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Regular paper

Synthesis and biological evaluation of 4′-*O*-acetylisoxanthohumol and its analogues as antioxidant and antiproliferative agents

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Isoxanthohumol (2) and its 4′-*O*-monoacylated (3) and 7,4′-*O*-diacetylated (4) derivatives were synthesized and evaluated *in vitro* for their cytotoxic activity against several cancer cell lines of various origins: MCF-7 (breast), A549 (lung), MESSA (uterine sarcoma), LoVo (colon), drug-resistant human cancer cells (MESSA/DX and LoVo/DX), glioblastoma (U-118 MG), and also towards the non-cancerous cell line MCF-10A (normal breast cells). An antiproliferative assay indicates that 7,4′-di-*O*-acylisoxanthohumol (4) has similar cytotoxicity to its precursor, isoxanthohumol (2), against selected cell lines (A549, MES-SA, MES-SA/5DX, and U-118 MG). Compound 4 was only slightly more cytotoxic to lung, colon, breast (cancerous and normal) and uterine sarcoma (drug sensitive and drug resistant) cell lines compared to its monoacylated derivative (3). Both acylated isoxanthohumols showed preferential activity against tumor cells (MCF-7) and low cytotoxicity to normal cells (MCF-10A), which suggests selectivity of the acylated isoxanthohumols towards cancer cells. Additionally, the activity of the acylated isoxanthohumols was higher than for 2. To the best of our knowledge this is the first report on bioactivity of monoacylated isoxanthohumol (3) and its ester derivatives as antiproliferative compounds in drug resistant cell cultures. Acylation of 2 decreased the antioxidant activity determined by the DPPH method in the order isoxanthohumol (2) >4′-*O*-acetylisoxanthohumol (3) >7,4′-di-*O*-acetylisoxanthohumol (4).

Key words: *O*-acylated flavanones, antiproliferative effect, isoxanthohumol, hop flavonoids

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Abbreviations: DEPT, distortionless enhancement by polarization transfer; DMPC, dimyristoylphosphatidylcholine; DMSO dimethyl sulfoxide; DPPC, dipalmitoylphosphatidylcholine; DX, doxorubicin; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NMR, nuclear magnetic resonance; PBS, phosphatebuffered saline; SRB, sulfrhodamine B; TLC, thin layer chromatography; TMS, tetramethylsilane; HSQC, heteronuclear single quantum correlation

INTRODUCTION

Isoxanthohumol (**2**) is a natural aglycone flavonoid found together with xanthohumol (**1**, XN) and 8-prenylnaringenin (8PN) in hop (*Humulus lupulus*, *Cannabaceae*) and in hop waste as by-products after extraction with supercritical carbon dioxide. Isoxanthohumol (**2**, IXN) is also one of the three major flavonoids most abundant in beer (Stevens *et al*., 1999). However, during the beer-making process, **2** is obtained through thermal isomerisation of its precursor – the corresponding chalcone XN. Like many flavonoids, **2** has been found to possess numerous biological activities with potentially beneficial effects on the human body. Reported health promoting functions of **2** include antioxidant, anti-inflammatory, antibacterial, antiviral, antitumor and proestrogenic properties (Mizobuchi & Sato, 1984; Miranda *et al*., 1999). Recent studies have shown that **1** can be used as an adjuvant in cancer therapy, due to its ability to increase *in vivo* cytostatic activity of paclitaxel, a commonly used cytostatic agent (Krajnović *et al*., 2016).

It is known that the biological activity of flavonoids is determined by their chemical structures and on their physicochemical properties such as size of molecules, lipophilicity and solubility (Kiekow *et al.,* 2016; Lim *et al.,* 2017). Many reports suggest that prenylation increases bioavailability and bioaccumulation of flavonoids and may be a promising tool for applying the biological functions of flavonoids for clinical use (Mukai *et al.,* 2013; Terao & Mukai, 2014; Venturelli *et al.,* 2016). C-isoprenylated derivatives of flavonoids are potential P-glycoprotein (Pgp) modulators in tumor cells. These properties make **2** a promising and easily accessible bioactive ingredient that can potentially be applied to many nutraceutical and pharmaceutical products. However, like many flavonoids, **2** is poorly soluble in both aqueous and non-aqueous systems thus limiting its processability and application potential. Many studies have concluded that despite the fact that hop flavonoids are practically insoluble in water in their basic form and poorly bioavailable as aglycones, they possess wide range of biological activity. Hence, there have been many studies aimed at improving water-solubility of flavonoids by acylation (Saik *et al.,* 2017). The mode of action of prenylated flavonoids is not yet fully understood. In the literature, there are only few reports describing synthesis of **2** and 8PN derivatives. To date, there are also no reports describing the chemical synthesis of mono-acylated derivatives of **2**. Also, there is no information in literature about the research comparing the antioxidant and antiproliferative activity of **2**, the monoacylated and diacylated derivatives of **2,** against cancer cell lines of various origin.

