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Limonoids and coumarin derivatives in *Citrus trifoliata* and *Foeniculum vulgare*

Ph.D. Thesis

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LIST OF PUBLICATIONS RELATED TO THE THESIS

I. **Kerekes D**, Horváth A, Kúsz N, Borcsa B. L; Szemerédi N, Spengler G, Csupor D. Coumarins, furocoumarins and limonoids of Citrus trifoliata and their effects on human colon adenocarcinoma cell lines
Heliyon **2022**, 8(9): e10453
Scopus – Multidisciplinary; SJR: Q1 IF: 4

II. **Kerekes D**, Csorba A, Gosztola B, Németh-Zámbori É, Kiss T, Csupor D. Furocoumarin content of Fennel – below the safety threshold
Molecules **2019,** 24(15): 2844
Scopus – Pharmaceutical Science; SJR: Q1 IF: 3.267

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TABLE OF CONTENTS

ABBREVIATIONS AND SYMBOLS

1D	one-dimensional
2D	two-dimensional
AcNi	acetonitrile
ATCC	American Type Culture Collection
C.	*Citrus*
CCCP	carbonyl cyanide *m*-chlorophenylhydrazine
CI	combination index
COSY	correlated spectroscopy
COX	cyclooxygenase
DMSO	dimethyl sulfoxide
EB	ethidium bromide
ED_{50}	median effective dose
EDTA	ethylenediaminetetraacetic acid
ELS	evaporative light scattering
EMA	European Medicines Agency
EMEM	Eagle's Minimal Essential Medium
EtOAc	ethyl acetate
F.	*Foeniculum*
FACS	fluorescence-activated cell sorting
FAR	fluorescence activity ratio
FL-1	mean fluorescence intensity
GRP78	78-kDa glucose-regulated protein
HCl	hydrochloric acid
HMBC	heteronuclear multiple-bond correlation spectroscopy
HSQC	heteronuclear single-quantum coherence spectroscopy
HPLC	high-performance liquid chromatography
IL	interleukin
iNOS	inducible nitric oxide synthase
JMOD	J-modulated spin-echo experiment
LB	Luria–Bertani
MeOH	methanol
MIC	minimum inhibitory concentration
Mill.	Miller
MPLC	medium pressure liquid chromatography
MS	mass spectroscopy
MTT	3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NMR	nuclear magnetic resonance
NOESY	nuclear Overhauser enhancement spectroscopy
OD	optical density
P.	*Poncirus*
PANC	pancreatic cancer
PARP	poly (ADP-ribose) polymerase
PBS	phosphate buffered saline
PTFE	polytetrafluoroethylene
RF	relative fluorescence

R_f	retention factor
RP	reversed-phase
RSD	relative standard deviation
SDS	sodium dodecyl sulphate
SRM	single reaction monitoring mode
subsp.	subspecies
TSB	tryptic soy broth
TLC	thin-layer chromatography
TNF	tumor necrosis factor
UV	ultraviolet
var.	varietas
VIS	visible light spectrophotometry

1. INTRODUCTION

One of the most important areas of pharmacognosy is the investigation of specific metabolites. Such investigations may be part of basic or applied research projects, but the analysis of plant metabolites serves common interests regardless of the goals of researchers. Phytochemical analysis can be qualitative or quantitative, and it can focus on identifying or quantifying active components that are responsible for beneficial benefits (as a food or a medicinal plant) or that play a role in toxic or adverse effects.

Certain groups of compounds deserve special attention, particularly in light of the discovery of novel bioactivities that may be related to therapeutic usage or safety concerns. Such compounds belong to limonoids, that are common compounds in *Citrus* species, and furocoumarins, which are found in a variety of food plants (for example, fennel). Limonoids are notable for their antibacterial and antiproliferative properties, but furocoumarins, in addition to their impact on cancer cells, may be responsible for deleterious effects. There are several studies showing that furocoumarins have toxic effects, the most common of which is phototoxicity, which has been demonstrated in human studies and is also exploited for medicinal purposes (PUVA therapy) [1]. Due to the potential toxic effects, the European Medicines Agency (EMA) restricts the use of certain furocoumarin-containing plants, especially in pregnancy and childhood.

Fennel fruits are widely used to treat a variety of gastrointestinal symptoms, even in children and infants, and are also used as a galactogogue [2]. They are most frequently consumed as tea. Although it is known that fennel fruits contain furocoumarins, the rational safety assessment is problematic due to a lack of extensive information on furocoumarin content. The aim of our research was to quantitatively measure the furocoumarin content of various fennel fruit samples in order to provide relevant information for risk assessment, considering all of the theoretically possible risks associated with consuming this furocoumarin-containing plant.

Citrus trifoliata has been used by Korean Oriental Medicine practitioners to treat various cancer types [3]. Fruits were also traditionally used in Asian folk medicine as antiphlogistic and to treat dysentery, gastritis, and digestive ulcers. Experimental data support many of these uses. In activated human mast cells, the fruit extract reduced the expression of pro-inflammatory cytokines [4]. *In vitro* testing has proven that plant extracts have anti-cancer activity against various cancer cell lines. These bioactivities may be associated with the furocoumarins and limonoids of the fruits, given that these substances have previously been shown to have cytotoxic and antiproliferative effects [5], [6]. However, other types of compounds may also

play a role in the overall effect of the fruit. Therefore, our aim was to systematically investigate the chemical composition the peel and seed of *Citrus trifoliata* with special focus on furocoumarins and limonoids, including the bioactivities which may be related to the supposed anticancer activity of the plant.

The present work is a summary of phytochemical and pharmacological investigations conducted on two plants, *Foeniculum vulgare* and *Citrus trifoliata*. Although the scientific methodologies and approaches used are varied, the main goals, namely the quantification, isolation, and bioactivity assessment of plant metabolites, are all related to improving the rational and safe use of food and medicinal plants.

2. AIMS OF THE STUDY

The aim of this work was to:

- review the literature of *Foeniculum vulgare* and *Citrus trifoliata*, from aspects of its botany, phytochemical characteristic and pharmacological properties of the plants
- measure the furocoumarin content of the different fennel samples with chromatographic technics,
- preparation of the seed and the peel of *Citrus trifoliata* extracts,
- isolation of limonoids and coumarins from the seed and the peel of *Citrus trifoliata* using a combination of different chromatographic methods,
- investigate the pharmacological activity of the isolated compounds.

3. LITERATURE OVERVIEW

3.1 *Citrus trifoliata*

3.1.1 Botany of *Citrus trifoliata*

Citrus trifoliata L. also known as Japanese or Chinese bitter orange belongs to the order of Sapindales, Rutaceae family, Aurantioideae subfamily, Citreae tribe. Its name was first published by *Carl Linné* in 1763 in his book Species Plantarum [7]. The taxon was named as *Poncirus trifoliata* by Carl Peter Thunberg in 1784 in his book that described the Japanese flora [8].

Trifoliate orange (*Poncirus*) was for many years considered a mono-typic genus, with trifoliate leaves unique among the true citrus fruit trees. Moreover, *Citrus trifoliata* has the highest degree of cold hardiness among the *Citrus* species, is tolerant to several diseases of Citrus species (including Citrus tristeza virus). In the 1980's, a new species, *Poncirus polyandra*, was classified as the second member of the genus. *P. polyandra* differs from *P. trifoliata* by its larger leaves, its flowers being evergreen [9].

Figure 1. Fruits of *Citrus trifoliata* (Source: Botond Lajos Borcsa)

The division of *Citrus* and *Poncirus* genera has been debated. One important argument for the inclusion of *Poncirus* into the *Citrus* genus is their sexual compatibility. Many fertile hybrids have been produced between *Citrus* species and *P. trifoliata*. Citrange, citrumelo, citremon, citradia, and citrandarin result, respectively, from hybridization between sweet orange,

grapefruit, lemon, sour orange, and mandarin with *P. trifoliata*. This fact, together with the high collinearity and synteny between genetic maps [10] and cytogenetic maps [11] of *Poncirus* and *Citrus* species support the proposal of Mabberley [12] to integrate *Poncirus* into the *Citrus* genus. However, although early analysis of plastids placed *Poncirus* within the *Citrus* genus [13], consistent with a single genus, the sequencing of the genome revealed a divergency that may justify the existence of the separate *Poncirus* taxon [14]. This contradiction may be explained by ancient hybridization between a *Citrus* and an unidentified more distant relative [15]. The authors of the most recent taxonomical work on *Citrus* species agree that the majority of data suggest that *P. trifoliata* belongs to the *Citrus* species and therefore its accepted name is *C. trifoliata* [9].

C. trifoliata is deciduous shrub or small tree, exhibits an impressive stature, reaching heights of 2.5 to 6 meters and often spreading as wide. Its branches are smooth, green, and characterized by their crooked and angular nature. The shrub boasts spines measuring 2.5 to 5 centimeters in length, known for their exceptional stiffness, straightness, and sharpness. The leaves typically consist of three, occasionally five, leaflets that are obovate in shape. The middle leaflet spans 4 to 5 centimeters in length, while the side leaflets are half as large. The leaf-stalks have a winged appearance. Prior to the emergence of leaves, sweetly scented flowers bloom from the axils of the spines. These pure white flowers, measuring 4 to 5 centimeters across, exhibit four or five concave obovate petals. The stamens are pink and separate. The flowers are hermaphrodite and are pollinated by insects. The whole plant, but especially the flowers, is strongly aromatic. The fruit resembles a small orange in both color and shape, spanning approximately 4 centimeters in diameter and covered with downy fuzz [16], [17].

This species originates from Korea and Northern China [17]. While fruit ripening is infrequent at colder climate, the plant is frost resistant and consistently produces flowers in spring. Optimal conditions for *C. trifoliata* include a sunny location and deep, moderately rich soil. Citrus grafted onto *C. trifoliata* tend to be more resilient than grown on their own roots because of the relative hardiness of the plant. *C. trifoliata* is an easily grown, extremely thorny plant, which makes it an excellent impenetrable hedge [17], [18]. A cultivar called "Flying Dragon" has twisted, contorted stems making an even more excellent barrier hedge [19].

3.1.2 Uses of *Citrus trifoliata*

The fruits have a flavor that is somewhat akin to lemon, although they are quite bitter and seedy. Fruits are not edible fresh, but can be used to make marmalade or a cool beverage. In dried and

powdered form, the fruits can be used as a condiment. However, the fruits are typically kept on the tree where they last far into the winter and are attractive [17], [18].

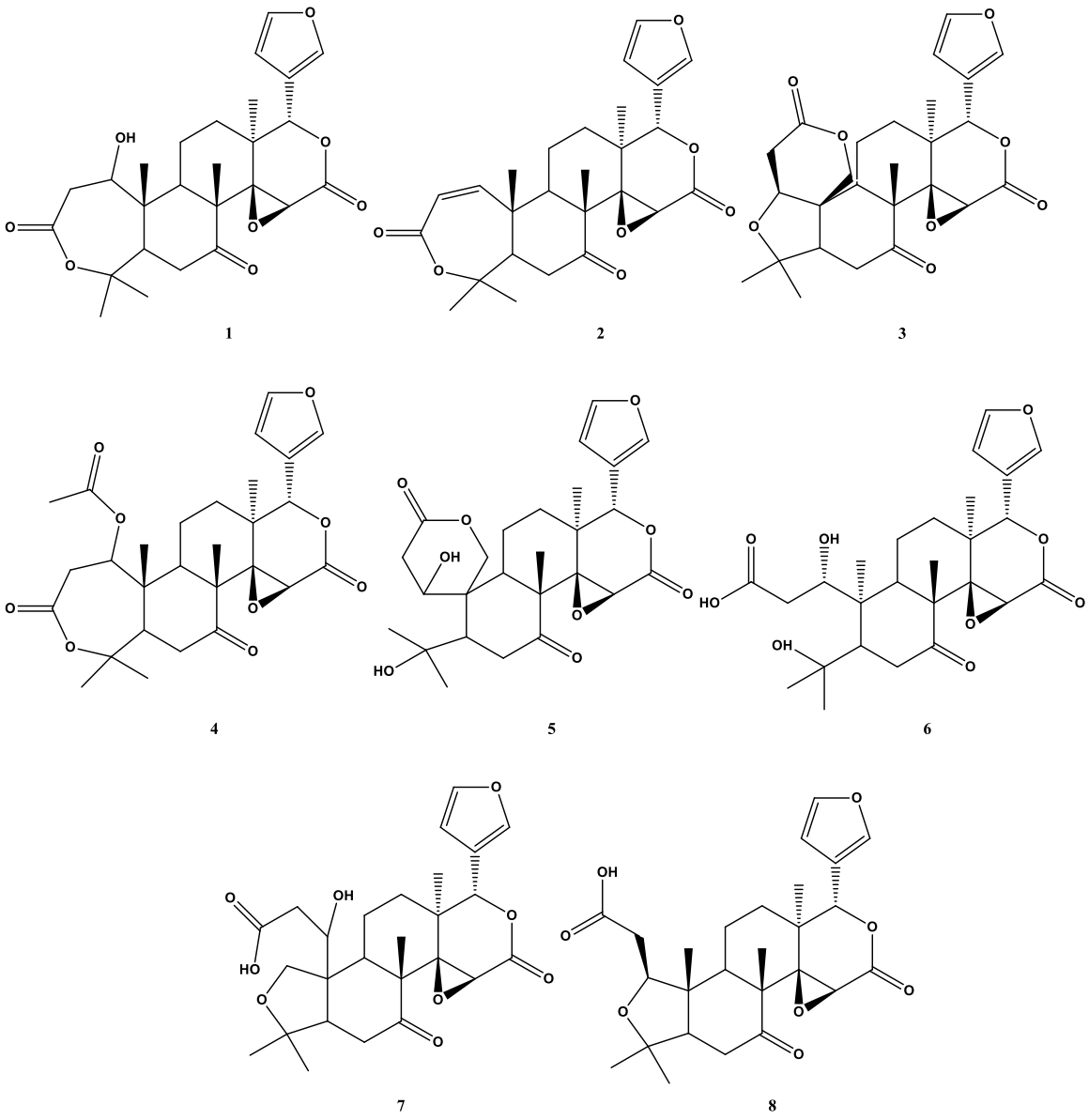
Ponciri Fructus is widely used in Asian traditional medicine as a prokinetic agent to correct the abnormal contraction of the uterus, to enhance blood flow, and to treat gastric reflux illness [20]. The use of the fruit is most widespread in China and Korea.

