Eur J Nutr
DOI 10.1007/s00394-015-0910-5

ORIGINAL CONTRIBUTION

Fruit peel polyphenols demonstrate substantial anti-tumour effects in the model of breast cancer

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Received: 13 February 2015 / Accepted: 22 April 2015

Abstract
Purpose Fruit and vegetable intake is inversely correlated with cancer; thus, it is proposed that an extract of phytochemicals as present in whole fruits, vegetables, or grains may have anti-carcinogenic properties. Thus, the anti-tumour effects of fruit peel polyphenols (Flavin7) in the chemoprevention of *N*-methyl-*N*-nitrosourea-induced mammary carcinogenesis in female rats were evaluated.
Methods Lyophilized substance of Flavin7 (F7) was administered at two concentrations of 0.3 and 3 % through diet. The experiment was terminated 14 weeks after carcinogen administration, and mammary tumours were removed and prepared for histopathological and immunohistochemical analysis. In addition, using an in vitro cytotoxicity assay, apoptosis and proliferation after F7 treatment in human breast adenocarcinoma (MCF-7) cells were performed.
Results High-dose F7 suppressed tumour frequency by 58 % ($P < 0.001$), tumour incidence by 24 % ($P < 0.05$), and lengthened latency by 8 days ($P > 0.05$) in comparison with the control rats, whereas lower dose of F7 was less effective. Histopathological analysis of tumours showed significant decrease in the ratio of high-/low-grade carcinomas after high-dose F7 treatment. Immunohistochemical analysis of rat carcinoma cells in vivo found a significant increase in caspase-3 expression and significant decrease in Bcl-2, Ki67, and VEGFR-2 expression in the high-dose group. Both doses demonstrated significant positive effects on plasma lipid metabolism in rats. F7 significantly decreased survival of MCF-7 cells in vitro in MTT assay by dose- and time-dependent manner compared to control. F7 prevented cell cycle progression by significant enrichment in G1 cell populations. Incubation with F7 showed

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Published online: 01 May 2015

significant increase in the percentage of annexin V-/PI-positive MCF-7 cells and DNA fragmentation.

Conclusions Our results reveal a substantial tumour-suppressive effect of F7 in the breast cancer model. We propose that the effects of phytochemicals present in this fruit extract are responsible for observed potent anti-cancer activities.

Keywords Mammary carcinogenesis · Rat · Fruit polyphenols · Angiogenesis · Apoptosis · Cell proliferation · MCF-7

Abbreviations

BrdU	5-Bromo-20-deoxyuridine
FCM	Flow cytometry analysis
FLAV 0.3/FLAV 3	Experimental group with dietary-administered Flavin7 in a concentration of 0.3 and 3 %
F7	Flavin7
HG	High grade
LDL	Low-density lipoprotein
LG	Low grade
MCF-7	Human adenocarcinoma cell lines, oestrogen receptor-positive
MDA-MB-231	Human breast adenocarcinoma cell line, oestrogen receptor-negative
NMU	*N*-methyl-*N*-nitrosourea
p.o.	Per os
VEGF	Vascular endothelial growth factor
VEGFR-2	Vascular endothelial growth factor receptor-2
VLDL	Very low-density lipoprotein

Introduction

The consumption of non-nutrient bioactive plant components in fruit, vegetables, grains, and other plant foods is associated with a decrease in the risk of major chronic diseases [1–3]. There are many studies, suggesting that phytochemicals display many beneficial biological roles, e.g. anti-tumour, neuroprotective, anti-allergic, and anti-aggregatory effects [4]. The mechanisms of action of phytochemicals may involve an ability to target multiple molecular pathways in carcinogenesis (e.g. proliferation, apoptosis, angiogenesis, inflammation, and cell infiltration) without eliciting undesirable side effects in cells. Due to promising epidemiological, preclinical, and clinical results, a number of phytochemicals and whole plant foods serve as candidates for development of anti-cancer agents.

The substance Flavin7® (F7) is a nutritional supplement frequently taken by cancer patients during radiation therapy or chemotherapy mainly in Central Europe and Canada. F7 is a lyophilized mixture of peels from seven fruits—red grape, blackberry, blackcurrant and redcurrant, black cherry, elderberry, plum, and blackthorn—with high concentration of polyphenols (2.77 % from total amount). F7 contains a high concentration of the following flavonoids—myricetin, quercetin, kaempferol, rutin, isorhamnetine, catechin, epicatechin, malvidin-3-glucoside, caffeic acid, chrysin, galangin, apigenin, fisetin, luteolin, morin—as well as several anthocyanidins and stilbene resveratrol (*trans*-form, *cis*-form, and glycosides) (Vita Crystal Ltd., Budapest, Hungary). Regular consumption of flavonoids in humans led to a significant decrease in the incidence of different neoplasia including breast cancer [5–7]. In preclinical research, flavonoids suppressed mammary carcinogenesis in vitro and in vivo [8, 9]. The important role of flavonoids as growth inhibitors on different cancer cell lines by induction of G1 cell cycle arrest [10], G2/M arrest, and apoptosis [11] was demonstrated. Flavonoids may have anti-angiogenesis activity via inhibitory effects on cell proliferation, cell migration, cell adhesion, and tube formation [12]. However, a key question remains whether a purified phytochemical has the same anti-carcinogenic effects as the whole functional food containing the same phytochemical in the mixture with other phytochemicals.

The main aim of this study was to evaluate the preventive effects of long-term F7 administration in a model of *N*-methyl-*N*-nitrosourea (NMU)-induced mammary carcinogenesis in female rats. As well, the mechanism of action after F7 treatment was determined in vivo. A histopathological analysis of carcinomas was performed to determine whether F7 effected the differentiation and prognosis of the tumours. The effects of F7 on parameters of oxidative damage in the cell and on plasma lipid metabolism in animals after long-term treatment were determined. Moreover, an in vitro study with cytotoxicity data, parameters of apoptosis and proliferation in human adenocarcinoma cell lines (MCF-7) after F7 treatment were also performed.

Materials and methods

The experiment was approved by the Ethical Commission of the Jessenius Faculty of Medicine of Comenius University (Protocol No. EK1125/2012) and by the State Veterinary and Food Administration of the Slovak Republic (Accreditation No. Ro-1759/11-221).

