interesting that castalagin-type *C*-glucosidic ETs have higher activities than vescalagin-type *C*-glucosidic ETs. It is generally known, that vescalagin-type ETs are more reactive than castalagin-type ETs (Quideau et al., 2003) and derivatives of vescalagin-type ETs can be easily synthesized even in mild reaction conditions (**III**; Quideau et al., 2003, 2005). In fact, the derivatives of castalagin-type ETs have only rarely been found in nature (Ishimaru et al., 1987) and they can be synthesized only with poor yields (Ishimaru et al., 1987) or not at all (Quideau et al., 2005). In this context the higher activity of castalagin-type ETs is peculiar. However, these results could be explained by the fact that in castalagin-type ETs an intramolecular hydrogen bond is formed (Quideau et al., 2005; Section 8.1.3.), which lowers the basicity of the O-1 atom (Quideau et al., 2005). In the alkaline conditions used in the pH 10 assay this would mean that the OH group at C-1 would be more easily deprotonated than in the vescalagin-type ETs.

The structural variety of ETs was later extended with eight previously unexamined structures: grandinin, roburin E, punicalagin, tellimagrandin II, chebulanin, chebulagic acid, geraniin and carpinusin (**IV**). The activity of roburin E (11.25×10^{-3} abs s^{-1} mM^{-1}) corresponded quite well with the calculated activity (13.2×10^{-3} abs s^{-1} mM^{-1}), but the activity of grandinin (8.1×10^{-3} abs s^{-1} mM^{-1}) deviated from the calculated. The calculated and measured activities of tellimagrandin II corresponded also each other well (calculated: 2.0×10^{-3} abs s^{-1} mM^{-1}, measured: 1.48×10^{-3} abs s^{-1} mM^{-1}). In conclusion, the results obtained in **III** and **IV** show clearly that glucopyranose-based ETs are not as prone to oxidation as are *C*-glucosidic ETs and that those ETs, which have a DHHDP group or its modification in their structure are particularly resistant to oxidation at high pH.