Ponciri Fructus, according to Traditional Chinese Medicine philosophy, can modify the appropriate flow of stagnated qi energy, remove unwanted food accumulation, reduce excess mucus, and eradicate unwanted mass formations in the body, therefore it is commonly used to treat chest pain, chest fullness, bronchitis, and asthma [21]. In Traditional Chinese Medicine, the fruit is applied as when the endocarp and seeds are removed. It is believed that it works more effectively to release food stagnation and blockages to vital energy in the spleen and stomach than the immature fruit since it has a softer effect [22]. The unripe fruit is used for its antidiarrheic, antiemetic, antispasmodic, digestive, diuretic, laxative, stimulant, and vasoconstrictor properties [23]. The fruits are used for the management of allergic diseases [24]. In China, the thorns are applied as a toothache remedy, whereas the stem bark is used to treat common colds [25].

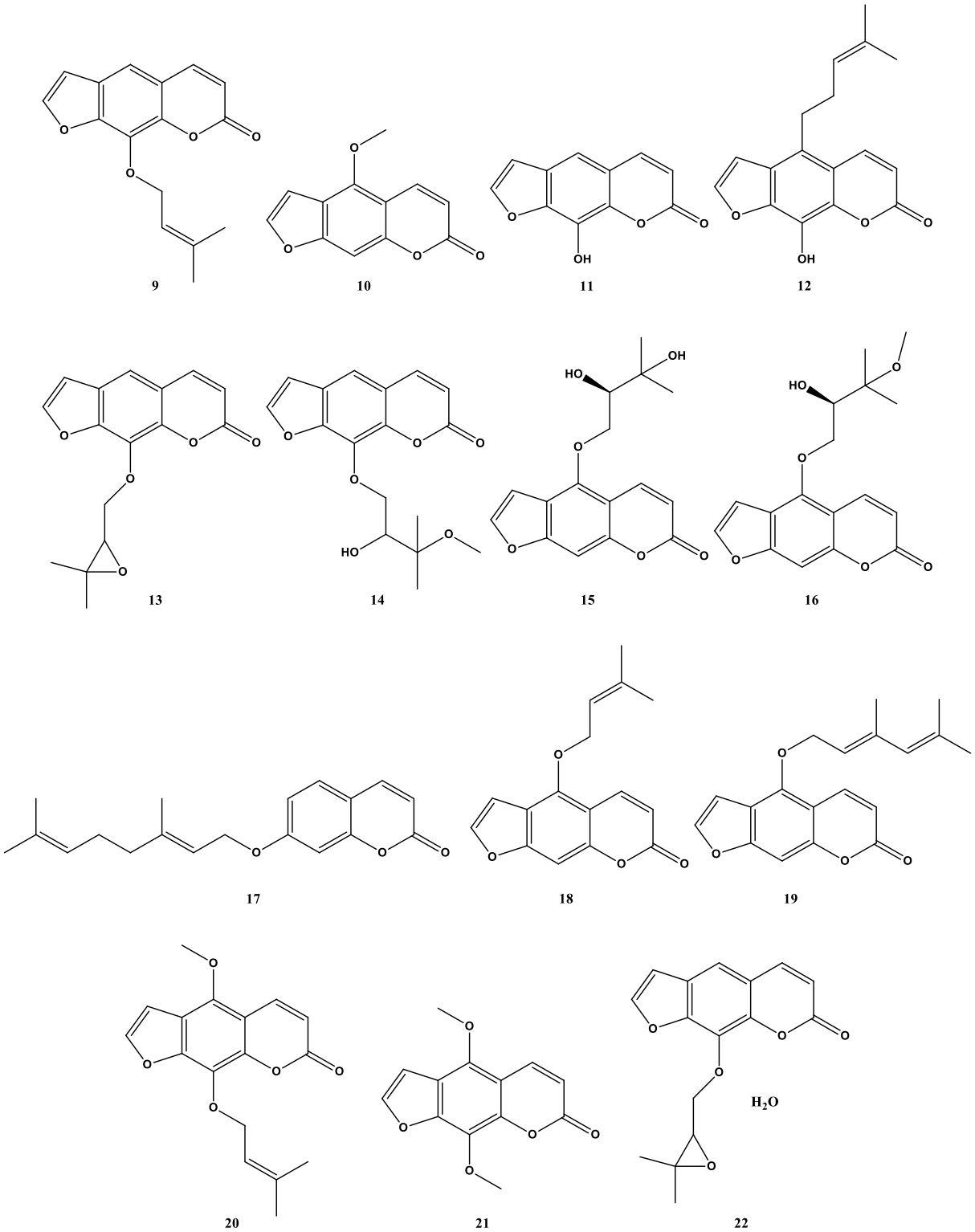
The extract of the fruits is used in Korea to treat a variety of gastrointestinal pathologies. The dried immature fruits are often used as folk treatments for hernia difficulties, sinusitis, sleeplessness, and to remove sputum [26], [27]. The fruits are incorporated in several Chinese and Korean formulations that are widely used for various diseases [20].

3.1.3 Phytochemical characteristics

The bitter taste of the fruit is due to the presence of limonoid compounds. Deacetyl nomilin (**1**) was the first limonoid discovered in *C. trifoliata* seeds [28]. Obacunone (**2**), limonin (**3**), and nomilin (**4**) have also been identified later [29]. The major limonoids in the seeds are deacetylnomilin (230 ppm, **1**) and limonin (390 ppm, **3**), followed by ichangin (20 ppm, **5**) and the acidic limonoids deacetyl nomilinic acid (2 ppm, **6**), isolimonic acid (2 ppm, **7**), and isoobacunoic acid (trace quantity, **8**) [30].

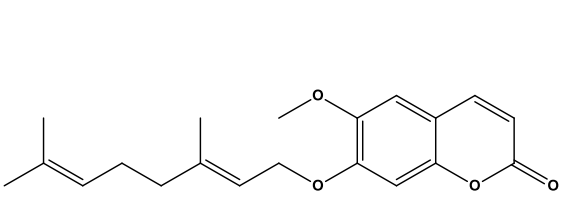


Furocoumarins are further characteristic compounds of the species, the first members of this group (namely imperatorin (**9**), bergapten (**10**), xanthotoxol (**11**) and alloimperatorin (**12**) reported in the 1960's from the plant [28], [29]. Heraclenin (**13**), heraclenol 3′-methyl ester (**14**), (R)-oxypeucedanin hydrate (**15**), oxypeucedanin methanolate (**16**), 7-geranyloxycoumarin (**17**), isoimperatorin (**18**), bergamottin (**19**), epoxyimperatorin (**13**) and phellopterin (**20**) are also present in the fruits [24], [31]–[36]. In the seeds of the plant, imperatorin 0.15% was reported as the major furocoumarin, followed by bergapten (**9**) 0.06%, whereas isopimpinellin (**21**), prangenin (**13**) and prangenin hydrate (**22**) were found in the order of less than 0.01% [37].



The coumarins auraptene (**17**), and 6-methoxy-7-geranyloxycoumarin (**23**) were also reported [38]. Poncimarin (**24**), isoponcimarin (**25**), isoschininallylol (**26**), 7-(3′-methyl-2′,3′-epoxybutyloxy)-8-(3″-methyl-2″,3″-epoxybutyl)coumarin (**27**) and the bis- and di-isoprenylated coumarins O-methyltriphasiol A (**28**), triphasiolene A (**29**), O-methylponciol A (**30**), O-methylponciol B (**31**) and 7-(6S-hydroxy-3,7-dimethyl-2,7-octadienyl)oxy-6-

methoxycoumarin (**32**) were identified as new compound from the fruits, whereas triphasiol (**33**), ponciol (**34**), isoschinilenol (**35**), scopoletin (**36**), umbelliferon (**37**), 7-(6S-hydroperoxy-3,7-dimethyl-2E, 7-dienyloxy)coumarin (**38**), 7-O-(7′-peroxygeranyl)coumarin (**39**) and 7-(6R-hydroxy-3,7-dimethyl-2E, 7-octadienyloxy)coumarin (**40**) were described previously from other taxa [24], [32], [35], [39], [40]. Heraclenol 3′-methyl ester (**14**) , phellopterin (**20**), (R)-oxypeucedanin hydrate (**15**) and oxypeucedanin methanolate (**16**) were isolated from the fruits [34]–[36].



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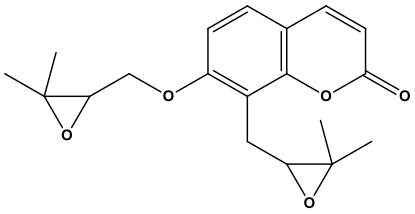

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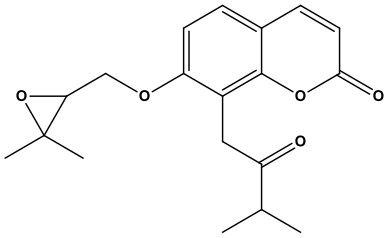

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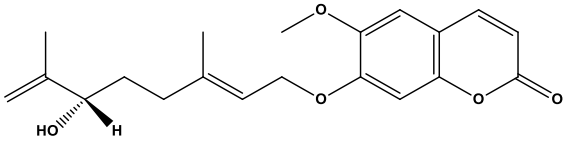

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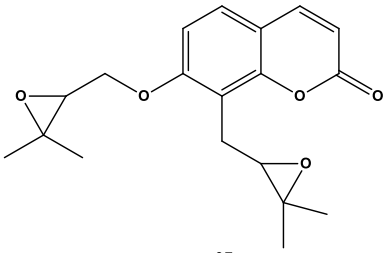

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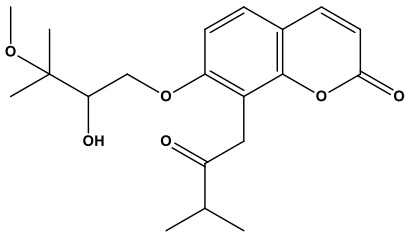

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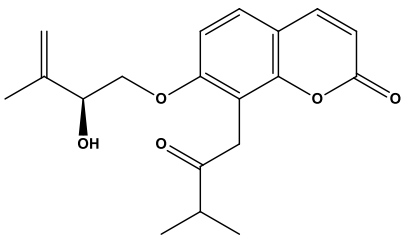

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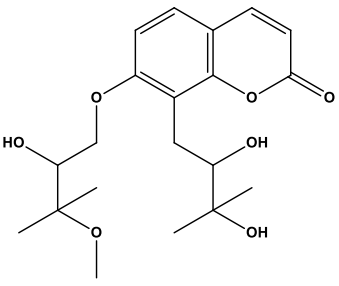

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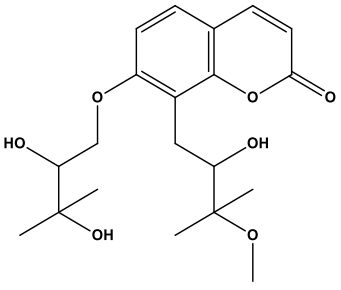