Animals and induction of mammary carcinogenesis, design of experiment

Female rats of the Sprague–Dawley strain (Charles River Laboratories, Sulzfeld, Germany) aged 31–35 days were

used in the experiment. The animals were adapted to standard vivarium conditions with temperature 23 ± 2 °C, relative humidity 40–60 %, artificial regimen (L/D 12:12 h). During the experiment, the animals were fed the Ssniff diet (Soest, Germany) and water ad libitum. Mammary carcinogenesis was induced by NMU (Sigma, Deisenhofen, Germany) administered intraperitoneally in one dose of 50 mg/kg body weight on average on the 42nd post-natal day.

Chemoprevention with Flavin7 (Vita Crystal Ltd., Budapest, Hungary; fruit origin—Central Europe) began 1 week before carcinogen administration and lasted until the end of the experiment (14 weeks after NMU administration). Lyophilized substance F7 was administered in the diet at two concentrations of 3 g/kg (0.3 %) and 30 g/kg (3 %). Animals were randomly assigned to one of three experimental groups: group 1—control group without chemoprevention; group 2—chemoprevention with F7 at a concentration of 0.3 % (FLAV 0.3); and group 3—chemoprevention with F7 at a concentration of 3 % (FLAV 3). Each group consisted of 25 animals. The lower dose of F7 in this experiment was based on the maximal clinical dose (approx. 6 g). In general, rats demonstrate different pharmacokinetics and pharmacodynamics of many drugs compared to humans; therefore, it was necessary to use high doses of F7 to prove its potential anti-neoplastic effect in this experiment (the average daily dose of F7 per rat was 57.51 mg in lower dose and 587.7 mg in higher dose). The animals were weighed and palpated weekly in order to register the presence, number, location, and size of each palpable tumour. Food intake per cage during 24 h was monitored in the 7th and 13th week of the experiment; the measurements were taken four times (twice in each week). The F7 doses per animal and day were calculated in accordance with the amount of chow consumed. In the last (14th) week of the experiment, the animals were quickly decapitated, the blood from each animal was collected, mammary tumours were excised, and the tumour size was recorded. Macroscopic changes in selected organs (liver, spleen, kidney, stomach, intestine, and lung) were evaluated at autopsy.

Histopathological and immunohistochemical analysis of rat tumours

A tissue sample of each mammary tumour was routinely formalin-fixed and paraffin-embedded. The tumours were classified according to the criteria for the classification of rat mammary tumours [13]. The additional parameter—grade of invasive carcinomas—was used. Tumour samples were divided into low-grade (LG) and high-grade (HG) carcinomas. The criteria for categorization (solidization, cell atypia, mitotic activity index, and necrosis) were chosen according to the standard diagnostic method of classification. HG carcinomas were considered to be tumours with $\geq$2 positive criteria; LG carcinomas were tumours with $\leq$1 positive criterion. Serum lipid parameters were evaluated using an Olympus AU640 (Olympus Optical, Tokyo, Japan) automatic biochemical analyser.

The paraffin block with the most representative tumour area of each mammary tumour was chosen for immunohistochemical analysis. The detection of selected proteins was carried out by indirect immunohistochemical method on whole paraffin sections, utilizing commercially available rat-specific antibodies (Santa Cruz Biotechnology, Paso Robles, CA, USA; Dako, Glostrup, Denmark; Bioss, Woburn, MA, USA). After deparaffinization, endogenous peroxidase activity was blocked by incubation with 0.3 % hydrogen peroxide in methanol for 30 min. Sections were pretreated in a microwave generator for 15 min in 10 mM citrate buffer (pH 6.0) and incubated with the primary antibody in PBS containing 1 % BSA, for 60 min at room temperature. The primary antibodies were visualized by a secondary staining system (EnVision, Dual Link System-HRP, cat. No. K4061, Dako North America, Carpinteria, CA, USA) using diaminobenzidine tetrahydrochloride (DAB) as a substrate. The sections were counterstained with haematoxylin, dehydrated, and mounted in Canadian balsam. Negative controls included omission of primary antibody. Immunohistochemically, detected antigen expression was evaluated by precise morphometric method. Sections were screened, and digital images of microscopic views at magnifications of 200× were taken with an Olympus Evolt E-420 installed in an Olympus BX41 N microscope. Expression of VEGF and caspase-3 was analysed in the cytoplasm of tumour cells. Bax and Bcl-2 are membrane-associated oncoproteins. Ki67 was detected within the nucleus. Receptors for VEGF were observed in the cell membrane. Expression of proteins was quantified as the average percentage of antigen-positive area in standard fields (0.5655 mm^2) of tumour hotspot areas. Morphometric analysis of the digital images was done using QuickPhoto Micro software, version 2.3 (Promicra, Prague, Czech Republic). The antigen-positive area was evaluated by phase analysis with standard thresholds for weak, mild, and strong intensities of immunoreactivity. The values of protein expression were compared between treated (FLAV 0.3 and FLAV 3) and non-treated (control) tumour cells of female rats; at least 60 images for one protein were analysed (in total 360 images for six proteins).

Homogenization, fluorescence assay of oxidative protein damage and ELISA method for antioxidant enzyme and 3-nitrotyrosine levels evaluation

For fluorescence-based determination of the parameters reflecting oxidative protein damage, the dissected cancer tissue was homogenized in five volumes of homogenization

buffer (100 mM Tris, 1 mM EDTA, 0.1 % Triton-100) and protease inhibitor cocktail (Sigma-Aldrich, Germany, pH 7.4), and supernatant obtained by following centrifugation (10,000×*g*, 4 °C, 10 min.) was collected and stored at −80 °C until further use. The protein content in homogenates was estimated by Bradford assay using bovine serum albumin as a standard. The relative levels of dityrosine and conjugates of lipid peroxidation products with lysine were estimated by measuring the intensities of their natural fluorescence [14]. Intensity of dityrosine fluorescence was measured at $\lambda_{ex} = 325$ nm (slit = 5 nm) and $\lambda_{em} = 410$ nm (slit = 10 nm) for solution containing 50 μg of homogenate proteins per 1 ml of buffer (50 mM KH_2PO_3/K_2HPO_3, pH 7.0; 10 mM KCl). Relative level of conjugates of lipid peroxidation products with lysine was estimated as intensity of fluorescence measured at $\lambda_{ex} = 365$ nm (slit = 5 nm) and $\lambda_{em} = 440$ nm (slit = 10 nm) for solution containing 50 μg of homogenate proteins per 1 ml of buffer (50 mM KH_2PO_3/K_2HPO_3, pH 7.0; 10 mM KCl). All measurements were taken on spectrofluorometer PerkinElmer LS-55.

