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

Summary of Ph.D. Thesis

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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). 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. 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. 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. *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. 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 seeds 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.

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.

MATERIALS AND METHODS

To investigate *Foeniculum vulgare* samples, 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, nonrelated progenies of former breeding work and three registered varieties (Berfena, Groβfrüchtig, and Soroksári).

As a result of recovery experiment, the most effective solvent (acetonitrile) was used for the extraction. 5.00 mL acetonitrile for 0.5000 mg plant material was used as the extracting solvent to extract plant samples. The extracts were filtered through 0.45 µM polytetrafluoroethylene (PTFE) syringe filters. The determination and quantification of furocoumarins - psoralene, 5-methoxypsoralene, and imperatorin - in Fennel extracts was performed by LC-MS. A Kinetex® 2.6 µm C18 100 Å, 100 x 2.1 mm column was used for separation, and the eluents were water and acetonitrile, both of which contained 0.1% formic acid. 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).

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. The seeds and the peels of *Citrus trifoliata* were crushed using a grinder and were ultrasonically extracted with MeOH at room temperature for 15 minutes. The crude MeOH extract were produced when the solvent evaporated at decreased pressure. Coumarins, furocoumarins and limonoids were isolated using multistep chromatographic methods like open column chromatography, medium pressure liquid chromatography, preparative thin layer chromatography, high pressure liquid chromatography, flash chromatography. The isolated compounds were identified by nuclear magnetic resonance spectroscopy (NMR).

During the investigation of the antiproliferative and cytotoxic effects of the isolated compounds, 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.

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. 50% of the growth inhibition dose (ED_{50}) were calculated by using CompuSyn software (ComboSyn, Inc., USA).

By flow cytometry evaluation of rhodamine 123 retention, multidrug resistant COLO 320 and doxorubicin-sensitive (parental) COLO 205 colonic adenocarcinoma cells were adjusted to a density of 2 x 10^6/mL. 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.

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 (tryptic soy broth) and LB (Luria-Bertani), respectively, until an optical density (OD) of 0.6 at 600 nm was reached. The relative fluorescence index (RFI) of the EB accumulation assay's final time point was calculated. 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.