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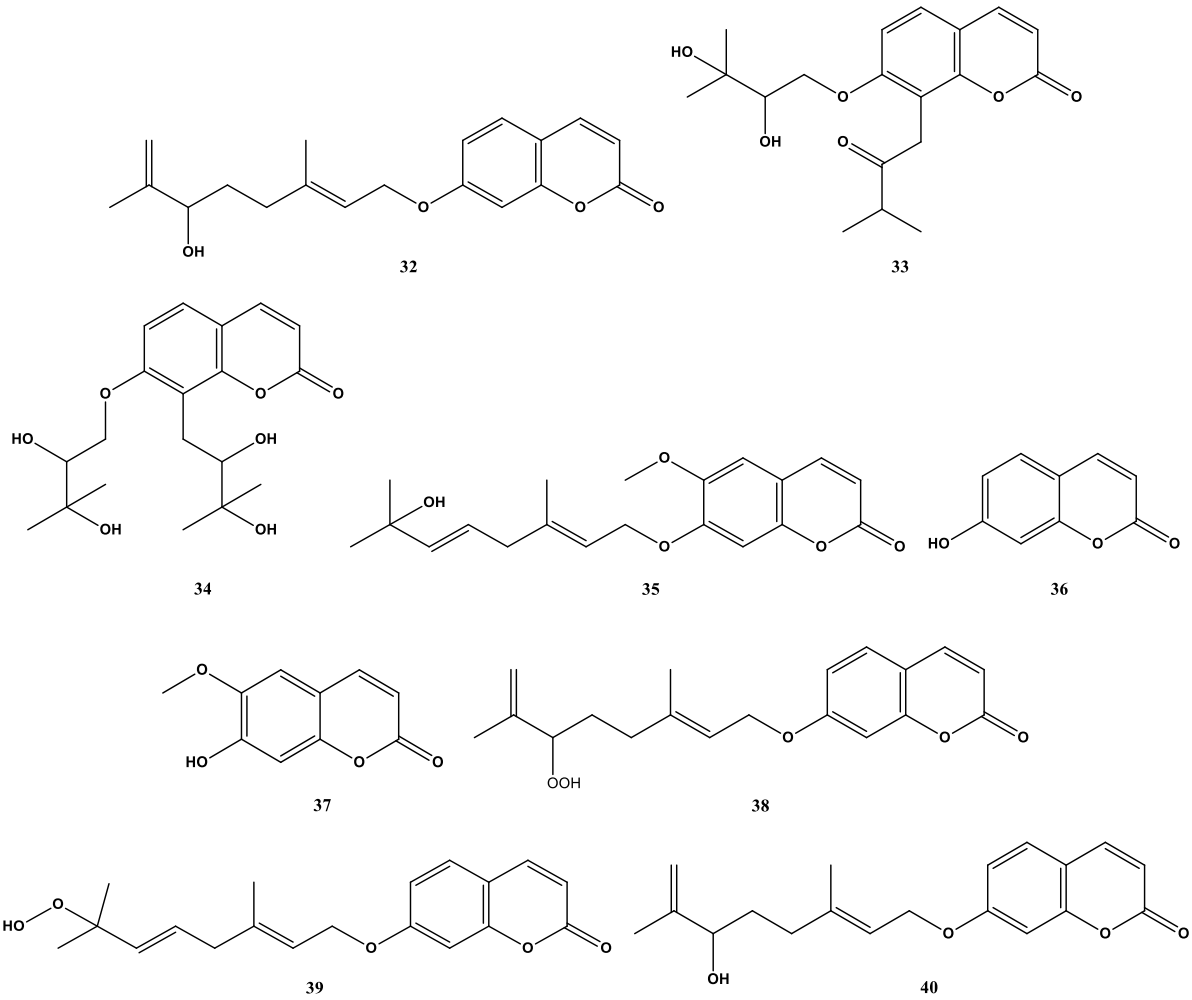
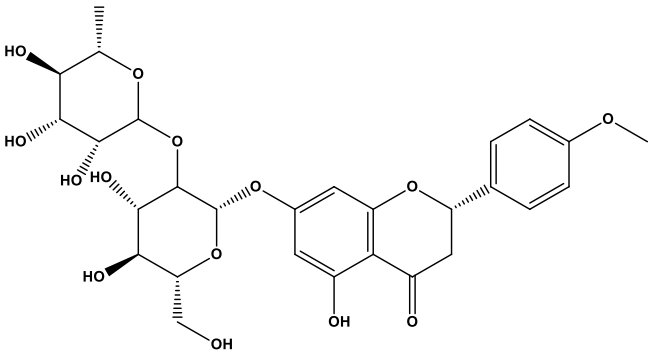


Flavonoids are the predominant special metabolites in the fruit, with poncirin (**41**) as the main compound, which accounts for the 6% of the dry weight of the fruit. Hesperidin (**42**), neohesperidin (**43**), nobiletin (**43**), sinensetin (**45**) [41]–[43], narirutin (**46**), naringin 4′-glucoside (**47**) [44], [45], naringin (**48**), neoponcirin (**49**) [46], [47] poncirenin (**50**) [48], and hesperidin methyl chalone (**51**) [34] have also been reported.



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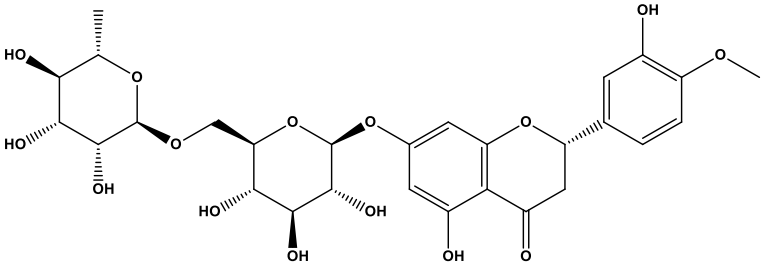

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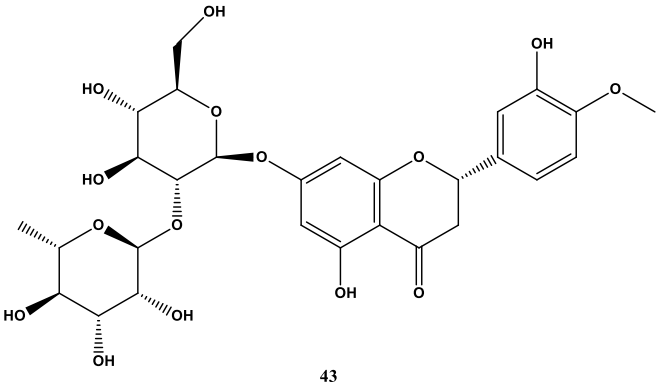

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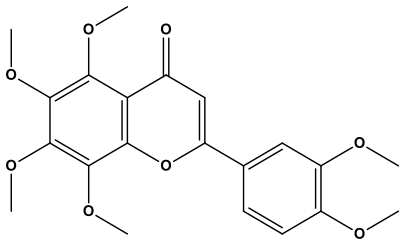

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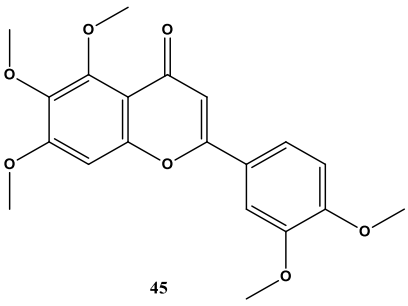

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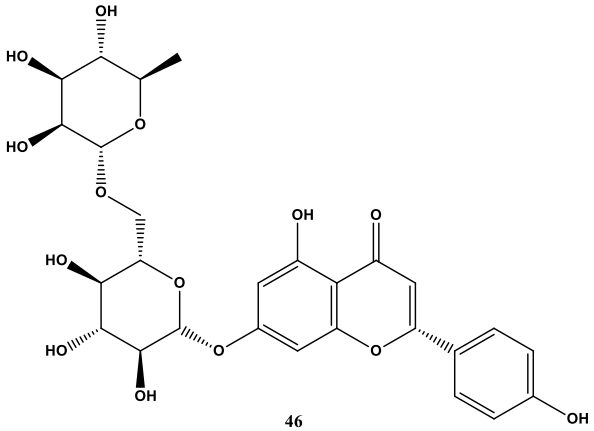

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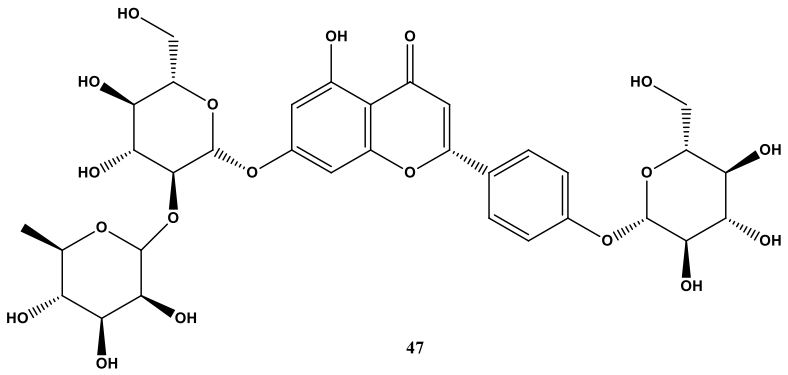

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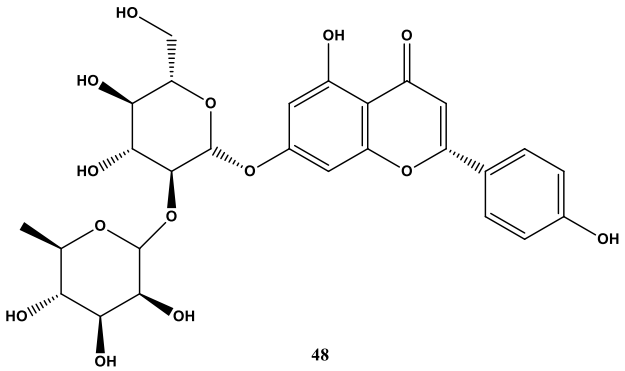

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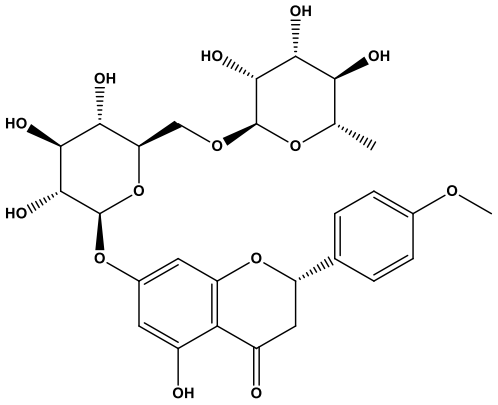

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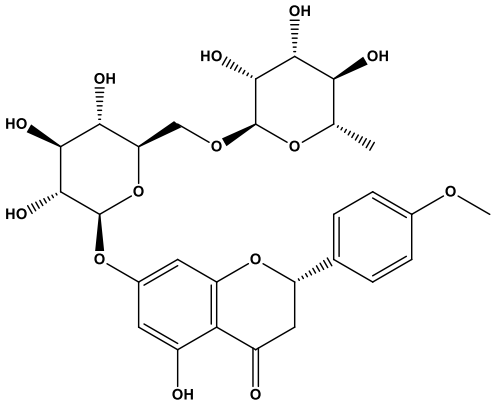

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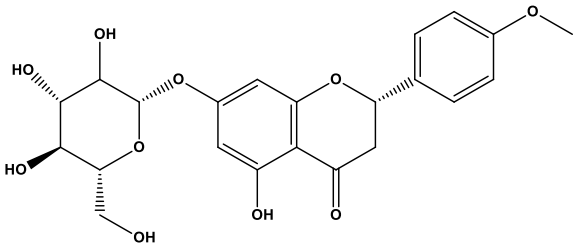

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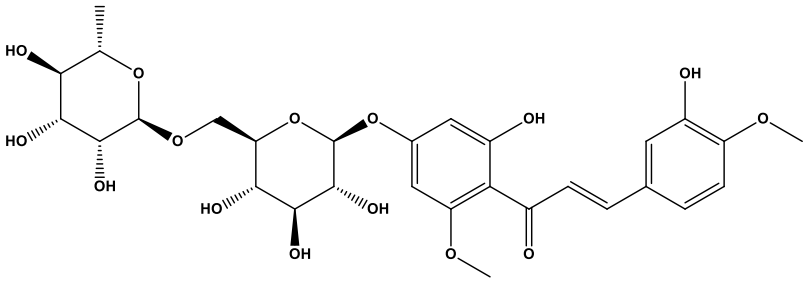

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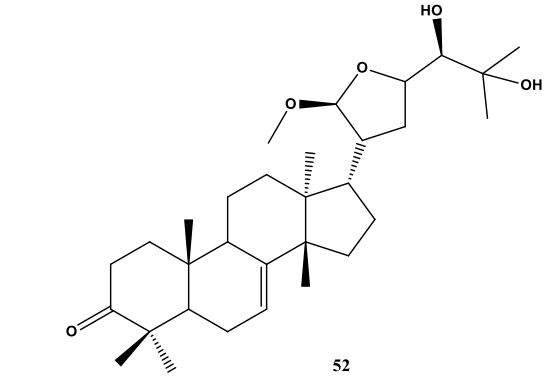
The fruit is also source of triterpenes (21β-methylmelianodiol (**52**), 21α-methylmelianodiol (**53**), hispidol β 25-methyl ether (**54**), hispidol α 25-methyl ether (**55**), 25-methoxyhispidol A (**56),** pancastatin A (**57**) and B (**58**) [24], [35], [49], [50].



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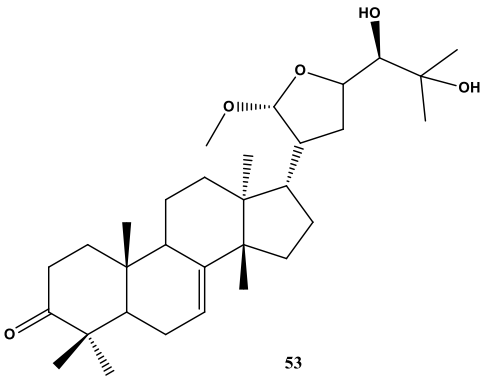

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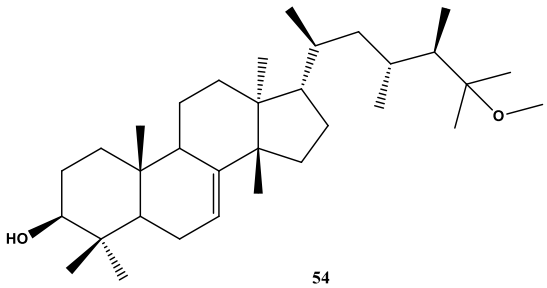

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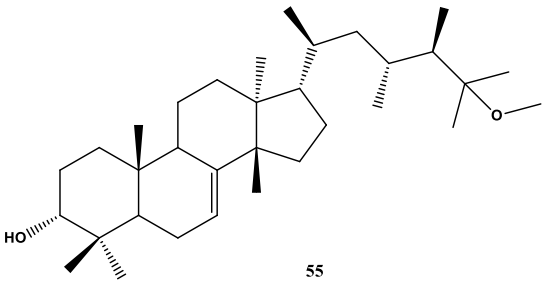

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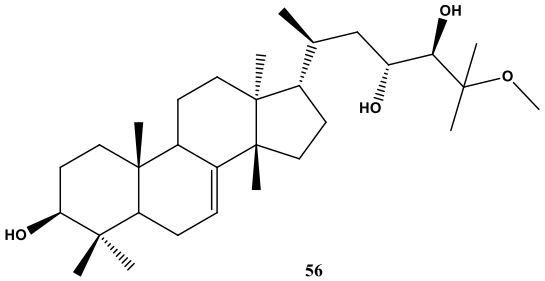