Keeping in mind the valuable properties of hop flavonoids, the objective of this study was to obtain **2**, its mono- and diacetate derivatives, and to investigate the

growth inhibitory effects of these compounds on cancer cell lines of various origin. The cell lines tested include breast, lung, uterine sarcoma, colon, glioblastoma, the drug-resistant human cancer cells (MESSA/DX and LoVo/DX), and non-cancerous breast cells. We hypothesize that since antiproliferative properties of **2** have been reported, structural modifications of this compound may lead to analogs with improved growth inhibitory effects.

In this paper we report the synthesis, characterization, spectroscopic properties, antioxidant and antiproliferative activities of 4'-*O*-acetylisoxanthohumol (**3**). To the best of our knowledge, the antiproliferative activity of compounds **2**-**4** toward drug resistant cancer cell lines are reported here for the first time.

The described research is a continuation of our previous work on chemical synthesis of analogues of **1** and assessment of their biological activity (Stompor & Żarowska, 2016). This study may be also helpful in utilization of by-products obtained after extraction of hop cones with supercritical carbon dioxide in the brewing industry. This waste material is rich in **1**, which we used as a substrate for the synthesis of **2** and its derivatives.

MATERIALS AND METHODS

Commercial solvents and reagents were of analytical grade. The reagents were purchased from Sigma-Aldrich Chemical Co. (Steinheim, Germany). As a raw material for isolation of **1**, by-products obtained after extraction of hops with supercritical CO_2 in industrial installations located in the New Chemical Syntheses Institute, in Puławy, Poland were used. Gradient grade purity methanol was purchased from Merck (Darmstadt, Germany). 2,2-Diphenyl-1-picrylhydrazyl radical (DPPH) was purchased from Sigma-Aldrich Chemical Co. (Steinheim, Germany). Analytical thin-layer chromatography was carried out on silica gel 60 F_{254} plates (Merck) with chloroform:methanol (95:5 v/v) for **1** and **2** and hexane:acetone (5:2 v/v) as the developing solvent for derivatives of **2**. Visualization of the compounds was accomplished with a solution of 10 g $Ce(SO_4)_2$ and 20 g phosphomolybdic acid in 1 L of 10% H_2SO_4, followed by heating. Silica gel (Kiesel 60, 230–400 mesh, Merck) was used as a stationary phase for column chromatography and a mixture of dichloromethane and methanol in different concentrations as eluents. NMR spectra (^{1}H, ^{13}C, HSQC, DEPT 135) were recorded with a Bruker 500 MHz Ultra Shield TM Plus instrument with acetone-d_6 and chloroform-d_1 as solvents and TMS as an internal standard. High-resolution mass spectra (HRMS) were measured with a Bruker micrOTOF-Q spectrometer located in the Department of Chemistry of the University of Wroclaw.

Chemical synthesis. Xanthohumol (**1**), the substrate for the synthesis of isoxanthohumol (**2**), was isolated from carbon dioxide-extracted hops. It was purified and transformed into **2** according to a modified procedure described by Wilhelm and Wessjohann (2006). Acetyl derivatives of **2** (compounds **3** and **4**) were synthesized using different amounts of acetic anhydride in 19.7% and 54% yield, respectively. Chemical structures of the compounds were confirmed by ^{1}H and ^{13}C NMR, HSQC, IR and HR ESI-MS spectroscopy.

Chemical synthesis of isoxanthohumol (2) and its derivatives (3 and 4). Xanthohumol (**1**) (5.08 mmol) was dissolved in 1 L of 1% NaOH solution and stirred at 0°C for 4 h. The reaction progress was monitored by TLC using chloroform-methanol as an eluent. Acidification with 50% H_2SO_4 gave light yellow precipitate. After filtration, the precipitate was carefully washed with water until neutral and dried. The crude product was purified by column chromatography (chloroform:methanol 95:5 v/v) to give **2** as a light yellow powder in 93.3% yield. The spectral data corresponds to that described by Potaniec and coworkers (2014).

4'-*O*-acetylisoxanthohumol (3). The acetic acid esters of **2**, compounds **3** and **4** were prepared by the reaction of **2** with acetic anhydride in pyridine using the modified method described by Zhu and coworkers (2014).

To a solution of **2** (0.2 g, 0.56 mmol) in pyridine (20 mL), acetic anhydride (0.07 mL, 0.56 mmoL) was added dropwise. The reaction mixture was stirred at room temperature for 24 h, then the solvent was removed using rotary evaporation. The residue was redissolved in CH_2Cl_2, washed with 1 M HCl (3×15 mL) and then with saturated $NaHCO_3$ solution (15 mL) and water to neutralize. The organic phase was separated, dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. The residue was purified by column chromatography on silica gel, eluting with hexane:acetone (5:2 v/v) to give the product as light yellow crystals.

