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ISOLATION AND IDENTIFICATION OF NEW BIOLOGICALLY ACTIVE COMPOUNDS FROM PLANTS

Ph.D. Thesis

1501 V BIOLOGY - BOTANY

Supervisor: Mgr. Karel Doležal, Dr.

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Abstract:

The presented doctoral thesis focuses on the isolation, identification, quantification and investigation of new biologically active natural compounds. The thesis is divided into three main parts: (1) Isolation and identification of anticancer active compounds from *Acalypha alopecuroidea*; (2) Geranylated flavanones from *Morus alba* and *Paulownia tomentosa* and their anticancer potential; (3) Miniaturized fast isolation and quantification of phytohormones from biological material.

The phytochemical studies, isolation and identification of biologically active compounds are very complicated and time-consuming processes. During the last period, we examined anticancer potential of Euphorbiaceous plant*, Acalypha alopecuroidea,* which is one of the plant species widely used in traditional Mayan medicine in Central America. The anticancer activity of prepared extracts was studied *in vitro* using the Calcein AM cell viability assay as cytotoxic effect against human breast adenocarcinoma cell line, human leukemic lymphoid cells and human cervical carcinoma cells. The highest antiproliferative activity was found in methanol-tetrahydrofurane (1:1) root extract which was fractionated by solid-phase extraction and two-dimensional high performance liquid chromatography. Finally, four fractions cytotoxic to the tested cancer cell lines were isolated and two possible structures of occurring compounds were suggested according to MS and NMR spectra as 9-(3,6-dimethylhepta-2,6-dienyl)-hypoxantine and 1,3,7,9-tetraethyluric acid. On the basis of designed structures, series of *N*9-prenylated derivatives of adenine and hypoxanthine and *N*-ethyl derivatives of uric acid were synthesised and their cytotoxic activity was tested against different cell types.

The next objective of the phytochemical approach was isolation of nine *C*-geranylated flavanones from the fruits of *Paulownia tomentosa* and two from the roots of *Morus alba.* Anticancer activity of these compounds was studied *in vitro* against five selected human cancer cell lines and normal human fibroblast*,* using the Calcein AM assay. The obtained EC_{50} values were compared with those of known simple flavanone standards (taxifolin, naringenin, hesperetin) and standard antineoplastic compounds (olomoucine II, diaziquon, oxaliplatin). Some of the isolated *C*-geranyl flavanones exhibited interesting cytotoxic activity towards the tested human cancer cell lines and showed effects of the structural modifications on activity. Comparison of the cytotoxicity against normal cell line and cancer cell lines revealed a very narrow therapeutic window of all the compounds tested.

The different groups of phytohormones have also shown activity towards human cancer cell lines, e.g. cytokinins, brassinosteroides, and jasmonates. The method development of a novel plant hormone isolation was the last aim of this thesis. A miniaturized purification method for cytokinin analysis performed in microtubes was designed for milligram amounts of biological samples. The purification protocol was based on a microextraction and a pipet-tip micropurification system named StageTips

(STop and Go Extraction Tips). Subsequently, the study of nature occurrence of phytohormones (cytokinins and indole-3-acetic acid) in beaver plasma samples was realized using the precipitation of plasma samples in acetonitrile, which was followed by mixed-mode cation exchange solid-phase extraction and immunoaffinity chromatography. The qualitative and quantitative phytohormone analysis was performed by an ultra performance liquid chromatography (Acquity™ UPLC, Waters) coupled to a triple quadrupole mass spectrometer (Xevo™ TQ MS, Waters) equipped with an electrospray interface.

In summary, the results of this doctoral thesis contribute to the phytochemical research of plant extracts, extend the knowledge about cytotoxic activity of plant metabolites as well as their closely related synthetic analogues against human cancer cells. The thesis improved the methods for purification of biologically interesting analytes. The thesis represents a progress in discovery of new highly bioactive compounds, which can be further investigated as potential therapeutics.

Keywords: *Acalypha alopecuroidea,* anticancer activity, Calcein AM cytotoxicity assay, *Castor fiber,* cytokinins, geranylated flavanones, high performance liquid chromatography, immunoaffinity extraction, mass spectrometry, *Morus alba,* nuclear magnetic resonance, *Paulownia tomentosa,* purine derivatives, solid-phase extraction, StageTips

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Number of appendices: 3

Language: English

Declaration I

Hereby I declare that this Ph.D. thesis is my original work. The literature used in this work is listed in the References section.

Brno, April 1, 2011 Jana Svačinová

List of papers

The thesis is based on the following papers. These are referred in the text as Supplement I-III and enclosed in the Supplement section:

Suppl. I Madlener S., **Svačinová J.**, Kitner M., Kopecký J., Eytner R., Lackner A., Vo T.P.N., Frisch R., Grusch M., De Martin R., Doležal K., Strnad M., Krupitza G. (2009) In vitro anti-inflammatory and anticancer activities of extracts of *Acalypha alopecuroidea* (Euphorbiaceae). *International Journal of Oncology* 35 (4): 881-891.

Suppl. II Šmejkal K., **Svačinová J.,** Šlapetová T., Schneiderová K., Dall'Acqua S., Innocenti G., Závalová V., Kollár P., Chudík S., Marek R., Julínek O., Urbanová M., Kartal M., Csöllei M., Doležal K. (2010) Cytotoxic activities of several geranyl-substituted flavanones. *Journal of Natural Products* 73 (4): 568-572.

Suppl. III **Svačinová J.**, Novák O., Lenobel R., Plačková L., Hanuš J., Strnad M., Doležal K. A new miniaturized analytical approach for cytokinin isolation from plant tissues using pipette tip solid-phase extraction (manuscript in preparation).

Declaration II

I declare that my role in preparation of the papers listed above was as following:

I. Co-author: preparation of *Acalypha* extracts and their fractions, testing of the cytotoxic activity by Calcein AM assay, manuscript preparation

II. Co-author: testing of the cytotoxic activity by Calcein AM assay, manuscript preparation

III. Joined first author: development, optimization and validation of StageTip purification method, UPLC-MS/MS measurements, manuscript preparation

Contents

Abbreviations

2-Cl-9-GA	2-chloro-9-geranyladenine
2-Cl-N6-Me-9-GA	2-chloro-*N*6-methyl-9-geranyladenine
2D	two-dimensional
9-DMAA	9-dimethylallyladenine
9-DMAH	9-dimethylallylhypoxanthine
9-GA	9-geranyladenine
9-GH	9-geranylhypoxanthine
9-IPA	9-isopentenyladenine
A549	human lung adenocarcinoma epithelial cell line
AcCoA	acetyl-coenzyme A
ADP	adenosine-5'-diphosphate
AHK	cytokinin histidine kinase receptors
AK	adenosine kinase
AMP	adenosine-5'-monophosphate
APCI	atmospheric pressure chemical ionization
API	atmospheric pressure ionization
APPI	atmospheric pressure photo-ionization
APRT	adenine phosphoribosyltransferase
ATP	adenosine-5'-triphosphate
BA	*N*6-benzyladenine
BA9G	*N*6-benzyladenine-9-glucoside
BAR	*N*6-benzyladenosine
BARMP	*N*6-benzyladenosine-5'-monophosphate
BEH	bridged ethylsiloxane/silica hybrid technology
BJ	human normal fibroblasts
C4H	cinnamate 4-hydroxylase
cAMP	adenosine-3',5'-cyclophosphate
Cdc25A	cyclin division cycle 25 homolog A (dual-specificity phosphatase)
CDK	cyclin-dependent kinase
CEM	human acute T-lymphoblastic leukaemia
CK	cytokinin
CKX	cytokinin oxidase/dehydrogenase
CoA	coenzyme A
COSY	correlation spectroscopy
COX-2	cyclooxygenase-2
CYC202	Seliciclib, (*R*)-roscovitine
CYP735A	cytochrome P450 monooxygenase
cZ	*cis*-zeatin
cZR	*cis*-zeatin riboside
DHZ	dihydrozeatin

DHZ9G	dihydrozeatin-9-glucoside
DHZOG	dihydrozeatin *O*-glucoside
DHZR	dihydrozeatin riboside
DMAPP	dimethylallyl diphosphate
DMEM	Dulbecco's modified Eagle's cell culture medium
DMSO	dimethylsulfoxide
DV41	human uterine fibroblasts
DW	dry weight
EMP	electromagnetic pulse
ESI	electrospray ionization
FAD	flavin adenine dinucleotide
FTICR	Fourier transform ion cyclotron resonance
FW	fresh weight
GC	gas chromatography
GC/MS	gas chromatography-mass spectrometry
Gln	glutamine
Gly	glycine
GMP	guanosine-5'-monophosphate
GTP	guanosine-5'-triphosphate
HeLa	human cervical carcinoma cells
HIV	human immunodefficiency virus
HL-60	human promyelocytic leukaemia cells
HMBC	heteronuclear multiple bond coherence
HMBDP	hydroxymethylbutenyl diphosphate
HMQC	heteronuclear correlation through multiple quantum coherence
HPLC	high performance liquid chromatography
HPLC/MS	high performance liquid chromatography with mass spectrometric detection
HSQC	heteronuclear single quantum correlation
HSV	herpes simplex virus
HUVEC	human umbilical vein endothelial cells
CHI	chalcone isomerase
CHS	chalcone synthase
IAA	indole-3-acetic acid
IAC	immunoaffinity chromatography
IAE	immunoaffinity extraction
IAG	immunoaffinity gel
IMP	inosine-5'-monophosphate
iP	*N*6-isopentenyladenine
iP9G	*N*6-isopentenyladenine-9-glucoside
iPR	*N*6-isopentenyladenosine
iPRDP	*N*6-isopentenyladenosine-5'-diphosphate
iPRMP	*N*6-isopentenyladenosine-5'-monophosphate

iPRTP	*N*6-isopentenyladenosine-5'-triphosphate
IPT	adenosine phosphate-isopentenyltransferase
IT	ion trap
K	kinetin
LOD	limit of detection
LOG	cytokinin nucleoside-5'-monophosphate phosphoribohydrolase (lonely guy)
m/z	mass-to-charge ratio
MCF-7	human breast adenocarcinoma cell line
MCF-7AL	human breast carcinoma cell line adriamycin resistant
MDA-MB-468	estrogen independent human breast carcinoma
MeOH	methanol
MEP	methylerythritol phosphate pathway
MNP	superparamagnetic iron nanoparticle
MRM	multiple reaction monitoring mode
MS	mass spectrometry
MS/MS	tandem mass spectrometry
mT	*meta*-topolin
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MVA	mevalonate pathway
N^{10}-formyl THF	N^{10}-formyltetrahydrofolate
NAD	nicotinamide adenine dinucleotide
NGT	*N*-glucosyltransferase
NMR	nuclear magnetic resonance spectroscopy
oT	*ortho*-topolin
PAL/TAL	phenylalanine/tyrosine ammonia lyase
PBS	phosphate-buffered saline
PDA	photodiode array detector
PRAT	phosphoribosylpyrophosphate amidotransferase
PRPP	phosphoribosyl-1-pyrophosphate
PT-SPE	pipette tip solid-phase extraction
qQ	single quadrupole mass spectrometer
QqQ	triple quadrupole mass spectrometer
QqTOF	quadrupole time-of-flight mass spectrometer
RPMI 8226	human multiple myeloma
SPE	solid-phase extraction
StageTip	STop And Go Extraction Tip
TEUA	1,3,7,9-tetraethyluric acid
THF	tetrahydrofurane
TNF-α	tumour necrosis factor-alpha
TOF	time-of-flight mass spectrometer
t_R	retention time
tRNA	transfer ribonucleic acid

tRNA-IPT	tRNA-isopentenyltransferase
tZ	*trans*-zeatin
tZ9G	*trans*-zeatin-9-glucoside
tZOG	*trans*-zeatin *O*-glucoside
tZR	*trans*-zeatin riboside
tZRMP	*trans*-zeatin riboside-5'-monophosphate
tZROG	*trans*-zeatin riboside *O*-glucoside
U266	human multiple myeloma cells
UHPLC	ultra-high performance liquid chromatography
UPLC-ESI-MS/MS	ultra performance liquid chromatography with electrospray interface and tandem mass spectrometric detection
UPLC-MS/MS	ultra performance liquid chromatography with tandem mass spectrometric detection
UV	ultraviolet
UV-DAD	ultraviolet-diode-array detector
XMP	xanthosine-5'-monophosphate
ZOGT	zeatin *O*-glycosyltransferase
ZR	zeatin reductase
λ_{max}	wavelength of maximum absorbance

1. Introduction

From the beginning of human existence, people have been searching for plants useful in a prevention and treatment of various diseases. The ancient knowledge of traditional healing remedies has been kept in memory till this time and herbal medicines are still popular and used in a practice. Although the utilization of a whole plant or a crude plant extract for therapeutic purposes is advantageous, because of the complex action of the plant compounds and interactions between individual substances, there can be some difficulties and drawbacks though. The coexistence of more than one plant constituent can result in not only synergistic, but also antagonistic effects and undesirable changes of the bioactivity of individual compounds. The amount of the active constituents and the level of their activity are often dependent on the plant habitat, geographic area, climatic conditions, seasonal changes and the plant organ and morphology. The biological activity may be changed or lost depending on the method of plant collection and manipulation, the storage conditions of plant material and the method of extract preparation. The disadvantage of using the whole plant or crude plant extracts can be overcome by the identification and isolation of pure compounds. Treatment with an individual bioactive compound has several advantages, such as the ability to accurately control dose rates and reproducibility.

Today plants are collected and planted not only for therapeutic purposes, but also for deeper investigation of the pharmacologically active constituents. The analysis of plant metabolites can also lead to a better understanding of plant growth and development. The isolation of a compound is the start of the process leading to substance identification, elucidation of structure and determination of structure-activity relationships. The identification of the active compound is essential for the development of analytical methods for its determination and quantification. Afterwards, the particular analytical method can be used for an evaluation of the quality of plant drugs and therapeutic formulations in relation to the presence of the target compound. Substances isolated from plants often function as lead structures for a drug discovery and development of new pharmacologically active compounds with similar or higher pharmacological activity and lower side effects. Hence plants are one of the important sources for finding new drugs. A wide variety of plants used in traditional herbal medicine have still not been phytochemically investigated and they represent a hidden and potentially important source of future natural bioactive compounds.

2. Aims and scopes

The screening of new plant metabolites helps to discover new interesting pharmaceuticals from plant sources as well as to understand the plant growth and development. In general, the isolation, purification and analyses of biologically active natural compounds are complicated for occurrence of interfering substances in the complex plant matrix. Furthermore, the levels of some target compounds such as phytohormones are very low (in concentration range 0.01-100 $pmol \cdot g^{-1}$ of fresh weight) and the use of high efficient purification protocols with modern analytical methods is necessary. Non-specific isolations accompanied by the application of different biological assays and analytical techniques are reasonable approaches for screening of unknown substances linked with known biological activity.

This doctoral thesis focused mainly on the method development of the different procedures for characterization of biologically active compounds. The modern phytochemical methods were applied to isolation and identification of plant secondary metabolites responsible for the anticancer activity, studied *in vitro* as cytotoxicity towards human cancer cell lines. Furthermore, a novel analytical approach for miniaturized purification of plant growth substances was designed for milligram amounts of biological samples.

The three main objectives elaborated and discussed in this doctoral thesis were as follows:

1. Isolation and identification of anticancer active compounds from *Acalypha alopecuroidea.*
2. Geranylated flavanones from *Morus alba* and *Paulownia tomentosa* and their anticancer potential.
3. Miniaturized fast isolation and quantification of phytohormones from biological material.

Each of the main topics has several goals and steps. Part of the work undertaken by the author of this thesis and some of the results included in objectives 1-3 are fully described in the multi-author papers attached as Supplements I-III. The author's personal contributions to these papers, together with details of non-published experiments, modifications, improvements, data and results, are discussed in Results and discussion.

3. Literature review

3.1 Phytochemistry

Phytochemistry is a branch of chemistry, which studies and classifies chemical compounds produced in plants called phytochemicals. In a narrower sense, the term is often used to describe the large number of secondary metabolic compounds found in plants (Matsumoto, 2008), which are known as secondary metabolites, secondary products, or natural products (Taiz et al., 2006). These plant constituents, firstly thought as functionless metabolic wastes, have important ecological functions. They protect plants against pathogens and herbivores, diseases or insect attack, they serve as attractants for pollinators, agents of competition between plants, agents of symbioses with microbes (Taiz et al., 2006), they can act as antioxidants or as signals in plants (Seigler, 1998). As a consequence these compounds exert interesting biological effects on other organism and they can exhibit beneficial effects for human consumers, they have significant ecological, economic and medicinal value (Hanson, 2003; Acamovic et al., 2005). Natural products have attracted interest, they have found use as essences, soaps, folk remedies and they have been studied as potential therapeutics (Hanson, 2003; Hopkins et al., 2003). Different methods of extraction, isolation and structure elucidation of natural products as well as various chromatography techniques are used in the field of phytochemistry to describe the large number of biologically active compounds produced by plants (Matsumoto, 2008).

3.2 Plants with potential therapeutic use in medicine

Three phytochemically interesting plant species, namely *Acalypha alopecuroidea*, *Morus alba* and *Paulownia tomentosa* were focused and studied in this thesis mainly for the anticancer activity of their extracts and constituents.

Acalypha alopecuroidea Jacq. commonly named foxtail copperleaf, tree-seeded mercury, yerba del cancer (Huerta et al., 2007), or rabo de gato, is a herbaceous annual weed, which belongs to the family Euphorbiaceae and is very similar to *A. arvensis*. *A. alopecuroidea* is traditionally used in Mayan medicine. In contrast to the rich knowledge of its use in traditional medicine, phytochemical studies of this species have never been performed. The plant is native to Dominican Republic, Guatemala, Haiti, Venezuela (Johnson, 1999) and is also found in Bermuda, Mexico, Central America, and the region from the West Indies to Venezuela. It can be found occasionally in South Florida as a weed in nursery liners. The plant is a weed of waste areas, open fields, rock flats, and disturbed soils (Artaud, 1995).

Morus alba L., commonly named as white mulberry, silkworm mulberry, "Shahtoot" (Urdu, Persian, Hindi) and known as "Tuta" in Sanskrit, is the dominant species of the genus *Morus* and with other over 150 species belongs to the family Moraceae. *M. alba* is medicinally important plant and it is a source of interesting bioactive natural compounds (Butt et al., 2008; Venkatesh et al., 2008; Dugo et al., 2009). A fruitless variety and a weeping cultivar of white mulberry "Pendula" are popular ornamental plants and roadside trees (Tutin et al., 1964; Zelený, 2005; Plants, 2010). *M. alba* is widely cultivated and even naturalized in many regions all over the warm temperate world (Butt et al., 2008; Venkatesh et al., 2008; Dugo et al., 2009). It is native to northern China, hilly areas of Himalaya (up to 3,300 m) and the region from India to Japan. It has been cultivated throughout Europe from the 12th century for leaves to feed silkworms (Tutin et al., 1964; Zelený, 2005; Venkatesh et al., 2008).

Paulownia tomentosa (Thunb.) Steud. commonly named as Empress tree, Royal Princess tree, Foxglove tree or "Pao tong" in Chinese and "Kiri" in Japanese, is an attractive, fast-growing, spreading, deciduous, popular ornamental tree belonging to family Scrophulariaceae (Rodd et al., 2008). *P. tomentosa* is native to central and western China and widely distributed throughout China, Korea, Japan, the United States and Brazil on waste places and disturbed areas including forests, streambanks, and steep rocky slopes (Leopold et al., 2003; Remaley, 2009; Rodd et al., 2008).

3.2.1 Taxonomic classification

Acalypha alopecuroidea (Hendrych, 1977)

Kingdom:	Plantae	Plants
Subkingdom:	Cormobionta	Vascular plants
Superdivision:	Spermatophyta	Seed plants
Division:	Magnoliophyta	Flowering plants
Class:	Magnoliopsida	Dicotyledons
Subclass:	Dileniidae	
Order:	Euphorbiales	
Family	Euphorbiaceae	Spurge family
Genus:	*Acalypha* L.	copperleaf
Species:	*A. alopecuroidea* Jacq.	foxtail copperleaf

Figure 1. *Acalypha alopecuroidea* Jacq. The photo was obtained from Dr. Richard Frisch (Institute for Ethnobiology and Maya Research, Guatemala).

Morus alba (Hendrych, 1977; Butt et al., 2008)

Kingdom:	Plantae	Plants
Subkingdom:	Cormobionta	Vascular plants
Superdivision:	Spermatophyta	Seed plants
Division:	Magnoliophyta	Flowering plants
Class:	Magnoliopsida	Dicotyledons
Subclass:	Hamamelididae	
Order:	Urticales	
Family:	Moraceae	Mulberry family
Genus:	*Morus* L.	mulberry
Species:	*M. alba* L.	white mulberry

Figure 2. *Morus alba* L. Photo by Luis Fernández García with Creative Commons Attribution-Share Alike 2.1.Spain Licence, http://commons.wiki media.org/wiki/File:Morus_alba.jpg.

Paulownia tomentosa (Hendrych, 1977)

Kingdom:	Plantae	Plants
Subkingdom:	Cormobionta	Vascular plants
Superdivision:	Spermatophyta	Seed plants
Division:	Magnoliophyta	Flowering plants
Class:	Magnoliopsida	Dicotyledons
Subclass:	Asteridae	
Order:	Scrophulariales	
Family:	Scrophulariaceae	Figwort family
Genus:	*Paulownia* Siebold & Zucc.	paulownia
Species:	*P. tomentosa* (Thunb.) Steud	princesstree

Figure 3. *Paulownia tomentosa* (Thunb.) Steud. Photo by Meneerke Bloem with GNU Free Documentation Licence, http://cs.wikipedia.org/wiki/Soubor:Paulownia_tomentosa.jpg.

3.2.2 Morphology

Acalypha alopecuroidea Jacq. is a 10-70 cm high herb with a glandular pubescent stem and with wide-spreading branches from near the base. Leaves are simple and alternate, petioles are nearly as long or longer than the blade. The blade is broadly ovate, 3-7 cm long and 2-4.5 cm wide, prominently five-nerved, truncate to slightly cordate at base, the margins of the blade are serrate. Glandular trichomes are present on most shoot organs. Individual flowers are arranged in an inflorescence, which is a dense spike. Staminate flowers are minute, light green, in 3-9 mm long axillary spikes. Pistillate flowers are enclosed in a bract with bristle-like lobed appendages and they form cylindrical, catkin-like terminal or axillary spikes, which are 5-20 mm long (Artaud, 1995; Hayden, 2008).

Morus alba L. is small to medium size, short-living, fast-growing, deciduous tree, which grows up to 10-20 m and has large, brown, rough stems and slender smooth branches. Leaves are 6-18 cm long, not lobed on older trees, with ovate, rounded or obliquely cordate shape at the base and dentate, serrated or lobed margins. The leaves are usually smooth at the adaxial side, glabrous or pubescent only on the veins beneath. On young, vigorous shoots, the leaves may be up to 30 cm long, deeply and intricately lobed, with lobes rounded. The flowers are single-sex catkins, male catkins are 2-3.5 cm long and female catkins 1-2 cm long. Catkins of both sexes are present on each tree. The syncarp (infructescence of achenes enclosed in pulpy perigone) has a length of 1-2.5 cm and a deep purple colour in the wild species, in many cultivated plants it varies from white to pinkish or purplish colour. The fruit is edible long before it is ripe and it is sweet but bland. Seeds are widely dispersed by birds, which eat the fruits and excrete the seeds (Tutin et al., 1964; Hendrych, 1977; Bean, 1978).

Paulownia tomentosa (Thunb.) Steud. grows rapidly up to 10-25 m. This tree has large, broad, heart-shaped, three- to five-lobed leaves, which are 15-40 cm long, at maturity thick, dark green and nearly smooth above, paler and tomentose beneath, arranged in opposite pairs on the stem. The flowers have a tubular lilac blue to light purple corolla 4-6 cm long resembling a foxglove flower and are arranged on panicle inflorescences 10-30 cm long. The fruit is a dry, leathery, olive brown to bronze egg-shaped capsule 3-4 cm long, containing numerous tiny, dark, oval, lace-winged seeds ripening in autumn and dispersed by wind and water. Opened capsules remain on branches throughout the winter season. Pollard trees do not produce flowers (Leopold et al., 2003; Levy-Yamamori et al., 2004; Remaley, 2009; Rodd et al., 2008).

3.2.3 Phytochemical constituents

Acalypha alopecuroidea has not been investigated phytochemically till this time and only limited data concerning this plant are available. Constituents mentioned in the literature are neolignans, phenoles and organic acids (Martínez, 2003). In general, euphorbiaceous plants contain latex, oil, gum, polyphenols, alkaloids, cyanogenic compounds and highly toxic polypeptides in the seeds (Novák, 1961; Zelený, 2005). The presence of alkaloids, tannins, saponins, cardenolides (Soladoye et al., 2008), cyanopyridone glucosides (acalyphin) (Nahrstedt et al., 1982; Hungeling et al., 2009), terpenes (cycloartane type) (Gutierrez-Lugo et al., 2002), diterpenes (Siems et al., 1996), tetraterpenes (acalyphaser A) (Kambara et al., 2006), polyphenols and tannins (acalyphidins) (Amakura et al., 1999), flavonoids (Nahrstedt et al., 2006), anthocyanins (Bailoni et al., 1998; Reiersen et al., 2003), labdane derivatives in the oil (Meccia et al., 2006) and amides (Siems et al., 1996) was reported in *Acalypha* species. The bioactive constituents of *Acalypha* sp. might be responsible for the frequent use of these plants in traditional herbal medicine.

Morus alba is very rich source of natural phenolic compounds. The most abundant are anthocyanins such as cyanidin and its glucosides (Chen et al., 2006) and flavonoids such as quercetin, kaempferol and their conjugates (Kim et al., 1999a; Dugo et al., 2009), astragalin, isoquercitrin, skopolin (Doi et al., 2001), albanol A and B (Kikuchi et al., 2010) and mulberrofurane Q (Venkatesh et al., 2008; Kikuchi et al., 2010). *M. alba* is known for isoprene substituted flavonoids (prenylflavanes) (Doi et al., 2001). The ones which have attracted researches the most are kuwanon G and H (Nomura et al., 1994; Nomura et al., 1998; Nomura, 2001; Nomura et al., 2009). Other representatives of isolated prenylflavanes are kuwanon C, kuwanon J, chalcomoracin (Nomura, 2001), morusin (Nomura et al., 1998), moralbanone, leachianone G (Butt et al., 2008). Except flavonoids, a broad spectrum of other compounds was isolated from *M. alba*, e.g. a glycoprotein Moran 20K (Kim et al., 1999b), a polyhydroxylated piperidine alkaloid 1-deoxynojirimycin (Kim et al., 2003; Kimura et al., 2004; Kimura et al., 2007; Vichasilp et al., 2009), arylbenzofuran derivatives (moracin V-Y, mulberroside C) (Piao et al., 2009; Tian et al., 2010; Yang et al., 2010a), coumarin and stilbene glycosides (Piao et al., 2009), sterols (Fujimoto et al., 2000), chalcone derivatives (morachalcones B, C) (Yang et al., 2010b) and triterpenes (Böszörményi et al., 2009). The study of nutritional quality of leaves and fruits of *M. alba* showed that proteins, carbohydrates, fatty acids (linoleic acid, palmitic acid, oleic acid), fiber, tannic acid, minerals (iron, zinc, calcium, potassium, phosphorous and magnesium) and some vitamins or their precursors (ascorbic acid and beta-carotenes) are present in appreciable amount (Srivastava et al., 2003; Srivastava et al., 2006; Ercisli et al., 2007).

Paulownia tomentosa represents another species studied for the presence of phenylpropanoid compounds, e.g. lignans such as asarinin, sesamin, paulownin, isopaulownin, piperitol (Takahashi et al., 1966; Ina et al., 1987; Park et al., 1992); phenolic glycosides such as acteoside, coniferin, syringin (Sticher et al., 1982) and phenylpropanoid phenols such as verbascoside, isoverbascoside (Schilling et al., 1982). Epimeric phenylpropanoid (Kim et al., 2007) and phenylethanoid glycosides (isoilicifolioside A, ilicifolioside A, campneoside II and isocampneoside II) were detected in the wood (Si et al., 2008a). From the group of flavonoids, flavan-3-ols, catechins, naringenin, taxifolin, apigenin, luteolin, kaempferol and quercetin were isolated (Si et al., 2008b; Si et al., 2009). The most intensively studied group of phenylpropanoids occurring in *P. tomentosa* are geranylated flavanones represented by diplacone, diplacol, tomentodiplacone, hydroxydiplacone, mimulone, acteoside and isoacteoside, which have been extracted either from flowers, fruits, or as a secretion from the surface of immature fruits (Du et al., 2004; Jiang et al., 2004; Šmejkal et al., 2007a; Asai et al., 2008; Si et al., 2008b; Šmejkal et al., 2008a; Wollenweber et al., 2008). The group of iridoids and iridoid glycosides (7-hydroxytomentoside and tomentoside) was also found in *P. tomentosa* (Adriani et al., 1981; Damtoft et al., 1993). Several sesquiterpenes were isolated from the flowers (Cao et al., 2009), and furanoquinone compounds were extracted from the stem (Kang et al., 1999; Park et al., 2009). Chemical investigation of the glandular trichome exudates of the leaves resulted in identification of thirty acylglycerols (Asai et al., 2009). Other compounds such as triterpenic ursolic acid and its glycosides, alkaloids, monosacharides, tannic acid, fatty acid and phenolic acids were also isolated from *P. tomentosa* (Si et al., 2008c; Cao et al., 2009).

3.2.4 Biological activity

Acalypha alopecuroidea displays many biological effects. Consequently, this plant is used in traditional herbal medicine. Plant extracts have anodyne, carminative, diuretic, sedative, vulnerary and energizing effects (Johnson, 1999). The plant is recommended in case of flatulence and inflammation. Because of the latex content, it can cause contact dermatitis (Martínez, 2003). It has been proven that an aqueous extract of *A. alopecuroidea* exhibited antiinflammatory and antiarthritic effects. It acted both in the acute and chronic phase of inflammation and inhibited the growth of some enterobacteria (Zavala-Sánchez et al., 2009).

Morus alba is of a great therapeutic worth and potentially can be used for prevention and treatment of some diseases. Active ingredients need further *in vivo* exploration, which will enhance chances of commercial use of isolated compounds in medicine (Butt et al., 2008). *M. alba* possesses antibacterial (Nomura et al., 1988; Nomura et al., 2001; Park et al., 2003; Sohn et al., 2004), antifungal (Nomura et al., 1988) and antiviral activity against herpes simplex type 1 virus (HSV-1) (Du et al., 2003; Butt et al., 2008) and human immunodeficiency virus (HIV) (Venkatesh et al., 2008). Flavonoids and anthocyanins from *M. alba* have the potential to act as immuno-stimulating nutrients and they can play an important role as in the prevention and cure of disorders such as inflammation and cancer. Cyanidin 3-rutinoside and cyanidin 3-glucoside exert inhibitory effect on the invasion of human lung cancer cells (Chen et al., 2006), prenylated flavanones exhibit cytotoxic activity against rat hepatoma cells (Kofujita et al., 2004), a water extract shows cytotoxic activity on human leukaemia and mouse melanoma cells (Nam et al., 2002). Leaf extracts possess antiinflammatory and chemopreventive potential as a consequence of suppression of inflammatory mediators, inhibition of cyclooxagenase-2 activity, nitric oxide production and production of tumour necrosis factor-alpha (TNF-α) (Chung et al.,

2003; Sohn et al., 2004; Choi et al., 2005). Some investigation examined the antiallergic potential of hot water extracts of mulberry leaves (Chai et al., 2005, Butt et al., 2008).

A high content of phenolic compounds with radical scavenging and antioxidant activity in leaves, root bark, fruits and stem supports a possible application of white mulberry extracts or their functional components to reduce *in vitro* and *in vivo* harmful oxidation processes (Doi et al., 2000; Du et al., 2003). Flavonoids such as quercetin and its glycosides (Sývacý et al., 2004; Enkhmaa et al., 2005; Katsube et al., 2006; Chen et al., 2007), prenylflavanes and prenylflavane glycosides (Kim et al., 1999a; Doi et al., 2001), anthocyanins (cyanidin 3-rutinoside and cyanidin 3-*O*-β-D-glucopyranoside) (Seeram et al., 2001; Konczak-Islam et al., 2003; Serraino et al., 2003; Chen et al., 2006; Ercisli et al., 2007) and oxyresveratrol (Oh et al., 2002) display antioxidant potential. It has been shown that diet supplementation of antioxidants from white mulberry can enhance lipid resistance to oxidative modification (Seifried et al., 2007) and decrease the development of atherosclerosis and mortality from coronary heart disease (Enkhmaa et al., 2005; Katsube et al., 2006; Shibata et al., 2006). Free radical production is often associated with neurodegenerative disorders, hence mulberry extracts protect the brain against cerebral ischemia (Kang et al., 2006) and endothelial dysfunction (Seeram et al., 2001; Serraino et al., 2003) and may also provide a treatment for Alzheimer's disease (Niidome et al., 2007).

The hypolipidemic effect of the total flavonoid fraction from white mulberry leaves was confirmed (Chen et al., 2007; El-Beshbishy et al., 2006). Hypotensive compounds were found in the ethyl acetate extract of root bark (Venkatesh et al., 2008). The leaves, roots and root bark have antidiabetic potential (Singab et al., 2005; Lee et al., 2008). Much of the antidiabetic action can be attributed to the alkaloid 1-deoxynojirimycin, which is one of the most potent α-glycosidase inhibitors (Sývacý et al., 2004; Oku et al., 2006; Kimura et al., 2007) and moran 20K, which is similar to insulin and decreases the blood glucose level (Kim et al., 1999b; Butt et al., 2008; Venkatesh et al., 2008). Antityrosinase activity, which prevents the overproduction of melanin and which may be beneficial for skin depigmentation (Iozumi et al., 1993), was detected with mulberroside F and in extracts from leaves and root bark (Lee et al., 2002; Andallu et al., 2003; Khan et al., 2003; Butt et al., 2008).

Paulownia tomentosa and its extracts have been studied by many research groups to investigate their biological activity, which can be attributed to mainly flavonoid content. Investigations led to the detection of the antibacterial activity of geranylflavonoids (Šmejkal et al., 2008a; Šmejkal et al., 2008b) and sesamin (Cao et al., 2009); the antiviral activity of furanoquinone (Kang et al., 1999); the antiinflammatory activity of fruits (Tunon et al., 2009) and apigenin (Jiang et al., 2004); the antiasthmatic activity (Jiang et al., 2004; Cao et al., 2009; Chen et al., 2009); the cytotoxic and antiproliferative activity of geranylflavonoids (Šmejkal et al., 2008b) and apigenin (Jiang et al., 2004); the antioxidant and free radical scavenging activity of geranylflavanones such as acteoside, isoacteoside, diplacone, diplacol (Šmejkal et al., 2007a; Šmejkal et al., 2007b; Asai et al., 2008), gallic and caffeic acid isolated from the leaves (Si et al., 2008c; Cao et al., 2009); the antihepatotoxic; vasculo-protected; vasorelaxant; spasmolithic activity and the antidiarrhoetic activity of apigenin (Jiang et al., 2004).

3.2.5 Practical applications

Acalypha alopecuroidea is used in traditional Mayan medicine in the form of a decoction or infusion to wash severe skin conditions and in large quantities per day as a tea for various stomach and urinary complaints (Arvigo et al., 1993). The plant is also recommended in the case of indigestion,

dyspepsia, diarrhoea, flatulence, asthma, bruises, sprains, infection, acute and chronic inflammations and cancer (Argueta, 1994; Johnson, 1999; Martínez, 2003).

Morus alba is cultivated through Europe for leaves, which are preferred feedstock for caterpillars of *Bombyx mori* (silkworms). The leaves are cut as food for livestock in some areas during dry seasons. Fleshy perianth of white, rose or red infructescences is consumable and sweet in taste. The fruits are eaten fresh or dried, made into juice, squashed and fermented to yield a spirit, added into dishes, pastry and desserts (Tutin et al., 1964; Bean, 1978; Venkatesh et al., 2008). The high quality proteins of mulberry leaves can be used with wheat flour to make parathas (Indian flat bread). Furthermore, a supplementation of mulberry powder improves the storage stability of wheat (Srivastava et al., 2003; Butt et al., 2008). The Chinese Pharmacopoeia from 1985 lists the leaves, root bark, branches, sap and fruits of *M. alba* as ingredients in medicinal preparations (Venkatesh et al., 2008). The fruits of white mulberry have cooling and laxative properties, they are used in case of digestive disorders (dyspepsia, loss of appetite, flatulence, constipation, intestinal worms) and urinary diseases (chronic nephritis, kidney asthenia), to tonify the blood and to promote urination, to treat fever, headache and melancholia, to reduce high blood pressure and high cholesterol, to prevent thrombus formation, to treat cerebral arteriosclerosis, heart diseases, diabetes and nasopharyngeal cancer. The fruits are also used to improve eyesight, to help in case of sore and dry eyes, to prevent alopecia areata, prematurely grey hair and aging (Doi et al., 2001; Venkatesh et al., 2008; Dugo et al., 2009). Mulberry tea made from leaves is claimed to be an antidiabetic drink in various Asian countries (Lee et al., 2008). The root is astringent and is one of the components of an oriental antidiabetic remedy (Butt et al., 2008). The bark is anthelmintic and used against chronic bronchitis, emphysema and asthma, to treat cough, wheezing, oedema and to promote urination (Venkatesh et al., 2008).

Paulownia tomentosa is an ornamental tree with a tropical appearance, which is grown for its large showy fragrant flowers and large leaves (Leopold et al., 2003). Pollard trees with characteristic large size, production of massive leaves and vigorous new growth every year are exploited by gardeners. These trees are popular features in modern gardens. *P. tomentosa* is very robust tree, it can survive wildfires and its roots can regenerate new, very fast-growing stems, but can not thrive when shaded by other taller trees. It functions ecologically as a pioneer plant, because it is tolerant to pollution and different soil types. Its leaves rich in nitrogen provide a good fertilizer and fodder and its roots prevent soil erosion (Hall, 2008; Fast, 2010). Carving the wood of *P. tomentosa* is an art form in Japan and China. The wood is strong, but soft and light and it is prized for its utilitarian and ornamental characteristics. It is very useful in the manufacture of furniture, toys, and crates, its outstanding resonant qualities make it suitable for musical instruments such as the Asian string instruments, the koto and the zither, which are made from *P. tomentosa* (Rodd et al., 2008). The timber is fire resistant and prevents clothes from being eaten by moths (Levy-Yamamori et al., 2004). The soft, lightweight seeds were used by exporters as a packing material for Chinese porcelain in the 19th century, before the development of polystyrene packaging. Parts of *P. tomentosa* have been used in traditional Chinese herbal medicine for a treatment of enteritis, tonsillitis, bronchitis and dysentery. The flowers are considered as the most important plant part (Chen et al., 2009). The stem bark of *P. tomentosa* has been used as a component of several remedies for treatment of infectious diseases such as gonorrhoea and erysipelas (Kang et al., 1999).

3.3 Plant secondary metabolites

Primary plant metabolites comprise the basic molecules of the cell (sugars, amino acids, proteins, lipids, nucleic acids) and are directly produced and involved in primary processes (photosynthesis, respiration). Other diverse organic molecules, which are produced by pathways derived from primary metabolic routes and which have no direct function in plant growth and development, are referred to as plant secondary metabolites. In practice, the distinction between primary and secondary metabolites is not easily made and there is an overlap between these two terms. In contrast with primary metabolites, secondary metabolites occur in relatively low quantities and they usually have restricted distribution among the plants. Each plant species produces a characteristic series of secondary metabolites, therefore these compounds can be used as taxonomic characteristic of plants (Seigler, 1998; Hopkins et al., 2003; Taiz et al., 2006).

Plant secondary metabolites can be classified according to different characteristics such as the composition, the chemical structure, the solubility in solvents or the metabolic pathway of their biosynthesis. The three major classes of plant secondary metabolites are terpenes (terpenoids, isoprenoids), phenolics (phenylpropanoids, polyphenols) and nitrogen-containing compounds (Hopkins et al., 2003; Taiz et al., 2006). Two significant metabolic pathways leading to both primary and secondary metabolites are the mevalonate pathway (mevalonic acid pathway, MVA) and shikimic acid pathway. Mevalonate pathway produces five-carbon compounds (isopentenyl pyrophosphate and dimethylallyl pyrophosphate), which are the basis for terpenoids. Shikimic acid pathway gives rise to aromatic acids (phenylalanine, tyrosine and tryptophan), which are the basis for phenolics (phenylalanine) and some alkaloids (tyrosine and tryptophan) (Hopkins et al., 2003). A scheme of the major pathways involved in the biosynthesis of secondary metabolites and their connections with primary metabolism are shown in Figure 4 (Taiz et al., 2006).

Terpenes represent the largest class of secondary metabolites, which are composed of five-carbon units (C5 units, isoprene units). Compounds of this class are generally insoluble in water. Terpenes are classified according to the number of present C5 units to monoterpenes (two C5 units), sesquiterpenes (three C5 units), diterpenes (four C5 units), triterpenes (six C5 units), tetraterpenes (eight C5 units) and polyterpenoids (more than eight C5 units) (Taiz at el., 2006). Terpenes include essential oils, pigments (carotene, xanthophyl), hormones (giberellins, abscisic acid), sterols (ergosterol, sitosterol, steroid glycosides), sterol derivatives (cardiac glycosides) and latex. Cytokinins and chlorophyll, which do not belong to the class of terpenes, contain terpenoid side chain (Hopkins et al., 2003). Terpenes are biosynthesized in the mevalonate pathway from acetyl-coenzyme A (AcCoA) or in the methylerythritol phosphate pathway (MEP) from glycolytic intermediates (Taiz at el., 2006).

Phenolics represent a chemically heterogenous group of compounds, which contain a hydroxyl functional group on an aromatic ring. Some compounds of this class are water-soluble (carboxylic acids, glycosides), some are soluble only in organic solvents and polymers are insoluble. Phenolic compounds are classified according to their structure to simple phenolics (phenylpropanoids, phenylpropanoid lactones and benzoic acid derivatives), flavonoids, which contain two aromatic rings connected by a C3 bridge (flavones, flavonols, isoflavones, anthocyanins) and phenolic polymers (lignin, tannins). Phenolics are biosynthesized mainly by shikimic acid pathway from carbohydrate precursors via phenylalanine and cinnamic acid and by malonic acid pathway, which is significant in fungi and bacteria, but less important in plants (Hopkins et al., 2003; Taiz et al., 2006).

Nitrogen-containing compounds include alkaloids, cyanogenic glycosides, non-protein amino acids and sulfur-containing glucosinolates (thiglucosides). The majority of these compounds is biosynthesized from common amino acids (Taiz et al., 2006). Highly bioactive alkaloids are soluble in water and possess at least one nitrogen atom, which is usually part of heterocyclic ring. The exceptions are some aliphatic nitrogen compounds, which are also considered as alkaloids. Alkaloids are classified according to the predominant ring system presented in the molecule (quinoline, isoquinoline, indole, pyrrolizidene, quinolizidene, tropane, piperidine, and purine). Most alkaloids are biosynthesized from a few common amino acids (tyrosine, tryptophan, ornithines, arginine, and lysine), some representatives are generated from nicotinic acid (nicotine) and some are purine derivatives (caffeine) (Hopkins et al., 2003).

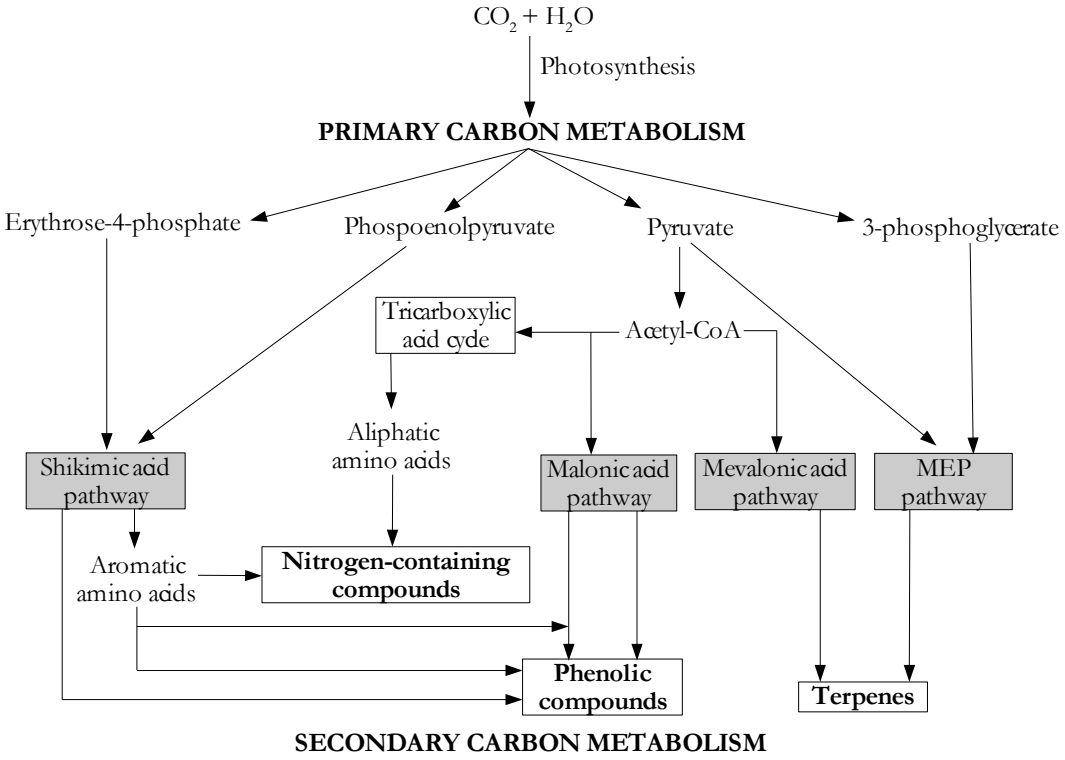


Figure 4. A scheme of the major pathways involved in secondary metabolism and their interconnections with primary metabolism (Taiz et al., 2006).

Plant secondary metabolites have always been constituents of vegetarian diets of animals and men. They have shown to have significant biological effects when ingested (Acamovic et al., 2005) and their metabolites have been investigated and searched in animal kingdom (Le Page-Degivry et al., 1986; Schram, 1998; Schoch et al., 2000). Hence, the application of modern phytochemical methods on an animal material could be considered as one of the directions in the field of phytochemistry, which fade into animal biology and pharmacology. For example, the typical high fibre diet of beaver (Castor) can be the basic presumption of the presence of plant derived metabolites in beaver body fluids. Beavers are the second largest type of rodents (Nowak, 1999). They consume bark, sapwood, shoots and leaves of twigs and small branches of trees (Wilsson, 1971; Godin, 1977), such as poplar, willow, birch and alder (Nowak, 1999; Burton et al., 2002).

The following chapters are focused on the groups of phytochemicals, which were studied in this doctoral thesis.

3.3.1 Flavonoids and prenylated flavonoid derivatives

Flavonoids are one of the largest groups of plant secondary metabolites and play an important role not only in plants, but also in human and animal health, because they are an integral part of the diet. The multiple structural varieties of the basic flavonoid skeleton result in an extremely large number of compounds. More than 4,000 flavonoids, comprising 12 subclasses, have been identified in plants (Cook et al., 2006; De Rijke et al., 2006). Plant flavonoids are the goal of many scientists, because they exert a wide range of biochemical and pharmacological activities, they have obvious positive effects on human health and disease prevention and therefore they are investigated as treatment options against many chronic human diseases (Skibola et al., 2000).

3.3.1.1 Chemical structure

Flavonoids are low molecular weight polyphenolic substances. The structure is the determining factor of their biological activities and it is based on the phenylpropanoid flavan nucleus (C6-C3-C6 carbon skeleton) composed of three rings assigned as A, B, and C as shown in Figure 5. In fact, it is the chroman type skeleton with a phenyl substituent in the *C*2 or *C*3 position.

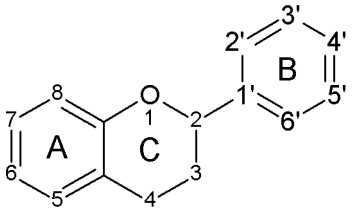


Figure 5. The basic structure and numbering system of flavonoids.

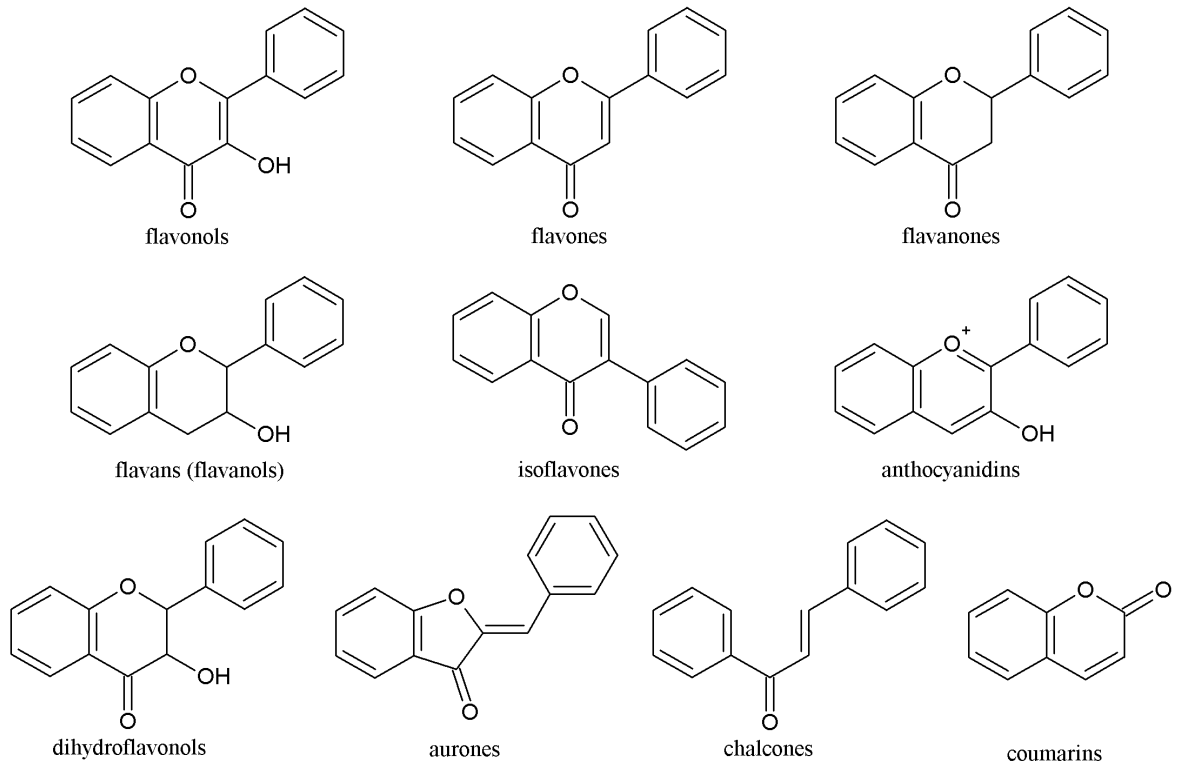


Figure 6. The structures of classes of flavonoids.

Flavonoids are classified into groups according to the structural feature around the heterocyclic ring (bond saturation, hydroxylation and ring position) (Peterson et al., 1998; Cook et al., 2006). Flavonoids can be diversified also within each group by prenylation. Differences in the prenylated position on aromatic rings, differing lengths of prenyl chain and further modifications of the prenyl moiety (cyclisation, hydroxylation) has resulted in approximately 1,000 prenylated flavonoids occurring in plants (Yazaki et al., 2009). The six major classes (Figure 6) of flavonoids are flavonols, flavones, flavanones, flavans (also called flavanols, catechins, leucoanthocyanidins, proanthocyanidins and tannins), isoflavonoids and anthocyanidins. Isoflavonoids differ from the common flavonoids in B ring orientation. Other flavonoid classes include dihydroflavonols, aurones, chalcones and coumarins (Peterson et al., 1998; Merken et al., 2000; Cook et al., 2006). The possible modifications of flavan nucleus are hydroxylation (in positions 3, 5, 7, 3', 4', 5'), hydrogenation, reduction, oxidation, alkylation, malonylation, sulphation, and glycosylation (Fowler et al., 2009). One or more of the hydroxyl groups can be methylated, acetylated, prenylated or sulphated (De Rijke et al., 2006).

Many natural flavonoids occur in the form of *O*-glycosides with sugar substituents bound to a hydroxyl group of the aglycone, usually located in position *C*3 or *C*7. Less frequent *C*-glucosides have the sugar moiety attached directly to the carbon of the aglycone usually *C*6 or *C*8. The most common sugars are glucose, rhamnose, glucorhamnose, galactose, arabinose and lignin (Cook et al., 2006). The sugars can be further substituted by acyl residues such as malonate and acetate. Flavonoid glycosides from the diet are hydrolysed by animal intestinal flora to biologically active aglycones (Peterson et al., 1998; Cook et al., 2006; De Rijke et al., 2006). Flavonoids can be monomeric, dimeric, oligomeric or polymeric. Polymeric flavonoids (condensed tannins, non-hydrolysable tannins, proanthocyanidins) are polymers of flavan-3-ol subunits (mainly epicatechin and catechin) attached by carbon-carbon bonds, which can not be cleaved by hydrolysis. They give characteristic colours, flavours, and astringency to plant drugs and extracts (Hagerman, 1998; Cook et al., 2006; Zhang et al., 2008). The group of prenylated flavonoids is rich in structural variety and biological activity and is characterized by the presence of isoprenoid side chain attached to the heterocyclic ring. An isoprenyl (3,3-dimethylallyl), a geranyl (*E*-3,7-dimethyl-2,6-octadienyl), a 1,1-dimethylallyl or a lavandulyl (5-methyl-2-isoprephenyl-hex-4-enyl) moiety is a part of the flavonoid structure (Chi et al., 2001; Sohn et al., 2004).

3.3.1.2 Natural occurrence in plants

Flavonoids are naturally occurring substances in fruits, vegetables, nuts, seeds, flowers and bark of many plant species (Cook et al., 2006). Flavonoids in plants have several functions as defence and signalling compounds in reproduction, pathogenesis and symbiosis (Maxwell et al., 1990; Stafford, 1990). They are involved in responses to and protection against oxidative injury (Swain, 1986), stress caused by UV radiation (Reuber et al., 1996; Heldt, 1997; Olsson et al., 1998; Zand et al., 2002), infection by fungal parasites (Harborne, 1988) and other pathogens and herbivore attack (Wang et al., 1998a). Flavonoids act in the production of root nodules after infection by *Rhizobium* in leguminous plants (Etherington, 1983), they give colour and odour to plants (Coultate, 1990), attract and guide pollinating insects (Biggs et al., 1978, Harborne, 1988; De Rijke et al., 2006) and produce stimuli to assist in pollination (Harborne, 1986; Cook et al., 2006).

Flavanones are the most abundant class of flavonoids and are found in citrus fruits (Peterson et al., 2006a; Peterson et al., 2006b), where they contribute to the flavour of citrus (naringin, hesperidin).

They occur also in chick peas, cumin, peppermint (hesperidin), hawthorn berry, rowanberry (narirutin and naringenin), licorice (liquiritigenin) (Kuhnau, 1976; Horowitz, 1986; Peterson et al., 1998). The group of flavones (apigenin, luteolin, nobiletin, neodismin) contributes to the taste of herbs (parsley, rosemary, and thyme), grains and vegetables (Herrman, 1976). From the group of flavonols, quercetin, kaempferol, myricetin and isorhamnetin, which occur in fruits and vegetables, are the best known (Herrman, 1976; Peterson et al., 1998). Isoflavonoids (genistein, diadzein, biochanin A, formononetin, coumesterol) occur predominantly in legumes, soya bean is the major source of genistein and daidzein (Franke et al., 1994; Mazur et al., 1996).

The next group of flavonoids is represented by coloured anthocyanins (delphinidin, cyanidin, petunidin, peonidin and malvidin) giving the blue and red colour to fruits and flowers. Anthocyanins may be complexed with flavones and metal ions such as iron and magnesium in flowers. The colour of anthocyanins depends on pH (red in case of low pH, shifting to blue with increasing pH (Herrman, 1976; Pierpoint, 1986). Flavans are rarely glycosylated, but can be polymerized or esterified with gallic acid. Monoflavans occur in ripe fruits and fresh leaves, biflavans and triflavans in fruits (Pierpoint, 1986; Peterson et al., 1998; Cook et al., 2006). Prenylated flavonoids have been isolated from a limited number of plant families especially Leguminosae, Moraceae, Asteraceae, Cannabaceae with few reports of pharmacological activities available to date. Prenylated polyphenols act in plants as protectants against pathogenic microorganisms and herbivores, they may serve as protection against abiotic environmental stresses like oxidative stress (Sohn et al., 2004).

