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Biological Screening of Plant Coumarins

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(née Kummala)

ACADEMIC DISSERTATION

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PREFACE

This work was carried out at the Division of Pharmacognosy, Department of Pharmacy, University of Helsinki during the years 1993-1999. Part of the work was performed at the Minerva Foundation Institute for Medical Research, Helsinki and at the Division of Pharmacognosy, Department of Pharmacy, Uppsala University, Sweden.

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ABSTRACT

The variety of physiological effects of natural coumarins is extensive. Humans are exposed to them in everyday life through the handling of garden plants like *Aegopodium podagraria* or ingestion of fruit, vegetables or spices (*e.g*. *Petroselinum crispum*). Based on the ethnopharmacological background and reported biological activities, properties of some of the natural coumarins have been investigated more thoroughly using either pure compounds or extracts from plants containing them.

The anti-inflammatory screening of twenty coumarin compounds studied with an *in vitro* model for elastase secretion in human neutrophils using PAF and fMLP as stimuli revealed that the ethnobotanical use of *Angelica archangelica*, *Petroselinum crispum* and *Ruta graveolens* as a relief for cough, colds and arthritis might be explained by the presence of linear furanocoumarins psoralen and xanthotoxin.

Coumarins are considered as phytoalexins since plants produce them as defence substances when wounded or attacked by other organisms. The antimicrobial effects of methanol extracts prepared from seven plants growing in Finland, namely *Aegopodium podagraria*, *Anethum graveolens*, *A. archangelica*, *Levisticum officinalis*, *P. crispum*, and *Peucedanum palustre*, and *R. graveolens*, and pure coumarins occurring in them were merely observed against plant pathogens which supported the role of coumarins and furanocoumarins as defensive compounds. The agar-diffusion methods used are suitable for the bioassay-guided isolation of active substances.

The phototoxicity of linear furanocoumarins (also referred to as psoralens) has been turned into a useful property when combined with controlled UVA irradiation, this PUVA treatment has been widely used for psoriasis. For screening phototoxic compounds and extracts, also others than coumarins, a microwell plate test with *Artemia salina* as test organism was developed. It is noteworthy, that with this test system it is possible to investigate both toxicity and phototoxicity at the same time with the same concentrations.

Furthermore, osthol, a simple coumarin from *Angelica archangelica*, is suggested to be a useful compound for investigations on ligand-receptor interactions and for receptor-mediated regulations of intracellular free calcium concentrations.

Coumarins can be suggested to be beneficial for the plants themselves as natural biocontrolling antipathogenic compounds, and for humans as remedy for hyperproliferative skin diseases and as reference compounds in various bioactivity tests. Furthermore, coumarin-containing plants are valuable as dietary supplements on the basis of their mild antimicrobial and anti-inflammatory effects.

LIST OF ORIGINAL PUBLICATIONS

I Kummala T., Vuorela P., Johansson S., Bohlin L., Vuorela H. and Vasänge M. Inhibitory activity of a series of coumarins on neutrophil elastase secretion induced by platelet activating factor (PAF) and the chemotactic peptide fMLP. *Pharm. Pharmacol. Lett.* 8: 144-147, 1998.

II Ojala T., Remes S., Haansuu P., Vuorela H., Hiltunen R., Haahtela K. and Vuorela P. Antimicrobial activity of some coumarin containing herbal plants growing in Finland. *J. Ethnopharm.* 73: 299-305, 2000.

III Ojala T., Vuorela P., Kiviranta J., Vuorela H. and Hiltunen R. A bioassay using *Artemia salina* for detecting phototoxicity of plant coumarins. *Planta Med.* 65: 715-718, 1999.

IV Kummala T., Vuorela H., Vuorela P., Hiltunen R. and Törnquist K. Actions of natural coumarins on calcium entry in rat thyroid FRTL-5 cells. *Pharm. Pharmacol. Lett.* 6: 1-4, 1996.

V Ojala T., Vuorela P., Vuorela H. and Törnquist K. The coumarin osthol attenuates the binding of thyrotropin-releasing hormone in rat pituitary GH_4C_1 cells. *Planta Med.* 67: (in press), 2001.

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

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LIST OF ABBREVIATIONS

AA	arachidonic acid
$[Ca^{2+}]_i$	intracellular concentration of free calcium
$\Delta[Ca^{2+}]_i$	change in intracellular concentration of free calcium
$^{45}Ca^{2+}$	radiolabelled calcium
CICR	calcium induced calcium release
conc.	concentration
COX	cyclooxygenase
DAG	1,2-diacylglycerol
DMSO	dimethylsulfoxide
Fluo-3-AM	indicator for intracellular calcium
fMLP	*N*-formyl-L-methionyl-L-leucyl-L-phenylalanine
FRTL-5 cells	cultivated cells from the rat thyroid (Fischer Rat Thyroid cells in Low serum)
GH_3 cells	cultivated cells from the rat pituitary gland
GH_4C_1 cells	cultivated cells from the rat pituitary gland, clone 1
HIV	human immunodeficiency virus
IC_{50}	concentration yielding 50 % inhibition
IP_3	inositol-1,4,5-trisphosphate
IPs	inositol phosphates
KB	human rhinopharynx cancer cell line
LC_{50}	concentration yielding 50 % lethality
5-LO	5-lipoxygenase
LTC_4	leukotriene C_4
MK-1	human gastric adenocarcinoma cell line
NSAID	non-steroidal anti-inflammatory drugs
NSCLC-N6	human bronchial epidermoid carcinoma cell line
PAF	platelet activating factor
PGE_2	prostaglandin E_2
PLC	phospholipase C
PRL	prolactin
PUVA	psoralen + UVA
SOCC(s)	store-operated calcium channel(s)
TG	thapsigargin
TLC	thin layer chromatography
TMP	trimethylpsoralen
TPA	12-O-tetradecanoylphorbol-13-acetate
TRH	thyrotropin-releasing hormone
[^{3}H]TRH	[^{3}H]-labelled thyrotropin-releasing hormone
UVA	long wave ultraviolet radiation (320-400 nm)
VOCC(s)	voltage-operated calcium channel(s)

1. INTRODUCTION

Natural products are typically secondary metabolites, produced by organisms in response to external stimuli such as nutritional changes, infection and competition (COTTON, 1996; STROHL, 2000). Natural products produced by plants, fungi, bacteria, insects and animals have been isolated as biologically active pharmacophores. Approximately one-third of the top-selling drugs in the world is natural products or their derivatives often with ethnopharmacological background. Moreover, natural products are widely recognised in the pharmaceutical industry for their broad structural diversity as well as their wide range of pharmacological activities.

New medicines have been discovered with traditional, empirical and molecular approaches (HARVEY, 1999). The traditional approach makes use of material that has been found by trial and error over many years in different cultures and systems of medicine (COTTON, 1996). Examples include drugs such as morphine, quinine and ephedrine that have been in widespread use for a long time, and more recently adopted compounds such as the antimalarial artemisinin. The empirical approach builds on an understanding of a relevant physiological process and often develops a therapeutic agent from a naturally occurring lead molecule (VERPOORTE, 1989, 2000). Examples include tubocurarine and other muscle relaxants, propranolol and other β-adrenoceptor antagonists, and cimetidine and other histamine H_2 receptor antagonists. The molecular approach is based on the availability or understanding of a molecular target for the medicinal agent (HARVEY, 1999). With the development of molecular biological techniques and the advances in genomics, the majority of drug discovery is currently based on the molecular approach.

The major advantage of natural products for random screening is the structural diversity provided by natural products, which is greater than provided by most available combinatorial approaches based on heterocyclic compounds (CLAESON and BOHLIN, 1997; HARVEY, 1999). Bioactive natural products often occur as a part of a family of related molecules so that it is possible to isolate a number of homologues and obtain structure-activity information. Of course, lead compounds found from screening of natural products can be optimised by traditional medicinal chemistry or by application of combinatorial approaches. Overall, when faced with molecular targets in screening assays for which there is no information about low molecular weight leads, use of a natural products library seems more likely to provide the chemical diversity to yield a hit than a library of similar numbers of compounds made by combinatorial synthesis. Since only a small fraction of the world’s

biodiversity has been tested for biological activity, it can be assumed that natural products will continue to offer novel leads for novel therapeutic agents, if the natural products are available for screening.

The search for bioactive chemicals from the unstudied part of the plant kingdom can be conducted essentially with three methods (COTTON, 1996): the random method involves the collection of all plants found in a given area of study, phylogenetic targeting means the collection of all members of those plant families which are known to be rich in bioactive compounds, and the ethnobotanical approach is based on the traditional knowledge of medicinal plant use. COX (1994) suggests that the ethno-directed sampling is most likely to succeed in identifying drugs used in the treatment of gastrointestinal, inflammatory and dermatological complaints.

Due to their specialised biochemical capabilities, plants are able to synthesise and accumulate a vast array of primary and secondary chemicals useful for the plant itself as protecting against environmental stress factors. These compounds have made many plants useful also for humans for instance as spices, medicines *etc*. Natural coumarins, like other unsaturated lactones, may exert various effects on living organisms, both in plants and in animals. In view of their established low toxicity, relative cheapness, presence in the diet and occurrence in various herbal remedies, it appears important to evaluate the properties and applications of coumarins further utilising an ethnobotanical approach.

2. REVIEW OF THE LITERATURE

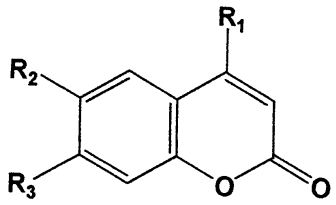
2.1. Coumarins in plants

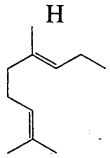
2.1.1. Phytochemistry of coumarins

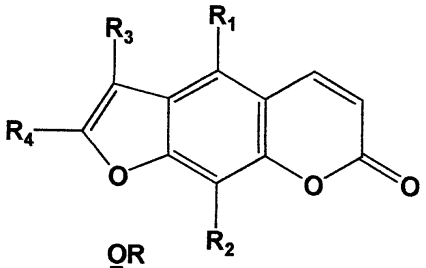
Coumarins owe their class name to ’coumarou’, the vernacular name of the tonka bean (*Dipteryx odorata* Willd., Fabaceae), from which coumarin itself was isolated in 1820 (BRUNETON, 1999). Coumarins belong to a group compounds known as the benzopyrones, all of which consist of a benzene ring joined to a pyrone. Coumarin and the other members of the coumarin family are benzo-α-pyrones, while the other main members of the benzopyrone group – the flavonoids – contain the γ-pyrone group (KEATING and O’KENNEDY, 1997). Coumarins may also be found in nature in combination with sugars, as glycosides. The coumarins can be roughly categorised as follows (MURRAY *et al*., 1982; see **Fig. 1** for coumarins used in this study):

- simple – these are the hydroxylated, alkoxylated and alkylated derivatives of the parent compound, coumarin, along with their glycosides
- furanocoumarins – these compounds consist of a five-membered furan ring attached to the coumarin nucleus, divided to linear and angular types with substituents at one or both of the remaining benzenoid positions
- pyranocoumarins – members of this group are analogous to the furanocoumarins, but contain a six-membered ring
- coumarins substituted in the pyrone ring.

