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Research article

Coumarins, furocoumarins and limonoids of *Citrus trifoliata* and their effects on human colon adenocarcinoma cell lines

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ABSTRACT

Citrus trifoliata L. (Chinese or Japanese bitter orange) is a medicinal plant with furocoumarins and limonoids as characteristic secondary metabolites. The bitter taste of the fruit limits its use as food, however, it is applied in Asian traditional medicine for its antiphlogistic effect, to treat digestive ulcers and different gastrointestinal disorders and cancer. The phytochemical composition and pharmacological characteristics of this species have not been fully discovered, nevertheless its potential antiproliferative or cytotoxic effects might be related to furocoumarins or limonoids.

Our aim was to isolate and identify secondary metabolites from *C. trifoliata* peel and seeds and to investigate their bioactivities that might be related to the supposed anticancer effect of the plant. By using different chromatographic methods, six pure compounds (phellopterin (**2**), scoparone (**3**), myrsellin (**4**), triphasiol (**6**), umbelliferone (**7**) and citropten (5,7-dimethoxycoumarin (**8**)) were isolated from the peel and four (imperatorin (**1**), auraptene (**5**), limonin (**9**) and deacetyl nomilin (**10**)) from the seeds of *C. trifoliata* fruits. These compounds are furocoumarin (**1**, **2**), coumarin (**3**–**8**), and limonoid derivatives (**9**, **10**). Scoparone (**3**) has been detected in this species for the first time.

The furocoumarins (**1**–**2**) showed moderate activity on the human colorectal adenocarcinona tumor cell line COLO 320 in antiproliferative assays and **2** also had remarkable P-glycoprotein inhibitory activity and synergistic effect with doxorubicin. The coumarin **5** showed significant activity on the COLO 320 cell line in antiproliferative assays and P-glycoprotein inhibitory activity in the FACS (fluorescence activated cell sorting) assay.

1. Introduction

The group of true citrus fruit trees was traditionally divided to six genera, *Citrus, Clymenia, Fortunella, Eremocitrus, Microcitrus,* and *Poncirus*. All of the species belonging to these genera have unifoliolate leaves except for the genus *Poncirus*, which has trifoliolate, deciduous leaves (Ruiz and Asins, 2003). *Citrus trifoliata* L., also known as Chinese or Japanese bitter orange, is a species native to northern China and Korea. *C. trifoliata* has pubescent fruits and characteristic palmately 3- or 5-foliolate leaves. Previously it was classified in the *Poncirus* taxon, however, today it is widely accepted as a *Citrus* species (WFO, 2021). The fruits of the plant have been used in traditional medicine in Asia, however the very bitter taste of the fruits limits their use as food.

The bitter taste of the fruit is related to its limonoid content. The first limonoid detected in *C. trifoliata* seeds was deacetylnomilin (Dreyer, 1965). Obacunone, limonin and nomilin were also reported (Dreyer, 1966). Miyake et al. quantified the limonoids of the seeds. Deacetylnomilin (230 ppm) and limonin (390 ppm) were the major constituents, followed by ichangin (20 ppm) and the acidic limonoids deacetylnomilinic acid (2 ppm), isolimonic acid (2 ppm) and isoobacunoic acid (trace amount) (Miyake et al., 1991).

Dreyer et al. also reported the presence of furocoumarins: imperatorin, bergapten and smaller amounts of xanthotoxol and

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alloimperatorin (Dreyer, 1965, 1966). The presence of imperatorin was confirmed by Weinstein et al., together with the isolation of heraclenin (Weinstein et al., 1972). In the 1970's, several furocoumarins and coumarins were isolated and identified by Guiotto et al. In the seeds of the plant, imperatorin 0.15% was reported as the major furocoumarin, followed by bergapten 0.06%, whereas isopimpinellin, prangenin and prangenin hydrate were found in the order of less than 0.01% (Guiotto et al., 1973).

