Eur Food Res Technol (2017) 243:1137–1148
DOI 10.1007/s00217-016-2827-5

ORIGINAL PAPER

Evaluation of cytotoxicity of 5-*n*-alkylresorcinol homologs and fraction on mouse fibroblast cell line L929

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Received: 27 July 2016 / Revised: 28 October 2016 / Accepted: 26 November 2016 / Published online: 9 December 2016

Abstract Cytotoxic effect of natural 5-*n*-alkylresorcinol fraction, individual homologs and some related compounds (resorcinol, orcinol and olivetol) was tested in vitro against mouse fibroblast cell line L929 in terms of their safety for use according to PN-EN ISO 10993-5:2009 method. Alkylresorcinol-rich fraction and five known individual alkylresorcinol homologs (C17:0, C19:0 C21:0, C23:0 and C25:0) were obtained from spelt (*Triticum spelta* L.) bran. The structures of these compounds were elucidated and confirmed by using two spectroscopic techniques: MS and NMR. Hydroquinone, positional isomer of resorcinol, was used as cytotoxic positive control. Cytotoxicity was expressed as IC_{50} and was estimated in the range of 171–2142 μM for individual homologs depending on alkyl chain length. Alkylresorcinol-rich fraction, individual homologs, orcinol and olivetol exhibited cytotoxicity lower than hydroquinone but higher than resorcinol on normal cells. The highest cytotoxicity among isolated compounds was evaluated for C17:0. Structure–activity relationship was dose dependent and can be described by quadratic function. This study extends our knowledge about potential safety of use of alkylresorcinols in diet supplements, cosmetics or pharmaceutical products.

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Keywords Alkylresorcinols · Resorcinol · Spelt · Cytotoxicity

Introduction

Wheat (*Triticum aestivum* L.) from family Poaceae is the elementary cereal in the human diet in many countries. However, products from other cereal species from this family, e.g., spelt (*Triticum spelta* L.) or rye (*Secale cereale* L.), are becoming more and more popular. Whole-grain food is rich in fiber, vitamins, minerals, phytosterols and phenolics. Phenolic compounds are the most prevalent group of phytochemicals in plants. Among them are 5-*n*-alkylresorcinols (ARs). These compounds are localized in the outer layer (bran) and protect the kernel, e.g., from pathogens. Their content in bran is about 0.2% [1]. The high amounts of ARs are found in rye, wheat, triticale and barley. ARs have antibacterial, antifungal, antiparasitic properties and prevent from oxidative stress (proteins, lipids and DNA oxidation) occurring in many diseases [2]. Their antimutagenic properties were confirmed few years ago [3]. ARs fraction isolated from wheat bran inhibited α-glucosidase in vitro, which may indicate that ARs may suppress postprandial blood glucose which is desired in type 2 diabetes management [4]. Wheat bran lipophilic extract containing ARs has also been proven to formulate nanoemulsion inhibiting tyrosinase activity involved in the browning of food products and melanosis in humans [5]. Previous studies on cereal ARs have evaluated them as active constituent in colon cancer prevention. Their antiproliferative activity was tested on human colon cancer cell lines HCT-116 and HT-29 [6, 7].

Separation of ARs from cereal source was usually performed by column chromatography combined with preparative HPLC and/or TLC [1, 6, 8]. Dey and Mikhailopulo

optimized method of isolation of ARs from rye bran applying SFE extraction combined with prep-HPLC [9]. No attempt was undertaken to isolate ARs from spelt.

New possibilities for the use of ARs as active ingredients in diet supplements, cosmetics and pharmaceutical products are suggested [5]. For this reason, estimation of safe ARs level for normal cells is necessary. Fibroblasts are the most common type of cell found in connective tissue, e.g., skin. The aim of the study was to evaluate the cytotoxicity of ARs fraction and individual homologs on mouse fibroblast cell line L929. The evaluation of ARs cytotoxicity in comparison with structurally related compounds as hydroquinone and resorcinol, which had previously been used in pharmacy and cosmetic industry, is necessary step for development of their new applications.

Materials and methods

Plant material

Spelt (*Triticum spelta* L.) bran and rye (*Secale cereale* L.) bran were from Mlyny Wodne (Poland) purchased commercially available on Polish market.

Solvents and chemicals

Organic solvents and reagents used in the experimental section were of analytical grade. Acetonitrile and formic acid were from JT Baker (USA), whereas all other solvents (methanol, acetone, chloroform) were from Chempur (Poland). Water was glass-distilled and deionized. Methanol for UHPLC-ESI-MS was from Sigma-Aldrich (Poland). DMSO (dimethyl sulfoxide) was from Sigma-Aldrich (Poland). Silica gel (0.063-0.200 mm) was from Merck (Germany), while octadecyl Bakerbond (40 µm) from JT Baker (USA).

Standards and standard solutions

Hydroquinone was from Fluka, Switzerland. Resorcinol, orcinol and olivetol (ARs-related compounds) were from Sigma-Aldrich, USA. Stock standard solutions (1 mg/mL) were prepared by dissolving an accurate amount of individual compound in methanol and filtered through a 0.45-µm membrane filter (Millipore, USA). The standards solutions were stored at −18 °C and were brought to the room temperature before use.

Extraction and isolation by column chromatography

Plant material (1 kg of spelt bran) was extracted with two solvents with different polarities. First, 70% methanol (*v/v*) was used for extraction in ultrasound bath at temperature below 40 °C (5 × 15 min, in total 10.5 L of solvent). Then, acetone (5 × 15 min, 10.5 L) was used. Extraction was monitored by TLC on silica gel plates with mobile phase described later. All extracts were filtered, pooled according to solvent used for extraction and concentrated under reduced pressure at 40 °C (Büchi, Switzerland). 70% Methanolic extract was left for further different constituent isolation and analysis. Acetone extract (30.4 g) was chromatographed over silica gel column (320 × 40 mm, 200 g). ARs were eluted with chloroform–acetone solvent gradient (10 + 0, 9.5 + 0.5, 9 + 1, 8 + 2, 7 + 3, 5 + 5, 0 + 10; *v/v*) (250 eluates, 50 mL each) which yielded 22 fractions (Ts1–Ts22) after TLC analyses. Ts1 to Ts8 were eluted with (10 + 0), Ts9 to Ts14 with (9.5 + 0.5), Ts15 (9 + 1), Ts16 to Ts19 with (8 + 2), Ts20 with (7 + 3), Ts21 with (5 + 5) and Ts22 with (0 + 10). Fractions were concentrated under reduced pressure at 40 °C. Fraction Ts11 (1.70 g, light beige powder), containing ARs mixture (**AR-Ts**), was dissolved in 10 mL of methanol in ultrasound bath at temperature below 40 °C and applied on octadecyl column (40 × 265 mm, 170 g). Column was connected to vacuum pump and fractionated using methanol yielding 60 eluates (50 mL each) which were combined together according to similar HPLC-PDA chromatographic profile to ten main subfractions (Ts11/1-Ts11/10) with **C21:0** homolog (573 mg) from Ts11/4, **C23:0** homolog (116 mg) from Ts11/6 and **C25:0** homolog (42 mg) from Ts11/8. Subfraction Ts11/2 (301 mg), with main compound **C19:0** and admixture of **C17:0**, was left for further analyses. Fractions were crystallized from methanol at room temperature.