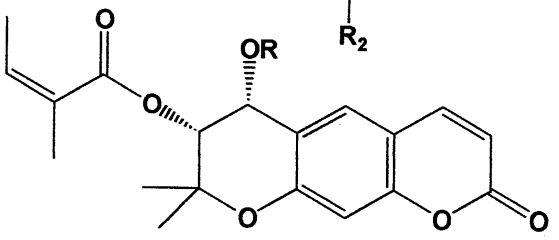
Like other phenylpropanoids, coumarins arise from the metabolism of phenylalanine via a cinnamic acid, *p*-coumaric acid (BRUNETON, 1999; MATERN *et al*., 1999). The specificity of the process resides in the 2’-hydroxylation, next comes the photocatalysed isomerisation of the double bond followed by spontaneous lactonisation. In some rare cases, glucosylation of cinnamic acid occurs, precluding lactonisation. In such cases, coumarin only arises after tissue injury and enzymatic hydrolysis. The formation of di- and trihydroxycoumarins and of their ethers involves the hydroxylation of umbelliferone rather than the lactonisation of the corresponding cinnamic acids. Prenylation of the benzene ring by dimethylallyl pyrophosphate in the 6-position of a 7-hydroxycoumarin yields the so-called linear furano- and pyranocoumarins, in the 8-position it affords the angular homologues. The formation of furanocoumarins includes two successive steps:

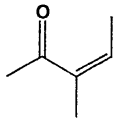
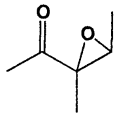


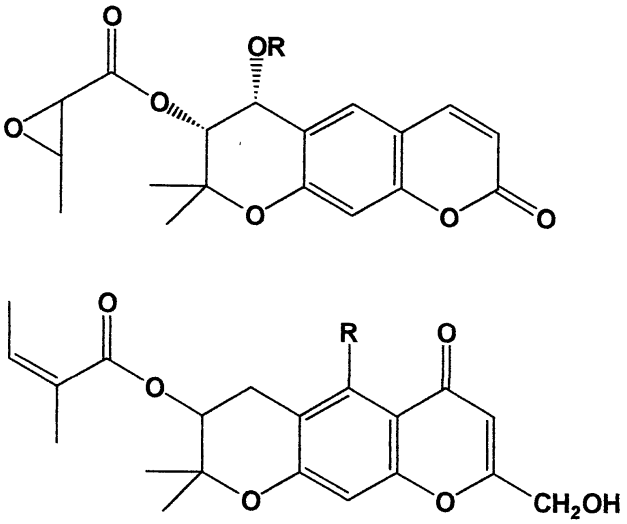
	R_1	R_2	R_3
Coumarin	H	H	H
Herniarin	H	H	OCH_3
Methyl-umbelliferone	CH_3	H	OH
Scopoletin	H	OCH_3	OH
Umbelliferone	H	H	OH
Ostruthin	H		OH



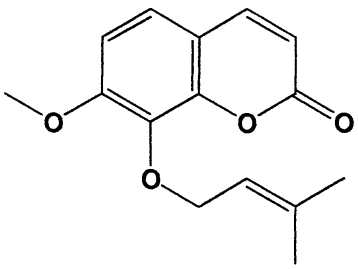
	R_1	R_2	R_3	R_4
Bergapten	OCH_3	H	H	H
Isopimpinellin	OCH_3	OCH_3	H	H
Peucedanin	H	H	OCH_3	$CH(CH_3)_2$
Psoralen	H	H	H	H
Xanthotoxin	H	OCH_3	H	H



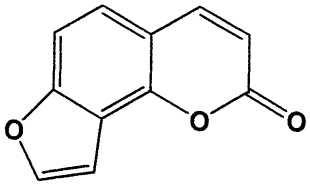
	R
Xanthalin	
Peuarenarin	
Peuarenin	"



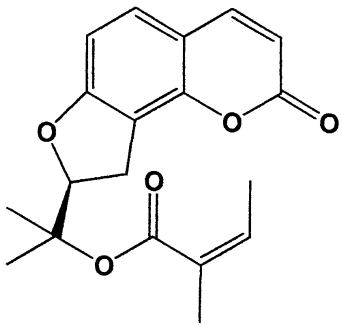
	R
Ledebouviellol	OH
2-Methylchromone derivative of ledebouviellol	OCH_3



Osthol

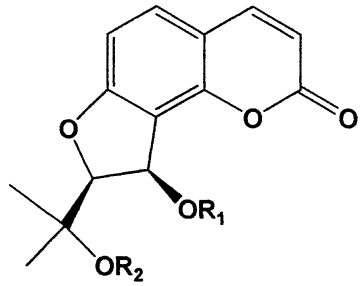


Angelicin

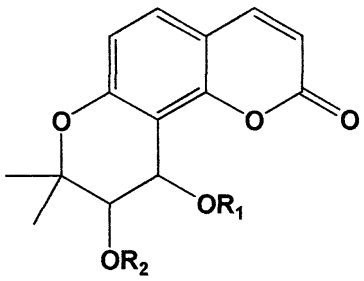


Columbianadin

Figure 1 Chemical structures of the coumarin compounds examined.



	R_1	R_2
Apterin	H	β-glucosyl
Athamantin	iso-valeryl	iso-valeryl



	R_1	R_2
Anomalin	valeryl	valeryl

Figure 1 Chemical structures of the coumarin compounds examined. (cont.)

stereospecific oxidation in the 4'-position and elimination of the hydroxyisopropyl residue in the 5'-position by retroaldol condensation. Substitution in the 5- or 8-position or in both positions of furanocoumarins occurs later and is catalysed by oxidases and *O*-methyltransferases.

The primary site of synthesis of coumarins is suggested to be the young, actively growing leaves, with stems and roots playing a comparatively minor role (MURRAY *et al.*, 1982). However, one should not forget the possibility of species and compound variation, for example furanocoumarins in *Pastinaca sativa* are formed in the fruits where they also accumulate, and furanocoumarins in *Angelica archangelica* are formed in the leaves with the exception of osthenol, a simple coumarin, which is probably formed in the roots.

2.1.2. Botanical aspects

Coumarins are found free or as heterosides in many dicotyledonous families, including the Apiaceae, Asteraceae, Fabiaceae, Moraceae, Rosaceae, Rubiaceae, Rutaceae and Solanaceae (WEINMANN, 1997; MATERN *et al.*, 1999). Many monocotyledonous plants, especially the Gramineae and orchids, also contain large amounts of coumarins. Although mainly synthesised in

the leaves, coumarins occur at the highest levels in the fruits, followed by the roots and stems. In addition, seasonal changes and environmental conditions may affect the occurrence in various parts of the plant.

The distribution of biologically active coumarins in a wide range of plants seems to correlate with their ability to act as phytoalexins, *i.e*. they are formed as a response to traumatic injury, during the wilting process, by plant diseases or through drying, they accumulate on the surface of the leaves, fruits and seeds, and they inhibit the growth and sporulation of fungal plant pathogens and act as repellents against beetles and other terrestrial invertebrates (WEINMANN, 1997; MATERN *et al*., 1999). Coumarins are leached from the roots of some plants, such as wild *Avena*, into the soil, where they provide a defence tool against hostile micro-organisms.

Coumarins are also active in plant metabolism, taking part in growth regulation (WEINMANN, 1997; MATERN *et al*., 1999). In particular furanocoumarins, are known to inhibit root tip growth and seem to induce membrane disturbances, and their excretion on seed surfaces might be a means to delay germination.

In this study, leaves of six Apiaceae plants, *Aegopodium podagraria* L., *Anethum graveolens* L., *Angelica archangelica* L., *Levisticum officinalis* Koch, *Petroselinum crispum* (P. Mill.) A. W. Hill., and *Peucedanum palustre* (L.) Moench, as well as of one from Rutaceae, *Ruta graveolens* L., were investigated. These plants are interesting due to their aromatic constituents (among others coumarins, see **Table 1**, and essential oils), they all grow in Finland (HÄMET-AHTI *et al*., 1998), and all except *P. palustre* have been cultivated successfully mostly for flavouring and culinary purposes (HÄLVÄ, 1988; B. GALAMBOSI, personal communication).

In nature, *A. podagraria* and *P. palustre* are common in southern Finland, more infrequent in the northern parts. They can be found in groves, brook sides, wet and moist meadows and peat-covered areas in the outer archipelago of Finland. *A. archangelica* is frequently observed growing by the streams and lakes in Lapland. *A. graveolens*, native to southwest Asia and India, and *P. crispum*, native of southern-east Europe and western India, are widely grown in Finland. *L. officinale*, native of southern Europe, has become naturalised in many places in the southern and middle parts of the Finnish countryside. Originally Mediterranean *R. graveolens* is cultivated as a spice herb in Finland. (HÄMET-AHTI *et al*., 1998).

Table 1 Reports in the literature of the coumarins in the plants investigated in this study.

Compound	Plant	Occurrence	Reference
Aesculetin	*Anethum graveolens* L.	fruit	DRANIK and PROKOPENKO, 1969
Angelicin	*Aegopodium podagraria* L.		SALGUES, 1963
	Angelica archangelica L.	fruit, leaves, roots	SPÄTH and PESTA, 1934
2'-Angeloyl-3'-isovaleryl vaginate	*Angelica archangelica* L.	roots	HÄRMÄLÄ *et al*., 1991
Apterin	*Aegopodium podagraria* L.	roots	FISCHER and BAERHEIM-SVENDSEN, 1976
	Angelica archangelica L.	roots	FISCHER and BAERHEIM-SVENDSEN, 1976
	Levisticum officinale (Hill) Koch	roots	FISCHER and BAERHEIM-SVENDSEN, 1976
	Peucedanum palustre (L.) Moench	roots	FISCHER and BAERHEIM-SVENDSEN, 1976
Archangelicin	*Angelica archangelica* L.	roots	NOGUCHI and KAWANAMI, 1940
Archangelin	*Angelica archangelica* L.	roots	CHATTERJEE and SEN GUPTA, 1964
Bergapten	*Anethum graveolens* L.	fruit	DRANIK and PROKOPENKO, 1969
	Angelica archangelica L.	fruit, leaves, roots	SPÄTH and VIERHAPPER, 1938
	Levisticum officinale (Hill) Koch	roots	NAVES, 1943
	Petroselinum crispum (Mill.) A.W. Hill		MUSAJO *et al*., 1954
	Peucedanum palustre (L.) Moench	roots	LESKOVA and ANANICHEV, 1969
	Ruta graveolens L.	stems, leaves	RODIGHIERO *et al*., 1952
(-)-Byakangelicin	*Ruta graveolens* L.	roots	REISCH *et al*., 1969
Byakangelicin angelate	*Angelica archangelica* L.	roots	HÄRMÄLÄ *et al*., 1992
Byakangelicin-2'-O-isovalerate	*Angelica archangelica* L.	roots	SUN and JAKUPOVIC, 1986
Chalepensin	*Ruta graveolens* L.	roots	REISCH *et al*., 1968c
Columbianadin	*Peucedanum palustre* (L.) Moench	fruit, roots	NIELSEN and LEMMICH, 1964
Columbianadinoxide	*Peucedanum palustre* (L.) Moench	fruit	NIELSEN and LEMMICH, 1965
Coumarin	*Levisticum officinale* (Hill) Koch	leaves, roots	NAVES, 1943
	Petroselinum crispum (Mill.) A.W. Hill		CASPARIS and MANELLA, 1944
	Ruta graveolens L.	leaves	SPÄTH, 1937
Daphnoretin	*Ruta graveolens* L.	aerial parts	REISCH *et al*., 1968a
Daphnoretin methyl ether	*Ruta graveolens* L.		MURRAY *et al*., 1982
Daphnorin	*Ruta graveolens* L.	roots	VARGA *et al*., 1974a
Gravelliferone	*Ruta graveolens* L.	roots	REISCH *et al*., 1968b
Gravelliferone methyl ether	*Ruta graveolens* L.	roots	REISCH *et al*., 1968c
Graveolone	*Anethum graveolens* L.	aerial parts	APLIN and PAGE, 1967
Heraclenol	*Petroselinum crispum* (Mill.) A.W. Hill	stems	KATO *et al*., 1978
Heraclenol-2'-O-isovalerate	*Angelica archangelica* L.	roots	SUN and JAKUPOVIC, 1986
Heraclenol-2'-O-seneciote	*Angelica archangelica* L.	roots	SUN and JAKUPOVIC, 1986
Herniarin	*Ruta graveolens* L.		REINHARD *et al*., 1968
8-Hydroxybergapten	*Angelica archangelica* L.	fruit	PATRA *et al*., 1976
Imperatorin	*Angelica archangelica* L.	fruit, leaves, roots	SPÄTH and VIERHAPPER, 1937
	Peucedanum palustre (L.) Moench	fruit, roots	LESKOVA and ANANICHEV, 1969
Isobergapten	*Angelica archangelica* L.	roots	CHATTERJEE and DUTTA, 1968

Table 1 Reports in the literature of the coumarins in the plants investigated in this study. (cont.)