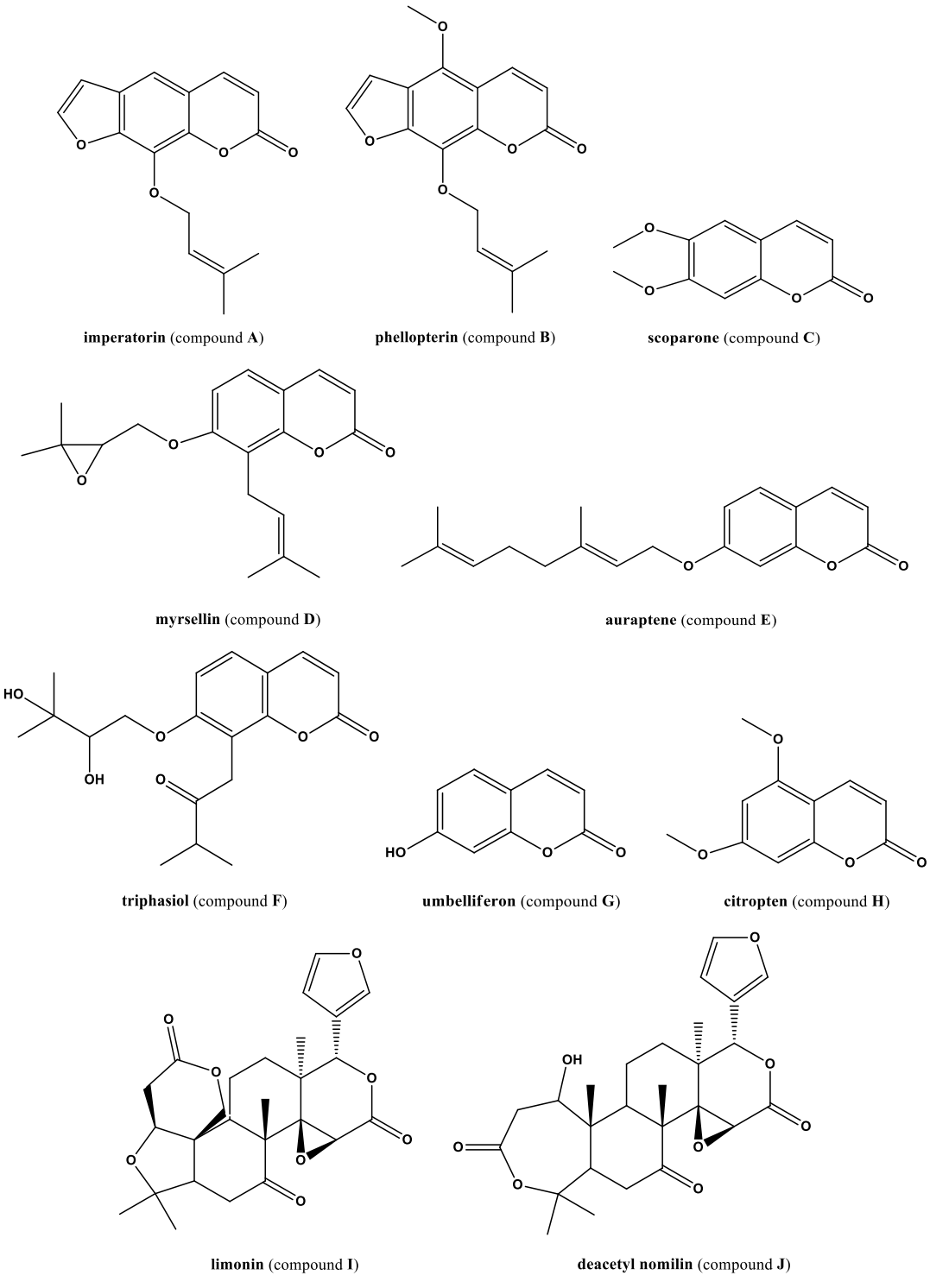
RESULTS

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*.



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**)

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

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 (µM)	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

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.

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

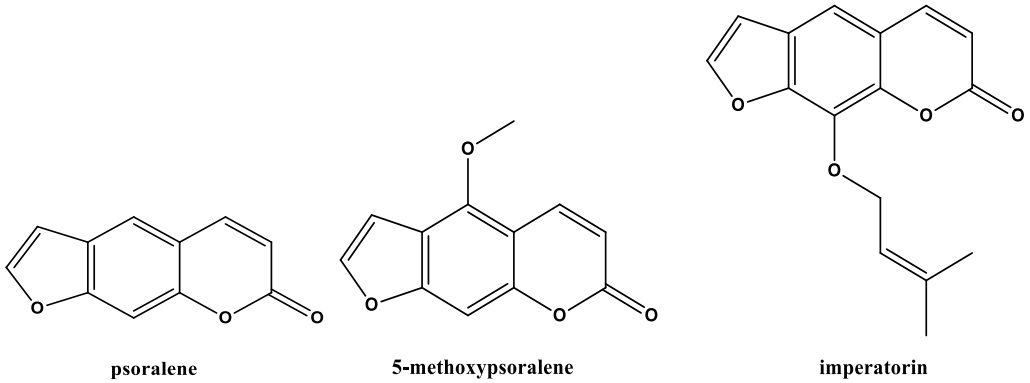
Compound	Concentration (µM)	FL-1*	FAR
tariquidar	0.2	88.20	11.44
imperatorin	2	11.30	1.47
	20	9.82	1.27
phellopterin	2	11.10	1.44
	20	20.30	2.63
scoparone	2	8.01	1.04
	20	15.60	2.02
myrsellin	2	10.90	1.41
	20	37.50	4.86
aurapene	2	13.60	1.76
	20	30.80	4.00
triphasiol	2	14.20	1.84
	20	14.30	1.85
limonin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

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.



psoralene 5-methoxypsoralene imperatorin

According to the results of extraction optimization, acetonitrile was the most effective solvent for the maximal extraction of furocoumarins *(Figure 1)*. Therefore, acetonitrile was used in our experiments.