Later, the coumarins aurapten, and 6-methoxy-7-geranyloxycoumarin were also reported (Guiotto et al., 1974). The latter, together with 7-geranyloxycoumarin, isoimperatorin, bergamottin, epoxyimperatorin and phellopterin was also present in the fruits (Guiotto et al., 1977; Jayaprakasha et al., 2012; Xu et al., 2008). The coumarins poncimarin, isoponcimarin, isoschininallylol, 7-(3′-methyl-2′,3′-epoxybutyloxy)-8-(3″-methyl-2″,3″-epoxybutyl)coumarin and 7-(3′-methyl-2′,3′-epoxybutyloxy)-8-(3″-methyl-2″-oxobutyl)coumarin and the bis- and di-isoprenylated coumarins *O*-methyltriphasiol A, triphasiolene A, *O*-methylponciol A, *O*-methylponciol B and 7-(6S-hydroxy-3,7-dimethyl-2E, 7-octadienyloxy)coumarin were identified as new compound from the fruits, whereas triphasiol, ponciol, isoschinilenol, scopoletin, umbelliferon, 7-(6S-hydroperoxy-3,7-dimethyl-2E, 7-dienyloxy)coumarin, 7-O-(7′-peroxygeranyl)coumarin and 7-(6R-hydroxy-3,7-dimethyl-2E, 7-octadienyloxy)coumarin were described previously from other taxa (Guiotto et al., 1977; Guiotto et al., 1975; Guiotto et al., 1976; J. S. Park et al., 2020; Xu et al., 2008). Heraclenol 3′-methyl ester, phellopterin, (R)-oxypeucedanin hydrate and oxypeucedanin methanolate were isolated from the fruits (Nizamutdinova et al., 2008; J. S. Park et al., 2020; Pokharel et al., 2006).

C. trifoliata fruits have been widely used in Asian folk medicine as antiphlogistic, to treat digestive ulcers, gastritis and dysentery. Many of these uses are supported by experimental data. The extract of the fruits decreased the expression of pro-inflammatory cytokines in activated human mast cells (Shin et al., 2006). 21-Methylmelianodiol, a triterpene of the fruits has key role in the anti-inflammatory effect, since it was not only effective in the carrageenan-induced paw edema model, but its mechanism of action was also partly revealed [attenuation of expression of iNOS (inducible nitric oxide synthase), COX-2 (cyclooxygenase-2), TNF-α (tumor necrosis factor alpha) and IL-1β (interleukin-1 beta)] (Zhou et al., 2007). Poncirin inhibited the lipopolysaccharide-induced prostaglandin E_2 and interleukin-6 production in murine macrophage cells (Han et al., 2007). This flavonoid inhibited the growth of *Helicobacter pylori* as well (Kim et al., 1999). In an animal experiment, the aqueous extract of the immature fruit accelerated the intestinal transit (Lee et al., 2005), whereas the hexane extract stimulated the motility of rat distal colon (Choi et al., 2010), referring to its prokinetic activity. Neohesperidin and poncirin significantly inhibited the HCl/ethanol-induced gastric lesions, and increased the pH and mucus content in the stomachs of rats (Lee et al., 2009).

The fruit has also been used for the treatment of various cancers among Korean Oriental Medical doctors (Yi et al., 2004). *In vitro* activity against cancer cells was observed for crude extracts and triterpenes and furocoumarins as well. In an *in vitro* experiment on HL-60 human leukemia cells, the extract of the fruit was cytotoxic in a concentration- and time-dependent manner. It induced apoptosis accompanied by the activation of caspase-3 and the specific proteolytic cleavage of PARP [poly (ADP-ribose) polymerase]. However, the extract was not cytotoxic in normal peripheral blood mononuclear cells (Yi et al., 2004). The extract of the immature fruit inhibited the proliferation of CT-26 colorectal cancer cells and it induced mitochondrial autophagy and apoptosis through the protein kinase B/mammalian target of rapamycin and 5′-AMP-activated protein kinase pathways (Kim et al., 2020). Bergamottin, imperatorin, isoimperatorin and epoxyimperatorin inhibited the growth of pancreatic cancer cells (Panc-28) by inducing apoptosis in culture models, epoxyimperatorin being the most active (Jayaprakasha et al., 2012).

Considering the folk medicinal use of *C. trifoliata* and the potential antitumor effects observed in preclinical studies, our goal was to isolate secondary metabolites, including furocoumarins and limoinoids from the seeds and peels of the plant and to investigate their antiproliferative, cytotoxic and anti-MDR (anti-multidrug resistance) effects.