Compound	Plant	Occurrence	Reference
Isobyakangelicin angelate	*Peucedanum palustre* (L.) Moench	roots	VUORELA *et al*., 1988
Isoimperatorin	*Angelica archangelica* L.	fruit, leaves, roots	CHATTERJEE *et al*., 1967
	Peucedanum palustre (L.) Moench	fruit, roots	NIELSEN and LEMMICH, 1964
	Ruta graveolens L.	roots, stems	ANDON *et al*., 1972
Isooxypeucedanin	*Peucedanum palustre* (L.) Moench	fruit	NIELSEN and LEMMICH, 1965
Isopeulustrin	*Peucedanum palustre* (L.) Moench	fruit	NIELSEN and LEMMICH, 1965
Isopimpinellin	*Angelica archangelica* L.	fruit, leaves	STECK and BAILEY, 1969
		roots	HÄRMÄLÄ *et al*., 1992
	Petroselinum crispum (Mill.) A.W. Hill		INNOCENTI *et al*., 1976
	Ruta graveolens L.		STECK *et al*., 1971
Isorutarin	*Ruta graveolens* L.	roots	VARGA *et al*., 1974b
Marmesin	*Ruta graveolens* L.	roots	NÓVAK *et al*., 1972
Marmesinin	*Ruta graveolens* L.	roots	NÓVAK *et al*., 1972
8-Methoxy-gravelliferone	*Ruta graveolens* L.		REISCH *et al*., 1970
5-Methoxy-heraclenol isovalerate	*Angelica archangelica* L.	roots	SUN and JAKUPOVIC, 1986
8-[2-(3-Methyl-butyroxy)-3-hydroxy-3-methylbutoxy]-psoralen	*Angelica archangelica* L.	roots	HÄRMÄLÄ *et al*., 1992
Oroselone	*Angelica archangelica* L.	fruit, roots	BAERHEIM-SVENDSEN, 1954
Osthenol	*Angelica archangelica* L.	fruit, roots	BÖCKER and HAHN, 1911
Osthol	*Angelica archangelica* L.	fruit, roots	BÖCKER and HAHN, 1911
Ostruthol	*Angelica archangelica* L.	fruit, roots	CHATTERJEE *et al*., 1967
		roots	HÄRMÄLÄ *et al*., 1992
	Peucedanum palustre (L.) Moench	roots	NIELSEN and LEMMICH, 1964
(+)-Oxypeucedanin	*Angelica archangelica* L.	fruit, leaves, roots	CHATTERJEE *et al*., 1967
	Peucedanum palustre (L.) Moench	roots	NIELSEN and LEMMICH, 1964
(+)-Oxypeucedanin hydrate	*Angelica archangelica* L.	fruit, roots	CHATTERJEE *et al*., 1967
	Peucedanum palustre (L.) Moench	roots	NIELSEN and LEMMICH, 1964
Oxypeucedanin methanolate	*Angelica archangelica* L.	roots	VISHWAPAUL and WEYERSTAHL, 1987
Pangeline	*Ruta graveolens* L.		GONZÁLEZ *et al*., 1974
Peucedanin	*Peucedanum palustre* (L.) Moench	fruit, roots	LESKOVA and ANANICHEV, 1969
Peulustrin	*Peucedanum palustre* (L.) Moench	fruit, roots	NIELSEN and LEMMICH, 1965a
Phellopterin	*Angelica archangelica* L.	fruit	BEYRICH, 1965
		roots	HÄRMÄLÄ *et al*., 1992
Pimpinellin	*Angelica archangelica* L.	fruit	CISOWSKI *et al*., 1987
Psoralen	*Angelica archangelica* L.	roots	HÄRMÄLÄ *et al*., 1992
	Ruta graveolens L.	roots, stems	ANDON *et al*., 1972
Rutacultin	*Ruta graveolens* L.	roots	REISCH *et al*., 1972b
Rutamarin	*Ruta graveolens* L.	aerial parts, roots, stems	REISCH *et al*., 1967
Rutamarin alcohol	*Ruta graveolens* L.	roots	REISCH *et al*., 1972b
Rutaretin	*Ruta graveolens* L.		SCHNEIDER *et al*., 1967
Rutarin	*Ruta graveolens* L.	aerial parts, roots	SCHNEIDER and MÜLLER, 1967
Scopoletin	*Anethum graveolens* L.	aerial parts, fruit, roots	APLIN and PAGE, 1967
	Ruta graveolens L.		REINHARD *et al*., 1968
Suberenon	*Ruta graveolens* L.	roots	REISCH *et al*., 1972a

Table 1 Reports in the literature of the coumarins in the plants investigated in this study. (cont.)

Compound	Plant	Occurrence	Reference
Umbelliferone	*Anethum graveolens* L.	fruit, roots	DRANIK and PROKOPENKO, 1969
	Angelica archangelica L.	fruit, roots	SOMMER, 1859
	Levisticum officinale (Hill) Koch	leaves, roots	SOMMER, 1859
	Ruta graveolens L.	roots	ANDON and DENISOVA, 1974
Umbelliprenin	*Anethum graveolens* L.	fruit	DRANIK and PROKOPENKO, 1969
	Angelica archangelica L.	fruit, roots	SPÄTH and VIERHAPPER, 1938a
	Peucedanum palustre (L.) Moench	fruit	NIELSEN and LEMMICH, 1965
Xanthotoxin	*Anethum graveolens* L.	fruit	CESKA *et al*., 1987
	Angelica archangelica L.	fruit, leaves, roots	SPÄTH and VIERHAPPER, 1938
	Petroselinum crispum (Mill.) A.W. Hill		INNOCENTI *et al*., 1976
	Ruta graveolens L.	stems, leaves	RODIGHIERO *et al*., 1954
Xanthotoxol	*Angelica archangelica* L.	fruit, roots	SPÄTH and VIERHAPPER, 1937
Xanthyletin	*Ruta graveolens* L.	roots	REISCH *et al*., 1969

2.1.3. Ethnobotany/Ethnopharmacology

There has been major changes in the definitions of ethnobotany as more and more disciplines have become involved. Nowadays, it is understood to comprise all studies (concerning plants) which describe local people's interaction with the natural environment. One part of ethnobotany, ethnopharmacology is considered as the scientific evaluation of traditional medicinal plants. (COTTON, 1996).

Several members of the plant families Apiaceae and Rutaceae are used as spices and vegetables in human nutrition or for medicinal purposes. *A. podagraria*, *A. graveolens*, *A. archangelica*, *L. officinale*, *P. crispum* and *R. graveolens* are known as spices and vegetables, their medicinal uses as well as those of *P. palustre*'s are presented in **Table 2**. The most common use of these coumarin containing species seems to be different kinds of gastric disorders.

Table 2 Ethnobotanical usage of the plants studied.

Plant	**Usage**	**Reference**
A. podagraria	as a remedy for gout	LINDMAN 1964; HOPPE, 1975; HÄNSEL *et al*., 1992
	as a remedy for rheumatism, haemorrhoids	HOPPE, 1975; HÄNSEL *et al*., 1992
A. graveolens	as a remedy for gastric disorders, as a stimulant for milk secretion	HOPPE, 1975
A. archangelica	as a remedy for gastric disorders, lack of appetite, insomnia, rheumatism	HOPPE, 1975; BLASCHEK *et al*., 1998
	as a remedy for cough	BLASCHEK *et al*., 1998
L. officinale	as a remedy for gastric disorders, lack of appetite, cough	HOPPE, 1975; HÄNSEL *et al*., 1993
	as an emmenagogue	HOPPE, 1975
	as a remedy for oedema	HÄNSEL *et al*., 1993
P. crispum	as a remedy for gastric disorders, colds against lice	HOPPE, 1975
	as a remedy for gastric disorders, jaundice, as an emmenagogue agent	HÄNSEL *et al*., 1994
	as a remedy for hypertension	ZIYYAT *et al*., 1997
	as an antidiabetic agent	TUNALI *et al*., 1999
P. palustre	as a remedy for cough, cramps, epilepsy	HOPPE, 1975
	P. sp.: as a remedy for rheumatism, fever	HIERMANN and SCHLANTL, 1998
	P. sp.: as a remedy for colds, cough, gout	HSIAO *et al*., 1998
R. graveolens	as a remedy for rheumatism, gout	HOPPE, 1975
	as a remedy for cramps	HOPPE, 1975; HÄNSEL *et al*., 1994
	as a remedy for gastric disorders	HOPPE, 1975; CONWAY and SLOCUMB, 1979; HÄNSEL *et al*., 1994
	as an emmenagogue agent	HOPPE, 1975; CONWAY and SLOCUMB, 1979
	as a remedy for colds, pains, epilepsy	HÄNSEL *et al*., 1994
	as a remedy for stiff neck, dizziness, headache	CONWAY and SLOCUMB, 1979; HÄNSEL *et al*., 1994
	as an abortifacient agent	CONWAY and SLOCUMB, 1979

2.2. Biological effects of plant coumarins

Coumarins have a variety of bioactivities including anticoagulant, estrogenic, dermal photosensitising, antimicrobial, vasodilator, molluscacidal, antithelmintic, sedative and hypnotic, analgesic and hypothermic activity (SOINE, 1964; O'KENNEDY and THORNES, 1997). Recent reports of the biological effects of coumarins are presented in **Table 3**, and some of the effects are discussed in more detail.

Table 3 Biological effects of coumarins reported in the literature.

Biological effect	**References**
Anti-inflammatory activity, *in vitro*, *in vivo*	CHEN *et al*., 1995; OKADA *et al*., 1995; LINO *et al*., 1997; HIERMANN and SCHLANTL, 1998; HSIAO *et al*., 1998; GARCIA-ARGAEZ *et al*., 2000
Antifungal activity	SARDARI *et al*., 1999
Antimalarial activity, *in vitro*, *in vivo*	YANG *et al*., 1992
Antimicrobial activity	DINI *et al*., 1992; KWON *et al*., 1997; KAYSER and KOLODZIEJ, 1997
Antitumor-promoting activity, *in vitro*	OKUYAMA *et al*., 1990; MARSHALL *et al*., 1993, 1994; MIZUNO *et al*., 1994; SELIGER, 1997; KOFINAS *et al*., 1998; FUJIOKA *et al*., 1999
Antiviral activity, *in vitro*	FULLER *et al*., 1994
Calcium antagonistic activity, *in vitro*, *in vivo*	VUORELA, 1988; YAMAHARA *et al*., 1989; TÖRNQUIST and VUORELA, 1990; HÄRMÄLÄ, 1991; CHIU and FUNG, 1997
Cytostatic effect, *in vivo*	EGAN *et al*., 1990
Inhibition of blood coagulation	EGAN *et al*., 1990
Inhibition of 5-lipoxygenase, *in vitro*	HOULT *et al*., 1994; LIU *et al*., 1998; RESCH *et al*., 1998
Inhibition of enzyme activity in the liver, *in vitro*	SCHIMMER *et al*., 1991; MÄENPÄÄ *et al*., 1993; LAKE *et al*., 1994; KOENIGS *et al*., 1997; BROCKMEYER *et al*., 1998
Inhibition of monoamine oxidase, *in vitro*	HUONG *et al*., 1999
Inhibition of protein kinases, *in vitro*	YANG *et al*., 1999
Photosensitising activity, *in vivo*	LEWIS, 1994; McNEELY and GOA, 1998

2.2.1. Anti-inflammatory activity

ROCHA e SILVA (1994) has presented various definitions for inflammation, starting from the description of the main four signs of inflammation, redness, swelling, heat, pain and loss of function, and ending to 'a multi-mediated phenomenon, of a pattern type in which all mediators would come and go at the appropriate moment to play their roles in increasing vascular permeability, attracting leukocytes, producing pain, local oedema and necrosis, in which the predominance of any one would be fortuitous or depending on its specific capabilities of producing symptoms, some directly, some indirectly, some by potentiating or by releasing other agents'. A

controversy about the activity of the coumarins as anti-inflammatory agents exists, since some authors reported (HOULT and PAYÁ, 1996) that coumarins do not exert potent activity in conventional short-term tests. Nevertheless, various coumarins have been reported to possess anti-inflammatory activity as shown in carrageenan-induced inflammation and cotton pellet granuloma tests.