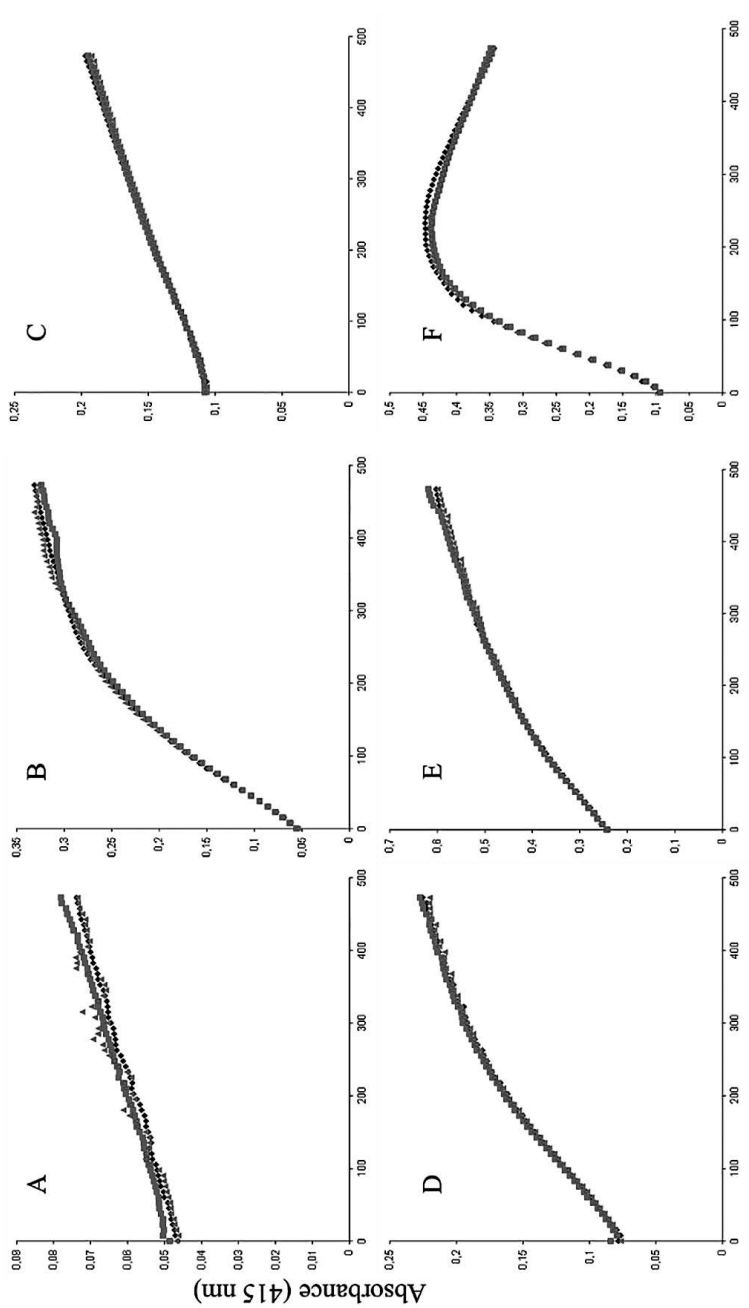


Figure 12. Examples of oxidation curves of selected compounds at 1.5 mM concentration. A = pentagalloyl glucose, B = gallic acid, C = geraniin, D = casuarinin, E = casuarictin and F = vescavaloninic acid.

8.3.2 Antioxidative activity with DPPH assay

Antioxidative activity of ETs used in **III** was measured with DPPH assay (Table 6). In this assay, the difference between IC_{50} values (i.e., concentration in which 50 % of the radicals are reduced) of the most active (lambertianin C) and inactive compound (isostrictinin) was ~4-fold. Similar structure-activity comparisons were made as for pH 10 assay. However, the effects of different structural features on the antioxidative activity were not as clear as was for the oxidative activity, i.e., the antioxidative activities of ETs could not be grouped into distinct structural features. The most active compounds were oligomeric ETs (lambertianin C, sanguiin H-6, cocciferin D_2, gemin A, agrimoniin and the *C*-glucosidic dimer). Moderate inhibition activity was obtained for ETs with acyclic glucose cores, i.e., *C*-glucosidic ETs, and low activity for glucopyranose-based ETs. The results show good correlation (Fig. 13) between the IC_{50} value and the number of phenolic OH groups in the structure.

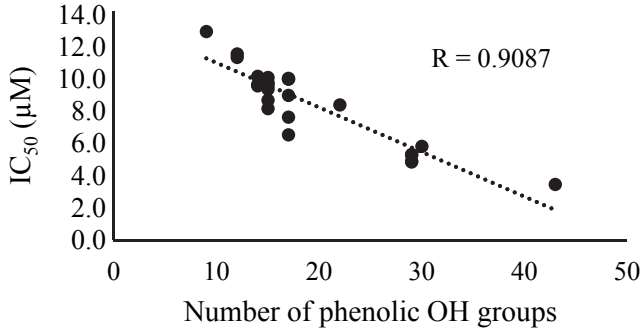


Figure 13. Correlation between IC_{50} value and the number of phenolic hydroxyl groups in the ellagitannin structure.

Thus, the more there are OH groups in the structure (oligomeric > monomeric compounds) the more efficiently the compound can reduce the DPPH radical. It has been suggested that the 1,2,3-trihydroxyl structure of gallic acid is responsible for the radical scavenging activity of ETs (Hatano et al., 1989b; Yokozawa et al., 1998) as resonance-stabilized radicals are formed (Yoshida et al., 1989). When the galloyl groups are replaced by, e.g., a DHHDP

group, the activity is suggested to decrease because there are fewer OH groups in the structure, or because of spatial hindrance (Yokozawa et al., 1998).

8.3.3 Activity of ellagitannins with deoxyribose assay

To be able to compare the activities of ETs in different biological conditions, the activities of 13 ETs were measured with 2-deoxyribose (2-DR) assay (Table 6). This assay is based on the degradation 2-DR caused by hydroxyl ($OH^{\cdot}$) radicals at pH 7.4. The radicals are generated from the Fe^{3+}/ascorbate/EDTA/H_2O_2 system (Fig. 14), i.e., by the Fenton reaction, which is the main source of hydroxyl radicals *in vivo* (Soberon et al., 2009).