The effect of F7 on relative levels of antioxidant enzymes and 3-nitrotyrosine levels was measured by indirect ELISA method. Microtiter plate wells (Maxisorp, Sigma-Aldrich) were coated for 2 h at room temperature with 100 μl of homogenate of mammary carcinoma tissues at 50 μg/ml in buffer, pH 7.6. After coating the antigen to microplate, the coating solution was removed and the plate was washed twice in PBS. The solutions or washes were removed by flicking the plate over a sink. The remaining protein-binding sites were blocked in the coated wells by adding blocking buffer (5 % BSA in PBS) for 30 min at room temperature. The primary antibodies against 3-nitrotyrosine (1:2500, PNK: sc-55256, Santa Cruz), manganese superoxide dismutase Mn-SOD (1:5000, SOD-2, A-2: sc-133134, Santa Cruz), glutathion reductase (1:2500, H-120: sc-32886, Santa Cruz), or catalase (1:5000, N-17: sc-34280, Santa Cruz) in blocking buffer were added and incubated overnight at 4 °C. The plate was washed three times with PBS and blocked with blocking buffer for 30 min. at room temperature. The appropriate secondary alkaline phosphatase-conjugated antibodies (1:5000, Novex, England) were added and incubated for 1 h at room temperature. The plate was washed three times with PBS following with addition of a chromogenic substrate solution (5 mM 4-nitrophenyl phosphate, 0.05 mM $MgCl_2$ in PBS). After sufficient yellow colour development, we added stop solution (2 M NaOH) to the wells and read at 405 nm.

Anti-proliferative activity: tumour cell lines and cytotoxicity assay

MCF-7 (human breast adenocarcinoma, oestrogen receptor-positive), MDA-MB-231 (human breast adenocarcinoma cell lines derived from metastatic site, oestrogen receptor-negative), and 3T3 fibroblasts (non-cancerous cell line) were routinely maintained in Dulbecco's modified Eagle's medium with Glutamax-I and sodium pyruvate supplemented with 10 % foetal calf serum, penicillin (100 IU/ml), and streptomycin (100 μg/ml, all from Invitrogen, Carlsbad, CA, USA). The cells (obtained from ECACC, Porton Down, Salisbury, UK) were maintained under standard tissue culture conditions of 37 °C, 95 % air/5 % CO_2. Cell viability, estimated by trypan blue exclusion, was >95 % before each experiment.

The cytotoxic effects of F7 were determined using colorimetric microculture assay with the MTT end-point [15]. Briefly, 3×10^3 cells were plated per well in 96-well polystyrene microplates (Sarstedt, Nümbrecht, Germany) in the culture medium containing tested compound at final concentrations of 0.05–0.2 mg/ml. After 72 h of incubation, 10 μl of MTT (5 mg/ml) (Sigma, Germany) was added to each well. After an additional 4 h, during which insoluble formazan was produced, 100 μl of 10 % sodium dodecyl sulphate was added to each well and another 12 h were allowed for the formazan to dissolve. The absorbance was measured at 540 nm using the automated uQuant™ Universal Microplate Spectrophotometer (BioTek, Winooski, VT, USA). The blank-corrected absorbance of the control wells was taken as 100 %, and the results were expressed as a percentage of the control. All experiments were performed in triplicate.

Analysis of cell cycle

For flow cytometric analysis of the cell cycle, floating and adherent cells were harvested together 24, 48, and 72 h after treatment (150 μg/ml), washed in cold PBS, fixed in cold 70 % ethanol, and kept at −20 °C overnight. Prior to analysis, cells were washed twice in PBS, resuspended in staining solution (final concentration of 0.1 % Triton X-100, 0.5 mg/ml ribonuclease A, and 0.025 mg/ml propidium iodide—PI), incubated in the dark at RT for 30 min and analysed using a FACSCalibur flow cytometer (Becton–Dickinson, San Jose, CA, USA).

Annexin V/PI staining

Early stage/phase of apoptosis was analysed using the annexin V/propidium iodide apoptosis kit (BD Biosciences Pharmingen, San Diego, CA, USA)—according to the manufacturer's recommendation. MCF-7 cells were harvested 24, 48, and 72 h after treatment (150 μg/ml) and stained with annexin V-FITC in binding buffer for 15 min, washed, stained with propidium iodide for 5 min, and analysed using a BD FACSCalibur flow cytometer.

DAPI staining

Final apoptosis morphology was evaluated by fluorescence microscopy. MCF-7 cells grown on cover slips for 24, 48, and 72 h after treatment (150 μg/ml) were fixed with 2 % paraformaldehyde for 20 min. at 4 °C. After incubation, the cells were washed briefly with PBS and incubated at room temperature with SlowFade® Gold anti-fade reagent with 4′,6-diaminidino-2-phenylindole, dihydrochloride (DAPI) (Invitrogen, USA) for nuclear visualization. The slides were analysed using fluorescence microscope Leica DMI6000 B (Leica Microsystems, Inc., Bannockburn, IL, USA) and evaluated as percentages of cells with a fragmented nucleus from a minimum of 300 cells.

DNA fragmentation assay

MCF-7 cells were harvested (24, 48, and 72 h after treatment) and lysed in a lysis buffer containing 10 mmol/l EDTA, 0.5 % Triton X-100. Proteinase K (1 mg/ml) was added, and cells were incubated at 37 °C for 1 h followed by 10-min incubation at 70 °C. RNase (200 μg/ml) was added, and cells were incubated for 1 h at 37 °C. Samples were transferred to 2 % agarose gel and run with 40 V for 3 h. DNA fragments were visualized by a UV illuminator.

