Division of Pharmacognosy
Department of Pharmacy
Faculty of Science
University of Helsinki

# The search for biological activity in Finnish plant extracts containing phenolic compounds

**Jussi-Pekka Rauha**

## ACADEMIC DISSERTATION

To be presented with the permission of the Faculty of Science of the University of Helsinki,
for public criticism in Sipi Siintola Auditorium of Biocentre Viikki,
on August 10th, 2001, at 12 o'clock noon.

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**Supervisor:** Prof. Heikki Vuorela, Ph.D.
Division of Pharmacognosy
Department of Pharmacy
University of Helsinki
Finland

**Reviewers:** Prof. Heikki Kallio, Ph.D.
Department of Biochemistry and Food Chemistry
University of Turku
Finland

Docent Riitta Törrönen, Ph.D.
Institute of Applied Biotechnology
University of Kuopio
Finland

**Opponent:** Prof. Kurt Hostettmann, Ph.D.
Institute of Pharmacognosy and Phytochemistry
School of Pharmacy
University of Lausanne
Switzerland

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**ORIGINAL COMMUNICATIONS**

# PREFACE

This work was carried out at the Division of Pharmacognosy, Department of Pharmacy, University of Helsinki during the years 1997–2001. Part of the work was performed at the Division of Food Chemistry, Department of Applied Chemistry and Microbiology, University of Helsinki, at the Institute of Pharmacognosy and Phytochemistry, University of Lausanne, Switzerland, and at the Laboratory of Physical Chemistry, Department of Chemistry, University of Turku.

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Helsinki, June 2001

## ABSTRACT

Plants from the Finnish flora were extracted and studied for their biological activity *in vitro*, especially in relation to phenolic compounds, which are known to have several beneficial health effects.

The content of total phenolic compounds in 92 crude plant extracts was determined according to the Folin-Ciocalteu procedure, and the antioxidant activities of the extracts were measured as inhibition of the autoxidation of methyl linoleate. The respective values showed good correlation, and *Andromeda polifolia*, *Thymus vulgaris*, *Filipendula ulmaria*, *Calluna vulgaris,* and leaves of *Rubus chamaemorus* had the best antioxidative activities.

The calcium antagonistic activity of 51 extracts was investigated as the inhibition of depolarisation-induced $^{45}Ca^{2+}$ uptake in rat pituitary $GH_4C_1$ cells. The most effective plants were *Humulus lupulus*, *Epilobium angustifolium*, *Lythrum salicaria*, and *Lysimachia vulgaris*. The extracts of *Allium cepa*, *Carum carvi*, *Malus pumila*, and *Thymus vulgaris* had an adverse effect *i.e.* promoted fluxes into the cells. These effects did not correlate with the content of total phenolics.

Diffusion methods were utilised to screen the antimicrobial effects of 13 phenolic compounds and 29 plant extracts. Among the standards, the widest antimicrobial activity was achieved with flavone, quercetin, morin, and naringenin. In the class of plant extracts, *Epilobium angustifolium* and *Filipendula ulmaria* showed the most effective bactericidic effects, whereas the extract of *Lythrum salicaria* was effective against both *Escherichia coli* and the yeast, *Candida albicans*. Antimicrobial effects were also noted for *Betula pubescens* and *Pinus sylvestris*, and berries of plants belonging to the genus *Rubus*.

In order to study the chemical basis of the results obtained, the polyphenolic composition of the crude extract of *Lythrum salicaria* was characterised after fractionation using HPLC/DAD-UV and two HPLC/MS methods. The presence of flavone-*C*-glucosides was determined by a positive ion LC/APCI-MS$^3$ method, and a negative ion LC/ESI-MS method was used to identify the tannins and phenolic acids. Based on UV and MS spectral data and reference material, a total of six compounds (vescalagin, pedunculagin, valoneic acid dilactone, 1,6-di-*O*-galloylglucose, 1-*O*-galloylglucose and 6-*O*-galloylglucose) were identified from *Lythrum salicaria* for the first time. The detection of four flavonoids and six other phenolics confirmed the information presented in the literature.

In order to further develop the applicability of LC/MS techniques in phytochemical characterisation, the effect of nine different eluent compositions on the ionisation efficiency of five flavonoids was studied using ionspray (IS), atmospheric pressure chemical ionisation (APCI), and the novel atmospheric pressure photoionisation (APPI). The optimal ionisation conditions were achieved in positive ion IS and APCI using 0.4% formic acid as buffer (pH 2.3), and in negative ion IS and APCI using ammonium acetate buffer adjusted to pH 4.0. For APPI work, the eluent of choice was a mixture of organic solvent and 5 mM aqueous ammonium acetate. The limits of detection (LOD) were determined for the analytes using each interface. Negative ion IS showed the lowest LODs (0.8–13 μM) with an eluent system consisting of acidic ammonium acetate buffer, so this is why this method provides the best conditions for the detection of flavonoids in MS mode.

In conclusion, the material studied in the present work contained several plant species possessing notable biological activity *in vitro*. The composition of phenolic compounds can be characterised using modern LC/MS techniques which, in combination with computer-assisted scale-up of the isolation procedure, can lead to identification of the active components.

## LIST OF ORIGINAL PUBLICATIONS

**I** Kähkönen, M.P., Hopia, A.I., Vuorela, H.J., Rauha, J.-P., Pihlaja, K., Kujala, T.S. and Heinonen, M. (1999): Antioxidant activity of plant extracts containing phenolic compounds.
*Journal of Agricultural and Food Chemistry* 47(10): 3954–3962.

**II** Rauha, J.-P., Tammela, P., Summanen, J., Vuorela, P., Kähkönen, M., Heinonen, M., Hopia, A., Kujala, T., Pihlaja, K., Törnquist, K. and Vuorela, H. (1999): Actions of some plant extracts containing flavonoids and other phenolic compounds on calcium fluxes in clonal rat pituitary $GH_4C_1$ cells.
*Pharmaceutical and Pharmacological Letters* 9(2): 66–69.

**III** Rauha, J.-P., Remes, S., Heinonen, M., Hopia, A., Kähkönen, M., Kujala, T., Pihlaja, K., Vuorela, H. and Vuorela, P. (2000): Antimicrobial effects of Finnish plant extracts containing flavonoids and other phenolic compounds.
*International Journal of Food Microbiology* 56(1): 3–12.

**IV** Rauha, J.-P., Wolfender, J.-L., Salminen, J.-P., Pihlaja, K., Hostettmann, K. and Vuorela, H. (2001): Characterization of the polyphenolic composition of purple loosestrife (*Lythrum salicaria*).
*Zeitschrift für Naturforschung* 56c(1/2): 13–20.

**V** Rauha, J.-P., Vuorela, H. and Kostiainen, R. (2001): Effect of eluent on the ionisation efficiency of flavonoids by ionspray, atmospheric pressure chemical ionisation, and atmospheric pressure photoionisation mass sprectrometry.
*Journal of Mass Spectrometry* (submitted).

These publications will be referred to in the text by their Roman numerals.

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## ABBREVIATIONS

| | |
|---|---|
| ABTS$^{\cdot+}$ | 2,2’-azinobis(3-ethylbenzothiazoline-6-sulphonate) |
| ACN | acetonitrile |
| APCI | atmospheric pressure chemical ionisation |
| API | atmospheric pressure ionisation |
| APPI | atmospheric pressure photoionisation |
| CE | high performance capillary electrophoresis |
| CHD | coronary heart disease |
| DAD | diode array detector |
| DPPH | 2,2-diphenyl-1-picrylhydrazyl |
| ESI | electrospray ionisation |
| $GH_4C_1$ cells | cultivated cells from rat pituitary gland, clone 1 |
| HHDP | hexahydroxydiphenoyl |
| HPLC | high performance liquid chromatography |
| IE | ionisation energy |
| IS | ionspray ionisation |
| LC | liquid chromatography |
| LOD | limit of detection |
| MPLC | medium pressure liquid chromatography |
| MS | mass spectrometry |
| NIST | The National Institute of Standards and Technology |
| NMR | nuclear magnetic resonance spectroscopy |
| PA | proton affinity |
| PEEK | polyetheretherketone |
| ROCC(s) | receptor-operated calcium channel(s) |
| RP | reversed phase |
| S/N | signal to noise |
| SPE | solid phase extraction |
| THF | tetrahydrofuran |
| TIC | total ion chromatogram |
| TLC | thin layer chromatography |
| UV | ultraviolet |
| UV/Vis | ultraviolet / visible |
| VIS | visible |
| VOCC(s) | voltage operated calcium channel(s) |
| XIC | extracted ion chromatogram |

# 1. INTRODUCTION

Flavonoids attracted growing global interest during the last decade and, as a result of this upsurge in research, the number of known flavonoids has increased dramatically: About 800 different flavonoids were known when Joachim Kühnau published, in 1976, one of the most cited articles dealing with flavonoids (KÜHNAU 1976). At the start of the 1990s, the number of reported flavonoid structures had increased to 4000 (HARBORNE 1994), and currently almost 6500 different flavonoids are known (HARBORNE and BAXTER 1999).

One important phenomenon affecting the increased interest in flavonoids has undoubtedly been the "French paradox". The name refers to France, where the mortality rate from coronary heart disease (CHD) is much lower than that in other industrialised countries – despite the fact that intakes of saturated fat and concentrations of serum cholesterol are similar to those in the other countries (RENAUD and de LORGERIL 1992). The phenomenon was explained on the basis of the antioxidative phenolics in red wine (FRANKEL et al. 1993), the most important evidence being published by a Dutch group (HERTOG et al. 1993). The report by Hertog et al. (1993) is one of the most famous research articles dealing with flavonoid consumption, and its inverse correlation with CHD mortality. Concurrently, it was demonstrated that plant-derived foodstuffs that had already been considered healthy contained relatively high levels of flavonoids. Flavonoid intake and its effects on CHD and cancer mortality were investigated in a number of epidemiological studies that have been summarised in review articles (*e.g.* HOLLMAN and KATAN 1999, BÖHM et al. 1998).

During the 1990s, flavonoids were shown to possess several biological effects, some of which were also related to human health (HARBORNE and WILLIAMS 2000). Thus it is only natural to search for even more effects from among the sources of these phenolic compounds in the plant kingdom. There is also a possibility that the new compounds could possess a noted but stronger activity in comparison to known substances. As is known, the structural diversity provided by natural products is greater than that provided by most synthetic techniques (HARVEY 1999). Furthermore, the new flavonoid structures can be used as pharmacologically unspecific leads for molecular design (NAHRSTEDT 1997).

In addition to the new candidate molecules for medical purposes, the investigation of crude plant extracts, *i.e.* ethnopharmacological research, may to some extent lead to elucidation of the pharmacological mechanisms of plants with folk-medicinal or nutritional value. The result of successful screening of plant material can thus not only be a new drug, but also a processed extract for food technological purposes or a guideline to better food consumption.

# 2. REVIEW OF THE LITERATURE

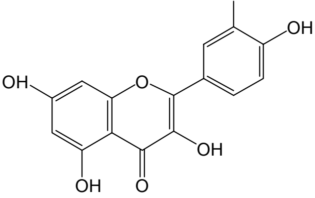
## 2.1. Phenolic compounds

Phenolic compounds are a large, heterogeneous group of secondary plant metabolites that are widespread in the plant kingdom (STRUBE et al. 1993). Phenolics display a vast variety of structures; here only flavonoids, tannins and phenolic acids are reviewed (**Figure 1**). The structural basis for all flavonoids is the flavone nucleus (2-phenyl-benzo-ν-pyrane) but, depending on the classification method, the flavonoid group can be divided into several categories based on hydroxylation of the flavonoid nucleus as well as the linked sugar (KÜHNAU 1976). Essential flavonoid structures divided into eleven classes are presented in **Figure 2** (HARBORNE and BAXTER 1999). "Tannins" is a general name for phenolic substances capable of tanning leather or precipitating gelatin from solution (HASLAM 1996). They can be divided into condensed proanthocyanidins, where the fundamental structural unit is the phenolic flavan-3-ol (catechin) nucleus, and into galloyl and hexahydroxydiphenoyl esters and their derivatives, gallotannins and ellagitannins (HASLAM 1998), as shown in **Figure 1b**. The essential two groups of phenolic acids are hydroxybenzoic acids and hydroxycinnamic acids (**Figure 1c**), both of which are derived from nonphenolic molecules benzoid and cinnamic acid, respectively (MACHEIX and FLEURIET 1998).

### 2.1.1. Occurrence of flavonoids in Finnish plants

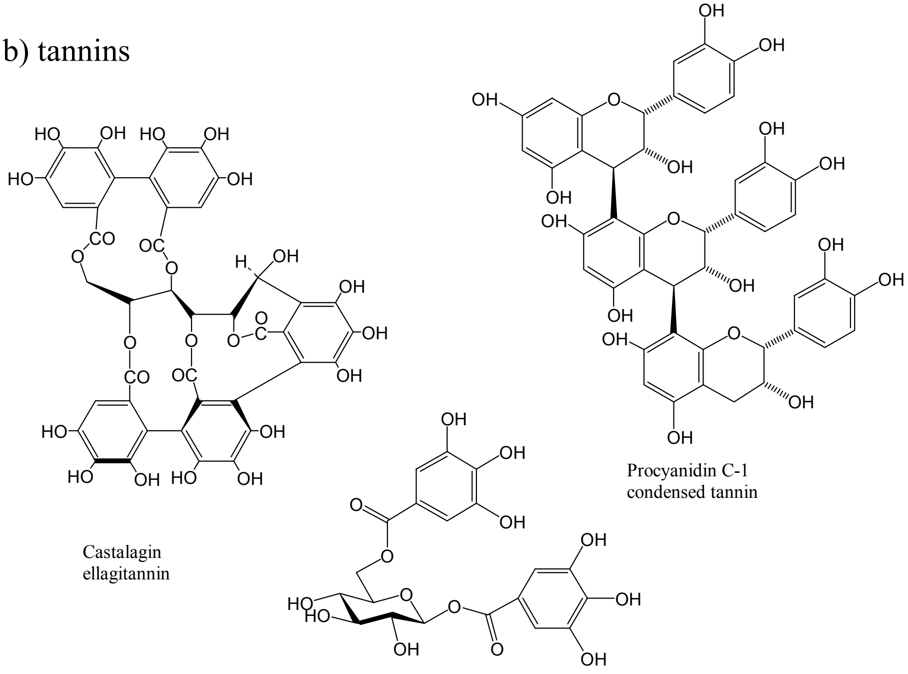
Flavonoids generally occur in all higher plants (HARBORNE and BAXTER 1999). **Table 1** shows a selection of plants belonging to the Finnish flora (**III**), and the flavonoid subclasses reported to be present in the species. A more detailed list of compounds is presented in **Appendix 1**. The most common flavonoid class in this material seems to be flavonols and their *O*-glycosides. Methyl ethers of flavones and flavonols have been reported most frequently from the families Asteraceae and Lamiaceae, which are also families that contain considerable amounts of essential oils (WOLLENWEBER 1994, BRUNETON 1995). Isoflavonoids, widely known as phytoestrogens, have been shown to be absent in Finnish berries (MAZUR 2000), but present in leguminous plants (Fabaceae) (GAGNON et al. 1995, HE et al. 1996).

a) flavonoids



Quercetin
flavonol

b) tannins



Castalagin
ellagitannin

Procyanidin C-1
condensed tannin

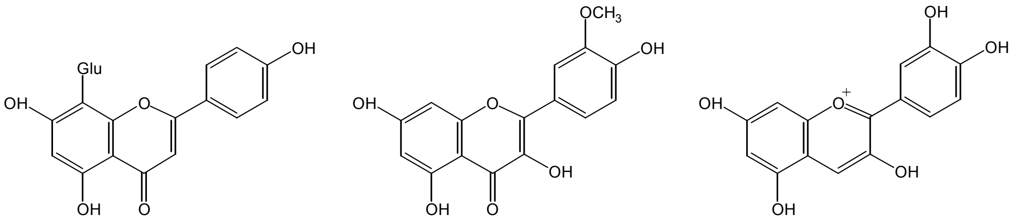
η-1,6-digalloyl-D-glucose
gallotannin

c) phenolic acids

Gallic acid
3,4,5-trihydroxybenzoic acid

Caffeic acid
3,4-dihydroxycinnamic acid

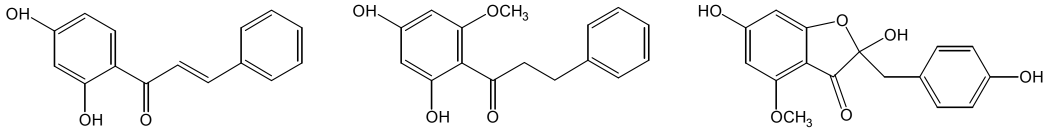
**Figure 1** Typical structures of different groups of plant phenolics: **a**) flavonoids, **b**) tannins, **c**) phenolic acids.