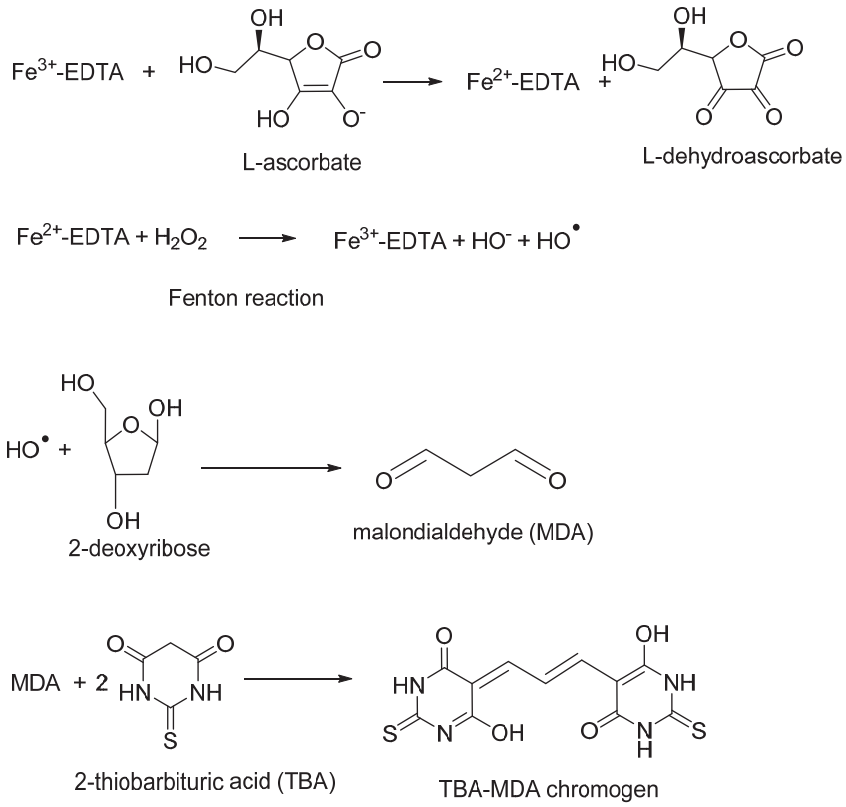


Figure 14. Reaction scheme for deoxyribose assay.

Although the chemistry of 2-DR assay is complicated and differentiates from that used in pH 10 assay, the results are surprisingly similar between these two methods. *C*-glucosidic ETs exhibited pro-oxidant activity also at neutral pH. This can be seen from both the $OH^{\bullet}$ scavenging activity and the pro-oxidant activity results (Table 6), which are negative in their sign (i.e., the degradation of 2-DR is enhanced by the presence of these ETs). However, the activity is dependent on the concentration of ET studied (Fig. 2 in **IV**): at lower concentrations (1–3 mM) most *C*-glucosidic ETs exhibit pro-oxidant effects, but as the concentration increases (5 mM) the pro-oxidant effect decreases and the ETs begin to exhibit antioxidative activity. Nevertheless, their overall activity has more of pro-oxidative character than antioxidative.

From glucopyranose-based ETs, tellimagrandin II is the only one which exhibits antioxidative activity in both assays, and also in all concentrations (Fig. 2 in **IV**). Punicalagin, which exhibits relatively good $OH^{\bullet}$ scavenging activity at all concentrations, exhibits pro-oxidant activity at low concentrations with the pro-oxidant assay (Fig. 2 in **IV**).

The dimeric ETs (agrimoniin and gemin A) are the only ones that show strong antioxidative activity in both assays and thorough the studied concentration range (Fig. 2 in **IV**). The two dehydroETs studied (geraniin and carpinusin) exhibit concentration-dependent antioxidant activity in $OH^{\bullet}$ scavenging assay, but in pro-oxidant assay they exhibit more pro-oxidant activity than antioxidative activity (Fig. 2 in **IV**). ETs that contain a chebuloyl group, i.e., chebulanin and chebulagic acid, also possess both antioxidative and pro-oxidative activities. However, these activities are not dependent on concentration (Fig. 2 in **IV**). This implies that they may react in some other way in the prevailing reaction conditions than the other ETs do.

It has been suggested that the antioxidative activity of ETs is not related to their ability to scavenge radicals, but instead on their ability to chelate metal ions (Gyamfi and Aniya, 2002), and similar conclusions have been drawn from studies in which different types of phenolic and polyphenolic compounds have been studied (e.g., Andrade et al., 2005, 2006; Lopes et al., 1999). As the results show (Table 6), the studied ETs possess good ability to chelate Fe ions (except chebulanin). This is further supported by the fact that the measurements of chelating ability needed to be performed at concentrations ten times more dilute

than $OH^{\bullet}$ scavenging and pro-oxidant activity measurements. The most effective chelators are dimeric ETs. Their chelating abilities are roughly two-fold compared to those of other tested ETs. For the rest of the ETs, the chelating abilities are in the range of 20–30 %. The only exception is chebulanin, whose chelating ability is the lowest, only about 4 %.