Another isolation (1 kg of rye bran) was performed again in the same manner with small modifications of solvent in order to compare their utilities for isolation. Acetone extract (32.8 g) was applied on silica gel column (310 × 60 mm, 220 g) and eluted with petroleum ether–acetone solvent gradient (10 + 0, 9.5 + 0.5, 9 + 1, 8 + 2, 7 + 3, 5 + 5, 0 + 10; *v/v*) (254 eluates, 50 mL each) yielding 49 main fractions according to similar TLC chromatographic profile (Sc1–Sc49). Sc1 was eluted with (10 + 0), Sc2 to Sc10 with (9.5 + 0.5), Sc11 to Sc17 with (9 + 1), Sc18 to Sc22 with (8 + 2), Sc23 to Sc25 with (7 + 3), Sc26 to Sc28 with (5 + 5) and Sc29 to Sc49 with (0 + 10). Fractions were concentrated under reduced pressure at 40 °C. Sc15 (2 g), containing ARs mixture (**AR-Sc**), was used for further analysis. AR-Sc (light beige powder) was dissolved in 10 mL of methanol in ultrasound bath (<40 °C) and applied on first octadecyl suspended in methanol column (265 × 40 mm, 140 g). Column was connected to vacuum pump and fractionated using methanol yielding 168 eluates (10 mL each) which were combined together according to similar HPLC-PDA chromatographic profile to ten main subfractions (Sc15/1–Sc15/10). **C21:0** homolog (403 mg)

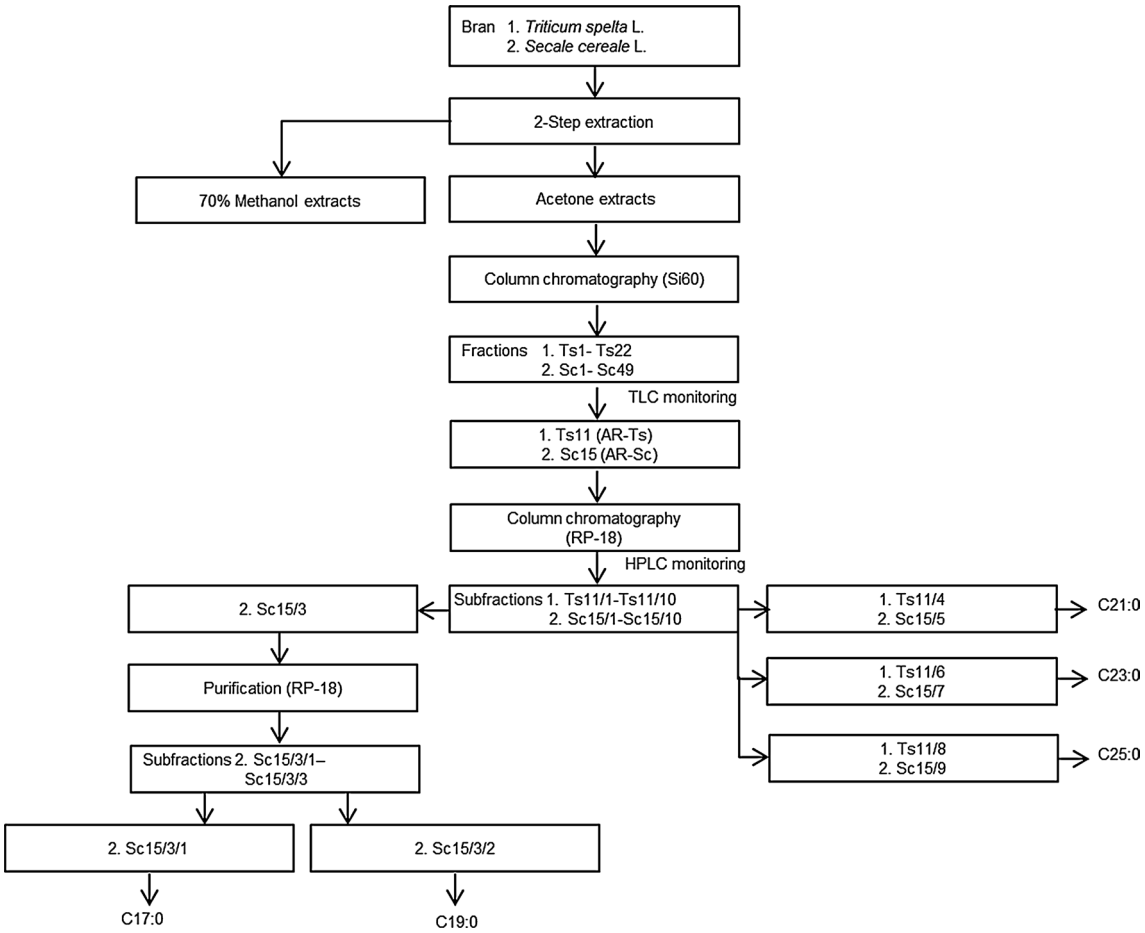

Fig. 1 Flowchart of ARs isolation from spelt and rye bran

was obtained from fraction Sc15/5, **C23:0** homolog (169 mg) from fraction Sc15/7 and **C25:0** homolog (88 mg) from fraction Sc15/9. Subfraction Sc15/3 (1.0 g) was used for second octadecyl column (375 × 30 mm, 128 g) to separate alkylresorcinol single homologs C17:0 and C19:0. It was dissolved in 2 mL of methanol, applied on octadecyl and eluted with methanol–water solvent solutions (9 + 1, 9.5 + 0.5; *v/v*). Eluates (206, 10 mL each) have been collected and combined together according to similar HPLC-PDA chromatographic profile to yield 3 subfractions with compound **C17:0** (285 mg) from Sc15/3/1 and **C19:0** (428 mg) from Sc15/3/2.

Process of isolation of ARs is depicted in flowchart (Fig. 1).

Structures of isolated compounds were confirmed on UHPLC-ESI-MS and NMR analyses.