FLAVONES
Vitexin

FLAVONOLS
Isorhamnetin

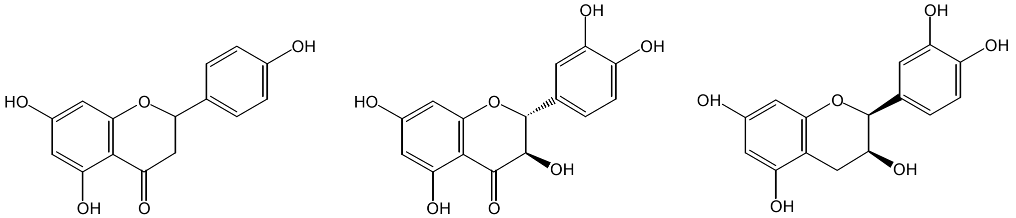
ANTHOCYANINS
Cyanidin



CHALCONES
2',4'-Dihydroxychalcone

DIHYDROCHALCONES
Uvangoletin

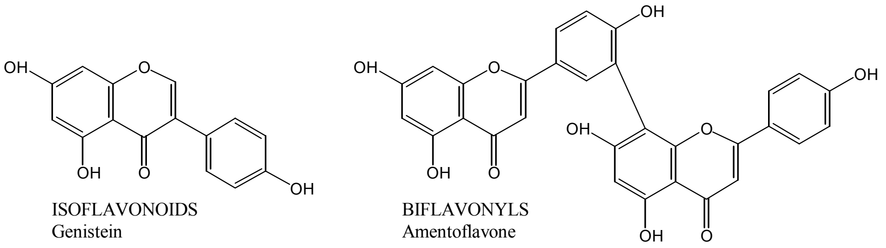
AURONES
Carpusin



FLAVANONES
Naringenin

DIHYDROFLAVONOLS
(*2R,3R*)-Taxifolin

FLAVANS
Epicatechin



ISOFLAVONOIDS
Genistein

BIFLAVONYLS
Amentoflavone

**Figure 2** Typical structures of flavonoids belonging to the different subgroups.

**Table 1** The flavonoid subclasses occurring in the Finnish vegetables, berries, wild plants and medicinal plants studied in this work (**III**). Classification is based on a semi-systematic, flavan-type nomenclature according to HARBORNE and BAXTER (1999).

| **Latin name** | **English name** | **Flavonoid types** | **Reference** |
|---|---|---|---|
| **Asteraceae** | | | |
| *Matricaria recutita* L. | Camomile | Flavones<br>Flavonols | SCHILCHER 1987, MULINACCI et al. 2000 |
| *Tanacetum vulgare* L. | Tansy | Flavones<br>Flavonols | OGNYANOV and TODOROVA 1983, WILLIAMS et al. 1999 |
| **Betulaceae** | | | |
| *Betula pubescens* Ehrh. | White birch | Flavones<br>Flavonols<br>Flavanones<br>Flavans | OSSIPOV et al. 1996, KEINÄNEN and JULKUNEN-TIITTO 1998 |
| **Chenopodiaceae** | | | |
| *Beta vulgaris* L. var. *rubra* | Red beet | Flavones<br>Flavonols | DIJOUX et al. 1995 |
| **Empetraceae** | | | |
| *Empetrum nigrum* L. | Crowberry | Flavonols<br>Chalcones<br>Dihydrochalcones | VASILETS et al. 1988, WOLLENWEBER et al. 1992, HÄKKINEN and AURIOLA 1998 |
| **Ericaceae** | | | |
| *Andromeda polifolia* L. | Bog rosemary | Flavonols | PACHALY and KLEIN 1987 |
| *Calluna vulgaris* (L.) Hull | Heather | Flavones<br>Flavonols<br>Flavanones<br>Dihydroflavonols<br>Anthocyanins | ALLAIS et al. 1991, ALLAIS et al. 1995, ERSÖZ et al. 1997 |
| *Vaccinium myrtillus* L. | Bilberry | Flavonols<br>Anthocyanins<br>Flavans | GERHARDT et al. 1989, WILSKA-JESZKA et al. 1992, SMOLARZ et al. 2000, HUOPALAHTI et al. 2000 |
| *Vaccinium oxycoccos* L. | Cranberry | Flavonols<br>Anthocyanins<br>Flavans | HÄKKINEN and AURIOLA 1998, WANG et al. 1978, HUOPALAHTI et al. 2000, ARTS et al. 2000 |
| *Vaccinium uliginosum* L. | Bog bilberry | Flavonols<br>Anthocyanins | ANDERSEN 1987, GERHARDT et al. 1989, CUI et al. 1992, HÄKKINEN and AURIOLA 1998, |
| *Vaccinium vitis-idaea* L. | Cowberry | Flavonols<br>Anthocyanins | HÄKKINEN and AURIOLA 1998, SMOLARZ et al. 2000, ANDERSEN 1985 |
| **Grossulariaceae** | | | |
| *Ribes nigrum* L. | Blackcurrant | Flavonols<br>Anthocyanins<br>Flavans | KOEPPEN and HERRMANN 1977, CALAMITA et al. 1983, da COSTA et al. 1998, ARTS et al. 2000 |
| **Lamiaceae** | | | |
| *Thymus vulgaris* L. | Thyme | Flavones<br>Flavonols<br>Flavanones | MIURA and NAKATANI 1989, MORIMITSU et al. 1995, WANG et al. 1998 |
| **Liliaceae** | | | |
| *Allium cepa* L. | Onion | Flavonols | BILYK et al. 1984, KIVIRANTA et al. 1988, PRICE and RHODES 1997 |
| **Lythraceae** | | | |
| *Lythrum salicaria* L. | Purple loosestrife | Flavones<br>Anthocyanins | PARIS and PARIS 1964, PARIS 1967 |
| **Onagraceae** | | | |
| *Epilobium angustifolium* L. | Willow herb | Flavonols | DUCREY et al. 1995, HIERMANN 1995 |
| **Pinaceae** | | | |
| *Picea abies* L. (Karsten) | Norway spruce | Flavones<br>Flavonols | SLIMESTAD et al. 1995, SLIMESTAD and HOSTETTMANN 1996, |

| | | | |
|---|---|---|---|
| | | Flavanones<br>Dihydroflavonols<br>Anthocyanins<br>Flavans | SLIMESTAD et al. 1996, SLIMESTAD et al. 1999 |
| *Pinus sylvestris* L. | Scot's pine | Flavonols<br>Flavanones<br>Dihydroflavonols<br>Flavans<br>Chalcones | IVANOVA et al. 1978, LARACINE-PITTET and LEBRETON 1988, FLIEGMANN et al. 1992, JUNGBLUT et al. 1995, PAN and LUNDGREN 1996, BENINGER and ABOU-ZAID 1997 |
| **Poaceae** | | | |
| *Avena sativa* L. | Oat | Flavones | POPOVICI et al. 1977, SALEH et al. 1988 |
| *Secale cereale* L. | Rye | Flavones<br>Flavonols<br>Anthocyanins | TROJNA et al. 1976, STRACK et al. 1982, SCHULZ and WEISSENBÖCK 1987 |
| **Rosaceae** | | | |
| *Aronia melanocarpa* (Michx.) Elliot | Black chokeberry | Flavonols<br>Flavans<br>Anthocyanins | OSZMIANSKI and SAPIS 1988, SZEPCZYNSKA 1989,WILSKA-JESZKA et al. 1992, HÄKKINEN and AURIOLA 1998 |
| *Filipendula ulmaria* (L.) Maxim. | Meadowsweet | Flavonols | SCHEER and WICHTL 1987, LAMAISON et al. 1992 |
| *Malus pumila* Mill. | Apple | Flavonols<br>Dihydroflavonols<br>Anthocyanins<br>Flavans<br>Dihydrochalcones | KANETA et al. 1979, LISTER et al. 1994, ARTS et al. 2000 |
| *Rubus chamaemorus* L. | Cloudberry | Flavonols<br>Anthocyanins | HÄKKINEN et al. 1999, KÄHKÖNEN et al. 2001 |
| *Rubus idaeus* L. | Raspberry | Flavonols<br>Anthocyanins<br>Flavans | HENNING 1981, ROMMEL and WROLSTAD 1993, GUDEJ and RYCHLINSKA 1996, ARTS et al. 2000, KÄHKÖNEN et al. 2001 |
| *Sorbus aucuparia* L. | Rowanberry | Flavonols<br>Anthocyanins<br>Flavans | VATULINA 1970, PYYSALO and KUUSI 1974, EDER et al. 1991, WILSKA-JESZKA et al. 1992, HÄKKINEN and AURIOLA 1998 |
| **Salicaceae** | | | |
| *Salix caprea* L. | Willow | Flavones<br>Flavonols<br>Flavanones<br>Dihydroflavonols<br>Flavans | NASUDARI et al. 1970, MALTERUD et al. 1985 |
| **Solanaceae** | | | |
| *Solanum tuberosum* L. | Potato | Flavonols<br>Flavanones<br>Anthocyanins<br>Flavans | LEWIS et al. 1998 |

## 2.2. Biological activities and health effects of flavonoids and other phenolic compounds

Flavonoids have certain health effects, even though they are non-nutritive compounds (HERTOG et al. 1992a). A considerable amount of research has been directed towards their activity as antioxidants and radical scavengers, as well as their anti-mutagenic and anti-carcinogenic properties. However, the most interest has been paid to their potential in the prevention of coronary heart disease (MELZER and MALTERUD 1997). Tannins seem to have no metabolic function (MEHANSHO et al. 1987) but, during the last decade, there has been increasing interest in the antimutagenic and antitumourigenic properties of tannic acid especially (MUKHTAR et al. 1988, WANG et al. 1999). The antioxidative properties of phenolic acids play an important role in the stability of food products, as well as in the antioxidative defence mechanisms of biological systems (MACHEIX and FLEURIERT 1998).

The health effects of dietary flavonoid intake that have been epidemiologically studied are collated in **Table 2**. Even though inverse relationships have been noted between vegetable and fruit intake and lung cancer (VOORRIPS et al. 2000), and between onion consumption and stomach carcinoma (DORANT et al. 1996), only a slight preventive relationship has been found between flavonoid intake and cancer mortality, primarily due to isoflavonoids (ADLERCREUTZ 1995). In contrast, evidence points to a protective effect of antioxidant flavonoids in cardiovascular disease – although this is not conclusive (HOLLMAN and KATAN 1998). Other advantageous effects of flavonoids, such as antiaggregatory activity, are not expressed after dietary intake of flavonoids. However, this result was obtained with concentrations that cannot be attained *in vivo* (JANSSEN et al. 1998).

**Table 2** Epidemiological evidence on the health effects of flavonoids according to the literature (HOLLMAN and KATAN 1998, BÖHM et al. 1998). Q = quercetin, K = kaempferol, M = myricetin, A = apigenin, L = luteolin.

| **Health effect** | **Flavonoids** | **References** |
|---|---|---|
| *Cardiovascular diseases* | | |
| Reduced risk of coronary heart disease | Q, K, M, A, L | HERTOG et al. 1993, |
| " | Q, K, M, A, L | KNEKT et al. 1996, |
| " | Q, K, M | HERTOG et al. 1997a, |
| " | Q, K, M, A, L | YOCHUM et al. 1999 |
| No association between flavonoid intake and CHD | Q, K, M, A, L | RIMM et al. 1996 |
| No association between quercetin intake and CVA | Q | KNEKT et al. 2000 |
| Increased risk of ischaemic heart disease | Q, K, M | HERTOG et al. 1997b |
| Reduced risk of myocardial infarction | Q, K, M, A, L | HERTOG et al. 1993 |
| Reduced risk of stroke | Q, K, M, A, L | KELI et al. 1996 |
| No association between flavonoid intake and stroke | Q, K, M, A, L | YOCHUM et al. 1999, |
| " | Q, K, M, A, L | HIRVONEN et al. 2000 |
| *Cancer* | | |
| No association between flavonoid intake and cancer | Q, K, M, A, L | HERTOG et al. 1994, |
| " | Q, M, K, L | GOLDBOHM et al. 1995 |
| No association between flavonoid intake and lung cancer | Q, K, M, L | GARCIA-CLOSAS et al. 1998 |
| Inverse relation between flavonoid intake and lung cancer | Q, K, M, A, L | KNEKT et al. 1997, |
| " | Q, naringin | le MARCHAND et al. 2000 |
| Inverse relation between flavonoid intake and gastric cancer | Q, K, M, L | GARCIA-CLOSAS et al. 1999 |
| Reduction of breast-cancer development | equol | INGRAM et al. 1997 |

The positive findings presented in **Table 2** have contributed to the dramatic increase in the use of plant flavonoids in large quantities by health-conscious consumers. However, the excessive and uncontrolled intake of flavonoids may lead to mutagenic or pro-oxidative activity or, furthermore, to the inhibition of key enzymes of hormone metabolism (SKIBOLA and SMITH 2000).

The next three chapters deal with the biological activities of phenolics observed *in vitro*, which may be related to the health effects also noted in epidemiological studies.

### 2.2.1. Antioxidative activity

An antioxidant is a substance that, when present at a concentration low compared to that of an oxidisable substrate, significantly delays or prevents oxidation of that substrate (HALLIWELL 1990). Even though plant phenols are not always treated as real antioxidants in the literature, many *in vitro* studies have demonstrated the antioxidant potential of phenols as direct aqueous phase radical scavengers and as agents capable of enhancing the resistance to oxidation of low density lipoproteins implicated in the pathogenesis of coronary heart disease (RICE-EVANS et al. 1995). It is admitted that a part of the antioxidant capacity of many fruits and berries is derived from flavonoids (WANG et al. 1996, HEINONEN et al. 1998a) and, in fact, all the major polyphenolic constituents of food show greater efficacy in these systems as antioxidants on a molar basis than the antioxidant nutrients vitamin C, vitamin E, and η-carotene (VINSON et al. 1995). Differences between the antioxidant potential of selected compounds can be measured using many different techniques. Because most phytochemicals are multifunctional, a reliable antioxidant protocol requires the measurement of more than one property relevant to either foods or biological systems (FRANKEL and MEYER 2000).

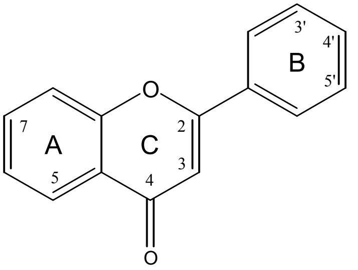


**Figure 3** Numbering of the flavone nucleus.

Certain structure-antioxidant activity relationships of flavonoids can be derived on the basis of the literature. Comparison between quercetin, catechin and cyanidin demonstrates the importance of unsaturation in the C ring (**Figure 3**), allowing electron localisation across the molecule for stabilisation of the aryloxyl radical (RICE-EVANS et al. 1995). The 3-OH group attached to the 2,3-double bond, and its location adjacent to the 4-carbonyl in the C ring, are required for the maximum effectiveness of radical scavenging (SHAHIDI and WANASUNDARA 1992). The weaker antioxidative potential of catechins can be enhanced to the stage of quercetin by incorporation of the 2,3-double bond and 4-oxo function, by ester linkage via the 3-OH group to gallic acid, and incorporation of an additional 5'-OH group in the B ring (SALAH et al. 1995, BOUCHET et al. 1998, GUO et al. 1999). This results in (-)-epigallocatechin-3-*O*-gallate being one of the most efficient scavengers of the superoxide radical (JOVANOVIC et al. 1995).

A monophenolic ring is not an effective hydrogen donor. Thus the antioxidant activity is at its maximum when the B ring is substituted by two hydroxyl groups in the *ortho*-diphenolic arrangement. The presence of a third OH group in the B ring does not enhance the effectiveness against aqueous phase radicals, except in the case of catechins; contributions to the antioxidant activity from hydroxyl groups on the A ring are present in the absence of the dihydroxy structure in the B ring, predominantly (RICE-EVANS et al. 1996).

Generally, *O*-methylation of the hydroxyl substitutions inactivates the antioxidant activities of flavonoids (CAO et al. 1997, YOKOZAWA et al. 1998). The 3-glycosylation of flavonoids also reduces their activity compared to the corresponding aglycones (HOPIA and HEINONEN 1999), but the glycolysation of the 7-OH group in a structure with a saturated heterocyclic ring and with a lone hydroxyl group on the B ring, even has a suppressive influence on the antioxidant activity (SHAHIDI and WANASUNDARA 1992, KAPIOTIS et al. 1997).

In fats, the 3',4'-orthodihydroxy configuration in the B ring, and the 4-carbonyl group and 3-OH group in the C ring, are still associated with optimum antioxidant activity. In contrast with aqueous phase interactions, however, the 2,3-double bond is considered to be less important (HUDSON and LEWIS 1983). This is only one consequence of the studied hypothesis that the antioxidant activity of flavonoids appears to be not only due to their structural features but also to their location in the biological membrane (SAIJA et al. 1995).

Procyanidin and polygalloylglucose are much more effective $ABTS^{\cdot+}$ radical scavengers than Trolox or flavonoid monomers (HAGERMAN et al. 1998). This is in proportion to their activity as DPPH radical scavengers: on a molar basis all the tannins are more effective than flavonoids or phenolic acids. Among the gallotannins and ellagitannins, compounds with large molecules show a stronger free-radical scavenging action than those with small molecules, but this difference is cancelled, when the activity is calculated on a mg/ml basis. Gallotannins are slightly more active than ellagitannins of corresponding size (YOKOZAWA et al. 1998). The level of antioxidative activity of procyanidins and condensed tannins can vary considerably depending on the test methods used. In a study of six tannins isolated from *Vaccinium vitis-idaea* L., the most active compound in a cytochrome C test (superoxide scavenger activity test), proanthocyanidin A-1, was the least active substance in a thiobarbituric acid test, and showed lower anti-lipid peroxidation activity than the tested condensed tannins (HO et al. 1999).

Phenolic acids and their esters have antioxidative activity that is dependent on the number of hydroxyl groups in the molecule. The electron-withdrawing properties of the carboxylate group, which have a negative influence on the H-donating abilities of hydroxy benzoates, can be prevented by steric hindrance (STERN et al. 1996, RICE-EVANS et al. 1996).

The monohydroxy benzoic acids act as very weak antioxidants: owing to the electronegative potential of a single carboxyl group, only *m*-hydroxy bezoic acid has antioxidative activity. This activity increases considerably in the case of dihydroxy substituted benzoic acids, whose antioxidant response is dependent on the relative positions of the hydroxyl groups in the ring. Gallic acid (3,4,5-trihydroxy benzoic acid) is the most potent antioxidant of all hydroxybenzoic acids (RICE-EVANS et al. 1996).

Insertion of an ethylenic group between a phenyl ring carrying a hydroxyl group and the carboxylate group, has a highly favourable effect on the reducing properties of the OH group. The total antioxidant activities of hydroxycinnamic acids are higher than those of the respective hydroxybenzoic acids. The activity can be increased by certain methoxylation substitutions, but glycosylation of the carboxylate group has no influence on this property (RICE-EVANS et al. 1996). In lipids, the antioxidative efficacy of monophenols is increased substantially by one or two methoxy substitutions in *o*-positions to the OH (CASTELLUCCIO et al. 1995, CUVELIER et al. 1992, RICE-EVANS et al. 1996). At least two, or even three, neighbouring phenolic hydroxyl groups and a carbonyl group are the essential molecular features required to achieve a high level of antioxidative activity (DZIEDZIC and HUDSON 1983).

### 2.2.2. Calcium channel activity

Calcium is a secondary messenger responsible for regulating a wide range of cellular processes. Paradoxically, while it is clearly capable of stimulating many cellular processes, it is also very toxic. It has been argued that the non-lethal effects of elevated calcium can lead to a variety of pathological conditions including hypertension, arteriosclerosis, transformation, malignant hyperthermia and possible neural disorders such as spreading depression and manic depressive illness (BERRIDGE 1994). Calcium homeostasis is regulated by an extracellular cycle, which controls calcium fluxes between the cytosol and extracellular space, and an intracellular cycle, which controls calcium fluxes between the cytosol and intracellular stores in the sarcoplasmic reticulum (KATZ 1997). The extracellular cycle is controlled by either receptor-operated $Ca^{2+}$ channels (ROCCs) or voltage-operated channels (VOCCs). According to their electrophysiological and pharmacological characteristics, the VOCCs can be divided into L, N, T and P types, the first of which being present especially in the heart and blood vessels (VUORELA et al. 1997).

Essential hypertension is the most typical consequence of an increased intracellular calcium level. Lymphocyte cytosolic calcium has been found to be significantly increased in hypertensive patients (RIVERA et al. 1996), and an excessive build-up of intracellular free $Ca^{2+}$ plays an important role in the pathogenesis of cardiac necrosis and congestive heart failure (LI et al. 1995), whereas low ionised $Ca^{2+}$ levels in the serum impair cardiovascular function (ZALOGA et al. 1985). Platelet activation leading to thrombosis and myocardial infarction is also a result of the elevation of free cytoplasmic calcium. However, the accumulation of calcium in platelets is due to greater $Ca^{2+}$ releasability from internal stores rather than to a greater $Ca^{2+}$ influx (KATOPODIS 1997). In clinical use, the drugs with calcium channel blocking activity, *e.g.* verapamil, nifedipin and diltiazem, have been widely accepted in the treatment of hypertension, dysrhytmia, and *angina pectoris* (RANG and DALE 1991).