From the herbivores' point of view, the results are interesting. For example, the metal chelating results suggest that herbivores might be able to use ETs as antioxidants to prevent Fenton-type reactions to take place in their digestive tracts. These reactions have been shown to occur in the prevailing iron concentrations that herbivores have in their gut (Barbehenn et al., 2005a). On the other hand, it has been shown that ETs cause oxidative stress in the guts of herbivores (Barbehenn et al., 2005b, 2008b), and that although the herbivore would be able to compensate (e.g., by antioxidants) and/or to take advantage on ETs it consumes during feeding, markers indicating oxidative stress are still observed (Barbehenn et al., 2001). Thus, it seems that ETs possess more negative features concerning larval performance than positive ones.

8.3.4 In vivo *activity of ellagitannins on* Amphipyrea pyramidea *larvae*

It is clear that the evalution of the biological effects that ETs have (or might have) on herbivores is not an easy task. Thus, it was of interest to perform some *in vivo* experiments as well. The idea for the experiment came from the results of Roslin and Salminen (2008). In their study, two generalist moth species and two oak-specialist moth species were fed with artificial diets containing an ET (vescalagin), PAs (mixture from mountain birch) or a mixture of them. The most notable finding of the study was that the larvae of the generalist species that were fed with a diet containing vescalagin were significantly affected: all utilized nutritional indices (the amount of food ingested, the approximate digestibility of food, the efficiency of conversion of ingested food to body substance, and the efficiency of conversion of digested food to body substance) were significantly affected and resulted in clearly reduced larval weight development. On the other hand, the diet containing PAs had no effect on any of the species. Thus, our aim was to repeat these results, but with a wider selection of structurally different ETs and by using leaf diet.

In this experiment bird cherry (*Prunus padus*) leaves were selected to be fed to the larvae. The feed choice was based on preliminary experiments and on the fact that bird cherry leaves do not naturally contain ETs (**II**). The generalist moth species, copper underwing (*Amphipyra pyramidea*), was chosen based on the results obtained by Roslin and Salminen (2008). ETs (chebulagic acid, chebulanin, cocciferin D_2, punicalagin, vescalagin and vescavaloninic acid) were selected so that they represented varying oxidative activities (Table 6).

The results of the 72 h experiment are presented in Table 7 and the statistical analyses of the results in Table 8. As can be seen, the results are highly non-significant, i.e., no effects on the nutritional indices or growth were observed (Table 8). There is one almost significant result, which indicates that the initial weight of the larvae may have an effect on the growth efficiency of the larvae, i.e., the heavier the larvae are at the beginning, the greater their growth efficiency is.

The results indicate that no negative effects on the larvae of the generalist moth species are observed when they feed on diet containing ETs painted on bird cherry leaves. There are several possible explanations for this. First and the most obvious explanations is that the compounds in bird cherry leaves have suppressed the activity of ETs. The total polyphenol content of bird cherry leaves is low (55 mg g^{-1} of the crude extract) and these polyphenols consist of PAs (25 mg g^{-1} of the crude extract), chlorogenic acid (14 mg g^{-1} of the crude extract) and flavonoid glycosides (12 mg g^{-1} of the crude extract). It has been shown, that PAs decrease the amount of semiquinone radicals produced by ETs *in vitro* (Barbehenn et al., 2006b) and that the negative effects of consuming ETs were absent when PAs were added to the food along with an ET (Roslin and Salminen, 2008). Thus, it is possible that the oxidative activity of ETs was suppressed by the presence of PAs in the leaves. However, there is another option to explain the results. Barbehenn et al. (2009b) have concluded that painting of compounds on the leaf surface does not influence the overall leaf chemistry. In their study, Barbehenn et al. (2009b) found no overall effects of leaf resistance to *M. dispar* larvae when they were fed with red oak leaves coated with HTs and, on the other hand, when fed with sugar maple leaves coated with PAs. It was expected that, e.g., in the case of sugar maple, which produces high concentrations of semiquinone radicals *in vivo* (Barbehenn et al.,

2005b), the levels of radicals would decrease when PAs are painted on the leaf surface, but this was not observed. Since only nutritional indices were taken into account in our study, it is impossible to evaluate the effects of ETs inside the digestive tract. Thus, it is possible that ETs enhanced the production of radicals and oxidative stress in the midgut tissues of these larvae to some extent, but these implications were not sufficient enough to have effects on the growth of the larvae. Another point is that often the feeding experiments last only for few days or throughout one or a couple of instars. Thus, it would be interesting to see, would the results be different if the experiment lasted throughout the whole life-cycle of larvae to adultery, and whether the consumption of ETs have effects e.g., on the fecundity and reproductivity of the adults.