2. Results

2.1. Secondary metabolites isolated from the peel and seeds of C. trifoliata fruits

As a result of the chromatographic purification of the investigated plant materials, 6 pure compounds (**2–4, 6–8**) were isolated from the peel and 4 (**1, 5, 9, 10**) from the seeds of *C. trifoliata* fruits. The structure elucidation process of these compounds was carried out by 1D (^{1}H, ^{13}C JMOD) and 2D (HSQC, HMBC, ^{1}H–^{1}H COSY, and NOESY) NMR experiments. According to the reported literature data, the isolated compounds were identified as imperatorin (**1**) (Ngwendson et al., 2003), phellopterin (**2**) (Bergendorff et al., 1997), scoparone (6,7-dimethoxycoumarin, **3**) (Gunther et al., 1975), myrsellin (**4**) (Hifnawy et al., 1977), auraptene (**5**) (Askari et al., 2009), triphasiol (**6**) (Desilva et al., 1981), umbelliferone (**7**) (Osborne, 1989), citropten (5,7-dimethoxycoumarin, **8**) (Singh et al., 2010), limonin (**9**) (Breksa et al., 2008), and deacetyl nomilin (**10**) (Mandadi et al., 2007). The obtained compounds are furocoumarin (**1**, **2**), coumarin (**3**–**8**), and limonoid derivatives (**9**, **10**) (Figure 1). Furomoumarins and coumarins were isolated both from the seeds and peel, however, limonoids were detected only in the seeds. All compounds but scoparone have previously been described from *C. trifoliata* (Nizamutdinova et al., 2008; J.-S. Park et al., 2020; Rahman et al., 2012) (Dreyer, 1966; Mercolini et al., 2013).

2.2. Antiproliferative and cytotoxic activity

The tested compounds exerted no activity on normal (MRC-5) and doxorubicin-sensitive colon carcinoma (COLO 205) cell lines, neither in the cytotoxicity nor in the antiproliferative assay (Tables S1 and **S2**). However, on resistant COLO 320 cell lines, some compounds were found to be effective. Compound **5** showed strong antiproliferative activity on COLO 320 cells with an IC_{50} value of 25.28 ± 0.42 μM, and compounds **1, 2** and **4** had also exerted moderate effect with IC_{50} values of 40.47 ± 1.22, 43.71 ± 1.78, 47.94 ± 1.11 μM, respectively.

2.3. Checkerboard combination assay

In the combination studies, the interactions of the tested compounds with doxorubicin were investigated at different concentrations (Table 1). Some compounds, such as compounds **3, 4** and **10** have synergistic effects with doxorubicin at certain concentrations. This might refer to their potential use in combination with standard anticancer therapies to improve their efficacy. However, for example, compounds **2, 3** and **5** have shown antagonistic effects at particular concentrations. Such interactions might decrease the efficacy of chemotherapy.

2.4. Effect on efflux pumps

In the rhodamine 123 retention assay, it is conventionally accepted that if the FAR value is above 2, the substance can be considered a good Pgp inhibitor. As positive control, tariquidar was used (FAR value: 11.44 at 0.2 μM). Four of the tested compounds were effective at 20 μM. The FAR values of compounds **2, 3, 4,** and **5** were 2.63, 2.02, 4.86, and 4.00, respectively (Table 2).

The ethidium bromide (EB) accumulation assay provides information about the intracellular accumulation of the general efflux pump (EP) substrate EB. A potential efflux pump inhibitor increases the fluorescence level of EB because of its accumulation within the bacterial cell. The EP inhibiting activity of the compounds was compared based on the relative fluorescence index (RFI) of the real-time accumulation curves. Two