(44.1 mg, 19.7%) ^{1}H NMR (500 MHz, $CDCl_3$) δ (ppm): 1.57 (s, 3H, CH_3-4"), 1.65 (s, 3H, CH_3-5"), 2.32 (s, 3H, C-4'-$COOCH_3$), 2.83 (dd, 1H, *J*=16.5 Hz, *J*=13.0 Hz, CH-3), 3.03 (dd, 1H, *J*=16.5 Hz, *J*=13.0 Hz, CH-3), 3.18 (d, *J*=7.0 Hz, CH_2-1"), 3.86 (s, 3H, C-5-O-CH_3), 5.06 (t_{sept}, 1H, *J*=7.0Hz, *J*=1.3 Hz, CH-2"), 5.36 (dd, 1H, *J*=13.0 Hz, *J*=2.9 Hz, CH-2), 6.28 (s, 1H, CH-6), 6.87 (d, 2H, *J*=8.6 Hz, CH-3' and CH-5'; AA'BB'), 7.29 (d, 2H, *J*=8.5 Hz, CH-2' and CH-6'; AA'BB'). ^{13}C NMR (125 MHz, $CDCl_3$) δ (ppm): 190.8 (C=O), 168.8 (-O<u>C</u>OCH_3), 162.0, 159.4, 156.2, 154.6, 132.0, 130.5, 127.7 (C-2', C-6'), 121.5 (C-2"), 115.5 (C-3', C-5'), 115.2, 109.6, 99.1 (C-6), 78.8 (C-2'), 56.2 (-OCH_3), 45.3 (C-3), 25.7 (C-5"), 22.9 (C-1"), 21.0 (-CO<u>C</u>H_3), 17.7 (C-4"). HR ESI-MS *m/z*: 419. 1463 $[M+Na]^+$; calcd: 419.1465 $[C_{23}H_{24}O_6+Na]^+$.

7,4'-*di*-*O*-acetylisoxanthohumol (4). The reaction was carried out in the same way as described for compound **3**, using 0.13 mL (1.4 mmoL) of acetic anhydride as the acyl group donor. The product was obtained as a white solid.

(134 mg, 54%) ^{1}H NMR (500 MHz, $CDCl_3$) δ (ppm): 1.59 (s, 3H, CH_3-4"), 1.65 (s, 3H, CH_3-5"), 2.31 (s, 3H, C-4'-$COOCH_3$), 2.32 (s, 3H, C-7-$COOCH_3$), 2.85 (dd, 1H, *J*=16.4 *J*=3.1 Hz, CH-3), 3.00 (dd, 1H, *J*=16.4 Hz, *J*=13.2 Hz, CH-3), 3.20 (d, 2H, *J*=7.0 Hz, CH_2-1"), 3.90 (s, 3H, C-5-O-CH_3), 5.07 (t_{sept}, 1H, *J*=7.0 Hz, *J*=1.2 Hz, CH-2"), 5.43 (dd, 1H, *J*=13.2 Hz, *J*=3.0 Hz, CH-2), 6.30 (s, 1H, CH-6), 7.14 (d, 2H, *J*=8.6 Hz, CH-3' and CH-5'; AA'BB'), 7.46 (d, 2H, *J*=8.5 Hz, CH-2' and CH-6'; AA'BB'). ^{13}C NMR (125 MHz, $CDCl_3$) δ (ppm): 189.9 (C=O), 169.4 (-O<u>C</u>OCH_3), 168.7, 161.6, 159.4, 154.6, 150.7, 136.3, 132.0, 127.1 (C-2', C-6'), 121.9 (C-3', C-5'), 121.5 (C-2"), 115.2, 109.7, 99.3 (C-6), 78.5 (C-2), 56.2 (-OCH_3), 45.6 (C-3), 25.7 (C-5"), 22.9 (C-1"), 21.1 (C-7-$COCH_3$), 21.0 (C-4'-CO<u>C</u>H_3), 17.8 (C-4"). HR ESI-MS *m/z*: 439.1755 $[M+H]^+$; calcd: 439.1751 $[C_{25}H_{26}O_7+H]^+$.

Assay for antiproliferative activity. Cell culture and determination of IC_{50}. Antiproliferative tests were performed on human cancer cell lines: A-549 (non-small cell lung), MCF-7 (breast), LoVo (colon), LoVo/DX (colon drug-resistant), MES-SA (uterine sarcoma), MES-SA/DX5 (drug-resistant uterine sarcoma) U-118 MG (glioblastoma) and normal MCF-10A breast cell line. The cell lines were obtained from the American Type Culture Collection (Rockville, Maryland, U.S.A.) or from

