

**Phytochemical and pharmacological analysis of certain plants
applied in the Iranian and European folk medicine**

Ph.D. Thesis

Javad Mottaghipisheh

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Department of Pharmacognosy

PHYTOCHEMICAL AND PHARMACOLOGICAL ANALYSIS OF CERTAIN PLANTS
APPLIED IN THE IRANIAN AND EUROPEAN FOLK MEDICINE

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Javad Mottaghipisheh

Supervisor:

Assoc. Prof. Dr. Dezső Csupor

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University of Szeged
Doctoral School of Pharmaceutical Sciences

Pharmacognosy Ph.D programme
Programme director: Prof. Dr. Judit Hohmann

Pharmacognosy Department
Supervisor: Assoc. Prof. Dr. Dezső Csupor

Javad Mottaghipisheh

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Defence Board:

Chair: Prof. Dr. Judit Hohmann

Member: Dr. Attila Hunyadi,

Dr. Gábor Janicsák

Assessment Board:

Chair: Prof. Dr. Zsolt Szakonyi, DSc

Opponents: Dr. Gábor Janicsák, PhD

Dr. Sándor Gonda, PhD

Members: Dr. Györgyi Horváth, PhD

Dr. Tamás Sovány, PhD

Dedication

I wish to dedicate this work to my wife, Nasim who her patience that cannot be underestimated, my parents, who have always urged me, the soul of my brother, and my nephews. This thesis would not have been possible without their love, encouragement, and appreciation for higher education.

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ABBREVIATIONS AND SYMBOLS

1D	one-dimensional
2D	two-dimensional
AAPH	2,2'-azobis(2-amidinopropane) dihydrochloride
ABCB1	ATP-binding cassette sub-family B member 1
ANOVA	analysis of variance
BDNF	brain-derived neurotrophic factor
CA	cluster analysis
CC	open-column chromatography
CCA	canonical correspondence analysis
CEM/C1	lymphoblastic leukemia
CHCl ₃	chloroform
CH ₂ Cl ₂	dichloromethane
CI	combination index
COSY	correlated spectroscopy
CPTLC	centrifugal preparative thin-layer chromatography
CREB	cyclic-AMP response element binding protein
DAD	diode array detector
DMSO	dimethyl sulfoxide
DPPH	2,2-diphenyl-1-picrylhydrazyl
EGCG	epigallocatechin gallate
ELISA	enzyme-linked immunosorbent assay
ELS	evaporative light scattering
EO	essential oil
EPG85.257RDB	MDR1 overexpressing human gastric adenocarcinoma cell line
ESIMS	electron spray ionization mass spectrometry
EtOAc	ethyl acetate
FAR	fluorescence activity ratio
FC	flash chromatography
FL-1	fluorescence intensity of the cells
FSC	forward scatter
FST	forced swimming test
GC	gas chromatography
GC-FID	gas chromatography-flame ionization detector
GC-MS	gas chromatography-mass spectrometry
GFC	gel filtration chromatography
GPS	global positioning system
H ₃ PO ₄	phosphoric acid
HEp-2	human epithelial type 2 cell line

HES-EI	high efficiency ion source
HPLC	high-performance liquid chromatography
HPLC-DAD	high-performance liquid chromatography-diode array detector
HSQC	heteronuclear single-quantum coherence spectroscopy
ICH	International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use
J	coupling constant
JMOD	<i>J</i> -modulated spin-echo experiment
K.G.	kaempferol-3- <i>O</i> -glucoside
K.S.	kaempferol-3- <i>O</i> -sophoroside
LC	liquid chromatography
LoD	limit of detection
LoQ	limit of quantification
MCF-7	Michigan Cancer Foundation-7 cell line
MCF7MX	BCRP overexpressing human epithelial breast cancer cell line
MDR	multiple drug resistance
MeOH	methanol
MPLC	medium pressure liquid chromatography
MS	mass spectrometry
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
<i>n</i> -BuOH	normal butanol
NMDA	<i>N</i> -methyl-D-aspartate
NMR	nuclear magnetic resonance
NO	nitric oxide
NOESY	nuclear Overhauser effect spectroscopy
OD	optical density
ORAC	oxygen radical absorbance capacity
PAR	proteinase-activated receptor
PBS	phosphate-buffered saline
PCA	principle component analysis
PTFE	polytetrafluoroethylene
PTLC	preparative thin-layer chromatography
Q.S.	quercetin-3- <i>O</i> -sophoroside
RPTLC	reverse-phase thin-layer chromatography
RP	reversed phase
RSD	relative standard deviation
SD	standard deviation
SDS	sodium dodecyl sulphate
SEM	standard error of the mean
SSC	side scatter

syn.	synonym
TI-2	topoisomerase-II
TLC	thin-layer chromatography
VLC	vacuum liquid chromatography
UV	ultraviolet
UV-Vis	ultraviolet-visible
δ	chemical shift
ρ -CREB	phosphor-cyclic-AMP response element binding protein

1. INTRODUCTION

Although medicinal plants belong to the most ancient tools of medicine, with several species having confirmed use for millennia, the first scientific data on their active constituents and mechanisms of action are not much older than 200 years. The discovery of secondary plant metabolites started in the 19th century, whereas the majority of pharmacological data was achieved in the 20th century. Compared to the huge number of plant taxa used in traditional medicine worldwide, the number of plants with confirmed efficacy, safety, elucidated mechanism of action and active constituents is infinitesimal. Plants of the European folk medicine have been studied relatively thoroughly, however, in case of several herbal drugs fundamental phytochemical and pharmacological data are missing.

The aim of the present work was to provide phytochemical and pharmacological data on some medicinal plants to support their rational use. The use of flowers of Roman chamomile (*Chamaemelum nobile*) for the symptomatic treatment of mild spasmodic gastrointestinal complaints including bloating has been acknowledged by the European Medicines Agency recommended the extract [1]. This indication is based on the long-standing use of this plant for the listed purposes in folk medicine, [2] however, no experimental data are available on its spasmolytic activity. Our goal was to experimentally analyse the spasmolytic effect of the plant, its essential oil and to determine the active components responsible for this effect. In case of *Matricaria chamomilla*, several metabolites responsible for the diverse biological activities have been identified, however, the systematic data on their amounts in different geographical regions are missing. Our goal was to study the phytochemical variation of samples collected in Iran, in my homeland.

In Iran, folk medicine is still a living tradition. Iranian flora is very diverse, comprising more than 8,000 vascular plant taxa, 2,597 of which are endemic or subendemic species [3]. Several species are used for therapeutic purposes without any scientific data on their active constituents. As part of my work, we examined the secondary metabolites of two native, previously slightly studied Iranian plants, *Eremurus persicus* and *Ducrosia anethifolia*. *E. persicus* is one of the 50 species of the genus [4], applied in Iranian folk medicine to treat skin diseases [5–11], as antidiabetic [11,12], and to treat gastrointestinal disorders and rheumatism [13]. From the six species of the *Ducrosia* genus, *D. anethifolia* is the most widely used in Iran. In folk medicine this plant is used in case of insomnia and anxiety [14], irregularities of menstruation, and for its carminative effect [15]. Since based on chemotaxonomic considerations we assumed that the latter species contains furocoumarins, our goal was to study the pharmacological effects of these compounds.

Crocus sativus is the most important aromatic plant of Iran, with increasing importance as an aromatic plant. The stigma of *C. sativus* (saffron) has been used for the treatment of depression and anxiety in folk medicine. Its efficacy has been confirmed in several clinical trials as well. However, the price of the raw material is a major obstacle that prevents its widespread use as medicinal drug. Other plant parts, including tepal and stamen are considered as by-products of saffron production and are not utilized industrially. Recent studies refer to antidepressant activities of these plant parts [16–19]. Although their major components of tepal and stamen have already been reported [20–25], there are

no systematic data on the variations and ranges of these compounds. Such data are indispensable if saffron by-products will be considered as medicinally valuable industrial raw materials.

2. AIMS OF THE STUDY

The Iranian flora comprises a wide range of plants that have been widely applied in folk medicine. Although some of these have been previously subjected to phytochemical and pharmacological analysis, many medicinal plants are still un-investigated. The plants applied in the European folk medicine have generally been investigated more in detail, however, the active components and mechanisms of action of many plants of great importance are still undiscovered. The goals of our study were to:

- study the phytochemical composition and certain bioactivities of some Iranian and European medicinal plants
- review the literature on the studied plants, especially in case of the less studied Iranian medicinal plants
- isolate and identify of secondary metabolites of *Ducrosia anethifolia* and study their antiproliferative/cytotoxic effect
- isolate and identify of secondary metabolites of *Eremurus persicus*,
- study the chemodiversity of *Crocus sativus* stamens and tepals
- study the spasmolytic activity of *Chamaemelum nobile* and discover the mechanism of activity and its active components
- study the chemodiversity of *Matricaria chamomila* essential oils of Iranian origin

3. LITERATURE OVERVIEW

3.1. *CHAMAEMELUM NOBILE* (L.) ALL.

Chamaemelum nobile (L.) All. (Roman chamomile) is a perennial herb native to South-Western Europe, and it is grown as a medicinal plant throughout Europe and Africa. In the European Pharmacopoeia, the dry flowers of the double-flowered variety are official [26].

The application of the plant in traditional herbal medicinal products has been accepted by the European Medicines Agency. The crushed herbal material (as tea) and a liquid extract of the plant (extraction solvent: ethanol 70% v/v) can be used for the symptomatic treatment of mild, spasmodic gastrointestinal complaints including bloating and flatulence [27].

3.1.1. *Taxonomy of C. nobile*

The *Chamaemelum* genus belongs to the Compositae (syn. Asteraceae) family, subfamily of Asteroideae, tribe of Anthemideae, order of Asterales, Asterids class, subclass of Asteridae, Spermatophyta subdivision, Magnoliopsida division [28].

3.1.2. *Phytochemistry of C. nobile*

The aliphatic esters of the EO (essential oil) [29], sesquiterpene lactones [30] and flavonoids [31–33] are the most characteristic phytoconstituents of *C. nobile*. The polysaccharide content of the dried flower was also found to be significant [34].

3.1.3. *Folk medicinal use of C. nobile*

Chamaemelum nobile has been applied as a medicinal plant from the middle ages. The beginning of cultivation of the species was in England in the 16th century [35]. The double variety of the flower has certainly been known since the 18th century [36]. The plant is called “nobile” (Latin, noble) to distinguish its outstanding therapeutic efficacy compared to *Matricaria recutita* L. (German chamomile) [35].

In the different regions of Europe, *C. nobile* has been used for several ailments, including dysmenorrhoea, flatulence, nausea and vomiting, dyspepsia, anorexia, and specifically in case of flatulent dyspepsia and gastrointestinal pain associated with mental stress [37–41]. The use of *C. nobile* as tea was also described in the Mediterranean region to improve appetite and to prevent indigestion after meal [42–44]. Many of the abovementioned traditional applications of *C. nobile* might be related to its supposed smooth muscle-relaxant effect.

3.1.4. *Bioactivities of C. nobile*

Most of the studies on *C. nobile* were performed with its EO. Antimicrobial effects of *C. nobile* EO against different bacterial and fungal strains [45–48] and the antifungal activity of the aqueous extract [49] have been reported.

The heat shock protein modulating effects and anti-inflammatory capacity of the flavonoids apigenin and quercetin (the main flavonoids of the species), along with the anti-inflammatory activities of α -bisabolol, guaiazulene, and chamazulene as the major EO contents have been reported

in preclinical studies [50–53]. *In vivo* antiphlogistic effect of the polysaccharides of *C. nobile* was also described [34].

It is worth noting that no *in vitro* and *in vivo* studies have been carried out to evaluate the smooth muscle relaxant effect of the plant, although the use of *C. nobile* extract for gastrointestinal disorders seems to be related to this bioactivity.

Nevertheless, an *in vitro* study was conducted to assess the vasorelaxant effect of an aqueous extract of *C. nobile* and the results showed an effect through the NO-cGMP pathway or possibly through a combination of Ca²⁺-channel inhibition plus NO-modulation and phosphodiesterase inhibition [54]. A significant hypotensive effect (*in vivo*) of *C. nobile* aqueous extract after the oral administration was reported on spontaneously hypertensive rats [55], which was supposed to be associated to the flavonoid content of the plant [56].

Only the effects after external application [57] and aromatherapeutic use [58] of *C. nobile* have been studied clinically.

Since apigenin and luteolin exert remarkable smooth muscle relaxant activities on guinea pig ileum [59], and these flavonoids are the main constituents of *C. nobile*, the recommended smooth muscle-relaxant activity of the plant may be attributed to its flavonoid content.

3.2. MATRICARIA CHAMOMILLA L.

Matricaria chamomilla L. (syn. *Chamomilla recutita* L. Rauschert, German chamomile), is one of the renowned medicinal plants with long-standing folk medicinal use throughout Europe. The name chamomile derives from a Greek word meaning "ground apple" due to its aroma being similar to some apple varieties. The name of *Matricaria* originates from the Latin (matrix means uterus), because of the use of chamomile by women for gynaecological disorders and abnormalities of the menstrual cycle [60].

3.2.1. Taxonomy of *M. chamomilla*

The *Matricaria* genus belongs to the Asteraceae family, subfamily of Asteroideae, suborder of Asteridae, order of Asterales, Magnoliopsida class, Asteridae subclass, Spermatophyta subdivision, and division of Magnoliophyta [61]

3.2.2. Phytochemistry of *M. chamomilla*

Sesquiterpenes are reported as the most dominant constituent of *M. chamomilla* EO. α -Bisabolol oxide A (7.9–62.1%) [62–65], α -bisabolol (56.9%) [66], β -farnesene (52.73%) [67], (*E*)- β -farnesene (42.59%) [68], α -bisabolol oxide B (25.56%) [65], β -cubebene (27.8%) [69], chamazulene (27.8–31.2%) [70], trans- β -farnesene [71,72], and spathulenol (12.50%) [73] were reported as the major EO compounds.

Non-volatile secondary metabolites of *M. chamomilla* have also been explored. The major phytochemicals consist of polyphenols, especially the flavonoids apigenin, luteolin, patuletin, quercetin and their glucosides [74–76]; among them apigenin is reported as one of the most bioactive phenolics [75–77].

3.2.3. *Folk medicinal use of M. chamomilla*

The flower of *M. chamomilla* is consumed around the world as herbal tea in an amount of over 1 million cups daily [78,79]. While *M. chamomilla* is mainly used internally to facilitate digestion and as antispasmodic, it is also used externally to treat minor wounds. In traditional medicine, its use extends from the relief of various pains to the facilitation of menstruation [80]. *M. chamomilla* EO has been frequently applied in Iranian folk medicine as an anti-inflammatory, antispasmodic, anti-peptic ulcer, antibacterial and antifungal agent [81,82].

3.2.4. *Bioactivities of M. chamomilla*

In preclinical studies several biological activities have been reported for *M. chamomilla*, for instance anti-inflammatory, analgesic, and antispasmodic effects [83–86]. The wound healing effect of different extract gastrointestinal effects of the extracts and pure constituents have been confirmed in animal experiments. The aqueous extracts of flowers and the EO exerted central nervous system effects, including sleep-inducing, anxiolytic, and sedative activities [87]. Many of the former studies focused on the pharmacological effects of the EO and its constituents (α -bisabolol, bisabolol oxides, chamazulene) [88]. Moreover, antibacterial activities of various extracts and EO of *M. chamomilla* indicated a significant potential [60].

The following indications are partly based on the results of clinical studies: treatment of minor gastro-intestinal, relief of common cold symptoms, remedy of minor ulcers and inflammations of the mouth and throat, and healing of minor superficial wounds and small boils (furuncles) [87].

3.3. *DUCROSIA ANETHIFOLIA* (DC.) BOISS.

D. anethifolia is one of the three wildy growing species of the genus in several areas of Iran, Afghanistan, Syria, Pakistan, Iraq, Lebanon, and some other Arab states and countries alongside of the Persian Gulf [89–91].

3.3.1. *Taxonomy of D. anethifolia*

The *Ducrosia* genus belongs to the Apiaceae Lindl. (Umbelliferae Juss.) family, sub-family Apioideae, order of Apiales, superorder of Rosidae, subclass of Dicotyledonae, Magnoliopsida class, Tracheophyta division [92]. The genus *Ducrosia* (Apiaceae) consists of six species: *Ducrosia ismaelis* Asch., *D. flabellifolia* Boiss., *D. assadii* Alava., *D. areysiana* (Deflers) Pimenov & Kljuykov, *D. inaccessa* (C.C.Towns.) Pimenov & Kljuykov and *D. anethifolia* (DC.) Boiss.

3.3.2. *Phytochemistry of D. anethifolia*

3.3.2.1. *Coumarin derivatives as the major phytochemicals of Ducrosia spp.*

Coumarins belong of benzopyrones (1,2-benzopyrones or 2H-1-benzopyran-2-ones). These compounds form an important class of oxygen-containing heterocycles extensively distributed in the nature [93].

The first natural coumarin was isolated in 1820 from the seeds of *Dipteryx odorata* (syn. *Coumarouna odorata*) (Fabaceae) [94]. The name of coumarin originates from the Portuguese term

“coumarou” used for the seeds of this plant [95]. It is assumed that these secondary metabolites have a protective role against predators and herbivores [96,97].

3.3.2.2. Chemotaxonomy of coumarins

Coumarins have been isolated from hundreds of plants species distributed in more than 40 different families with diversity of 1300 types. Families with occurrence numbers of > 100 are identified as Apiaceae (Umbelliferae), Rutaceae, Asteraceae (Compositae), Fabaceae (Leguminosae), Oleaceae, Moraceae, and Thymelaeaceae, respectively [98]. Natural coumarins can be classified as simple coumarins, furocoumarins (linear and angular), dihydro-furocoumarins, pyranocoumarins (linear and angular), phenyl-coumarins, and bis-coumarins [93]. Apiaceae is the major and most diverse source of coumarins, containing five different types of coumarin derivatives including simple coumarins, linear and angular furocoumarins, linear and angular pyranocoumarins [98,99].

3.3.2.3. Furocoumarins

Furocoumarins contain a furan ring fused with a coumarin nucleus. Depending on the attachment of furan group to the coumarin skeleton, they are structurally divided into linear and angular groups [100]. They are thought to be synthesized as phytoalexins [101].

Furocoumarins have been identified in several plant families, including Meliaceae, Rosaceae, Pittosporaceae and Asteraceae. The plants that with the highest furocoumarin contents are mostly members of the Fabaceae, Moraceae, Apiaceae and Rutaceae families [102].

Psoralen, bergapten, xanthotoxin, and imperatorin belong to the most widely distributed linear furocoumarins, whereas angelicin is the simplest angular compound. In combination with UV radiation in a dose- and time-dependent manner, linear furocoumarins have phototoxic and photogenotoxic effects [103]. Furthermore, other class of furocoumarins have also been described. Marmesin and colombianin are examples of dehydro-linear and angular furocoumarins, respectively.

Consumption of furocoumarin-rich foods combined with UV exposure (320–400 nm) has been correlated with phototoxic skin reactions, while long-term oral exposure to high doses of certain pure furocoumarins in PUVA (psoralen + UVA treatment for eczema, psoriasis, etc.) therapy may lead to higher occurrence of some types of skin tumors. Clinical evidence supports that intake of excessive amounts of furocoumarins might cause kidney and liver toxicity [104]. The most common phototoxic furocoumarins are psoralen, bergapten, angelicin, bergaptol, xanthotoxin, sphondin, and pimpinellin [105].

3.3.2.4. Chemistry of *D. anethifolia*

The phytochemical profile of *D. anethifolia* has only been partly explored. Furocoumarins and terpenoids are characteristic components of the *Ducrosia* genus. From the seeds of *D. anethifolia*, two new terpenoids, the monoterpene ducrosin A and the sesquiterpene ducrosin B were isolated along with stigmasterol and the furocoumarins heraclenin and heraclenol [106]. Psoralen, 5-methoxypsoralen, 8-methoxypsoralen, imperatorin, isooxypeucedanin, pabulenol, pangelin, oxypeucedanin methanolate, oxypeucedanin hydrate, 3-*O*-glucopyranosyl- β -sitosterol and 8-*O*-debenzoylpaeoniflorin were also isolated from the extract of *D. anethifolia* [14,107]. GC analysis of the fatty acids showed high percentages of elaidic acid and oleic acid [106], beside 58.8% petroselinic

acid in the seed oil of *D. anethifolia* [108]. Apart from *D. anethifolia*, furocoumarins (psoralen, isopsoralen) have been reported only from *D. ismaelis* from this genus [109].

In the literature, most of papers deal with the composition of the EO. As major constituents, α -pinene (11.6% [110], 70.3% [111], 59.2% [112]); *n*-decanal (1.4–45% [113], 45.06% [114], 70% [115], 57% [116], 25.6–30.3% [117], 18.8% [118]), dodecanal (28.8% [119]), and *cis*-chrysanthenyl acetate (72.28%) [120,121] have been reported.

3.3.3. Folk medicinal use of the *Ducrosia* genus and *D. anethifolia*

Ducrosia species belong to rare plants, hence their medicinal use is not widespread. *D. flabellifolia* Boiss. is traditionally used for its pain relieving and sedative properties in Jordan [122]. *D. assadi* has been consumed as a flavouring in food, and as antispasmodic, soporific, anti-inflammatory, antiseptic and carminative agent in the Iranian folk medicine [123,124].

In Iranian folk medicine, the whole herb of *D. anethifolia* (especially its aerial part) has been consumed as an analgesic and in case of insomnia and anxiety [14]. The aerial part, including the seed was described as carminative and useful for irregularities of menstruation [15]. Moreover, the herb is added to a variety of Persian foods for flavouring and spice [89,125].

3.3.4. Bioactivities of the *Ducrosia* genus and *D. anethifolia*

The ethanolic extract of *D. flabellifolia* exerted antiproliferative activity against MCF-7 and HEp-2 cell lines [126]. The EO of *D. flabellifolia* showed antimicrobial activity against *Candida albicans* and *Staphylococcus aureus* [122]. The EOs obtained from *D. assadi* was characterized by moderate antioxidant capacity *in vitro* [123].

The extracts of aerial parts of *D. anethifolia* have been assessed for its bioactivities *in vitro* and *in vivo*. Moderate anti-radical scavenging [111,127] and antibacterial effects [116,128] have been reported. The furocoumarin pangelin isolated from *D. anethifolia* showed effect against a panel of fast growing mycobacteria [107]. The EO of the seeds and the methanol extract of *D. anethifolia* showed a weak antibacterial effect against 14 Gram positive and negative bacteria [121,129].

The EO of *D. anethifolia* demonstrated significant to moderate cytotoxic activity on three human cancer cell lines (K562, LS180 and MCF-7), while EO of *D. flabellifolia* possessed less remarkable effect [119]. The sesquiterpene ducrosin B isolated from *D. anethifolia* also exerted remarkable *in vitro* cytotoxicity against the human colon HCT-116 and ovary SKOV-3 cancer cell lines [106].

The crude extract of *D. anethifolia* and the isolated furocoumarins demonstrated *in vivo* antidiabetic activities [14]. The following activities of the EO have been described in *in vivo* experiments: anxiolytic [115,130,131], sedative [115], analgesic and anti-inflammatory [132], and anti-locomotor effect [131]. An *in vivo* study demonstrated that intra-peritoneal administration of the EO improved spatial learning and memory in adult male rats [133]. The reduction of pentylenetetrazole-induced seizure manifestations in male Wistar rats was observed after intra-peritoneal injection of the hydroalcoholic extract [134]. The extract of *D. anethifolia* reduced the level of testosterone and spermatogenesis, and number of germ cells in male Wistar rats [135].

3.4. EREMURUS PERSICUS (JAUB. & SPACH) BOISS.

The genus *Eremurus* comprises 50 species, mainly distributed in Central and Western Asia [136–139].

3.4.1. Taxonomy of *E. persicus*

The *Eremurus* genus belongs to the Xanthorrhoeaceae (syn. Asphodelaceae) family, subfamily of Asphodeloideae, order of Asparagales, Liliopsida class, Tracheophyta division [140].

3.4.2. Phytochemistry of the *Eremurus* genus and *E. persicus*

The investigation of non-volatile phytoconstituents of *Eremurus* spp. extracts has been rarely carried out. The following compounds have been previously isolated from *Eremurus* species: aloesaponol III 8-methyl ether, chrysophanol 8-methyl ether, 2-acetyl-1-hydroxy-8-methoxy-3-methylnaphthalene [141–143], methyl linolenate, β -sitosterol, isoorientin, inosine [144], chrysophanol [141–144], altaicusin A, emodin [142], a bi-anthraquinone glycoside, 2-acetyl-1,8-dimethoxy-3-methylnaphthalene, a pre-anthraquinone, 10-(chrysophanol-7'-yl)-10-hydroxychrysophanol-9-anthrone [141], aloesaponol III, and (R)-aloechryson [143]. By using Folin-Ciocalteu's method, total phenolic contents of various extract of *E. spectabilis* [10,145,146] were also assessed.

The phytochemistry of *E. persicus* has been scarcely studied. Only four compounds have been identified from extracts of *E. persicus*. (R)-(-)-Aloesaponol III 8-methyl ether [147], 2-acetyl-1-hydroxy-8-methoxy-3-methylnaphthalene, helminthosporin [148], and 5,6,7-trimethoxy-coumarin [4] were isolated from the leaves of *E. persicus*.

3.4.3. Folk medicinal use of the *Eremurus* genus and *E. persicus*

Some species of the genus *Eremurus* have traditionally been used in Iranian, Turkish, and Chinese folk medicine. The roots of *E. chinensis* Fedtsch and *E. anisopterus* (Ker. et Kir) Regel. have been used in China for medicinal purposes [138,141], whereas the leaves of *E. spectabilis* (Bieb.) Fedtsch. have been used in Iranian and Turkish folk medicine as food additive [149] and as remedy of various disorders [4,7,15,146,150–152].

The studied species *E. persicus* (Jaub. & Spach) Boiss. is broadly spread out in Iran [4]. In Turkish folk medicine, its root is consumed for relieving gastrointestinal disorders and rheumatism [13], against inflammatory skin conditions [5,6], and scabies [7]. The leaves are traditionally eaten in Iran with rice [153], also used as a remedy of diabetes and constipation, and to treat of different disorders of stomach, liver, and the genitourinary system [11,12], and as diuretic [154], against fungal skin diseases, as a remedy of atherosclerosis, as well as for inflammation-related diseases [8–11].

3.4.4. Bioactivities of the *Eremurus* genus and *E. persicus*

The bioactivities of different extracts of *E. spectabilis* populations have been extensively studied. Antioxidant and antiradical activities of leaves and roots [10,145,146,155,156], anti-proliferative effects [10,151,156,157], cytotoxic, nitric oxide (NO) production inhibitory activity [144], gastroprotective effect [158], and antibacterial potential [139,145,156] of *E. spectabilis* extracts have been documented in the literature. Analysis of antioxidant potency of root extract of *E. chinensis*

Fedtch. [157], *in vivo* hypoglycaemic effect of methanolic extracts of *E. himalaicus* [159], and protein tyrosine phosphatase inhibitory activity of *E. altaicus* (Pall.) Stev. [142] have also been reported.

Different extracts of leaves and roots of *E. persicus* were previously indicated to possess anti-inflammatory [136], antibacterial and cytotoxic activities [12], dihydrofolate reductase inhibitory [160], antiglycation [4], antiradical, anti-inflammatory, anti-proliferative [10], antileishmanial [147], and antifungal effects [9]. The antioxidant, anticancer, acetylcholinesterase inhibitory, and antimicrobial activities and anti-dermatophyte effects of the EO of *E. persicus* were also studied [161].

3.5. CROCUS SATIVUS L.

The genus of *Crocus* includes 85–100 species distributed around the globe, mostly in Western Asia and the Mediterranean region. Phytogeographically, *Crocus* species usually occur within the Mediterranean floristic region, extending eastward into the Irano-Turanian region.

Crocus flowers, having monocot characteristic structure, consist of stamen, stigma and tepal. However numerous studies refer to perianth as “sepal” and “petal.” In some cases the investigated flower part is not clearly named, but the description narrows it to tepals [24,25,162]. Some of the studies are referring to the investigated sample as “petals” without any further description [18,19,22,163], while some further studies refer to saffron flower without stigma [20,164–166].

From the *Crocus* genus, *C. sativus* (saffron crocus) has the highest industrial importance. The flowers of the plant compose 6 tepals, 3 yellow stamens, and a white filiform style ending in a stigma composed of 3 threads [167]. The tepals are fragrant, have deep lilac purple colour with darker veins and a darker violet stain in the throat; the throat is white or lilac, pubescent. The stamen includes 7–10 mm long filament, and yellowish, 15–20 mm long anther. The style is divided into three deep red clavate branches, each branch is 25–32 mm long. Capsules and seeds are rarely produced [168].

The stigma of *C. sativus* is famed as saffron and well-known as the priciest spice in the world. At the moment, the stigma is the only utilized plant part. To obtain 1 kg of dried stigma, 300,000 flowers are approximately needed [167]. The analysis of alternative plant parts (the industrial by-products tepal and stamen) that are available in larger amounts and considered as waste seems to be promising topic for the research, due to increasing scientific interest for the bioactivities of saffron crocus.

3.5.1. Taxonomy of saffron crocus

Crocus genus belongs to Iridaceae family, tribe of Croceas, order of Liliales, Liliidae sub-class, class of Liliopsida, sub-division of Spermatophyta, and Magnoliophyta division [169].

3.5.2. Folk medicinal application of saffron crocus stigma

The stigma of saffron crocus is a popular spice and has also been consumed in the traditional Arabic and Islamic medicine for several purposes, especially to facilitate difficult labour, as cardiac and liver tonic and hepatic deobstruent, and for the treatment of female genito-urinary system disorders and male impotence. Its effect on the central nervous system have also been studied. Regarding to Sayyed Esmail Jorjani (1042-1136 A.D.): "saffron is astringent and resolvent and its fragrance can strengthen

these two effects. Hence, its action on enlivening the essence of the spirit and inducing happiness is great". This suggested activity can be interpreted as a positive effect on mood or as an antidepressant effect [170].

3.5.3. *Phytochemistry of saffron crocus*

3.5.3.1. *Chemistry of stigma of saffron crocus*

The characteristic constituents of saffron crocus stigma consist of crocin, crocetin, picrocrocin and safranal. The carotenoids of crocin and crocetin are responsible for its color, the specific bitterish taste is attributed to the monoterpene glycoside picrocrocin, while safranal (an aromatic aldehyde) provides the aroma [170].

3.5.3.2. *Phytoconstituents of saffron crocus by-products*

Several papers report the chemical composition of sepal and petal samples, however, botanically these plant parts should be defined as tepal. Therefore, in case of previous papers we refer to the plant parts used by the authors.

After removing the stigma from flowers of saffron crocus, tepal and stamen parts are considered as waste. Many papers studied the major phytoconstituents of saffron crocus by-products. The petal contained total phenolic content with 95.3 ± 0.8 mg kaempferol-3-glucoside equivalent (KE)/g of dry weight (dw), flavonoids (63.9 ± 1.1 mg KE/g dw), and anthocyanins (16.6 ± 0.1 mg malvidin-3-glucoside equivalent/g dw). The stamen comprised lower contents of phenolic, flavonoid, and anthocyanin [167]. A mixture of petal and stamen materials possessed the total flavonoid content of 55.8 mg rutin equivalent (RE)/g after application of ultrasonic extraction [171]. In another study, by using Folin-Ciocalteu method the methanolic extract of petal contained the highest content of phenolics and flavonoids with 65.34 ± 1.74 mg of GAE/g of extract and 60.64 ± 2.71 mg of catechin equivalent (CE)/g of dry plant material, respectively, compared to the style and stamen parts [172].

The flavonol glycosides including kaempferol-3-*O*-sophoroside, kaempferol-3-*O*-rutinoside, quercetin-3-*O*-glucoside-7-*O*-rhamnoside, and isorhamnetin-3-*O*-sophoroside were identified as the petal major compounds [165]. Kaempferol-3-*O*-sophoroside was isolated as the major compound from the flower material of saffron crocus (except stigma) [20]. Several non-flavonoid compounds including crocetin derivatives, crocusatin B and C, safranal, picrocrocin, and sinapic acid derivatives were previously identified from saffron crocus flowers [173–175].

Several glycosylated flavonoids including kaempferol-3-*O*- β -*D*-glucopyranoside, kaempferol-7-*O*- β -*D*-glucopyranoside, kaempferol-3-*O*-sophoroside, kaempferol-3-*O*- β -*D*-(2-*O*- β -*D*-6-*O*-acetylglucosyl)-glucopyranoside, isorhamnetin-3-*O*- β -*D*-glucopyranoside, isorhamnetin-3,7-di-*O*- β -*D*-glucopyranoside, and quercetin-3-*O*- β -*D*-glucopyranoside were isolated from the methanolic extract of the petal. By applying LC-MS/MS, quercetin-3,7-di-*O*- β -*D*-glucopyranoside (27.6 mg/g of dry petal), and kaempferol-3,7-di-*O*- β -*D*-glucopyranoside (20.2 mg/g of dry petal) were quantitatively identified as the main constituents of the petal [166].

In a further study, the most abundant natural compounds from petal were characterized as kaempferol 3-*O*- α -(2-*O*- β -glucosyl)-rhamnoside-7-*O*- β -glucoside and quercetin 3-*O*- α -(2-*O*- β -glucosyl)-rhamnoside-7-*O*- β -glucoside [176]. Kaempferol and crocin were isolated from methanolic

extract of petal with yields of 12.6 and 0.6% (w/w), respectively [164]. In an LC-MS study, kaempferol, naringenin, and quercetin, some flavanone and flavanol glycosides and derivatives esterified with phenylpropanoic acids were also detected [177]. The presence of 3-hydroxy- γ -butyrolactone, kinsenoside, and goodyeroside A was also reported from the petal [163].

Three new monoterpenoids crocusatin-J, -K, and -L, a new naturally occurring acid (3S),4-dihydroxybutyric acid and kaempferol glycosides including kaempferol 3-glucoside (astragalin), kaempferol 3-*O*-sophoroside, and kaempferol 3-*O*- β -*D*-(2-*O*- β -*D*-6-acetylglucosyl) glucopyranoside were furtherly isolated from methanolic extract of petal as the major compounds [22]. Several other known compounds including 6-hydroxy-3-(hydroxymethyl)-2,4,4-trimethyl-2,5-cyclohexadien-1-one 6-*O*- β -*D*-glucoside, 3-formyl-6-hydroxy-2,4,4-trimethyl-2,5-cyclohexadien-1-one, 4-hydroxy-3,5,5-trimethylcyclohex-2-enone, picrocrocin, crocusatin-C, -D, -E, and -I, methylparaben, 4-hydroxyphenethyl alcohol, *p*-coumaric acid, 4-hydroxybenzoic acid, protocatechuic acid methyl ester, protocatechuic acid, vanillic acid, methylvanillate, 3-hydroxy-4-methoxybenzoic acid, kaempferol, kaempferol 3-*O*- β -*D*-(6-*O*-acetyl) glucopyranoside, kaempferol 3-*O*- β -*D*-(2-*O*- β -*D*-6-*O*-acetylglucosyl)-glucopyranoside, kaempferol 7-*O*- β -*D*-glucopyranoside, kaempferol 3,7-di-*O*- β -*D*-glucopyranoside, kaempferol 3-*O*- β -*D*-(6-*O*-acetyl)-glucopyranoside-7-*O*- β -*D*-glucopyranoside, nicotinamide, tribulusterine, harman, 1-(9H- β -carbolin-1-yl)-3,4,5-trihydroxypentan-1-one, adenosine were also isolated and identified from the petal matters [22].

By applying LC-UV-Vis-DAD-MS, the dried petal samples were analysed and three aglycone flavonoids kaempferol, quercetin, and isorhamnetin and 17 glycosylated derivatives of them including kaempferol 7-*O*-sophoroside, kaempferol 3,7-di-*O*-glucoside, kaempferol 7-*O*-gentobioside, kaempferol 3-*O*-sophoroside, kaempferol 3-*O*-neohesperidoside, kaempferol 3-*O*-sophoroside acetate, kaempferol 3-*O*-glucoside, isorhamnetin 3-*O*-sophoroside, isorhamnetin 3-*O*-gentobioside, isorhamnetin 3-*O*-neohesperidoside, isorhamnetin 3-*O*-glucoside, quercetin 3-*O*-neohesperidoside, and quercetin 3-*O*-sophoroside were characterized [24].

In a HPLC fingerprinting study on perianth segments (sepal and petal) of 70 *Crocus* species, numerous glycosylated anthocyanins, including delphinidin 3,5-di-*O*- β -glucoside, delphinidin 3-*O*- β -rutinoside, delphinidin 3-*O*- β -glucoside-5-*O*- β -(6-*O*-malonyl)-glucoside, petunidin 3,5-di-*O*- β -glucoside petunidin 3,7-di-*O*- β -(6-*O*-malonyl)-glucoside, petunidin 3-*O*- β -rutinoside, malvidin 3,7-di-*O*- β -(6-*O*-malonyl)-glucoside, and flavonoids with quantity ratio of >20% for quercetin 3-*O*- β -sophoroside and kaempferol 3-*O*- β -sophoroside were identified in saffron crocus [25]. In a comparison research performed by HPLC-DAD, different parts of saffron crocus were qualitatively and quantitatively analysed. The results indicated that the major phytoconstituents of tepal are the following: kaempferol di-hexoside > kaempferol 3-*O*-glucoside > kaempferol tri-hexoside > quercetin 3,4'-di-*O*-glucoside. Moreover, delphinidin 3,5-di-*O*- β -glucoside was the main anthocyanin from the petal materials harvested from diverse origins. The comparison of phytoconstituents of stamen and stigma samples revealed that crocin, picrocrocin, safranal, 4-hydroxy-2,6,6-trimethyl-3-oxocyclohexa-1,4-diene-1-carboxaldehyde, 4-hydroxy-2,6,6-trimethyl-1-carboxaldehyde-1-cyclohexene, and 4-hydroxy-3,5,5-trimethyl-2-cyclohexen-1-one are not present in stamen, whilst they are predominant components of stigma sample [178].

In two samples harvested from different regions of Italy, flavonol derivatives (6–10 mg/g) were characterized as the major compounds of stamen and sepal. In this study, sepals mainly contained kaempferol-3-*O*-sophoroside (6.41–8.30 mg/g of fresh sample), quercetin and methylquercetin glycosides, whereas the stamens mainly contained kaempferol-3-*O*-sophoroside (1.70–0.37 mg/g of fresh sample) [179].

3.5.4. *Pharmacological activities of saffron crocus*

The efficacy of saffron crocus has been clinically investigated in diabetes [180], asthma [181,182], cognitive impairment [183–186], glaucoma [187], macular degeneration related to age [188,189], sexual dysfunction in women [190] and men [191], and premenstrual syndrome [192]. These effects are out of the scope of the present thesis, therefore clinical and preclinical studies not related to the antidepressant activity are not discussed here.

3.5.4.1. *Antidepressant activity of saffron crocus stigma*

The majority of the preclinical and clinical [18,19,201,202,193–200] studies dealing with saffron crocus (stigma) focus on its antidepressant activity. The efficacy in mild to moderate depression (daily dosage: 30–100 mg) has been verified by a recent meta-analysis as well [203].

Bioactivity-guided studies identified crocin as the active component by means of behavioural models of depression [204–206]. The efficacy of crocin was confirmed by a clinical study (30 mg per day for 8 weeks); it reduced the symptoms of depression compared to the placebo group in case of metabolic syndrome [207]. Furthermore, crocin increased the efficacy of selective serotonin reuptake inhibitors in patients with depression [208]. The *in vivo* anxiolytic effect of crocin was also analysed [209,210]. Another study reported anxiolytic effect of safranal [211]. The antidepressant effects of crocin and crocetin were assessed on mice after acute and sub-acute administration and crocetin was more effective than crocin in the forced swimming and tail suspension tests [212].

As observed in an animal experiment, the mechanism of the antidepressant effect might be partly mediated by safranal and crocin by inhibiting the uptake inhibition of norepinephrine, serotonin, and dopamine [213]. Although crocin is a weak inhibitor of monoamine oxidase, safranal did not possess this effect [214]. An animal experiment confirmed that the antidepressant activity of crocin may be related to the suppression of oxidative stress and neuroinflammation [206]. The levels of brain-derived neurotrophic factor (BDNF), cyclic-AMP response element binding protein (CREB), phospho-CREB (p-CREB), and VGF neuropeptide were increased by the aqueous extract of saffron crocus in rat hippocampus, where it can be related to the antidepressant effect [215]. The effect on CREB might have only insignificant function in the antidepressant activity of crocin, since its level changed only slightly in the rat cerebellum, while no change was observed in the levels of BDNF and VGF neuropeptide [216]. Nevertheless, a research described that crocin prevented the reducing effect of malathion on BDNF in the rat hippocampus [217]. Moreover, crocetin showed antidepressant activity in animals exposed to chronic stress, also demonstrated a neuroprotective effect by decreasing oxidative damage in their brains [218].

Trans-crocetin and a hydro-ethanolic extract of saffron crocus stigma possessed antagonistic effect on the *N*-methyl-D-aspartate receptor in rat cortical brain slices, although only the extract was

active on kainate receptors [219]. Crocetin and saffron crocus stigma extracts showed affinity at the phencyclidine binding site of the NMDA receptor and at the sigma-1 receptor, whilst crocin and picrocrocin were inactive [220]. Since a connection between depression and hyper-homocysteinemia is assumed, the reduction of homocysteine level in patients with high depression by saffron crocus might be a part of its mechanism of action; however, the components responsible for this bioactivity have not been identified [221]. It is presumed that the clinical effect of the stigma is partly correlated with the antioxidant capacity [207].

3.5.4.2. Antidepressant activity of saffron crocus by-products

Since the exact mechanism of action and the full spectrum of active components is still undiscovered, other parts of saffron crocus than stigma might be considered as industrially perspective substituents of the expensive stigma. Based on this approach, by-products of saffron crocus, including petal (tepal), stamen, and corm have also been subjected to scientific studies.

In an animal experiment, the ethanolic and aqueous saffron crocus extracts of stigma and petal samples were studied for their antidepressant effect using FST in mice; both the aqueous and ethanolic extracts of stigma and petal decreased immobility time in comparison with normal saline [17]. The dichloromethane and petroleum ether fractions gained from the aqueous-ethanol extract of *C. sativus* corms exerted significant antidepressant-like activities in dose-dependent manner in animal behavioural models of depression [204]. Kaempferol (one of the major flavonoid of the petal) was recorded to possess antidepressant activity on rats and mice in the FST [16].

In two clinical trials, the efficacy of petals has been furtherly validated. In a study 40 patients with mild to moderate depression were treated with capsules containing 15 mg dried ethanolic extract of saffron crocus petal or 20 mg fluoxetine for 8 weeks, in a randomized, double-blind trial. The petal was similarly effective to fluoxetine with remission rates of 25% in both groups [18]. In another similar trial, the efficacy of saffron crocus petals (same product as above) was compared to placebo in 40 patients. After 6 weeks of treatment, the petal was more effective than placebo in improving the severity of depression as determined by using the Hamilton Depression Rating Scale [19].

4. MATERIALS AND METHODS

4.1. SCIENTIFIC DATA COLLECTION

Literature search for phytochemical and pharmacological data was carried out in the databases Scopus, SciFinder Scholar, Web of Science, Science Direct, and PubMed.

4.2. IDENTIFICATION AND COLLECTION OF THE PLANT MATERIALS

4.2.1. Chamaemelum nobile

The flowers of *Chamaemelum nobile* (L.) All. were purchased from Pál Bobvos (Hungary). The identity of the plant material was verified according to the requirements of the European Pharmacopoeia. A voucher specimen (voucher no.: 886/1) is stored for verification purposes in the herbarium of the Department of Pharmacognosy, University of Szeged. The EO of *C. nobile* was purchased from Aromax Ltd. (Hungary).

4.2.2. *Matricaria chamomilla*

The flowers of *Matricaria chamomilla* L. (300 g/sample) were individually harvested from different regions of Iran in the flowering period (April) of 2017. The collected populations “Mollasani”, “Gotvand”, “Izeh”, “Masjed Soleyman”, “Bagh Malek”, “Lali”, and “Saleh Shahr” from Khuzestan, while “Abdanan”, “Murmuri”, “Sarableh”, and “Darreh Shahr” from Ilam province were compared with “Bodgold”, which was cultivated at the botanical garden of Department of Horticultural Sciences, Shahid Chamran University of Ahvaz, Ahvaz, Iran. The identification of the plants was carried out by Dr. Mehrangiz Chehrazi (the same department), and a voucher specimen of each sample is stored in the herbarium of the Department. The voucher codes and geographic coordinates including the latitude, longitude, altitude using the GPS, along with meteorological data [222] are shown in Table S1. The flowers were dried in the shade and finely crushed.

4.2.3. *Ducrosia anethifolia*

3 kg of the aerial parts of *Ducrosia anethifolia* (DC.) Boiss. were collected from the southern part of Iran (Fars, Neyriz) in April 2016. The plant was identified by Dr. Mohammad Jamal Saharkhiz (Department of Horticultural Science, Faculty of Agriculture, Shiraz University, Iran), and a voucher specimen is stored in the Herbarium of Department of Pharmacognosy, University of Szeged (voucher no.: 880).

4.2.4. *Eremurus persicus*

1.8 kg aerial parts of *Eremurus persicus* (Jaub. & Spach) Boiss. were collected in south of Iran (Neyriz, Far) in July 2018. A voucher specimen is kept in the Herbarium of Department of Pharmacognosy, University of Szeged (voucher no.: 887/1).

4.2.5. *Crocus sativus*

Saffron crocus (*Crocus sativus* L.) tepal and stamen samples were collected from 40 different locations of Iran during November 2018 at the same harvesting period. All were dried under shade, then the tepals were accurately separated from stamens. The sealed plastic bags were used for packing the plant samples individually and they were kept at room temperature. The growth locations, altitudes, and coordinates of the harvested plant materials are shown in Table S2. For comparison, a commercial saffron crocus stigma sample was also analysed (Bahraman Co., Mashhad, Iran). Voucher specimens are deposited in the Herbarium of Department of Pharmacognosy, University of Szeged (voucher no.: 888/1-80).

4.3. PHYTOCHEMICAL AND BIOACTIVITY ANALYSIS

4.3.1. *Chamaemelum nobile*

4.3.1.1. Preparation of herbal extracts and isolation of reference standards

The crude extract of *C. nobile* was prepared according to the description of the European Medicines Agency monograph [223], for the pharmacological studies. The plant material (10 g) was extracted via ultrasonic bath with 70% EtOH (3 × 100 mL), then evaporated in vacuum and lyophilized (yield:

3.169%). To obtain *C. nobile* extract fractions with different compositions, a part of the *C. nobile* crude extract was fractionated. VLC on polyamide with elution by MeOH–H₂O (20:80, 40:60, 60:40, 80:20, 100:0) was used to gain fractions from the crude extract as follows: F20, F40, F60, F80, and F100.

Four marker compounds (used as reference standards in further experiments) were isolated from the methanolic extract of 200 g *C. nobile* flowers, by using MPLC (silica gel 60, 0.045–0.063 mm, Merck, Darmstadt, Germany), GFC (Sephadex® LH-20, 25–100 mm, Pharmacia Fine Chemicals), RP-PTLC (Silica gel 60, Merck, Darmstadt, Germany), HPLC, and VLC on polyamide (ICN Polyamide for column chromatography).

4.3.1.2. HPLC experiments

HPLC experiments were performed on a Shimadzu LC-20AD Liquid Chromatograph (SPD-M20A diode array detector, CBM-20A controller, SIL-20AC_{HT} autosampler, DGU-20A_{5R} degasser unit, CTO-20AC column oven) using a Kinetex 5 µm C-18 100 Å column (150 mm × 4.6 mm) with a gradient of 0.01% trifluoroacetic acid in H₂O (A) and acetonitrile (B) as follows: 0–5 min 25% B, 14 min 28% B, 15 min 70% B, 16 min 70% B, 16.5 min 25% B, and 20 min 25% B. The flow was 1.2 mL/min, column oven temperature was 55 °C. Detection was carried out within the range of 190–800 nm. For quantification, chromatograms were integrated at 344 nm. The reference standards and the evaporated extracts were dissolved in MeOH, filtered through a PTFE syringe filter and injected in volumes of 5 or 10 µL. Calibration curves were established for all the four reference standards.

4.3.1.3. GC and GC-MS experiments

The GC analysis was performed with an HP 5890 Series II gas chromatograph (FID), using a 30 m × 0.35 mm × 0.25 mm HP-5 fused silica capillary column. The temperature program ranged from 60 °C to 210 °C at 3 °C/min, and from 210 °C to 250 °C (2 min hold) at 5 °C/min. The temperatures of detector and injector were set to 250 °C, while the carrier gas was N₂, with split sample introduction. Quantities of the individual components of the EO were stated as the percent of the peak area relative to the total peak area from the GC-FID analysis.

The GC-MS analysis was carried out with a Finnigan GCQ ion trap bench-top mass spectrometer. All conditions were same with GC's except that the carrier gas was He at a linear velocity of 31.9 cm/s equipped with the capillary column was DB-5MS (30 m × 0.25 mm × 0.25 µm). The positive ion electron ionization mode was used, with ionization energy of 70 eV, and the mass range of 40–400 amu.

The compound identification was based on comparisons with published MS data [224], with a computer library search (the database was delivered together with the instrument), and by comparing their retention indices with literature values [224]. Retention indices were calculated against C₈–C₃₂ *n*-alkanes on a CB-5 MS column [225]. A mixture of aliphatic hydrocarbons was injected in *n*-hexane (Sigma-Aldrich, St. Louis, MO, United States) by using the same temperature program that was used for analysing of the EO.

4.3.1.4. Experiments on smooth muscles

The effects of extracts and essential oil were tested on ileum, jejunum and colon preparations of animal and human origin and on guinea pig urinary bladder or rat gastric fundus. These experiments

were carried out in the Department of Pharmacology and Pharmacotherapy, University of Pécs, Medical School, Pécs, Hungary (Prof. Loránd Barthó et al.). Guinea pigs and Wistar rats were used to obtain segments of the ileum or distal colon. Macroscopically intact segments of human jejunum which were removed during the surgical treatment of pancreatic cancer, were used. The preparations of smooth muscle were used in a traditional organ bath arrangement, movements of the preparations were recorded with isotonic transducers. Experiments were carried out at basal tone or after inducing spasm by histamine. Certain experiments were performed in the presence of the muscarinic receptor antagonist atropine or tetrodotoxin (blocker of voltage sensitive Na⁺ channels) together with histamine. The methods used throughout these experiments are presented in detail elsewhere [226].

4.3.2. *Matricaria chamomilla*

4.3.2.1. *Hydro-distillation of volatile oils and GC-FID and GC-MS experiments*

From each sample 60 g was individually powdered and subjected to hydro-distillation using a Clevenger apparatus for 3 h. The obtained EOs were dehydrated over anhydrous sodium sulphate and stored in refrigerator at 4 °C till analysis. Diethyl ether was also applied to elute the whole content of EOs from the apparatus, and after evaporating the solvent, the weighing process was performed.

In the GC-FID measurement, a Shimadzu GC-17A (Kyoto, Japan) equipped with an FID detector and SGE™ BP5 capillary column (Trajan Scientific and Medical, Victoria, Australia) (30 m × 0.25 mm column with a 0.25 µm film thickness) was applied. The split ratio was 1:100 in GC. The temperatures of injector and FID detector were set at 280 and 300 °C, respectively, with 0.2 µL of injection volume. The oven temperature was kept at 60 °C for 1 min and then raised to 250 °C at 5.0 °C/min and held for 2 min, while the ambient oven temperature range was +4 to +450 °C. Helium gas was applied as a carrier gas at a flow rate of 1 mL/min.

In case of GC-MS analysis an Agilent 7890B gas chromatograph (Agilent Technologies, Inc., Santa Clara, CA, USA) equipped with a 5977B mass spectrometry detector was utilized. The GC instrument was equipped with a 30 m × 0.25 mm HP-5MS capillary column with a 0.25 µm film thickness and 0.25 µm particle size (temperature range: -60 to +320/340 °C), and a split inlet ratio of 100:1. The injection port temperature was 250 °C. The oven temperature was kept at 60 °C for 1 min and then planned from 60 to 250 °C at 5 °C/min; then, the temperature was kept at 250 °C for 2 min. As a carrier gas Helium (99.999%) was used (flow rate: 1 mL/min) and inlet pressure was 35.3 kPa. The mass spectrometer was operated in the electron impact mode at 70 eV, and the inert ion source (HES-EI) temperature was set to 350 °C; the temperature of the quadrupole was set at 150 °C, while the MS interface was set to 250 °C. A scan rate of 0.6 s (cycle time: 0.2 s) was applied, covering a mass range from 35 to 600 amu.

The identification of the most compounds were performed by two different analytical approaches: (a) comparison of Kovats indices of *n*-alkanes (C₈–C₂₄) [224] and (b) comparison of mass spectra (using authentic chemicals and Wiley spectral library collection). Identification was considered tentative when based on mass spectral data alone. In GC-FID and GC-MS, data acquisition and analysis were carried out applying Chrom-card™ (Scientific Analytical Solutions, Zurich, Switzerland, version

DS) and Xcalibur™ software (Thermo Fisher Scientific, Waltham, MA, USA, 4.0 Quick Start), respectively.

4.3.2.2. Preparation of extracts and HPLC analysis of apigenin and luteolin contents

The extraction was done using 5 g of each sample, individually, with MeOH (3 × 75 mL) in an ultrasonic bath (VWR-USC300D) for 10 min, at 40 °C under power grade 9. The concentrated extracts were subjected to evaluate the antiradical assays and HPLC analysis, after evaporation of the solvent under reduced pressure at 50 °C (Rotavapor R-114, Büchi, Switzerland).

20 µL of each extract (1 mg/mL) was separately injected into an analytical HPLC (Knauer, Berlin, Germany) by using an end capped Eurospher II 100-5 C18, Vertex Plus Column (Knauer, Berlin, Germany) (250 × 4.6 mm with precolumn, particle size: 5 µm, pore size: 100 Å) in temperature of 30 °C; coupled to UV detector (Knauer GmbH-Smart line 2600, Berlin, Germany) at a wavelength range of 190 to 500 nm (quantification at 330 nm), while MeOH/H₂O was used as the mobile phase with a gradient system, increasing MeOH from 30% to 70% within 40 min, with a flow rate of 1 mL/min, at ambient temperature. Analysis was carried out using SAS software, version 9.2 (SAS Institute Inc., Cary, NC, USA).

4.3.2.3. Evaluation of antiradical activity via DPPH and ORAC assays

- DPPH assay

DPPH measurement was done on 96-well microtiter plates. In brief, microdilution series of samples (1 mg/mL, dissolved in MeOH) were prepared starting with 150 µL. To gain 200 µL of sample, 50 µL of DPPH reagent (100 µM) was added to each sample. The microplate was stored at room temperature under dark conditions. The absorbance was measured after 30 min at 550 nm using a microplate reader. MeOH (HPLC grade) and ascorbic acid (0.01 mg/mL) were used as a blank control and standard, respectively. Antiradical activity was calculated using the following equation: $I\% = [(A_0 \times A_1/A_0) / 100]$ (A_0 is the absorbance of the control and A_1 is the absorbance of the sample). Anti-radical activity of the samples was expressed as EC₅₀ (concentration of the compounds that caused 50% inhibition).