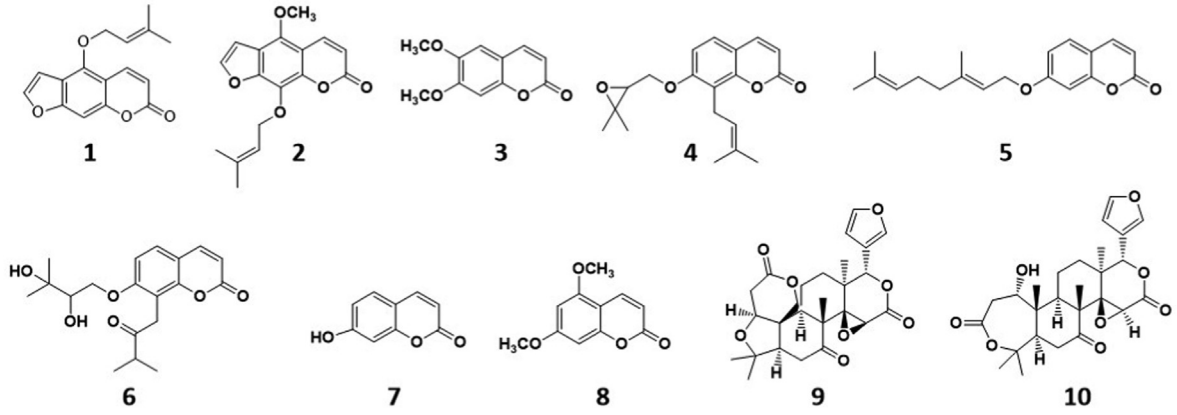

Figure 1. Compounds isolated from *Citrus trifoliata:* imperatorin (**1**), phellopterin (**2**), scoparone (**3**), myrsellin (**4**), auraptene (**5**), triphasiol (**6**), umbelliferone (**7**), citropten (5,7-dimethoxycoumarin (**8**)), limonin (**9**) and deacetyl nomilin (**10**)).

Table 1. Checkerboard combination assay results of selected *C. trifoliata* compounds to assess potential interactions with doxorubicin.

Compound	Starting concentration	Ratio	Combination index (CI)	SD	Type of interaction
imperatorin (1)	150 μM	17.42:1	1.29	0.11	moderate antagonism
		34.84:1	1.06	0.07	additive effect
		69.68:1	0.75	0.06	moderate synergism
		139.36:1	1.24	0.05	moderate antagonism
		278.72:1	0.99	0.04	additive effect
		557.44:1	0.99	0.11	additive effect
phellopterin (2)	150 μM	17.42:1	2.31	0.46	Antagonism
		34.84:1	1.40	0.17	moderate antagonism
		69.68:1	1.33	0.15	moderate antagonism
		139.36:1	1.54	0.26	antagonism
		278.72:1	1.05	0.12	additive effect
		557.44:1	1.03	0.23	additive effect
scoparone (3)	200 μM	23.2:1	2.06	0.39	antagonism
		46.4:1	1.22	0.18	moderate antagonism
		92.8:1	0.81	0.05	moderate synergism
		185.6:1	0.75	0.03	moderate synergism
		371.2:1	0.62	0.05	synergism
		742.5:1	0.60	0.07	synergism
myrsellin (4)	150 μM	17.42:1	1.17	0.09	slight antagonism
		34.84:1	0.72	0.05	moderate synergism
		69.68:1	0.83	0.04	moderate synergism
		139.36:1	0.58	0.11	synergism
		278.72:1	0.64	0.12	synergism
		557.44:1	0.73	0.21	moderate synergism
auraptene (5)	80 μM	9.2:1	3.16	0.85	slight syn.
		18.4:1	8.37	1.03	strong antagonism
		36.8:1	1.13	0.20	slight antagonism
		73.6:1	1.72	0.24	antagonism
		147.2:1	2.06	1.00	antagonism
		294.4:1	5.30	1.60	strong antagonism
triphasiol (6)	200 μM	23.2:1	0.86	0.06	slight synergism
		46.4:1	0.76	0.08	moderate synergism
		92.8:1	0.82	0.03	moderate synergism
		185.6:1	0.78	0.02	moderate synergism
		371.2:1	0.81	0.10	moderate synergism
deacetyl nomilin (10)	200 μM	23.2:1	1.18	0.19	nearly additive
		46.4:1	1.02	0.08	nearly additive
		92.8:1	0.67	0.04	synergism
		185.6:1	0.66	0.07	synergism
		371.2:1	0.62	0.02	synergism
		742.5:1	0.82	0.32	moderate syn.

Table 2. Efflux pump inhibitory activities of compounds isolated from *C. trifoliata*.