Chromatographic conditions

HPLC-PDA analysis of alkylresorcinols for monitoring of isolation procedure was performed on the Smartline Manager 5000 system version (Knauer, Germany) equipped with the Smartline Pump 1000, degasser, sample injector, column thermostat (Jetstream 2), Smartline PDA Detector 2800 and a Hypersil GOLD column (250 × 4.6 mm i.d., octadecyl 5 µm; Thermo Scientific, UK) with precolumn (10 × 4.5 mm i.d., octadecyl 5 µm; Thermo Scientific, UK). Detection was carried out using UV/Vis diode detector. The compounds were monitored at 202, 220 and 280 nm, and UV spectra from 200 to 600 nm were recorded for peak characterization. Solvent solutions were vacuum degassed with ultrasonification prior to usage. Composition of mobile phases and profile of gradient for HPLC-PDA analyses were as follows: solvent A—water; solvent B—acetonitrile; commencing with 10% B in A, rising to 70% after 25 min and then to 100% after 30 min. Total run time was 55 min. The flow rate was 1.3 mL/min. The injection volume was 20 µL. All HPLC-PDA experiments were performed at 22 ± 2 °C.

UHPLC-ESI-MS system (Shimadzu) equipped with Discovery HS C18 (Supelco) 7.5 cm × 2.1 mm × 3 µm column operated at 30 °C was used for preliminary structure identification. The mobile phase consisted of methanol

Table 1 Linearity parameters of HPLC-PDA method for ARs at λ = 280 nm ($n = 2 \times 11$)

Compound	Olivetol	C17:0	C19:0	C21:0	C23:0	C25:0
a (slope)	132	30	69	63	47	35
b (intercept)	1	1	0	0	0	0
r (correlation coefficient)	0.997	0.995	0.997	1.000	1.000	0.998
Range (mg/mL)	0.01–0.8	0.01–1.0	0.01–1.0	0.01–1.0	0.01–1.0	0.01–1.0
LOD (μg/mL)	2	10	4	5	6	9
LOQ (μg/mL)	675	33	15	16	21	29

Table 2 Repeatability parameters of HPLC-PDA method for ARs at λ = 280 nm ($n = 6$)

Compound	Olivetol[a]	C17:0[b]	C19:0[b]	C21:0[b]	C23:0[b]	C25:0[b]
Retention time t_R (min)	8.25	24.98	28.65	31.64	35.41	39.86
SD	0.23	0.67	0.76	1.36	1.29	0.93
CV (%)	2.79	2.69	2.64	4.30	3.65	2.34
Min (min)	7.79	24.33	27.80	29.30	33.35	38.23
Max (min)	8.57	25.70	29.48	33.07	37.05	40.67

[a] For olivetol at concentration 0.8 mg/mL

[b] For ARs homologs in AR-Ts fraction

(eluent C) and water (eluent D), and the following gradient program was used: 10% C in D (5 min), 47% C in D (5 min), 85% C in D (15 min), 100% C (20 min). Total run time was 55 min. The injection volume was 2 μL; total flow was 0.2 mL/min. ARs were monitored at 202, 220 and 280 nm. In addition, UV–Vis spectra were recorded in the range 200–600 nm. Molecules were detected on positive ion mode. Detector voltage was 1.2 kV, while interface voltage was 4 kV. Desolvation line and heat block temperature were 250 and 200 °C, respectively. Nebulizing gas flow was 1.5 mL/min, drying gas flow was 16 L/min, and event time was 0.5 s. Positive ion mass spectra were recorded in the range of *m/z* 100–500 in SIM mode [10].

TLC analyses were carried out on 10 × 20 cm silica gel plates Si60 (0.25 mm) obtained from Merck. Extracts, fractions from column chromatography and standard solutions were separated in mobile phase: chloroform–methanol–formic acid (96:3:1; *v/v/v*) at a distance of 9 cm. Chromatograms were developed in horizontal Teflon DS chambers (Chromdes, Poland). Colored compounds were detected as orange-red bands in visible light after spraying with visualizing reagent (0.5 mL anise aldehyde, 10 mL acetic acid, 85 mL methanol, 0.5 mL sulfuric acid). All TLC procedures were performed at room temperature.

Validation of HPLC-PDA method and quantitative measurement

Measurements of ARs content in alkylresorcinol fraction (AR-Ts) were taken using HPLC-PDA and the external standards for calibration. Olivetol and isolated homologs were used for this purpose. Calibration equations for ARs were assessed at 11 concentration levels, and duplicate injections were performed for each concentration (0.01, 0.04, 0.06, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.8 and 1.0 mg/mL). The linearity of standard curves was confirmed by plotting the peak areas (*y*, mAU/s) and the corresponding concentration (*x*, mg/mL). Limits of detection (LOD) and quantitation (LOQ) were calculated from calibration equations based on the signal-to-noise ratio (S/N ≥ 3:1 and S/N ≥ 10:1, respectively) and expressed as the concentration of examined compound (μg/mL) in Table 1. Repeatability and intermediate precision were calculated from retention time and peak areas, respectively. Olivetol (0.8 mg/mL) and AR-Ts (1 mg/mL) were used for this purpose (Tables 2, 3).

The content of individual ARs (milligrams per gram or μM) was estimated as the mean of three independent samples using the HPLC-PDA method. The sum of ARs was calculated as the sum of saturated homologs which were present in the analyzed fractions from their average values. Slope (a), intercept (b) and correlation coefficient (r) were also calculated.

Structure elucidation of isolated alkylresorcinol homologs

To assure the structures based on previously obtained MS data and to observe possible impurities, ^{1}H-, ^{13}C-NMR, COSY and HSQC were measured for all compounds. 1D and 2D spectra were recorded on Avance II NMR apparatus

Table 3 Intermediate precision parameters of HPLC-PDA method for ARs at λ = 280 nm ($n = 6$)

Compound	Olivetol[a]	C17:0[b]	C19:0[b]	C21:0[b]	C23:0[b]	C25:0[b]
Peak area (mAU × min)	102.04	2.46	14.72	19.64	4.80	1.66
SD	3.53	0.10	0.52	0.65	0.21	0.09
CV (%)	3.46	4.12	3.56	3.31	4.33	5.13
Min (mAU × min)	99.55	2.27	14.19	19.01	4.64	1.54
Max (mAU × mi)	104.54	2.54	15.43	20.52	5.19	1.76

[a] For olivetol at concentration 0.8 mg/mL

[b] For ARs homologs in AR-Ts fraction

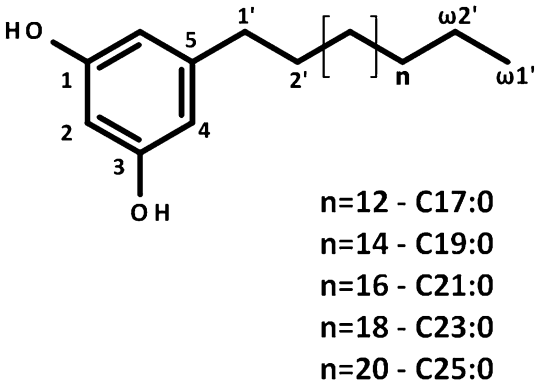

Fig. 2 Structures of ARs

(Bruker Biospin, Rheinstetten, Germany), with 300 MHz for proton and 75 MHz for carbon. Because of non-complete solubility of obtained compounds in $CDCl_3$, samples were finally dissolved and measured in deuterated acetone and its internal signal was used as a standard for chemical shifts correction.