- ORAC assay

Twelve extracts were subjected to ORAC assay [227] with slight modifications. Microtiter plates (96-well) were used for measurement of the samples. Briefly, 20 µL of extracts (0.01 mg/mL) was mixed with 60 µL of AAPH (peroxyl free radical generator) (12 mM) and 120 µL of fluorescein solution (70 mM). Then, the fluorescence was measured for 3 h at 1.5 min cycle intervals with the microplate reader. The standard Trolox® was used. Activity of all samples was compared with rutin and EGCG as positive controls. Antioxidant capacities were presented as µmol TE (Trolox® equivalent)/g of dry matter.

4.3.2.4. Statistical Analysis

All the tests were carried out in triplicate, and the results are expressed as means ± SD. The data were assessed with one-way analysis of variance (ANOVA) using SAS software (version 9.2, SAS institute Inc., Cary, NC, USA) and GraphPad Prism version 6.05. The means were compared using Duncan's comparisons test ($p < 0.05$).

4.3.3. *Ducrosia anethifolia*

4.3.3.1. Isolation and identification of the major phytochemicals of *D. anethifolia*

3 kg of aerial parts of *D. anethifolia* (including flower, leaves and stem) were dried in shade at room temperature and crushed, then extracted with methanol (40 L). To yield the crude extract, the filtrate was concentrated under reduced pressure. The extract (464.1 g) was dissolved with methanol–water 1:1 (1.5 L) and successively partitioned with *n*-hexane (4 × 1 L), CHCl₃ (4 × 1 L), EtOAc (4 × 1 L) and *n*-BuOH (4 × 1 L). The solvents of each extract were evaporated to gain the *n*-hexane, CHCl₃, EtOAc and *n*-BuOH soluble-extracts.

Initially, the CHCl₃-soluble fraction (20.6 g) was subjected to CC with a gradient system consisting of increasing concentration of MeOH in CHCl₃ (0–80%); column fractions with similar TLC patterns were combined to get six major fractions D1, D2, D3, D4, D5 and D6. D1 was chromatographed by MPLC, first eluting with *n*-hexane–CH₂Cl₂ (50:50; 0:100), then adding MeOH to CH₂Cl₂ (0–100%), to afford four subfractions (D11, D12, D13 and D14). D11 was separated to 49 subfractions using CPTLC with an isocratic eluting system *n*-hexane–EtOAc–MeOH (10:3:1), which resulted in the isolation of the pure compound **5** (82.8 mg).

The RP-HPLC purification of D11 subfractions with MeOH–H₂O (1:1) afforded compound **6** (1.7 mg). D12 was chromatographed by MPLC applying a gradient solvent system with increasing EtOAc in *n*-hexane (5–100%) to get eight major subfractions (D121–D128). From D123, the pure compound **7** (3.1 mg) was isolated by using CPTLC with EtOAc in *n*-hexane (5–100%). D124 was successively separated to 81 fractions by CPTLC (same system), then subfractions 49–54 was subjected to RP-HPLC with MeOH–H₂O (15–50% H₂O in MeOH) yielding compound **8** (2.56 mg).

D13 was separated by MPLC with increasing ratio of EtOAc in *n*-hexane (5–100%) to get seven fractions (D131–D137). D133 was subjected to MPLC with the same solvent system to gain 19 subfractions. Finally, subfractions 1–2 were purified by using CPTLC with toluene–EtOAc (90:10, 80:20, 70:30, 60:40, 50:50) as eluents to gain compound **9** (100.4 mg). Subfraction 3 from D133 was subjected to CPTLC by eluting with toluene–EtOAc (90:10, 80:20, 70:30, 60:40, 50:50) to yield 32 subfractions. Subfractions 18–19 were separated by PTLC with toluene–EtOAc (1:1) to get compound **10** (35.3 mg). Besides, subfractions 23–32 were chromatographed by RP-HPLC (MeOH–H₂O 1:1) and then by PTLC with toluene–EtOAc (1:1) to yield compounds **11** (1.78 mg) and **12** (1.02 mg). By using CPTLC with increasing concentration of EtOAc in toluene (5–100%) as eluent, subfraction 6 from D133 was chromatographed to get 43 subfractions. Subfractions 24–40 were separated by PTLC with CHCl₃–MeOH–*n*-hexane (5:1:5) to retrieve compounds **13** (2.5 mg) and **14** (21.7 mg), respectively.

D3 was separated to five major fractions (D31–D35) by MPLC with a solvent system containing increasing ratio of MeOH in CHCl₃ (0–100%). D33 was chromatographed by CPTLC with raising the concentration of MeOH (0–20%) in the mixture of cyclohexane–EtOAc (1:1) to afford 70 subfractions. Subfractions 21–23 contained the pure compound **15** (1.0 mg).

The main fraction D4 was separated by MPLC to seven subfractions (D41–D47) by increasing the ratio of MeOH (0–100%) in acetone–toluene (1:1). Subfraction D42 was subsequently chromatographed by PTLC with eluting cyclohexane–EtOAc–MeOH (4.75:4.75:0.5) and compound **16**

(2.7 mg) was isolated. Furthermore, D46 was purified with CPTLC by increasing ratio of MeOH (0–100%) in acetone–toluene (1:1); then compound **17** (2.9 mg) was purified by using RP-PTLC (MeOH–H₂O 1:1) from subfractions 35–37 (**Figure 1**).

Thin layer chromatography (TLC) (aluminium sheets coated with silica gel 60 F₂₅₄, 0.25 mm, Merck 5554 and silica gel 60 RP-C₁₈ F_{254S}, Merck) were used for monitoring in whole separation procedure.

Open column chromatography (CC) (Silica gel 60, 0.063–0.2 mm, Merck, Darmstadt, Germany)

Medium pressure liquid chromatography (MPLC) (glass column: length of 460 mm, inner diameter of 26 mm, BÜCHI, Switzerland; pump: BÜCHI C-605, Switzerland; silica gel 60, 0.045–0.063 mm, Merck, Darmstadt, Germany)

Gel filtration chromatography (GFC) (Sephadex® LH-20, Pharmacia, Uppsala, Sweden)

Centrifugal PTLC (CPTLC) (Chromatotron®, Harrison Research, USA; Silica gel 60 GF₂₅₄, Merck, Darmstadt, Germany).

Preparative thin layer chromatography was performed by **normal** (Silica gel 60, Merck, Darmstadt, Germany) and **reverse phase** (Silica gel 60 RP-18 F_{254S}, Merck, Darmstadt, Germany) (**PTLC and RP-PTLC**, respectively).

High pressure liquid chromatography (HPLC) experiments were carried out on reverse phase (**RP-HPLC**) (Kinetex® 5 mm C-18 100 Å, 150 × 4.6mm Phenomenex, Torrance, CA); while the HPLC flow was 1.2 mL/min, column oven temperature was 24 °C. Detection was carried out within the range of 190–800 nm. The HPLC system comprised of Waters 600 pump, Waters 2998 PDA detector, Waters in/line degasser AF degasser unit connected with Waters 600 control module using Empower Pro 5.00 software.

Eluents:

CC: MeOH–CHCl₃ [0–80%]

MPLC I: *n*-hexane–CH₂Cl₂ [50:50, 0:100], MeOH–CH₂Cl₂ [0–100%]

CPTLC I: *n*-hexane–EtOAc–MeOH [10:3:1]

RP-HPLC I: MeOH–H₂O [1:1]

MPLC II: EtOAc–*n*-hexane [5–100%]

CPTLC II: EtOAc–*n*-hexane [5–100%]

RP-HPLC II: MeOH–H₂O [15–50%]

CPTLC III: toluene–EtOAc [90:10, 80:20, 70:30, 60:40, 50:50]

PTLC I: toluene–EtOAc [1:1]

CPTLC IV: EtOAc–toluene [5–100%]

PTLC II: CHCl₃–MeOH–*n*-hexane [5:1:5]

MPLC III: MeOH–CHCl₃ [0–100%]

CPTLC V: MeOH [0–20%] in cyclohexane–EtOAc [1:1]

MPLC IV: MeOH [0–100%] in acetone–toluene [1:1]

PTLC III: cyclohexane–EtOAc–MeOH [4.75:4.75:0.5]

CPTLC VI: MeOH [0–100%] in acetone–toluene [1:1]

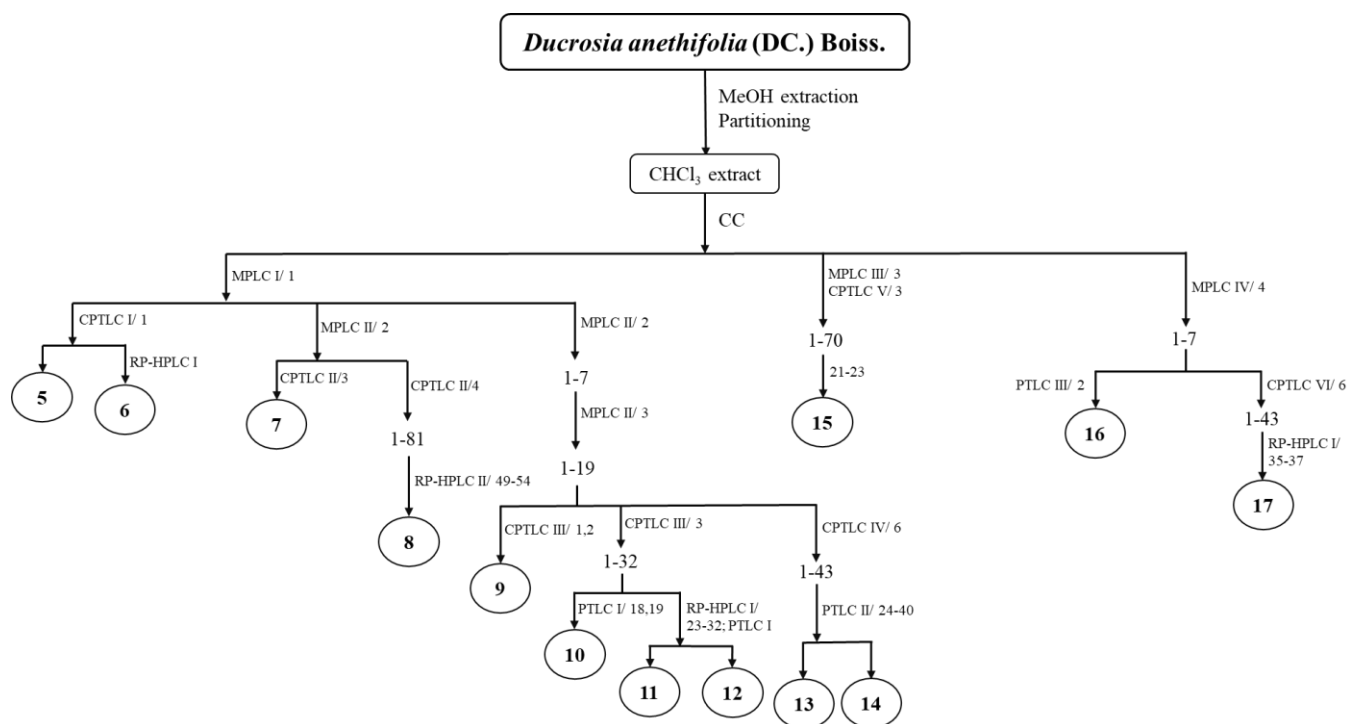


Figure 1. Isolation of pure compounds from *Ducrosia anethifolia*

4.3.3.2. Biological activities of furocoumarins from *D. anethifolia*

4.3.3.2.1.1. Assay for anti-proliferative effect

The effects of increasing concentrations of the analysed compounds on cell proliferation were tested in 96-well flat-bottomed microtiter plates [228]. The compounds were diluted in 100 μL of McCoy's 5A medium. 6×10^3 mouse T-cell lymphoma cells (PAR or MDR) in medium (100 μL) were added to each well, except for the medium control wells. The culture plates were further incubated at 37 $^\circ\text{C}$ for 72 h; at the end of the incubation period, 20 μL of MTT solution (Sigma, St. Louis, MO) (from a 5 mg/mL stock) was added to each well.

After incubation at 37 $^\circ\text{C}$ for 4 h, 100 μL of SDS (Sigma, St. Louis, MO) solution (10% in 0.01 M HCl) was added to each well and the plates were further incubated at 37 $^\circ\text{C}$ overnight. The cell growth was determined by measuring the OD at 540 nm (ref. 630 nm) with a Multiscan EX ELISA reader (Thermo Labsystems, Waltham, MA). IC_{50} values were calculated via the following equation:

$$\text{IC}_{50} = 100 - \left[\frac{\text{OD}_{\text{sample}} - \text{OD}_{\text{medium control}}}{\text{OD}_{\text{cell control}} - \text{OD}_{\text{medium control}}} \right] \times 100$$

4.3.3.2.2. Assay for cytotoxic effect

The effects of increasing concentrations of compounds on cell growth were tested in 96-well flat-bottomed microtiter plates [228]. The compounds were diluted in a volume of 100 μL medium. Then, 1×10^4 cells in 100 μL of medium were added to each well, except for the medium control wells. The culture plates were incubated at 37 $^\circ\text{C}$ for 72 h; at the end of the incubation period, 20 μL of MTT

solution (from a 5 mg/mL stock) were added to each well. After incubation at 37 °C for 4 h, 100 µL of sodium dodecyl sulphate (SDS, Sigma, USA) solution (10% in 0.01 M HCl) was added to each well and the plates were further incubated at 37 °C overnight. Cell growth was determined by measuring the optical density (OD) at 550 nm (ref. 630 nm) with a Multiscan EX ELISA reader. Inhibition of the cell growth was determined according to the formula:

$$IC_{50} = 100 - \left[\frac{OD_{sample} - OD_{\text{medium control}}}{OD_{cell\ control} - OD_{\text{medium control}}} \right] \times 100$$

Results are expressed as IC₅₀ values: the inhibitory dose that reduces by a 50% the growth of the cells exposed to the tested compound.

4.3.3.2.3. Assay for multidrug resistance reversing activity

The inhibition of the cancer MDR efflux pump ABCB1 by the tested compounds was assessed by flow cytometry measuring the retention of rhodamine 123 by ABCB1 (*P*-glycoprotein) in MDR mouse T-lymphoma cells, as the L5178Y human ABCB1-gene transfected mouse T-lymphoma cell line (MDR) overexpress *P*-glycoprotein [229]. This method is a fluorescence-based detection system which uses verapamil as reference inhibitor. Briefly, cell number of L5178Y MDR and PAR cell lines were adjusted to 2 × 10⁶ cells/mL, re-suspended in serum-free McCoy's 5A medium and distributed in 0.5 mL aliquots into Eppendorf centrifuge tubes. The tested compounds were added at different concentrations and the samples were incubated for 10 min at room temperature. Verapamil (Sigma, USA) and tariquidar (Sigma, USA) were applied as positive controls. Next, 10 µL (5.2 µM final concentration) of the fluorochrome and ABCB1 substrate rhodamine 123 (Sigma, USA) were added to the samples and the cells were incubated for 20 min at 37 °C, washed twice and re-suspended in 0.5 mL PBS for analysis. The fluorescence of the cell population was measured with a Partec CyFlow® flow cytometer (Partec, Germany). The percentage of mean fluorescence intensity was calculated for the treated MDR cells as compared with the untreated cells. A fluorescence activity ratio (FAR) was calculated based on the following equation which relates the measured fluorescence values:

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

The results attained from a representative flow cytometry experiment in which 10,000 individual cells of the population were evaluated for amount of rhodamine 123 retained with the aid of the Partec CyFlow® flow cytometer, are first presented by the histograms and this data converted to FAR units that define fluorescence intensity, standard deviation, peak channel in the total- and in the gated-populations. Parameters calculated are: SSC (of cells in the samples); FL-1; FSC (of cells in the samples or cell size ratio); and FAR, whose values were calculated using the equation given above.

4.3.3.2.4. Checkerboard combination assay

A checkerboard microplate method was used to evaluate the effect of drug interactions between furocoumarins and the chemotherapeutic drug doxorubicin [230]. This assay was carried out by multidrug resistant mouse T-lymphoma cells overexpressing the ABCB1 transporter. Doxorubicin is classified in the anthracycline antitumor agents, and it exerts anticancer activity as a topoisomerase-II (TI-2) inhibitor. The dilutions of doxorubicin (Teva, Hungary, stock solution: 2 mg/mL) were made in

a horizontal direction in 100 μL (final concentration: 17.242 μM), and the dilutions of the test compounds vertically in the microtiter plate in 50 μL volume.

The plates were incubated for 72 h at 37 $^{\circ}\text{C}$ in 5% CO_2 atmosphere. The cells were re-suspended in McCoy's 5A culture medium and distributed into each well in 50 μL containing 6×10^3 cells each. The cell growth rate was determined after MTT staining. 20 μL of MTT solution (from a stock solution of 5 mg/mL) was added to each well, at the end of the incubation period. After incubation at 37 $^{\circ}\text{C}$ for 4 h, 100 μL of SDS solution (10% in 0.01 M HCl) was added to each well and the plates were further incubated at 37 $^{\circ}\text{C}$ overnight. OD was measured at 540/630 nm with Multiscan EX ELISA reader (Thermo LabSystems, USA) as described elsewhere [230]. CI values at 50% of the growth inhibition dose (ED_{50}), were determined using CompuSyn software (ComboSyn, Inc., USA) to plot four to five data points to each ratio. CI values were calculated by means of the median-effect equation, where $\text{CI} < 1$, $\text{CI} = 1$, and $\text{CI} > 1$ represent synergism, additive effect (or no interaction), and antagonism, respectively [231,232].

4.3.4. *Eremurus persicus*

1.8 kg of the aerial part materials (flowers, stems and leaves) were shade-dried at room temperature and finely ground. Methanol (50 L) was applied for the extraction procedure. After filtration and concentration under reduced pressure, 127.75 g crude extract was obtained. The extract was dissolved with methanol-water 1:1 (1 L), subsequently by using separating funnel partitioned successively with *n*-hexane (4×1 L), CHCl_3 (4×1 L), and EtOAc (4×1 L). Then, the solvents were evaporated to achieve the *n*-hexane, CHCl_3 and EtOAc extracts.

The EtOAc-soluble extract (3.5 g) was initially subjected to flash chromatography with a gradient solvent system by increasing the ratio of CHCl_3 -MeOH (1:1) from 20% to 100% in *n*-hexane. Column fractions with similar TLC patterns were combined to obtain six major fractions E_1 , E_2 , E_3 , E_4 , E_5 and E_6 . By applying flash chromatography with increasing concentration of CHCl_3 -MeOH (9:1) from 10% to 100% in *n*-hexane, E_1 was separated to six fractions (E_{11} , E_{12} , E_{13} , E_{14} , E_{15} , E_{16}). E_{15} was further chromatographed by flash chromatography applying the same solvent system to afford four subfractions (E_{151} - E_{154}). E_{153} was separated to 60 subfractions with CPTLC by increasing MeOH in toluene (0-100%). Subfraction 39-49 was also purified to get compound **18** (1.41 mg) by RP-PTLC (MeOH- H_2O 1:1).

E_{16} was subjected to CPTLC with two gradient solvent systems, first with increasing ratio of solvent mixture of EtOAc-acetone (1:1) 50-100% in *n*-hexane, then increasing MeOH (0-50%) in EtOAc-acetone (1:1). From the obtained six fractions (E_{161} - E_{166}), GFC (MeOH as eluent) was developed to separate E_{166} and compound **19** (1.24 mg) was finally isolated.

MPLC was exploited for further separation of the major fraction E_2 with increasing concentration of MeOH (0-100%) in EtOAc-*n*-hexane (1:2). From eight gained fractions (E_{21} - E_{28}), E_{24} was subsequently chromatographed to 40 subfractions by GFC (MeOH as eluent). Subfractions 25 and 26 contained the pure compound **20** (22.22 mg).

The CHCl_3 -soluble fraction (4.7 g) was also separated to 7 major fractions (C_1 - C_7) by applying MPLC eluting with EtOAc in *n*-hexane (0-50%), then increasing ratio of MeOH (0-100%) in EtOAc-*n*-

hexane (1:1). By using GFC (eluent: MeOH), C₄ was also separated to 50 sub-fractions, and sub-fractions 22-24 contained the pure compound **21** (1 mg). C₇ was fractionated by GFC (eluent: CH₂Cl₂–MeOH 1:1) to get three major fractions (C₇₁, C₇₂ and C₇₃). Then, C₇₂ was purified with the same method to yield five fractions (C₇₂₁–C₇₂₅). By applying CPTLC with MeOH in toluene (10–100%) as a gradient solvent system, 38 subfractions were afforded. Subfraction 1-12 was chromatographed by PTLC with toluene–MeOH (9.8:0.2), and pure compounds **22** (1.03 mg) and **23** (0.95 mg) were isolated (**Figure 2**).

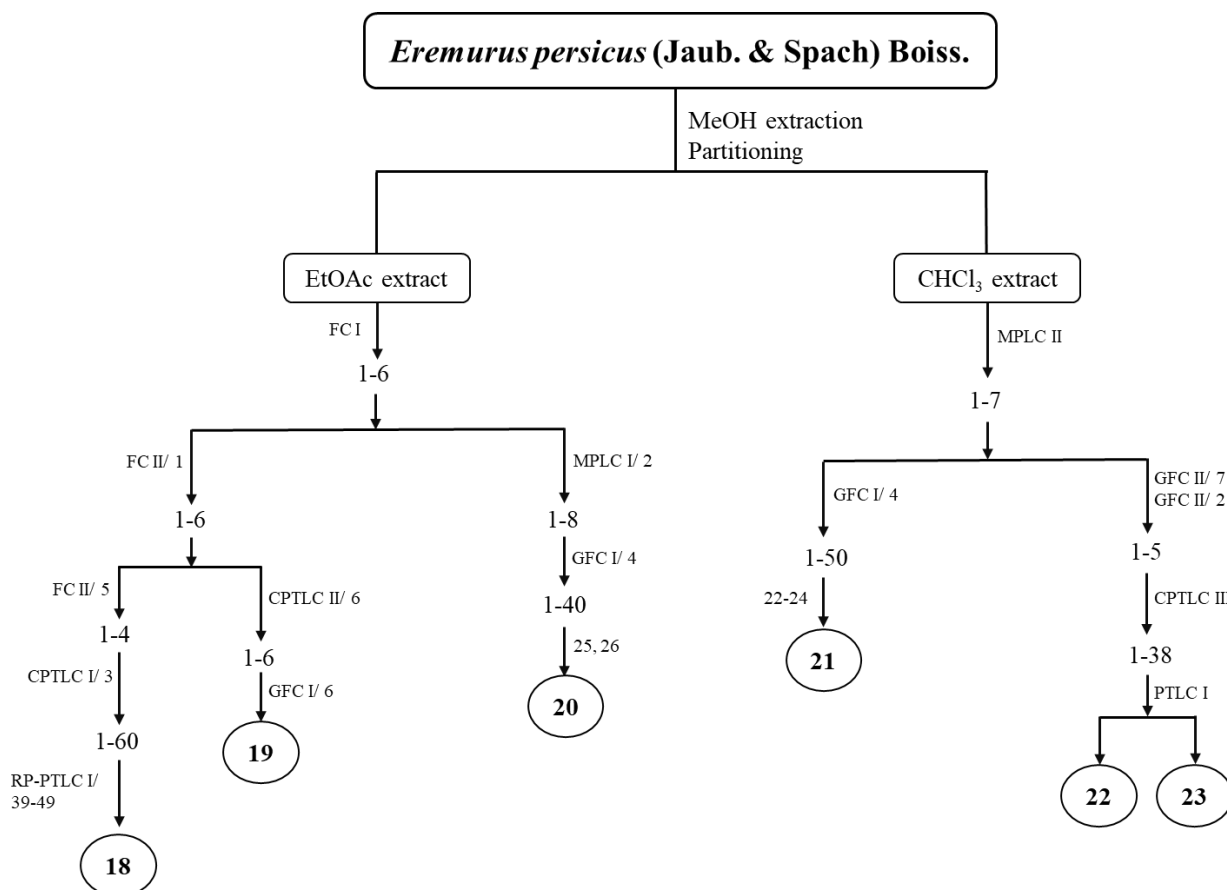


Figure 2. Isolation of the compounds from *Eremurus persicus*

Flash chromatography (FC) Biotage® Instrument (Isolera™ Spektra Systems with ACI™ and Assist) with integrated UV, UV-Vis, and ELS detection using RediSep R_f Gold normal phase flash columns (10, 50 and 80 g) (Biotage® SNAP cartridge, KP-Sil)

Thin layer chromatography (TLC) (aluminium sheets coated with silica gel 60 F₂₅₄, 0.25 mm, Merck 5554 and silica gel 60 RP-C₁₈ F_{254S}, Merck) was applied to monitor in whole separation procedure.

Medium pressure liquid chromatography (MPLC) (glass column: length of 460 mm, inner diameter of 26 mm, BÜCHI, Switzerland; pump: BÜCHI C-605, Switzerland; silica gel 60, 0.045–0.063 mm, Merck, Darmstadt, Germany)

Gel filtration chromatography (GFC) (Sephadex® LH-20, Pharmacia, Uppsala, Sweden)

Centrifugal PTLC (CPTLC) (chromatotron®, Harrison Research, USA; Silica gel 60 GF₂₅₄, Merck, Darmstadt, Germany).

Preparative thin layer chromatography was performed by **normal** (Silica gel 60, Merck, Darmstadt, Germany) and **reverse phase** (Silica gel 60 RP-18 F₂₅₄S, Merck, Darmstadt, Germany) (**PTLC and RP-PTLC**, respectively).

Eluents:

FC I: CHCl₃–MeOH [1:1] (20–100%) in *n*-hexane

FC II: CHCl₃–MeOH [9:1] (10–100%) in *n*-hexane

CPTLC I: MeOH–toluene (0–100%)

GFC I: MeOH as eluent

RP-PTLC I: MeOH–H₂O [6:4]

RP-PTLC II: MeOH–H₂O [1:1]

CPTLC II: EtOAc–acetone [1:1] (50–100%) in *n*-hexane; MeOH (0–50%) in EtOAc–acetone [1:1]

MPLC I: MeOH (0–100%) in EtOAc–*n*-hexane [1:2]

MPLC II: EtOAc–*n*-hexane (0–50%); MeOH (0–100%) in EtOAc–*n*-hexane [1:1]

GFC II: MeOH–CH₂Cl₂ [1:1] as eluent

CPTLC III: MeOH–toluene (10–100%)

PTLC I: MeOH–toluene [0.2:9.8]

4.4. CHARACTERIZATION AND STRUCTURE ELUCIDATION

NMR spectra were recorded in CD₃OD and CDCl₃ on a Bruker Avance DRX 500 spectrometer at 500 MHz (¹H) and 125 MHz (¹³C). The peaks of the residual solvent (δ_{H} 3.31 and 7.26, δ_{C} 49.0 and 77.2, respectively) were taken as reference. The data were acquired and processed with MestReNova v6.0.2e-5475 software. Chemical shifts are expressed in parts per million and *J* values are reported in Hz. All solvents were used in analytical grade (Molar Chemicals Kft, Halásztelek, Hungary). By applying a Perkin-Elmer 341 polarimeter, optical rotation was also established in CHCl₃ at room temperature.

4.5. ANALYSIS OF *C. SATIVUS L.* SAMPLES

4.5.1. Extract preparation

20 mg of tepal, 10 mg of stigma, and 50 mg of stamen samples were separately extracted by ultrasonic bath for 15 min with mixture of solvents of EtOH–H₂O (1:1), then diluted with the above solvents to 10.0 mL (tepal) and 5.0 mL (stigma and stamen) in volumetric flasks, respectively.

In case of filtered samples, the extracts were filtered via a filter membrane (PTFE-L syringe filter, hydrophilic, FilterBio®, diameter: 13 mm, pore size: 0.45 μm), the first 1 mL was unused, and the rest 1.5 mL was analysed by HPLC-DAD. The samples were centrifuged for 1 min at 7000 rpm. In case of all samples, three extracts were prepared and analysed in triplicate.

4.5.2. *HPLC apparatus and measurement conditions*

HPLC-DAD analysis was performed on a Shimadzu SPD-M20A (Shimadzu Corporation, Kyoto, Japan), equipped with a Shimadzu SPD-M20A photodiode array detector, an on-line degasser unit (Shimadzu DGU-20A5R), a column oven (Shimadzu CTO-20AC column oven) and autosampler (Shimadzu SIL-20A8HT) using a RP Kinetex® C8 column (5 µm, 100 Å, 150 × 4.6 mm, Phenomenex, Torrance, USA) at 30 °C. Chromatographic elution of the samples was accomplished with a gradient solvent system by increasing the ratio of MeOH in H₂O (containing 0.066% of H₃PO₄) from 0 to 30% (0–1 min), 30 to 57% (1–7 min), 57 to 76% (7–12 min), and 76 to 100% (12–13 min) at a flow rate of 1.5 mL/min. UV-Vis range of 190–800 nm was used to detect the compounds. The samples were monitored at the UV_{max} of the standards (picrocrocin: 247 nm, quercetin-3-*O*-sophoroside: 360 nm, kaempferol-3-*O*-sophoroside: 354 nm, kaempferol-3-*O*-glucoside: 348 nm, crocin: 441 nm, safranal: 316 nm, and crocetin: 427 nm). Data assessment and acquisition was performed with LabSolutions (Version 5.82) software (Shimadzu, Kyoto, Japan). The volume of 10 and 20 µL of tepal and stamen samples were injected, respectively.

4.5.3. *System validation*

Validation of the analytical method was developed by us, done according to the ICH Harmonised Guideline [233], and completed with further experiments. Validation was accomplished by establishing the calibration curves of 7 reference compounds, determining the LoD and LoQ values, assessing system suitability, accuracy, precision, repeatability, stability and filter compatibility of the extracts.

5. RESULTS

5.1. CHAMAEMELUM NOBILE

5.1.1. Chemical characterization of the extracts

Four characteristic peaks in the HPLC were detected in the crude *C. nobile* extract and its fractions F40–F100 at retention time range of 4–9 min. The corresponding components were isolated from the plant material and identified by ^1H and ^{13}C NMR experiments as the flavonoids apigenin, eupafolin, hispidulin, and luteolin (**Figure 3**, and **Figures S1–S6**). These compounds were further used as reference standards to characterize the *C. nobile* extracts. The identification of reference compounds in different extracts was based on matching the retention times and UV spectra. Retention times of luteolin, eupafolin, apigenin, and hispidulin were 4.5, 5.0, 7.5, and 8.5 min, respectively (**Figure 4**). The baseline separation of these compounds allowed their reliable quantification in different extracts.

The crude extract of *C. nobile* comprised eupafolin as the main flavonoid, followed by luteolin, hispidulin, and apigenin (**Table 1**). The fractionation on polyamide resulted in subfractions F20–F100 with different compositions, as demonstrated by the differences in their flavonoid content. In F20, the quantities of flavonoids were below the level of quantification. The flavonoid content of the fractions increased with increasing MeOH content of the eluting solvent. The highest flavonoid levels were measured in F80, except for luteolin and apigenin, which were mainly concentrated in F100.

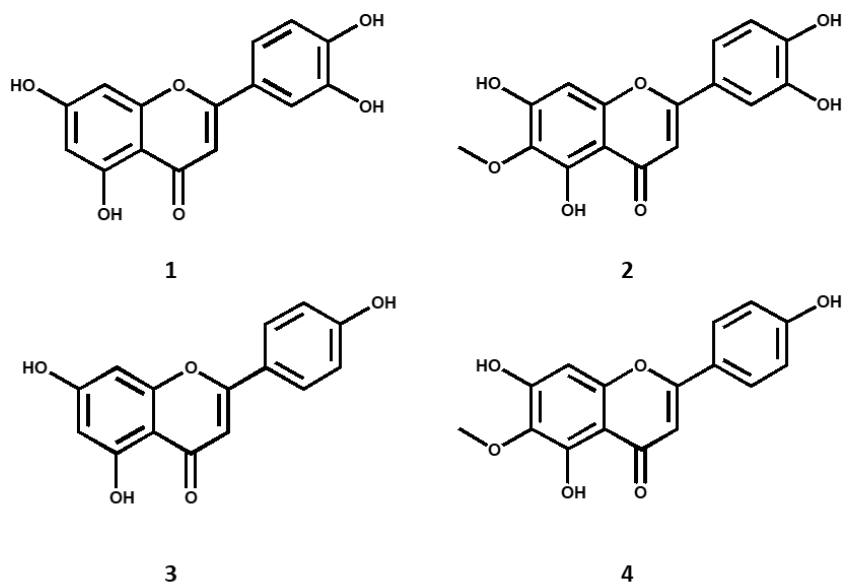


Figure 3. The chemical structures of the isolated flavonoids from *Chamaemelum nobile*; **1:** luteolin; **2:** eupafolin; **3:** apigenin; **4:** hispidulin

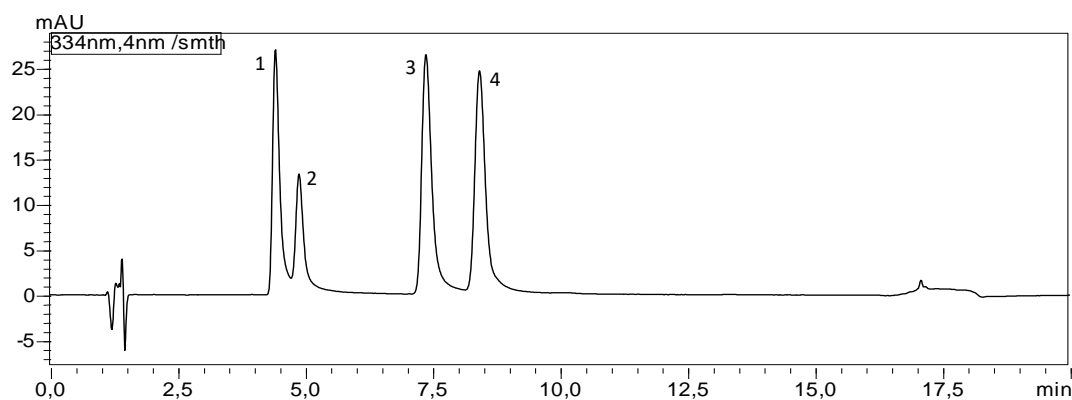


Figure 4. HPLC chromatogram of the crude *C. nobile* extract with the peaks of luteolin (1), eupafolin (2), apigenin (3), and hispidulin (4) (344 nm).

Table 1. Flavonoid content of *C. nobile* crude extract and its fractions as determined by HPLC

Sample	Luteolin (mg/g extract)	Eupafolin (mg/g extract)	Apigenin (mg/g extract)	Hispidulin (mg/g extract)
Crude extract	4.167 ± 0.616	18.756 ± 2.121	0.298 ± 0.027	1.584 ± 0.181
F20	Not detected	Not detected	Not detected	Not detected
F40	0.578 ± 0.001	1.800 ± 0.001	0.179 ± 0.001	0.231 ± < 0.001
F60	1.904 ± 0.001	62.591 ± 0.025	0.151 ± < 0.001	5.951 ± 0.004
F80	22.605 ± 0.001	223.488 ± 0.036	0.859 ± < 0.001	17.060 ± < 0.006
F100	55.305 ± 0.002	150.206 ± 0.005	2.055 ± < 0.001	4.983 ± < 0.001

5.1.2. Chemical characterization of the essential oil

Based on their retention times and mass spectrometric data, methyl angelate, 3-methyl pentyl angelate, and 3-methylamylisobutyrate were identified as the major constituents of *C. nobile* EO (19.0, 18.2, and 10.4%, respectively) (**Table 2**). The identified components comprised 97% of the EO.

Table 2. Chemical composition of *C. nobile* essential oil

Compounds ^a	RI ^b	% in samples
Methyl tiglate	867	tr
<i>n</i> -Hexanol	875	1.1
2-Methylbutyl acetate	897	0.4
Isobutyl isobutyrate	925	0.8
Acetylacetone	932	0.7
α-Pinene	935	2.4
Camphene + allyl methacrylate	958	0.6
Thuja-2,4(10)-diene	960	1.1
Isoamyl propionate	966	tr
β-Pinene	969	0.3
Myrcene	973	0.6
Propyl angelate	993	1.1
Isobutyl 2-methylbutyrate	998	tr

Compounds ^a	RI ^b	% in samples
Isoamyl isobutyrate	1004	1.5
2-Methylbutyl isobutyrate	1006	2.7
1,8-Cineol	1035	tr
Isoamyl methacrylate	1037	1.1
Isobutyl angelate	1058	4.9
Methallyl angelate	1068	19.0
2-Butenyl angelate	1119	tr
3-Methylamyl isobutyrate	1122	10.4
3-Methylamyl methacrylate	1150	6.6
trans-Pinocarveol + isoamyl angelate	1153	8.6
2-Methylbutyl angelate	1168	8.3
Pinocarpone	1177	3.9
Prenyl angelate	1213	1.5
Myrtenal	1217	1.2
3-Methyl pentyl angelate	1264	18.2
Identified components		97.0

^aCompounds listed in sequence of elution from a DB-5 MS column.

^bRetention indices calculated against C₈ to C₃₂ *n*-alkanes on a DB-5MS column. tr, in traces.

5.1.3. *Effects on smooth muscles*

The crude extract of *C. nobile* induced a transient longitudinal contraction on guinea pig ileum preparations. Both atropine (an antagonist of acetylcholine at the muscarinic receptors) and tetrodotoxin (an inhibitor of voltage-sensitive Na⁺ channels; hence, of neuronal axonal conduction) inhibited the contractile effect of *C. nobile* crude extract. The functional blockade of capsaicin-sensitive neurons did not inhibit, whereas the cyclooxygenase inhibitor indomethacin moderately inhibited the contraction in response to the *C. nobile* extract. Subfractions of the crude extract also possessed contracting activity.

After transient contraction, smooth muscle relaxing activity was observed. On histamine-precontracted preparations (in the presence of atropine and tetrodotoxin), concentration-dependent relaxation was observed in response to treatment with *C. nobile* crude extract (60–200 µg/mL). The highest tested concentration induced full relaxation. The relaxation induced by the 60 µg/mL extract was not significantly altered by the adrenergic β-receptor antagonist propranolol or by the NO synthase inhibitor N^G-nitro-L-arginine. Different fractions of the *C. nobile* extract demonstrated distinct relaxant effects. F20 and F40 (60 or 200 µg/mL) produced no relaxation, whereas F60, F80 and F100 had remarkable and dose-dependent spasmolytic activity in this concentration range (up to 100%). This observation refers to the potential role of flavonoids in the relaxant effect, and experiments with four flavonoids isolated from the plant material reassured this hypothesis. The four major flavonoids of the extracts (hispidulin, luteolin, eupafolin and apigenin) had relaxant activities at 2 µM ranging between 18.2–24.2%, whereas at 20 µM between 64.5–81.9%. The EO (0.1–10 µg/mL) induced 12.8–69.7% relaxation in dose-dependent manner with no pre-contraction.

5.2. MATRICARIA CHAMOMILLA

5.2.1. Essential oil yields

The studied plant samples were characterized mainly with similar EO yields. Populations “B” ($1.03 \pm 0.003\%$) and “BM” ($0.78 \pm 0.017\%$) contained the highest and lowest amount of EO, respectively, as shown in **Table 3**. As the plant sample “B” was cultivated in the university’s garden and it was regularly irrigated, the highest EO content can be predicted.

5.2.2. Chemical profiles of volatile oils

Seventeen compounds were detected in these twelve populations. The sesquiterpene α -bisabolone oxide A (45.64–65.41%) was the major EO constituent in the samples except “B” and “S”, whereas its concentration was the highest in population “Mu” (**Table 3**).

The cultivated sample “B” was rich in α -bisabolol oxide B (21.88%) and chamazulene (19.22%), while the percentages of these compounds were lower under the wild growth conditions. The blue colour of EOs in all samples except “S” represented their chamazulene contents, whereas, “S” due to lack of this compound showed a yellowish green colour. Accordingly, oxygenated sesquiterpenes (53.31–74.52%) were the predominant chemical group of EO constituents in all studied samples, excluding “S”, which was rich in sesquiterpene hydrocarbons (**Figure 5**).

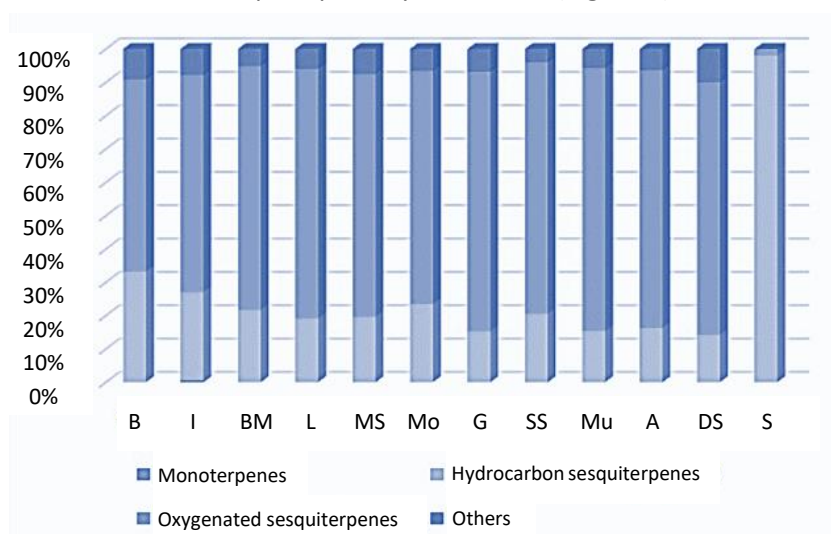


Figure 5. Volatile oil components from *M. chamomilla* populations in percentage of total identified compounds

Table 3. Essential oil constituents and yields of twelve harvested *M. chamomilla* populations

No.	RI ^A	RT ^B	Populations Compounds ^C	B	I	BM	L	MS	Mo	G	SS	Mu	A	DS	S
				Amount (%)											
1	1063	7.79	Artemisia ketone	nd	0.65	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
2	1423	17.75	<i>Trans</i> -caryophyllene	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	0.5
3	1454	18.75	(<i>E</i>)- β -Farnesene	8.51 ^b	16.68 ^a	9.58 ^{ab}	10.60 ^{ab}	13.92 ^{ab}	13.91 ^{ab}	9.82 ^{ab}	6.61 ^b	10.33 ^{ab}	15.29 ^a	12.37 ^{ab}	5.25 ^b
4	1484	19.45	Germacrene D	1.31	nd	nd	nd	nd	nd	nd	nd	nd	0.55	nd	2.21
5	1500	20	Bicyclo-germacrene	2.01	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
6	1506	20.21	(<i>Z</i>)- α -Bisabolene	nd	nd	nd	nd	nd	0.81	nd	nd	nd	nd	nd	nd
7	1514	20.3	(<i>Z</i>)- γ -Bisabolene	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	40.08
8	1529	20.7	(<i>E</i>)- γ -Bisabolene	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	42.76
9	1561	21.65	(<i>E</i>)-Nerolidol	1.76	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
10	1577	22.1	(+)-Spathulenol	1.53	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
11	1630	23.18	(γ)-Eudesmol	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	1.78
12	1656	24.16	α -Bisabolol oxide-B	21.88 ^a	1.55 ^{bc}	1.52 ^{bc}	1.59 ^{bc}	1.39 ^c	1.65 ^{bc}	1.68 ^{bc}	1.80 ^b	1.58 ^{bc}	1.64 ^{bc}	1.37 ^c	nd
13	1685	24.56	α -Bisabolol	nd	nd	nd	nd	1.32	2.86	2.07	nd	nd	nd	nd	nd
14	1693	24.9	α -Bisabolone oxide A	11.36 ^d	47.91 ^{bc}	53.28 ^b	52.14 ^{bc}	46.98 ^c	46.74 ^c	45.64 ^c	51.87 ^{bc}	65.41 ^a	47.7 ^{bc}	49.18 ^{bc}	nd
15	1730	25.76	Chamazulene	19.22 ^a	8.29 ^{bc}	9.74 ^b	4.74 ^e	8.44 ^{bc}	6.14 ^d	8.02 ^c	9.3 ^{bc}	4.18 ^e	2.58 ^f	6.06 ^d	nd
16	1748	26.18	α -Bisabolol oxide A	16.78 ^{bc}	14.03 ^c	13.93 ^c	19.35 ^b	16.75 ^{bc}	19.41 ^b	24.02 ^a	22.26 ^{ab}	7.53 ^d	20.25 ^b	17.22 ^{bc}	nd
17	1890	26.31	(<i>E</i>)-Spiroether	8.37 ^a	7.51 ^{ab}	4.70 ^c	5.75 ^{bc}	7.12 ^{ab}	6.41 ^b	6.49 ^b	3.73 ^c	5.31 ^{bc}	5.96 ^b	7.26 ^{ab}	nd
Total identified compounds %				92.73	96.64	92.76	94.2	95.27	96.51	97.71	95.59	94.35	94.53	93.47	93.08
EOs yield %				1.03 \pm	0.84 \pm	0.78 \pm	0.88 \pm	0.94 \pm	0.79 \pm	0.88 \pm	0.91 \pm	0.9 \pm	0.89 \pm	0.83 \pm	0.98 \pm
				0.003	0.006	0.17	0.012	0.035	0.006	0.021	0.009	0.023	0.006	0.009	0.021

^A Relative retention index to C₈–C₂₄ *n*-alkanes on HP-5MS column; ^B Retention times; ^C Compounds listed in order of elution from HP-5MS column; nd: not detected; the means were compared using Duncan's comparisons test ($p < 0.05$); small letters (a, b, c) in each row show the significant difference of related component among various populations

5.2.3. Apigenin and luteolin quantification of methanolic extracts

Methanolic extracts of samples “L” and “A” contained the highest amounts of apigenin, with 1.19 ± 0.01 mg/g and 1.02 ± 0.01 mg/g, respectively. Luteolin was present in higher concentrations in “BM” (2.20 ± 0.0 mg/g) and “A” (1.01 ± 0.02 mg/g) extracts (**Figure 6**).

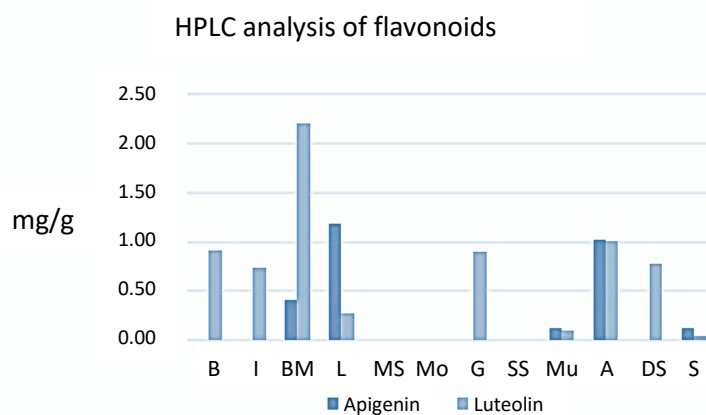


Figure 6. Apigenin and luteolin contents of twelve plant samples (mg/g of dry weight)

5.2.4. Classification of *M. chamomilla* populations

To characterize and identify the different chemotypes of Iranian *M. chamomilla* populations, their EO compositions and main flavonoids (apigenin and luteolin) were subjected to CA and PCA. As shown in **Figures 7** and **8**, the dendrograms allowed the separation of *M. chamomilla* populations into three main groups, each representing a distinct chemotype.

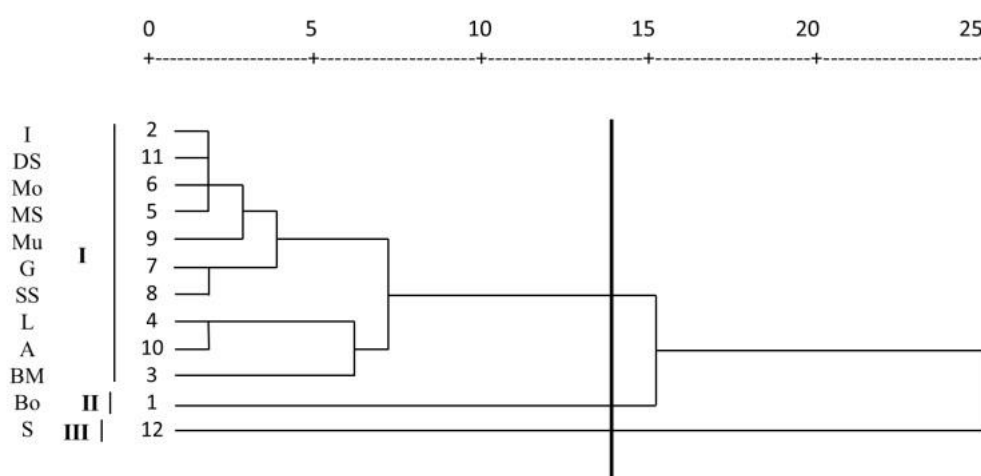


Figure 7. Dendrogram of the *M. chamomilla* populations resulting from the cluster analysis (based on Euclidean distances) of the volatile oil components. chemotype I (α -bisabolone oxide A and α -bisabolol oxide A), chemotype II (chamazulene and α -bisabolol oxide B), chemotype III ((Z) and (E)- γ -bisabolene).

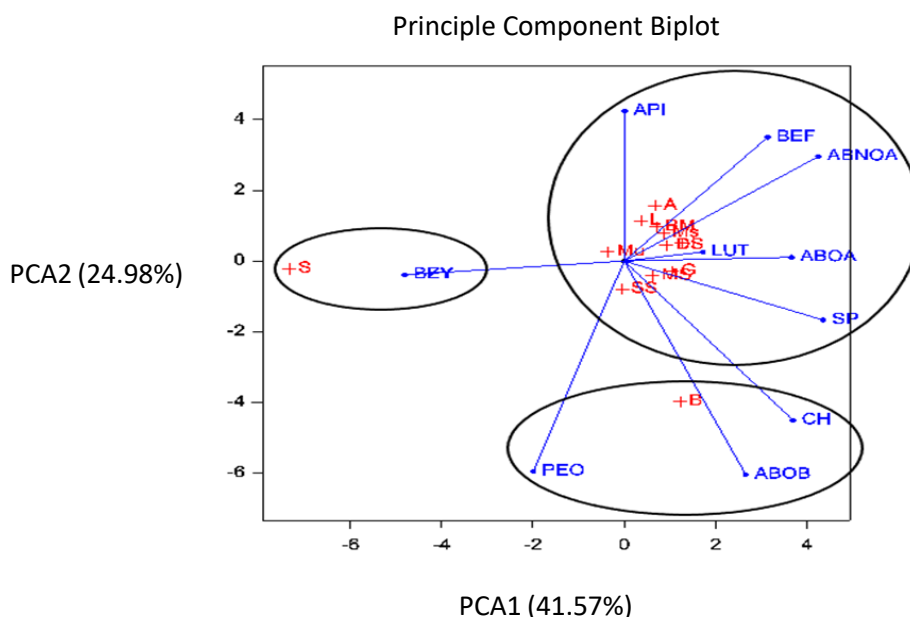


Figure 8. Principal component analysis (PCA) of the *M. chamomilla* populations. ABOA: α -bisabolol oxide A, ABOB: α -bisabolol oxide B, PEO: percentage of essential oil, CH: chamazulene, SP: (*E*)-spiroether, ABNOA: α -bisabolone oxide A, BEF: (*E*)- β -farnesene, BZY: (*Z*)- γ -bisabolene, BEY: (*E*)- γ -bisabolene, LUT: luteolin, API: apigenin.

PCA is a mathematical procedure that transforms several correlated variables into various uncorrelated variables called principal components (PC). PC1, PC2, and PC3 showed the highest variation of phytochemicals among the studied populations. PC1 explained 41.57% of total variation and had a positive correlation with α -bisabolol oxide A, (*E*)- β -farnesene, α -bisabolone oxide A and (*E*)-spiroether, and negative correlation with (*Z*)- γ -bisabolene and (*E*)- γ -bisabolene. The second PC (PC2), with 24.98% of variance, demonstrated positive correlation with chamazulene, α -bisabolol oxide B, α -bisabolone oxide A and the EO content. Furthermore, PC3 represented positive correlation in the case of apigenin and luteolin, which accounted for 12.02% of the total variance (**Table 4**).

Table 4. Eigenvalues, variance and cumulative variance for three principal components

Major phytochemicals	Principal components		
	PC1	PC2	PC3
Chamazulene	0.377	0.881	0.023
α -Bisabolol oxide A	0.760	0.126	0.114
(<i>E</i>)- β -Farnesene	0.679	-0.293	-0.004
α -Bisabolol oxide B	0.044	0.961	0.011
α -Bisabolone oxide A	0.742	0.525	0.104
(<i>E</i>)-Spiroether	0.849	0.377	-0.032
(<i>Z</i>)- γ -Bisabolene	-0.965	-0.119	-0.130
(<i>E</i>)- γ -Bisabolene	-0.965	-0.119	-0.130
Essential oil content	-0.480	0.600	-0.348
Apigenin	0.002	-0.295	0.638
Luteolin	0.160	0.235	0.857

Eigenvalues	4.57	2.74	1.32
Variance (%)	41.57	24.98	12.02
Cumulative variance (%)	41.57	66.55	78.57

Regarding a significant contribution of phytochemical variation in PC1 and PC2, the scatter plot of PC1 and PC2 was used to specify phytochemical distance. The studied populations were classified in three groups, which confirmed the CA results.

In accordance with the CA, the populations “I”, “DS”, “Mo”, “MS”, “Mu”, “G”, “SS”, “L”, “A” and “BM” were classified into the same category, while, “B” and “S” were grouped into the individual subclasses. The first group possessed α -bisabolone oxide A and α -bisabolol oxide A as the major constituents as well as apigenin and luteolin (chemotype I). The second chemotype (II), was characterized by high amounts of chamazulene and α -bisabolol oxide B. The chemotype (III) was the richest in (Z) and (E)- γ -bisabolene.

5.2.5. Effect of environmental factors on secondary metabolite production

In order to assess the effect of environmental factors on EO components, along with apigenin and luteolin contents, CCA was applied based on a matrix of three environmental factors including altitude, mean annual temperature (MAT), and mean annual precipitation (MAP) [222] and major EO compounds, along with apigenin and luteolin contents (**Figure 9**).

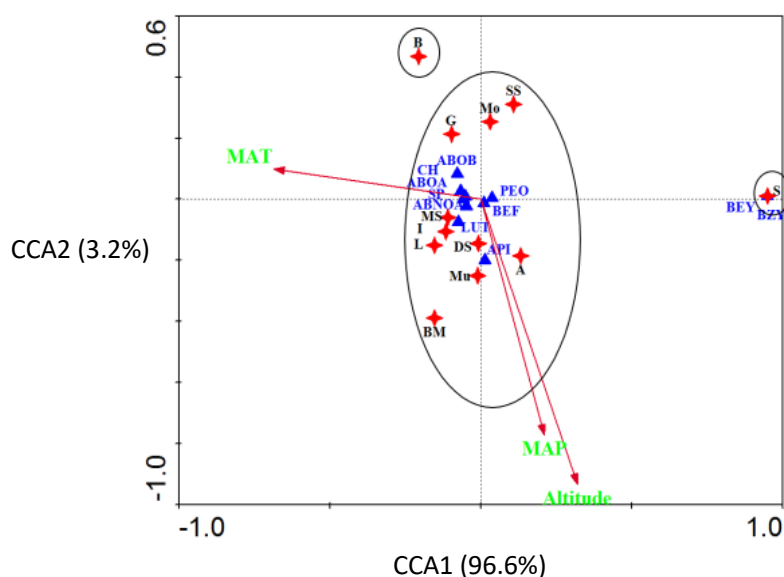


Figure 9. Canonical correspondence analysis (CCA) biplot of *M. chamomilla* populations, linking percentages of the major constituents, collected from different environmental conditions. Populations B: Bodgold, I: Izeh, BM: Bagh Malek, L: Lali, MS: Masjed Soleyman, Mo: Mollasani, SS: Saleh Shahr, Mu: Murmuri, A: Abdanan, DS: Darreh Shahr, and S: Sarableh, MAT: mean annual temperature; MAP: mean annual precipitation; ABOA: α -bisabolol oxide A, ABOB: α -bisabolol oxide B, PEO: essential oil percentage, CH: chamazulene, SP: (*E*)-spiroether, ABNOA: α -bisabolone oxide A, BEF: (*E*)- β -farnesene, BZY: (*Z*)- γ -bisabolene, BEY: (*E*)- γ -bisabolene, LUT: luteolin, API: apigenin.