Compound	Concentration (μM)	FL-1	FAR
Tariquidar	0.2	88.20	11.44
imperatorin (1)	2	11.30	1.47
	20	9.82	1.27
phellopterin (2)	2	11.10	1.44
	20	20.30	2.63
scoparone (3)	2	8.01	1.04
	20	15.60	2.02
myrsellin (4)	2	10.90	1.41
	20	37.50	4.86
auraptene (5)	2	13.60	1.76
	20	30.80	4.00
triphasiol (6)	2	14.20	1.84
	20	14.30	1.85
limonin (9)	2	2.92	0.38
	20	10.00	1.30
deacetyl nomilin (10)	2	4.61	1.01
	20	3.53	0.78

FL-1: mean fluorescence intensity of the cells; FAR: fluorescence activity ratio.

compounds, **2** and **4** showed remarkable activity with 5.49 and 5.51 RFI values, respectively. These values were higher than that of the positive control, reserpine (2.77). Besides, compound **3** showed even moderate activity with an RFI value of 1.04. The results suggest that these substances can be considered as good EP inhibitors.

In a subsequent experiment, three compounds (**1, 4** and **5)** showed moderate activity on *E. coli* AG100 with 0.26, 0.39 and 0.34 RFI values, respectively. The positive control was CCCP, with an RFI value of 1.34.

3. Discussion

C. trifoliata has been used by Korean Oriental Medical doctors to treat various cancerous diseases. The activity of plant extracts against different cancer cell lines has been confirmed *in vitro*. These bioactivities might be related to the furocoumarin and limonoid content of the fruits, since these types of compounds were found to have antiproliferative and cytotoxic activities previously (Ahmed et al., 2020; Shi et al., 2020). Such activities were also reported for certain furocoumarins and limonoids of *C. trifoliata*, as presented in the Introduction section. However, other types of compounds may also play a role in the overall effect of the fruits. Therefore, our aim was to systematically investigate the bioactivities of secondary metabolites of *C. trifoliata* peel and seeds that might be related to the supposed anticancer effect of the plant.

None of the isolated 10 compounds exerted strong antiproliferative and cytotoxic effects on the investigated cell lines. Although the compounds we tested have been previously identified from the plant, similar bioactivity studies with these compounds have not been made before. The isolated furocoumarins (imperatorin (**1**) and phellopterin (**2**)) showed moderate activity on the tumor cell line COLO 320 in antiproliferative assays. In addition, phellopterin (**2**) has good P-glycoprotein inhibitory activity as well. Furthermore, the investigated furocoumarins were also shown to have good efflux pump inhibitory activity on different bacterial cell lines by the ethidium bromide accumulation assay. Among the furocoumarins, phellopterin (**2**) in combination with doxorubicin had antagonistic effects at different concentrations, but a moderate synergistic effect was also observed with imperatorin (**1**).

The antiproliferative activity of imperatorin has been described in several in vitro studies on different cell lines, and its effect is partly related to the induction of apoptosis and cell cycle arrest (Deng et al., 2020). The antitumor effect of phellopterin has been less extensively studies, however its anti-melanoma effect was observed in an animal experiment (Sumiyoshi et al., 2014).

Of the coumarins, auraptene (**5**) showed significant activity on the COLO 320 cell line in antiproliferative assays, but triphasiol (**6**) and scoparone (**3**) showed no activity. In addition, auraptene (**5**) was also shown to be a good P-glycoprotein inhibitor in the FACS assay. Of the studied compounds, auraptene is promising for further studies. This compounds exerted chemopreventive and antiproliferative effects on several cell lines via targeting different cell signalling pathways, and it has also an excellent safety profile based on animal studies (Tayarani-Najaran et al., 2021).

The results of the checkerboard assay reveal different interactions with the concurrently applied doxorubicin, ranging from synergism to antagonism. These results draw the attention to the potential interactions of these compounds with chemotherapeutic agents, however, no conclusion can be drawn for the clinical relevance of these interactions.

Although the results presented here do not hold the promise of the use of *C. trifoliata* extracts of secondary metabolites in cancer treatment, the scientific value of these data is supported by the extensive consumption of *Citrus* fruits. The compounds analyzed by us are present in other species as well and thus are ingested in significant amounts when consuming *Citrus* fruits as food or as medicinal plants.

4. Materials and methods

4.1. Plant material

Citrus trifoliata L. fruits were collected by Botond Lajos Borcsa in Tompa, Hungary, in 2018. Seeds and peels were dried and stored at room temperature before processing. A voucher specimen (No. 893) is stored in the herbarium of the Department of Pharmacognosy, University of Szeged.