Carrageenan stimulates the release of several inflammatory mediators such as histamine, serotonin, bradykinin and prostaglandins (LINO *et al*., 1997). Non-steroidal anti-inflammatory drugs (NSAID) block the synthesis of prostaglandins by inhibiting cyclooxygenase (COX). COX and 5-lipoxygenase (5-LO) catalyse peroxidation of arachidonic acid, and polyphenols like coumarins and flavonoids might be expected to interfere with this process (HOULT *et al*., 1994b). Actually, fraxetin, esculetin, 4-methylesculetin, daphnetin and 4-methyldaphnetin inhibited generation of leukotriene B4 (a 5-LO product) (HOULT *et al*., 1994a). In an other experiment, coumarin and umbelliferone were found to have a mechanism of action similar to NSAID in a carrageenan-induced inflammation, and the effect lasted for at least 3 h, which is the time for the maximum effect of carrageenan (LINO *et al*., 1997). Coumarin was also effective in the rat paw oedema induced by dextran. Osthol, isolated from *Angelica archangelica*, *A. pubescens* f. *biserrata* and *Atractyloides lancea*, turned out to be a selective inhibitor of 5-LO *in vitro* (ROOS *et al*., 1997; LIU *et al*., 1998; RESCH *et al*., 1998). Since 5-LO is activated by calcium influx, this effect was suggested to be due to its calcium antagonistic properties (HÄRMÄLÄ *et al*., 1992). Seselin from the aerial parts of *Decatropis bicolor* was active in the carrageenan-induced inflammation assay in rats (GARCIA-ARGAEZ *et al*., 2000). Carrageenan-induced rat paw oedema has been inhibited also by ethanol extract of the roots of *Peucedanum ostruthium* (HIERMANN and SCHLANTL, 1998), 6-(3-carboxybut-2-enyl)-7-hydroxycoumarin being the most important anti-inflammatory compound in the plant. Carrageenan-induced inflammation was also suppressed by seselin isolated from *Seseli indicum* (TANDAN *et al*., 1990) and by ethanol extract of the aerial parts of *Ruta chalepensis* (AL-SAID *et al*., 1990). Columbianadin, columbianetin acetate, bergapten and umbelliferone isolated from *Angelica pubescens* demonstrated both anti-inflammatory and analgesic activities at 10 mg/kg in mice (CHEN *et al*., 1995). Osthole and xanthotoxin revealed only anti-inflammatory activity, and isoimperatorin only analgesic effect. Interestingly, coumarins can also possess pro-inflammatory effects: lower doses of psoralen and imperatorin have shown an anti-inflammatory effect but at higher doses they have a pro-inflammatory effect (GARCIA-ARGAEZ *et al*., 2000).

Chronic inflammation induced by cotton pellet granuloma was inhibited by ethanol extracts of *Apium graveolens* and *Ruta graveolens* (ATTA and ALKOFAHI, 1998) and *R. chalepensis* (AL-SAID *et al*., 1990). These extracts showed also an anti-nociceptive effect against both acetic acid-induced writhing and hot plate-induced thermal stimulation in mice indicating central and peripheral effects. According to PILLER (1997), coumarin, or its metabolic products, have the potential to become the treatment of scalds and other forms of thermal wounding because it facilitates the removal of extravasated protein through proteolytic breakdown by stimulated macrophages.

2.2.2. Antimicrobial properties

There has been a dramatic increase in pathogen resistance to both pharmaceutical and agrochemical antimicrobial agents. New prototype compounds are needed to address this situation. Successful discovery of novel natural product antimicrobials has necessitated the development of new bioassay techniques and protocols that allow for the detection of small amounts of biologically active chemicals, which should be selective enough to determine optimum target pathogens, and amenable to the analysis of complex mixtures.

Antimicrobial activities have been evaluated with diverse settings often difficult to compare. There are reports on efficacies of pure coumarins against Gram-positive and Gram-negative bacteria as well as fungi (**Table 4**), also extracts have shown activities, *e.g*. methanol extract from *Mitracarpus scaber* against *Staphylococcus aureus* and *Candida albicans* (BISIGNANO *et al*., 2000) and water extract from *Pelargonium sisoides* against *Escherichia coli*, *Klebsiella pneumoniae, Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Streptococcus pneumoniae* (KAYSER and KOLODZIEJ, 1997). Free 6-OH in the coumarin nucleus has been found to be important for antifungal activity, while the free hydroxyl group at position 7 is important for antibacterial activity (SARDARI *et al*., 1999).

Table 4 Antimicrobial properties of coumarin compounds.

Microbe	Coumarin	Activity	Method	Reference
Gram-positive bacteria				
Staphylococcus aureus	psoralen	inhibitory	disc-diffusion	BISIGNANO *et al*., 2000
	6,8-dihydroxy-5,7-dimethoxycoumarin	bacteriostatic	agar dilution	KAYSER and KOLODZIEJ, 1997
	scopoletin	”	”	”
	umckalin	”	”	”
Streptococcus pneumoniae	6,8-dihydroxy-5,7-dimethoxycoumarin	”	”	KAYSER and KOLODZIEJ, 1997
	5,6,7-trimethoxycoumarin	”	”	”
	umckalin	”	”	”
Gram-negative bacteria				
Acaligenes faecalis	herniarin	inhibitory	Lederberg’s replica plating	JURD *et al*., 1970
Bacillus cereus	herniarin	”	”	”
Escherichia coli	scopoletin	bacteriostatic	agar dilution	KAYSER and KOLODZIEJ, 1997
Haemophilus influenzae	5,6,7-trimethoxycoumarin	”	”	”
Klebsiella pneumoniae	scopoletin	”	”	”
Proteus mirabilis	scopoletin	”	”	”
Pseudomonas aeruginosa	scopoletin	”	”	”
Sarcina lutea	herniarin	inhibitory	Lederberg’s replica plating	JURD *et al*., 1970
Fungi				
Alternaria alternata	scopoletin	inhibitory		SHUKLA *et al*., 1999
Aspergillus sp.	angelicin	inhibitory	microwell plates	SARDARI *et al*., 1999
	5,8-di(2,3-dihydroxy-3-methylbutoxy)-psoralen	”	”	KWON *et al*., 1997
	herniarin	inhibitory	Lederberg’s replica plating	JURD *et al*., 1970
Byssochlamys fulva	herniarin	inhibitory	”	”
	scopoletin	”	”	JURD *et al*., 1971
Candida sp.	angelicin	inhibitory	microwell plates	SARDARI *et al*., 1999
	herniarin	”	Lederberg’s replica plating	JURD *et al*., 1970
	psoralen	”	disc-diffusion	BISIGNANO *et al*., 2000
Cladosporium sp.	byakangelicin	inhibitory	microwell plates	KWON *et al*., 1997
	oxypeucedanin	”	TLC	MARSTON *et al*., 1995
	oxypeucedanin hydrate	”	”	”
Cryptococcus neoformans	angelicin	inhibitory	microwell plates	SARDARI *et al*., 1999
Hanseniaspora melligeri	herniarin	inhibitory	Lederberg’s replica plating	JURD *et al*., 1970
Hansenula anomala	herniarin	inhibitory	”	”
Penicillium chrysogenum	scopoletin	inhibitory	”	JURD *et al*., 1971
Pichia chodati	herniarin	inhibitory	”	JURD *et al*., 1970
Saccharomyces sp.	angelicin	inhibitory	microwell plates	SARDARI *et al*., 1999
	herniarin	”	Lederberg’s replica plating	JURD et al., 1970
Zygosaccharomyces sp.	herniarin	inhibitory	”	”

Interestingly, coumarins have also inhibitory effect on DNA gyrase which may be linked to the anti-HIV (human immunodeficiency virus) activity (MATERN *et al*., 1999), for example calanolide A isolated from *Calopyllum lanigerum* var. *austrocoriaceum* (FULLER *et al*., 1994) and a related coumarin, costatolide, from the latex of *C. teysmanii* var. *inophylloide*. Recently collinin, isolated from *Zanthoxylum schinifolium*, has been exhibiting anti-HBV (hepatitis B virus) activity (TSAI *et al*., 2000). Antimalarial activity has been addressed to daphnetin, extracted *e.g*. from the plants of the genus *Daphne* (YANG *et al*.,1992), as well as dentatin and clausarin, isolated from *Clausena harmandiana* (YENJAI *et al*., 2000).

2.2.3. Phototoxicity

Psoriasis is a common skin disease affecting ~2 % of the population (DISEPIO *et al*., 1999). While the appearance of the psoriatic skin can vary, each form of psoriasis is characterised by epidermal keratinocyte hyperproliferation, abnormal keratinocyte differentiation and immune-cell infiltration. Psoriasis is often difficult to treat owing to its sporadic course, variable response to treatments and adverse effects (ASHCROFT *et al*., 2000). When searching for suitable therapies for such a complex disease, the effectiveness of the screening method is of outmost importance.

Linear furanocoumarin xanthotoxin purified from *Ammi majus* was first introduced in the treatment of vitiligo over 50 years ago (el MOFTY, 1948). Investigations of dermatologists A. Lerner and T. Fitzpatrick led to further development of this therapy for the treatment of psoriasis (PARRISH *et al*., 1974). Administration of oral or topical psoralens (such as xanthotoxin) followed by irradiation with long wave ultraviolet radiation in the 320-400 nm range (UVA) is now a widely used, frequently convenient and effective systemic treatment of psoriasis with well-characterised and controllable side effects (LEWIS *et al*., 1994; McNEELY and GOA, 1998). PUVA suppresses the accelerated proliferation of the keratinocytes, another mechanism of action in psoriasis is suggested to be a result of its direct lymphotoxic effects. In case of major acute adverse reactions associated with PUVA and xanthotoxin (nausea, vomiting, pruritus and erythema), xanthotoxin can be replaced with bergapten. Xanthotoxin may be applied topically as bath before exposure to UVA, advantages of such administration are shorter irradiation times and a lack of gastrointestinal, hepatic or other systemic adverse effects. Trimethylpsoralen (TMP) and its derivatives have been found to inhibit lymphocyte proliferation to a greater extent than xanthotoxin (BERGER *et al*., 1985;

COVEN *et al*., 1999), and could provide one of the safest and most effective treatment for psoriasis. Angular furanocoumarins, or angelicins, were long thought to be unable to form crosslinks because of their geometry. The amount of crosslinks formed correlates to the skin-photosensitization (DALL'ACQUA *et al*., 1974). However, it has been shown that 4,6,4'-trimethylangelicin induces crosslinks (CHEN *et al*., 1994; BORDIN *et al*., 1994).

2.2.4. Effects on calcium fluxes

The entry of calcium into the cell occurs through various channels: *e.g*. voltage operated calcium channels (VOCCs), receptor operated channels, and calcium release activated channels (CASTALDO and CAPASSO, 1996). So far, six types of VOCCs (N, T, L, P, Q, R) have been identified (ALEXANDER and PETERS, 1998; DENYER *et al*., 1998). The channels are transmembrane proteins with an ion-selective aqueous pore that, when open, extends across the membrane (DENYER *et al*., 1998). Channel opening and closing ('gating') is controlled by a voltage-sensitive region of the protein containing charged amino acids that move within the electric field. The movement of these charged groups leads to conformational changes in the structure of the channel resulting in conducting (open/activated) or nonconducting (closed/inactivated) states. Depolarisation, ligands and mechanical factors control the calcium influx by regulating how long the calcium channel is open (NAYLER, 1993).

A working model of the modulation of $[Ca^{2+}]_i$ in GH_4C_1 cells is presented in **Fig. 2** (modified from WAGNER *et al*., 1993). In general, inositol phospholipids are broken down by phosphoinositide specific phospholipase C (PLC) in response to many agonists, *e.g*. thyrotropin-releasing hormone (TRH) or ATP, (*e.g*. WAGNER *et al*., 1993; BERRIDGE, 1995; BERRIDGE *et al*., 2000). The generated products, inositol-1,4,5-trisphosphate (IP_3) and 1,2-diacylglycerol (DAG), serve as second messengers and play a role in intracellular Ca^{2+} mobilisation and in the activation of protein kinase C, respectively. At normal physiological stimulation levels IP_3 may increase the sensitivity of the IP_3 receptor to Ca^{2+}, resulting in a process called Ca^{2+} induced Ca^{2+} release (CICR). In most cells, stimulation with agonists leads to emptying the Ca^{2+} stores, which in turn activates the store-operated Ca^{2+} channels (SOCCs) leading to Ca^{2+} entry through an unknown mechanism. A conformational-coupling mechanism has been suggested, which proposes that IP_3 receptors in the

endoplasmic reticulum are directly coupled to SOCCs. Once Ca^{2+} has carried out its signalling functions, it is rapidly removed from the cytoplasm by various pumps and exchangers.