Outside the cardiovascular system, the level of extracellular calcium in psoriatic lesions was lowered, whereas cells had an increased calcium content. Psoriatic suprabasal cell layers also displayed higher than normal concentrations of calcium, indicating loss of the normal calcium gradient that programmes terminal differentiation, thereby accounting for the differentiation defects that occur in psoriasis (MENON and ELIAS 1991). Chronic renal failure is associated with elevated basal levels of cytosolic $Ca^{2+}$ in hepatocytes (KLIN et al. 1995), and the amount of circulating calcium is an essential factor in nephrolithiasis (HESS 1998). Furthermore, some disorders of the endocrine glands are very sensitive to differences in calcium homeostasis. Such illnesses are parathyroidism (LOCKER et al. 1997) and pancreatitis (ZHOU et al. 1996). Following hyperglycemia and insulin deficiency, increased intracellular free calcium can also be observed in diabetes mellitus, which easily leads to arterial hypertension and enhances the risk of cardiovascular morbidity and mortality (DUNER et al. 1997, GOYAL 1999). In the gastrointestinal tract, calcium also regulates the balance between absorption and secretion across the intestinal mucosa (BERRIDGE 1984), as well as expressing migrating contractions that are related to the antidiarrhoeal effects of $Ca^{2+}$ channel blockers (LEE et al. 1997).

Abnormalities of calcium homeostasis have also been implicated in a number of diseases of the central nervous system. High cytosolic $Ca^{2+}$ contents have been reported in epileptic activity (IKEGAYA 1997), and increased serum $Ca^{2+}$ levels have been detected in major depression (JOFFE et al. 1996) and in bivalent psychoses (CARMAN and WYATT 1979). In recent years, the effect of calcium fluxes on the development of dementia and Alzheimer's disease has been investigated, together with the option of using calcium channel blockers in the prevention of these diseases (FORETTE et al. 1998, LOPEZ-ARRIETA and BIRKS 2000).

Calcium channel activity can be tested using experiments with animals in which blood pressure or blood flow is measured, using isolated heart muscle, vascular or other smooth muscle preparations, or with cell lines. Cultured cell lines, *e.g.* thyreoidea cells and pituitary cells, have increased their importance as tools for preliminary screening *in vitro*, since relatively homogeneous populations are easily obtainable, are rather stable, and do not have the difficulties associated with the breeding of laboratory animals (VUORELA et al. 1997).

A very common flavonoid, quercetin has been reported to have a spasmolytic action on aortic smooth muscle, the effect being expressed as a vasodilatory effect in isolated rat aorta (MORALES et al. 1994, MORALES and LOZOYA 1994), and to have inhibitory activity on intestinal motility in mice (MELI et al. 1990). Both effects have been explained as calcium antagonistic activity. Quercetin inhibited both contractions induced by increases in extracellular $Ca^{2+}$ in KCl-depolarised muscles and protein kinase C (PKC), which plays a key role in the maintenance of tonic contractions of vascular smooth muscle (DUARTE et al. 1993a). Flavones inhibited $Ca^{2+}$ induced contractions in $K^+$ depolarised smooth muscles (van den BROUCKE and LEMLI 1983), and a more general calcium antagonistic action has also been noted for flavonoids (quercetin, kaempferol and genistein), although showing a different behaviour toward the calcium channel blockers, nifedipine and verapamil, in studies on their effect in rat uterus (REVUELTA et al. 1997).

Few studies have been carried out to elucidate the differences in calcium channel activity among the family of flavonoids. In rat aortic smooth muscle, a comparison between flavonols and flavones showed that catechins were the least effective agents against the contractile responses induced by vasoconstrictors (DUARTE et al. 1993b). In a screening of simple phenolic compounds on calcium fluxes in clonal rat pituitary $GH_4C_1$ cells, some noticeable differences were found between groups of phenolics, which is contradictory with the results of earlier studies (SUMMANEN et al. 2001). Flavones and phenylmetanes had a clear inhibiting effect on calcium fluxes, whereas caffeic acid, ferulic acid and flavonols increased the $Ca^{2+}$ intake of cells (**Table 3**). The effects were smoothed by glycolysation of the flavonoid nucleus; the inhibitory effect of gallates was potented by the presence of a lipophilic chain. The unexpected behaviour of flavonols was studied further, and it was concluded that the enhanced entry of $^{45}Ca^{2+}$ evoked by quercetin is the result of an increase in cAMP and concomitant activation of protein kinase A (PKA), leading to upmodulation of the VOCCs.

**Table 3** Effects of selected phenolic compounds on $^{45}Ca^{2+}$ uptake (%) in clonal rat pituitary $GH_4C_1$ cells (SUMMANEN et al. 2001).

| **Compound (20 σg/ml)** | **Systematic name** | **Effect on $Ca^{2+}$ uptake** | **Mw** |
|---|---|---|---|
| *Flavones* | | | |
| Apigenin | 5,7,4'-trihydroxyflavone | -29.3 | 270.2 |
| Luteolin | 5,7,3',4'-tetrahydroxyflavone | -51.4 | 286.2 |
| Acacetin | 5,7-dihydroxy-4'-methoxyflavone | -1.39 | 284.3 |
| Flavone | flavone | -63.5 | 222.2 |
| Vitexin | 5,7,4'-trihydroxyflavone-8-glucoside | -2.12 | 432.4 |
| Vitexin-2''-*O*-rhamnoside | 5,7,4'-trihydroxyflavone-8-glucoside-2''-rhamnoside | -14.6 | 587.5 |
| Luteolin-7-glucoside | 5,7,3',4'-tetrahydroxyflavone-7-glucoside | -16.3 | 448.4 |
| Luteolin-3',7-diglucoside | 5,7,3',4'-tetrahydroxyflavone-7,3'-diglucoside | -14.6 | 610.5 |
| *Flavonols* | | | |
| Quercetin | 3,5,7,3',4'-pentahydroxyflavone | 54.1 | 302.2 |
| Rhamnetin | 3,5,3',4'-tetrahydroxy-7-methoxyflavone | 6.63 | 316.3 |
| Isorhamnetin | 3,5,7,4'-tetrahydroxy-3'-methoxyflavone | 52.4 | 316.3 |
| Morin | 3,5,7,2',4'-pentahydroxyflavone | 48 | 302.2 |
| Quercitrin | 3,5,7,3',4'-pentahydroxyflavone-3-rhamnoside | 20.1 | 448.4 |
| Rutin | 3,5,7,3',4'-pentahydroxyflavone-3-rutinoside | -3.88 | 610.5 |
| *Flavanones* | | | |
| Naringenin | 5,7,4'-trihydroxyflavanone | -56.3 | 272.3 |
| Naringin | 5,7,4'-trihydroxyflavanone-7-rhamnoglucoside | 6.5 | 580.5 |
| *Isoflavones* | | | |
| Daidzein | 7,4'-dihydroxyisoflavone | -26.2 | 254.2 |
| Genistein | 5,7,4'-trihydroxyisoflavone | -54.6 | 270.2 |
| Daidzin | 7,4'-dihydroxyisoflavone-7-glucoside | 7.6 | 416.4 |
| Genistin | 5,7,4'-trihydroxyisoflavone-7-glucoside | -3.39 | 432.4 |
| *Phenylmetanes* | | | |
| Benzoic acid | benzoic acid | -9.82 | 122.1 |
| Gallic acid | 3,4,5-trihydroxybenzoic acid | -5.33 | 170.1 |
| Syringic acid | 4-hydroxy-2,5-dimethoxybenzoic acid | -10.9 | 198.2 |
| Methyl gallate | methyl ester of 3,4,5-trihydroxybenzoic acid | -21.2 | 184.1 |
| Propyl gallate | propyl ester of 3,4,5-trihydroxybenzoic acid | -37.9 | 212.2 |
| Octyl gallate | octyl ester of 3,4,5-trihydroxybenzoic acid | -92.2 | 282.3 |
| Dodecyl gallate | dodecyl ester of 3,4,5-trihydroxybenzoic acid | -40.4 | 338.4 |
| *Phenylpropanes* | | | |
| Caffeic acid | 3,4-dihydroxycinnamic acid | 9.71 | 180.2 |
| Ferulic acid | 4-hydroxy-3-methoxycinnamic acid | 9.23 | 194.2 |

Apart from Summanen et al. (2001), there are practically no reports available concerning the calcium channel effects of phenolic acids. Neither has the calcium channel activity of tannins been studied very much. Rugosin E, an ellagitannin that was a notably more effective platelet aggregating agent than eight other ellagitannins studied (including grandinin, vescalagin and pedunculagin), induced an increase in the intracellular calcium concentration in rabbit platelets (TENG et al. 1997). This also correlated with the $Ca^{2+}$ homeostasis *in vivo*, owing to certain similarities between platelets and vascular smooth muscle, especially in their ability to respond to vasoactive hormones (RIVERA et al. 1996).

### 2.2.3. Antimicrobial activity

Flavonoids are known to be synthesised by plants in response to microbial infection (DIXON et al. 1983). It is therefore logical that they have been found *in vitro* to be effective antimicrobial substances against a wide array of micro-organisms (RECIO et al. 1989, COWAN 1999). Most of the available research reports deal with antibacterial or antifungal properties, but antiviral activity has also been reviewed (BENAVENTE-GARCÍA et al. 1997).

The structure-activity relationships of the antimicrobial activity of flavonoids are contradictory. It has been shown that less polar compounds, *i.e.* flavonoids lacking hydroxyl groups on their B ring, are more active against microorganisms than those with -OH groups (CHABOT et al. 1992). This is supported by the finding that methylation of the flavonoid nucleus increases antibacterial activity against *Staphylococcus aureus* (IBEWUIKE et al. 1997). In the case of methicillin-resistant *S. aureus*, aliphatic side chains on the A flavone ring (6 or 8) make the molecule more lipophilic and increase its antibacterial activity compared with unsubstituted flavones (TSUCHIYA et al. 1996). It has been shown with cariogenic bacteria (*Actinomyces viscosus* and *A. naeslundii*, several *Streptococci*) that polyhydroxyl groups on the A and B rings (5-hydroxylation is essential), plus aliphatic substitution on the A ring, are determinants of the antibacterial activity of flavones (SATO et al. 1996). However, it has also been claimed that lipophilic flavonoids (methoxylated) are not very good protective agents against microorganisms (MENDOZA et al. 1997). To support this, the glycosides of quercetin and quercetagetin have shown significant antimicrobial activity against several pathogenic microorganisms (WAAGE and HEDIN 1985, TERESCHUK et al. 1997).

A free 3',4',5'-trihydroxy B ring and a free 3-OH have been found to be necessary for antibacterial activity against *Staphylococcus aureus* and *Proteus vulgaris* (MORI et al. 1987). This is supported by the result of Puupponen-Pimiä et al. (2001), in which the broadest antibacterial activity of the tested flavonoids was achieved using myricetin against *Lactobacilli* and *E. coli*. The same requirement was also verified in a Japanese study in which catechins of tea, especially (+)-gallocatechin and (-)-epigallocatechin, were found to inhibit the growth of *Streptococcus mutans*, a cariogenic bacterium responsible for tooth decay (SAKANAKA et al. 1989). (-)-Epigallocatechin has also been reported to exhibit antibacterial activity against *Staphylococcus epidermidis* (G+), *Proteus vulgaris* (G-) and *S. aureus* (G+) (NISHINO et al. 1987). Theaflavin, a catechin isolated from *Camellia sinensis* L., inhibited *Shigella spp.* at non-cytotoxic concentrations (VIJAYA et al. 1995). On the other hand, a common (+)-catechin has been found to be ineffective against several gram-positive and gram-negative bacteria compared to coumarins and gallic acid derivatives (KAYSER and KOLODZIEJ 1997).

One of the molecular actions of tannins is to complex with proteins through so-called non-specific forces such as hydrogen bonding and hydrophobic effects, as well as by covalent bond formation (HASLAM 1996, STERN et al. 1996). Thus their mode of antimicrobial action may be related to their ability to inactivate microbial adhesins, enzymes, cell envelope transport proteins etc. (COWAN 1999). Tannins are reported to have antibacterial, antifungal, and antiviral activity (SCALBERT 1991, NONAKA et al. 1990).

Procyanidin inhibited the growth of Gram-negative *Pseudomonas maltophilia* (WAAGE et al. 1984), and epicatechin-(4η⇓ 8)-epicatechin-(4η⇓ 8, 2η⇓ *O*⇓ 7)-catechin has shown strong antimicrobial activity against the periodontal Gram-negative *Porphyromonas gingivalis* and *Prevotella intermedia* (HO et al. 2001). However, condensed tannins have also been reported to be active against Gram-positive *Streptococcus mutans* (HADA et al. 1989). An antibacterial assay also showed that gallotannins had activity against both main groups of bacteria (NISHIZAWA et al. 1990). Gallotannins inhibited the glucosyltransferase reactions of *S. mutans*, whereas ellagitannins were rather stimulative. The number of galloyl residues was proportional to the inhibitory potency of gallotannins (KAKIUCHI et al. 1986), thereby permitting the development of some structural activity theories. When tannic acid inhibited the growth of *Photobacterium phosphoreum* (G-), one of the inhibitory sites was assumed to be the respiratory chain, another terminal oxidase (KONISHI et al. 1987).

Even though tannins are widely known as antimicrobial compounds, there are still several species that can grow and develop on tannin-rich materials (SCALBERT 1991). Eucaryotic microbial species are especially numerous in this respect. The resistance mechanism could involve the secretion of tannin-binding polymers, tannin oxidation or siderophores. One unusual finding is that some microorganisms (*e.g. Penicillium* sp., *Candida* sp., and especially *Aspergillus niger*) can grow on tannins as their sole source of carbon.

Both tannins, which are large phenolic molecules, and the more simple phenols and phenolic acids are antimicrobial (COWAN 1999). The mechanisms thought to be responsible for phenolic toxicity to microorganisms include enzyme inhibition by the oxidised compounds, possibly through reaction with sulphhydryl groups or through more non-specific interactions with the proteins (MASON and WASSERMAN 1987).

Gallic acid and its methyl ester had a clear inhibitory effect on several harmful intestinal bacteria (AHN et al. 1998), and six other simple phenolic acids were found to be antimicrobially active against a variety of bacteria and moulds (AZIZ et al. 1998). Vanillic and caffeic acids completely inhibited both the growth and aflatoxin production of *Aspergillus flavus* and *A. parasiticus*. When the causes of the antimicrobial activity of honey were investigated, phenolic acids appeared to have a potential importance (WAHDAN 1998).

## 2.3. Analytical methods in research of phenolic compounds

The analysis of phenolic compounds is very challenging due to the great variety and reactivity of these compounds (BRONZE and BOAS 1998). On the other hand, polyphenolics are suitable compounds for analysis using modern separation and detection methods, such as hyphenated techniques of high-performance liquid chromatography (HPLC) with mass spectrometry (MS), ultraviolet-visible light (UV/Vis), or nuclear magnetic resonance (NMR) spectroscopy.

Group-selective chemical reactions, paper chromatography (PC), and gas chromatography (GC) have been important methods in the qualitative analysis of phenolics (PARIS 1967, BHATIA and BAJAJ 1975, HARBORNE 1975) – however, the latter only after derivatisation (ROBARDS and ANTOLOVICH 1997). Thin layer chromatography (TLC) has its own advantages (*e.g.* rapidity and inexpensiveness), and modern densitometric and video-camera detection techniques have further increased its versatility as a widely used analysis method for phenolic compounds (SUMMANEN et al. 1998, SUMMANEN 1999).

Phenolic acids of natural origin are weak acids and, owing to their phenolic hydroxyl groups, flavonoids and tannins also have a slightly acidic nature. They are therefore ionisable in alkaline conditions, which has led to successful applications of different types of capillary electrophoresis (CE) in the analysis of flavonoids (PIETTA et al. 1991, MARKHAM and McGHIE 1996, LIANG et al. 1997), tannins (BRONZE et al. 1997), and phenolic acids (SEITZ et al. 1991, BJERGEGAARD et al. 1992, HIERMANN and RADL 1998).

### 2.3.1. High-performance liquid chromatography (HPLC)

In the early days of high-performance liquid chromatography, it was stated that: "While LC gives accurate, specific results, it is slow relative to total phenol assay procedures, requires expensive equipments and specialized skills. Moreover, in many cases, the details provided by this method (*i.e.* relative concentrations of each isomer) are not needed." (WALTER and PURCELL 1979). Even though some of those claims are basically still valid, the introduction of enhanced resolution and increased automation has resulted in HPLC (also known as high-pressure liquid chromatography) becoming the most popular analysis method for plant phenolics (ROBARDS and ANTOLOVICH 1997, WAKSMUNDZKA-HAJNOS 1998).

The phenolic compounds of natural origin have the positive property of being soluble in polar solvents. This leads to the possibility of using reversed phase HPLC (RP-HPLC) in their analysis, sufficient retention being achieved by using acidic conditions in order to avoid the presence of ionised forms of the analytes (WAKSMUNDZKA-HAJNOS 1998). Octadecylsilane (ODS, C18, RP-18) is by far the most popular of the stationary phases, both generally and for phenolics (MAJORS 2001). Other RP materials (*e.g.* C8) are chosen only seldom (ROBARDS and ANTOLOVICH 1997), which is at least partly due to the fact that these products are to a lesser extent available on the market (MAJORS 2001). However, polymeric condensed tannins cannot be analysed using RP-HPLC because of too strong sorption to the stationary phases (SCHOFIELD et al. 1998). Condensed tannins therefore have to be analysed using the normal phase HPLC technique, which is not so widely used since it has much poorer resolution compared to RP-HPLC (WAKSMUNDZKA-HAJNOS 1998).

The eluents used in the RP-HPLC analysis of phenolics are mixtures of aqueous pH modifiers with a polar, water-soluble organic solvent: methanol (MeOH), acetonitrile (ACN) or tetrahydrofurane (THF). Even though THF has its own advantages as regards selectivity, it is not used very frequently because of its degradability, long equilibration time, toxicity, and high UV background absorbance below 240 nm (DOLAN 2000). However, some methods have been published in which THF has been selected as the only organic solvent in the mobile phase (DICK et al. 1987, de LAURENTIS et al. 1997). 2-propanol, n-butanol or other organic solvents appear only rarely in the mobile phase and, when used, predominantly for selectivity reasons (BRONNER and BEECHER 1995). However, as low-concentration organic modifiers they can also have a dramatic effect on the retention of phenolic compounds (ARÍN et al. 1995). pH is a major factor especially in the separation of ionisable compounds such as phenolic acids. The most frequently used pH modifiers in RP-HPLC methods for phenolic compounds are the acids, formic, acetic, trifluoroacetic and phosphoric acid, as well as phosphate buffers adjusted to acidic pH in order to achieve the unionised form of the phenolic analytes (ROBARDS and ANTOLOVICH 1997, WAKSMUNDZKA-HAJNOS 1998).