3.3.1.3 Biosynthesis and metabolism

Flavanones as direct precursors of each flavonoid class are synthesized from the amino acids, phenylalanine or tyrosine. The enzyme phenylalanine/tyrosine ammonia lyase (PAL/TAL) converts phenylalanine and tyrosine into phenylpropanoic acids. Cinnamate 4-hydroxylase (cytochrome P450 enzyme, C4H) adds a 4'-hydroxyl group to phenylalanine aromatic ring. The coenzyme A (CoA) esters of aminoacids are subsequently synthesized from phenylpropanoic acids by the action of phenylpropanoyl-CoA ligases. The chalcone synthase (type III polyketide synthase, CHS) then catalyses the condensation of three malonyl-CoA moieties with one CoA-ester molecule to form chalcones. This is the first step leading to flavonoid biosynthesis. The chalcones are stereospecifically isomerised into (2*S*)-flavanones by chalcone isomerase (CHI) in an alkali environment and form the final flavanone structure (Figure 7). The generation of over 8,000 different chemical structures is the result of subsequent functionalisation and alteration of the flavan skeleton by the action of numerous enzymes (Fowler et al., 2009).

Prenylated flavonoids, namely kuwanons G and H, are considered to be formed through an enzymatic Diels-Alder reaction of a chalcone and a dehydro-kuwanon C or its equivalent. About forty kinds of Diels-Alder type adducts (e.g. kuwanon J, chalcomoracin) structurally similar to kuwanon G has been isolated from moraceous plants. A novel way of isoprenoid biosynthesis of prenylflavonoids through the junction of glycolysis and pentose-phosphate cycle was proposed in *Morus alba* callus tissue (Nomura, 2001). The biosynthetic pathway of prenylated flavonoids represents the crucial coupling reaction of two major metabolic pathways, the shikimate or polyketide (acetate/malonate) pathway providing an aromatic moiety (Knaggs, 2003) and the isoprenoid pathway derived from the mevalonate or methylerythritol phosphate pathway providing the isoprenoid side chain. Membrane-bound

flavonoid-specific prenyltransferases catalyse the key step of flavonoid prenylation in plants (Yazaki et al., 2009).

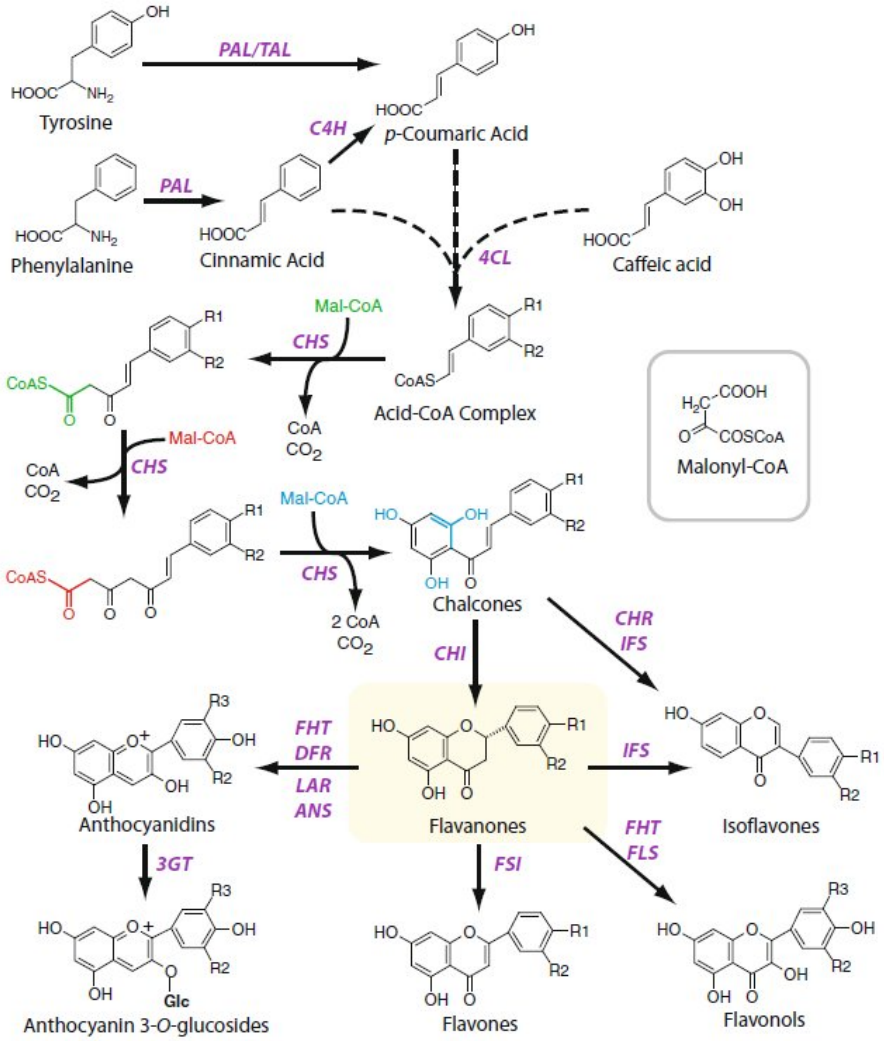


Figure 7. Detailed biosynthetic steps for flavanones and the diversification of flavonoids (Fowler et al., 2009). Abbreviations are defined in the text or as follows: 4CL, 4-coumaryl:CoA ligase; CHR, chalcone reductase; IFS, isoflavanone synthase; FHT, flavanone hydroxytransferase; FLS, flavonol synthase; FSI, flavon synthase; DFR, dihydroflavanone reductase; LAR, leucoanthocyanidin reductase; ANS, anthocyanidin synthase; 3GT, uridine flavanone-3-glucoside transferase.

3.3.1.4 Biological activity and benefits for humans

Flavonoids have been reported to exhibit a wide range of biological effects, including antibacterial, antiviral, antiinflammatory, analgesic, cytostatic, antiallergic, estrogenic (isoflavonoids) and vasodilatory (Cook et al., 1996; Bravo, 1998). Flavonoids also reduce capillary permeability and fragility, inhibit lipid peroxidation, platelet aggregation and the activity of enzymes (cyclooxygenase, lipooxygenase, protein kinase, tyrosine protein kinase and topoisomerase II). Flavonoids also act as antioxidants, free radical scavengers and chelators of divalent metal ions (Cook et al., 1996; Cao et al., 1997; Heim et al., 2002; Williams et al., 2004). Flavonoids may prevent *in vivo* free radical mediated cytotoxicity and lipid peroxidation, which is associated with cell aging and chronic diseases, and are proposed as anticarcinogens and cardioprotective agents on account of their positive effects on the human body (Cook et al., 1996; Manthey et al., 2001; Kinghorn et al., 2004).

Flavonoids are believed to be non-toxic at the usual levels of dietary intake, but they are often consumed by human in dietary supplements in doses exceeding the normal concentrations in vegetarian diets. Despite of beneficial effect, a potential toxicity can be connected with excessive flavonoid intake. Several studies have shown mutagenic, genotoxic, pro-oxidant, DNA damaging activity of flavonoids in a number of *in vitro* assays, when using high doses in experimental system (Peterson et al., 1998; Skibola et al., 2000). Flavonoids are common components of natural medicines. Preparations containing rutin and diosmin are used to increase vascular tone, anthocyanins from bilberry improve vision. The whole family Labiaceae is a source of traditional herbal medicines where, with the exception of flavonoids, terpenes and saponins also play an active role (Peterson et al., 1998).

Prenylated flavonoids also act as active compounds in medicinal plants and a few reports of pharmacological activities of prenylated flavonoids is available (Iinuma et al., 1992; Kang et al., 2000; Chi et al., 2001; Son et al., 2001). Proven biological activities include anticancer, antibacterial and antiparasitical (Wang et al., 1998b; Sohn et al., 2004; Shakil et al., 2007), antileishmania (Yazaki et al., 2009), antiandrogen, antioxidant, and antiinflammatory activity, inhibition of cyclooxygenases, lipooxygenases, tyrosinases and glucosidases (Kimura et al., 1986; Chi et al., 2001; Kim et al., 2002; Yazaki et al., 2009), cytochrome P450 interactions (Hodek et al., 2002), prevention of osteoporosis and enhancement of collagen synthesis in the skin (Tielens et al., 2008). Prenylated flavonoids contribute to the antiinflammatory effect by reduction of TNF-α, suppression of nitric oxide production and cytotoxic protein kinase C, and inhibition of platelet aggregation (Yazaki et al., 2009). Prenylated flavonoids are more hydrophobic than the non-prenylated ones. Consequently they may more easily penetrate through the cell membrane and skin barrier when used topically. Plants containing various types of prenylated flavonoids as the major constituents have been used as antiinflammatory agents in Chinese medicine (Chi et al., 2001). The prenyl moiety often plays a crucial role in divergent biological activities of these prenylated compounds (Yazaki et al., 2009).

3.3.2 Purine derivatives

The heterogenous group of purines is the most widely distributed group of *N*-heterocycles in nature. As a consequence of many possible modifications to the purine skeleton, this group includes a broad range of biologically active compounds, ensuring many essential functions and involved in numerous metabolic processes. Purine alkaloids, purine bases of nucleic acids, cytokinins, and purine nucleoside antibiotics can be named as representatives of this group of natural compounds. Many synthetic derivatives have also been prepared and examined for their biological activities. The name purine was first used by Emil Fischer, who synthesized a colourless crystalline weak purine base for the first time in 1899 from uric acid isolated from gallstones by C.W. Scheele in 1776 (Rosemeyer, 2004).

3.3.2.1 Chemical structure

The basic chemical structure of purines (imidazo[4,5-d]pyrimidine) is shown in Figure 8. Many of purine derivatives were isolated long before their structures were elucidated and are generally described by trivial names in the literature. The compounds like adenine, hypoxanthine, guanine, isoguanine, xanthine, and uric acid are the basic structures, which can be further modified by methylation, alkylation, glycosylation to give the active natural components as demonstrated in Figure 9 (Rosemeyer, 2004).

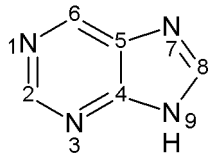


Figure 8. The basic chemical structure and numbering system of purines.

3.3.2.2 Natural occurrence in plants

Purines can occur in nature in the form of free bases, nucleosides (*N*-glycosides) and nucleotides. The unsubstituted purine base does not occur in the nature. The simplest natural purine form is the ribonucleoside nebularine (9-*β*-D-ribofuranosylpurine), which is an antibiotic isolated from the mushroom *Agaricus nebularis* (Rosemeyer, 2004). Adenine derivatives are of the highest importance in living organisms and they are essential molecules found in all cell. Adenosine-5'-triphosphate (ATP) is a storage form of energy, adenosine-3',5'-cyclophosphate (cAMP) acts as a second messenger regulating the activation of protein kinases in the cell as well as in transcription and other metabolic processes. Nicotinamide adenine dinucleotide (NAD) and flavin adenine dinucleotide (FAD) participate as coenzymes in cellular reduction/oxidation processes. Acetyl-coenzyme A, which possesses high C2-group transfer potential, is another example of a molecule containing purine with biological importance. Furthermore, nucleic acids are composed of equal amounts of pyrimidine and purine nucleotides, which form the double helical structure of nucleic acids (Rosemeyer, 2004).

3.3.2.3 Biosynthesis and metabolism

The biosynthesis of the purine ring and nucleotide metabolism are basic metabolic functions in all living organisms, and are essential processes in plant development. Nucleotides can be supplied to a plant organism by salvage reactions, which convert corresponding free nucleobases and nucleosides into nucleotides and thus recycle nucleotides for everyday needs (Zrenner et al., 2006). As a consequence, the requirement for *de novo* purine biosynthesis in differentiated cells is small and the activity of this metabolic pathway is relatively low. New purine rings are only synthesised when DNA is replicated (Smith et al., 2002; Boldt et al., 2003).

The first basic step for *de novo* purine biosynthesis is catalysed by phosphoribosylpyrophosphate amidotransferase (PRAT) which uses phosphoribosyl-1-pyrophosphate (PRPP) and glutamine (Gln) as substrates (Figure 9). Simple molecules such as CO_2, Gln, glycine (Gly), N^{10}-formyltetrahydrofolate (N^{10}-formyl THF) are substrates for subsequent reactions. Inosine-5'-monophosphate (IMP) is the final product and also the starting purine nucleotide providing the basic purine nuclei for further structural modifications and synthesis of other purine nucleotides, xanthosine-5'-monophosphate (XMP), adenosine-5'-monophosphate (AMP) and guanosine-5'-monophosphate (GMP) (Smith et al., 2002; Boldt et al., 2003; Stasolla et al., 2003).

AMP and GMP provide a purine base for the formation of nucleic acids (DNA and RNA), nicotinamide and flavine coenzymes, coenzyme A, and for the signalling molecule (cAMP). AMP is also the precursor of the phytohormones, cytokinins. ATP and guanosine-5'-triphosphate (GTP) participate in the energy metabolism of the cell, ATP providing energy for many chemical reactions. Both xanthosine and xanthine are used as precursors for secondary metabolites such as purine alkaloids (caffeine, theobromine, theofyline), and their further oxidation give rise to ureides, allantoin and allantoic acid. Purines also regulate the synthesis of amino acids, phospholipids, glycolipids, sugars and polysac-

charides. Nucleotides are degraded to simple metabolites and permit the recycling of phosphate, nitrogen, and carbon into central metabolic pools (Smith et al., 2002; Boldt et al., 2003; Stasolla et al., 2003; Zrenner et al., 2006).

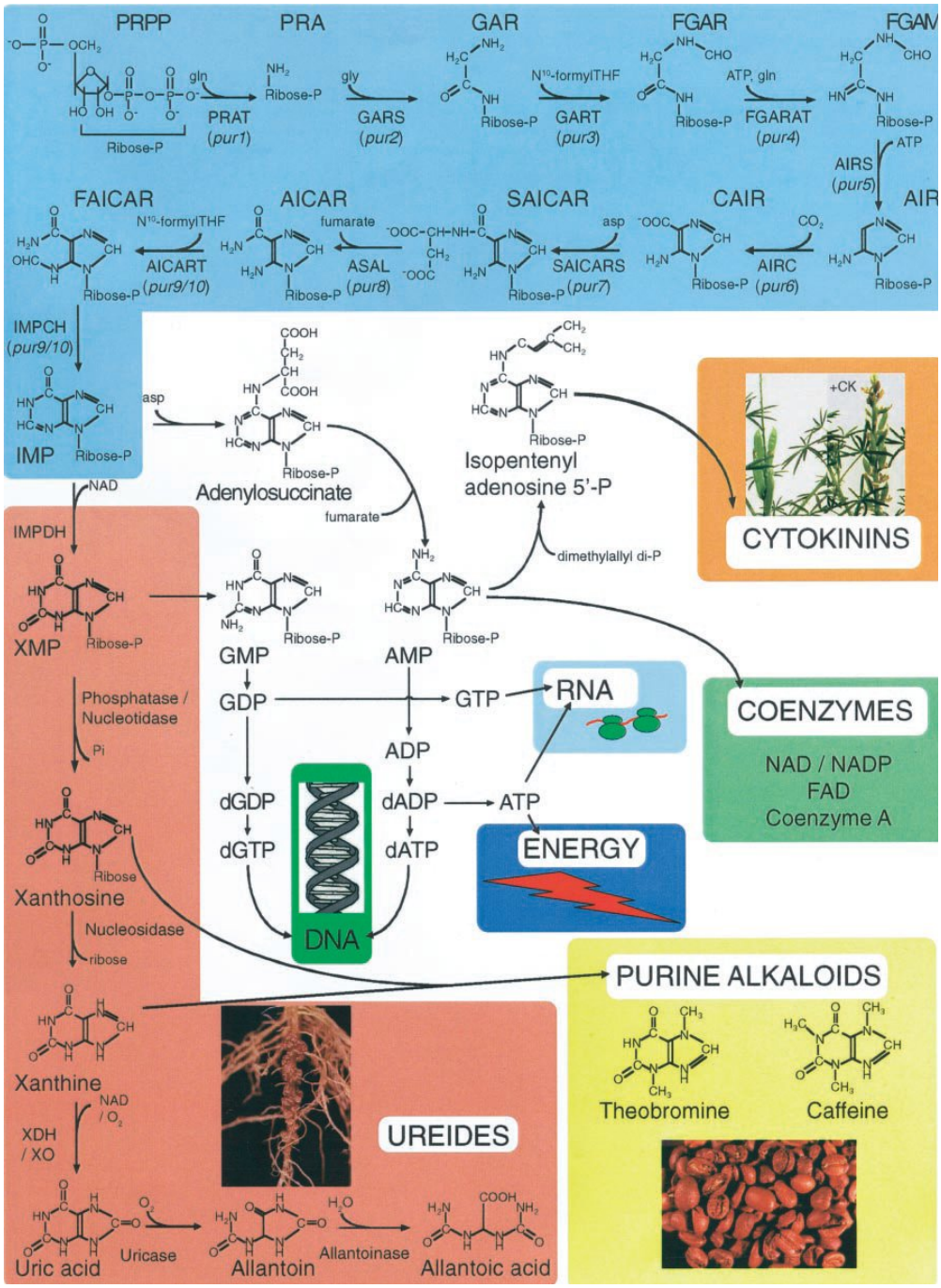


Figure 9. Diagram of *de novo* purine biosynthesis and metabolism in plant cells (Smith et al., 2002). Abbreviations are defined in the text or as follows: PRA, phosphoribosylamine; GARS, glycinamide ribonucleotide synthetase; GAR, glycinamide ribonucleotide; GART, glycinamide ribonucleotide transformylase; FGAR, formylglycinamide ribonucleotide; FGARAT, formylglycinamide ribonucleotide amidotransferase; FGAM, formylglycinamidine ribonucleotide; AIRS, aminoimidazole ribonucleotide synthetase; AIR, aminoimidazole ribonucleotide; AICR, aminoimidazoleribonucleotide carboxylase; CAIR, carboximideaminoimidazole ribonucleotide; SAICARS, succinoaminoimidazolecarboximide ribonucleotide synthetase; SAICAR, succinoaminoimidazolecarboximide ribonucleotide; ASAL, adenylosuccinate-AMP lyase; AICAR, aminoimidazolecarboximide ribonucleotide; AICART, aminoimidazolecarboximide ribonucleotide transformylase; FAICAR, formylaminoimidazolecarboximide ribonucleotide; IMPCH, inosine monophosphate cyclohydrolase; Pi - inorganic phosphate; XDH/XO, xanthine dihydrogenase/oxidase; NADP, nicotinamide adenine dinucleotide phosphate.

3.3.2.4 Biological activity and benefits for humans

Some nucleosides have therapeutic potential in human nutrition and diseases. As an example, the combination of inosine and the *p*-(acetylamino) benzoate salt of 1-(dimethylamino) propan-2-ol (Inosiplex®) is administered as a stimulating agent for normal human T-cells and human leukocytes. The sodium salts of inosine- and guanosine-5'-monophosphate are widely used as flavour enhancers in food industry (Rosemeyer, 2004).

Modern purine research makes efforts to find new agonists and antagonists of the purine receptors with pharmacological potential and use in medical practice. Adenosine receptors in the human body (sub-types A1, A2A, A2B, A3 are currently known) play an important role in regulation of blood pressure, they are involved in regulation of pain signal transmission. A3 receptors are over-expressed in certain tumour tissues. A3 receptor stimulation using A3 agonists can inhibit growth of some cancer cells. New A2A agonists are helpful in the treatment of asthma as an alternative to traditionally used glucocorticoides (Rosemeyer, 2004). Different types of modified purines and nucleosides have been found and isolated as marine metabolites and display potent bioactivites, such as the antifungal (phidolopine), hypotensive (1-methylisoguanosine, known as doridosine), and cytotoxic activity in the case of mycalisines. Spongothymidine and spongouridine served as models for the development of marine-derived commercially available drugs, such as adenine-arabinoside used for treatment of *Herpes simplex* infections and cytosine-arabinoside for treatment of leukaemia (Lindsay et al., 1999a).

3.3.2.5 Selected groups of naturally occurring purines

The vast majority of common natural purines and their nucleosides occur as mono- or poly-methylated derivatives. The methylation reactions are very important metabolic pathways (Rosemeyer, 2004).

N-methylated purines and purine nucleosides

The most widespread methylated purines are methyl derivatives of xanthine (3,9-dihydro-purine-2,6-dione). 1-methylxanthine together with uric acid is present in human urine in very high concentrations (Figure 10).

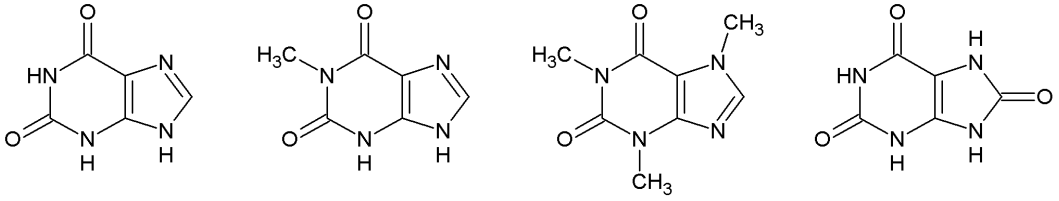


Figure 10. Chemical structure of xanthine, methylated derivatives of xanthine and uric acid.

Caffeine (1,3,7-trimethylxanthine) is the most important purine alkaloid occurring in coffee beans and in tea leaves. Caffeine is linked by non-covalent bonds to chlorogenic acid in unroasted coffee beans and to tannic acids (gallic and ellagic acid) in tea leaves. Caffeine is an adenosine antagonist and influences the human central nerve system by inhibition of a cAMP-specific phosphodiesterase. Therefore, the cAMP is not converted to AMP and prolongs the effect of adrenalin-mediated cAMP. Caffeine has addictive effects and a dose of 100 mg stimulates metabolism, heart function and respiration, increases blood pressure and body temperature and promotes blood vessel constriction in the intestines and vasodilatation in brain (Waldvogel, 2003; Rosemeyer, 2004). Theophylline (1,3-

dimethylxanthine) is found together with caffeine in tea plants. Both compounds have diuretic effect. Theophylline has bronchodilatant effects and is an active component of antiasthmatic medicines. Theobromine (3,7-dimethylxanthine) is the main xanthine component in cocoa bean. These compounds were used as lead structures for synthesis of modern medicine such as 1-(5-oxohexyl)theobromine, which is a potent inhibitor of TNF-α, decreases blood viscosity and is prescribed for patients suffering from tinnitus. *N*-methyladenine, *N,N*-dimethyladenine (Figure 11) and methylated adenosines are regular components of transfer-RNA (tRNA) (Rosemeyer, 2004).

A relatively wide range of simple methylated purine derivatives was isolated from marine organisms as secondary metabolites with no obvious benefit for the producing organisms, some of these compounds exhibited pharmacological activity. Herbipolin (7,9-dimethylguanine with betaine structure, Figure 11) was isolated from giant silica sponge and it is a representative of purines carrying several methyl groups on the imidazole ring (Rosemeyer, 2004). 1,3-dimethylisoguanine (Figure 11) is active against human ovarian cancer cells. 3,7-dimethylisoguanine has antibacterial potential (Lindsay et al., 1999b; Rosemeyer, 2004). 1-methylherbipolin (1,7,9,-trimethylguanine) is an inhibitor of collagenase (Yagi et al., 1994). 1,3,7-trimethylguanine and 1,9-dimethylhypoxanthine (Figure 11) have also been isolated from marine sponges (Capon et al., 2000).

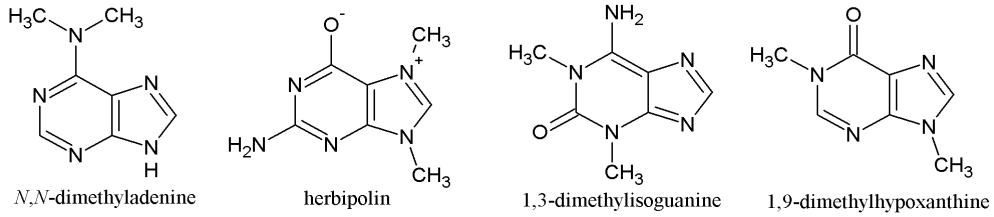


Figure 11. Chemical structure of *N,N*-dimethyladenine, herbipolin, 1,3-dimethylisoguanine and 1,9-dimethyl-hypoxanthine.

Heteromines A, B and C (Figure 12) similar to herbipolin are used to treat certain tumours in traditional Taiwan medicine (Lin et al., 1996). The group of alkaloids agelasines (7,9-dialkylpurinium salts) is structurally related to herbipolines, the diterpenoid side chain is attached to *N*7 of adenine nucleus. Agelasines (Figure 12) are associated with antimicrobial and cytotoxic activity, contrastive responses of the smooth muscles and inhibition of sodium-potassium pump (Na,K-ATPase) (Nakamura et al., 1984; Fathi-Afshar et al., 1988; Vik et al., 2007).

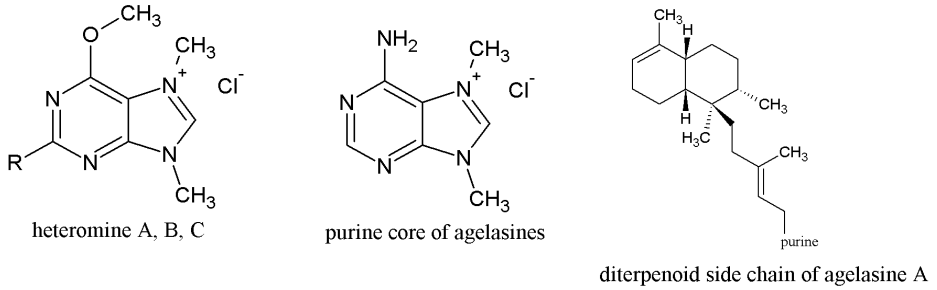


Figure 12. Chemical structure of heteromines (R = -NH_2 (A), -NHMe (B), -NMe_2 (C)) and agelasines.

8-oxopurines were also isolated from marine organisms and are mainly thought to be the main products of oxidative damage to DNA purine bases. This group is represented by 6-methoxy-7-methyl-8-oxoguanine, phosmidosine and aplidiamine with labile *N*-glycosidic bond of a purine 2'-deoxyribonucleoside (Itaya et al., 1999; Lindsay et al., 1999a).

Only a few naturally occurring methylated purine nucleosides have been detected besides the methylated adenosines. 7-methylguanosine is the 5'-terminal nucleoside in mRNA and has an important role in mRNA splicing procedures (Rosemeyer, 2004). Doridosine (1-methylisoguanosine) extracted from the marine sponges acts as an A1-receptor agonist, binds to the human benzodiazepine receptor and has blood pressure reducing and heart rate slowing properties (Fuhrman et al., 1980).

_N_-alkylated purines and purine nucleosides

Besides methylated purines a large number of higher alkylated purines can be found in the nature. The most important of them is the group of phytohormones cytokinins described in more details in another section of this thesis. Discadenine (Figure 13) isolated from the slime mold *Dictyostelium discoideum* also possesses cytokinin activity and is an inhibitor of slime mold spore germination (Obata et al., 1973; Abe et al., 1976).

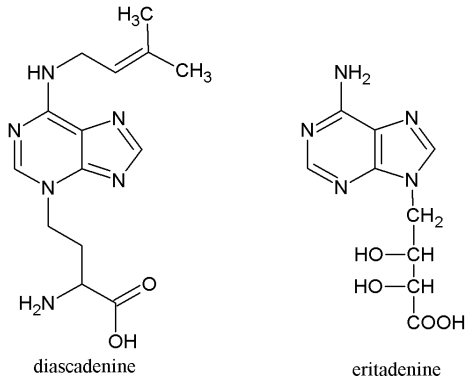


Figure 13. Chemical structure of discadenine and eritadenine.

*N*9-alkylated adenines were isolated from the Japanese Shii-take mushroom *Lentinus edodes*, which is consider as a life elixir. Eritadenine (Figure 13) showed hypocholesteremic effect and improves the cholesterol transport along the blood vessels. *N*9-(3-oxoprop-1-enyl)purines as well as *N*3-(3-oxoprop-1-enyl)pyrimidines are cytotoxic to a variety of tumour cells. Other example of higher-alkylated purine system is higher-alkylated adenine alkaloids (Rosemeyer, 2004). Alkaloid triacanthine (6-amino-3-(γ,γ-dimethylallyl)purine) was the first detected and isolated from the tree *Gleditsia triacanthos* (Leonard et al., 1962). A group of interesting tricyclic purine alkaloids asmarines contain [1,4]diazepino[1,2,3-*gh*]purine skeleton and was isolated from the Red Sea sponge *Raspailia*. Asmarines exhibited significant toxicity towards various tumour cell lines (Yosief et al., 1998; Ohba et al., 2002).

_N_-glycosylated purines

N-glycosylated purines are mainly represented by natural purine nucleoside antibiotics and antimycotics. Many of them were isolated a long time ago, but the structure was fully elucidated only recently. Some of these compounds were used as leading structures for the development of new chemotherapeutic agents from the group of purine antimetabolites, which are characterised by extraordinary structural features. The sinefungins (Figure 14) are active against fungi, viruses, and some tumour cell lines. Compounds of this group contain an ornithine side chain at the 5'-deoxyadenosine nuclei. The side chain may simulate the *S*-adenosylmethionine and hence inhibit methyltransferase reactions (Boeck et al., 1973; Fuller et al., 1978). Amipurimycin (Figure 14) (Harada et al., 1977) and miharamycin contain 2-aminopurine which is otherwise not found in nature (Rosemeyer, 2004).

The sugar moiety was recognized as heptuluronic acid (Fairbanks et al., 1995). Septacidin (Figure 14) is unusually glycosylated by aminoaldoheptose with a coupled branched fatty acid at the exocyclic amino group of adenine (Fasman, 1975). Agrocin (Figure 14) is the only known *N6*-phosphoramidate with bound glucofuranose and is an inhibitor of the induction of crown gall tumours by *Agrobacterium tumefaciens* (Roberts et al., 1977; Suhadolnik, 1979).

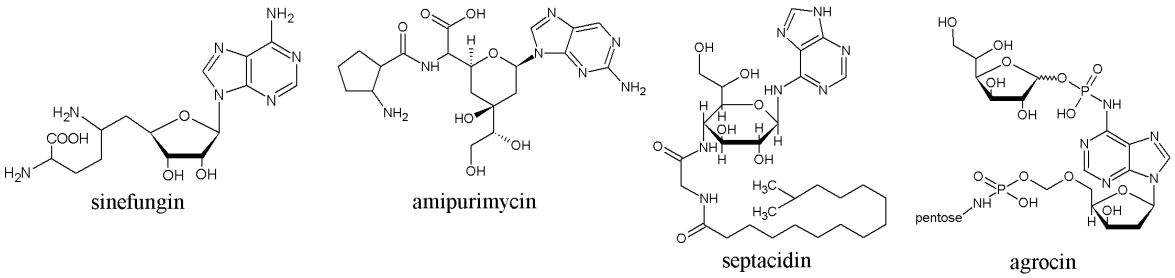


Figure 14. Chemical structure of sinefungin, amipurimycin, septacidin and agrocin.

Crotonoside (isoguanosine) was isolated from *Croton tiglium* and later from the butterfly *Prioneris thestylis* and from the slug *Diaulula sandiegensis* (Fuhrman et al., 1981; Rosemeyer, 2004). Isoguanosine and 2-aminoguanosine are the only known natural guanine nucleoside antibiotics. Di- and triphosphates of isoguanosine strongly inhibit glutamic acid dehydrogenase (Mantsch et al., 1975).

The series of purine nucleoside antibiotics acting as inhibitors of protein biosynthesis can be found in nature with following examples: puromycin, nucleocidin, ascamycin and its dealanyl derivative, trachycladine A, kumusine (Rosemeyer, 2004). Cordyceptin (3'-deoxyadenosine) inhibits the phosphoribosylpyrophosphate amidotransferase and hence acts as a feedback inhibitor of purine nucleotide biosynthesis (Rottman et al., 1964). Thuringiensin (Figure 15) is a potent insecticide that acts as an inhibitor of DNA-dependent RNA polymerases (Šebesta et al., 1969a; Šebesta et al., 1969b).

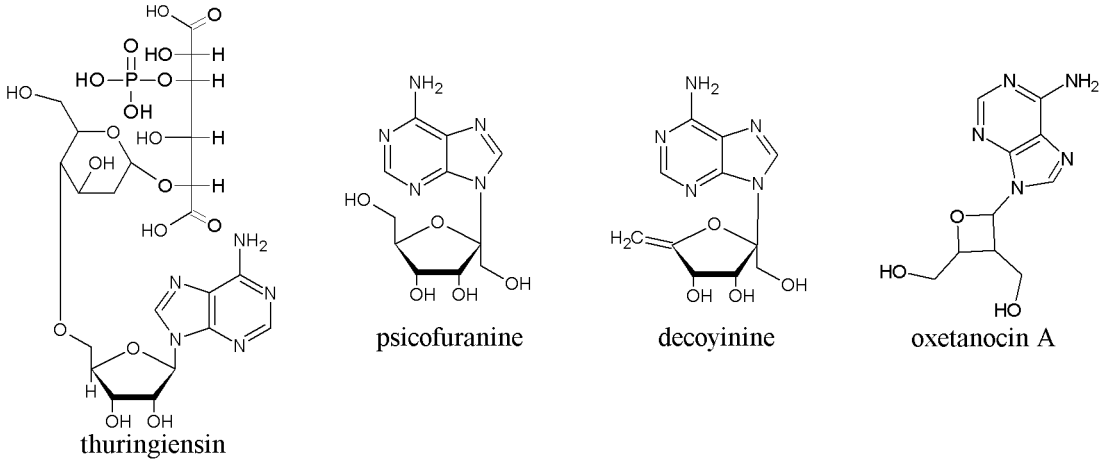


Figure 15. Chemical structure of thuringiensin, psicofuranine, decoyinine and oxetanocin A.

Spongosine (2-methoxyadenosine) represents one of the rare cases in which the 2'-deoxyribonucleoside is present (Suhadolnik, 1979). Nebularine (9-2'-deoxy-β-ribofuranosylpurine) is a highly toxic nucleoside from *Agaricus nebularis* which exerts its toxicity as a very potent inhibitor of adenosine deaminase (Suhadolnik, 1979; Evans et al., 1970; Brown et al., 1994). The only currently known ketose adenine nucleoside antibiotics found in nature are psicofuranine and decoyinine (Suhadolnik, 1979) (Figure 15), which act as non-competitive inhibitors of xanthosine-5'-monophosphate aminase and therefore inhibitors of GMP synthesis. Oxetanocin A (Figure 15) is

the natural tetrose nucleoside, which exhibits both antibiotic and anti-HIV activity (Hoshino et al., 1987; Hambalek et al., 1990).

The group of griseolic acids (Figure 16) isolated from *Streptomyces griseoaurantiacus* contain a bicyclic 1,4:3,6-dianhydrohexitol derivative as sugar moiety and show inhibitory activity against cyclic nucleotide phosphodiesterase (Nakagawa et al., 1985; Pickering et al., 1997). A powerful herbicide herbicidin B (Figure 16) is substituted by the more complicated tricyclic undecose as sugar substituent (Ichikawa et al., 1999). The adenophostines (Figure 16) are potent agonists of the inositol-1,4,5-triphosphate receptor and possess a high potential as regulators of many physiological processes (Takahashi et al., 1994; Rosemeyer, 2004). One big disappointment in purine nucleoside antibiotic research is the fact that these compounds are too toxic and none of them exhibits an acceptable therapeutic gap to be used clinically. One of the most toxic non-protein substances is a reduced purine derivative saxitoxin (Figure 16), which is produced by a group of dinoflagellates (*Alexandrium cantenella*). It is a potent and extremely selective sodium channel blocker having no effect on potassium or calcium channels. The danger could be in poisoning of humans via the food chain resulting in a syndrome called paralytic shellfish poisoning (PSP) (Schantz et al., 1966; Scheuer, 1977).

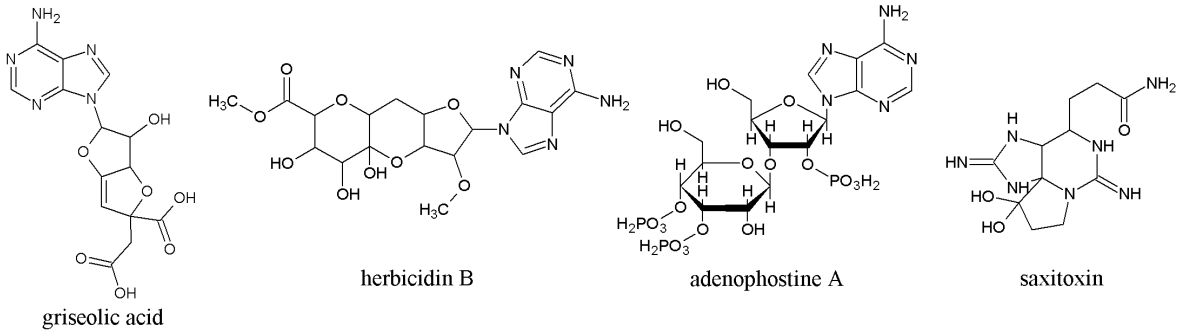


Figure 16. Chemical structure of griseolic acid, herbicidin B, adenophostine A and saxitoxin.

3.3.2.6 Synthetic purine derivatives

Synthetic purine analogues have been prepared and studied by many research groups in an attempt to find new potent compounds for cancer therapy and as agents against viral, bacterial and fungal diseases. Also synthetic purine analogues can be incorporated in normal cell metabolism and then they act like false metabolites called antimetabolites. Particular groups of antimetabolites differ in the type of chemical modification of the basic purine core. Some examples of selected synthetic purine derivatives and their biological and pharmacological effects are given in this section. The focus is given to *N9*-substituted purines, which were also studied in the experimental section of this thesis.

The group of acyclic analogues exhibit high biological activity and significant resistance against chemical and biological degradation due to absence of a glycosidic bond. The flexibility of the acyclic chain enables the compounds to adapt with suitable conformation for interaction with an active site of the enzyme or receptor. For this reason several α-branched acyclic adenosine analogues with side chain attached at *N*7- or *N*9- position were synthesised and can be used as starting materials for other acyclic adenosine analogues (Hocková et al., 1999; Marek et al., 2002). *N*9-polymethylene derivatives of adenine and hypoxanthine substituted with functional groups in the ω-position of the alkyl substituent were prepared and the effects on the HIV reverse transcriptase and human DNA topoisomerase I, which are the targets of most of the known antiviral and antitumour drugs, were examined (Makinsky et al., 2001). *N*9-pentylarginin-hypoxanthine derivative increases immune functions both *in vitro* and *in*

vivo experiments (De Simone et al., 1993). 3,9-diemthylhypoxanthines (Itaya et al., 1977; Itaya et al., 1985) and 7- and 9-methoxymethyl-hypoxanthines, as well as bismethoxymethyl-hypoxanthins were synthesized (Madre et al., 1986). The activity of hypoxanthine, adenine, and 6-chloropurine substituted at the *N*9-position with alkyl and cycloalkyl groups against human epidermal carcinoma cell line (either sensitive to 6-mercaptopurine or resistant to 6-mercaptopurine) were tested and it was found that some of the tested compounds showed an inhibitory effect on the cell lines used (Kelley et al., 1962).

The reason for synthesis of acyclic nucleoside analogues was the fact that a guanosine analogue, acyclovir, was found to be active against herpes viruses (especially HSV-1 and HSV-2) and is used to treat virus infections. Ganciclovir is other acyclic guanosine anologue active against human cytomegalovirus infection. 6-(alkylthio)purines and 9-alkyl-2-aminopurine-6-thiols expressed activity against adenocarcinoma and leukaemia cells. Some alkenyl purines, such as 2-penten-1-yl and 3-methyl-2-buten-1-yl derivatives of 5-aminothiazolo(4,5-d)pyrimidine-2,7,-dione, have been found to be more active against cytomegalovirus than ganciclovir in similar experiments *in vitro* (Rao et al., 1995). 4-aminopyrazolo[3,4-d]pyrimidine exhibited significant inhibition of TNF-α production in the human monocytic leukaemia cells together with poor toxicity profile (Rao et al., 1997). Serotonine-like 9-substituted hypoxanthine derivatives possess unexpected pharmacological effects such as inhibition of the monoamineoxidases activity, positive effects on obesity, increase of the serum cholesterol and the level of high density lipoprotein (Glasky, 1999). The synthesized 9-(3-oxoprop-1-enyl) derivatives of adenine and guanine displayed high cytotoxicity towards a variety of tumour cell lines and inhibited macromolecular synthesis in cultured human cervical carcinoma cells (HeLa). Also this group of compounds can be further studied as a new class of cytotoxic agents (Johnson et al., 1984).

3.3.3 Purine-type plant hormones

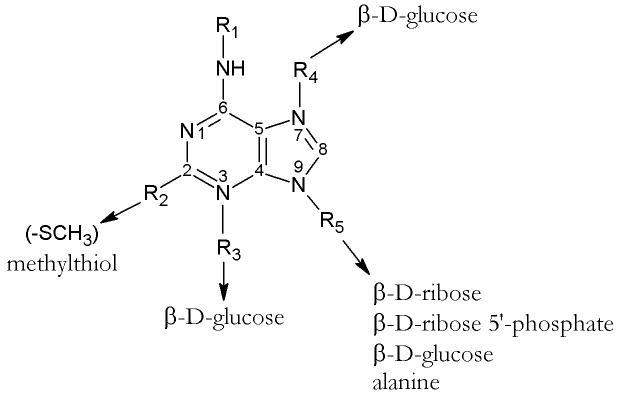
Phytohormones are natural organic compounds with an ability to affect physiological processes in plants at concentration far below those of nutrients and vitamins. Phytohormones do not fulfil the same requirements as mammalian hormones, but the essentiality and crucial role in controlling growth and development are common. Auxin was the first identified plant hormone, isolated as a compound which caused coleoptile cell elongation. Indole-3-acetic acid (IAA) is the major natural auxin and produces a response at a distance from the place of synthesis (Hooykaas et al., 1999; Davies, 2004). To date, plant hormones have been studied in different plant species, for instance, in *Arabidopsis thaliana*, which is used as a model plant in molecular biology and plant physiology. *Arabidopsis* is a small, spring, annual, flowering plant with a relatively short and rapid life cycle (six weeks). The genome of *A. thaliana* is one of the smallest plant genomes and it became the first completely sequenced plant genome (Arabidopsis Genome Initiative, 2000). These properties are advantageous for research and *A. thaliana* has become the model organism of choice for research in plant biology, plant molecular biology, and genetics (Meyerowitz et al., 1994; Meinke et al., 1998; Bomblies et al., 2010).

Purine-type plant hormones, cytokinins (CKs), can be briefly characterized as a group of phytohormones that promote a cell growth, division and differentiation in plant tissue cultures in the presence of another plant hormone, auxin (Davies, 2004). Nowadays, this group includes a large set of natural and synthetic compounds which are adenine or phenylurea derivatives. CKs are of great interest to many scientists and both natural and synthetic compounds exhibiting an cytokinin activity are widely studied. The first described cytokinin, kinetin, was isolated by Skoog and Miller in 1955

from autoclaved products of herring sperm DNA (Miller et al., 1955) and is present in human urine (Barciszewski et al., 2000). Zeatin was the first isolated plant cytokinin from *Zea mays* (Letham, 1963). Its *trans*- and *cis*-isomers differ in their biological activity and function (Spiess, 1975). Isopentenyladenine was isolated as the free base from *Corynebacterium fasciens* (Hall et al., 1971). Interestingly, CKs have been previously found also in animals, e.g. in the salivary glands of the moths *Stigmella argyropeza* and *S. argentipedellacose* (Rosemeyer, 2004). Isopentenyladenine is an atypical base present in the tRNA of mammals, which is released into the cytosol and body fluids as a result of tRNA turnover (Chheda et al., 1972; Schram, 1998).

3.3.3.1 Chemical structure

The naturally occurring cytokinins are adenine derivatives which can be classified according to the configuration of their side chain at the *N*6-terminus as either isoprenoid or aromatic cytokinins. Both are naturally occurring (Figure 17). Small variations in side chain structure such as the presence of hydroxyl groups occur in both CK type, affect the interaction with CK receptors and signify the functional specificity (Mok et al., 2001; Sakakibara, 2006).



Structure of CKs	CK-type	R_1	R_6
	*N*6-isopentenyladenine (iP)		---
	trans-zeatin (tZ)	OR_6	β-D-glucose β-D-xylose
	cis-zeatin (cZ)	OR_6	β-D-glucose
	dihydrozeatin (DHZ)	OR_6	β-D-glucose β-D-xylose
	*N*6-benzyladenine (BA)		---
	ortho-topolin (oT)	OR_6	β-D-glucose
	meta-topolin (mT)	OR_6	β-D-glucose
	para-topolin (pT)	OR_6	β-D-glucose

Figure 17. Cytokinins and their conjugates with sugars, sugar phosphates, and others, *O*-glycosylation of the side chain (Sakakibara, 2006; Novák et al., 2008). Studied cytokinin derivatives are displayed in Supplement III.

Cytokinins with isoprenoid side chain are classified into four basic molecules: N6-isopentenyl-adenine (iP), *trans*-zeatin (tZ), *cis*-zeatin (cZ) and dihydrozeatin (DHZ) (Kamada-Nobusada et al., 2009). Especially those with an unsaturated *trans*-hydroxylated N6-side chain, such as tZ, iP and their

sugar conjugates, are by far the most represented. DHZ with a saturated side chain has been identified in many species, while cZ is generally a minor component (Mok et al., 2001; Sakakibara, 2006).

Kinetin (K) and *N*6-benzyladenine (BA) are the representatives of aromatic cytokinins with ring substitutions at the *N*6-position of adenine nucleus. *Meta*-topolin (mT) and *ortho*-topolin (oT) are hydroxylated forms of BA which occur naturally, together with their nucleosides, nucleotides, and *O*-glucosides (Strnad, 1997). Their methoxy-derivatives (*ortho*-methoxytopolin, *meta*-methoxytopolin) as well as BA were detected only in some plant species (Horgan et al., 1973; Ernst et al., 1983; Nandi et al., 1989; Strnad, 1997; Baroja-Fernández et al., 2002; Hauserová et al., 2005).

The third group of phenylurea derivatives represents synthetic highly active cytokinins, not occurring naturally in plant tissues. Diphenylurea was the first phenylurea derivative with cytokinin activity discovered. Although it was first detected in liquid coconut endosperm (Shantz et al., 1955), it is now considered to be a contaminant from prior chemical analysis (Mok et al., 2001). The discovery was followed by synthesis of a number of potent analogues such as *N*-phenyl-*N*′-[2-chloro-4-pyridyl]urea and thidiazuron with cytokinin activity and stability exceeding zeatin. The major metabolites are glucosyl derivatives (Shudo, 1994; Mok et al., 2001).

3.3.3.2 Natural occurrence in plants

Cytokinins appear in plants mainly as free bases, nucleosides (ribosides), glycosides (*O*- and *N*-glucosides) and nucleotides (ribotides) and are generally present at very low concentrations (pmol·g^{-1} fresh weight) (Hauserová et al., 2005). The occurrence, distribution and variation of individual cytokinins depend on plant species, tissue, and developmental stage. The group of isoprenoid cytokinins is the most abundant. It is not clear yet if the distribution of aromatic cytokinins is limited to only a few species or if they are more ubiquitous in plants (Mok et al., 2001). Although CKs are probably present in every tissue and they have been found in roots, stems, leaves, flowers, fruits and seeds, traditionally the root tips were considered to be the main site of cytokinin biosynthesis (Letham et al., 1983). However, according to recent research, the roots are not the only place of cytokinin biosynthesis in the plant (Miyawaki et al., 2004; Kudo et al., 2010).

3.3.3.3 Biosynthesis and metabolism

The molecular mechanism of biosynthesis and the signal transduction of CKs have been elucidated recently due to the identification and characterization of key pathway genes encoding enzymes and proteins with control function in CK biosynthesis and signalling. On the basis of these findings a basic model for the CK biosynthesis pathway was proposed (Sakakibara, 2006; Hirose et al., 2008; Kamada-Nobusada et al., 2009; Werner et al., 2009; Kudo et al., 2010) and is shown in Figure 18.

The initial step in the biosynthesis of isoprenoid CKs is catalysed by two classes of isopentenyltransferases, adenosine phosphate-isopentenyltransferase (IPT) and tRNA-isopentenyltransferase (tRNA-IPT). IPT (Kakimoto, 2001) is responsible for the synthesis of iP- and tZ-type CKs (Miyawaki et al., 2006), when adenosine-5'-phosphates (AMP, ADP, or ATP) are *N*-prenylatated at the *N*6-terminus by dimethylallyl diphosphate (DMAPP) or hydroxymethylbutenyl diphosphate (HMBDP). DMAPP is synthesized via the methylerythritol phosphate pathway (MEP) and mevalonate pathway (MVA) located in the cytosol of eukaryotes. HMBDP is a metabolic intermediate of the MEP pathway, which is formed only in plastids of higher plants. The isoprenoid side chain of iP-and tZ-type CKs predominantly originates from the MEP pathway (Kakimoto, 2001; Sakakibara, 2006), whereas

a substantial portion of the cZ side chain in *Arabidopsis* is provided by the MVA pathway (Kasahara et al., 2004). The plant IPTs are believed to utilize predominantly DMAPP and ADP or ATP as precursors to form *N*6-isopentenyladenosine-5'-diphosphate (iPRDP) and *N*6-isopentenyladenosine-5'-triphosphate (iPRTP) as reaction products (Kakimoto, 2001; Hirose et al., 2008).

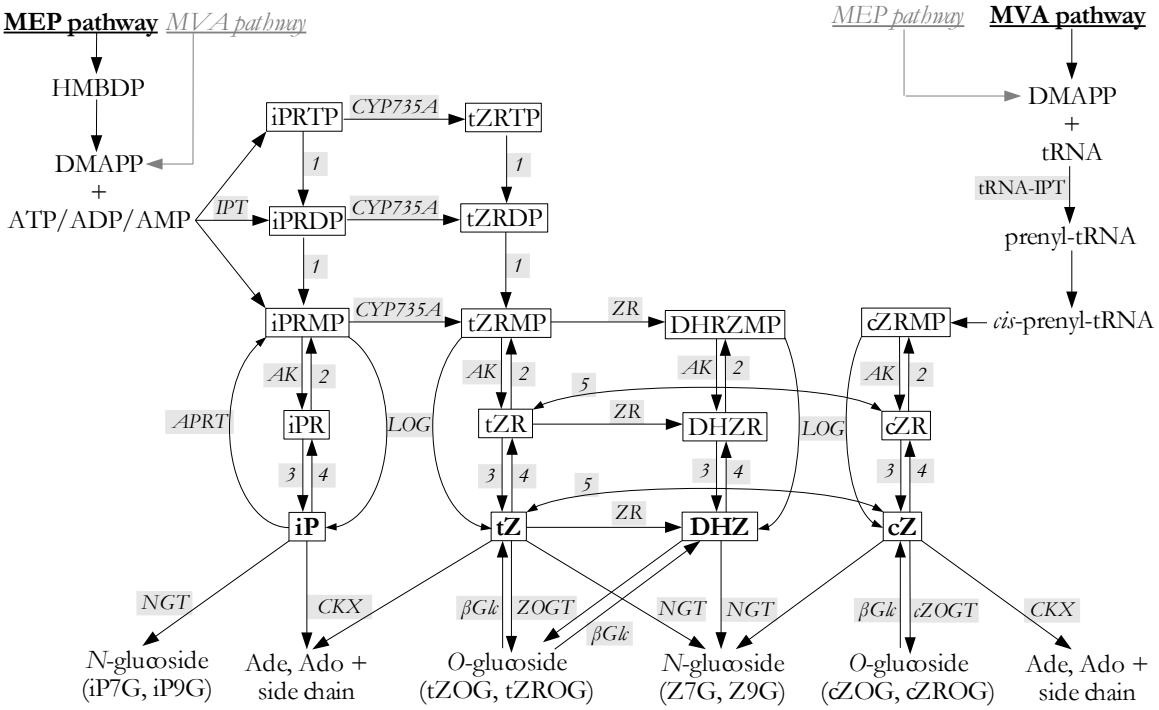


Figure 18. Current model of isoprenoid cytokinin biosynthesis pathways in *Arabidopsis* (Sakakibara, 2006; Hirose et al., 2008; Kamada-Nobusada et al., 2009; Werner et al., 2009). Abbreviations are defined in the text or as follows: 1, phosphatase; 2, 5'-ribonucleotide phosphohydrolase; 3, adenosine nucleosidase; 4, purine nucleoside phosphorylase; 5, zeatin *cis-trans* isomerase; tZRTP, *trans*-zeatin riboside-5'-triphosphate; tZRDP, *trans*-zeatin riboside-5'-diphosphate; iP7G, *N*6-isopentenyladenine-7-glucoside; iP9G, *N*6-isopentenyladenine-9-glucoside; Ade, adenine; Ado, adenosine; βGlc, β-glucosidase; t/cZOG, *trans*/*cis*-zeatin *O*-glucoside; t/cZROG, *trans*/*cis*-zeatin riboside *O*-glucoside; Z7G, zeatin-7-glucoside; Z9G, zeatin-9-glucoside.

The tZ-type compounds can be formed by two possible pathways, the iP nucleotide-dependent and the iP necleotide-independent (Sakakibara, 2006). In the iP nucleotide-dependent pathway, *trans*-hydroxylation of the side chain of iP nucleotides is catalysed by the cytokinin *trans*-hydroxylases (cytochrome P450 monooxygenases) CYP735As in *Arabidopsis* (Takei et al., 2004). In the iP nucleotide-independent pathway, tZ nucleotides are produced directly by IPT using a hydroxylated side-chain precursor HMBDP (Åstot et al., 2000). In summary, if DMAPP is a substrate for the IPT, the product is iP nucleotide, when HMBDP is taken as substrate, tZ is formed (Sakakibara, 2006). The cZ-type CKs are generated by tRNA-dependent pathway from tRNA species containing prenylated adenine core. The prenylation of tRNA using DMAPP from the MVA pathway is catalysed by tRNA-IPT. Subsequent hydrolysis of *cis*-hydroxy isopentenyl tRNAs is capable of forming cZRMP (Kasahara et al., 2004; Miyawaki et al., 2006). It was proven that the degradation of prenylated tRNA is the main pathway to supply cZ in *Arabidopsis* (Miyawaki et al., 2006).

CK nucleotides are converted to their active free-base form by two possible pathways. In the two step pathway the nucleotides are dephosphorylated to the ribosides (Chen et al., 1981a) and then

deribosylated to free bases (Chen et al., 1981b). The cytokinin nucleoside-5'-monophosphate phosphoribohydrolase (LONELY GUY, LOG) (Kurakawa et al., 2007) directly converts CK nucleotides to the free bases. Reversely, nucleotides can be formed by the phosphorylation of nucleosides catalysed by adenosine kinase (AK), or directly from free bases by the phosphoribosylation catalysed by adenine phosphoribosyltransferase (APRT). APRTs have broad substrate specificity and act both on CK bases and on adenine (Mok et al., 2001; Kamada-Nobusada et al., 2009).

The main enzymes of CK degradation are cytokinin oxidase/dehydrogenases (CKXs), which selectively catalyse the irreversible cleavage of unsaturated *N*6-isoprenoid side chains and convert active cytokinins to adenine. CK bases and nucleosides are sensitive, nucleotides are not susceptible. Different CKX gene expression, regulated by biotic and abiotic factors, contributes to specification of the catalytic properties of individual CKX enzymes and their localization and is important for regulation of cytokinin catabolism and functions (Galuszka et al., 2000; Bilyeu et al., 2003; Schmülling et al., 2003; Galuszka et al., 2005; Werner et al., 2006). Aromatic CKs and cZ are resistant to CKXs which recognize the double bond of the isoprenoid side chain (Mok et al., 2001; Sakakibara, 2006).

An important CK modification reaction is glycosylation, which has been observed at the hydroxyl group of the prenyl side chain of tZ, cZ, and DHZ (*O*-glucosides or *O*-xylosides), at the *N*3, *N*7 and *N*9 position of adenine core (*N*-glucosides) and at the ribose moiety of cytokinin nucleosides (Mok et al., 2001; Sakakibara, 2006). *O*-glucosylation is reversible and serves as an important reaction in regulating the level of active cytokinins. *O*-glucosides represent stable, inactive storage forms of CKs, because they are resistant to CKX attack, but they are ready to be converted to active aglycone by *β*-glucosidase (Brzobohatý et al., 1993; Sakakibara, 2006). The existence of *trans*- and *cis*-specific zeatin *O*-glycosyltransferases (ZOGTs) fortifies the precision of metabolic regulation (Mok et al., 2001; Sakakibara, 2006). *N*-glucosylation is catalysed by *N*-glucosyltransferases (NGT), which recognize quite a large number of adenine derivatives as substrates, and is irreversible. *N*-glucoconjugates are not efficiently cleaved by any of plant *β*-glucosidases (Entsch et al., 1979; Brzobohatý et al., 1993).