the European Collection of Authenticated Cell Cultures (MCF-7 cell line; ECACC, Salisbury, UK). The cell lines are maintained in the Institute of Immunology and Experimental Therapy, Wroclaw, Poland. The A549 and MES-SA cell lines were cultured in a mixture of RPMI 1640+Opti-MEM (1:1) (both from IIET, Wroclaw, Poland) supplemented with 2 mM L-glutamine, 5% fetal bovine serum (all from Sigma-Aldrich Chemie GmbH, Steinheim, Germany). LoVo and LoVo/DX cells were cultured in the mixture of RPMI 1640+Opti-MEM (1:1), supplemented with 2 mM L-glutamine and 5% fetal bovine serum (all from Sigma-Aldrich Chemie GmbH, Steinheim, Germany) adjusted to contain 1.0 mM sodium pyruvate. MCF-7 cells were cultured in Eagle medium (IIET, Wroclaw, Poland), supplemented with 10% fetal bovine serum, 1% non-essential amino acid solution, 0.8 mg/L of insulin and 2 mM L-glutamine (all from Sigma-Aldrich Chemie GmbH, Steinheim, Germany). MCF-10A cells were cultured in the F-12 nutrient mixture (Gibco, Scotland, UK), supplemented with 5% horse serum (Gibco, Scotland, UK), 10 μg/mL of cholera toxin (*Vibrio cholerae)*, 10 μg/mL of hydrocortisone and 20 ng/mL of human epidermal growth factor (all from Sigma-Aldrich, Chemie GmbH, Steinheim, Germany). The LoVo/DX cell culture was supplemented with 0.1 μg/mL and the MES-SA/DX5 cell culture with 0.58 μg/mL of doxorubicin hydrochloride (Accord Healthcare Limited, UK). All culture media were supplemented with penicillin (100 units/mL), and streptomycin (100 μg/mL) (both from Polfa Tarchomin S.A., Warsaw, Poland). U-118 MG cells were cultured in the Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin (100U/mL) and streptomycin (100 μg/mL) solutions (all from American Type Culture Collection (ATCC, Manassas, VA, USA). All cell lines were grown at 37°C in a 5% CO_2 humidified atmosphere.

Antiproliferative effects of the tested compounds was examined after a 72 h-exposure of the cultured cells to varying concentrations of the compounds tested (total plate incubation time: 96 h), using the sulforhodamine B (SRB) assay for A549, MCF-7, MCF-10A, LoVo, LoVo/Dx, MES-SA, MES-SA/DX5 cells or XTT and NR assay for glioblastoma (U-118 MG). The details of SRB technique is described by Sidoryk and coworkers (2014). The results were calculated as an IC_{50} (inhibitory concentration 50); the concentration of the tested agent that inhibits proliferation of the cell population by 50%. IC_{50} values were calculated for each experiment separately (Nevozhay, 2014) and mean values ±S.D. are presented in Tables 1 and 2. Each compound in each concentration was tested in triplicate in a single experiment, which was repeated 3–5 times. The activity of the tested compounds was compared to the activity of cisplatin and doxorubicin used as reference agents.

Using the obtained IC_{50} values, the resistance indexes (RI) for LoVo/DX and MES-SA/DX cells were calculated by dividing the IC_{50} values of the compounds tested against the drug-resistant cells by respective values obtained for the drug-sensitive cells. According to Harker and Sikic (1985), three categories of cells could be distinguished: (a) drug-sensitive cells – if the ratio approaches 0–2; (b) moderately drug-resistant cells – if the ratio ranges from 2 to 10; (c) markedly drug-resistant cells – if the ratio is higher than 10.

Neutral Red assay (NR). Glioblastoma cells were seeded in flat bottom 96-well culture plates in triplicate at a density of 5×10^3 cells per well (100 μL cell suspension per well). The cells were allowed to attach for 24 h. Working solutions of **2**, **3** or **4** (0.78–50 μM) were prepared in culture media with FBS from the 100 mM stock solutions of the tested compounds in DMSO. The DMSO concentration was adjusted to 0.05% in all samples, which had no significant effect on treated cell lines (not shown). Cell monolayers were treated with working solutions of **2**, **3** or **4** (150 μL/well) for 72 h at 37°C. Afterwards, the media were removed and 100 μL of NR solution (2% of the culture medium volume) was added to each well and the cells were incubated for 1 h in 5% CO_2 at 37°C. After washing once with PBS, 100 μL/well of the fixative (50% ethanol, 49% H_2O, and 1% glacial acetic acid) was added and the plates were shaken at 450 rpm for 15 min. Absorbance was measured at 540 nm against 620 nm in a microtiter plate reader (μQuant – BioTek, Winooski, Vermont, USA) against blank (fixative mixture). The assays were performed in triplicate in three independent experiments. Neutral red solution and 3-amino-m-dimethylamino-2-methyl-phenazine hydrochloride (0.33%) were provided by Sigma-Aldrich Co. (Steinheim, Germany).

XTT assay. Cells were cultured as described earlier. After exposure to the tested compounds, the medium was removed and a mixture of 5 mM of XTT and 25 μM of PMS diluted three times in the complete medium was added (100 μL/well) and the plates were returned to the incubator for 1 h. Then absorbance was measured at 450 nm against 620 nm against a blank sample (100 μL of complete growth medium containing XTT and PMS), using a microplate reader (μQuant – BioTek, Winooski, VT, USA). The assays were performed in triplicates in three independent experiments. XTT sodium salt (2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide inner salt) and phenazine methosulfate (PMS), N-methyldibenzopyrazine methyl sulfate were provided by Sigma-Aldrich Co. (Steinheim, Germany).