Spectra measured for all compounds were similar. Signals typical for 1,3-diphenol substituted with an aliphatic chain at C-5 position were clearly assigned: doubled singlet signal of two symmetrical protons at 6.94 ppm (H-4 and H-6) correlated with carbon signal at 107.7 ppm (C-4 and C-6), proton signal at 6.93 ppm (s, H-2) correlated with 100.9 ppm (C-2), additionally—quaternary carbons signals at 159.3 ppm (phenolic, doubled, C-1, C-3) and at 145.8 (C-5). Chain methylene signals were typically as follows: triplet at 3.19 ppm (ring connected H-1′, $J = 7.5$ Hz, 2H) correlated with 36.6 ppm (C-1′), multiplet at 2.31 ppm (H-2′, 2H) correlated with 32.1 ppm (C-2′), multiplet at 2.25–1.93 ppm correlated with carbon signals at 31–28 ppm (unresolved, middle chain signals), 1.64 ppm (H-ω2′, 2H) at 23.3 ppm (C-ω2′) and methyl group signal of chain end at 1.64 ppm (H-ω1′) correlated with 14.4 ppm (C-ω1′). ω Indication was per analogy to fatty acids (Fig. 2). The experimental results were in good accordance with the literature data [11, 12] and predicted values.

In vitro cytotoxicity test

Mouse fibroblast cell line L929 was obtained from American Type Culture Collection (ATCC CCL-1). The cells were cultured in MEM (minimum essential medium Eagle) supplemented with 10% inactivated fetal bovine serum, 100 U/mL penicillin, 100 µg/mL streptomycin and 2 mM L-glutamine at 37 °C, in a humidified atmosphere containing 5% CO_2. All reagents, cell culture media and supplements used in cytotoxicity study were obtained from Sigma-Aldrich except MEM medium which was from IITD (Poland).

Cytotoxicity of AR-Ts, isolated homologs, compounds with related structures and hydroquinone (positional isomer of resorcinol), as positive control, were studied on mouse fibroblast cell line L929 according to standard method (PN-EN ISO 10993-5:2009). Tested compounds were dissolved in DMSO (2500 µg/mL), but serial dilutions (19.5–1250 µg/mL) of each compound were prepared in the culture medium. Additionally, DMSO was also checked for toxicity by preparing serial dilution in medium and microscopic observation (Blank).

100 µl of 2 × 10^4 cells was seeded in 96-well plates (Nunc) and left for 24 h. Then, 100 µl various doses (ranged from 19.5 to 2500 µg/mL) of tested compounds were added to the cells and cultivated for 72 h at 37 °C in air containing 5% carbon dioxide and 95% humidity. Then, cultures were examined under microscope and pictures were taken. Cell growth, cell morphology and cell viability were used as parameters to determine the cytotoxicity of these substances. For cell viability, trypan blue staining assay was used. 10 µL of a cell suspension was incubated at room temperature with 10 µL of 0.4% trypan blue for 15 min. Viability of the cells was measured in a Bürker chamber. Dead cells were navy blue and live cells remained unstained. The degeneration of cells was assessed using an inverted microscope at 400 × magnification.

MTT assay was performed to determine IC_{50} (minimal concentration toxic to approximately 50% of cells). 25 µL of MTT solution (2 mg/mL) was added to each well. After 3-h incubation, content was removed from wells and 80 µL

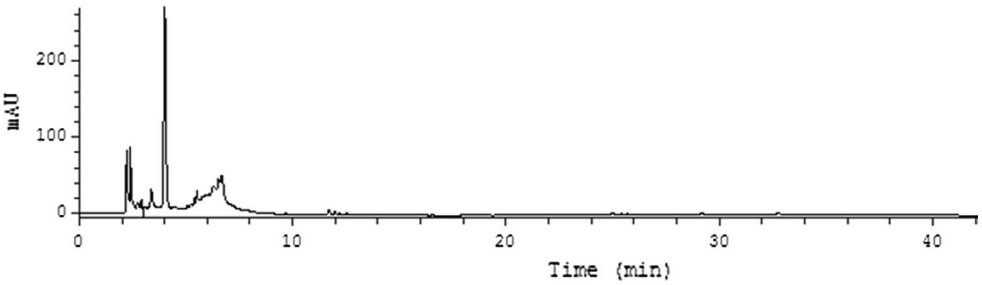

Fig. 3 HPLC-PDA chromatogram of 70% methanolic extract (λ 280 nm)

of saturated SDS solution was added to each well and shaken. Absorbance was read at λ 550/630 nm. Viability of cells was calculated by dividing optical density of tested substance (ts) by control (c) and multiplying by 100% (viability% = $OD_{ts}/OD_c \times 100\%$). IC_{50} was calculated from concentration curve equation and was expressed as μg/mL and μM.

Results and discussion

The main saturated alkylresorcinol homologs of natural origin were isolated for biological tests. The following cereal bran was used: spelt for homologs C19:0 to C25:0 and rye for isolation of shorter chain homolog: C17:0 and also others. First, the two-step ultrasound-assisted extraction (UAE) with solvents of different polarities (70% methanol and acetone) was carried out. Use of 70% methanol (first step) allowed the separation of more polar ballast compounds prior to ARs which were extracted with acetone (second step). ARs have been detected in traces amounts in 70% methanolic extract (Fig. 3). Usage of UAE shortens the extraction time compared to 24-h maceration protocol [1, 13]. Supercritical fluid extraction applied to separate ARs fraction described by Dey et al. and Athukorala et al. [8, 9] also accelerated the extraction process but requires advanced apparatus. Proposed simple two-step UAE method gave similar extraction yield in a similar duration time compared to SFE—about 2 g of ARs from 1 kg rye bran. An alternative way of obtaining ARs is chemical synthesis, e.g., by Grignard or alkyl-lithium methods. They are time-consuming and require special conditions and the yields vary. However, modification of Wittig reaction by microwaves use greatly improved the synthesis [14].

Acetone extracts from spelt and rye bran rich in ARs were subjected to series of column chromatography (Fig. 1). First, extracts were chromatographed over silica gel to afford alkylresorcinol fractions (AR-Ts from spelt and AR-Sc from rye) as light beige powders. ARs separation was performed twice with two different eluents in order to verify their utility: with increasing percentage of acetone in a nonpolar organic solvent (chloroform and petroleum ether for spelt and rye bran, respectively). Usage of chloroform with acetone accelerates separation compared to eluent composed of petroleum ether and acetone (22 vs. 49 fractions). The collected fractionations were monitored by TLC on silica gel plates. UHPLC-ESI-MS was used for preliminary identification of alkylresorcinols (Table 4) while HPLC-PDA for their quantification (Table 5; Fig. 4).