The highly specific zeatin reductase (ZR) catalyses the reduction of the *trans*-zeatin side chain to dihydrozeatin, which is resistant to cytokinin oxidases. This may consequently conserve CK activity, especially in tissues with high levels of oxidases (Martin et al., 1989). c*is*-Zeatin and *trans*-zeatin can be enzymatically interconverted by zeatin *cis-trans* isomerase (Bassil et al., 1993). This represent a possible conversion of the *cis*-isomer, derived from the indirect tRNA-dependent cytokinin biosynthesis pathway, to the active *trans*-zeatin and hence the contribution to the synthesis of tZ from cZ (Taller, 1994; Prinsen et al., 1997).

The biosynthetic and degradation pathway of aromatic cytokinins remains unknown. No enzymes mediating the substitution of adenine with a benzyl ring have been identified up to date. The enzymes and receptors of isoprenoid CKs recognize aromatic CKs. The glycosylation of aromatic CKs and their interaction with the cellular signalling system appear to be shared with isoprenoid CKs. A precursor suggested for topolin is the amino acid phenylalanine (Mok et al., 2001; Hirose et al., 2008).

De novo cytokinin biosynthesis is tissue and cell specific. The expression of key pathway genes is up-regulated or down-regulated by internal and external factors such as phytohormones including CKs themselves, auxin, and abscisic acid and by inorganic nitrogen sources (Nordström et al., 2004; Takei et al., 2001; Takei et al., 2002). The interdependent regulation of phytohormones is responsible for morphogenetic responses of plants to environmental stimuli (Sakakibara, 2006).

3.3.3.4 Biological activity in plants

Cytokinins play a crucial role in plant grow and development. They act at various sites in the plant body and coordinate plant functions as long-distance messengers as well as local paracrine signals. The action of CKs is often masked by interaction with other phytohormones, either synergistically or antagonistically (Mok, 1994; Galinha et al., 2009). In plants, CK free bases are considered to be the most active forms, which may be related to rapid uptake and high intrinsic activity. CK ribosides display almost the same biological effects as free bases, whilst CK nucleotides and glucosides show only weak or no activity (in bioassays based on bonding of substrate on CK receptors) (Spíchal et al., 2004; Yonekura-Sakakibara et al., 2004). The levels of active CK in plants are determined by the rate of their degradation, inactivating conjugation and release of free bases from their conjugates representing translocation or depot forms. Local CK composition in organs and tissues at particular stages of development may be more important than total CKs in plants (Mok et al., 2001; Sakakibara, 2006).

The three forms of CK are transported to the target cells differentially. Selective transport is based on the capability of plant cells to absorb CK bases and nucleosides, and on the accumulation of tZ-type CK in xylem and iP- and cZ-type in phloem (Sakakibara, 2006; Hirose et al., 2008). The major CK forms in the xylem are nucleosides (mainly tZR), which act as a root-to-shoot acropetal signal (Sakakibara, 2006; Hirose et al., 2008). Phloem CKs play role as a basipetal or systemic signal (Hirose et al., 2008). Free bases are present in xylem and phloem (Sakakibara, 2006). Transport of CK bases across the plasma membrane shares the same proton-coupled multiphasic transport systems with purine derivatives and nucleosides (Cedzich et al., 2008). Some members of the purine permease family with broad substrate specificity (Gillissen et al., 2000; Bürkle et al., 2003) and equilibrative nucleoside transporter family (Hirose et al., 2005; Hirose et al., 2008) appear to be involved in the selective transport of CK nucleosides (Kudo et al., 2010). The identification of CK receptors and detailed analysis of their ligand specificity led to a better understanding of the relative activity of different CKs (Kakimoto, 2003). At least three CK histidine kinase receptors (AHKs) were identified in *Arabidopsis* (Suzuki et al., 2001; Yamada et al., 2001). Structural modifications of the side chain or modifications of adenine moiety impute specificity of the CK-receptor interaction. CK bases are the primary ligands for receptors, sugar conjugates are less active or inactive (Spíchal et al., 2004; Yonekura-Sakakibara et al., 2004; Mizuno et al., 2010).

CKs, in conjunction with auxin, regulate the plant cell proliferation and differentiation (Galinha et al., 2009; Nieuwland et al., 2009), control essential processes in plant growth and development such as delay of senescence, apical dominance, root and shoot branching, chloroplast development and phyllotaxis (Mok, 1994; Werner et al., 2001; Hirose et al., 2008). Cytokinins control balance between shoot and root, transport nutritional signals such as nitrogen nutrient signal from roots to shoot (Takei et al., 2001; Takei et al., 2002) and regulate reproductive competence (Ashikari et al., 2005). It is also known, that CKs participate in the maintenance of meristem function (Werner et al., 2003; Nieuwland et al., 2009) and in the modulation of metabolism and morphogenesis in response to environmental stimuli (Werner et al. 2001; Sakakibara 2006, Hirose et al., 2008).

3.3.3.5 Synthetic cytokinin derivatives and benefits for humans

Both natural CKs and their synthetic analogues have been tested in various assays for cytokinin activity, cyclin dependent kinase (CDK) inhibitory activity and anticancer activity (among others) with the aim of discovering useful substances for further CK research, and with applications in agriculture

or medicine. Cytokinins can be conceivably used to optimize agronomic characteristics (Mok et al., 2001; Eckardt et al., 2003) and for further improvement of agricultural outputs (Kudo et al., 2010). Besides cytokinin activity, the natural cytokinins can affect growth and differentiation of human cells. They were found to inhibit various protein kinases such as CDKs, which play an essential role in the regulation of the cell division cycle and their alterations are associated with human tumour development (Veselý et al., 1994; Vermeulen et al., 2002a). The proapoptotic and anticancer activities of natural CKs have been demonstrated in a variety of cultured tumour cell lines (Voller et al., 2010).

During recent decades, many compounds derived from the natural cytokinin structures have been prepared and intensively studies. Screening of chemically synthesized aromatic cytokinin-derived inhibitors of CDK activity led to the discovery of olomoucine (6-(benzylamino)-2-[(2-hydroxyethyl)amino]-9-methylpurine), (*R)*-roscovitine (6-(benzylamino)-2(*R*)-[[1-(hydroxymethyl)-propyl]amino]-9-isopropylpurine), bohemine, purvalanols, olomoucine II and other *C*2-, *C*6-, *N*9-trisubstituted adenines, which exerted different levels of inhibitory activity on CDK1 and CDK2 (Havlíček et al., 1997; Meijer et al., 1997; Kryštof et al., 2002; Vermeulen et al., 2002a; Vermeulen et al., 2002b; Kryštof et al., 2005). The discovered antiproliferative and proapoptotic effects of CK derived CDK inhibitors suggest that these compounds are a perspective class of cytostatic agents and that they may find an application in the chemotherapy of cancer, as reviewed recently (Diaz-Padilla et al., 2009; Wesierska-Gadek et al., 2009; Galons et al., 2010; Kryštof et al., 2010). (*R)*-roscovitine (CYC202, Seliciclib) exhibited the best profile both *in vitro* studies and *in vivo* pharmacokinetic behaviour (Fischer et al., 2005; Raynaud et al., 2005) and showed highly selective inhibitory effects for rapidly proliferating cells in comparison with normal ones (McClue et al., 2002). As a consequence, (*R*)-roscovitin was chosen as a lead CDK inhibitor drug, which selectively inhibits multiple cyclin-dependent kinases (CDK2/E, CDK2/A, CDK7 and CDK9). Nowadays, it is being evaluated in the APPRAISE trial (Phase 2b randomized, double-blinded, placebo-controlled study) as a third line treatment in patients with non-small cell lung cancer, and in a randomized Phase 2 study as a single agent in patients with nasopharyngeal cancer (Cyclacel, 2010a; Cyclacel, 2010b; Cyclacel 2010c).

A group of synthetically prepared *N*6-benzyladenine and *N*6-benzyladenosine derivatives with different phenyl ring substitutions were found to be very active in different cytokinin bioassays, but to a much lower extent they were able to interact with the cytokinin receptors. Some of the prepared derivatives displayed high cytotoxic activity against various cancer cell lines. This effect may be caused by the inhibition of CDK activity in these cells. The results from the antitumour assay and human recombinant CDK2 inhibition assay were helpful to design novel trisubstituted compounds potent as CDK inhibitors and inducers of apoptosis in cancer cells (Doležal et al., 2006; Doležal et al., 2007). Kinetin (6-furfuryladenine) has been intensively studied as a potent anticancer, antioxidant and antiaging natural substance (Minorsky, 2003; Griffaut et al., 2004; Barciszewski et al., 2007; Rattan, 2008). Nowadays, the improved chemical modification has a significant growth-stimulating and antisenescence activity towards human skin fibroblasts and is commercially distributed under the name Pyratine™ by Senetek PLC as antiaging skin care (Pyratine-6, 2010; PyratineXR, 2010).

3.4 Phytochemical methods

The basic challenging steps in investigation of pure natural products are the isolation of individual substances from the plant matrix, chemical analysis and identification of the compound, as well as testing its biological activity. The phytochemical approach to the isolation and analysis of a plant metabolite can vary according to a character of plant material, group of targeted compounds as well as availability of methods and instruments. The isolation procedure has been described in many publications and articles both from the general point of view (Starmans et al., 1996; Sarker et al., 2006; Colegate et al., 2008) and from the point of particular groups of plant metabolites (Hooykaas et al., 1999; Davies, 2004).

The procedure leading from the plant material to the elucidated structure will be explained in this theoretical part in relation to the protocols and methods used in the experimental part of this thesis. Preparation of the plant material and extraction procedure may be assigned as the first step of compound isolation. Subsequently, the crude extract is cleaned-up and fractionated to isolate a group of structurally related compounds, which are further separated to obtain pure individual compounds. Finally, the individuals are identified and/or quantified.

3.4.1 Extraction

Extraction from plant material is a procedure in which different solvents with ability to extract plant components are used under specific conditions such as the time and temperature. The purpose of extraction is to remove desired compounds from the plant material to the solvent. The selection of an optimal solvent can maximize yields of target compounds and minimize the extraction of unwanted balast. The number of possible solvents is very broad, starting with polar solvents such as water and methanol on one side, ending with non-polar solvents such as hexane and heptane on the other side. The extraction can be further improved by the use of a solvent mixture and specific extraction conditions. The chemical character and solubility of the compounds of interest are the main factors influencing the choice of solvent. The quotation "similia similibus solvuntur" (like dissolves like) should be kept in mind, polar compounds can be dissolved and extracted in polar solvents and non-polar compounds in non-polar solvents.

Prior to the extraction procedure, antioxidants may be added to prevent enzymatic reactions and decomposition of analytes. Internal (stable isotope) standards (^{2}H, ^{15}N, ^{13}C) are often used as internal tracers to control losses at each stage of extraction and purification and to increase the precision during quantification of isolated target compounds. It may be useful to perform several trial extractions using different solvents and conditions and then compare total yield of extract or target compounds or intensity of biological activity to see which method gives the best results. The plant material used for extraction can be fresh, air-dried of freeze-dried, and should be well homogenized in mill or with a mortar and pestle to improve the efficiency of extraction. The simplest way of extraction in case of small amounts of plant material (0.01-10 g) is maceration. It is recommended to repeat the extraction with a new portion of fresh solvent. The plant material should be almost completely exhausted after three solvent changes. Then the collected supernatants are evaporated if necessary prior to further purification (Sarker et al., 2006; Colegate et al., 2008).

3.4.2 Fractionation and purification methods

The target analytes are extracted from the plant material usually as a mixture of compounds with similar physical and chemical properties and they need to be separated to individuals and then identified. Isolation of a pure compound from crude extract or fraction is often time consuming, expensive and requires several separation steps involving different chromatographic techniques. Generally, conventional methods such as low pressure column chromatography (using silica gel, polyamide, Sephadex LH-20) and preparative reversed-phase high pressure liquid chromatography are the most often used to fractionate crude extract and isolate pure substances (Cannel et al., 1998; Colegate et al., 2008).

3.4.2.1 Solid-phase extraction

Solid-phase extraction (SPE) is a very efficient method for isolation, purification and concentration of analytes from relatively large volumes of liquid mixture. SPE is based on affinity of solutes dissolved or suspended in a liquid for a solid sorbent. It can offer initial fractionation of a crude extract, separation of target analytes from other substances present in extract and also sample clean-up and concentration prior to high performance liquid chromatographic separation. SPE is more efficient than liquid/liquid extraction. The selection of the most suitable SPE product for target analytes depends on the physical and chemical properties of analytes (Hennion, 1999; Poole et al., 2000; Sarker et al., 2006; Colegate et al., 2008).

SPE extraction cartridges and disks are produced by many companies and they are commercially available in a wide variety of chemistries, sorbents, retention mechanisms and sizes. The basic types of stationary phases are the reversed-phase, normal-phase, ion-exchange and mixed-mode phase, which differ in their retention mechanisms. The majority of stationary phases are based on silica with bound specific functional groups, including hydrocarbon chains of variable lengths, e.g. C8, C18 (reversed-phase), quarternary ammonium or amino groups (anion exchange) and sulfonic acid or carboxyl groups (cation exchange) (Hennion, 1999; Poole et al., 2000; Sarker et al., 2006; Colegate et al., 2008). DEAE-Sephadex (cross linked dextran) can also be used as SPE sorbent. It combines the advantages of cellulose and resin ion exchangers, the diethylaminoethyl (DEAE) functional group remains charged and maintains a high capacity over the pH range 2-9 ensuring a weak basic anion exchange retention mechanism (DEAE, 2007).

Except the chemically modified silicas, highly cross-linked copolymers and functionalized copolymers are available in different functionalities such as mixed-mode sorbents, restricted access matrix sorbents, immunosorbents and molecular imprinted polymers (Hennion, 1999). More modern copolymer-based stationary phases overcome some of the disadvantages of the traditional silica-based sorbents. The copolymer-based sorbents bring new benefits such as high retention and capacity for a wide spectrum of analytes. The desired adsorption properties of these sorbents are kept also when the sorbent pores run dry, whilst the retention of analytes by classical silica-based sorbents in this case is reduced and the resulting recovery is very poor. The general procedure consists of conditioning and equilibration of the SPE sorbent and subsequent loading the liquid sample (extract dissolved in a weak solvent) onto sorbent. Then balast components are washed with weak solvent, the desired analytes are eluted with strong solvent and the eluate is evaporated to dryness (Waters, 2010a).

3.4.2.2 Immunoaffinity extraction

Immunoaffinity extraction (IAE) is a highly effective type of SPE, which enables a specific isolation of analytes of interest from the complex liquid sample. The method is based on a specific molecular recognition and a selective, highly affinity antibody-antigen interaction. Specific antibodies against the target molecules are immobilized to a solid support and bind analytes by specific non-covalent interactions. After washing non-bound compounds away, the captured analytes are released from the interaction with antibodies. IAE provides selective sample enrichment and is useful in the field of biomedical and biological analysis where co-extraction of analytes at trace concentrations with interfering substances at much higher concentration is a serious problem (Yarmush et al., 1992; Hage, 1999; Hennion et al., 2003). IAE can be performed in different ways; as immunoaffinity extraction in a batch configuration (Hauserová et al., 2005), or immunoaffinity chromatography (IAC) using a column arrangement (Novák et al., 2003). Immunoaffinity extraction was developed for different groups of plant metabolites such as plant hormones occurring at very low levels in plants, where a highly selective purification step is necessary (Novák et al., 2003; Hradecká et al., 2007; Pěnčík et al., 2009).

Immuno-based extraction sorbent can be characterised by several parameters which need to be controlled and optimised when developing a new purification method. The bonding density of the immunosorbent is defined by the number and density of antibodies linked to the surface of the sorbent, and is usually expressed in $mg \cdot ml^{-1}$ of sorbent bed or $mg \cdot g^{-1}$ of sorbent. The value of this parameter determines the future antigen-binding capacity. The capacity corresponds to the total number of accessible binding sites. The specificity and affinity of the antibodies used determines the group of analytes which an antibody can bind, and is usually not the same towards different members of the analyte group. The stronger the affinity of the immobilized antibodies for a particular analyte, the higher is the extraction recovery for a given sample volume. Extraction recovery through the purification procedure gives information about the specificity of antibodies and is determined with help of a mixture of labelled standards added in exact amount to the sample. From the amount of standards added and yielded after the IAE, the percentage recovery is calculated (Novák et al., 2008).

Cross-linked reactivity of antibodies ensures that all compounds with the same binding epitopes can be bound, extracted from the liquid sample and then separated by analytical techniques. A loss of analytes can occur due to the overloading of the capacity or due to an insufficient retention. The design of antibodies defines the potential of immunosorbent (Hennion et al., 2003). Polyclonal and monoclonal antibodies differ in their interaction with antigens. Polyclonal antibodies are a mixture of many antibody varieties, each responding to different epitopes by slightly different mechanism. Slightly different elution conditions are required to remove all the antigen molecules from polyclonal antibodies. Monoclonal antibodies always bind one specific epitope by the same combination of non-covalent bonds and the effective elution can be achieved by one type of elution solvent.

Appropriate buffer conditions for binding and elution of antigen from antibody vary and also need optimisation. Antibody-antigen binding is usually most efficient in aqueous buffers at physiological pH and ionic strength such as phosphate-buffered saline (PBS), subsequent elution can be achieved by the change in pH or in the ionic state to disrupt the binding interaction without irreversibly denaturing or inactivating antibodies or antigens (Optimize, 2004). The already developed basic method for IAE of cytokinins (Vaňková et al., 1998; Novák et al., 2003) was modified and applied in this thesis.

3.4.2.3 High performance liquid chromatography

High performance liquid chromatography (HPLC), sometimes called high pressure liquid chromatography, is now one of the most powerful tools in preparative and analytical scale separations, identifications, and quantifications of soluble analytes present in complex samples. The method is used for compounds that are not volatile, often thermolabile and not suitable for gas chromatography (GC). The components to be separated are selectively distributed between two immiscible phases: a mobile phase, which is flowing through the column, and a stationary phase inside the column. The chromatographic process can be described as a series of repeated sorptions and desorptions during the movement of analytes along the stationary phase. The separation is due to difference in distribution of the individual analytes between the mobile phase and stationary phase (Niessen, 1999). In classical phytochemical approach of isolation of new natural compounds also low pressure column liquid chromatography and medium pressure liquid chromatography are used to separate components in a crude plant extract or in an isolated fraction (Siems et al., 1996; Amakura et al., 1999; Kambara et al., 2006; Nahrstedt, et al., 2006; Šmejkal et al., 2007a; Šmejkal et al., 2008a).

The basic difference between the high pressure and low pressure liquid chromatography is that in HPLC the size of particles of stationary phase is much smaller (3-10 μm), and the flow of the solvent through the stationary phase is achieved by pumping it through the column under pressure. Generally, the smaller the particles of the sorbent, the higher the pressure of the mobile phase required (exceeding 10 MPa in HPLC) and the better the separation efficiency and chromatographic resolution. The stationary phase (chromatographic packing material) is usually packed into a column made from a rigid material such as stainless steel to withstand the high pressures required. In general, HPLC columns are 20-500 mm long and have an internal diameter in the range of 1-100 mm. Most HPLC applications are done in reversed-phase mode, where the stationary phase is non-polar and the mobile phase is polar. This is ideally suited for the analysis of polar and ionogenic analytes. The non-polar stationary phases are usually chemically-modified silicas (octadecyl, octyl, phenyl), styrene-divinylbenzene copolymers (XAD, PRP) or hybrid silicon-carbon particles (XTerra), and are used with aqueous-organic solvent mixtures (water or aqueous buffers and an organic modifier such as methanol, acetonitrile, or tetrahydrofuran) as the mobile phase. Conventional chemically-modified silica materials are stable in organic and aqueous solvents (pH range 2.5-8) whilst the styrene-divinylbenzene copolymers and the XTerra material can be used over a wider pH-range. Buffers are frequently used in real applications to reduce the protolysis of ionogenic analytes, which in ionic form show little retention in reversed-phase mode (Niessen, 1999; Waters, 2010b).

Several basic parameters can be used to describe the chromatographic process. The degree to which two compounds are separated by an HPLC column is called chromatographic resolution (also called separation power) and is determined by both mechanical separation power (efficiency), created by the column length, particle size, and packed-bed uniformity, and chemical separation power (selectivity), created by a combination of particle chemistry and mobile phase composition. The most powerful means of creating a separation is to optimize selectivity by the choice among a multiplicity of phase combinations (stationary and mobile phase) and retention mechanisms (modes of chromatography) (Waters, 2010b). The choice of the most suitable chromatographic conditions is based on the sample type, the complexity of the biological matrix and on the required sensitivity. These influence the time spent on sample pretreatment, the chromatographic resolution and the detection method.

Further advances in instrumentation and column technology led to the development of ultra-high performance liquid chromatography (UHPLC), which has recently become a widely used analytical technique. The UHPLC is characterized by further reduction of the particle size of the stationary phases (sub-2-micron particles), smaller diameter and length of analytical columns, and specially designed instruments capable to deliver mobile phase at 100 MPa. These improvements resulted in higher separation power and resolution, higher sensitivity, increased speed of analysis and much lower solvent consumption in liquid chromatography (Nguyen et al., 2006; Nováková et al., 2009).

3.4.3 Identification and quantification methods

With increasing interest in identification and structure elucidation of phytochemicals, HPLC is often combined with sophisticated analytical instrumentation including ultraviolet-diode-array detection (UV-DAD), mass spectrometric detection and nuclear magnetic resonance. The development of mass spectrometry combined online with efficient HPLC separation has enabled more reliable and precise identification and quantification to be realized in one analysis of a complex samples containing low levels of analytes (Crews et al., 1998; Silverstein et al., 2005; Niessen, 2006).

3.4.3.1 Ultraviolet detection

Ultraviolet (UV) detectors, mainly photodiode array detectors (PDAs), are nowadays the most widely used detectors in HPLC separation. The detection is based on the absorption of photons by a chromophore (double bond, aromatic ring, hetero-atom) and it is a concentration sensitive device. PDAs can operate at a wide range of wavelengths and enable determination of the basic characteristic of the analysed compounds such as the retention time (t_R) and wavelength of maximum absorbance (λ_{max}) (Niessen, 1999). The problem while using the UV profile of a compound as an identification parameter is that structurally related compounds may give very similar UV spectra and therefore their applicability in identification is very limited. Whilst HPLC/UV often gives good quantitative results, more reliable data, with better selectivity and lower detection limits can be achieved using HPLC combined with mass spectrometric detection (HPLC/MS) (Niessen, 1999; Waters, 2010b).

3.4.3.2 Mass spectrometry

Mass spectrometry (MS) is a powerful analytical technique for the determination of molecular weight and elemental composition of a molecule (ion), which can be used both for qualitative and quantitative purposes. These include the identification of unknown compounds and help to elucidate the chemical structure of analytes, or quantification of the amount of a known compound present in the sample. The method is based on the production of gas-phase ions that are subsequently separated or filtered according to their mass-to-charge ratios (m/z) and finally detected.

The mass spectrometer basically consists of five major parts: (1) sample introduction is performed by direct infusion or direct insertion methods; (2) ionization source converts sample molecules into ions; (3) mass analyser sorts ions according to their mass-to-charge ratio (m/z), in either time or space, by applying either static or dynamic, magnetic or electric fields.; (4) ion detector transforms the ions which passed through into a usable signal, usually an electric current, that is proportional to the ion abundance; (5) data system converts the signal from a detector to a digital information and displays it as a mass spectrum. All mass spectrometers must operate under high vacuum to allow ions to reach the detector without undergoing collision with other gaseous molecules, which can result in deviation

of the ion trajectory or loss of its charge. Some techniques use controlled collision in specific regions of the mass spectrometer (Niessen, 2006; De Hoffman et al., 2007).

A wide variety of ionization techniques is available. The most used are atmospheric pressure ionization methods (APIs), such as electrospray ionization (ESI), atmospheric pressure chemical ionization (APCI) and atmospheric pressure photo-ionization (APPI). APIs are classified as soft techniques generating mainly even electron protonated molecules in positive ion mode or deprotonated molecules in negative ion mode. APIs represent the liquid-phase ion sources, when the analyte is in solution. In solid-state ion sources, the analyte is in an non-volatile deposit and the matrix, which can be either a solid or viscous liquid and play an important role in ionization process. Matrix-assisted laser desorption ionization, plasma desorption, field desorption use this strategy for production of ions (Niessen, 2006; De Hoffman et al., 2007; Waters, 2010c).

As in the case of ion sources, many types of mass analysers are available, each with their advantages and limitations. The five main parameters characterizing the performance of mass analyser are the mass range limit, the speed of analysis, the transmission, the mass accuracy and the resolution. Magnetic sector instruments and linear quadrupoles (qQ) are scanning analysers, which transmit ions of different mass successively along the time scale. Other analysers such as time-of-flight mass analyser (TOF), ion trap (IT), Fourier transform ion cyclotron resonance (FTICR) and orbitrap instruments allow simultaneous transmission of all ions (Niessen, 2006; De Hoffman et al., 2007; Waters, 2010c).

Very useful are instruments, which combine two or more mass analysers to perform tandem mass spectrometry (MS/MS), e.g. triple quadrupole (QqQ) and hybrid instruments such as quadropole time-of-flight instrument (QqTOF) and IT-FTICR. MS/MS involve at least two steps of mass analysis connected with a controlled fragmentation (collision-induced dissociation), which causes a change in the mass or charge of an ion. MS/MS allow to obtain a mass spectrum of fragments (product ions) of a precursor ion, which is selected and isolated in the first analyser, decomposed through collision with another molecule (typically a gas like argon), and analysed (as product ions) by a second analyser (De Hoffman et al., 2007). The general aim is to improve the selectivity, specificity and sensitivity of detection over single-stage-instruments. MS/MS can be performed in a number of scan modes, in which either a product ion (daughter scan), a precursor ion (parent scan), a loss of neutral fragment (neutral loss scan) or a selected fragmentation reaction (selected-reaction monitoring, multiple-reaction monitoring) can be monitored. Except mass analysers in series, also two stages of mass analysis in a single instrument, e.g. IT and FTICR, can be used for MS/MS experiments (Waters, 2010c).

MS is the most often used in online combination with HPLC or GC. The complex sample is separated and the products are introduced one after another either in a solution (HPLC/MS) or in gaseous state (GC/MS) into the mass spectrometer. The most obvious advantage of coupling both HPLC/MS and GC/MS is the obtained mass spectrum for identifying each of isolated products. Several coupling methods to connect HPLC with MS were developed. Atmospheric pressure ionization sources (ESI, APCI, APPI) are the interfaces of choice, because they can tolerate low concentration of volatile buffers and flow rates up to 1 $ml \cdot min^{-1}$ without requiring a flow split, therefore the detection sensitivity is increased. The sensitivity of analysis can be improved by precolumn derivatization of non-volatile thermolabile compounds. Nowadays, the HPLC coupled with MS is the analytical technique of choice for most tasks both in plant metabolite analysis and discovery of new drugs (Willoughby et al., 1998; Niessen, 1999; Niessen, 2006; De Hoffman et al., 2007).

3.4.3.4 Nuclear magnetic resonance spectroscopy

Nuclear magnetic resonance (NMR) spectroscopy is one of the most direct and general tools for identifying the structure of compounds, which allows physical, chemical, electronic and structural information about molecules to be obtained. The technique can provide detailed information on the topology, dynamics and three-dimensional structure of molecules, either in solution or in the solid state. NMR spectroscopy is also used to study molecular physics of crystals and non-crystalline materials and in advanced medical imaging techniques, such as magnetic resonance imaging (Akitt et al., 2000; Lambert et al., 2004; Keeler, 2005; Tyszka et al., 2005; Edwards et al., 2009; Hornak, 2009).

The NMR measurements are performed on the atomic nuclei. Structural and dynamic information is obtainable from NMR studies of stable isotopes with non-zero spins, which contain an odd number of protons and neutrons and have their intrinsic magnetic moment. The most commonly studied nuclei are ^{1}H and ^{13}C. NMR is a property that nuclei of certain atoms have when immersed in a static magnetic field and exposed to a second oscillating magnetic field after application of an electromagnetic pulse (EMP) or pulses. The EMP causes the nuclei to absorb energy from the EMP and to radiate this energy back out at a specific resonance frequency, which depends mainly on the nuclei under observation and the strength of the static magnetic field. Different protons in a molecule resonate at slightly different frequencies. The resonant frequency, energy of the absorption and the intensity of the signal are proportional to the strength of the magnetic field. The NMR experiment usually involves two sequential steps; the polarization of the magnetic nuclear spins in an applied constant magnetic field, and interruption of this alignment of the nuclear spins by employing an electromagnetic radio-frequency pulse. Chemical shift, coupling constant and integration of proton signals can be read from a one-dimensional spectrum and give information not only about the chemical environment of the nuclei, but also the number of neighbouring NMR active nuclei within the molecule (Lambert et al., 2004; Keeler, 2005; Edwards et al., 2009; Hornak, 2009).

The process of identifying the compound using NMR spectroscopy often involves performing several NMR experiments to deduce the molecular structure from the magnetic properties of the atomic nuclei and surrounded electrons. The basic one-dimensional technique for proving the probable structure is the recording of hydrogen spectra (^{1}H spectrum). The ^{13}C spectra are also useful in structure determination. Frequency and intensity serve as the axis of the standard one-dimensional spectrum and represent the characterisation of the nuclei in terms of chemical shifts and couplings (Lambert et al., 2004; Hornak, 2009). Two-dimensional (2D) spectra provide more information about a molecule than one-dimensional. The second dimension refers to an additional frequency axis, where also magnetic interactions between nuclei are considered. 2D-experiments can be homonuclear such as COSY (correlation spectroscopy) observing proton-proton correlations, and heteronuclear providing correlation, e.g. between the ^{13}C and ^{1}H resonances. HSQC (heteronuclear single quantum correlation) spectrum contains a peak for each proton attached to the heteronucleus which is observed. HMQC (heteronuclear correlation through multiple quantum coherence) and HMBC (heteronuclear multiple bond coherence) are 2D inverse correlation techniques where the ^{13}C-response is observed in ^{1}H spectrum and allow the determination of connectivity between two different nuclear species. HMQC is selective for direct coupling whereas HMBC detects longer range couplings through 2-4 bonds (Martin et al., 1988; Lambert et al., 2004).

3.5 Cytotoxicity testing

The testing of cytotoxic activity of substances usually serves for screening and development of new therapeutic agents. The *in vitro* cytotoxicity tests can detect compounds that target rapidly dividing cancer cells or compounds exerting unwanted cytotoxic effects in normal cells, prior to their subsequent *in vivo* testing as pharmaceuticals. Cytotoxicity and cytotoxicity-correlated cell viability can be monitored by various chemical and biological reagents. Cell viability is the most often measured by assessing cell membrane integrity after cell treatment, because cytotoxically active compounds often compromise the cell membrane integrity (Invitrogen, 2010).

3.5.1 Basic principles of cytotoxicity assays

Cytotoxicity and cell viability assays are used to enumerate the proportion of live and dead cells in a population. These assays can be based on the measurement of enzymatic activity (using esterase substrates), membrane permeability and oxidation-reduction (redox) potential. Each of the approaches has advantages and limitations. Fluorescence based cell viability assays are generally more sensitive than colorimetric techniques and less hazardous and less expensive than radioisotopic methods (Invitrogen, 2010).

Cytotoxicity assays using esterase substrates

The fluorogenic esterase substrates, mainly acetates or acetoxymethylester (AM) derivatives (calcein AM, bis-carboxyethyl-carboxyfluorescein AM, fluorescein diacetate derivatives) can be passively loaded both into adherent and non-adherent cells. These cell-permeable esterase substrates serve as viability probes to measure enzymatic activity, which is required to activate the fluorescence of substrate, as well as membrane integrity, which is required for intracellular retention of fluorescent products. The non-fluorescent substrates are converted by non-specific intracellular esterases into fluorescent products inside the live cell. The products are retained by cells with intact membranes, but the unhydrolyzed substrates leak rapidly from dead or damaged cells even when the cells retain some residual esterase activity (Wang et al., 1993; Kolber et al., 1988).

Cytotoxicity assays using membrane permeability

Viability assessments of cells frequently use polar (cell-impermeant) nucleic acid stains to detect the dead cell population (Roth et al., 1997). Nucleic acid stains (probes) are often used in combination with the intracellular esterase substrates mentioned above, and with membrane-permeant nucleic acid stains, membrane potential sensitive probes, organelle probes or cell-permeant indicators, to detect the live cell population. Many other cell-impermeant dyes could be used to detect dead or dying cells such as the red, fluorescent, cell-impermeant ethidium and propidium dyes (ethidium bromide, propidium iodide) and trypan blue. These vital dyes are excluded from the inside of live cells, but freely cross the membrane and stain intracellular component in case of cell membrane damage (Beletsky et al., 1990; Riss et al., 2004). Another way to assess membrane integrity is to monitor the passage of substances, that are normally present inside cells (such as lactate dehydrogenase) to the external environment (Decker et al., 1988).

Cytotoxicity assays that measure oxidation and reduction

Live cells can oxidise or reduce a variety of substrates, which then provide a measure of cell viability or cell health differently from the assays based on detection of esterase activity and cell permeability. Some oxidation-reduction indicators such as rezaurin and dodecylresazurin provide the products, which are retained by a single cell and the developed fluorescence is proportional to cell number (De Fries et al., 1995). Dihydrofluorescein, dihydrorhodamine, dihydroethidium, dihydrocalcein derivatives are readily oxidized back to the parent dye by reactive oxygen species, which are produced only by live cells. Fluorescent oxidation products are retained in cells and serve as fluorogenic probes for detecting oxidative activity in metabolically active cells (Burghardt, 1994).

Tetrazolium salts are widely used for detecting redox potential of cells. The water soluble colorless compounds are reduced in viable cells to uncharged brightly coloured non-fluorescent formazans and after solubilization in an organic solvent they are quantified by standard spectrophotometric techniques. The most common assay for tetrazolium salt-based viability testing is the MTT test, which measures the reducing potential of the cell on the bases of colorimetric reaction. Several types of tetrazolium salts are used: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT); 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide; 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy-phenyl)-2-(4-sulfophenyl)-2H-tetrazolium (Imamura et al., 1994).

Other cytotoxicity assays

Every viable cell contains ion pumps and channels that maintain intracellular ion concentrations and transmembrane potentials. The loss of activity in maintenance of ion gradients in a dead cell can be determined using potentiometric dyes (methyl and ethyl esters of tetramethylrhodamine, styryl dyes, oxonol and carbocyanine dyes), acidotropic stains (lysosomal stain neutral red, Acridine orange), Ca^{2+} indicators and pH indicators. Measurements of glucose uptake using fluorescent glucose analogs can be used to assess cell viability. Fluorescent phosphatase substrate can be utilized as a marker for membrane integrity and the intracellular phosphatase activity of membrane-intact cells is detected (Invitrogen, 2010). Protease biomarkers are another example of biomarkers, which allow measurement of the relative numbers of live and dead cells within one cell population (Niles et al., 2007). Assays, which use ATP content as a marker of viability, are also available. Bioluminiscent assays use ATP as the limiting reagent for the luciferase reaction (Fan et al., 2007). Electric cell-substrate impedance sensing is a label-free approach, which monitors the cytotoxic response of adherent animal cells in real time and therefore provides the kinetics of the cytotoxic cell response. The cells are grown on gold coated electrodes and the electric impedance is measured (Heijink et al., 2010; Xiao et al., 2010).

3.5.2 Calcein AM cell viability assay

Basic principle

The Calcein AM cell viability assay is a simple, rapid and accurate method to determine cell viability and cytotoxicity of tested compounds. Calcein acetoxymethylester (calcein AM) is a non-fluorescent, hydrophilic compound that posses the ability to easily permeate intact, live cells. Calcein AM is then hydrolysed by intracellular esterases and produces calcein (fluorescein complexon, 2,7-bis(*N*,*N*-bis(carboxymethyl) aminomethylene) fluorescein) (Figure 19). Calcein is a hydrophilic, strongly fluorescent compound that is well-retained in the cell cytoplasm, it has better retention and brightness

compared to other fluorescent compounds such as fluorescein (BD Calcein, 2006; Calcein, 2007; Calcein, 2008). Tested cells are usually grown in 96-well microplates, after which they are stained and quantified in less than two hours. The generated fluorescent signal, which is proportional to the number of living cells in the sample (well), is monitored using 485 nm excitation wavelength and 530 nm emission wavelength.

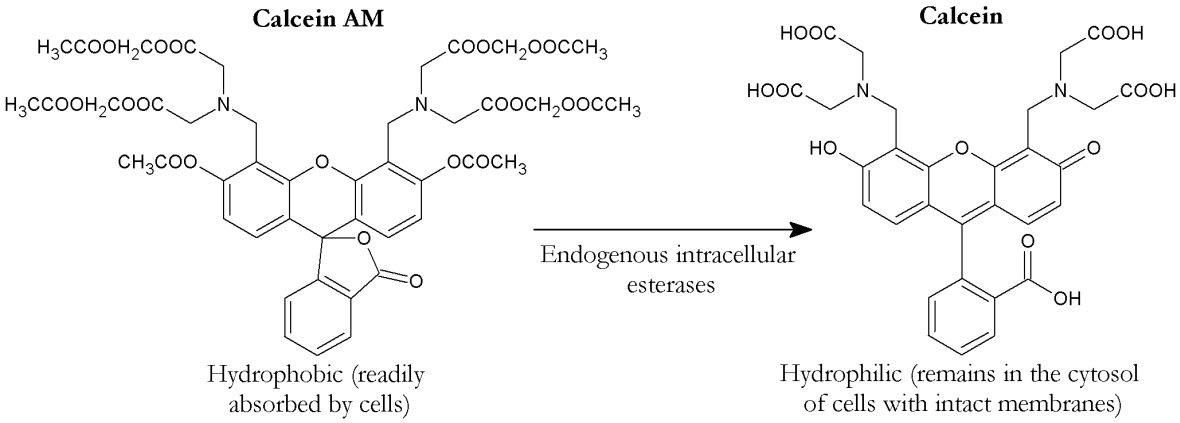


Figure 19. Formation of fluorescent calcein from calcein acetoxymethylester in living cells (Calcein, 2008).

There are two options for quantification; measurement of the relative differences, or determination of absolute cell number. When calculating the relative changes in the cell number, the system does not have to be calibrated and the results are presented as the percentage change in fluorescence intensity relative to an experimental control. Calibration is required when the absolute cell numbers are used. The calibration involves determination of cell numbers in a sample (well) and preparation of dilution in triplicate across the range of $1 \cdot 10^3$ cells·ml^{-1} to $5 \cdot 10^5$ cells·ml^{-1} in 50 µl of medium. After performing the assay, the averages of triplicates values are determined and the data are expressed as linear dependence of cell number per well versus intensity of fluorescence (BD Calcein, 2006; Calcein, 2007; Calcein, 2008).

Practical use

Calcein AM fluorescent dye can be used to fluorescently label viable cells, both proliferating and non-proliferating cells, whether in suspension or adherent cells. This labelling method is suitable for use with a wide variety of techniques, including microplate assays, immunocytochemistry, flow cytometry, and *in vivo* long-term cell tracing due to its lack of cellular toxicity (Wang et al., 1993; De Clerck et al., 1994; Papadopoulos et al., 1994). This assay is useful for studies of cell membrane integrity (Sroda et al., 2008) and determination of cell viability, cytotoxicity and apoposis (Moravcová et al., 2003; Kryštof et al., 2005; Malíková et al., 2008). It can be used to quantify cell number, to study cell adhesion (Braut-Boucher et al., 1995), chemotaxis, tumour cell invasion, endothelial cell migration (Weston et al., 1990), endothelial cell tubulogenesis (Arnaoutova et al., 2010) and multi-drug resistance (Eneroth et al., 2001).

4. Materials and methods

The following list of materials and methods contains a brief summary of all the experimental approaches used in this doctoral thesis. Full details are described in the following articles attached as Supplement I-III:

I Madlener S., **Svačinová J.**, Kitner M., Kopecký J., Eytner R., Lackner A., Vo T.P.N., Frisch R., Grusch M., De Martin R., Doležal K., Strnad M., Krupitza G. (2009) In vitro anti-inflammatory and anticancer activities of extracts of *Acalypha alopecuroidea* (Euphorbiaceae). *International Journal of Oncology* 35 (4): 881-891.

II Šmejkal K., **Svačinová J.,** Šlapetová T., Schneiderová K., Dall'Acqua S., Innocenti G., Závalová V., Kollár P., Chudík S., Marek R., Julínek O., Urbanová M., Kartal M., Csöllei M., Doležal K. (2010) Cytotoxic activities of several geranyl-substituted flavanones. *Journal of Natural Products* 73 (4): 568-572.

III **Svačinová J.**, Novák O., Lenobel R., Plačková L., Hanuš J., Strnad M., Doležal K. A new miniaturized analytical approach for cytokinin isolation from plant tissues using pipette tip solid-phase extraction (manuscript in preparation).

The materials and methods included in Supplement I-III are completed in this section with full description of non-published experimental approaches, which were also used to achieve the three main objectives of this thesis:

1. Isolation and identification of anticancer active compounds from *Acalypha alopecuroidea.*
2. Geranylated flavanones from *Morus alba* and *Paulownia tomentosa* and their anticancer potential.
3. Miniaturized fast isolation and quantification of phytohormones from biological material.

4.1 List of chemicals

- All chemicals and solvents were at least of analytical reagent grade from Lach-Ner (Neratovice, Czech Republic); Merck (Darmstadt, Germany) or Sigma-Aldrich (St. Louis, MO, USA)
- *N*9-substituted purine derivatives were synthesised by RNDr. Marek Zatloukal, Ph.D. in the Laboratory of Growth Regulators (Palacký University, Olomouc, Czech Republic) and the synthesis will be described elsewhere (1)
- Geranylated flavanones were obtained from Department of Natural Drugs (Faculty of Pharmacy, University of Veterinary and Pharmaceutical Sciences, Brno, Czech Republic) (II, 2)
- Authentic and deuterium-labelled CK standards were obtained from OlChemim Ltd (Olomouc, Czech Republic) (III, 3)
- Tritium-labelled CK standards ([^{3}H]tZR, 0.851 TBq·mmol^{-1}; [^{3}H]cZ, 0.777 TBq·mmol^{-1}; [^{3}H]iPR, 1.184 TBq·mmol^{-1}; [^{3}H]BA, 1.0 TBq·mmol^{-1}) with radiochemical purity >98% were obtained from the Isotope Laboratory (Institute of Experimental Botany AS CR, Prague, Czech Republic) (III, 3)
- [$^{13}C_6$]IAA was a generous gift from Dr. Karin Ljung (Umeå Plant Science Center, Department of Forest Genetics and Plant Physiology, Umeå, Sweden) (3)
- Deionised (Milli-Q) water was obtained from Simplicity 185 system (Millipore, Bedford, MA, USA)
- Scintillation cocktail Ultima Gold™ from Packard BioScence (Groningen, The Netherlands) (III, 3)

4.2 List of biological materials

- *Acalypha alopecuroidea* plants collected in the Botanical garden of the Institute for Ethnobiology (Playa Diana, San José, Petén, Guatemala) were mechanically cleaned and lyophilized. Extracts of individual plant parts were produced at the Laboratory of Growth Regulators (Palacký University, Olomouc) (I, 1).
- *Arabidopsis thaliana* (ecotype Colombia) were grown *in vitro* in Petri dishes containing Murashige & Skoog medium (Duchefa Biochemie B.V., Haarlem, The Nederland) including vitamins. 10-day old seedlings, or roots and shoots were harvested, weighted, immediately plunged into liquid nitrogen and stored at -70 ˚C (III).
- *Castor fiber* blood plasma samples in syringes were delivered by Mgr. Jaroslav Maloň from the Department of Ecology & Environmental Sciences (Faculty of Science, Palacký University, Olomouc). Samples were stored at -70 °C until precipitation and phytohormone purification. Blood was obtained either from beavers from the rescue stations or beavers trapped in Southern Moravia region Lanžhot area (flooded forests) and in Czech forest around Přimda (piedmont brooks and grasslands). Blood was taken in full consciousness (with no full anaesthesia) from the veins on upper part of the hind footpad. Afterwards, beavers were set free in the same territory (3).

4.3 List of solid-phase extraction materials

- Agarose gel (Affi-Gel 10, Bio-Rad Labs, Hercules, USA) (3)
- C18 cartridge AccuBond SPE ODS-C18, 500 mg/6 ml (Agilent Technologies, Santa Clara, California, USA) (I, 1)
- C18 cartridge Sep-Pak C18, 0.5 × 1.5 cm, 360 mg (Waters, Milford, MA, USA) (I, 1)
- DEAE-Sephadex A25 (Sigma-Aldrich, St. Lous, MO, USA) for preparation of activated DEAE-Sephadex column (1.0 × 5.0 cm) (I, 1)
- Empore™ High Performance Extraction Disks, sorbent type C18 (Octadecyl), SDB-RPS and Cation-SR (3M Center, St. Paul, MN, USA) (III)
- Chitosan coated magnetic nanoparticles with immobilized group-specific monoclonal anticytokinin antibodies NZRD or 1G6 (magnetic nanoparticles were from the Centre of Nanomaterial Research, Palacký University, Olomouc; preparation of antibodies and immobilization was done in the Laboratory of Growth Regulators, Palacký University, Olomouc) (3)
- Immunoaffinity agarose gel Affi-Gel 10 (Bio-Rad Labs, Hercules, USA) with bound group-specific monoclonal anticytokinin antibodies NZRD or 1G6 (prepared in the Laboratory of Growth Regulators, Palacký University, Olomouc) (3)
- Oasis MCX cartridges 30 mg/1 ml (Waters, Milford, MA, USA) (3)

4.4 List of experimental equipment

- Centrifuge Avanti™ (Beckman Coulter, Brea, CA, USA)
- Dynal MPC®-S magnetic particle concentrator (Dynal A.S, Oslo, Norway) (III)
- HPLC systems
  - preparative HPLC (I)

    - LabAlliance™ system, DeltaChrom™ Prep 100 pumps (Watrex, Prague, Czech Republic)
    - diode array detector Agilent 1100 DAD and ion trap mass spectrometer MSD Ion Trap SL (Agilent Technologies, Santa Clara, SA, USA)
    - reversed-phase column Reprosil 100, C8, 5 μm, 25 × 250 mm (Dr. Maisch GmbH, Ammerbuch-Entringen, Germany)
  - semipreparative 2D-HPLC: 1st fractionation (1)
    - Beckman System Gold 125 Solvent Module (Beckman Coulter, Brea, CA, USA)
    - photodiode array detector Beckman 168 Detector (Beckman Coulter, Brea, CA, USA)
    - reversed-phase column Symmetry Shield RP-18, 7 μm, 8 mm × 250 mm
  - semipreparative 2D-HPLC: 2nd fractionation (1)
    - Alliance 2695 Separations Module (Waters, Milford, MA, USA)
    - photodiode array detector PDA 2996 (Waters, Milford, MA, USA)
    - reversed-phase column Symmetry C18, 5 μm, 2.1 × 150 mm (Waters)
    - reversed-phase column Synergi 4u Fusion-RP, 4 μm, 10 × 250 mm (Phenomenex, Torrance, California, USA)
- HPLC (UHPLC)/MS systems
  - System HPLC-ESI-qQ (1)
    - Alliance 2695 Separations Module (Waters, Milford, MA, USA)
    - photodiode array detector PDA 2996 (Waters, Milford, MA, USA)
    - ZMD 2000 single quadrupole mass spectrometer with an electrospray interface (Micromass, Manchester, UK)
    - reversed-phase column Symmetry C18, 5 μm, 2.1 × 150 mm (Waters, Milford, MA, USA)
  - System HPLC-ESI-QqTOF (1)
    - Aliance 2695 Separations Module (Waters, Milford, MA, USA)
    - photodiode array detector PDA 2996 (Waters, Milford, MA, USA)
    - Q-TOF micro™ hybrid tandem mass analyzer with an electrospray interface (Micromass, Manchester, UK)
    - reversed-phase column Symmetry C18, 5 μm, 2.1 × 150 mm (Waters, Milford, MA, USA)
  - System UPLC-ESI-MS/MS (III, 3)
    - Acquity UPLC™ System (Waters, Milford, MA, USA)
    - 2996 PDA detector (Waters, Milford, MA, USA)
    - Xevo™ TQ MS mass analyzer with an electrospray interface (Waters MS Technologies, Manchester, UK)
    - reversed-phase column Acquity UPLC™ BEH C18, 1.7 μm, 2.1 × 150 mm (Waters)
    - reversed-phase column Acquity UPLC™ BEH C18, 1.7 μm, 2.1 × 50 mm (Waters)
    - reversed-phase column BetaMax Neutral, 5 μm, 1.0 × 150 mm with Uniguard™ column protection Hypurity advance, 5 μm, 1.0 mm × 10 mm (Thermo Fisher Scientific, Waltham, MA)
  - MS data were processed by MassLynx™ software with QuanLynx™ program (version 4.0) or TargetLynx™ program (version 4.2, Waters, Milford, MA, USA)
- Multi-purpose scintillation counter LS 6500 (Beckman, Ramsey, MN, USA) (III, 3)

- NMR spectrometers Varian Unity Inova 700 MHz and 500 HMz, Varian Mercury 400 MHz (Varian, Walnut Creek, CA, USA) (1)
- pH/mV/°C meter (Cole Parmer, East Bunker Court Vernon Hills, Illinois, USA)
- Rotary evaporator Rotavapor® R-200 equipped with Vacuum Controler V-805 and Heating Bath B-490 (Büchi Labortechnik AG, Postfach, Switzerland)
- Solid-phase extraction vacuum manifold Visiprep™ (Supelco, Bellefonte, PA, USA) (3)
- Sonicator Transsonic 310 (Elma, Singen, Germany)
- Speed-Vac concentrator RC1010 (Jouan, Winchester, UK)
- Vacuum centrifuge concentrator Univapo 150 H (UniEquip, Munich, Germany)
- Vibration mill MM 301 (Retsch GmbH & Co. KG, Haan, Germany), 12 mm stainless steel grinding balls, 25 ml stainless steel grinding jars, 3 mm tungsten carbide beads (Retsch GmbH & Co. KG, Haan, Germany)
- Vortex mixer Wizard X (Velp Scientifica, Usmate, Italy)

4.5 Sample preparation and SPE purification

4.5.1 *Acalypha alopecuroidea* extract preparation

Lyophilized plants *A. alopecuroidea* were divided into roots (0.5 g or 2.0 g), stems (1.0 g), leaves (1.0 g) and inflorescences (0.5 g), homogenized using the vibration mill and extracted separately in different extraction solvents, e.g. 20 ml of ethanol 70% (v/v), methanol-tetrahydrofurane (MeOH-THF, 1:1), with addition of antioxidant (sodium diethyldithiocarbamate), 400 $\mu g \cdot g^{-1}$ DW). After overnight extraction and centrifugation, the sediments were re-extracted for 1 h in the same way. The supernatants were pooled and evaporated to dryness under vacuum at 35 °C prior to the cytotoxicity testing (Supplement I).

According to the results from the Calcein AM cytotoxicity assay (see the section Results and discussion), the subsequent fractionation (see Figure 20, p. 66) focussed only on the MeOH-THF extracts from the roots (2.0 g DW), which were immediately without evaporation purified by passage through AccuBond C18 cartridges and then concentrated to the aqueous phase. This was diluted in 20 ml of ammonium acetate buffer (40 mM, pH 6.5) and purified by DEAE-Sephadex coupled to two Sep-Pak C18 cartridges in tandem. The Sep-Pak cartridges were separately eluted with 5 ml methanol 80% (v/v) (fraction B and C) after decoupling and washing with 10 ml of distilled water. The load through fraction and water used to rinse the Sep-Pak cartridges was collected as fraction A. Afterwards, the DEAE-Sephadex column was coupled to third Sep-Pak cartridge and washed with 10 ml of formic acid 6% (v/v). Sep-Pak was eluted (fraction E) the same way as the first and second Sep-Pak cartridges. The liquid (acidified water) which passed through and water used to rinse the third Sep-Pak was collected as fraction D. The last fraction F was obtained from the precipitate that did not dissolve in the ammonium acetate buffer prior to the SPE purification. All six of these fractions (A-F) were evaporated to dryness and stored at -20 °C (Supplement I).

On the bases of the results from Calcein AM assay, only fraction B was used for the routine isolation of active compounds from the MeOH-THF root extract. In this case, the SPE was optimized and performed with gradient elution of the first Sep-Pak cartridge and without the second and third

Sep-Pak. The first Sep-Pak was eluted with 5 ml methanol 40% (v/v) and subsequently with 5 ml methanol 70% (v/v). Only the methanol 70% (v/v) eluate, which contained the targeted biologically active compounds, was collected, evaporated to dryness and further purified by HPLC (Objective 1).

4.5.2 Liquid microextraction and pipette tip SPE

Samples of 10-day old *Arabidopsis thaliana* seedlings, roots or shoots (1.0, 2.0 and 5.0 mg FW) were placed into 2.0 ml microcentrifuge tubes with Bieleski buffer (60% methanol, 25% $CHCl_3$, 10% formic acid and 5% H_2O) in proportion 50 µl of buffer per 1 mg FW. Stable isotope-labelled cytokinin internal standards (see Supplement III) at concentration 0.5 pmol of each compound per 50 µl of Bieleski buffer were added to the samples to determine the endogenous cytokinin levels and to check the recoveries during the purification procedure. Tritium-labelled standards ([^{3}H]iPR, [^{3}H]tZR, [^{3}H]cZ; $1 \cdot 10^5$ dpm per sample) were added for the method development and the selectivity, affinity, and capacity tests of different sorbents; non-labelled standards (1 pmol) were used for spiking samples to verify the analytical precision and accuracy of the whole method. Plant material was extracted using a vibration mill (27 Hz, 3 min) after adding 3 mm tungsten carbide bead to each tube. The tube contents were ultrasonicated for 3 min, then stirred for 30 min at 4 ºC and centrifuged (10 min; 15,000 rpm; 4 ºC). The supernatants (50 µl aliquots) were immediately purified by pipette tip SPE (PT-SPE). Samples containing only selected CK standards in 50 µl of Bieleski buffer without plant matrices were prepared during the method optimization and directly purified (Supplement III).

The PT-SPE was performed using self-packed solid-phase extraction microcolumns in pipette tips termed StageTips (STop And Go Extraction Tips), which were prepared manually from the Empore™ High Performance Disks as described by Rappsilber et al. (2007). Briefly, very small disks (ca. 1.0 mm diameter, 0.5 mm thickness) were cut from Empore disks using a hollow blunt-ended syringe needle and placed inside a pipette tip with help of a plunger that ideally fitted into the needle (Rappsilber et al., 2007). C18, SDB-RPS and Cation-SR Empore disk sorbents were used individually or in combinations. The prepared StageTip was inserted into a hole at the centre of the lid of a microcentrifuge tube (1.5 ml). Solutions (in 50 µl portions) were loaded from the top of the tip using a pipette and the liquid was pressed through the column by centrifugation of the StageTip. The StageTip sorbent was activated with isopropanol and equilibrated with 50% MeOH + 2% formic acid and Bieleski buffer. Then the sample was loaded and the sorbent was washed with methanol. Elution of the sample was performed with 60% MeOH + 0.5 M NH_4OH to the new clean microcentrifuge tube. Eluate was either immediately mixed with scintillation cocktail prior to measurement of radioactivity of samples containing tritium-labelled CK standards, or evaporated to dryness in a Speed-Vac concentrator prior to UPLC-MS/MS analyses (Supplement III).

4.5.3 Blood plasma precipitation and SPE

Samples of *Castor fiber* plasma, stored at -70 ºC in syringes, were defrosted, transferred to glass test tubes, ultrasonicated for 5 min and divided into three similar aliquots of approximately 500 µl. At the beginning of the extraction, the following stable isotope-labelled standards were added as internal tracers for recovery studies and quantification: [$^{13}C_6$]IAA (5 pmol); CK bases (3 pmol): [$^{13}C_5$]cZ, [$^{13}C_5$]tZ, [$^2H_5$]tZR, [$^2H_5$]tZ9G, [$^2H_3$]DHZ, [$^2H_3$]DHZR, [$^2H_3$]DHZ9G, [$^2H_6$]iP, [$^2H_6$]iPR, [$^2H_6$]iP9G, [$^2H_7$]BA, [$^2H_7$]BAR, [$^2H_7$]BA9G, [$^{15}N_4$]mT, [$^{13}C_5$]oT, [$^{15}N_4$]K; CK nucleotides (5 pmol): [$^2H_5$]tZRMP,

[$^2H_3$]DHZRMP, [$^2H_6$]iPRMP, [$^2H_7$]BARMP and CK *O*-glucosides (5 pmol): [$^2H_5$]tZOG, [$^2H_5$]tZROG, [$^2H_7$]DHZOG. Samples containing only authentic standards in 100 µl of water (IAA, iPR, oT) and mammalian plasma samples (200 µl) spiked with stable isotope-labelled standards ([$^{13}C_6$]IAA, [$^2H_6$]iPR, [$^{13}C_5$]oT) at concentrations 0.1, 1.0 or 10.0 pmol in both cases, were prepared and used for optimization of the extraction and purification procedures. For protein precipitation, samples were mixed with the first portion of acetonitrile (420 µl) and vortexed. Then the second portion of acetonitrile (2400 µl) was added, samples were vortexed and centrifuged (3,000 rpm, 10 min, 4 ºC). Supernatant was transferred to glass test tubes and evaporated to dryness (Objective 3).