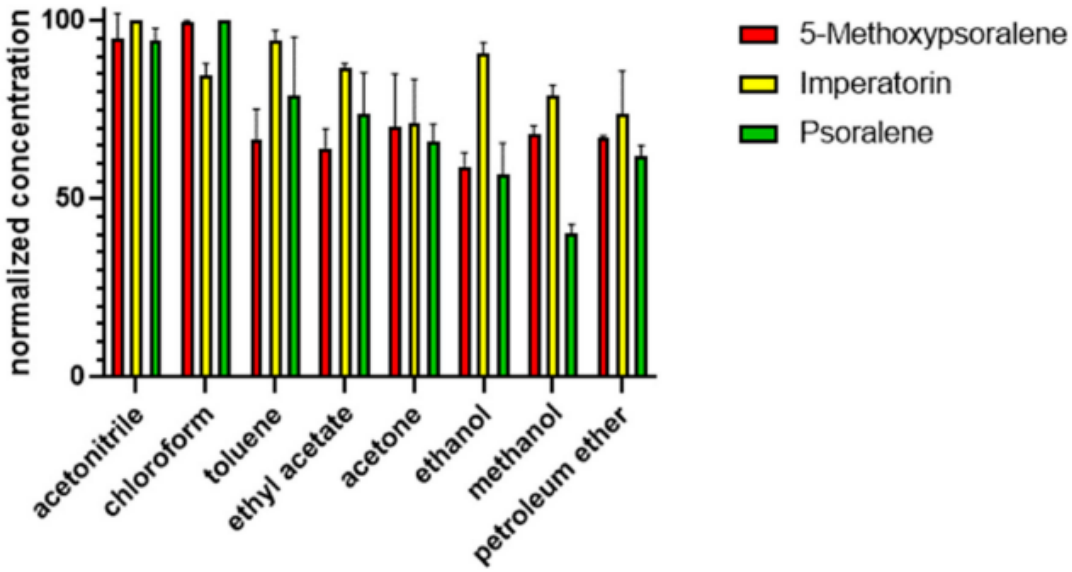


Figure 1. Different solvent extraction efficiencies expressed as normalized concentrations of the analytes

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

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 2).* 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.

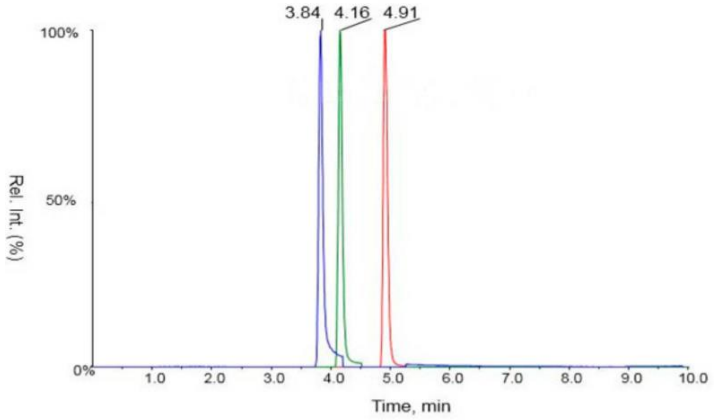


Figure 2. LC-MS chromatograms of the mixture of psoralene (Rt = 3.84 min), 5-methoxypsoralene (Rt = 4.16 min) and imperatorin (Rt = 4.91 min)

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, 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.

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). Although the compounds we investigated had previously been identified from the species, similar bioactivity studies with these compounds had not yet been conducted. None of these isolated compounds showed a strong antiproliferative effect and had a cytotoxic effect on normal (MRC-5) or doxorubicin-sensitive colon carcinoma (COLO 205) cell lines. 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. 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. 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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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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