Another positive property of phenolic compounds is their conjugated C-C double bonds which act as chromophores. Thanks to them, a combination of RP-HPLC and an ultraviolet/visible light (UV/Vis) detector is commonly used in both qualitative and quantitative analysis of nature-derived samples containing phenolics (MARKHAM and BLOOR 1998). The UV wavelength of 280 nm has proved to be suitable for the universal detection of all phenolics, ranging from simple hydroxybenzoic acids to larger tannins (WAKSMUNDZKA-HAJNOS 1998, MARKHAM 1982, SALMINEN et al. 1999). However, other wavelengths are frequently used for more selective detection of certain subgroups of phenolic compounds. On the basis of their absorption maxima, ellagic acid and *p*-hydroxybenzoic acid can be detected at 260 nm, gallic acid and catechins at 280 nm, hydroxycinnamic acids at 320 nm, flavonols at 360 nm, and anthocyanins at 530 nm (HÄKKINEN et al. 1998, PHIPPEN and SIMON 1998). The sensitivity of UV detectors with a detection limit of about 0.2 pg/flavonoid/injection is adequate for normal analytical work with plant samples, or for the analysis of biological fluids in pharmacokinetic investigations with enlarged flavonoid doses (GAGNON et al. 1992, MORRICA et al. 1998). Nevertheless, HPLC methods with UV detection do not meet the requirements of sensitivity and specificity for body fluids after the consumption of a normal diet. Therefore, the ability of flavonols to form fluorescent chelates with metal ions has been utilised (HOLLMAN et al. 1996). The detection limit of some flavonols has thereby been reduced to under 3 fg/injecton. A number of papers have described the advantages of electrochemical detectors that have been reported to be 10- to 25-fold more sensitive than UV (MIKAMO et al. 1999). These benefits in sensitivity have also led to applications with chemiluminescence detection (CL-HPLC) and coulometric electrode array detection of (-)-epigallocatechin-3-gallate in human plasma samples (MIYAZAWA et al. 1999, FRIEDRICH et al. 1999).

### 2.3.2. HPLC/Mass spectrometry (LC/MS)

Mass spectrometric detection (MS) combined with chromatography provides, in addition to mass selective detection, a wealth of structural information (COVEY et al. 1986). Older hyphenated MS techniques were associated with a number of problems in the analysis of phenolic compounds, such as the need of derivatisation for GC/MS, the technical complexity of moving belt (MB) and continuous-flow fast atom bombardment (CF-FAB), and the strong fragmentation of electron impact mass spectrometry (EI-MS) for flavonoid glycosides (GAMES and MARTÍNEZ 1989, WOLFENDER et al. 1993, HEDIN and PHILLIPS 1992). Following the introduction of atmospheric pressure ionisation (API) techniques, electrospray ionisation (ESI) and atmospheric pressure chemical ionisation (APCI), these problems have been resolved and the hyphenated technique of HPLC and mass spectrometry (HPLC/MS) has become a powerful tool in the chemical characterisation of plant extracts and other samples of biological origin (COVEY et al. 1986, JOOS 1995, WOLFENDER and HOSTETTMANN 1996).

ESI, where the effluent flows through a capillary kept at a potential of several kilovolts, allows flow rates of 5–10 σl/min, whereas APCI having a heated pneumatic nebulizer provides routine operation using HLPC flow rates up to 2 ml/min (COVEY et al. 1986). ESI, ionspray (IS), *i.e.* pneumatically assisted ESI, and APCI are nowadays the most widely used interfaces (NIESSEN 1998, STOBIECKI 2000). These techniques together permit the analysis of a wide range of compounds of different molecular size and polarity. They are also easy to use, and considerable flexibility in the form of different LC/MS constructions, ion traps and quadrupole analysers, is possible (BRUINS et al. 1987, COVEY et al. 1986, WOLFENDER et al. 1995). Matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS) has also been used successfully in the analysis of flavonoids (WANG and SPORNS 2000). It is a sensitive technique but, up until now, it has not been possible to use it on-flow in connection with HPLC separation.

To date, LC/(API)MS techniques have been widely accepted for the analysis of flavonoids, including a wide variety of different RP-HPLC conditions (**Table 4**). The most frequent application of LC/MS in the analysis of flavonoids is a full scan over the selected *m/z* range during the course of an HPLC run (ANDLAUER et al. 1999, da COSTA et al. 2000, HÄKKINEN and AURIOLA 1998, JUSTESEN et al. 1998). Being a complementary detection method to DAD-UV/Vis, this provides a basis for the identification of compounds and for resolving the problem of co-eluted peaks. In single API-MS mode, the flavonoid glycosides produce protonated or deprotonated molecules and ions corresponding to the loss of sugar moieties. The relationship between the flavonoid structure, such as the location of hydroxyl groups as well as sugar moieties, and mass spectra have been studied using ESI-MS (MAURI et al. 1999) and APCI-MS (GRAYER et al. 2000). The limit of detection for flavonoids in full scan MS mode has been reported to vary from 0.1 ng to 40 ng per injection by ESI (CARERI et al. 1999, HÄKKINEN and AURIOLA 1998), which is reported to be more sensitive than APCI (ROBARDS et al. 1997). However, when APCI is used in the single ion monitoring mode, a limit of quantitation of as low as 10 pg per injection has been reached (NIELSEN et al. 2000).

**Table 4** Selected LC/MS methods used for the analysis of flavonoids from different matrices.

| **Compounds** | **Sample** | **Solvent system** | **Ionisation** | **Analyser** | **Reference** |
|---|---|---|---|---|---|
| Flavones, Flavonols, Flavanones, Dihydroflavonols | standards | A: 5% $CH_3COOH$ (aq)<br>B: MeOH (40:60) | pos MB | VG 7070E double focusing | GAMES and MARTÍNEZ 1989 |
| Flavonols | *Epilobium* species | A: 0.05% TFA (aq)<br>B: ACN + 0.05% TFA, postcolumn add. of 0.5 M $NH_4OAc$ | pos TSP | Finnigan MAT TSQ-700 triple quadrupole | DUCREY et al. 1995 |
| Flavones, Flavanones, Isoflavones, Pterocarpans | *Medicago sativa, Cicer arietinum* | A: 97.9% water + 2% glycerol + 0.1% TFA<br>B: 97.9% ACN + 2% glycerol + 0.1% glycerol | pos/neg CF-LSIMS | VG ZAB2-SE double-focusing | SUMNER et al. 1996 |
| Flavones, Catechins | rosemary, sage, barley | A: 1% $CH_3COOH$ (aq)<br>B: MeOH:water:ACN 3:1:1 | pos APCI | Trio 1000 quadrupole | MAILLARD et al. 1996 |
| Flavonols, Flavones, Flavanones, Isoflavones | *Eugenia jambos* | A: water<br>B: ACN | neg ESI | HP 5989B quadrupole | CONSTANT et al. 1997 |
| Flavanones, Flavones | citrus juice, sweet orange | A: 2 mM $NH_4OAc$ (aq)<br>B: ACN:water 70:30 | pos/(neg) ESI/(APCI) | VG Trio-2 quadrupole | ROBARDS et al. 1997 |
| Catechins | tea | A: 2 mM $NH_4OAc$ (aq)<br>B: ACN | neg ESI | Finnigan TSQ 7000 quadrupole | MIKETOVA et al. 1998 |
| Flavonols, Flavones, Flavanones | fruits, vegetables, beverages | A: 1% HCOOH (aq)<br>B: ACN | neg APCI | VG Platform II quadrupole | JUSTESEN et al. 1998 |
| Flavonols | berries | A: 1% HCOOH (aq)<br>B: ACN | pos ESI | Finnigan MAT LCQ ion trap | HÄKKINEN and AURIOLA 1998 |
| Flavonols, Isoflavones, Catechins | onion, soy bean | A: ACN:water:HCOOH 10:90:5<br>B: ACN:water:HCOOH 90:10:5 | neg ESI | Micro Mass Platform II quadrupole | ANDLAUER et al. 1999 |

|  |  |  |  |  |  |
|---|---|---|---|---|---|
| Flavonols | tomato, plasma | A: 0.05% TFA (aq)<br>B: 0.05% TFA in ACN | pos/neg ESI | HP 5989A quadrupole | MAURI et al. 1999 |
| Flavanones, Flavones, Flavonols | standards | A: HCOOH (aq) pH 2.4<br>B: ACN | neg Turbo IS | PE Sciex 150EX single quadrupole | CARERI et al. 1999 |
| Flavanones | root bark of *Maclura pomifera* | A: 0.05% TFA (aq)<br>B: 0.005% TFA in ACN | pos APCI | HP 1100 single quadrupole | da COSTA et al. 2000 |
| Flavanones, Dihydrochalcones, Flavonols | urine | A: 0.5% HCOOH (aq)<br>B: ACN<br>C: MeOH | neg APCI | HP 1100 single quadrupole | NIELSEN et al. 2000 |
| Flavonols, Flavones | *Ocimum gratissimum* var. *gratissimum* | A: 1% $CH_3COOH$ (aq)<br>B: MeOH | pos/neg APCI | Finnigan LCQ quadrupole ion trap | GRAYER et al. 2000 |
| Isoflavonoids | *Astragalus* species | A: 0.25% $CH_3COOH$ (aq)<br>B: 0.25% $CH_3COOH$ in ACN | pos ESI | HP 5989B quadrupole | LIN et al. 2000a |

### 2.3.3. Computer-assisted mobile phase optimisation in HPLC

Although method optimisation is one of the essential steps in chromatographic analysis, it is relatively frequently neglected. During the last two decades, a number of computer-assisted method development programs, which make the optimisation process easier and more profitable, have become available (DEMING et al. 1989, RAUHA et al. 1997). Most optimisation strategies are based on the resolution ($R_s$) equation, in which the three basic parameters, retention factor ($k$), separation factor ($\zeta$) and theoretical plate number ($N$), can be optimised separately (OUTINEN et al. 1995). Commercially available computer-based optimisation programs have followed in the wake of microcomputers; laboratory-written programs were used successfully up until the mid 1990s (JINNO and KAWASAKI 1984, HATRÍK et al. 1994, WANG et al. 1994, OUTINEN et al. 1998).

A tool that is especially suitable for searching for the right selectivity point of the HPLC eluent in isocratic work are gridding experiments; this refers to the repeated separation of a sample mixture while varying two or more variables in small steps (SNYDER and LOMMEN 1991). Computer-based programs of this kind are PRISMA, "Osiris" and Turbo Method Development (NYIREDY et al. 1991, HEINISCH et al. 1997). The results of the programs are illustrated after the calculations as triangular resolution maps. The commercial version of Turbo Method Development, former PESOS (Perkin-Elmer, Norwalk, USA), is sold in connection with the HPLC system and allows series of analyses to be carried out in an automated fashion (RAUHA et al. 1997). TMD interprets the results and selects the best analysis carried out, as well as helps to adjust the conditions experimentally. PRISMA and Osiris (ATLAS, Lyon, France) also predict the best resolution for conditions that have not been tested experimentally, and the optimisation itself does not necessarily require any further runs (NYIREDY et al. 1991, HEINISCH et al. 1997).

Some other computer-based optimisation programs worth mentioning are ChromSword and CHROM. The ChromSword program (Merck, Darmstadt, Germany) predicts elution conditions and the chromatographic separation based solely on the structural formulae of the analytes. The prediction can be improved by entering retention data obtained from experimental runs (GALUSHKO 1996, RAUHA et al. 1997). The CHROM software (Marcel Dekker, New York, USA) optimises the complementary mobile phases for optimum resolution of each analyte, based on only a small number of experiments followed by matrix calculations (VIVÓ-TRUYOLS et al. 2000).

The DryLab™ (LC Resources, Walnut Creek, USA) simulation and optimisation program is based on the retention data of 2–16 preliminary input runs and system variables. In addition to the usual gradient profile modelling, the software can be used for the optimisation of operating temperature, pH, concentration of eluent additive, and column parameters (SNYDER et al. 1989, DOLAN et al. 1989, DryLab 2000). HPLC methods have been developed using DryLab™ for flavonoids and phenolic acids (DZIDO et al. 1991, DZIDO and SMOLARZ 1994, MARKOWSKI et al. 1998). In the best case, computer-assisted optimisation programs can be used not only for the development of analytical methods, but also to transfer the separation system to another, *e.g.* to scale up a method from analytical HPLC to preparative MPLC separation, as demonstrated in a recent report by Wennberg et al. (2001).

## 2.4. *Lythrum salicaria*

Owing to the large number of species studied in the present work, it would be impossible to characterise each plant in depth. The pharmacognostic profile of one of the essential plants investigated, *Lythrum salicaria*, has been selected as an example of a species with considerable biological activity and commercial value.

### 2.4.1. Botany

The plant family Lythraceae (loosestrife) consists of about 500 different species, and has been divided into 21 genus. The plants, the majority of which grow in tropical South America, are mostly herbs, but some non-European genera include trees and shrubs. The genus *Lythrum*, with almost 30 species, is spread throughout the world. Most of them are wetland plants, and about ten species are met in Europe. Out of the whole Lythraceae family, only two species, *Lythrum salicaria* L. and *Peplis portula* L. occur in Finland (KALELA and VÄÄNÄNEN 1960, JALAS 1980).

**Figure 4** *Lythrum salicaria* L. (Lythraceae)

Purple loosestrife (*L. salicaria*) is a 50 to 150-cm tall perennial herb, the stems of which are pubescent and distinctly four-sided, with a tendency to appear woody at the base of large plants (Flora Europaea 1968). Its impressive inflorescences are long, terminal spikes formed from trimorphic flowers with 5–7 reddish-purple petals, the flowering season in Finland being from July to August (HÄMET-AHTI et al. 1984). The leaves are mostly opposite or in whorls of 3, lanceolate, and up to 10 cm long and 1.5 cm wide with an obtuse or cordate leaf base (Flora Europaea 1968). Purple loosestrife (**Figure 4**) grows on fens, riversides and other damp places throughout almost the whole of Europe except for the extreme north. In Finland it grows as a common plant up to Kainuu and North-Ostrobothnia, but it has also been met to the north of the Arctic Circle (JALAS 1980). The plant is originally Eurasian, but during the 19th century it spread via the ballast of European ships also into North and South America, as well as to Australia. In the northern parts of the United States and Canada it is one of the most prominent aggressive wetland plants (Flora of North America 1993). *Lythrum salicaria* L. is also called in English "blooming sally", "purple willow-herb" and "rainbow weed", in German

"Blutweiderich", in French "salicaire", and in Swedish "fackelblomster". The Finnish name of *L. salicaria* is "rantakukka".

### 2.4.2. Phytochemistry

Relatively little work has been done on the phytochemistry of *Lythrum salicaria* in recent years, with four previously unidentified tannins being reported by a Chinese group in 1996 (MA et al.). The only other study concerning the phytochemistry of this plant during the last 20 years was published in Yugoslavia in 1988 (MIHAJLOVIC). Thus almost all the information about the chemical constituents of purple loosestrife has been published up to the 1960's and 1970's. Compounds found in *Lythrum salicaria*, based on the literature, are listed in **IV**.

*Lythrum salicaria* is especially rich in tannins. It contains a notable amount of flavonoids, represented by anthocyanins and flavone-*C*-glycosides (PARIS and PARIS 1964, PARIS 1964). Even though extraction with acetone or some other non-reactive solvents is nowadays favoured, the raw extraction in all the surveys presented in Table 1 (**IV**) has been made with aqueous MeOH or EtOH. The family Lythraceae has many alkaloid-rich species, *e.g. Decodon verticillatus* (L.) Ell., *Heimia salicifolia* and *Lythrum anceps* Makino (FERRIS et al. 1966a, FERRIS et al. 1966b, FUJITA et al. 1971). The compounds found in these species, lythraceous alkaloids, fall into two categories: the piperidine and quinolizidine types. However, only traces of alkaloids have been detected in *L. salicaria* (FUJITA et al. 1972), and none of them have been isolated or identified (STEINFELD 1969).

### 2.4.3. Ethnopharmacology

*Lythrum salicaria* was known as a medicinal plant already in ancient Greek and Roman times (CADAVID and CALLEJA 1980), and in Finland *Salicariae herba* has been an important drug for centuries, not only in folk medicine but also in the organised pharmaceutical system (ILMONIUS 1837, ERVAST 1840). The plant was used in archaic times for the treatment of dysentery and for moderating menstrual flow, while its effects were known to be cicatrizant and antihemorrhagic. The drug was used up until the 1900's in the treatment of diarrhoea; and typhoid has also been mentioned (LIST and HÖRHAMMER 1976). The astringent and antidiarrhoeal effects are supposed to be due to tannins. The role of tannins was demonstrated when the effect of a registered extract of *Lythrum salicaria*, Salicairine™ (by Laboratoires Legras, France), was studied against diarrhoea in mice (BRUN et al. 1998). According to this study, the antidiarrhoeal activity of Salicairine™ is related, at least in part, to an increase in colon net fluid absorption or a decrease in net fluid secretion, but the main mechanism of activity has not yet been clarified.

In antimicrobial tests, an extract of *Lythrum salicari*a has been reported to have activity against *Staphylococcus aureus* and *Candida albicans* (AL-SHAMMA and MITSCHER 1979). The antimicrobial activity spectrum has been shown to be even wider (MOSKALENKO 1986), although in some tests no noticeable activity has been found (DORNBERGER and LICH 1982).

Hypoglychemic activity of *Lythrum salicaria* was proposed already in the 1920's, but animal tests to prove this were not carried out until the 1980's (CADAVID and CALLEJA 1980, LAMELA et al. 1985). Flower and stem extracts were found to be active in both normoglycemic and hyperglycemic rats, the mode of action probably being enhancement of the circulating insulin levels (LAMELA et al. 1986).

Schistosomiasis, a widespread parasitic disease in tropical and subtropical countries, can be limited by *Lythrum salicaria* in community participation. The molluscicidal activity of the plant extract has been observed to be due to the tannins present in the plant (SCHAUFELBERGER and HOSTETTMANN 1983).

## 3. AIMS OF THE STUDY

The overall aim of the study was to investigate Finnish plants rich in phenolics as a potential source of compounds possessing beneficial biological activities. The selection of plant material was based on the known use of the species as food or herbal remedies, or on their availibility in vast quantities as process waste or as undesirable wild plants.

The specific aims of the study were:

- to identify the most promising plant materials expressing a functional character in tests on antioxidative activity (**I**), calcium channel activity (**II**), and antimicrobial activity (**III**).

- to characterise the phenolic composition of *Lythrum salicaria* L., a species with the most marked biological activity, by applying the most promising LC/MS methods available (**IV**).

- to further increase the applicability of LC/MS in the analysis of plant phenolics by studying the novel atmospheric pressure photoionisation (APPI) of selected flavonoids, and evaluating this method against other frequently used, atmospheric pressure mass spectrometric (LC/API-MS) techniques: electrospray (ESI) and atmospheric pressure chemical ionisation (APCI) (**V**).

# 4. EXPERIMENTAL

Detailed descriptions of the materials and methods can be found in the original publications. Studies **I–III** were a joint research project between the Division of Pharmacognosy, Division of Food Chemistry (University of Helsinki) and the Department of Chemistry (University of Turku).

## 4.1. Material and equipment

### 4.1.1. Phenolic compounds

The flavonoids and phenolic acids used in this study are described in **Table 5**. For the antimicrobial activity tests the phenolics were used as 1 mg/ml solutions in methanol (**III**).