The solid-phase extraction was performed as described by Dobrev et al. (2002), with slight modifications, in Oasis MCX extraction columns (3 ml/60 mg). Evaporated samples were diluted in 2 ml of 1 M formic acid and immediately passed through a MCX column, which was activated with 2 ml of methanol and equilibrated with 2 ml of 1 M formic acid. After applying the sample, the column was washed with 2 ml 1 M formic acid followed by 2 ml 100% MeOH. This methanol wash was collected as the fraction containing indole-3-acetic acid (fraction IAA). Subsequently, the cytokinin ribonucleotides (fraction NT) were eluted using 2 ml of 0.35 M NH_4OH. Finally, cytokinin free bases, ribosides and *O*-glucosides (fraction B+OG) were eluted with 2 ml of 0.35 M NH_4OH in MeOH 60% (v/v). All samples were evaporated to dryness in a Speed-Vac concentrator and further purified by immunoaffinity chromatography (Objective 3).

4.6 HPLC fractionation

Preparative HPLC separation of the *A. alopecuroidea* fraction B was carried on a LabAlliance™ system linked to an UV-DAD (Agilent 1100) and ion trap mass spectrometer. The column thermostat was set to 30 °C. Fraction B was injected on a preparative reversed-phase column Reprosil 100 and eluted using a linear water-methanol gradient of 30-100% methanol in water (v/v) (0-30 min) at a flow rate of 15 ml·min^{-1}. One-minute fractions were collected and evaporated to dryness (Supplement I).

Later, during the routine isolation of active compounds, semipreparative 2D-HPLC separation was used for fractionation of fraction B (see Figure 21, p. 68). The first HPLC fractionation was carried on a Beckman System linked to a Beckman 168 Detector. Fraction B was injected on a Symmetry Shield RP-18 column. The elution was performed at a flow rate of 2.5 ml·min^{-1} with a linear water-methanol gradient rising from 70:30 to 0:100 (water:methanol, v/v) over 30 min. Either one-minute fractions or the range of 22nd-25th min as one fraction (assigned B22-25) were collected and evaporated to dryness. Fraction B23 and fraction B22-25 selected according to the Calcein AM assay was submitted to the second HPLC fractionation carried out on an Alliance 2695 Separation Module, linked simultaneously to a Waters PDA 2996 detector. Fraction B23 was repeatedly injected onto an analytical Symmetry C18 column and eluted with a linear methanolic gradient rising from 50:50 to 40:60 (A:B) over 30 min at a flow rate of 0.25 ml·min^{-1}. Solvent A was composed of water + 2% methanol + 0.05% formic acid, solvent B was 100% methanol + 0.05% formic acid (see Figure 22, p. 68). Half-minute fractions were collected and evaporated to dryness. Fraction B22-25 was repeatedly injected onto a semipreparative Synergi Fusion-RP column and eluted with a 30-min isocratic elution of water + 2% methanol:methanol (30:70) at a flow rate of 2.0 ml·min^{-1} (see Figure 23, p. 69). Fractions corresponding to individual chromatographic peaks (B22-25/1-4) were collected and evaporated to dryness (Objective 1).

4.7 Cytokinin immunoaffinity extraction

4.7.1 Immunoaffinity extraction on magnetic nanoparticles

The batch immunoaffinity extraction of cytokinins on magnetic nanoparticles was performed in 1.5 ml microcentrifuge tubes using group-specific monoclonal antibodies against cytokinins (Novák et al., 2003). The antibodies were immobilized onto superparamagnetic iron oxide nanoparticles (MNPs) coated with chitosan (Belessi et al., 2008) using two different methods, the glutaraldehyde method (Kluchová et al., 2009) and the carbodiimide method (Aslam et al., 1998). Prepared nanocomposites were used as immunosorbent in the batch immunoaffinity extraction with magnetic separation.

The method was tested using samples containing cytokinin standards in 150 µl of PBS buffer. Following selected standards were used either individually or in a mixture: tritium-labelled standards ([^{3}H]iPR, [^{3}H]tZR, [^{3}H]BA; $1 \cdot 10^5$ dpm per sample); non-labelled standards (iPR, cZR, oT, oTR, mT, mTR, pT, pTR; 0.1 or 0.5 pmol of each compound per sample). Defined amount of antibodies bound on magnetic nanoparticles (100-300 µg) was added in 50 µl of PBS buffer to each sample prior to the IAE. The total samples volume was then 200 µl. The PBS buffer contained 7.8 g $NaH_2PO_4 \cdot 2H_2O \cdot l^{-1}$ (pH 7.2). Samples were homogenized for 5 s every 5 min using sonicator during the 60 min incubation at laboratory temperature (21 ºC). The supernatant (PBS fraction) was easily removed after putting the microcentrifuge tubes with samples on the magnetic particle concentrator for ca. 20 s. Cytokinin bases, ribosides and 9-glucosides (fraction B) were retained by antibodies and eluted with ice cold methanol (200 µl twice) after washing the nanocomposites in the microcentrifuge tubes with PBS buffer and water (200 µl twice of each solvent). The eluate was evaporated to dryness in a Speed-Vac concentrator. The regeneration of antibodies after elution was performed as follows: 200 µl of water twice, 200 µl of MeOH, 200 µl of water twice and 200 µl of PBS twice. Antibodies in nanocomposites were stored in PBS with 0.02% natrium azide at 4 ºC (Objective 3).

4.7.2 Immunoaffinity chromatography

IAC of cytokinins in column format was performed in plastic cartridges using the generic monoclonal antibodies against cytokinins bound on an immunoaffinity gel (IAG). IAG was prepared, used and stored as described previously in more detail (Vaňková et al., 1998; Novák et al., 2003). Briefly, the monoclonal antibodies were raised against *ortho*-topolin riboside by hybridoma technology and purified by affinity chromatography on protein G. Purified antibodies were used for preparation of an IAG based on the coupling of antibodies to *N*-hydroxysuccinimide-ester activated agarose Affi-Gel 10. Each immunoaffinity column contained 0.5 ml of IAG packed in 3 ml-polypropylene cartridge. The pre-immune gel was prepared by coupling rabbit IgG to Affi-Gel 10 (25 mg ml·1-1). The pre-immune and IAC columns were stored in PBS buffer (50 mM NaH_2PO_4, 15 mM NaCl, pH 7.2) with 0.1% natrium azide at 4 ºC.

The IAC was performed as described previously (Novák et al., 2003). Briefly, fraction B+OG from the solid-phase extraction was dissolved in 50 µl ethanol 70% (v/v) and 450 µl PBS buffer and purified using pre-immune gel (0.5 ml). Afterwards, the flow through fraction together with a 0.5 ml PBS wash was repeatedly (five times) applied to an immunoaffinity column and then collected as the fraction OG and evaporated to dryness. The elution (fraction B) was performed with 3 ml ice-cold 100% methanol (-20 ºC). Evaporated fraction OG was dissolved in 200 µl of 100 mM CH_3COONH_4 (pH 5.0), treated

with 0.1 mg of β-glucosidase (4.0 U·mg^{-1}) and immunopurified again as described above. Fraction NT from the solid-phase extraction was dissolved in 20 µl of ethanol 70% (v/v) and 180 µl of $NaHCO_3$ (420 mg·100 ml^{-1}, pH 9.6), treated with 50 µl of alkaline phosphatase solution (5 µl of alkaline phosphatase in 2.5 ml $NaHCO_3$) and purified using pre-immune gel and IAG. Finally, all samples were evaporated to dryness and stored at -20 ºC until further UPLC-MS/MS analysis. According to this purification protocol, ribonucleotides (fraction NT) and *O*-glucosides (fraction OG) were analysed after enzyme treatment as appropriate ribosides and bases, respectively (Objective 3).

4.8 Instrument conditions for substance identification (Objective 1)

4.8.1 Nuclear magnetic resonance spectroscopy

NMR spectra were recorded on a Varian Unity Inova 700 MHz, a Varian Unity Inova 500 HMz (^{1}H: 500.13 MHz ^{13}C: 125.77 MHz), or a Varian Mercury 400 MHz spectrometer (^{1}H: 399.880 MHz ^{13}C: 100.559 MHz) at 25 ºC. The ^{1}H and ^{13}C chemical shifts were recorded in parts per million relative to the residual solvent signal (δ_H 3.31 and δ_C 49.0 ppm for CD_3OD, δ_H 2.49 and δ_C 39.51 ppm for DMSO-d_6). For an accurate measurement of the coupling constants, the one-dimensional ^{1}H NMR spectra were transformed at 64K points (digital resolution 0.09 Hz). Homonuclear ^{1}H connectivities were determined by ^{1}H-^{1}H COSY (mixing time 100 ms) experiments. One-bond heteronuclear ^{1}H-^{13}C connectivities were determined with 2D reverse single-quantum heteronuclear correlation pulse sequence (^{1}H-^{13}C HSQC) with an interpulse delay set for $^1J_{CH}$ of 145 Hz. Two and three bond heteronuclear ^{1}H-^{13}C connectivities were determined with 2D multiple-bond heteronuclear correlation experiments (^{1}H-^{13}C HMBC) optimised for $^{2,3}J_{CH}$ of 8 Hz (Objective 1).

4.8.2 HPLC/MS analysis

HPLC/MS experiments were carried out on an Alliance 2695 Separations Module linked simultaneously to a photodiode array detector (PDA 2996, Waters) and either a ZMD 2000 single quadrupole mass spectrometer (HPLC-ESI-qQ) or a hybrid mass analyser Q-TOF micro™ (HPLC-ESI-QqTOF) both equipped with an electrospray interface. Samples were injected onto an analytical Symmetry C18 column, the column/sample thermostat was set at 30/4 °C. Elution was performed with a linear methanolic gradient composed of water + 2% methanol + 0.05% formic acid (A) and 100% methanol + 0.05% formic acid (B) at a flow rate of 0.25 ml·min^{-1}. The following binary gradient was used: 0 min, 50:50 (A:B); 0-30 min, 40:60 (A:B); 30-32 min, 0:100 (A:B). At the end of the gradient the column was washed with 100% methanol and re-equilibrated to initial conditions for 11 min. Effluent was simultaneously introduced to the UV-DAD (scanning range 210-400 nm, resolution 1.2 nm, sampling rate 1.0 spectra·s^{-1}). For Fullscan mode performed at both ZMD 2000 and Q-TOF micro™ mass spectrometer, MS conditions were as follows: source/desolvation gas temperature 100/350 °C, capillary voltage 2.5 kV, cone voltage 25 V, mass monitoring interval 50-1000 amu. Nitrogen was used both as desolvation gas (550 l·h^{-1}) and as cone gas (50 l·h^{-1}). Collision energy for MS/MS experiments performed at Q-TOF micro™ was ranging 15.0-30.0 eV, scan time was 1.5 s. Argon was used as collision gas with an optimized pressure of 5·10^{-3} mbar (Objective 1).

4.9 Instrument conditions for phytohormone quantification (Supplement III, Objective 3)

4.9.1 Radioactivity measurement

The radioactivity of tritium-labelled cytokinin standards was measured by scintillation counting using the Beckman LS 6500 scintillation counter after addition of liquid scintillation cocktail Ultima Gold™ (1 ml of cocktail per 50 µl or 200 µl of liquid sample). Calculations of cytokinin content were performed using Microsoft Excel 2000 software.

4.9.2 Cytokinin UPLC-ESI(+)-MS/MS analysis

All cytokinins were analysed and quantified according to the modified method described by Novák et al. (2008). Briefly, an Acquity™ UPLC System, including a 2996 PDA detector, was coupled to a triple quadrupole mass spectrometer, Xevo™ TQ MS, equipped with an electrospray interface and a ScanWave™ collision cell. The samples were dissolved in 20 µl of the mobile phase used for quantitative analysis (10% methanol). 10 ul of each sample was injected onto an Acquity UPLC™ BEH C18 column (column/sample temperature 40/4 ºC) and eluted with a binary gradient composed of methanol as solvent A and 15 mM ammonium formate pH 4.0 as solvent B (Supplement III, Objective 3).

Slightly different conditions were used for UPLC analysis of samples purified by PT-SPE and for analysis of immunopurified samples. The samples pre-purified by PT-SPE were injected onto a BEH C18 column (1.7 µm; 2.1 × 150 mm) and eluted with a 24-min gradient (0-7 min, 5:95 (A:B); 7-16 min, 20:80 (A:B); 16-23 min, 50:50 (A:B); 23-24 min, 50:50 (A:B); flow rate of 0.25 ml·min^{-1}). At the end of the gradient the column was washed with 100% methanol and re-equilibrated to initial conditions for 10 min (Supplement III). The samples pre-purified by IAC were injected onto a BEH C18 column (1.7 µm; 2.1 × 50 mm) and eluted with a 4.2-min gradient (0-0.5 min, 10:90 (A:B); 0.5-4.0 min, 45:55 (A:B); 4.0-4.2 min, 100:0 (A:B); flow rate of 0.5 ml·min^{-1}). At the end of the gradient the column was washed with 100% methanol and re-equilibrated to initial conditions for 1.3 min (Objective 3).

The effluent from UPLC column was passed through an UV-DAD (scanning range 200-400 nm, resolution 1.2 nm, sampling rate 5 points·s^{-1}) and the tandem mass spectrometer without post-column splitting. Cytokinins were quantified by multiple reaction monitoring (MRM) of $[M+H]^+$ and the appropriate product ion. For selective MRM experiments, optimal conditions, dwell times, cone voltages, and collision energies in the collision cell corresponding to exact diagnostic transitions were optimized earlier for each cytokinin with few modifications to maximize the sensitivity (Novák et al., 2008). Quantification was performed by TargetLynx™ software using a standard isotope dilution method. Briefly, the ratio of endogenous cytokinin to the appropriate labelled standard was determined and used to quantify the level of endogenous compound in the original extract according to the knowledge of the quantity of an added internal standard (Novák et al. 2003) (Supplement III, Objective 3).

4.9.3 Indole-3-acetic acid UPLC-ESI(+)-MS/MS analysis

Indole-3-acetic acid was analysed using the same instruments as cytokinins, but with conditions optimized for IAA according to the modified method described by Malá et al. (2009). The samples purified by MCX column (fraction IAA) were dissolved in 25 µl of ACN:water (10:90, v/v) and

filtered through a Micro-spin® filter tube (0.2 μm, Grace, USA) 3 min at 8,000 rpm. 15 μl of each sample was injected onto a BetaMax Neutral column with a Uniguard™ column protection. The samples were eluted with a 20-min gradient composed of water with 1% ACN and 0.1% formic acid (solvent A) and 95% ACN with 0.1% formic acid (solvent B) at a flow rate of 0.05 ml·min^{-1}, column/sample temperature of 40/4 ºC. The following binary gradient was used: 0-5 min, 90:10 (A:B); 5-10 min, 80:20 (A:B); 10-18 min, 70:30 (A:B); 18-19 min, 50:50 (A:B); 19-20 min, 10:90 (A:B). At the end of the gradient the column was equilibrated to initial conditions for 5 min. Under these conditions, retention times for the monitored IAA was 13.44 min and for [$^{13}C_6$]IAA 13.41 min (Malá et al., 2009).

The effluent was passed through an UV-DAD (scanning range 200-400 nm, resolution 1.2 nm, sampling rate 20 points·s^{-1}) and the tandem mass spectrometer without post-column splitting. Indole-3-acetic acid was quantified by selective MRM experiments (IAA transition: 175.95 > 129.93; [$^{13}C_6$]IAA transition: 181.95 > 135.93). Optimized conditions were as follows: capillary voltage 1.5 kV; cone voltage 20 V; collision energy 15.0 eV; source/desolvation gas temperature 150/350 ºC; cone/desolvation gas flow rates 2.0/350 l·hr^{-1}; LM/HM resolution 3.0/15.0; ion energy 1/2 0.5 V; entrance, exit and multiplier voltages of 0.5 V, 0.5 V and 490.60 eV, respectively. Argon was used as the collision gas with an optimized pressure of $5 \cdot 10^{-3}$ mbar. The dwell times (1.09 s) for diagnostic transitions were calculated to provide 16 scan points per peak and to maximize the sensitivity. Quantification was performed by MassLynx software using a standard isotope dilution method (Malá et al., 2009) (Objective 3).

4.10 Calcein AM cell viability assay

The Calcein AM assay was used to determine the anticancer potential of the plant extracts, extract fractions, and isolated and synthetic compounds in Objectives 1 and 2 and is described in detail in Supplement I and II. The brief summary is given in this chapter.

Anticancer potential was determined as cytotoxic effects on the growth of malignant human cancer cell lines in 96-well microtitre plates. Each of the tested substance was characterized by the value of IC_{50} (inhibitory concentration 50%, Supplement I) or EC_{50} (effective concentration 50%, Supplement II). These values represents the quantity of the starting lyophilized plant material (mg·ml^{-1}) or the concentration of the compound (μM) required for inhibition of the growth of 50% of the cells in the tested cell lines. Two different sample preparation protocols were used, one for the plant extracts and extract fractions (Supplement I) and the modified one for the pure compounds (Supplement II).

Sample preparation

Each of the extract and/or purified fraction was dissolved in 1 ml of 20% methanol in 0.1 M Tris buffer (pH 7.2, Serva Electrophoresis, Heidelberg, Germany). Six 3-fold dilutions were prepared by subsequent dilutions in the 96-well microplates. It can be calculated that the resulting concentrations in the wells with cell suspension ranged from 0.8 to 200.0 mg·ml^{-1} cell culture medium in case of extract/fraction prepared from 1 g of lyophilized plant material (Supplement I). In case of tested pure compounds, 100 mM stock solutions were prepared in dimethylsulfoxide (DMSO) and then diluted to 15 mM with DMSO. The first concentration to be applied to the cells was prepared by diluting 15 mM DMSO solution distilled water to 500 μM. Starting with the 500 μM concentration, serial 3-fold dilutions (six in total) ranging from 2 to 500 μM were prepared by diluting the solution

with water. The highest final concentration of a tested compound in the wells with cell suspension was 100 μM and the lowest 0.4 μM. The final concentration of DMSO in the reaction mixture never exceeded 0.6% (Supplement II).

Cell cultures

For the basic screening of the cytotoxic activity against human cancer cell lines, the breast adenocarcinoma (MCF-7) and the acute lymphoblastic leukaemia (CEM) cancer cell lines were used. For deeper investigations of cytotoxicity and anticancer potential of selected compounds the human breast carcinoma cell line adriamycin resistantant (MCF-7AL), the human estrogen independent breast carcinoma (MDA-MB-468), the human multiple myeloma (RPMI 8226 and U266), the human cervical carcinoma cells (HeLa), the human lung adenocarcinoma epithelial cell line (A549) as well as normal human uterine fibroblasts (DV41) and normal human fibroblasts (BJ) were also used. The cell cultures were purchased from the American Type Culture Collection (ATCC, Rockvill, Maryland, USA) and maintained in plastic tissue culture flasks.

Treatment of cell cultures

The cells were grown on DMEM medium (Gibco BRL) supplemented with 10% (v/v) foetal bovine serum, 0.3 $g \cdot l^{-1}$ L-glutamine, 100 $U \cdot ml^{-1}$ penicillin and 100 $\mu g \cdot ml^{-1}$ streptomycin. The cultures were maintained at 37 °C in a fully humidified atmosphere containing 5% CO_2. Cells were subcultured two or three times a week using the standard trypsinization procedure. The cell suspensions were counted in a Bürker chamber and diluted in DMEM to an approximate density of $1.25 \cdot 10^5$ $cells \cdot ml^{-1}$. Then 80 μl of cell suspension was placed into each well of a 96-well microtitre plate and stabilized for 3 h. Afterwards, six prepared 3-fold diluted concentrations of extracts or compounds to be tested were added in triplicates in 20 μl aliquots to the appropriate microtitre plate wells. The cell suspensions in wells were incubated for 72 h at 37 °C in an atmosphere containing 5% CO_2 and then treated and incubated with 100 μl of Calcein AM solution (2 μM) for further 1 h. This resulted in fluorescent staining of viable cells by means of the free calcein produced.

Fluorescence measurement and cytotoxicity estimations

The fluorescence from viable cells was measured and quantified using a 485 nm excitation filter and a 538 nm emission filter in a Fluoroskan Ascent reader (Labsystems, Finland). The data measured by fluoroskan were processed by Ascent Calcein, Origin 6.0 and Microsoft Excel 2000 software. The percentage of surviving cells in each well was calculated as the ratio of the fluorescence obtained from each well with exposed cells/the mean fluorescence obtained from control wells multiplied by 100%. Finally, cytotoxic effective concentrations were calculated from the generated dose-response curves and expressed as IC_{50} or EC_{50} values (the concentrations causing a 50% reduction in the number of live cells).

5. Results and discussion

All the obtained results are summarized and discussed in this section separately according to the three main objectives of this doctoral thesis:

1 Isolation and identification of anticancer active compounds from *Acalypha alopecuroidea.* (Chapter 5.1: Investigation of *Acalypha alopecuroidea*.)
2 Geranylated flavanones from *Morus alba* and *Paulownia tomentosa* and their anticancer potential. (Chapter 5.2: Anticancer potential of geranylated flavanones.)
3 Miniaturized fast isolation and quantification of phytohormones from biological material. (Chapter 5.3: Miniaturized methods for phytohormone analysis.)

The majority of results is described in detail in publications attached as Supplement I-III.

5.1 Investigation of *Acalypha alopecuroidea*

Acalypha alopecuroidea (Euphorbiaceae) belongs to a group of plants widely used in traditional Mayan medicine in Central America to treat various diseases, among them acute and chronic inflammation and cancer. This plant has not been investigated phytochemically, hence the isolation and identification of active compounds may result in the discovery of potentially useful new compounds. On the basis of the above mentioned facts, this doctoral thesis was mainly focused on a search for anticancer active compounds in *A. alopecuroidea* extracts and fractions and the subsequent isolation and identification of compounds with detected anticancer potential. Anticancer activity was determined as a cytotoxic effect on the growth of malignant human cancer cells by a Calcein AM cytotoxicity assay in 96-well plates. Each extract and fraction was characterized by the IC_{50} value. This value indicated the quantity (mg) of the starting lyophilized plant material per ml cell culture medium, which inhibits the growth of 50% of the cells. Human breast adenocarcinoma cell line (MCF-7) and human leukaemic lymphoid cells (CEM) were used for the basic cytotoxicity screening. On the basis of the results from Calcein AM assay, the most active extract or extract fraction was chosen at each step of the whole isolation procedure and further purified. The phytochemical research on *A. alopecuroidea* was started by Prof. Georg Krupitza (Institute of Clinical Pathology, Medical University of Vienna, Austria) and Dr. Richard Frisch (Institute for Ethnobiology and Maya Research, Guatemala) who provided and delivered the plant material to the Laboratory of Growth Regulators.

5.1.1 Compound isolation and anticancer activity of isolated fractions

The first step in the isolation of compounds from *Acalypha alopecuroidea* with potential anticancer activity was a determination of the anticancer potential of individual plant parts extracted in different extraction solvents. *A. alopecuroidea* roots, leaves, stems and inflorescences were extracted in either ethanol 70% (v/v) or a mixture of methanol-tetrahydrofurane (MeOH-THF, 1:1). Polar organic solvents such as ethanol serve mainly to extract polar compounds, while the mixture of solvents with different polarity ensured extraction of as broad spectrum of compounds as possible in one extraction procedure. In this case, methanol extracted polar compounds while tetrahydrofurane extracted non-polar ones. As determined by Calcein AM assay, the stem extracts were the least effective and the root extracts the most effective. In case of 70% ethanol root extract, IC_{50} concentration for MCF-7 and

CEM cell line was 2.5 mg·ml^{-1} and less than 0.4 mg·ml^{-1}, respectively. In case of MeOH-THF root extract, IC_{50} concentration for MCF-7 and CEM cells was 1.1 mg·ml^{-1} and 0.9 mg·ml^{-1}, respectively (Supplement I). The MeOH-THF root extract was considered as the most active. After this initial cytotoxicity testing, only roots were extracted in a variety of different solvents and examined towards four cell lines to prove the highest effectivity of the MeOH-THF extraction solvent (Table 1, Objective 1). Slight differences in IC_{50} values of one extract type determined towards the same cell line are probably incurred by different batch of plant extract as well as the actual health condition and variability of cells as living organisms.

Table 1. Cytotoxic activities, towards different cell lines, of extracts prepared from *A. alopecuroidea* roots.

Extracted DW (g)	Extraction solvent	Extr. temp.	IC_{50} (mg·ml^{-1})			
			MCF-7	CEM	HeLa	DV41
1.0	MeOH-THF (1:1)	-20 ºC	0.65	0.51	34.50	ND
1.0	methanol	Lab t.	7.87	0.41	ND	ND
1.0	methanol 80% (v/v)	Lab t.	2.75	0.41	ND	ND
1.0	ethanol	Lab t.	6.90	0.90	ND	ND
1.0	ethanol 70% (v/v)	Lab t.	2.73	0.98	24.50	6.10
2.0	H_2O	4 ºC	>50.00	27.33	>50.00	ND
2.0	H_2O	Lab t.	>50.00	25.00	>50.00	ND
2.0	H_2O H^+	4 ºC	>50.00	28.00	>50.00	ND
2.0	H_2O H^+	Lab t.	>50.00	27.00	>50.00	ND
2.0	H_2O OH^-	4 ºC	>50.00	13.50	32.00	ND
2.0	H_2O OH^-	Lab t.	>50.00	18.50	>50.00	ND

Cytotoxicity was determined by Calcein AM cytotoxicity assays towards MCF-7, CEM, HeLa and DV41 cells. The IC_{50} values in the table indicate the quantity (mg) of the lyophilized starting plant material per ml cell culture medium lethal to 50% cancer cells. Water was acidified with 0.3% formic acid (pH = 2.91), or alkalined with 0.3% NH_4OH (pH = 9.94). ND, not determined.

The most potent MeOH-THF root extract was taken for the further investigation. In the next step of the compound isolation, root samples (5.0 or 2.0 g DW) extracted in MeOH-THF were purified by solid-phase extraction on AccuBond C18, DEAE-Sephadex and Sep-Pak C18 columns (Figure 20). This purification resulted in six fractions (A-F), which were evaluated by Calcein AM assay. Fractions A, B, and E expressed significant cytotoxicity towards MCF-7 and CEM cell lines in contrast with fractions C, D and F, which had negligible effects on MCF-7 cell line and minimal effects on CEM cells. Fraction A ($IC_{50\ (CEM)}$ = 46.0 mg·ml^{-1}, $IC_{50\ (MCF\text{-}7)}$ = 215.0 mg·ml^{-1}) was the buffer flow through solution of the coupled DEAE-Sephadex and first Sep-Pak C18 column. Fraction B ($IC_{50\ (CEM)}$ = 15.3 mg·ml^{-1}, $IC_{50\ (MCF\text{-}7)}$ = 127.5 mg·ml^{-1}) was collected as the methanolic eluate of the first Sep-Pak. Fraction E ($IC_{50\ (CEM)}$ = 130.0 mg·ml^{-1}, $IC_{50\ (MCF\text{-}7)}$ = 550.0 mg·ml^{-1}) was the methanolic eluate of the third Sep-Pak coupled to the DEAE-Sephadex column (Supplement I).

The cytotoxicity data published in Supplement I were completed with cytotoxicity testing of fractions A-F towards human cervical carcinoma cells (HeLa) and normal human uterine fibroblasts (DV41) (Table 2). These additional data confirmed that fraction B was the most active one. Therefore, fraction B was used for further studies and fractionated by two-dimensional high performance liquid chromatography. During the routine isolation of active compounds, the optimized SPE with gradient elution of the first Sep-Pak cartridge was performed to reduce the background of co-eluting

compounds prior to the HPLC fractionation. The second and third Sep-Pak cartridges were not used. This modified SPE was repeated many times to obtain active fraction B in amount sufficient for identification of individual content substances (Objective 1).

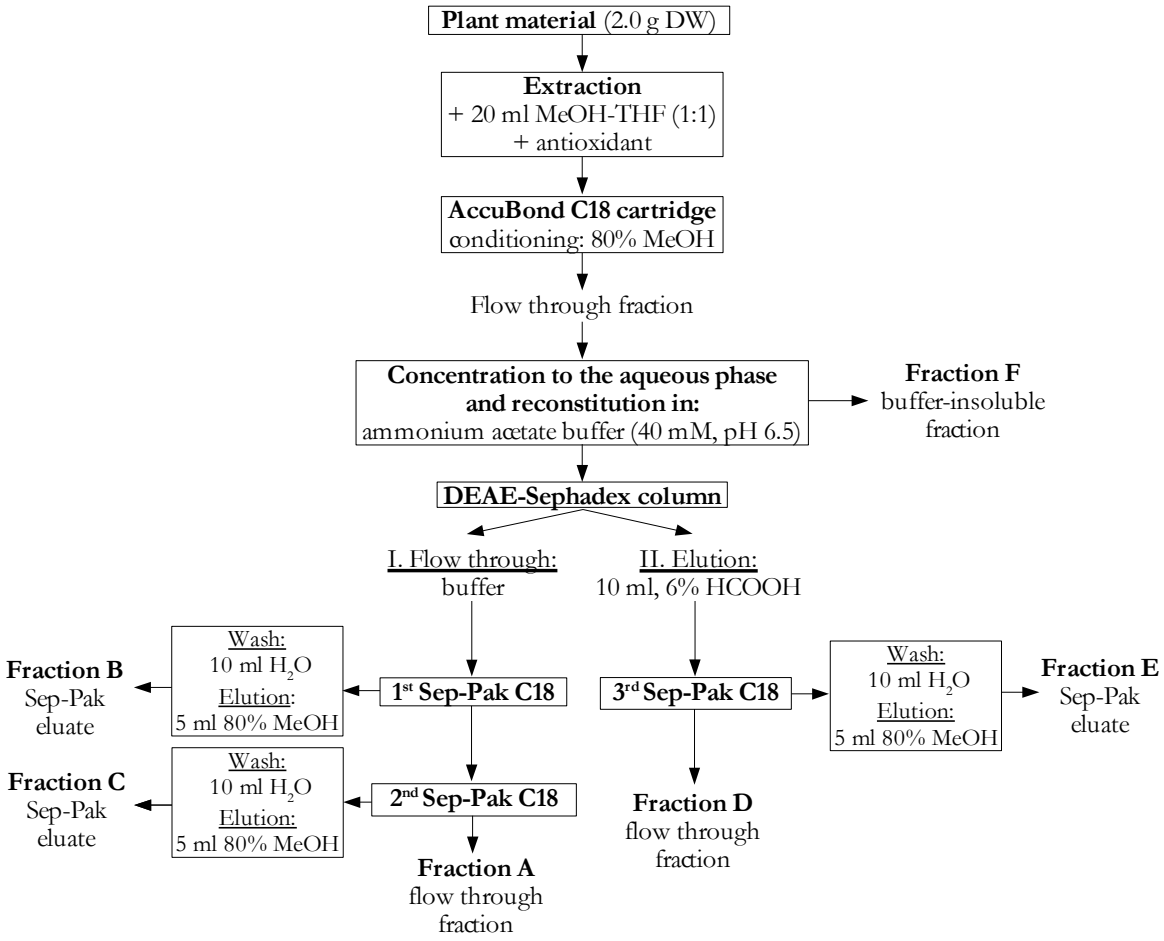


Figure 20. Initial protocol of root extraction and purification of the MeOH-THF extract of *A. alopecuroidea*. Plant material (2.0 g DW) is homogenized and extracted in 20 ml of MeOH-THF (1:1) with addition of antioxidant. Supernatant is immediately applied on a pre-conditioned AccuBond C18 column and the flow through fraction is concentrated to the aqueous phase. This is dissolved in ammonium acetate buffer (40 mM, pH 6.5) and purified by application on a DEAE-Sephadex column coupled with two Sep-Pak cartridges in tandem. Afterwards, the DEAE-Sephadex is coupled to the third Sep-Pak and washed with formic acid 6% (v/v). The flow through portions are collected as fractions A and D. The compounds retained on Sep-Pak cartridges are eluted with 5 ml methanol 80% (v/v) as fractions B, C and E. Collected fractions are evaporated to dryness and stored at -20 ºC.

Table 2. Cytotoxic activities of SPE-fractions derived from the MeOH-THF root extract of *A. alopecuroidea*.

Fraction	Extracted DW (g)	IC_{50} (mg·ml^{-1})	
		HeLa	DV41
A	2.0	179.0	>200.0
B	2.0	59.0	>200.0
C	2.0	>200.0	>200.0
D	2.0	>200.0	>200.0
E	2.0	177.0	199.0
F	2.0	81.0	84.0

Cytotoxicity was determined by Calcein AM assays towards HeLa and DV41 cells. The IC_{50} values in the table indicate the quantity (mg) of the lyophilized starting plant material per ml cell culture medium lethal to 50% cancer cells.

The initial HPLC fractionation of *A. alopecuroidea* fraction B was performed on a preparative reversed-phase column Reprosil 100. Twenty-seven one-minute fractions were collected and their cytotoxicity was determined by Calcein AM assay. The fraction with the highest activity had a retention time of 25 min and showed comparable biological activity as fraction B of the MeOH-THF extract towards CEM cells (Table 3). A weaker activity was detected in fractions B7, B17 and B26. Consequently, fractions B7, B25 and B26 were used in different biological assays, which are described in details in Supplement I.

Table 3. Cytotoxic activities of HPLC fractions obtained by separating fraction B of the MeOH-THF root extract of *A. alopecuroidea*.

Fraction	IC_{50} (mg·ml^{-1})			
	Preparative column		Semipreparative column	
	MCF-7	CEM	MCF-7	CEM
B1	> 200.0	148.0	> 200.0	151.5
B2	> 200.0	> 200.0	> 200.0	164.5
B3	> 200.0	> 200.0	> 200.0	130.5
B4	> 200.0	> 200.0	> 200.0	146.5
B5	> 200.0	> 200.0	> 200.0	117.0
B6	> 200.0	> 200.0	> 200.0	174.0
B7	> 200.0	89.0	> 200.0	160.0
B8	> 200.0	> 200.0	> 200.0	152.0
B9	> 200.0	> 200.0	> 200.0	167.0
B10	> 200.0	> 200.0	> 200.0	138.0
B11	> 200.0	173.0	> 200.0	131.5
B12	> 200.0	> 200.0	167.0	97.0
B13	> 200.0	> 200.0	> 200.0	200.0
B14	> 200.0	> 200.0	> 200.0	160.0
B15	> 200.0	> 200.0	> 200.0	196.0
B16	> 200.0	> 200.0	> 200.0	163.0
B17	175.0	153.0	> 200.0	156.5
B18	200.0	166.0	> 200.0	140.5
B19	> 200.0	> 200.0	> 200.0	183.5
B20	> 200.0	174.0	> 200.0	200.0
B21	> 200.0	> 200.0	> 200.0	200.0
B22	> 200.0	> 200.0	30.5	13.5
B23	> 200.0	125.0	86.5	0.5
B24	ND	ND	92.5	52.5
B25	32.0	12.0	> 200.0	130.5
B26	> 200.0	73.0	> 200.0	145.5
B27	> 200.0	140.0	> 200.0	134.5
B28	ND	ND	> 200.0	145.0
B29	ND	ND	> 200.0	167.5
B30	ND	ND	> 200.0	190.0

Cytotoxicity was determined by Calcein AM assays. The IC_{50} values in the table indicate the quantity (mg) of the lyophilized starting plant material per ml cell culture medium lethal to 50% cancer cells. ND, not determined.

The routine HPLC fractionation and purification (Objective 1) in order to isolate individual active compounds present in fraction B and to maximize the yields of active components was performed by a semipreparative 2D-HPLC separation using reversed-phase columns (Figure 21). The first HPLC fractionation on a Symmetry Shield RP-18 column resulted in thirty one-minute fractions. The cytotoxicity profile of collected one minute fractions was similar to that in case of the preparative C8 Reprosil 100 column. Using the Symmetry Shield RP-18 column, fraction B23 was the most active one (Table 3) and displayed similar biological activity to the whole fraction B. The isolated fraction

B23 was repeatedly fractionated by the second HPLC (Figure 22) to half-minute fractions using an analytical Symmetry C18 column and utilized for optimization of the second HPLC step. Collected half-minute fractions were subsequently tested by Calcein AM assay (Table 4). Fractions B23/17-18, which displayed the highest cytotoxic activity, corresponded to one chromatographic peak. Therefore, these fractions were combined together and submitted to MS and NMR analysis (Objective 1).

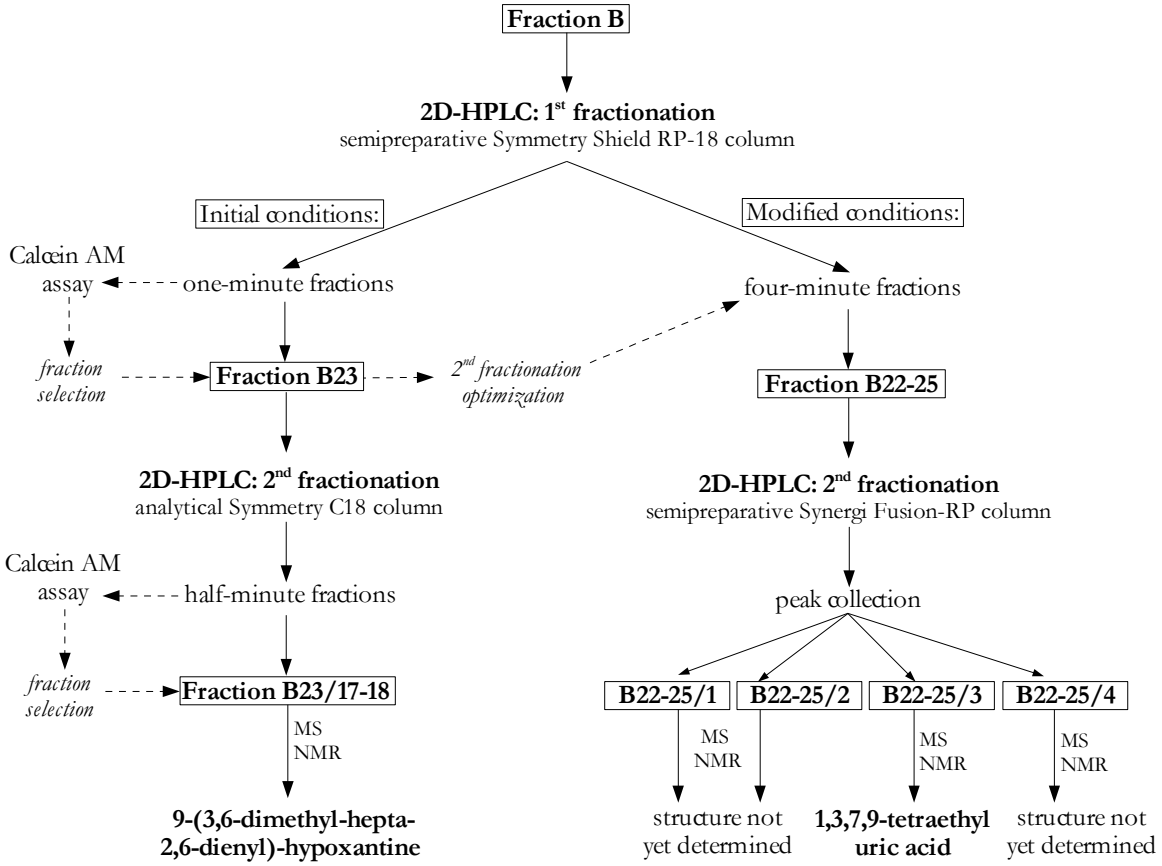


Figure 21. Scheme of 2D-HPLC purification of fraction B of the MeOH-THF root extract of *A. alopecuroidea*. Active HPLC fractions obtained from fraction B were selected according to the Calcein AM assay and subsequently isolated in higher amounts using two slightly modified HPLC conditions. The structure of isolated fractions was determined by means of MS and NMR experiments.

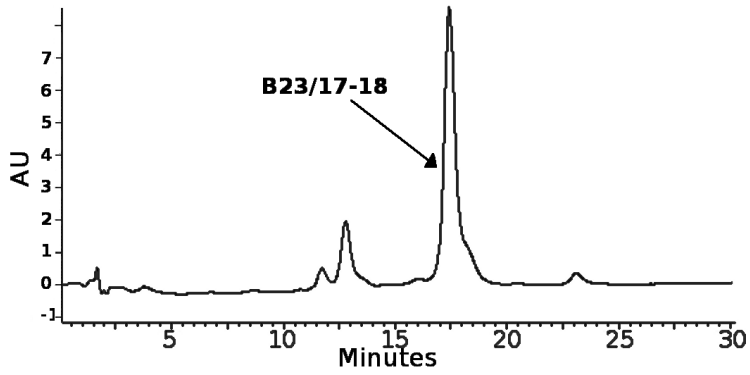


Figure 22. Chromatographic separation of partially purified fraction B23 of the MeOH-THF root extract of *A. alopecuroidea* on an analytical Symmetry C18 column. UV chromatogram is monitored at 250 nm. Half-minute fraction were collected and examined by Calcein AM assay, MS and NMR.

Table 4. Cytotoxic activities of selected HPLC fractions obtained by separating fraction B23 of the MeOH-THF root extract of *A. alopecuroidea*.

Fraction	Extracted DW (g)	IC_{50} (mg·ml-1)	
		MCF-7	CEM
B23/16.0	2.8	>280.0	169.0
B23/16.5	2.8	>280.0	133.0
B23/17.0	2.8	69.0	17.0
B23/17.5	2.8	101.0	4.7
B23/18.0	2.8	74.0	18.0
B23/18.5	2.8	264.0	61.0
B23/19.0	2.8	>280.0	243.0
B23/19.5	2.8	>280.0	>280.0
B23/20.0	2.8	>280.0	>280.0

Cytotoxicity was determined by Calcein AM assays. The IC_{50} values in the table indicate the quantity (mg) of the lyophilized starting plant material per ml cell culture medium lethal to 50% cancer cells.

The 2D-HPLC separation of fraction B was later modified (see Figure 21) to ensure the highest yields of the desired fraction B23 necessary for successful structure elucidation. For this purpose, the crude fraction B corresponding to 1.0-2.0 g of dry plant material was re-applied (ca. 240 injections in total) onto the first column (Symmetry Shield RP-18) and not only fraction B23, but the range of fractions B22-25 was collected. The total yield of fraction B22-25, isolated from 436.0 g of lyophilized *A. alopecuroidea* roots by means of SPE and the first HPLC, was 31.47 mg. Isolated fraction B22-25 was repeatedly submitted to the second HPLC fractionation using a semipreparative Synergi Fusion-RP column (instead of the analytical Symmetry C18 column used for fractionation of fraction B23). The second HPLC step allowed a separation of four major individual chromatographic peaks, assigned with numbers 1-4 (Figure 23). The total fraction (peak) yield after ca. 72 injections, peak retention times (t_R), wavelength of maximum absorbance (λ_{max}), and IC_{50} values are summarized in Table 5 (Objective 1).

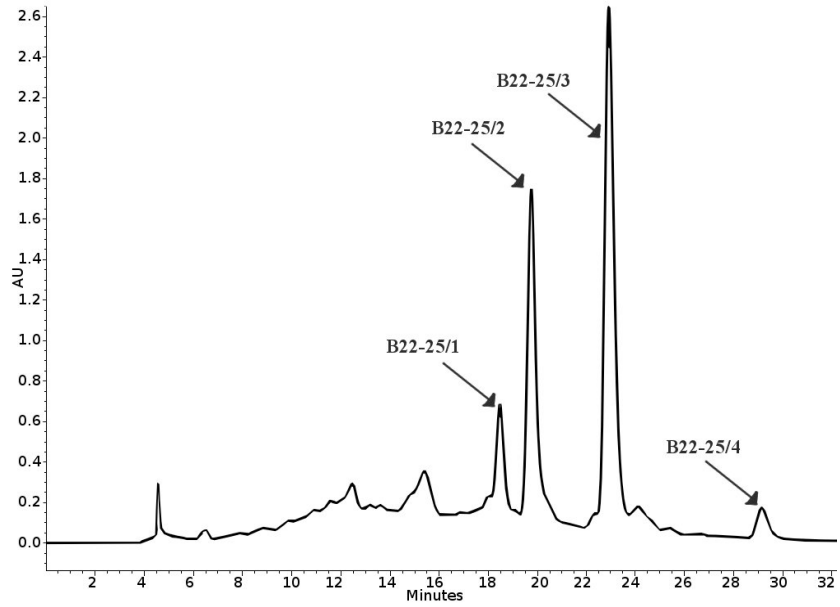


Figure 23. Chromatographic separation of partially purified fraction B22-25 of the MeOH-THF root extract of *A. alopecuroidea* on a semipreparative Synergi Fusion-RP column. UV chromatogram is monitored at 250 nm. Four individual chromatographic fractions (B22-25/1-4) were isolated.

Table 5. Chromatographic, UV and cytotoxicity characteristics of four chromatographic fractions (peaks) B22-25/1-4 separated on a semipreparative Synergi Fusion-RP column.

Fraction	Isolated amount (mg)	t_R (min) UV-DAD	λ_{max} (nm)	IC_{50} (mg·ml^{-1})	
				MCF-7	CEM
B22-25/1	1.94	18.51	277.2	98.0	59.0
B22-25/2	2.78	19.89	279.5	84.0	16.0
B22-25/3	4.18	22.96	279.5	78.0	1.3
B22-25/4	0.41	29.17	215.8	>400.0	358.0

The fractions were isolated from the MeOH-THF root extract of *A. alopecuroidea* by means of the SPE and 2D-HPLC using semipreparative reversed-phase columns. Cytotoxicity was determined by Calcein AM assays. The IC_{50} values in the table were calculated as the quantity (mg) of the lyophilized starting plant material per ml cell culture medium lethal to 50% cancer cells.

5.1.2 Biological activity of *Acalypha* extracts

In addition to the cytotoxicity tests, other biological assays were used to discriminate between the effects of *Acalypha alopecuroidea* extracts on the cell cycle machinery (growth inhibition) and cell death (apoptosis). All the other assays were performed by Sibylle Madlener and Prof. Georg Krupitza (Institute of Clinical Pathology, Medical University of Vienna, Austria). Briefly, the root extract inhibited TNF-α-induced E-selectin (CD62E) expression and c-Jun activation (phosphorylation) in HUVECs (human umbilical vein endothelial cells). The activation of c-Jun probably plays a role in hepatic inflammation and in arteriosclerosis. Another mediator of inflammatory reaction is the enzyme cyclooxygenase-2 (COX-2). It was found out that COX-2 enzymatic activity was not inhibited by MeOH-THF extract of *A. alopecuroidea* (Supplement I).

Fraction B25 obtained from the initial preparative HPLC fractionation displayed cell cycle inhibitory activity by inactivation of cyclin D1 and Cdc25A and induction of cyclin A. The inhibition of the cell cycle progression and cell proliferation appeared to be mediated by down-regulation and suppression of the positive cell cycle regulators cyclin D1 and Cdc25A, which causes the G1/S phase arrest and abrogation of mitotic signalling. Together with the rapid induction of cyclin A, which accelerates the G2/M transition, the controlled cell cycle arrest is disturbed. Furthermore, fraction B25 inhibited the cell proliferation in chemoresistant MCF-7 clones (resistant to tamoxifen and cytosine-arabinoside) and may be a potent candidate for the treatment of breast cancer. The apoptotic response rate of MCF-7 clones (dependent on caspase 7) to fraction B25 was rather low, whereas the apoptosis of human promyelocytic leukaemia cells (HL-60), dependent on activation of caspase 3, was induced. These findings indicate that constituents of fraction B25 may also posses potential value as caspase 3 specific apoptosis-inducing agents.

All the results are fully described in Supplement I and confirm the high prospects for the isolation of biologically active constituents from *Acalypha alopecuroidea* and the use of purified compounds for *in vivo* preclinical studies.

5.1.3 Identification of isolated compounds

Isolated active fractions, namely combined fraction B23/17-18, which was collected during optimization of the purification procedure, and isolated individual fractions B22-25/1-4, were submitted to mass spectrometry and nuclear magnetic resonance spectroscopy in order to identify the structure of present compounds (Objective 1). The MS identification of the fractions based on

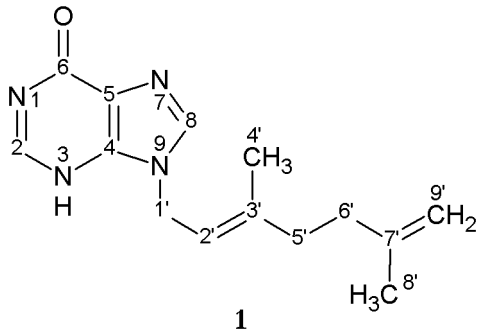
measurement of precursor and product ions was performed by HPLC/MS analyses using a single quadrupole or hybrid tandem (QqTOF) mass spectrometers. The samples were injected onto an analytical Symmetry C18 column and monitored simultaneously by a photodiode array detector and mass spectrometric analysers. The summary of detected retention times (t_R), wavelength of maximum absorbance (λ_{max}) and exact masses of protonated and sodium adduct ions are shown in Table 6.

Table 6. Chromatographic, UV and MS characteristics of isolated active fraction B23/17-18 and four chromatographic fractions (peaks) B22-25/1-4.

Fraction	t_R (min) UV-DAD	t_R (min) MS	λ_{max} (nm)	Exact mass (m/z)		
				$[M+H]^+$	$[M+Na]^+$	$[2M+Na]^+$
B23/17-18	17.42	17.16	279.93	281.0875	303.0722	583.1599
B22-25/1	12.61	12.73	275.93	236.0989	258.0828	ND
B22-25/2	13.64	13.71	279.93	265.1039	287.0844	551.1890
B22-25/3	18.41	18.50	279.93	281.0948	303.0731	583.1682
B22-25/4	24.39	24.54	217.93	297.0805	319.0623	615.1388

The fractions were isolated from the MeOH-THF root extract of *A. alopecuroidea* by means of the SPE and 2D-HPLC using initial (fraction B23/17-18) or modified (fractions B22-25/1-4) conditions. Fractions were injected onto a Symmetry C18 column and analysed by UV-DAD and Q-TOF micro™ mass analyser. ND, not detected.

Moreover, structures of isolated compounds were proposed with help of the second method for structure elucidation. The NMR measurements were successfully applied on fractions B23/17-18 and B22-25/3 purified by HPLC on the analytical Symmetry C18 and semipreparative Synergi Fusion-RP columns, respectively. All the NMR data were measured by Prof. Virginia Lanzotti, Elisa Barile and Adriana Romano at the Department of Chemistry of Natural Substances (Pharmaceutical Faculty, University of Naples Federico II, Italy) using the 400 MHz, 500 MHz and 700 MHz spectrometers. Two structures (Figure 24) were finally designed according to one- (^{1}H) and two-dimensional (COSY, HSQC, HMBC) NMR spectra, full-scan mass spectra and the measured accurate mass of protonated or sodium adduct ions in positive MS mode (Figure 25).

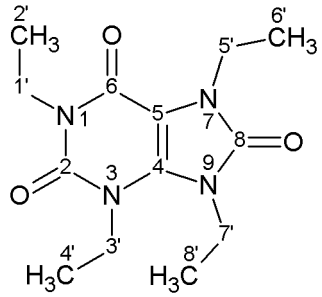


1

9-(3,6-dimethylhepta-2,6-dienyl)-hypoxanthine

Exact mass = 258.1481

Elemental composition = $C_{14}H_{18}N_4O$



2

1,3,7,9-tetraethyluric acid

Exact mass = 280.1535

Elemental composition = $C_{13}H_{20}N_4O_3$

Figure 24. Designed structures of isolated compounds from fractions B23/17-18 (Compound **1**) and B22-25/3 (Compound **2**).

Under the same chromatographic conditions, compound **1** (fraction B23/17-18) had comparable retention time, absorption maximum and mass spectra to compound **2** (fraction B22-25/3, see Table 6, Figure 25). Although both compounds gave corresponding molecular weights, different structures and elemental compositions were predicated according to their divergent NMR spectra (Figure 26 and 27).

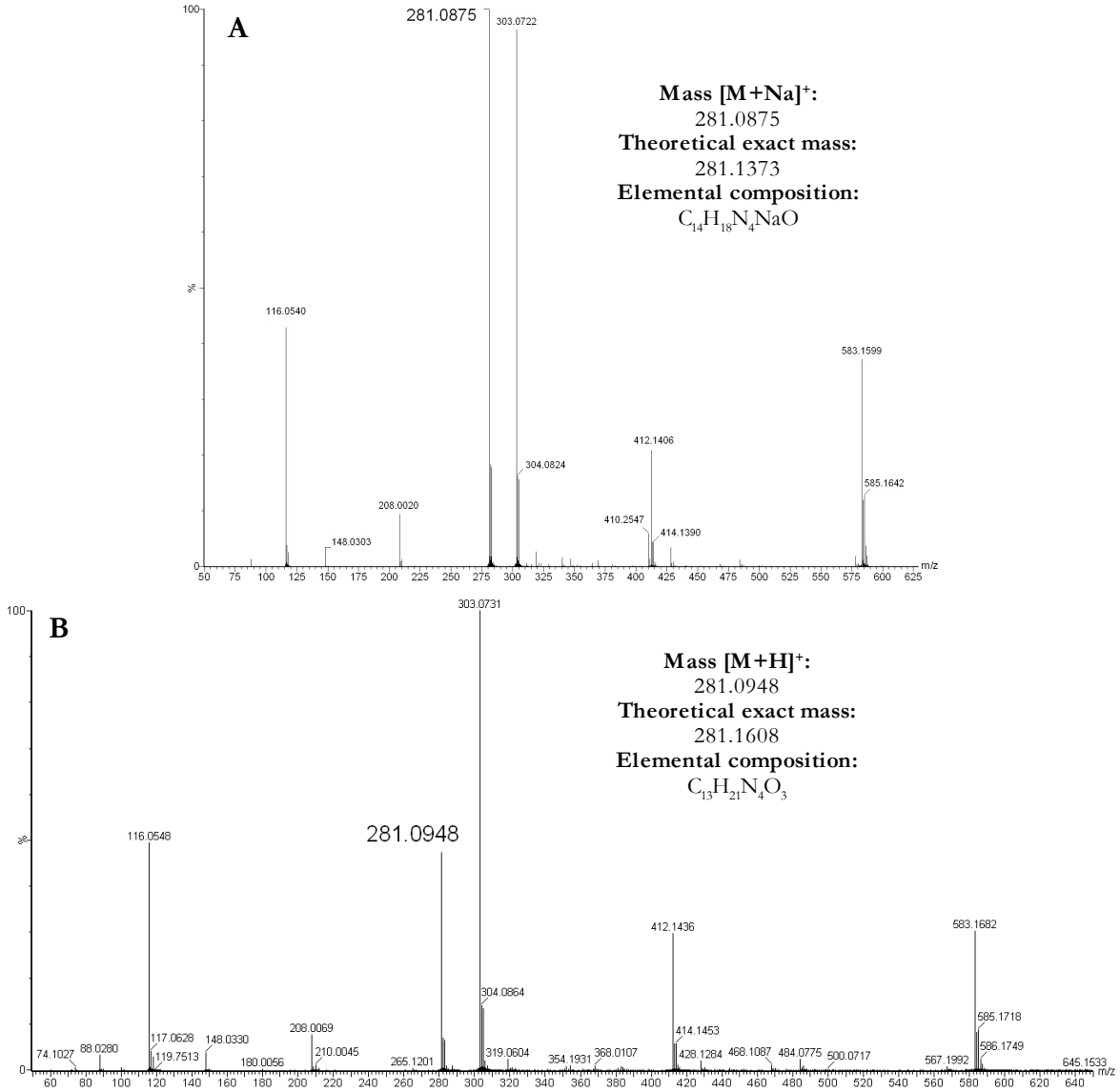


Figure 25. Full scan mass spectra of compound **1** (A) and compound **2** (B) isolated form the MeOH-THF root extract of *Acalypha alopecuroidea*.