In rat thyroid FRTL-5 cells, the regulation of Ca^{2+} entry occurs via receptor- and store-operated pathways (TÖRNQUIST, 1992, 1993). GH_4C_1 cells contain at least two functionally distinct intracellular Ca^{2+} stores (WAGNER *et al.*, 1993). The first store (marked as I in **Fig. 2**) is IP_3-sensitive and releases Ca^{2+} in response to TRH. This store is sensitive to emptying by thapsigargin, but maintains its ability to respond to TRH in the absence of extracellular Ca^{2+}. The second pool (marked as II in **Fig. 2**) is not sensitive to emptying by thapsigargin. Antagonists like nifedipine decrease the fluctuations in intracellular concentration of free calcium ($[Ca^{2+}]_i$) by inhibiting Ca^{2+} influx through L-type VOCCs. GH_4C_1 cells are able to decrease $[Ca^{2+}]_i$ by at least three different mechanisms: Ca^{2+} uptake into intracellular stores, Ca^{2+} efflux via Na^+/ Ca^{2+} exchanger, and Ca^{2+} efflux via plasma membrane Ca^{2+}–ATPase.

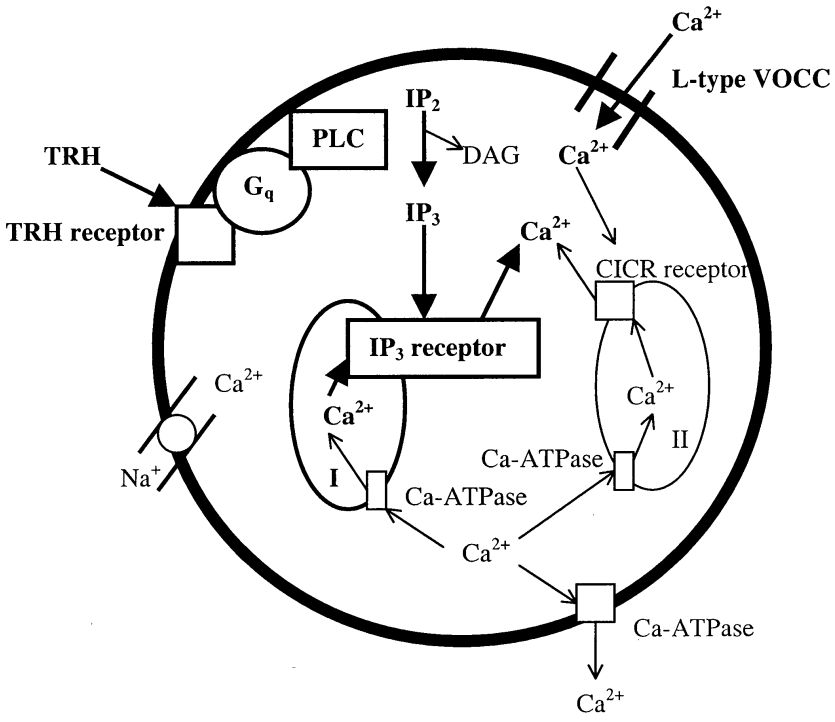


Figure 2 Working model of the modulation of $[Ca^{2+}]_i$ in GH_4C_1 cells (modified from WAGNER *et al.*, 1993).

There are many plant-derived compounds active on calcium channels, which have been extensively reviewed by VUORELA and co-workers (1997). For example, coumarins have a possible calcium blocking activity studied mostly with vascular preparates, *e.g.* dihydropyranocoumarin visnadin

isolated from *Ammi visnaga* fruits (RAUWALD *et al*., 1994a; DUARTE *et al*., 1997), 6,7-dimethoxycoumarin scoparone isolated from *Artemisia capillaris* (YAMAHARA *et al*., 1989), columbianadin isolated from *Peucedanum palustre* (TÖRNQUIST and VUORELA, 1990; VUORELA, 1988), ostruthol isolated from *Peucedanum ostruthium* (RAUWALD *et al*., 1994b), osthol isolated from *Angelica archangelica* (HÄRMÄLÄ *et al*., 1992). Aqueous extract of common rue (*Ruta sp*.) has shown positive chronotropic and inotropic effects on isolated right atria of normotensive rats (CHIU and FUNG, 1997). It also relaxed KCl preconstricted rat tail artery strips probably by a direct effect on the vascular smooth muscle. Extract from *R. graveolens* proved to, besides K^+-currents, also block Na^+-currents, although to a lesser extent, in intact myelinated nerve fibres (BETHGE *et al*., 1991).

The interference at different levels of the cellular calcium regulation demonstrates that many of natural calcium antagonists represent a promising field of research for identifying derivatives which are more effective or able to react on structures not sensitive to synthetic calcium antagonists (CASTALDO & CAPASSO, 1996). It has been suggested that these drugs could be useful tools to better understand channel kinetics and calcium mobilisation from intracellular deposits and to proceed to the synthesis of new molecules with calcium antagonistic action.

2.2.5. Other biological effects and toxicity

Linear furanocoumarin xanthotoxin is capable of inactivating human P450 2A6, the major coumarin 7-hydroxylase present in human liver, at physiologically relevant concentrations (KOENIGS *et al*., 1997) and bergapten against intestinal CYP3A4 (HO *et al*., 2000), and therefore these compounds carry the potential of causing a serious drug-drug interaction with any drug, compound or toxin whose clearance is largely dependent on these enzymes. Psoralen, xanthotoxin and sphondin proved to be inhibitors of coumarin 7-hydroxylase activity both in mice and in human liver microsomes (MÄENPÄÄ *et al*., 1993). WOO and co-workers (1983) investigated the effects of coumarins from *Angelica koreana* on the drug-metabolising enzymes and found imperatorin, isoimperatorin, oxypeucedanin, isooxypeucedanin, and oxypeucedanin methanolate (in decreasing order) to retard the drug metabolism both *in vitro* and *in vivo*. Praeruptorin A, xanthotoxin, psoralen and bergapten isolated from chloroform extract of the root of *Peucedanum japonicum* inhibited monoamine

oxidase (mouse brain) (HUONG *et al*., 1999), and daphnetin proved to be a protein kinase inhibitor in human hepatocellular carcinoma HepG2 cells (YANG *et al*., 1999).

In sensitive tumour cells, coumarin and its derivatives cause significant changes in the regulation of immune responses, cell growth and differentiation (SELIGER, 1997). Coumarins appear to act either directly on tumour cells, or via modulation of the host's immune system, thereby stimulating immune reactivity which leads to protection against recurrence of a particular tumour or even to activation of host defence mechanisms which also help to eliminate small tumour burdens (ZLABINGER, 1997). Direct (*i.e.* non-immunologically mediated) antitumor effects have been shown in a number of studies demonstrating a growth inhibitory capacity for a number of malignant cell lines *in vitro*, *e.g*. extract of the root of *Angelica japonica* (containing scopoletin, japoangelone, oxypeucedanin methanolate, xanthotoxin, bergapten) against human gastric adenocarcinoma MK-1 cell growth (FUJIOKA *et al*., 1999), methanol extract of *Tordylium apulum* (containing umbelliferone, isoimperatorin and an angelicin derivative) against the KB (human rhinopharynx cancer) and NSCLC-N6 (human bronchial epidermoid carcinoma) cancer cell lines (KOFINAS *et al*., 1998).

The toxicological profile of coumarin has been somewhat ambiguous, therefore the US Food and Drug Administration and the National Cancer Institute have nominated coumarin for toxicity and carcinogenicity studies (WEINMANN, 1997). The report states that organ-specific toxicity occurs in species and strains only, that metabolise coumarin qualitatively and quantitatively different from man, and in rodents, chronic lesions and tumorigenesis might be seen after overdosing of the compound for months to years. According to LAKE (1999), the majority of tests for mutagenic and genotoxic potential suggest that coumarin is not a genotoxic agent, and exposure to coumarin from food and/or cosmetic products poses no health risk to humans. However, the possibility for phototoxic effects of furanoderivatives of coumarin as well as hepatotoxic aflatoxins, metabolites from *Aspergillus* species, should be born in mind.

2.3. Use of coumarins in pharmaceutical and chemical industry

The bioactivities of phototoxic psoralens and of dicoumaroul derivatives are well known and several of these compounds are used in antipsoriatic and anticoagulant therapy, respectively (HÖNIGSMANN *et al.*, 1989; MATERN *et al.*, 1999). Besides psoriasis, skin diseases like cutaneous T-cell lymphoma, atopic dermatitis, alopecia areata, urticaria pigmentosa and lichen planus (OLIVER and WINKELMANN, 1993; GOODMAN and GILMAN, 1996) are treated with the photochemotherapy with linear furanocoumarins (also referred to as psoralens) and UVA. The most widely used compound is xanthotoxin (CONCONI *et al.*, 1998). Bergapten is considered a valuable alternative for chemotherapy of psoriasis, since its clinical efficacy is comparable to that of xanthotoxin, although bergapten requires significantly higher cumulative UVA doses. Since skin phototoxicity and genotoxicity seem to be related to the formation of diadducts to DNA, several monofunctional compounds have been synthesised. The introduction of methyl groups at positions 3 or 4 and 4' of the tricyclic structure of xanthotoxin led to compounds such as 3,4'-dimethyl xanthotoxin, entirely monofunctional, which is not genotoxic and phototoxic, although it shows an elevated antiproliferative activity. These features also appeared in methylangelicins.

Coumarin is the parent molecule of warfarin, which acts as a vitamin K antagonist. Warfarin is a clinically useful anticoagulant and widely employed rodenticide whose discovery was based on the studies of the bleeding tendency of cattle suffering from 'sweet clover disease' (sweet clover = *Melilotus officinalis*) (HOULT and PAYÁ, 1996). In the treatment of small-cell lung cancer, the use of warfarin, in conjugation with standard chemotherapy, produces a higher response rate than chemotherapy alone (*e.g.* ZACHARSKI, 1994).

The usefulness of coumarins and coumarin derivatives has been shown in various areas of analysis (COOKE *et al.*, 1997). The inherent fluorescent properties of many coumarins are a key factor in many applications. Areas where coumarins are widely used include estimation of enzymatic activity (*e.g.* derivatives of 7-hydroxycoumarin as fluorigenic enzyme substrates; EGAN *et al.*, 1990), labelling of proteins, antibodies, DNA and lipids (*e.g.* aminomethyl coumarin acetic acid fluorescent labelling antibodies and lectins for staining; EGAN *et al.*, 1990), derivatising agents in chromatography, dyes for tuning lasers in ion analysis (*e.g.* 7-amino-4-methylcoumarin, 4-methylumbelliferone; EGAN *et al.*, 1990), intracellular ion indicators, pH and gas detection, measurement of drug/ion transport, studies on bioreactor characterisation, chemical markers in

kerosene, food adulterant detection and in sensors. 3,4-Dichloroisocoumarin is a commercially available, relatively non-toxic inhibitor, which shows good reactivity with a large number of serine proteases (BEYNON & BOND, 1989).

Coumarin has a wide variety of uses in industry, mainly due to its strong fragrant odour (EGAN *et al*., 1990). Its uses include that of a sweetener and fixative of perfumes (*e.g.* 3,4-dihydrocoumarin), an enhancer of natural oils, such as lavender, a food additive in combination with vanillin, a flavour/odour stabiliser in tobaccos, an odour masker in paints and rubbers, and, finally, it is used in electroplating to reduce the porosity and increase the brightness of various deposits, such as nickel. 6-methylcoumarin is mainly used as a flavour enhancer, and 7-hydroxycoumarin in sunscreens.

3. AIMS OF THE STUDY

This study is based on ethnobotanical knowledge of coumarins and coumarin containing plants *Aegopodium podagraria*, *Anethum graveolens*, *Angelica archangelica*, *Levisticum officinalis*, *Petroselinum crispum*, *Peucedanum palustre* and *Ruta graveolens* growing in Finland. For the scientific evaluation of traditional use of these plants as drugs, the biological activities of the coumarin containing materials were studied with the biological tests, aiming

- to study the anti-inflammatory potential of coumarins in PAF- and fMLP-stimulated neutrophils (**I**)
- to investigate the antimicrobial activity of plant crude extracts and coumarins in agar diffusion tests (**II**)
- to test phototoxicity in a newly developed microwell test which measures phototoxicity and toxicity at the same time (**III**), and
- to increase our knowledge about the mode of action of coumarins on cellular calcium regulation by evaluating the effects of selected plant coumarins on receptor-regulated and store-operated changes in intracellular free calcium concentrations (**IV**, **V**).