Statistical analysis

Statistical analyses were conducted using GraphPad Prism, version 5.01 (GraphPad Software, La Jolla, CA, USA). The Mann–Whitney and Kruskal–Wallis tests and one-way analysis of variance were the statistical methods used in data evaluation. P values of 0.05 or less were considered statistically significant. Tumour volume was calculated according to the formula: $V = \pi.(S_1)^2 \cdot S_2/12$ (S_1, S_2 are tumour diameters; $S_1 < S_2$).

Results

Rat mammary carcinogenesis

F7 inhibited rat mammary carcinogenesis in a dose-dependent manner. In the FLAV 3 group, an apparent decrease in tumour frequency (the most sensitive parameter of rat breast cancer model) by 58 % ($P = 0.0002$), decrease in incidence by 24 % ($P = 0.041$), and lengthening of tumour latency by 8 days ($P = 0.12$) were observed compared to control. The FLAV 3 group also showed a tendency to decrease tumour volume. Average tumour volume significantly correlated ($r = 0.020$, $P = 0.329$) with the treatment efficacy (tumour frequency) in the FLAV 3 group. In the FLAV 0.3 group, a decrease in tumour frequency by 28 % was observed ($P = 0.054$, Table 1).

Pathology of rat tumours

The most frequently occurring lesions in all experimental groups were mixed papillary/cribriform and cribriform/papillary carcinomas, and cribriform carcinomas. A significant shift in the rate of poorly differentiated (high grade, HG) and well-differentiated (low grade, LG) mammary carcinomas after the treatment with F7 in higher dose was found [control group: 30/48 (HG/LG); FLAV 0.3: 16/41 ($P = 0.23$ vs control); FLAV 3: 7/29 ($P = 0.049$ vs control)]. A significant positive correlation between histological grade and tumour volume ($r = 0.46$; $P = 0.004$) was also observed. A histopathological classification of all mammary tumours is summarized in Table 2.

Immunohistochemical evaluation of rat tumours

Figures 1 and 2 represent the immunohistochemical analysis of rat mammary tumour cells. In the FLAV 3 group, caspase-3 expression increase by 45 % ($P = 0.0003$) and Bcl-2 expression decrease by 26.5 % ($P = 0.03$) were observed compared to control. A tendency to increase Bax expression (by 4.5 and 10 % vs control) in treated groups was observed. Dose-dependent decrease in Ki67 expression in tumour cells of both treated groups by 15 % ($P > 0.05$) and 28 % ($P = 0.042$) was found. VEGFR-2 significantly decreased by 29 % ($P = 0.0006$) in cells treated with higher F7 dose compared to control. No changes in VEGF expression in treated carcinoma cells (Figs. 1, 2) were observed compared to the control cells.

Oxidative damage of proteins, antioxidant enzymes, and 3-nitrotyrosine levels

The higher dose of F7 in the diet appeared to reduce dityrosine levels (by 15 %, $P > 0.05$) when compared to control (Fig. 3a). F7 supplementation in diet had no effect on level of conjugates of lysine with the products of lipid peroxidation. F7 also appeared to decrease the relative 3-nitrotyrosine levels (a product of tyrosine nitration) by 32 % (FLAV 0.3) and by 28 % (FLAV 3) in comparison with control ($P > 0.05$, Fig. 3b).

ELISA and western blot analysis were used to investigate the expression of the enzymes with anti-oxidative capacity. The both methods showed that supplementation of F7 in the diet in any of the tested parameters had no significant effect on the expression of superoxide dismutase type 2, catalase, and glutathione reductase (data not shown).

In vitro analysis in MCF-7 cells

F7 significantly inhibited metabolic activity and viability of the MCF-7 and MDA-MB-231 cells by a dose- and

Table 1 Effects of fruit peel polyphenols (Flavin7) in *N*-methyl-*N*-nitrosourea-induced mammary carcinogenesis in female Sprague–Dawley rats at the end of experiment

Group	CONT	FLAV 0.3	FLAV 3
All animals/tumour bearing animals	25/25	25/23	25/19
Tumour frequency per group*	3.40 ± 0.39	2.44 ± 0.29 (−28 %)	1.44 ± 0.28 (−58 %)[a,b]
Tumour incidence (%)	100.0	92.0 (−8 %)	76.0 (−24 %)[c]
Tumour latency (days)*	66.64 ± 3.38	70.91 ± 3.12 (+4 days)	74.42 ± 3.44 (+8 days)[a]
Average tumour volume (cm^3)*	0.63 ± 0.11	0.67 ± 0.17 (+6 %)	0.54 ± 0.16 (−14 %)
Cumulative tumour volume (cm^3)**	49.69	36.82 (−26 %)	20.00 (−60 %)

CONT control group, *FLAV 0.3* group with administered F7 at a concentration of 3 g/kg in diet (0.3 %), *FLAV 3* group with administered F7 at a concentration of 30 g/kg in diet (3 %)

* Data are expressed as mean ± SEM

** Data are expressed as a sum of volumes per group. Values in brackets are calculated as %-ual deviation from the 100 % of non-influenced control group (with exception of latency). Significantly different

[a] $P < 0.001$ versus CONT

[b] $P < 0.02$ versus FLAV 0.3

[c] $P < 0.05$ versus CONT

Table 2 Histopathological classification and number of mammary tumours

Mammary tumours	CONT	FLAV 0.3	FLAV 3
Malignant lesions			
P, C	28	29	9
C, P	26	16	12
C	19	11	14
C, CO	7	2	–
P	3	3	–
P, C, CO	1	–	–
C, P, CO	1	–	1
Precancerous lesion			
IDP	1	–	–
Total number	85	61	36

Dominant type in mixed tumours is the first in order

Type: invasive carcinoma (*C* cribriform, *P* papillary, *CO* comedo), *IDP* intraductal proliferation

time-dependent manner as detected by MTT assay ($P < 0.05$, $P < 0.01$, $P < 0.001$, Fig. 4). 3T3 fibroblast non-cancerous control cell lines showed only minimal effect after high-dose F7 treatment. For further in vitro studies in MCF-7 cells, we selected concentration of 150 µg/ml.