As demonstrated in Figure 24, the structure of compound **1** was determined as 9-(3,6-dimethylhepta-2,6-dienyl)-hypoxantine. On the bases of QqTOF analysis ($[M+Na]^+$, m/z 281.0875, see Figure 25) and NMR spectral data (Figure 26), the molecular formula of **1** was deduced as $C_{14}H_{18}N_4O$. The ^1H NMR spectrum (500 MHz, CD_3OD) displayed signal for a proton at *C*2 (δ = 8.49 ppm, 1H) and *C*8 (δ = 6.98 ppm, 1H), which revealed the probable presence of hypoxanthine skeleton. Signals for protons attached to carbons in the unsaturated side chain at position *N*9 of hypoxanthine were detected as follows: δ = 4.01 ppm (2H, *C*1'-H), 5.60 ppm (1H, *C*2'-H), 1.61 ppm (3H, *C*4'-H), 2.37 ppm (2H, *C*5'-H), 2.37 ppm (2H, *C*6'-H), 1.72 ppm (3H, *C*8'-H). Signals observed for protons at *C*9' (δ = 6.09 ppm, 1H; δ = 5.45 ppm, 1H) indicated the presence of terminal methylene group. Homonuclear COSY experiments showed correlations between hydrogen atoms. Heteronuclear HSQC and HMBC experiments showed one-bond and long-range correlations between hydrogen and carbon atoms. These 2D experiments disclosed the position of atoms in the molecule and enabled to design the complete structure of compound **1**.

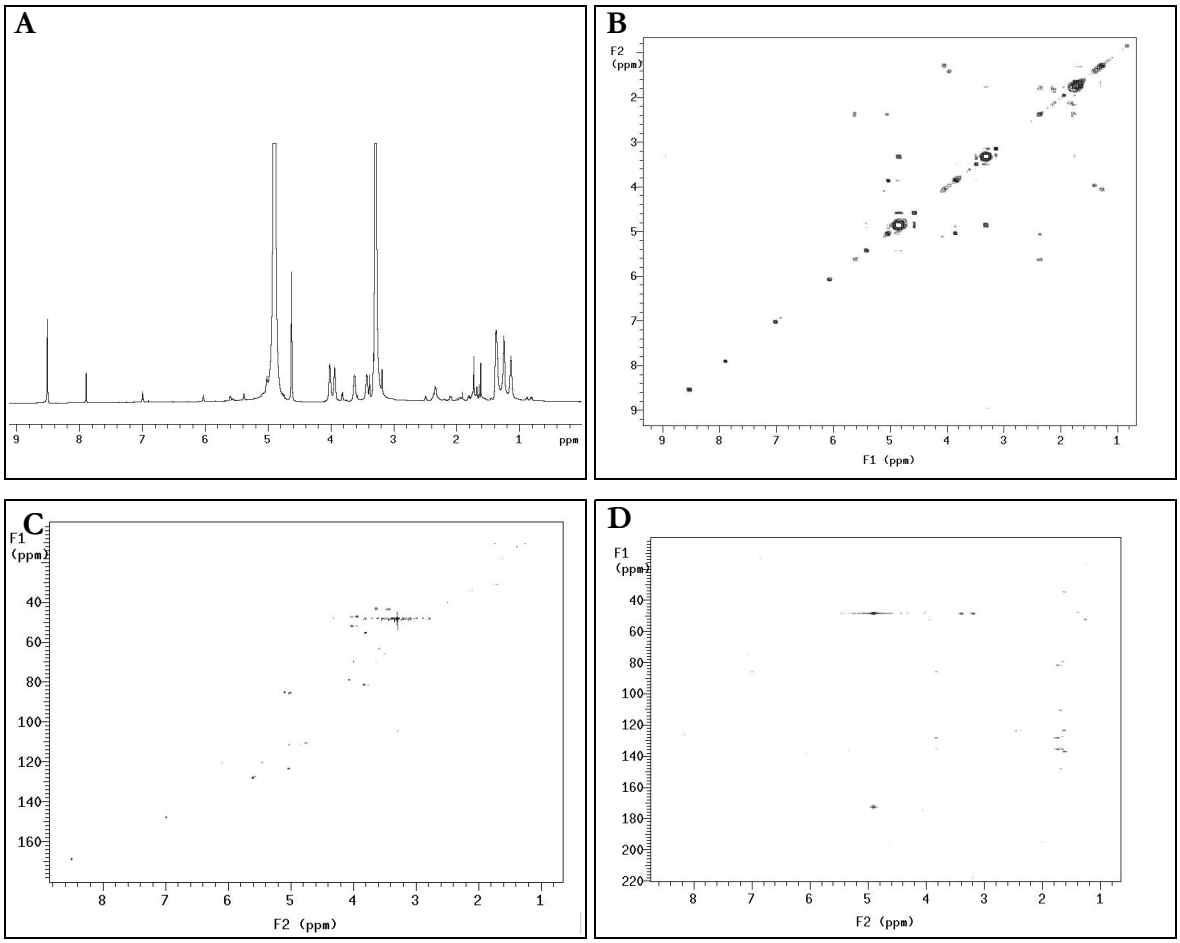


Figure 26. NMR spectra of compound **1** isolated form the MeOH-THF root extract of *Acalypha alopecuroidea*. A) ^{1}H NMR spectra (500 MHz, CD_3OD); B) ^{1}H-^{1}H COSY (400 MHz, CD_3OD); C) ^{1}H-^{13}C HSQC (700 MHz, CD_3OD); D) ^{1}H-^{13}C HMBC (700 MHz, CD_3OD).

On the other hand, the NMR spectra interpretation of compound **2** resulted in tentative elucidation of 1,3,7,9-tetraethyluric acid (1,3,7,9-tetraethyl-7,9-dihydro-1*H*-purine-2,6,8(3*H*)-trion; TEUA). The structure of TEUA is shown in Figure 24. On the bases of QqTOF analysis ($[M+H]^+$, m/z 281.0948, see Figure 25) and NMR spectral data (Figure 27), the molecular formula of **2** was determined as $C_{13}H_{20}N_4O_3$. Chemical shifts in ^{1}H NMR spectra were attributed to hydrogen atoms present in the molecule. In the ^{1}H NMR spectrum (500 MHz, DMSO-d_6), signals for methyl groups were observed at δ = 1.06 ppm (bs, 2H), 1.19 ppm (t, J = 6.8 Hz, 3H), 1.27 ppm (bs, 2H) and 1.32 ppm (t, J = 6.9 Hz, 3H). Signals for methylene groups were detected at δ = 3.53 ppm (bs, 2H), 3.91 ppm (q, J = 7.0 Hz, 2H) and 3.96 ppm (q, J = 7.0 Hz, 2H). The interpretation of ^{1}H and 2D NMR spectra (COSY, HSQC, HMBC) disclosed the presence of ethyl groups in the structure of compound **2**, which were attached at position *N*1, *N*3, *N*7 and *N*9 of the basic skeleton of uric acid.

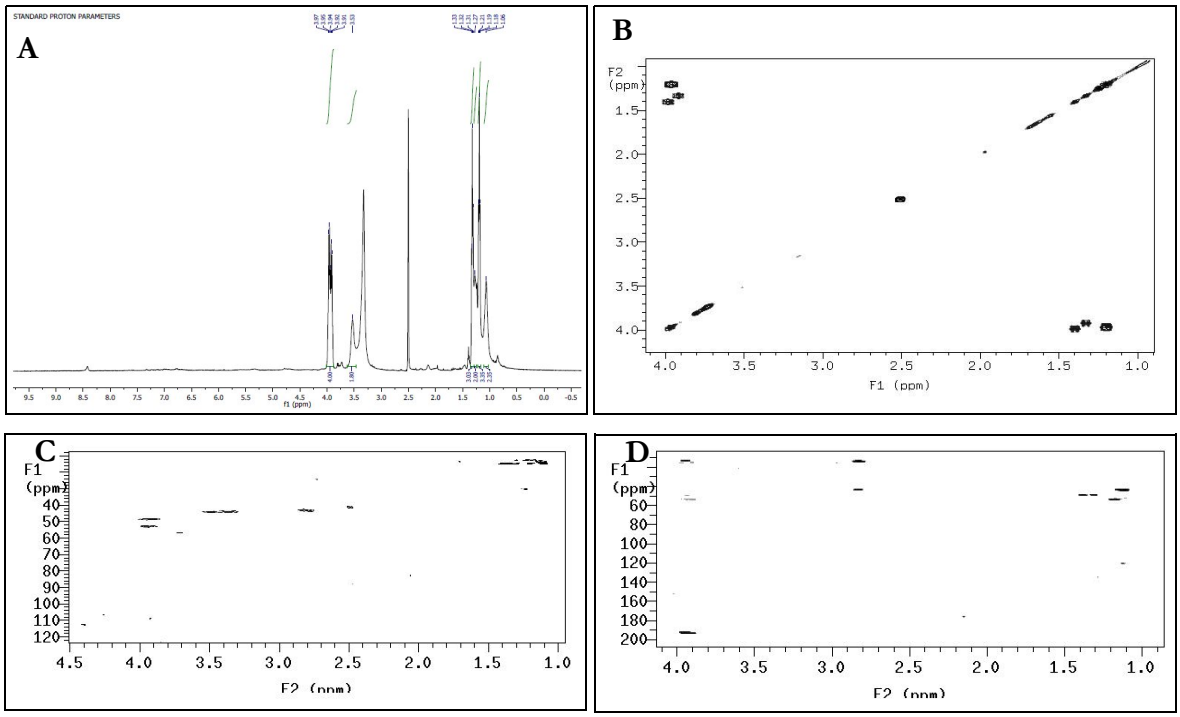


Figure 27. NMR spectra of compound **2** isolated form the MeOH-THF root extract of *Acalypha alopecuroidea*. A) ^{1}H NMR spectra (500 MHz, DMSO-d_6); B) ^{1}H-^{1}H COSY (400 MHz, DMSO-d_6); C) ^{1}H-^{13}C HSQC (700 MHz, DMSO-d_6); D) ^{1}H-^{13}C HMBC (700 MHz, DMSO-d_6).

Although the amount of lyophilized plant material used for compound isolation was almost 500 g, the final 0.5-4.5 mg fraction weight of isolated individual peaks was not very high and probably not sufficient for the nuclear magnetic resonance spectroscopy measurements and generation of high-quality spectra. It was not possible to completely elucidate the structure of B22-25/1, because of the complexity of this fraction and probable presence of several compounds. Unfortunately, compounds occurring in fractions B22-25/2 and B22-25/4 were also not identified. Therefore, the structures shown above are only one of the structural possibilities to the detected compounds and they need to be further verified. It is also not clear, why fractions B23/17-18 and B22-25/3 with similar mass spectra provided different nuclear magnetic resonance spectra resulting in propose of two different structures. The main difference in those two fractions was that they were isolated from different batches of plant material and purified by slightly modified HPLC fractionation (Figure 21). In the first case, the analytical C18 column was used and the fraction at 17-18 min, which exhibited the highest cytotoxic activity, was collected and analysed (Figure 22). In the second case, the semipreparative C18 column was applied after optimization of HPLC conditions and the individual chromatographic peaks were isolated (Figure 23). Despite an incomplete structure elucidation, the designed structures give some insight to the novel possible chemical modification of natural substances.

5.1.4 Synthetic analogues

The suggested chemical structures of isolated compounds served as templates for the synthesis of a group of new *N*9-prenylated derivatives of adenine and hypoxanthine and the group of *N*-alkyl derivatives of uric acid (Objective 1). The structures shown in Figure 28 were synthesized by RNDr. Marek Zatloukal, Ph.D. at the Laboratory of Growth Regulators and detailed procedures of the syntheses as well as structural confirmation will be published elsewhere.

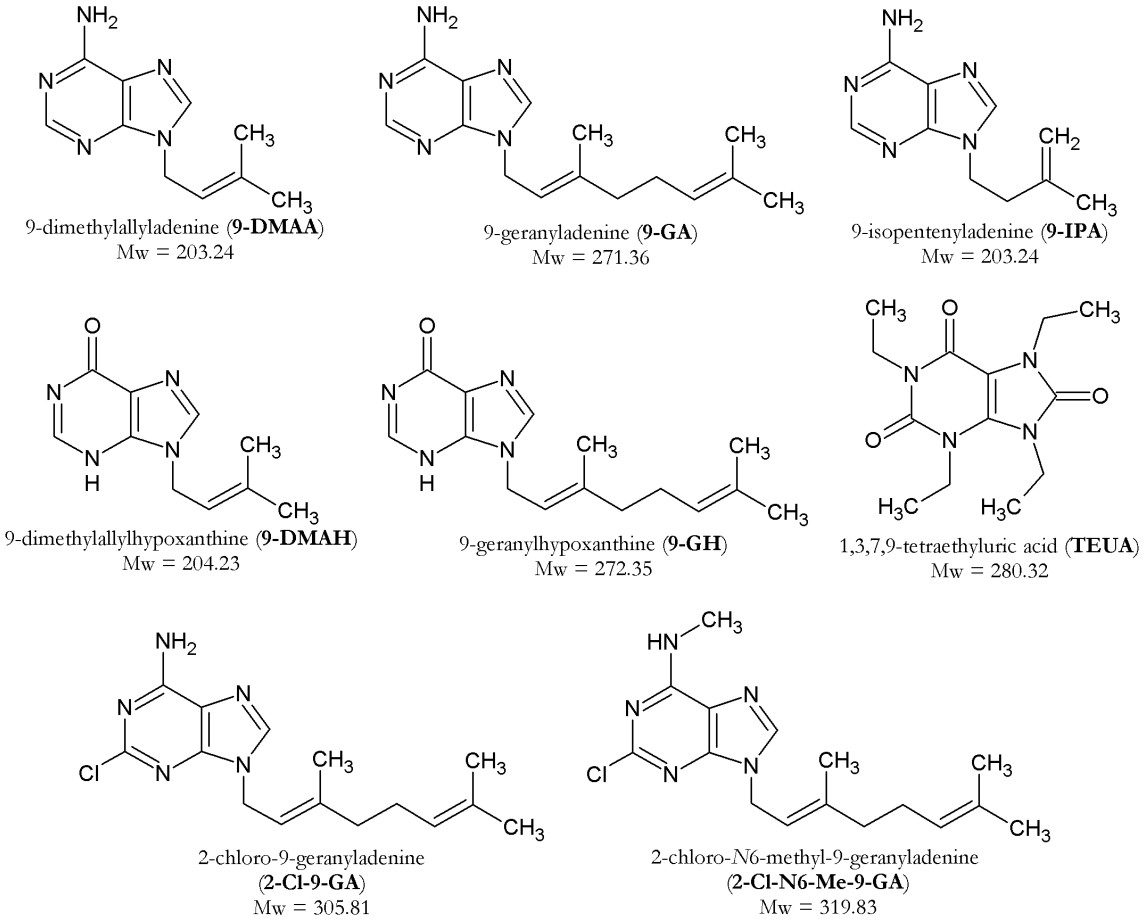


Figure 28. Synthetically prepared *N*9-prenylated derivatives of adenine and hypoxanthine and *N*-alkyl derivative of uric acid tested in Calcein AM assay.

The structure-activity relationship of these synthesized compounds became an object of further investigation. Cytotoxic activity was tested using Calcein AM cytotoxicity assay and the obtained IC_{50} values are summarized in Table 7. Isopentenyl (dimethylallyl) derivatives were less active than geranyl derivatives of both adenine and hypoxanthine. Therefore, the presence of geranyl side chain can be considered as the crucial factor of the higher cytotoxicity. Presence of chlorine as the heteroatom bound on *C*2 of adenine core of 9-geranyladenine brought further rise in cytotoxic activity against human cancer cell lines. On the other hand, these chlorine derivatives displayed some level of cytotoxicity also towards normal human fibroblasts. Usually, compounds with IC_{50} less than 10 μM are considered as interesting structures in relation to their cytotoxic effects. Compounds with higher IC_{50} values can be further chemically modified to obtain compounds with selective anticancer activity and lower side effects on normal cells.

Table 7. Cytotoxic activities of synthetic *N*9-prenylated derivatives of adenine and hypoxanthine and *N*-alkyl derivatives of uric acid.

Compound	IC_{50} (μM)									
	MCF-7	**CEM**	**HeLa**	**DV41**	**MCF 7AL**	**A549**	**BJ**	**U266**	**RPMI 8226**	**MDA MB-468**
9-DMAA	>100	99.1	>100	>100	ND	ND	ND	ND	ND	ND
9-DMAH	>100	>100	>100	>100	ND	ND	ND	ND	ND	ND
9-GA	78.8	44.2	24.7	>100	ND	ND	ND	ND	ND	ND
9-GH	80.7	43.9	45.9	>100	ND	ND	ND	ND	ND	ND
9-IPA	>100	>100	>100	>100	ND	ND	ND	ND	ND	ND
2-Cl-9-GA	16.3	27.1	41.0	ND	45.8	ND	30.6	ND	ND	ND
cis-2-Cl-N6-Me-9-GA	41.1	27.1	38.8	ND	26.6	84.1	87.5	ND	ND	ND
trans-2-Cl-N6-Me-9-GA	36.3	33.4	32.1	ND	31.5	60.0	88.6	ND	ND	ND
TEUA	>600	>600	178.9	ND	>600	>600	ND	>600	>600	>600

Cytotoxicity was determined by Calcein AM assays towards MCF-7, CEM, HeLa, DV41 cells. The IC_{50} values in the table indicate the concentration (μM) of tested compounds lethal to 50% cancer cells. ND, not determined.

For the first time, according to our current knowledge, the group of several *N*9-prenylated derivatives of adenine and hypoxanthine is described in this thesis. The exception is 9-dimethylallyl-adenine (9-(3-methylbut-2-en-1-yl)adenine; 9-DMAA), which was synthesised in 1970 (Miyaki et al., 1970; Shimizu et al., 1970) and later isolated from a plant material, the leaves of *Bridelia balansae* (Tsai et al., 2003). The biological activity of 9-DMAA has not been examined. Some previous studies have shown other compounds with similar structure and investigated their biological activity. Cytotoxicity assays and the way of calculating IC_{50} values differ in each publication and it is not possible to compare adequately the results. Vik et al. (2007) synthesized *N*6-aminopurine, *N*6-alkoxypurine and 3-methyladenine derivatives with diterpenoid (geranylgeranyl) side chain attached in the position *N*7 or *N*9 as analogues of agelasine and agelasimine (Vik et al., 2007). This type of compounds was originally isolated form marine sponges as natural products (Nakamura et al., 1984; Fathi-Afshar et al., 1988). The synthesized derivatives exhibited excellent activity against cancer cells (CEM, RPMI 8226) with IC_{50} values lower than 10 μM (Vik et al., 2007).

Rao et al. (1997) have reported the synthesis of two *N*9-alkenyl derivatives of adenine (2-penten-1-yl and 3-methyl-2-buten-1-yl) and evaluated their *in vitro* cytotoxicity against human monocytic lekemia cells. Both compounds had IC_{50} less than 2.34 $\mu g \cdot ml^{-1}$ (Rao et al., 1997). Synthetic 9-(3-oxoprop-1-enyl) derivatives of adenine and guanine exerted cytotoxicity towards HeLa cells with IC_{50} values 2 μM and 24 μM, respectively (Johnson et al., 1984). Kelley et al. (1962) performed a systematic study of the activity of hypoxanthine, adenine, and 6-chloropurine with alkyl and cycloalkyl substitutions at the 9-position. The butyl, pentyl, cyclopentyl, hexyl, cyclohexyl, heptyl and octyl derivatives displayed an inhibitory effect on a human epidermal carcinoma cell line. The compounds were tested at 10 $\mu g \cdot ml^{-1}$ and the decrease in cell population was calculated. In this case, no IC_{50} values are available. The cycloalkyl derivatives were the most effective (decrease in tested cell population up to 5%), the alkyl derivatives of hypoxanthine were more active than the corresponding derivatives of adenine and 6-chloropurine (Kelley et al., 1962).

One of the disappointments was the fact that 1,3,7,9-tetraethyluric acid displayed no activity towards the majority of tested cell lines in this thesis, except its slight toxicity towards HeLa cells (IC_{50} = 178.9 μM). There are no reports in the literature focused on anticancer activity of urate derivatives, in contrast with numerous studies concerning potent antioxidant activity of uric acid (Ames et al., 1981; Simic et al., 1989) and *N*-alkylated urates (Smith et al., 1987; Fraisse et al., 1993).

5.2 Anticancer potential of geranylated flavanones

Geranylated flavonoids are often in focus for their interesting chemical properties and great therapeutic potential in the field of treatment of inflammation (Kimura et al., 1986; Chi et al., 2001; Kim et al., 2002; Yazaki et al., 2009), cancer, and bacterial infections (Wang et al., 1998b; Sohn et al., 2004; Shakil et al., 2007). The type of the modification of basic flavonoid structure as well as the type of prenyl substitution affects the biological activity. In contrast to the phytochemical research on *A. alopecuroidea*, directed towards the isolation of unknown compounds according to their determined cytotoxic activity, the second aim of this doctoral thesis was focused on cytotoxic activity of compounds already isolated from plants with fully elucidated structure, the geranylated flavanones. Isolation of these compounds was the main research field of scientists at the Department of Natural Drugs (Faculty of Pharmacy, University of Veterinary and Pharmaceutical Sciences, Brno). Screening of the anticancer activity of isolated compounds was done at the Laboratory of Growth Regulators and is included in this doctoral thesis. All the results are completely described in Supplement II.

Fruits of the Chinese medicinal plant *Paulownia tomentosa* (Scrophulariaceae) served as a source for the isolation of eight geranylated flavanones (Šmejkal et al., 2007b; Šmejkal et al., 2008a) and a geranylated substance called schizolaenone C (Supplement II). In addition, two *C*-geranylflavanones were obtained by HPLC fractionation of chloroform fraction of the root extract of *Morus alba* (Moraceae) and their structures were determined as kuwanon E, which was also previously isolated from *M. alba* (Nomura et al., 1978), and a new compound 4'-*O*-methylkuwanon E (Supplement II). The cytotoxicity of the flavonoid compounds isolated from *P. tomentosa* and *M. alba* was tested using Calcein AM cell viability assay against five selected human cancer cell lines to determine the anticancer potential as well as normal human fibroblast to detect the non-specific cytotoxicity.

The cell lines used for the cytotoxicity screening were as follows: MCF-7, CEM, RPMI 8226, U266, HeLa and BJ. Each compound was diluted to six different concentrations and EC_{50} value were determined. Simple non-geranylated flavanones (taxifolin, naringenin and hesperetin) and standard anticancer lead compounds (olomoucine II, diaziquon, oxaliplatin) were used to compare the level of cytotoxic effects. From the EC_{50} values determined for each compound it can be concluded that the geranylation of the flavanone skeleton significantly increases cytotoxicity. Among the geranylated compounds, the activity was higher, when the *C*-geranyl side chain was attached at the position *C*6 on the ring A compared to the attachment of a geranyl side chain at *C*3' position of the ring B. The positive effect of the geranyl substitution in the *C*6 position (ring A) on the cytotoxic activity was also observed by Phommart et al. (Phommart et al., 2005). Modification of *C*-geranyl side chain by hydroxylation resulted in reduced cytotoxic activity. Decreased cytotoxicity for similar flavanones with hydroxylated geranyl substituent was also shown in previous study (Šmejkal et al., 2008b).

The effect of the presence of hydroxy group at position *C*3 of ring C was observed as a reduction in activity of hydroxy-substituted flavanones. The effect of differing hydroxy-substitutions on flavanone ring B of geranylated derivatives was deduced from the EC_{50} values and is discussed in detail in Supplement II. In summary, mono-, dihydroxy-, and dihydroxy-methoxy-substitution of flavanone ring B resulted in more cytotoxically active compounds in comparison with hydroxy-methoxy- and hydroxy-dimethoxy-substituted compounds. In addition to human cancer cell lines, normal human fibroblasts were also tested. All compounds expressed significant toxicity to BJ comparable to those of the standards used. This suggests a very narrow therapeutic window of all the compounds tested (Supplement II).

5.3 Miniaturized methods for phytohormone analysis

Cytokinins are naturally occurring adenine derivatives. In addition to their physiological functions *in planta*, they can also act on animal cells. Therefore, the study of cytokinins may bring the better knowledge of plant growth and development as well as investigation of new potent pharmacologically active compounds. Because of the low levels of naturally occurring phytohormones, selective and efficient purification and sensitive analytical methods are necessary. That is why the third goal of this doctoral thesis was focused on the development of new methods for isolation of selected phytohormones, which can also be applied for other interesting biologically active compounds. Firstly, a new miniaturized method for isolation of cytokinins from very small amounts of plant material (1-5 mg) was developed. Secondly, the previously developed method of isolation of phytohormones was modified and optimized for beaver blood samples (plasma) to investigate phytohormone profile in mammalian blood. Both isolation procedures represented a sample pre-treatment prior to an efficient ultra-high performance liquid chromatography (Acquity^{TM} UPLC, Waters) coupled to a sensitive fast scanning triple quadrupole mass spectrometer (Xevo^{TM} TQ MS, Waters) equipped with an electrospray interface (UPLC-ESI(+)-MS/MS), which was applied to measure the phytohormone concentrations.

5.3.1 Isolation of cytokinins by new miniaturized method

(Supplement III, Objective 3)

The new micromethod was developed for fast and simple isolation and quantification of a wide range of naturally occurring isoprenoid cytokinin metabolites (bases, ribosides, *N*- and *O*-glucosides and nucleotides) in samples containing very small amount of plant tissue, only 1-5 mg fresh weight. The development of the miniaturized method was based mainly on the principles of the current method of cytokinin analysis reported by Novák et al. (2008) with the main aim of its further miniaturization. On this account, the purification step was successfully optimized by utilization of self-packed StageTips microcolumns as a novel potential PT-SPE. As described in Supplement III, this simplified one-step purification was finally completed with efficient UPLC-MS/MS analysis, which was modified to single 24.5-min chromatographic run on a C18 analytical column with fast sensitive and selective mass spectrometric detection in optimized multiple reaction monitoring mode. The possible two-step modification of the developed micropurification, which might be obtained by an introduction of a miniaturized immunoaffinity extraction, is discussed in this thesis as a part of Objective 3.

5.3.1.1 StageTip purification method (Supplement III)

The newly developed miniaturized analytical approach for cytokinin isolation was composed of two equiponderant parts, isolation and quantification procedures. The isolation was performed with help of self-packed microcolumns in pipette tips termed StageTips (STop And Go Extraction Tips), which are often used as a desalting microdevice or as fractionation columns and has been reported mainly for sample enrichment and clean-up prior to an HPLC/MS analysis in proteomics (Ishihama et al., 2006; Rappsilber et al., 2007). In Supplement III, the StageTip solid-phase extraction was applied in phytohormone analysis for the first time to achieve fast one-step isolation of cytokinins from minute amounts of plant material. During the StageTip method development, three sorbent types (C18, SDB-RPS and Cation-SR) were inserted individually (single-StageTip) or in combinations (multi-StageTip) in

the tips. The sorbent parameters (affinity and capacity) were determined in samples without and with plant matrix with help of selected tritium-labelled cytokinin standards with different polarities. In the tests on single-StageTips, recovery of ^{3}H-CKs in fraction elute was in average 80% when using the cation-exchange sorbent (SDB-RPS, Cation SR) and 0.5% in case of reversed-phase sorbent. In the tests on multi-StageTips (C18/SDB-RPS, C18/Cation-SR, C18/SDB-RPS/Cation-SR), recovery of ^{3}H-CKs in samples without plant extract was 70-99%. The recovery values decreased with increasing amount of plant matrix. The combination of three sorbents (two reverse phases and one cation-exchange phase) displayed the highest efficiency in the purification of spiked 1 and 2 mg FW plant extracts with recoveries 99 ± 4% and 92 ± 3% for [^{3}H]cZ, 72 ± 6% and 32 ± 1% for [^{3}H]tZR**,** 97 ± 3% and 91 ± 3% for [^{3}H]iPR, respectively. Hence, the triple combination of sorbents in StageTips was considered as the best tool in the new one-step PT-SPE approach, with the total extraction recovery higher than 80% and reproducibility with a RSD less than 10%.

The fast, simple, one-step PT-SPE method might bring an increased number of pre-purified samples per one day. High number of samples to be analysed, small amount of sample, low content of target molecules to be analysed and the complexity of the plant matrix require very powerful techniques for sensitive detection and quantification of all present analytes. Novák et al. (2008) reported the UPLC-MS/MS analysis of 32 CK derivatives in samples pre-purified by SPE and batch IAE, which was based on two baseline chromatographic separations using short C18 column (2.1 × 50 mm) packed with sub-2-micron bridged ethylsiloxane/silica hybrid (BEH) particles and a MS quantification in MRM modes. In the simplified one-step PT-SPE method described in Supplement III, the immunoaffinity step was replaced by the modified UPLC-MS/MS analysis using a longer (2.1×150 mm) BEH C18 column. This optimized 24.5-min analyses provided a baseline separation and direct estimation of 25 isoprenoid cytokinin metabolites (*trans*-zeatin-, *cis*-zeatin-, dihydrozeatin-, and isopentenyladenine-types) during one analysis without necessity of enzyme treatment or separate analysis of *O*-glucosides and nucleotides. Limits of detection (signal to noise ratios 3:1) were ranging between 50 amol-1 fmol and limits of quantification (signal to noise ratios 10:1) between 100 amol-5 fmol. The method could be also applied for analysis of aromatic CKs, because the optimized conditions enabled baseline separation of 17 aromatic CKs (benzyladenine-, topolin-, and kinetin-types) and their corresponding derivatives. In summary, the modified UPLC method might be suitable for a routine analysis of 42 cytokinins in single chromatographic run.

The whole developed approach was tested and verified using extracts prepared from three different amounts of plant material (*A. thaliana* seedlings; 1.0, 2.0 and 5.0 mg FW), which were spiked with authentic and stable isotope-labelled cytokinin standards. Recoveries of cytokinin standards in samples reached on average 77 ± 17%, 46 ± 17% and 10 ± 6% for examined 1.0, 2.0, and 5.0 mg FW, respectively. The results indicated that the effect of plant matrix grew rapidly with increasing amount of applied plant tissues. The highest recovery in case of all tested amounts was obtained for CK-bases, followed by *N*-glucosides, *O*-glucosides, and ribosides. The lowest recovery was detected for nucleotides. The potential of the newly developed and validated StageTip purification linked with optimized UPLC-ESI(+)-MS/MS method was demonstrated on isolation and analysis of endogenous cytokinins in minute amounts of 10-day old *A. thaliana* seedling, roots and shoots (1.0, 2.0 and 5.0 mg FW). Fifteen isoprenoid cytokinin metabolites were quantified in the range 0.15-65.76 pmol·g^{-1} FW. No endogenous CK nucleotides were detected The cytokinin profile was consistent with previously

published data (Novák et al., 2008; Jones et al., 2010; Gajdošová et al., 2011). Additionally, the negative factors, which influenced the analysis of CK nucleotides as well as the effect of the plant tissue type (seedlings, shoots, roots) on recovery values are discussed more deeply in Supplement III.

In conclusion, the isolation based on one-step solid-phase extraction in StageTips represents a new miniaturized modification of previous isolation methods. The effectiveness of the StageTip purification depends on the right choice of the sorbent type in relation to the sample type, amount of plant tissue and sensitivity of the detection method. The StageTip purification can be easily modified and used for fast, effective and cheap isolation of not only cytokinins, but also other biologically active target compounds from milligram amounts of plant material. In combination with single optimized UPLC-MS/MS analysis, the whole developed procedure allow fast, effective, selective and sensitive quantification of phytohormones in very limited amounts of plant material (Supplement III).

5.3.1.2 Future modification of the miniaturized method (Objective 3)

A modification of the developed one-step micropurification of cytokinins using PT-SPE can be achieved by addition of a suitable miniaturized immunopurification. For this purpose, a batch IAE based on monoclonal anticytokinin antibodies immobilized on superparamagnetic iron nanoparticles (MNPs) was designed and separately examined in this thesis. The IAE is performed in microcentrifuge tubes with small samples volumes (50-200 μl), therefore it ideally fits as a selective purification step, that can follow the StageTip SPE. The optimization of the complete two-step micropurification method will be performed in the future. As a consequence the two-step protocol shares the same principles with the method described previously (Novák et al., 2008), it will enable selective separation of cytokinin bases with ribosides and 9-glucosides (fraction B) and *O*-glucosides with ribonucleotides (fraction OG+NT) into individual fractions. Finally, the analysis of CKs will be completed with two fast chromatographic separations using a short reversed-phase column (1.7 μm, 2.1 × 50 mm) and accurate, sensitive quantification by MS/MS as described by Novák et al. (2008).

Immunoaffinity extraction on magnetic nanoparticles

The immunoaffinity extraction provides highly specific sample enrichment and is often used in combination with conventional SPE as an advantageous final pre-treatment step during the analysis of complex biological samples (Hage, 1999; Hennion et al., 2003). The application of IAC in column format for production of highly purified cytokinin metabolites was published by Novák et al. (2003). Later, Hauserová et al. (2005) and Novák et al. (2008) explained the batch IAE as a sensitive and relatively cheap method for purifying of cytokinins from biological samples. In the batch configuration, the amount of antibodies is decreased and the sample volume, consumption of solvents and extraction time is reduced. The batch configuration was further modified and tested for purification of cytokinins in this work. For this purpose, the group-specific monoclonal anticytokinin antibodies were immobilized to a solid support of chitosan coated magnetic nanoparticles of magnetite (synthetic and bacterial Fe_3O_4, particle size 20-50 nm) and tested as fast and simple type of IAE with magnetic separation, which was designed to be used in combination with StageTip purification.

Non-toxic, biocompatible and biodegradable MNPs of iron oxides are used in biomedical and biotechnological applications, such as a negative contrast agents in magnetic resonance imaging, targeted drug delivery, magnetic cell separation (Alexiou et al., 2006) or a magnetic solid support to immobilize different types of biomolecules (proteins, oligonucleotides) (Bílková et al., 2006; Rotková et

al., 2009). Magnetite MNPs with diameters 20-50 nm have ferromagnetic behaviour, large surface area and they can be controlled by an external magnetic field. It is necessary to modify the surface of these MNPs by coating biocompatible surfactant, especially naturally occurring polysaccharides such as chitosan or dextran, to protect aggregation and to introduce functional groups. Subsequently, the agent of interest (drug, protein, enzyme, or antibody) can be either chemically conjugated or ionically bound to the outer layer of the polymer coating (Belessi et al., 2008; Kluchová et al., 2009). Hence, magnetic nanocomposites are made up of magnetite as a core and chitosan as a shell. In this work, monoclonal anticytokinin antibodies were used to functionalize the surface of MNPs.

Immobilization of antibodies on amine group of chitosan coating of MNPs was performed with help of two methods and the success of each approach was checked. In glutaraldehyde method, the antibodies and the chitosan coating of nanoparticles are linked via glutaraldehyde cross-linking agent by their amino groups (Kluchová et al., 2009). This method is less specific in comparison with the one-step carbodiimide active ester method, where the immobilization is based on specific peptide bond between carboxylic group of antibodies and amine group of chitosan MNPs, which is formed due to the activation of carboxylic groups in presence of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride and *N*-hydroxysulfosuccinimide (Aslam et al., 1998). With help of anticytokinin antibodies immobilized to the surface of MNPs, cytokinins are captured on magnetic nanocomposites and can be then selectively separated from the solution of small volume (50-200 μl) by attraction of magnetic nanocomposites with use of external magnetic filed. The process can be assigned as batch immunoaffinity extraction on magnetic nanoparticles, which represents a possible practical use of magnetic nanoparticles in relation to cytokinin analysis.

For the initial testing of the IAE on MNPs, the binding affinity and specificity of the immobilized antibodies towards selected CKs was determined as the extraction recovery using cytokinin standards and different amount of immobilized antibodies. Unfortunately, antibodies immobilized by glutaraldehyde method did not capture the tested cytokinins (iPR, cZ, ZR). The zero recovery values pointed at no cytokinins in the desired elute fraction. In comparison, a carbodiimide method of immobilization was more successful and the resulted recovery was higher (Table 8).

Table 8. Recovery (%) of cytokinins from standard solutions without plant matrices following the batch immunoaffinity extraction on magnetic nanoparticles.

CKs	100 μg Ab	300 μg Ab	CKs	200 μg Ab		
	one STD	one STD		0.1 pmol, one STD	0.5 pmol, one STD	0.1 pmol, STD mixture
[^{3}H]iPR	0.14 ± 0.03	0.25 ± 0.05	iPR	ND	0.69 ± 0.40	ND
[^{3}H]tZR	0.09 ± 0.02	0.40 ± 0.05	cZR	ND	0.32 ± 0.11	ND
[^{3}H]BA	ND	0.79 ± 0.06	oT	31.60 ± 6.22	22.85 ± 4.56	21.35 ± 1.45
			oTR	39.49 ± 15.24	18.11 ± 1.43	26.91 ± 1.74
			mT	ND	ND	0.70 ± 0.10
			mTR	ND	ND	1.03 ± 0.15
			pT	ND	ND	1.78 ± 0.08
			pTR	ND	ND	0.70 ± 0.02

Standard solutions were prepared in PBS buffer and purified using antibodies bound on magnetic nanoparticles. Values are means ± SD (n=3). CKs, cytokinins; Ab, antibody; STD, standard; ND, not detected.

Interestingly, the recovery of *ortho*-topolins was significantly higher (ca. 30%) compared to other cytokinin groups (benzyladenine, isopentenyladenine, zeatin, *meta*-topolin, *para*-topolin), where the recovery was below 2%. Therefore, the immobilization of antibodies resulted in increased selectivity towards the *ortho*-topolin group of cytokinins (oT and oTR), which was used for preparation of anticytokinin antibodies. No cross-linked reactivity for related cytokinin species (*meta*-topolin and *para*-topolin) was observed. The opposite situation was in the case of antibodies bound to the *N*-hydroxysuccinimide ester activated agarose Affi-Gel 10, where the cross-linked reactivity played significant role in purification of *ortho*-topolins as well as other cytokinin groups (*meta*-topolin, *para*-topolin, benzyladenine, isopentenyladenine, zeatin) with recoveries higher then 50% (Novák et al., 2003; Hauserová et al., 2005). Increasing of anticytokinin antibodies immobilization on MNPs led to the idea of the use of other method of immobilization. The plan for the future is to perform the immobilization through the avidin-biotin interaction and to test the extraction recovery and capacity of newly prepared immunoaffinity nanocomposites.

Complete protocol of the miniaturized two-step purification

In the future, successfully prepared nanocomposites will be finally used in the IAE of samples pre-purified on the StageTips and the efficiency of the two-step purification method will be determined. The proposed complete purification protocol is illustrated in Figure 29.

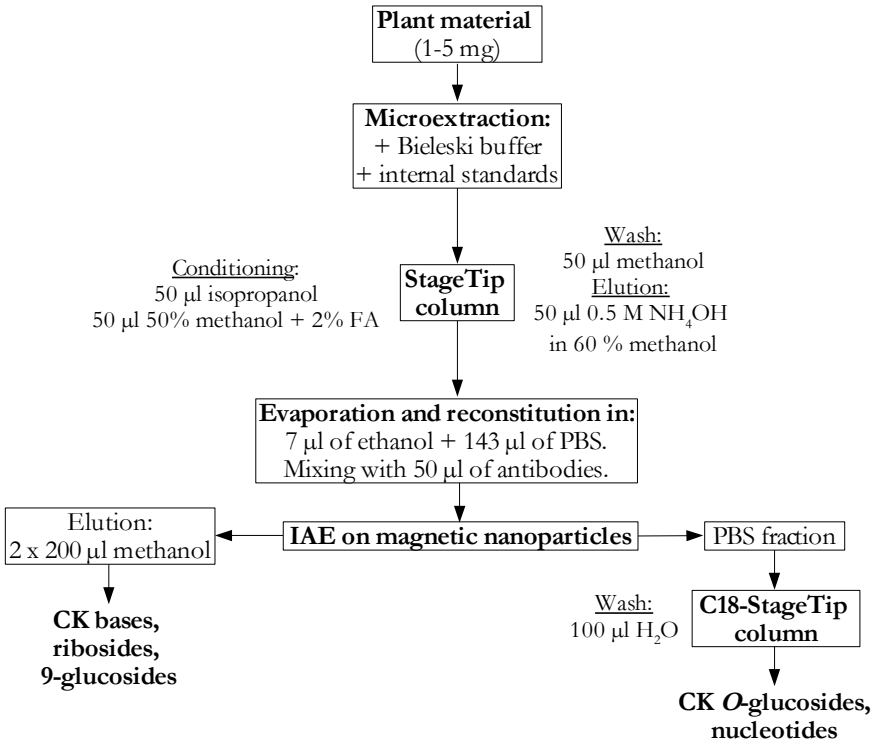


Figure 29. Proposed two-step protocol of CK extraction and micropurification. Plant material (1-5 mg F.W.) is homogenized and extracted in Bieleski buffer with addition of isotope-labelled internal standards. Supernatant (50 µl) is immediately applied on a pre-conditioned multi-StageTip and CKs are eluted with solvent after washing the tip. The eluate is evaporated to dryness, dissolved in 150 µl of ethanol:PBS (5:95, v/v), mixed with antibodies immobilized on MNPs (100 µg/50 µl) and incubated for 1 h at room temperature. Afterwards, the PBS fraction (containing *O*-glucosides and nucleotides) is collected and desalted using C18-StageTip. Compounds retained by antibodies are eluted with 200 µl methanol twice. All fractions are evaporated to dryness and stored at -20 ºC.

The sample after StageTip purification is immunopurified on the MNPs and hence separated into two fractions, fraction B as the eluate, and fraction OG+NT as the PBS fraction. The PBS fraction is desalted using C18-StageTip prior to the UPLC-ESI(+)-MS/MS analysis, fraction B is directly analysed after its evaporation. The time needed for the batch immunoaffinity extraction as the additional clean-up step is compensated with application of two shortened (less than 10-min) UPLC separations of cytokinin metabolites, where CK bases, ribosides and 9-glucosides (fraction B) are analysed in the first one and O-glucosides and nucleotides (fraction OG+NT) in the second as described by Novák et al. (2008). In summary, the StageTip purification followed by IAE on magnetic nanoparticles will represent new, easy, time and cost-saving selective two-step micropurification method for routine isolation of cytokinins from milligram amount of plant material after its successful optimization.

5.3.2 Isolation of phytohormones from beaver blood samples (Objective 3)

Except intensive studies of cytokinins in plants, some attempts to detect these phytohormones in human plasma were performed. In 1998, Schram et al. detected iPR in human blood. Later, other groups of CKs were successfully identified (Strnad et al., unpublished), but the obtained results were unfortunately not reproducible and varied according to the blood donor and year season. This indicated that CKs in human plasma could partially originate from human diet (Strnad et al., unpublished). In the work presented here, beavers were chosen as the model mammals for screening of plant hormones in blood, because of their stable and homogeneous diet rich in phytohormones, which consists mainly of trees such as poplar, willow, and birch. Although the beaver diet can be influenced by the actual season, there is a presumption of more consistent phytohormone levels.

As a consequence of the fact that mammalian blood is completely different matrix in comparison with plant tissues, diverse and simplified purification protocol was used for isolation of adenine and indole derivatives from beaver blood samples. The purification procedure was based on protein precipitation of blood plasma samples and a combination of SPE on Oasis MCX columns and cytokinin immunoaffinity extraction. SPE was performed as described previously by Dobrev et al. (2002) with slight modifications, the applied immunoaffinity extraction was documented in details by Novák et al. (2003).

The Oasis MCX sorbent was chosen for the purification of precipitated beaver plasma due to its beneficial properties and facilitation of the more complex phytohormone analysis compared to the classical methods of CK purification (Hoyerová et al., 2006), such as the multi-step DEAE-Sephadex RP-C18 method (Redig et al., 1996; Novák et al., 2003). DEAE-Sephadex has been successfully used for the separation of CK nucleotides from CK bases, ribosides and *O*-glucosides. The retention of CK nucleotides is quite poor and the method does not provide the separation of the anionic IAA from anionic CK nucleotides (Hoyerová et al., 2006). The mixed-mode reversed-phase and cation-exchange mode of the Oasis MCX sorbent enable the increased retention of the cationic CK free bases, ribosides and *O*-glucosides (fraction B+OG) as well as the anionic CK nucleotides (fraction NT) and indole-3-acetic acid and abscisic acid (fraction IAA). Retained compounds can be sequentially eluted using different concentrations of methanol and ammonium hydroxide. Furthermore, the method is characterised by high recoveries of analytes and their sufficient purity for HPLC/MS quantification (Dobrev et al., 2002; Hoyerová et al., 2006). In summary, the use of Oasis MCX sorbent allow simple

and fast separation and purification of different classes of phytohormones in one pre-purification step on a single Oasis MCX column from the liquid sample. The need of only one sample for isolation of both CKs and IAA is beneficial for unique material, such as beaver blood.

The purification of phytohormones from beaver plasma was optimized using standard phytohormone water solutions and mammalian plasma containing a mixture of indole-3-acetic acid and selected cytokinin authentic and/or stable isotope-labelled standards. After conjunct Oasis MCX purification, CKs (fraction B+ OG and fraction NT) where further selectively purified by IAC and afterwards analysed by UPLC-ESI(+)-MS/MS using Acquity UPLC™ BEH C18 column. Fraction IAA was directly injected onto BetaMax Neutral column and analysed by UPLC-ESI(+)-MS/MS without immunopurification step. The recoveries for IAA and selected cytokinin types were calculated during the method optimization (Table 9). The recoveries higher than 40% were obtained for IAA in the concentration range 1.0-10 pmol. The recoveries in the range 35-73% were obtained for iPR, the recoveries were the lowest (23-58%) for oT in the concentration range 0.1-10 pmol.

Table 9. Recoveries (%) of indole-3-acetic acid and selected cytokinins from standard solutions with and without blood plasma matrix following the purification on Oasis MAX columns.

STD conc (pmol)	Recovery (%), STD			Recovery (%), plasma matrix		
	IAA	**iPR**	**oT**	**[$^{13}C_6$]IAA**	**[$^{2}H_6$]iPR**	**[$^{13}C_5$]oT**
0.1	ND	72.9 ± 6.7	26.9 ± 2.8	ND	53.8 ± 3.8	23.4 ± 10.4
1.0	50.1 ± 3.6	59.6 ± 5.1	57.8 ± 6.0	85.6 ± 0.5	46.2 ± 7.8	46.1 ± 8.0
10.0	40.4 ± 1.1	44.3 ± 4.4	34.1 ± 1.2	71.0 ± 2.3	35.6 ± 8.8	26.3 ± 10.0

Samples containing 100 µl of water or 200 µl of mammalian plasma were spiked with phytohormone standards, precipitated with acetonitrile and purified by Oasis MCX columns (IAA, CKs) and by IAC (CKs). Finally, phytohormones were measured by UPLC-ESI(+)-MS/MS. Mean values ± SD from triplicate measurements of samples are shown. STD, standard; conc, concentration; ND, not detected.

The levels of indole-3-acetic acid and cytokinins in real beaver blood plasma samples were finally determined and the results are summarized in Table 10. Cytokinins not displayed in the table were below the limit of detection (LOD; based on 3:1 signal to noise ratios). Very high content of indole-3-acetic acid (93.5-2,579.3 pmol·ml^{-1}) found in beaver plasma correlates with higher endogenous concentration of IAA in the plant material (up to hundreds pmol·g^{-1} of fresh weight; Hellgren et al., 2004; Pěnčík et al., 2009) compared to lower cytokinin content (less than 50 pmol·g^{-1} of fresh weight; Novák et al., 2003). On the other hand, only three cytokinin ribosides, cZR, iPR and oTR, respectively, were detected in all studied samples. Furthermore, the majority of CK derivatives (t/cZ(R), iP, BA, mT(R), oT, and pT) determined in samples with the different levels underline the probable variability between beaver individuals. No cytokinin 9-glucosides, *O*-glucosides and nucleotides as well as DHZ-type cytokinins were found in beaver plasma. This indicate, that cytokinin glucoconjugates and riboside-5'-monophosphates are the most probably metabolised or cleaved during the passage through beaver digestive system. Moreover, the higher concentrations of *ortho*-topolin (757.9-957.0 fmol·ml^{-1}), *meta*-topolin (202.9-586.9 fmol·ml^{-1}), and iPR (129.8-631.1 fmol·ml^{-1}) in comparison with the lower levels of other CKs (7.1-167.6 fmol·ml^{-1}) were measured in beaver plasma. The results correlate with previously published iPR levels (Novák et al., 2003; Chen et al., 2010), which showed up to ten times higher content of iPR, varying from 3.4 ± 0.2 pmol·g^{-1} to 25.7 ± 1.7 pmol·g^{-1} FW, in contrast with

other isoprenoid cytokinins presented in poplar. Furthermore, mature poplar leaves are known to be a rich source of aromatic cytokinins (Horgan et al., 1973). Strnad et al. (1994; 1997) published the endogenous levels of topolin-type cytokinins in poplar leaves, 20.5 ± 4.1, 35.7 ± 4.7, and 48.7 ± 9.3 pmol·g^{-1} FW for oT, oTR, and oT9G, as well as 22.2 ± 5.3, 37.1 ± 6.3, and 13.2 ± 3.4 pmol·g^{-1} FW for mT, mTR, and mT9G, respectively. This data support the statement, that the content of cytokinins is influenced by the beaver diet rich in poplar. Finally, the general question of phytohormone metabolization when ingested by mammals still remains open and requires deeper investigation and collaboration with animal physiologists in the future.

Table 10. Levels of indole-3-acetic acid (pmol·ml^{-1}) and cytokinins (fmol·ml^{-1}) in beaver blood plasma

	Auxin content (pmol·ml^{-1})								
	5486	5487	5488	5489	5490	5491	5492	5493	5494
IAA	134.0 ± 5.4	169.3 ± 11.7	1562.4 ± 81.5	853.5 ± 22.7	2579.3 ± 173.7	727.9 ± 37.7	332.7 ± 22.4	257.5 ± 24.3	93.5 ± 3.9
	Cytokinin content (fmol·ml^{-1})								
	5486	5487	5488	5489	5490	5491	5492	5493	5494
tZ	8.1 ± 1.6	22.5 ± 2.3	15.6 ± 7.9	13.8 ± 4.5	35.3 ± 19.2	ND	ND	ND	ND
tZR	ND	9.6 ± 0.3	9.0± 0.6	7.5 ± 0.5	7.1 ± 0.5	ND	ND	ND	ND
cZ	ND	122.4 ± 9.3	ND	ND	46.0 ± 3.8	ND	ND	ND	ND
cZR	67.6 ± 13.4	97.4 ± 1.2	90.0 ± 24.6	44.8 ± 16.1	74.1 ± 2.6	82.8 ± 9.2	167.6 ± 10.0	106.2 ± 9.6	25.4 ± 5.5
iP	ND	166.8 ± 3.3	52.2 ± 7.2	24.1 ± 5.0	72.7 ± 13.8	39.6 ± 7.5	ND	25.4 ± 8.8	ND
iPR	160.6 ± 14.8	452.0 ± 40.9	495.3 ± 9.5	253.4 ± 11.2	631.1 ± 41.4	453.9 ± 30.4	365.4 ± 9.9	320.9 ± 37.3	129.8 ± 9.9
BA	ND	116.0 ± 9.4	45.4 ± 1.3	38.2 ± 4.3	45.1 ± 12.4	ND	ND	ND	ND
mT	227.2 ± 14.2	586.2 ± 39.1	ND	212.9 ± 29.4	202.9 ± 29.5	ND	ND	ND	ND
mTR	ND	4.6 ± 2.1	2.6 ± 0.6	4.0 ± 0.4	3.6 ± 0.6	ND	ND	ND	ND
oT	ND	957.0 ± 35.1	757.9 ± 53.8	813.4 ± 47.2	775.9 ± 57.7	ND	ND	ND	ND
oTR	37.6 ± 5.4	57.3 ± 14.6	54.0 ± 5.6	34.9 ± 7.3	98.2 ± 4.8	70.3 ± 14.3	41.3 ± 6.6	37.1 ± 8.9	32.2 ± 9.8
pT	ND	112.4 ± 18.1	41.7 ± 20.4	46.0 ± 10.7	61.6 ± 6.9	ND	ND	ND	ND

Samples of beaver plasma were divided into triplicates of approximately 500 µl and precipitated with acetonitrile. The obtained supernatant was evaporated to dryness and purified by Oasis MCX columns (IAA, CKs) and IAC (CKs). Phytohormone levels were measured by UPLC-ESI(+)-MS/MS. Mean values ± SD from triplicate measurements are shown; ND, not detected.

6. Conclusions and perspectives

The study of plant endogenous metabolites can lead to a better understanding of plant growth and development and to the discovery of phytochemicals with antioxidant, antimicrobial, antiinflammatory or chemopreventive potential. Therefore, it can subsequently reveal new compounds with possible therapeutical applications, which can be further studied as potential new medicines. Although working with isolated pure natural compounds brings many advantages for further investigations due to defined structure and molecular weight, and the fact that exact amounts and concentrations can be used, the isolation of pure natural substance is very difficult and requires development and use of effective and selective purification and analytical tools. Among the plant kingdom many species used in traditional herbal medicine have not been yet sufficiently studied from the chemical point of view and they represent a hidden source of new potentially biologically-active compounds. On the basis of the above mentioned facts, this doctoral thesis focused on cytotoxic and antiproliferative activities of plant metabolites with the aim of isolating and identifying bioactive compounds as well as developing and applying new methods of isolating the phytohormones and quantifying them. Hence, the main goals are connected with the common aim of isolating, identifying and quantifying plant metabolites and investigating their anticancer potential.

The whole work dealt with various features and was divided into three main fields of research:

1. Isolation and identification of anticancer active compounds from *Acalypha alopecuroidea.*
2. Geranylated flavanones from *Morus alba* and *Paulownia tomentosa* and their anticancer potential.
3. Miniaturized fast isolation and quantification of phytohormones from biological material.

All the obtained findings are described and discussed in the section Results and Discussion and in the attached articles (Supplement I-III) and can be briefly summarized in several main points, which are appointed in the following pages.

1. Isolation and identification of anticancer active compounds from *Acalypha alopecuroidea*.

- The MeOH-THF root extract prepared from the euphorbiaceous plant, *Acalypha alopecuroidea,* was fractionated by solid-phase extraction and two-dimensional high performance liquid chromatography. All the procedure was performed according to the results obtained by Calcein AM cytotoxicity assay. Cytotoxic activity testing was essential for following the bioactive extract constituents and targeting their isolation.

- Biological activity and mechanism of action of the *A. alopecuroidea* MeOH-THF extract and the HPLC extract fraction B23 was studied in more depth to discriminate between the effects on cell cycle machinery (growth inhibition) and on cell death (apoptosis). The effect of the isolated fraction on cell proliferation, expression of cyclins, activation of the Chk 2 kinase (DNA damage response pathway) and induction of chromatin condensation was studied. All the results suggested that there were good prospects for purifying biological active compounds from *A. alopecuroidea* and for their use for *in vivo* preclinical studies.

- Four fractions (chromatographic peaks) cytotoxic to the human breast adenocarcinoma cell line and the human leukaemic lymphoid cells, were isolated by means of SPE and 2D-HPLC purification from the MeOH-THF root extract from *A. alopecuroidea*.

- The structures of two isolated compounds were designed according to MS and NMR spectra as 9-(3,6-dimethylhepta-2,6-dienyl)-hypoxantine and 1,3,7,9-tetraethyluric acid. However, the presumable structures still need the further verification using complementar analytical methods.

- A series of *N9*-prenylated derivatives of adenine and hypoxanthine and *N*-ethyl derivatives of uric acid were synthesised as new compounds according to the natural template represented by the designed structure of isolated compounds.

- The biological activity of synthetic purine derivatives was examined against different cell types using Calcein AM assay. The geranyl-substituted derivatives of adenine and hypoxanthine and chlorine-derivatives of geranyladenine exhibited some interesting cytotoxic effects.

- The cytotoxicity of extracts prepared from *A. alopecuroidea* was studied against human breast adenocarcinoma cell line (MCF-7) and human leukaemic lymphoid cells (CEM) using the Calcein AM cell viability assay. Extracts were characterized by an IC_{50} value, which represents the quantity of the starting lyophilized plant material per ml cell culture medium ($mg \cdot ml^{-1}$) required for inhibition of the growth of 50% cells in the tested cell line. The highest antiproliferative activity was found in methanol-tetrahydrofurane (1:1) root extract (IC_{50} = 1.1 $mg \cdot ml^{-1}$ for MCF-7; 0.9 $mg \cdot ml^{-1}$ for CEM) and in extract fractions designated B (IC_{50} = 127.5 $mg \cdot ml^{-1}$ for MCF-7; 15.3 $mg \cdot ml^{-1}$ for CEM) and B23 (IC_{50} = 86.5 $mg \cdot ml^{-1}$ for MCF-7; 0.5 $mg \cdot ml^{-1}$ for CEM).