According to the CCA, phytochemicals of populations in first group were significantly affected by ecological parameters (altitude, temperature and precipitation) while the main oil composition and flavonoids of “Sarableh” and “Boldgold” were changed by genetic factors. Therefore, the “Sarableh” population can be introduced as a new chemotype.

5.2.6. Antiradical activity of the extracts

In the evaluation of the antioxidant potential of the twelve selected populations, “S” showed the most significant antiradical capacity, with $EC_{50} = 7.76 \pm 0.3 \mu\text{g/mL}$ and $6.51 \pm 0.63 \text{ mmol TE/g}$ measured by DPPH and ORAC assays, respectively. However, the extracts showed lower activity compared to ascorbic acid ($EC_{50} = 0.3 \pm 0.02 \mu\text{g/mL}$) in the DPPH and rutin ($20.22 \pm 0.63 \text{ mmol TE/g}$) and EGCG ($11.97 \pm 0.02 \text{ mmol TE/g}$) in the ORAC assay (Figures 10 and 11).

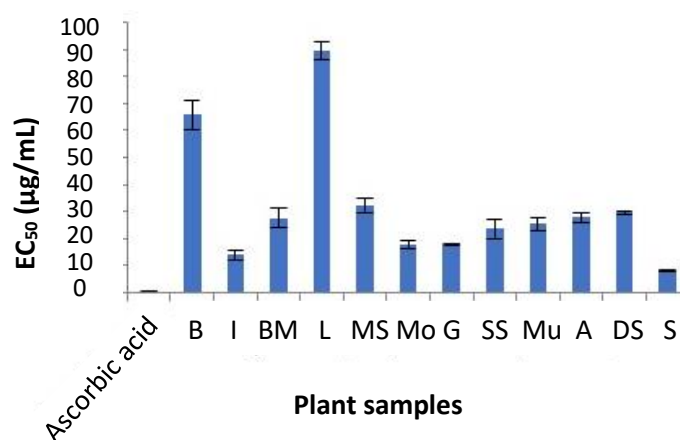


Figure 10. Antiradical scavenging activity of twelve plant samples of *M. chamomilla* in the DPPH assay

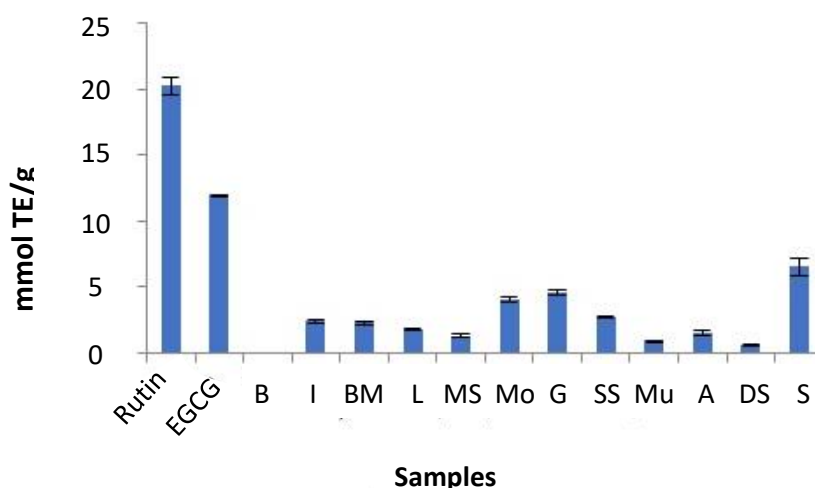


Figure 11. Antiradical capacity of *M. chamomilla* selected populations in the ORAC assay

5.3. DUCROSIA ANETHIFOLIA

5.3.1. Isolation of the pure compounds

Repeated column chromatography of the bioactive fractions resulted in the isolation of 13 compounds. The compounds were identified by careful interpretation of NMR data and comparison of ^1H and ^{13}C chemical shifts with those reported in literature. Nine linear furocoumarin derivatives, namely imperatorin (**5**) [234], oxypeucedanin (**7**) [235], heraclenol (**8**) [236], (+)-oxypeucedanin hydrate (aviprin) (**9**) [235], heraclenin (**10**) [237], pabulenol (**11**) [235], oxypeucedanin methanolate (**13**) [238], isogospherol (**14**) [239], (–)-oxypeucedanin hydrate (prangol) (**16**) [240]; along with vanillic aldehyde (**6**) [241], 3-hydroxy- α -ionone (**12**), harmine (**15**), and 2-C-methyl-erythrytol (**17**) were identified (^1H -NMR spectra see in Supporting Information **Figure S7**) (**Figure 12**).

The diastereomers (+)-oxypeucedanin hydrate and (–)-oxypeucedanin hydrate were distinguished by determining their optical rotations and comparing with literature [242]. The ^1H and ^{13}C -NMR spectral data of **12**, **15**, and **17** in CD_3OD are reported here for the first time.

Compound **12** (3-hydroxy- α -ionone): ^1H -NMR (500 MHz, CD_3OD) δ = 6.67 (H-7, dd, J = 15.8 Hz, 10.3 Hz), 6.13 (H-8, d, J = 15.8 Hz), 5.60 (H-4, br s), 4.22 (H-3, br s), 2.58 (H-6, d, J = 10.3 Hz), 2.27 (H3-10, s), 1.80 (H-2b, dd, J = 13.2 Hz, 5.9 Hz), 1.63 (H3-13, s), 1.38 (H-2a, dd, J = 13.2 Hz, 7.2 Hz), 1.01 (H3-11, s), 0.90 (H3-12, s); ^{13}C -NMR (125 MHz, CD_3OD) δ = 200.8, 149.8, 135.9, 134.7, 127.3, 65.9, 55.6, 45.0, 35.0, 29.8, 27.1, 24.5, 22.8.

Compound **15** (harmine): ^1H -NMR (500 MHz, CD_3OD) δ = 8.11 (H-3, d, J = 5.5 Hz), 8.02 (H-5, d, J = 8.7 Hz), 7.86 (H-4, d, J = 5.5 Hz), 7.06 (H-8, d, J = 1.9 Hz), 6.89 (H-6, dd, J = 8.7 Hz, 1.9 Hz), 3.92 (s, 7- OCH_3), 2.80 (s, 1- CH_3); ^{13}C -NMR (125 MHz, CD_3OD) δ = 162.9, 144.6, 141.6, 137.0, 136.2, 130.7, 123.7, 116.3, 113.5, 111.4, 95.4, 56.0, 19.1.

Compound **17** (2-C-methyl-erythrytol): ^1H -NMR (500 MHz, CD_3OD) δ = 3.80 (H-4a, dd, J = 10.4 Hz, 2.5 Hz), 3.61 (H-3, m), 3.59 (H-4b, m), 3.52 (H-1a, d, J = 11.1 Hz), 3.44 (H-1b, d, J = 11.1 Hz), 1.11 (2- CH_3 , s); ^{13}C -NMR (125 MHz, CD_3OD) δ = 76.2, 75.0, 68.5, 63.8, 19.7.

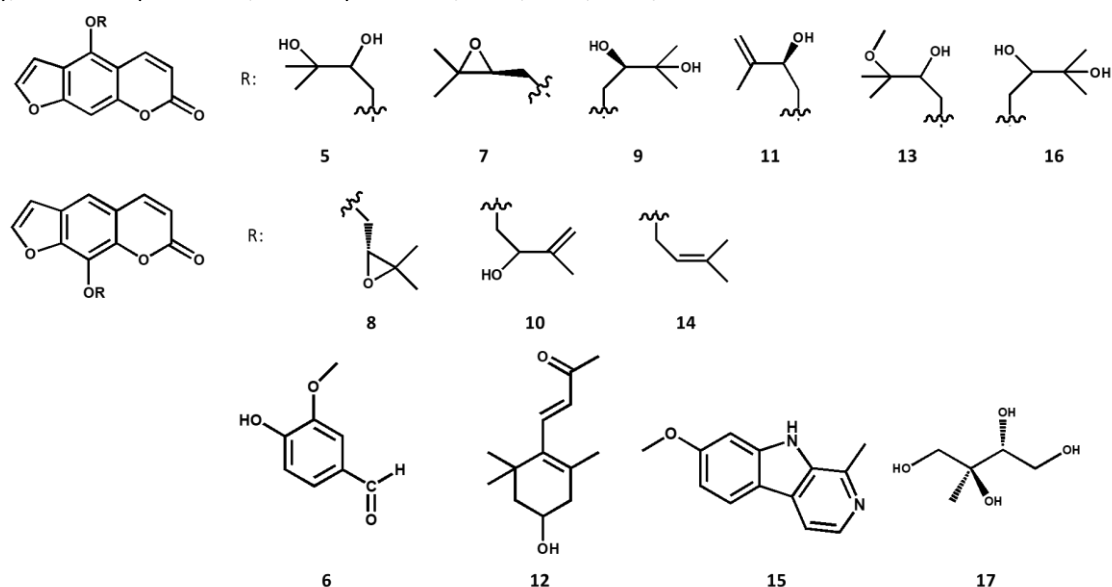


Figure 12. Secondary metabolites isolated from *D. anethifolia*

5.3.2. Antiproliferative and cytotoxic activities on cancer cell lines

Furocoumarins isolated from *D. anethifolia* were subjected to bioassay for cytotoxic and antiproliferative activity against cancer cell lines. All compounds exerted potent antiproliferative effect on sensitive and resistant mouse T-lymphoma cells (**Table 5**).

Table 5. Antiproliferative (AA) and cytotoxic activities (CA) of the furocoumarins against PAR, MDR and NIH/3T3 cells presented as IC₅₀ values

Compounds	AA on PAR cells (μM)	AA on MDR cells (μM)	CA on PAR cells (μM)	CA on MDR cells (μM)	CA on NIH/3T3 cells (μM)
imperatorin (5)	36.12 ± 0.91	42.24 ± 0.88	52.56 ± 4.19	> 100	47.16 ± 1.28
oxypeucedanin (7)	25.98 ± 1.27	28.89 ± 0.73	40.33 ± 0.63	66.68 ± 0.00	57.18 ± 3.91
heraclenol (8)	52.31 ± 2.12	46.57 ± 0.47	> 100	> 100	70.91 ± 4.26
aviprin (9)	41.96 ± 0.88	60.58 ± 2.74	> 100	> 100	83.55 ± 0.57
heraclenin (10)	32.73 ± 2.40	46.54 ± 1.22	65.81 ± 1.00	83.94 ± 1.68	54.82 ± 1.99
pabulenol (11)	30.47 ± 0.47	29.28 ± 0.45	51.32 ± 3.32	> 100	54.09 ± 3.83
oxypeucedanin methanolate (13)	35.88 ± 0.96	33.23 ± 0.51	56.42 ± 5.23	> 100	65.78 ± 0.46
isogospherol (14)	46.53 ± 0.47	48.75 ± 0.28	> 100	> 100	92.41 ± 2.80
doxorubicin	0.054 ± 0.005	0.468 ± 0.065	0.377 ± 0.02	7.152 ± 0.358	5.71 ± 0.50

Data were expressed as mean ± standard deviation ($n = 3$). Different letters represent significant differences ($p < 0.05$).

However, they did not show any selectivity towards the resistant cell line. The most potent compound was oxypeucedanin (**7**) on both cell lines. Some compounds had no toxic effects heraclenol (**8**), ((+)-oxypeucedanin hydrate (**9**), isogospherol (**14**)); furthermore, imperatorin (**5**), pabulenol (**11**), oxypeucedanin methanolate (**13**) and were more toxic on the sensitive PAR cell line (IC₅₀ between 52 and 57 mM) without any toxicity on MDR cells (**Table 5**).

Oxypeucedanin (**7**) and heraclenin (**10**) exhibited cytotoxic activity; however, they were more potent on the sensitive PAR cell line (**Table 5**). Using NIH/3T3 normal murine fibroblast cells, the cytotoxic activity of furocoumarins was evaluated. Some compounds indicated slight toxic effect on normal fibroblasts, namely (+)-oxypeucedanin hydrate (**9**), heraclenol (**8**) and isogospherol (**14**) with IC₅₀ values of 83.55, 65.78 and 54.82 mM, respectively. Pabulenol (**11**) possessed similar activity on fibroblast and parental mouse lymphoma cells. In addition, oxypeucedanin (**7**), heraclenin (**10**), and oxypeucedanin methanolate (**13**) exhibited mild toxicity on fibroblasts and parental lymphoma cells. Imperatorin (**5**) had no toxic activity on fibroblasts.

5.3.3. Multidrug resistance reversing activity

Regarding the efflux pump inhibiting activity of the compounds on ABCB1 overexpressing MDR mouse T-lymphoma cells, only oxypeucedanin (**7**) showed moderate ABCB1 inhibiting effect (FAR: 2.22); however, this inhibition was lower than in case of the positive controls tariquidar (FAR: 100) and verapamil (FAR: 8.2) (**Table 6**, **Figure S8** in Supporting Information).

Table 6. Efflux pump inhibiting activities of furocoumarins

Samples	Conc. μ M	FSC	SSC	FL-1	FAR
PAR	-	2069	658	98.20	-
MDR	-	2152	725	1.79	-
		MDR mean		1.182	-
tariquidar	0.02	2156	719	119	100.68
verapamil	20	2143	740	9.69	8.20
imperatorin (5)	2	2165	749	0.727	0.62
	20	2161	750	0.783	0.66
oxypeucedanin (7)	2	2190	749	0.9	0.76
	20	2164	763	2.62	2.22
heraclenol (8)	2	2300	742	0.995	1.37
	20	2317	740	0.348	1.30
(+) -oxypeucedanin hydrate (9)	2	2325	725	0.715	0.98
	20	2305	769	0.583	0.80
heraclenin (10)	2	2318	723	0.942	1.29
	20	2294	764	0.57	0.78
pabulenol (11)	2	2324	728	0.596	0.82
	20	2323	750	0.544	0.75
oxypeucedanin methanolate (13)	2	2310	737	0.58	0.80
	20	2290	766	0.499	0.68
isogospherol (14)	2	2305	741	0.531	0.73
	20	2326	741	0.548	0.75
DMSO	2% (V/V)	2308	762	0.497	0.68
MDR	-	2301	746	0.535	-

5.3.4. Combination assay results on MDR cells

The two most promising compounds in the previous assays were investigated in combination with the standard chemotherapeutic drug doxorubicin. Oxypeucedanin (**7**) and heraclenin (**10**) showed slight synergistic effect with doxorubicin, for this reason, they might be potential adjuvants in combined chemotherapy applying standard anticancer drugs with compounds that can act synergistically (**Table 7**).

Table 7. Checkerboard combination assay of selected compounds with doxorubicin

Compound	Best ratio	CI at ED ₅₀	Interaction	SD (\pm)
oxypeucedanin	1:50	0.85537	Slight synergism	0.07800
heraclenin	4:100	0.88955	Slight synergism	0.06334

5.4. ISOLATION OF THE MAJOR PHYTOCHEMICALS FROM *EREMURUS PERSICUS*

The successive chromatography techniques were led to isolate six pure compounds. A rare glucoside aliphatic alcohol corchoionoside A (**18**) [243,244], 4-amino-4-carboxychroman-2-one (**19**) [245,246], a C-glycosyl flavone isoorientin (**20**) [247], ziganein 5-methyl ether (**21**) [248], and two coumarin derivatives namely auraptene (**22**) [249,250], and imperatorin (**23**) [251]. Except isoorientin (**20**) all the compounds were isolated for the first time in the *Eremurus* genus. The chemical structures of the isolated compounds are given in **Figure 13**. The ¹H-NMR spectra of the isolated compounds are illustrated in **Figure S9**.

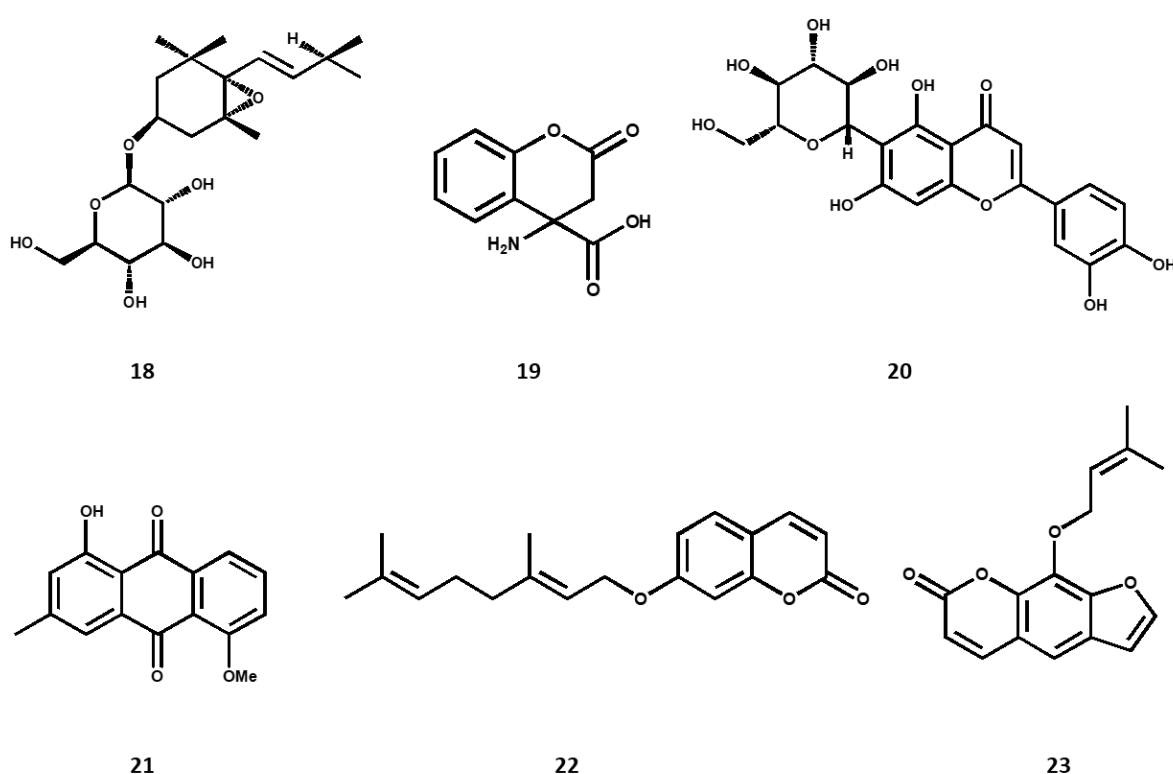


Figure 13. The chemical structures of the isolated phytochemicals of *Eremurus persicus*

5.5. HPLC-DAD FINGERPRINTING OF SAFFRON CROCUS SAMPLES

5.5.1. Method validation

Our aim was to validate and develop an analytical method that is suitable for the analysis of saffron crocus stigma and by-products samples. Marker compounds, that were used as reference standards during our experiments were chosen based on literature data as follows: safranal (**24**), picrocrocin (**25**), crocetin (**26**), crocin (**27**), kaempferol-3-*O*-glucoside (K.G.) (**28**), quercetin-3-*O*-sophoroside (Q.S) (**29**), and kaempferol-3-*O*-sophoroside (K.S.) (**30**). During validation, tepal samples and the mixture of the reference compounds were used. Validation was carried out for all the analytes, where possible. The validation was partial for crocin, crocetin, picrocrocin, and safranal, due to these compounds did

not contain in saffron crocus tepal. The chemical structures of the marker compounds are displayed in **Figure 14**.

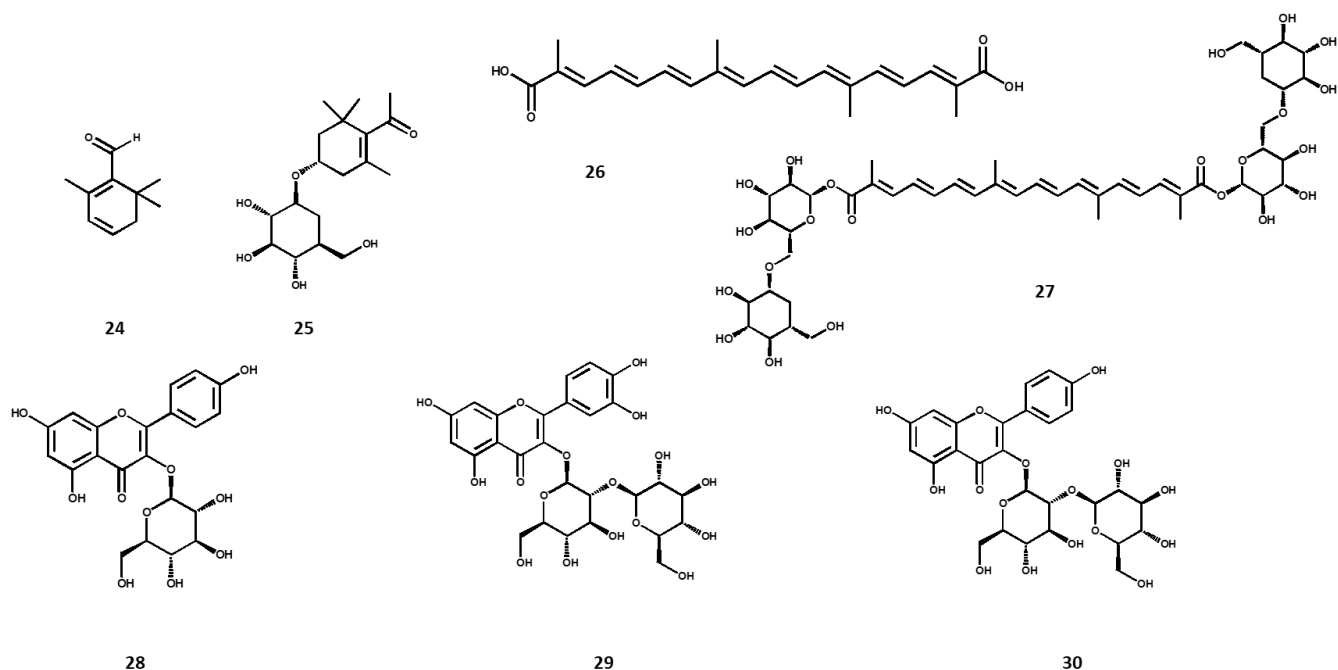


Figure 14. The chemical structures of the reference compounds of *Crocus sativus* L.; **24**: safranal; **25**: picrocrocin; **26**: crocetin; **27**: crocin; **28**: kaempferol-3-*O*-glucoside; **29**: quercetin-3-*O*-sophoroside; **30**: kaempferol-3-*O*-sophoroside

5.5.1.1. Calibration and linearity

To establish calibration curves and limit of detection (LoD) and limit of quantitation (LoQ) values, seven major components of saffron crocus including kaempferol-3-*O*-sophoroside, kaempferol-3-*O*-glucoside, quercetin-3-*O*-sophoroside, crocetin, picrocrocin, safranal, and crocin were utilized (**Table 8**). Calibration curves are based on 8–11 calibration points. The correlation coefficient of the calibration curves was at least 0.998, while these curves covered 2 orders of magnitude of analyte concentration.

Table 8. Calibration curve characteristics and limit of detection and quantification values

Standard	LoD ($\mu\text{g/inj}$)	LoQ ($\mu\text{g/inj}$)	Calibration points	Range covered ($\mu\text{g/inj}$)	Regression equations	R ²
Picrocrocin	0.00245	0.00741	8	0.03-0.9	$y = (1.10320 \times 10^9)x - 10690.0$	0.9997394
Q.S.	0.02554	0.07739	10	0.082-6.15	$y = (6.55841 \times 10^8)x - 5275.02$	0.9996089
K.S.	0.02277	0.06901	9	0.076-3.04	$y = (5.96019 \times 10^8)x - 20062.4$	0.9996702
K.G.	0.06763	0.20495	9	0.1075-4.3	$y = (1.36039 \times 10^8)x - 4682.70$	0.9984738
Crocin	0.00128	0.00386	10	0.0145-0.58	$y = (2.92163 \times 10^9)x - 13570.0$	0.9994265
Safranal	0.00177	0.00536	8	0.08-2.4	$y = (2.06147 \times 10^9)x + 37991.1$	0.9982921
Crocetin	0.00032	0.00097	11	0.00172-0.129	$y = (9.35271 \times 10^9)x - 5778.24$	0.9999475

5.5.1.2. Filter compatibility

In order to choose the best method for sample preparation, one tepal specimen was extracted by ultrasonic bath, then filtered or centrifuged. The sample preparation by filtration or centrifugation had no major impact on quantitative results, however in case of crocetin, the amount of the analyte decreased with 17.3% as the result of filtration. For the other analytes, slighter higher values were measured in filtered samples (picrocrocin: 0.311 ± 0.0011 mg; Q.S.: 1.51 ± 0.0047 mg; K.S.: 0.935 ± 0.0027 mg; K.G.: 1.148 ± 0.0161 mg; safranal: 1.046 ± 0.0143 mg).

5.5.1.3. Stability

The stability of the solutions of reference compounds was assessed. For this case, the standard mixtures were prepared by filter and centrifuge methods, stored at 4 °C and room temperature (23 °C), then injected at day 0, 1, 3, 5, and 7 (**Table 9**). In case of picrocrocin and the flavonoids (K.S., K.G., and Q.S.), temperature and storage time did not influence the concentration of the analytes, whereas in case of crocin and safranal, decomposition was observed especially at room temperature. Interestingly, after one day, the concentration of crocetin significantly decreased, while temperature had no major impact on this process.

Table 9. Stability of the dissolved reference compounds after 1, 3, 5 and 7 days (values compared to 100% day 0 values)

Sample	Days	Picrocrocin	Q.S.	K.S.	K.G.	Crocin	Safranal	Crocetin
23 °C	1	100.97	101.08	100.92	101.77	101.18	99.84	86.28
	3	104.06	100.59	100.69	101.80	95.93	87.89	76.71
	5	100.37	99.97	100.53	102.74	91.29	85.89	76.47
	7	100.59	99.70	101.12	105.00	90.65	83.71	78.94
4 °C	1	100.51	100.66	100.56	101.05	100.84	100.29	86.52
	3	104.09	100.66	100.56	101.82	100.57	99.74	85.92
	5	100.69	100.41	100.67	102.30	100.30	96.44	84.48
	7	100.77	100.89	101.27	104.98	100.50	94.64	85.44

5.5.1.4. System suitability

5 times of injection was carried out for the mixture of the reference standards to investigate suitability of the analytical system. The low RSD% values of the AUCs and retention times, together with the tailing factors below 2 confirm that the system is suitable for the measurement of these compounds (**Table 10**).

Table 10. System suitability of the standards compared to the prepared mixture of standards

System suitability characteristic	Picrocrocin	Q.S.	K.S.	K.G.	Crocin	Safranal	Crocetin
RSD% of AUC	0.47	0.18	0.43	2.53	0.38	0.41	1.11
Tailing factor	1.139 - 1.145	1.043 - 1.067	1.113 - 1.127	1.356 - 1.408	1.177 - 1.910	1.171 - 1.184	1.310 - 1.334
RSD% of RT	0.66	0.62	0.36	0.22	0.19	0.14	0.10

RT: retention time

5.5.1.5. Accuracy

By the determination of recoveries, the accuracy of the method was evaluated (**Table 11**). Recoveries of the marker compounds was assessed by adding known amounts of the standards to a tepal (or in case of crocin, picrocrocin, crocetin, and safranal to stigma) sample at three different concentrations (50, 100, and 150% of the previously determined amounts). Three independent samples were prepared for each concentration levels and injected in triplicates. The recovery values ranged between 96.09–111.92%, 83.69–112.25%, and 89.40–116.26% in case of 50%, 100%, and 150% of concentration, respectively, therefore the accuracy of the method was considered to be acceptable.

Table 11. Mean recovery values (%) of the controls

Level	Picrocrocin (stigma)		Q.S.		K.S.		K.G.		Crocine (stigma)		Safranal (stigma)		Crocetin (stigma)	
	%	RSD%	%	RSD%	%	RSD%	%	RSD%	%	RSD%	%	RSD%	%	RSD%
50%	111.92	0.27	106.54	0.12	100.95	0.29	96.09	0.62	-	-	-	-	99.54	1.33
100%	101.80	0.07	112.25	0.10	99.60	0.06	103.36	2.03	97.89	0.27	-	-	83.69	1.73
150%	110.32	1.32	116.26	0.30	101.54	0.12	97.57	2.77	108.10	0.06	-	-	89.40	0.42

5.5.1.6. Precision

One tepal and one stigma sample were individually extracted and injected 10 times, to determine the precision of the analytical method. Precision was established by calculating RSD% values of the AUCs (**Table 12**). The RSD% values below 1% confirm the precision of the method.

Table 12. Precision of the analytical method as determined by RSD%

Samples	Picrocrocin	Q.S.	K.S.	K.G.	Crocine	Crocetin
Tepal	-	1.72	0.07	0.53	-	-
Stigma	1.86	0.30	2.26	2.68	0.58	1.86

5.5.1.7. Repeatability

The repeatability of the experiment was carried out by analysis of six tepal sample extracts within 24 h (intra-assay precision). Repeatability is described by RSD% values of the whole datasets for Q.S., K.S., and K.G., where RSD% values were 3.38%, 3.71% and 3.44%, respectively.

5.5.1.8. Intermediate precision

The same tepal sample was examined by two analysts and intermediate precision was determined as RSD% of the means, to assess intermediate precision. For Q.S., K.S., and K.G. the RSD% values were 3.81%, 2.29%, and 6.85%, respectively.

5.5.2. HPLC-DAD analysis of tepal and stamen samples

40 different tepal and stamen samples were subjected to HPLC-DAD analysis to qualitatively and quantitatively analyse their selected markers. Our developed HPLC method allowed the good separation and hence the reliable analysis of these compounds in saffron crocus samples.

In tepal and stamen samples only three glycosylated flavonols including quercetin-3-O-sophoroside, kaempferol-3-O-sophoroside, and kaempferol-3-O-glucoside were detected. We analysed a saffron stigma sample as well. From this sample picrocrocin, crocin, and crocetin were identified, which is in line with previous data.

The amounts of reference standards in tepal and stamen samples are presented in **Table S3** (Supplementary materials) as average (mg/g plant material) and standard deviation values. The tepal samples comprised Q.S., K.S. and K.G., in the ranges of 6.20–10.82, 62.18–99.48, and 27.38–45.17 mg/g, respectively, whereas the amount of these compounds was lower in stamen (1.72–6.07, 0.89–6.62 and 1.72–7.44 mg/g, respectively). Sample 1 contained K.S. (99.48 mg/g) as the major constituent of tepal. Moreover, the tepal sample 23 possessed the highest amount of Q.S. (10.82 mg/g), while the tepal sample 5 contained the lowest amounts of Q.S. and K.S. with 6.21 and 62.19 mg/g, respectively. In tepal samples the K.G. content ranged between 27.74 to 45.18 mg/g in samples 40 and 1, respectively. In general, the stamen samples contained less flavonoids than tepals. The highest amount of Q.S. in stamens was observed in sample 29 with 6.08 mg/g, whilst sample 3 contained the lowest concentration (1.63 mg/g). The quantity of K.S. ranged from 6.62 to 0.93 mg/g in stamen samples 40 and 3, respectively. The quantity of the flavonoid K.G. was also variable, the highest and lowest amounts determined in sample 31 and 8 with 7.44 and 1.72 mg/g, respectively.

5.5.3. *Classification of saffron crocus populations*

CA and PCA were applied to characterize and classify saffron crocus samples (tepal and stamen) collected from different locations in Iran according to their Q.S., K.S., and K.G. contents. In accordance with the CA analysis, the saffron crocus samples harvested from various locations were categorized in three major groups showing three distinct chemotypes based on their flavonoid contents. Chemotype I was characterized with the highest Q.S. content of in tepal samples, including the populations 2, 3, 6-12, and 39, samples belonging to chemotype II contained high quantity of K.G. in tepal samples (populations 4, 5, 13, 14, 16–18, 22, 24, 26–30, 32-34, and 37), whereas chemotype III was the richest in Q.S., K.S., and K.G. in stamen, and K.S. in tepal (populations 1, 15, 16, 19-21, 23, 25, 31, 35, 36, 38, 40). The PCA results are given on a dendrogram in **Figure 15** and PCA biplot which is reported in **Figure 16**.

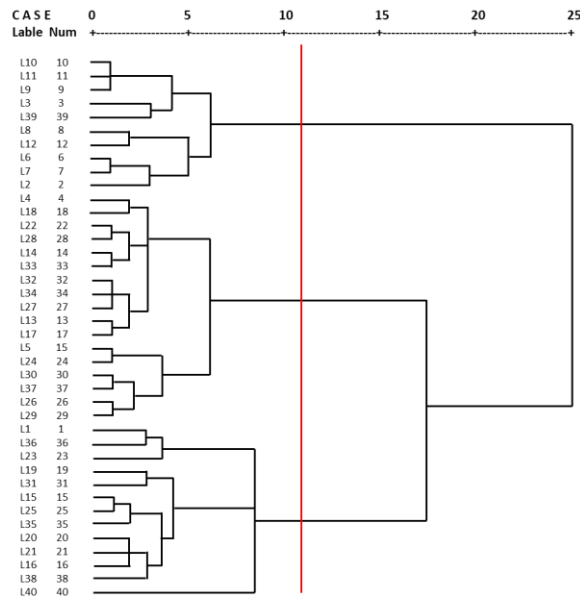


Figure 15. Dendrogram of cluster analysis (based on Euclidean distances) of the saffron crocus samples: chemotype I (Q.S. in tepal), chemotype II (K.G. in tepal), chemotype III (Q.S., K.S., and K.G. in stamen, and K.S. in tepal).

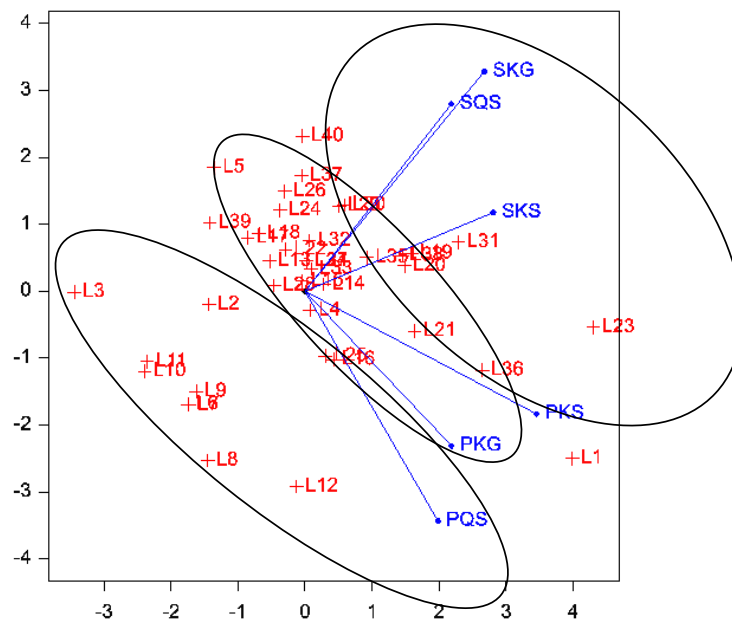


Figure 16. Principal component biplot (PCA) of the saffron crocus samples; L: Location; PQS: quercetin-3-*O*-sophoroside in tepal; PKS: kaempferol-3-*O*-sophoroside in tepal; PKG: kaempferol-3-*O*-glucoside in tepal; SQS: quercetin-3-*O*-sophoroside in stamen; SKS: kaempferol-3-*O*-sophoroside in stamen; SKG: kaempferol-3-*O*-glucoside in stamen

6. DISCUSSION

6.1. SMOOTH MUSCLE-RELAXANT ACTIVITY OF *CHAMAEMELUM NOBILE*

Our experiments aimed at the analysis of the effect of *C. nobile* extracts, pure compounds and essential oil on smooth muscle cells. Previous studies reported sesquiterpenes, coumarin derivatives as characteristic compounds of the flower, flavonoids being the qualitatively most abundant compounds [223]. Taking into account the spasmolytic effect of some flavonoids (eg. that of apigenin and luteolin on guinea pig ileum [59]), we hypothesized that the assumed spasmolytic effect of the plant may be related to its flavonoid contents.

As a result of our work, we isolated the flavonoids apigenin, luteolin, eupafolin, and hispidulin using an activity-guided approach. The EtOH extract of *C. nobile* possessed both stimulatory and relaxant effects on small intestine of guinea-pig and on the urinary bladder, the stimulatory effect being transient. Based on experiments with the concurrent use of tetrodotoxin and atropine, it can be suggested that the site of action for the relaxant effect on the smooth muscle itself, and it was assumed that the excitatory activity was related to capsaicin-sensitive sensory [252]. The relaxant effect was confirmed in experiments on human jejunal preparations as well. The transient contraction was independent from the flavonoid content of fractions, whereas for relaxant activity there was a clear-cut correlation. The EO produced no contraction on the test preparations. A consequent relaxant effect was detected with the oil.

We analysed the chemical composition of the EO as well. Previous articles reported isobutyl angelate as the major constituent of *C. nobile* EO (21.6–38.5%), followed by 2-methylbutyl angelate (11.6–20.3%) [48,253–256], and propyl tiglate (10.8–13.1%) [255,256] or isobutyl isobutyrate (3.3%) [48] or 2-butenyl angelate (7.9–8.4%) [253] or 2-methyl-2-propenyl angelate (9.1%) [254]. However, several commercial samples have similar compositions to the sample analysed by us, with methallyl angelate and 3-methyl pentyl angelate as the major components [29].

6.2. PHYTOCONSTITUENTS OF *MATRICARIA CHAMOMILLA* POPULATIONS, AND BIOACTIVITIES OF VOLATILE OILS

The results of the carried-out study confirm the impact of variation of ecological conditions on the variability of EO contents, phenolic profile and antiradical activity.

The abiotic factors (e.g. moisture, topography, temperature, and edaphic factors) affect the variation of phytoconstituents and/or biotic factors significantly influenced the chemotype profiles and biosynthesis pathways of terpenes [257]. Nevertheless, the role of other ecological effects including physiographic factors (e.g. steepness and sunlight on vegetation and direction of slopes), edaphic factors (soil attributes), climatic factors (e.g. light, wind, temperature, humidity, and atmosphere gases), and biotic factors (e.g. the existence of parasites, epiphytes, lianas, etc.) can also be impressive in variation of chemotypes.

According to our findings, *M. chamomilla* samples were rich in oxygenated sesquiterpenes (53.31–74.52%) as the most dominant EO compounds, which corroborates the former reports. In case of EO composition, the wild populations were significantly different compared with the cultivated

sample (Bodgold). Among the populations, α -bisabolone oxide A was identified as the major EO constituent.

α -Bisabolone oxide A was previously detected from *M. chamomilla*; for instance, the EO, dichloromethane and diethyl ether fractions of EO contained α -bisabolone oxide A with 47.7, 50.5 and 57.7%, respectively [63]; whereas high bisabolone oxide A content (13.9%) was characterized in the EO of an Estonian *M. chamomilla* sample [79]. Also in *M. chamomilla* samples harvested from Italy (9.2–11.2%) [258], Iran (53.45%) [259], India (20.4 and 8.9%) [260] and Turkey (47.7%) [62] this compound was detected as the main EO constituent. In a study the daily fluctuation of α -bisabolol oxide A content in *M. chamomilla* EOs was determined and the highest content was measured between 16:00–18:00 (55.41%) [261].

The sample “Sarableh” collected from the highest altitude with lowest minimum and maximum annual temperatures was identified as a new chemotype with the highest content of the isomers of γ -bisabolene (82.84%), whilst these sesquiterpenes were not detected in other plant populations. This plant sample also exerted the most potent antiradical capacity compared with those populations.

γ -Bisabolene was previously identified as the major characteristic EO constituent of several plant species [262–264]. In Iranian populations, the main EO constituents of *M. chamomilla* were formerly identified as α -bisabolol oxide A (17.14%) [265], α -bisabolol (7.27%) [266], (*Z,Z*)-farnesol (39.70–66.00%), and (*E*)- β -farnesene (24.19%) [267]. EOs containing significant quantities of γ -bisabolene exhibited anti-inflammatory properties and anti-proliferative activities on human prostate cancer, breast, lung carcinoma, glioblastoma carcinoma, human oral squamous cell lines and colon adenocarcinoma [268–271].

In a former study, apigenin, luteolin, and their derivatives, along with cynaroside, and chlorogenic acid were identified as the significant phytoconstituents of *M. chamomilla* extract [272]. Our results showed the plant samples were much richer in luteolin than in apigenin. Interestingly, in populations “MS” and “Mo” no luteolin and apigenin was detected. Due to the low concentration of apigenin and luteolin in “Sarableh”, the high free radical scavenging capacity of this sample is obviously correlated to other polyphenolics.

6.3. ISOLATION OF SECONDARY METABOLITES FROM *DUROSIA ANETHIFOLIA* AND ANTIPROLIFERATIVE POTENTIAL OF ISOLATED FUROCOUMARINS

Chromatographic separation of *D. anethifolia* extract led to the isolation of 13 compounds, including 9 furocoumarins. Compounds **6**, **7**, **9**, **12**, **14–17** were identified for the first time from *Ducrosia* genus.

In literature, the furocoumarin derivatives including psoralen, xanthotoxin, bergapten, imperatorin, iso-oxypeucedanin, pabulenol, pangelin, heraclenin, heraclenol, oxypeucedanin methanolate, oxypeucedanin hydrate have been isolated as major components of the *D. anethifolia* [14,106,107]. Furocoumarins iso-psoralen and psoralen have been also isolated from *D. ismaelis* [109].

The analysed furocoumarins exerted antiproliferative activities on sensitive and resistant mouse T-lymphoma cells with no selectivity towards the resistant cell line. This is the first comprehensive study on this plant and its furocoumarins on these cells. Among the isolated

furocoumarins, oxypeucedanin (**7**) possessed the most significant activity on both tested cell lines (PAR and MDR). Insignificant toxicity on normal fibroblast cells and sensitive parental mouse lymphoma cells was measured in case of the most effective furocoumarins oxypeucedanin (**7**) and heraclenin (**10**), also they showed less toxicity on multidrug resistant lymphoma cells. From the studied compounds, only oxypeucedanin (**7**) demonstrated moderate multidrug resistance reversing activity. Oxypeucedanin (**7**) and heraclenin (**10**) also showed slight synergistic effect with doxorubicin in the checkerboard assay. These compounds can improve the cytotoxic effect of the standard chemotherapeutic drug doxorubicin.

In the literature, several papers report the antiproliferative, and cytotoxicity potencies of furocoumarins. For instance, imperatorin isolated from *Angelica dahurica* exhibited antiproliferative effect on human hepatoma HepG2 cells [273]; besides, induction of apoptosis in Jurkat leukemia cells was observed in this compound and in heraclenin. In Jurkat cells treated with heraclenin and imperatorin for 72 h, most of the DNA fragmentation occurred at the G2/M and G1/S phases of the cell cycle, respectively [274]. Xanthotoxin showed an inhibition against the growth of neuroblastoma ($IC_{50} = 56.3 \mu\text{M}$) and metastatic colon cancer cells ($IC_{50} = 88.5 \mu\text{M}$) by triggering both extrinsic and intrinsic apoptotic pathways, independently of photoactivation [275]. Moreover, in a dose-dependent manner, imperatorin, isoimperatorin, oxypeucedanin, cnidicin, byakangelicol, and oxypeucedanin hydrate exhibited a significant inhibition on cell proliferation, particularly oxypeucedanin against HCT-15 (colon cancer) cells with $ED_{50} = 3.4 \pm 0.3 \mu\text{g/mL}$ [276].

Furocoumarins affect MDR, beside direct antiproliferative and cytotoxic activities. Among twenty selected furocoumarin derivatives, phellopterin ($IC_{50} = 8.0 \pm 4.0 \mu\text{M}$) and isopimpinellin ($IC_{50} = 26.0 \pm 5.7 \mu\text{M}$) demonstrated the highest activity against CEM/C1 and HL-60/MX2 (MDR) cell lines, respectively [277]. A novel furocoumarin feroniellin A reverted MDR in A549RT-eto lung cancer cells [278]. Also, furocoumarins xanthotoxin ($IC_{50} = 1.10 \pm 0.91 \text{ nM}$) and bergapten ($IC_{50} = 40.29 \pm 0.30 \text{ nM}$) indicated remarkable anticancer activity against MCF7MX, and EPG85.257RDB, respectively [279].

The results of the comprehensive analysis of furocoumarins on tumor cells reassure previous findings and highlight the need of further investigations of these compounds with the perspective of potential use against cancer cells, possibly as additional treatment.

6.4. ISOLATION OF MAJOR CONSTITUENTS FROM EREMURUS PERSICUS

The phytochemical composition of *Eremurus* species have been rarely investigated. In general, isoorientin and methylnaphthalene derivatives have been identified as the predominant phytoconstituents of the plants in this genus [141–144]. In our study, by extracting of CHCl_3 and EtOAc soluble fractions, six pure compounds including corchoionoside A (**18**), a rare dihydro-coumarin 4-amino-4-carboxychroman-2-one (**19**), C-glycosyl flavone isoorientin (**20**), a very scarce anthraquinone ziganein 5-methyl ether (**21**), auraptene (**22**), and imperatorin (**23**) were isolated from *E. persicus*.

All the isolated compounds are new in *E. persicus*. Although isoorientin (**20**) was isolated from leaves of *E. spectabilis* [144], the other identified constituents are reported for the first time in *Eremurus* genus. 4-Amino-4-carboxychroman-2-one (**19**) was isolated only in two studies from *Centipeda minima* and *Prunus domestica* L. [245,246]. Ziganein 5-methyl ether (**21**) was isolated for

the first time from *Aloe hijazensis* as a new natural product and here we isolated for the second time in the nature [248].

Isoorientin (**20**), a C-glycosyl flavone was identified as the major compound of the plant species with significant amount, thus they can be considered as a rich source of this compound. Many reports have been reported the pharmacological activities of it; for instance, antinociceptive, anti-inflammatory [280], antiproliferative [281,282], and gastroprotective effects [158]. Therefore, *E. persicus* is a promising raw material to supply this valuable flavonoid for application in the related phytopharmaceutical industries.

6.5. HPLC-DAD ANALYSIS OF THE MAIN COMPOUNDS OF SAFFRON CROCUS BY-PRODUCTS

In stamen and tepal samples, three flavonol glycosides, namely Q.S., K.S., and K.G. were detected as major components. For comparison, we analyzed a stigma sample as well. From this sample picrocrocin, crocin, and crocetin were identified, which is in line with previous data. There is only one report on the presence of crocin in saffron crocus tepal, however, very low amount (0.6%) was reported in hydrolysed extracts compared to kaempferol (12.6%) [164]. A comparative study of the stamen and stigma revealed that crocin, picrocrocin, and safranal are not present in stamen, whilst they are major components of stigma sample [178].

Our results showed tepal and stamen samples contained Q.S., K.S. and K.G. In general, the stamen samples contained less flavonoids than tepals. The content of Q.S., K.S. and K.G. and stamen samples were in the ranges of 6.20–10.82, 62.19–99.48, and 27.38–45.17 mg/g in tepal, and 1.72–6.07, 0.89–6.62 and 1.72–7.44 mg/g for stamens, respectively. K.S., was quantified as the major constituent of tepal, while sample 1 (99.48 mg/g) was the richest sample. Also, the tepal sample 23 contained the highest amount of Q.S. (10.82 mg/g), while the tepal sample 5 contained the lowest amounts of Q.S. and K.S. with 6.21 and 62.19 mg/g, respectively. The content of K.G. in tepal samples (27.74 to 45.18 mg/g) was significantly different in samples 40 and 1, respectively.

Our results confirmed some previous findings. K.S., K.G., and Q.S. were characterized as major or characteristic flavonoid components of flowers, sepals or tepals by some authors [20,22,24,25,163,165,166,178,179]. Nevertheless, from two different regions of Italy, flavonol derivatives (6–10 mg/g) were characterized as the major compounds of stamen and sepal samples. K.S. was the major compound (6.41–8.30 mg/g of fresh sepals and 1.70–0.37 mg/g of fresh stamen) [179].

The analysis of a large sample set presented here might be a starting point for the determination of quality specifications for these so far not utilized, but industrially perspective plant parts of saffron crocus.

7. SUMMARY

The work presented here was aimed at investigating the secondary metabolites of Iranian and European medicinal plants. As a part of the research, we verified the folk medicinal use of *Chamaemelum nobile* for its spasmolytic effect in gastrointestinal disorders. For this purpose, the smooth muscle-relaxant effect of various extracts, essential oil, and isolated flavonoids (luteolin, apigenin, hispidulin, and eupafolin) *in vitro* was analyzed. Our results suggest that *C. nobile* extract has a direct smooth muscle-relaxant effect related to its flavonoid content. The essential oil also has a remarkable smooth muscle relaxant effect in this setting. Our study is the first report on the activity of this plant on smooth muscles that may reassure the rationale of the traditional use of this plant in spasmodic gastrointestinal disorders.

The impact of growth environmental factors on chemodiversity and antiradical potential of twelve Iranian populations of *Matricaria chamomilla* was furtherly investigated. Among seventeen identified volatile components, representing more than 90% of the total oil, α -bisabolone oxide A (45.64–65.41%) was the major constituent, except the sample “Sarableh”, a new chemotype, where (*E*)- and (*Z*)- γ -bisabolene (42.76 and 40.08%, respectively) were the predominant components. Oxygenated sesquiterpenes (53.31–74.52%) were the most abundant compounds in the samples excluding “Sarableh”. “Sarableh” also possessed the most potent antioxidant capacity. In addition, populations “Lali” and “Bagh Malek” contained the highest amounts of apigenin and luteolin with 1.19 ± 0.01 mg/g and 2.20 ± 0.0 mg/g of plant material, respectively. Our findings depict a correlation between phytochemical profiles and antiradical potential of *M. chamomilla* and geographical factors.

From the Iranian species *Ducrosia anethifolia*, nine linear furocoumarin derivatives, namely imperatorin (**5**), oxypeucedanin (**7**), heraclenol (**8**), (+)-oxypeucedanin hydrate (aviprin) (**9**), heraclenin (**10**), pabulenol (**11**), oxypeucedanin methanolate (**13**), isogospherol (**14**), (–)-oxypeucedanin hydrate (prangol) (**16**); along with vanillic aldehyde (**6**), 3-hydroxy- α -ionone (**12**), harmine (**15**), and 2-*C*-methyl-erythrytol (**17**) were identified. Compounds **7**, **9**, **12**, **14–17** were reported for the first time from the *Ducrosia* genus. Furthermore, we measured *in vitro* antiproliferative and cytotoxic activities of the furocoumarins on multidrug resistant and sensitive L51787Y mouse T-lymphoma cell lines. A checkerboard microplate method was applied to study the interactions of furocoumarins and doxorubicin. Oxypeucedanin (**7**) demonstrated a promising potency and may be considered for further anticancer effects investigations.

A rare Iranian plant species, *Eremurus persicus* was also subjected to phytochemical analysis. Six pure compounds, including corchoionoside A (**18**), 4-amino-4-carboxychroman-2-one (**19**), isoorientin (**20**), ziganein 5-methyl ether (**21**), auraptene (**22**), and imperatorin (**23**) were isolated. Except isoorientin (**20**), which is considered as the predominant secondary metabolite of the *Eremurus* genus and previously isolated from *E. spectabilis* [144], all the other compounds were isolated and characterized for the first time in this genus. Among the identified phytochemicals, 4-amino-4-carboxychroman-2-one (**19**) has been previously isolated in only two species, including *Centipeda minima* and *Prunus domestica* L. [245,246]. An anthraquinone derivative ziganein 5-methyl ether (**21**)

was isolated for the first time from *Aloe hijazensis* as a new natural product and here we isolated for the second time in the nature [248].

The stigma of *Crocus sativus* (known as saffron) has been used in Iranian traditional folk medicine as antidepressant and anxiolytic agent, along with its application in the cuisine as spice. Several studies (preclinical and clinical) reported the antidepressant effect of saffron, however, recently these bioactivities have been reported for other parts of the flower as well. We collected 40 different tepal and stamen samples growing from Iran and quantitatively and qualitatively analysed their main components of flower parts (kaempferol-3-*O*-sophoroside, kaempferol-3-*O*-glucoside, quercetin-3-*O*-sophoroside, picrocrocin, crocin, crocetin, and safranal) by HPLC-DAD using a validated method. Tepal and stamen samples contained three flavonol glycosides. Kaempferol-3-*O*-sophoroside (62.19–99.48 mg/g) and kaempferol-3-*O*-glucoside (1.72–7.44 mg/g) were identified as the most dominant phytochemicals in tepal and stamen samples, respectively. Crocin, crocetin, picrocrocin, and safranal were not detected in any of the analysed samples (except stigma). Our results point out that *C. sativus* by-products, particularly tepals might be considered as rich sources of flavonol glycosides. The data presented here can be useful in setting quality standards for plant parts of *C. sativus* that might be used for medicinal purposes in the future.