4.2. Extraction and isolation of secondary metabolites from the peel of Citrus trifoliata

The dried and crushed peel (236.2 g) was extracted with 6500 mL MeOH by ultrasonication at room temperature for 15 min. The solvent was evaporated under reduced pressure to yield 70.4 g of crude MeOH extract. The extract was redissolved in 100 mL of methanol and was subjected to solvent-solvent partitioning with dichloromethane (4 × 250 mL). The weight of the crude dichloromethane fraction was 23.7 g. This fraction was chromatographed by open column chromatography on silica gel (Silica gel 60 (0.040–0.063 mm)) with a gradient system of *n*-hexane-chloroform-acetone (2:1:0, 1:1:0, 0:1:0, 0:9:1, 0:8:2, 0:7:3, 0:6:4, 0:1:1, 0:0:1, 1000 mL each), gaining 49 fractions (O1–O49).

Fractions O21–27 were combined (14.61 g) and subjected first to medium pressure liquid chromatography on silica gel (Silica gel 60 (0.040–0.063 mm)) with a gradient system of *n*-hexane-EtOAc (9:1 to 0:1) with flow rate 50 mL/min, gaining 16 fractions (M1-M16). The merged fractions M1–7 (5 g) were separated into 15 subfraction by open column chromatography on silica gel (Silica gel 60 (0.040–0.063 mm)) and eluted with cyclohexane-dichloromethane 7:3 with increasing acetone concentration (from 0% to 25%). Subfractions 5–7 (428 mg) of this separation were purified by normal-phase preparative TLC (eluent: cyclohexane-acetone 7:3). Finally, compounds **2, 4, 7, 8** were obtained by using high pressure liquid chromatography (HPLC) (method I).

Merged fractions M9-10 (3 g) were subjected first to open column chromatography on silica gel (Silica gel 60 (0.040–0.063 mm)) by eluting with cyclohexane-dichloromethane 1:1 with increasing acetone concentration (0.1, 0.3, 0.5, 1, 2, 5, 10, 20, 50%), resulting in 14 subfractions. Subfraction 5 was purified by reverse-preparative TLC with MeOH-water 7:3. This last purification step afforded the isolation of compound **3**.

Fraction M12 (1.07 g) was separated by reverse-phase medium pressure liquid chromatography (MPLC) on RP18ec (40–60 μm, 40 × 150 mm) eluting with MeOH-water (3:7 to 1:0) with a flow rate 20 mL/min 15 fractions were collected. Combined fractions 8–9 (248.3 mg) were

further purified by normal-phase open column chromatography (Silica gel 60 (0,040–0,063 mm)) by eluting with chloroform-acetone (9:1) and finally the subfractions 4–6 were purified by normal phase preparative TLC (with dichloromethane-methanol (9:1) as eluent), obtaining compound **6** in pure form.

4.3. Extraction and isolation of secondary metabolites from the seed of Citrus trifoliata

Dried and crushed citrus seeds (170 g) were extracted with 4000 mL MeOH by ultrasonication at room temperature for 25 min. This extract was evaporated under vacuum to 100 mL. Coumarins were extracted by solvent-solvent partitioning with 4 × 200 mL dichloromethane. The extracts were combined and evaporated under reduced pressure at room temperature.

The dried and crude dichloromethane extract (24 g) was chromatographed on a silica column with gradient elution (*n*-hexane-dichloromethane-acetone 2:1:0, 1:1:0, 0:1:0, 0:9:1, 0:8:2, 0:7:3, 0:3:2, 0:1:1, 0:2:3, 0:3:7, 0:1:1) resulting in 32 fractions. The merged fraction O3 was also subjected to normal-phase preparative TLC with the same eluent system to provide compound **1**. Subfraction O7–9 was purified by preparative TLC on silica gel and eluted with toluene-acetone 95:5 to yield compound **5**. Merged fractions O13–15 (867.3 mg) was further separated by flash chromatography with method I, resulting in 76 subfractions. Subfractions OF68–76 (178.5 mg) were purified also by flash chromatography with method II, resulting in 33 subfractions. Finally, from merged OFF18–26 subfractions, compounds **9** and **10** were obtained by using high pressure liquid chromatography with method II.