Significant enrichment in G_0/G_1 (24, 48, and 72 h) and occurrence of sub-G_0 population (72 h) were observed after F7 treatment (150 µg/ml), as compared with untreated cells. As a consequence, a significant reduction in the S and G_2/M phase cell population was observed ($P < 0.01$; $P < 0.05$, respectively, Table 3).

Analysis of phosphatidylserine externalization, shortly (24 h) after F7 incubation, showed the significant increase in the percentage of annexin V-positive MCF-7 cells. Simultaneously, percentage of annexin V-/PI-positive cells increase from 2.4 % (untreated cells) to 15.4 % (F7-treated cells after 72-h incubation, Table 4).

The nuclear morphological changes of MCF-7 cells were analysed using DAPI staining. F7 at concentration of 150 µg/ml significantly increased percentage of cells with condensed chromatin after 48 and 72 h of incubation ($P < 0.05$, $P < 0.01$, Fig. 5a). Furthermore, as shown in Fig. 5b, treatment with F7 at the same concentration resulted in the formation of DNA fragments (characteristic ladder pattern).

Side effects in animals

Compared to the control animals, F7 at higher dose significantly decreased serum levels of triacylglycerols and VLDL cholesterol by 20.5 %. F7 in lower dose significantly reduced serum total cholesterol levels by 13.5 % (data not shown). Compared to control animals, there was no difference in final body weight. Animals from both treated groups exhibited a significant increase in food intake by approximately 2.5 g compared to control. The phytotherapy was well tolerated by animals (in both doses); no macroscopic changes due to F7 administration in the selected organs (e.g. liver steatosis, hepato/splenomegaly, ovarian cysts, gastritis, and apparent hematopoietic disorders) were observed.

Discussion

A plethora of plant products are commercially available as dietary supplements. However, only limited data about

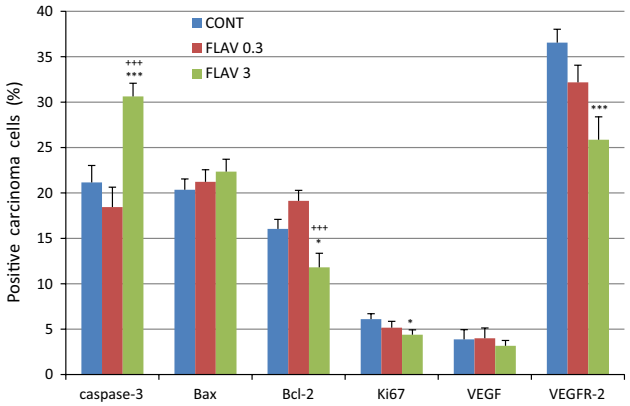

Fig. 1 Immunohistochemical evaluation of caspase-3, Bax, Bcl-2, Ki67, VEGF, and VEGFR-2 expression in rat mammary carcinoma cells after the treatment with F7 in two doses. Data are expressed as mean ± SEM. Significantly different, $^{*}P < 0.05$, $^{***}P < 0.001$ versus CONT, $^{+++}P < 0.001$ versus FLAV0.3. Figure represents the expression of proteins quantified as the average percentage of antigen-positive area in standard fields (0.5655 mm^2) of tumour hotspot areas. The values of protein expression were compared between treated (FLAV 0.3, FLAV 3) and non-treated (control) carcinoma cells of rat females; at least 60 images for one protein were analysed

their efficacy and mechanism of action are known. Well-defined animal studies demonstrate high validity model for the anti-carcinogenic evaluation of possible new drugs. In this regard, we have evaluated the effects of fruit peel polyphenols (constituents of F7) in a well-established mammary carcinoma model in female rats. In this study, dietary-administered F7 significantly inhibited rat mammary carcinomas in a dose-dependent manner. F7 is a commercial source of phytochemicals, especially flavonoids and stilbenoid resveratrol, which play an important role in carcinogenesis [8, 9]. We believe that the additive and synergistic effects of many flavonoids present in F7 were responsible for its potent anti-carcinogenic effects in this study, as we previously shown that resveratrol administered alone did not show any tumour-suppressive activities in rat mammary carcinogenesis [16, 17]. It remains unknown whether a purified phytochemical has the same anti-carcinogenic effects as the whole plant-based food or mixture of foods [18]. A number of papers have been published regarding the role of resveratrol in cardiovascular protection, chemoprevention, and anti-ageing agent [19]. One of the main phenolic compounds in F7 is resveratrol. Based on the analysis of the manufacturer (Vita Crystal Ltd., Budapest, Hungary), lyophilized F7 consists of 0.014 % of resveratrol alone and 2.77 % of total polyphenol. Using the daily pellet food consumption, the daily doses of resveratrol per rat in treated groups were 8 μg (FLAV 0.3) and 82.3 μg (FLAV 3), or daily total polyphenol consumption per rat was 1.6 and 16.3 mg, respectively. In our recent study (using the same model), resveratrol administered alone (p.o.) in a daily dose of approximately 27 mg per rat did not present any effects on rat mammary carcinogenesis when compared to control [16]. In our earlier study, similar results without any effect of resveratrol in rat mammary carcinogenesis (daily dose of 2 mg/rat, dietary administration) were found [17]. The lack of in vivo efficacy of resveratrol alone was also reported by others [20]. In our recent study, mixture of carotenoids and flavonoids present in *Chlorella pyrenoidosa* (concentration of 3 % in the diet) has caused distinct reduction in tumour frequency by 61 % and significantly lengthened tumour latency by 12.5 days in comparison with the control [21]. Young barley leaf powder (0.3 % in the diet), rich in many flavonoids, demonstrated 37 % reduction in tumour frequency using the same rat model (Kubatka et al. submitted manuscript). Distinct differences in our results using single phytochemical or mixture of phytochemicals in whole foods propose that the single phytochemical (e.g. resveratrol in relatively high dose) cannot replace the combination of natural phytochemical mixture in fruits (e.g. several polyphenols in relatively low dose, such as that in F7).