Antioxidant activity. The antioxidant activity was determined by the DPPH method according to the modified procedure described by Potaniec and coworkers (2014). The absorbance was measured using a microplate reader (TECAN Infinite 200). All experiments were performed in triplicate. The radical solution was prepared by dissolving 3.94 mg DPPH in 50 mL metthanol (0.2 mM). For the photometric assay 100 μL DPPH solution and 100 μL antioxidant solution (0.05 mg of compound/1 mL methanol) were mixed. The study used the 96-well microtiter plates. After 30 min, the absorbance was measured at 517 nm. The antioxidant ability of the tested compound was assessed according to the following formula: % DPPH inhibition=$100\times(A_0-A_c)/A_0$, where A_0 is the absorbance of DPPH solution, A_c is the average absorbance value of the tested solution containing antioxidant at a known concentration.

Statistical analysis. Statistical analysis showed a non-normal distribution of data in experimental groups (Shapiro–Wilk test). Therefore, to estimate the differences between **2**, **3** or **4** treated and non-treated control samples a statistical analysis was performed using the nonparametric Kruskal–Wallis test. Statistical analysis was performed with Statistica 12.5 software (StatSoft).

RESULTS AND DISCUSSION

Chemistry

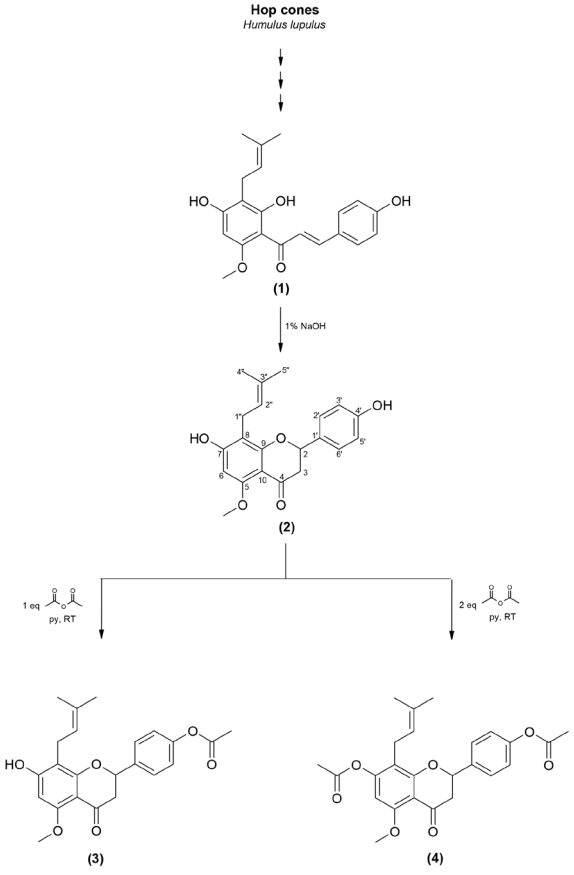
Based on the known biological characteristics of **2** and taking into account the antiproliferative activity of

its precursor **1**, the anticipated antiproliferative activity of the hop-derived prenylflavonoids **3** and **4** was tested on several human cancer cell lines.

The percentage content of **2** in the dry weight of the hop flowers is 0.008%, however, the content of **2** in hop products, including the extract resulting from extraction with supercritical carbon dioxide, is below the detection limit (Magalhães *et al.,* 2007). For this reason, in our research, the main hop chalcone **1**, which is a precursor of **2**, was extracted from the by-product waste obtained with supercritical CO_2. The spectral characteristics of the obtained substance was in agreement with literature data for **1** (Stompor *et al.,* 2013). Next, **2** was prepared by base-catalyzed cyclization of **1** using 1% NaOH at 0°C, followed by acidification of the solution with 50% H_2SO_4, to give yields up to 100%.

Synthesis of monosubstituted isoxanthohumol (**3**) containing the acyl group at C-4' was achieved by acylation of the substrate **2** with one equivalent of acetic anhydride (Scheme 1). This reaction resulted in 19.7% conversion of substrate after 24 h of stirring at room temperature. The structure of the obtained product **3** was established based on the spectral data.

In the ^{1}H NMR spectrum of 4'-*O*-acetylisoxanthohumol (**3**) protons of the aromatic ring B are visible as two signals, integrating for two protons each, as an AA'BB' system at δ=7.29 ($J_{2'6'}$=8.5 Hz) and 6.87 ppm ($J_{3'5'}$=8.6 Hz). The only proton present in ring A in compound **3** gives a singlet at δ=6.28 ppm (CH-6). The methylene protons of ring C in **3** give two signals in the shape of wide one-proton doublets of doublets at δ=3.03 ppm (H-3_{ax}) and 2.83 ppm (H-3_{eq}), with coupling constants J=16.4 Hz and 13.0 Hz. Two three-proton singlets at δ=3.86 and δ=2.32 ppm indicate the presence of two alkoxy groups at C-5 and C-4' positions, respectively. A singlet of 3H at δ_H=3.86 ppm, corresponding to the carbon atom at δ_c=56.2 ppm in the ^{13}C NMR was assigned to the methoxy group, whereas the second singlet in this region (δ=2.32 ppm) to the alkoxy group at C-4' (δ=20.9 ppm). Methyl protons of the prenyl group were observed as two three-proton singlets at δ_H=1.65 ppm (H-4") and δ_H=1.59 ppm (H-5"), which show strong correlation with the carbon atoms at δ_c=25.7 ppm and δ_c=17.8 ppm, respectively. The HR ESI-MS analysis of the pure product **3** (positive ionization mode) showed the presence of the base peak $[M+Na]^+$ at m/z 419.1465 which suggests a monoacetate-**2** derivative. The calculated m/z value for $[C_{23}H_{24}O_6+Na]^+$ is 419.1465. The structure of compound **3** was found to be 4'-*O*-acetylisoxanthohumol, and to the best of our knowledge, it has not been previously reported in the literature.