4.4. Chromatographic methods

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

For the preparative reversed-phase HPLC, a Waters 600 HPLC system (Waters Corporation, Milford, USA) was used, equipped with a 2998 photodiode array detector, on-line degasser and autosampler. Separation was carried out on a Kinetex C18 (5 μm, 100 Å, 150 × 4.6 mm) column (Phenomenex, Torrance, USA). Elution was accomplished by a gradient solvent system consisting of methanol and water (0–1 min: water-MeOH 1:1; 10 min: 0:1; 11 min: 0:1; 11.5 min: 1:1; 15 min: 1:1) with a flow rate of 1.5 mL/min. Coumarins and furocoumarins were detected at 254 and 366 nm.

For isolating limonoids (HPLC method II), we used a Waters 600 HPLC system (Waters Corporation, Milford, USA) equipped with a 2998 photodiode array detector, on-line degasser and autosampler. Separation was carried out on a Kinetex C18 (5 μm, 100 Å, 150 × 4.6 mm) column (Phenomenex, Torrance, USA). Gradient elution was performed using a solvent system consisting of acetonitrile and water (0–2 min: water-AcNi 3:2; 10 min: 0:1; 11–16 min: 3:2) with a flow rate of 1 mL/min. Limonoids were detected at 210 and 254 nm.

For flash chromatographic separation, we used a Biotage® instrument (Isolera™ Spektra Systems with ACI™ and Assist) with integrated UV, UV-VIS, and ELS detection. In the case of method I, separation was carried out on a normal phase SNAP ultra 50 g column. Elution was accomplished by a gradient solvent system consisting of cyclohexane (A) and ethyl acetate (B) (198 mL: B 1%; 660 mL: 1%–50%; 132 mL: 50%–70%; 66 mL: 70%–100%; 132 mL: 100%) with a flow rate of 100 mL/min. Separation was detected at 265 and 366 nm. In the case of method II, separation was achieved on a reverse phase column (SNAP C18 12 g) using gradient elution. The solvent system consisting of water (A) and acetonitrile (B) (30 mL: B 0%–10%; 150 mL: 10%–100%; 30 mL: 100%) was pumped with a flow rate of 12 mL/min. Separation was detected at 200 and 210 nm.

4.5. NMR experiments

NMR spectra were recorded on a Bruker Avance DRX 500 spectrometer in $CDCl_3$ and acetone-d_6 at 500 MHz (^{1}H) and 125 MHz (^{13}C JMOD). The residual peaks of the deuterated solvents were taken as reference points. Two-dimensional (2D) experiments were performed with a standard Bruker software. In the ^{1}H–^{1}H COSY, HSQC, and HMBC experiments, gradient-enhanced versions were applied. The data were acquired and processed with MestReNova *v*6.0.2−5475 software.

4.6. Cell lines

MRC-5 human embryonal lung fibroblast cell lines (ATCC CCL-171) were obtained from LGC Promochem (UK). This cell line was cultured in Eagle's Minimal Essential Medium (EMEM) with a 4.5 g/L glucose content, supplemented with 10% heat-inactivated fetal bovine serum, non-essential amino acids and vitamins. Human colon adenocarcinoma cell lines (COLO 320/MDR-LRP multidrug resistant overexpressing ABCB1 (MDR1)-LRP and COLO 205 doxorubicin-sensitive), CCL-222 (COLO 205) and ATCC-CCL-220.1 (COLO 320) were obtained from LGC Promochem (UK). The cell lines were cultured in an RPMI 1640 medium supplemented with 1 mM Na-pyruvate, 2 mM L-glutamine, 10% heat-inactivated fetal bovine serum, and 100 mM Hepes. For the detachment of the semi-adherent human colon cancer cells Trypsin-Versene (EDTA) solution was applied at 37 °C for 5 min. All the cell lines were incubated in a 5% CO_2, 95% air atmosphere at 37 °C.

4.7. Assay for antiproliferative and cytotoxic effects

The effect of increasing concentrations of tested compounds on cell growth and proliferation was determined on doxorubicin-sensitive COLO 205 and multidrug resistant COLO 320 colonic adenocarcinoma cells in 96-well flat-bottomed microtiter plates.