4. EXPERIMENTAL

A detailed presentation of the materials and methods can be found in the original publications.

4.1. Materials

4.1.1. Coumarins, sample preparation

The sources of the coumarin compounds used in this study are presented in **Table 5**.
For the anti-inflammatory tests, compounds were used as 10 and 100 μM solutions in 20 % DMSO (**I**), while for the phototoxicity tests, they were dissolved/dispersed in DMSO and saline solution (1 mg of a coumarin in 10 μl of DMSO and 1 ml of saline solution) by sonication for 1 h in an ultrasonic bath (**III**). For the evaluation of the antimicrobial activity, coumarins were dissolved in a suitable solvent (methanol or acetone) to give a concentration of 1 mg/ml (**II**). In the calcium flux experiments, they were dissolved in DMSO to make a stock solution of 10 mg/ml (**IV**, **V**).

4.1.2. Plant material, sample preparation

Leaves of *Aegopodium podagraria* L., *Anethum graveolens* L., *Angelica archangelica* L., *Levisticum officinalis* Koch, *Petroselinum crispum* (P. Mill.) A. W. Hill., and *Ruta graveolens* L. were supplied from Dr. B. Galambosi (senior research scientist at Agricultural Centre, Mikkeli, Finland) in summer 1995. Leaves of *Peucedanum palustre* (L.) Moench were collected from Laajalahti, Espoo, Finland (identified by Docent K. Fagerstedt, Division of Plant Physiology, University of Helsinki, Finland) in 1992. Voucher specimens are deposited at the Division of Pharmacognosy, Department of Pharmacy, University of Helsinki, Finland. Plant material was dried at ambient temperature, and stored in a dry and dark place until use.

Air-dried and mill-powdered plant material was extracted with methanol (**II, III**). Combined extracts from the three consecutive extractions were lyophilised after which they were dissolved/dispersed in DMSO and saline solution by vortex (**III**).

Table 5 Sources of the coumarin compounds used in this study (chemical structures in **Fig. 1**).

Compound	Source
Simple coumarins	
Coumarin	Carl Roth GmbH & Co, Germany; E. Merck KgaA, Germany
Herniarin	Carl Roth GmbH & Co, Germany
Methylumbelliferone	Carl Roth GmbH & Co, Germany
Osthol	isolated[4] from *Angelica archangelica* L.
Ostruthin	isolated[1] from *Peucedanum sp.*
Scopoletin	Sigma Chemical, MO, USA; Carl Roth GmbH & Co, Germany
Umbelliferone	Carl Roth GmbH & Co, Germany
Linear furanocoumarins	
Bergapten	Fluka Chemie AG, Switzerland
Isopimpinellin	isolated[3] from *Angelica archangelica* L.; Carl Roth GmbH & Co, Germany
Peucedanin	isolated[1] from *Peucedanum sp.*
Psoralen	isolated[3] from *Angelica archangelica* L.; Carl Roth GmbH & Co, Germany
Xanthotoxin	isolated[3] from *Angelica archangelica* L.; Carl Roth GmbH & Co, Germany
Linear pyranocoumarins	
Ledebouviellol	isolated[1] from *Peucedanum sp.*
2-Methylchromone derivative of ledebouviellol	isolated[1] from *Peucedanum sp.*
Peuarenarin	isolated[1] from *Peucedanum sp.*
Peuarenin	isolated[1] from *Peucedanum sp.*
Xanthalin	isolated[1] from *Peucedanum sp.*
Angular furanocoumarins	
Angelicin	Carl Roth GmbH & Co, Germany
Apterin	isolated[1] from *Peucedanum sp.*
Athamantin	isolated[1] from *Peucedanum sp.*
Columbianadin	isolated[2] from *Peucedanum palustre* (L.) Moench
Angular pyranocoumarins	
Anomalin	isolated[1] from *Peucedanum sp.*

[1] by Dr. F. Hadacêk (Vienna, Austria); [2] by Prof. H. Vuorela (University of Helsinki);
[3] by Docent P. Vuorela (University of Helsinki); [4] by Docent P. Vuorela and T. Ojala (University of Helsinki)

4.2. Methods

4.2.1. Assay for anti-inflammatory activity (**I**)

Coumarin compounds were tested for their *in vitro* anti-inflammatory potential with human neutrophils. The assay used has been described earlier by TUOMINEN and co-workers (1992). The cells were stimulated with platelet activating factor (PAF) and the chemotactic peptide fMLP, which, together with cytochalasin B, leads to the release of the proteolytic enzyme elastase and makes these intracellular processes measurable extracellularly. After terminating the reaction with citric acid, the inhibitory activity of the test compounds was calculated as the percentage decrease in absorbance measured with a spectrophotometer as compared to vehicle (20 % DMSO).

4.2.2. Assays for antimicrobial activity (**II**)

A hole plate diffusion method was used for the antimicrobial screening of plant extracts and pure coumarin compounds against selected Gram-positive and Gram-negative bacteria, yeasts and mold. After a 1 h period of diffusion at room temperature (or +8 °C for fungi) and 24 h incubation in darkness at +35 °C (or +22 °C for fungi), the effect was evaluated by measuring the diameters of the inhibitory zones. For the plant pathogenic fungi, a disc diffusion method was employed. After prediffusion at +8 °C for 1 h, the Petri dishes were incubated in darkness at room temperature till the mycelium had completely overgrown the control disc, that is 3-11 days, and the evaluation of the effect was classified as retarding, inhibiting or promoting the growth.

4.2.3. Assay for phototoxic activity (**III**)

The dried brine shrimp (*Artemia salina*) eggs were bred as described earlier (EPPLEY, 1974; KIVIRANTA and ABDEL-HAMEED, 1994). One-day-old *A. salina* were pipetted into framed 8-well strips along with the samples of coumarins or lyophilised plant extracts dissolved/dispersed in DMSO and saline solution. Bergapten served as standard compound. After 10-15 min, the larvae were exposed to the UV radiation at 366 nm for 0-20 h. The strips measuring toxicity, which also served as controls for phototoxicity testing, were kept in dark throughout the study. After 24 h from the start, the number of both dead and living larvae was counted under a substage microscope.

4.2.4. Assays for calcium-antagonistic activity (**IV, V**)

The effects of the test compounds on ATP- and thapsigargin-induced changes in intracellular free calcium $[Ca^{2+}]_i$ were investigated in Fluo-3-AM loaded rat thyroid FRTL-5 cells (grown as described by TÖRNQUIST, 1992; **IV**), while the influence of the test compounds on $[Ca^{2+}]_i$ changes evoked by TRH was studied in clonal rat pituitary GH_4C_1 cells (TASHJIAN, 1979, and TÖRNQUIST and TASHJIAN, 1989; **V**). In both assays, fluorescence was measured with fluorometer using an excitation wavelength of 506 nm and that of emission 526 nm. FRTL-5 cells were used for the evaluation of $^{45}Ca^{2+}$ uptake and efflux (methods according to TÖRNQUIST and TASHJIAN, 1989; **IV**), the results were further confirmed with [^{3}H]TRH binding (KARHAPÄÄ

and TÖRNQUIST, 1995), inositol phosphates production (OLDHAM, 1990) and $^{45}Ca^{2+}$ uptake experiments (TÖRNQUIST and TASHJIAN, 1989) in GH_4C_1 cells (**V**). The radioactivity of the samples was detected by liquid scintillation counting. The protein content in binding experiments was determined according to LOWRY *et al*. (1951).

4.2.5. Statistical evaluation

In phototoxicity assays, LC_{50} values and 95 % confidence intervals were determined from the 24-hour counts using the probit analysis method described by FINNEY (1971) (**III**).

Statistical analysis of the data from first calcium assays (**IV**) was made using analysis of variance. Each $[Ca^{2+}]_i$ experiment was repeated 10-13 times, with 2-8 different batches of cells. The $^{45}Ca^{2+}$ experiments were made in triplicate and repeated 2-5 times. Student's t-test was used for the analysis of data from further calcium tests (**V**), and the analysis of [^{3}H]TRH binding data was performed with Prism® (version 3.0; GraphPad Software Inc., San Diego, CA, USA).

5. RESULTS AND DISCUSSION

5.1. Anti-inflammatory activity (I)

Neutrophils play a central role in acute inflammation. Their main function is host protection by generating and releasing agents that destroy invading micro-organisms. Two of the chemoattractants that are involved in the migration of neutrophils from the circulation to the tissue are the phospholipid metabolite platelet activating factor (PAF) and the bacterial product N-formyl-Met-Leu-Phe (fMLP). The functional responses that follow the recognition of these compounds include phagocytosis, secretion of proteolytic enzymes such as elastase, and the production of free oxygen radicals. One approach to measure these functional responses is via PAF induced exocytosis of elastase method developed by TUOMINEN and co-workers (1992). The reaction of elastase with its substrate leads to formation of a coloured product, which can be quantified by UV measurement.

In this work, the effects of twenty coumarins on elastase secretion in human neutrophils were evaluated with the above-mentioned test. Four compounds, namely psoralen, xanthotoxin, ledebouviellol and athamantin, showed significant activity (**Table 6**). When the dose-response curves were determined, the potency of athamantin and ledebouviellol was found to be of the same magnitude as that of the reference compound BN 52021, which is a PAF antagonistic gingkolide isolated from the Chinese fossil tree *Gingko biloba* (BOHLIN *et al*., 1997). None of the four coumarins was selective towards PAF or fMLP receptor because there was no difference between the inhibition against PAF or fMLP stimulated response. One possible target for the compounds may lie in the early phases of intracellular signalling since a common pathway is used in the transduction of signals from both of these receptors. An additional possibility could be interference in calcium regulation of the cell, *e.g.* inhibition of the receptor-mediated influx of calcium – however, this has not been proven yet. Furthermore, inhibition of the enzyme elastase would give positive results in the assay used. Direct effects on elastase activity were tested and none of the coumarins were identified as elastase inhibitors. Besides the inhibition of neutrophil function studied in this work, coumarin compounds have been found to possess also other anti-inflammatory effects (**Table 6**).

Due to the low number of compounds found to be active, it is not possible to draw conclusions about the structural features essential for their effect. However, some structural specificity was seen

in their action, since some of their structural relatives that were also tested were not capable of inhibiting elastase release: linear furanocoumarins psoralen and 8-methoxypsoralen (xanthotoxin) were active while 5-methoxypsoralen (bergapten) was not; the activity of ledebouviellol disappeared when hydroxylgroup was changed to methoxylgroup (the 2-methylchromone derivative of ledebouviellol); and angular furanocoumarin athamantin lost its activity when the two iso-valeryl-substituents were changed to β-glucosyl and hydrogen (apterin) (chemical structures in **Fig. 1**; results in **I**, Table 1, and **Table 6**).

Table 6 Activities of the coumarin compounds in anti-inflammatory tests reported here and in the literature.