Carcinogenesis is a process characterized by reduced apoptosis and uncontrolled proliferation. There are many experimental data that prove that different phytochemicals are involved in the induction of programmed cell death and in the inhibition of proliferation. Activation of caspases is recognized as a key element in the apoptotic process. Caspase-3 has been implicated as an "effector" caspase associated with the initiation of the cell death. Moreover, apoptosis regulator proteins, e.g. pro-apoptotic Bax or anti-apoptotic Bcl-2, are important parameters of programmed cell death. There is evidence that flavonoids effectively trigger the activation of caspase-3 [22, 23] or increase the Bax/Bcl-2 ratio [24] in cancer cell lines. Our study indicates that F7 in higher dose significantly increased caspase-3 expression in rat mammary tumour cells. These findings are similar to our previous results reporting the pro-apoptotic effects of young barley characterized by the increased expression of both "executioner" caspases—caspase-3 and caspase-7—in rat mammary carcinoma cells (Kubatka et al. submitted manuscript). In addition, higher dose of F7 significantly increased the Bax/bcl-2 pro-apoptotic ratio in rat mammary carcinoma cells in this in vivo study. To confirm whether F7 could induce cellular changes leading to cell death on MCF-7 cell line, we performed annexin V/PI staining. This method detects an early stage of apoptosis and combined staining with PI detects a late stage of apoptosis or necrosis. Moreover, analysis of final morphology changes (nuclei condensations and DNA fragmentation) confirmed pro-apoptotic potential of F7 in MCF-7 cells.

Recent in vitro and in vivo studies on various cell lines have shown that flavonoids have growth inhibitory potential [25, 26]. Ki67 is considered a good tumour marker,

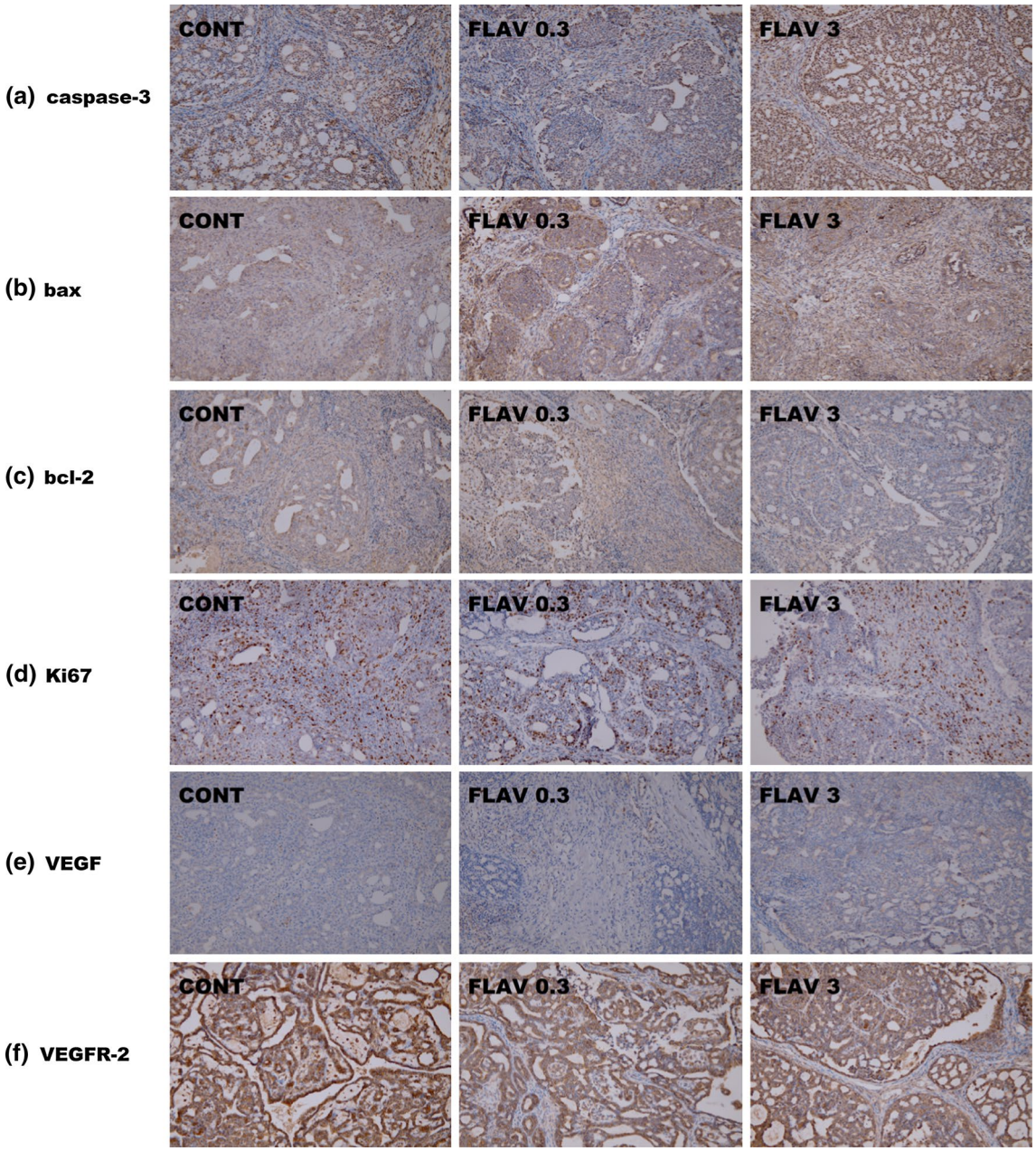

Fig. 2 Representative images of caspase-3, Bax, Bcl-2, Ki67, VEGF, and VEGFR-2 expression in rat mammary carcinoma cells after the treatment with F7 in two doses using the immunohistochemical analysis. Images were selected according to results summarized in Fig. 1. For detection polyclonal caspase-3 antibody (Bioss, Woburn, USA), polyclonal Bax and Bcl-2 antibody (Santa Cruz Biotechnology, Paso Robles, CA, USA), monoclonal Ki67 antibody (Dako, Glostrup, Denmark), monoclonal VEGF, and VEGFR-2 antibodies (Santa Cruz Biotechnology, Paso Robles, CA, USA) were used; final magnifications: ×200

which is strictly associated with cell proliferation [27]. In this study, we observed a significant decrease in the expression of Ki67 in rat mammary carcinoma cells in vivo from the group with higher F7 dose compared to control. Mixture of flavonoids demonstrated anti-proliferative effects also in our previous study—young barley significantly reduced Ki67 expression in rat mammary carcinoma cells in vivo (Kubatka et al. submitted manuscript). In our parallel in vitro study performed in human breast adenocarcinoma cells (MCF-7 and MDA-MB-231), F7 significantly decreased (in a dose- and time-dependent manner) metabolic activity and viability of this cell lines in MTT assay. F7 treatment showed minimal effect on 3T3 fibroblasts used as non-cancerous cell control. Regarding the oestrogen receptor positivity/negativity (ER+, ER−), we demonstrated that ER+ MCF-7 cells were more sensitive