**Table 5** Sources of the flavonoids and phenolic acids used in this study.

| Compound | Source |
|---|---|
| *Flavonoids* | |
| Flavone | Carl Roth GmbH & Co, Karlsruhe, Germany |
| Vitexin (apigenin-8-*C*-glucoside) | Extrasynthése S.A., Genay, France |
| Luteolin-3',7-diglucoside | Extrasynthése S.A., Genay, France |
| Kaempferol | Carl Roth GmbH & Co, Karlsruhe, Germany |
| Morin | Carl Roth GmbH & Co, Karlsruhe, Germany |
| Isorhamnetin | Extrasynthése S.A., Genay, France |
| Quercetin | E. Merck KgaA, Darmstadt, Germany |
| Isoquercitrin (quercetin-3-glucoside) | Extrasynthése S.A., Genay, France |
| Rutin (quercetin-3-rutinoside) | Carl Roth GmbH & Co, Karlsruhe, Germany |
| (+)-Catechin | Sigma Chemical Co., St.Louis, USA |
| (±)-Catechin | Sigma Chemical Co., St.Louis, USA |
| Naringenin | Carl Roth GmbH & Co, Karlsruhe, Germany |
| Naringin (naringenin-7-neohesperidoside) | Carl Roth GmbH & Co, Karlsruhe, Germany |
| *Phenolic acids and derivatives* | |
| Caffeic acid | Sigma Chemical Co., St.Louis, USA |
| Gallic acid | E. Merck KgaA, Darmstadt, Germany |
| Methyl gallate | Sigma Chemical Co., St.Louis, USA |
| Protocatechuic acid | Carl Roth GmbH & Co, Karlsruhe, Germany |

### 4.1.2. Plant material

The plants investigated in the individual studies are presented in **Table 6**. All the samples were of Finnish origin, being collected from different parts of the country in 1997–1998. Voucher samples are deposited at the Division of Pharmacognosy, Department of Pharmacy, University of Helsinki, Finland. Dried and powdered plant materials were extracted with aqueous methanol (except for the berries, fruits, and leaves of trees that were extracted with aqueous acetone), and the extracts were cold-stored. The extractions for activity tests were performed at test-tube scale (**I–III**), and for phytochemical characterisation using a medium pressure solid/liquid extraction (MPSLE) technique (**IV**) according to Mousa (1995).

**Table 6** Plants investigated in studies **I–IV**. C&E = collection and extraction of plants, P = made according to 4.1.2. by the Division of Pharmacognosy.

| Latin name | English name | Plant parts investigated | C&E | I | II | III | IV |
|---|---|---|---|---|---|---|---|
| **Aceraceae** | | | | | | | |
| *Acer plantanoides* L. | maple | leaf | | Δ | | | |
| **Apiaceae** | | | | | | | |
| *Carum carvi* L. | caraway | seed | P | Δ | Δ | | |
| *Daucus carota* subsp. *sativus* (Hoffm.) Arcangeli | carrot | root, peel, leaf | | Δ | | | |
| **Asteraceae** | | | | | | | |
| *Achillea millefolium* L. | yarrow | herb | P | Δ | Δ | | |
| *Matricaria matricarioides* (Less.) Porter | pineapple weed | herb | P | Δ | Δ | | |
| *Matricaria recutita* L. | camomile | flower | P | Δ | Δ | Δ | |
| *Solidago virgaurea* L. | goldenrod | herb | P | Δ | Δ | | |
| *Tanacetum vulgare* L. | tansy | herb | P | Δ | Δ | Δ | |
| *Tripleurospermum inodorum* Schultz Bip. | | herb | P | Δ | Δ | | |
| **Betulaceae** | | | | | | | |
| *Betula pendulas* Roth | silver birch | leaf, phloem, bark | | Δ | | | |
| *Betula pubescens* Ehrh. | white birch | leaf | | Δ | | Δ | |
| **Brassicaceae** | | | | | | | |
| *Armoracia rusticana* P. Haertner, B. Meyer & Scherb. | horseradish | root | P | Δ | Δ | | |
| *Brassica napus* var. *napobrassica* (L.) Reichenb. | swede | peel of tuber | | Δ | | | |
| **Cannabaceae** | | | | | | | |
| *Humulus lupulus* L. | hop | strobulus | P | Δ | Δ | | |
| **Caprifoliaceae** | | | | | | | |
| *Viburnum opulus* L. | guelder rose | berry | P | | Δ | | |
| **Chenopodiaceae** | | | | | | | |
| *Beta vulgaris* L. var. *altissima* | sugar beet | peel of tuber | | Δ | | | |
| *Beta vulgaris* L. var. *rubra* | red beet | peel of tuber | | Δ | Δ | Δ | |
| **Cichoriaceae** | | | | | | | |
| *Sonchus arvensis* L. | field milk thistle | herb | P | Δ | Δ | | |
| *Taraxacum officinale s.l.* L. | dandelion | root | P | | Δ | | |
| **Cucurbitaceae** | | | | | | | |
| *Cucumis sativus* L. | cucumber | leaf | | Δ | | | |
| **Dipsacaceae** | | | | | | | |
| *Succisa pratensis* Moench | Devil's bit scabious | herb | P | Δ | Δ | | |
| **Elaeagnaceae** | | | | | | | |
| *Hippophaë rhamnoides* L. | sea buckthorn | leaf | P | | Δ | | |
| **Empetraceae** | | | | | | | |
| *Empetrum nigrum* L. | crowberry | berry | | Δ | Δ | Δ | |
| **Ericaceae** | | | | | | | |
| *Andromeda polifolia* L. | bog rosemary | herb | P | Δ | Δ | Δ | |
| *Calluna vulgaris* (L.) Hull | heather | herb | P | Δ | Δ | Δ | |
| *Vaccinium myrtillus* L. | bilberry | berry | | Δ | Δ | Δ | |
| *Vaccinium oxycoccos* L. | cranberry | berry | | Δ | Δ | Δ | |
| *Vaccinium uliginosum* L. | bog bilberry | berry | | Δ | Δ | Δ | |
| *Vaccinium vitis-idaea* L. | cowberry | berry | | Δ | Δ | Δ | |
| **Fabaceae** | | | | | | | |
| *Lupinus angustifolius* L. | blue lupin | herb | P | Δ | Δ | | |
| *Pisum sativum* L. | pea | legume | | Δ | | | |
| *Trifolium hybridum* L. | alsike clover | herb | P | Δ | Δ | | |
| *Trifolium pratense* L. | red clover | herb | P | Δ | Δ | | |
| *Trifolium repens* L. | white clover | herb | P | Δ | Δ | | |
| **Grossulariaceae** | | | | | | | |
| *Ribes uva-crispa* L. | gooseberry | berry | | Δ | | | |
| *Ribes nigrum* L. | blackcurrant | berry | | Δ | Δ | Δ | |

**Table 6** Plants investigated in studies **I–IV.** (cont.)

| **Latin name** | **English name** | **Plant parts investigated** | **C&E** | **I** | **II** | **III** | **IV** |
|---|---|---|---|---|---|---|---|
| *Ribes* Δ*pallidum* Otto & Dietr. | red currant | berry | | Δ | | | |
| **Lamiaceae** | | | | | | | |
| *Hyssopus officinalis* L. | hyssop | herb | P | | Δ | | |
| *Thymus vulgaris* L. | thyme | herb | P | Δ | Δ | Δ | |
| **Liliaceae** | | | | | | | |
| *Allium cepa* L. | onion | bulb | P | Δ | Δ | Δ | |
| *Allium cepa* var. *rubra* L. | red onion | bulb | | Δ | | | |
| **Linaceae** | | | | | | | |
| *Linum usitatissimum* L. | flax | seed | | Δ | | | |
| **Lythraceae** | | | | | | | |
| *Lythrum salicaria* L. | purple loosestrife | herb | P | Δ | Δ | Δ | Δ |
| **Onagraceae** | | | | | | | |
| *Epilobium angustifolium* L. | willow herb | herb | P | Δ | Δ | Δ | |
| **Pinaceae** | | | | | | | |
| *Picea abies* L. (Karsten) | Norway spruce | needle | | Δ | Δ | Δ | |
| *Pinus sylvestris* L. | Scot's pine | phloem | | Δ | Δ | Δ | |
| *Pinus sylvestris* L. | Scot's pine | needle, bark | | Δ | | | |
| **Poaceae** | | | | | | | |
| *Avena sativa* L. | oat | seed | | Δ | | | |
| *Avena sativa* L. | oat | seed (bran) | | Δ | Δ | Δ | |
| *Hordeum vulgare* L. | barley | seed | | Δ | | | |
| *Phragmites australis* (Cav.) Trin. ex Steudel | lake reed | leaf, stalk | | Δ | | | |
| *Secale cereale* L. | rye | seed (bran) | | Δ | Δ | Δ | |
| *Secale cereale* L. | rye | flour | | Δ | | | |
| *Triticum aestivum* L. | wheat | seed (bran) | | Δ | | | |
| **Polygonaceae** | | | | | | | |
| *Fallopia convolvulus* (L.) Á. Löve | black bindweed | herb | P | Δ | Δ | | |
| *Rumex acetosa* L. | sorrel | herb | P | Δ | Δ | | |
| **Primulaceae** | | | | | | | |
| *Lysimachia vulgaris* L. | yellow loosestrife | herb | P | Δ | Δ | | |
| **Ranunculaceae** | | | | | | | |
| *Ranunculus repens* L. | creeping buttercup | herb | P | Δ | Δ | | |
| **Rosaceae** | | | | | | | |
| *Aronia melanocarpa* (Michx.) Elliot | black chokeberry | berry | | Δ | Δ | Δ | |
| *Filipendula ulmaria* (L.) Maxim. | meadowsweet | herb | P | Δ | Δ | Δ | |
| *Fragaria* Δ*ananassa* Duchesne | strawberry | berry | | Δ | | | |
| *Malus pumila* Mill. | apple | fruit | | Δ | Δ | Δ | |
| *Potentilla anserina* L. | silverweed | herb | P | Δ | Δ | | |
| *Prunus domestica* subsp. *insititia* (L.) C.K. Schneider | damson | fruit | P | Δ | Δ | | |
| *Rosa rugosa* Thunb. | rose | fruit, seed | P | Δ | Δ | | |
| *Rubus chamaemorus* L. | cloudberry | berry | | Δ | Δ | Δ | |
| *Rubus chamaemorus* L. | cloudberry | leaf | P | Δ | Δ | Δ | |
| *Rubus idaeus* L. | raspberry | berry | | Δ | Δ | Δ | |
| *Sorbus aucuparia* L. | rowanberry | berry | | Δ | Δ | Δ | |
| **Salicaceae** | | | | | | | |
| *Populus tremula* L. | aspen | leaf, bark | | Δ | | | |
| *Salix alba* L. | silverwillow | leaf, bark | | Δ | | | |
| *Salix caprea* L. | goat willow | leaf | | Δ | | | |
| *Salix caprea* L. | goat willow | bark | | Δ | Δ | Δ | |
| **Solanaceae** | | | | | | | |
| *Lycopersicon esculentum* Miller | tomato | fruit | | Δ | | | |
| *Solanum tuberosum* L. | potato | tuber (peel) | | Δ | Δ | Δ | |
| **Typhaceae incl. Sparganiaceae** | | | | | | | |
| *Typha latifolia* L. | bulrush | leaf, stalk | | Δ | | | |

### 4.1.3. Instrumentation

The instrumentation used in the respective studies was:

Perkin-Elmer Lambda15 UV-vis spectrophotometer (Norwalk, CT, USA) (**I**).
1214 Rackbeta liquid scintillation counter (Wallac, Turku, Finland) (**II**).

Perkin Elmer Series 200 LC Pump and Autosampler (Norwalk, CT, USA), Perkin Elmer LC 235C Diode Array Detector at a scanning wavelength of 195–365 nm (Norwalk, CT, USA), and PE Nelson 600 Series Link (Norwalk, CT, USA) (**II**, **IV**, **V**).

Waters M-6000A Chromatography pump (Waters Inc., Milford, MA, USA) (**IV**).
Kontron T-414 LC pump (Tegimenta AG, Rotkreuz, Switzerland) (**IV**).

Hewlett-Packard Series 1100 Pump, Column thermostat and Autosampler (Hewlett Packard GmbH, Waldbronn, Germany), Series 1100 Degasser and Diode array detector at a scanning wavelength of 190–500 nm (Hewlett Packard, Japan) connected to a Finnigan MAT LCQ ion trap mass spectrometer (San Jose, CA, USA) and APCI interface (**IV**).

HPLC/UV system consisting of two Perkin-Elmer Series 200 micro LC pumps (Perkin-Elmer, Norwalk, CT, USA), Series 200 autosampler (Perkin-Elmer, Norwalk, CT, USA) and a 785A UV/Vis detector (Perkin-Elmer, Norwalk, CT, USA) connected to a PE Sciex API 365 triple quadrupole mass specrometer (Sciex, Toronto, Canada) equipped with a Turbo Ion Spray interface (**IV**).

HPLC/UV system consisting of two Perkin-Elmer Series 200 micro LC pumps, Perkin Elmer Series 200 Autosampler (Norwalk, CT, USA), an effluent flow splitter (Acurate, LC Packings, Zürich, Switzerland) connected to a PE Sciex API 300 LC/MS/MS triple quadrupole mass spectrometer (Sciex, Concord, ON, Canada) equipped with an Ion Spray (IS), Heated Nebulizer (APCI), and Atmospheric Pressure Photoionisation (APPI) interfaces. A Rheodyne 7725 injector (Rheodyne, Cotati, CA, USA) and a Harvard Appratus micro syringe pump (Harvard Apparatus, So.Natick, MA, USA) were also used in connection with the MS with IS and APPI (**V**).

### 4.1.4. Columns

The columns used for extraction, fractionation, and analyses in the respective studies were:

LiChrosorb Hypersil ODS (5 µm), 250 mm Δ 4 mm i.d. (Merck, Darmstadt, Germany) (**II**).
Symmetry C18 (4 µm), 250 mm Δ 4.6 mm i.d. (Waters Associates, Milford, MA, USA) equipped with a Guard-Pak C18 (Waters) pre-column (**IV**).
Superspher 100 RP-18 (4µm), 75 mm Δ 4 mm i.d. (Merck, Darmstadt, Germany) (**IV**).
Prodigy ODS (3) (3 µm), 100 mm Δ 4.6 mm i.d. (Phenomenex, Torrance, CA, USA) (**IV**, **V**)
Büchi 17980 (460 mm Δ 49 mm i.d., Büchi Laboratoriums-Technik AG, Flawil, Switzerland) (**IV**).

Büchi 17982 (460 mm Δ 26 mm i.d., Büchi Laboratoriums-Technik AG, Flawil, Switzerland) packed with 145 g of LiChrospher RP-18 (15–25 µm, Merck, Darmstadt, Germany) (**IV**).
Open chromatography column (700 mm Δ 35 mm i.d.) packed with Sephadex LH-20 (Pharmacia LKB Biotechnology AB, Uppsala,Sweden) yielding a gel layer of 600 ml in methanol (**IV**).

### 4.1.5. Computer programs

The programs used for HPLC and MPLC method optimisation were DryLab for PE Nelson (LC Resources, Walnut Creek, CA, USA) (**IV**) and Turbo Method Development 1.0 (Perkin-Elmer, Norwalk, CT, USA) (**V**). The $pK_a$ values of analytes were calculated using ACD/ILab version 4.0 (ACD Labs, Toronto, ON, Canada) (**V**), and the statistical significance of the results tested using Systat 6.0.1. (SPSS Inc., Chicago, IL, USA) (**I**, **II**).

## 4.2. Methods

### 4.2.1. Assay for total phenolic content (**I**)

The work was carried out at the Division of Food Chemistry (Department of Applied Chemistry and Microbiology, University of Helsinki). The amount of total phenolics in 92 plant extracts was determined according to the Folin-Ciocalteu procedure, based on complex formation of molybdenum-tungsten blue (SINGLETON and ROSSI 1965). The samples were allowed to react with Folin-Ciocalteu's reagent and sodium carbonate solution. Absorption at 765 nm was measured and the total phenolic content calculated as gallic acid equivalents (GAE).

### 4.2.2. Assay for antioxidant activity (**I**)

The work was carried out at the Division of Food Chemistry (Department of Applied Chemistry and Microbiology, University of Helsinki). Antioxidant testing of the 92 extracts was carried out by oxidising methyl linoleate (MeLo) in the presence of the extracts according to the method used earlier for samples rich in phenolic compounds (HEINONEN et al. 1998b). The extracts were tested at a concentration of 500 ppm (based on the dry weight of the extract) as well as at a level representing 5000 ppm of original plant material using ζ-tocopherol (20 ppm) as a reference antioxidant. The oxidation of MeLo was carried out in the dark at 40°C for 72 hours. The sample aliquot was diluted in isooctane, and the conjugated diene absorption of the sample was measured spectrophotometrically at 234 nm. The amount of hydroperoxides was calculated using absoptivity of 26 000 (CHAN and LEVETT 1977), and the antioxidant activity was expressed as percentual inhibition of formation of MeLo hydroperoxides compared to the absorption value of control sample not containing an antioxidant at the 72-h time point.

### 4.2.3. Assay for calcium-antagonistic activity (**II**)

The calcium antagonistic activity of 51 extracts was investigated as the inhibition of depolarisation-induced $^{45}Ca^{2+}$ uptake accomplished by potassium in rat pituitary $GH_4C_1$ cells using the method described in detail by Törnquist and Tashjian (1989). The dry extracts were dissolved in DMSO at a concentration of 20 mg/ml.

The preliminary tests were made on Petri dishes at concentrations of 0.4, 4 and 40 µg/ml, and the final study at a concentration of 20 µg/ml. The cell-associated radioactivity was measured by liquid scintillation counting, and the result was calculated in comparison to the value of the blank sample, verapamil acting as the positive control.

### 4.2.4. Assays for antimicrobial activity (**III**)

Antimicrobial screening of 13 phenolic substances and 29 plant extracts was conducted using agar-diffusion methods (OJALA et al. 2000) with four to nine microbial species and a sample size of 0.5 mg of dry extract. The extracts were dissolved in methanol in a concentration of 1 mg/ml. The pure compounds were tested using the hole-plate diffusion method, and the extracts using the cylinder diffusion method. The bacterial plates were incubated at 23°C for 1 h to facilitate diffusion, and then incubated at 35°C for 24 h. The antifungal test plates were refrigerated at 8°C for 1 h and then incubated at 25°C for 72 h, except for *Aspergillus niger* which was incubated at 25°C for 6 days. The effect was evaluated by measuring the diameter of the inhibitory zones.

### 4.2.5. Study on the phenolic composition of *Lythrum salicaria* (**IV**)

The crude extract of *Lythrum salicaria* L. was fractionated using an RP MPLC column into five fractions, and using an open column gel chromatography system yielding nine fractions. All the samples were analysed using an HPLC/DAD-UV method. The HPLC/APCI-MS$^n$ method in positive ion mode was used to elucidate the flavonoid glycosides present, and the HPLC/ESI-MS method in negative ion mode to assess the composition of tannins and phenolic acids.

### 4.2.6. LC/MS study on flavonoids (**V**)

The mass spectrometric behaviour of five flavonoid standards, (+)-catechin, isorhamnetin, vitexin, isoquercitrin and luteolin-3’,7-diglucoside, in different HPLC eluent conditions was studied using an LC/MS instrument equipped with three different API interfaces: IS, APCI and APPI. The analysis conditions were optimised in both ionisation modes for the IS and APCI interfaces using HPLC runs, and for the APPI interface using direct sample inlet analysis. The limits of detection were determined for each method, and comparisons made between the optimised conditions.

# 5. RESULTS AND DISCUSSION

## 5.1. Total phenolic content and antioxidative activity (I)

Potential sources of antioxidant compounds have been searched from several types of plant material (RAMARATHNAM et al. 1997), and using a number of different methods. Flavonoids and other plant phenolics are especially common in leaves, flowering tissues and woody parts such as the stem and bark (LARSON 1988). The antioxidant activity of phenolics is mainly due to their redox properties, which allow them to act as reducing agents, hydrogen donors, and singlet oxygen quenchers (RICE-EVANS et al. 1995). The purpose of this study was to screen the total phenolic content and antioxidant activity of a large number of extracts made from plants of Finnish origin. The herbs and medicinal plants were the materials of interest.