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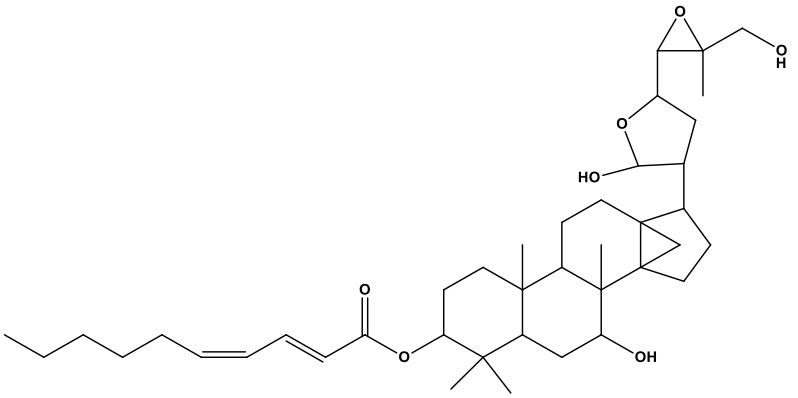

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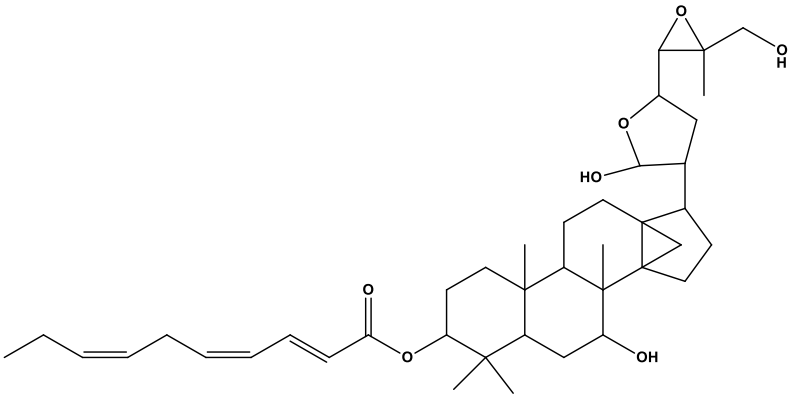

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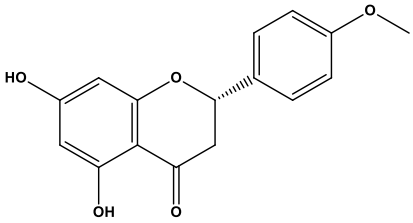
3.1.4 Pharmacological activities related to ethnomedicinal uses

C. trifoliata fruits have been widely used in Asian folk medicine as antiphlogistic, to treat digestive ulcers, gastritis and dysentery. Many of these uses are supported by experimental data. The extract of the fruit exerted anti-inflammatory activity in vitro on Raw 264.7 cells, by inhibiting nitric oxide, tumor necrosis factor alpha and interleukin-6 production and matrix metallopeptidase activity [51]. The fruit extract decreased the expression of pro-inflammatory cytokines in activated human mast cells [4].

The triterpenoid hispidol α 25-methyl ether (**55**) inhibited bacterial infection-induced neuroinflammation in mice by inhibiting the production of proinflammatory cytokines, and also exhibited anti-inflammatory activity in a carrageenan-induced paw edema model of mice [52], [53]. 21 (α,β)-methylmelianodiol (**52, 53**), a triterpene of the fruits has key role in the anti-inflammatory effect, since it was not only effective in the carrageenan-induced paw edema model, but its mechanism of action was also partly revealed [attenuation of expression of iNOS (inducible nitric oxide synthase), COX-2 (cyclooxygenase-2), TNF-α (tumor necrosis factor alpha) and IL-1β (interleukin-1 beta)] [54]. Poncirin (**41**) inhibited the lipopolysaccharide-induced prostaglandin E2 and interleukin-6 production in murine macrophage cells [48]. The

coumarins also may play role in the anti-inflammatory effect, since imperatorin (**9**) and phellopterin (**20**) were shown to have strong anti-inflammatory effects in an in vivo experiment [55].

The extract of the fruit improves mucus secretion and accelerates gastric emptying, thereby may ameliorate gastroesophageal reflux disease [56]. Poncirin (**41**, and its metabolite, ponciretin, **59**) inhibited the growth of Helicobacter pylori, which may contribute to the gastrointestional effects of the plant [47]. In an animal experiment, the aqueous extract of the immature fruit accelerated the intestinal transit [26], whereas the hexane extract stimulated the motility of rat distal colon [57], referring to its prokinetic activity. Neohesperidin (**43**) and poncirin (**41**) significantly inhibited the HCl/ethanol-induced gastric lesions, and increased the pH and mucus content in the stomachs of rats [42].



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The fruit has also been used for the treatment of various cancers among Korean Oriental Medical doctors [3]. In vitro activity against cancer cells was observed for crude extracts and triterpenes and furocoumarins as well.

Ponciri Fructus methanolic extract has been shown to specifically induce apoptosis in glucose-deprived pancreatic cancer (PANC-1) cells. The extract decreased the production of GRP78, a marker that boosts cell survival and lowers apoptosis during stress response [58]. The triterpenoids pancastatin A (**57**) and B (**58**) exerted cytotoxicity and reduced the expression of the GRP78 protein in PANC-1 cells [35]. In an in vitro experiment on HL-60 human leukemia cells, the extract of the fruit was cytotoxic in a concentration- and time-dependent manner. It induced apoptosis accompanied by the activation of caspase-3 and the specific proteolytic cleavage of PARP [poly (ADP-ribose) polymerase]. However, the extract was not cytotoxic in normal peripheral blood mononuclear cells [3]. The extract of the immature fruit inhibited the proliferation of CT-26 colorectal cancer cells and it induced mitochondrial autophagy and apoptosis through the protein kinase B/mammalian target of rapamycin and 5′-AMP-activated protein kinase pathways [59]. Different exctracts exerted antiproliferative activities on pituitary

tumor cells (GH3) [60], triple-negative breast cancer by increasing apoptosis through kinase enzymes [61].

Bergamottin (**19**), imperatorin (**9**), isoimperatorin (**18**) and epoxyimperatorin (**13**) inhibited the growth of pancreatic cancer cells (Panc-28) by inducing apoptosis in culture models, epoxyimperatorin (**13**) being the most active [33]. Imperatorin (**9**) and limonin (**3**) inhibited he growth of liver cancer (SNU 449), and colon cancer (HCT-15) cells by cell cycle arrest and apoptosis promotion [62]. The triterpenoid 25-methoxyhispidol A (**56**) exerted anticancer activity against human hepatocarcinoma cells (SK-HEP-1) by induction of apoptosis and by cell cycle arrest in the G1 phase [49].

3.2 *Foeniculum vulgare*

3.2.1 Botany of *Foeniculum vulgare*

Foeniculum vulgare Mill. (fennel) belongs to the order of Apiales, Apiaceae family [63]. The name *Foeniculum vulgare* is accredited to Philip Miller, who published it in the "Gardeners Dictionary" in 1768 [64]. In the European Pharmacopoeia, *Foeniculum vulgare* Miller subsp. *vulgare* var. *vulgare* and *Foeniculum vulgare* Miller subsp. *vulgare* var. *dulce* (Mill.) Batt. & Trab. are the official names of bitter and sweet fennel [65]. These taxa are also classified as *Foeniculum vulgare* subsp. *capillaceum* var. *vulgare*, and *F. vulgare* subsp. *capillaceum* var. *dulce* [66], however, more recently these taxa are generally mentioned as *Foeniculum vulgare* var. *vulgare* Mill and *Foeniculum vulgare* var. *dulce* Mill. in the literature.

The plant originated in the southern Mediterranean region and has spread wild throughout Asia, North America, and Europe, thanks to naturalization and cultivation. Fennel is an essential component in current French and Italian cuisine. It is often planted in vegetable and herb gardens for its anise-flavored bulb, leaf and seeds, all of which are used in cooking [63].

F. vulgare is a 2 m tall, branching perennial herb with soft, feathery, almost hair-like leaves. The leaves can grow to be up to 40 cm long and are finely divided, with the last segments filiform (threadlike) and around 0.5 mm broad. In July and August, the beautiful golden flowers appear in huge, flat terminal umbels with thirteen to twenty rays. Fruits are 3-5 mm length and 1.5-2.0 mm wide, with an oblong to ovoid shape. The stylopodium is still present on the fruit. Because the seed in the fruit is linked to the pericarp, the entire fruit is frequently referred to as "seed." The fruits are slightly curved, and slightly obtuse at the ends, greenish-yellow in colour, similar to hay, from which the name fennel is derived. The seeds ripen from September through October [63].

Fennel is widely cultivated using three varieties: *Foeniculum vulgare* var. *dulce* Mill. named sweet fennel, *Foeniculum vulgare* var. *vulgare* Mill., known as bitter fennel and *Foeniculum vulgare* var. *azoricum* Mill., also called bulbing fennel or Florence fennel [67]. Bitter fennel has slightly pungent and bitter-sweet fruits, sweet (or Roman) has sweet-tasting fruits, whereas bulbing fennel is cultivated for its fleshy leaf sheaths at the base of the stems [68]. Florence fennel plants are smaller than the other two varieties. There are some botanical distinctions between the varieties *vulgare* and *dulce*. The stem height of var. *vulgare* can reach 2 m while var. *dulce* just 1.3 m. The bitter fennel has 6-7 stems, whereas the sweet fennel has one stem. The quantity of umbels also varies; the sweet fennel has fewer umbels but weighs more than the bitter fennel. Sweet fennel has a vegetation period of 150-160 days, and bitter fennel has a vegetation period of 190-200 days, or longer depending on the climatic circumstances, therefore it may not reach maturity in some years. However, bitter fennel has a stronger root than sweet fennel, so it can withstand colder weather better [69].

3.2.2 Uses of *Foeniculum vulgare*

Ancient Romans, Egyptians, Indians, and Chinese were all familiar with fennel. The ancient Greeks and Romans valued fennel and used it as medicine, nourishment, and bug repellent. Fennel tea was said to give warriors courage before battle. Prometheus, according to Greek mythology, used a large stalk of fennel to transport fire from Mount Olympus to Earth. It was grown by the Romans for its aromatic seeds, and the delicious fleshy shoots are still a popular vegetable in southern Italy. It is said that Emperor Charlemagne fostered its development in Central Europe [70].

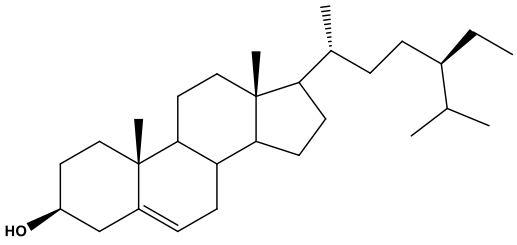
Foeniculum vulgare has been used in not only in traditional systems of medicine of Unani, Siddha and Ayurveda, but also in folk medicine throughout Europe, Asia and the Americas. The fruits, stems, leaves, and the whole plant have all been used for different purposes. Its major uses are the treatment of respiratory diseases (cough, common cold) and gastrointestinal disorders, however, its use comprises application in more severe diseases as well (eg. cancer, arthritis, cancer, conjunctivitis, fever, insomnia, kidney ailments, liver pain) [63]. Fennel fruit has been used in some areas to relieve painful menstruation and to relieve symptoms of climacteric [71]. According to the Pharmacopoeia of the People's Republic of China, in Traditional Chinese Medicine it is used for lower abdominal pain with cold sensation, dysmenorrhoea; distending pain in the epigastrium with reduced appetite, vomiting and diarrhoea; hydrocele testis [72]. In different parts of the world, leaf, stem, and fruit have been

extensively used as galactagogues for increasing the quantity and quality of milk of breastfeeding mothers [73], [74].

In the European official medicine Foeniculi dulcis fructus, Foeniculi amari fructis and the essential oil of the latter can be used as traditional herbal medicinal product for the symptomatic treatment of mild, spasmodic gastro-intestinal complaints including bloating and flatulence, for the symptomatic treatment of minor spasm associated with menstrual periods and as an expectorant in cough associated with colds [75], [76]. The fruit is used after crushing, sweet fennel being more broadly used. Crushed fennel fruits gradually lose their essential oil content.

3.2.3 Phytochemical characteristics

The taste, odor, and pharmacological effects of fennel are related to its essential oil content. The major components of the fruits are proteins, cellulose, triglycerides, phospholipids, pectin, lignin. Its major specific metabolites belong to phytosterols (beta-sitosterol **60**, and stigmasterol **61**), flavonoids, other phenolics, coumarins, furanocoumarins and essential oil components [65]. Flavonoids like eriodictyol-7-rutinoside (**62**), quercetin-3-rutinoside (**63**), quercetin-3-glucuronide (**64**), quercetin-3-arabinoside (**65**), kaempferol-3-glucuronide (**66**) and kaempferol-3-arabinoside (**67**), isoquercitrin (**68**), and isorhamnetin glucoside (**69**) and their aglycons have been reported from the fruits [77]–[79]. Fennel seeds contain rosmarinic acid (**70**) and chlorogenic acids (**71**) as major phenolic compounds (14.9% and 6.8%, respectively) [80].