Scheme 1. Chemical synthesis of isoxanthohumol (2) and its derivatives (3 and 4).

Treatment of **2** with two equivalents of acetic anhydride in the presence of pyridine led to 7,4'-di-*O*-acetoxylisoxanthohumol (**4**). In the ^{1}H NMR spectrum of product **4**, the most shielded H-2', H-6' and H-3', H-5' protons in ring B were observed at δ=7.46 and 7.14 ppm as two multiplets similar to a doublet, showing a typical pattern for the AA'BB' system. The presence of a methoxy group is proved by a three-proton singlet at δ_H=3.90 ppm in the ^{1}H NMR spectrum and the signal at δ_C=56.2 ppm in the ^{13}C NMR spectrum. A combined analysis of ^{13}C and DEPT 135° NMR indicates the presence of five primary, seven tertiary and ten quaternary carbon atoms in compound **4**. Two signals of methylene groups at δ_c=22.9 ppm (C-1") and δ_c=45.6 ppm (C-3) were negative. In the ^{1}H NMR of **4** we observed the signals of alkoxy groups, which appeared at δ=2.31 ppm and δ=2.32 ppm, integrating for three protons each. The HSQC spectrum showed a strong coupling correlation between the alkoxy protons and carbon atoms at δ_c=21.1 and δ_c=21.0 ppm, respectively. High-resolution electrospray ionization mass spectral data (HR ESI-MS) of compound **4** showed the molecular ion $[M+H]^+$ at m/z 439.1755 which established the molecular formula as $C_{25}H_{26}O_7$ (calcd for $[C_{25}H_{26}O_7+H]^+$: 439.1751), demonstrating that we obtained isoxanthohumol diacetate. Compound **4** has been synthesized and is known in the literature (Aniol *et al.,* 2012).

Antiproliferative activity

Because the biological activity of natural flavonoids and their synthetic derivatives depend on their chemical structures and on relative orientation of various moieties present in the molecule, the aim of our study was at first to obtain **2**, one of the hop flavanones, and in the next step, to synthesize two derivatives of **2** and to compare their growth inhibitory effect on cancer cell lines (MCF-7, LoVo, LoVo/DX, MESSA, MESSA/DX and U-118 MG) with parent **2**. Keeping in mind that the solubility of free flavonoids is low in water and moderate in organic solvents, in our investigations comprising isolation of the polyphenol compounds from spent hops and derivatization of **2** by chemical methods we were looking for such a method of determination of antiproliferative activity that would be suitable for both hydrophilic and hydrophobic compounds. The most commonly used are MTT, NR, SBR and XTT techniques.

In our research, we used three of the above methods (XTT, NR, and SRB) based on spectrophotometric measurements involving different dyes. The XTT technique

Table 1. IC_{50} values (μM) representing the antiproliferative activity of compounds 2-4 against human cancer cell lines determined using the SRB assay.

Compound	IC_{50} ± S.D. [μM][a]						
	Cell line						
	MCF-7	MCF-10A	A-549	LoVo	LoVo/DX	MES-SA	MES-SA/DX5
2	30±3.8	37.1±3.8	29.7 ±4.2	8.96±1.5	26.8±4.0	16±3.6	30.4±4.1
3	32.1±4.8	49.6±14.5	29.2±3.7	11.1±4.6	25.4±4.7	22.1±7.9	32.3±1.5
4	29±2.65	38.7±4.4	26.3±3.4	9.8±3.6	29.3±2.4	15.7±6.9	28.6±2.1
cisplatin[b]	9.0±1.0	9.0±1.7	6.8±2.7	4.9±0.7	3.5±0.3	1.3±0.1	2.0±0.3
doxorubicin[b]	–	–	–	0.086±0.05	6.2±2.9	0.02±0.0074	4.9±0.8

[a]Data are the means ±S.D. of three independent experiments. [b]Cisplatin and doxorubicin were employed as positive controls.

utilizes tetrazolium salts which allow an evaluation of the impact of the tested compounds on the activity of mitochondrial enzymes. The NR test utilizes neutral red, a dye which is incorporated into lysosomes of viable, uninjured cells. Lastly, we used the SRB cytotoxicity assay utilizing the dye sulforhodamine B, which at certain pH values has the ability to bind electrostatically to proteins in living cells and therefore is used for cell density determination based on measurements of the cellular protein content. Because, as described earlier, anticancer effects induced by **1** towards lung cancer cells at concentrations ranging from 0.1–100 μM increased at longer exposure time (Young *et al.,* 2015), we used 72-h tests in our study.