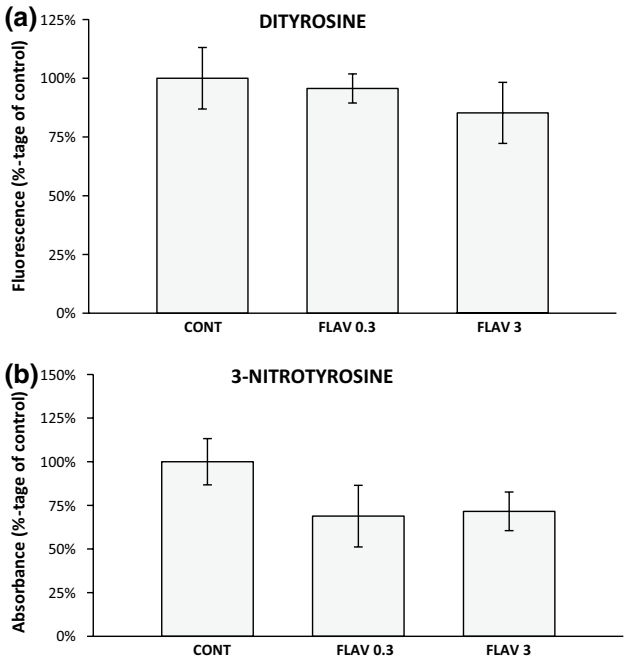

Fig. 3 Effect of F7 on dityrosines **a** and 3-nitrotyrosine **b** levels as markers of cell damage and oxidative stress in mammary cancer tissue. The mammary carcinoma tissues were homogenized, and intensities of natural fluorescence signals reflecting the levels of dityrosines, respectively, levels of 3-nitrotyrosine using indirect ELISA method were measured. Data are presented as a mean ± SEM. One-way analysis of variance was first carried out to test for the differences between groups. Between individual groups, comparisons were made using a Student–Newman–Keuls multiple comparisons test. A value of $P < 0.05$ was considered to be statistically significant

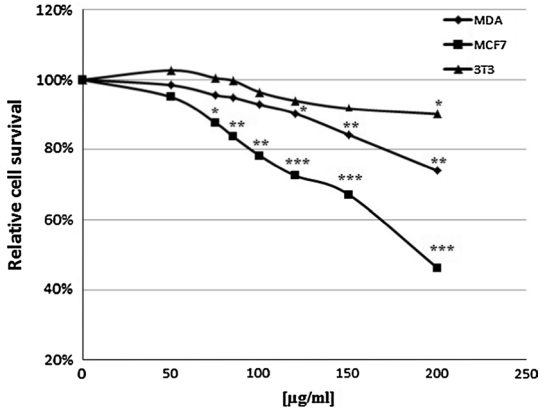

Fig. 4 MTT assay showing the percentage of viable MCF-7, MDA-MB-231, and 3T3 cells after 72-h incubation with F7 at 0.05–0.2 mg/ml concentrations. MTT is a membrane permeable dye, which is metabolized to dark blue crystals of formazan by mitochondrial dehydrogenases of living cells. After lysis of the cell and solubilization of the formazan crystals, the absorbance was measured at 540 nm using the automated uQuant™ Universal Microplate Spectrophotometer. The blank-corrected absorbance of the control wells was taken as 100 %, and the results were expressed as a percentage of the control. Data are expressed as means. Data were obtained from three independent replicate experiments with at least three wells per treatment group in each individual replicate. Significantly different, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ versus control cells (untreated)

to F7 treatment as ER− MDA-MB-231 cells. We suggested that the presence of oestrogen receptor could be one factor associated with cell growth inhibition after F7 dietary supplementation. Our results showed that F7 treatment negatively affected cell metabolism that can lead to cell proliferation changes. Moreover, to evaluate the effect of F7 treatment on MCF-7 cell cycle progression, we performed flow cytometry analysis on cells treated with 150 µg/ml F7 for 24, 48, and 72 h. Our results suggest that F7 could prevent cell cycle progression and lead to inhibition of proliferation in MCF-7 cells.

Angiogenesis, which involves a complex cascade of events, is a good target for cancer chemoprevention. The VEGF-kinase ligand/receptor signalling system plays a key role in angiogenesis. Recent in vivo studies showed that flavonoids could inhibit breast cancer growth and neoangiogenesis accompanying with suppressed VEGF/VEGFR-2 signalling [28, 29]. In accordance with these results, flavonoids present in F7 significantly decreased expression of VEGFR-2 in this study. Mojžiš et al. [30] found that F7 could prevent tumour formation by inhibiting angiogenesis through the suppression of endothelial cell motility, proliferation, and tube formation in human umbilical vein

Table 3 Flow cytometric analysis of cell cycle distribution in MCF-7 cells treated with Flavin7 (in %)

Treatment	Time (h)	Sub-G_0/G_1	G_0/G_1	S	G_2/M
Control		0.18 ± 0.09	62.69 ± 1.50	17.36 ± 1.31	19.76 ± 2.21
F7	24	1.94 ± 1.13	70.93 ± 2.29*	14.30 ± 1.44	12.83 ± 1.66*
F7	48	2.89 ± 1.03	73.67 ± 2.21**	9.73 ± 1.13*	13.71 ± 1.34*
F7	72	5.95 ± 1.84*	76.63 ± 3.20**	8.28 ± 1.45**	9.72 ± 1.76**

Cells were treated with F7 (150 µg/ml) for 24, 48, and 72 h. The distribution of cell cycle was assessed by flow cytometry. Each value is the mean ± SD of three independent experiments