Then, AR-Ts (1.7 g) and AR-Sc (2 g) were chromatographed over octadecyl columns. Individual homologs: C17:0, C19:0, C21:0, C23:0 and C25:0 were eluted with methanol–water system and methanol. All steps were monitored with HPLC-PDA. The structures of isolated ARs were elucidated by ^{1}H-, ^{13}C-NMR, COSY and HSQC and compared with the literature [11, 12]. 1D and 2D NMR spectra were used for structure confirmation. All spectra were almost identical; thus, only example (C21:0) is given here: ^{13}C NMR (75 MHz, acetone) δ 159.29 (C-1, C-3), 145.84 (C-5), 107.69 (C-4, C-6), 100.91 (C-2), 36.60 (C-1′), 32.08 (C-2′), 31–28 (unres., chain), 23.33 (C-ω2′), 14.36 (C-ω1′) and ^{1}H NMR (300 MHz, acetone) δ 6.94 (H-4, H-6, 2H), 6.93 (H-2, 1H), 3.19 (H-1′, *t*, $J = 7.5$ Hz, 2H), 2.31 (H-2′, m, 2H), 2.25–1.93 (chain), 1.64 (H-ω1′, 3H) (Fig. 5).

The contents of main individual homologs and sum of them in AR-Ts were calculated and expressed as commonly used olivetol equivalents and as original compounds (Table 5). Chromatographic analyses of AR-Ts fraction (Tables 4, 5) revealed 28 alkylresorcinols with five saturated homologs as major components, which constituted 86% mass weight (2174 μM). Additionally, in small amounts, unsaturated alkylresorcinol homologs containing 1, 2 or 3 double bonds in alkyl chain (compounds 3, 6–8, 10–12, 14, 16–17, 19–22, 24, 26–27) and monounsaturated homologs with an additional –OH group in the alkyl chain (compounds 4–5, 9, 15) were identified (Table 4; Fig. 5) which is in agreement with literature data [10].

ARs have been suggested as foodstuff, cosmetic or pharmaceutical ingredients, and therefore, examination of their cytotoxicity on normal cells is essential. The cytotoxic activity of AR-Ts, isolated homologs and

Table 4 Identification of ARs by UHPLC-ESI-MS

Compound	t_R (min)	λ_{max} (nm)	$[M + H]^+$ *m/z*	–R (alkyl chain)	Identity
Resorcinol	3.6	199, 218, 273, 281	111	–H	Standard
Olivetol	10.6	199, 226, 273, 281	181	C5:0	Standard
1	12.8	199, 232, 273, 281	279	unknown	Unknown
2	**14.1**	**198, 220, 273, 281**	**321**	**C15:0**	**5-*n*-Pentadecylresorcinol**
3	14.7	+	371	C19:3	Nonadecatrienylresorcinol
4	14.8	+	391	C19:1OH	Hydroxynonadecenylresorcinol
5	15.8	+	363	C17:1OH	Hydroxyheptadecenylresorcinol
6	16.0	198, 217, 262, 281	371	C19:3	Nonadecatrienylresorcinol
7	16.2	198, 219, 262, 280	429	C23:2	Tricosadienylresorcinol
8	19.0	+	371	C19:3	Nonadecatrienylresorcinol
9	19.6	199, 230, 273, 281	391	C19:1 OH	Hydroxynonadecenylresorcinol
10	19.8	199, 271, 281	347	C17:1	Heptadecenylresorcinol
11	20.3	199, 272, 281	373	C19:2	Nonadecadienylresorcinol
12	21.2	+	399	C21:3	Heneicosatrienylresorcinol
13	**21.8**	**199, 223, 274, 281**	**349**	**C17:0**	**5-*n*-Heptadecylresorcinol**
14	21.9	199, 227, 273, 281	431	C23:1	Tricosenylresorcinol
15	22.0	199, 224, 274, 281	419	C21:1OH	Hydroxyheneicosenylresorcinol
16	22.1	199, 227, 274, 281	459	C25:1	Pentacosenylresorcinol
17	22.5	199, 230, 272, 281	401	C21:2	Heneicosadienylresorcinol
18	**23.9**	**199, 224, 274, 282**	**377**	**C19:0**	**5-*n*-Nonadecylresorcinol**
19	23.9	199, 224, 274, 282	399	C21:3	Heneicosatrienylresorcinol
20	24.0	201, 223, 274, 282	431	C23:1	Tricosenylresorcinol
21	24.1	199, 223, 274, 281	403	C21:1	Heneicosenylresorcinol
22	24.3	199, 230, 274, 281	403	C21:1	Heneicosenylresorcinol
23	**25.9**	**199, 223, 274, 281**	**405**	**C21:0**	**5-*n*-Heneicosylresorcinol**
24	26.0	199, 230, 274, 281	431	C23:1	Tricosenylresorcinol
25	**27.6**	**199, 223, 274, 281**	**433**	**C23:0**	**5-*n*-Tricosylresorcinol**
26	27.6	199, 223, 274, 281	431	C23:1	Tricosenylresorcinol
27	29.5	199, 225, 274, 281	431	C23:1	Tricosenylresorcinol
28	**29.5**	**199, 225, 274, 281**	**461**	**C25:0**	**5-*n*-Pentacosylresorcinol**

+ Present in very small amount; main compounds bolded

Table 5 Concentration of 5-*n*-alkylresorcinol homologs in AR-Ts

Compound	Concentration in mg/g (μM)	
	Olivetol eq.	Original compound
C17:0	7 [37]	56 [162]
C19:0	139 [773]	287 [763]
C21:0	168 [931]	372 [921]
C23:0	25 [141]	103 [239]
C25:0	1 [4]	42 [89]
Sum	340 [1886]	860 [2174]

related compounds such as resorcinol, orcinol and olivetol were tested by reference method in mouse fibroblast cell line L929 (PN-EN ISO 10993-5:2009). DMSO was also checked for toxicity by preparing its serial dilution in medium and microscopic observation (Blank).

Hydroquinone was used as a cytotoxic positive control. Hydroquinone has been used in skin-lightening products for almost 50 years. Many ambivalent opinions have been attributed to this compound because of its cytotoxicity. Nowadays, only prescription-based medications with hydroquinone are allowed in EU [15]. In our experiment, hydroquinone inhibited cell growth completely at all concentrations. No agglutination, vacuolization, separation from the medium or cell membrane lyses were observed for cell control or blank (Fig. 6a). The cell culture after contact with all compounds up to 39 μg/mL also showed good proliferation and no toxic effect (Fig. 6b). Cells were damaged and dead after the contact with tested substances at different concentrations depending on structure (Fig. 6c).