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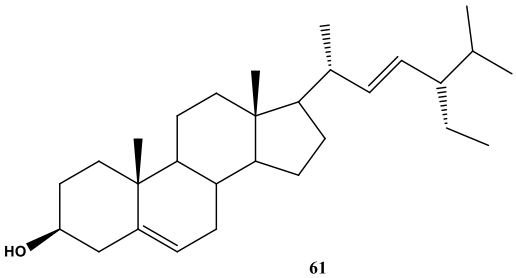

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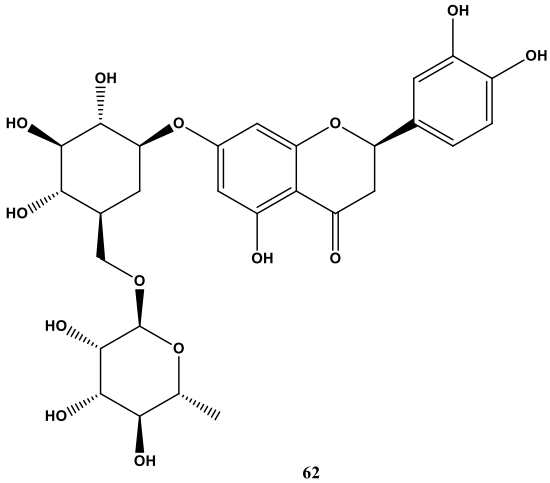

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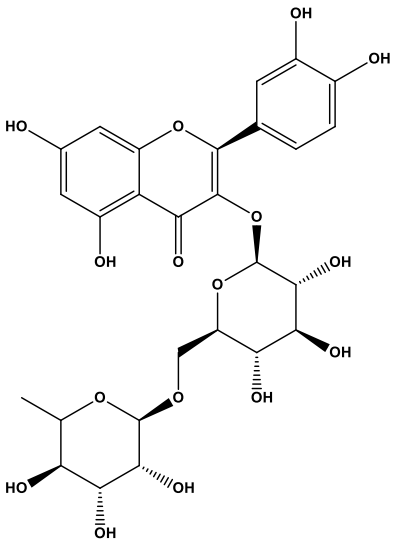

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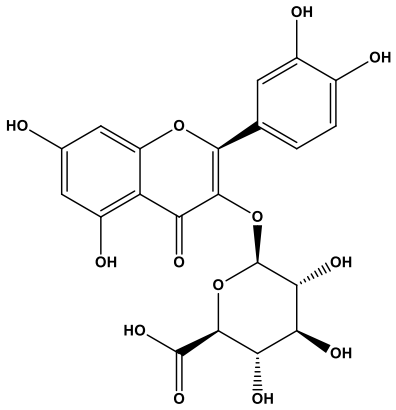

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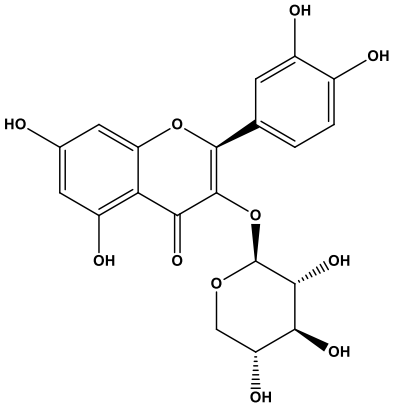

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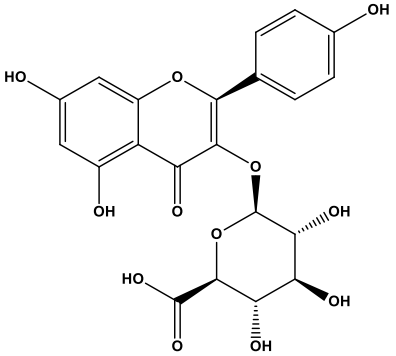

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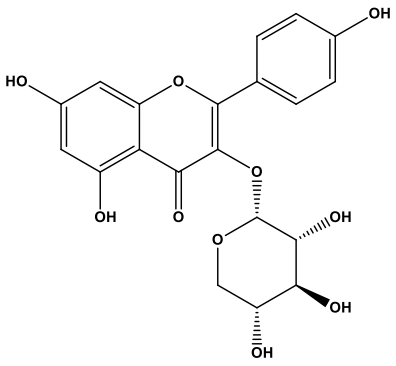

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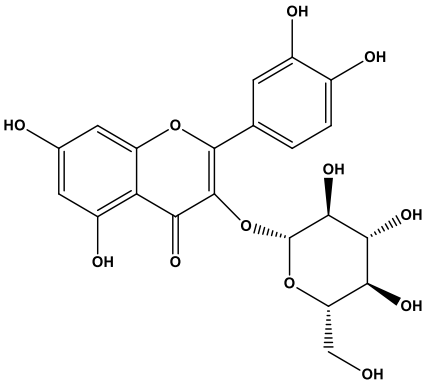

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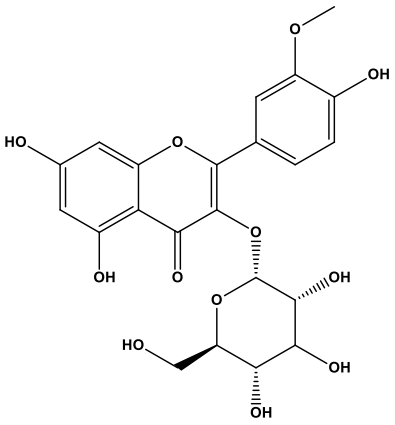

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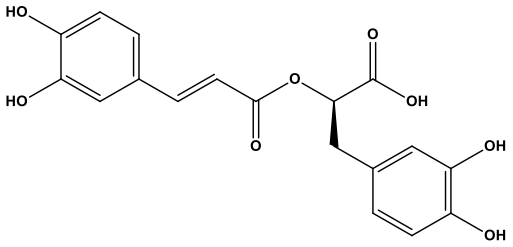

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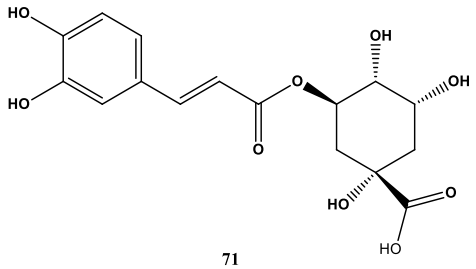

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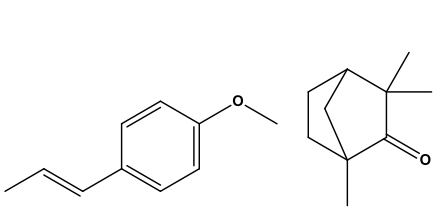
The composition of the essential oil is variable, the main components being trans-anethole (**72**), fenchone (**73**), estragole (**74**) and limonene (**75**) [63]. According to the European Pharmacopoeia, sweet fennel fruit consists is characterized by a content of essential oil not lower than 20 ml per kg anhydrous fruit with a 80.0% minimum content of anethole (**72**) in its essential oil, whereas bitter fennel fruit contains not less than 40 ml per kg anhydrous fruit of essential oil that contains not less than 60.0% of anethole (**72**) and not less than 15.0% of fenchone (**73**) [81]. Although trans-anethole (**72**) may trigger hypersensitivity, more concern is related to the estragole (**74**) content of fennel. This compound may be present in a concentration as high as 5% in bitter fennel and 10% in sweet fennel essential oils, and it is claimed to have genotoxic and carcinogenic effects [82], [83].



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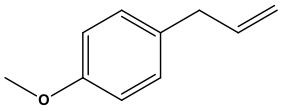

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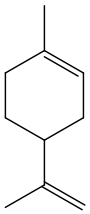

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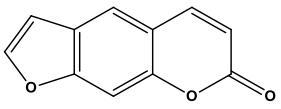
Another group of compounds that may pose a risk to fennel consumers include furocoumarins. Linear furocoumarins are carcinogenic in combination with UV light exposure [84]. The stem base of fennel was reported to contain 5.24 mg/kg of 5-methoxyspsoralen (**10**) and 2.8 mg/kg of isoimperatorin (**18**) [85]. From the stems of the plant bergapten (**10**), imperatorin (**9**) and

psolaren (**76**) were isolated (70, 10, and 35 mg from 600 g of dry stem, respectively) [86]. From fennel fruits, 5-methoxypsoralen (**10**) was reported, but its concentration is not known) [87]. An LC-MS based study did not report the presence of furocoumarins in fennel teas; however, the identification of this type of compounds was out of the focus of the research [88].



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3.2.4 Pharmacological activities related to ethnomedicinal uses

The spasmolytic effect of an alcoholic extracts of *F. vulgare* fruits was evaluated utilizing an experimental model of guinea pig ileum using the spasmogens acetylcholine and histamine. In vitro, the alcoholic extract reduced acetylcholine and histamine-induced guinea pig ileal contractions [89]. The addition of anethole (**72**) to isolated mouse intestinal jejunum in saline resulted in relaxation at higher doses [90]. The addition of 0.5% powdered fennel seeds to rats' diets for six weeks reduced food transit time by 12% [91]. Fennel at 24 mg/kg b.w. increased spontaneous stomach movement in unanaesthetized rabbits and reduced the inhibition of stomach movement caused by sodium pentobarbitone [92]. In rats, an aqueous extract of fennel demonstrated a strong antiulcerogenic activity against ethanol-induced stomach ulcers. The pretreatment greatly reduced ethanol-induced stomach injury. This impact of aqueous extract was greatest and statistically significant in the 300 mg/kg group of animal compared to the control [93].

F. vulgare ethanol extract and essential oil displayed bronchodilatory action in guinea pig constricted tracheas [94]. This activity may be based on the structural similarity of anethol (**72**) to catecholamines [95]. The essential oil content of fennel seeds increase ciliary motility in the respiratory tract and improve exogenous corpuscle and sputum movement [96]. Inhalation of fenchone (**73**) by rabbits resulted a dose-dependent augmentation of the volume output of a respiratory tract fluid with a decreased density, referring to the secretolytic effect of this treatment. In case of anethol no such effect was observed [97].

Fennel oil has been associated with estrogenic activity, promotion of menstruation, and reduction of climacteric symptoms [95]. As a confirmation of this observation, oral administration of fennel extract to female rats for 10 days resulted in vaginal cornification and oestrus cycle, as well as an increase in organ weight, indicating estrogenic action [98]. The estrogenic effect was reassured in a clinical trial as well. The ethanolic extract of *F. vulgare*

fruits was assessed against idiopathic hirsutism in creams containing 1 and 2% of extract. Both creams had more pronounced antihirsutic affect than placebo, and the 2% cream proved to be the most potent. The mean hair diameter reduction values were 7.8%, 18.3%, and −0.5% in patients treated with the creams containing 1%, 2%, and 0% extract, respectively [99].

The galactogogue effect of fennel might be related to the main constituent of its essential oil, anethole (**73**), which is structurally similar to dopamine. Since dopamine inhibits the secretion of the milk-producing hormone, prolactin, dopamine antagonist effect of anethol may explain the clinical effect of the plant [95]. Preclinical studies suggest that the actual pharmacologically active agents are polymers of anethole, rather than anethole itself [100].

4. MATERIALS AND METHODS

4.1 Plant material

4.1.1 *Citrus trifoliata* L.

In 2018, Botond Lajos Borcsa collected *Citrus trifoliata* L. fruits in Tompa, Hungary. Before processing, seeds and peels were dried and kept at room temperature. In the herbarium of the Institute of Pharmacognosy at the University of Szeged, a voucher specimen (No. 893) is kept.

4.1.2 *Foeniculum vulgare* Mill.

For the analysis, ripe fruits of 33 *F. vulgare* Mill. subsp. *vulgare* var. *vulgare* were used from the collection of the Genebank of the Department of Medicinal and Aromatic Plants, Szent István University, Hungary. Among the examined bitter fennel accessions there were cultivated and wild growing populations of unknown origin (samples 1–25), nonrelated progenies of former breeding work (samples 26–30), and three registered varieties (Berfena, Groβfrüchtig, and Soroksári). For comparison, a caraway commercial fruit sample was also investigated.

4.2 Extraction and isolation from *Citrus trifoliata*

4.2.1 Extraction and isolation of specialised metabolites from the peel of *Citrus trifoliata*

The dried peels were crushed using a grinder (Retsch Grindomix GM 200). 236.2 g of this crushed peel was ultrasonically extracted with 6500 mL of MeOH at room temperature for 15 minutes. 70.4 g of crude MeOH extract were produced when the solvent evaporated at decreased pressure. After being redissolved in 100 mL of methanol, the extract underwent solvent-solvent partitioning using 4,250 mL of dichloromethane. The crude dichloromethane fraction weighed 23.7 g in total. This fraction was separated into 49 fractions (O1-O49) by open column chromatography using silica gel 60 (0.040–0.063 mm) and a gradient of *n*-hexane chloroform–acetone (2:1:0, 1:1:0, 0:9:1, 0:8:2, 0:7:3, 0:6:4, 0:1:1, 0:0:1, 1000 mL each).

The combined fractions O21–27 were subjected to medium pressure liquid chromatography on silica gel (Silica gel 60 (0.040–0.063 mm)) using a gradient system of *n*-hexane–EtOAc (9:1 to 0:1), 50 mL/min flow rate, gaining 16 fractions (M1–M16). By using open column chromatography on silica gel (Silica gel 60 (0.040-0.063 mm)), the combined fractions M1–7 (5 g) was divided into 15 subfractions and eluted with cyclohexane–dichloromethane 7:3 with increasing acetone concentration (from 0% to 25%). Purification of the subfractions 5-7 (428 mg) of this separation was done using normal-phase preparative TLC with cyclohexane-acetone

as the eluent. Finally, using high pressure liquid chromatography (HPLC method I.), compounds **B**, **D**, **G**, and **H** were obtained.

Combined fractions M9-10 (3 g) were initially subjected to open column chromatography on silica gel (Silica gel 60 (0.040-0.063 mm)), which produced 14 subfractions after eluting with cyclohexane-dichloromethane 1:1 with increasing acetone content (0.1, 0.3, 0.5, 1, 2, 5, 10, 20, 50%). Using reverse-preparative TLC and a MeOH-water 7:3 ratio, subfraction 5 was purified. This final purification step afforded the isolation of compound **C**.

Reverse-phase medium pressure liquid chromatography (MPLC) was used to separate fraction M12 (1.07 g) on RP18ec (40-60 μm, 40x150 mm), eluting with MeOH-water (3:7 to 1:0), and collecting 15 fractions in overall.