2. Geranylated flavanones from *Morus alba* and *Paulownia tomentosa* and their anticancer potential.

- Anticancer activity of nine *C*-geranylated flavanones isolated from the fruits of *Paulownia tomentosa* and two from the roots of *Morus alba* was studied towards five selected human cancer cell lines and normal human fibroblast *in vitro,* using the Calcein AM cytotoxicity assay. Each compound was characterized by an EC_{50} value for each cell line and the values for these compounds were compared with those of known simple flavanone standards and standard antineoplastic compounds.

- The isolated *C*-geranyl flavanones showed interesting cytotoxic activity towards the tested human cancer cell lines and normal human fibroblast used as a control to distinguish between the specific and non-specific action towards tumour cells. Comparison of the cytotoxicity against normal cell line and cancer cell lines revealed a very narrow therapeutic window of all the compounds tested.

- The effects of structural modifications on activity were discussed. The presence of a geranyl side chain was an important factor for cytotoxic activity. Compounds substituted with the *C*-geranyl chain at ring B of the flavanone core were less cytotoxic than compounds with the side chain attached on ring A. Mono- and dihydroxy- substitution and the dihydroxy methoxy- substitution of flavanone ring B resulted in compounds with the most potent activity.

3. Miniaturized fast isolation and quantification of phytohormones from biological material.

- A new miniaturized approach was developed for fast isolation and quantification of biologically active compounds (cytokinins) from minute amount of plant material. The method combines a simple one-step purification and an ultra-high pressure liquid chromatography-fast scanning tandem mass spectrometry.

- For the first time, plant tissue samples (1-5 mg fresh weight), were purified in one step by STop And Go Extraction Tips (StageTips). The triple combination of sorbents (two reversed-phases and one cation-exchange phase) was found to be the best tool in the StageTip purification with the extraction recoveries higher than 80% for tritium-labelled cytokinin standards.

- The process was completed with single chromatographic analysis of naturally occurring cytokinins (bases, ribosides, *O*-, *N*-glucosides, and nucleotides) using sub-2-micron particle packed analytical column in 24.5 minutes and a mass spectrometric detection in multiple reaction monitoring mode.

- The potential of the micromethod was demonstrated with the isolation of isoprenoid cytokinins from seedlings, roots and shoots of *Arabidopsis thaliana*. Fifteen compounds were successfully determined in the concentration range 0.15-65.76 pmol·g^{-1}.

- Improvement in the developed miniaturized approach might be achieved by addition of selective immunoaffinity extraction on magnetic nanoparticles, where specific monoclonal anticytokinin antibodies are bound onto magnetic iron nanoparticles. This additional step requires further optimization.

- A previously developed method for phytohormone isolation, which was based on mixed-mode cation exchange solid-phase extraction, was applied to blood samples (plasma). The optimized method was finally used for purification of cytokinins together with indole-3-acetic acid from beaver plasma samples in order to study the occurrence of phytohormones in beaver blood.

- The samples of beaver plasma were precipitated in acetonitrile and purified on Oasis MCX columns. The purification allowed separation of the cytokinin bases with ribosides and *O*-glucosides, cytokinin nucleotides and indole-3-acetic acid in individual fractions. Cytokinin metabolites were further specifically purified by immunoaffinity chromatography using monoclonal anticytokinin antibodies bound on an immunoaffinity gel.

- Significantly higher levels of indole-3-acetic acid (93.5-2,579.3 pmol·ml^{-1}), *ortho*-topolin (757.9-957.0 fmol·ml^{-1}), *meta*-topolin (202.9-586.9 fmol·ml^{-1}) and *N*6-isopentenyladenosine (129.8-631.1 fmol·ml^{-1}) in comparison with other detected cytokinin types were found in beaver plasma. Cytokinin 9-glucosides, *O*-glucosides and nucleotides as well as DHZ-type cytokinins were not detected in beaver plasma.

- The newly developed qualitative and quantitative UPLC-ESI(+)-MS/MS analysis of phytohormones was performed by ultra-performance liquid chromatography (Acquity™ UPLC, Waters) coupled to a triple quadrupole mass spectrometer (Xevo™ TQ MS, Waters) equipped with an electrospray interface.

In summary, this doctoral thesis comprises the first phytochemical study of the herb *Acalypha alopecuroidea,* which is widely used in traditional Mayan medicine, but which has not previously been investigated from the chemical point of view. The knowledge about the anticancer activity of the compounds extracted from this herb, as well as their antiinflammatory potential and interference with cell cycle is now extended. There is a high prospect that the isolated fractions and compounds will undergo further *in vivo* testing and preclinical studies and be used as lead structures. This group of naturally-based synthetic compounds represents a novel type of structural modification of the adenine and hypoxanthine cores and provides promising adepts for further structural modifications and *in vitro* biological activity testing. The group of natural anticancer active geranylated flavanones, occurring in *Morus alba* and *Paulownia tomentosa*, is an example of an other potent natural product class. The screening of biological activity of these compounds may bear fruit in the discovery of highly bioactive compound and the further exploration of potential therapeutic usage in the future is of great interest.

In the field of phytohormone analysis, the new approaches of isolation from small amount of the plant material and/or blood samples, were developed, optimized and used for real applications. The miniaturization is necessary for small samples size (less than 5 mg FW), the specific immunoaffinity step provide selective enrichment of samples and increase the effectiveness of purification of minute quantities of phytohormones from large amounts of interfering material. Except the progress in the field of miniaturization, the simple-based micromethod coupled with powerful analytical techniques such as fast and efficient ultra-high performance liquid chromatography separation and potent sensitive and selective tandem mass spectrometric detection already provides great improvements in terms of time reduction, cost effectiveness, robustness, through-put, sensitivity and selectivity for plant samples. Modern phytochemical methods can be easily modified and applied to isolate and determine phytochemicals in matrices different from plant material, such as animal or human blood. Mammalian body fluids might contain plant metabolites, such as plant hormones, originating from the vegetarian diet, as well as their derivatives incurred from the mammalian metabolism. Hence, the study of phytohormones in blood samples can extend the knowledge of phytohormones and their action in relation with animal or human metabolism, health and longevity.

7. References

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8. Supplements

The author's personal contribution to the papers listed below includes the work described and discussed in the section Results and discussion.

Supplement I

Madlener S., **Svačinová J.**, Kitner M., Kopecký J., Eytner R., Lackner A., Vo T.P.N., Frisch R., Grusch M., De Martin R., Doležal K., Strnad M., Krupitza G. (2009) In vitro anti-inflammatory and anticancer activities of extracts of *Acalypha alopecuroidea* (Euphorbiaceae). *International Journal of Oncology* 35 (4): 881-891.

Supplement II

Šmejkal K., **Svačinová J.,** Šlapetová T., Schneiderová K., Dall'Acqua S., Innocenti G., Závalová V., Kollár P., Chudík S., Marek R., Julínek O., Urbanová M., Kartal M., Csöllei M., Doležal K. (2010) Cytotoxic activities of several geranyl-substituted flavanones. *Journal of Natural Products* 73 (4): 568-572.

Supplement III

Svačinová J., Novák O., Lenobel R., Plačková L., Hanuš J., Strnad M., Doležal K. A new miniaturized analytical approach for cytokinin isolation from plant tissues using pipette tip solid-phase extraction (manuscript in preparation).

Supplement I

Supplement II

Supplement III

MADLENER S., **SVAČINOVÁ J.**, KITNER M., KOPECKÝ J., EYTNER R., LACKNER A., VO T.P.N., FRISCH R., GRUSCH M., DE MARTIN R., DOLEŽAL K., STRNAD M., KRUPITZA G. (2009) In vitro anti-inflammatory and anticancer activities of extracts of *Acalypha alopecuroidea* (Euphorbiaceae). *International Journal of Oncology* 35 (4): 881-891.

In vitro anti-inflammatory and anticancer activities of extracts of *Acalypha alopecuroidea* (Euphorbiaceae)

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Abstract. More than 60% of conventional drugs are derived from natural compounds, some of the most effective pharmaceuticals (e.g. aspirin, quinine and various antibiotics) originate from plants or microbes, and large numbers of potentially valuable natural substances remain to be discovered. Plants with considerable medicinal potential include members of the genus *Acalypha*. Notably, extracts of *A. platyphilla*, *A. fruticosa*, *A. siamensis*, *A. guatemalensis* and *A. wilkesiana* have been recently shown to have antioxidant, antimicrobial and cytotoxic effects. In the study presented here we investigated the anti-inflammatory, anti-proliferative and pro-apoptotic activities of *A. alopecuroidea*, which is endemic in parts of Central America and is traditionally used by the Mopan- and Itza-Maya in the form of decoctions to treat skin conditions, and as a tea to treat stomach and urinary complaints. We demonstrate here that extracts of *A. alopecuroidea* can inhibit TNFα-induced E-selectin production, providing a mechanistic validation of its traditional use against inflammatory diseases. Furthermore, a fraction of *A. alopecuroidea* root extracts purified by solid phase extraction and separated by HPLC displayed strong cell cycle inhibitory activity by down-regulating and inactivating two proto-oncogenes (cyclin D1 and Cdc25A), and simultaneously inducing cyclin A, thereby disturbing orchestrated cell cycle arrest, and thus (presumably) triggering caspase 3-dependent apoptosis. The results of this study indicate that there are high prospects for purifying an active principle from *A. alopecuroidea* for further *in vivo* and preclinical studies.

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Key words: Acalypha alopecuroidea, cancer, anti-inflammatory activity, anticancer activity, MCF-7 cells, CEM cells

Introduction

There is increasing interest in traditional plant-based medicines (e.g. Ayurveda and traditional Chinese medicines) as potential sources of new anticancer drugs, partly because many conventional drugs originate from plant sources (1). For example, both ginger and an active principle obtained from it, [6]-gingerol, have been shown to have antibacterial and anti-angiogenic effects *in vitro* and *in vivo* (2). Further, plant extracts frequently contain various compounds, such as the polyhydroxyphenol gallic acid, that have high radical and/or reactive oxygen species (ROS) scavenging activities. These substances may have profound health effects, since radicals and ROS are capable of severely damaging proteins and nucleic acids, leading to cell or tissue injury. ROS are also involved in the development of inflammation, various cancers and several major degenerative diseases, such as arteriosclerosis, liver injury, Alzheimer's disease, diabetes, Parkinson's disease and coronary heart pathologies (3-5). We have previously shown that the radical scavenging properties of gallic acid make it an effective inhibitor of the enzyme ribonucleotide reductase (RR; EC1.17.4.1), which is frequently overexpressed in cancer cells and catalyses the rate-limiting step for dNTP synthesis and cell division (6).

Gallic acid and several other compounds (including geraniin, corilagin and cycloartane-type triterpenoids) obtained from various members of the genus *Acalypha* of the Euphorbiaceae (including *A. wilkesiana*, *A. hispidia* and *A.*

communis) have been isolated and shown to have biological activities (7,8). In addition, extracts of the *Acalypha* species *A. platyphilla, A. fruticosa*, *A. siamensis*, *A. wilkesiana* and *A. guatemalensis* have been recently shown to have anti-oxidant, antimicrobial and cytotoxic activities (9-13). These encouraging studies with various *Acalypha* taxa prompted the investigation reported here of the species *A. alopecuroidea*, which is endemic in parts of Central America and traditionally used (together with *A. arvensis*) by the Mopan- and Itza-Maya in the form of decoctions as washes to treat severe skin conditions (deep sores, ulcers, blisters, rashes, fungal infections and inflammations) and as teas (in large quantities per day) to treat stomach and urinary complaints (14). Hyper-proliferative disorders and cancer of the uterus are also treated by *A. alopecuroidea* decoctions. Interestingly, the extracts of foliage and twigs of *A. arvensis* have been found to be inactive towards colon cancer cells (15). In the investigation presented here we studied the anti-inflammatory, anti-proliferative and pro-apoptotic properties of fractionated *A. alopecuroidea* extracts. The findings show that extracts prepared from *A. alopecuroidea* roots were highly active against selected cancer cell lines.

Materials and methods

Plant material and chemicals. Acalypha alopecuroidea plants were collected in the Botanical garden of the Institute for Ethnobiology, Playa Diana, San José, Petén, Guatemala. The plants were mechanically cleaned and lyophilised at the Institute and samples were then imported from Guatemala into the Czech Republic and extracts of specific parts were produced, as described below, at the Laboratory of Growth Regulators, Palacky University, Olomouc. Glutaraldehyde (25%), Sigma*Fast*-OPD, Hoechst 33258 and propidium iodide were purchased from Sigma.

Extract preparation. The plant material was divided into shoots, leaves and inflorescences, which were separately ground to a fine powder at laboratory temperature and homogenised. Portions of the ground roots (0.5 g), shoots (1.0 g), leaves (1.0 g) and inflorescences (0.5 g) were then extracted separately in 70% (v/v) ethanol and/or methanol-tetrahydrofurane (MeOH:THF, 1:1). After 16 h (overnight) extraction at -20˚C, the resulting homogenates were centrifuged (15,000 rpm, 4˚C, 20 min), the sediments were re-extracted for 1 h in the same way, and centrifuged. The supernatants were pooled and dried *in vaccuo* at 35˚C, then dissolved in 200 μl of methanol and 800 μl of 0.1 M Tris buffer (pH 7.2). The cytotoxic activity of extracts was subsequently screened in Calcein AM cytotoxicity assays as described below. After screening the biological activity of extracts prepared from each of the plant parts using the two procedures, further analyses focussed solely on the root samples. For these assays each sample (5.0 g) was extracted in 40 ml of MeOH:THF (1:1) with 400 μg/g dried weight of the antioxidant sodium diethyldithiocarbamate. After 16 h (overnight) extraction at -20˚C, the homogenate was centrifuged (15,000 rpm, 4˚C, 20 min), then the sediment was re-extracted for 1 h in the same way, centrifuged, supernatants were pooled and further purified by solid phase extraction, as described below.

Purification of root extracts by solid phase extraction (SPE). The extracts (from 5 g of each sample) were initially purified by passage through octadecylsilica columns (AccuBond SPE ODS-C18 cartridges, 500 mg/6 ml activated with 80% methanol) and concentrated to the aqueous phase by rotary evaporation *in vaccuo* at 35˚C. The aqueous phase was diluted in 20 ml of ammonium acetate buffer (40 mM, pH 6.5) and purified by applying it to a DEAE-Sephadex (1.0x5.0 cm) column coupled to two octadecylsilica (Sep-Pak C18, 0.5x1.5 cm) cartridges in tandem. After washing the coupled columns with a further 10 ml of the ammonium acetate buffer the columns were decoupled, the reversed-phase Sep-Pak cartridges were separately washed with 10 ml of distilled water, and the compounds retained by each cartridge were eluted in 5 ml 80% (v/v) methanol. The buffer wash solution that passed through the columns and water used to rinse the Sep-Pak cartridges was pooled and designated fraction A, while the fractions eluting from the first Sep-Pak C18 cartridge (coupled to the DEAE-Sephadex column) and the second cartridge were designated fractions B and C, respectively. The DEAE-Sephadex column was then coupled to another Sep-Pak C18 cartridge and eluted with 10 ml of 6% HCOOH. Compounds retained on this cartridge were eluted in 5 ml of 80% (v/v) methanol (fraction E) after washing the column with 10 ml of water. The liquid (acidified water) which passed through, and water used to rinse this third Sep-Pak C18 was pooled and designated fraction D. A final fraction, designated fraction F, was prepared from the material that did not dissolve in the ammonium acetate buffer prior to purification. All six of these fractions (A-F) were evaporated to dryness in a Speed-Vac concentrator (UniEquip) and stored at -20˚C until subsequent analysis.

High-performance liquid chromatography separation and fractionation. Fraction B had the strongest anticancer activity of fractions A-F according to Calcein AM cytotoxicity assays (see below). Therefore, this fraction was further purified by a preparative HPLC system-including Prep 100 HPLC pumps, a column thermostat and LabAlliance™ gradient controller from Watrex, Prague, a DeltaChrom™ processor and a Reprosil 100, C8 reversed-phase column (5 μm, 250x25 mm)-linked to a diode array detector (DAD Agilent 1100) and ion trap mass spectrometer (MSD Ion Trap SL, Agilent). The column thermostat was set to 30˚C, and injected samples were eluted using a linear methanol gradient rising from 70:30 to 0:100 water:methanol (v/v) over 30 min at a flow rate of 15 ml.min^{-1}. Twenty-seven one-minute fractions of the eluate were collected after the void fraction had cleared, each of which was evaporated to dryness *in vaccuo*, stored at -20˚C, and its cytotoxicity was then tested in Calcein AM assays (following dilution just prior to the assays), as described below.

Cytotoxicity testing. The anticancer activity of the extracts and each of the fractions described above was assessed by measuring their cytotoxic effects on malignant human cancer cell lines in Calcein AM cytotoxicity assays in 96-well microtitre plates, as follows. Each of the fractions was dissolved in 1 ml of 20% methanol in 0.1 M Tris buffer (pH 7.2) and six 3-fold dilutions were prepared. In addition, cultures of human breast adenocarcinoma MCF-7 and acute lymphoblastic leukemia CEM cancer lines (purchased from the

American Type Culture Collection, ATCC) were cultivated in DMEM medium (Gibco-BRL) supplemented with 10% (v/v) foetal bovine serum, L-glutamine (0.3 g/l), 100 U/ml penicillin and 100 μg/ml streptomycin at 37˚C in a humidified atmosphere containing 5% CO_2 to densities of ca. Cells/ml ($1.25x10^5$) in wells of a 96-well plate. After 3 h of stabilization 20 μl portions of each of the tested fractions and extracts were added in triplicate at six concentrations ranging from 0.4 to 100.0 mg.well^{-1} (5 g root extract) and from 0.08 to 20.00 mg.well^{-1} (1 g root extract) of the original extract, respectively. The cells were then incubated at 37˚C in an atmosphere containing 5% CO_2 for three days, after which Calcein AM solution (Molecular Probes) was added for 1 h according to the manufacturer's instructions. The fluorescence of viable cells was quantified using a Fluoroscan Ascent (Labsystems) reader. Cytotoxic effective concentrations were calculated and expressed as IC_{50} values from dose-response curves. The IC_{50} value represents the quantity of the starting lyophilised plant material lethal to 50% of the cancer cells used.

CD62E (E-selectin, ELAM)-induction assays. Human umbilical vein endothelial cells (HUVECs) were isolated and cultured in M199 medium supplemented with 20% foetal calf serum (FCS), antibiotics, endothelial cell growth supplement and heparin as previously described (16). Each well of a 96-well plate was coated with gelatine by applying 200 μl of 1% gelatine for 10 min at room temperature. Outer wells (A1-A12, H1-H12, A1-H1 and A12-H12) contained only 200 μl/well medium and served as an evaporation barrier. HUVECs ($1x10^4$) were seeded in each of the other wells in 200 μl medium and grown for 48 h to optimal confluence. Prior to treatment with plant extracts the cultivation medium was removed and collected in a reagent reservoir. Then, 100 μl medium was back-transferred to each well (no fresh medium was added, since that would have stimulated further growth of the HUVECs). Plant extracts (corresponding to 1.5, 2.0, 2.5, 3.0, 3.5 mg dried root weight per ml culture medium) were then added to the HUVEC-containing wells, in triplicate, and the cells were incubated with the extracts for an hour, after which 10 ng/ml TNFα was added per well to stimulate NFκB, and thus CD62E. After a further 4 h incubation the levels of CD62E in each of the HUVEC-containing wells were determined by enzyme-linked activity assays (ELISAs) as described below.

Cell-surface CD62E ELISAs. The extract/fraction and TNFα-treated HUVECs were washed once with PBS and fixed with 100 μl 25% glutaraldehyde per well for 15 min at room temperature. The cells were then washed with 3x200 μl PBS/0.05% Tween-20, blocked with 200 μl 5% BSA/PBS for 1 h, and washed again 3 times with 200 μl/well PBS/0.05% Tween-20. Then, anti-ELAM-antibody (clone BBA-1, R&D Systems) diluted 1:5,000 in 0.1% BSA/PBS (100 μl/well) was added, the cells were incubated for a further hour at room temperature and subsequently washed 5 times with 200 μl/well PBS/0.05% Tween-20. Goat anti-mouse-HRP conjugated antibody (Sigma Aldrich) diluted 1:10,000 in 0.1% BSA/PBS (100 μl/well) was then applied, the cells were incubated for a further hour in the dark at room temperature for 1 h and, after decanting, washed 5 times with 200 μl/well PBS/0.05% Tween-20. The HRP-activity of the cells in each of the wells was estimated using Fast-OPD (o-phenylene-diamine dihydrochloride) assays, as follows. An OPD tablet (silver foil) and a urea hydrogen peroxide tablet (gold foil, Sigma) were vortex-mixed in 20 ml H_2O until dissolved, then 200 μl of the resulting substrate mixture was added to each well and the plates were incubated for 30 min in the dark. The reaction was stopped by adding 50 μl of 1 M H_2SO_2 per well and the absorbance was measured at $OD_{492\ nm}$ in a vertical spectrophotometer.

Proliferation assays. MCF-7 cells that had been transfected with mutant p53 cDNA (MCF-7^{mtp53}) using Lipofectamin 2000 (Invitrogen) and acquired specific resistance to AraC (17) or tamoxifen (Vo *et al,* unpublished) were grown in low glucose DMEM medium supplemented with 10% heat inactivated FCS, 1% each of penicillin and streptomycin, and 400 μg/ml G418, at 37˚C in a humidified atmosphere containing 5% CO_2. The cells were then seeded at a density of $1x10^5$ cells/ml in 24-well plates and grown to 30% confluence. To determine its anti-proliferative effects on the cells, HPLC fraction B-25 (i.e. the 25th fraction collected from HPLC separations of fraction B, see above) at concentrations corresponding to 1.5 and 3.5 mg dried root weight per ml medium was then added to separate wells. After 24 and 72 h triplicates of treated MCF-7^{mtp53} cells were washed with PBS, trypsinised, and counted with a semi-automatic cell counter (Sysmex Corp., Japan). The degree of cell division inhibition was calculated in terms of numbers of cells in treated wells relative to controls, in percentages, as follows:

$[(C_{72\ h\ +\ drug} - C_{24\ h\ +\ drug})/(C_{72\ h\ -\ drug} - C_{24\ h\ -\ drug})]$ x100 = % cell division

$C_{72\ h\ +\ drug}$ ... cell number after 72 h of drug treatment

$C_{24\ h\ +\ drug}$ ... cell number after 24 h of drug treatment

$C_{72\ h\ -\ drug}$ ... cell number after 72 h without drug treatment

$C_{24\ h\ -\ drug}$ ... cell number after 24 h without drug treatment

Determination of cell death by Hoechst 33258/propidium iodide (HOPI) double staining. Two types of cell lines (MCF-7 and HL60) were used in cell death assays, as follows. MCF-7 cells were seeded at a density of $1x10^5$ cells/ml in a 24-well plate (1 ml medium per well) and incubated as described above for 24 h. HL-60 promyeloic leukaemia cells, purchased from the ATCC, were grown in RPMI-1640 medium supplemented with 10% heat inactivated FCS plus 1% penicillin and streptomycin at 37˚C in a humidified atmosphere containing 5% CO_2, then seeded at a density of $1x10^5$ cells/ml in 25 cm^2 cell culture flasks (5 ml medium per flask). HPLC-fraction B-25, derived from SPE-purified fraction B of the MeOH:THF root extract (concentrations referring to dried root material per ml cell culture medium are given in the text and in respective figures) was then added, in a series of concentrations to triplicates of the HL60 and MCF-7 cell preparations, which were incubated for 72 and 120 h, respectively, at 37˚C in a 5% CO_2 atmosphere. Then, Hoechst 33258 and propidium iodide were added (to final concentrations of 5 and 2 μg/ml, respectively) directly to the culture medium, and after further cultivation for 1 h the stained

Table I. Cytotoxic activities, towards MCF-7 and CEM cells, of extracts from indicated parts of *A. alopecuroidea* according to Calcein AM cytotoxicity assays.

Plant part	D.W. extracted (g)	Sample extraction procedure		IC_{50} (mg/ml) MCF-7	IC_{50} (mg/ml) CEM
Roots	0.5	3594	70% EtOH	2.5	<0.4
	0.5	3594K	MeOH:THF	1.1	0.9
Leaves	1.0	3595	70% EtOH	7.9	2.3
	1.0	3595K	MeOH:THF	2.7	1.0
Inflorescence	0.5	3596	70% EtOH	3.2	1.2
	0.5	3596K	MeOH:THF	3.6	1.2
Stems	1.0	3597	70% EtOH	8.0	3.9
	1.0	3597K	MeOH:THF	16.1	2.6

Extracts in six 3-fold dilutions (20 μl) were added to wells containing 80 μl of MCF-7 and CEM cell cultures for 72 h. The number of viable cells in each well was then quantified using the Calcein AM method and IC_{50} values were determined from dose-response curves. The IC_{50} values in the table indicate the quantity (mg) of the lyophilised starting plant material per ml cell culture medium lethal to 50% cancer cells. MeOH, methanol; THF, tetrahydrofurane and D.W., dried weight in g.

cells were examined under a fluorescence microscope with a DAPI filter, photographed, analysed and counted.

FACS cell cycle distribution analysis. MCF-7^{mtp53} cells were seeded in 6-well plates and incubated with portions of fraction B-25 corresponding to 3.5 mg dried root material/ml cell culture medium for 0.5, 2, 8, 24 and 48 h. The cells were then harvested, washed with 5 ml cold PBS, centrifuged (600 rpm for 5 min), re-suspended and fixed in 3 ml cold ethanol (70%) for 30 min at 4˚C. After two further washing steps with cold PBS, RNAse A and propidium iodide were each added to a final concentration of 50 μg/ml and incubated at 4˚C for 1 h. The cell cycle distribution of the cells was then analysed using a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA) in conjunction with ModFit LT software (Verity Software House, Topsham, ME, USA).

Western blotting. MCF-7^{mtp53} cells were seeded in 6-well plates and HL60 cells were seeded at a density of $1x10^5$ cells/ml in 25 cm^2 cell culture flasks. Both cell lines were incubated with portions of fraction B-25 corresponding to 3.5 mg dried root material/ml cell culture medium for 0.5, 2, 8, 24 and 48 h. Then the cells were harvested, washed twice with ice cold PBS, centrifuged at 1,000 rpm for 5 min, and finally lysed in a buffer containing 150 mM NaCl, 50 mM Tris pH 8.0, 1% Triton X-100, 1 mM phenylmethylsulfonylfluoride (PMSF) and protease inhibitor cocktail (PIC; from a 100x stock). The lysates were subsequently centrifuged at 12,000 rpm for 20 min at 4˚C, and the resulting supernatants were stored at -20˚C until further analysis. Equal amounts (validated by staining membranes with Poinceau S) of protein samples were separated by polyacrylamide gel electrophoresis (PAGE) and electro-blotted onto PVDF-membranes (Hybond, Amersham) overnight at 4˚C. After washing with phosphate-buffered saline/Tween-20 (PBS/T, pH 7.2) or Tris-buffered saline/Tween-20 (TBS/T, pH 7.6), membranes were blocked for 1 h in blocking solution (5% non-fat dry milk in PBS containing 0.5% Tween-20 or TBS containing 0.1% Tween-20). To visualise and evaluate protein and phospho-protein expression levels of selected cell cycle regulators and apoptosis effectors in the treated cells the membranes were each incubated with one of the primary antibodies listed below in blocking solution (dilution, 1:500-1:1,000) with gentle rocking at 4˚C, overnight. The membranes were then washed with PBS/T or TBS/T and further incubated with a corresponding secondary antibody (peroxidase-conjugated goat anti-rabbit IgG or anti-mouse IgG, dilution 1:2000-1:5000 in PBS/T or TBS/T) for 1 h. Antibodies directed against ph(Tyr15)-Cdc2, ph(Ser63)c-Jun, ph(Ser345)-Chk1, Chk1, ph(Thr68)-Chk2, caspase 3, caspase 7 and Chk2, were obtained from Cell Signaling, against ph(Ser177)-Cdc25A from Abgent, against cyclin D1, cyclin A, cyclin E, Cdc25A (F6), c-Jun, and PARP from Santa Cruz, and against Cdc2 and ß-actin from Sigma. Anti-mouse IgG was from Dako, and anti-rabbit IgG from GE-Healthcare. Chemiluminescence signals from the secondary antibody conjugates were generated and detected by an ECL detection kit (Amersham, UK) then membranes were exposed to Amersham Hyperfilm.

COX-2 inhibition assays. An ELISA kit supplied by IBL products (Hamburg, Germany) was used to determine COX-2 activities of cells treated with the B-25 fraction at a range of concentrations. This assay quantitatively determines prostaglandins F, E and D and thromboxane B-type prostaglandins produced in cyclooxygenase reactions. The measured COX-2 activities were then used to determine IC_{50} values, i.e. the concentration of the fraction that inhibited 50% of the COX-2 activity in the cells.

Statistics. Data regarding the activities of the extracts and fractions obtained from all of the experiments described above were analysed using GraphPad Prism 4.0 software.

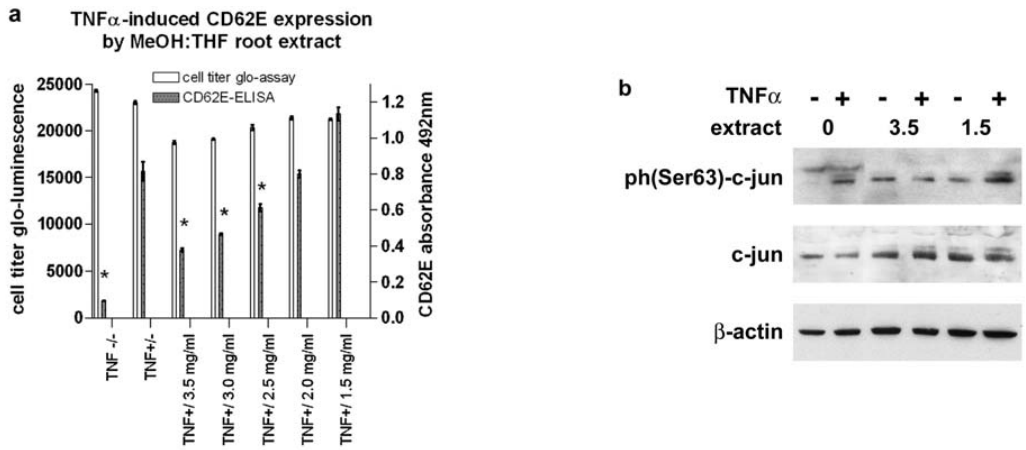


Figure 1. (a) Inhibition of TNFα-induced E-selectin expression. HUVEC cells were pre-treated with MeOH:THF extracts of *A. alopecuroidea* roots in dilutions corresponding to 3.5, 3.0, 2.5, 2.0 and 1.5 mg/ml dried plant material for an hour, then incubated with 10 ng/ml TNFα for a further 4 h, after which their CD62E levels were analysed. Extract (3.0-3.5 mg/ml) suppressed TNFα-induced CD62E induction by ~50%. Error bars indicate SEMs and asterisks significant differences from controls at the 0.05 probability level. Experiments were performed in triplicate. (b) Analysis of *c-Jun* phosphorylation. HUVECs were pre-stimulated with 10 ng/ml TNFα for 10 min then incubated with MeOH:THF root extract at concentrations corresponding to 3.5 and 1.5 mg/ml dried plant material for another 60 min. Extract (3.5 mg/ml) prevented further Ser63-*c-Jun* phosphorylation by TNFα. ß-actin was used as loading control.

Table II. Cytotoxicity of partially purified MeOH:THF extract fractions towards MCF-7 and CEM cells determined by Calcein AM assays.

Fraction	D.W. extracted (g)	IC_{50} (mg/ml) MCF-7	CEM
A	5.0	215.0	46.0
B	5.0	127.5	15.3
C	5.0	750.0	575.0
D	5.0	>1000.0	750.0
E	5.0	550.5	130.0
F	10.0	1051.0	346.0

Twenty microliter portions of fractions of the MeOH:THF root extract purified by C18-DEAE Sephadex-C18 chromatography were added in six 3-fold dilutions to wells containing 80 μl of MCF-7 and CEM cell cultures for 72 h. The number of viable cells in each well was then determined using the Calcein AM method and compared to untreated controls. The numbers in the Table indicate mg amounts of dried roots per ml cell culture medium (mg/ml) which inhibit Calcein AM uptake by 50% (IC_{50}). D.W., dried weight in g.

Table III. IC_{50} values of selected HPLC factions obtained by separating SPE fraction B of the MeOH:THF root extract.

Fraction	Extracted from g dried roots	IC_{50} (mg/ml) MCF-7	CEM
B-6	1.0	>200.0	>200.0
B-7	1.0	>200.0	89.0
B-17	1.0	175.0	153.0
B-25	1.0	32.0	12.0
B-26	1.0	>200.0	73.0

Twenty microliter portions of fractions of the HPLC-separated MeOH:THF root extract were added in six 3-fold dilutions to wells containing 80 μl of MCF-7 and CEM cell cultures for 72 h, then the number of viable cells was determined using the Calcein AM method and compared to numbers in untreated controls. The numbers indicate the concentrations (in mg amounts of dried roots per ml cell culture medium) that inhibit Calcein AM uptake by 50% (IC_{50}).

Results

Cytotoxic activity of A. alopecuroidea extracts. The cytotoxic activities towards MCF-7 and CEM cancer cell lines of ethanol (70%, v/v) and methanol-tetrahydrofurane (MeOH:THF, 1:1) extracts of the roots, leaves, inflorescences and stems of *A. alopecuroidea* were initially examined in Calcein AM cytotoxicity assays. Generally the stem extracts were the least effective (Table I), and root extracts the most effective of the tested extracts, for which IC_{50} concentrations against the CEM cell line were less than 0.4 mg/ml (70% ethanol extract) and 0.9 mg/ml (MeOH:THF extract), respectively. However, extracts of the leaves and inflorescence were also generally able to inhibit growth of all the cancer cell lines at low concentrations, the MeOH:THF extracts proving to be the most active in this respect (Table I). Further, the *A. alopecuroidea* root extracts were found to be strongly active against all of the tested cancer cell lines, especially those (such as the CEM cell line) bearing mutations and deletions affecting the expression of cell cycle-associated proteins. These findings indicate that root extracts are likely to be effective against tumours with various alterations of tumour suppressor genes such as p53 and pRb. Growth of the normal

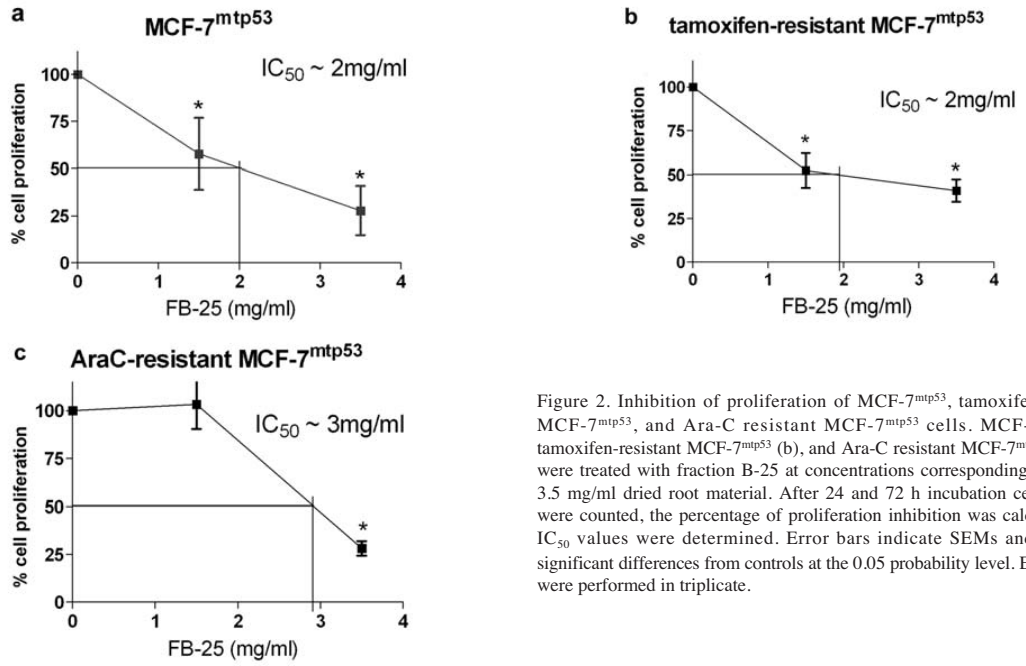


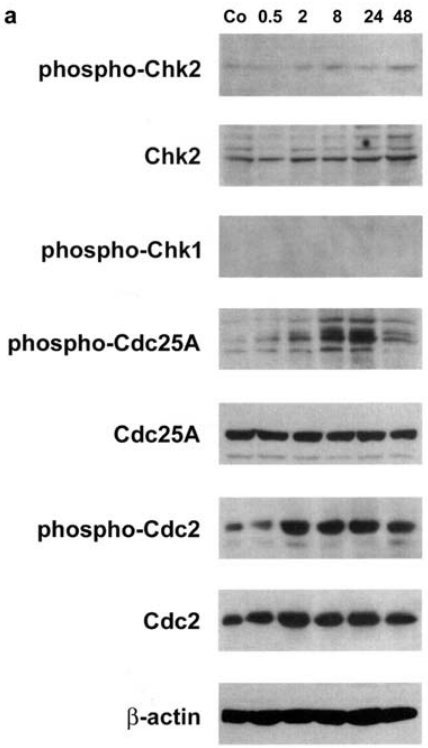
Figure 2. Inhibition of proliferation of MCF-7^{mtp53}, tamoxifen-resistant MCF-7^{mtp53}, and Ara-C resistant MCF-7^{mtp53} cells. MCF-7^{mtp53} (a), tamoxifen-resistant MCF-7^{mtp53} (b), and Ara-C resistant MCF-7^{mtp53} cells (c) were treated with fraction B-25 at concentrations corresponding to 1.5 and 3.5 mg/ml dried root material. After 24 and 72 h incubation cell numbers were counted, the percentage of proliferation inhibition was calculated and IC_{50} values were determined. Error bars indicate SEMs and asterisks significant differences from controls at the 0.05 probability level. Experiments were performed in triplicate.

mouse and human fibroblasts was not affected by at least at 10-fold higher concentrations (data not shown).

MeOH:THF extracts of A. alopecuroidea roots inhibit TNFα-induced inflammatory responses. TNFα is a prominent inducer of inflammation (which plays an important role in the development of cancer and is generally prominent in both cancerous tissues and adjacent stroma) and is involved in the development of rheumatoid arthritis (18), Crohn's disease (19), psoriasis and psoriatic arthritis (20). TNFα dysregulation and overexpression have also been found to increase the incidence of lung tumours (21) and liver metastases (22) in mouse models, and have been implicated in increased risks of the development of cervical neoplasia in papillomavirus 16-seropositive women (23). In addition, TNFα contributes to a variety of other human cancers (24) and targeted anti-TNFα therapy has proved to be effective in the treatment of renal cancer (25). TNFα induces NF-κB and, thus, NF-κB is also involved in inflammation and tumourigenesis. E-selectin (CD62E) is up-regulated by TNFα and NF-κB (26,27), and is overexpressed in inflammatory tissues (28,29). Since *A. alopecuroidea* is used by the Maya as a remedy to treat inflammations we investigated the possibility that MeOH:THF extracts of the plants' roots may inhibit CD62E expression in TNFα-induced HUVEC cells, and found that they dose-dependently suppressed TNFα-induced CD62E induction (extracts corresponding to 3.0-3.5 mg dried root weight per ml medium inhibited CD62E induction by 50%; Fig. 1a). We also examined the phosphorylation state of serine 63 (Ser63) of *c-Jun*, which is enhanced in response to TNFα-treatment, in HUVECs incubated with and without TNFα and MeOH:THF extracts of *A. alopecuroidea* roots at two concentrations. The results show that TNFα-treatment strongly induced *c-Jun*(Ser63) phosphorylation (Fig. 1b), and the *A. alopecuroidea* extracts also induced *c-Jun*(Ser63) phosphorylation at both tested concentrations, but only half as intensely as the TNFα-treatment. Co-treatment with TNFα increased the phosphorylation level in cells treated with extracts at a concentration equivalent to 1.5 mg dried root/ml, but not in those treated with 3.5 mg/ml. These findings imply that *A. alopecuroidea* extract dose-dependently inhibited TNFα-induced *c-Jun*(Ser63) phosphorylation, and thus its activation.

The inhibitory effects of *A. alopecuroidea* extracts on COX-2 enzymatic activity were also analysed in a COX-2 inhibition ELISA, because COX-2 (but not COX-1) has been shown to contribute to tumourigenesis. However, incubations with *A. alopecuroidea* MeOH:THF extracts with concentrations ranging from 0.5 to 3.5 mg/ml had no effect on COX-2 activity (data not shown). Thus, *A. alopecuroidea* did not inhibit COX-2-mediated effects, but interfered with the TNFα-induced inflammatory cell response *in vitro*. These findings provide mechanistic evidence for the empirical observation that *A. alopecuroidea* could be an effective remedy against severe inflammation.

Cytotoxic activities of A. alopecuroidea SPE-fractions derived from MeOH:THF root extracts. The A-F fractions of the MeOH:THF *A. alopecuroidea* root extract were tested for cytotoxicity towards selected cancer cell lines (MCF-7 and CEM). As shown by the data in Table II, fractions A (the buffer wash solution of the coupled DEAE-Sephadex and Sep-Pak C18 columns), B (the methanolic eluate of the 1st Sep-Pak) and E (the methanolic eluate containing substances flushed by 6% HCOOH from the DEAE-Sephadex column but retained by the third Sep-Pak C18) all showed significant cytotoxic activity towards the CEM line, with low IC_{50} values, and weaker activity towards the MCF-7 line. In contrast, fractions C, D and F had almost negligible effects against the MCF-7 cell line and minimal effects on CEM cells. Therefore,



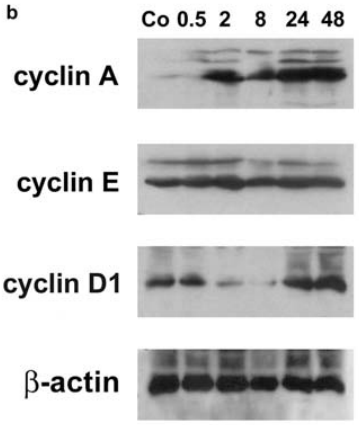


Figure 3. Effects of fraction B-25 on cell cycling. MCF-7^{mtp53} cells were treated with fraction B-25 at a concentration corresponding to 3.5 mg/ml dried plant material for 0.5, 2, 8, 24 and 48 h, then harvested for Western blot analyses of activation of the Chk-2 pathway and expression of selected cyclins. (a) Increasing levels of phosphorylation at Chk2-threonine 68 were observed with time, correlating with induced increases in phosphorylation at Cdc25A-serine 177 and (hence) Cdc2-tyrosine 15 phosphorylation, leading to cell cycle arrest. (b) Expression levels of the indicated cyclins. ß-actin was used as a loading control. (c) MCF-7^{mtp53} cells harvested at the all the time points mentioned above except 48 h were subjected to FACS analysis to investigate cell cycle phase specific inhibition. Fraction B-25 induced G1-G0 arrest in 70% of the cells after 24 h treatment. Error bars indicate SEMs and asterisks significant differences from controls at the 0.05 probability level. Experiments were performed in triplicate.

in further studies we solely used the most active fraction, B, obtained after partial purification of the MeOH:THF *A. alopecuroidea* root extract.

Cytotoxic activities of HPLC-separated fractions of the MeOH:THF fraction B A. alopecuroidea root extract. The cytotoxicity of one-minute HPLC fractions of fraction B of the MeOH:THF root extract were tested using the highly specific cytotoxic MCF-7 cell assay. As shown by the illustrative histogram in Table III, the strongest cytotoxicity peak (with respect to both cells lines) had a retention time of 25 min (fraction B-25) and a further, slightly weaker, activity peak was detected in fraction B-17 (Table III). B-26 and a more polar peak, B-7, also exhibited strong cytotoxicity towards the CEM cells, which seemed to be more sensitive than the MCF-7 cells. A few other fractions (e.g. B-6) also had weak activity, but most of them yielded IC_{50} values exceeding 200 mg/ml. Hence, fractions B-7, B-25 and B-26 were used in subsequent biological testing. Interestingly, B-25 showed comparable biological activity to fraction B of the MeOH:THF extract towards CEM, but not MCF-7 cells (cf. Tables II and III).

The Calcein AM cytotoxicity assay is based on the retention of the dye in vital cells, since the dye is washed out of dead cells, so it measures the combined effects of mortality and growth inhibition. To discriminate between the effects of *A. alopecuroidea* extracts on cell cycle machinery and cell death/apoptosis we analysed the cells at two time points at which information can be obtained on the relative proportions of cells affected in these ways. MCF-7 cells are particularly useful in this respect because apoptosis is not generally initiated in them until at least 72 h after drug treatment, thus any reductions in their numbers before this point (relative to controls) must be induced by cell cycle arrest. Hence, we distinguished cycle inhibitory effects of *A. alopecuroidea* from apoptotic and necrotic effects by calculating proliferation inhibition in the time window between 24 and 72 h of treatment (when no apoptotic phenotypes are observed), and determining levels of apoptosis after 120 h of treatment (as described in the Materials and methods section).

Fraction B-25 inhibits cell proliferation. To investigate the effects of fraction B-25 on cell proliferation, naïve MCF-7^{mtp53}- and tamoxifen- and/or Ara-C- resistant MCF-7^{mtp53} cells were exposed to the fraction at a series of concentrations. The cell numbers were measured after 24 and 72 h of treatment and

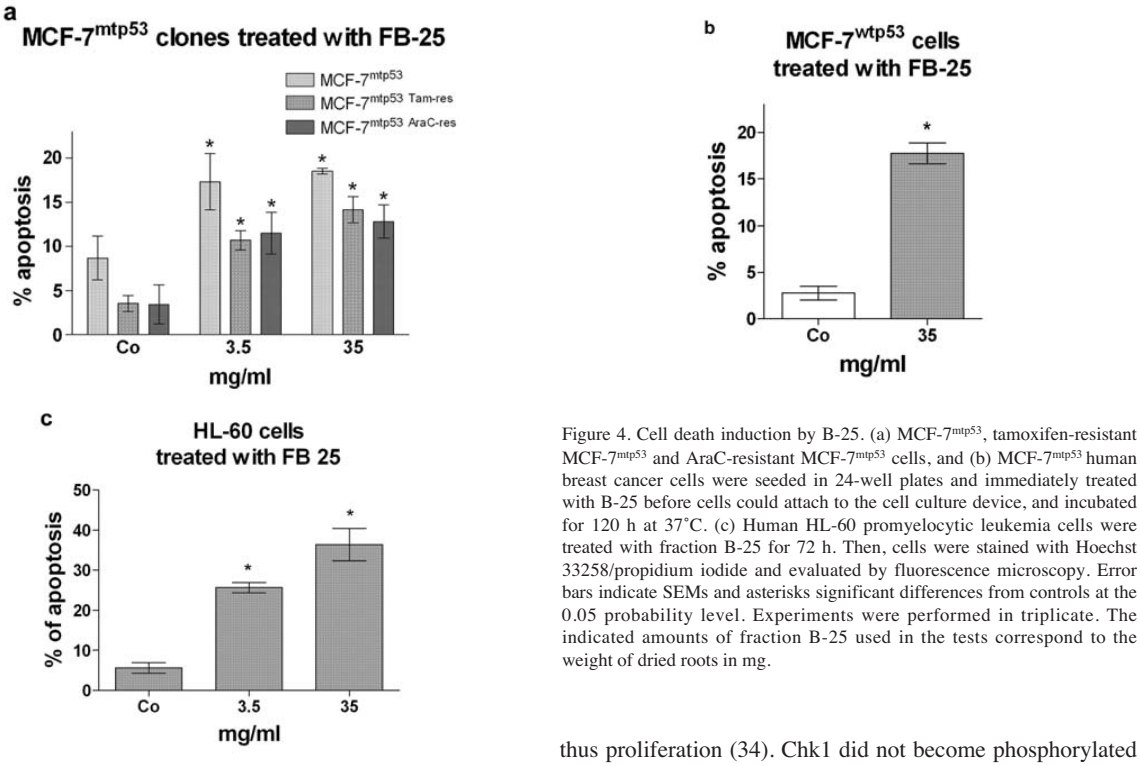


Figure 4. Cell death induction by B-25. (a) MCF-7^{mtp53}, tamoxifen-resistant MCF-7^{mtp53} and AraC-resistant MCF-7^{mtp53} cells, and (b) MCF-7^{mtp53} human breast cancer cells were seeded in 24-well plates and immediately treated with B-25 before cells could attach to the cell culture device, and incubated for 120 h at 37˚C. (c) Human HL-60 promyelocytic leukemia cells were treated with fraction B-25 for 72 h. Then, cells were stained with Hoechst 33258/propidium iodide and evaluated by fluorescence microscopy. Error bars indicate SEMs and asterisks significant differences from controls at the 0.05 probability level. Experiments were performed in triplicate. The indicated amounts of fraction B-25 used in the tests correspond to the weight of dried roots in mg.

the results were used to calculate the percentage inhibition of cell proliferation during this period, as described in Materials and methods. The concentration inhibiting cell proliferation by 50% (I_PC_{50}) ranged between 2 and 3 mg/ml (Fig. 2a-c), demonstrating that fraction B-25 suppressed cell division at approximately 10-15x lower concentration than the IC_{50} cytotoxicity concentration determined by the Calcein AM assay (32 mg/ml; see Table III). However, unlike the MeOH: THF extract, fraction B-25 did not inhibit TNFα-induced CD62E expression (data not shown).

Fraction B-25 interferes with the expression of cyclins and activates the Chk 2 pathway. MCF-7^{mtp53} cells were incubated with the B-25 fraction at concentrations corresponding to 3.5 mg dried root material/ml cell culture medium for 0.5, 2, 8, 24 and 48 h. Phosphorylation levels of selected cell cycle regulatory proteins (checkpoint kinases 1 and 2, Cdc25A and Cdc2) were analysed by Western blotting after each of these times. Checkpoint kinase 2 (Chk2) is part of a DNA damage-sensing mechanism that inactivates downstream cell cycle regulators, including the dual-specificity phosphatase Cdc25A-which it phosphorylates at serine 177-following genotoxic stress (30-32). This results in cell cycle arrest, allowing DNA to be repaired or, if the damage is too great for repair, apoptosis to be initiated (33). B-25 treatment induced Chk2 phosphorylation within 2 h, peaking after 8 h, and thus increasing Ser177-Cdc25A phosphorylation. Due to the inactivating phosphorylation of Cdc25A phosphatase, the downstream target molecule of Cdc25A, Cdc2 (Cdk1), became hyper-phosphorylated at tyrosine 15 (also an inactivating phosphorylation), which inhibited cell cycle progression and thus proliferation (34). Chk1 did not become phosphorylated (Fig. 3a).

B-25 also rapidly induced cyclin A expression (which facilitates G2/M transition) and repressed cyclin D1 (which is specifically involved in passage through G1; Fig. 3b). The expression of cyclin E was unchanged. FACS analysis showed that the proportion of cells in G2/M transition was reduced in B-25-treated MCF-7^{mtp53} cells (in accordance with cyclin A induction), while the proportion in the G1 phase was increased (in accordance with cyclin D1 repression and inactivation of Cdc25A; Fig. 3c), relative to controls.

Fraction B-25 induces chromatin condensation. Increasing concentrations of fraction B-25 corresponding to 3.5 and 35 mg dried root material/ml cell culture medium induced chromatin condensation (a typical feature of both type 1 and type 2 apoptosis) (35) in MCF-7^{mtp53} cells after 120 h of treatment. However, cell death was only elicited when fraction B-25 was administrated to detached MCF-7 cells (which readily detach after cell splitting); it did not trigger apoptosis when cells were already attached to the cell culture dish. These findings imply that anti-apoptotic survival signals, generated by cell attachment (e.g. integrins), interfered with the pro-apoptotic signal triggered by fraction B-25, reminiscently of classical anoikis type cell death induction (36,37). In chemo-resistant MCF-7 clones death induction was further reduced (Fig. 4a). To test whether the weak apoptotic response was due to the p53 mutation, we examined the effects of B-25 on MCF-7 cells harbouring wild-type p53 (MCF-7^{wtp53}). The results showed that the presence of intact p53 did not increase the cells' sensitivity to B-25-induced apoptosis (Fig. 4b). Further, we investigated whether human HL-60 promyeloic leukemia cells, which are very sensitive to various apoptotic triggers, were also sensitive to B-25. HL-60 cells were exposed

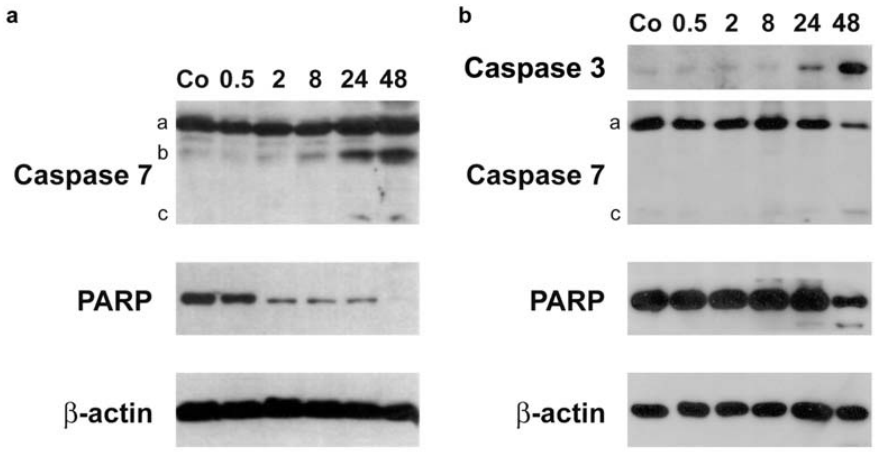


Figure 5. After incubation of MCF-7^{mtp53} cells (left images) and HL60 cells (right images) with fraction B-25 at a concentration corresponding to 3.5 mg/ml dried plant material cells were harvested and protein lysates were analysed by Western blotting. Left images: Increased levels of pre-activated Caspase 7 [b], but only minute levels of fully activated caspase 7 [c], were observed in MCF-7^{mtp53} cells after 24 h of treatment. The non-active form of caspase 7 is indicated as [a]. Right images: High levels of activated caspase 3, but only minute levels of fully activated caspase 7 [c] were observed in HL-60 cells after 24 h. PARP was not cleaved to the 85 kDa fragment characteristically found in apoptotic events in MCF-7^{mtp53} cells, but an 85 kDa fragment appeared in HL60 cells after 24 h of treatment. ß-actin was used as loading control.

to B-25 and after 72 h a significant increase (40%) in numbers of apoptotic cells was observed (Fig. 4c). Thus, HL-60 cells are also sensitive to fraction B-25.

The possibility that fraction B-25 treatment may activate caspase 3 and caspase 7, and the cleavage of PARP, was investigated by applying it to HL-60 and MCF-7^{mtp53} cells. Increased levels of pre-activation of caspase 7 were detected in MCF-7^{mtp53} cells, which are caspase 3-deficient (Fig. 5a). However, full activation of caspase 7 was marginal and PARP, which is a direct target of caspase 7, did not become degraded to the 85 kDa fragment that is a hallmark of apoptotic cell death (38), while in HL-60 cells B-25 treatment activated caspase 3 after 24 h and PARP (also a target of caspase 3) was cleaved into the 85 kDa fragment (Fig. 5b).

Discussion

In this study potential medical uses of extracts of *A. alopecuroidea*, a member of the Euphorbiaceae family that is endemic in Central America, were investigated. Other *Acalypha* species have been recently shown to have anti-oxidant, antimicrobial and cytotoxic activities (10-13), and *A. alopecuroidea* is used in traditional Maya medicine to treat severe skin conditions, stomach, urinary and endometrial complaints (14). Since *A. alopecuroidea* is used against severe inflammation we tested the anti-inflammatory potential of extracts of the plant *in vitro* and obtained evidence that MeOH:THF root extracts inhibited TNFα-induced E-selectin (CD62E) expression and c-Jun activation (phosphorylation) in HUVECs. Activation of c-Jun seems to play a role in hepatic inflammation (39) and is indicative of JNK2 signalling, which plays a role in arteriosclerosis (40). In contrast, COX-2, which is another mediator of inflammatory reactions, was not inhibited. This prompted us to investigate whether the tested extracts also have anticancer properties, since anti-inflammatory formulations (both purified compounds and complex mixtures) can interfere with malignant cell signalling. It is also well known that chronic inflammation plays a critical role in cancer development.

According to this rationale we tested the properties of various *A. alopecuroidea* extracts and fractions, discovering that the MeOH:THF root extract, especially a specific HPLC fraction (designated B-25, see above), exhibited the strongest anti-proliferative activity towards both MCF-7 breast and CEM leukaemia cancer cells. However, it is worth noting that the B-25 fraction did not exhibit anti-inflammatory properties.