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9. SUPPLEMENTARY MATERIALS

Table S1. Geographic locations and climatic conditions of the studied *Matricaria chamomilla* populations from Iran

Population Name	Voucher's Code	Abbreviated Name	Altitude (m)	Latitude	Longitude	MAP (mm/year)	MAT (°C)	MMaxAT (°C)	MminAT (°C)
Bodold (Ahvaz)	KHAU_236	B	16	31°18' N	48°39' E	9820	22.62	35.77	9.47
Izeh	KHAU_237	I	428	31°57' N	48°49' E	472.80	18.35	31.28	5.42
Bagh Malek	KHAU_238	BM	907	31°19' N	50°05' E	285.90	20.28	32.17	8.4
Lali	KHAU_239	L	373	32°20' N	49°05' E	280.60	21.26	33.57	8.95
Masjed Soleyman	KHAU_240	MS	250	32°02' N	49°11' E	241.30	22.67	34.27	11.07
Mollasani	KHAU_241	Mo	51	31°39' N	48°57' E	100.80	22.26	36.35	8.17
Gotvand	KHAU_242	G	70	32°14' N	48°48' E	159.70	21.08	34.96	7.21
Saleh Shahr	KHAU_243	SS	65	32°04' N	48°40' E	164.30	20.69	34.07	7.32
Murmuri	KHAU_244	Mu	530	32°46' N	47°37' E	213.90	23.35	35.17	11.53
Abdanan	KHAU_245	A	740	32°55' N	47°31' E	363.60	19.62	30.44	8.8
Darreh Shahr	KHAU_246	DS	629	33°05' N	47°28' E	463.00	17.85	30.9	4.8
Sarableh	KHAU_247	S	1037	33°47' N	46°35' E	345.80	15.01	27.31	2.71

MAP: mean annual precipitation; MAT: mean annual temperature; MmaxAT: mean maximum annual temperature; MminAT: mean minimum annual temperature

Table S2. Geographical, coordinate, and locations of the harvested saffron plant samples

Code	location	Altitude (m)	Latitude	Longitude
1T, 1S	Dehouye, Meshkan, Neyriz, Fars	1567	29° 15' N	53° 56' E
2T, 2S	Dehouye, Meshkan, Neyriz, Fars	1567	29° 15' N	53° 56' E
3T, 3S	Dehouye, Meshkan, Neyriz, Fars	1567	29° 15' N	53° 56' E
4T, 4S	Dehouye, Meshkan, Neyriz, Fars	1567	29° 15' N	53° 56' E
5T, 5S	Dehouye, Meshkan, Neyriz, Fars	1567	29° 15' N	53° 56' E
6T, 6S	Meshkan, Neyriz, Fars	2167	29° 28' N	54° 20' E
7T, 7S	Meshkan, Neyriz, Fars	2167	29° 28' N	54° 20' E
8T, 8S	Meshkan, Neyriz, Fars	2167	29° 28' N	54° 20' E
9T, 9S	Meshkan, Neyriz, Fars	2167	29° 28' N	54° 20' E
10T, 10S	Meshkan, Neyriz, Fars	2167	29° 28' N	54° 20' E
11T, 11S	Meshkan, Neyriz, Fars	2167	29° 28' N	54° 20' E
12T, 12S	Meshkan, Neyriz, Fars	2167	29° 28' N	54° 20' E
13T, 13S	Meshkan, Neyriz, Fars	2167	29° 28' N	54° 20' E
14T, 14S	Meshkan, Neyriz, Fars	2167	29° 28' N	54° 20' E
15T, 15S	Meshkan, Neyriz, Fars	2167	29° 28' N	54° 20' E
16T, 16S	Dehchah, Neyriz, Fars	2188	29° 13' N	54° 26' E
17T, 17S	Dehchah, Neyriz, Fars	2188	29° 13' N	54° 26' E
18T, 18S	Dehchah, Neyriz, Fars	2188	29° 13' N	54° 26' E
19T, 19S	Dehchah, Neyriz, Fars	2188	29° 13' N	54° 26' E
20T, 20S	Dehchah, Neyriz, Fars	2188	29° 13' N	54° 26' E
21T, 21S	Abadeh Tashk, Neyriz, Fars	1608	29° 48' N	53° 43' E
22T, 22S	Abadeh Tashk, Neyriz, Fars	1608	29° 48' N	53° 43' E
23T, 23S	Neyriz, Fars	1606	29° 11' N	54° 19' E
24T, 24S	MahmoudAbad, Neyriz, Fars	1579	29° 14' N	54° 17' E
25T, 25S	MahmoudAbad, Neyriz, Fars	1579	29° 14' N	54° 17' E
26T, 26S	MahmoudAbad, Neyriz, Fars	1579	29° 14' N	54° 17' E
27T, 27S	Roniz, Estahban, Fars	1593	29° 11' N	53° 46' E
28T, 28S	Roniz, Estahban, Fars	1593	29° 11' N	53° 46' E
29T, 29S	HasanAbad, Neyriz, Fars	1579	29° 44' N	53° 54' E
30T, 30S	Estahban, Fars	1797	29° 7' N	54° 2' E
31T, 31S	Estahban, Fars	1797	29° 7' N	54° 2' E
32T, 32S	Estahban, Fars	1797	29° 7' N	54° 2' E
33T, 33S	Estahban, Fars	1797	29° 7' N	54° 2' E
34T, 34S	Estahban, Fars	1797	29° 7' N	54° 2' E
35T, 35S	Estahban, Fars	1797	29° 7' N	54° 2' E
36T, 36S	Estahban, Fars	1797	29° 7' N	54° 2' E
37T, 37S	Ghaen, Khorasan e Jonoubi	1445	33° 43' N	59° 11' E
38T, 38S	Torbatejam, Khorasan e Razavi	894	35° 14' N	60° 37' E
39T, 39S	Dehdez, Khoozestan	1439	31° 42' N	50° 17' E
40T, 40S	Dehdez, Khoozestan	1439	31° 42' N	50° 17' E

MAT: mean annual temperature; MAP: mean annual precipitation; T: tepal; S: stamen

Figure S1. ¹H-NMR spectrum of luteolin (**1**) (500 MHz, CD₃OD)

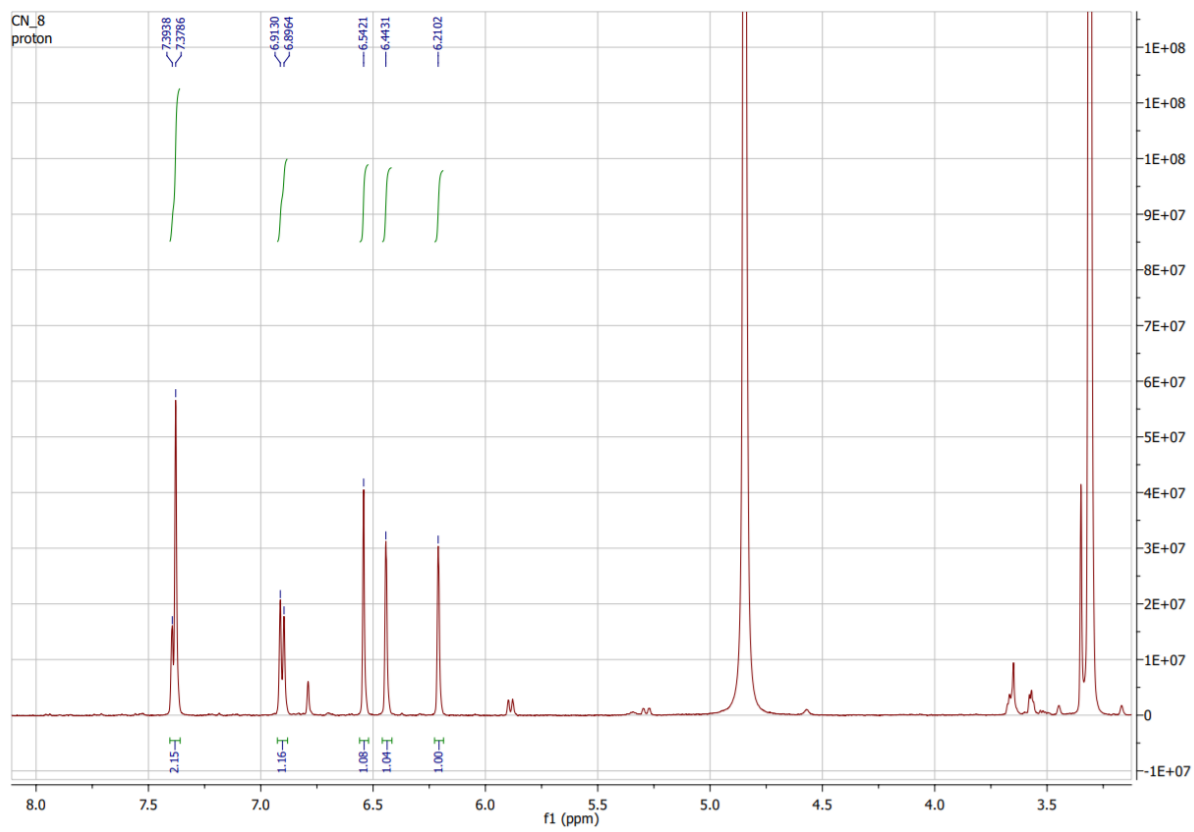


Figure S2. ¹H-NMR spectrum of eupafolin (**2**) (500 MHz, CD₃OD)

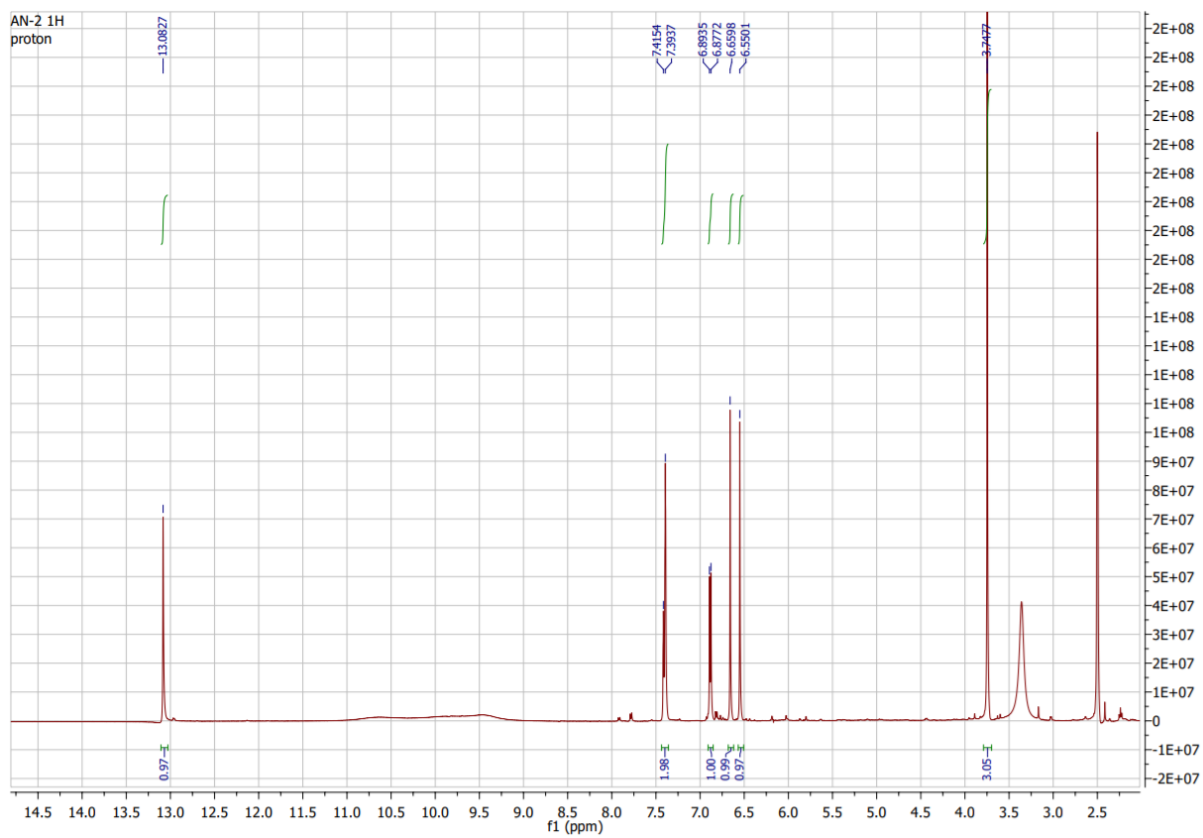


Figure S3. JMOD spectrum of eupafolin (2) (125 MHz, CD₃OD)

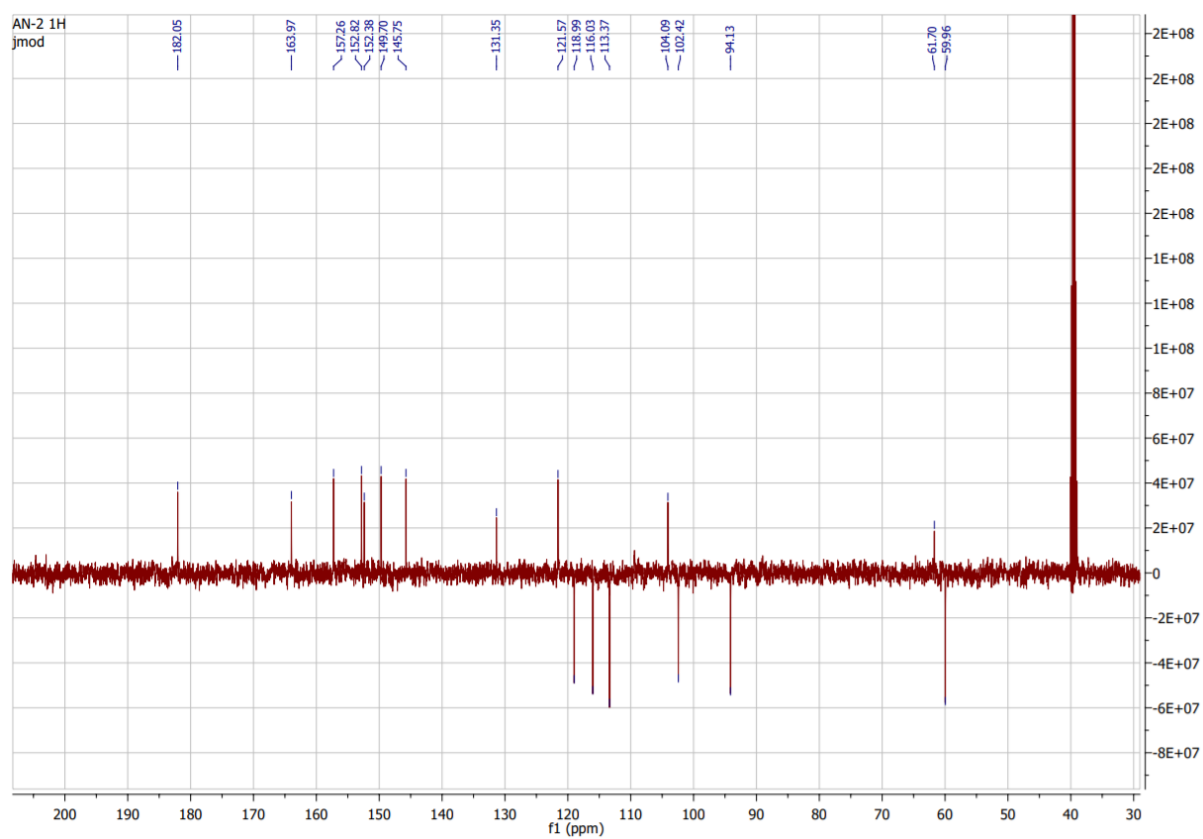


Figure S4. $^1\text{H-NMR}$ spectrum of apigenin (**3**) (500 MHz, CD_3OD)

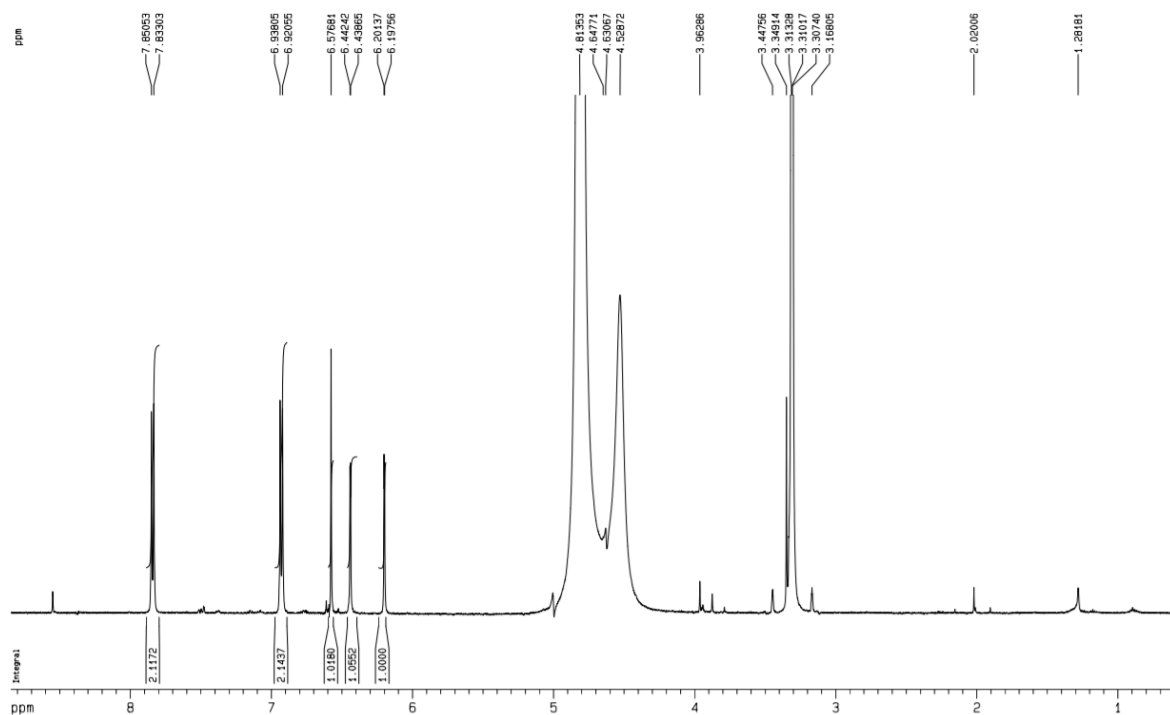


Figure S5. JMOD spectrum of apigenin (**3**) (125 MHz, CD₃OD)

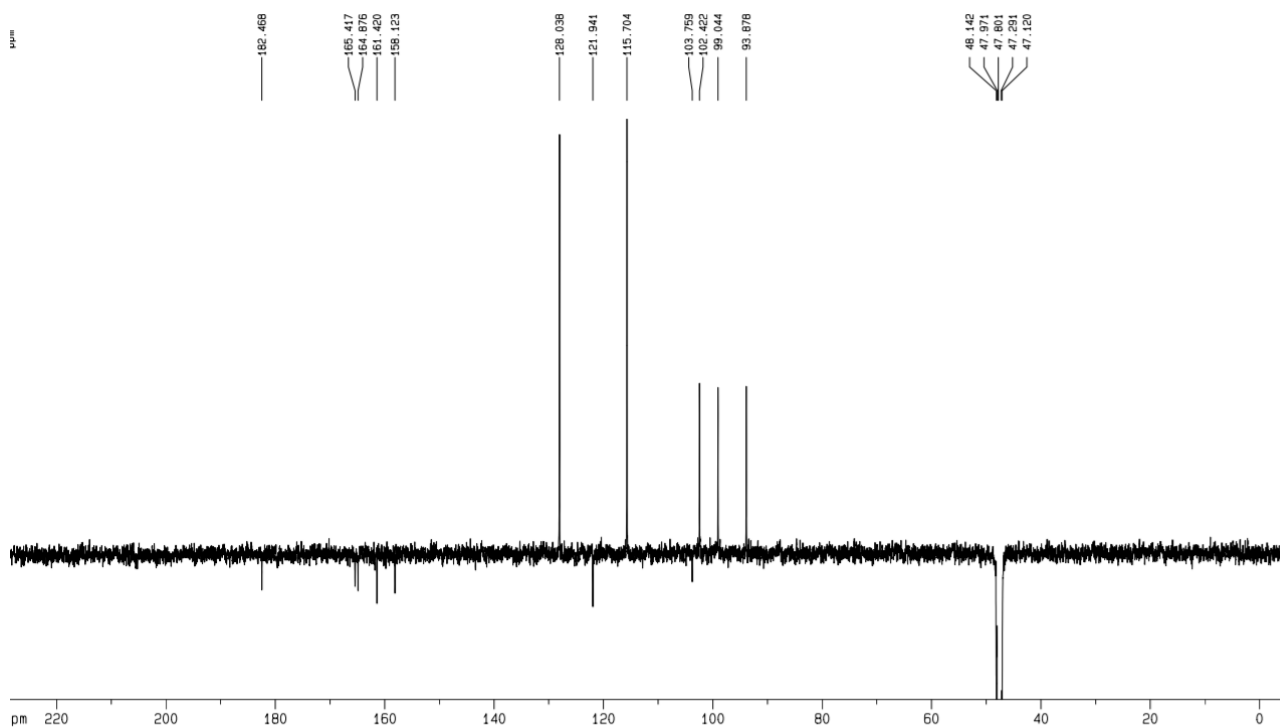


Figure S6. ¹H-NMR spectrum of hispidulin (**4**) (500 MHz, CD₃OD)

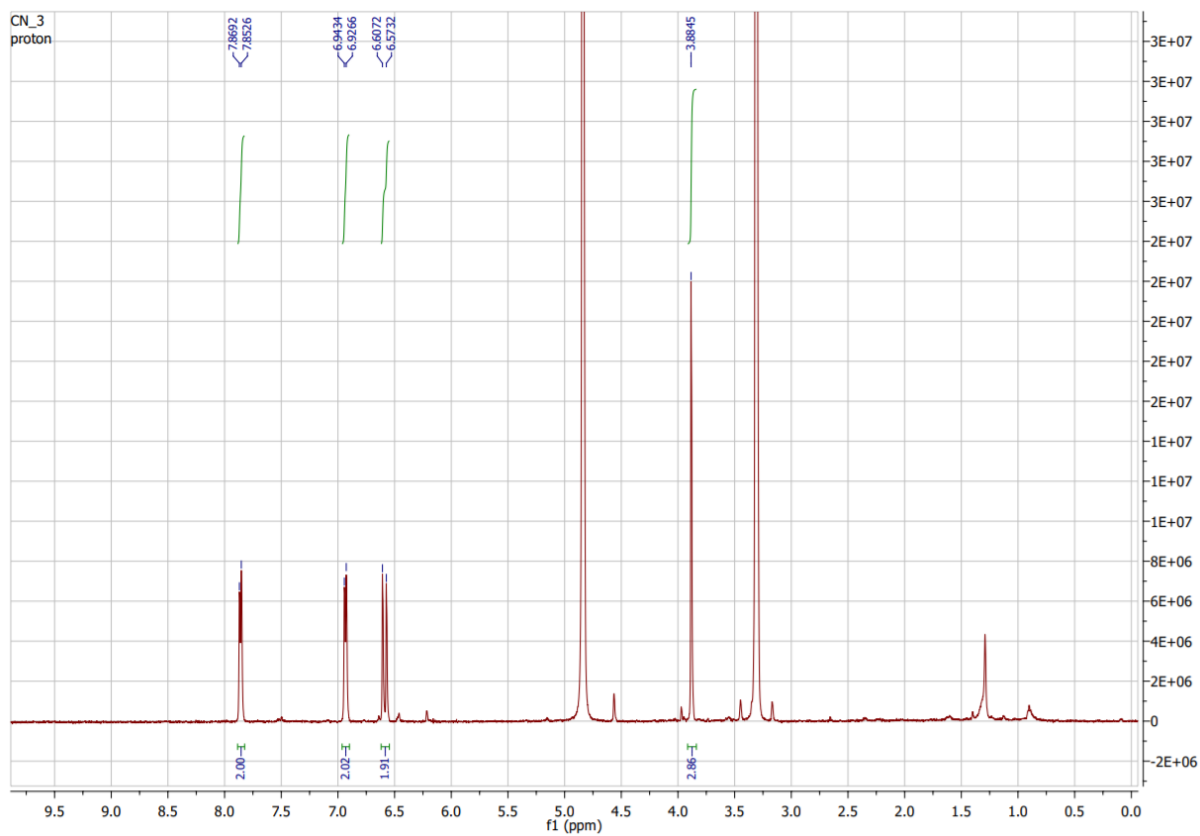
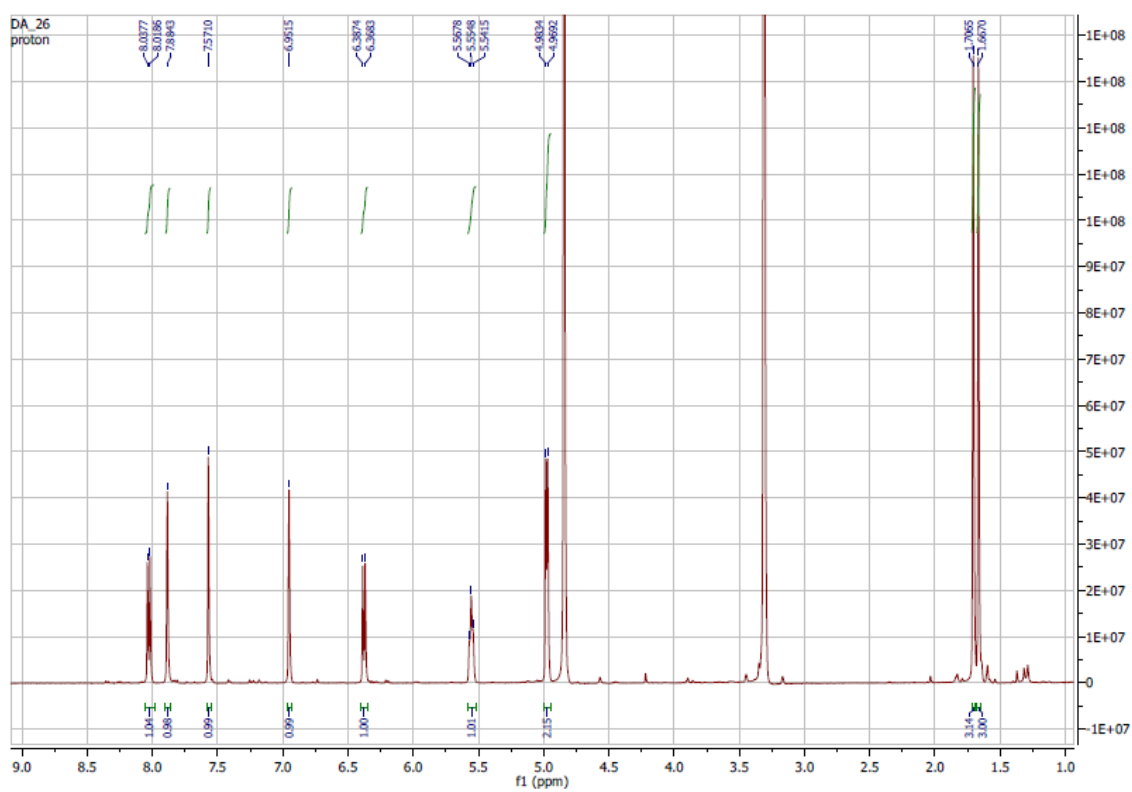
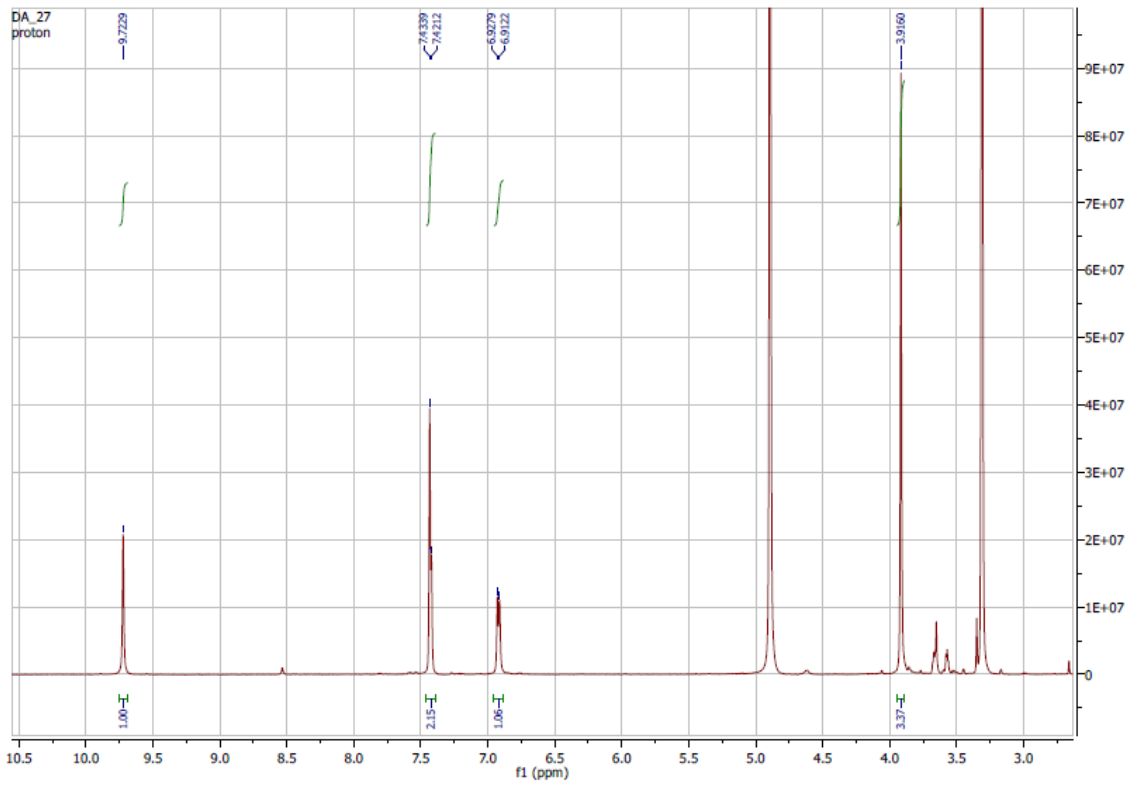


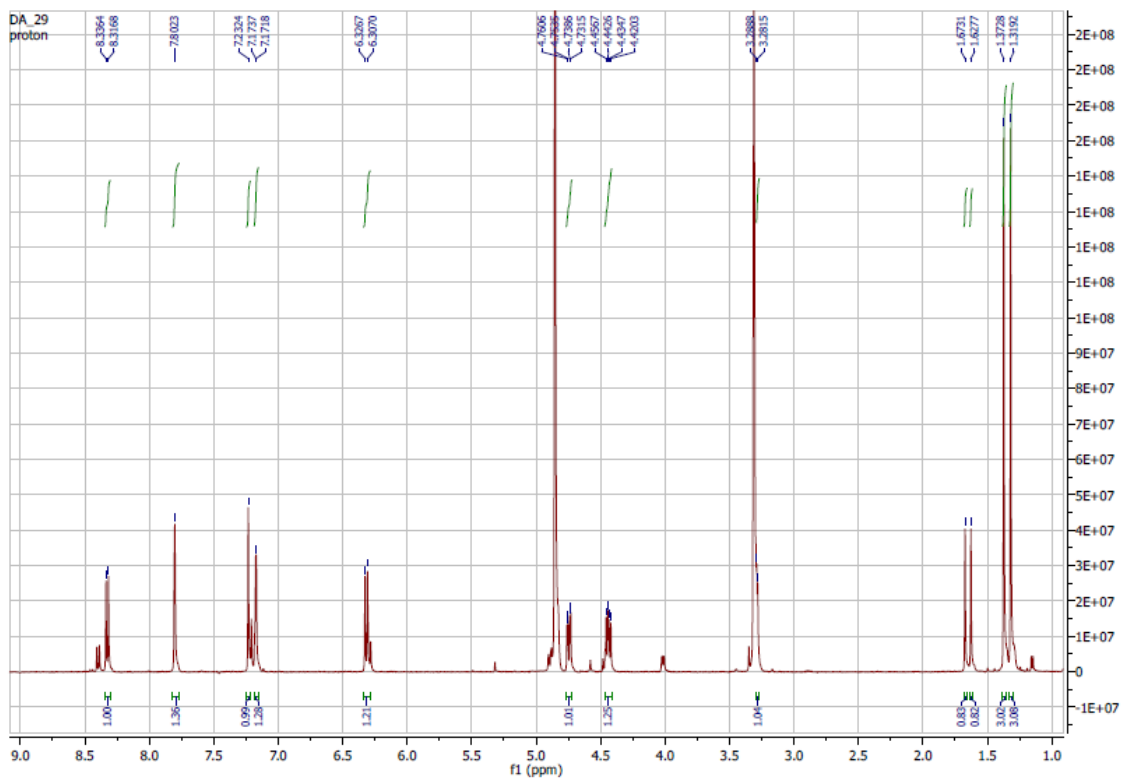
Figure S7. ¹H-NMR spectra of the compounds isolated from *Ducrosia anethifolia*



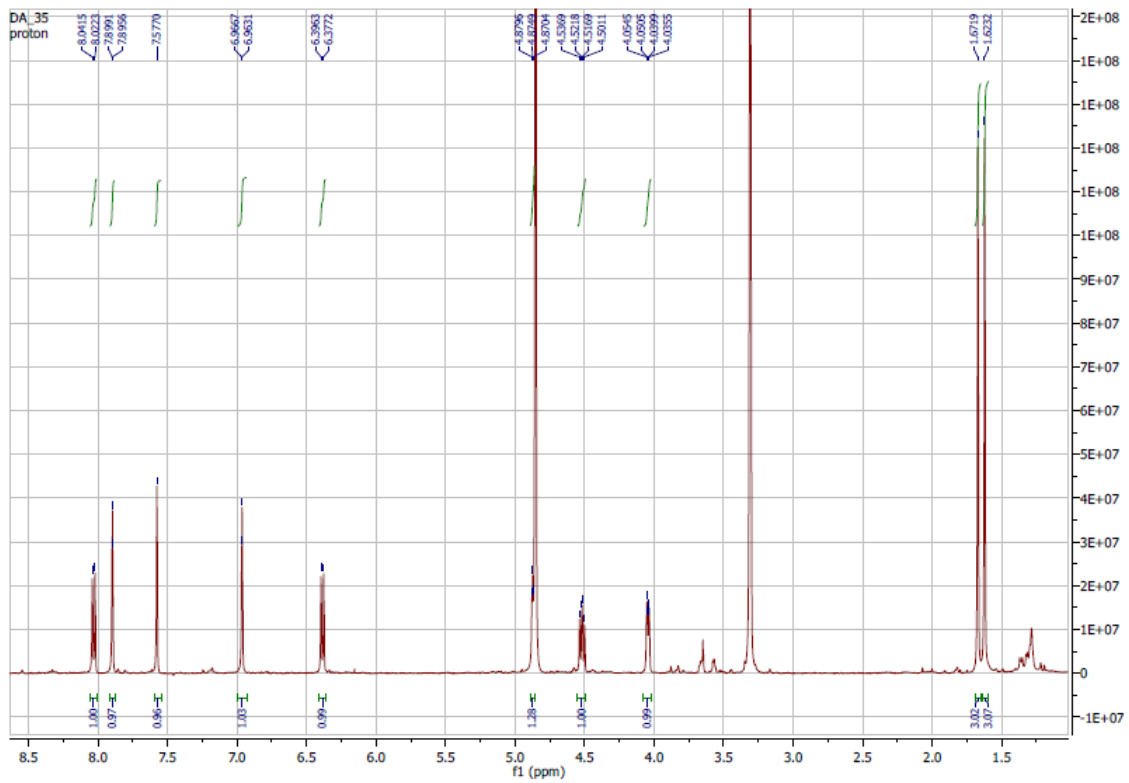
Imperatorin (5)



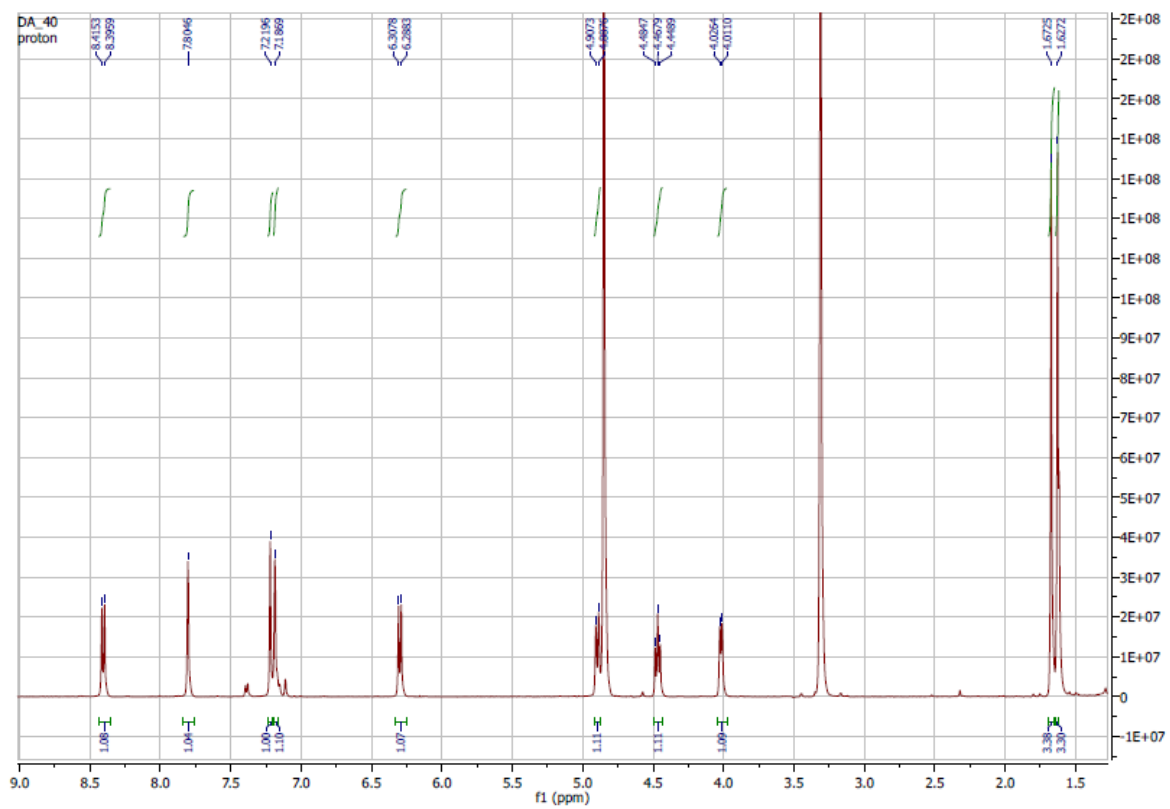
Vanillic aldehyde (6)



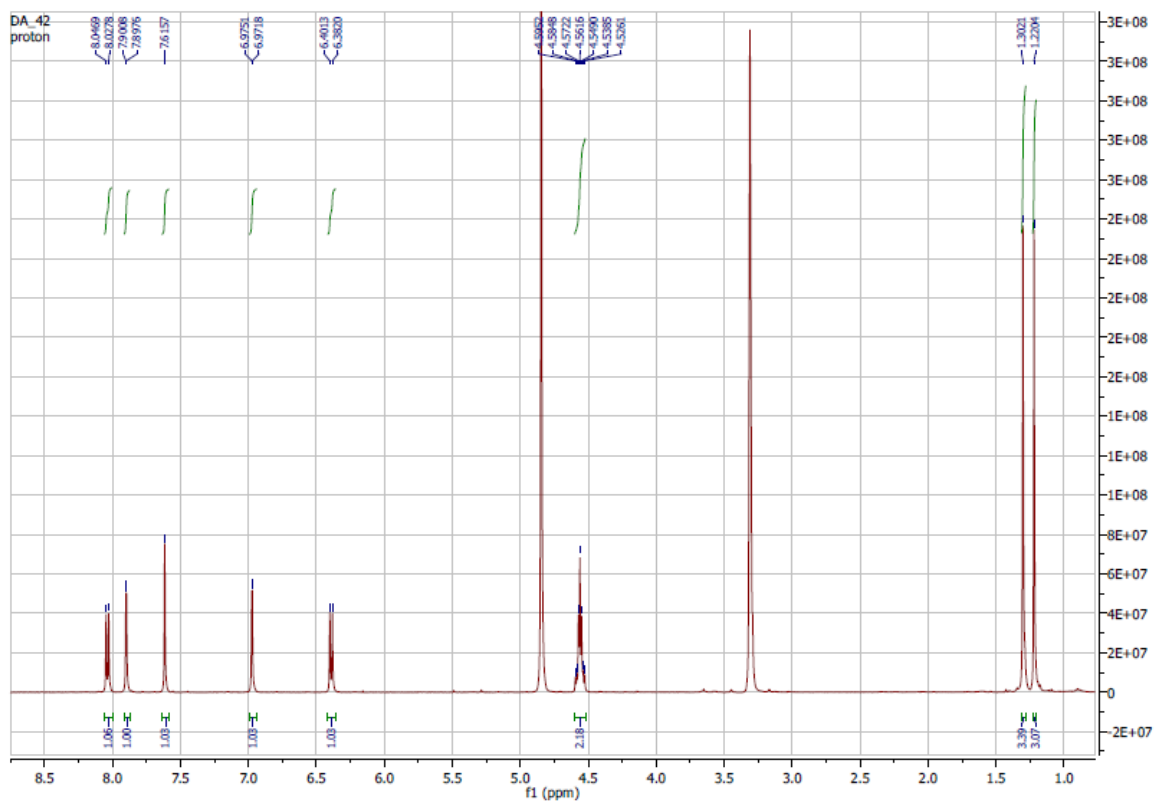
Oxypeucedanin (7)



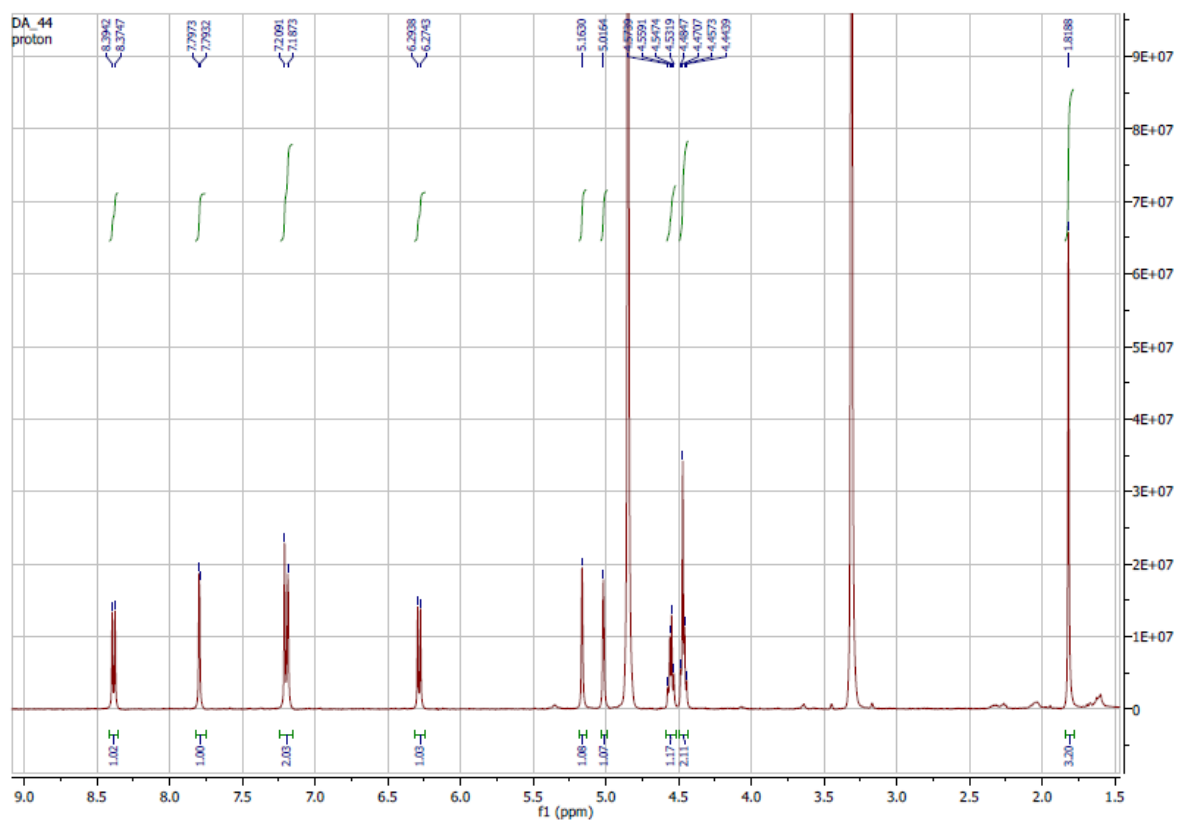
Heraclenol (8)



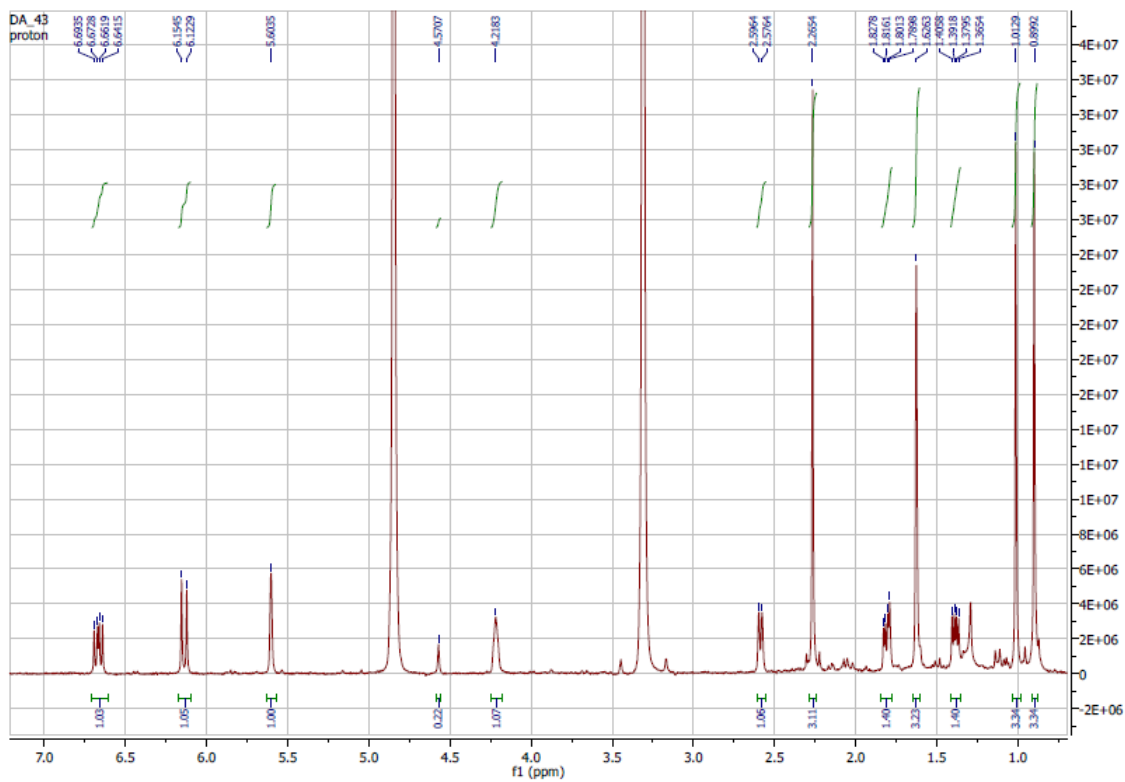
Aviprin (9)



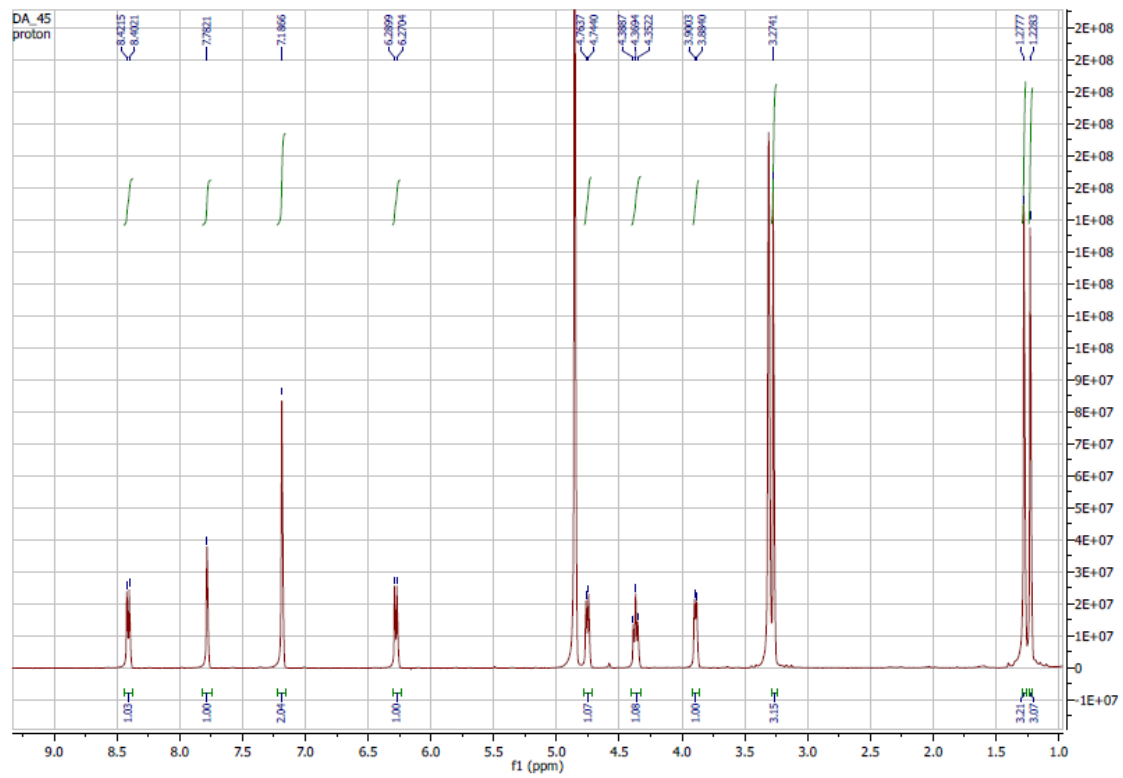
Heraclein (10)



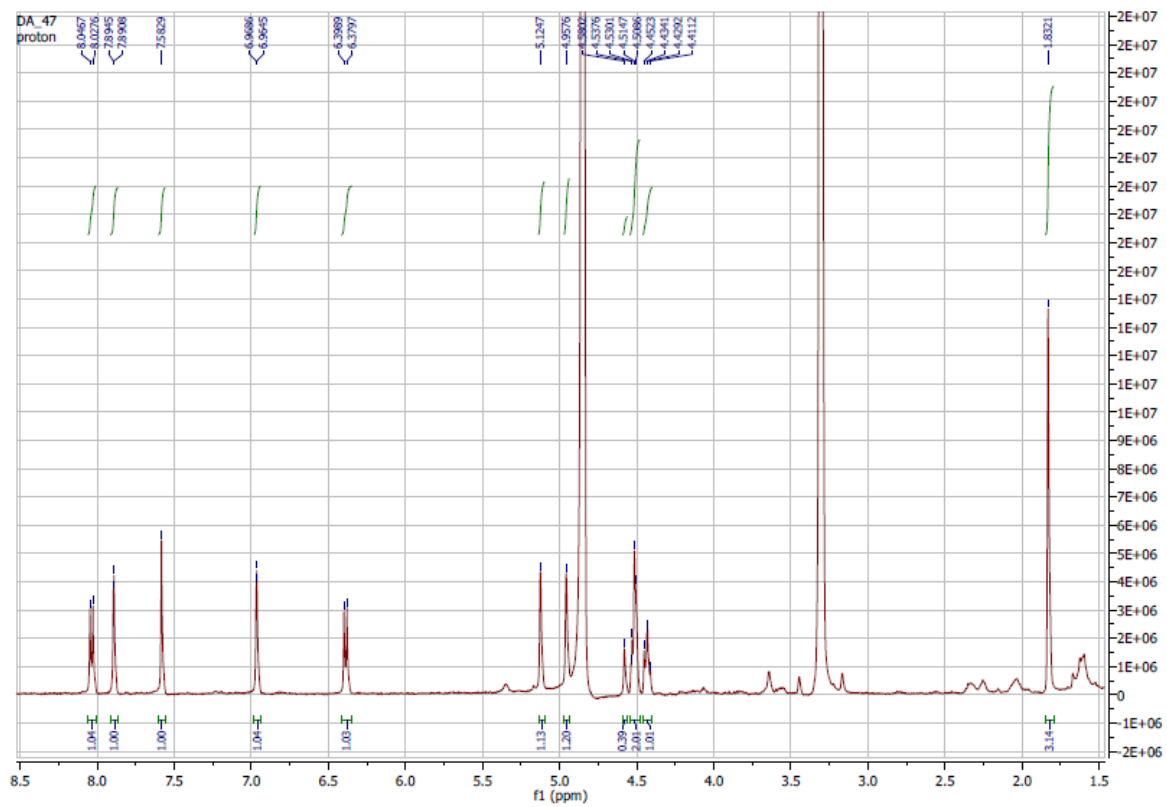
Pabulenol (11)



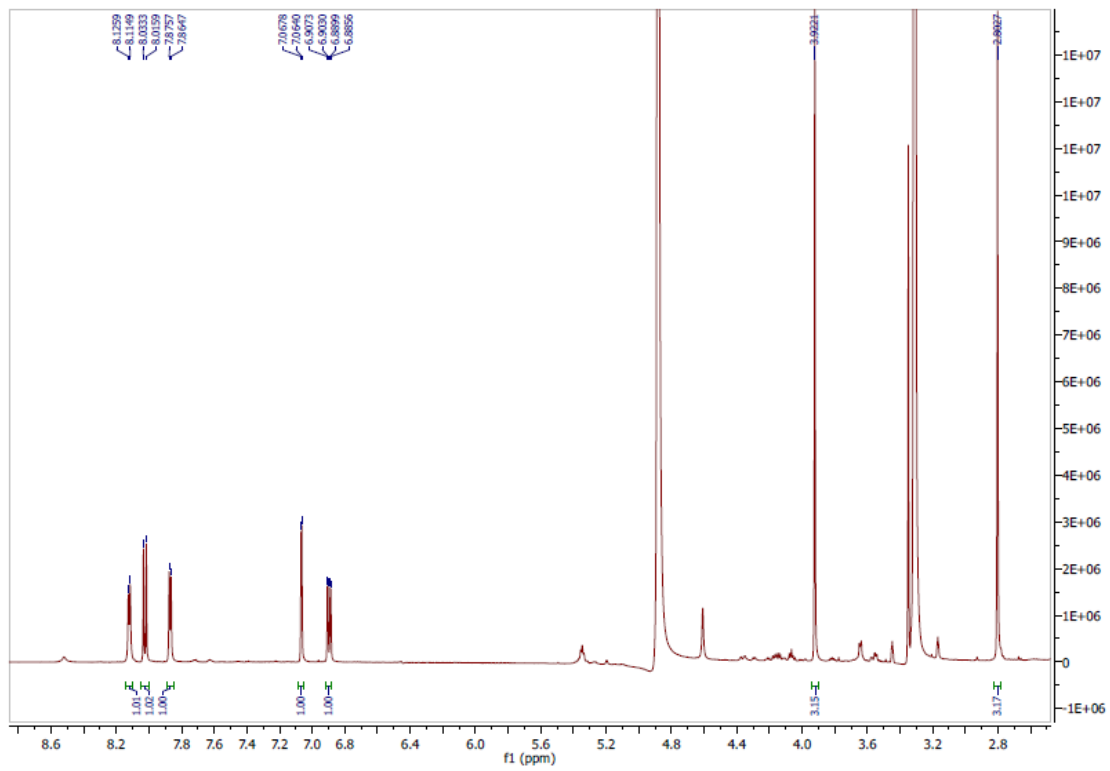
3-Hydroxy- α -ionone (12)