Compound	Dose or conc.			Inhibition [%]		Test system	Reference
Angelicin	100	µM		17.1		COX-1; preparation from sheep seminal vesicles	ROOS *et al*., 1997
	100	µM		3.9		5-LO; intact porcine leucocytes	”
Athamantin	61	µM		50		PAF induced elastase secretion	I
	87	µM		50		fMLP induced elastase secretion	I
Bergapten	10	mg/kg	i.p.	39	5 h	carrageenan induced rat paw oedema	CHEN *et al*., 1995
	10	mg/kg	i.p.	27	1 h	formalin induced rat paw oedema	”
	100	µM		15.9		COX-1; preparation from sheep seminal vesicles	ROOS *et al*., 1997
	50	µM		6.3		5-LO; intact porcine leucocytes	ROOS *et al*., 1997
Coumarin	20	mg/kg	p.o.	34		carrageenan induced rat paw oedema	LINO *et al*., 1997
	20	mg/kg	p.o.	44		carrageenan induced rat paw oedema	LEAL *et al*., 2000
	20	mg/kg	p.o.	22		dextran induced rat paw oedema	LINO *et al*., 1997
	20	mg/kg	p.o.	5.8		dextran induced rat paw oedema	LEAL *et al*., 2000
	4100	µM		50		PAF induced rabbit platelet aggregation	OKADA *et al*., 1995
	3400	µM		50		AA induced rabbit platelet aggregation	”
	3400	µM		50		collagen induced rabbit platelet aggr.	”
Herniarin	100	µM		65		inhibition of PGE_2-release from mouse peritoneal macrophages	SILVÁN *et al*., 1996
	100	µM		2.9		inhibition of LTC_4-release from mouse peritoneal macrophages	”
Ledebouviellol	121	µM		50		PAF induced elastase secretion	I
	134	µM		50		fMLP induced elastase secretion	I
Psoralen	100	µM		33		PAF induced elastase secretion	I
	100	µM		34		fMLP induced elastase secretion	I
	0.5	mg	/ ear	41.8		TPA induced inflammation in mice	GARCIA-ARGAEZ *et al*., 2000
Scopoletin	231	µM		50		inhibition of prostaglandin synthetase	FARAH &
	10	µg	/ ear	50		ethyl phenylpropiolate-induced oedema in rats	SAMUELSSON, 1992
	2600	µM		50		PAF induced rabbit platelet aggregation	OKADA *et al*., 1995
	500	µM		50		AA induced rabbit platelet aggregation	”
	1000	µM		50		collagen induced rabbit platelet aggr.	”
	100	µM		77		inhibition of PGE_2-release from mouse peritoneal macrophages	SILVÁN *et al*., 1996
	100	µM		8.8		inhibition of LTC_4-release from mouse peritoneal macrophages	”
Umbelliferone	10	mg/kg	i.p.	44	5 h	carrageenan induced rat paw oedema	CHEN *et al*., 1995
	10	mg/kg	i.p.	30	1 h	formalin induced rat paw oedema	”
	20	mg/kg	p.o.	38		carrageenan induced rat paw oedema	LINO et al., 1997
	3100	µM		50		PAF induced rabbit platelet aggregation	OKADA *et al*., 1995
	2500	µM		50		AA induced rabbit platelet aggregation	”
	1900	µM		50		collagen induced rabbit platelet aggr.	”
Xanthotoxin	100	µM		31		PAF induced elastase secretion	I
	100	µM		33		fMLP induced elastase secretion	I
	10	mg/kg	i.p.	40	5 h	carrageenan induced rat paw oedema	CHEN *et al*., 1995
	10	mg/kg	i.p.	25	2 h	formalin induced rat paw oedema	”
	100	µM		5.0		COX-1; preparation from sheep seminal vesicles	ROOS *et al*., 1997
	100	µM		3.5		5-LO; intact porcine leucocytes	”

5.2. Antimicrobial activity (II)

The use of higher plants and preparations made from them to treat infections is an age-old practice and in past possibly the only method available. Diffusion and dilution methods have been employed to study the antimicrobial activity of medicinal plants. A number of modifications have been made in the technique in order to obtain better results. Since the information given in many of the reports is very variable, for instance the amount of the sample on the agar (*e.g*. LAMIKANRA *et al*., 1990; LI *et al*., 1997) or the results of the negative control are not mentioned (*e.g*. MARTINEZ *et al*., 1996; TERESCHUK *et al*., 1997), it is difficult to compare these methods. Therefore certain standardisation of antimicrobial testing would be advantageous, as already suggested by Brantner and co-workers (BRANTNER, 1997; BRANTNER *et al*., 1994).

In these investigations, the agar-diffusion method described in the European Pharmacopoeia was further developed to serve as a screening procedure when searching for antimicrobial agents. On the basis of the solvent tolerance tests, the 40:60 mixture of methanol and 0.1 M phosphate buffer solution pH 8.0 used in the European Pharmacopoeia was replaced with methanol or acetone depending on the type of plant extracts and compounds examined. These solvents are frequently used in plant extracts, nearly all of the identified components from plants active against micro-organisms are aromatic or saturated organic compounds and as such most often obtained through ethanol or methanol extraction (COWAN, 1999), and they did not differ significantly from the buffer solution with respect to the antibacterial properties. However, in plant pathogenic fungi tests, the samples had to be absorbed on paper discs and left to dry properly before application to the Petri dish in order to avoid the solvent toxicity. The advantage of large volumes of the hole-plate diffusion method utilised for bacteria, yeasts and mold was necessary for the low-soluble compounds. The method used in this study is qualitative, but needs a lot of compound. However, it is discriminatory for the bioassay-directed isolation of active components. It will serve as a reference method when we are aiming to more effectiveness by using microwell plates (unpublished results HAANSUU *et al*.).

Coumarins are considered phytoalexins since they are produced by the plant as a defence mechanism against attack by other organisms (BERENBAUM, 1991, WEINMANN, 1997). The selected plant species had total coumarin contents of between 5 – 834 μg/ml in their methanol leaf extracts calculated from the bergapten standard curve and determined with an HPLC method

developed by VUORELA and co-workers (1989), and, as they and the pure coumarins studied were merely active against plant pathogens (**II**, Tables 1 and 2), this study supports the role of coumarins and furanocoumarins as defensive compounds. Extract of *P. palustre* possessed inhibition against the widest spectrum of plant pathogenic fungi. Extracts of *P. crispum* and *R. graveolens* are suggested either to possess good antimicrobial potency or to contain active principle(s), which could belong to the group of coumarins, essential oils or flavonoids. Substances could also be present in the extract that stimulate the growth of the micro-organisms, as was especially evident in the case of *Phytophtora (cactorum)*, thus counteracting the effect of inhibitory substances.

5.3. Phototoxicity (III)

The phototoxic properties of furanocoumarins and related compounds have been assayed using fungi (*e.g*. DANIELS, 1965; SLUIS *et al*., 1981; GIBBS, 1987), green algae (SCHIMMER *et al*., 1980), bacteria (ASHWOOD-SMITH *et al*., 1983), laboratory animals (NILSSON *et al*., 1993) and cultured human skin systems (EDWARDS *et al*., 1994; DAMOUR *et al*., 1998). The early methods were basically similar to those used for testing the antimicrobial properties of compounds, further coupled with irradiation of the samples by UV light at 366 nm in the presence of the test organism on agar dishes. Later, a thin layer chromatographic procedure was combined with detection of the antimicrobial properties of components in the sample that were separated (SLUIS *et al*., 1981), and nowadays cultured human skin systems are available (*e.g*. EDWARDS *et al*., 1994; DAMOUR *et al*., 1998). Here, a new method employing *Artemia salina* was developed for phototoxicity testing in order to screen quickly the possibly phototoxic compounds and extracts. In addition, toxicity can be measured at the same time.

Artemia salina (brine shrimp) has been successfully used for toxicity testing (KIVIRANTA *et al*., 1991; KIVIRANTA and ABDEL-HAMEED, 1994). The advantages of *A. salina* as a test organism are that it is easy to grow from dried eggs viable for several years and easily available, it is small enough to be contained in a small liquid volume (EPPLEY, 1974), and no complex instrumentation is needed. *A. salina* larvae are more sensitive to toxicants than adults, it is not necessary to feed them, *i.e*. to change the saline solution prior or during the test, and they are in a similar physiological condition therefore reducing the variation (van STEERTEGEM and PERSOONE, 1993).

For the development of this method, test compounds were chosen to represent different structural types of coumarins: umbelliferone, a simple coumarin, exhibited no phototoxicity, while the linear furanocoumarins bergapten, psoralen and xanthotoxin showed phototoxic activity (**Table 7**), this effect was abolished in the absence of radiation. These results are in accordance with the results of other phototoxicity tests (SLUIS *et al*., 1981; ASHWOOD-SMITH *et al*., 1983; GIBBS, 1987; EDWARDS *et al*., 1994), and they were the basis for choosing the radiation times for the examination of the plant extracts. Conveniently, *A. salina* method can be used for simultaneous toxicity studies, and peucedanin was found to be toxic to the larvae irrespective of the irradiation time.

Suitability of the *A. salina* method for the phototoxicity testing of plant material was tested with leaf extracts of six plants from Apiaceae and one from Rutaceae (**III**, Table 2). Leaf extracts containing one or more above mentioned linear coumarins exhibited phototoxic activity with the exception of the extract of *Anethum graveolens*, which was poor in coumarin content. Leaf extracts of *Levisticum* did not contain any of these three linear coumarins, and neither toxic nor phototoxic effects were observed, while *Ruta graveolens* exhibited both phototoxicity and toxicity. In order to investigate the influence of the changes in total coumarin content during the growth period, *A. salina* tests were performed at different stages of flowering. *Aegopodium podagraria* showed the most remarkable changes: the coumarin content decreased but toxic effects appeared, which indicated an increase of some other compounds responsible for this phenomenon.

With the method described here it is not possible to distinguish active and inactive components in a mixture or in an extract. However, the overall procedure is rapid: one can screen active plant extracts, isolate compounds from them for structural elucidation, and finally screen the compounds for activity. *A. salina* screening test supplement animal bioassays with which there can be problems caused by differences for example in skin penetration and the presence of hair. Active compounds verified by the *A. salina* screening test could later be subjected to more elaborate bioassays like laboratory-grown human dermis and epidermis.

Table 7 Summary of the phototoxic effect of the coumarin compounds used in this study reported here and in the literature.

Compound	Concentration			Effect	Wavelength	Test system	Reference
Athamantin	1000	µg/ml		not phototoxic	366 nm	*Artemia salina*	III
Bergapten	11	µg/ml	4 h	phototoxic	366 nm	*A. salina*	III
	0.008	µg		”	”	TLC; *Penicillium expansum*	SLUIS *et al*., 1981
	10	µg		”	320-380 nm	*Candida albicans*,	ASHWOOD-SMITH
	5	µg		”	”	*Escherichia coli*,	*et al*., 1983
	0.5	µg		”	”	*Micrococcus luteus*,	”
	0.5	µg		”	”	*Saccharomyces cerevisiae*	”
	0.001	µg		”	”	TLC, *E. coli*,	”
	0.008	µg		”	”	TLC, *P. expansum*	”
	5	mM		”	366 nm	*C. albicans*	GIBBS, 1987
Isopimpinellin	96	µg/ml	4 h	not phototoxic	366 nm	*A. salina*	III
	2	µg		”	”	TLC; *P. expansum*	SLUIS *et al*., 1981
	> 10	µg		”	320-380 nm	*C. albicans*,	ASHWOOD-SMITH
	> 10	µg		”	”	*E. coli*,	*et al*., 1983
	> 10	µg		”	”	*M. luteus*,	”
	> 10	µg		”	”	*S. cerevisiae*	”
Peucedanin	17	µg/ml	0 h	toxic	366 nm	*A. salina*	III
Psoralen	0.04	µg/ml	4 h	phototoxic	366 nm	*A. salina*	III
	0.008	µg		”	”	TLC; *P. expansum*	SLUIS *et al*., 1981
	0.5	µg		”	320-380 nm	*C. albicans*,	ASHWOOD-SMITH
	1	µg		”	”	*E. coli*,	*et al*., 1983
	0.5	µg		”	”	*M. luteus*,	”
	0.5	µg		”	”	*S. cerevisiae*	”
	0.05	ng		”	”	TLC, *E. coli*,	”
	0.008	µg		”	”	TLC, *P. expansum*	”
Umbelliferone	1000	µg/ml		not phototoxic	366 nm	*A. salina*	III
Xanthotoxin	25	µg/ml	4 h	phototoxic	366 nm	*A. salina*	III
	0.004	µg		”	”	TLC; *P. expansum*	SLUIS *et al*., 1981
	0.5	µg		”	320-380 nm	*C. albicans*,	ASHWOOD-SMITH
	5	µg		”	”	*E. coli*,	*et al*., 1983
	1	µg		”	”	*M. luteus*,	”
	0.1	µg		”	”	*S. cerevisiae*	”
	0.001	µg		”	”	TLC, *E. coli*,	”
	0.004	µg		”	”	TLC, *P. expansum*	”
	5	mM		”	366 nm	*C. albicans*	GIBBS, 1987
	5	%		”	280-420 nm	Skin[2]	EDWARDS, 1994

5.4. Effects on calcium fluxes (IV, V)

Columbianadin, isolated from *Peucedanum palustre* roots, has been shown to possess calcium channel blocking activity in the test with rabbit aortic rings, the test system measuring calcium-dependent prolactin release with rat pituitary GH_3 cells, and depolarisation-induced uptake of calcium with a potency comparable to verapamil (VUORELA *et al.*, 1988b; TÖRNQUIST and VUORELA, 1990). Athamantin, isolated from *P. oroselinum*, showed the highest activity in inhibition of depolarisation induced uptake of $^{45}Ca^{2+}$ in rat pituitary GH_4C_1 cells when athamantin and four linear coumarins were tested (HADACÊK *et al.*, 1991). Archangelicin, osthol, isoimperatorin, imperatorin and phellopterin, all isolated from *Angelica archangelica* roots, inhibited the uptake of $^{45}Ca^{2+}$ in GH_4C_1 cells (HÄRMÄLÄ, 1991). These results lead to further investigations presented in this thesis, and a simple coumarin, osthol, a linear furanocoumarin, xanthotoxin, and an angular furanocoumarin, columbianadin, were chosen to represent different structures of coumarins. FRTL-5 rat thyroid cells were chosen as the test system because they are non-excitable cells with both receptor- and store-operated calcium entry pathways (TÖRNQUIST, 1992, 1993). The rationale for using GH_4C_1 cells is that the calcium channels in these cells have been thoroughly characterised and the cells used in a multitude of studies on calcium channels (*e.g.* KOSHIYAMA and TASHJIAN, 1991; TÖRNQUIST, 1991).