Merged fractions 8–9 (248.3 mg) were further purified using normal-phase open column chromatography (Silica gel 60 (0,040–0,063 mm)) with chloroform-acetone (9:1) eluent system. As the last step the subfractions 4–6 were purified using normal phase preparative TLC and dichloromethane-methanol (9:1) as the eluent, and that final purification step afforded the isolation of compound **F**.

4.2.2 Extraction and isolation of specialised metabolites from the seed of *Citrus trifoliata*

Dried *Citrus* seeds were crushed also with a grinder Retsch Grindomix GM 200. After that, 170 g of this crushed seed were ultrasonically extracted with 4000 mL MeOH at room temperature for 25 min. This extract was reduced to 100 mL by vacuum evaporation. By solvent-solvent partitioning with 4 x 200 mL of dichloromethane, coumarins were extracted. At room temperature, the extracts were combined and evaporated under reduced pressure. The 24 g dried and crude dichloromethane extract was chromatographed on a silica column using gradient elution (*n*-hexane-dichloromethane-acetone 2:1:0, 1:1:0, 0:9:1, 0:8:2, 0:7:3, 0:3:2, 0:1:1, 0:2:3, 0:3:7, 0:1:1) to collect 32 fractions.

Compound **E** was produced after subfraction O7-9 was purified using preparative TLC on silica gel and eluted with toluene-acetone 95:5. Combined fraction O13-15, was further separated (867.3 mg) by using flash chromatography technic (Flash method I) to gain 76 subfractions. As a next step, subfractions OF 68-76 (178.5 mg) were further purified by flash chromatography using Flash method II, yielding 33 subfractions. Compounds **I** and **J** were isolated from the merged subfractions OFF18-26 using high pressure liquid chromatography (HPLC method II).

4.3 Furocoumarin content of *Foeniculum vulgare* samples

4.3.1 Recovery experiment – extraction optimization

First, to optimize extraction, the most effective solvent was selected from methanol, ethanol, petroleum ether, chloroform, acetonitrile, acetone, toluene, and ethyl acetate. All solvents came from VWR International LLC in Radnor, Pennsylvania, USA, and were of HPLC gradient grade quantity. After being extracted for 10 minutes in an ultrasonic bath with 4.95 ml of extracting solvent and 0.500 g of freshly ground dry fruits, the mixture was spiked with 0.05 mL of a solution containing psoralene, 5-methoxypsoralene, and imperatorin in 100 µM concentrations each. The extracts were filtered through 0.45 µM polytetrafluoroethylene (PTFE) syringe filters. A total of 3.00 mL of the extracts were evaporated to dryness under nitrogen stream, re-dissolved in 1 mL methanol:water 3:1, and were filtered again.

Two experiments were conducted to determine whether sample preparation had an impact on quantitative determination. To evaluate whether sample preparation affected quantitative determination, two experiments were carried out. First, 0.500 g plant material was spiked with 10 µL stock solution containing known amounts of psoralene, 5-methoxypsoralene, and imperatorin, and then 4.99 mL acetonitrile was added. Extraction was carried out as previously described. In the second experiment, after extraction and filtering, the sample was spiked. The same processing steps were used on one sample, but no spikes were used.

4.3.2 Determination and quantification of furocoumarins in fennel extracts

As stated in the extraction section, acetonitrile (5.00 for 0.5000 g plant material) was used as the extracting solvent to extract plant samples. The determination and quantification of furocoumarins in fennel extracts was performed by LC-MS (LC-MS method).

4.4 Chromatographic techniques

MPLC system:

A Büchi medium pressure liquid chromatography system equipped with a Büchi Pump Manager C-615 and with two Büchi Pump Modules C-605 was used for the MPLC separation.

HPLC method I:

A Waters 600 HPLC system (Waters Corporation, Milford, USA) with a 2998 photodiode array detector, on-line degasser, and autosampler was used for the preparative reversed-phase HPLC. For the separation a Kinetex C18 (5 µm, 100Å, 150 x 4.6 mm) column from Phenomenex,

Torrance, USA was used. For elution, a gradient solvent system was used with methanol and water (0-1 min: water-MeOH 1:1; 10 min: 0:1; 11 min: 0:1; 11.5 min: 1:1; 15 min: 1:1) with a flow rate of 1.5 mL/min. Detection of coumarins and furocoumarins was at 254 and 366 nm.

HPLC method II:

A Waters 600 HPLC system (Waters Corporation, Milford, USA) equipped with a 2998 photodiode array detector, on-line degasser and autosampler was used for isolating limonoids. For separating, a Kinetex C18 (5 µm, 100Å, 150 x 4.6 mm) column was used from Phenomenex, Torrance, USA. We used a gradient elution with the solvent system acetonitrile and water (0–2 min: water-AcNi 3:2; 10 min: 0:1; 11–16 min: 3:2) with a flow rate of 1 mL/min. Limonoids were detected at 210 and 254 nm.

Flash method I:

We used a Biotage® instrument (Isolera™ Spektra Systems with ACI™ and Assist) with integrated UV, UV-VIS, and ELS detection for flash chromatographic separation. For this separation process, we used a normal phase SNAP ultra 50 g column. Elution was carried out using a gradient solvent system with cyclohexane (A) and ethyl acetate (B) at a flow rate of 100 mL/min (198 mL: B 1%; 660 mL: 1%-50%; 132 mL: 50%-70%; 66 mL: 70%-100%; 132 mL: 100%). Separation was detected at 265 and 366 nm.

Flash method II:

In the case of this method, we also used the same Biotage® instrument (Isolera™ Spektra Systems with ACI™ and Assist) with integrated UV, UV-VIS, and ELS detection for flash chromatographic separation. It was accomplished using gradient elution on a reverse phase column (SNAP C18 12 g). The solvent system was made up of water (A) and acetonitrile (B) (30 mL: B 0%-10%; 150 mL:10%-100%; 30mL: 100%) with a flow rate of 12 mL/min. Limonoids were detected at 200 and 210 nm.

LC-MS method:

As stated in the extraction section above, acetonitrile (5.00 for 0.5000 g plant material) was used as the extracting solvent to extract plant samples. A Kinetex® 2.6 µm C18 100 Å, 100 x 2.1 mm column was used for separation, and the eluents were water (A) and acetonitrile (B) both of which contained 0.1% formic acid (HPLC gradient grade, VWR International Ltd.). The column temperature was set to 40 °C, and the flow rate was 0.5 mL/min throughout the separation procedure. Within five minutes, the ratio of B went from 5% to 95%, followed by

the washing and equilibrating phases (each lasting 2.5 minutes). Analysis was carried out on a Shimadzu HPLC system (Shimadzu Corporation, Kyoto, Japan) comprising an LC-20AD pump, a DGU-20A5R degasser, an SIL-20ACH autosampler (tempered to 21 °C), a CTO-20AC column oven, and SPD-M20A photodiode array detector modules (connected with CBM-20A control module) coupled to an AB Sciex API 2000 triple quadrupole mass spectrometer (AB Sciex LLC, Redwood City, CA, USA). Furocoumarins were detected using the single reaction monitoring mode (SRM). Precursor ions were detected at m/z values of 187.4, 217.3, and 271.2, whereas product ions were detected at m/z 131.2, 202.2, and 203.3 in the cases of psoralene, 5-methoxypsoralene, and imperatorin, respectively. Collision energies were 36, 31, and 19 eV, respectively. Based on the quantifications of six solutions that contained mixtures of the three analytes, calibration curves were created. The limit of detection (three times the noise) and the limit of quantitation (ten times the noise) were expressed using the signal-to-noise ratio. Intraday precisions were calculated from data acquired during a three-day validation. Relative standard deviation (RSD %) was used to express precision.

4.5 NMR experiments

NMR spectra were recorded on a Bruker Avance DRX 500 spectrometer in $CDCl_3$ and acetone-d_6 at 500 MHz (^1H) and 125 MHz (^{13}C JMOD). As a point of reference, the residual peaks of the deuterated solvents were used. With the help of typical Bruker software, two-dimensional (2D) experiments were carried out. Gradient-enhanced versions were used in the ^1H-^1H COSY, HSQC, and HMBC experiments. MestReNova v6.0.25475 software was used to collect and process the data.

4.6 Pharmacological tests

4.6.1 Cell lines

LGC Promochem (UK) provided the MRC-5 human embryonal lung fibroblast cell lines (ATCC CCL-171). This cell line was grown in Eagle's Minimal Essential Medium (EMEM), with a 4.5 g/L glucose content, supplemented with 10% heat-inactivated fetal bovine serum, non-essential amino acids and vitamins.

Human colon adenocarcinoma cell lines (COLO 320/MDR-LRP multidrug resistant overexpressing ABCB1 (MDR1)-LRP and COLO 205 doxorubicin-sensitive), CCL-222 (COLO 205) and ATCC-CCL-220.1 (COLO 320) were also obtained from LGC Promochem (UK). The cell lines were cultured in RPMI 1640 medium that also contained 10% heat-

inactivated fetal bovine serum, 100 mM hepes, 1 mM Na-pyruvate, and 2 mM L-glutamine. Trypsin-Versene (EDTA) solution was applied at 37 °C for 5 min. to detach the semi-adherent human colon cancer cells. All of the cell lines were incubated in a 5% CO2, 95% air atmosphere at 37 °C.

4.6.2 Assay for antiproliferative and cytotoxic effects

Doxorubicin-sensitive COLO 205 and multidrug resistant COLO 320 colonic adenocarcinoma cells were used to test the effects of increasing concentrations of tested compounds on cell growth and proliferation, in 96-well flat-bottomed microtiter plates.

The dilutions of the compounds were prepared in 100 µL of RPMI 1640, horizontally, for the colonic adenocarcinoma cells. Trypsin-Versene (EDTA) solution was used to treat the semi-adherent colonic adenocarcinoma cells.

In 96-well flat-bottomed microtiter plates, adherent human embryonal lung fibroblast cells were cultured using EMEM supplemented with 10% heat-inactivated fetal bovine serum. After 24 hours of seeding at 37 °C, the medium with the cells was removed, and the diluted test compounds were added to the cells.

The density of the cells was adjusted to 1 x 10^4 cells (in the cytotoxicity assay) and 6 x 10^3 cells (in the antiproliferative assay). The plates were incubated at 37 °C for 24 h (cytotoxicity test) or 72 h (antiproliferative test). At the end of this incubation period, 20 µL of MTT (thiazolyl blue tetrazolium bromide) solution were added to each well. After an incubation for 4 h at 37 °C, 100 µL of sodium dodecyl sulfate solution (10% in 0.01 M HCI) was added to the wells and the incubation was continued at 37 °C overnight. Cell growth was measured based on optical density (OD) determination at 540/630 nm with Multiscan EX ELISA reader (Thermo Labsystems, USA). The following formula was used to calculate cell growth inhibition [101]:

$$\text{Inhibition \%} = 100 - \left[\frac{OD\,sample - OD\,medium\,control}{OD\,cell\,control - OD\,medium\,control} \right] \times 100$$

4.6.3 Checkerboard combination assay

Using the checkerboard microplate method and the multi-drug resistant COLO 320 colonic adenocarcinoma cells overexpressing the ABCB1 transporter, the interactions between the tested substances and doxorubicin were evaluated. In earlier antiproliferative assays, the dosage of doxorubicin used in this combination experiment was established. Doxorubicin dilutions were made in a horizontal direction in a volume of 100 µL, and the dilutions of the tested

compounds were made in a vertical direction in a microtiter plate in a volume of 50 µL. $6x10^3$ cells were distributed among the resuspended cells in each well. The plates were then incubated for 72 hours at 37 °C in a 5% CO_2 atmosphere. MTT staining was used to determine cell growth rate. 20 µL of MTT (thiazolyl blue tetrazolium bromide) solution was added to each well at the end of the incubation period. 100 µL of SDS solution (10% in 0.01 M HCl) was added to each well after another 4 hours of incubation at 37 °C, and incubation was carried out at the same temperature overnight. A Multiscan EX ELISA reader (Thermo Labsystems, USA) was used to measure optical density (OD) at 540/630 nm with. Combination index (CI) values at 50% of the growth inhibition dose (ED_{50}) were calculated by using CompuSyn software (ComboSyn, Inc., USA). CI values were calculated by using of the median-effect equation, according to the Chou-Talalay method, where CI < 1, CI = 1, and CI > 1 represent synergism, additive effect (or no interaction), and antagonism, respectively [101].