The Folin-Ciocalteu colorimetric assay applied in this study is a simple method and requires few reagents, thus being suitable for crude estimation of the content of total phenols, even though it is limited by the low specificity toward polyphenols, and relies on the use of a standard compound (MOSCA et al. 2000). The highest total phenolic contents occurred in the extracts of purple loosestrife (*Lythrum salicaria* L.), heather (*Calluna vulgaris* L.), bog rosemary (*Andromeda polifolia* L.), and willow herb (*Epilobium angustifolium* L.) (**Table 7**).

**Table 7** Ten highest contents of total phenolics in the sample group of herbs and medicinal plants (**I**). GAE = gallic acid equivalents, % In = inhibition of methyl linoleate oxidation at 5000 ppm calculated using the dry weight of the original sample.

| Plant | GAE | % In |
|---|---|---|
| *Lythrum salicaria* | 42.1∂0.9 | 41 |
| *Calluna vulgaris* | 36.0∂1.2 | 93 |
| *Andromeda polifolia* | 32.8∂1.1 | 98 |
| *Epilobium angustifolium* | 32.2∂0.9 | 88 |
| *Filipendula ulmaria* | 26.8∂1.3 | 87 |
| *Humulus lupulus* | 23.1∂0.8 | -24 |
| *Rubus chamaemorus* | 17.2∂0.5 | 97 |
| *Thymus vulgaris* | 17.1∂0.2 | 97 |
| *Succisa pratensis* | 16.4∂0.4 | 10 |
| *Tanacetum vulgare* | 14.2∂0.6 | -12 |

Most of the examined plant extracts showed weak or moderate antioxidative activity. However, there were some herbs and medicinal plants possessing a strong antioxidant response (**I**, Table 1). In accordance with the dry weight of the extract, the inhibition of formation of methyl linoleate hydroperoxides by these plant extracts decreased in the order *Andromeda polifolia* = *Thymus vulgaris* > *Calluna vulgaris* = *Filipendula ulmaria* > leaf of *Rubus chamaemorus* > *Epilobium angustifolium.* The strong antioxidant activity of thyme (*Thymus vulgaris* L.) was expected on the basis of the literature (TAKÁCSOVÁ et al. 1995, NAKATANI 1997, HIRASA and TAKEMASA 1998). Thymol and carvacrol are major aroma components of the essential oil of thyme, and both show high antioxidant and antimicrobial activity. Biphenyl compounds, dimerisation products of thymol and carvacrol, and a flavonoid (eriodictyol), have also been isolated as efficient antioxidants inhibiting superoxide anion production in the xanthine/xanthine oxidase system and mitochondrial and microsomal lipid peroxidation (HARAGUCHI et al. 1996). Highly methylated flavonoids with antioxidant activity in the linoleic acid oxidation system have been found in the less polar fraction (MIURA and NAKATANI 1989). Bog rosemary, heather, meadowsweet (*Filipendula ulmaria* (L.) Maxim.) and willow herb, the most active medicinal plants in this study, all accumulate large amounts of phenolic compounds. Quantitative data on the phenolics in these medicinal plants are not available, but the main consitutents are suggested to be flavonol aglycones and glycosides, as shown in **Appendix 1** (PACHALY and KLEIN 1987, ALLAIS et al. 1991, ALLAIS et al. 1995, LAMAISON et al. 1992, DUCREY et al. 1995, HIERMANN 1995).

Camomile (*Matricaria recutita* L.) and hop (*Humulus lupulus* L.) were practically inactive, although their total phenol contents were rather high (12.7 and 23.1 mg/g GAE). Hop even showed moderate pro-oxidant activity in MeLo. Xanthohumol, a prenylated chalcone, is the principal flavonoid in hop, constituting 80–90% of the total flavonoids. Minor amounts of other prenylchalcones and prenylnaringenins have also been reported (STEVENS et al. 1999). Xanthohumol has high antioxidant activity, but obviously the pro-oxidative effects of nonprenylated chalcones and flavanones compensate this action (MIRANDA et al. 2000). The present results thus do not suggest that the crude extract of hop would have significant antioxidant activity.

The results were tested using a linear correlation coefficient, where the null hypothesis $H_0$ was $\psi=0$, and the correlation coefficient an esimate of the normal distribution parameter $\psi$ (LENTNER 1982). The content of total phenolics and the antioxidant activity, calculated using the dry weight of the original samples, correlated significantly with each other. This was the case when the correlation was determined for the entire material ($r=0.553$, $p<0.001$), and for the herbs and medicinal plants ($r=0.495$, $p<0.01$). In contrast, the dry weight of the extract did not correlate with total phenolics in the respective subgroup, but it did in the whole material ($r=0.214$, $p<0.05$).

The results obtained in the determination of total phenolics and the test of antioxidative activity were used as a basis for selecting the plant material used in further studies on the biological activity.

### 5.2. Effects on calcium fluxes (II)

The rationale for using the thoroughly characterised $GH_4C_1$ cells is that they posses to a marked extent the same L-type VOCCs as cardiovascular muscles (WAGNER et al. 1993, KATZ 1997), the channels of which can therefore be blocked with the clinically used calcium antagonists. A study carried out on flavonoids and other phenolics showed that the different groups of phenolic compounds have different inhibitory properties on calcium fluxes as a result of variations in their structural details (SUMMANEN et al. 2001). For instance, flavones clearly inhibit the $Ca^{2+}$ intake of the cells, while flavonols may even act as activators of $Ca^{2+}$ entry. A conjugated sugar makes the effect of pure aglycone considerably weaker. Similarly, the plant extracts had both calcium flux-inhibiting and -increasing effects (**II**, Table 1). Extracts that effectively inhibited $Ca^{2+}$ fluxes into the cells were hop (*Humulus lupulus* L., 94.7 % inhibition), willow herb (*Epilobium angustifolium* L., 36.9 %), purple loosestrife (*Lythrum salicaria* L., 36.3 %), cloudberry leaves (*Rubus chamaemorus* L., 35.7 %), and yellow loosestrife (*Lysimachia vulgaris* L., 33.8 %). According to the paired Student's t-test between the values for the extracts and blank samples in the same cell batch, these inhibitions were statistically significant.

The marked inhibitory effect of hop can be explained on the basis of its high content of prenylflavonoids (STEVENS et al. 1997). The effect of pure standards of these compounds on calcium fluxes in clonal rat pituitary $GH_4C_1$ cells has not been tested so far. However, Summanen *et al.* (2001) showed that the length of the side chain contributes to some extent to the degree of calcium activity. The homologic series of gallic acid derivatives has correlation between the side chain length and calcium-antagonistic activity. Based on the results obtained with pure flavonoid standards, willow herb extract, which contains flavonols and their glycosides (DUCREY et al. 1995), should increase calcium fluxes into the cells. However, it has in fact the opposite effect. The same phenomenon occurred with the flavonol-containing extract of yellow loosestrife that has been used for the treatment of high blood pressure in Chinese folk medicine (YASUKAWA and TAKIDO 1988). However, purple loosestrife, which contains mostly *C*-glycosylflavones (MIHAJLOVIC 1988), has the expected inhibiting effect on calcium fluxes. This mechanism could also be one possible mode of action of the commercially available antidiarrhoeal preparation of the plant, Salicairine™ (BRUN et al. 1998).

Extracts which effectively promoted $Ca^{2+}$ fluxes into the cells were onion (*Allium cepa* L., 31.2 %), caraway (*Carum carvi* L., 26.8 %), apple (*Malus pumila* L., 18.2 %) and thyme (*Thymus vulgaris* L., 18.0 %), all of which were statistically significant. Onion and apple have been shown to be important sources of flavonoids in the diet of Western Europeans (HERTOG et al. 1992b). As they contain considerable amounts of flavonols, it is not surprising that their extracts increased calcium fluxes into animal cells. Despite the fact that the extraction method was intended to favour phenolics, the extracts of thyme and caraway most probably contained small amounts of essential oils. The flavonoids of thyme, most of which are flavones, have been shown to inhibit $Ca^{2+}$ mediated contractions in $K^+$ depolarised smooth muscles of the guinea-pig ileum and trachea and of the rat *vas deferens* (van den BROUCKE and LEMLI 1983). However, our experiments showed a clear increase in calcium intake in the cells. This could be partly due to the essential oils (KOSHITA and OBA 1989) present in these plants (PANIZZI et al. 1993, GALAMBOSI and PEURA 1996).

Most of the representatives of the family *Asteraceae* seemed to inhibit $Ca^{2+}$ fluxes, while in the families *Ericaceae*, *Fabaceae* and *Rosaceae* the situation was relatively unclear (**II**, Table 1.). No correlation was found between $Ca^{2+}$ channel activity and the content of total phenols in the extracts studied in **I**. Based on these findings, it is clear that the effect of an extract is always the sum of the inhibiting and activating components with respect to their individual effectivities and concentrations in the extract.

## 5.3. Antimicrobial activity (III)

Diffusion methods are extensively used to investigate the antibacterial activity of natural substances and plant extracts. In these methods, the agar gel is treated with an appropriate microbial suspension, and the antimicrobial activity of compounds that penetrate into the agar by diffusion is measured. The assays are based on the use of discs or holes as reservoirs containing the solutions of substances to be examined (BRANTNER 1994). In the case of solutions with a low activity, however, a larger concentration or volume is needed. The limited capacity of discs means that holes or cylinders are preferably used when investigating plant extracts.

All of the microbial strains used were non-invasive species of their genera and thus applicable for analytical work, but as opportunists they can all also cause infections. *Escherichia coli* and *Staphylococcus aureus* are common bacteria causing food-poisoning (MADIGAN et al. 2000), and in addition to the food spoiling property of *Aspergillus niger*, this fungus and *Candida albicans* are the most usual fungi responsible for clinical infections (BODEY et al. 1992). The sense of *S. epidermidis*, *Bacillus subtilis*, *Micrococcus luteus*, *Pseudomonas aeruginosa*, and *Saccharomyces cerevisiae* in this study was essentially diagnostic. However there are some pharmaceutical challenges: there is no drug against *P. aeruginosa* (MADIGAN et al. 2000), *S. aureus* has resistent strains (TSUCHIYA et al. 1996), and *S. cerevisiae* has been reported to act as an important pathogen also in healthy people (PONTÓN et al. 2000).

In the study on pure substances, very clear differences were found between the effects of the individual phenolics (**III**, Tables 3 and 4). The growth of *Staphylococcus aureus* was inhibited very effectively in our study by flavone, flavonols and naringenin. The inhibition was stronger than that described by Mori et al. (1987). The results for flavonoids and *Staphylococcus epidermidis* are in agreement with those reported in earlier studies (NISHINO et al. 1987). Catechins, +/- -catechin and (+)-catechin, were inactive against *S. epidermidis*. Categoristically, flavonoid glycosides failed to show any activity against the microbial strains investigated. This is supported by the survey carried out by Waage and Hedin (1985), in which only low effectivity was achieved using different glycosides of quercetin. No other clear structure-activity relationship was found. The pure nonhydroxylated flavone slowed the growth of the yeasts, but the addition of hydroxylic groups decreased its activity against *Saccharomyces cerevisiae* and *Candida albicans*.

Plant extracts generally contain flavonoids in glycosidic form. This may be the reason why the plant extracts did not produce as marked inhibition as many of the pure compounds did (**III**, Table 5). Furthermore, the concentrations used were not as high as those reported in some references. The best results were obtained with herbal extracts of willow herb (*Epilobium angustifolium* L.), meadowsweet (*Filipendula ulmaria* (L.) Maxim.) and purple loosestrife (*Lythrum salicaria* L.). Willow herb and meadowsweet had a bactericidical effect on the growth of the bacteria, *Staphylococcus aureus* and *Eschericia coli*. This is in agreement with the literature (MOSKALENKO 1986, KÄLLMAN 1994), but the effect may be due to compounds other than flavonoids: willow herb contains a wide variety of flavonoids but they are in the form of glycosides (DUCREY et al. 1995). The antibacterial effects of meadowsweet have been attributed to salicylic acid. This is not an impossible assumption, because the extract of goat willow (*Salix caprea*), which also contains salicylic acid, was slightly active against the bacteria. Purple loosestrife has not earlier been tested against yeasts, but in this study it showed fungicidic activity against *Candida albicans*. The plant contains *C*-

glycosides of flavonoids and tannins (PARIS 1967, MIHAJLOVIC 1988), but its effectivity is presumably not due to these compounds. Even though tannins are known to have good antimicrobial activity, paradoxically some organisms, *e.g. Candida sp.*, are capable of using tannins as a source of carbon (SCALBERT 1991, IROBI et al. 1994).

The growth of the gram-positive bacterium, *S. aureus*, was strongly inhibited by extracts of white birch (*Betula pubescens* Ehrh.) and pine (*Pinus sylvestris* L.). The same plant part of pine has earlier given similar results (MOSKALENKO 1986), but leaves from the *Betula* genus have not been reported to have any significant antimicrobial activity (MOSKALENKO 1986, KLINGER et al. 1989). Neither has a potato-peel extract (*Solanum tuberosum* L.) earlier been reported to have antimicrobial activity. However, bacterisidical activity against *S. aureus* was found in these tests. Samples originating from tree and cereal material or vegetable waste all had some activity against the bacteria. However, almost all of them failed to inhibit the growth of yeast and fungus.

Of the berry and fruit extracts, the widest bacterisidical activity was expressed by berries belonging to the genus *Rubus*, as was recently reported by Puupponen-Pimiä et al. (2001). Cloudberry (*Rubus chamaemorus* L.) and raspberry (*Rubus idaeus* L.) especially inhibited the growth of *Bacillus subtilis* and *Micrococcus luteus*, but they had only a slight effect on the other bacterial strains studied. The difference between the inhibition of cloudberry against *S. epidermidis* and *S. aureus* shows that it has no selective effect on the genus *Staphylococci*. The reason for the activity of the *Rubus* genus may be due to either the main flavonol, quercetin, or to their exceptionally high content of ellagic acid (HÄKKINEN et al. 1999). The antimicrobial active compounds in crowberry have been shown to be bibenzyls and a phenantrene derivative (MATSUURA et al. 1995). In this study, the extract of crowberry (*Empetrum nigrum* L.) was active against all the bacteria. However, no significant activity was noted against eucaryotic species. None of the berry extracts inhibited the growth of the food-poison mold, *Aspergillus niger,* and the effect of raspberry and bilberry (*Vaccinium myrtillus* L.) against the yeast, *Candida albicans*, was only moderate.

### 5.4. Polyphenols of *Lythrum salicaria* L. (IV)

*Lythrum salicaria* L. had the highest content of total phenolics among the herbal samples studied in this work (**I**). Its extract, which also had some antioxidant potential (**I**), was found to possess marked calcium channel activity (**II**), and to show inhibition against *E. coli* and *C. albicans* in the antimicrobial tests (**III**). *L. salicaria* has been the subject of a number of phytochemical studies during the 1960's and 1970's. The phytochemical information published in the literature was collated in this study (**IV**, Table I), and partly confirmed using hyphenated HPLC techniques.

The crude extract was fractionated in two different ways in order to characterise and localise the different constituents of the plant precisely in the HPLC chromatogram. Based on the UV spectra obtained from the HPLC/DAD-UV chromatograms (**IV**, Method A), four peaks were classified as flavone-*C*-glycosides (RATH et al. 1995), and several compounds with short retention times as ellagitannins (SALMINEN et al. 1999). Positive ion HPLC/APCI-MS$^n$ analyses using a broad ACN-water gradient were carried out to determine the nature of the flavone-*C*-glycosides (**IV**, Method B). Peaks producing ions *m/z* 449 and 433,

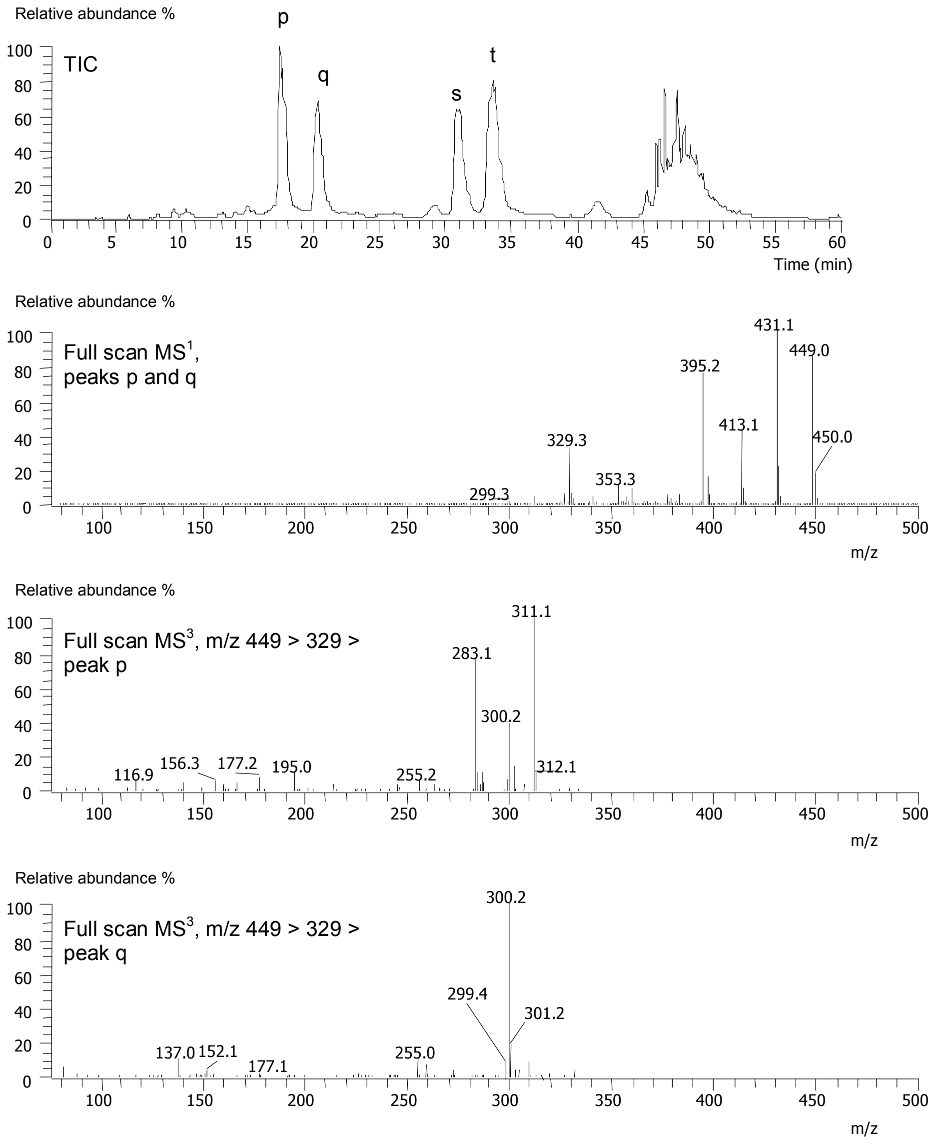


**Figure 5** Total ion chromatogram (TIC) for MPLC fraction 3 (R3) detected at *m/z* 75–800, MS$^1$ of isoorientin (p) and orientin (q), MS$^3$ of isoorientin, and MS$^3$ of orientin (**IV**).

corresponding to the values expected for orientin and vitexin and their isomers, were isolated in the ion trap mass analyser, selectively excited, and their fragments analysed (MS$^2$). This step was repeated on the fragments in order to generate MS$^3$ spectra. It has been shown that, under these conditions, a clear distinction can be made between isomeric pairs of *C*-glycosyl flavones (RATH et al. 1995). The spectra of isomers produced in this manner are presented in **Figure 5**. The identities of the *C*-glycosyl flavones were determined (**IV**, Fig. 2), and were in agreement with the literature (PARIS 1967, MIHAJLOVIC 1988).