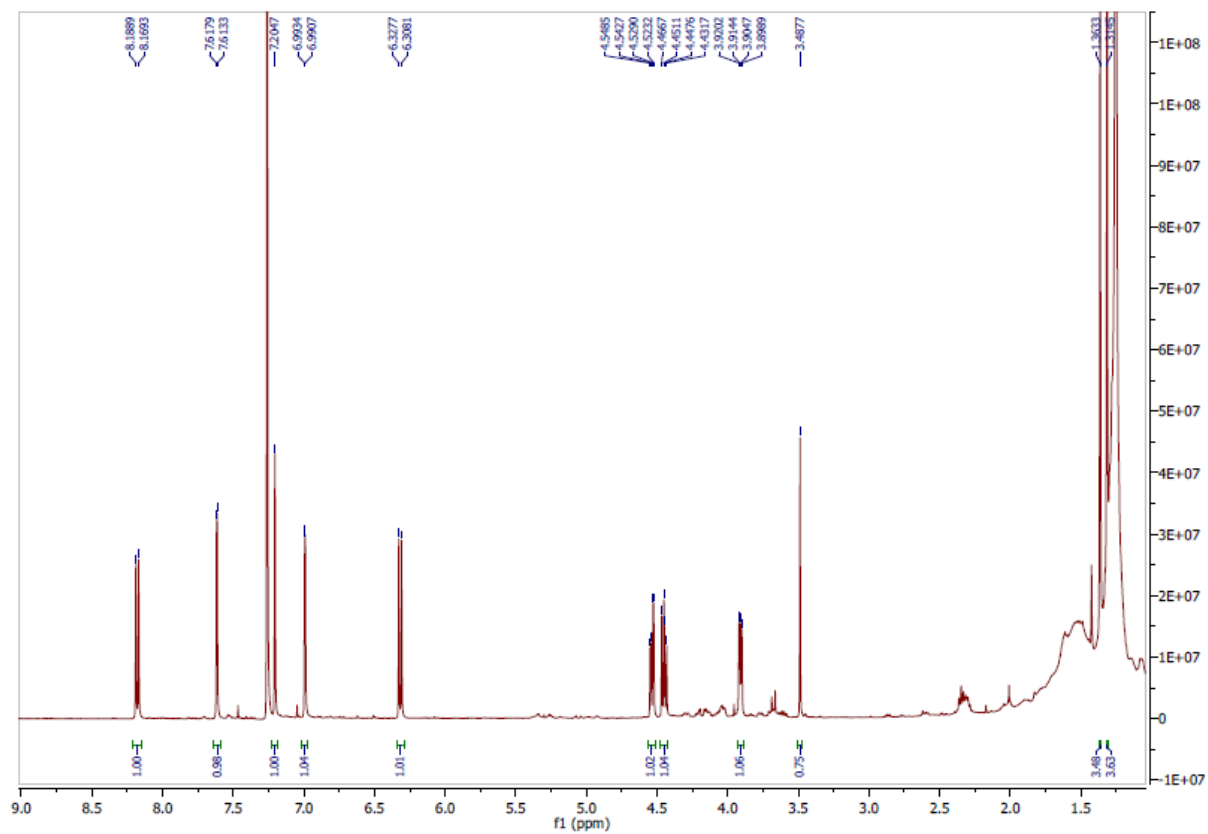
Oxypeucedanin methanolate (**13**)



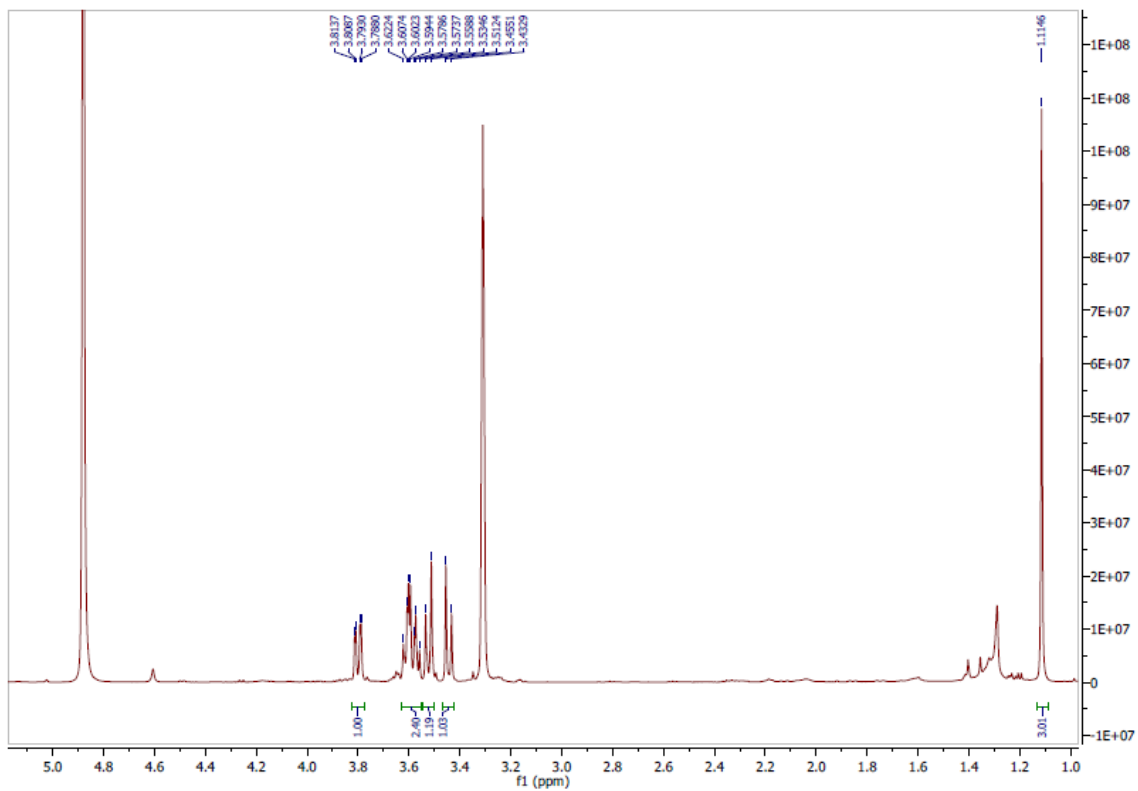
Isogospherol (14)



Harmine (15)

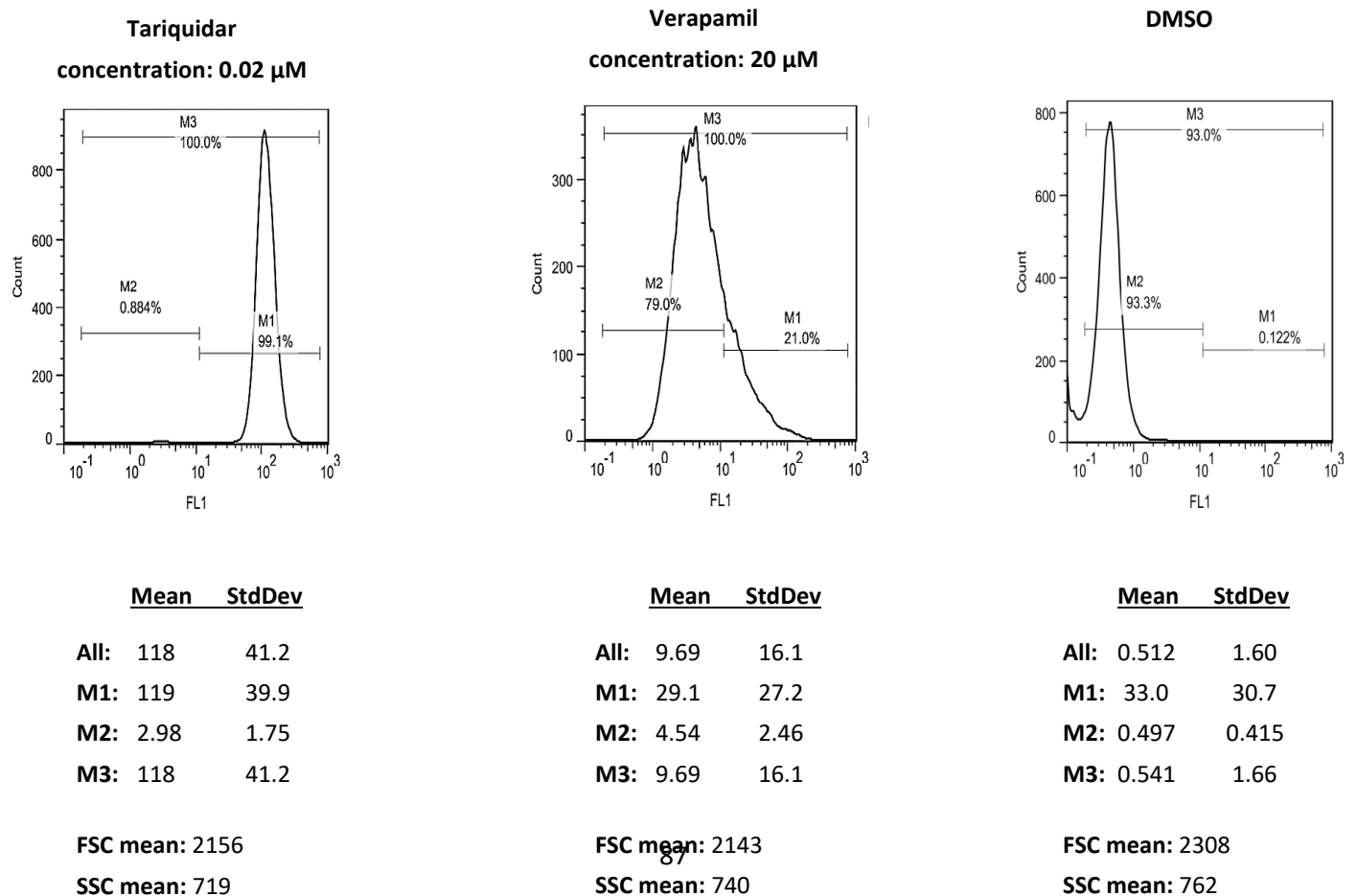
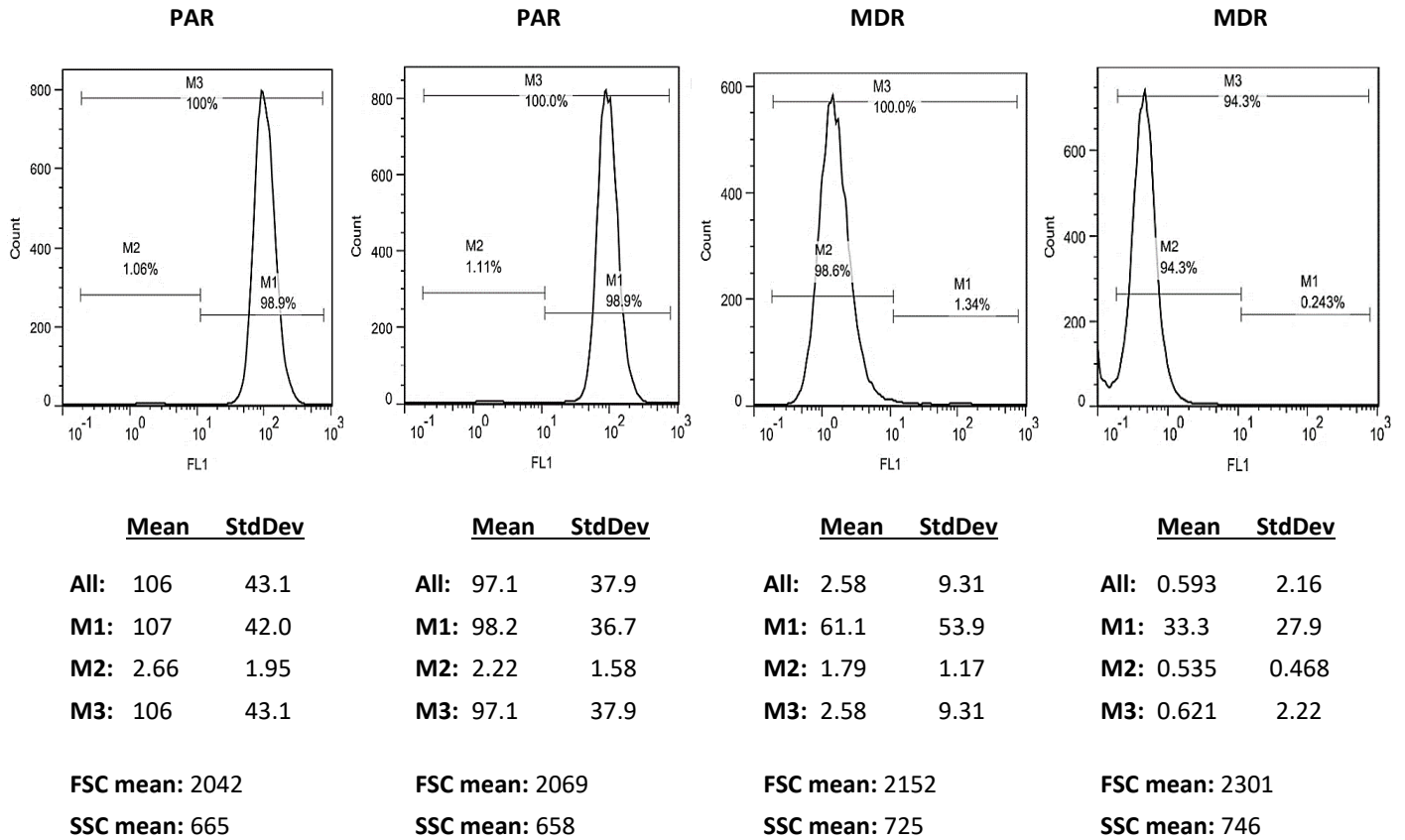


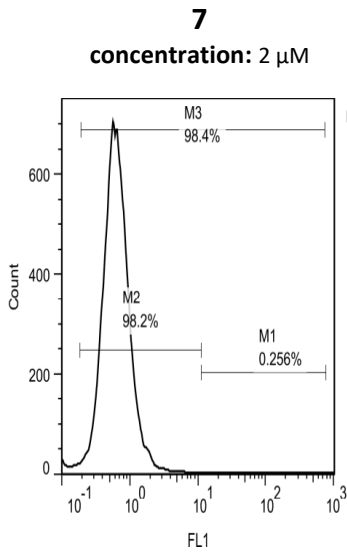
Prangol (16)



2-C-methyl-erythritol (17)

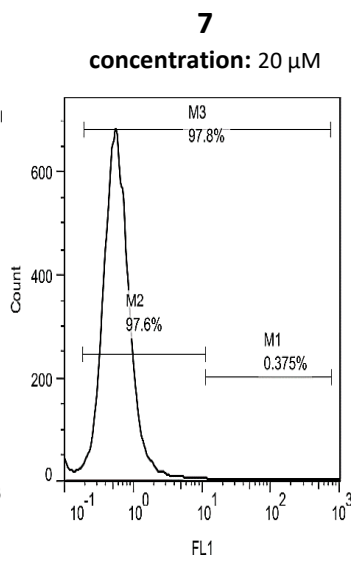
Figure S8. Inhibition of the ABCB1 transporter on multidrug resistant (MDR) L51787Y mouse T-lymphoma cells by flow cytometry





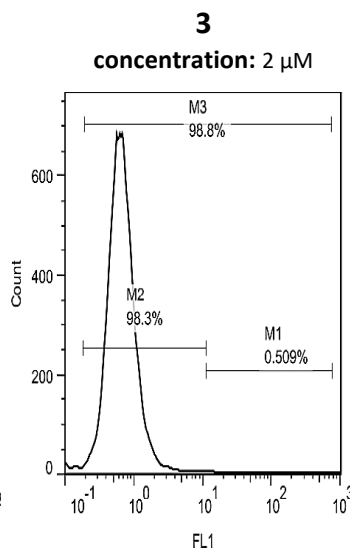
	Mean	StdDev
All:	0.796	2.01
M1:	31.0	23.5
M2:	0.727	0.568
M3:	0.807	2.03

FSC mean: 2165
SSC mean: 749



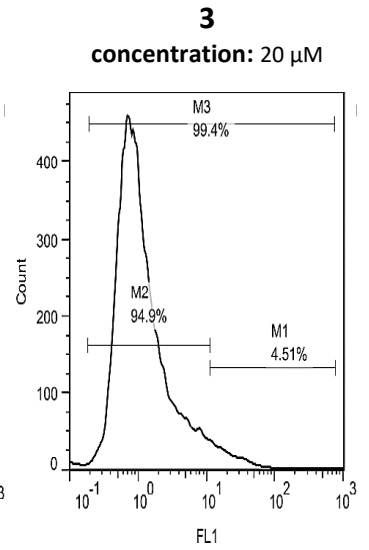
	Mean	StdDev
All:	0.783	2.14
M1:	28.0	19.4
M2:	0.692	0.633
M3:	0.798	2.16

FSC mean: 2161
SSC mean: 750



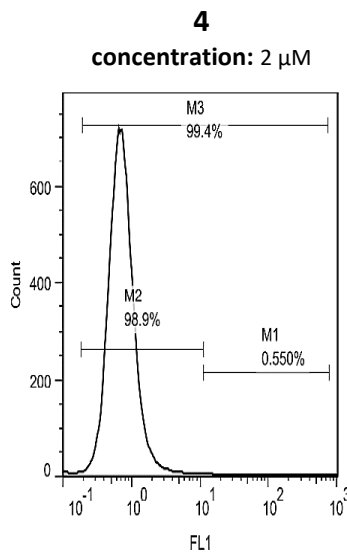
	Mean	StdDev
All:	0.900	2.54
M1:	28.5	20.8
M2:	0.766	0.613
M3:	0.910	2.56

FSC mean: 2190
SSC mean: 749



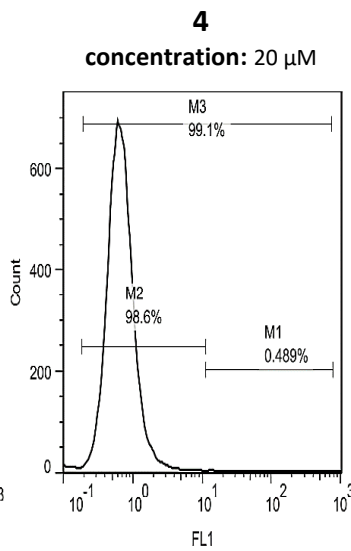
	Mean	StdDev
All:	2.62	6.64
M1:	25.6	19.0
M2:	1.55	1.74
M3:	2.64	6.66

FSC mean: 2164
SSC mean: 763



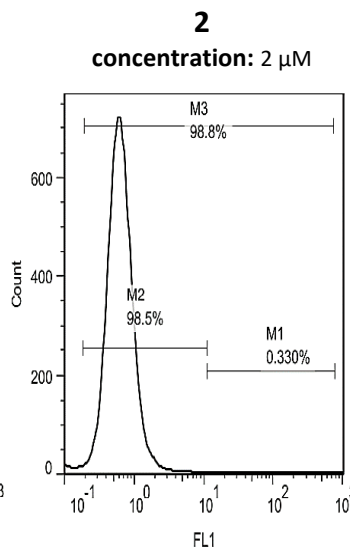
	Mean	StdDev
All:	0.995	3.70
M1:	31.4	38.5
M2:	0.832	0.681
M3:	1.00	3.71

FSC mean: 2300
SSC mean: 742



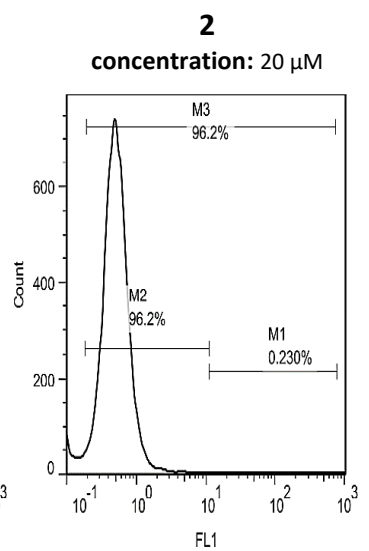
	Mean	StdDev
All:	0.948	3.55
M1:	38.0	33.7
M2:	0.771	0.609
M3:	0.955	3.56

FSC mean: 2317
SSC mean: 740



	Mean	StdDev
All:	0.825	2.77
M1:	35.9	31.8
M2:	0.715	0.545
M3:	0.833	2.79

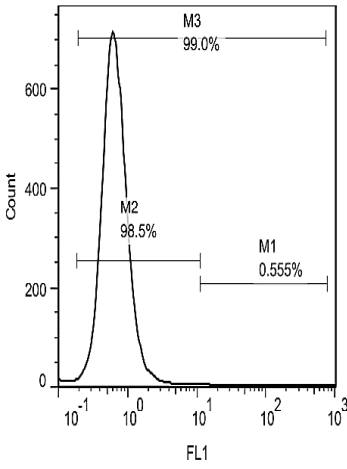
FSC mean: 2325
SSC mean: 725



	Mean	StdDev
All:	0.640	1.83
M1:	32.7	17.8
M2:	0.583	0.508
M3:	0.660	1.86

FSC mean: 2305
SSC mean: 769

9
concentration: 2 µM



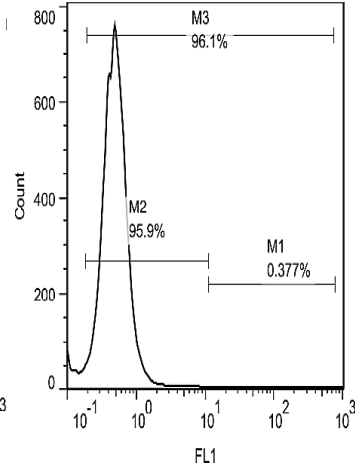
Mean StdDev

All: 0.942 3.64
M1: 35.5 33.5
M2: 0.755 0.614
M3: 0.950 3.66

FSC mean: 2318

SSC mean: 723

9
concentration: 20 µM



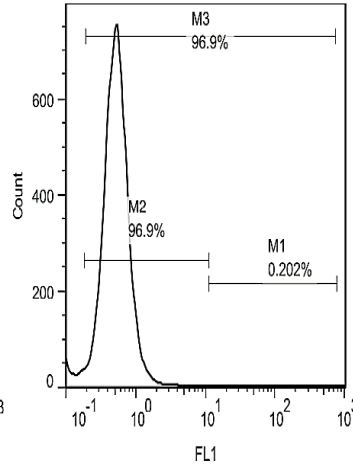
Mean StdDev

All: 0.669 2.32
M1: 31.1 20.9
M2: 0.570 0.525
M3: 0.690 2.37

FSC mean: 2294

SSC mean: 764

1
concentration: 2 µM



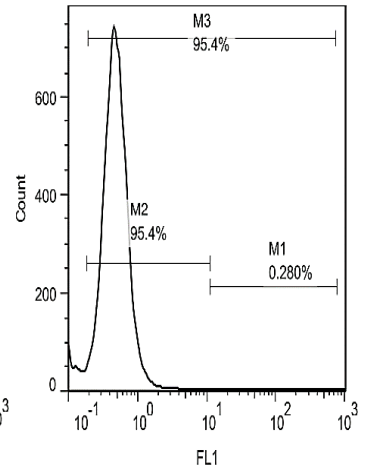
Mean StdDev

All: 0.663 2.37
M1: 40.6 32.9
M2: 0.596 0.488
M3: 0.680 2.40

FSC mean: 2324

SSC mean: 728

1
concentration: 20 µM



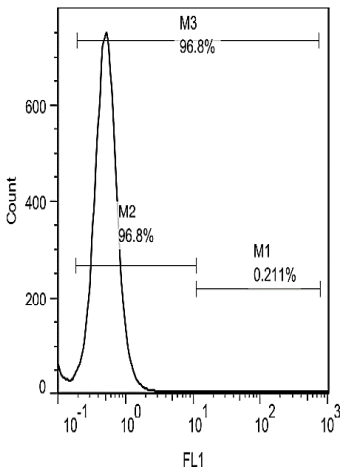
Mean StdDev

All: 0.607 1.82
M1: 29.5 17.1
M2: 0.544 0.433
M3: 0.630 1.86

FSC mean: 2323

SSC mean: 750

5
concentration: 2 µM



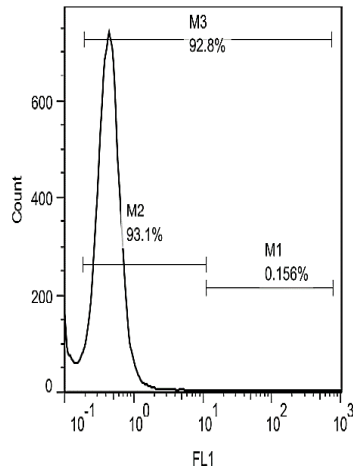
Mean StdDev

All: 0.646 2.29
M1: 38.0 31.5
M2: 0.580 0.478
M3: 0.663 2.32

FSC mean: 2310

SSC mean: 737

5
concentration: 20 µM



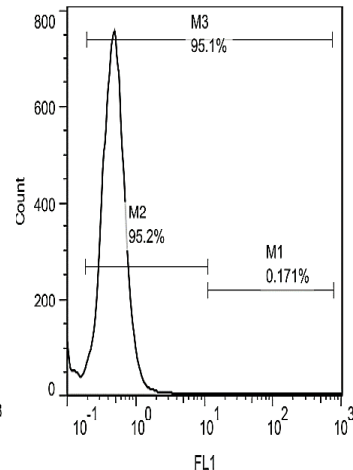
Mean StdDev

All: 0.517 1.33
M1: 28.2 16.5
M2: 0.499 0.426
M3: 0.547 1.38

FSC mean: 2290

SSC mean: 766

8
concentration: 2 µM



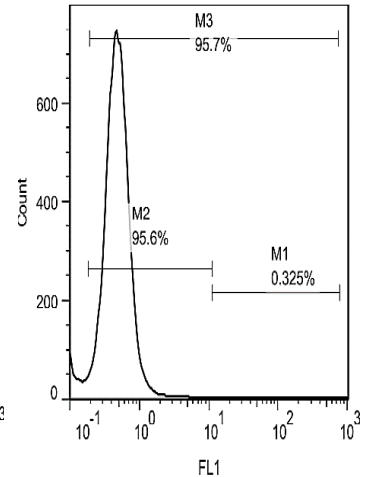
Mean StdDev

All: 0.575 1.89
M1: 36.9 26.2
M2: 0.531 0.392
M3: 0.598 1.93

FSC mean: 2305

SSC mean: 741

8
concentration: 20 µM



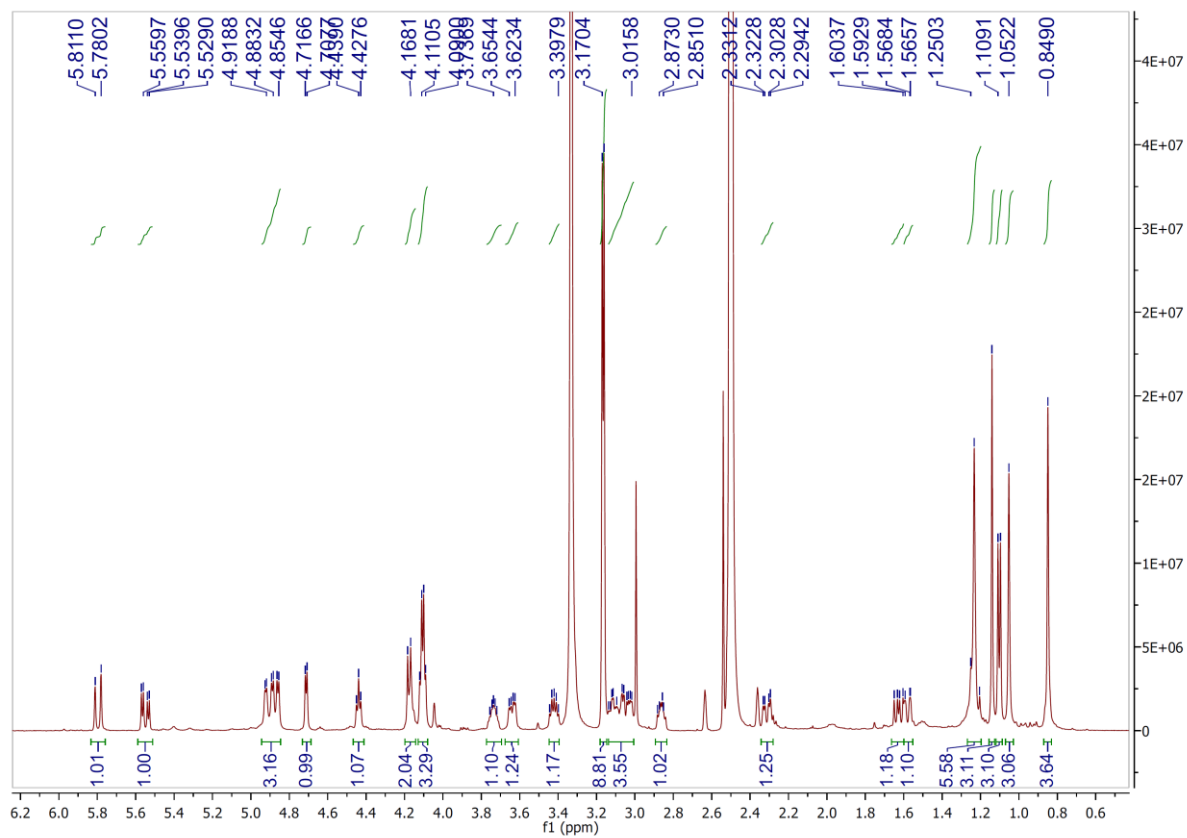
Mean StdDev

All: 0.644 2.63
M1: 35.6 29.1
M2: 0.548 0.443
M3: 0.667 2.68

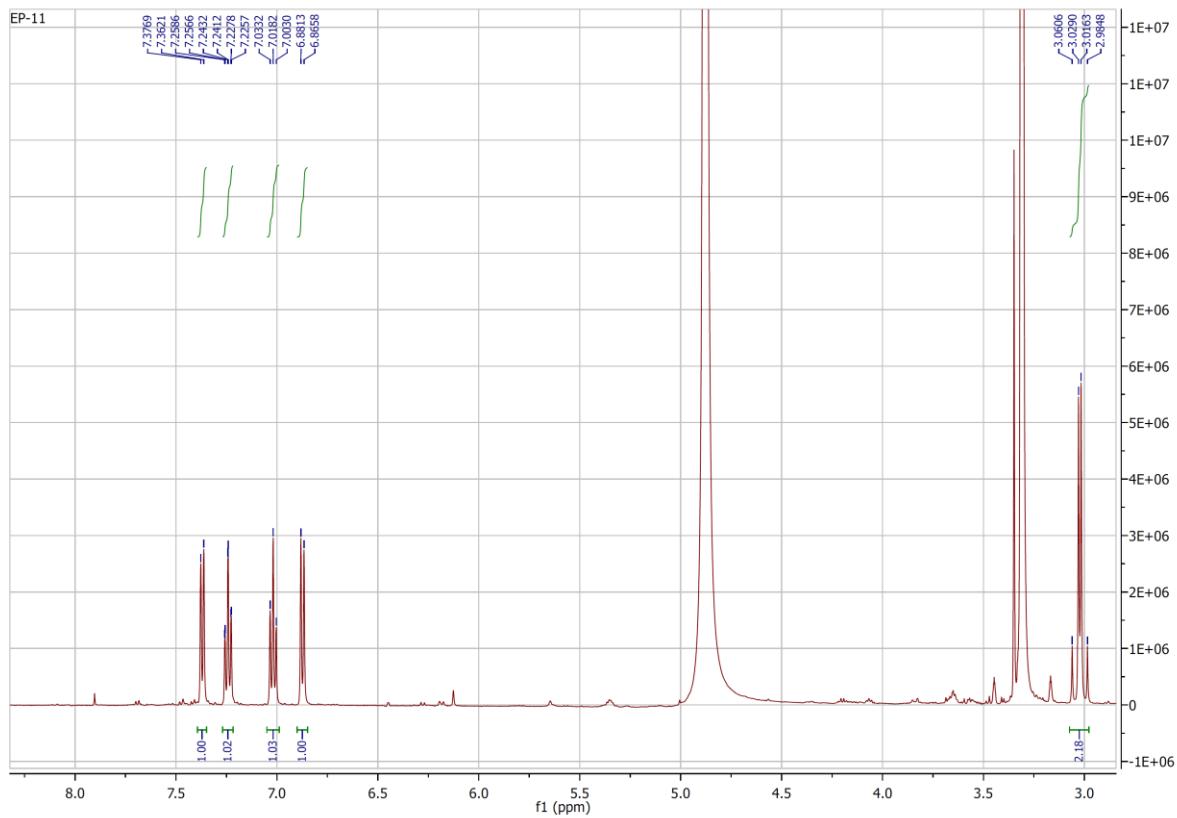
FSC mean: 2326

SSC mean: 741

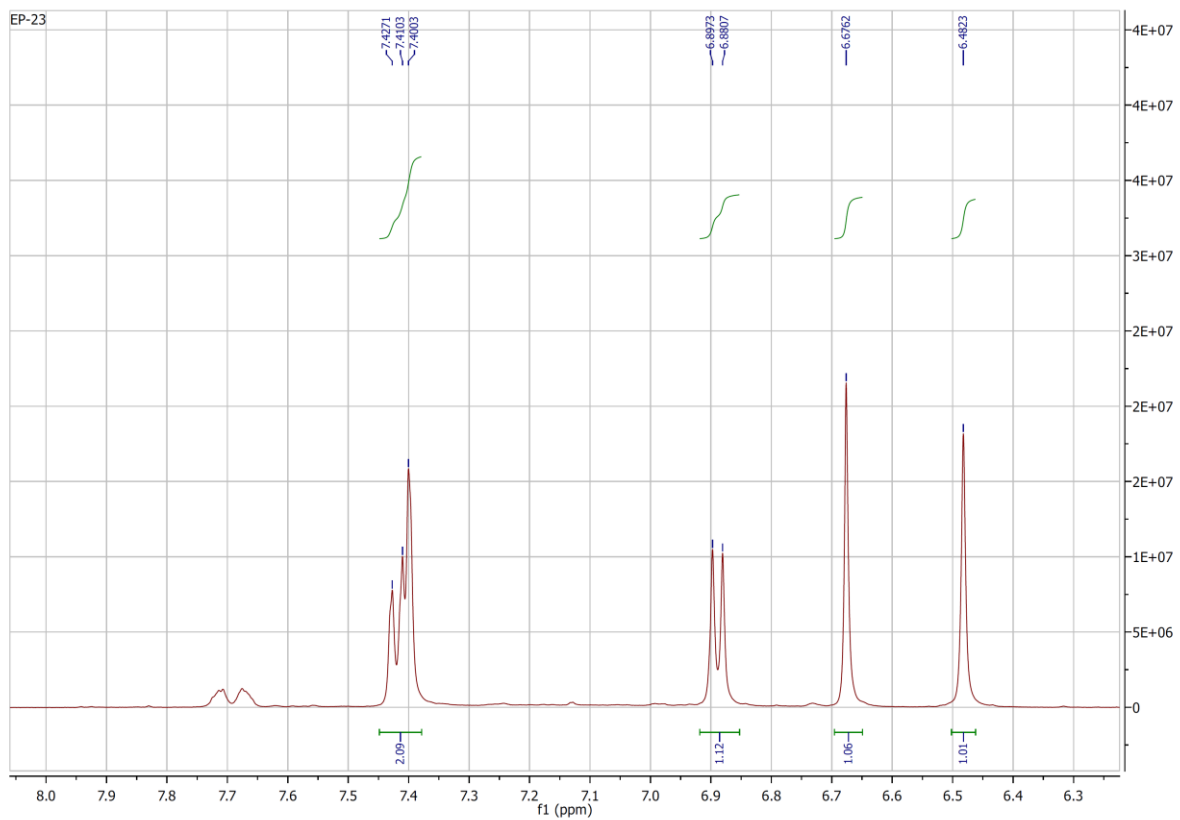
Figure S9. ¹H-NMR spectra of the compounds isolated from *Eremurus persicus*



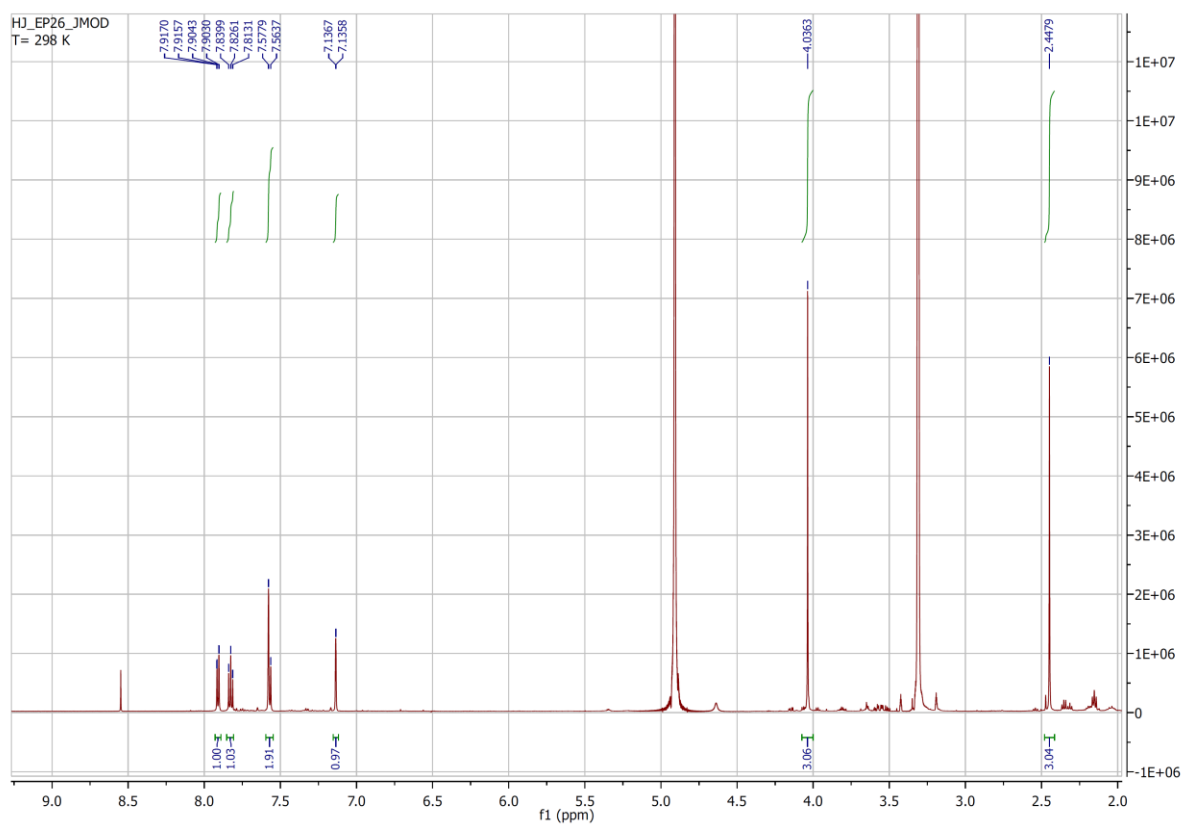
Corchoionoside A (18)



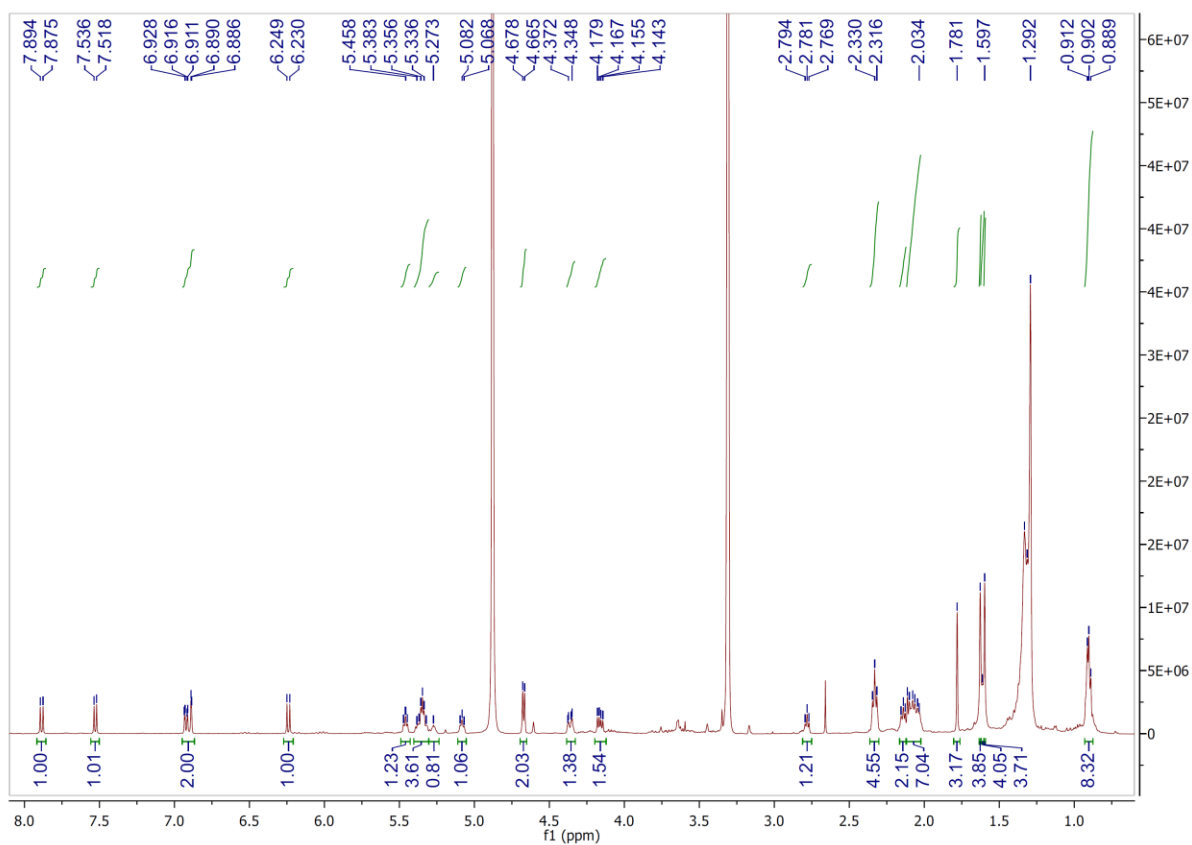
4-Amino-4-carboxychroman-2-one (**19**)



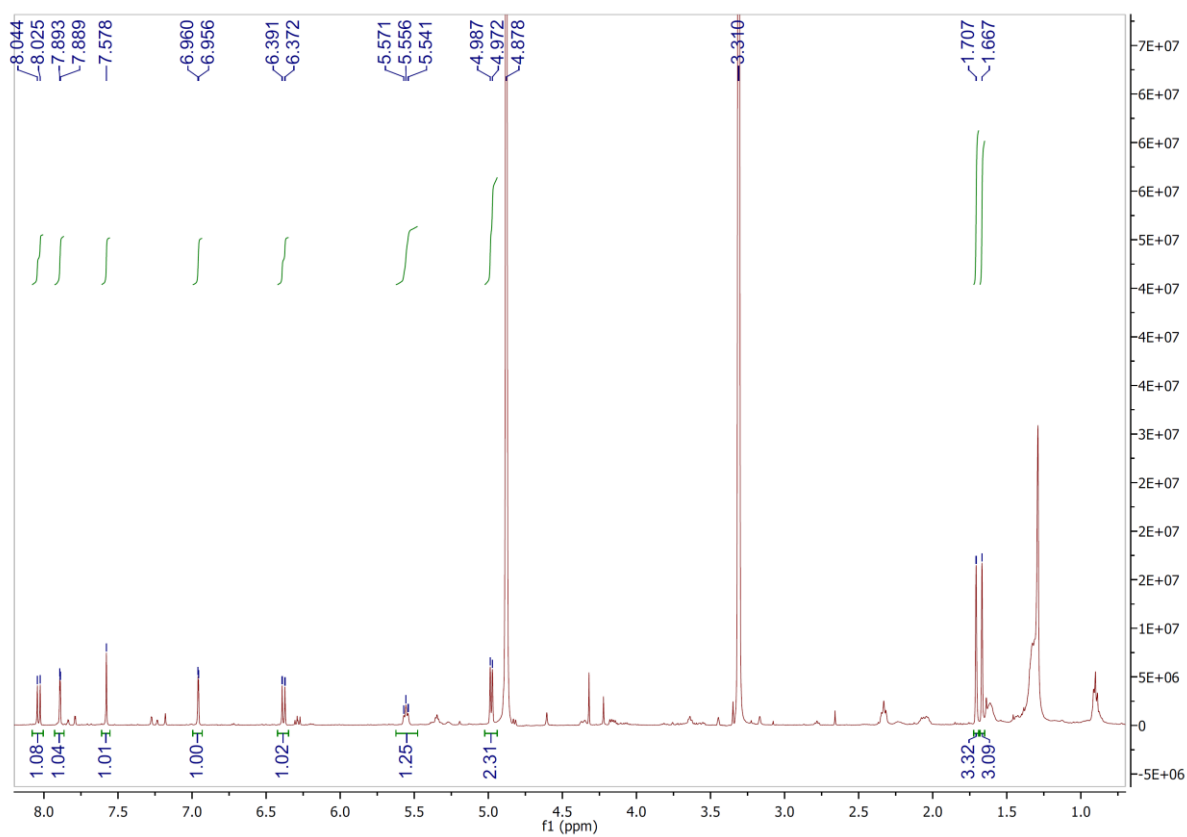
Isoorientin (20)



Ziganein 5-methyl ether (21)



Auraptene (22)



Imperatorin (23)

Table S3. Levels of marker compounds in *Crocus sativus* L. samples

Sample	Q.S.		K.S.		K.G.	
	T	S	T	S	T	S
1	10.24 ± 0.23	4.23 ± 0.14	99.48 ± 0.07	1.82 ± 0.10	45.18 ± 0.24	3.65 ± 0.21
2	6.29 ± 0.04	3.49 ± 0.14	76.40 ± 1.37	1.47 ± 0.06	37.31 ± 0.77	2.47 ± 0.12
3	6.23 ± 0.15	1.63 ± 0.18	62.41 ± 1.41	0.93 ± 0.02	33.71 ± 0.98	2.42 ± 0.46
4	9.19 ± 0.32	5.60 ± 0.20	74.82 ± 3.32	1.53 ± 0.26	36.01 ± 1.60	3.69 ± 0.15
5	6.21 ± 0.20	4.56 ± 0.20	62.19 ± 2.06	2.21 ± 0.09	35.73 ± 1.23	4.88 ± 0.29
6	7.92 ± 0.34	2.11 ± 0.12	71.81 ± 3.67	1.03 ± 0.03	39.25 ± 2.27	2.05 ± 0.01
7	7.61 ± 0.30	1.72 ± 0.02	77.35 ± 3.96	1.03 ± 0.06	37.38 ± 1.73	2.01 ± 0.11
8	9.43 ± 0.23	1.98 ± 0.06	75.00 ± 0.55	0.90 ± 0.03	37.30 ± 0.46	1.72 ± 0.12
9	8.68 ± 0.29	1.80 ± 0.06	74.88 ± 2.84	1.20 ± 0.03	34.09 ± 1.09	2.72 ± 0.24
10	8.26 ± 0.10	1.79 ± 0.04	71.45 ± 1.51	1.03 ± 0.00	32.08 ± 0.07	2.17 ± 0.09
11	8.51 ± 0.38	2.02 ± 0.08	68.55 ± 1.88	1.20 ± 0.04	32.08 ± 0.82	2.33 ± 0.05
12	10.26 ± 0.03	2.15 ± 0.10	82.91 ± 2.41	1.07 ± 0.07	38.75 ± 0.44	2.76 ± 0.09
13	8.24 ± 0.04	4.43 ± 0.15	72.94 ± 2.02	2.49 ± 0.13	32.58 ± 0.61	4.00 ± 0.08
14	8.37 ± 0.34	5.16 ± 0.06	79.83 ± 0.13	1.78 ± 0.04	35.34 ± 0.56	4.30 ± 0.06
15	7.10 ± 0.08	4.65 ± 0.20	77.79 ± 0.33	2.24 ± 0.25	38.77 ± 0.47	3.72 ± 0.22
16	9.14 ± 0.48	4.45 ± 0.11	86.50 ± 0.85	1.64 ± 0.06	34.83 ± 0.06	3.45 ± 0.09
17	8.34 ± 0.41	4.77 ± 0.11	72.03 ± 3.65	1.68 ± 0.06	30.28 ± 1.62	4.39 ± 0.23
18	7.47 ± 0.28	5.95 ± 0.60	72.28 ± 3.47	1.51 ± 0.01	34.49 ± 1.82	3.55 ± 0.10
19	7.56 ± 0.07	3.54 ± 0.11	83.01 ± 0.74	4.63 ± 0.26	38.17 ± 0.30	5.94 ± 0.01
20	7.91 ± 0.02	5.32 ± 0.14	86.22 ± 2.77	3.08 ± 0.12	37.44 ± 1.21	5.00 ± 0.14
21	9.25 ± 0.17	5.08 ± 0.19	85.59 ± 2.04	3.40 ± 0.02	37.59 ± 0.82	4.16 ± 0.14
22	7.45 ± 0.21	4.10 ± 0.23	79.01 ± 2.93	2.09 ± 0.13	33.23 ± 1.12	4.67 ± 0.22
23	10.82 ± 0.38	4.53 ± 0.19	98.99 ± 2.85	5.21 ± 0.22	36.44 ± 0.88	7.31 ± 0.05
24	7.24 ± 0.04	4.82 ± 0.03	67.80 ± 2.72	2.07 ± 0.00	37.28 ± 0.19	5.34 ± 0.53
25	8.25 ± 0.22	4.55 ± 0.24	79.30 ± 1.54	1.99 ± 0.09	40.96 ± 1.40	3.11 ± 0.09
26	7.58 ± 0.17	5.52 ± 0.13	71.07 ± 0.07	2.04 ± 0.07	33.31 ± 0.43	5.19 ± 0.10
27	8.63 ± 0.07	4.43 ± 0.08	75.66 ± 2.47	2.06 ± 0.06	33.22 ± 1.11	5.15 ± 0.24
28	7.83 ± 0.30	3.95 ± 0.22	76.59 ± 1.52	2.02 ± 0.03	34.77 ± 0.66	4.03 ± 0.17
29	7.44 ± 0.20	6.08 ± 0.36	75.61 ± 1.07	2.20 ± 0.05	36.73 ± 0.73	5.23 ± 0.41
30	7.39 ± 0.23	5.28 ± 0.28	80.46 ± 4.01	2.01 ± 0.02	34.76 ± 1.79	5.96 ± 0.43
31	7.16 ± 0.06	4.29 ± 0.21	87.52 ± 1.50	3.09 ± 0.19	42.51 ± 1.15	7.44 ± 0.31
32	8.37 ± 0.26	5.14 ± 0.30	74.16 ± 1.03	2.33 ± 0.13	33.61 ± 1.30	4.83 ± 0.01
33	7.83 ± 0.06	4.43 ± 0.08	75.77 ± 1.56	2.24 ± 0.13	36.99 ± 0.63	4.71 ± 0.15
34	8.35 ± 0.10	4.49 ± 0.30	74.16 ± 1.12	2.45 ± 0.17	34.69 ± 0.42	4.80 ± 0.25
35	7.83 ± 0.33	5.74 ± 0.09	75.86 ± 0.83	2.19 ± 0.08	41.03 ± 0.47	5.13 ± 0.05
36	9.43 ± 0.36	4.77 ± 0.13	88.63 ± 3.41	3.34 ± 0.19	43.68 ± 1.13	5.10 ± 0.11
37	6.75 ± 0.04	5.40 ± 0.31	76.00 ± 1.31	1.83 ± 0.10	34.32 ± 0.39	5.70 ± 0.05
38	8.96 ± 0.12	5.13 ± 0.15	81.03 ± 3.35	1.96 ± 0.10	36.87 ± 1.00	6.78 ± 0.04
39	6.87 ± 0.11	2.87 ± 0.06	74.44 ± 1.20	2.59 ± 0.15	29.04 ± 1.19	4.30 ± 0.22
40	6.42 ± 0.21	2.86 ± 0.12	73.91 ± 2.85	6.62 ± 2.06	27.74 ± 1.22	5.26 ± 1.03

The amounts are presented as mg of samples; Q.S.: quercetin-3-O-sophoroside; K.S.: kaempferol-3-O-sophoroside; K.G.: kaempferol-3-O-glucoside; T: tepal; S: stamen.



Evidence Supports Tradition: The *in Vitro* Effects of Roman Chamomile on Smooth Muscles

Zsolt Sándor^{1†}, Javad Mottaghipisheh^{2†}, Katalin Veres², Judit Hohmann², Tímea Bencsik³, Attila Horváth², Dezső Kelemen⁴, Róbert Papp⁴, Loránd Barthó¹ and Dezső Csupor^{2,5*}

¹ Department of Pharmacology and Pharmacotherapy, University of Pécs, Medical School, Pécs, Hungary,

² Department of Pharmacognosy, University of Szeged, Szeged, Hungary, ³ Department of Pharmacognosy, University of Pécs, Pécs, Hungary, ⁴ Department of Surgery, Clinical Center, University of Pécs, Medical School, Pécs, Hungary,

⁵ Interdisciplinary Centre for Natural Products, University of Szeged, Szeged, Hungary

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Edited by:

Luc Pieters,
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Christian W. Gruber,
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Liselotte Krenn,
Universität Wien, Austria

*Correspondence:

Dezső Csupor
csupor.dezso@pharm.u-szeged.hu;
csupor.dezso@pharmacognosy.hu

[†] These authors have contributed
equally to this work as first authors.

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The dried flowers of *Chamaemelum nobile* (L.) All. have been used in traditional medicine for different conditions related to the spasm of the gastrointestinal system. However, there have been no experimental studies to support the smooth muscle relaxant effect of this plant. The aim of our research was to assess the effects of the hydroethanolic extract of Roman chamomile, its fractions, four of its flavonoids (apigenin, luteolin, hispidulin, and eupafolin), and its essential oil on smooth muscles. The phytochemical compositions of the extract and its fractions were characterized and quantified by HPLC-DAD, the essential oil was characterized by GC and GC-MS. Neuronally mediated and smooth muscle effects were tested in isolated organ bath experiments on guinea pig, rat, and human smooth muscle preparations. The crude herbal extract induced an immediate, moderate, and transient contraction of guinea pig ileum via the activation of cholinergic neurons of the gut wall. Purinoceptor and serotonin receptor antagonists did not influence this effect. The more sustained relaxant effect of the extract, measured after pre-contraction of the preparations, was remarkable and was not affected by an adrenergic beta receptor antagonist. The smooth muscle-relaxant activity was found to be associated with the flavonoid content of the fractions. The essential oil showed only the relaxant effect, but no contracting activity. The smooth muscle-relaxant effect was also detected on rat gastrointestinal tissues, as well as on strip preparations of human small intestine. These results suggest that Roman chamomile extract has a direct and prolonged smooth muscle-relaxant effect on guinea pig ileum which is related to its flavonoid content. In some preparations, a transient stimulation of enteric cholinergic motoneurons was also detected. The essential oil also had a remarkable smooth muscle relaxant effect in this setting. Similar relaxant effects were also detected on other visceral preparations, including human jejunum. This is the first report on the activity of Roman chamomile on smooth muscles that may reassure the rationale of the traditional use of this plant in spasmodic gastrointestinal disorders.

Keywords: Roman chamomile, *Chamaemelum nobile*, Asteraceae, organ bath experiment, gastrointestinal preparations, spasmolytic effect, contractile action

INTRODUCTION

Chamaemelum nobile (L.) All. (Asteraceae), widely known as Roman chamomile, is a perennial herb native to South-Western Europe, but it is cultivated as a medicinal plant all over Europe and in Africa as well. Dried flowers of the cultivated, double-flowered variety of the species are official in the European Pharmacopoeia (European Pharmacopoeia, 2008). Incorporating the plant in traditional herbal medicinal products has been acknowledged by the European Medicines Agency. The comminuted herbal substance (as tea) and a liquid extract of the plant (extraction solvent: ethanol 70% v/v) may be used for the symptomatic treatment of mild, spasmodic gastrointestinal complaints including bloating and flatulence. The British Herbal Pharmacopoeia indicates its use as carminative, anti-emetic, antispasmodic, and sedative (British Herbal Pharmacopoeia, 1971).