In the first step of our study, a screening test was performed on the MCF-7 human breast cancer cell line and the MCF-10A normal cell line (Table 1). All tested compounds proved to be antiproliferative agents towards MCF-7 cancer cells, with IC_{50}<33 μM. At the same time, these compounds have a lower antiproliferative activity with respect to normal cells relative to MCF-10A. The solvent used (DMSO) in the highest concentration of 1% had no significant effect on cell growth (not shown).

In the next step, compounds **2**, **3** and **4** were tested for antiproliferative activity toward six human cancer cell lines as follows: A549 (lung), MES-SA (uterine sarcoma), LoVo (colon), doxorubicin-resistant colon cancer LoVo/DX (P-gp-dependent, MRP-, LRP-dependent multidrug resistance), MES-SA/DX5 (P-gp-dependent resistance to doxorubicin), and also toward glioblastoma (U-118 MG).

Our results indicate that the compounds are selective in their activity towards various cell lines. According to our results, all compounds have a preference for colon (LoVo) and uterine sarcoma (MES-SA) cell lines ($8 \leq IC_{50} \leq 22$). We observed that almost all the synthesized compounds were cytotoxic to the tested cancer cell lines and that cytotoxic activity of the acyl derivatives was dependent on the position of the acyl group in the backbone of the flavone. In previous studies, diacyl derivatives of **2** showed low activity against selected human cell lines (Aniol *et al.,* 2012). In this study, the diacyl derivative **4** showed slightly higher activity towards all newly tested cell lines, except for LoVo and LoVo/DX, compared to the monoacylated isoxanthohumol **3**. This is consistent with a previous report that indicated prenylated hop flavonoids can suppress invasion and genotoxicity colon cancer cells (Caco-2) (Allsopp *et al.,* 2013). According to these authors, the described mechanism of action appears to be independent of the estrogenic activity of these compounds.

To date, **2** was tested *in vitro* as a potential inhibitor of cancer cell growth toward only few human tumor lines (Hudcová *et al.,* 2014; Miranda *et al.,* 1999). Many results show that the determined antiproliferative activity may also depend on the method of analysis and the concentration of tested compounds. For this reason, in our study we used three independent tests: SRB, NR and XTT. IC_{50} values for all the prenylated compounds on tested cell lines using the SRB assay are summarized in Table 1. Table 2 presents the results obtained in two separate *in vitro* studies utilizing the XTT and the NR assay in the U-118 MG cell line. Using both XTT and NR methods, compound **2** showed about 1.5-1.8-fold higher activity toward glioblastoma cells (U-118 MG) than **3** and **4** indicating that exchange of 7- and 4'-hydroxy groups on A and B-rings, respectively, with *O*-acetoxy groups has a considerable influence on the cytotoxicity profile (Table 2).

Similarly, slightly better results were obtained for MCF-7, A-549, MES-SA and MES-SA/DX5 cell lines. Considering the effect of hydroxyl groups on the activity of the hydroxyflavanones studied, it was observed that starting compound **2** was slightly more active than derivatives **3** and **4** towards U-118 MG cancer cells both in the NR and XTT assay. **2** was also found active against the PC-3 and DU145 human prostate cancer cells using the WST-1 assay, with the reported IC_{50} value of 45 and 47 μM, respectively (Delmulle *et al.,* 2006).

Previous research shows that some prenylated isoflavones improve the stability of the phospholipid bilayer (Wesolowska *et al.,* 2014). Their accumulation close to biological membranes increases membrane resistance to oxidative agents. Therefore, investigation of anticancer activity of the tested compounds requires a detailed analysis of many factors which influence this process.

It has been suggested that the biological activity of the compounds found in hops (*Humulus lupulus*) may be attributed to changes in nature and permeability of cell membranes. Wesolowska and coworkers (2014) and Arczewska and coworkers (2013) carried out research on major hop flavonoids **1** and **2**. The results indicate that these hop flavonoid compounds have strong affinity to biological membranes (DPPC, DMPC). It was shown that the presence of **2** in the cell membrane changes its

Table 2. IC_{50} values for 2, 3 and 4 after 72 h of treatment of U-118 MG cells. IC_{50} was calculated from medians of the results obtained in the NR and XTT assays.

Compound	NR IC_{50} [μM]	XTT IC_{50} [μM]
2	10.5	15.6
3	15.4	27.7
4	17.5	20.5

Table 3. Resistance index (RI) values of compounds 2–4.

Compounds	RI*	
	MES-A/DX5/MES-SA	LoVo/DX/LoVo
2	1.9	2.99
3	1.46	2.29
4	1.82	2.99
cisplatin	1.54	0.71
doxorubicin	245	72.1

conformational state in the hydrophobic region, mainly due to the strong affinity of the membrane to the prenyl group. Hydrocarbon chains of the membrane lipids change their shape and methylene group conformation, which results in a drop of the main-phase transition temperature and conversion of the membrane into the liquid-crystalline state. Among the tested flavonoids, the strongest capability of fluidization of the lipid bilayer was observed for the relatively planar, lipophilic molecule **1**. Its strong affinity to phosphatidylcholine acyl chains allows deeper penetration into the lipid bilayer. Elucidation of the mechanisms of interaction between biologically active flavonoids or new synthetic derivatives with particular elements of the cytoplasmic membrane bilayer may be helpful in the design of novel therapeutic agents.