Table 7. Results of different ellagitannin treatments on the performance (weight, growth, excretion, consumption and pupal weight) and nutritional indices (digestability, growth efficiency and metabolic efficiency) of *Amphipyrea pyramidea* larvae.

Treatment	Initial weight (mg)	Final weight (mg)	Growth (mg)	Defecated (mg)	Cons. (mg)	Digestability	GE	ME	Pupal weight (mg)
Control	24.0 ± 3.8	84.9 ± 11.3	60.9 ± 9.5	22.3 ± 3.0	38.8 ± 5.8	0.4 ± 0.1	1.6 ± 0.1	3.8 ± 0.7	469.5 ± 90.3
Chebulagic acid	24.1 ± 2.0	88.8 ± 8.0	64.8 ± 8.0	24.7 ± 3.0	40.6 ± 5.7	0.4 ± 0.1	1.6 ± 0.1	4.2 ± 0.7	485.2 ± 84.8
Chebulanin	24.9 ± 3.0	88.1 ± 7.1	63.1 ± 6.9	23.4 ± 2.7	39.0 ± 6.5	0.4 ± 0.1	1.6 ± 0.1	4.2 ± 0.7	459.6 ± 78.5
Cocciferin	23.8 ± 2.9	84.9 ± 8.6	61.1 ± 8.7	22.6 ± 3.2	37.8 ± 6.4	0.4 ± 0.1	1.6 ± 0.1	4.2 ± 0.9	467.5 ± 65.6
Punicalagin	25.0 ± 3.7	88.8 ± 9.8	63.8 ± 8.6	24.0 ± 3.8	41.0 ± 5.7	0.4 ± 0.1	1.6 ± 0.1	3.9 ± 0.6	465.0 ± 71.6
Vescalagin	23.4 ± 2.6	87.6 ± 6.2	64.2 ± 5.8	23.7 ± 3.1	39.1 ± 5.2	0.4 ± 0.1	1.7 ± 0.1	4.4 ± 0.9	495.8 ± 83.8
Vescavalo-ninic acid	24.3 ± 2.8	90.5 ± 12.2	66.2 ± 10.9	24.8 ± 4.1	40.8 ± 6.0	0.4 ± 0.1	1.6 ± 0.1	4.4 ± 0.6	478.9 ± 87.1

Cons. = consumption, GE = growth efficiency, ME = metabolic efficiency

Table 8. Results of the analysis of treatment and initial weight on the different metrics of larval performance.

	Treatment	Initial weight
Growth	F = 0.82, p = 0.55	F = 0.36, p = 0.55
Consumption	F = 0.63, p = 0.70	F = 0.24, p = 0.62
Defecated	F = 1.48, p = 0.19	F = 0.13, p = 0.72
Digestability	F = 1.33, p = 0.25	F = 0.00, p = 0.098
Growth efficiency	F = 1.68, p = 0.13	F = 3.78, p = **0.05**
Metabolic efficiency	F = 1.66, p = 0.14	F = 0.43, p = 0.51
Pupal weight	F = 0.33, p = 0.92	F = 2.52, p = 0.12

9 CONCLUSIONS

The main aim of this thesis work was to study ellagitannins. On one part, the aim was to enhance the characterization of ETs from complex samples without having to isolate every ET for individual characterization. Thus, a thorough representation of how ETs having different substructures can be effectively characterized from complex plant samples was given (**I**). The characterization patterns presented in **I** should be useful for phytochemists in their task of finding out what kind of ETs their plant samples contain. The results presented in **I** significantly aided the characterization of ETs in a screening study (**II**) that was done to obtain information about the distribution and content of ETs in the Finnish flora. This was first study that investigated individual ETs from Finnish plants at this extent. In addition, the taxonomic distribution of ETs in the studied plant species was investigated, and was found to correlate well with their classification.

The second aim was to study the biological activities of individual ETs focusing on their role in plant-herbivore interactions. The results showed that ETs possess varying oxidative activities both at high pH (**III**) and at neutral pH (**IV**), and that their activity is dependent on the structure. Moreover, the results showed that some of the ETs possess oxidative activity even at neutral pH. Despite of these encouraging results, no effects on the performance of *Amphipyrea pyramidea* larvae were found in the feeding experiment, which included also some of the most active ETs. Thus, there is still work to be done to find out if ETs are produced for defensive purposes by plants, and if they are, by which mechanism(s) they affect the herbivores.

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