Roman chamomile (RC) has been used as a medicinal plant from the middle ages. The cultivation of the plant started in England in the 16th century (Hiller and Melzig, 1999). The double variety of the flower, which now serves as the main commercial drug, has certainly been known since the 18th century (Evans, 1989). The plant gained the name “nobile” (Latin, noble) to illustrate its superior therapeutic efficacy over *Matricaria recutita* L. (German chamomile) (Hiller and Melzig, 1999). The plant was first listed in the Pharmacopoeia of Württemberg (1741) as a carminative, painkiller, diuretic, and digestive aid (Lukacs, 1990). In the folk medicine of different regions of Europe, RC has been used for numerous conditions, including dyspepsia, flatulence, nausea and vomiting, anorexia, vomiting of pregnancy, dysmenorrhoea, and specifically for gastrointestinal cramps and flatulent dyspepsia associated with mental stress (Augustin et al., 1948; Rápóti and Romváry, 1974; Melegari et al., 1988; Rossi et al., 1988; Bradley, 1992). In the Mediterranean region, RC tea is consumed to improve appetite and also after meal to prevent indigestion (Rivera and Obon, 1995; Menendez-Baceta et al., 2014; Alarcün et al., 2015). Traditional use of RC is largely related to its supposed smooth muscle-relaxant activity.

The majority of the secondary metabolites described from the plant belong to the aliphatic esters (essential oil) (Fauconnier et al., 1996), sesquiterpene lactones (Bisset, 1994) and flavonoids (Herisset et al., 1971, 1973; Abou-Zied and Rizk, 1973; Pietta et al., 1991). The polysaccharide content of the dried flower is noteworthy, 3.9% (Lukacs, 1990). The supposed smooth muscle-relaxant activity of the plant might be attributed to its flavonoid content. Apigenin and luteolin possess remarkable smooth muscle relaxant effects on guinea pig ileum (Lemmens-Gruber et al., 2006).

Although several studies on the bioactivities of RC are available, the majority of these studies were carried out using the essential oil, which is not used medicinally, or the observed activities are not related to the traditional use of the plant. Several studies demonstrate the antimicrobial effects of RC essential oil against different bacterial and fungal strains (Hänsel et al., 1993; Piccaglia et al., 1993; Chao et al., 2000; Bail et al., 2009), and antifungal activity was demonstrated also for the aqueous extracts

of RC (Magro et al., 2006). The anti-inflammatory capacity and heat shock protein modulating effects of the flavonoids apigenin and quercetin, as well as the anti-inflammatory activities of α -bisabolol, guajazulene, and chamazulene have been reported in preclinical studies (Viola et al., 1995; Baghalian et al., 2008, 2011; Hernández-Ceruelos et al., 2010). The polysaccharides of RC exerted antiphlogistic effect *in vivo* (Lukacs, 1990).

Although the use of RC extract for gastrointestinal problems seems to be related to its presumptive smooth muscle-relaxant effect, interestingly no *in vitro* or *in vivo* studies have been carried out so far to assess this bioactivity. However, in an *in vitro* study an aqueous extract of *C. nobile* was demonstrated to induce a vasorelaxant effect through the NO-cGMP pathway or possibly through a combination of Ca^{2+} channel inhibition plus NO-modulating and phosphodiesterase inhibitory mechanisms. After the oral administration of RC aqueous extract, significant hypotensive effect was observed in an animal study on spontaneously hypertensive rats (Zeggwagh et al., 2009), which may be related to the flavonoid content of the plant (Jouad et al., 2001). The clinical efficacy of orally applied preparations has not been studied yet, only the effects of external application (Schrader et al., 1997) and aromatherapeutic use (Wilkinson et al., 1999) have been reported.

The aim of the current study was to assess the effects of a hydroethanolic RC extract, its fractions and essential oil on gastrointestinal and urogenital smooth muscles, including preparations of human jejunum, in order to clarify the rationale for the use of this plant for smooth muscle relaxation. Experiments were carried out with an extract conforming to the monograph of the European Medicines Agency (see in section “Preparation of Herbal Extracts and Isolation of Reference Standards”), and also with the fractions of this extract and with the essential oil of the plant.

MATERIALS AND METHODS

Plant Material

Roman chamomile flowers were purchased from Pál Bobvos (Hungary). The identity of the plant material was confirmed according to the requirements of the European Pharmacopoeia. A voucher specimen is stored for verification purposes in the herbarium of the Department of Pharmacognosy, University of Szeged. RC essential oil was purchased from Aromax Ltd. (Hungary).

Chemicals

For the pharmacological experiments the following drugs were used: atropine sulfate (Sigma), α,β -methylene ATP lithium salt (Tocris), capsaicin, histamine dihydrochloride, indomethacin, isoprenaline hydrochloride, N^G -nitro-L-arginine, papaverine hydrochloride, prostaglandin $\text{F}_{2\alpha}$ (Sigma), methysergide (Sandoz), (\pm)-propranolol hydrochloride (Sigma), pyridoxalphosphate-6-azophenyl-2',4'-disulfonic acid tetrasodium salt (PPADS), tetrodotoxin (Tocris), 8-amino-7-chloro-2,3-dihydro-1,4-benzodioxan-5-carboxylic acid, 1'-butyl-4'-piperidinylmethyl ester (SB204070), N-(1-azabicyclo

[2.2.2]oct-3-yl)-6-chloro-4-methyl-3-oxo-3,4-dihydro-2H-1,4-benzoxazine-8-carboxamide hydrochloride (Y25130) (all from Tocris). For dissolving and diluting of these drugs the following solvents were used: 96% ethanol for capsaicin, indomethacin, and prostaglandin F_{2α}, DMSO for SB204070 and isotonic NaCl solution for the rest of the drugs. Solutions in organic solvents were administered at a maximum of 1 μl/ml, aqueous solutions at 1 or 3 μl/ml bathing solution.

Millipore Direct-Q UV3 clarifier (Molsheim, France) was used to produce purified water for the HPLC measurements. Methanol and ethanol (LiChrosolv® HPLC grade) was obtained from Merck (Darmstadt, Germany). For chromatographic separation, polyamide (ICN Polyamide for Column Chromatography), Sephadex LH-20 (25–100 μm, Pharmacia Fine Chemicals), SiO₂ (Silica gel 60 G, 15 μm, Merck), and SiO₂ plates (20 cm × 20 cm Silica gel 60 F₂₅₄, Merck) were used.

Preparation of Herbal Extracts and Isolation of Reference Standards

For the pharmacological experiments, RC crude extract was prepared according to the description of the European Medicines Agency monograph (EMA-HMPC, 2012). Ten grams of plant material was extracted with 70% EtOH (3 × 100 ml) via ultrasonic bath, evaporated in vacuum and lyophilized (yield, 3.169%). Part of the RC crude extract was fractionated to gain RC extract fractions with different compositions for further experiments. Vacuum liquid chromatography on polyamide with elution by MeOH-H₂O (20:80, 40:60, 60:40, 80:20, 100:0) was used to gain fractions from the crude extract as follows: F20, F40, F60, F80, and F100.

From the methanolic extract of 200 g RC flowers, four marker compounds (used as reference standards in further experiments) were isolated using medium pressure liquid chromatography (SiO₂ as stationary phase), gel chromatography (Sephadex LH-20), preparative HPLC (RP), and preparative TLC (SiO₂). The purity and identity of these compounds were analyzed by NMR. NMR spectra were recorded in MeOD on a Bruker Avance DRX 500 spectrometer (Bruker, Fallanden, Switzerland) at 500 MHz (¹H) or 125 MHz (¹³C).

HPLC Experiments

HPLC experiments were carried out on a Shimadzu LC-20AD Liquid Chromatograph (SPD-M20A diode array detector, CBM-20A controller, SIL-20AC_{HT} autosampler, DGU-20A_{5R} degasser unit, CTO-20AC column oven) using a Kinetex 5 μm C-18 100A (150 mm × 4.6 mm) with a gradient of 0.01% trifluoroacetic acid in H₂O (A) and acetonitrile (B) as follows: 0–5 min 25% B, 14 min 28% B, 15 min 70% B, 16 min 70% B, 16.5 min 25% B, and 20 min 25% B. The flow was 1.2 ml/min, column oven temperature was 55°C. Detection was carried out within the range of 190–800 nm. For quantification, chromatograms were integrated at 344 nm. The reference standards and the evaporated extracts were dissolved in MeOH, filtered through a PTFE syringe filter and injected in volumes of 5 or 10 μl. Calibration curves were established for all the four reference standards.

GC and GC-MS Experiments

The GC analysis was carried out with an HP 5890 Series II gas chromatograph (FID), using a 30 m × 0.35 mm × 0.25 μm HP-5 fused silica capillary column. The temperature program ranged from 60°C to 210°C at 3°C min⁻¹, and from 210°C to 250°C (2 min hold) at 5°C min⁻¹. The detector and injector temperature was set to 250°C and the carrier gas was N₂, with split sample introduction. Quantities of the individual components of the essential oil were expressed as the percent of the peak area relative to the total peak area from the GC/FID analysis.

The GC-MS analysis was performed with a Finnigan GCQ ion trap bench-top mass spectrometer. All conditions were as above except that the carrier gas was He at a linear velocity of 31.9 cm s⁻¹ and the capillary column was DB-5MS (30 m × 0.25 mm × 0.25 μm). The positive ion electron ionization mode was used, with ionization energy of 70 eV, and the mass range of 40–400 amu.

Identification of the compounds was based on comparisons with published MS data (Adams, 2007) and with a computer library search (the database was delivered together with the instrument) and also by comparing their retention indices with literature values (Adams, 2007). Retention indices were calculated against C₈–C₃₂ *n*-alkanes on a CB-5 MS column (Kovats, 1965). A mixture of aliphatic hydrocarbons was injected in hexane (Sigma-Aldrich, St. Louis, MO, United States) by using the same temperature program that was used for analyzing the essential oil.

Guinea Pig and Rat Preparations

Guinea pigs (short-haired, colored, 350–450 g) or Wistar rats (220–300 g) of either sex were killed by a blow to the head and exsanguination. Three centimeter segments of the ileum or distal colon were placed into the organ bath in a vertical position, under a constant tension of 7 mN. Longitudinal strip preparations (approximately 2–3 cm in length) of guinea pig urinary bladder or rat gastric fundus were fixed under a tension of 5 mN.

This study was carried out in accordance with University guidelines. The protocol was approved by the Animal Welfare Committee, University of Pécs (registration number, BA02/2000-1/2012) with the understanding that isolated organ studies after stunning the animal cannot be regarded as animal experiments.

Organ Bath Experiments

Smooth muscle preparations were used in a traditional organ bath arrangement. The preparations were suspended in Krebs–Henseleit solution of 5 or 7 ml, kept at 37°C, and aerated with a mixture of 95% O₂ and 5% CO₂. The solution contained 119 mM NaCl, 25 mM NaHCO₃, 2.5 mM KCl, 1.5 mM MgSO₄, 2.5 mM CaCl₂, 1.2 mM KH₂PO₄, and 11 mM glucose (pH = 7.4). Movements of the preparations were recorded with isotonic transducers (Hugo Sachs Elektronik-Harvard Apparatus, March, Germany) on ink writers or stored online on a personal computer.

The guinea pig ileum is a preparation of a low spontaneous tone. In part of the experiments the effects of RC extract,

its fractions, essential oil, or flavonoids were studied at basal tone. All experiments commenced with determining the maximal longitudinal spasm by adding histamine (10 μM) to the organ bath for 2 min. This response served as reference for determining the relative amplitude of any contractile effect that the samples to be tested might have had.

In another set of experiments the possible relaxant effect of the materials was tested. For this reason, a tonic, approximately half-maximal contraction of the ileum was provoked by a moderate concentration of histamine (0.5 μM , added for 15 min). In preliminary experiments, no pretreatment was used. In the bulk of the experiments, however, the muscarinic receptor antagonist atropine, as well as tetrodotoxin, an inhibitor of nerve axonal conduction (blocker of voltage-sensitive Na^+ channels) was added prior to the histamine administration. This was done to avoid the interference with a possible contractile effect of the material to be examined, i.e., to create a methodologically clear situation.

All experiments commenced after an equilibration period of 45 min. Relaxant effects were studied on pre-contracted preparations (see below). Maximal spasm of the tissue was evoked to serve as a basis for the comparisons to evaluate the responses obtained by our test materials. The materials examined were administered in a single concentration (i.e., in a non-cumulative manner).

Human Jejunal Preparations

Macroscopically intact segments of human jejunum, removed during the surgical treatment of pancreatic cancer were used. Strips of either longitudinal or circular orientation were prepared (the mucosa and submucosa were removed) and fixed as described above, under a tension of 10 mN. Freeze-dried extracts of RC, as well as the essential oil of the plant were mixed in and further diluted with DMSO. The amounts of DMSO administered to the jejunum preparations are indicated in the Section "Results".

This study was carried out in accordance with the Declaration of Helsinki and the guidelines set by the Research Ethics Committee, Scientific Council of the Ministry of Health, Hungary. This Committee agreed to the use of discarded human tissue for the experimental work. The protocol was approved by the ETT-TUKEB (Scientific Council of the Ministry of Health, Research Ethical Committee) [No. 17861-0/2010-1018EKU (749/PI/10)]. All subjects gave written informed consent in accordance with the Declaration of Helsinki.

Statistical Analysis

Results of the pharmacological experiments are given as mean \pm SEM, with N denoting the number of preparations used for testing efficacy. At most 2 preparations from the same animal were used for each type of experiment. Results are provided in relative values, where 100% contraction means maximal spasm of the preparations from the basal tone and, in pre-contracted preparations, 100% relaxation means relaxation of the preparation to the level before adding the precontracting agent. The Mann–Whitney U test was used for the comparisons of two independent groups, more than two independent samples were compared by the Kruskal–Wallis test, and two dependent

samples were compared by the Wilcoxon test. The statistical program GraphPad Prism 5 was used throughout. A probability of $P < 0.05$ or less indicated statistical significance.

RESULTS

Chemical Characterization of RC Extracts

In the HPLC retention time range of 4–9 min, four characteristic peaks were detected in the crude RC extract and its fractions F40–F100. The corresponding components were isolated from the plant material and were identified as the flavonoids apigenin, eupafolin, hispidulin, and luteolin by ^1H and ^{13}C NMR experiments (see Supplementary Figures S1–S6). These compounds were further used as reference standards to characterize the RC extracts. The identification of reference compounds in different extracts was based on matching the retention times and UV spectra. Retention times of luteolin, eupafolin, apigenin, and hispidulin were 4.5, 5.0, 7.5, and 8.5 min, respectively (**Figure 1**). The baseline separation of these compounds allowed their reliable quantification in different extracts.

The crude RC extract contained eupafolin as the main flavonoid, followed by luteolin, hispidulin, and apigenin (**Table 1**). The fractionation on polyamide resulted in subfractions F20–F100 with different compositions, as demonstrated by the differences in their flavonoid content. In F20, the quantities of flavonoids were below the level of quantification. The flavonoid content of the fractions increased with increasing MeOH content of the eluting solvent. The highest flavonoid levels were measured in F80, except for luteolin and apigenin, which were mainly concentrated in F100.

Chemical Characterization of RC Essential Oil

Based on their retention times and mass spectrometric data, methallyl angelate, 3-methyl pentyl angelate, and 3-methylamyl isobutyrate were identified as the major constituents of RC essential oil (19.0, 18.2, and 10.4%, respectively) (**Table 2**). The identified components comprised 97% of the essential oil. Previous articles reported isobutyl angelate as the major constituent of RC essential oil (21.6–38.5%), followed by 2-methylbutyl angelate (11.6–20.3%) (Antonelli and Fabbri, 1998; Farkas et al., 2003; Omidbaigi et al., 2003, 2004; Bail et al., 2009), and propyl tiglate (10.8–13.1%) (Omidbaigi et al., 2003, 2004) or isobutyl isobutyrate (3.3%) (Bail et al., 2009) or 2-butenyl angelate (7.9–8.4%) (Antonelli and Fabbri, 1998) or 2-methyl-2-propenyl angelate (9.1%) (Farkas et al., 2003). However, several commercial samples have similar compositions to the sample analyzed by us, with methallyl angelate and 3-methyl pentyl angelate as the major components.

Effects on Guinea Pig Ileum

The crude RC extract induced a transient longitudinal contraction on guinea pig ileum preparations (**Figure 2**).

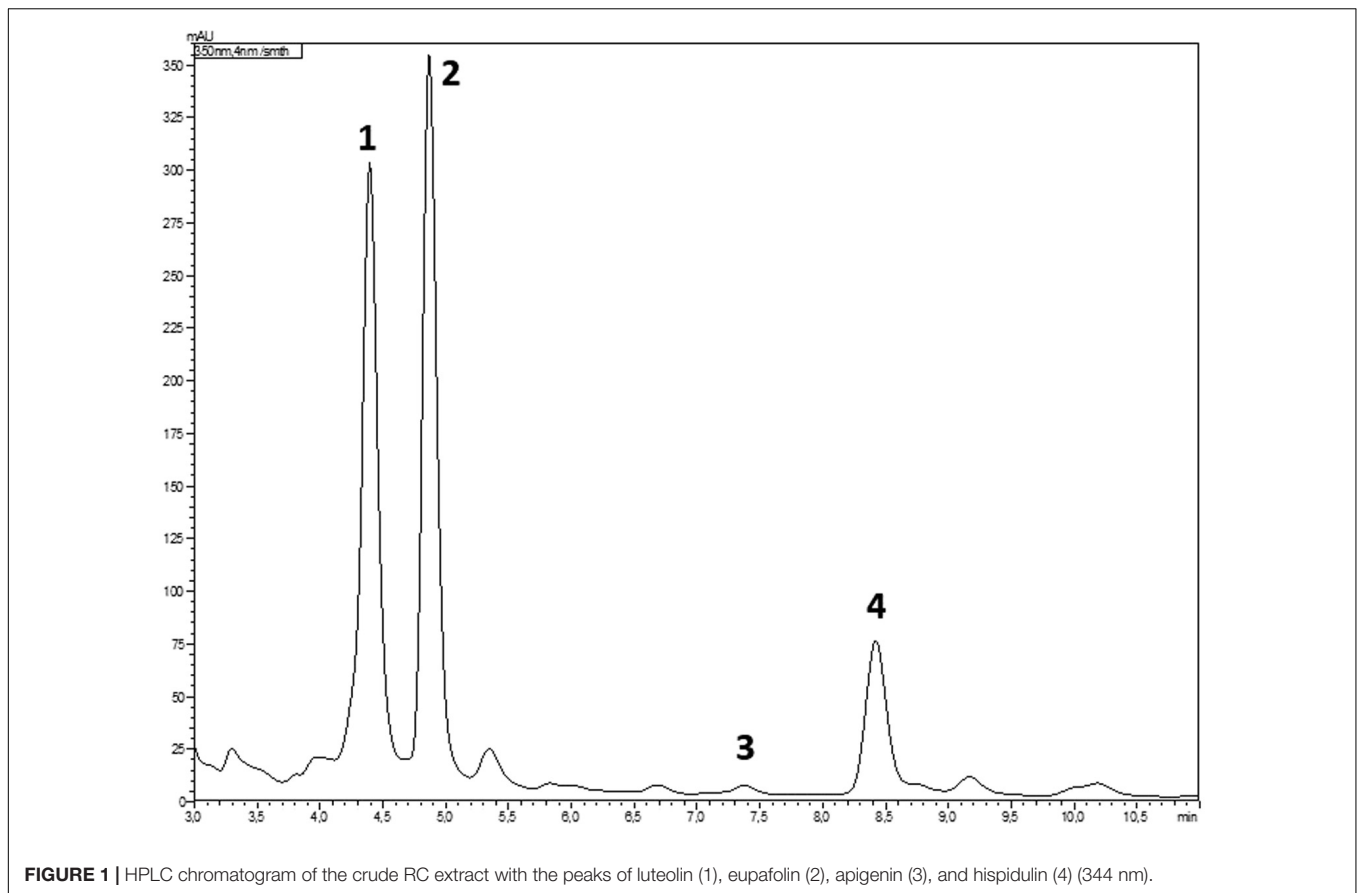


TABLE 1 | Flavonoid content of RC crude extract and its fractions as determined by HPLC.

Sample	Luteolin (mg/g extract)	Eupafolin (mg/g extract)	Apigenin (mg/g extract)	Hispidulin (mg/g extract)
Crude extract	4.617 ± 0.616	18.756 ± 2.121	0.298 ± 0.027	1.584 ± 0.181
F20	Not detected	Not detected	Not detected	Not detected
F40	0.578 ± 0.001	1.800 ± 0.001	0.179 ± 0.001	0.231 ± < 0.001
F60	1.904 ± 0.001	62.591 ± 0.025	0.151 ± < 0.001	5.951 ± 0.004
F80	22.605 ± 0.001	223.488 ± 0.036	0.859 ± < 0.001	17.060 ± 0.006
F100	55.305 ± 0.002	150.206 ± 0.005	2.055 ± < 0.001	4.983 ± < 0.001

The amplitudes of the contractions were related to the maximal longitudinal spasm evoked with 10 μ M of histamine at the beginning of the experiments. The threshold concentration for this effect was equal to or below 20 μ g/ml of the extract (which was the lowest concentration tested) and reached a plateau with 60 and 200 μ g/ml. Quantitative results were as follows (expressed as % of the maximal spasm): 18.8 ± 3.1% at 20 μ g/ml ($N = 6$), 40.1 ± 3.3% at 60 μ g/ml ($N = 11$), and 36.3 ± 4.9% at 200 μ g/ml ($N = 7$). A second administration of the same concentration after a 40-min washout period usually had a qualitatively similar effect. Yet, because of variable reproducibility, we examined the effects on separate preparations with only one administration of the extract. The solvent of the extract (DMSO; 0.3 or 1 μ l/ml) caused no or minimal contraction (on average, 0 and 2%, respectively; $N = 12$).

The 60 μ g/ml concentration of the extract was used for pharmacological analysis. Both atropine (0.5 μ M), an antagonist of acetylcholine at the muscarinic receptors and tetrodotoxin (0.5 μ M), an inhibitor of voltage-sensitive Na^+ channels (hence, of neuronal axonal conduction) inhibited the contractile effect of RC crude extract. In contrast, the purinoceptor antagonist PPADS (50 μ M) or a combination of serotonin (5-HT) receptor antagonists methysergide (0.3 μ M), SB204070 (1 μ M) and Y25130 (1 μ M) failed to influence the contractile effect of the extract (**Table 3**). This combination of 5-HT antagonists is suitable for blocking the contractile effect of 5-HT (Sandor et al., 2016).

The functional blockade of capsaicin-sensitive neurons did not inhibit the contractile effect of the RC extract (Barthó et al., 2004) as compared to time-matched, solvent-treated controls (**Table 3**). The cyclooxygenase inhibitor indomethacin (3 μ M)

TABLE 2 | Chemical composition of RC essential oil.

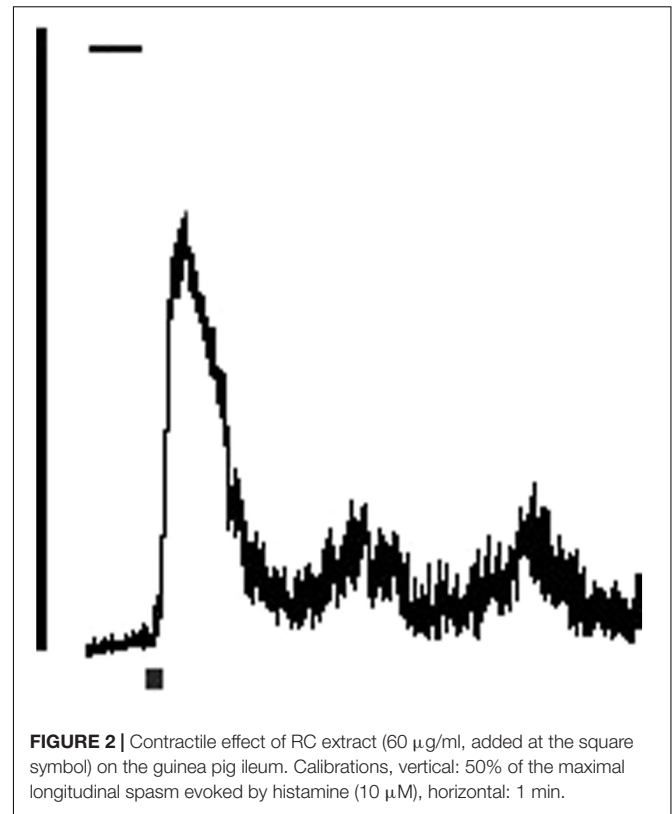
Compounds ^a	RI ^b	% in samples
Methyl tiglate	867	tr
<i>n</i> -Hexanol	875	1.1
2-Methylbutyl acetate	897	0.4
Isobutyl isobutyrate	925	0.8
Acetonyl acetone	932	0.7
α -Pinene	935	2.4
Camphene + allyl methacrylate	958	0.6
Thuja-2,4(10)-diene	960	1.1
Isoamyl propionate	966	tr
β -Pinene	969	0.3
Myrcene	973	0.6
Propyl angelate	993	1.1
Isobutyl 2-methylbutyrate	998	tr
Isoamyl isobutyrate	1,004	1.5
2-Methylbutyl isobutyrate	1,006	2.7
1,8-Cineol	1,035	tr
Isoamyl methacrylate	1,037	1.1
Isobutyl angelate	1,058	4.9
Methylallyl angelate	1,068	19.0
2-Butenyl angelate	1,119	tr
3-Methylamyl isobutyrate	1,122	10.4
3-Methylamyl methacrylate	1,150	6.6
<i>trans</i> -Pinocarveol + isoamyl angelate	1,153	8.6
2-Methylbutyl angelate	1,168	8.3
Pinocarvone	1,177	3.9
Prenyl angelate	1,213	1.5
Myrtenal	1,217	1.2
3-Methyl pentyl angelate	1,264	18.2
Identified components		97.0

^aCompounds listed in sequence of elution from a DB-5 MS column. ^bRetention indices calculated against C8 to C32 *n*-alkanes on a DB-5MS column. tr, in traces.

moderately but significantly inhibited the contraction in response to the RC extract.

Fractions of the RC extract were also tested for contracting activity. Fraction F20 induced a moderate contraction (approximately 20% of the maximum) (Table 4). Similar results were obtained with F40, F60, F80, and F100, although the extent of contraction tended to decline with F60, F80, and F100.

Our experiments revealed the smooth muscle relaxing activity of RC extract, fractions and essential oil in this experimental setting. On histamine-precontracted preparations (treated with 0.5 μ M histamine for 15 min, in the presence of atropine and tetrodotoxin, both 0.5 μ M), concentration-dependent relaxation was observed in response to treatment with RC crude extract (60–200 μ g/ml) (Figure 3 and Table 5). The highest concentration tested induced full relaxation. The relaxation detected with the 20 μ g/ml test sample did not exceed the changes evoked by the solvent itself (1 μ l/ml DMSO; see below). In several cases the relaxation was preceded by a little contraction. The relaxation induced by the 60 μ g/ml extract was not significantly altered by the adrenergic β -receptor antagonist propranolol (1 μ M; 51.3 \pm 7.8% relaxation, N = 6) or by the NO synthase inhibitor N^G-nitro-L-arginine (100 μ M; 47.4 \pm 7.4% relaxation,

**FIGURE 2** | Contractile effect of RC extract (60 μ g/ml, added at the square symbol) on the guinea pig ileum. Calibrations, vertical: 50% of the maximal longitudinal spasm evoked by histamine (10 μ M), horizontal: 1 min.**TABLE 3** | Effects of drugs on the contractile response to RC crude extract (60 μ g/ml) on guinea pig small intestine (mean \pm SEM).

Pretreatment	Contraction (% of maximal spasm)	N
No pretreatment (control)	40.1 \pm 3.3	11
Tetrodotoxin (0.5 μ M)	15.0 \pm 1.8*	6
Atropine (0.5 μ M)	8.2 \pm 2.0*	6
PPADS (50 μ M)	49.7 \pm 2.7	9
5-HT receptor antagonists [#]	52.1 \pm 3.0	11
Solvent for capsaicin	51.1 \pm 5.2	7
Capsaicin ^{&}	46.9 \pm 6.0	9
Solvent for indomethacin	46.4 \pm 4.0	6
Indomethacin (3 μ M)	31.2 \pm 5.6*	10

Values significantly different from the respective control group are indicated by asterisks (*). [#]methysergide (0.3 μ M), SB204070 (1 μ M) and Y25130 (1 μ M). [&]10 μ M of capsaicin for 10 min, followed by a 60-min washout period.

N = 10) (both compared with the group indicated in Table 5). The solvent DMSO (0.3 or 1 μ l/ml) had a slight relaxant effect in this experimental arrangement (histamine-precontracted ileum pretreated with atropine and tetrodotoxin); it amounted to 3.0 \pm 1.6% at 0.3 μ l/ml and 7.9 \pm 1.4% at 1 μ l/ml; N = 11 and 17, respectively.

On histamine-precontracted, atropine- and tetrodotoxin-treated ileum, different fractions of RC extract showed distinct relaxant effects (Table 6). F20 and F40 (60 or 200 μ g/ml) practically produced no relaxation (N = 5 for both concentrations). This observation refers to the potential role of flavonoids in the relaxant effect, and experiments with

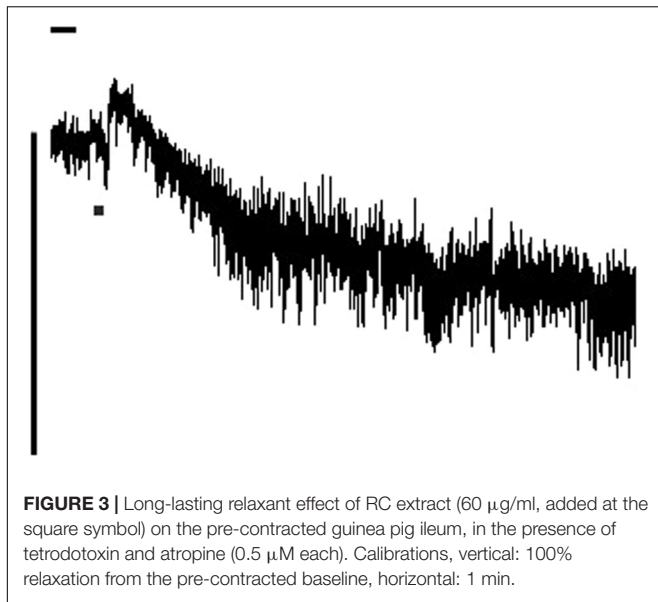


FIGURE 3 | Long-lasting relaxant effect of RC extract (60 µg/ml, added at the square symbol) on the pre-contracted guinea pig ileum, in the presence of tetrodotoxin and atropine (0.5 µM each). Calibrations, vertical: 100% relaxation from the pre-contracted baseline, horizontal: 1 min.

TABLE 4 | Contractile effects of RC extract fractions on guinea pig ileum (longitudinally oriented preparations, mean ± SEM).

Bath concentration	Contraction (% of the maximal spasm)	N
F20		
2 µg/ml	5.2 ± 2.4%	5
20 µg/ml	18.2 ± 6.1%	5
60 µg/ml	18.5 ± 4.4%	6
200 µg/ml	22.2 ± 3.1%*	5
F40		
2 µg/ml	2.5 ± 1.2%	5
20 µg/ml	18.2 ± 5.9%	5
60 µg/ml	15.5 ± 3.3%	5
200 µg/ml	22.8 ± 8.4%*	5
F60		
2 µg/ml	8.4 ± 1.3%	6
20 µg/ml	21.8 ± 6.4%*	5
60 µg/ml	20.9 ± 8.2%*	5
200 µg/ml	4.0 ± 2.7%	6
F80		
2 µg/ml	0.4 ± 0.4%	5
20 µg/ml	13.0 ± 3.2%	5
60 µg/ml	12.0 ± 4.0%	5
200 µg/ml	2.5 ± 1.5%	5
F100		
20 µg/ml	12.4 ± 2.1%	6
60 µg/ml	26.4 ± 5.8%*	5
200 µg/ml	15.1 ± 2.7%	6

Asterisks denote values significantly different from a pooled group of solvent effects (Kruskal–Wallis test for several unrelated samples).

four flavonoids isolated from the plant material reassured this hypothesis. All the flavonoids exerted a dual effect on the guinea pig ileum, i.e., a short-lived contraction at basal tone and a long-lasting relaxation on the histamine-pre-contracted, atropine- and tetrodotoxin-pretreated preparations (Table 7). At

TABLE 5 | Relaxing effect of RC crude extract and essential oil on the pre-contracted ileum.

Bath concentration of RC crude extract	Relaxation (% of the maximum)	N
20 µg/ml	18 ± 5.2%	6
60 µg/ml	76.2 ± 8.5%*	9
200 µg/ml	100%*	5
Bath concentration of RC essential oil	Relaxation %	N
0.1 µg/ml	12.8 ± 3.5%	6
1 µg/ml	30.8 ± 5.9%*	9
10 µg/ml	69.7 ± 5.6%*	6

Atropine and tetrodotoxin (0.5 µM each) were present in the medium in order to inhibit the contractile effect of the RC extract. Tonic submaximal contraction was evoked with histamine (0.5 µM for 15 min). Values are given in %, where 100% means full relaxation reaching the pre-histamine values (mean ± SEM). Asterisks denote values significantly different from a pooled group of solvent effects (0.3 or 1 µl/ml of DMSO, see text) (Kruskal–Wallis test for several unrelated samples).

TABLE 6 | Relaxant effects of RC extract fractions on pre-contracted guinea pig ileum (mean ± SEM).

Bath concentration	Relaxation %	N
F60		
20 µg/ml	18.7 ± 5.7%	5
60 µg/ml	96.0 ± 3.0%*	6
200 µg/ml	93.5 ± 4.9%*	6
F80		
20 µg/ml	47.2 ± 7.7%	5
60 µg/ml	93.0 ± 5.9%*	6
200 µg/ml	100%*	4
F100		
6 µg/ml	12.5%	4
20 µg/ml	61 ± 13.7%	5
60 µg/ml	69.4 ± 7.5%*	6
200 µg/ml	100%*	4

100% relaxation denotes that tone returns to baseline. Asterisks denote values significantly different from a pooled group of solvent effects (0.3 or 1 µl/ml DMSO) (Kruskal–Wallis test for several unrelated samples).

a concentration of 1 µM, the flavonoids did not exert contractile activities, however, at 10 µM, a slight effect (34.6 ± 7.4% for hispidulin, 32.0 ± 3.1% for luteolin, 27.1 ± 4.2% for eupafolin, and 20.5 ± 5.1% for apigenin, N = 5 each) was observed. The relaxant activities at 2 µM ranged between 18.2 ± 5.4% and 24.2 ± 3.7%, whereas at 20 µM between 64.5 ± 4.1% and 81.9 ± 5.3%.

The essential oil of RC (0.1, 1, 10, or 30 µg/ml) showed no contractile effect on the ileum (N = 6–8). RC oil (1 or 10 µg/ml) induced considerable relaxation on histamine-precontracted, atropine- and tetrodotoxin-pretreated preparations (Table 5). Similar results were obtained on preparations without atropine and tetrodotoxin pretreatment (N = 6–8, data not shown).

Papaverine was used as positive control. As with other experiments for studying relaxation, the drug was administered to histamine-precontracted, atropine- and tetrodotoxin-treated

TABLE 7 | Relaxant effects of flavonoids on pre-contracted guinea pig ileum (mean \pm SEM).

Bath concentration	Relaxation %	N
Hispidulin		
2 μ M	19.4 \pm 3.5%	5
20 μ M	64.5 \pm 4.1%	6
Luteolin		
2 μ M	19.6 \pm 1.6%	5
20 μ M	80.0 \pm -4.5%	6
Eupafolin		
2 μ M	18.2 \pm 5.4%	5
20 μ M	68.7 \pm 6.8%	6
Apigenin		
2 μ M	24.2 \pm 3.7%	5
20 μ M	81.9 \pm 5.3%	6

100% relaxation denotes that tone returns to baseline. The solvent contained a maximum of 10% DMSO and had no relaxant effect in the volumes used.

TABLE 8 | Contractile effect of RC extract on guinea pig urinary bladder strip, without pretreatment and following treatment with capsaicin or its solvent (mean \pm SEM).

Bath concentration of RC crude extract	Contraction (% of the maximal spasm)	N
20 μ g/ml No pretreatment	8.0 \pm 4.2%	7
200 μ g/ml No pretreatment	20.0 \pm 5.1%	7
200 μ g/ml Ethanol pretreatment [#]	21.6 \pm 4.9%	8
200 μ g/ml Capsaicin (10 μ M) pretreatment ^{&}	9.1 \pm 1.3%*	8

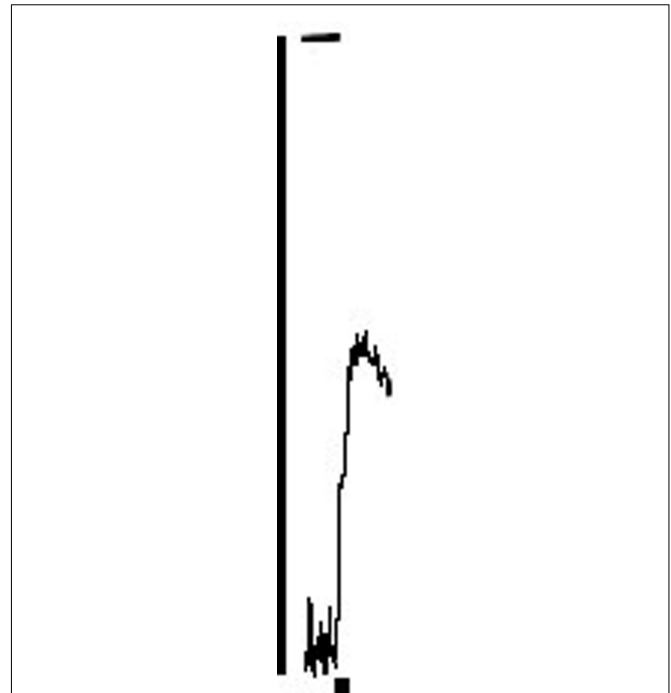
[#]Solvent and time control for capsaicin. [&]Capsaicin was added and incubated for 10 min. This was followed by a 90-min washout period. *Significantly different from the ethanol control.

ileum preparations, in a non-cumulative manner. The relaxant responses obtained were as follows, 0.3 μ M: 19.7 \pm 4.7% ($N = 6$); 1 μ M: 29.1 \pm 3.5% ($N = 6$); 3 μ M: 30.1 \pm 4.8% ($N = 7$); 10 μ M: 92.9 \pm 4.2% ($N = 6$), where, as also elsewhere in this paper, relaxation to the pre-histamine baseline was taken as 100%.

Effects on Guinea Pig Urinary Bladder

The effects of the RC extract and the essential oil were tested on guinea pig urinary bladder strips as well. RC crude extract evoked concentration-dependent contraction at 20 and 200 μ g/ml (Table 8 and Figure 4 for 200 μ g/ml). The solvent of the extract had no contractile effect on the bladder ($N = 4$). The effect of the 200 μ g/ml extract was diminished (approximately halved) by *in vitro* capsaicin pretreatment (Table 8). Contractile responses were compared to the maximal spasm evoked by 100 mM of KCl at the end of the experiment.

To study the relaxant effect of the crude extract, the bladder strips were sub-maximally pre-contracted with histamine (1–3 μ M). Atropine and tetrodotoxin were present in the bathing fluid. Since excitatory purinergic mechanisms are present in the bladder, α , β -methylene ATP desensitization (10 + 10 μ M for 10 min each) was also performed. RC crude extract had no

**FIGURE 4** | Contraction, in response to 200 μ g/ml RC extract (added at the square symbol), on the guinea pig urinary bladder detrusor strip. Calibrations, vertical: 50% of the maximal spasm in response to 100 mM KCl, horizontal: 1 min.

effect at a concentration of 20 μ g/ml, whereas a concentration of 200 μ g/ml induced 58.3 \pm 4.6% relaxation ($N = 7$ and 8, respectively). The solvent itself (1 μ l/ml DMSO) exerted no effect.

Roman chamomile essential oil caused no contraction in a concentration of 10 μ g/ml. Similarly to the extract, RC oil exerted a moderate relaxing effect on the pre-contracted bladder: 17.9 \pm 6.1% and 34.1 \pm 5.8% relaxation was evoked with 1 and 10 μ g/ml, $N = 7$ and 6, respectively.

Effects on Rat Gastrointestinal Preparations

The relaxant effect of RC essential oil was confirmed on longitudinally oriented preparations of the rat gastrointestinal tract. Rat whole ileum and distal colon preparations are characterized by high intrinsic tone, therefore no pre-contraction was needed. RC oil (10 μ g/ml) induced relaxation (41.4 \pm 7.4% and 21.8 \pm 3.7%, $N = 7$ and 10, respectively, on these preparations, where 100% means the relaxant response to 3 μ M isoprenaline, given at the end of the experiment). A concentration of 1 μ g/ml was barely effective. No contractile effect was observed. Rat stomach fundus strips were pre-contracted with 0.1–0.3 μ M acetylcholine. RC oil produced relaxation (on average 44 and 70.3% relaxation in response to concentrations of 1 and 10 μ l/ml, $N = 4$ each, where 100% means relaxation to the pre-acetylcholine level).

Effects on Human Jejunal Preparations

In untreated longitudinally oriented human jejunal preparations RC crude extract induced transient contraction amounting to $12.4 \pm 3.5\%$ and $30.5 \pm 0.6\%$ at 20 and 200 $\mu\text{g/ml}$ concentrations, respectively ($N = 8$). Atropine- and tetrodotoxin-pretreated preparations were pre-contracted with $\text{PGF}_2\alpha$ (1 or 2 μM). RC extract showed a concentration-dependent relaxant effect ($25.8 \pm 4.3\%$ at 20 $\mu\text{g/ml}$, $N = 8$; $68.5 \pm 8.5\%$ at 60 $\mu\text{g/ml}$, $N = 7$; $50.8 \pm 3.7\%$ at 200 $\mu\text{g/ml}$, $N = 9$). RC essential oil produced no contraction. A lasting relaxant effect was detected on pre-contracted preparations (in the presence of atropine and tetrodotoxin). The relaxant effect reached $26.9 \pm 5.2\%$ at 1 $\mu\text{g/ml}$ and $81.4 \pm 8.2\%$ at 10 $\mu\text{g/ml}$ concentrations ($N = 8$ and 7, respectively).

In untreated circularly oriented human jejunal preparations the RC crude extract evoked transient contraction ($43.4 \pm 10\%$ at 200 $\mu\text{g/ml}$, $N = 5$), while the 20 $\mu\text{g/ml}$ concentration sample had a negligible effect (2.8% contraction on 5 preparations). RC extract induced relaxation in $\text{PGF}_2\alpha$ -precontracted preparations (in the presence of atropine and tetrodotoxin). This effect reached 45% with 60 $\mu\text{g/ml}$ concentration ($N = 4$), while a concentration of 200 $\mu\text{g/ml}$ produced full relaxation ($N = 6$), where a relaxation to the pre-prostaglandin level was taken as 100%.

The solvent itself (1 $\mu\text{l/ml}$ DMSO) did not evoke either contraction or relaxation on this preparation ($N = 4-6$).

DISCUSSION

The experiments presented here have been carried out with RC, which has been used traditionally for the symptomatic treatment of mild, spasmodic and other gastrointestinal complaints, however, the presumed smooth muscle-relaxant effect has not been confirmed experimentally. The aim of our study was to investigate the effect of a traditionally used extract of the plant, its fractions and the essential oil of the plant, as well as of four of its flavonoid components.

The phytochemical analysis of the extract revealed the presence of flavonoids in the plant, four of which (apigenin, luteolin, eupafolin, and hispidulin) were isolated and identified. The hydroethanolic extract was fractionated on polyamide to gain fractions with different flavonoid content in order to examine the role of these compounds in the effect on smooth muscles. The flavonoid content of the extract and fractions was analyzed by HPLC. The flavonoid content of the extract was fractionated successfully, fractions F80, F100 (and to some extent F60) containing higher amounts of flavonoids than the crude extracts. The flavonoid pattern of fractions also differed. The essential oil analyzed by us belongs to the chemotype characterized by the predominance of methallyl angelate.

Pharmacological experiments were carried out with the hydroethanolic extract, its fractions (F20, F40, F60, F80, and F100), four flavonoids (apigenin, eupafolin, hispidulin, and luteolin) and the essential oil of the plant on different smooth muscles *in vitro*. The hydroethanolic extract of RC has both stimulatory and relaxant effects on guinea pig

ileum. The moderate, transient stimulatory activity results from the activation of cholinergic neurons, as confirmed by its inhibition by tetrodotoxin (an inhibitor of neuronal voltage-sensitive Na^+ channels) and atropine (antagonist on muscarinic receptors for acetylcholine). Although capsaicin, a stimulant of a certain class of sensory receptors (through which it evokes a "local efferent" response) shows a similar cholinergic, neurogenic effect in guinea pig small intestine preparations (see Barthó et al., 2004 for review), a functional blockade of capsaicin-sensitive nerve endings by capsaicin pretreatment failed to inhibit the contractile action of RC extract. The contractile action of RC extract was moderately, yet significantly reduced by the cyclooxygenase inhibitor indomethacin, which may indicate a modulatory role of endogenous prostanoids. Other pharmacological inhibitors tested had no contraction-reducing effect, therefore it is proposed that neither endogenous serotonin, nor PPADS-sensitive purinergic mechanisms play a role in the excitatory action of RC extract. It should be noted that both serotonin and ATP or the P_2X receptor agonist α,β -methylene ATP are able to evoke cholinergic contractions in guinea pig ileum (Benko et al., 2005; Barthó et al., 2006; Sandor et al., 2016 for recent data).

Following the transient and mild-to-moderate stimulant effect, the RC crude extract exhibited a sustained relaxant effect on the guinea pig ileum in the presence of tetrodotoxin and atropine, and preliminary experiments indicated a similar relaxant effect also in the absence of atropine and tetrodotoxin. Yet, atropine and tetrodotoxin were included into these experiments for creating a methodologically clear situation, where an initial, neuronally mediated excitatory action would hardly interfere with the relaxant one. The concentrations of the extract causing relaxation were roughly the same as those causing contraction. Based on these data we propose that the site of action for the relaxant effect of RC is on the smooth muscle itself. Neither the adrenergic β -receptor antagonist propranolol nor the NO synthase inhibitor N^G -nitro-L-arginine significantly reduced the relaxant effect of the RC extract, hence, no evidence was found for these mechanisms to be involved in the relaxant response. In fact, a direct relaxant effect of the extract was demonstrated on all gastrointestinal preparations tested, including human and rat gastrointestinal preparations, as well as in guinea pig bladder. In preparations with high intrinsic tone (rat ileum and rat colon), relaxant activity was practically the only response to be seen.

Different RC extract fractions evoked both contraction and relaxation on guinea pig ileum. While there was a clear-cut tendency correlation between the flavonoid content and the relaxant effect, in case of contractile action such correlation was not observed (on the contrary, fractions with lower flavonoid content exerted slightly higher contractile activities). Fractions with high flavonoid content (F60, F80, and F100) had remarkable relaxant effects, whereas fractions with no (F20) or low (F40) flavonoid content exerted no such activity. The pure flavonoids of the extract showed dual effects in the guinea pig ileum, much similarly to the crude extract. Moreover, there was no substantial

difference between the potencies of the flavonoids. This means that any of these flavonoids may contribute to the sustained relaxant and the transient contractile effect of the extract. The essential oil of RC caused no contraction on the test preparations; nevertheless, a consistent relaxant effect was detected. This seems to indicate that (i) chemical components responsible for the stimulant effect may be absent in the essential oil; (ii) it may be that several types of compounds present in the extract are responsible for the smooth muscle-relaxant action. This point needs to be clarified in subsequent experiments.

The contraction of guinea pig bladder in response to the RC extract proved to be special, in that it was reduced by half following *in vitro* capsaicin pretreatment. This indicates that capsaicin-sensitive sensory nerves of the bladder wall are in some way involved in the excitatory effect of the RC extract (see Barthó et al., 2004). Nevertheless, the RC extract and oil also induced bladder relaxation in pre-contracted preparations.

Roman chamomile extract was effective in human jejunal preparations as well. Both an excitatory and a tetrodotoxin- and atropine-resistant relaxing effect were demonstrated, again, the smooth muscle relaxant one being more sustained than the excitatory one. Preliminary experiments have shown that this early contraction is reduced by atropine. Due to limited access to human tissue, no further analysis of these responses could be performed.

In terms of possible medical uses it may be noted that a combination of nerve-mediated, mild-to moderate excitatory effect and a smooth muscle-relaxant action may even be advantageous for some gastrointestinal problems, e.g., diminished peristaltic activity, while for spasms in the stomach or large intestine it is obviously the smooth muscle relaxing activity that offers benefits.

CONCLUSION

Our results support the overall smooth muscle relaxant effect of a hydroethanolic RC extract and of the essential oil of RC. The

predominant effect of the extract, its fractions and flavonoids is relaxation, being more sustained than the transient contraction observed in some cases. This activity is in correlation with the flavonoid content of the extract. Since the components of the essential oil are partly extracted with alcohols, the constituents of the oil also contribute to the overall effect of the hydroethanolic RC extract. The extract used in our experiments was prepared in accordance with the European Medicines Agency monograph for this plant, therefore our study contributes to the body of evidence relating the traditional use of this plant.

AUTHOR CONTRIBUTIONS

ZS, DK, TB, and RP carried out the organ bath experiments. JM, KV, and AH performed the phytochemical studies. JH, LB, and DC designed and co-ordinated the experiments and wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fphar.2018.00323/full#supplementary-material>

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RESEARCH ARTICLE



Antiproliferative and cytotoxic activities of furocoumarins of *Ducrosia anethifolia*

Javad Mottaghipisheh^a, Márta Nové^b, Gabriella Spengler^b, Norbert Kúsz^{a,c}, Judit Hohmann^{a,c} and Dezső Csupor^{a,c}

^aDepartment of Pharmacognosy, Faculty of Pharmacy, University of Szeged, Szeged, Hungary; ^bDepartment of Medical Microbiology and Immunobiology, Faculty of Medicine, University of Szeged, Szeged, Hungary; ^cInterdisciplinary Centre for Natural Products, University of Szeged, Szeged, Hungary

ABSTRACT

Context: Phytochemical and pharmacological data on *Ducrosia anethifolia* (DC.) Boiss. (Apiaceae), an Iranian medicinal plant, are scarce; however, furocoumarins are characteristic compounds of *D. anethifolia*.

Objective: Our experiments identify the secondary metabolites of *D. anethifolia* and assess their antitumor and anti-multidrug resistance activities.

Materials and methods: Pure compounds were isolated from the extract of aerial parts of the plant by chromatographic methods. Bioactivities were tested on multidrug resistant and sensitive mouse T-lymphoma cell lines. The inhibition of the cancer MDR efflux pump ABCB1 was evaluated by flow cytometry (at 2 and 20 μ M). A checkerboard microplate method was applied to study the interactions of furocoumarins and doxorubicin. Toxicity was studied using normal murine NIH/3T3 fibroblasts.

Results: Thirteen pure compounds were isolated, nine furocoumarins namely, pabulenol (**1**), (+)-oxypeucedanin hydrate (**2**), oxypeucedanin (**3**), oxypeucedanin methanolate (**4**), (–)-oxypeucedanin hydrate (**5**), imperatorin (**6**), isogospherol (**7**), heraclenin (**8**), heraclenol (**9**), along with vanillic aldehyde (**10**), harmine (**11**), 3-hydroxy- α -ionone (**12**) and 2-C-methyl-erythrytol (**13**). Oxypeucedanin showed the highest *in vitro* antiproliferative and cytotoxic activity against parent (IC₅₀ = 25.98 \pm 1.27, 40.33 \pm 0.63 μ M) and multidrug resistant cells (IC₅₀ = 28.89 \pm 0.73, 66.68 \pm 0.00 μ M), respectively, and exhibited slight toxicity on normal murine fibroblasts (IC₅₀ = 57.18 \pm 3.91 μ M).

Discussion and conclusions: Compounds **2**, **3**, **5**, **7**, **10–13** were identified for the first time from the *Ducrosia* genus. Here, we report a comprehensive *in vitro* assessment of the antitumor activities of *D. anethifolia* furocoumarins. Oxypeucedanin is a promising compound for further investigations for its anti-cancer effects.

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Introduction


The genus *Ducrosia* (Apiaceae) consists of six species: *Ducrosia ismaelis* Asch., *D. flabellifolia* Boiss., *D. assadii* Alava., *D. areysiana* (Deflers) Pimenov & Kljuykov, *D. inaccessa* (C.C.Towns.) Pimenov & Kljuykov and *D. anethifolia* (DC.) Boiss. *D. anethifolia* is one of the three species growing wild in several areas of Iran, Afghanistan, Pakistan, Syria, Lebanon, Iraq, and some other Arab states and countries along the Persian Gulf (Aynehchi 1991; Ghahreman 1993; Mozaffarian 1996). The whole herb, especially its aerial part, has been used in Iranian folk medicine as an analgesic and in case of anxiety and insomnia (Shalaby et al. 2014). The aerial part, including the seed was reported to be carminative and useful for irregularities of menstruation and galactagogue (Amiri and Joharchi 2016). The herb is added to a variety of Persian foods for flavouring (Aynehchi 1991; Haghi et al. 2004).

The phytochemical profile of *D. anethifolia* has only been partly explored. In the literature, the majority of papers deal with the composition of the essential oil (EO). As major constituents, α -pinene (11.6% (Mostafavi et al. 2008), 70.3% (Mottaghipisheh et al. 2014), 59.2% (Janssen et al. 1984)); *n*-decanal (1.4–45% (Karami and Bohlooli 2017), 45.06%

(Vazirzadeh et al. 2017), 70% (Hajhashemi et al. 2010), 57% (Mahboubi and Feizabadi 2009), 25.6–30.3% (Mazloomifar and Valian 2015), 18.8% (Sefidkon and Javidtash 2002)), dodecanal (28.8% (Shahabipour et al. 2013)), *cis*-chrysanthenyl acetate (72.28%) (Ashraf et al. 1979; Habibi et al. 2017) have been reported.

Furocoumarins and terpenoids are characteristic components of the *Ducrosia* genus. From the seeds of *D. anethifolia*, two new terpenoids, the monoterpene ducrosin A and the sesquiterpene ducrosin B were isolated along with stigmaterol and the furocoumarins heraclenin and heraclenol (Queslati et al. 2017). Psoralen, 5-methoxy-psoralen, 8-methoxy-psoralen, imperatorin, isooxypeucedanin, pabulenol, pangelin, oxypeucedanin methanolate, oxypeucedanin hydrate, 3-*O*-glucopyranosyl- β -sitosterol and 8-*O*-debenzoyl-paeoniflorin were also isolated from the extract of *D. anethifolia* (Stavri et al. 2003; Shalaby et al. 2014). GC analysis of the fatty acids showed high percentages of elaidic acid and oleic acid (Queslati et al. 2017), beside 58.8% petroselinic acid in the seed oil of *D. anethifolia* (Khalid et al. 2009). Apart from *D. anethifolia*, furocoumarins (psoralen, isopsoralen) have been reported only from *D. ismaelis* from this genus (Morgan et al. 2015).

CONTACT Dezső Csupor  csupor.dezso@pharmacognosy.hu  Department of Pharmacognosy, University of Szeged, Szeged, Hungary

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The bioactivities of the extracts of aerial parts of *D. anethifolia* have been studied *in vitro* and *in vivo*. Different extracts of the plant exerted moderate anti-radical scavenging (Mottaghipisheh et al. 2014; Shahat et al. 2015); and antibacterial effects (Syed et al. 1987; Mahboubi and Feizabadi 2009). Pangelin isolated from *D. anethifolia* demonstrated activity against a panel of fast growing mycobacteria (Stavri et al. 2003). Essential oil of the seeds and methanol extract showed a weak antibacterial effect against 14 Gram positive and negative bacteria (Javidnia et al. 2009; Habibi et al. 2017). In an experiment on three human cancer cell lines (K562, LS180 and MCF-7), *D. anethifolia* EO demonstrated remarkable to moderate cytotoxic activity, while EO of *D. flabellifolia* showed less pronounced activity (Shahabipour et al. 2013). Ducrosin B exerted remarkable cytotoxicity against the human colon HCT-116 and ovary SKOV-3 cancer cell lines *in vitro* (Queslati et al. 2017).