The negative ion HPLC/ESI-MS method (**IV**, C) and the data of reference material (SALMINEN et al. 2001) were used to assess the composition of the tannins and phenolic acids. Molecular weight assignment was based on the strong $[M-H]^-$ ion obtained, as well as on the presence of respective $[M-2H]^{2-}$ and $[2M-H]^-$ ions. In this mode, almost no fragmentation was observed, except for the peaks *m/z* 169 and 301 (deprotonated gallic acid and ellagic acid, respectively), and the peaks $[M-H-170]^-$ and $[M-H-302]^-$, which were successfully utilised in identification. As a result, vescalagin, pedunculagin, valoneic acid dilactone, 1,6-di-*O*-galloylglucose, 1-*O*-galloylglucose and 6-*O*-galloylglucose were identified from *Lythrum salicaria* for the first time (**IV**, Table I), and the detection of six other phenolics confirmed the information reported in the literature. Only partial structure assignment was performed, based on LC/MS data, for the other phenolic compounds reported in the study. Information on the nature of the sugars and linkage positions of galloyl or HHDP could not be ascertained using the respective methods and reference material available. A more comprehensive structural investigation would require the isolation of all the compounds of interest. No evidence of lythrines (MA et al. 1996) was detected in our plant material.

The noted calcium antagonistic and antimicrobial activities of *L. salicaria*, combined with the phytochemical information obtained, create a basis for further investigations concerning the substances responsible for the effects. Based on the available evidence, flavone-*C*-glycosides can still be considered as one potential group of calcium channel active compounds, especially if the effects of compounds closely related to vitexin can be shown to be parallel; the simultaneous presence of vitexin, isovitexin, orientin and isoorientin is still an exceptional phytochemical situation among the plants investigated (**Appendix 1**). These findings could co-explain, together with the astringent effect of the tannin-rich extract and the optionally beneficial microbisidic effect of the extract, the antidiarrhoeal effects of Salicairine™.

### 5.5. Atmospheric pressure ionisation in the LC/MS of flavonoids (V)

Although a considerable amount of new information about the phenolic composition of *L. salicaria* was obtained in **IV**, the available methods were not sensitive enough to detect all the minor compounds. In order to enhance the applicability of LC/MS methods in future phytochemical studies, the mass spectrometric ionisation of flavonoids was studied and optimised in relation to the HPLC eluent composition, using three API-MS techniques and a mixture of flavonoid compounds found to be typical in the respective Finnish plants. The compounds in the test mixture (**V**, Figure 1) were selected such that each of the ions produced by the mixture was derived from only one compound. The eluent compositions were selected for this study on the basis of their suitability for LC/MS (**V**, Table 1).

Modern LC/MS methods are still inferior in their limits of detection compared to *e.g.* UV detectors, and therefore new, more sensitive MS techniques are constantly under development. The novel atmospheric pressure photoionisation (APPI) interface has recently been reported to express an enhanced ionisation effeciency for selected analytes compared to APCI (ROBB et al. 2000). In our study, APPI was used for the first time in the LC/MS analysis of flavonoids. The interface is based on the same configuration as APCI, but the discharge needle is displaced by a photoionisation lamp. Dopant liquid (*e.g.* toluene) is introduced into the vaporiser coaxially at a flow rate about 1/10th that of the effluent flow rate. The ionisation energy of the dopant must be lower than the energy of the photons (10 eV) emitted by the photoionisation lamp. The initial reaction in APPI is formation of a radical cation of the dopant, which may ionise the analyte through a charge exchange reaction if the recombination energy of the radical cation of the dopant (8.83 eV for toluene) is higher than that of the neutral analyte. Alternatively, the dopant radical cation initialises a process that produces protonated eluent molecules, followed by a proton transfer reaction with the analyte (ROBB et al. 2000).

The APPI-MS spectra of flavonoids showed no adduct formation in either positive ion or negative ion modes, and the only fragmentation, which was stronger in positive than in negative mode, indicated the loss of sugar moieties from the glycosidic analytes (**V**, Figure 6). The positive ion mode produced a protonated and the negative ion mode a deprotonated molecule, indicating that the main ionisation mechanism in APPI-MS is a gas-phase proton transfer reaction.

In order to study the effect of dopant, all the measurements were made with and without toluene as a dopant. The sensitivity was 10–100 times better with the dopant than without, verifying that the initial reaction in APPI is the formation of a radical cation of the dopant. Tetrahydrofuran (ionisation energy = 9.4 eV, NIST) was also tested as a dopant at a concentration of 10%. The results indicated that THF is suitable for LC/APPI-MS, but the sensitivity for flavonoids with toluene was 30–100% better than that with THF. According to this result, if tetrahydrofuran is used as an eluent at a concentration of 5–10%, an additional dopant would be unnecessary. For safety reasons, the PEEK capillaries should be substituted with stainless steel tubing, and the air used as nebulising gas replaced with nitrogen.

The effect of the solvent on the ionisation efficiency of flavonoids in APPI was studied with five different eluent compositions. The mixture of organic solvents selected utilising a computer-based optimisation program Turbo Method Development, methanol and acetonitrile (3:4), was kept the same and only the composition of the aqueous phase changed. The results of positive ion APPI show that the best sensitivity was obtained with pure water as the aqueous phase (**V**, Figure 2c and Table 1). The addition of ammonium acetate decreased the sensitivity slightly, and the addition of formic acid or ammonium hydroxide significantly. The protonated $CH_3CN$ is obviously the main reactant ion in the case of the eluent without a pH modificator, since the PA of $CH_3CN$ (779.2 kJ/mol) (NIST) is the highest among the components in the eluent and lower than that of flavonoids (ALEMÁN 2000). This means that proton transfer is exothermic and efficient, which explains the good sensitivity. The addition of ammonium acetate changes the composition of the reactant ions in the gas phase, with the result that the initially formed protonated methanol, acetonitrile and water transfer their protons to the weakest acid, *i.e.* ammonia (PA = 854 kJ/mol, NIST), and the role of ammonium ions in the ionisation process increases. Thus, the diminished exothermicity of the proton transfer reaction makes the ionisation less efficient. The decreased sensitivity with formic acid and ammonium hydroxide may be due to the formation of $[COO]^-$ and $[OH]^-$ gas phase ions, which neutralise positive charges.

In negative ion APPI, the highest responses for the aglycones were obtained with an organic solvent and pure water (**V**, Figure 3c). The addition of ammonium acetate decreased the sensitivity, while significantly higher responses were obtained for glycosides with ammonium hydroxide than without. This may indicate the deprotonation of hydroxide groups in the glycoside moiety by hydroxide ions under basic conditions. However, with basic eluents the flavonoids are ionised and the retention on the reversed-phase C18 column is poor, leading to significantly decreased resolution in LC. The decreased sensitivity under acidic conditions may be due to neutralisation of the negative charges by hydronium ions.

Additional LC/APPI-MS runs were carried out in both ion modes using 5, 10 and 20 mM ammonium acetate buffers, and an increase in sensitivity occurred as the buffer concentration decreased. It was therefore concluded that, in order to compromise between chromatographic behaviour and sensitivity, aqueous ammonium acetate at a concentration of 5 mM (pH 6.7) represented the most suitable eluent system.

To optimise the ionisation conditions of older API techniques, HPLC/MS runs of the test mixture were performed using IS and APCI interfaces and 7–9 eluent compositions. The best conditions were achieved in positive ion IS and APCI using 0.4% formic acid as a buffer, and in negative ion IS and APCI using ammonium acetate buffer adjusted to pH 4.0 (**V**, Table 1 and Figures 2 and 3).

According to the results, with IS in acidic conditions the ionisation mechanism of flavonoids is not the ion evaporation process, but protonation and deprotonation occur at the liquid-gas inteface of the droplet, *i.e.* in the incipient gas phase, or by proton transfer reaction in the gas phase. The ionisation efficiency is dependent on the PA of the flavonoid. Compared to the respective main reactant molecules, this means that in positive ion mode the PAs of flavonoids are high enough for efficient protonation, and in negative ion mode the PAs of flavonoids compared to reactant anions are low enough for efficient deprotonation. Thus the proton transfer reaction is exothermic and efficient with all the eluents studied. These reactions are also the valid ionisation mechanism of flavonoids in analyses carried out using the APCI interface.

The six LC/MS methods were compared with respect to detection sensitivity (S/N ratio of 3) and their applicability for the analysis of flavonoids with optimised eluent compositions (**V**, Figure 5 and Tables 3 and 4). Since the selected conditions represent a compromise between several factors, such as chromatographic behaviour, individual properties of the analytes, and instrumental settings, broad generalisations must be avoided. Data obtained from extracted ion chromatograms (XIC) show that there are no significant differences in ionisation efficiencies between the positive and negative ion modes (**V**, Table 4). However, the detection limits in the total ion chromatogram (TIC) were about 2–9 times lower in negative ion modes than in positive ion modes, irrespective of the ionisation technique tested, which shows that the chemical background noise in positive ion mode is stronger than that in negative ion mode.

The limits of detection determined from the TICs and XICs in positive ion mode do not differ essentially between the techniques, but in negative ion mode the TIC and XIC limits of glycosidic analytes are clearly lower using IS than APCI or APPI. Although none of the optimised conditions can be ideal for all the analytes, and the results therefore cannot be generalised, the results show that negative ion IS with an eluent system containing acidic ammonium acetate buffer provides the best conditions for the detection of flavonoids in MS mode.

## 6. CONCLUSIONS

Finnish flora growing in the stress conditions set by the northern climate, provide an interesting source of plants for studying their chemical composition and its effects in biological systems and man. Several serviceable *in vitro* tests that illuminate the property of interest are available for screening plants and their constituents in order to find the most effective materials and components for further investigations. This is the main function of the *in vitro* tests, even if they cannot predict the effects *in vivo*.

The efficiency of antioxidants depends strongly on the oxidation conditions and lipid substrate, and thus the MeLo bulk oil method used in the current study gives only an approximation of the possibilities of an extract acting as an antioxidant. In the respective conditions, however, the amount of total phenolics determined well predicts the obvious antioxidative activity of the extract. The obtained results highlight several herbal extracts that possess strong antioxidant potential, including species such as *Andromeda polifolia*, *Thymus vulgaris*, *Filipendula ulmaria* and *Calluna vulgaris* and, lying behind them, *Lythrum salicaria* with its pronounced antioxidant activity.

Several plant species having marked effects on the calcium homeostasis of rat pituitary cells were identified in our experiments, the most effective plants being *Humulus lupulus*, *Epilobium angustifolium* and *Lythrum salicaria*. The basis of the effect could not be attributed to either the total phenolic content of the extract or to the family of the species. The first assumption is supported by the finding that no correlation was found either between the total phenol content and Ca-flux inhibition value, or between the total phenol content and the modulus values of the Ca-flux effect .

Many plant extracts showed antimicrobial activity in this study. Even though flavonoids inhibited the growth of bacteria, some more active phytoalexins present in the plant extracts may be responsible for the antibacterial effects. This may also be the situation in *Epilobium angustifolium* and *Filipendula ulmaria*, both of which were found to possess antibacterial activity, and *Lythrum salicaria* which had an antifungal effect. Special attention should be paid to the tannin fraction of *L. salicaria*, the components of which have now been identified but not tested as pure compounds in relation to their antifungal activity.

The protocol and LC/MS techniques used for identification of the phenolic composition of *Lythrum salicaria* are effective means of performing the most essential step when studying the background of the biological activity of a plant extract. In this study 11 compounds, not detected in earlier studies, were found in *L. salicaria*. This approach for determining the chemical composition can also therefore be used for other extracts containing plant phenolics.

The HPLC eluent composition has a significant effect on the ionisation of flavonoids using all the respective LC/API-MS techniques. The effect is greater with APPI than with IS and APCI, but the differences in ionisation sensitivity are evened out when optimised eluent compositions are utilised. In such optimised eluent conditions, there is no marked difference in absolute ionisation efficiency between the positive and negative ion modes. However, the stronger background noise in positive ion mode leads to higher limits of detection. The LODs achieved using the novel APPI could not be reduced below the LODs attained with IS.

Further experiments should be carried out with isolated constituents in order to identify the compounds responsible for the activity detected in the crude extracts. In the coming years, computer-aided optimisation of preparative HPLC separation should be carried out in order to isolate, in *deep well plates*, the compounds in *L. salicaria* and other plants of interest, and to determine the bioactivity of the compounds using the high throughput testing approach. However, these biological activities may not have a direct relationship with the polyphenolic fraction of the extracts.

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## APPENDICES

**Appendix 1** Flavonoids reported from the selected plant species investigated. Further references can be found in the Literature.

| Latin name | Flavonoid types | Literature |
|---|---|---|
| **Apiaceae** | | |
| *Carum carvi* L. | *Flavonols:* kaempferol-3-glucoside, isoquercitrin, quercetin-3-glucuronide, quercetin-3-caffeoylglucoside | KUNZEMANN and HERRMANN 1977 |
| **Asteraceae** | | |
| *Matricaria recutita* L. | *Flavones:* apigenin, apigenin-7-glucoside, apigenin-7-apiosyl-glucoside, apigenin-7-glucosyl-6"-acetate, apigenin-7-glucosyl-2"-acetate, apigenin-7-glucosyl-2",3"-diacetate, apigenin-7-glucosyl-3",4"-diacetate, luteolin, luteolin-7-glucoside, luteolin-4'-glucoside, luteolin-7-rutinoside, 6-hydroxyluteolin-7-glucoside, chrysoeriol-7-*O*-glucoside<br>*Flavonols:* quercetin, quercetin-7-glucoside, quercetin-3-rutinoside, quercetin-3-galactoside, patuletin, patuletin-7-glucoside, isorhamnetin, isorhamnetin-7-glucoside, jaceidin, chrysosplenol, eupatoletin, spinacetin, axillarin, eupalitin, chrysosplenetin, astragalin | SCHILCHER 1987,<br>MULINACCI et al. 2000 |
| *Matricaria matricarioides* (Less.) Porter | *Flavones:* apigenin, cosmosiin, luteolin, luteolin-7-glucoside, cynaroside<br>*Flavonols:* kaempferol, quercetin, quercimeritrin, gossypetin-7-glucoside, isorhamnetin | PROSOVSKII and OLESHKO 1985,<br>CHETVERNYA 1986 |
| *Tanacetum vulgare* L. | *Flavones:* apigenin, apigenin-7-glucoside, apigenin-7-glucuronide, luteolin, luteolin-7-glucoside, luteolin-7-glucuronide, 6-hydroxyluteolin-7-glucoside, nepetin, jaceosidin, eupatorin, hispidulin, chrysoeriol, diosmetin, diosmetin-7-glucuronide<br>*Flavonols:* quercetin, isorhamnetin, axillarin, jaceidin, bonanzin | OGNYANOV and TODOROVA 1983,<br>WILLIAMS et al. 1999 |
| **Betulaceae** | | |
| *Betula pubescens* Ehrh. | *Flavones:* apigenin, acacetin<br>*Flavonols:* kaempferol-3-glucoside, kaempferol-3-rhamnoside, kaempferol-3-glucuronide, quercetin-3-glucoside, quercetin-3-galactoside, quercetin-3-arabinofuranoside, quercetin-3-glucuronide, quercetin-3-rhamnoside, quercetin-3-(4"-acetyl)-rhamnoside, quercetin-3-arabinopyranoside, myricetin-3-glucoside, myricetin-3-galactoside, myricetin-3-glucuronide, myricetin-3-arabinopyranoside, myricetin-3-arabinofuranoside, myricetin-3-rhamnoside, myricetin-3-(acetyl)-rhamnoside<br>*Flavanones:* naringenin<br>*Flavans:* (+)-catechin | OSSIPOV et al. 1996,<br>KEINÄNEN and JULKUNEN-TIITTO 1998 |
| **Cannabaceae** | | |
| *Humulus lupulus* L. | *Flavones:* apigenin, apigenin-7-glucoside<br>*Flavonols:* astragalin, kaempferol-3-rhamnoside, kaempferol-3-rutinoside, guaijaverin, avicularin, quercetin-3-galactoside, quercetin-3-rhamnoside, isoquercetin, quercetin-7-glucoside, isoquercitrin, rutin | BHANDARI 1964,<br>HUBACEK et al. 1970,<br>van SUMERE et al. 1989,<br>STEVENS et al. 2000 |

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| | *Flavanones:* isoxanthohumol, 7-*O*-methyl-6-prenylnaringenin, 7-*O*-methyl-8-prenylnaringenin, 5,7-di-*O*-methyl-8-prenylnaringenin, 5,7-di-*O*-methylnaringenin, 6-prenylnaringenin, 8-prenylnaringenin, 6,8-diprenylnaringenin, 6-geranylnaringenin, 8-geranylnaringenin<br>*Anthocyanins:* cyanidin-3,5-diglucoside<br>*Flavans:* catechin, epicatechin<br>*Chalcones:* xanthohumol, xanthogalenol, 4'-*O*-methylxanthohumol, 4',6'-di-*O*-methylchalconaringenin, desmethylxanthohumol, 3'-geranylchalconaringenin, 3',5'-diprenylchalconaringenin, 5'-prenylxanthohumol, xanthohumol B, xanthohumol C, xanthohumol D, xanthohumol E | |
| **Chenopodiaceae** | | |
| *Beta vulgaris* L. var. *rubra* | *Flavones:* 2''-xylosylisovitexin, 2''-rhamnosylisovitexin<br>*Flavonols:* 3-glucopyranosyl(1⇓ 6)-glucopyranosylisorhamnetin | DIJOUX et al. 1995 |
| **Cichoriaceae** | | |
| *Sonchus arvensis* L. | *Flavones:* luteolin, luteolin-7-glucoside, sonchoside, acacetin, chrysoeriol<br>*Flavonols:* kaempferol, kaempferol-3,7-dirhamnoside, quercetin, quercetin-7-glucoside, quercetin-3-rhamnoside, isorhamnetin, isorhamnetin-3-glucoside | BONDARENKO et al. 1976,<br>BONDARENKO et al. 1978,<br>QU et al. 1995,<br>QU et al. 1996 |
| **Empetraceae** | | |
| *Empetrum nigrum* L. | *Flavonols:* quercetin, hyperoside, isoquercitrin, avicularin, myricetin<br>*Chalcones:* 2',4'-dihydroxychalcone, 2',4'-methoxychalcone<br>*Dihydrochalcones:* 2',4'-dihydrochalcone, 4'-hydroxy-2'methoxy-dihydrochalcone, 2'-hydroxy-4'methoxy-dihydrochalcone, uvangoletin, 4'-methoxy uvangoletin | VASILETS et al. 1988,<br>WOLLENWEBER et al. 1992,<br>HÄKKINEN and AURIOLA 1998 |
| **Ericaceae** | | |
| *Andromeda polifolia* L. | *Flavonols:* quercetin, hyperoside, guaijaverin, avicularin, polifolioside | PACHALY and KLEIN 1987 |
| *Calluna vulgaris* (L.) Hull | *Flavones:* isoscutellarein, apigenin 7-(2-acetyl)glucuronic acid methyl ester<br>*Flavonols:* kaempferol, kaempferol-3-glucoside, kaempferol-3-arabinoside, kaempferol-3-galactoside, kaempferol 3-triacetylarabinosyl(1⇓ 6)glucoside, quercetin, quercetin-3-glucoside, quercetin-3-galactoside, quercetin 3-triacetylarabinosyl(1⇓ 6)glucoside, quercetin 3-triacetylarabinosylgalactoside, isorhamnetin-3-galactoside, myricetin, herbacetin, herbacetin-8-glucoside, herbacetin-4'-glucoside, herbacetin-8-gentiobioside, gelidolin, galangin 3-methyl ether 7-glucoside<br>*Flavanones:* 3-desoxycallunin<br>*Dihydroflavonols:* callunin, 2''-acetylcallunin, taxifolin-3-glucoside<br>*Anthocyanins:* cyanidin 3-glucoside | ALLAIS et al. 1991,<br>ALLAIS et al. 1995,<br>ERSÖZ et al. 1997 |