In rat thyroid FRTL-5 cells, osthol prevented the ATP-induced influx of extracellular calcium (**IV**; results here in **Table 8**). The effect of osthol on the ATP-evoked increase in $[Ca^{2+}]_i$ was probably due to modification of the IP_3 pathway, because the calcium transient was decreased in medium containing calcium and medium without calcium. Osthol also decreased the thapsigargin evoked change in $[Ca^{2+}]_i$ in buffer containing calcium, suggesting that osthol inhibited the thapsigargin evoked influx of extracellular calcium (*i.e.* store-operated calcium entry). Furthermore, part of the decreased ATP response was probably also due to an attenuation of store-operated calcium entry, as the thapsigargin evoked increase in $[Ca^{2+}]_i$ also was attenuated. Columbianadin had no effect on the ATP-evoked increase in $[Ca^{2+}]_i$.

The mechanism of action of osthol and xanthotoxin on agonist-evoked changes in $[Ca^{2+}]_i$ were further delineated in rat pituitary GH_4C_1 cells (**V**; results here in **Table 8**, see also **Fig. 1**). Especially the effects of these compounds on the ligand-receptor interaction were possible to test as the binding of TRH to its receptors is well characterised and relatively easy to measure (HINKLE,

1989; GERSHENGORN and OSMAN, 1996). The results show that osthol, but not xanthotoxin, interferes with the binding of TRH to its receptor, and thus alters receptor-evoked intracellular signals, *i.e*. the production of IPs and the release of sequestered calcium. The mechanism through which osthol interacted with the receptor is not clear at present. It may function as a competitive antagonist, or it could modulate the binding indirectly, *i.e*. by modulating receptor-G protein interactions, or it may activate intracellular signalling pathways which could modulate receptor function. However, it is clear that the effect on the receptor is rather compound-specific than a non-selective feature of all coumarins.

Table 8 Summary of the effects on the calcium fluxes and calcium-mediated effects of the coumarin compounds used in this study reported here and in the literature.

Compound	Conc.		Effect	Test system	Reference
Columbianadin	61	µM	no effect on ATP-evoked $\Delta[Ca^{2+}]_i$	FRTL-5 cells	IV
	61	µM	enhancement of TG-evoked $\Delta[Ca^{2+}]_i$	”	”
	55	µM	50 % inhibition of barium induced contractions	guinea pig ileum	THASTRUP *et al.*, 1983
	3.6	µM	50 % inhibition of KCl-induced contractions	rabbit aorta rings	VUORELA, 1988b
	100	µM	inhibition of TRH-induced release of PRL	GH_3 cells	VUORELA *et al.*, 1988b
Osthol	77	µM	inhibition of ATP-induced influx of extracellular calcium,	FRTL-5 cells	IV
	77	µM	decrease in TG-evoked $\Delta[Ca^{2+}]_i$	”	”
	77	µM	decrease in TRH-evoked $\Delta[Ca^{2+}]_i$	GH_4C_1 cells	V
	77	µM	decrease in the formation of IPs in TRH-stimulated cells,	”	”
	77	µM	modulation of the binding of TRH to its receptor,	”	”
	77	µM	decrease in depolarisation-evoked uptake of extracellular calcium	”	”
	2200	µM	50 % inhibition of spontaneous cell beating,	mouse myocardial cells	NAMBA *et al.*, 1988
	500	µM	modest reduction in calcium uptake	”	”
	500	µM	inhibition of ouabain-induced	”	”
	500	µM	inhibition of ouabain-induced calcium uptake	”	”
	16.4	µM	50 % inhibition of depolarisation-induced uptake of extracellular calcium	GH_4C_1 cells	HÄRMÄLÄ *et al.*, 1992
Xanthotoxin	93	µM	no effect on agonist-evoked $\Delta[Ca^{2+}]_i$	FRTL-5 cells	IV
	93	µM	no effect on TRH-evoked $\Delta[Ca^{2+}]_i$,	GH_4C_1 cells	V
	93	µM	no effect on the formation of IPs,	”	”
	93	µM	no effect on the binding of TRH to its receptor,	”	”
	93	µM	no effect on depolarisation-evoked uptake of extracellular calcium	”	”
	93	µM	41 % inhibition of depolarisation-induced uptake of extracellular calcium	GH_4C_1 cells	HÄRMÄLÄ *et al.*, 1992
	551	µM	50 % inhibition	guinea pig papillary muscle	NEUHAUS-CARLISLE *et al.*, 1996

5.5. Verification of traditional use of coumarin containing plants as drugs

In this study, coumarin containing plants were tested for their antimicrobial and phototoxic activities. As the traditional uses presented in **Table 2** does not correlate directly to these activities (perhaps with the exception of *P. crispum* as a repellent against lice), results of the coumarins found in these plants with respect to their relevance in the traditional use of these plants are discussed.

- *Aegopodium podagraria*

In our investigations, *A. podagraria* showed only modest activity. Pure coumarins found in it (**Table 1**), angelicin and apterin, failed to show any anti-inflammatory activity, and the total coumarin content was low, therefore the traditional use described in the literature as a remedy for rheumatism (~ arthritis) (**Table 2**) is not likely to be explained with the presence of coumarins in this plant.

- *Anethum graveolens*

A. graveolens contains, among other compounds, xanthotoxin, scopoletin, umbelliferone and bergapten (**Table 1**). Xanthotoxin was active in the anti-inflammatory tests. Xanthotoxin, scopoletin and umbelliferone exhibited strong inhibition against the plant pathogen *Fusarium culmorum*. Phototoxicity of the linear furanocoumarins bergapten and xanthotoxin was proven with the new *Artemia salina* method. However, extracts of *A. graveolens* had only a modest activity in the antimicrobial tests and were inactive in the phototoxicity which might be attributed to the low coumarin content.

- *Angelica archangelica*

A. archangelica was the second richest in total coumarin content, it contains for instance psoralen and xanthotoxin (**Table 1**), active in our anti-inflammatory tests, which might, at least in part, explain its ethnobotanical use as a remedy for cough, colds and rheumatism (**Table 2**). The strong phototoxic effect seen with *A. archangelica* can be ascribed to the presence of psoralens (bergapten, psoralen, xanthotoxin). The antimicrobial effect of *A. archangelica* was more pronounced against the plant pathogens.

- *Levisticum officinale*

As the coumarins found in *L. officinale*, apterin, bergapten, coumarin and umbelliferone (**Table 1**), were not active in the anti-inflammatory test, and the amount of coumarins was low, the antitussive and antiedematous properties utilised in the folk medicine (**Table 2**) are likely to be due to compounds of other type. However, a clear dependence of the phototoxicity on the coumarin content was proven: the phototoxicity of *L. officinale* observed before the flowering period was abolished as the coumarin content in the leaves decreased after flowering.

- *Petroselinum crispum*

Methanol extract of *P. crispum* possessed inhibitory activity against the widest spectrum of microbes, and furthermore, it contains xanthotoxin (**Table 1**), active in our anti-inflammatory tests, which might partly explain its ethnobotanical use as a remedy for cough, colds and rheumatism (**Table 2**). Xanthotoxin was not active in calcium flux tests suggesting that other mechanisms may explain its use as a remedy for hypertension (**Table 2**). Although the total amount of coumarins in *P. crispum* was not high, the compounds present (*e.g*. bergapten and xanthotoxin) were highly phototoxic ones.

- *Peucedanum palustre*

While *P. palustre* was the richest in total coumarin content, the coumarins *e.g*. bergapten and peucedanin (**Table 1**), were not active in the anti-inflammatory test, the folk medicinal use as a treatment for cough, rheumatism, fever and colds (**Table 2**) is therefore likely due to other substances and/or mechanisms. Additionally, the antimicrobial effects of the leaf extract were more specifically against plant pathogenic fungi. Strongly phototoxic coumarins seem to appear on the later stages of plant development since the phototoxic effect strengthens although the amount of coumarins in leaves decreases during the vegetative period.

- *Ruta graveolens*

Xanthotoxin and psoralen found in *R. graveolens* (**Table 1**) might, at least to some extent, explain its ethnobotanical use as a remedy for cough, colds and rheumatism (**Table 2**). This was further supported by the inhibitory activity of the leaf extract against the widest spectrum of microbes which might, at least partly, be addressed to xanthotoxin, umbelliferone, herniarin and scopoletin present in this plant, especially the latter two (**Table 4**). Besides phototoxic, *R. graveolens* was also toxic to *A. salina* larvae.

6. CONCLUSIONS

Natural coumarins, like other unsaturated lactones, may exert various effects on living organisms. The extensive range of physiological effects, both in plants and in animals, in addition to low toxicity (with the exception of phototoxicity, which, however, with careful dosimetry is one of the most important indications to this group), relative cheapness, presence in the daily groceries as spices, fruit and vegetables and occurrence in various herbal remedies of coumarins, led us to investigate more thoroughly properties of some natural coumarins and coumarin containing extracts with ethnopharmacological background.

Twenty coumarin compounds were studied for their anti-inflammatory properties in an *in vitro* model for elastase secretion in human neutrophils using PAF and fMLP as stimuli. The activities of the compounds used were relatively modest and non-specific, psoralen, xanthotoxin, ledebouviellol and athamantin being the most active ones. The anti-inflammatory activity may be involved in their ethnobotanical use as remedies for cough, cold and rheumatism (arthritis). Besides anti-inflammatory activity, psoralen and xanthotoxin were active also in the phototoxicity tests, and both of these activities may support their use in the treatment of psoriasis.

Antimicrobial screening against selected Gram-positive and Gram-negative bacteria, yeasts, mold, as well as plant pathogenic fungi, with emphasis on method optimisation was carried out on methanol extracts prepared from seven plants growing in Finland. The selected plant species were merely active against plant pathogens which supports the role of coumarins and furanocoumarins as defensive compounds rather for the plant itself. The agar-diffusion methods (discs, holes) utilised serve as reference methods when developing a more effective microwell plate test.

Microwell technique was already applied in developing an easy-to-perform and inexpensive method, the *Artemia salina* (brine shrimp) larvae test, for screening phototoxic compounds. Linear furanocoumarins bergapten, psoralen and xanthotoxin were phototoxic and umbelliferone as a simple coumarin was not, as expected along with earlier observations. Methanol extracts from seven coumarin containing plants were also studied, and the clearest phototoxic activity was observed with *Angelica archangelica* throughout the vegetative period and with *Peucedanum palustre* at later stages of growth. Additionally, it is possible to investigate also toxicity at the same time with the same concentrations, as seen with peucedanin and the extract of *Ruta graveolens*.

Based on earlier observations (VUORELA, 1988; TÖRNQUIST and VUORELA, 1990; HÄRMÄLÄ *et al.*, 1992), columbianadin, osthol and xanthotoxin were used to evaluate further the mode of action on calcium fluxes in rat thyroid FRTL-5 cells as well as in rat pituitary GH_4C_1 cells. The results suggest that osthol may prove to be a useful compound for investigations on ligand-receptor interaction, and some coumarins may have a dual mechanism of action: in addition to their earlier documented effects on VOCCs, they may also interact with receptor-mediated signalling events.

In conclusion, coumarin compounds can be suggested to be beneficial for the plants themselves as natural biocontrolling antipathogenic compounds, and for human beings as dietary supplements on the basis of their mild antimicrobial and anti-inflammatory effects, and as reference compounds in various bioactivity tests. The use of these compounds as medicinal agents is of importance in the case of hyperproliferative skin diseases like psoriasis.

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