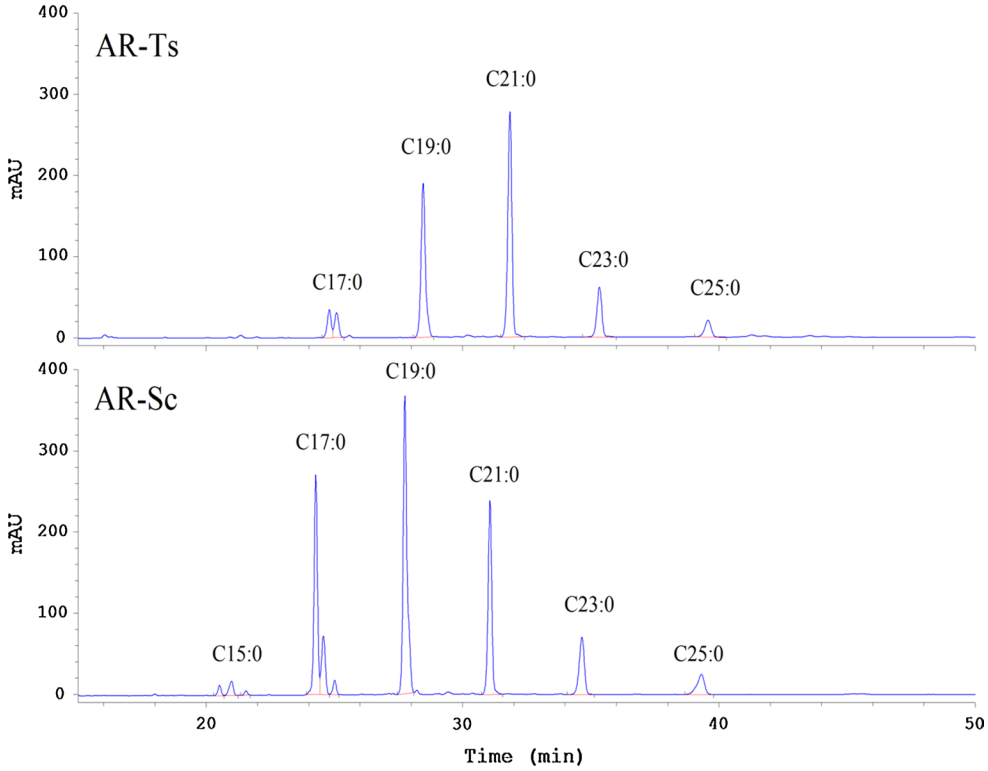

Fig. 4 HPLC-PDA chromatogram of alkylresorcinol fractions (λ 280 nm): AR-Ts (*upper*) and AR-Sc (*lower*)

Cytotoxicity in a dose-dependent manner was observed for each tested compound and IC_{50} was also determined (Table 6; Fig. 6d).

Homolog with the short alkyl chain (C17:0) had the strongest inhibitory effect on the growth of mouse fibroblast cell line L929 expressed as IC_{50} (171 µM) among all tested ARs. It was followed by homolog C19:0 (330 µM) and C21:0 (511 µM) and then olivetol (632 µM). Homologs with longer alkyl chain, C23:0 and C25:0, had IC_{50} at levels much higher (1 965 and 2142 µM, respectively). The highest IC_{50} values and the lowest cytotoxicity were observed for orcinol (6918 µM) and resorcinol (11,043 µM). Cytotoxicity of AR-Ts fraction was at level between C17:0 and C19:0 homologs (C17:0 > AR-Ts > C19:0) (Table 6; Fig. 6d) and is probably a consequence of high content of the sum of these homologs in fraction (40% mass weight). For experimental point 156 µg/mL, concentration of C17:0 and C19:0 homologs in AR-Ts fraction were 10 and 52 µg/mL (12 and 55 µM), respectively. These concentrations were lower than cytotoxic concentration levels of single homologs. It may indicate that the presence of unsaturated homologs in fraction should be also considered. Their content is much lower, but the activity is much higher as it was observed for colon cancer cells [12]. Effective tyrosinase inhibitory concentration of ARs estimated as 10 µg/mL [5] was lower than concentration evaluated in our experiment as cytotoxic (Table 6; Fig. 6d).

Cytotoxic activity of resorcinol and orcinol is much lower than ARs homologs with side chain from 17 to 25 carbon atoms. Higher activity of ARs may be due to their amphiphilic properties and ability to integrate with bilayer membrane [2]. By comparing cytotoxic activity of hydroquinone and resorcinol, which are positional isomers, it was found that position of –OH groups in benzene ring of tested compounds is crucial for activity on mouse fibroblast cell line L929. Isomer *p* is cytotoxic in the whole range of concentration tested while isomer *m* only at high concentration (Table 6). IC_{50} for resorcinol and its 5-methyl derivative (orcinol) had the highest values. 5-*n*-Pentylresorcinol (olivetol) was less cytotoxic than 5-*n*-heneicosylresorcinol, 5-*n*-nonadecylresorcinol and 5-*n*-heptadecylresorcinol (C21:0, C19:0 and C17:0). Thus, activity of ARs-related compounds increases with carbon number at –R but is lower than homologs with 17–21 carbon atoms in alkyl chain.

Based on results from Table 6, increasing length of the ARs alkyl chain is diminishing the cytotoxic activity which is in agreement with results from Zhu and co-workers studies [6, 7] where alkylresorcinols were evaluated for growth inhibition of human colon cancer cell lines HCT-116 and HT-29. IC_{50} values of ARs in our study are two, three or more times higher than values reported for these compounds by Zhu et al. [6]. Higher IC_{50} values to normal than to cancer cells are favorable features of possible candidates for future therapeutic agents and food ingredients to prevent the process of carcinogenesis.