In case of the colonic adenocarcinoma cells, the dilutions of compounds were prepared in 100 μL of RPMI 1640, horizontally. The semi-adherent colonic adenocarcinoma cells were treated with Trypsin-Versene (EDTA) solution.

The adherent human embryonal lung fibroblast cells were cultured using EMEM supplemented with 10% heat-inactivated fetal bovine serum in 96-well flat-bottomed microtiter plates. The cells were seeded at 37 °C for 24 h, and then after the removal of the medium with the cells, the diluted tested compounds were added to the cells.

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

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

4.8. Checkerboard combination assay

The interactions of the tested compounds and doxorubicin were assessed by the checkerboard microplate method with multidrug resistant COLO 320 colonic adenocarcinoma cells overexpressing the ABCB1 transporter. The concentration of doxorubicin used in this combination experiment was determined in previous antiproliferative assays. The

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

4.9. Evaluation of rhodamine 123 (R123) retention by flow cytometry

The doxorubicin-sensitive (parental) COLO 205 and the multidrug resistant COLO 320 colonic adenocarcinoma cells were adjusted to a density of 2×10^6/mL, resuspended in serum-free RPMI 1640 and distributed into Eppendorf centrifuge tubes in 0.5 mL aliquots. The tested compounds were added into tubed at 0.02–0.2 μM final concentrations, and then incubated at room temperature for 10 min. 10 μL (5.2 μM final concentration) of rhodamine 123 was added, which was followed by an incubation of 20 min at 37 °C. Cells were washed twice with phosphate buffered saline (PBS) and resuspended in 1 mL PBS. A Partec CyFlow flow cytometer (Partec, Germany) was used to determine fluorescence intensity. Tariquidar was applied as positive control at 0.2 μM final concentration, the fluorescence intensity of the untreated cells was taken as baseline. During this experiment, FL-1 (mean fluorescence intensity of the cells) was determined. The following equation was used to calculate fluorescence activity ratio (FAR):

$$FAR = \frac{MDR \text{ treated}/MDR \text{ control}}{parental \text{ treated}/ parental \text{ control}}$$

4.10. Real-time ethidium bromide accumulation assay

The effect of tested compounds on ethidium bromide (EB) accumulation was measured by using a CLARIOstar Plus plate reader (BMG Labtech, UK). *Escherichia coli* AG100 was incubated in LB and *Staphylococcus aureus* ATCC was incubated in TSB until an optical density (OD) of 0.6 at 600 nm was reached. After washing with PBS and a centrifugation at 13.000 × g for 3 min, the cell pellet was resuspended in PBS. The tested compounds were added at ½ MIC concentration (except >100 where the concentration was 100 μM) to PBS containing 2 μg/mL EB. Then, 50 μLs of this mixture were transferred into 96-well black microtiter plates (Greiner Bio-One Hungary, Hungary), and 50 μL of bacterial suspension (OD_{600} 0.6) were added to the each well. The fluorescence was monitored by using a CLARIOstar plate reader, at excitation and emission wavelengths of 525 and 615 nm in every minute for 1 h. From these data, the elative fluorescence index (RFI) of the last time point (minute 60) of the EB accumulation assay, were calculated according to the following formula:

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

where $R_{Funtreated}$ is the RF at the last time point of the EB retention curve of the untreated control with the solvent control (DMSO) and $RF_{treated}$ is the relative fluorescence (RF) at the last time point of EB retention curve in the presence of an inhibitor. In the case of *S. aureus*, reserpine (25 μM) was used as positive control, for *E. coli*, CCCP (50 μM) was used. Bacteria and EB solution only served as negative control.

Declarations

Author contribution statement

Dezső Csupor: Conceived and designed the experiments, Contributed reagents, materials, analysis tools or data, Wrote the paper. Diána Kerekes: Performed the experiments, Wrote the paper. Attila Horváth, Norbert Kúsz: Performed the experiments. Botond Lajos Borcsa: Contributed reagents, materials, analysis tools or data, Wrote the paper. Nikoletta Szemerédi: Performed the experiments, Analyzed and interpreted the data. Gabriella Spengler: Conceived and designed the experiments, Performed the experiments, Analyzed and interpreted the data, Contributed reagents, materials, analysis tools or data.

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Data availability statement

Data will be made available on request.

Declaration of interest statement

The authors declare no conflict of interest.

Additional information

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