The inhibition of proliferation appeared to be mediated by down-regulation and inactivation of the positive cell cycle regulators cyclin D1 and Cdc25A, and thus Cdk1, thereby abrogating mitotic signalling. Cyclins are essential for the activity of cyclin-dependent kinases (Cdks), which are rate-limiting for cell cycle progression. The D-type family of cyclins has been associated with a variety of proliferative diseases. Cyclin D1 appears in early G1 of the cell cycle and is required for the activation of Cdk4 and Cdk6 (41,42). Furthermore, cyclin D1 is frequently overexpressed in human neoplasias and has oncogenic effects (43). Therefore, suppression of cyclin D1 is a powerful measure to combat malignancies. Cyclin D1 was transiently down-regulated after treatment with the B-25 fraction, which caused G1/S arrest. In contrast, cyclin A was rapidly induced, accelerating G2/M transition. The only examined cyclin whose expression was not significantly changed by B-25 treatment was cyclin E.

Cdc25A is a phosphatase that plays an essential role in cell-cycle entry (G1/S), it is overexpressed in many human tumours and classified as an oncogene (34). Upon stress Cdc25A becomes inactivated by checkpoint kinases Chk1 or Chk2 (32,44). Fraction B-25 activated Chk2, thereby inducing Ser177-phosphorylation and inhibiting Cdc25A phosphatase activity. Consequently, the inhibitory phosphorylated form

of Tyr15-Cdk1, which is otherwise de-phosphorylated by Cdc25A (34), accumulated and thus inhibited cell cycle progression and cell proliferation.

Following chemotherapeutic treatment cancer cells frequently acquire both specific and non-specific drug resistance. Therefore, novel drugs are needed to provide further treatment options. Agents in the B-25 fraction may be potent candidates since the fraction inhibited the cell proliferation of chemoresistant MCF-7 clones (resistant to tamoxifen and AraC) with similar efficiency, a finding of high possible significance since breast cancer is the second most common type of cancer after lung cancer, globally accounting for 10.4% of all cancers in the total, male and female population, and the fifth most common cause of cancer death. Further, the ability of tumour cells to evade apoptosis is a major cause of the development and progression of cancer and plays a significant role in its resistance to conventional therapeutic regimens (45). Therefore, we examined the apoptosis-inducing properties of the B-25 fraction. The apoptotic response rate of MCF-7 clones was rather low. This was not due to the mutated p53 present in the MCF-7 clone panel (46), because MCF-7 cells expressing intact p53 responded similarly weakly. However, apoptosis was induced by fraction B-25 in HL-60 cells, which are also p53-negative. MCF-7 cells do not express caspase 3, instead caspase 7 is responsible for initiating apoptotic cell death. Interestingly, in MCF-7 cells caspase 7 was only weakly activated following B-25 treatment, whereas in HL-60 cells caspase 3 was strongly activated. This was most likely the reason for the differences in apoptotic effects between the two cell types. These results indicate that the B-25 fraction stimulated the pathway leading to caspase 3 activation, but not the pathway leading to caspase 7 activation. Analysis of caspase-specific proteolysis of PARP, which generates an 85 kDa cleavage fragment, supported this interpretation, because PARP was degraded in a caspase-typical manner in HL-60 cells, but not in MCF-7 cells. Nevertheless, the nuclear chromatin condensation was reminiscent of an apoptotic phenotype in B-25-treated MCF-7 clones. Thus, these findings indicate that substances present in fraction B-25 may also have potential value as apoptosis-inducing agents, at least in some cases.

In conclusion, *A. alopecuroidea*, which is used in traditional Maya medicine in Central America, especially a specific HPLC fraction of root extracts (designated B-25), contains a strongly cytostatic agent that targets cell cycle regulators and activates caspase 3-specific apoptosis. Therefore, further tests are required to identify and characterise the agent, which could be a potent lead for a new therapeutic drug.

Acknowledgements

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Supplement I

Supplement II

Supplement III

Šmejkal K., **Svačinová J.,** Šlapetová T., Schneiderová K., Dall'Acqua S., Innocenti G., Závalová V., Kollár P., Chudík S., Marek R., Julínek O., Urbanová M., Kartal M., Csöllei M., Doležal K. (2010) Cytotoxic activities of several geranyl-substituted flavanones. *Journal of Natural Products* 73 (4): 568-572.

Cytotoxic Activities of Several Geranyl-Substituted Flavanones

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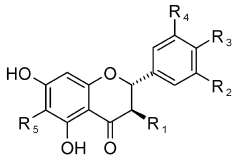
Nine geranylated flavanones isolated from the fruits of *Paulownia tomentosa* (**4**−**12**) and two from the roots of *Morus alba* (**13** and **14**) were examined for cytotoxicity to selected human cancer cell lines and normal human fibroblasts. Cytotoxicity was determined in vitro using a calcein AM cytotoxicity assay. Cytotoxicity for the THP-1 monocytic leukemia cell line was tested using erythrosin B cell staining. The geranylated compounds tested were compared with the known simple flavanone standards taxifolin (**1**), naringenin (**2**), and hesperetin (**3**) and with the standard anticancer drugs olomoucine II, diaziquone, and oxaliplatin and the antineoplastic compound camptothecin, and showed different levels of cytotoxicity. The effects of structural changes on cytotoxic activity, including geranyl substitution of the flavanone skeleton and the oxidation pattern of ring B of the flavanones, are discussed.

Polyphenolic compounds occur in nature as secondary metabolites in many plant species. According to their basic skeleton, they can be divided into several categories with one common structural feature, namely, the presence of phenolic hydroxy groups. Aromatic organic acids, coumarins, phthalides, quinones, stilbenes, xanthones, and especially flavonoids are included.[1] The biological activities of plant phenols vary and are often modified by the presence of different substituents on the basic skeleton. One possible structural modification is substitution by a prenyl side chain.

Prenylated flavonoids resulting from the combination of the phenolic and terpenoid metabolic pathways are often the focus of phytochemists and experimental biologists because of their interesting chemical properties and biological activities. The structural characteristic of prenyl flavonoids combines the lipophilic properties of prenyl or geranyl side chains with the hydrophilicity of the phenolic skeleton. The type of prenyl substitution and other modifications affect the biological activity of modified phenolic compounds.[2−4] Prenylated phenols can exhibit a broad spectrum of biological effects, including antioxidative, antiphlogistic, anticarcinogenic, and estrogenic properties, as described, for example, for the prenyl xanthones[5] and for prenyl compounds isolated from hops.[6,7] The ability of prenylated phenols to inhibit growth significantly or to induce cell death of bacteria and cancer cells arises from this wide spectrum of biological activities. The antibacterial activity of prenylated chalcones,[8] prenyl flavonoids,[9] or pterocarpans,[10] the effect of prenylation on the cytotoxic potential of flavonoids,[2,3,11,12] the cancer-related activities of prenylated phenols derived from hops,[13] and the potent cytotoxic properties of prenylated stilbenes[14] have been described previously. Due to the increasing interest in geranylated flavanones and their possible biological effects in cancer treatment and their antibacterial effects, we isolated eight geranylated flavanones (**4**−**9**, **11**, and **12**) from fruits of the Chinese medicinal plant *Paulownia tomentosa* Baill. (Scrophulariaceae).[15] We isolated subsequently and identified another geranylated substance called schizolaenone C (**10**), previously detected only in the fruits of *Schizolaena hystrix* Capuron (Sarcolaenaceae), from *P. tomentosa*.[16] Two *C*-geranyl flavanones (**13** and **14**) were obtained by HPLC fractionation of a root extract of *Morus alba* L. (Moraceae), as described in this work. On the basis of the analysis of spectroscopic data and comparison with published values, compound **13** was identified as kuwanon E.[17] Compound **14**, 4′-methoxykuwanon E, was isolated as a new compound.

On the basis of previous findings, the cytotoxicity of flavanones **4**−**14** was tested against five human cancer cell lines and on normal human fibroblasts using the calcein AM cytotoxicity assay. Cell viability and cytotoxicity were established also by staining THP-1 monocytic leukemia cells using erythrosin B. The cytotoxic effects were then compared with those of the known simple nongeranylated flavanones taxifolin (**1**), naringenin (**2**), and hesperetin (**3**), as well as the standard anticancer drugs olomoucine II, diaziquone, and oxaliplatin, and the antineoplastic lead compound camptothecin.



	R_1	R_2	R_3	R_4	R_5
1	OH	OH	OH	H	H
2	H	H	OH	H	H
3	H	OH	OMe	H	H
4	OH	OMe	OH	H	geranyl
5	H	OMe	OH	H	geranyl
6	H	OMe	OH	OMe	geranyl
7	H	OMe	OH	OH	geranyl
8	H	OH	OH	H	geranyl
9	H	H	OH	H	geranyl
10	H	OH	H	OH	geranyl

Results and Discussion

Compounds **4**−**9**, **11**, and **12** were isolated from *P. tomentosa* as reported recently,[15] with compound **10** obtained as described in the Experimental Section. Compounds **13** and **14** were isolated as

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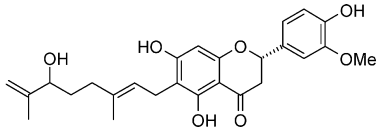
brownish, amorphous powders from the $CHCl_3$ fraction of *M. alba* extract by exhaustive HPLC fractionation. Compound **13** was determined to be identical with a geranyl flavanone previously isolated from *M. alba* and assigned as kuwanon E.[17] Circular dichroism (CD) spectroscopic analysis and comparison with previous results were used to establish the absolute configuration at the C-2 stereogenic center of **13**.[15] The absence of a Cotton effect for n→π* electronic transitions at ca. 340 nm and for π→π* at ca. 290 nm led to the conclusion that **13** was obtained as a racemic mixture.



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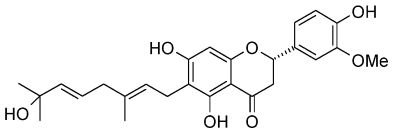

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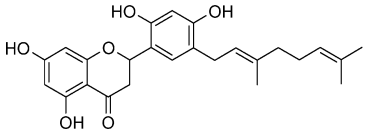
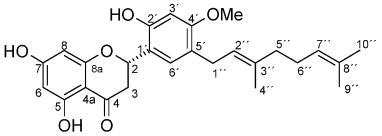


13



14

Compound **14** was isolated as a new compound, and its structure elucidation is described. The structure of compound **14** was determined to be 4′-*O*-methylkuwanon E. On the basis of HRAP-ITOFMS analysis ($[M - H]^-$ *m*/*z* 437.1867), the molecular formula of **14** was established as $C_{26}H_{30}O_6$. Chemical shifts similar to those obtained for **13** were observed in the ^1H and ^{13}C NMR spectra of **14** (Table 1). However, several minor differences observed between **13** and **14** are highlighted below. In the ^1H NMR spectrum, a signal was detected for a methoxy group at C-4′ (δ 3.73 ppm, 3H). The HMBC experiment confirmed the presence of this structural feature from interactions of C-4′ with H-1″ and CH_3O. The assignments of protons H-3α and H-3β were analogous to those obtained for compound **13** [H-3α (*cis*): δ 2.62 ppm, J = 3.1 Hz; H-3β (*trans*): δ 3.24 ppm, J = 12.8 Hz)]. A positive Cotton effect for the n→π* electronic transition at ca. 330 nm and a negative Cotton effect for the π→π* electronic transition at ca. 290 nm were observed in the CD spectrum of **14**. Therefore, a 2*S* configuration was assigned to this compound.[15]

Previous reports have described some effects of flavanones in the prevention of cancer development and their cytotoxicity for cancer cell lines.[18] For *C*-geranyl compounds, for example, analysis of propolis from Taiwan and Brazil showed the presence of cytotoxic geranyl flavanones.[19] Propolin C [a synonym for diplacon (**8**)] has been demonstrated to be cytotoxic to human melanoma cells and to be a potent trigger of apoptosis.[19] Propolin H showed similar properties in a subsequent study by Weng et al.,[20] while propolin G induced apoptosis in brain cancer cell lines.[21] A *C*-geranyl compound with an unsubstituted flavanone B-ring showed some activity against the human pancreatic cancer cell line PANC-1.[22] Several geranyl flavanones isolated from hops induced apoptosis in the human Burkitt's lymphoma BJAB cell line.[23]

Therefore, the flavonoid compounds isolated from *P. tomentosa* (**4**−**12**) and *M. alba* (**13** and **14**) were tested against seven different human cell lines: breast carcinoma (MCF-7), T-lymphoblastic leukemia (CEM), multiple myeloma (RPMI 8226 and U266), cervical cancer cells (HeLa), THP-1, a monocytic leukemia cell line, and a normal BJ fibroblast cell line. Six different concentrations of the compounds were used to obtain EC_{50} values, which were comparable with previous results (Table 2).[24,25] Evaluation of the EC_{50} data showed that the geranylated derivatives exhibited significantly greater cytotoxicity than the corresponding simple flavanones. This suggests that the presence of a geranyl side chain is a crucial structural requirement for the cytotoxic effect of flavanones on the cancer cell lines tested. Comparison of the EC_{50} values of compounds **5**, **11**, and **12** demonstrated that hydroxylation of the geranyl substituent in compounds **11** and **12** decreased the cytotoxic activity for all lines tested. A previous study has also shown reduced cytotoxicity for similar flavanone compounds with the *C*-geranyl side chain modified by hydroxylation.[25] The effect of the position of the *C*-geranyl group was clearly visible when the cytotoxicity of compounds **8**, **10**, **13**, and **14** was compared. A significant increase in activity was observed when the *C*-geranyl side chain occurred at position 6 on the A-ring (**8** and **10**), when compared to position 3′ in the B-ring (**13** and **14**) of the flavanone skeleton.

The effect of the presence of a hydroxy group at position C-3 of ring C was compared for compounds **4** and **5**. The present results confirmed previous reports indicating that OH-3 substitution of flavanones reduces their cytotoxicity for almost all cell lines.[25] Consequently, the effect of substitution of flavanone ring B was compared for compounds **4**−**10**, **13**, and **14** on cancer cell cytotoxicity in order to investigate the differing sensitivities of the cell lines to the compounds. For the MCF-7 line, compounds **7** (OCH_3-3′, OH-4′, OH-5′) and **10** (OH-3′, OH-5′) displayed the least potent cytotoxicity. The CEM line showed the highest sensitivity to **8** (3′,4′-di-OH, EC_{50} 3.2 μM). This was followed by **9** (OH-4′,

Table 1. NMR Spectroscopic Data for Compound **14** at 303 K

position	δ_C	δ_H (J in Hz)
2	73.9	5.61 dd (3.0, 12.7)
3	41.1	2.62 dd (3.1, 17.1)
		3.24 dd (12.8, 17.1)
4	196.6	
4a	101.7	
5	163.6	12.14 (OH)
6	95.8	5.86 dd (2.1, 4.5)
7	166.7	9.0 brs
8	95.0	5.86 dd (2.1, 4.5)
8a	163.3	
1′	115.9	
2′	153.9	9.0 brs
3′	99.0	6.47 s
4′	157.7	
OCH_3-4′	55.3	3.73 s
5′	119.7	
6′	127.6	7.11 s
1″	27.4	3.15 d (7.1)
2″	122.8	5.21 t (6.9)
3″	134.9	
4″	15.9	1.64 s (CH_3)
5″	39.2	1.99 m
6″	26.2	2.03 m
7″	124.1	5.06 t (6.9)
8″	130.7	
9″	17.5	1.53 s (CH_3)
10″	25.5	1.59 s (CH_3)

Table 2. Cytotoxic Activities of Simple and *C*-Geranyl-Substituted Flavanones Isolated from *P. tomentosa* and *M. alba* on Different Cell Lines[a]

	cell line tested, EC_{50}[b] [μM]						
compound	MCF-7	CEM	RPMI8226	U266	HeLa	BJ	THP-1
5	<10	<10	<10	<10	9.2 ± 4.2	<10	<10
7	<10	<10	7.3 ± 2.4	5.5 ± 4.0	7.4 ± 8.0	4.7 ± 2.0	<10
8	<10	3.2 ± 2.3	<10	2.4 ± 5.8	<10	5.9 ± 1.9	<10
10	<10	<10	7.1 ± 0.7	1.9 ± 1.1	6.3 ± 0.5	7.5 ± 1.1	8.5 ± 2.9
olomoucine II	4.6 ± 3.8	8.1 ± 1.7	6 ± 2.5	7.2 ± 3.0	9.9 ± 2.2	5.4 ± 4.5	
diaziquone	4 ± 1.3	0.86 ± 0.7	3.4 ± 2.1	2.8 ± 2.9	6.4 ± 1.8	<10	
oxaliplatin	<10	1.2 ± 1.4	1.7 ± 6.4	<10	<10	<10	
camptothecin							0.16 ± 0.07

[a] The calcein AM assay was used for MCF-7, CEM, RPMI8226, U266, HeLa, and BJ cells, and erythrosin B staining for THP-1 cells. The EC_{50} values and corresponding SD were calculated from the dose−response curves of three independent measurements. Compounds with $EC_{50} > 10$ μM (or $EC_{50} > 50$ μM for THP-1) were not cytotoxic in the range of concentrations used in these cytotoxicity assays. [b] Compounds **1**−**4, 6**, **9**, and **11**−**14** were inactive ($EC_{50} > 10$ μM) for all cell lines in which they were evaluated.

EC_{50} 11.6 μM) and **10** (3′,5′-di-OH, EC_{50} 12.3 μM). Compound **8** showed a higher cytotoxicity than olomoucine II. For the RPMI 8226 cell line, the most cytotoxic were compounds **7** (OCH_3-3′, OH-4′, OH-5′, EC_{50} 7.1 μM) and **10** (3′,5′-di-OH, EC_{50} 7.1 μM), which had a cytotoxicity similar to that of olomoucine II. Compounds **8** and **9**, with 3′,4′-di-OH and OH-4′, respectively, exhibited EC_{50} values close to 10 μM. Three compounds, **7** (OCH_3-3′, OH-4′, OH-5′), **8** (3′,4′-di-OH), and **10** (3′,5′-di-OH), showed high cytotoxicity, with EC_{50} values in the range 1.9−5.5 μM for the U266 cell line. It is interesting to note that EC_{50} values of the standards were greater in this case. The HeLa cell line was the most sensitive of those used in the experiments, and compounds **5**, **7**, and **10** showed comparable activities to those of olomoucine II and diaziquone and greater activity than that of oxaliplatin. The BJ human fibroblast cell line was also used for testing; compounds **7**, **8**, and **10** showed the highest cytotoxicities, with EC_{50} values in the range 4.7 to 7.5 μM, again comparable to those of the standards used.

For partial comparison, a different method based on erythrosin B staining was used on the monocyte THP-1 cell line. The antineoplastic compound camptothecin was shown to be more than 50 times as active as **10**, the most cytotoxic geranylated flavanone.

These results partially confirm the results published previously for the A2780 ovarian cancer cell line, for which compounds **8** and **9** showed EC_{50} values of 10 and >10 μM, respectively.[16] In contrast, for the same cancer cell line, Murphy et al.[26] have reported EC_{50} values for **8** and **9** of 5.5 and >10 μM, respectively.

It may be concluded from the present data that selected *C*-geranyl flavanones show cytotoxic activity for cancer cell lines. The geranyl substituent is an important factor for flavanone cytotoxicity and should not be modified by hydroxylation. Compounds having a *C*-geranyl side chain attached to ring B of a flavanone skeleton showed less activity than those with the same chain on ring A. Evaluating the effect of B-ring substitution showed that mono- or dihydroxy substitution of flavanones (**8**−**10**) led to compounds with the most potent activities; strong cytotoxicity was also displayed by the dihydroxy methoxy-substituted flavanone **7**. Comparison of cytotoxicity against normal cells and against cancer cell lines revealed a very narrow therapeutic window for all of the compounds tested.

Experimental Section

General Experimental Procedures. A JASCO P 2000 digital polarimeter (cell volume 1 mL, 10 cm) was used for optical rotation measurement. UV spectra of samples were recorded in MeOH on a Synergy HT multiplate reader. CD spectra were recorded on a JASCO J-810 spectrometer (MeOH; the molar ellipticity θ_λ values are reported). IR spectra were determined using the ATR method on a Nicolet Impact 400D FT-IR spectrophotometer. NMR spectra were recorded using a Bruker Avance 300 spectrometer operating at frequencies of 300.13 MHz (^{1}H) and 75.48 MHz (^{13}C). The spectra were measured in DMSO-d_6 or $CDCl_3$ at 303 K. The ^{1}H and ^{13}C NMR chemical shifts (δ in ppm) were referenced to the signals of the solvent [2.50 (^{1}H) and 39.43 (^{13}C) for DMSO-d_6 and 7.26 (^{1}H) and 77.00 (^{13}C) for $CDCl_3$]. 2D NMR, gs-COSY, gs-HSQC, and gs-HMBC were used to assign the individual ^{1}H and ^{13}C resonances. The HSQC experiment was adjusted for the coupling $^1J_{HC} = 150$ Hz and the HMBC experiment for long-range couplings of 7.5 Hz. ESIMS results for samples dissolved in MeOH were collected on an Agilent HP 1100 LC/MSD Trap VL Series, using direct infusion with a linear pump (K & D Electronics) at a flow rate of 300 μL min^{-1}. The spectra were collected in the negative mode, with the nebulizing and drying gas N_2 ($t = 300$ °C) flowing at a rate of 10 L min^{-1}, a nebulizer pressure of 80 psi, and a capillary voltage of 3.5 kV. The full mass scan covered the range from *m*/*z* 200 to 1500. A Mariner PE Biosystem Workstation (Tekas) with APITOF was used to collect the HRMS. These spectra were collected also in the negative mode. An Agilent 1100 apparatus equipped with a diode-array detector was used for chromatographic semipreparative (Supelcosil ABZ+Plus, 250 mm × 10 mm i.d., particle size 5 μm) and analytical (Supelcosil ABZ+Plus, 150 mm × 4.6 mm i.d., particle size 3 μm) separation. Preparative HPLC was carried out on a LCP 4100 instrument, with loop injection of 100 μL, column block LCO 101, UV detector LCD 2084 (Ecom, CR), and column Supelcosil ABZ+Plus, 250 mm × 21.2 mm i.d., particle size 5 μm.

The simple flavanones **1**−**3** were acquired from Sigma (Czech Republic). The purity of all compounds always exceeded 95%, as determined by analytical HPLC. The geranylated flavonoids **4**−**9**, **11**, and **12** used in the cytotoxicity testing were isolated from *P. tomentosa* according to the method described previously.[15]

Plant Material. The roots of *M. alba* (Moraceae) were collected in Konya, Turkey, in April 2007. A voucher specimen (MA-07A) was deposited at the herbarium of the Department of Natural Drugs, Faculty of Pharmacy, University of Veterinary and Pharmaceutical Sciences Brno, Brno, Czech Republic. The plant material was identified by Assoc. Prof. Murat Kartal (Faculty of Pharmacy, Ankara University, Turkey) and Assoc. Prof. Petr Babula (Faculty of Pharmacy, University of Veterinary and Pharmaceutical Sciences Brno, Brno, Czech Republic). The fruit of *Paulownia tomentosa* was collected in the area of the University of Veterinary and Pharmaceutical Sciences Brno, Brno, Czech Republic, during October 2004. A voucher specimen (PT-04O) has been deposited at the herbarium of the Department of Natural Drugs, Faculty of Pharmacy, University of Veterinary and Pharmaceutical Sciences Brno, Brno, Czech Republic.

Extraction and Isolation. Schizolaenone C (**10**) was obtained from the MeOH part of fraction K (fraction 197−199)[15] of the *P. tomentosa* extract, using flash chromatography and subsequent semipreparative HPLC. Flash chromatography of fraction K was carried out on silica gel Merck 60 (particle size 0.040−0.063 mm), the mobile phase was composed of C_6H_6−$CHCl_3$−MeOH (v/v/v, 5:85:10, with the addition of 0.1% HCOOH), and 150 mL fractions were collected. Fraction K-8, containing geranylated flavonoids, was subsequently separated by analytical and semipreparative HPLC. Fractions were collected on the basis of the UV detector response at $\lambda = 280$ nm. The identity of compound **10** was confirmed by comparison of the ^{1}H and ^{13}C NMR, HRMS, IR, and UV data with those in the literature.[16]

The dried root of *M. alba* (537 g) was crushed into small pieces and then extracted by maceration with ethanol (3 × 5 L). The dried ethanol extract (20 g) was dissolved in 90% MeOH (500 mL), and the

resulting uniform suspension was extracted with 3 × 250 mL of hexane to remove strongly nonpolar compounds. One liter of water was added to the MeOH residue, and the resulting solution was extracted with $CHCl_3$ (3 × 500 mL). The $CHCl_3$ extracts were combined and dried in vacuo to give a brownish mass (15 g). This was dissolved in a sufficient volume of MeOH and separated using reversed-phase preparative HPLC. The gradient elution used 0.2% HCOOH and a mixture of MeCN and MeOH, 8:2 (v/v) (A). The gradient of the mobile phase initially consisted of 20% A and reached 100% A in the 40th minute. Flow rate was 25 mL min^{-1}. Fractions were acquired according to the detector response (λ = 280 nm). After removing the organic solvent and precipitation, fractions with t_R of 22−23 and 26−27 min, respectively, yielded compounds **13** and **14** (138 and 182 mg, respectively).

Schizolaenone C (10): yellowish powder; UV (MeOH) λ_{max} (log ε) 340 (sh) (3.55), 285 (4.08), 235 (sh) (4.10), 210 (4.76) nm; ^1H and ^{13}C NMR data in accord with ref 16; HRAPITOFMS $[M - H]^-$ *m/z* 423.1789 (calcd for $C_{25}H_{27}O_6^-$ 423.1807); ESIMS $[M - H]^-$ *m/z* 423.5.

Kuwanon E (13): yellowish powder; UV (MeOH) λ_{max} (log ε) 331 (sh), 288 (4.26), 231 (sh), 201 (4.76) nm; ^1H and ^{13}C NMR in accord with ref 17; ESIMS $[M - H]^-$ *m/z* 423.

4′-Methoxykuwanon E (14): brownish powder; $[\alpha]^{25}_D$ −14.8 (*c* 0.29, MeOH); UV (MeOH) λ_{max} (log ε) 201 (4.85), 224 (sh), 288 (4.36), 334 (sh) nm; CD (MeOH) θ_{331} +5904, θ_{291} −24 703, θ_{227} 10 595; IR (ATR) ν_{max} 3318, 2966, 2915, 1636, 1604, 1506, 1449, 1356, 1258, 1154, 1107 cm^{-1}; ^1H and ^{13}C NMR data, see Table 1; ESIMS $[M - H]^-$ *m/z* 437; HRAPITOFMS $[M - H]^-$ *m/z* 437.1940 (calcd for $C_{26}H_{29}O_6^-$, 437.1964).

Calcein AM Cytotoxicity Assay. The cell lines used in the cytotoxicity assays, human breast carcinoma (MCF-7), human T-lymphoblastic leukemia (CEM), human multiple myeloma (RPMI 8226 and U266), human HeLa cervical cancer cells, and human normal fibroblasts (BJ), were purchased from the American Type Culture Collection (ATCC). The cells were grown on Dulbecco's modified Eagle's cell culture medium (DMEM, Gibco BRL) supplemented with 10% (v/v) fetal bovine serum, L-glutamine (0.3 g/L), 100 U/mL penicillin, and 100 μg/mL streptomycin. The cell cultures were maintained at 37 °C in a fully humidified atmosphere containing 5% CO_2. Cells were subcultured two or three times a week using a trypsinization procedure.[27]

To determine the anticancer potential of the individual compounds, their cytotoxic effect on the growth of malignant human cancer cell lines was determined by calcein AM cytotoxicity assays in 96-well microtiter plates. For cytotoxicity estimations, 100 mM stock solutions were prepared in dimethylsulfoxide (DMSO) from each compound and diluted to 15 mM with DMSO. Then, 8 μL of this solution was diluted in 232 μL of distilled water just before use to obtain the first concentration (500 μM) to be applied to the cell cultures. Starting with the 500 μM concentration, six 3-fold dilutions of the intended test concentration (ranging from 2 to 500 μM) were prepared by adding 160 μL of distilled water to 80 μL of the solution with the corresponding higher concentration. The cell suspensions were counted in a Bürker chamber, diluted in DMEM to an approximate density of 1.25 × 10^5 cells/mL, then placed into each well of a 96-well plate and stabilized for 3 h. Afterward, six serial 3-fold dilutions of the tested compounds were added to the appropriate microtiter plate wells already containing 80 μL of the cell suspension in 20 μL aliquots. Each concentration tested was added in triplicate; the highest final concentration in the wells was 100 μM and the lowest 0.4 μM. The final concentration of DMSO in the reaction mixture never exceeded 0.6%. After 72 h of incubation at 37 °C and 5% CO_2, the addition of 100 μL of 2 μM calcein AM solution (Molecular Probes) to the microtiter plate wells and incubation for 1 h resulted in the staining of viable cells. The fluorescence of these viable cells was measured and quantified at 485/538 nm (ex/em) using a Fluoroscan Ascent (Labsystems) reader. Each compound was characterized by an EC_{50} value, the concentration of the compound lethal to 50% of the tumor cells, as calculated from the dose−response curves obtained. Calculations were performed using MS Excel 2000.[27]

Cellular Proliferation and Viability Assay. Human monocytic leukemia THP-1 cells were obtained from the European Collection of Cell Cultures (ECACC). These cells were routinely cultured in RPMI medium supplemented with 10% fetal bovine serum, 2% L-glutamine, and 1% penicillin and streptomycin, at 37 °C with 5% CO_2. The compounds tested were dissolved in DMSO and added in five increasing concentrations to the cell suspension in culture medium. Subsequently, the cells were incubated for 72 h at 37 °C with 5% CO_2. The cell number and viability were determined following staining with erythrosin B (Sigma, Germany). The staining solution (0.1% erythrosin B (w/v) in phosphate-buffered saline (PBS), pH 7.2−7.4) was mixed with an equal amount of cell suspension, and the number of viable and nonviable cells was counted manually using a hemocytometer and a light microscope. Cells that remained unstained were considered viable and light red cells as nonviable. The cytotoxic EC_{50} concentrations of the compounds tested were determined by combining the data from the equation generated by the KURV+ Version 4.4b software with statistical analysis using STAT+ software (used for the EC_{50} data derived from the values plotted on the graph). The two methods were compared, and the final EC_{50} values obtained.

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Supporting Information Available: NMR spectra for compound **14** and table of HMBC and NOESY for this substance. This information is available free of charge via the Internet at http://pubs.acs.org.

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Supplement I

Supplement II

Supplement III

Svačinová J., Novák O., Lenobel R., Plačková L., Hanuš J., Strnad M., Doležal K. A new miniaturized analytical approach for cytokinin isolation from plant tissues using pipette tip solid-phase extraction (manuscript in preparation).

A new miniaturized analytical approach for cytokinin isolation from plant tissues using pipette tip solid-phase extraction

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Abstract

We have developed a new analytical approach for the isolation and quantification of cytokinins (CK) in minute amounts of fresh plant material, which combines a simple one-step purification with ultra-high pressure liquid chromatography–fast scanning tandem mass spectrometry. For the first time, plant tissue samples (1-5 mg FW) were purified by stop-and-go-microextraction (StageTip purification), which previously has only been applied for clean-up and pre-concentration of peptides. We found that a combination of three sorbents, two reverse phases and one cation-exchange phase, was the best tool in the new miniaturized one-step purification, giving a total extraction recovery higher than 80% for tritium-labelled standards. The process was completed by a single chromatographic analysis of a wide range of naturally occurring cytokinins (bases, ribosides, *O*- and *N*-glucosides, and nucleotides) in 24.5 minutes using an analytical column packed with sub-2-microne particles. In multiple reaction monitoring mode, the detection limits ranged from 0.05 to 5 fmol and the linear ranges for most cytokinins were at least five orders of magnitude. The accuracy and precision of the method was validated using samples spiked with 1pmol of authentic cytokinin standards. The StageTip purification was optimized using samples from 10-day-old *Arabidopsis thaliana* seedlings, roots and shoots where fifteen CKs were successfully determined in concentrations ranging from 0.2 to 65.8 pmol g^{-1} FW.

Keywords

Arabidopsis thaliana, cytokinins, pipette tip solid-phase extraction, StageTip, tandem mass spectrometry (MS/MS), ultra-high performance liquid chromatography (UPLC)

1. Introduction

The plant hormone cytokinin (CKs) is essential to promote cell growth and differentiation in plant tissues in the presence of another phytohormone, auxin (Miller et al., 1955; Mok et al., 2001; Sakakibara, 2006). Natural cytokinins are adenine derivatives, which are substituted at the N^6 position by either an isoprenoid or an aromatic side chain (Table 1). The presence of a hydroxyl group, double bonds and other structural variations affect cytokinin-receptor interactions and determine the functional specificity of individual CKs (Spíchal et al., 2004; Werner et al., 2009). Cytokinins occur in plants as free bases, nucleosides (ribosides), glycosides (*O*- and *N*-glycosides) and nucleotides. The occurrence, distribution and variation of individual CKs depends on plant species, tissue, and developmental stage (Mok et al., 2001; Sakakibara, 2006).

The analysis of cytokinins is difficult because of their presence in plants at very low concentrations (pmol·g^{-1} fresh weight: Strnad et al., 1997; Baroja-Fernández et al., 2002; Novák et al., 2003; Stirk et al., 2003; Hauserová et al., 2005; Bairu et al., 2011). Therefore, isolation and quantification of endogenous levels of cytokinins from the complex multi-component plant matrix requires efficient, selective and sensitive analytical approaches. Modern analytical procedures for the determination of cytokinins consist of sample pre-treatment and subsequent instrumental measurement of individual CK metabolites (Tarkowski et al., 2009). Extraction of analytes from the plant material by an organic solvent such as Bieleski buffer (methanol/chloroform/water/formic acid, 12/5/2/1) (Bieleski, 1964), 80% (v/v) methanol or modified Bieleski buffer (methanol/water/formic acid, 15/4/1) (Hoyerová et al., 2006) is usually followed by solid-phase extraction (SPE) using reversed-phase, cation- and anion-exchange sorbents (Dobrev et al., 2002; Novák et al., 2003; Takei et al., 2003; Hoyerová et al., 2006). The SPE step effectively removes ultraviolet absorbing contaminants in cases in which the analytes have greater affinity for the solid phase than for the sample matrix (Nováková et al., 2009). The final step for isolation of biologically active compounds from plant extracts often involves an immunoaffinity chromatography (IAC), which provides selective sample enrichment and is required in cases when co-extraction of analytes at trace concentrations together with interfering substances at much higher concentrations is problematic (Yarmush et al., 1992; Hage et al., 1999; Hennion et al., 1999; Hennion et al., 2003). Antibodies against an analyte immobilized to a solid support bind the target molecule by specific non-covalent interactions. As a result, a highly purified cytokinin fraction free of contaminating sample constituents can be obtained (Hauserová et al., 2005; Novák et al., 2008). Nowadays, lower levels of naturally occurring cytokinins can be analysed as more powerful analytical instruments are being developed. As a result, during the last decade, the starting amount of plant material has decreased from gram- (1-5 g) to milligram quantities (50-100 mg). Miniaturized purification methods (Novák et al., 2008) are more efficient, less time consuming and cheaper in comparison with classical extraction approaches (van Rhijn et al., 2001; Zhang et al., 2001; Novák et al., 2003). To minimize the required amount of biological tissues (<5 mg FW), the pipette tip SPE (PT-SPE) method is a useful tool for purification, concentration, and selective compound isolation. The PT-SPE method, in which chromatographic material is polymerized into pipette tips, is widely used in genomic, proteomic, metabolomic, pharmaceutical and biomedical

applications (van Hout et al., 2001; Pluskal et al., 2002; Keough et al., 2002; Kumazawa et al., 2007). The so-called StageTip (STop And Go Extraction Tip) purification has been published as a simple and extremely economical approach to desalting, concentration, purification and pre-fractionation of proteins and peptides prior to LC/MS analysis (Rappsilber et al., 2003; Ishihama et al., 2006; Saito et al., 2006). StageTips are ordinary pipette tips containing very small disks made of beads with reversed-phase, cation-exchange or anion-exchange surfaces embedded in a Teflon mesh (Rappsilber et al., 2007). These self-made micro-tips combine flexibility, ease and speed of use, small bed volume and high loading capacity, and provide excellent recovery, high reproducibility, robustness and versatility for even minute amounts of analyte. A further advantage for sample preparation is that the one-time usage eliminates the risk of carryover (Rappsilber et al., 2007). However, no previous applications of PT-SPE for phytohormone analysis have been reported.

High performance liquid chromatography (HPLC) combined with mass spectrometric (MS) detection has become the main technique of choice in cytokinin analysis after pre-cleaning samples (Prinsen et al., 1998; van Rhijn et al., 2001; Novák et al., 2003). The introduction of ultra-high performance liquid chromatography (UPLC), in which sub-2-microne particles (1.7 μm) of hybrid materials utilizing bridged ethyl/siloxane silica structures (BEH technology) are packed in a column, brought new improvements compared with HPLC in terms of separation efficiency, resolution, sensitivity, speed and sample throughput (Nguyen et al., 2006; Wu et al., 2007; Nováková et al., 2009). Fast chromatographic separation of complex samples and low concentration of analytes require a sensitive and selective MS detection. This can be achieved using a fast scanning tandem mass spectrometer (MS/MS) which provides a trace analysis of femtomolar concentrations. Novák et al. (2008) reported the application of a UPLC-MS/MS technique for 32 cytokinin derivatives pre-purified by SPE and batch immunoextraction procedures. The analytical approach was based on two baseline chromatographic separations using a short reversed-phase column (2.1×50 mm) and sensitive, accurate quantification by MS/MS in multiple reaction monitoring modes (MRM). The practical application of UPLC-MS/MS methods has also been reported for some other phytohormone groups (Hirano et al., 2007; Zentella et al., 2007; Kojima et al., 2009; Turečková et al., 2009).

The main aim of this study was further miniaturization of our current method of cytokinin isolation (Novák et al., 2008) with applicability to plant tissue samples weighing as little as 1-5 mg fresh weight. To achieve this, we have successfully optimized the purification step employing self-packed StageTips as a novel powerful PT-SPE approach to complex cytokinin analysis. Finally, we have developed UPLC-MS/MS methods requiring only a single chromatographic run. The improved method uses an efficient separation onto a C18 column packed with sub-2-microne particles, combined with sensitive fast scanning tandem mass spectrometric detection for measurement of naturally occurring isoprenoid cytokinin metabolites (bases, ribosides, *N*-glucosides, *O*-glucosides and nucleotides).

2. Experimental procedures

2.1 Reagents and materials

Authentic and deuterium-labelled CK standards were obtained from Olchemim Ltd (Olomouc, Czech Republic). Tritium-labelled CK standards ([^{3}H]*t*ZR, 0.851 TBq mmol^{-1}; [^{3}H]*c*Z, 0.777 TBq mmol^{-1}; [^{3}H]iPR, 1.184 TBq mmol^{-1}) with radiochemical purity >98% were synthesized in the Isotope Laboratory, Institute of Experimental Botany, AS CR (Prague, Czech Republic). Methanol (gradient grade), formic acid and ammonium hydroxide were from Merck (Darmstadt, Germany); chloroform, acetic acid and formic acid for LC/MS from Sigma-Aldrich (St. Louis, MO, USA); Murashige & Skoog medium from Duchefa Biochemie B.V. (Haarlem,The Netherlands); Empore™ High Performance Extraction Disks (sorbent type C18, SDB-RPS and Cation-SR) from 3M Center (St. Paul, MN, USA). Deionised (Milli-Q) water was obtained from Simplicity 185 system (Millipore, Bedford, MA, USA) and scintillation cocktail Ultima Gold™ was from Packard BioScence (Groningen, The Netherlands).

2.2 Biological material

Arabidopsis thaliana seedlings (ecotype Colombia) were grown *in vitro* in Petri dishes containing Murashige & Skoog medium including vitamins (4.4 g MS medium, 10 g of sucrose, 10 g of plant agar·l^{-1}, pH 5.7) at 23 ˚C in a 16 h photoperiod. 10-day-old seedlings, roots and shoots were harvested, weighed, immediately plunged into liquid nitrogen and stored at -70 ˚C.

2.3 Pipette tip SPE purification

2.3.1 Liquid microextraction

Bieleski buffer (60% methanol, 25% $CHCl_3$, 10% HCOOH and 5% H_2O) was used as extraction solvent (50 µl per sample). Tritium-labelled (10^5 dpm) or unlabelled (1 pmol) cytokinin standards were added to the sample extracts during the method optimization to determine the recoveries of the StageTip purification procedure. To validate the quantification of the endogenous cytokinin levels in 10-day old *A. thaliana* seedlings, roots and shoots, the following stable isotope-labelled cytokinin internal standards (IS) were added as internal tracers at a concentration 0.5 pmol of each compound per 50 µl of Bieleski buffer: [$^{13}C_5$]*c*Z, [$^{13}C_5$]*t*Z, [$^2H_5$]*t*ZR, [$^2H_5$]*t*Z7G, [$^2H_5$]*t*Z9G, [$^2H_5$]*t*ZOG, [$^2H_5$]*t*ZROG, [$^2H_5$]*t*ZMP, [$^2H_3$]DHZ, [$^2H_3$]DHZR, [$^2H_3$]DHZ9G, [$^2H_7$]DHZOG, [$^2H_3$]DHZMP, [$^2H_6$]iP, [$^2H_6$]iPR, [$^2H_6$]iP7G, [$^2H_6$]iP9G, [$^2H_6$]iPMP. The plant material was placed in 2.0 ml microcentrifuge tubes and extracted in Bieleski solvent using a MM 301 vibration mill (Retsch GmbH & Co. KG, Haan, Germany) at a frequency of 27 Hz for 3 min after adding 3 mm tungsten carbide beads (Retsch GmbH & Co. KG, Haan, Germany) to increase the extraction efficiency. The tube content was ultrasonicated for 3 min and then stirred for 30 min at 4 ºC. After centrifugation (10 min, 15,000 rpm, 4 ºC) the supernatants (50 µl aliquots) were immediately transferred onto StageTips and purified according to the following protocol.

2.3.2 Preparation of StageTips

The PT-SPE was performed in self-packed StageTips by placing a very small disk of matrix in an ordinary pipette tip. Commercially available matrix of poly-tetrafluoroethylene (PTFE, Teflon) containing reversed-phase octadecyl-bonded silica phase (C18) or poly(styrene-divinylbenzene) (SDB) copolymer modified with sulfonic acid groups to make it more hydrophilic (SDB-RPS Disk) was normally used. Alternatively, two ion-exchange sorbents including sulfonic acid as cation exchanger (Cation-SR Disk), or quarternary ammonium groups as anion exchanger (Anion-SR Disk), were also employed. The procedure shown in Supplement 1, was described by Rappsilber et al. (2003; 2007). Small disks (approximately 1.0 mm diameter, 0.5 mm thickness) were cut out manually from the Empore™ High Performance Extraction Disk placed on a clean surface (Petri dish) using a hollow tool cutter (blunt-ended syringe needle). The cutter was gently pressed into the Empore disk and the material penetrated to the inside of the needle. Subsequently, the cutter was placed inside a pipette tip (disposable GELoader® Tip, 100 µl, from Eppendorf). The small disk was then released using a plunger (rod) that fitted into the needle (both parts from Hamilton Company, Reno, NV, USA) and pressed gently repeatedly into place using the weight of the plunger. After removing the cutter and plunger, the single-StageTip was finished. Additional disks were added the same way to produce combined multi-StageTips.

2.3.3 Sample purification using StageTips

The Empore sorbents were tested individually (C18, SDB-RPS, and Cation-SR) or in combination (C18/SDB-RPS, C18/Cation-SR, C18/SDB-RPS/Cation-SR). All solutions were loaded from the top of the tip in a volume of 50-100 µl using a pipette. The prepared StageTip was inserted into a hole at the centre of the lid of the microcentrifuge tube (1.5 ml) and placed in a centrifuge after solvent pipetting (Figure 1). The liquid was forced through the column by centrifugation of the StageTip at a rate and time depending on the solvent viscosity and buffer volume.

Prior to loading the sample the StageTip sorbents were activated with 50 µl isopropanol (by centrifugation at 3,500 rpm, 10 min, 4 ºC) and equilibrated with 50 µl 50% MeOH + 2% FA (2,000 rpm, 15 min, 4 ºC) and 50 µl Bieleski solvent (2,000 rpm, 15 min). Afterwards, the samples were loaded in extraction buffer (30 min, 1,500 rpm, 4 ºC). The tips were washed using 50 µl methanol (15 min, 2,000 rpm, 4 ºC) and elution of samples was performed with 50 µl of 0.5M NH_4OH in 60% MeOH (30 min, 1,500 rpm, 4 ºC). Eluates were collected into new clean microcentrifuge tubes and directly mixed with scintillation buffer prior to measurement of radioactivity or evaporated to dryness in a Speed-Vac concentrator RC1010 (Jouan, Winchester, UK) and dissolved in 20 µl of mobile phase prior to UPLC-MS/MS analyses.

2.4 Instrumentation

The radioactivity of tritium-labelled cytokinin standards was measured after addition of 1 ml liquid scintillation cocktail Ultima Gold™ on a multi-purpose scintillation counter LS 6500 (Beckman Coulter, Brea, CA, USA). An Acquity UPLC® System (Waters, Milford, MA, USA), including a Binary solvent manager and Sample manager was linked simultaneously to a 2996 PDA detector (Waters) and a triple quadrupole mass spectrometer Xevo™ TQ MS (Waters MS

Technologies, Manchester, UK) equipped with an electrospray interface (ESI) and the unique performance of a collision cell (ScanWave™). This hyphenated technique was used for analysis of unlabelled and stable isotope-labelled cytokinins. All MS data were processed by MassLynx™ software with TargetLynx™ program (version 4.2., Waters, Milford, MA, USA).

2.5 UPLC-ESI(+)-MS/MS conditions

Cytokinin derivatives were quantified according to the modified method described by Novák et al. (2008). The samples pre-purified on StageTips were dissolved in 20 µl of 10% methanol and 10 µl of each sample was injected onto a reversed-phase column (Acquity UPLC® BEH C18, 1.7 µm, 2.1 × 150 mm, Waters). The samples were eluted with a 24-min gradient composed of methanol (A) and 15 mM ammonium formate pH 4.0 (B) at a flow rate of 0.25 ml min^{-1}, column temperature of 40 ºC and sample temperature of 4 ºC. The following binary gradient was used: 0 min, 5:95 (A:B) – 7.0 min isocratic elution, 5:95 (A:B) – 9.0 min linear gradient, 20:80 (A:B) – 7.0 min linear gradient, 50:50 (A:B) – 1 min isocratic elution, 50:50 (A:B). At the end of the gradient the column was washed with 100% methanol and re-equilibrated to initial conditions (6 min). The effluent was passed through an ultraviolet-diode array detector (scanning range 200–400 nm, resolution 1.2 nm, sampling rate 5 points s^{-1}) and the tandem mass spectrometer Xevo TQ MS without post-column splitting. Selective multiple reaction monitoring (MRM) mode using mass-to-charge (m/z) transitions of precursor and product ions was performed under MS conditions optimized earlier (Novák et al., 2008) with few modifications: capillary voltage 0.35 kV; source/desolvation gas temperature 120/575 ºC; cone/desolvation gas flow rates 70/1000 l hr^{-1}; collision gas (argon) pressure/flow 5 10^{-3} mbar/0.2 ml min^{-1}; LM/HM resolution 2.8/15.0; ion energy 1/2 1.0 V; entrance/exit voltages 0.5 V, respectively. The MS/MS parameters like dwell time (automatic mode for 16 scan points per peak), cone voltage (21-35 V) and collision energy (15-24 eV) were selected to maximize the sensitivity of exact diagnostic transitions. Quantification was conducted using TargetLynx™ programs and a standard isotope dilution method. The calibration curves ranged 100 amol to 100 pmol were constructed by serial dilutions of the CK standards listed in Table 1 and the known concentration of the appropriate internal labelled standards. The ratio of endogenous cytokinin to the appropriate labelled standard was determined and further used to quantify the level of endogenous compounds in the original extract, according to a known quantity of an added internal standard (Novák et al., 2003).

2.6 Validation of the analytical procedure

To test the selectivity, affinity, and capacity of different StageTip sorbent combinations, samples containing ^{3}H-labelled CK standards ([^{3}H]*c*Z, ^{3}H]*t*ZR, [^{3}H]iPR) were used. Firstly, to characterize the potential sorbents (C18, SDB-RPS, and Cation-SR), the single-StageTip experiments were performed by pipetting 50 µl of the selected ^{3}H-CK standard (10^{5} dpm) in Bieleski buffer directly onto a PT-SPE column. Secondly, different combinations of sorbents (multi-StageTips) were tested using the same spiked Bieleski buffer with and without plant matrices (1.0 and 2.0 mg FW) to monitor the loading capacity and extraction recovery. For all samples, the yields of ^{3}H-CKs after passage through StageTips filled with different sorbents and their combinations were determined.

Thirdly, non-spiked and spiked (with 1 pmol of unlabelled CK standards) samples of 10-day old *Arabidopsis thaliana* seedlings (1-5 mg FW) were used to verify the reproducibility, sensitivity and accuracy of the method. Aliquots of the extracts were processed using the developed purification procedure (Figure 1), then analyzed by UPLC-ESI(+)-MS/MS. The recovery of added authentic cytokinin standards were then determined from each series of extracts, based on the amounts of endogenous compounds, calculated from non-spiked samples, which subsequently served as reference levels. Finally, the endogenous cytokinin levels were determined in samples of *A. thaliana* seedlings, roots and shoots (1.0, 2.0 and 5.0 mg FW of each sample type).

3. Results and discussion

3.1 Development of StageTip purification method

The optimization of homogenization, extraction and purification, critical and rate-limiting steps in the isolation of natural compounds, is particularly important for small sample sizes, where losses and cross-contamination must be reduced to a minimum. Self-made StageTips represent a miniaturization of the SPE, which was previously described as an extremely economical sample enrichment and pre-fractionation in proteomics (Ishihama et al., 2006; Saito et al., 2006). Here, we employed the PT-SPE method for the first time in a phytohormone analysis. The presented micropurification protocol (Figure 1) is based on the same principles as the SPE methods previously described for cytokinin purification (Novák et al., 2003; 2008). As well as the decreased amount of the SPE sorbent in the pipette tip, the solvent volumes required were significantly lowered and the sample size was reduced to only 1-5 mg fresh weight of plant material. Hence, homogenization and extraction with Bieleski buffer was done in one microcentrifuge tube and accelerated by crushing the plant material in a vibration mill as described by Novák et al. (2008). The aliquots extracted from 1 mg of fresh plant material in 50 µl of extraction buffer were immediately purified using the StageTips.

In SPE, results depend strongly on the physical-chemical properties of the analytes, as well as on the kind of sample matrix and the choice of sorbent which, can control parameters such as selectivity, affinity and capacity (Nováková et al., 2009). The characteristics of different sorbents can be determined by measuring the recovery of labelled standards, a mixture of which is added to the sample during the purification procedure. For this purpose, we chose the tritium-labelled cytokinins ([^{3}H]*c*Z, [^{3}H]*t*ZR, or [^{3}H]iPR) representing compounds with different polarities and retention times under our chromatographic conditions (Figure 3a). The charge on cytokinin molecules may be positive or negative depending on the pH (changes from pH<3 to pH>11 result in the charge becoming progressively more negative). Moreover, the presence of an N^6-side chain adds hydrophobicity to molecules such as adenine (Dobrev et al., 2002). In the highly acidic extraction mixture, cytokinins should be predominantly presented as cations, and they should be completely retained on a cation-exchange sorbent from which they can be eluted with an alkaline solvent. The presence of an organic constituent in Bieleski buffer (60% MeOH) reduces the ability of non-polar compounds to be retained on a reversed-phase sorbent. These theoretical expectations were confirmed by a test using single-StageTips filled with different sorbents (C18, SDB-RPS, and

Cation-SR). The recoveries of added ^{3}H-CK standards (10^{5} dpm) in Bieleski buffer applied onto microcolumns are summarized in Table 2. In agreement with our previous findings, the most hydrophobic cytokinin tested, iPR, was not retained at all on C18 sorbent following the direct application of 50 µl acidic extract. This illustrates that higher concentrations of organic solvent (in combination with acidic conditions) may influence the selectivity of octadecyl-bonded silica Empore Disks. A high loading capacity was observed while using the second reversed-phase sorbent (SDB-RPS) based on poly(styrene-divinylbenzene) copolymer, which retains analytes by different mechanisms such as hydrophilic, hydrophobic and π-π interactions (Qureshi et al., 2011). Moreover, the extraction recoveries were comparable to the cation-exchange sorbent (Table 2).

Due to our presumption of the strong matrix effect and the possibility of easy preparation of various multi-StageTips, we also tested the combination of reversed-phase and/or cation-exchange sorbents, C18/SDB-RPS, C18/Cation-SR, C18/SDB-RPS/Cation-SR, respectively. The PT-SPE purification procedure was optimized using two types of sample in quadruplicate: (i) samples containing one of the selected ^{3}H-labelled standards (10^{5} dpm) in extraction buffer; and (ii) *A. thaliana* seedling extracts prepared from 1.0 and 2.0 mg FW with added ^{3}H-labelled cytokinin standards. For both sample types, the recoveries of ^{3}H-CKs were determined (Figure 2). By utilizing different types of stationary phase, cations as well as hydrophobic compounds should be retained in accordance with results published by Dobrev et al. (2002) who developed a method for separation and purification of cytokinins from auxin and abscisic acid using commercially available mixed-mode SPE columns. As demonstrated in Figure 2, more cytokinin standards were recovered from the samples without added plant extract. The presence of plant matrix in the sample caused slightly decreased yields of the added CK standards; however the values were still close to 80%. The effect of plant matrix was also obvious from the correlation between the increasing sample weight and decreasing recovery values. Under our experimental conditions, all tested tritium-labelled standards were eluted in the first elution step by 50 µl of 0.5M NH_4OH in 60% MeOH. For instance, the combination of C18/SDB-RPS sorbents used for the purification of spiked 1 and 2 mg plant extracts recovered 68 ± 2 % and 36 ± 1% for [^{3}H]*c*Z, 13 ± 1% and 5 ± 1% for [^{3}H]*t*ZR**,** 78 ± 4% and 70 ± 2% for [^{3}H]iPR, respectively (Figure 2a). Despite our reasoning and previous experience (Novák et al., 2003; 2008), the replacement of a polymer based sorbent (SDB-RPS) by cation-exchange phase (Cation-SR) in combination with plant matrices did not greatly improve the selectivity and retention of cytokinins on multi-StageTips (Figure 2b). Hence, the combination of all three sorbent types (C18/SDB-RPS/Cation-SR) in one multi-StageTip was examined and final recoveries for each of the tested ^{3}H-CK showed the highest efficiency in the purification procedure (Figure 2c). Moreover, radioactivity of tritium-labelled CKs was measured continuously at different steps in the tested purification protocols (load, wash and elution) to monitor the loading capacity and extraction recovery (Figure 2d). For the definite StageTip configuration, the high selectivity and acceptable retention of all analytes were demonstrated by minute losses in the methanolic wash and second elution step, respectively. However, the losses of all ^{3}H-CK standards in loading step depend on increasing sample amount and are in the range 7-31% and 21-53% of tritium-labelled CKs for 1.0 and 2.0 mg FW, respectively (see Figure 2d; Supplement 2). The results confirmed that the triple combination of sorbents in StageTips was the best tool in the new miniaturized one-step purification procedure. In conclusion, a total extraction recovery higher than

80% and reproducibility with an RSD of 10% (or less) was in good agreement with previously published data using PT-SPE for analysis of drugs in different biological matrices (van Hout et al., 2001; Kumazawa et al., 2007).