The crude *D. anethifolia* extract and the isolated furocoumarins exhibited *in vivo* antidiabetic activities (Shalaby et al. 2014). The *in vivo* anxiolytic (Hajhashemi et al. 2010; Shokri et al. 2013; Zamyad et al. 2016), sedative (Hajhashemi et al. 2010), analgesic and anti-inflammatory (Asgari Nematian et al. 2017) and also anti-locomotor activities (Zamyad et al. 2016) of *D. anethifolia* EO have been tested. Intra-peritoneal administration of the *D. anethifolia* EO improved spatial learning and memory in adult male rats (Abbasnejad et al. 2017). The intra-peritoneal injection of the hydroalcoholic extract of *D. anethifolia* effectively reduced the pentylenetetrazole-induced seizure manifestations in male Wistar rats (Nyasty et al. 2017). Moreover, *D. anethifolia* extract reduced the number of germ cells, the level of testosterone and spermatogenesis in male Wistar rats (Rahimi et al. 2016).

As presented above, furocoumarins are the most characteristic compounds of the *Ducrosia* and their activities against cancer cells seem to be promising. Imperatorin showed antiproliferative effect on human hepatoma HepG2 cells (Luo et al. 2011); furthermore, this compound and heraclenin induced apoptosis in Jurkat leukemia cells. In Jurkat cells treated for 72 h with heraclenin and imperatorin, most of the DNA fragmentation occurred at the G2/M and G1/S phases of the cell cycle, respectively (Appendino et al. 2004). 8-Methoxypsoralen inhibited the growth of neuroblastoma (IC₅₀ = 56.3 μM) and metastatic colon cancer cells (IC₅₀ = 88.5 μM) by triggering both extrinsic and intrinsic apoptotic pathways, independently of photoactivation (Bartnik et al. 2017). Isoimperatorin, cnidicin, imperatorin, oxypeucedanin, byakangelicol and oxypeucedanin hydrate exhibited a significant inhibition on cell proliferation in a dose-dependent manner, particularly oxypeucedanin against HCT-15 (colon cancer) cells with ED₅₀ = 3.4 ± 0.3 μg/mL (Kim et al. 2007).

Beside direct antiproliferative and cytotoxic activities, furocoumarins affect multidrug resistance (MDR) as well. Among 20 selected furocoumarin derivatives, phellopterin (IC₅₀ = 8.0 ± 4.0 μM) and isopimpinellin (IC₅₀ = 26.0 ± 5.7 μM) exhibited the highest activity against CEM/C1 (lymphoblastic leukaemia) and HL-60/MX2 (MDR) cell lines, respectively (Kubrak et al. 2017). Feroniellin A reverted MDR in A549RT-eto lung cancer cells (Kaewpiboon et al. 2014). Bergapten (IC₅₀ = 40.29 ± 0.30 nM) and xanthotoxin (IC₅₀ = 1.10 ± 0.91 nM) showed remarkable anticancer activity against EPG85.257RDB (MDR1 overexpressing human gastric adenocarcinoma cell line) and MCF7MX (BCRP overexpressing human epithelial breast cancer cell line), respectively (Mirzaei et al. 2017).

Our work explores the phytochemical composition of *D. anethifolia*, examines the complex *in vitro* anticancer activities, including antiproliferative, cytotoxic and anti-MDR effects of its

isolated compounds, and analyses the interaction of compounds possessing promising bioactivities with chemotherapeutics.

Materials and methods

General procedures

NMR spectra were recorded in CD₃OD and CDCl₃ on a Bruker Avance DRX 500 spectrometer at 500 MHz (¹H) and 125 MHz (¹³C). The peaks of the residual solvent (δ_H 3.31 and 7.26, δ_C 49.0 and 77.2, respectively) were taken as reference. The data were acquired and processed with MestReNova v6.0.2e-5475 software. Chemical shifts are expressed in parts per million and coupling constants (*J*) values are reported in Hz. All solvents were used in analytical grade (Molar Chemicals Kft, Halásztelek, Hungary).

Pure compounds were isolated by using open column chromatography (Silica gel 60, 0.063–0.2 mm, Merck, Darmstadt, Germany) (CC), medium pressure liquid chromatography (MPLC, silica gel 60, 0.045–0.063 mm, Merck, Darmstadt, Germany), gel chromatography (Sephadex[®] LH-20, Pharmacia, Uppsala, Sweden), normal (Silica gel 60, Merck, Darmstadt, Germany) and reverse phase (Silica gel 60 RP-18 F₂₅₄s, Merck, Darmstadt, Germany) preparative thin layer chromatography (PTLC and RP-PTLC, respectively), centrifugal PTLC (Silica gel 60 GF₂₅₄, Merck, Darmstadt, Germany) (CPTLC) and reverse phase preparative HPLC (Kinetex[®] 5 μm C-18 100 Å, 150 × 4.6 mm Phenomenex, Torrance, CA) (RP-HPLC). The HPLC flow was 1.2 mL/min, column oven temperature was 24 °C. Detection was carried out within the range of 190–800 nm. The HPLC system comprised of Waters 600 pump, Waters 2998 PDA detector, Waters in/line degasser AF degasser unit connected with Waters 600 control module using Empower Pro 5.00 software.

Plant material

The aerial parts of *Ducrosia anethifolia* were collected by JM from south of Iran (Fars, Neyriz, Iran) in April 2016. Identification of the plant was done by Dr. Mohammad Jamal Saharkhiz at Department of Horticultural Science, Faculty of Agriculture, Shiraz University, Iran, and a voucher specimen was deposited in the Herbarium of Department of Pharmacognosy, University of Szeged (voucher no.: 880).

Isolation of compounds

Aerial parts (flower, leaves and stem, 3 kg) were dried in shade at room temperature and powdered, then extracted with methanol (40 L). After filtration, the filtrate was concentrated under reduced pressure to yield the crude extract. The extract (464.1 g) was dissolved with methanol–water 1:1 (1.5 L) and then partitioned successively with *n*-hexane (4 × 1 L), CHCl₃ (4 × 1 L), EtOAc (4 × 1 L) and *n*-BuOH (4 × 1 L). The solvents were removed from each extract to yield the *n*-hexane extract, CHCl₃ extract, EtOAc extract and *n*-BuOH extract.

The CHCl₃-soluble fraction (20.6 g) was initially subjected to CC with a gradient system consisting of increasing concentration of MeOH in CHCl₃ (0–80%); column fractions with similar TLC patterns were combined to get six major fractions D₁, D₂, D₃, D₄, D₅ and D₆. D₁ was chromatographed by MPLC, first eluting with *n*-hexane–CH₂Cl₂ (50:50; 0:100), then adding MeOH to CH₂Cl₂ (0–100%), to afford four subfractions (D₁₁, D₁₂, D₁₃ and D₁₄). D₁₁ was separated to 49 subfractions using CPTLC with an isocratic eluting system *n*-hexane–EtOAc–MeOH (10:3:1), which

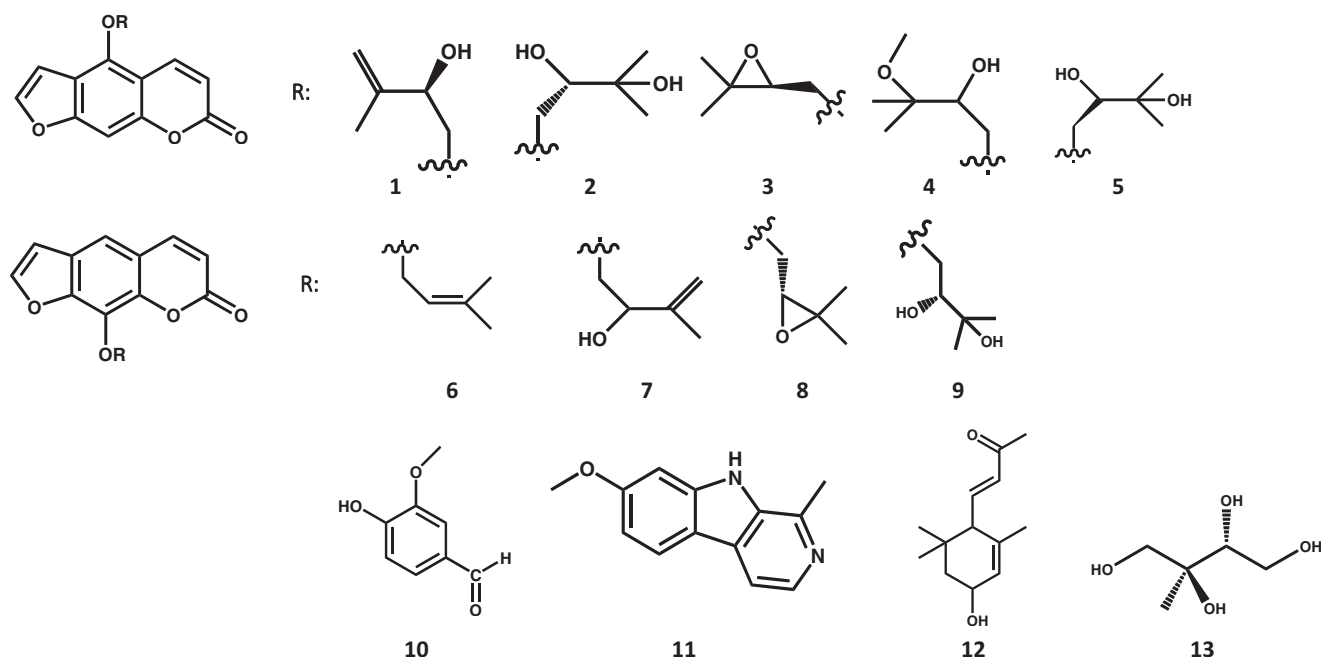


Figure 1. Secondary metabolites isolated from *Ducrosia anethifolia*.

resulted in the isolation of the pure compound **6** (82.8 mg). The RP-HPLC purification of D₁₁ subfractions with MeOH–H₂O (MeOH–H₂O 1:1) afforded compound **10** (1.7 mg). D₁₂ was chromatographed by MPLC applying a gradient solvent system with increasing EtOAc in *n*-hexane (5–100%) to get eight major subfractions (D₁₂₁–D₁₂₈). From D₁₂₃, the pure compound **3** (3.1 mg) was isolated by using CPTLC with EtOAc in *n*-hexane (5–100%). D₁₂₄ was successively separated to 81 fractions by CPTLC (same system), then subfractions 49–54 was subjected to RP-HPLC with MeOH–H₂O (15–50% H₂O in MeOH) yielding compound **9** (2.56 mg).

D₁₃ was separated by MPLC with increasing ratio of EtOAc in *n*-hexane (5–100%) to get seven fractions (D₁₃₁–D₁₃₇). D₁₃₃ was subjected to MPLC with the same solvent system to gain 19 subfractions. Finally, subfractions 1–2 were purified by using CPTLC with toluene–EtOAc (90:10, 80:20, 70:30, 60:40, 50:50) as eluents to gain compound **2** (100.4 mg). Subfraction 3 from D₁₃₃ was subjected to CPTLC by eluting with toluene–EtOAc (90:10, 80:20, 70:30, 60:40, 50:50) to yield 32 subfractions. Subfractions 18–19 of D₁₃₃ were separated by PTLC with toluene–EtOAc (1:1) to get compound **8** (35.3 mg). Besides, subfractions 23–32 were chromatographed by RP-HPLC (MeOH–H₂O 1:1) and then by PTLC with toluene–EtOAc (1:1) to yield compounds **12** (1.02 mg) and **1** (1.78 mg). By using CPTLC with increasing concentration of EtOAc in toluene (5–100%) as eluents, subfraction 6 from D₁₃₃ was chromatographed to get 43 subfractions. Subfractions 24–28 and 36–40 were separated by PTLC with CHCl₃–MeOH–*n*-hexane (5:1:5) to retrieve compounds **4** (2.5 mg) and **7** (21.7 mg), respectively.

D₃ was separated to five major fractions (D₃₁–D₃₅) by MPLC with a solvent system containing increasing ratio of MeOH in CHCl₃ (0–100%). D₃₃ was chromatographed by CPTLC with raising the concentration of MeOH (0–20%) in the mixture of cyclohexane–EtOAc (1:1) to afford 70 subfractions. Subfractions 21–23 contained the pure compound **11** (1.0 mg). The main fraction D₄ was separated by MPLC to seven subfractions (D₄₁–D₄₇) by raising the ratio of MeOH (0–100%) in acetone–toluene (1:1). Subfraction D₄₂ was subsequently chromatographed by PTLC

with eluting cyclohexane–EtOAc–MeOH (4.75:4.75:0.5) and compound **5** (2.7 mg) was isolated. Furthermore, D₄₆ was purified with CPTLC by increasing ratio of MeOH (0–100%) in acetone–toluene (1:1); then compound **13** (2.9 mg) was purified by using RP-PTLC [MeOH–H₂O (1:1)] from subfractions 35–37 (Figure 1).

Cell lines

L5178Y mouse T-cell lymphoma cells: parent, PAR cells (ECACC cat. no. 87111908, obtained from FDA, Silver Spring, MD) were transfected with pHa MDR1/A retrovirus. The ABCB1-expressing L5178Y cell line (MDR) was selected by culturing the infected cells in 60 ng/mL colchicine containing medium. L5178Y PAR mouse T-cell lymphoma cells and the L5178Y human ABCB1-transfected subline (MDR) were cultured in McCoy's 5A medium supplemented with 10% heat-inactivated horse serum, 200 mM L-glutamine, and penicillin–streptomycin mixture in 100 U/L and 10 mg/L concentration, respectively, at 37 °C and in a 5% CO₂ atmosphere.

NIH/3T3 mouse embryonic fibroblast cell line (ATCC CRL-1658) was purchased from LGC Promochem (Teddington, UK). The cell line was cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, St. Louis, MO), containing 4.5 g/L glucose, supplemented with 10% heat-inactivated foetal bovine serum (FBS). The cells were incubated at 37 °C, in a 5% CO₂, 95% air atmosphere.

Assay for antiproliferative effect

The effects of increasing concentrations of the analysed compounds on cell proliferation were tested in 96-well flat-bottomed microtiter plates (Poljarević et al. 2018). The compounds were diluted in 100 µL of McCoy's 5A medium. 6 × 10³ mouse T-cell lymphoma cells (PAR or MDR) in medium (100 µL) were added to each well, with the exception of the medium control wells. The culture plates were further incubated at 37 °C for 72 h; at the end of the incubation period, 20 µL of MTT solution

(thiazolyl blue tetrazolium bromide, Sigma, St. Louis, MO) (from a 5 mg/mL stock) was added to each well. After incubation at 37 °C for 4 h, 100 µL of sodium dodecyl sulphate (SDS, Sigma, St. Louis, MO) solution (10% in 0.01 M HCl) was added to each well and the plates were further incubated at 37 °C overnight. The cell growth was determined by measuring the OD at 540 nm (ref. 630 nm) with a Multiscan EX ELISA reader (Thermo LabSystems, Waltham, MA). IC₅₀ values were calculated via the following equation:

$$IC_{50} = 100 - \left[\frac{OD_{\text{sample}} - OD_{\text{mediumcontrol}}}{OD_{\text{cellcontrol}} - OD_{\text{mediumcontrol}}} \right] \times 100$$

Assay for cytotoxic effect

The effects of increasing concentrations of compounds on cell growth were tested in 96-well flat-bottomed microtiter plates (Poljarević et al. 2018). The compounds were diluted in a volume of 100 µL medium. Then, 1×10^4 cells in 100 µL of medium were added to each well, with the exception of the medium control wells. In case of NIH/3T3 cells, the compounds were added after seeding the cells at 37 °C overnight. The culture plates were incubated at 37 °C for 24 h; at the end of the incubation period, 20 µL of MTT solution (from a 5 mg/mL stock) was added to each well. After incubation at 37 °C for 4 h, 100 µL of SDS solution (10% in 0.01 M HCl) was added to each well and the plates were further incubated at 37 °C overnight. Cell growth was determined by measuring the optical density (OD) at 540 nm (ref. 630 nm) with a Multiscan EX ELISA reader. Inhibition of the cell growth was determined according to the formula:

$$IC_{50} = 100 - \left[\frac{OD_{\text{sample}} - OD_{\text{mediumcontrol}}}{OD_{\text{cellcontrol}} - OD_{\text{mediumcontrol}}} \right] \times 100$$

Results are expressed in terms of IC₅₀, defined as the inhibitory dose that reduces by a 50% the growth of the cells exposed to the tested compound.

Assay for multidrug resistance reversing activity

The inhibition of the cancer MDR efflux pump ABCB1 by the tested compounds was evaluated using flow cytometry measuring the retention of rhodamine 123 by ABCB1 (P-glycoprotein) in MDR mouse T-lymphoma cells, as the L5178Y human ABCB1-gene transfected mouse T-lymphoma cell line (MDR) overexpresses P-glycoprotein (Domínguez-Álvarez et al. 2016). This method is a fluorescence-based detection system which uses verapamil as reference inhibitor. Briefly, cell number of L5178Y MDR and PAR cell lines was adjusted to 2×10^6 cells/mL, re-suspended in serum-free McCoy's 5A medium and distributed in 0.5 mL aliquots into Eppendorf centrifuge tubes. The tested compounds were added at different concentrations and the samples were incubated for 10 min at room temperature. Verapamil (Sigma, St. Louis, MO) and tariquidar (Sigma, St. Louis, MO) were applied as positive controls. Next, 10 µL (5.2 µM final concentration) of the fluorochrome and ABCB1 substrate rhodamine 123 (Sigma, St. Louis, MO) were added to the samples and the cells were incubated for 20 min at 37 °C, washed twice and re-suspended in 0.5 mL PBS for analysis. The fluorescence of the cell population was measured with a Partec CyFlow® flow cytometer (Partec, Görlitz, Germany). The percentage of mean fluorescence intensity was calculated for the treated MDR cells as compared with the untreated cells. A fluorescence activity ratio

(FAR) was calculated based on the following equation which relates the measured fluorescence values:

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

The results obtained from a representative flow cytometry experiment in which 20,000 individual cells of the population were evaluated for amount of rhodamine 123 retained with the aid of the Partec CyFlow® flow cytometer, are first presented by the histograms and these data converted to FAR units that define fluorescence intensity, standard deviation, peak channel in the total- and in the gated-populations. Parameters calculated are: forward scatter (FSC, forward scatter count of cells in the samples or cell size ratio); side scatter (SSC, side scatter count of cells in the samples); FL-1 (mean fluorescence intensity of the cells) and FAR, whose values were calculated using the equation given above.

Checkerboard combination assay

A checkerboard microplate method was applied to study the effect of drug interactions between furocoumarins and the chemotherapeutic drug doxorubicin (Takács et al. 2015). This assay was carried out using multidrug resistant mouse T-lymphoma cells overexpressing the ABCB1 transporter. Doxorubicin is in the class of anthracycline antitumor agents, and it exerts anti-cancer activity as a topoisomerase-II (TI-2) inhibitor. The dilutions of doxorubicin (Teva, Debrecen, Hungary, stock solution: 2 mg/mL) were made in a horizontal direction in 100 µL (final concentration: 17.242 µM), and the dilutions of the test compounds vertically in the microtiter plate in 50 µL volume. The cells were re-suspended in McCoy's 5A culture medium and distributed into each well in 50 µL containing 6×10^3 cells each. The plates were incubated for 72 h at 37 °C in 5% CO₂ atmosphere. The cell growth rate was determined after MTT staining. At the end of the incubation period, 20 µL of MTT solution (from a stock solution of 5 mg/mL) was added to each well. After incubation at 37 °C for 4 h, 100 µL of SDS solution (10% in 0.01 M HCl) was added to each well and the plates were further incubated at 37 °C overnight. Optical density was measured at 540/630 nm with Multiscan EX ELISA reader (Thermo LabSystems, Waltham, MA) as described elsewhere (Takács et al. 2015). Combination index (CI) values at 50% of the growth inhibition dose (ED₅₀) were determined using CompuSyn software (ComboSyn, Inc., Paramus, NJ) to plot four to five data points to each ratio. CI values were calculated by means of the median-effect equation, where CI < 1, CI = 1 and CI > 1 represent synergism, additive effect (or no interaction) and antagonism, respectively (Chou and Martin 2005; Chou 2010).

Results

Isolated compounds

Repeated column chromatography of the bioactive fractions resulted in the isolation of 13 compounds. The compounds were identified by careful interpretation of NMR data and comparison of ¹H and ¹³C chemical shifts with those reported in literature. Nine linear furocoumarin derivatives, namely pabulenol (1) (Sbai et al. 2016), (+)-oxypeucedanin hydrate (aviprin) (2) (Sbai et al. 2016), oxypeucedanin (3) (Sbai et al. 2016), oxypeucedanin methanolate (4) (Fujioka et al. 1999), (-)-oxypeucedanin hydrate (prangol) (5) (Rahimifard et al. 2018), imperatorin (6) (Lv et al.

Table 1. Antiproliferative (AA) and cytotoxic activities (CA) of the furocoumarins against PAR, MDR and NIH/3T3 cells presented as IC₅₀ values.

Compounds	AA on PAR cells (μM)	AA on MDR cells (μM)	CA on PAR cells (μM)	CA on MDR cells (μM)	CA on NIH/3T3 cells (μM)
Pabulenol (1)	30.47 ± 0.47	29.28 ± 0.45	51.32 ± 3.32	>100	54.09 ± 3.83
(+)-Oxypeucedanin hydrate (2)	41.96 ± 0.88	60.58 ± 2.74	>100	>100	83.55 ± 0.57
Oxypeucedanin (3)	25.98 ± 1.27	28.89 ± 0.73	40.33 ± 0.63	66.68 ± 0.00	57.18 ± 3.91
Oxypeucedanin methanolate (4)	35.88 ± 0.96	33.23 ± 0.51	56.42 ± 5.23	>100	47.16 ± 1.28
Imperatorin (6)	36.12 ± 0.91	42.24 ± 0.88	52.56 ± 4.19	>100	92.41 ± 2.80
Isogospherol (7)	46.53 ± 0.47	48.75 ± 0.28	>100	>100	54.82 ± 1.99
Heraclenin (8)	32.73 ± 2.40	46.54 ± 1.22	65.81 ± 1.00	83.94 ± 1.68	70.91 ± 4.26
Heraclenol (9)	52.31 ± 2.12	46.57 ± 0.47	>100	>100	65.78 ± 0.46
Doxorubicin	0.054 ± 0.005	0.468 ± 0.065	0.377 ± 0.02	7.152 ± 0.358	5.71 ± 0.50

Data were expressed as mean ± standard deviation ($n = 3$). Different letters represent significant differences ($p < 0.05$).

Table 2. Efflux pump inhibiting activities of furocoumarins.

Samples	Conc. μM	FSC	SSC	FL-1	FAR
PAR	–	2069	658	98.20	–
MDR	–	2152	725	1.79	–
		MDR mean		1.182	–
Tariquidar	0.02	2156	719	119	100.68
Verapamil	20	2143	740	9.69	8.20
Pabulenol (1)	2	2324	728	0.596	0.82
	20	2323	750	0.544	0.75
(+)-Oxypeucedanin hydrate (2)	2	2325	725	0.715	0.98
	20	2305	769	0.583	0.80
Oxypeucedanin (3)	2	2190	749	0.9	0.76
	20	2164	763	2.62	2.22
Oxypeucedanin methanolate (4)	2	2310	737	0.58	0.80
	20	2290	766	0.499	0.68
Imperatorin (6)	2	2165	749	0.727	0.62
	20	2161	750	0.783	0.66
Isogospherol (7)	2	2305	741	0.531	0.73
	20	2326	741	0.548	0.75
Heraclenin (8)	2	2318	723	0.942	1.29
	20	2294	764	0.57	0.78
Heraclenol (9)	2	2300	742	0.995	1.37
	20	2317	740	0.348	1.30
DMSO	2% (V/V)	2308	762	0.497	0.68
MDR	–	2301	746	0.535	–

Table 3. Checkerboard combination assay of selected compounds with doxorubicin.

Compound	Best ratio	CI at ED ₅₀	Interaction	SD (±)
Oxypeucedanin	1:50	0.85537	Slight synergism	0.07800
Heraclenin	4:100	0.88955	Slight synergism	0.06334

2013), isogospherol (7) (Macias et al. 1990), heraclenin (8) (Poonkodi 2016), heraclenol (9) (Harkar et al. 1984); along with vanillic aldehyde (10) (Chung et al. 2011), harmine (11), 3-hydroxy- α -ionone (12) and 2-C-methyl-erythrytol (13) were identified (¹H NMR spectra see in Supporting Information). The diastereomers (+)-oxypeucedanin hydrate and (–)-oxypeucedanin hydrate were distinguished by determining their optical rotations and comparing with literature (Atkinson et al. 1974).

The ¹H and ¹³C NMR spectral data of 11, 12 and 13 in CD₃OD are reported here for the first time. Compound 11 (harmine): ¹H NMR (500 MHz, CD₃OD) δ = 8.11 (H-3, d, J = 5.5 Hz), 8.02 (H-5, d, J = 8.7 Hz), 7.86 (H-4, d, J = 5.5 Hz), 7.06 (H-8, d, J = 1.9 Hz), 6.89 (H-6, dd, J = 8.7 Hz, 1.9 Hz), 3.92 (s, 7-OCH₃), 2.80 (s, 1-CH₃); ¹³C NMR (125 MHz, CD₃OD) δ = 162.9, 144.6, 141.6, 137.0, 136.2, 130.7, 123.7, 116.3, 113.5, 111.4, 95.4, 56.0, 19.1. Compound 12 (3-hydroxy- α -ionone): ¹H NMR (500 MHz, CD₃OD) δ = 6.67 (H-7, dd, J = 15.8 Hz, 10.3 Hz), 6.13 (H-8, d, J = 15.8 Hz), 5.60 (H-4, br s), 4.22 (H-3, br s), 2.58 (H-6, d, J = 10.3 Hz), 2.27 (H₃-10, s), 1.80 (H-2 β , dd, J = 13.2 Hz, 5.9 Hz), 1.63 (H₃-13, s), 1.38 (H-2 α , dd, J = 13.2 Hz, 7.2 Hz), 1.01 (H₃-11, s), 0.90 (H₃-12, s); ¹³C NMR (125 MHz, CD₃OD) δ = 200.8, 149.8, 135.9, 134.7, 127.3, 65.9,

55.6, 45.0, 35.0, 29.8, 27.1, 24.5, 22.8. Compound 13 (2-C-methyl-erythrytol): ¹H NMR (500 MHz, CD₃OD) δ = 3.80 (H-4a, dd, J = 10.4 Hz, 2.5 Hz), 3.61 (H-3, m), 3.59 (H-4b, m), 3.52 (H-1a, d, J = 11.1 Hz), 3.44 (H-1b, d, J = 11.1 Hz), 1.11 (2-CH₃, s); ¹³C NMR (125 MHz, CD₃OD) δ = 76.2, 75.0, 68.5, 63.8, 19.7.

Antiproliferative and cytotoxic activities on cancer cell lines

Furocoumarins isolated from *D. anethifolia* were subjected to bioassay for cytotoxic and antiproliferative activity against cancer cell lines. All compounds exerted potent antiproliferative effect on sensitive and resistant mouse T-lymphoma cells (Table 1). However, they did not show any selectivity towards the resistant cell line. The most potent compound was oxypeucedanin on both cell lines. Some compounds had no toxic effects ((+)-oxypeucedanin hydrate (2), heraclenol (9), isogospherol (7)); furthermore, pabulenol (1), oxypeucedanin methanolate (4) and imperatorin (6) were more toxic on the sensitive PAR cell line (IC₅₀ between 52 and 57 μM) without any toxicity on MDR cells (Table 1). Oxypeucedanin (3) and heraclenin (8) exhibited cytotoxic activity; however, they were more potent on the sensitive PAR cell line (Table 1). The cytotoxic activity of furocoumarins was assessed using NIH/3T3 normal murine fibroblast cells. Some compounds showed slight toxic effect on normal fibroblasts, namely (+)-oxypeucedanin hydrate (2), heraclenol (4) and isogospherol (8) with IC₅₀ values of 83.55, 65.78 and 54.82 μM, respectively. Pabulenol (1) possessed similar activity on fibroblast and parental mouse lymphoma cells. In addition, oxypeucedanin (3), oxypeucedin methanolate (5) and heraclenin (9) exhibited mild toxicity on fibroblasts and parental lymphoma cells. Imperatorin (7) had no toxic activity on fibroblasts.

Multidrug resistance reversing activity

Regarding the efflux pump inhibiting activity of the compounds on ABCB1 overexpressing MDR mouse T-lymphoma cells, only oxypeucedanin (3) showed moderate ABCB1 inhibiting effect (FAR: 2.22); however, this inhibition was lower than in case of the positive controls tariquidar (FAR: 100) and verapamil (FAR: 8.2) (Table 2, figures see in Supporting Information).

Combination assay results on MDR cells

The two most promising compounds in the previous assays were investigated in combination with the standard chemotherapeutic drug doxorubicin. The compounds oxypeucedanin (3) and heraclenin (8) showed slight synergistic effect with doxorubicin, for this reason, they might be potential adjuvants in combined chemotherapy applying standard anticancer drugs with compounds that can act synergistically (Table 3).

Discussion

Chromatographic separation of the extract of *D. anethifolia* herbs resulted in the isolation of 13 compounds, among them were nine furocoumarins. Compounds **2**, **3**, **5**, **7**, **10–13** were identified for the first time from *Ducrosia* genus.

The tested furocoumarins exerted antiproliferative effects on sensitive and resistant mouse T-lymphoma cells with no selectivity towards the resistant cell line. This is the first comprehensive analysis of this plant and its furocoumarins on these cells. Oxypeucedanin (**3**) had the most remarkable activity on both cell lines. The most effective furocoumarins, oxypeucedanin (**3**) and heraclenin (**9**) exhibited marginal toxicity on normal fibroblast cells and sensitive parental mouse lymphoma cells; furthermore, they were less toxic on multidrug resistant lymphoma cells. From the tested compounds, only oxypeucedanin showed moderate MDR reversing activity. In the checkerboard assay, oxypeucedanin and heraclenin showed slight synergistic effect with doxorubicin. These compounds might improve the cytotoxic effect of the standard chemotherapeutic drug doxorubicin.

Disclosure statement

The authors declare no conflict of interest.

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Article

Chemo-Diversity and Antiradical Potential of Twelve *Matricaria chamomilla* L. Populations from Iran: Proof of Ecological Effects

Elahe Piri ¹, Mohammad Mahmoodi Sourestani ^{1,*}, Esmail Khaleghi ¹,
Javad Mottaghipisheh ², Zoltán Péter Zomborszki ², Judit Hohmann ² and Dezső Csupor ^{2,*}

¹ Department of Horticultural Science, Faculty of Agriculture, Shahid Chamran University of Ahvaz, Ahvaz 61357-43311, Iran; elahe.73.piri@gmail.com (E.P.); khaleghi2184@gmail.com (E.K.)

² Department of Pharmacognosy, University of Szeged, Eötvös u. 6, H-6720 Szeged, Hungary; Imanmottaghipisheh@pharmacognosy.hu (J.M.); zombozope@pharmacognosy.hu (Z.P.Z.); hohmann@pharm.u-szeged.hu (J.H.)

* Correspondence: mohammad.mahmoodi1360@gmail.com (M.M.S.); csupor.dezso@pharmacognosy.hu (D.C.); Tel.: +98-6133364053 (M.M.S.); +36-62545559 (D.C.)

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Abstract: *Matricaria chamomilla* L. is a popular medicinal herb that is used for healing various diseases and is widely distributed worldwide in temperate climate zones, and even in the subtropical climate of Southern and Western Iran. This study was aimed at comparing the volatile oil constituents, along with antiradical potential and HPLC analysis of methanolic extracts from twelve plant samples growing in Iran. The present research was carried out for the first time on these populations. Among seventeen identified volatile chemicals evaluated by GC/MS and GC/FID, representing 92.73–97.71% of the total oils, α -bisabolone oxide A (45.64–65.41%) was the major constituent, except in case of “Sarableh” as a new chemotype, where (*E*)- and (*Z*)- γ -bisabolene (42.76 and 40.08%, respectively) were the predominant components. Oxygenated sesquiterpenes (53.31–74.52%) were the most abundant compounds in the samples excluding “Sarableh” with 91.3% sesquiterpene hydrocarbons. “Sarableh” also exerted the most potent antioxidant capacity with $EC_{50} = 7.76 \pm 0.3 \mu\text{g/mL}$ and $6.51 \pm 0.63 \text{ mmol TE (Trolox}^{\text{®}} \text{ equivalents)/g}$. In addition, populations “Lali” and “Bagh Malek” contained the highest amounts of apigenin and luteolin with $1.19 \pm 0.01 \text{ mg/g}$ and $2.20 \pm 0.0 \text{ mg/g}$ of plant material, respectively. Our findings depict a clear correlation between phytochemical profiles and antiradical potential of *M. chamomilla* and geographical factors.

Keywords: *Matricaria chamomilla* L.; Iranian populations; ecological effects; volatile compounds; antiradical capacity

1. Introduction

Matricaria chamomilla L. (syn. *Chamomilla recutita* L. Rauschert, German chamomile), belonging to the Asteraceae family, is one of the well-known medicinal plant species which has been widely used for centuries. The herb is currently consumed around the world as herbal tea, with more than 1 million cups daily [1,2]. *M. chamomilla* is used in the treatment of many ailments and disorders; internally to facilitate digestion and as antispasmodic, externally to treat minor wounds. In folk medicine, its use spreads from the relief of various pains such as headaches and toothaches to the facilitation of menstruation [3]. Chamomile essential oil (EO) has been commonly applied in Iranian folk medicine as an anti-inflammatory, antispasmodic, anti-peptic ulcer, antibacterial and antifungal agent [4,5].

Several papers report sesquiterpenes as the most dominant constituent of *M. chamomilla* EO. Spathulenol (12.50%) [6], α -bisabolol oxide A (7.9–62.1%) [7–10], α -bisabolol oxide B (25.56%) [10],

β -farnesene (52.73%) [11], (*E*)- β -farnesene (42.59%) [12], β -cubebene (27.8%) [13], *trans*- β -farnesene [14,15], chamazulene (27.8–31.2%) [16], and α -bisabolol (56.9%) [17] were recently reported as the main EO compounds.

Non-volatile compounds of *M. chamomilla* have also been investigated. The major phytochemicals comprise polyphenols, particularly the flavonoids apigenin, patuletin, quercetin, luteolin and their glucosides [18–20]; among them apigenin is reported as one of the most bioactive phenolics [19,21].

In the present study, EO compositions of twelve Iranian *M. chamomilla* populations collected from diverse regions were qualitatively and quantitatively assessed. Since the main radical scavengers are phenolics, flavonoids being the major group within phenolics [22], our study was also designed to determine apigenin and luteolin contents, as its main flavonoid aglycons [19,23], by utilizing analytical HPLC. Antiradical capacities of the extracts with DPPH and ORAC assays were also compared. In order to make comparison, the plants were harvested at the same flourishing period. To the best of our knowledge, this is the first report of these populations.

2. Results

2.1. Essential Oil Contents

As shown in Table 1, the studied plant samples were characterized mostly with similar EO yields. Populations “B” ($1.03 \pm 0.003\%$) and “BM” ($0.78 \pm 0.017\%$) contained the highest and lowest amount of EO, respectively (Table 1). As the plant sample “B” was cultivated in the university’s garden and it was regularly irrigated, the highest EO content can be predicted.

2.2. Chemical Profiles of Volatile Oils

Seventeen compounds were detected in these twelve populations. The sesquiterpene α -bisabolone oxide A (45.64–65.41%) was the major EO constituent in the samples except “B” and “S”, whereas its concentration was the highest in population “Mu” (Table 1).

The cultivated sample “B” was rich in α -bisabolol oxide B (21.88%) and chamazulene (19.22%), while the percentages of these compounds were lower under the wild growth conditions. The blue colour of EOs in all samples except “S” represented their chamazulene contents, whereas, “S” due to lack of this compound showed a yellowish green colour. Accordingly, oxygenated sesquiterpenes (53.31–74.52%) were the predominant chemical group of EO constituents in all studied samples, excluding “S”, which was rich in sesquiterpene hydrocarbons (Figure 1).

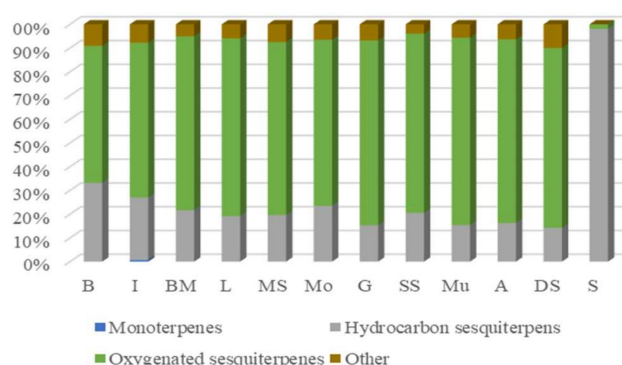


Figure 1. Volatile oil components from *Matricaria chamomilla* populations as a percentage of total identified compounds.

Table 1. Essential oil constituents and yields of twelve harvested *Matricaria chamomilla* populations.

No.	RI ^A	RT ^B	Populations Compounds ^C	B	I	BM	L	MS	Mo	G	SS	Mu	A	DS	S	
				Amount (%)												
1	1063	7.79	Artemisia ketone	nd	0.65	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
2	1423	17.75	<i>Trans</i> -caryophyllene	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	0.5
3	1454	18.75	(<i>E</i>)- β -Farnesene	8.51 ^b	16.68 ^a	9.58 ^{a,b}	10.60 ^{a,b}	13.92 ^{a,b}	13.91 ^{a,b}	9.82 ^{a,b}	6.61 ^b	10.33 ^{a,b}	15.29 ^a	12.37 ^{a,b}	5.25 ^b	2.21
4	1484	19.45	Germacrene D	1.31	nd	nd	nd	nd	nd	nd	nd	nd	0.55	nd	nd	2.21
5	1500	20	Bicyclo-germacrene	2.01	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
6	1506	20.21	(<i>Z</i>)- α -Bisabolene	nd	nd	nd	nd	nd	0.81	nd	nd	nd	nd	nd	nd	nd
7	1514	20.3	(<i>Z</i>)- γ -Bisabolene	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	40.08
8	1529	20.7	(<i>E</i>)- γ -Bisabolene	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	42.76
9	1561	21.65	(<i>E</i>)-Nerolidol	1.76	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
10	1577	22.1	(+)-Spathulenol	1.53	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
11	1630	23.18	(γ)-Eudesmol	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	1.78
12	1656	24.16	α -Bisabolol oxide-B	21.88 ^a	1.55 ^{b,c}	1.52 ^{b,c}	1.59 ^{b,c}	1.39 ^c	1.65 ^{b,c}	1.68 ^{b,c}	1.80 ^b	1.58 ^{b,c}	1.64 ^{b,c}	1.37 ^c	nd	nd
13	1685	24.56	α -Bisabolol	nd	nd	nd	nd	1.32	2.86	2.07	nd	nd	nd	nd	nd	nd
14	1693	24.9	α -Bisabolone oxide A	11.36 ^d	47.91 ^{b,c}	53.28 ^b	52.14 ^{b,c}	46.98 ^c	46.74 ^c	45.64 ^c	51.87 ^{b,c}	65.41 ^a	47.7 ^{b,c}	49.18 ^{b,c}	nd	nd
15	1730	25.76	Chamazulene	19.22 ^a	8.29 ^{b,c}	9.74 ^b	4.74 ^e	8.44 ^{b,c}	6.14 ^d	8.02 ^c	9.3 ^{b,c}	4.18 ^e	2.58 ^f	6.06 ^d	nd	nd
16	1748	26.18	α -Bisabolol oxide A	16.78 ^{b,c}	14.03 ^c	13.93 ^c	19.35 ^b	16.75 ^{b,c}	19.41 ^b	24.02 ^a	22.26 ^{a,b}	7.53 ^d	20.25 ^b	17.22 ^{b,c}	nd	nd
17	1890	26.31	(<i>E</i>)-Spiroether	8.37 ^a	7.51 ^b	4.70 ^c	5.75 ^{b,c}	7.12 ^{a,b}	6.41 ^b	6.49 ^b	3.73 ^c	5.31 ^{b,c}	5.96 ^b	7.26 ^{a,b}	nd	nd
Total identified compounds %				92.73	96.64	92.76	94.2	95.27	96.51	97.71	95.59	94.35	94.53	93.47	93.08	
EOs yield %				1.03 \pm 0.003	0.84 \pm 0.006	0.78 \pm 0.17	0.88 \pm 0.012	0.94 \pm 0.035	0.79 \pm 0.006	0.88 \pm 0.021	0.91 \pm 0.009	0.9 \pm 0.023	0.89 \pm 0.006	0.83 \pm 0.009	0.98 \pm 0.021	

^A Relative retention index to C₈–C₂₄ *n*-alkanes on HP-5MS column; ^B Retention times; ^C Compounds listed in order of elution from HP-5MS column; nd: not detected; the means were compared using Duncan's comparisons test ($p < 0.05$); small letters (^{a, b, c}) in each row show the significant difference of related component among various populations.

PCA is a mathematical procedure that transforms several correlated variables into various uncorrelated variables called principal components (PC). PC1, PC2, and PC3 showed the highest variation of phytochemicals among the studied populations. PC1 explained 41.57% of total variation and had a positive correlation with α -bisabolol oxide A, (*E*)- β -farnesene, α -bisabolone oxide A and (*E*)-spiroether, and negative correlation with (*Z*)- γ -bisabolene and (*E*)- γ -bisabolene. The second PC (PC2), with 24.98% of variance, demonstrated positive correlation with chamazulene, α -bisabolol oxide B, α -bisabolone oxide A and the EO content. Furthermore, PC3 represented positive correlation in the case of apigenin and luteolin, which accounted for 12.02% of the total variance (Table 2).

Table 2. Eigenvalues, variance and cumulative variance for three principal components.

Major Phytochemicals	Principal Components		
	PC1	PC2	PC3
Chamazulene	0.377	0.881	0.023
α -Bisabolol oxide A	0.760	0.126	0.114
(<i>E</i>)- β -Farnesene	0.679	−0.293	−0.004
α -Bisabolol oxide B	0.044	0.961	0.011
α -Bisabolone oxide A	0.742	0.525	0.104
(<i>E</i>)-Spiroether	0.849	0.377	−0.032
(<i>Z</i>)- γ -Bisabolene	−0.965	−0.119	−0.130
(<i>E</i>)- γ -Bisabolene	−0.965	−0.119	−0.130
Essential oil content	−0.480	0.600	−0.348
Apigenin	0.002	−0.295	0.638
Luteolin	0.160	0.235	0.857
Eigen values	4.57	2.74	1.32
Variance (%)	41.57	24.98	12.02
Cumulative variance (%)	41.57	66.55	78.57

Regarding a significant contribution of phytochemical variation in PC1 and PC2, the scatter plot of PC1 and PC2 was used to specify phytochemical distance. The studied populations were classified in three groups, which confirmed the CA results.

In accordance with the CA, the populations “I”, “DS”, “Mo”, “MS”, “Mu”, “G”, “SS”, “L”, “A” and “BM” were classified into the same category, while, “B” and “S” were grouped into the individual subclasses. The first group possessed α -bisabolone oxide A and α -bisabolol oxide A as the major constituents as well as apigenin and luteolin (chemotype I). The second chemotype (II), was characterized by high amounts of chamazulene and α -bisabolol oxide B. The chemotype (III) was the richest in (*Z*) and (*E*)- γ -bisabolene.

2.5. Effect of Environmental Factors on Phytochemicals

In order to assess the effect of environmental factors on EO components, along with apigenin and luteolin contents, canonical correspondence analysis (CCA) was applied based on a matrix of three environmental factors including altitude, mean annual temperature (MAT), and mean annual precipitation (MAP) [24] and major EO compounds, along with apigenin and luteolin contents (Figure 5).

According to the CCA, phytochemicals of populations in first group were significantly affected by ecological parameters (altitude, temperature and precipitation) while the main oil composition and flavonoids of “Sarableh” and “Boldgold” were changed by genetic factors. Therefore, the “Sarableh” population can be introduced as a new chemotype.

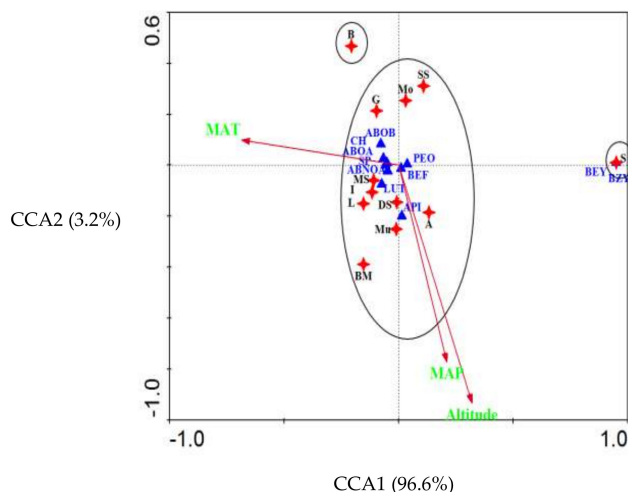


Figure 5. Canonical correspondence analysis (CCA) biplot of *Matricaria chamomilla* populations, linking percentages of the major constituents, collected from different environmental conditions. Populations B: Bodgold, I: Izeh, BM: Bagh Malek, L: Lali, MS: Masjed Soleyman, Mo: Mollasani, SS: Saleh Shahr, Mu: Murmuri, A: Abdanan, DS: Darreh Shahr, and S: Sarableh, MAT: mean annual temperature; MAP: mean annual precipitation; ABOA: α -bisabolol oxide A, ABOB: α -bisabolol oxide B, PEO: essential oil percentage, CH: chamazulene, SP: (*E*)-spiroether, ABNOA: α -bisabolone oxide A, BEF: (*E*)- β -farnesene, BZY: (*Z*)- γ -bisabolene, BEY: (*E*)- γ -bisabolene, LUT: luteolin, API: apigenin.

2.6. Antiradical Activity of the Extracts

In the evaluation of the antioxidant potential of the twelve selected populations, “S” showed the most significant antiradical capacity, with $EC_{50} = 7.76 \pm 0.3 \mu\text{g/mL}$ and $6.51 \pm 0.63 \text{ mmol TE/g}$ measured by DPPH and ORAC assays, respectively. However, the extracts showed lower activity compared to ascorbic acid ($EC_{50} = 0.3 \pm 0.02 \mu\text{g/mL}$) in the DPPH and rutin ($20.22 \pm 0.63 \text{ mmol TE/g}$) and EGCG ($11.97 \pm 0.02 \text{ mmol TE/g}$) in the ORAC assay (Figures 6 and 7).

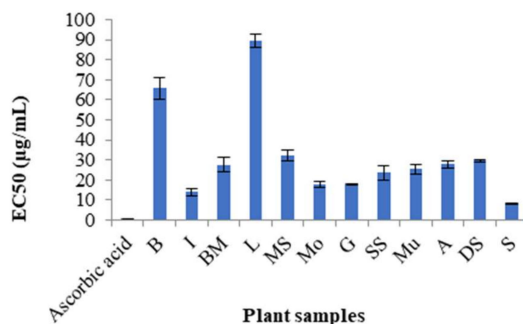


Figure 6. Antiradical scavenging activity of twelve plant samples of *Matricaria chamomilla* in the DPPH assay.

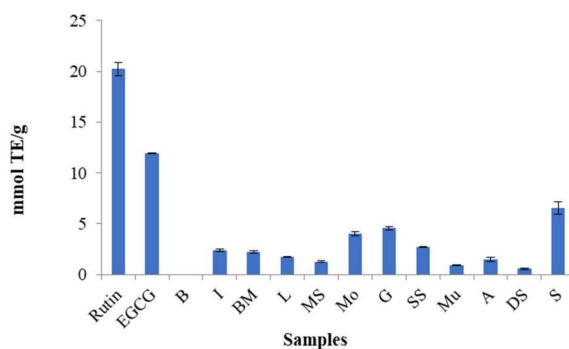


Figure 7. Antiradical capacity of selected *Matricaria chamomilla* populations in the ORAC assay.

3. Discussion

The results of the present study confirm the variability of EO composition, flavonoid profile and antiradical activity, which are significantly affected by a diversity of ecological conditions.

The abiotic factors (e.g., moisture, topography, temperature, and edaphic factors) highly impact the phytoconstituents variation, and/or biotic factors remarkably influenced the terpene biosynthesis pathways and chemotype profiles [25]. However, the role of other ecological effects such as climatic factors (e.g., humidity, wind, atmospheric gases, and light), physiographic factors (e.g., steepness and sunlight on vegetation and direction of slopes), edaphic factors (the soil attributes), and biotic factors (e.g., the existence of lianas, epiphytes, parasites etc.) may also be considerable in chemotype variations.

In accordance with our findings, oxygenated sesquiterpenes (53.31–74.52%) are the most dominant EO compounds of the selected *Matricaria chamomilla* L. samples, which corroborates previous reports. The wild populations were significantly different compared with the cultivated sample (Bodgold) in EO composition. α -Bisabolone oxide A was mostly the major EO constituent in the populations.

In the literature, α -bisabolone oxide A was previously identified from *M. chamomilla* by different groups; for instance, the EO, diethyl ether and dichloromethane fractions of EO contained α -bisabolone oxide A, with 47.7, 57.7 and 50.5%, respectively [8]; whereas the EO of an Estonian *M. chamomilla* sample was characterized by high bisabolone oxide A content (13.9%) [2]. This compound was the major EO constituent of *M. chamomilla* grown in Italy (9.2–11.2%) [26], Iran (53.45%) [27], India (20.4 and 8.9%) [28] and Turkey (47.7%) [7]. The evaluation of the daily α -bisabolol oxide A content in *M. chamomilla* EO revealed that the highest amount can be between 16:00–18:00 (55.41%) [29]. Moreover, this aromatic phytoconstituent was isolated with three extraction methods (7.9, 42.3 and 50.5%) in EO of the species [9].

It is noteworthy, that the sample “Sarableh” (with the highest altitude and least minimum and maximum annual temperatures) as a new chemotype was the richest in two geometric isomers of γ -bisabolene (82.84%), whilst these sesquiterpenes were not markedly detected in the other plant populations. This population also demonstrated the most potent antiradical capacity compared with those samples.

γ -Bisabolene was previously specified as the major characteristic constituent of *Pimpinella pruatjan* Mol. [30]. EO of *Ocimum africanum* Lour. was also rich in (*E*)- γ -bisabolene (2.6–9.5%) [31]. This sesquiterpene was isolated from seeds of *Ziziphus jujuba* var. *inermis*. [32]. The main EO compounds of *M. chamomilla* harvested in Iran were identified as α -bisabolol (7.27%), (*Z,Z*)-farnesol (39.70–66.00%) [33], (*E*)- β -farnesene (24.19%) [34] and α -bisabolol oxide A (17.14%) [35]. EOs containing remarkable amounts of γ -bisabolene exhibited anti-inflammatory properties and anti-proliferative activities in human prostate cancer, glioblastoma, lung carcinoma, breast carcinoma, colon adenocarcinoma and human oral squamous cell lines [36–39].

According to the results, the abundance of luteolin was much higher than the apigenin content. The lack of luteolin and apigenin as the selected flavones was reported in populations “MS” and “Mo”. These samples are most probably rich in other phenolic natural products.

The total phenolic and flavonoid contents of a *M. chamomilla* extract were 37.51 and 21.72 mg/g of dry weight, respectively; this report was formerly accomplished by using HPLC-DAD [40]. Moreover, chlorogenic acid, apigenin-7-glucoside, rutin, cynaroside, luteolin, apigenin and apigenin-7-glucoside derivatives were previously qualified as the significant phytochemical composition of *M. chamomilla* extract [23].

As apigenin and luteolin were present in “Sarableh” in low concentrations, the high free radical scavenging capacity of this sample is obviously related to other polyphenolic compounds.

In a similar study, methanol extracts of *M. chamomilla* yielded from different samples indicated more potent antiradical activity than EOs analysed by the DPPH test, due to polyphenols present in extracts; although, they possessed a moderate effect in comparison with the standards [6].

Furthermore, *in vitro* antioxidant activities of *M. chamomilla* extracts, along with its apigenin and apigenin-7-glycoside contents were previously characterized, with IC₅₀ of 18.19 ± 0.96 µg/mL, 2.0 ± 0.1 mg/g and 20.1 ± 0.9 mg/g, respectively [41], confirming the primary importance of apigenin

in the antioxidant capacity of *M. chamomilla*. Moreover, free radical scavenging activity (DPPH assay) of *M. chamomilla* volatile oil and its major components were formerly recorded in the following order: chamazulene > α -bisabolol oxide A > chamomile EO > (*E*)- β -farnesene > α -bisabolol [7].

4. Materials and Methods

4.1. Plant Material

M. chamomilla flowers (300 g/sample) were individually collected from different regions of Iran in the flowering period, in spring (April) 2017. The harvested populations “Izeh”, “Bagh Malek”, “Lali”, “Masjed Soleyman”, “Mollasani”, “Gotvand” and “Saleh Shahr” from Khuzestan and “Murmuri”, “Abdanan”, “Darreh Shahr” and “Sarableh” from Ilam province were compared with “Bodgold”, which was cultivated at the botanical garden of Department of Horticultural Sciences, Shahid Chamran University of Ahvaz, Ahvaz, Iran.

The plants were identified by Dr. Mehrangiz Chehrizi affiliated with the same department, and a voucher specimen of each sample was deposited in the herbarium of the department. The voucher’s codes and geographic coordinates including the latitude, longitude, altitude using the Global Positioning System (GPS), along with mean annual temperatures of the studied populations are given in Table 3. The meteorological data (MAP, MAT, MMaxAT, and MMinAT) were collected from September until May 2017 [24]. The flowers were shade dried and finely ground. Each powdered sample was individually well-mixed and subjected to analysis.

4.2. Chemicals and Spectrophotometric Measurements

Analytical grade 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,2'-azobis-2-methylpropionamide dihydrochloride (AAPH) and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox[®]) (Sigma-Aldrich, Steinheim, Germany), fluorescein (Fluka Analytical, Buchs, Germany); ascorbic acid, rutin and Na₂SO₄ (Merck, Darmstadt, Germany); epigallocatechin gallate (EGCG), apigenin ($\geq 99\%$) and luteolin ($\geq 97\%$) (Sigma-Aldrich, Germany) were purchased. Furthermore, all solvents of analytical grade were provided by the Merck company (Germany). Spectrophotometric measurements were carried out by a UV-VIS spectrophotometer (FLUOstar Optima, BMG Labtech, Ortenberg, Germany).

4.3. Extraction of Volatile Oils

To extract the EOs, 60 g of each powdered sample was individually subjected to Clevenger apparatus (hydro-distillation method) for 3 h. The obtained EOs were dried over anhydrous sodium sulphate and stored in refrigerator at 4 °C until analysis. Yields of extracted EOs were calculated by Equation (1):

$$\text{EO\%} = (\text{EOs weight}) / (\text{dried plants weight}) \times 100 \quad (1)$$

Diethyl ether was used to elute the whole amount of EOs from the apparatus, and the weighing process was performed after evaporating the solvent.