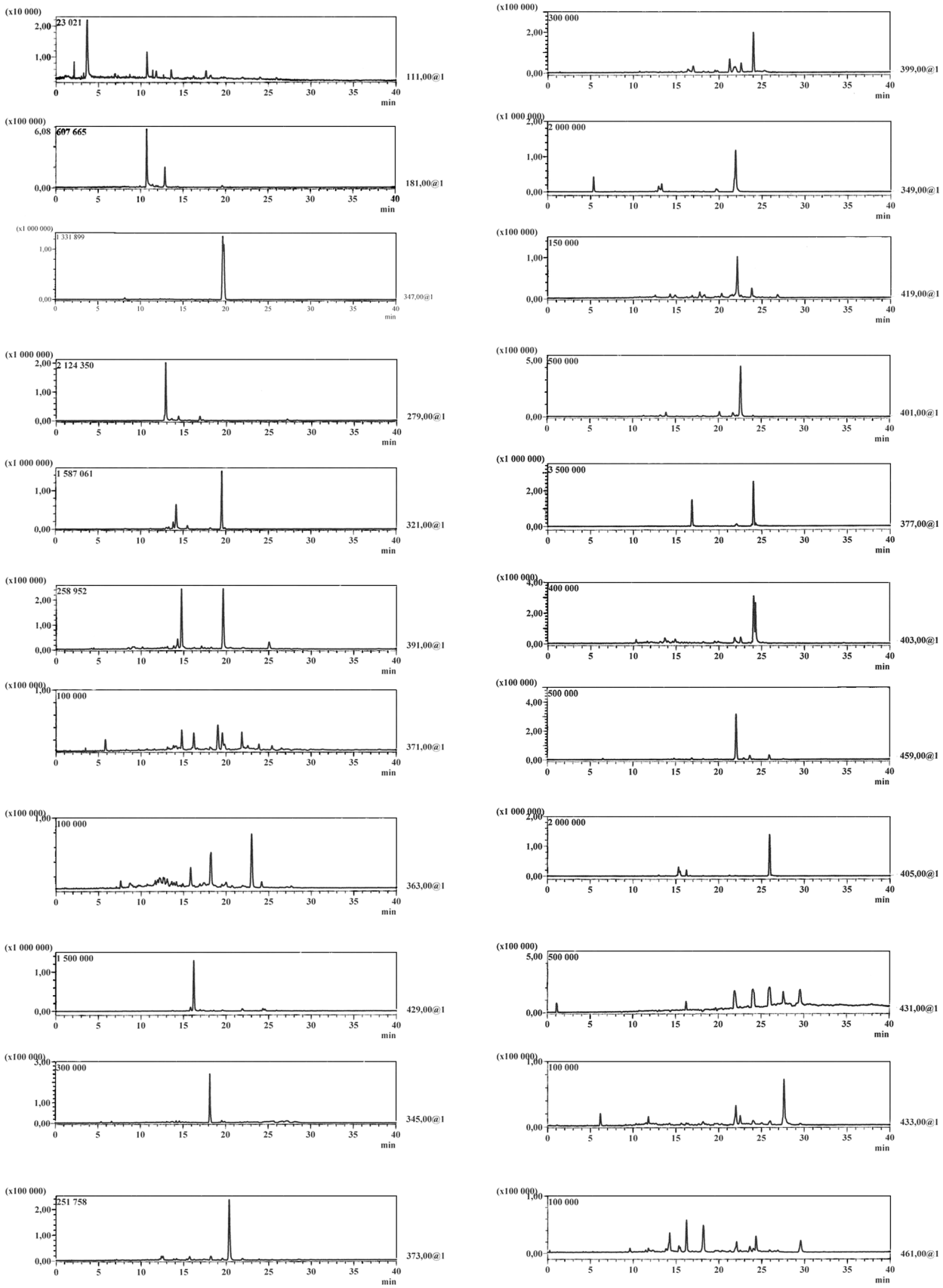

Fig. 5 UHPLC-ESI–MS spectra of alkylresorcinols protonated pseudomolecular ions detected in AR-Ts fraction conducted in the single ion monitoring mode (SIM)

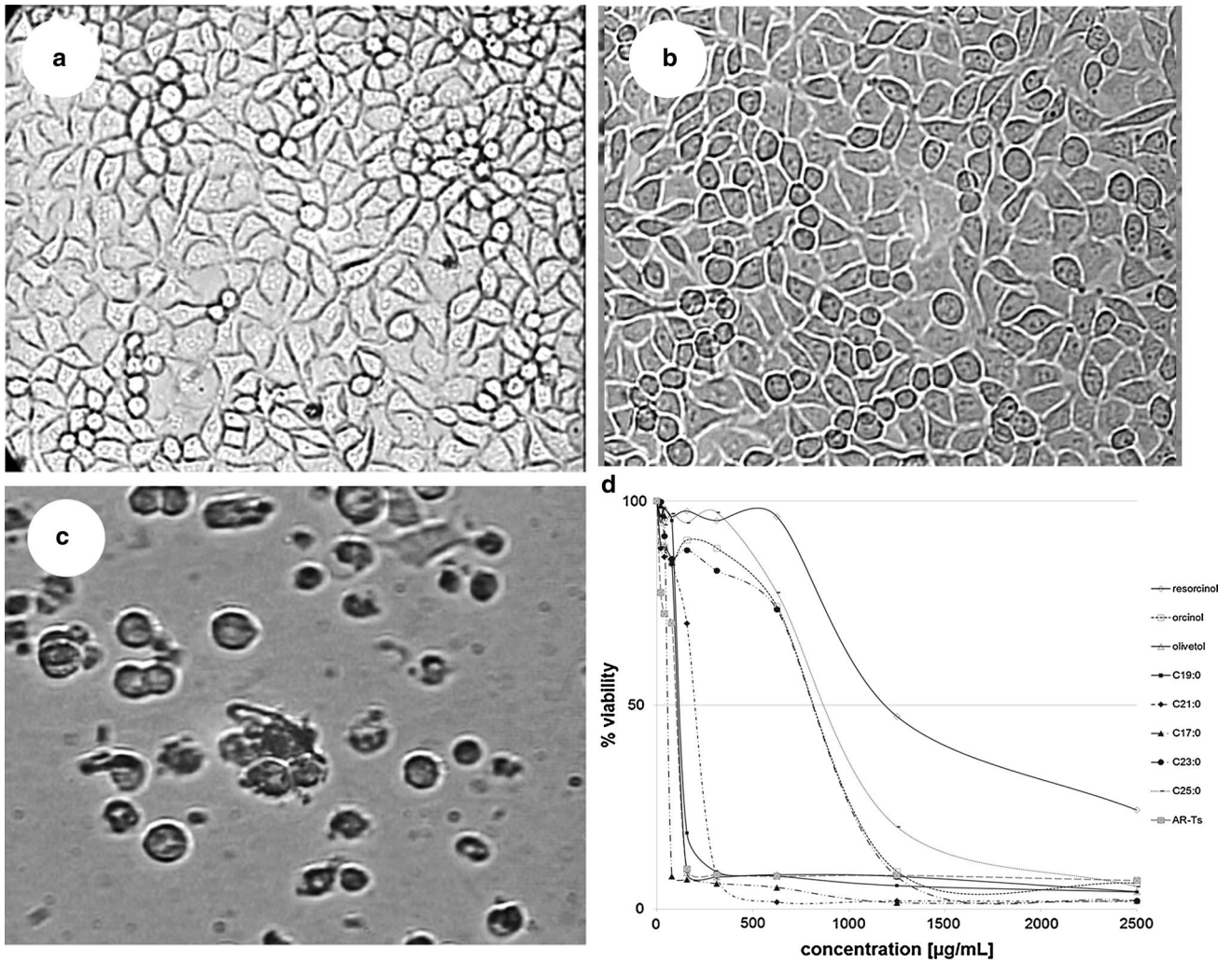

Fig. 6 **a** Mouse fibroblast cell line L929 that illustrates control. **b** Mouse fibroblast cell line L929 that illustrates non-toxic effects of tested compounds. **c** Mouse fibroblast cell line L929 that illustrates toxic effects of tested compounds. **d** Dose–response curves for alkylresorcinols and structurally related compounds. Response is expressed as % viability of mouse fibroblast cell line L929 after 72-h contact with tested substances