4.6.4 Evaluation of rhodamine 123 (R123) retention by flow cytometry

The multidrug resistant COLO 320 and the doxorubicin-sensitive (parental) COLO 205 colonic adenocarcinoma cells were adjusted to a density of 2 x 10^6/mL, resuspended in serum-free RPMI 1640 and distributed into Eppendorf centrifuge tubes in 0.5 mL aliquots. After that, the tested compounds were put into tubes at final concentrations ranging from 0.02-0.2 µM, and they were incubated at room temperature for 10 minutes. Rhodamine 123 was added to 10 µL (5.2 µM final concentration), and then the mixture was incubated for 20 min at 37 °C. Phosphate buffered saline (PBS) was used to wash the cells twice before resuspending them in 1mL of PBS. To measure fluorescence intensity, a Partec CyFlow flow cytometer (Partec, Germany) was employed. At a final concentration of 0.2 µM, tariquidar was used as a positive control, and the fluorescence of untreated cells was used for the baseline measurement. During this experiment, FL-1 (mean fluorescence intensity of the cells) was determined. The fluorescence activity ratio (FAR) was calculated using the following equation:

$$FAR = \frac{MDR_{treated} / MDR_{control}}{parental_{treated} / parental_{control}}$$

4.6.5 Real-time ethidium bromide accumulation assay

A CLARIOstar Plus plate reader (BMG Labtech, UK) was used to measure the impact of the tested compounds on ethidium bromide (EB) accumulation. *Staphylococcus aureus* ATCC and *Escherichia coli* AG100 were incubated in TSB and LB, respectively, until an optical density (OD) of 0.6 at 600 nm was reached. The cell pellet was resuspended in PBS after being washed

with PBS and centrifuged at 13.000 x g for 3 min. To PBS containing 2 g/mL EB, the tested compounds were added at a concentration of 1/2 MIC (apart from >100, where the concentration was 100 μM). After that, 50 μLs of this mixture were put into 96-well black microtiter plates made by Greiner Bio-One Hungary, and 50 μL of bacterial suspension (OD_{600} 0.6) was added into each well. Using a CLARIOstar plate reader, the fluorescence was observed for one hour at excitation and emission wavelengths of 525 and 615 nm every minute. From these data, the relative fluorescence index (RFI) of the EB accumulation assay's final time point (minute 60) was calculated using the formula:

$$RFI = (RF_{treated} - RF_{untreated}) / RF_{untreated}$$

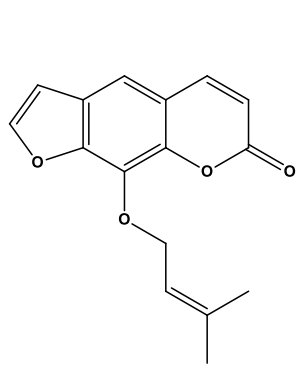
where $RF_{treated}$ is the relative fluorescence (RF) at the final time point of the EB retention curve in the presence of an inhibitor and $RF_{untreated}$ is the RF at the final time point of the EB retention curve of the untreated control with the solvent control (DMSO). Reserpine (25 μM) was used as a positive control in the case of *S. aureus*, and CCCP (50 μM) was used in the case of *E. coli*. Only the EB solution and bacteria were used as a negative control.

5. RESULTS

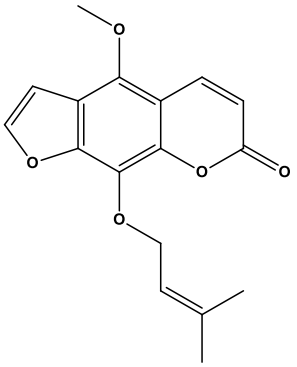
5.1 Isolated specialised metabolites from the seeds and peel of *C. trifoliata*

Following the chromatographic purification of the investigated plant materials, 4 pure compounds (compound **A, E, I, J**) were isolated from the seed and 6 (compound **B-D, F-H**) from the peel of *Citrus trifoliata*. The structure elucidation process of these compounds was carried out by 1D (^{1}H, ^{13}C JMOD) and 2D (HSQC, HMBC, ^{1}H–^{1}H COSY, and NOESY) NMR experiments.

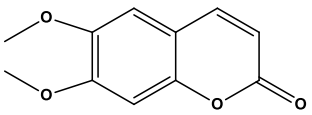
According to the reported literature data, the isolated compounds were identified as imperatorin (compound **A**), phellopterin (compound **B**), scoparone (6,7-dimethoxycoumarin, compound **C**), myrsellin (compound **D**), auraptene (compound **E**), triphasiol (compound **F**), umbelliferone (compound **G**), citropten (5,7-dimethoxycoumarin, compound **H**), limonin (compound **I**), and deacetyl nomilin (compound **J**). The obtained compounds are furocoumarin- (compound **A, B**), coumarin- (compound **C-H**), and limonoid derivatives (compound **I, J**). All compounds but scoparone have previously been described from *P. trifoliata* [29], [34], [102]–[104].



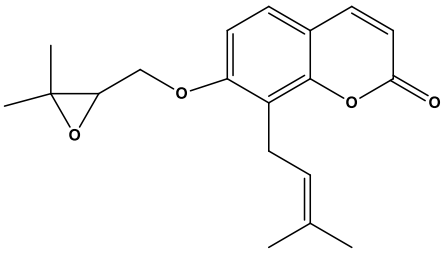
imperatorin (compound **A**)



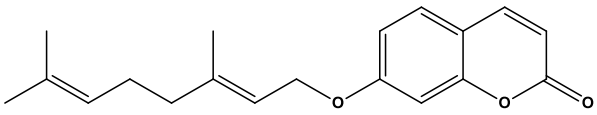
phellopterin (compound **B**)



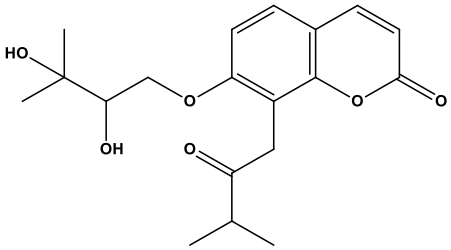
scoparone (compound **C**)



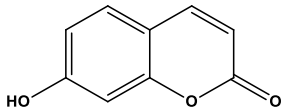
myrsellin (compound **D**)



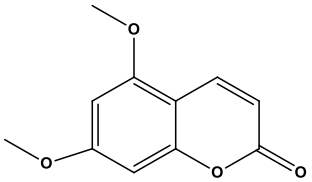
auraptene (compound **E**)



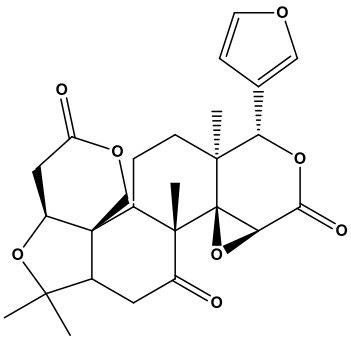
triphasiol (compound **F**)



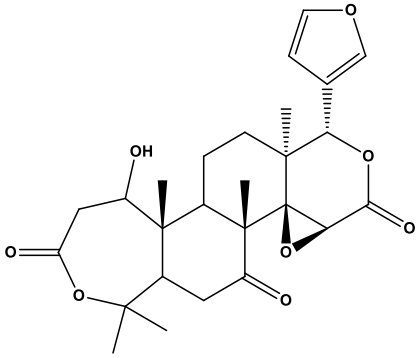
umbelliferon (compound **G**)



citropten (compound **H**)



limonin (compound **I**)



deacetyl nomilin (compound **J**)

5.2 Pharmacological activity of the isolated compounds from *C. trifoliata*

5.2.1 Antiproliferative and cytotoxic activity

Neither the cytotoxicity assay nor the antiproliferative assay showed any activity from the tested compounds on normal (MRC-5) or doxorubicin-sensitive colon carcinoma (COLO 205) cell lines. However, some substances were found to be effective on the resistant COLO 320 cell lines. With an IC_{50} value of 25.28 ± 0.42 µM, auraptene demonstrated potent antiproliferative activity on COLO 320 cells. Imperatorin, phelloterin, and myrsellin also had moderate effects, with IC_{50} values of 40.47 ± 1.22, 43.71 ± 1.78, and 47.94 ± 1.11 µM, respectively *(Table 1).*

Table 1. Antiproliferative activity

Compound	MEAN	SD
Colo 320 AP DOXO (control)	0.2	0.002
imperatorin	40.47	1.22
phellopterin	43.71	1.78
scoparone	50.24	2.33
myrsellin	47.94	1.11
auraptene	25.28	0.42
triphasiol	72.05	1.38

5.2.2 Checkerboard combination assay

In the combination studies, various doses of the examined drugs' interactions with doxorubicin were investigated. At certain doses, some substances, including scoparone, myrsellin, and

deacetyl nomilin show synergistic effects with doxorubicin. This could be a reference to their possible use in combination with established cancer treatments to increase their efficacy. Phellopterin, scoparone, and auraptene have, for instance, demonstrated antagonistic effects at specific concentrations *(Table 2).* Such interactions might make chemotherapy less effective.

Table 2. Results of checkerboard combination assay

Compound	Starting conc.	Ratio	Combination index (CI)	SD	Type of interaction
imperatorin	150 µM	17.42:1	1.29	0.11	moderate antagonism
		34.84:1	1.06	0.07	additive effect
		69.68:1	0.75	0.06	moderate synergism
		139.36:1	1.24	0.05	moderate antagonism
		278.72:1	0.99	0.04	additive effect
		557.44:1	0.99	0.11	additive effect
phellopterin	150 µM	17.42:1	2.31	0.46	antagonism
		34.84:1	1.40	0.17	moderate antagonism
		69.68:1	1.33	0.15	moderate antagonism
		139.36:1	1.54	0.26	antagonism
		278.72:1	1.05	0.12	additive effect
		557.44:1	1.03	0.23	additive effect
scoparone	200 µM	23.2:1	2.06	0.39	antagonism
		46.4:1	1.22	0.18	moderate antagonism
		92.8:1	0.81	0.05	moderate synergism
		185.6:1	0.75	0.03	moderate synergism
		371.2:1	0.62	0.05	synergism
		742.5:1	0.60	0.07	synergism
myrsellin	150 µM	17.42:1	1.17	0.09	slight antagonism
		34.84:1	0.72	0.05	moderate synergism
		69.68:1	0.83	0.04	moderate synergism
		139.36:1	0.58	0.11	synergism
		278.72:1	0.64	0.12	synergism
		557.44:1	0.73	0.21	moderate synergism
auraptene	80 µM	9.2:1	3.16	0.85	slight syn.
		18.4:1	8.37	1.03	strong antagonism
		36.8:1	1.13	0.20	slight antagonism
		73.6:1	1.72	0.24	antagonism
		147.2:1	2.06	1.00	antagonism
		294.4:1	5.30	1.60	strong antagonism
triphasiol	200 µM	23.2:1	0.86	0.06	slight synergism
		46.4:1	0.76	0.08	moderate synergism
		92.8:1	0.82	0.03	moderate synergism
		185.6:1	0.78	0.02	moderate synergism
		371.2:1	0.81	0.10	moderate synergism
deacetyl nomilin	200 µM	23.2:1	1.18	0.19	nearly additive
		46.4:1	1.02	0.08	nearly additive
		92.8:1	0.67	0.04	synergism
		185.6:1	0.66	0.07	synergism
		371.2:1	0.62	0.02	synergism
		742.5:1	0.82	0.32	moderate syn.

5.2.3 Effect on efflux pumps

According to conventional knowledge, if the FAR (fluorescence activity ratio) value in the rhodamine 123 retention assay is higher than 2, the substance can be considered as a potent Pgp inhibitor. Tariquidar was used as a positive control (FAR value: 11.44 at 0.2 μM). Four of the tested compounds were effective at 20 μM. The FAR values of phellopterin, scoparone, myrsellin and auraptene were 2.63, 2.02, 4.86, and 4.00, respectively *(Table 3).*

The ethidium bromide (EB) accumulation assay determines the intracellular accumulation of the general efflux pump (EP) substrate EB. Due to its accumulation inside the bacterial cell, a potential efflux pump inhibitor raises the level of EB's fluorescence. The relative fluorescence index (RFI) of the real-time accumulation curves was used to compare the compounds' EP inhibitory activity. Phellopterin and myrsellin, demonstrated remarkable activity with respective RFI values of 5.49 and 5.51. These values were higher than the reserpine (2.77), the positive control. Furthermore, scoparone showed even moderate activity, as demonstrated by its RFI value of 1.04. According to the results, these substances may be regarded as effective EP inhibitors.

Table 3. Efflux pump inhibitory activities of the isolated compounds

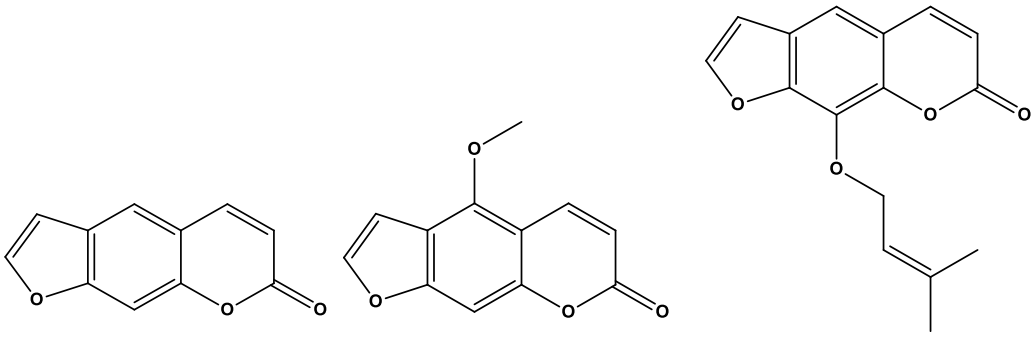
Compound	Concentration (μM)	FL-1*	FAR
tariquidar	0.2	88.20	11.44
imperatorin	2	11.30	1.47
	20	9.82	1.27
phellopterin	2	11.10	1.44
	20	20.30	2.63
scoparone	2	8.01	1.04
	20	15.60	2.02
myrsellin	2	10.90	1.41
	20	37.50	4.86
aurapene	2	13.60	1.76
	20	30.80	4.00
triphasiol	2	14.20	1.84
	20	14.30	1.85
limonin	2	2.92	0.38
	20	10.00	1.30
deacetyl nomilin	2	4.61	1.01
	20	3.53	0.78

*FL-1: mean fluorescence intensity of the cells

In a subsequent experiment, three compounds (imperatorin, myrsellin and auraptene) showed moderate activity on E. coli AG100 with RFI values of 0.26, 0.39, and 0.34, respectively. The positive control was CCCP, with an RFI value of 1.34.

5.3 Results of extraction optimization by *Foeniculum vulgare*

Three furocoumarins — psoralene, 5-methoxypsoralene, and imperatorin — that have been reported to be present in different parts of fennel were the focus of our experiments.



According to the results of extraction optimization, acetonitrile was the most effective solvent for the maximal extraction of furocoumarins *(Figure 2).* Therefore, acetonitrile was used in our experiments.