To summarize, all the compounds were moderately active antiproliferative agents and none of the modifications of **2** elicited significant changes in activity. This trend is similar to that demonstrated previously. These results confirm that new prenylated derivatives of xanthohumol have good cytotoxicity towards all tested cell lines. We observed that **2** exhibits higher activity than its esters against only few tested cell lines. After a 3-day treatment, the lowest concentration of **2** at which the growth of the cell lines was inhibited by 50% (IC_{50}) was 8.96 μM for colon cancer (LoVo).

We also calculated the resistance indexes (RI) by dividing the IC_{50} values of the compounds tested against the drug resistant cells LoVo/DX and MES-SA/DX5 by respective values obtained against the drug sensitive LoVo and MES-SA cell lines (Table 3).

All compounds were able to overcome the barrier of P-gp-dependent resistance. Among the obtained acylated derivatives of **2,** compound **3** has the highest ability to overcome the barrier of multidrug resistance (RI = 1.46), which is comparable to cisplatin used as a reference (RI=1.54). The LoVo/DX cells showed moderate resistance (RI≤3). In all cases, the degree of resistance to the three tested flavonoids was lower than that of the established anticancer drug, doxorubicin (RI=72), nevertheless they possess the potential to fight cancer multidrug resistance. Our results with cancer cell lines of various origins corroborate earlier observations. Potential application of natural flavonoids in the pharmaceutical industry can be further enhanced by simple structure modifications, such as selective esterification for developing new drugs for clinical use in the future.

Table 4. Antioxidant activity of isoxanthohumol (2) and its derivatives (3–4).

Compound	EC_{50} ± S.D. [mM]	$EC_{50}/EV_{50}AA$
Ascorbic acid (AA)	0.200442±0.000146	–
2	7.6006±0.458	37.19
3	59.701[a]±8.933	297.91
4	73.4538[a]±8.595	366.46

[a]Extrapolated

Antioxidant activity

Antioxidant activity shown in Table 4 is defined as a concentration of the antioxidant in mmol/L (mM) that causes 50% loss of the DPPH activity (EC_{50}). The data are presented as mean values ±S.D. (n=3). L-Ascorbic acid was used as a positive control. In the described method a small value indicates strong antioxidant properties and therefore represents small residual unreacted DPPH in the analyzed sample. As shown in Table 4, all investigated flavanones have antioxidant activity between 73.5 mM (very low activity) and 7.6 mM (the highest activity, observed for **2**). Ascorbic acid, a very good antioxidant, has been used as a reference standard with activity of 0.2004 mM.

Antioxidant activity depends on particular aspects of the molecular structures of the compounds. In this study we wanted to verify that the *O*-acylation of one or two of the hydroxyl groups present in **2** will not lower the antioxidant activity in comparison to the starting substrate. Our results indicate that **2** (EC_{50}=7.6 mM) is about 8-fold stronger antioxidant than its mono- (**3**) (EC_{50}=59.7 mM) and 10-fold stronger than diacylderivatives (**4**), (EC_{50}=73.5 mM) due to the presence of two hydroxyl groups in the molecule.

CONCLUSION

In the present study we demonstrated antiproliferative activity of flavanones **2–4** against drug-sensitive and drug-resistant cancer cell lines. For all compounds the highest activity was noted against the colon cancer cell line (IC_{50}≤11 μM). Some compounds were most active toward uterine sarcoma (MES-SA) and glioblastoma (U-118 MG) cell lines (10 < IC_{50}≤16). The highest ability to overcome the barrier of resistance (RI=1.56) against the drug-resistant MES-SA/DX5 cells compared to the parental drug-sensitive MES-SA cell line was found for the new compound 4'-*O*-acetoxyisoxanthohumol (**3**). Both acylated isoxanthohumols (**3** and **4**) showed higher activity against tumor cells and lower cytotoxicity against normal cells (MCF-10A) compared to **2**. The antioxidant activity of the tested compounds decrease in the order: **2>3>4**. Therefore, the developed acylation protocol can be used in the synthesis of bioactive hop prenylated derivatives as potential adjuvant drugs. Such flavonoids in combination with chemotherapeutics may improve the efficacy of these agents in induction of apoptosis in cancer cells. The potential usefulness of hop prenylated flavonoids and their derivatives as interesting anticancer targets is reinforced.

For better understanding of the mechanisms involved in the action of isoxanthohumol esters as therapeutic agents, it would be interesting to characterize their activity at the molecular level. It is possible to compare their tissue distribution and subcellular localization in various cancer cell lines using the fluorescently labeled derivatives.

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Conflict of interest

The authors declare that they have no competing interests.

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