3.2 Development of the UPLC-ESI(+)-MS/MS method

Introduction of ultra-high pressure liquid chromatography (UHPLC) enabled high-speed analyses of an increased number of analytes with high efficiency compared to the previously used high performance liquid chromatography (HPLC: Nováková et al., 2006). Using UHPLC, the run time for the analysis of a mixture of cytokinin bases, ribosides and 9-glucosides was reduced 4-times. The analysis of *O*-glucosides and nucleotides without the need for enzymatic treatment was possible with help of BEH column sorbent technology (Novák et al., 2008). A further improvement of the currently used methods described in this work was the direct estimation of all isoprenoid cytokinin metabolites (zeatin-, dihydrozeatin-, and isopentenyladenine-types) during a single UPLC-MS/MS analysis, without the necessity of enzyme treatment or separate analysis of *O*-glucosides and nucleotides. On the other hand, development of the new procedure, which includes only one-step high-throughput solid-phase extraction, also required the selection of appropriate analytical conditions for the separation of *cis*- and *trans*-zeatin isomers, their ribosides and nucleotides as well as *N*- and *O*-glycosides with identical precursor and basic product ions (Table 3). Other critical factors influence the accuracy and precision of quantitative MS detection. For instance, a signal for DHZ-type cytokinins in the mass spectra is still at least partially obscured by the second natural isotope (M+2) of *cis/trans*-zeatin derivatives. This phenomenon negatively affects the correct estimate of the natural cytokinins in different types of plant material with higher levels of zeatin metabolites and must be taken in account during the final calculation (Novák et al., 2003). Furthermore, the complex plant matrix, and presence of unidentified peaks in the individual mass chromatograms complicate the final high selectivity of tandem MS detection (van Rhijn et al., 2001). The effectiveness of the IAC to minimize the occurrence of interfering substances during isolation of CKs from plant tissue extracts has been described in previous studies (Novák et al., 2003; Hauserová et al., 2005). Due to simplification of the purification procedure, the IAC step was removed from the established purification protocol and replaced with the advantages of the UPLC-MS/MS technique, namely the higher separation efficiency and resolution of a UPLC system together with maximal selectivity and sensitivity of a tandem MS instrument (Figure 3).

The chromatographic conditions using methanol/15mM HCOOH (pH 4.0, adjusted by NH_4OH) for a fast cytokinin analysis were modified here to obtain the baseline separation of 25 isoprenoid N^6-adenine isomers onto reversed-phase column (Table 3). As described by Van Deemter equations, the efficiency of the chromatographic process is proportional to a decrease in particle size (Nováková et al., 2006). Moreover, a change in column length can also increase the resolution of the critical pair of isomer peaks. We have tested three different BEH C18 column lengths, viz. 50 mm, 100 mm, and 150 mm. The shorter column was unable to resolve all the compounds of interest, especially the closely eluted pairs such as *c*Z9G/*t*ZOG, *c*Z/*t*ZROG, and DHZ/DHZOG (data not shown). The 150-mm long Acquity UPLC BEH C18 column provided an adequate separation of isoprenoid CK derivatives as well as aromatic cytokinins (benzyladenine-, topolin-,

and kinetin-types), Figure 3. With the optimized isocratic steps and programme of gradients, we obtained a separation with acceptable resolution for the twenty-five *cis/trans*-zeatin, dihydrozeatin, and isopentenyladenine standards, including baseline separation of all CK isomers with identical MRM transitions (Figure 3a). For example, glycosylated zeatin-types (*t*Z7G, *t*Z9G, *t*ZOG, *c*Z9G, and *c*ZOG) were subsequently eluted with different retention times (12.26 ± 0.02, 14.23 ± 0.02, 15.13 ± 0.02, 14.83 ± 0.03, 15.79 ± 0.03 min), Table 3.

The higher efficiency of applied BEH technology using a long UPLC reversed-phase column was also demonstrated by the separation of racemic (±)-dihydrozeatin derivatives. Moreover, the (–)-antipode was isolated as a natural cytokinin from immature seeds of *Lupinus luteus* (Koshimiz et al., 1967). The different cytokinin activity of enantiomers of unnatural (R)- and naturally-occurring (S)- configurations in dihydrozeatin and their ribosides was reported by Matsubara et al. (1977). Compared with a previously published LC method (Witters et al., 1999), N_7-substituted glucosides, (±)-DHZ7G, were baseline separated into two peaks with retention times of 13.80 ± 0.02 and 14.13 ± 0.02 min respectively (Figure 3a). Moreover, (±)-DHZOG and (±)-DHZROG were partially separated in 16.5 and 19.2 min, respectively, while (±)-DHZ, (±)-DHZR, and (±)-DHZ9G were still eluted as identical peaks. Possibly, changes in the orientation of the side chain resulting from *N*- or *O*-glycosyl substitution of the dihydrozeatins might interact differently with the stationary phase by the regular non-polar interactions with the aliphatic chains under the chromatographic conditions used. In *Arabidopsis* extracts, DHZ7G was identified as the peak with the highest intensity using the selective MRM transition (384.2>222.1) and comparing the retention time (13.78 ± 0.03 min) with injected standard (13.80 ± 0.02) under the same chromatographic conditions (Supplement 3). In accordance with our results and the published natural occurrence of (S)-(–)-dihydrozeatins, we proposed that the first separated peak was (–)-DHZ7G with (S)-configuration. Moreover, we also confirmed the similar chromatographic behaviour for the other DHZ-*O*-glucoside derivatives. Finally, we compared the high resolution of the LC method with retention of the aromatic CK group, which is characterized by its higher hydrophobicity (Figure 3b). Under the optimized conditions, all bases, ribosides and 9-glucosides of *ortho*-, *meta*-, and *para*-topolins, as well as benzyladenine and kinetin with corresponding derivatives, were baseline separated. Although the final separation of seventeen aromatic cytokinins was not the main purpose of this work, the method we decribe here could be adapted for their analysis.

An additional issue which can further complicate the separation is that protonated CK metabolites ($[M+H]^+$) produce a typical fragmentation pattern after collision induced dissociation, characterised by the loss of the nucleosyl/glycosyl/nucleotyl group and the fragmentation of the N^6-substituent for the aglycons (Table 3). Moreover, since an in-source fragmentation of ribosides occurs, the signal of the aglycone is obscured by the signal of its riboside in cases where they co-elute (Tarkowski et al., 2010). In agreement with this observation, analogous in-source dissociations were observed for all CK derivatives. Therefore a separation with higher peak resolution was preferred to obtain accurate endogenous levels of free bases, nucleosides (ribosides), glycosides (*N*- and *O*-glucosides) and nucleotides. Taking all the above into account, the total run time of the UPLC method, including equilibration, was 30 min and might be acceptable for a routine single chromatographic analysis of 25 isoprenoid and 17 aromatic cytokinins in biological samples (Figure 3). Under these compromise conditions, the retention times for the monitored

compounds ranged from 12.0 to 24.5 min. Based on the excellent stability of the retention times, which ranged between 0.04% and 0.24% RSD (Table 3), the chromatographic run was split into five retention windows (11.0-13.0, 13.0-17.7, 17.7-20.0, 20.0-22.0, 22.0-24.5 min) which increased the sensitivity of the subsequent ESI-MS/MS measurements. Electrospray capillary and cone voltages were subsequently optimized to generate the required precursor ions in the positive ion mode and the collision energy was tuned to dissociate and produce characteristic fragments for each cytokinin. All precursor and product ions of unlabelled and labelled cytokinin types determined by UPLC-ESI(+)-MS/MS as well as cone voltages (21-35 V) and collision energies (15-24 eV) corresponded with the previously published data (Novák et al., 2008).

Further improvement of our developed MS method might be achieved by utilization of Scanwave collision cell technology, which significantly enhances the signal intensity of full scan product ion spectra. In MRM mode, it acts as a conventional collision cell whereas in the second mode, called PIC (product ion confirmation), the front of the cell is used to fragment and accumulate ions whilst the rear of the cell mass selectively delivers ions to the second resolving quadrupole (Kenny et al., 2010). This approach allows rapid switching between both modes of operation, hence a simultaneous quantification and a sensitive MS/MS full scan data collection for structural confirmation are enabled (Figure 3d,e). Undoubtedly, use of the PIC mode amplifies the accuracy and precision of trace component analysis in a complex plant matrix. Therefore we have examined the sensitivity and usability of the PIC mode for quantitative analysis of cytokinin derivatives. Under applied MS conditions, the lowest measurable concentrations were close to 10 fmol for most of the cytokinins analyzed (Figure 3d,e). However, in this paper, the qualified cytokinin levels mainly varied between 0.1 and 10 fmol mg^{-1} FW (Table 5) and the PIC scan could be used only for *tZ*7G and iP7G identification (data not shown). For these reasons, the usefulness of the PIC scanning mode is limited by amount of naturally occurring cytokinins in extracted samples.

3.3 Validation and application of the method

As mentioned above the purification steps in phytohormone analysis are the most important and most time-consuming parts of the sample preparation procedure. Extraction from minute amounts of plant tissue substantially reduces the matrix effect and minimizes the content of sample impurities. On the other hand, final quantities of analytes present in the sample are negatively influenced. Therefore both parts of the developed analytical approach (isolation and quantification procedures) have to be equiponderant. In the present study, the whole method was tested to examine the influence of the miniaturized one-step purification on subsequent UPLC-ESI(+)-MS/MS analysis, again using the 10-day-old Arabidopsis seedlings.

In the first instance, for the improved UPLC-ESI(+)-MS/MS method, the basic validation characteristics were calculated after repeatedly injecting solutions with varied concentrations of each unlabelled analyte and fixed concentrations of the corresponding IS. Limits of detection (LOD) and quantification (LOQ), based on 3:1 and 10:1 signal to noise ratios, ranged between 50 amol – 1 fmol and 100 amol – 5 fmol for all of the investigated analytes, respectively (Table 3). In accordance with our published fast UPLC-MS/MS method, the lowest LODs were obtained for DHZR and iPR (Figure 3c) as well as for DHZ9G and iP7G, respectively. The highest LODs for

cytokinin nucleotides correlated with the reduction in ionization efficiency associated with presence of phosphate groups on adenosine moiety (Berés et al., 2010). The calibration curves constructed from 14 points were obtained from separate injections of standard mixtures and gave a broader linear range, spanning at least four orders of magnitude. For most cytokinins, the response was linear up to 10 pmol injected with correlation coefficients (R^2) varying from 0.9983 to 0.9994 (Table 3). The use of UPLC-ESI(+)-MS/MS method with Scanwave technology provided 5–25-fold greater sensitivity in MRM mode in comparison with previously published UPLC-MS/MS techniques (Novák et al., 2008; Kojima et al., 2009). The linearity of the method was also in a good agreement with that of other authors using mass spectrometry for cytokinin analysis (Prinsen et al., 1998; Novák et al., 2003; Nordström et al., 2004; Hussain et al., 2010).

For verification of the sample matrix effects, the efficiency of whole developed approach was evaluated by spiking *A. thaliana* seedling extract with authentic and stable isotope-labelled cytokinin standards, 1.0 and 0.5 pmol added respectively (Table 4). The capacity of the StageTip microcolumns to isolate cytokinins from plant extracts was tested by purification of three different amounts of fresh plant weight (1.0, 2.0 and 5.0 mg) in quadruplicates. Recoveries were calculated as a percentage of defined amounts added to the sample prior to the StageTip purification procedure (Figure 1). After the PT-SPE step followed by chromatographic separation and mass spectrometric quantitative measurement, recoveries of samples with plant matrices reached, on average, 77 ± 17%, 46 ± 17% and 10 ± 6% for 1.0, 2.0, and 5.0 mg FW, respectively (Table 4). These data are consistent with results obtained from the preliminary experiment with ^{3}H-labelled CK standards (Figure 2). The results showed the increasing amount of the applied sample tissues as the main limitation for using of the multi-StageTips as a high-throughput purification step. On the other hand, the yields for 1.0 mg indicated a 2-fold higher capacity of the SPE step than the previously published batch immunoextraction purification procedure, for which average values of 40% and 60% were found for isoprenoid and aromatic cytokinins, respectively (Novák et al., 2008). However, behaviour of the cytokinins during the PT-SPE procedure was also highly dependent on their structural modification (conjugation) (Table 4). This could be related to combination of two different mechanisms, the reversed-phase and cation-exchange, which are responsible for the retention of cytokinins on mixed-mode sorbents (Dobrev et al., 2002). Independently on sample size, the highest recovery of CK-bases was obtained, followed by a lower yield of *N*-glucosides, *O*-glucosides, and ribosides, respectively. The lowest recovery was determined for CK-nucleotides which are relatively polar molecules with possibility to have one positive and one negative charge (Béres et al., 2010).

The precision and accuracy of the method, expressed as relative standard deviation (RSD) and as percentage bias, respectively, were also determined using acquired data from the non-spiked and spiked samples. The cytokinin analytes were quantified by standard isotope dilution analysis (Rittenberg et al., 1940), and subsequently the detected amounts of endogenous compounds were subtracted and the values were finally averaged for each compound (Table 4). The mean precision was 11.2% (range 4.3–18.1% RSD), and the mean accuracy was 3.0%, ranging from -18.2 to 17.6% bias. In general, the precision and accuracy tended to become worse when higher amounts of plant tissues were applied onto StageTips. Similar to our results published for UPLC-ESI-MS/MS analyses of the different phytohormone groups (Novák et. al., 2008; Turečková et al.

2009), the acquired data showed that the variations in recoveries, validated by the internal standardization procedure, facilitated the detection of natural cytokinins within ±20% of the true amounts. Hence, accuracy of the developed analytical approach is satisfactory for determining these trace components in a complex plant matrix (van Rhijn et al., 2001).

Finally, the newly developed and validated micropurification procedure, linked to highly selective and sensitive MS detection, was applied for cytokinin isolation and analysis from the model plant *A. thaliana*. Addition of stable isotope-labelled standards as internal tracers during the extraction procedure allowed calculation of the intrinsic levels of isoprenoid cytokinins in minute amounts of 10-day old *A. thaliana* seedlings, roots and shoots (1.0, 2.0, and 5.0 mg FW). Fifteen isoprenoid cytokinin metabolites were detected and quantified in a single unique UPLC-ESI(+)-MS/MS run after microextraction and StageTip purification of the samples prepared in quadruplicates. In Table 5, the data are summarized as average values obtained for each tissue, recalculated per gram of fresh plant material. The detailed results for different sample loads of the extracted *Arabidopsis* roots and shoots as well as whole seedlings are presented in Supplement 4. Non-significant variations were seen in the estimated levels of the analytes which were on average 2.1(±0.5)-fold and 5.7(±1.6)-fold higher for 2 and 5 mg samples, respectively, compared with CK amounts in 1 mg FW. The results corroborate the consequence of using specific labelled analogues as recovery markers for each hormone metabolite measured (Chiwocha et al., 2003). In summary, the measured amount of CKs for all tested plant tissues varied between 0.15 to 65.76 pmol g^{-1} FW. The wide spectrum of cytokinins obtained from the seedling samples was consistent with previously published profiles (Novák et al., 2008). A similar pattern was found in the shoots and roots in agreement with Jones et al. (2010). The ratio between *cis*- and *trans*-zeatin-type CKs was similar to previously reported levels in the youngest leaves of *A. thaliana* (Gajdošová et al., 2011). In agreement with the results published by Riefler et al. (2006) and Werner et al. (2010), we have found the iP-type and *trans*-Z-type metabolites as the major cytokinins, comprising ca. 50% and 40% of the total CK content in *A. thaliana* tissues, respectively. Conversely, the different proportion of *cis*-Z-type CKs were found in the roots compared with CK profile in shoot samples analyzed (Table 5). Furthermore, the 7-glucoside forms, iP7G and *t*Z7G, were the most abundant CK metabolites in whole 10-day old seedlings as well as in shoot and root parts of the studied plants, followed by *O*-glucoside and 9-glucoside forms, which were present at approximately 10-fold lower concentrations. In accordance with our findings, Gajdošová et al. (2011) noted the *N*- or *O*-glucosides as prevailing CK forms within more than 150 representative plant species analyzed. Moreover, the *Arabidopsis* roots contained higher (ca. 3-fold) levels of the bioactive CK forms (iP, *t*ZR, *c*ZR, DHZR, and iPR) than the shoots. Surprisingly, no endogenous CK nucleotides were detected in this experiment. This was caused by negative effect of low recovery of the PT-SPE step and higher LODs for these CK metabolites, as described above.

In addition, the results also showed that the MS signal acquired for each CK metabolite was proportional to the amount and the type of purified plant material (Figure 4). Both selected tissues, the root and shoot systems, represented the samples with different complexity, proportions of potentially interfering compounds, and concentrations of lipids and plant pigments. In relation to the increasing sample weight, the purification and analysis of the extracts resulted in more stable signal-to-noise ratio for the root extracts (Figure 4a,b,c) in comparison with decreasing signal intensity in

the shoot extracts (Figure 4d,e,f), respectively. This could be related to the higher presence of contaminants and impurities in some plant organs such as leaves, buds, stems, and flowers. Furthermore, the trends observed for recoveries of the internal standards added to the shoot samples (on average ca. 75% IS for 1.0 mg FW, 46% IS for 2.0 mg FW, and 16% IS for 5.0 mg FW recovered) agreed with our measured results for the spiked seedlings (Table 4). On the other hand, for *Arabidopsis* root extracts, the purification procedure yielded somewhat higher recoveries of ca. 98%, 58%, and 29% IS for 1.0 mg, 2.0 mg, and 5.0 mg FW, respectively. All data demonstrated that the effect of plant matrix grew rapidly with increasing amounts of plant tissues and showed the following progression among plant tissue types, seedlings > shoots >> roots. In conclusion, the advantages of using StageTip purification prior to MS analysis of CK derivatives, as well as other phytohormones, depend on the right choice of SPE sorbent used, in correlation with sample type, optimal amount of tissue extracted, and high sensitivity of the detection methods, respectively.

4. Conclusions

To our knowledge, this is the first time that a STop And Go Extraction Tip purification procedure has been described for the determination of biologically active compounds in plant extracts. The data presented in this study demonstrated that the most common problems in analysis of phytohormones, namely occurrence of the plant hormones in minute quantities together with large amounts of interfering compounds, can be solved by the application of a miniaturized purification method linked to the high separation efficiency and sensitivity of UPLC-MS/MS analysis. The combination of microextraction with one-step high-throughput solid-phase extraction in StageTips provides fast, effective and cheap sample purification prior to qualitative and quantitative measurements. The pipette tip SPE can be used not only for cytokinins, but after modification also for other phytohormones, depending on selectivity, affinity and capacity of the selected sorbents. The UPLC analysis presented in this work was developed to separate cytokinin metabolites, including CK bases, ribosides, *N*-glucosides, *O*-glucosides and nucleotides, in single chromatographic run in contrast to our previously published method. Furthermore, coupling with the fast scanning tandem mass spectrometer ensured sensitive and selective MRM detection of 42 cytokinins in 24.5 minutes. The applicability and reliability of this analytical approach was confirmed by method validation and successful analysis of 15 cytokinin metabolites in *A. thaliana* seedlings, roots and shoots (1-5 mg FW). Use of our procedure can allow the quantification of plant hormones in very limited amounts of material, and can be beneficial for samples such as root tips, meristems and embryos.

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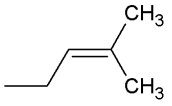
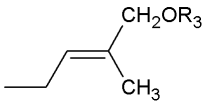
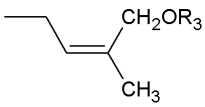
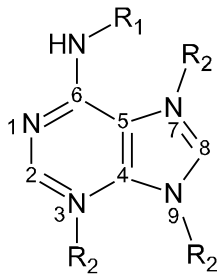
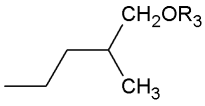
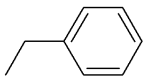
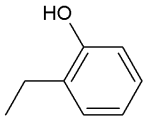
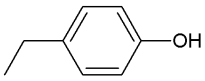
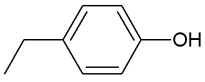
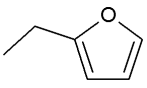
Tables, figures, supplements

Table 1. Structures, names and abbreviations of the studied cytokinins.

General structure of CKs	R_1	R_2	R_3	Compound	Abbreviation
		H	-	N^6-isopentenyladenine	iP
		N_9-R	-	N^6-isopentenyladenosine	iPR
		N_7-G	-	N^6-isopentenyladenine-7-glucoside	iP7G
		N_9-G	-	N^6-isopentenyladenine-9-glucoside	iP9G
		N_9-RP	-	N^6-isopentenyladenosine-5'-monophosphate	iPMP
		H	H	*trans*-zeatin	*t*Z
		N_9-R	H	*trans*-zeatin riboside	*t*ZR
		N_7-G	H	*trans*-zeatin-7-glucoside	*t*Z7G
		N_9-G	H	*trans*-zeatin-9-glucoside	*t*Z9G
		H	G	*trans*-zeatin *O*-glucoside	*t*ZOG
		N_9-R	G	*trans*-zeatin riboside *O*-glucoside	*t*ZROG
		N_9-RP	H	*trans*-zeatin riboside-5'-monophosphate	*t*ZMP
		H	H	*cis*-zeatin	*c*Z
		R	H	*cis*-zeatin riboside	*c*ZR
		N_9-G	H	*cis*-zeatin-9-glucoside	*c*Z9G
		H	G	*cis*-zeatin *O*-glucoside	*c*ZOG
		N_9-R	G	*cis*-zeatin riboside *O*-glucoside	*c*ZROG
		N_9-RP	H	*cis*-zeatin riboside-5'-monophosphate	*c*ZMP
		H	H	dihydrozeatin	DHZ
		N_9-R	H	dihydrozeatin riboside	DHZR
		N_9-G	H	dihydrozeatin-9-glucoside	DHZ9G
		H	G	dihydrozeatin *O*-glucoside	DHZOG
		N_9-R	G	dihydrozeatin riboside *O*-glucoside	DHZROG
		N_9-RP	H	dihydrozeatin riboside-5'-monophosphate	DHZMP
		H		N^6-benzyladenine	BA
		N_9-R	-	N^6-benzyladenosine	BAR
		N_3-G	-	N^6-benzyladenine-3-glucoside	BA3G
		N_7-G	-	N^6-benzyladenine-7-glucoside	BA7G
		N_9-G	-	N^6-benzyladenine-9-glucoside	BA9G
		N_9-RP		N^6-benzyladenosine-5'-monophosphate	BAMP
		H	-	*ortho*-topolin	*o*T
		N_9-R	-	*ortho*-topolin riboside	*o*TR
		N_9-G	-	*ortho*-topolin-9-glucoside	*o*T9G
		H	-	*meta*-topolin	*m*T
		N_9-R	-	*meta*-topolin riboside	*m*TR
		N_9-G	-	*meta*-topolin-9-glucoside	*m*T9G
		H	-	*para*-topolin	*p*T
		N_9-R	-	*para*-topolin riboside	*p*TR
		H	-	kinetin	K
		N_9-R	-	kinetin riboside	KR
		N_9-G	-	kinetin-9-glucoside	K9G

H = hydrogen, R = *β*-D-ribofuranosyl, G = *β*-D-glucopyranosyl, RP = *β*-D-ribofuranosyl-5'-monophosphate

Table 2 The loading capacity and extraction recovery of single-StageTip microcolumns. Recovery (%) of ^{3}H-labelled CK standards without added plant matrix.

	Recovery of ^{3}H-labelled CKs (%)					
	C18		SDB-RPS		Cation-SR	
	Load	Elution	Load	Elution	Load	Elution
[^{3}H]*c*Z	98.8 ± 3.3	0.6 ± 0.2	1.3 ± 0.1	96.6 ± 1.8	1.1 ± 0.1	100.7 ± 3.0
[^{3}H]*t*ZR	98.8 ± 4.4	0.6 ± 0.2	39.2 ± 2.2	29.8 ± 2.2	22.4 ± 0.6	64.7 ± 1.1
[^{3}H]iPR	102.9 ± 3.5	0.5 ± 0.3	4.9 ± 0.2	95.6 ± 1.4	4.7 ± 0.1	97.0 ± 2.8

Tritium-labelled standards (10^{5} dpm) were dissolved in Bieleski buffer and applied onto different Empore sorbents (C18, SDB-RPS, Cation-SR). Values are means ± SD (n = 4).

Table 3 List of precursor and product ions of the studied compounds (the most abundant product ion is shown in bold type). The retention time stability, limits of detection, dynamic linear range, linearity (correlation coefficients, R^2) are shown for UPLC-ESI(+)-MS/MS analysis of isoprenoid cytokinins.

Com-pounds	Pre-cursor	Products	Retention time[a] (min)	LOD[b] (fmol)	Dynamic range (mol)	R^2
t/cZ	220.1	**136.1**, 119.0	15.39 ± 0.04 / 16.82 ± 0.03	0.5	$1x10^{-15}$-$5x10^{-11}$	0.9989 / 0.9987
t/cZR	352.2	**220.1**, 136.1	19.06 ± 0.02 / 19.72 ± 0.01	0.1	$5x10^{-16}$-$5x10^{-11}$	0.9993 / 0.9986
tZ7G			12.26 ± 0.02	0.1	$5x10^{-16}$-$1x10^{-11}$	0.9989
t/cZ9G	382.2	**220.1**, 136.1	14.23 ± 0.02 / 15.13 ± 0.02	0.1	$5x10^{-16}$-$5x10^{-11}$	0.9993 / 0.9985
t/cZOG			14.83 ± 0.03 / 15.79 ± 0.03	0.5	$1x10^{-16}$-$1x10^{-11}$	0.9987 / 0.9988
t/cZROG	432.2	**382.2**, 220.1	18.08 ± 0.02 / 18.77 ± 0.02	1.0	$5x10^{-15}$-$1x10^{-11}$	0.9992 / 0.9984
t/cZMP	514.2	**220.1**, 136.1	13.72 ± 0.02 / 14.67 ± 0.02	5.0	$1x10^{-14}$-$5x10^{-11}$	0.9990 / 0.9985
DHZ	222.1	**136.1**, 119.0	16.15 ± 0.04	0.1	$5x10^{-16}$-$1x10^{-11}$	0.9991
DHZR	354.2	**222.1**, 136.1	19.61 ± 0.01	0.05	$1x10^{-16}$-$5x10^{-10}$	0.9989
DHZ7G			13.80 ± 0.02 / 14.13 ± 0.02	0.1	$5x10^{-16}$-$1x10^{-10}$	0.9994
DHZ9G	384.2	**222.1**, 136.1	15.00 ± 0.01	0.05	$1x10^{-16}$-$1x10^{-10}$	0.9992
DHZOG			16.37 ± 0.03	0.1	$5x10^{-16}$-$5x10^{-12}$	0.9992
DHZROG	434.2	**384.2**, 222.1	19.22 ± 0.03	1.0	$5x10^{-15}$-$1x10^{-11}$	0.9983
DHZMP	516.2	**222.1**, 136.1	14.34 ± 0.01	1.0	$5x10^{-15}$-$1x10^{-11}$	0.9992
iP	204.1	**136.1**, 119.0	23.21 ± 0.01	0.1	$5x10^{-16}$-$1x10^{-11}$	0.9991
iPR	336.2	**204.1**, 136.1	23.88 ± 0.01	0.05	$1x10^{-16}$-$1x10^{-11}$	0.9989
iP7G	366.2	**204.1**, 136.1	18.70 ± 0.01	0.05	$1x10^{-16}$-$1x10^{-11}$	0.9988
iP9G			21.50 ± 0.01	0.5	$1x10^{-15}$-$1x10^{-11}$	0.9992
iPMP	416.2	**204.1**, 136.1	21.31 ± 0.02	5.0	$1x10^{-14}$-$5x10^{-11}$	0.9993

[a] Values are means ± SD (n = 10). [b] Limit of detection, defined as a signal-to-noise ratio of 3:1.

Table 4. Method validation. Recovery (%) is shown for different amounts of plant matrix together with determinations of the analytical precision and accuracy of whole procedure.

CKs	Recovery (%)[a]			Determined spiked CK content(pmol)[b]	Method precision (% RSD)[b]	Method accuracy (% bias)[b]
	1 mg FW	2 mg FW	5 mg FW			
*t*Z	80 ± 10	63 ± 8	21 ± 4	0.99 ± 0.17	17.2	0.1
*t*ZR	72 ± 12	46 ± 7	8 ± 1	0.84 ± 0.09	10.4	16.1
*t*Z7G	88 ± 6	57 ± 4	6 ± 1	0.91 ± 0.13	14.6	8.6
*t*Z9G	59 ± 7	31 ± 3	8 ± 1	0.97 ± 0.09	9.6	2.5
*t*ZOG	85 ± 5	68 ± 6	11 ± 2	1.07 ± 0.19	18.1	-7.1
*t*ZROG	55 ± 4	30 ± 3	4 ± 1	0.82 ± 0.09	11.1	17.6
*t*ZMP	35 ± 6	11 ± 1	5 ± 1	0.85 ± 0.11	13.3	14.7
*c*Z	75 ± 9	65 ± 5	24 ± 4	0.83 ± 0.02	2.5	17.0
*c*ZR	81 ± 13	44 ± 9	8 ± 1	0.96 ± 0.12	12.8	4.1
*c*Z9G	74 ± 12	37 ± 5	5 ± 1	1.18 ± 0.13	11.3	-17.6
*c*ZOG	89 ± 6	66 ± 7	9 ± 2	1.09 ± 0.14	13.2	-9.1
*c*ZROG	52 ± 6	24 ± 2	3 ± 1	0.89 ± 0.11	12.9	11.2
*c*ZMP	32 ± 3	17 ± 1	2 ± 1	0.86 ± 0.15	17.9	13.9
DHZ	77 ± 13	61 ± 7	20 ± 3	0.90 ± 0.10	10.8	9.7
DHZR	88 ± 13	48 ± 8	12 ± 1	1.03 ± 0.06	5.6	-2.9
DHZ7G	89 ± 3	65 ± 3	8 ± 2	1.18 ± 0.05	4.3	-18.2
DHZ9G	78 ± 10	35 ± 6	6 ± 1	0.96 ± 0.07	7.2	3.7
DHZOG	77 ± 5	50 ± 7	9 ± 3	1.16 ± 0.06	4.9	-15.8
DHZROG	87 ± 8	42 ± 5	5 ± 1	0.90 ± 0.12	13.3	9.8
DHZMP	37 ± 1	12 ± 1	3 ± 1	0.96 ± 0.15	15.8	3.6
iP	76 ± 9	68 ± 3	26 ± 5	0.97 ± 0.06	5.9	3.3
iPR	84 ± 8	53 ± 4	17 ± 1	1.13 ± 0.06	5.6	-12.8
iP7G	83 ± 10	60 ± 5	7 ± 1	0.91 ± 0.15	16.5	9.4
iP9G	74 ± 8	49 ± 8	8 ± 2	0.97 ± 0.08	8.0	3.1
iPMP	78 ± 9	39 ± 9	9 ± 2	0.92 ± 0.16	17.0	8.4

Plant tissues (1-5 mg FW of *A. thaliana* seedlings spiked with 1 pmol of authentic CK standards) were extracted in Bieleski buffer, purified by multi-StageTip microcolumn chromatography and directly analysed by UPLC-ESI(+)-MS/MS. [a] Values are means ± SD (n = 4); [b] Values are means ± SD (n = 12).

Table 5. Cytokinin levels in Arabidopsis thaliana extracts determined by UPLC-ESI(+)MS/MS.

CKs	Cytokinin content (pmol g^{-1} FW)		
	Seedlings	**Shoots**	**Roots**
*t*Z	n.d.	n.d.	0.96 ± 0.12
*t*ZR	2.23 ± 0.37	0.88 ± 0.32	3.27 ± 0.52
*t*Z7G	37.99 ± 2.81	23.49 ± 3.07	6.54 ± 0.75
*t*Z9G	3.88 ± 1.22	4.32 ± 1.31	2.08 ± 0.52
*t*ZOG	9.25 ± 2.77	9.42 ± 1.75	7.10 ± 1.33
*c*ZR	0.80 ± 0.16	1.14 ± 0.37	3.71 ± 0.79
*c*Z9G	n.d.	n.d.	2.02 ± 0.47
*c*ZOG	0.90 ± 0.27	1.04 ± 0.33	2.89 ± 0.66
DHZR	0.64 ± 0.19	0.94 ± 0.27	0.97 ± 0.28
DHZ7G	5.48 ± 1.28	5.78 ± 1.51	2.07 ± 0.42
DHZ9G	n.d.	0.71 ± 0.22	0.29 ± 0.07
DHZOG	0.46 ± 0.13	0.28 ± 0.07	0.15 ± 0.05
iP	0.24 ± 0.10	0.15 ± 0.04	0.56 ± 0.17
iPR	1.96 ± 0.28	1.24 ± 0.26	2.18 ± 0.46
iP7G	53.87 ± 2.86	65.76 ± 12.47	30.16 ± 4.57

10-day-old *A. thaliana* seedlings, roots and shoots were extracted in Bieleski buffer, purified by multi-StageTip microcolumns and measured by UPLC-ESI(+)-MS/MS method. Values are meansf ± SD (n = 12); n.d. – not detected.

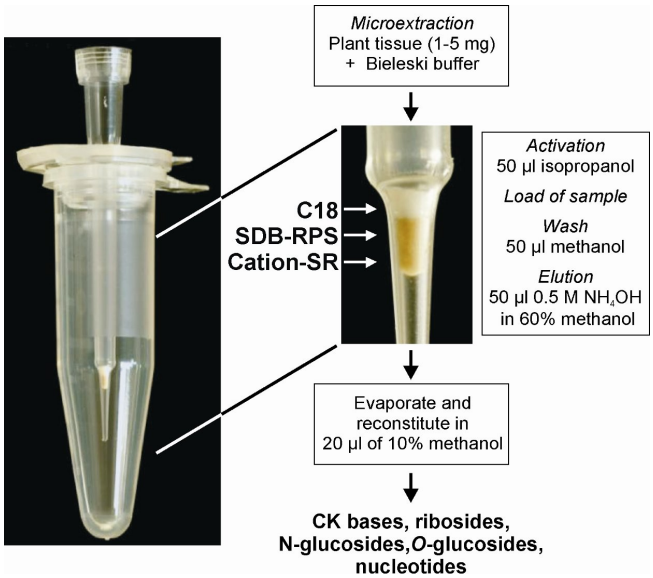


Figure 1. Purification protocol for cytokinins using multi-StageTips (STop And Go Extraction Tips), which combine three different sorbents (C18/SDB-RPS/Cation-SR), inserted into a microcentrifuge tube (1.5 ml). Plant material (1-5 mg) was homogenized and extracted in Bieleski buffer containing labelled internal standards. Pooled supernatant was applied (1 mg FW/50 µl) to a pre-conditioned multi-StageTip microcolumn, which was then washed and eluted with the indicated solutions. The eluate was evaporated to dryness, dissolved in 20 µl of 10% methanol and analyzed by UPLC-MS/MS method.

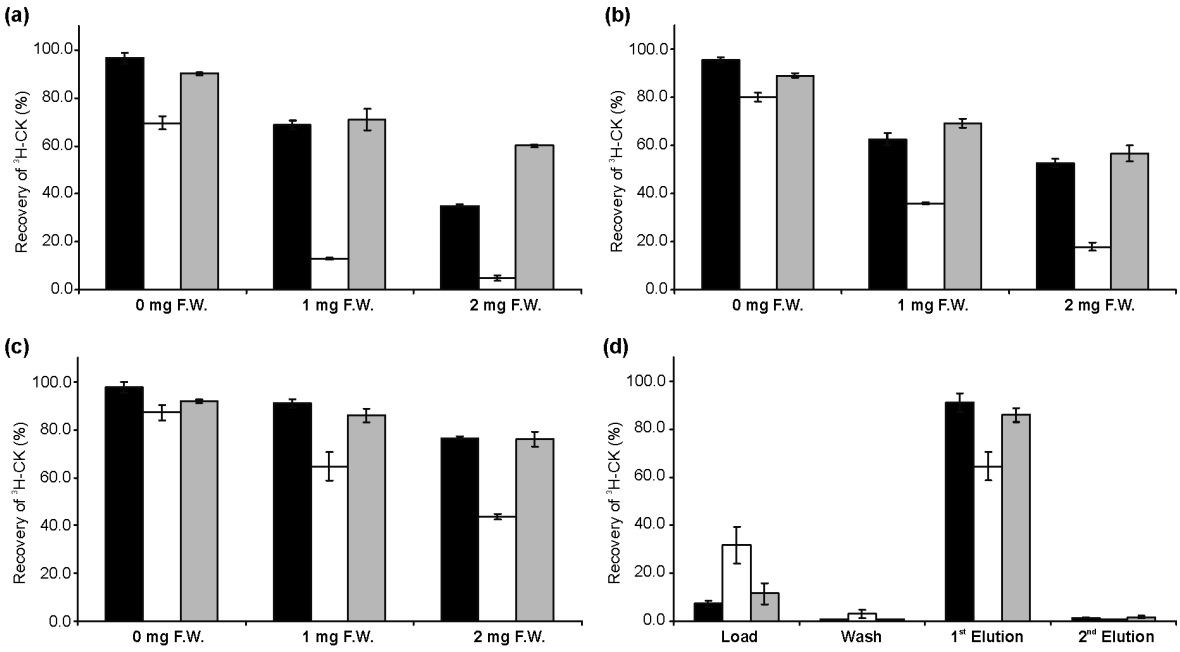


Figure 2. The loading capacity and extraction recovery of multi-StageTip microcolumns. (a-c) Recoveries (%) of ^{3}H-labelled CK standards in the first elution step (50 µl 0.5M NH_4OH in 60% MeOH) applied onto multi-StageTips using Empore sorbents in different combinations, C18/SDB-RPS (a), C18/Cation-SR (b), C18/SDB-RPS/Cation-SR (c) without (0 mg FW) and with plant matrices (1 or 2 mg FW). (d) Representative test of loading capacity and extraction recoveries at different steps during the purification protocol described in Figure 1 for 1 mg F.W of *A. thaliana* seedlings extracted. For all experiments, tritium-labelled standards (10^5 dpm) were dissolved and the extracts were prepared in Bieleski buffer. Values are means ± SD (n = 4); [^{3}H]*c*Z, black bars; [^{3}H]*t*ZR, white bars; [^{3}H]iP, grey bars.

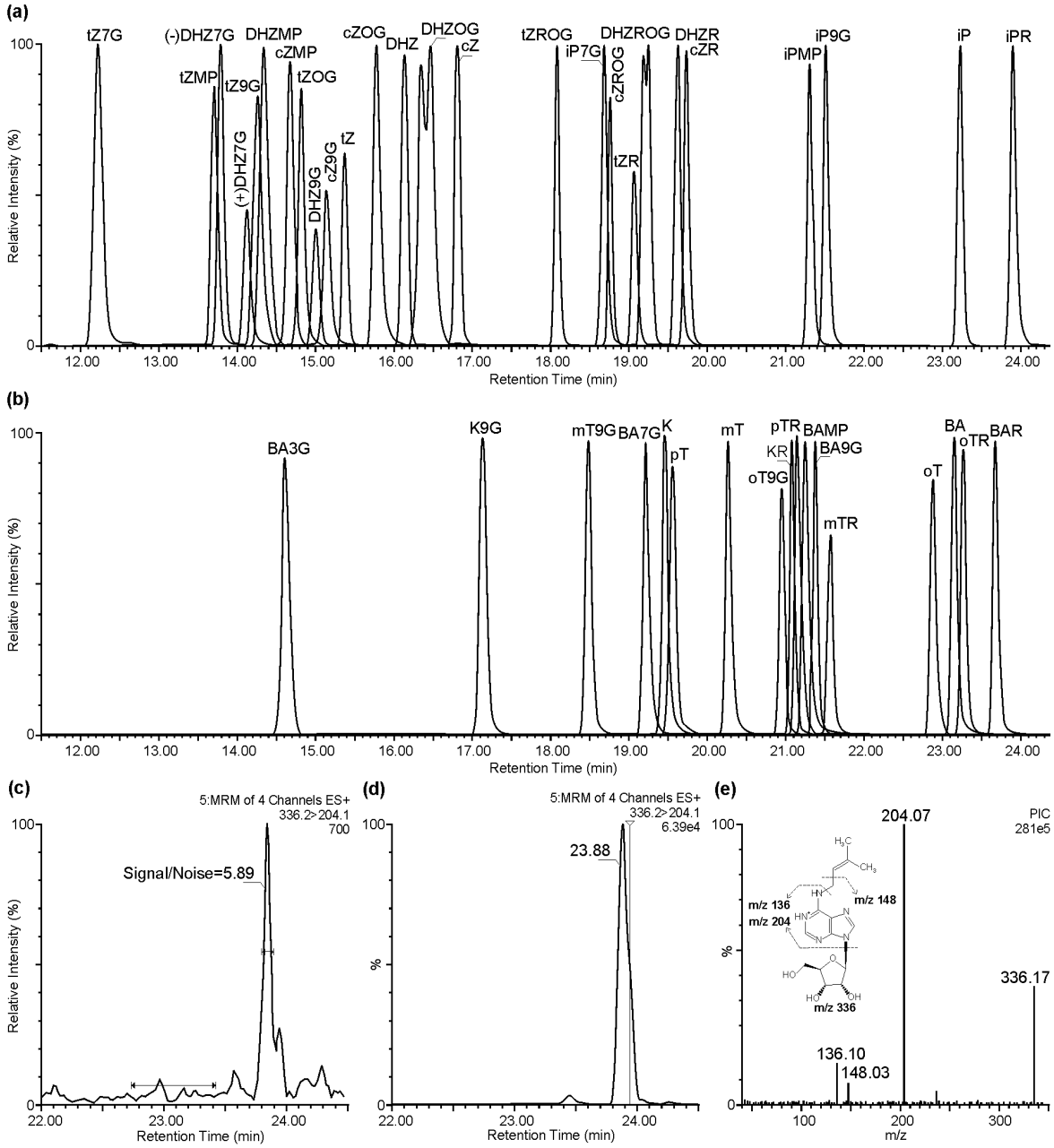


Figure 3 Separation of cytokinin standards by UPLC-ESI(+)-MS/MS method using an Acquity UPLC® BEH C18 2.1×150 mm column. (a,b) Multi-MRM chromatograms of 25 isoprenoid cytokinins (a) and 17 aromatic cytokinins (b) including bases, ribosides, *N*-/*O*-glucosides and nucleotides containing 1 pmol of each derivative per injection. (c) Limit of detection of iPR (50 amol injected onto column). (d,e) Representative product ion confirmation (PIC) experiment. The MRM chromatogram and full scan product ion spectrum were collected simultaneously for 10 fmol iPR injected onto column (switching between two MS scanning modes is marked by line in MRM window).

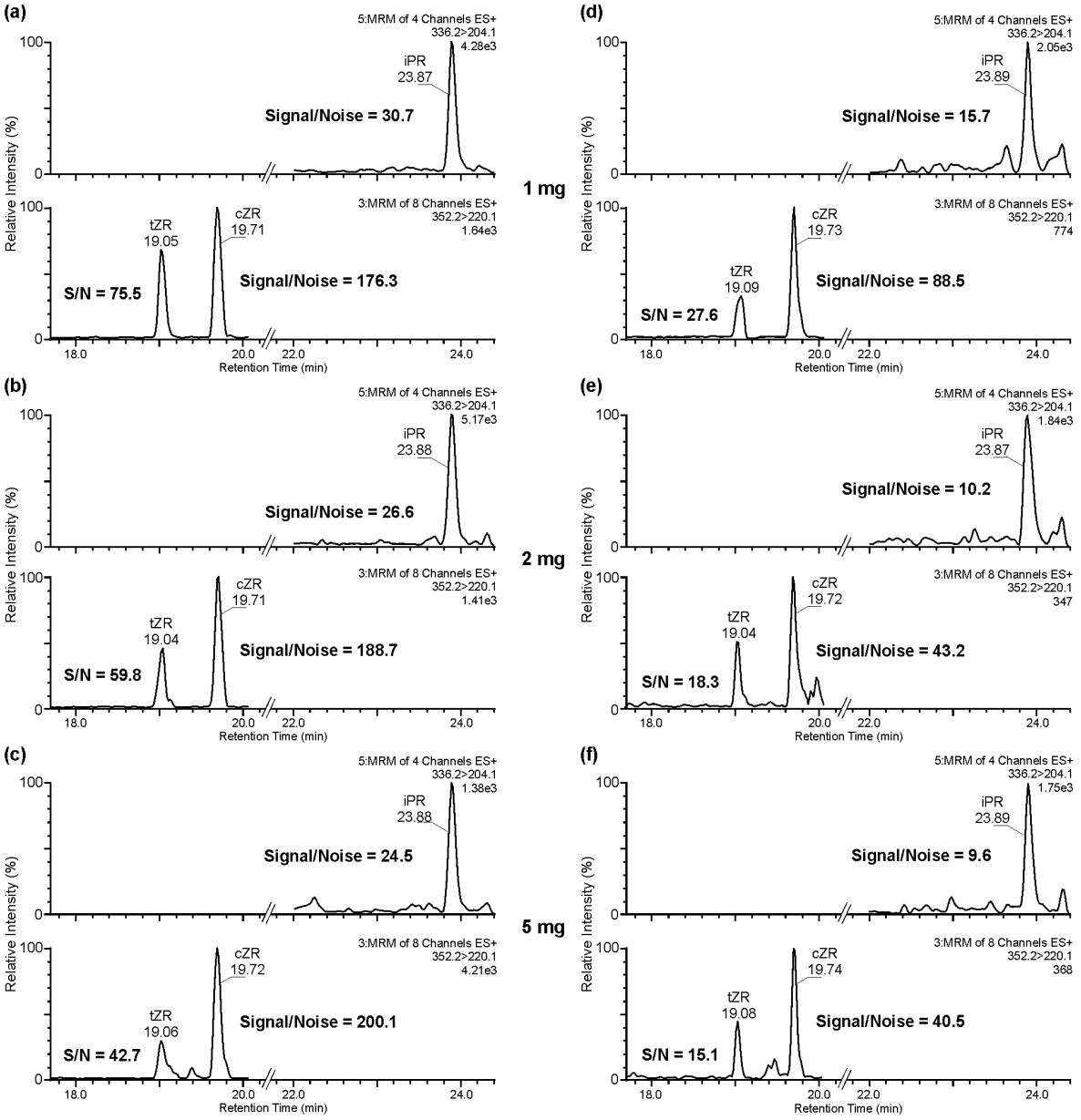
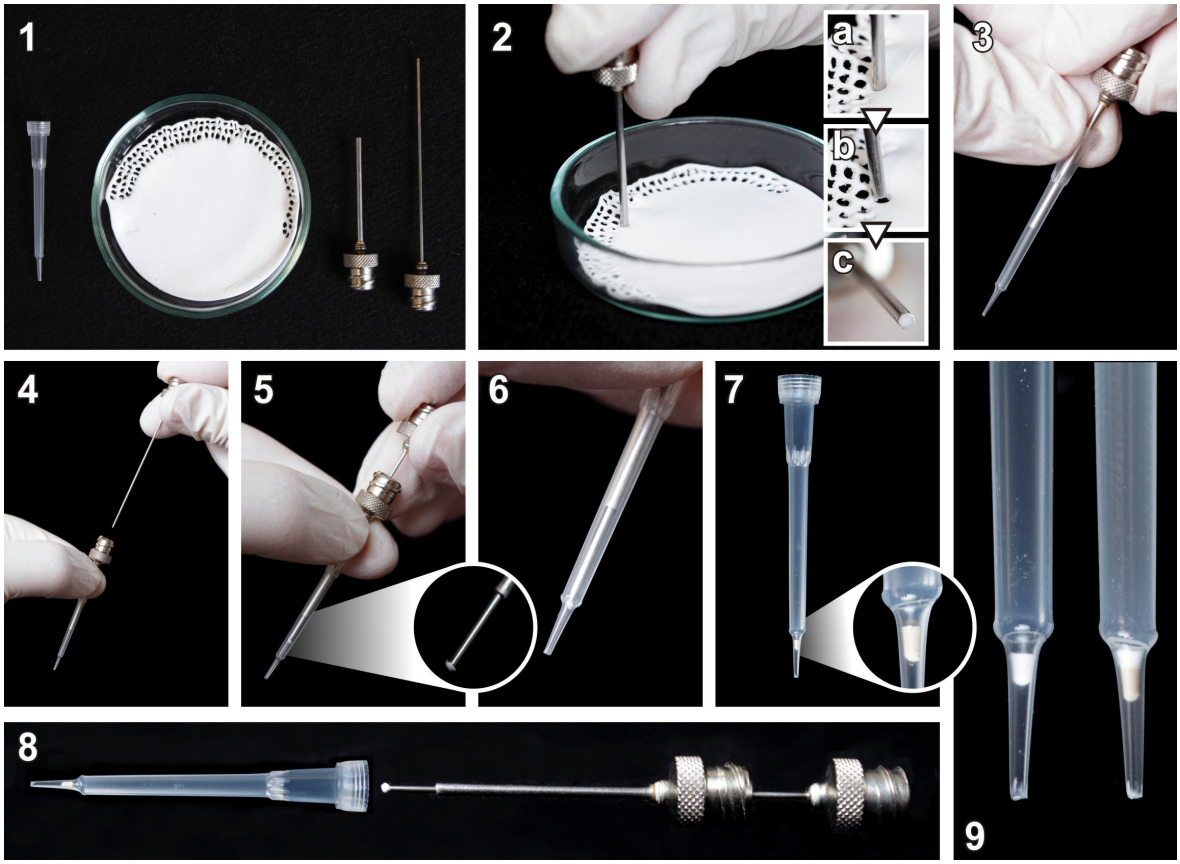
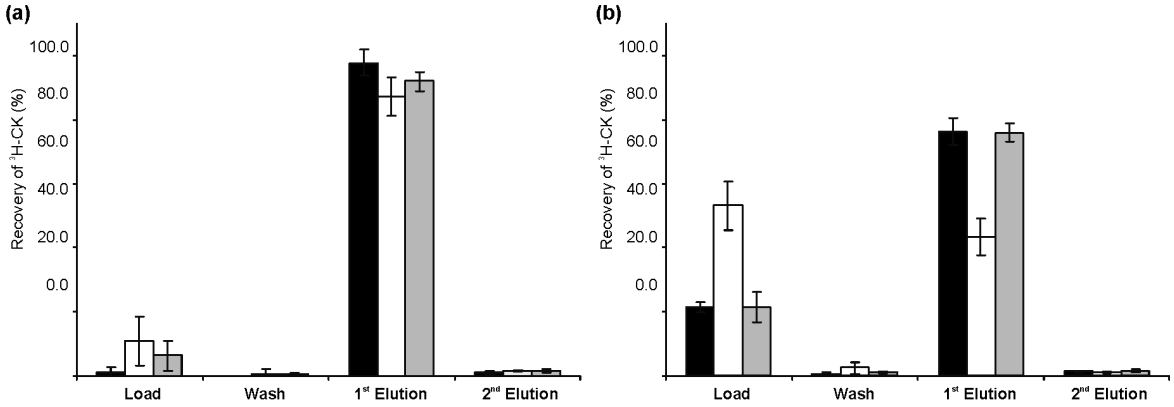


Figure 4 Matrix effects on selectivity and sensitivity of UPLC–ESI(+)-MS/MS method. MRM chromatograms of authentic *t*/*c*ZR and iPR show the change in signal sensitivity for different *A. thaliana* extracts, roots (a-c) and shoots (d-f), in minute amounts of plant tissue (1, 2, and 5 mg FW).

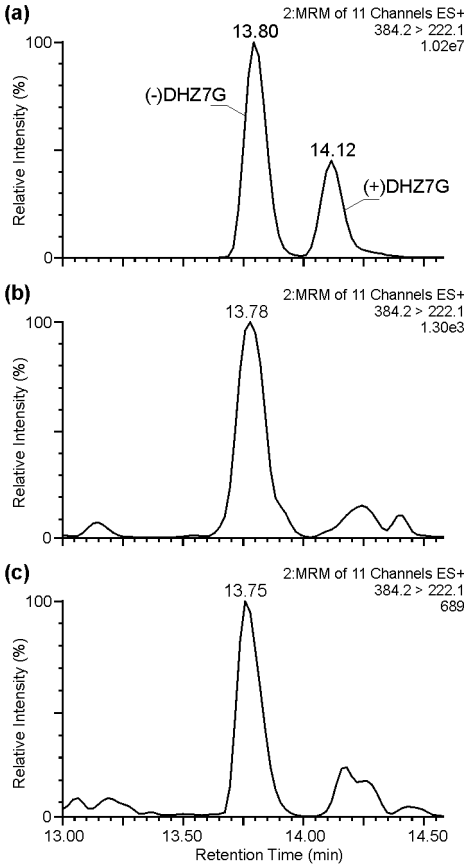
Supplement 1 Step-by-step guide on the manufacture of a StageTip. (1) The pipette tip, Empore™ High Performance Extraction Disk placed on a Petri dish, cutter (blunt-ended syringe needle) and plunger (rod needle); (2) cutting of the small disk (approximately 1.0 mm diameter, 0.5 mm thickness); (3-7) insertion of the disk into the pipette tip using the cutter and plunger fitted into the needle; (8) placing of additional disk onto the first disk; (9) single-StageTip (on the left) and multi-StageTips (on the right).



Supplement 2 The loading capacity and extraction recovery of multi-StageTip microcolumns (C18/SDB-RPS/Cation-SR) at different steps of purification protocol described in Figure 1. Tritium-labelled standards (10^5 dpm) were dissolved in Bieleski buffer and the extracts without (a; 0 mg FW) and with plant matrix (b; 2 mg FW) applied onto multi-StageTips. Values are means ± SD (n = 4); [^{3}H]*c*Z, black bars; [^{3}H]*t*ZR, white bars; [^{3}H]iP, grey bars.



Supplement 3 Identification of (–)-DHZ7G with (S)-configuration in *A. thaliana* extracts based on accordance with the peak retention time of reference standard mixure (a) and different plant extracts (b-c) injected onto an Acquity UPLC® BEH C18 2.1×150 mm column using the selective MRM transition (384.2>222.1). (a) Representative MRM chromatograms of racemic (±)-DHZ7G standard; (b) *Arabidopsis* shoot extract; (c) *Arabidopsis* root extract. The samples (2 mg F.W.) were extracted in Bieleski buffer, purified by StageTip protocol (Figure 1) and measured by UPLC-ESI(+)-MS/MS method under experimental conditions (Chapter 2.5).



Supplement 4 Levels of CK metabolites in varied amounts of *Arabidopsis thaliana* seedlings, roots and shoots. 10-day-old seedlings, roots and shoots were extracted in Bieleski buffer, purified by StageTip microcolumns and measured by UPLC-ESI(+)-MS/MS method. *t*/*c*Z, *t*/*c*ZROG, *t*/*c*ZMP, DHZ, DHZROG, DHZMP, iP9G, and iPMP were not detected in *Arabidopsis* extracts.

Weight (mg FW)	Cytokinin content (fmol in sample)														
	***t*Z**	***t*ZR**	***t*Z7G**	***t*Z9G**	***t*ZOG**	***c*ZR**	***c*Z9G**	***c*ZOG**	**DHZR**	**DHZ7G**	**DHZ9G**	**DHZOG**	**iP**	**iPR**	**iP7G**
Seedlings															
1	n.d.	n.d.	41.9±1.9	3.7±1.1	8.3±2.5	0.6±0.1	n.d.	1.0±0.3	n.d.	5.6±1.1	n.d.	0.5±0.1	n.d.	1.7±0.2	51.1±2.0
2	n.d.	3.4±0.4	70.2±9.0	8.2±2.6	18.7±5.0	1.5±0.9	n.d.	1.5±0.3	0.9±0.5	10.4±3.4	n.d.	0.8±0.2	n.d.	3.7±0.6	105.0±4.2
5	n.d.	13.9±2.9	184.8±10.0	n.d.	50.9±6.6	3.2±0.3	n.d.	5.1±2.0	3.5±1.2	28.3±5.4	n.d.	2.7±0.9	1.2±0.6	11.6±2.0	270.1±22.7
Shoots															
1	n.d.	0.8±0.3	26.8±2.8	4.5±1.5	8.1±2.1	1.1±0.4	n.d.	1.1±0.4	0.8±0.2	6.2±2.0	0.7±0.3	0.3±0.1	n.d.	1.1±0.2	58.0±11.5
2	n.d.	1.6±0.6	44.2±3.8	10.7±3.7	17.9±2.4	2.0±0.8	n.d.	1.7±0.5	1.9±0.7	10.4±1.9	1.7±0.6	0.6±0.2	n.d.	2.3±0.4	127.6±21.9
5	n.d.	5.5±1.7	107.9±22.8	15.5±2.8	55.9±9.9	4.5±1.5	n.d.	5.8±1.9	5.5±1.6	29.6±7.7	2.9±0.6	1.2±0.3	0.7±0.1	7.2±2.0	312.4±74.7
Roots															
1	n.d.	3.7±0.6	7.3±0.6	1.9±0.6	6.3±0.5	3.1±0.8	1.3±0.2	3.1±0.9	0.6±0.2	2.2±0.3	0.3±0.1	n.d.	0.4±0.2	2.0±0.3	26.2±4.8
2	2.1±0.7	6.1±1.3	11.4±0.7	4.1±1.4	13.6±2.0	6.8±1.3	4.9±0.7	5.9±1.1	2.0±0.7	3.7±0.6	0.6±0.1	0.4±0.1	0.7±0.1	4.3±0.9	61.9±7.5
5	4.2±1.1	15.4±1.8	32.9±6.8	11.5±1.4	37.8±8.2	23.4±4.9	14.3±4.3	13.2±2.4	6.3±2.1	10.9±3.2	1.3±0.2	n.d.	2.0±0.7	12.1±3.1	166.9±26.1

Values are means ± SD (n = 4); n.d. – not detected.