Significantly different, * $P < 0.05$, ** $P < 0.01$ versus untreated cells (control); *F7* Flavin7 (150 µg/ml); sub-G0/G1 fraction of cells identified as apoptotic population

Table 4 Induction of apoptosis after F7 treatment measured by annexin V/PI staining

Treatment	Time (h)	An^-/PI^-	An^+/PI^-	An^+/PI^+
Control		91.82 ± 1.83	5.74 ± 0.70	2.44 ± 0.57
F7	24	77.93 ± 2.18**	19.36 ± 1.26**	2.72 ± 0.95
F7	48	62.28 ± 1.20***	28.87 ± 1.37***	8.86 ± 2.24*
F7	72	58.48 ± 1.45***	26.08 ± 1.50***	15.44 ± 2.70**

MCF-7 cells were treated with F7 for 24, 48, and 72 h, stained with annexin V and PI and analysed by flow cytometry. The significant differences between control and F7-treated cells were signed as * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ versus untreated cells (control)

An−/PI−, live cells; An+/PI−, early apoptotic cells; An+/PI+, late apoptotic/necrotic cells

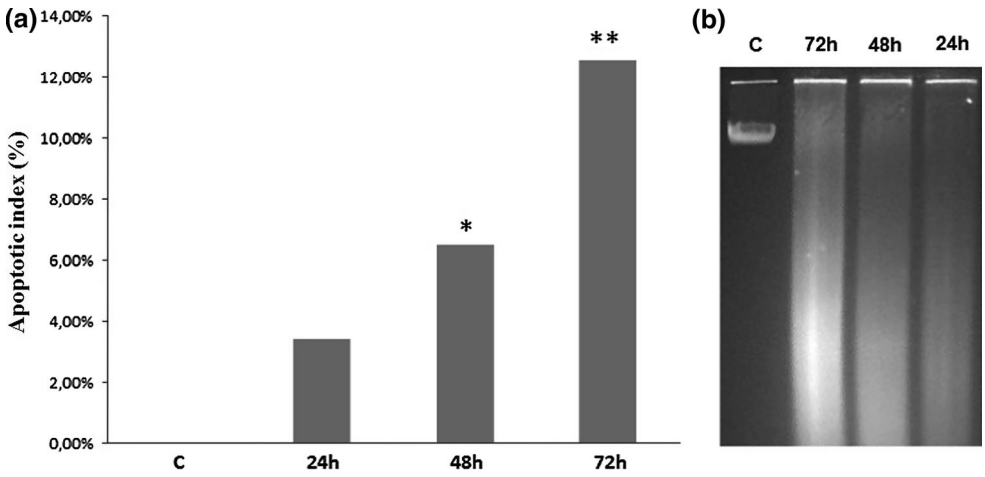

Fig. 5 Apoptotic effect of F7 on MCF-7 cells. **a** Apoptotic index of MCF-7 cells treated with 150 μg/ml of F7 for 24, 48, and 72 h. The results (mean ± SD) of three independent experiments are shown as the apoptotic index evaluated as a percentage of cells with fragmented nuclei from a total number of minimum 300 cells, *$P < 0.05$, **$P < 0.01$. **b** DNA fragmentation of MCF-7 cells after incubation with 150 μM μg/ml of F7 for 24, 48, and 72 h. *C* control (untreated cells)

endothelial cells (HUVECs). In the same study, F7 reduced MMP-9 (matrix metalloproteinase) and MMP-2 activity in HUVECs (MMP is supposed to have major regulatory role in tumour invasion and angiogenesis). Wide spectrum of phytochemicals present in plant-derived products may interfere with several processes involved in cancer angiogenesis; therefore further research on the benefits of phytopharmaceuticals is warranted.

Since free radicals are considered to play an important role during the etiopathogenesis of cancer [31], the putative effect of fruit peel polyphenols supplementation in diet on the products of oxidative damage of proteins and the expression of enzymes with anti-oxidative activity were evaluated. Supplementation of the diet with F7 at higher dose was linked with the tendency to decrease dityrosine levels. Dityrosines specifically reflect the oxidative damage of proteins by free radicals [32]. Our results suggest that molecular constituents of fruit peel polyphenols may support the anti-oxidative capacity of the cells. The expression levels of the anti-oxidative enzymes such as manganese superoxide dismutase, catalase, and glutathione reductase remained unchanged, suggesting that these constituents are most probably the molecular scavengers.

The results of this experiment showed significant antitumour effects of fruit peel polyphenols (from F7) in rat mammary carcinogenesis. The results obtained suggested significant pro-apoptotic, anti-proliferative, and anti-angiogenic effects of F7 in rat mammary tumour cells in vivo and significant pro-apoptotic and anti-proliferative effects in human breast adenocarcinoma cells in vitro. Moreover, F7 demonstrated significant positive effect on plasma lipids in rats. Despite the optimistic results from preclinical research, epidemiologic studies have not provided sufficient evidence regarding cancer preventing activities of phytochemicals and/or plant-derived whole foods. Future studies, especially carefully designed and mechanism-based animal studies, may facilitate better understanding of the potential health benefits of dietary phytopharmaceuticals. Moreover, the dosing of phytopharmaceuticals in oncology patients will depend solely on the results from clinical research. Based on our results with F7, it can be concluded that daily consumption of fruits could be beneficial for the prevention of breast cancer and cardiovascular disease.

Acknowledgments This work was supported by the Scientific Grant Agency of the Ministry of Education of the Slovak Republic under the Contract No. VEGA 1/0071/13, VEGA 1/0043/12 and by the Slovak Research and Development Agency under the Contract No. APVV-0325-07. This work was supported by the Grant "Martin Biomedical Centre" (ITMS: 26220220187), "Competence center for research and

development in diagnosis and therapy", code: 26220220153, and the Grant FNUSA-ICRC (No. CZ.1.05/1.1.00/02.0123), both projects co-funded from EU sources and European Regional Development Fund. This study was also supported by the project Medicínsky univerzitný park v Košiciach (MediPark, Košice) ITMS:26220220185 supported by Operational Programme Research and Development (OP VaV-2012/2.2/08-RO) (Contract No. OPVaV/12/2013). We thank to Eva Jakubovičová for technical support.

Conflict of interest On behalf of all authors, the corresponding author states that there is no conflict of interest.

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