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| *Vaccinium myrtillus* L. | *Flavonols:* quercetin-3-glucuronide, hyperin, isoquercitrin, myricetin<br>*Anthocyanins:* cyanidin-3-glucoside, cyanidin-3-arabinoside, cyanidin-3-galactoside, delphinidin-3-glucoside, delphinidin-3-arabinoside, delphinidin-3-galactoside, peonidin-3-glucoside, peonidin-3-arabinoside, peonidin-3-galactoside, petunidin-3-glucoside, petunidin-3-arabinoside, petunidin-3-galactoside, malvidin-3-glucoside, malvidin-3-galactoside<br>*Flavans:* catechin, epicatechin | GERHARDT et al. 1989,<br>WILSKA-JESZKA et al. 1992,<br>SMOLARZ et al. 2000,<br>HUOPALAHTI et al. 2000 |
| *Vaccinium oxycoccos* L. | *Flavonols:* quercetin-*O*-hexose, quercetin-*O*-deoxyhexose, quercetin-*O*-pentose, quercetin-*O*-dipentose, myricetin-*O*-glycosides<br>*Anthocyanins:* cyanidin-3-glucoside, cyanidin-3-arabinoside, cyanidin-3-galactoside, delphinidin-3-glucoside, delphinidin-3-galactoside, peonidin-3-glucoside, peonidin-3-arabinoside, peonidin-3-galactoside<br>*Flavans:* (+)-catechin, (-)-epicatechin | WANG et al. 1978,<br>HÄKKINEN and AURIOLA 1998,<br>HUOPALAHTI et al. 2000,<br>ARTS et al. 2000 |
| *Vaccinium uliginosum* L. | *Flavonols:* quercetin, quercetin-3-galactoside, quercetin-3-glucuronide, quercetin-*O*-pentoses, quercetin-*O*-hexose, myricetin, laricitrin<br>*Anthocyanins:* cyanidin-3-glucoside, cyanidin-3-arabinoside, cyanidin-3-galactoside, delphinidin-3-glucoside, delphinidin-3-arabinoside, delphinidin-3-galactoside, peonidin-3-glucoside, peonidin-3-arabinoside, peonidin-3-galactoside, petunidin-3-glucoside, petunidin-3-arabinoside, petunidin-3-galactoside, malvidin-3-glucoside, malvidin-3-arabinoside, malvidin-3-galactoside | ANDERSEN 1987,<br>GERHARDT et al. 1989<br>CUI et al. 1992,<br>HÄKKINEN and AURIOLA 1998, |
| *Vaccinium vitis-idaea* L. | *Flavonols:* quercetin, hyperin, isoquercitrin, avicularin<br>*Anthocyanins:* cyanidin-3-galactoside, cyanidin-3-arabinoside, cyanidin-3-glucoside, delphinidin-3-glucoside | ANDERSEN 1985,<br>HÄKKINEN and AURIOLA 1998,<br>SMOLARZ et al. 2000, |
| **Fabaceae** | | |
| *Trifolium hybridum* L. | *Flavones:* isopratol<br>*Flavonols:* astragalin, populnin, quercetin, isoquercitrin, hyperin, robinin<br>*Isoflavonoids:* vestitol, sativan, medicarpin, maackiain, methoxymaackiain, isosativan | SHALASHVILI 1974,<br>INGHAM 1976,<br>FRAISHTAT and VULFSON 1981 |
| *Trifolium pratense* L. | *Flavones:* apigenin, apigenin-7-glucoside, luteolin, luteolin-7-glucoside<br>*Flavonols:* kaempferol, quercetin, isoquercitrin, isoquercitrin-6"-malonate, hyperoside, 3-methylquercetin, 3-methylquercetin-7-glucoside, 3-methylquercetin-7-glucoside-6"-malonate, isorhamnetin<br>*Isoflavonoids:* daidzein, daidzin, genistein, genistin, genistin-6"-malonate, formononetin, ononin, ononin-6"-malonate, biochanin A, sissotrin, sissotrin-6"-malonate, trifoside, calycosin, calycosin-7-galactoside, calycosin-7-glucoside, calycosin-7-glucoside-6"-malonate, prunetin, trifoside-6"-malonate, glycitein, glycitin, irilone, irilone-4'-glucoside, irilone-4'-glucoside-6"-malonate, afrormosin, afrormosin-7-glucoside, pectolinaringenin, pratensein, pratensein-7-glucoside, pratensein-7-glucoside-6"-malonate, pseudobaptigenin, rothidin, madicarpin, medicarpin-3-glucoside, | HE et al. 1996,<br>LIN et al. 2000b |

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| | medicarpin-3-glucoside-6"-malonate, maackiain, trifolirhizin, trifolirhizin-6"-malonate | |
| *Trifolium repens* L. | *Flavonols:* kaempferol-3-galactoside, kaempferol-3-(6"-acetyl)-galactoside, quercetin-3-galactoside, quercetin-3-(6"-acetyl)-galactoside, myricetin-3-galactoside, myricetin-3-(6"-acetyl)-galactoside<br>*Flavans:* gallocatechin, epigallocatechin<br>*Isoflavonoids:* daidzein, genistein, genistein-7-(2"-coumaroyl)-glucoside, formononetin-7-(2"-*p*-hydroxybenzoyl)-glucoside, formononetin, biochanin A | SAXENA and JAIN 1989,<br>VETTER 1995,<br>FOO et al. 2000 |
| **Grossulariaceae** | | |
| *Ribes nigrum* L. | *Flavonols:* kaempferol, kaempferol-3-glucosylxyloside, kaempferol-3-glucosylrhamnoside, quercetin, rutin, isoquercitrin, quercetin-3-glucosylxyloside, quercetin-3-glucosylrhamnoside, astragalin, myricetin, myricetin-3-glucoside<br>*Anthocyanins:* cyanidin-3-glucoside, cyanidin-3-rutinoside, delphinidin-3-glucoside, delphinidin-3-rutinoside<br>*Flavans:* (+)-catechin, (-)-epicatechin | KOEPPEN and HERRMANN 1977, CALAMITA et al. 1983, da COSTA et al. 1998, ARTS et al. 2000 |
| **Lamiaceae** | | |
| *Thymus vulgaris* L. | *Flavones:* xanthomicrol, cirsimaritin, 5,4'-dihydroxy-6,7,8,3'-tetramethoxyflavone, cirsilineol, qenkwanin, 5-hydroxy-7,4'-dimethoxyflavone, thymonin, apigenin-7-rutinoside, vicenin-2, luteolin-7-glucoside<br>*Flavonols:* quercetin<br>*Flavanones:* eriodictyol, eriocitrin, hesperidin | MIURA and NAKATANI 1989,<br>MORIMITSU et al. 1995,<br>WANG et al. 1998 |
| **Liliaceae** | | |
| *Allium cepa* L. | *Flavonols:* kaempferol, kaempferol-3,4'-diglucoside, kaempferol-4'-glucoside, quercetin, quercetin-3,4'-diglucoside, quercetin-7,4'-diglucoside, quercetin-3,7-diglucoside, quercetin-4'-glucoside, quercetin-3-glucoside, quercetin-7-glucoside, isorhamnetin-4'-glucoside, isorhamnetin-3,4'-diglucoside | BILYK et al. 1984,<br>KIVIRANTA et al. 1988,<br>PRICE and RHODES 1997 |
| **Lythraceae** | | |
| *Lythrum salicaria* L. | *Flavones:* vitexin, isovitexin, orientin, isoorientin<br>*Anthocyanins:* cyanidin-3-galactoside, malvidin-3,5-diglucoside | PARIS and PARIS 1964,<br>PARIS 1967 |
| **Onagraceae** | | |
| *Epilobium angustifolium* L. | *Flavonols:* kaempferol-3-rhamnoside, kaempferol-3-arabinofuranoside, kaempferol-3-glucuronide, quercitrin, avicularin, guajaverin, hyperosid, isoquercitrin, quercetin-3-glucuronide, quercetin-3-(6"-galloyl)-galactoside, myricitrin, isomyricitrin, myricetin-3-galactoside, myricetin-3-glucuronide | DUCREY et al. 1995,<br>HIERMANN 1995 |
| **Pinaceae** | | |
| *Picea abies* L. (Karsten) | *Flavones:* apigenin-7-glucoside<br>*Flavonols:* kaempferol, kaempferol-3-glucoside, kaempferol-7-glucoside, kaempferol-3,4'-diglucoside, kaempferol-3-rutinoside, kaempferol-3-(6"-acetyl)-glucoside, kaempferol-3-(*p*-coumaroyl)-glucoside, quercetin, quercetin-3-glucoside, quercetin-7-glucoside, | SLIMESTAD et al. 1995,<br>SLIMESTAD and HOSTETTMANN 1996,<br>SLIMESTAD et al. 1996,<br>SLIMESTAD et al. 1999 |

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| | quercetin-3'-glucoside, quercetin-3-rutinoside, quercetin-3-(6"-acetyl)-glucoside, myricetin-3-glucoside, myricetin-7-glucoside, myricetin-3-rutinoside, myricetin-3,4'-diglucoside, myricetin-3-(6"-acetyl)-glucoside, isorhamnetin, isorhamnetin-3-glucoside, isorhamnetin-3-rutinoside, isorhamnetin-3-(6"-acetyl)-glucoside, laricitrin-3-glucoside, laricitrin-3-rutinoside, laricitrin-3-(6"-acetyl)-glucoside, syringetin-3-glucoside, syringetin-7-glucoside, syringetin-3-rutinoside, syringetin-3-(6"-acetyl)-glucoside<br>*Flavanones:* eriodictyol-7-glucoside, naringenin-7-glucoside<br>*Dihydroflavonols:* aromadendrin-7-glucoside, taxifolin, taxifolin-7-glucoside, taxifolin-3'-glucoside, ampelopsin-7-glucoside<br>*Anthocyanins:* pelargonidin-3-glucoside, cyanidin-3-glucoside, delphinidin-3-glucoside, peonidin-3-glucoside<br>*Flavans:* catechin, epicatechin, gallocatechin, 3'-methoxycatechin-7-glucoside | |
| *Pinus sylvestris* L. | *Flavonols:* kaempferol-3-rhamnoside, 3',6'-di-(4-coumaryl)-astragalin, quercetin, quercetin-3-glucoside, quercetin-3-rutinoside, 3',6'-di-(4-coumaryl)-isoquercetin, 6-methylkaempferol-3-glucoside, 3',6'-di-(4-coumaryl)-isorhamnetin-3-glucoside<br>*Flavanones:* pinocembrin<br>*Dihydroflavonols:* taxifolin, taxifolin-3'-glucoside, taxifolin-7-glucoside<br>*Flavans:* catechin, catechin-3-glucoside, 3'-methoxycatechin<br>*Chalcones:* 2',4',6'-trihydroxychalcone | IVANOVA et al. 1978, LARACINE-PITTET and LEBRETON 1988, FLIEGMANN et al. 1992, JUNGBLUT et al. 1995, PAN and LUNDGREN 1996, BENINGER and ABOU-ZAID 1997 |
| **Poaceae** | | |
| *Avena sativa* L. | *Flavones:* vitexin, isovitexin, vicenin-2, vitexin-2"-rhamnoside, isovitexin-2"-arabinoside, 7-*O*-methylvitexin-2"-rhamnoside, isoswertisin-2"-rhamnoside, isoorientin, 2"-arabinosyl-isoorientin, 2"-diglycosyl-isoorientin, isoorientin-7-glucoside, isoscoparin, tricin, tricin-5-glucoside, tricin-7-glucoside, tricin-7-rhamnoside, tricin-4'-arabinoside, tricin-4'-glucoside | POPOVICI et al. 1977, SALEH et al. 1988 |
| *Secale cereale* L. | *Flavones:* apigenin, luteolin, luteolin-3-glucoside, luteolin-7-diglucuronide, luteolin-7-diglucuronide-4'-glucuronide, vitexin-2"-rhamnoside, isovitexin-2"-galactoside, isoorientin-2"-arabinoside, chrysoeriol, tricin<br>*Flavonols:* quercetin-*O*-glycoside, isorhamnetin-*O*-glycoside<br>*Anthocyanins:* cyanidin-3-glucoside | TROJNA et al. 1976, STRACK et al. 1982, SCHULZ and WEISSENBÖCK 1987 |
| **Polygonaceae** | | |
| *Fallopia convolvulus* (L.) Á. Löve | *Flavones:* isovitexin<br>*Flavonols:* kaempferol-3-glucoside, kaempferol-3-galactoside, kaempferol-3-rhamnosylglucoside, quercetin, quercetin-3-glucoside, quercetin-3-galactoside, quercetin-3-arabinoside, quercetin-7-glucoside, quercetin-3-rhamnosylglucoside, myricetin-3-glucoside | KIM et al. 2000 |

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| **Primulaceae** | | |
| *Lysimachia vulgaris* L. | *Flavonols:* kaempferol-3-glucoside, kaempferol-3-rutinoside, quercetin, quercetin-3-glucoside, quercetin-7-glucoside, rutin, quercetin-7-sophoroside-3-glucoside, myricetin-3-glucorhamnoside, myricetin-3-rutinoside, quercetin-3-rhamnosyl-(1⇓ 2)-galactoside, isorhamnetin-3-galactoside, isorhamnetin-3-robinobioside, syringetin-3-galactoside | RZADKOWSKA-BODALSKA and OLECHNOWICZ-STEPIEN 1975,<br>YASUKAWA and TAKIDO 1988 |
| **Rosaceae** | | |
| *Aronia melanocarpa* (Michx.) Elliot | *Flavonols:* quercetin, quercetin-3-rutinoside, quercetin-3-galactoside, quercetin-3-arabinosyl-(1⇓ 6)-galactoside<br>*Anthocyanins:* cyanidin-3-glucoside, cyanidin-3-galactoside, cyanidin-3-arabinoside, cyanidin-3-xyloside, cyanidin-3,5-diglucoside, pelargonidin-3,5-diglucoside<br>*Flavans:* catechin, epicatechin | OSZMIANSKI and SAPIS 1988,<br>SZEPCZYNSKA 1989,<br>WILSKA-JESZKA et al. 1992,<br>HÄKKINEN and AURIOLA 1998 |
| *Filipendula ulmaria* (L.) Maxim. | *Flavonols:* kaempferol-4'-glucoside, quercetin, avicularin, spiraeoside, hyperoside, quercetin-3-glucuronide, rutin | SCHEER and WICHTL 1987,<br>LAMAISON et al. 1992 |
| *Malus pumila* Mill. | *Flavonols:* quercetin-3-glucoside, quercetin-3-galactoside, quercetin-3-rhamnoside, quercetin-3-rhamnoglucoside, quercetin-3-xyloside, quercetin-3-arabinopyranoside, quercetin-3-arabinofuranoside<br>*Dihydroflavonols:* taxifolin<br>*Anthocyanins:* cyanidin-3-galactoside<br>*Flavans:* catechin, epicatechin, gallocatechin<br>*Dihydrochalcones:* phloridzin | KANETA et al. 1979,<br>LISTER et al. 1994,<br>ARTS et al. 2000 |
| *Rubus chamaemorus* L. | *Flavonols:* kaempferol-*O*-glycosides, quercetin-*O*-glycosides, myricetin-*O*-glycosides<br>*Anthocyanins:* no identified substances | HÄKKINEN et al. 1999,<br>KÄHKÖNEN et al. 2001 |
| *Rubus idaeus* L. | *Flavonols:* kaempferol, kaempferol-3-glucoside, kaempferol-3-glucuronide, kaempferol-3-galactoside, kaempferol-3-xylosylglucuronide, kaempferol-3-xylosylglucoside, quercetin, quajaverin, quercetin-3-glucuronide, quercetin-3-glucoside, quercetin-3-galactoside, quercetin-3-sophroside, quercetin-3-xylosylglucuronide<br>*Anthocyanins:* no identified substances<br>*Flavans:* catechin, epicatechin | HENNING 1981,<br>ROMMEL and WROLSTAD 1993,<br>GUDEJ and RYCHLINSKA 1996,<br>ARTS et al. 2000,<br>KÄHKÖNEN et al. 2001 |
| *Sorbus aucuparia* L. | *Flavonols:* quercetin, isoquercetin, isoquercitrin, hyperin, rutin, meratin, kaempferol-*O*-glycosides<br>*Anthocyanins:* cyanidin-3-glucoside, cyanidin-3-galactoside, cyanidin-3,5-diglucoside<br>*Flavans:* catechin, epicatechin | VATULINA 1970,<br>PYYSALO and KUUSI 1974,<br>EDER et al. 1991,<br>WILSKA-JESZKA et al. 1992,<br>HÄKKINEN and AURIOLA 1998 |
| **Salicaceae** | | |
| *Salix caprea* L. | *Flavones:* diosmetin, luteolin-7-glucoside, chrysoeriol, chrysoeriol-7-galactoside, salicaprin<br>*Flavonols:* isoquercetin, isoquercitrin, isorhamnetin<br>*Flavanones:* naringenin, prunin<br>*Dihydroflavonols:* aromadendrin, dihydrokaempferide, taxifolin<br>*Flavans:* catechin, gallocatechin, leucocyanidin | NASUDARI et al. 1970,<br>MALTERUD et al. 1985 |
| **Solanaceae** | | |
| *Solanum tuberosum* L. | *Flavonols:* kaempferol, kaempferol-3-rutinoside, quercetin, quercetin-3-glucoside, rutin, myricetin<br>*Flavanones:* naringenin, eriodictyol<br>*Anthocyanins:* | LEWIS et al. 1998 |

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| | pelargonidin-*p*-coumaroyl-3-rutinoside-5-glucoside, cyanidin-*p*-coumaroyl-3-rutinoside-5-glucoside, delphinidin-*p*-coumaroyl-3-rutinoside-5-glucoside, peonidin-*p*-coumaroyl-3-rutinoside-5-glucoside, petunidin-*p*-coumaroyl-3-rutinoside-5-glucoside, malvidin-*p*-coumaroyl-3-rutinoside-5-glucoside,<br>*Flavans:* catehcin, epicatechin |