4.4. Gas Chromatographic Analysis (GC-FID)

In the case of GC-FID analysis, the EOs were analyzed by Shimadzu GC-17A (Kyoto, Japan) equipped with an FID detector and SGE[™] BP5 capillary column (Trajan Scientific and Medical, Victoria, Australia) (30 m \times 0.25 mm column with a 0.25 μ m film thickness). The split mode in GC was a ratio of 1:100. Injector and FID detector temperatures were set at 280 and 300 °C, respectively. The oven temperature was kept at 60 °C for 1 min and then raised to 250 °C at 5.0 °C/min and held for 2 min, while the ambient oven temperature range was +4 to +450 °C. Helium gas was used at a flow rate of 1 mL/min as a carrier gas.

Table 3. Geographic locations and climatic conditions of the studied *Matricaria chamomilla* populations from Iran.

Population Name	Voucher's Code	Abbreviated Name	Altitude (m)	Latitude	Longitude	MAP (mm/year)	MAT (°C)	MMaxAT (°C)	MMinAT (°C)
Bodold (Ahvaz)	KHAU_236	B	16	31°18' N	48°39' E	98.20	22.62	35.77	9.47
Izeh	KHAU_237	I	428	31°57' N	48°49' E	472.80	18.35	31.28	5.42
Bagh Malek	KHAU_238	BM	907	31°19' N	50°05' E	285.90	20.28	32.17	8.4
Lali	KHAU_239	L	373	32°20' N	49°05' E	280.60	21.26	33.57	8.95
Masjed Soleyman	KHAU_240	MS	250	32°02' N	49°11' E	241.30	22.67	34.27	11.07
Mollasani	KHAU_241	Mo	51	31°39' N	48°57' E	100.80	22.26	36.35	8.17
Gotvand	KHAU_242	G	70	32°14' N	48°48' E	159.70	21.08	34.96	7.21
Saleh Shahr	KHAU_243	SS	65	32°04' N	48°40' E	164.30	20.69	34.07	7.32
Murmuri	KHAU_244	Mu	530	32°46' N	47°37' E	213.90	23.35	35.17	11.53
Abdanan	KHAU_245	A	740	32°55' N	47°31' E	363.60	19.62	30.44	8.8
Darreh Shahr	KHAU_246	DS	629	33°05' N	47°28' E	463.00	17.85	30.9	4.8
Sarableh	KHAU_247	S	1037	33°47' N	46°35' E	345.80	15.01	27.31	2.71

MAP: mean annual precipitation; MAT: mean annual temperature; MMaxAT: mean maximum annual temperature; MMinAT: mean minimum annual temperature.

4.5. Gas Chromatography-Mass Spectrometric (GC-MS) Analysis

Analysis of the samples was carried out using an Agilent 7890B gas chromatograph (Agilent Technologies, Inc., Santa Clara, CA, USA) equipped with a 5977B mass spectrometry detector. The GC instrument was equipped with a split inlet, working in a split ratio of 100:1 mode with a 30 m × 0.25 mm HP-5MS capillary column with a 0.25 µm film thickness and 0.25 µm particle size (temperature range: −60 to +320/340 °C). The injection port temperature was 250 °C. The oven temperature was kept at 60 °C for 1 min and then programmed from 60 °C to 250 °C at 5 °C/min; then, the temperature was kept at 250 °C for 2 min. Helium (99.999%) was used as a carrier gas with a flow rate of 1 mL/min and inlet pressure 35.3 kPa. The mass spectrometer was operated in the electron impact mode at 70 eV, and the inert ion source (HES EI) temperature was set to 350 °C; the temperature of the quadrupole was set at 150 °C, while the MS interface was set to 250 °C. A scan rate of 0.6 s (cycle time: 0.2 s) was applied, covering a mass range from 35 to 600 amu.

4.6. Identification of Essential Oil Components

Most of the compounds were identified using two different analytical approaches: (a) comparison of Kovats indices of *n*-alkanes (C₈–C₂₄) [42] and (b) comparison of mass spectra (using authentic chemicals and Wiley spectral library collection). Identification was considered tentative when based on mass spectral data alone. In GC-FID and GC-MS, data acquisition and analysis were performed using Chrom-card™ (Scientific Analytical Solutions, Zurich, Switzerland, version DS) and Xcalibur™ software (Thermo Fisher Scientific, Waltham, MA, USA, 4.0 Quick Start), respectively.

4.7. Preparation of Solvent Extracts

Five g of each sample was individually extracted with MeOH (3 × 75 mL) in an ultrasonic bath (VWR-USC300D) for 10 min, at 40 °C under power grade 9.

After removing the solvent under reduced pressure at 50 °C (Rotavapor R-114, Büchi), the concentrated extracts were subjected to evaluate the antiradical assays and HPLC analysis.

4.8. HPLC Analysis of Apigenin And Luteolin

Twenty µL of each extract (1 mg/mL) was separately injected into an analytical high-performance liquid chromatography system (HPLC) (Knauer, Berlin, Germany) by using an end capped Eurospher II 100-5 C18, Vertex Plus Column (Knauer, Berlin, Germany) (250 × 4.6 mm with precolumn) with particle size: 5 µm, pore size: 100 Å and temperature 30 °C; coupled to UV detector (Knauer GmbH-Smartline 2600, Berlin, Germany) at a wavelength range 190 to 500 nm (quantification at 330 nm), while MeOH/H₂O was applied as the mobile phase with a gradient system, increasing MeOH from 30% to 70% within 40 min, with a flow rate of 1 mL/min, at ambient temperature. Analysis was performed using SAS software, version 9.2 (SAS Institute Inc., Cary, NC, USA).

4.9. Antiradical Capacity

4.9.1. DPPH Assay

Free radical scavenging activity of the plant extracts was assessed by DPPH assay [43]. The measurement was carried out on 96-well microtiter plates. In brief, Microdilution series of samples (1 mg/mL, dissolved in MeOH) were prepared starting with 150 µL. To gain 200 µL of sample, 50 µL of DPPH reagent (100 µM) was added to each sample. The microplate was stored at room temperature under dark conditions. The absorbance was measured after 30 min at 550 nm using the microplate reader. MeOH (HPLC grade) and ascorbic acid (0.01 mg/mL) were used as a blank control and standard, respectively. Antiradical activity was calculated using the following Equation (2):

$$I\% = [(A_0 - A_1/A_0) \times 100] \quad (2)$$

where A_0 is the absorbance of the control and A_1 is the absorbance of the standard sample. Anti-radical activity of the samples was expressed as EC_{50} (concentration of the compounds that caused 50% inhibition). EC_{50} ($\mu\text{g}/\text{mL}$) values were calculated using GraphPad Prism software version 6.05 (GraphPad Software, San Diego, CA, USA). Each sample was measured in triplicate.

4.9.2. ORAC Assay

Twelve extracts were subjected to ORAC assay [44] with slight modifications. Microtiter plates (96-well) were used for measurement of the samples. Briefly, 20 μL of extracts (0.01 mg/mL) was mixed with 60 μL of AAPH (peroxyl free radical generator) (12 mM) and 120 μL of fluorescein solution (70 mM). Then, the fluorescence was measured for 3 h at 1.5-min cycle intervals with the microplate reader. The standard Trolox[®] was used. Activity of all samples was compared with rutin and EGCG as positive controls. Antioxidant capacities were reported as $\mu\text{mol TE}$ (Trolox[®] equivalents)/g of dry matter.

4.10. Statistical Analysis

All the experiments were done in triplicate, and the results are expressed as means \pm SD. The data were assessed with one-way analysis of variance (ANOVA) using SAS software (version 9.2, SAS Institute Inc., Cary, NC, USA) and GraphPad Prism version 6.05. The means were compared using Duncan's comparisons test ($p < 0.05$).

5. Conclusions

According to our preliminary study, the chemo-diversity and antiradical potential of twelve studied *Matricaria chamomilla* populations were highly affected by a variety of ecological conditions. To acquire the best yield with a good profile of active principles, it is crucial to combine a good genotype with optimal environmental circumstances.

In accordance with our findings, oxygenated sesquiterpenes are the most dominant EO compounds of the selected *Matricaria chamomilla* samples. Almost all plant populations contained apigenin and luteolin. Since the plant sample "Sarableh" indicated the most potent capacity to scavenge free radicals and its apigenin and luteolin contents were insignificant, its activity undoubtedly refers to the presence of other polyphenolic compounds. Due to high amounts of apigenin and luteolin, populations "Lali" and "Bagh Malek" can be considered as a rich source of these compounds. Our results confirm the effects of growth conditions on the quantity and quality of aromatic phytochemicals. More investigations are required to study the amounts of other polyphenolic compounds in diverse populations and the correlations between secondary metabolite contents, bioactivities and ecological effects.

Author Contributions: E.P. harvested the plant materials and performed the essential oil extraction. M.M.S. designed the experiments and analyzed the essential oils. E.K. conducted the study. Bioactivity was analyzed by Z.P.Z., J.M. wrote, and J.H. and D.C. revised the manuscript.

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Comprehensive chemotaxonomic analysis of saffron crocus tepal and stamen samples, as raw materials with potential antidepressant activity

Javad Mottaghipisheh^a, Mohammad Mahmoodi Sourestani^b, Tivadar Kiss^a, Attila Horváth^a, Barbara Tóth^a, Mehdi Ayanmanesh^c, Amin Khamushi^d, Dezső Csopor^{a,*}

^a Department of Pharmacognosy, University of Szeged, Eötvös u. 6, H-6720, Szeged, Hungary

^b Department of Horticultural Science, Faculty of Agriculture, Shahid Chamran University of Ahvaz, Ahvaz, 61357-43311, Iran

^c Department of Horticultural Science, Islamic Azad University, Estahban Branch No. 69, Niroo Av., Satarkhan Str., 14536-33143, Tehran, Iran

^d Department of Horticultural Science, Faculty of Agriculture, University of Mashhad, Mashhad, Iran

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ABSTRACT

Saffron crocus (*Crocus sativus* L.) has been widely grown in Iran. Its stigma is considered as the most valuable spice for which several pharmacological activities have been reported in preclinical and clinical studies, the antidepressant effect being the most thoroughly studied and confirmed. This plant part contains several characteristic secondary metabolites, including the carotenoids crocetin and crocin, and the monoterpenoid glucoside picrocrocin, and safranal. Since only the stigma is utilized industrially, huge amount of saffron crocus by-product remains unused. Recently, the number of papers dealing with the chemical and pharmacological analysis of saffron is increasing; however, there are no systematic studies on the chemical variability of the major by-products.

In the present study, we harvested saffron crocus flowers from 40 different locations of Iran. The tepals and stamens were separated and subjected to qualitative and quantitative analysis by HPLC-DAD. The presence and amount of seven marker compounds, including crocin, crocetin, picrocrocin, safranal, kaempferol-3-*O*-sophoroside, kaempferol-3-*O*-glucoside, and quercetin-3-*O*-sophoroside were determined.

The analytical method was validated for filter compatibility, stability, suitability, accuracy, precision, intermediate precision, and repeatability. Tepal and stamen samples contained three flavonol glycosides. The main constituent of the tepals was kaempferol-3-*O*-sophoroside (62.19–99.48 mg/g). In the stamen, the amount of flavonoids was lower than in the tepal. The amount of kaempferol-3-*O*-glucoside, as the most abundant compound, ranged between 1.72–7.44 mg/g. Crocin, crocetin, picrocrocin, and safranal were not detected in any of the analysed samples.

Our results point out that saffron crocus by-products, particularly tepals might be considered as rich sources of flavonol glycosides. The data presented here can be useful in setting quality standards for plant parts of *C. sativus* that are currently considered as by-products of saffron production.

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1. Introduction

Saffron crocus (*Crocus sativus* L., Iridaceae) is cultivated in some Asian and European countries, in highest extent in Iran. The stigma of this plant (known as saffron) is a popular spice and has also been applied in the traditional Arabic and Islamic medicine for several purposes, especially as cardiac and liver tonic and hep-

atic deobstruent, to facilitate difficult labour and for the treatment of female genito-urinary disorders and male impotence. Its effects on the central nervous system have also been recorded; however, old texts have to be reinterpreted according to the current conceptions of medicine and pharmacology. According to Sayyed Esma'il Jorjani (1042–1136 A.D.) saffron is astringent and resolvent and its fragrance can strengthen these two effects. Hence, its action on enlivening the essence of the spirit and inducing happiness is great. This supposed activity might be translated as a positive effect on mood or as an antidepressant effect [1].

* Corresponding author.

E-mail address: csupor.dezso@pharmacognosy.hu (D. Csopor).

There is an overlap between the traditional uses and the evidence-based applications of saffron. The clinical efficacy of saffron has been studied in diabetes [2], age-related macular degeneration [3,4], cognitive impairment [5–8], glaucoma [9], sexual dysfunction in women [10] and men [11], and premenstrual syndrome [12]. However, most of the studies assessed its antidepressant activity and the efficacy in mild to moderate depression has been confirmed by a recent meta-analysis as well [13].

Crocin, crocetin, picrocrocin and safranal are the main characteristic compounds of saffron stigma. The colour of saffron is due to the carotenoids crocin and crocetin, the specific bitterish taste is attributed to the monoterpene glycoside picrocrocin, whereas safranal, an aromatic aldehyde of the volatile oil contributes to the aroma [1].

Saffron is known as the most expensive spice in the world, 300 000 flowers are approximately required to obtain 1 kg of dried stigma [14]. Considering its price and the increasing scientific interest for the bioactivities of saffron, the analysis of alternative plant parts (the industrial by-products tepal and stamen), that are available in larger amounts and hence are cheaper, seems to be promising approach. Several papers have reported the constituents of major saffron crocus by-products. It has to be noted, that several papers report the chemical composition of sepal and petal samples, however, botanically these plant parts should be defined as tepal. Therefore, in case of previous papers we refer to the plant parts used by the authors. The petals of the plant were characterized by a high total phenolic and flavonoid content compared to stamens and styles [14,15]. Both sepals and stamens of samples from different regions of Italy were characterized with kaempferol-3-*O*-sophoroside as main flavonoid constituent [16]. From the flower material of saffron crocus (except stigma) kaempferol-3-*O*-sophoroside was isolated as the major component [17]. Beside flavonols, anthocyanins are also present in the floral bioresidues of saffron crocus as major constituents, delphinidin 3,5-di-*O*-glucoside being the predominant compound [18]. From a methanolic extract of tepals, kaempferol glycosides comprising kaempferol 3-*O*-sophoroside, kaempferol 3-glucoside, and kaempferol 3-*O*- β -D-(2-*O*- β -D-6-acetylglucosyl)glucopyranoside-7-*O*- β -D-glucopyranoside were isolated as the major compounds [19]. Kaempferol 3-*O*-sophoroside was identified as main component by utilizing HR-MAS NMR spectroscopy as well [20]. Other studies have also reported flavonoids from tepals. Kaempferol-3-*O*-sophoroside, kaempferol-3-*O*-glucoside, and quercetin-3-*O*-sophoroside were reported in these studies [21,22].

The antidepressant activities of tepals have been studied in animal experiments. Both the aqueous and ethanolic extracts of stigma and tepal decreased immobility time in comparison with normal saline in the forced swimming test in mice [23]. Kaempferol, a flavonoid of the tepals was reported to have antidepressant activity on mice and rats in the same test [24].

The efficacy of tepals has been confirmed in two clinical trials. In a randomized, double-blind trial, 40 patients with mild to moderate depression were treated either with 30 mg saffron crocus tepal or 20 mg fluoxetine for 8 weeks. The herbal preparation was similarly effective as fluoxetine with remission rates of 25 % in both groups [25]. In a similar trial with 40 patients, the efficacy of the tepals (30 mg) was compared to placebo. After 6 weeks of treatment, the tepal was more effective than placebo in improving the severity of depression using the Hamilton Depression Rating Scale [26].

Considering the increasing scientific and industrial interest for saffron crocus by-products, the aim of our study was to systematically analyse the composition of tepal and stamen samples. We developed and validated an HPLC/DAD method for the analysis of seven marker compounds previously identified as the major constituents of saffron crocus stigma, tepal and stamen. Crocin, crocetin, picrocrocin, safranal and

flavonoids [kaempferol-3-*O*-sophoroside (K.S.), kaempferol-3-*O*-glucoside (K.G.), quercetin-3-*O*-sophoroside (Q.S.)] were assessed qualitatively and quantitatively in 40 tepal and stamen samples collected from different regions of Iran.

2. Materials and methods

2.1. Plant materials

Saffron crocus (*Crocus sativus* L.) tepal and stamen samples were collected from 40 different locations of Iran at the same harvesting period in November 2018. All were dried under shade, then the tepals were accurately separated from stamens. The plant samples were individually packed in the sealed plastic bags and stored at room temperature. The growth locations, altitudes, and coordinates of the harvested plant materials are shown in Table 1. For comparison, a commercial saffron stigma sample was also analyzed (Bahraman Co., Mashhad, Iran).

2.2. Chemicals and reagents

All solvents were of analytical grade. Kaempferol-3-*O*-glucoside (K.G.) (Santa Cruz Biotech, California, USA), quercetin-3-*O*-sophoroside (Q.S.), crocetin (trans-crocetin, 98 %), and picrocrocin (Carbosynth, Berkshire, UK), safranal, and crocin (crocetin digentio-biose ester, Sigma-Aldrich, St. Louis, Missouri, USA) were purchased in analytical grade. Kaempferol-3-*O*-sophoroside (K.S.) was isolated in our laboratory, its structure and purity was determined by NMR.

2.3. Extract preparation

20 mg of tepal, 10 mg of stigma, and 50 mg of stamen samples were extracted with the solvent mixture EtOH-H₂O 1:1, using an ultrasonic bath for 15 min, then diluted with the above solvents to 10.0 mL (tepal) and 5.0 mL (stigma and stamen) in volumetric flasks, respectively.

In case of filtered samples, the extracts were filtered via a filter membrane (PTFE-L syringe filter, hydrophilic, FilterBio[®], diameter: 13 mm, pore size: 0.45 μ m), the first 1 mL was unused, and the rest 1.5 mL was analysed by HPLC-DAD. In case of centrifuged samples, centrifugation was performed by using DLAB D1008 instrument (7000 rpm) for 1 min. For all samples, three extracts were prepared and analysed in triplicate.

2.4. HPLC apparatus and measurement conditions

HPLC-DAD analysis was carried out on a Shimadzu SPD-M20A HPLC (Shimadzu Corporation, Kyoto, Japan), equipped with a Shimadzu SPD-M20A photodiode array detector, an on-line degasser unit (Shimadzu DGU-20A5R), a column oven (Shimadzu CTO-20AC column oven) and autosampler (Shimadzu SIL-20A8HT) using a RP Kinetex[®] C8 column (5 μ m, 100 Å, 150 \times 4.6 mm, Phenomenex, Torrance, USA) at 30 °C. Chromatographic elution of the samples was accomplished with a gradient solvent system by changing the ratio of MeOH in H₂O (containing 0.066 % of H₃PO₄) as follows: 30 % (0–1 min), 30–57% (1–7 min), 57–76% (7–12 min), 76–100% (12–13 min), keeping at 100 % for 1 min, 100 to 30 % (14–15 min) and keeping at 30 % for 3 min at a flow rate of 1.5 mL/min. The samples were monitored in the UV-VIS range (190–800 nm) and at the UV_{max} of the standards (picrocrocin: 247 nm, Q.S.: 360 nm, K.S.: 354 nm, K.G.: 348 nm, crocin: 441 nm, safranal: 316 nm, and crocetin: 427 nm). Data assessment and acquisition were performed with the LabSolutions (Version 5.82) software (Shimadzu, Kyoto,

Table 1
Provenances of saffron samples.

Code	Location	Altitude (m)	Latitude	Longitude
1T, 1S	Dehouye, Meshkan, Neyriz, Fars	1567	29° 15' N	53° 56' E
2T, 2S	Dehouye, Meshkan, Neyriz, Fars	1567	29° 15' N	53° 56' E
3T, 3S	Dehouye, Meshkan, Neyriz, Fars	1567	29° 15' N	53° 56' E
4T, 4S	Dehouye, Meshkan, Neyriz, Fars	1567	29° 15' N	53° 56' E
5T, 5S	Dehouye, Meshkan, Neyriz, Fars	1567	29° 15' N	53° 56' E
6T, 6S	Meshkan, Neyriz, Fars	2167	29° 28' N	54° 20' E
7T, 7S	Meshkan, Neyriz, Fars	2167	29° 28' N	54° 20' E
8T, 8S	Meshkan, Neyriz, Fars	2167	29° 28' N	54° 20' E
9T, 9S	Meshkan, Neyriz, Fars	2167	29° 28' N	54° 20' E
10T, 10S	Meshkan, Neyriz, Fars	2167	29° 28' N	54° 20' E
11T, 11S	Meshkan, Neyriz, Fars	2167	29° 28' N	54° 20' E
12T, 12S	Meshkan, Neyriz, Fars	2167	29° 28' N	54° 20' E
13T, 13S	Meshkan, Neyriz, Fars	2167	29° 28' N	54° 20' E
14T, 14S	Meshkan, Neyriz, Fars	2167	29° 28' N	54° 20' E
15T, 15S	Meshkan, Neyriz, Fars	2167	29° 28' N	54° 20' E
16T, 16S	Dehchah, Neyriz, Fars	2188	29° 13' N	54° 26' E
17T, 17S	Dehchah, Neyriz, Fars	2188	29° 13' N	54° 26' E
18T, 18S	Dehchah, Neyriz, Fars	2188	29° 13' N	54° 26' E
19T, 19S	Dehchah, Neyriz, Fars	2188	29° 13' N	54° 26' E
20T, 20S	Dehchah, Neyriz, Fars	2188	29° 13' N	54° 26' E
21T, 21S	Abadeh Tashk, Neyriz, Fars	1608	29° 48' N	53° 43' E
22T, 22S	Abadeh Tashk, Neyriz, Fars	1608	29° 48' N	53° 43' E
23T, 23S	Neyriz, Fars	1606	29° 11' N	54° 19' E
24T, 24S	MahmoudAbad, Neyriz, Fars	1579	29° 14' N	54° 17' E
25T, 25S	MahmoudAbad, Neyriz, Fars	1579	29° 14' N	54° 17' E
26T, 26S	MahmoudAbad, Neyriz, Fars	1579	29° 14' N	54° 17' E
27T, 27S	Roniz, Estahban, Fars	1593	29° 11' N	53° 46' E
28T, 28S	Roniz, Estahban, Fars	1593	29° 11' N	53° 46' E
29T, 29S	HasanAbad, Neyriz, Fars	1579	29° 44' N	53° 54' E
30T, 30S	Estahban, Fars	1797	29° 7' N	54° 2' E
31T, 31S	Estahban, Fars	1797	29° 7' N	54° 2' E
32T, 32S	Estahban, Fars	1797	29° 7' N	54° 2' E
33T, 33S	Estahban, Fars	1797	29° 7' N	54° 2' E
34T, 34S	Estahban, Fars	1797	29° 7' N	54° 2' E
35T, 35S	Estahban, Fars	1797	29° 7' N	54° 2' E
36T, 36S	Estahban, Fars	1797	29° 7' N	54° 2' E
37T, 37S	Ghaen, Khorasan e Jonoubi	1445	33° 43' N	59° 11' E
38T, 38S	Torbatejam, Khorasan e Razavi	894	35° 14' N	60° 37' E
39T, 39S	Dehdez, Khoozestan	1439	31° 42' N	50° 17' E
40T, 40S	Dehdez, Khoozestan	1439	31° 42' N	50° 17' E

T: tepal; S: stamen.

Japan). 10 and 20 μ L of tepal and stamen extracts were injected, respectively.

2.5. System validation

Validation of our analytical method was carried out according to the ICH Harmonised Guideline [27] and completed with further experiments. Validation was performed by establishing the calibration curves of seven reference compounds, determining the limit of detection and quantification values, assessing system suitability, accuracy, precision, repeatability, stability and filter compatibility of the extracts or pure compounds.

3. Results and discussion

3.1. Validation

Our goal was to develop and validate an analytical method that is suitable for the analysis of saffron stigma samples and saffron crocus by-products. Marker compounds that were used as reference standards during our experiments were chosen based on literature data as follows: crocin, crocetin, picrocrocin, safranal, K.S., K.G., and Q.S. During validation, tepal samples and the mixture of the reference compounds were used, and where it was possible, validation was carried out for all the analytes. However, since saffron crocus tepal did not contain crocin, crocetin, picrocrocin, and safranal, validation was partial for these compounds.

3.1.1. Calibration curve and linearity

Seven major components of saffron crocus, K.S., K.G., Q.S., crocetin, picrocrocin, safranal, and crocin were used to establish calibration curves and limit of detection (LoD) and limit of quantitation (LoQ) values (Table 2). Calibration curves are based on 8–11 calibration points. The correlation coefficient of the calibration curves was at least 0.998. Calibration curves covered 2 orders of magnitude of analyte concentration.

3.1.2. Filter compatibility

To select the best method for sample preparation, one tepal specimen was extracted by ultrasonic bath, then filtered or centrifuged. Except for crocetin, sample preparation by filtration or centrifugation had no major impact on quantitative results; however, in case of this compound, the amount of the analyte decreased with 17.3 % as the result of filtration. For the other analytes, slight higher values were measured in filtered samples (picrocrocin: 100.86 ± 0.35 %; Q.S.: 101.14 ± 0.31 %; K.S.: 100.89 ± 0.29 %; K.G.: 100.20 ± 1.4 %; crocin: 101.21 ± 0.38 %; safranal: 100.27 ± 1.37 %).

3.1.3. Stability

To evaluate the stability of the solutions of reference compounds, the standard mixtures were prepared by filtration or centrifugation, stored at 4 °C and room temperature (23 °C), then injected at day 0, 1, 3, 5, and 7 (Table 3). In case of picrocrocin and the flavonoids, storage time and temperature did not affect the concentration of the analytes, whereas in case of crocin and

Table 2
Calibration curve characteristics and limit of detection and quantification values.

Standard	LoD ($\mu\text{g}/\text{inj}$)	LoQ ($\mu\text{g}/\text{inj}$)	Calibration points	Range covered ($\mu\text{g}/\text{inj}$)	Regression equations	R ²
Picrocrocin	0.00245	0.00741	8	0.03-0.9	$y = (1.10320 \times 10^9)x - 10690.0$	0.9997394
Q.S.	0.02554	0.07739	10	0.082-6.15	$y = (6.55841 \times 10^8)x - 5275.02$	0.9996089
K.S.	0.02277	0.06901	9	0.076-3.04	$y = (5.96019 \times 10^8)x - 20062.4$	0.9996702
K.G.	0.06763	0.20495	9	0.1075-4.3	$y = (1.36039 \times 10^8)x - 4682.70$	0.9984738
Crocin	0.00128	0.00386	10	0.0145-0.58	$y = (2.92163 \times 10^9)x - 13570.0$	0.9994265
Safranal	0.00177	0.00536	8	0.08-2.4	$y = (2.06147 \times 10^9)x + 37991.1$	0.9982921
Crocetin	0.00032	0.00097	11	0.00172-0.129	$y = (9.35271 \times 10^9)x - 5778.24$	0.9999475

Table 3
Stability of the dissolved reference compounds after 1, 3, 5 and 7 days (values compared to 100 % day 0 values).

Sample	Days	Picrocrocin	Q.S.	K.S.	K.G.	Crocin	Safranal	Crocetin
23 °C	1	100.97	101.08	100.92	101.77	101.18	99.84	86.28
	3	104.06	100.59	100.69	101.80	95.93	87.89	76.71
	5	100.37	99.97	100.53	102.74	91.29	85.89	76.47
	7	100.59	99.70	101.12	105.00	90.65	83.71	78.94
4 °C	1	100.51	100.66	100.56	101.05	100.84	100.29	86.52
	3	104.09	100.66	100.56	101.82	100.57	99.74	85.92
	5	100.69	100.41	100.67	102.30	100.30	96.44	84.48
	7	100.77	100.89	101.27	104.98	100.50	94.64	85.44

Table 4
Results of the system suitability tests.

	Picrocrocin	Q.S.	K.S.	K.G.	Crocin	Safranal	Crocetin
RSD% of AUC	0.47	0.18	0.43	2.53	0.38	0.41	1.11
Tailing factor	1.139-1.145	1.043-1.067	1.113-1.127	1.356-1.408	1.177-1.910	1.171-1.184	1.310-1.334
RSD% of RT	0.66	0.62	0.36	0.22	0.19	0.14	0.10

RT: retention time.

Table 5
Recovery values (%) of the controls.

Level	Picrocrocin (stigma)		Q.S.		K.S.		K.G.		Crocin (stigma)		Safranal (stigma)		Crocetin (stigma)	
	%	RSD%	%	RSD%	%	RSD%	%	RSD%	%	RSD%	%	RSD%	%	RSD%
50 %	111.92	0.27	106.54	0.12	100.95	0.29	96.09	0.62	-	-	-	-	99.54	1.33
100 %	101.80	0.07	112.25	0.10	99.60	0.06	103.36	2.03	97.89	0.27	-	-	83.69	1.73
150 %	110.32	1.32	116.26	0.30	101.54	0.12	97.57	2.77	108.10	0.06	-	-	89.40	0.42

safranal, decomposition was observed especially at room temperature. Interestingly, the concentration of crocetin decreased remarkably after one day, and the temperature had no major influence on this process.

3.1.4. System suitability

The mixture of the reference standards was injected 5 times to assess suitability of the analytical system. The low RSD% values of the AUCs and retention times, together with the tailing factors below confirm that the system is suitable for the measurement of these compounds (Table 4).

3.1.5. Accuracy

The accuracy of the method was assessed by the determination of recoveries (Table 5). Recoveries of the marker compounds was assessed by adding known amounts of the standards to a tepal (or in case of crocin, picrocrocin, crocetin, and safranal: stigma) sample at three different concentrations (50, 100, and 150 % of the previously determined amounts). Three independent samples were prepared for each concentration levels and injected in triplicates. The recovery values ranged between 96.09–111.92 %, 83.69–112.25 %, and 89.40–116.26 % in case of 50 %, 100 %, and 150 % amounts of added analytes, respectively.

3.1.6. Precision

In order to determine the precision of the analytical method, one tepal and one stigma sample was extracted individually and injected 10 times. Precision was determined by calculating RSD% values of the AUCs (Table 6). The RSD% values below 1% confirm the precision of the method.

3.1.7. Repeatability

The repeatability of the experiment was performed by analysis of six tepal sample extracts within 24 h (intra-assay precision). Repeatability was characterized by RSD% values of the whole datasets for Q.S., K.S., and K.G., RSD% values were 3.38 %, 3.71 % and 3.44 %, respectively.

3.1.8. Intermediate precision

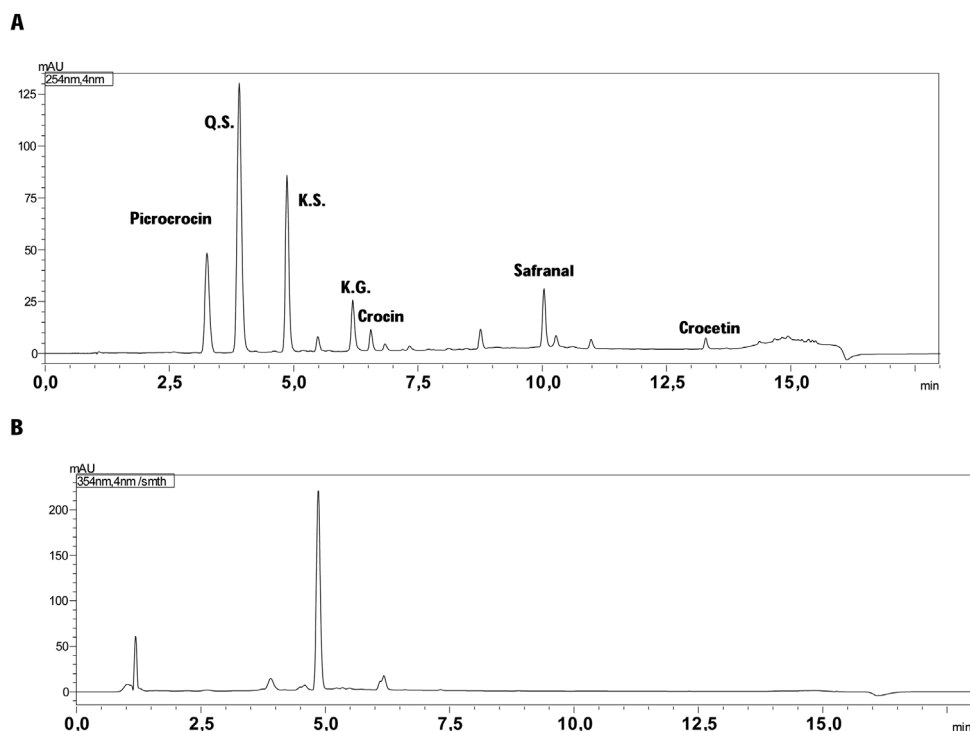
The same tepal sample was analysed by two analysts and intermediate precision was determined as RSD% of the means. For Q.S., K.S., and K.G. the RSD% values were 3.81 %, 2.29 %, and 6.85 %, respectively.

3.2. HPLC/DAD analysis of tepal and stamen samples

HPLC/DAD analysis of 40 different tepal and stamen samples was carried out to qualitatively and quantitatively analyse their selected marker compounds. Our newly developed HPLC method

Table 6
Precision of the analytical method as determined by RSD%.

Samples	Picrocrocin	Q.S.	K.S.	K.G.	Crocin	Crocetin
Tepal	–	1.72	0.07	0.53	–	–
Stigma	1.86	0.30	2.26	2.68	0,58	1.86

**Fig. 1.** HPLC chromatogram of the mixture of reference compounds (A), and of a tepal sample (B) (354 nm).

allowed good separation and hence reliable analysis of these compounds in saffron crocus samples (Fig. 1).

In stamen and tepal samples, three flavonols glycosides, namely Q.S., K.S., and K.G. were detected as major components. For comparison, we analysed a saffron stigma sample as well. From this sample picrocrocin, crocin, and crocetin were identified, which is in line with the previous data. There is only one report on the presence of crocin in saffron crocus tepal; however, very low amount (0.6 %) was reported in the hydrolysed extracts compared to kaempferol (12.6 %) [28]. A comparative study of the stamen and stigma revealed that crocin, picrocrocin, and safranal are not present in stamen, whilst they are major components of the stigma [29].

In Table 7, the amounts of marker compounds in tepal and stamen samples are presented as means (mg/g plant material) together with the standard deviation values. The tepal samples contained Q.S., K.S. and K.G., in the ranges of 6.20–10.82, 62.19–99.48, and 27.38–45.17 mg/g, respectively, whereas the amount of these compounds was lower in the stamen (1.72–6.07, 0.89–6.62 and 1.72–7.44 mg/g, respectively). K.S., as the major constituent of saffron crocus tepal was present in the highest amount in sample 1 with an amount of 99.48 mg/g. Tepal sample 23 contained the highest amount of Q.S. (10.82 mg/g), while tepal sample 5 contained the lowest amounts of Q.S. and K.S. with 6.21 and 62.19 mg/g, respectively. The content of K.G. in tepal samples was remarkably different from 27.74 to 45.18 mg/g analysed in samples 40 and 1, respectively. In general, the stamen samples contained less flavonoids than the tepals. The highest amount of Q.S. in stamens was observed in sample 29 with 6.08 mg/g, whilst sample 3 contained the lowest concentration (1.63 mg/g). The content of K.S.

ranged from 6.62 mg/g (sample 40) to 0.93 mg/g (sample 3) in the stamen. The quantity of K.G. was also variable, the highest and lowest amounts were determined in sample 31 and 8 with 7.44 and 1.72 mg/g, respectively.

Our results confirmed some former findings. K.S., K.G., and Q.S. were described as major or characteristic flavonoid components of flowers and tepals by several authors [16,17,19–20,21,22,29–31]. Flavonol derivatives (6–10 mg/g) were characterized as the major compounds of stamen and tepal samples harvested from two different region of Italy. K.S. was the major compound (6.41–8.30 mg/g of fresh tepals and 0.37–1.70 mg/g of fresh stamen) [16].

The comparison of our results to those published previously has some limitations. *Crocus* flowers, having monocot characteristic structure, consist of stamens, stigmas and tepal. However, numerous studies refer to perianth as “sepal” and “petal.” In some cases the investigated flower part is not clearly named, however, the description narrows it to tepals [21,22,32]. Some of the studies are referring to the investigated sample as “petals” without any further description [19,20,25,26], while some further studies refer to saffron flower without stigma [17,28,30,31].

3.3. Classification of saffron crocus populations

Cluster analysis (CA) and principal component analysis (PCA) were performed to characterize and classify saffron crocus tepal and stamen samples harvested from different locations in Iran according to their Q.S., K.S., and K.G. contents. According to the CA analysis, the saffron crocus samples collected from various locations were classified in three major groups demonstrating three distinct chemotypes based on their flavonoid contents. Chemotype

Table 7
Levels of marker compounds in saffron samples.

Sample	Q.S.		K.S.		K.G.	
	T	S	T	S	T	S
1	10.24 ± 0.23	4.23 ± 0.14	99.48 ± 0.07	1.82 ± 0.1	45.18 ± 0.24	3.65 ± 0.21
2	6.29 ± 0.04	3.49 ± 0.14	76.40 ± 1.37	1.47 ± 0.06	37.31 ± 0.77	2.47 ± 0.12
3	6.23 ± 0.15	1.63 ± 0.18	62.41 ± 1.41	0.93 ± 0.02	33.71 ± 0.98	2.42 ± 0.46
4	9.19 ± 0.32	5.60 ± 0.2	74.82 ± 3.32	1.53 ± 0.26	36.01 ± 1.6	3.69 ± 0.15
5	6.21 ± 0.2	4.56 ± 0.2	62.19 ± 2.06	2.21 ± 0.09	35.73 ± 1.23	4.88 ± 0.29
6	7.92 ± 0.34	2.11 ± 0.12	71.81 ± 3.67	1.03 ± 0.03	39.25 ± 2.27	2.05 ± 0.01
7	7.61 ± 0.30	1.72 ± 0.02	77.35 ± 3.96	1.03 ± 0.06	37.38 ± 1.73	2.01 ± 0.11
8	9.43 ± 0.23	1.98 ± 0.06	75.00 ± 0.55	0.90 ± 0.03	37.30 ± 0.46	1.72 ± 0.12
9	8.68 ± 0.29	1.80 ± 0.06	74.88 ± 2.84	1.20 ± 0.03	34.09 ± 1.09	2.72 ± 0.24
10	8.26 ± 0.10	1.79 ± 0.04	71.45 ± 1.51	1.03 ± 0.0	32.08 ± 0.07	2.17 ± 0.09
11	8.51 ± 0.38	2.02 ± 0.08	68.55 ± 1.88	1.20 ± 0.04	32.08 ± 0.82	2.33 ± 0.05
12	10.26 ± 0.03	2.15 ± 0.1	82.91 ± 2.41	1.07 ± 0.07	38.75 ± 0.44	2.76 ± 0.09
13	8.24 ± 0.04	4.43 ± 0.15	72.94 ± 2.02	2.49 ± 0.13	32.58 ± 0.61	4.00 ± 0.08
14	8.37 ± 0.34	5.16 ± 0.06	79.83 ± 0.13	1.78 ± 0.04	35.34 ± 0.56	4.30 ± 0.06
15	7.10 ± 0.08	4.65 ± 0.2	77.79 ± 0.33	2.24 ± 0.25	38.77 ± 0.47	3.72 ± 0.22
16	9.14 ± 0.48	4.45 ± 0.11	86.50 ± 0.85	1.64 ± 0.06	34.83 ± 0.06	3.45 ± 0.09
17	8.34 ± 0.41	4.77 ± 0.11	72.03 ± 3.65	1.68 ± 0.06	30.28 ± 1.62	4.39 ± 0.23
18	7.47 ± 0.28	5.95 ± 0.6	72.28 ± 3.47	1.51 ± 0.01	34.49 ± 1.82	3.55 ± 0.1
19	7.56 ± 0.07	3.54 ± 0.11	83.01 ± 0.74	4.63 ± 0.26	38.17 ± 0.3	5.94 ± 0.01
20	7.91 ± 0.02	5.32 ± 0.14	86.22 ± 2.77	3.08 ± 0.12	37.44 ± 1.21	5.00 ± 0.14
21	9.25 ± 0.17	5.08 ± 0.19	85.59 ± 2.04	3.40 ± 0.02	37.59 ± 0.82	4.16 ± 0.14
22	7.45 ± 0.21	4.10 ± 0.23	79.01 ± 2.93	2.09 ± 0.13	33.23 ± 1.12	4.67 ± 0.22
23	10.82 ± 0.38	4.53 ± 0.19	98.99 ± 2.85	5.21 ± 0.22	36.44 ± 0.88	7.31 ± 0.05
24	7.24 ± 0.04	4.82 ± 0.03	67.80 ± 2.72	2.07 ± 0.0	37.28 ± 0.19	5.34 ± 0.53
25	8.25 ± 0.22	4.55 ± 0.24	79.30 ± 1.54	1.99 ± 0.09	40.96 ± 1.4	3.11 ± 0.09
26	7.58 ± 0.17	5.52 ± 0.13	71.07 ± 0.07	2.04 ± 0.07	33.31 ± 0.43	5.19 ± 0.1
27	8.63 ± 0.07	4.43 ± 0.08	75.66 ± 2.47	2.06 ± 0.06	33.22 ± 1.11	5.15 ± 0.24
28	7.83 ± 0.3	3.95 ± 0.22	76.59 ± 1.52	2.02 ± 0.03	34.77 ± 0.66	4.03 ± 0.17
29	7.44 ± 0.2	6.08 ± 0.36	75.61 ± 1.07	2.20 ± 0.05	36.73 ± 0.73	5.23 ± 0.41
30	7.39 ± 0.23	5.28 ± 0.28	80.46 ± 4.01	2.01 ± 0.02	34.76 ± 1.79	5.96 ± 0.43
31	7.16 ± 0.06	4.29 ± 0.21	87.52 ± 1.5	3.09 ± 0.19	42.51 ± 1.15	7.44 ± 0.31
32	8.37 ± 0.26	5.14 ± 0.3	74.16 ± 1.03	2.33 ± 0.13	33.61 ± 1.3	4.83 ± 0.01
33	7.83 ± 0.06	4.43 ± 0.08	75.77 ± 1.56	2.24 ± 0.13	36.99 ± 0.63	4.71 ± 0.15
34	8.35 ± 0.1	4.49 ± 0.3	74.16 ± 1.12	2.45 ± 0.17	34.69 ± 0.42	4.80 ± 0.25
35	7.83 ± 0.33	5.74 ± 0.09	75.86 ± 0.83	2.19 ± 0.08	41.03 ± 0.47	5.13 ± 0.05
36	9.43 ± 0.36	4.77 ± 0.13	88.63 ± 3.41	3.34 ± 0.19	43.68 ± 1.13	5.10 ± 0.11
37	6.75 ± 0.04	5.40 ± 0.31	76.00 ± 1.31	1.83 ± 0.1	34.32 ± 0.39	5.70 ± 0.05
38	8.96 ± 0.12	5.13 ± 0.15	81.03 ± 3.35	1.96 ± 0.1	36.87 ± 1.0	6.78 ± 0.04
39	6.87 ± 0.11	2.87 ± 0.06	74.44 ± 1.2	2.59 ± 0.15	29.04 ± 1.19	4.30 ± 0.22
40	6.42 ± 0.21	2.86 ± 0.12	73.91 ± 2.85	6.62 ± 2.06	27.74 ± 1.22	5.26 ± 1.03

The amounts are presented as mg of samples; Q.S.: quercetin-3-O-sophoroside; K.S.: kaempferol-3-O-sophoroside; K.G.: kaempferol-3-O-glucoside; T: tepal; S: stamen.

I was characterized with the highest Q.S. content of tepal samples, including the populations 2, 3, 6–12, and 39, samples belonging to chemotype II contained high quantity of K.G. in tepal samples (populations 4, 5, 13, 14, 16–18, 22, 24, 26–30, 32–34, and 37), whereas chemotype III was the richest in Q.S., K.S., and K.G. in the stamens, and K.S. in the tepals (populations 1, 15, 16, 19–21, 23, 25, 31, 35, 36, 38, 40). The results of the PCA are presented on a dendrogram (Fig. 2).

Eigenvalues, variances and cumulative variances of the four PCs are listed in Table 8. In accordance with PCA analysis, four principal components (PC): PC1, PC2, PC3, and PC4 explained 89.17 % of total variation. As demonstrated in Table 8, PC1 described 25.17 % of total variation which had positive correlation with SQS (0.96 %) and SKG (0.75 %). Interestingly, a positive correlation between PC2 with SKS (0.92 %), SKG (0.57 %) and PKS (0.52 %) was recorded by 24.22 % of variance. Furthermore, PC3 and PC4 explained 22.09 % and 17.69 % of total variance, respectively, while the most positive correlations in PC3 and PC4 were observed with PQS (0.80 %) and PKG (0.96 %), respectively.

Biosynthesis of secondary metabolites may be influenced by different environmental factors (e.g. UV irradiation, temperature) at different altitudes. The impact of altitude on the composition of certain plant species has previously been studied. For example, we have confirmed that the apigenin and luteolin content of *Matri-*

Table 8
Eigenvalues, variance and cumulative variance for four principal components.

Flavonoids	Principal components			
	PC1	PC2	PC3	PC4
PQS	-0.02	-0.06	0.80	0.13
PKS	0.13	0.52	0.78	0.32
PKG	0.07	0.05	0.25	0.96
SQS	0.96	0.02	0.06	0.09
SKS	0.12	0.92	0.07	0.48
SKG	0.75	0.57	-0.05	-0.01
Eigenvalue	1.51	1.45	1.33	1.06
Variance (%)	25.17	24.22	22.09	17.69
Cumulative variance (%)	25.17	49.39	71.49	89.17

PQS: Quercetin-3-O-sophoroside in tepal; PKS: Kaempferol-3-O-sophoroside in tepal; PKG: Kaempferol-3-O-glucoside in tepal; SQS: Quercetin-3-O-sophoroside in stamen; SKS: Kaempferol-3-O-sophoroside in stamen; SKG: Kaempferol-3-O-glucoside in stamen.

caria chamomilla L. was significantly higher at higher altitude [33]. The diversity of total phenolic and flavonoid contents of different Iranian *Ferulago angulata* (Schltdl.) Boiss. populations also confirm the influence of altitude on the chemodiversity of plants [34]. The volatile oil composition may also be affected by altitude [33–36]. In case of saffron crocus, this is the first report on chemodiversity of samples from different geographical locations.

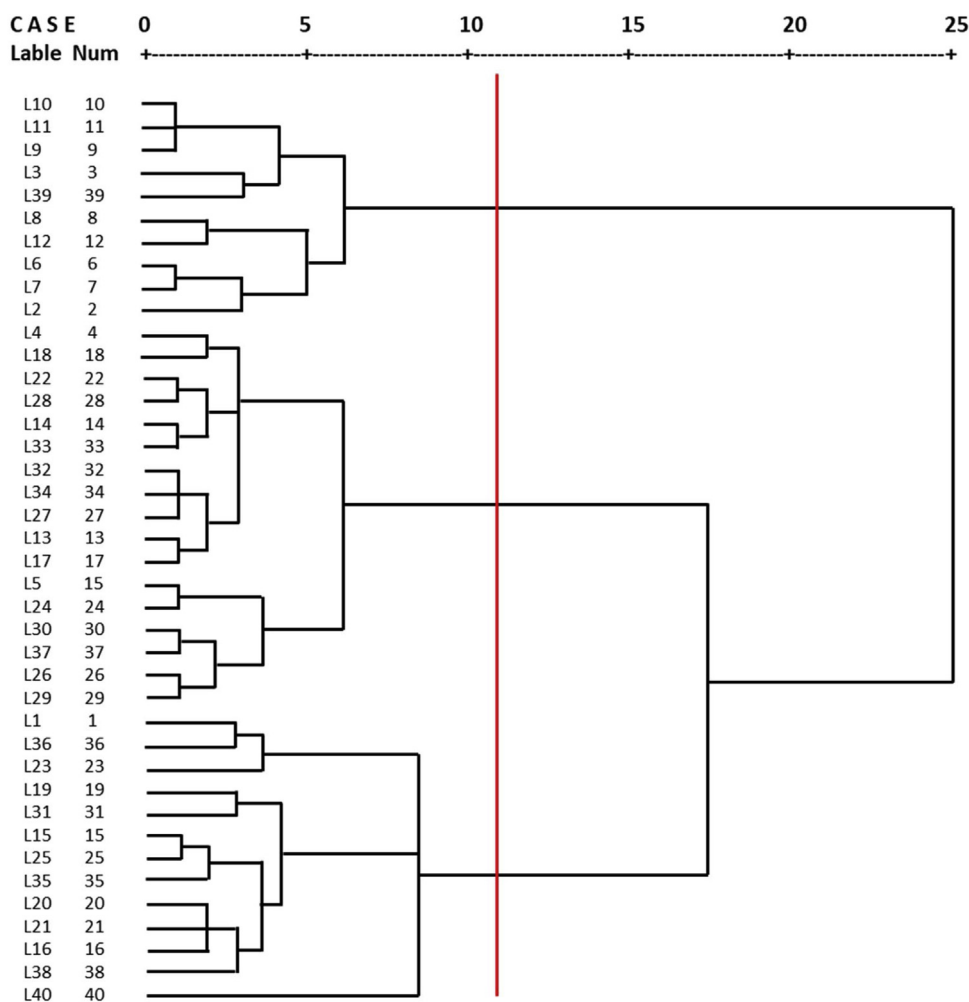


Fig. 2. Dendrogram of cluster analysis (based on Euclidean distances) of the saffron samples: chemotype I (Q.S in tepal), chemotype II (K.G in tepal), chemotype III (Q.S in stamen, K.S in stamen, K.G in stamen, K.S in tepal).

4. Conclusion

The industrial and scientific interests for saffron crocus by-products, including tepals and stamens have considerably been increased, due to the high price of saffron stigma and the new data on bioactivities of tepals. Here we report the development and validation of an HPLC-DAD method that allows the assessment of saffron crocus samples based on the analysis of seven marker compounds (K.S., K.G., Q.S., picrocrocin, crocin, crocetin, and safranal). A recent study reports the metabolomic fingerprinting of saffron by using LC-MS, which can be the basis of the authentication of this spice [37]. LC-MS was used also for the analysis of saffron crocus by-products, ie. tepals [31], tepals, stamens and flowers [32], leaves, tepals, spaths, corm, and tunics [38], however, this is the first report on the analysis of a series of samples of different geographic origin. The flavonol glycosides K.S., K.G., and Q.S. can be utilized as qualitative and quantitative marker compounds, their total amount in dry tepal and stamen samples ranged between 62.19–99.48 mg/g and 0.90–6.62 mg/g for K.S., 27.74–45.18 mg/g and 1.72–7.44 mg/g for K.G., and 6.21–10.82 mg/g and 1.63–6.08 mg/g for Q.S., respectively. These ranges were established based on the analysis of 40 different Iranian tepal and stamen samples. K.S. was the main component of tepals (62.19–99.48 mg/g), while K.G. (1.72–7.44 mg/g) was the predominant constituent of the stamen samples. Characteristic compounds of the stigma (picrocrocin, crocin, crocetin,

and safranal) could not be detected in tepal and stamen samples; however, our method allows their determination as well.

The biplot was prepared based on the two PCs by 89.17 % of variance. As it was shown in Fig. 3, saffron crocus samples were grouped in three classes that approved the CA analysis. According to the PCA and CA analyses, some of the samples from long distances, for example samples 40 and 38 deriving from locations with different climate, were classified in the same group (III), while others from the same location, Meshkan (population 12 in class I, Meshkan samples 13 and 14 in group II, Meshkan 15 in group III), were classified in separate groups. Saffron crocus is propagated by vegetative method which may affect plant diversity. It seems that the analyzed flavonoids were not affected by genotype and climate condition, however, edaphic factors, water and nutrition management might be responsible for the chemodiversity of the studied samples.

CRedit authorship contribution statement

Javad Mottaghipisheh: Data curation, Formal analysis, Investigation, Writing - original draft. **Mohammad Mahmoodi Sourestani:** Software, Investigation, Visualization, Writing - original draft. **Tivadar Kiss:** Data curation, Investigation, Project administration. **Attila Horváth:** Formal analysis, Investigation. **Barbara Tóth:** Data curation, Validation. **Mehdi Ayanmanesh:**

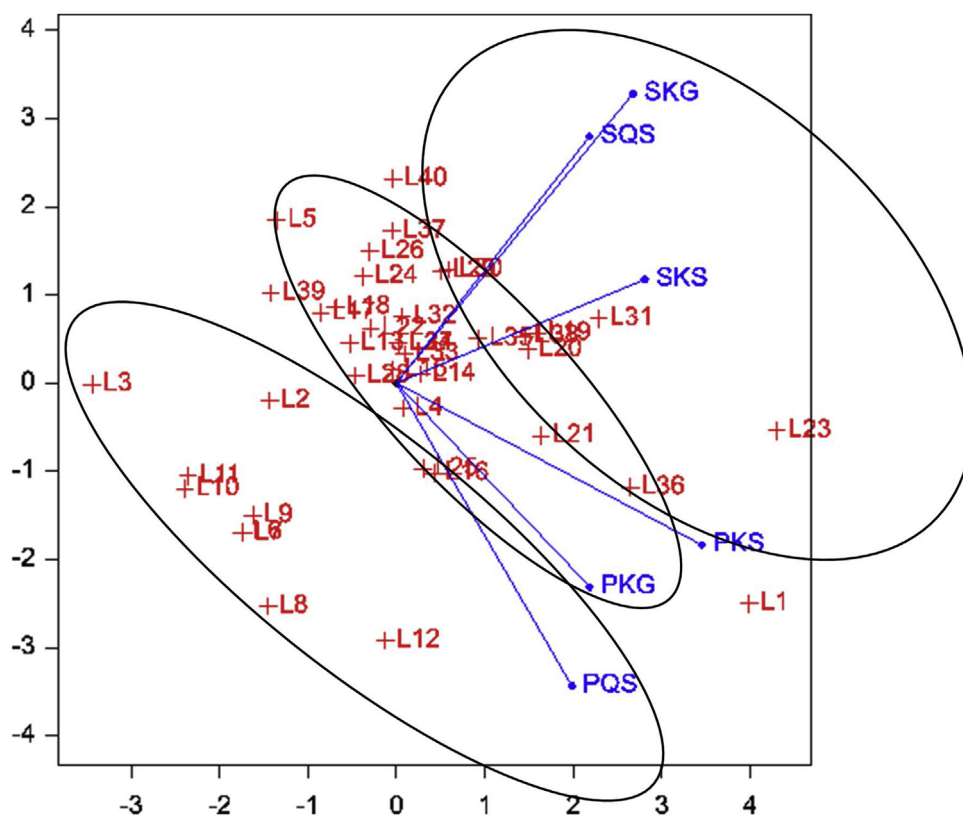


Fig. 3. Principal component biplot (PCA) of the saffron samples; L: Location; PQS: quercetin-3-O-sophoroside in tepal; PKS: kaempferol-3-O-sophoroside in tepal; PKG: kaempferol-3-O-glucoside in tepal; SQS: quercetin-3-O-sophoroside in stamen; SKS: kaempferol-3-O-sophoroside in stamen; SKG: kaempferol-3-O-glucoside in stamen.

Investigation. **Amin Khamushi:** Formal analysis. **Dezső Csopor:** Conceptualization, Funding acquisition, Methodology, Resources, Supervision, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.jpba.2020.113183>.

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