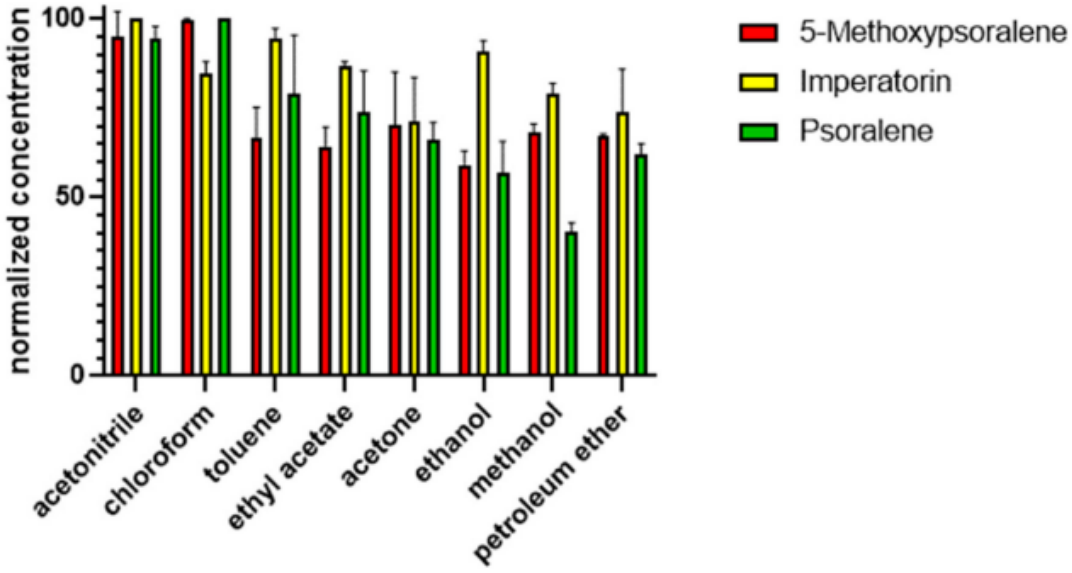


Figure 2. Different solvent extraction efficiencies expressed as normalized concentrations of the analytes *[105]*

5.4 Results of the recovery experiments

No remarkable differences in recovery values were found between samples spiked before and after extraction in these experiments, indicating that the analytes were stable during the extraction. Additionally, the relatively high (80.17%-133.44%) recovery values supported the validity of the sample preparation method *(Table 4)*.

Table 4. Recovery values of the analytes when added to the sample before and after extraction

Sample	Recovery (%)		
	psoralene	**5-methoxypsoralene**	**imperatorin**
Spiked before extraction	101.34	105.81	64.56
Spiked after extraction	133.44	80.17	92.42

5.5 Furocoumarin content of *Foeniculum vulgare* samples

In the used analytical system, psoralene, 5-methoxypsoralene, and imperatorin could all be detected with good resolution and selectivity *(Figure 3)*. Limits of quantification and detection ranged from 8.8 to 10.1 nM and 28.7 to 31.3 nM, respectively. The linear calibration curves of these compounds, which were determined in the concentration range of 10-1000 nM, were represented by the equations $y = 12.2x + 185$ ($R^2 = 0.9960$), $y = 38x + 159$ ($R^2 = 0.9991$), and $y = 96.3x - 1.25 \times 10^{-3}$ ($R^2 = 0.9997$) for psoralene, 5-methoxypsoralene, and imperatorin, respectively. For the reference standards (RSD%, $n = 12$), intraday precision ranged from 0.67% to 1.81%. The positive reproducibility of our method has been demonstrated by these results.

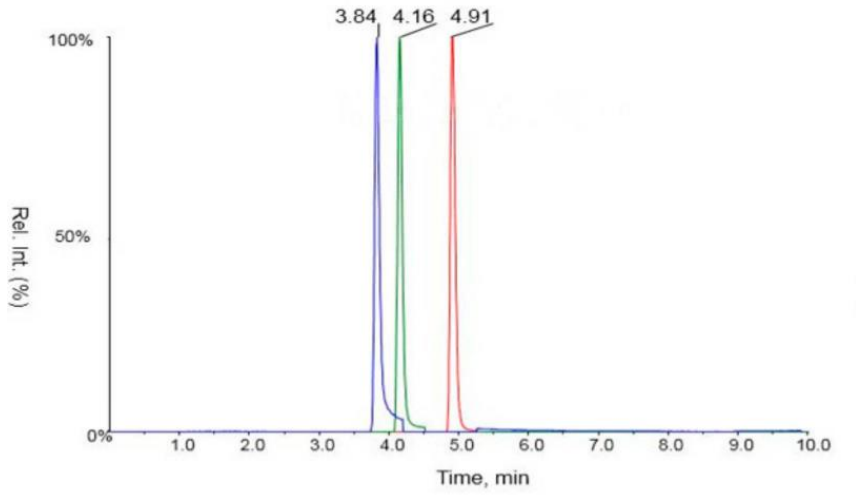


Figure 3. LC-MS chromatograms of the mixture of psoralene (Rt = 3.84 min), 5-methoxypsoralene (Rt = 4.16 min) and imperatorin (Rt = 4.91 min)

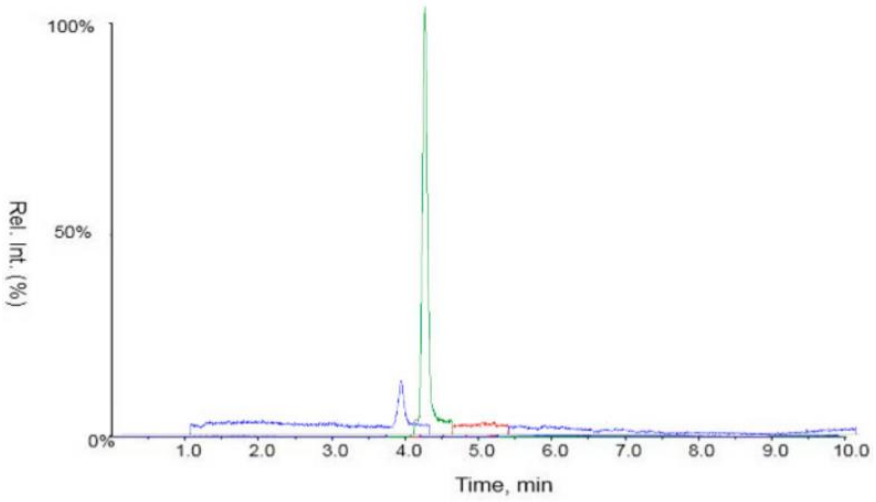


Figure 4. LC-MS chromatogram of a fennel extract

The level of imperatorin was below the limit of detection in all analyzed samples. 5-methoxypsoralene could not be detected in 7 samples, whereas psoralene could not be detected in 19 samples *(Table 5)*. Psoralene's concentration was about an order of magnitude lower than of 5-methoxypsoralene. In accordance with the European Medicines Agency's monographs [106], [107], the amounts of psoralene and 5-methoxypsoralene for the maximum therapeutic dose of fennel (7.5 g) were calculated. The total amount of furocoumarins was below the limit of detection in 7 out of the 33 samples. Samples that contained furocoumarin were identified by their total furocoumarin contents, which ranged from 0.0099 to 1.2209 µg/7.5 g *(Table 5).*

Table 5. Furocoumarin contents of fennel fruit samples

Sample	µg/7.5 g plant material (±SD)		
	psoralene	**5-methoxypsoralene**	**Total**
1	0.0551 ± 0.0015	0.6829 ± 0.0311	0.7380
2	0.0431 ± 0.0017	0.4830 ± 0.0172	0.5261
3	0.0504 ± 0.0011	0.6192 ± 0.0256	0.6696
4	0.0561 ± 0.0019	0.6419 ± 0.0301	0.6980
5	0.0646 ± 0.0031	0.6684 ± 0.0219	0.7330
6	0.0449 ± 0.0011	0.5239 ± 0.0092	0.5688
7	0.1072 ± 0.0050	1.1137 ± 0.0112	1.2209
8	0.0886 ± 0.0039	1.1052 ± 0.0216	1.1938
9	0.0938 ± 0.0045	1.0114 ± 0.0328	1.1052
10	0.071 ± 0.0018	0.8730 ± 0.0178	0.9440
11	0.0175 ± 0.0006	0.1165 ± 0.0033	0.1340
12	*	0.0220 ± 0.0007	0.0220
13	*	0.0236 ± 0.0009	0.0236
14	*	*	*

15	*	0.0183 ± 0.0008	0.0183
16	0.0142 ± 0.0002	0.0618 ± 0.0021	0.0760
17	*	*	*
18	*	*	*
19	*	*	*
20	0.0193 ± 0.0007	0.2360 ± 0.0072	0.2553
21	*	*	*
22	*	0.0116 ± 0.0003	0.0116
23	*	*	*
24	*	*	*
25	0.0133 ± 0.0005	0.1184 ± 0.0039	0.1317
26	0.0078 ± 0.0002	0.0321 ± 0.0007	0.0399
27	*	0.0188 ± 0.0006	0.0188
28	*	0.0426 ± 0.0018	0.0426
29	*	0.0111 ± 0.0003	0.0111
30	*	0.1045 ± 0.0061	0.1045
variety 'Berfena'	*	0.0634 ± 0.0028	0.0634
variety 'Grossfrüchtig'	*	0.0099 ± 0.0002	0.0099
variety 'Soroksár'	*	0.0745 ± 0.0011	0.0745
caraway commercial sample	*	0.1062 ± 0.0018	0.1062

* below the level of detection

Overall, based at least in part on the low furocoumarin content of the plant material, the results show that the therapeutic use of fennel fruits (not exceeding 7.5 g/day) can be regarded as safe for adults.

6. DISCUSSION

The aims of the present work were to isolate furocoumarins and limonoids from the seed and the peel of *Citrus trifoliata* and investigate their pharmacological activity, and to measure the furocoumarin content of *Foeniculum vulgare* samples. For the isolation, we used different chromatographic techniques, such as medium pressure liquid chromatography, flash chromatography, high performance liquid chromatography and open column chromatography. To determine the furocoumarin content of fennel samples, HPLC-MS was used after the sample preparation.

As previously described, *C. trifoliata* have been widely used by Korean Oriental Medicine doctors to treat various types of cancer, and also in Asian folk medicine to its antiphlogistic effect. Since the phytochemical composition and pharmacological characteristics of this species have not been fully explored, nevertheless its potential antiproliferative or cytotoxic effects may be related to furocoumarins or limonoids, we therefore aimed to isolate these compounds from *Citrus trifoliata* and investigate their pharmacological activity.

From the seed and the peel of *Citrus trifoliata* 10 compounds were isolated. The obtained compounds are furocoumarin (imperatorin, phellopterin), coumarin (scoparone, myrsellin, auraptene, triphasiol, umbelliferon, citropten), and limonoid derivatives (limonin, deacetyl nomilin). None of these isolated compounds showed a strong antiproliferative effect and had a cytotoxic effect on the tested cell lines. Although the compounds we investigated had previously been identified from the species, similar bioactivity studies with these compounds had not yet been conducted. The furocoumarins imperatorin and phellopterin had a moderate antiproliferative effect on the tumor cell line COLO 320. Additionally, phellopterin showed potent P-glycoprotein inhibitory activity. The ethidium bromide accumulation assay further demonstrated potent efflux pump inhibitory activity of the investigated furocoumarins on various bacterial cell lines. Phellopterin had antagonistic effects when combined with doxorubicin at various concentrations, but imperatorin also showed a modest synergistic effect. Auraptene, triphasiol, and scoparone were the only coumarins to demonstrate antiproliferative activity on the COLO 320 cell line. Additionally, the FACS assay demonstrated that auraptene is an effective P-glycoprotein inhibitor. The results of the checkerboard assay showed various interactions with the concurrently administered doxorubicin, ranging from synergism to antagonism.

In summary, these findings highlight the potential for these compounds to interact with chemotherapeutic agents, although no judgments concerning practical applicability can be drawn. The compounds examined by us are ingested in significant amounts when citrus fruits are eaten or used in the therapy, proving the scientific importance of these findings.

Furocoumarins are well known for their phototoxic effects. These compounds have been previously reported in the literature to be present in fennel, but no reliable information was available on their levels. This may be a risk, because fennel is widely used as a medicinal and aromatic plant. Using a sensitive liquid chromatography-mass spectrometry (LC-MS) technique, psoralene, 5-methoxypsoralene (bergapten), and imperatorin contents of 33 fennel samples were examined.

The estimated average overall intake of dietary furocoumarins in westernized populations is about 1.2-1.5 mg/day, with *Citrus* species such as grapefruit being the main sources. [108]. The European Medicines Agency's reflection paper suggests that a daily intake of 15 µg of furocoumarins in a herbal medicinal preparation does not pose any unacceptable risk [109]. In our experiments, the furocoumarin content of fennel fruits ranged up to 1.22 µg/day when used at the highest therapeutic dose stated in the monograph published by the European Medicines Agency. Therefore, the therapeutic use of fennel appears to be safe, at least for its furocoumarin content. Furthermore, because the values provided here were measured from extracts prepared using an organic solvent to ensure maximal extraction, it can be anticipated that the furocoumarin concentration of herbal infusions prepared with water, a less effective extracting solvent, may be much lower.

The work presented here was based on the combination of preparative and analytical phytochemical experiments. Although this thesis summarizes a methodologically diverse research project, the fundamental goals were similar: to contribute to the safe and rational use of medicinal plants and to identify specialized metabolites that might be used in therapy or drug discovery.

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APPENDIX

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II. **Kerekes D**, Csorba A, Gosztola B, Németh-Zámbori É, Kiss T, Csupor D. Furocoumarin content of Fennel – below the safety threshold
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