This is the first time that cytotoxicity of C17:0 to C25:0 alkylresorcinol homologs was evaluated on normal cell line. We observed a structure–cytotoxicity relationship of tested compounds expressed as carbon atom number in side chain and logIC_{50}, respectively ($r = 0.965$, Fig. 7). This relationship is as follows: hydroquinone ≫ C17:0 > C19:0 > C21:0 > olivetol > C23:0 and C25:0 ≫ orcinol > resorcinol and is similar to relationship reported by Arisawa et al. [16] for shorter homologs and their cytotoxic effect on oral carcinoma KB cells (C13:0 > C15:0 > C11:0 > C9:0 and C17:0 > C7:0 > olivetol). Unfortunately, we were not able to isolate homolog C15:0 because of low amount in bran, but its IC_{50} was calculated (152 μM) from quadratic equation determined experimentally on the base of our results for resorcinol derivatives (Fig. 7). This value is comparable with IC_{50} obtained for C17:0 homolog determined experimentally.

The median plasma total ARs concentrations among participants of European Prospective Investigation into Cancer and Nutrition (EPIC) cohort were assessed to be 41 nM in samples drawn from fasting participants in Scandinavian population which is known to consume high amounts of whole-grain food and below 23 nM in those of participants from the Mediterranean countries [17]. The levels of cytotoxicity of individual homologs and AR-Ts indicated in this study exceed at least 10^4 times the concentration of ARs identified in human plasma, and under conditions of regular diet are not possible to obtain. However, ARs can be accumulated in human adipose tissue. The concentration was in range from 0 to 1.5 μg/g and was correlated with long-term

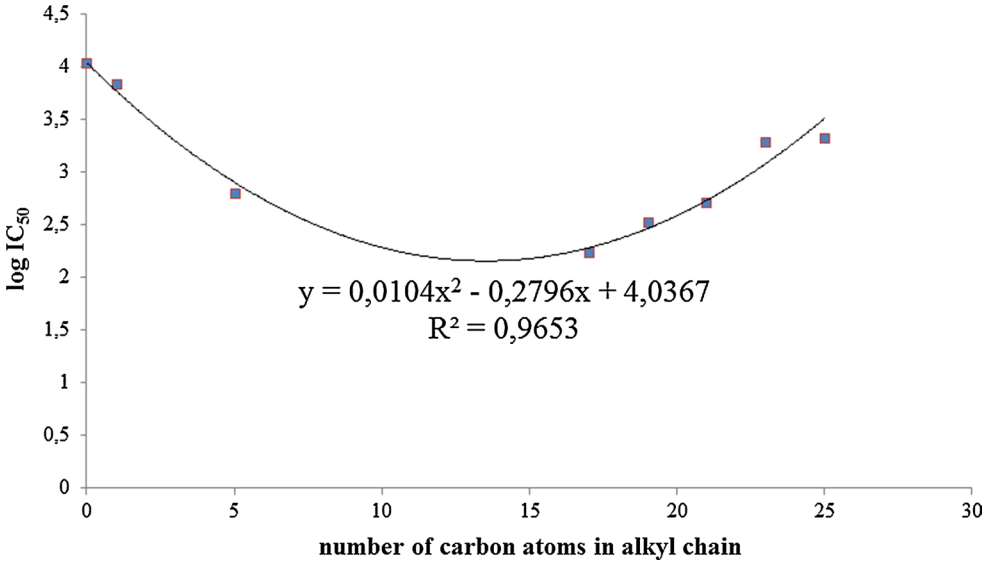

Fig. 7 Relationship between carbon atoms in side chain of ARs and related compounds and cytotoxicity to mouse fibroblast cell line L929 (as logIC_{50})

Table 6 Cytotoxicity of ARs homologs, fraction and related compounds on mouse fibroblast cell line L929

Compound	Molecular weight (g/mol)	IC_{50} (MTT assay)		Concentration in μg/mL (microscopic observation)							
		(μg/mL)	(μM)	19.5	39	78	156	312	625	1250	2500
Blank[a]		–	–	n	n	n	n	n	n	n	n
Hydroquinone[b]	110	<19.5	<177	t	t	t	t	t	t	t	t
Resorcinol	110	1215	11,043	n	n	n	n	n	n	t	t
Orcinol C1:0	124	858	6 918	n	n	n	n	n	n	t	t
Olivetol C5:0	180	114	632	n	n	n	t	t	t	t	t
Homolog C 17:0	348	59	171	n	n	t	t	t	t	t	t
Homolog C 19:0	376	124	330	n	n	n	t	t	t	t	t
Homolog C 21:0	404	206	511	n	n	n	n	t	t	t	t
homolog C 23:0	432	849	1965	n	n	n	n	n	n	t	t
Homolog C 25:0	460	925	2142	n	n	n	n	n	n	t	t
AR-Ts fraction		104	–	n	n	n/t	t	t	t	t	t

[a] Serial dilution of DMSO in MEM (without tested compound)

[b] Positive control

intake of whole-grain food [18]. It means that cytotoxicity IC_{50} values of C17:0 homolog, potentially the most cytotoxic component, and AR-Ts are more than 50 times higher than concentration in adipose tissue. However, high local concentrations of ARs from fortified food or skin-lightening products are possible to arise and need to be kept in mind.

ARs could be potentially used as food additive in order to prevent food browning or to fortification of bread and breakfast cereals, or as ingredients of diet supplements which could help in type 2 diabetes prevention. For such use of ARs, it is necessary to establish safe concentrations and the ADI (acceptable day intake) for them. In the present study, alkylresorcinols isolated from spelt and rye bran exhibited cytotoxicity lower than hydroquinone but higher than resorcinol on normal cells. The highest cytotoxic activity among isolated homologs was evaluated for C17:0 which level in bran, especially spelt, is low. This study extends our knowledge about potential safety of use of alkylresorcinols in food, cosmetic or pharmaceutical industry.

Acknowledgements Authors greatly acknowledged Maciej Wlodarczyk for support in NMR analysis.

Funding This work was partly supported by the research fellowship for Izabela Biskup from European Social Fund, Human Capital, National Cohesion Strategy under Development Program of Wroclaw Medical University [UDA-POKL.04.01.01-00-010/08-00] and

internal grant of Wroclaw Medical University for Young Scientists under Grant [Pbmn 128].

Compliance with ethical standards

Conflict of interest Authors declare no conflict of interest.

Human and animal rights This article does not contain any studies with human or animal subjects.

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