Original Article

Genistein and Selected Phytoestrogen-Containing Extracts Differently Modulate Antioxidant Properties and Cell Differentiation: an *in Vitro* Study in NIH-3T3, HaCaT and MCF-7 Cells

(*Trifolium pratense* L. / *Glycine max* L. / isoflavonoids / cytotoxicity / wound healing / tumour growth / breast cancer)

I. ŠUŠANÍKOVÁ[1], M. PUCHĽOVÁ[1], V. LACHOVÁ[1], E. ŠVAJDLENKA[2], P. MUČAJI[1], K. SMETANA JR.[3,4], P. GÁL[1,5,6,7]

[1]Department of Pharmacognosy and Botany, [2]Department of Chemical Theory of Drugs, Faculty of Pharmacy, Comenius University in Bratislava, Bratislava, Slovak Republic
[3]Institute of Anatomy, First Faculty of Medicine, Charles University, Prague, Czech Republic
[4]BIOCEV, First Faculty of Medicine, Charles University, Vestec, Czech Republic
[5]Laboratory of Cell Interactions, MediPark, Pavol Jozef Šafárik University in Košice, Košice, Slovak Republic
[6]Department of Biomedical Research, East-Slovak Institute of Cardiovascular Diseaes, Inc., Košice, Slovak Republic
[7]Burn Centre Prague, Third Faculty of Medicine, Charles University, Prague, Czech Republic

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Corresponding authors: Peter Gál, Department of Biomedical Research, East-Slovak Institute of Cardiovascular Diseases, Inc., Ondavská 8, 040 11 Košice, Slovak Republic. Fax: (+421) 55 789 1613; e-mail: pgal@vusch.sk or Ivana Šušaníková, Department of Pharmacognosy and Botany, Faculty of Pharmacy, Comenius University in Bratislava, Odbojárov 10, 832 32 Bratislava, Slovak Republic. e-mail: susanikova1@uniba.sk

Abbreviations: DCFH-DA – 2',7'-dichlorodihydrofluorescein diacetate, DMEM – Dulbecco's Modified Eagle's Medium, DMSO – dimethyl sulphoxide, ER-α and ER-β – oestrogen receptors α and β, respectively, FBS – foetal bovine serum, Gen – genistein, GlEt – ethanol extract from soybeans, GlW – water extract from soybeans, HRT – hormone replacement therapy, MTT – 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide, PBS – phosphate-buffered saline, SERMs – selective oestrogen receptor modulators, TrAlW – water extract from the flowers of Albanian red clover, TrSlEt – ethanol extract from the flowers of Slovak red clover, TrSlW – water extract from the flowers of Slovak red clover, TrAlEt – ethanol extract from the flowers of Albanian red clover.

Abstract. During the last decades, plant extracts containing phytoestrogens have increasingly been used as an alternative to oestradiol hormone replacement therapy. The aim of the present study was to compare the effects of genistein with those of different phytoestrogen-containing plant extracts (from red clover flowers and soybeans) on the proliferation and differentiation of NIH-3T3, HaCaT and MCF-7 cells. Our results showed poor correlations between direct anti/pro-oxidant effects and cytotoxicity of the tested samples. In contrast, genistein showed a direct correlation between significant pro-oxidative effects at cytotoxic concentrations and almost no pro-oxidative effects at non-cytotoxic concentrations. Moreover, the tested red clover extract and genistein induced keratin-8 (luminal and prognostic marker in breast cancer) expression only in MCF-7 cells, but this effect was not seen following treatment with the soybean extract. From this point of view, the effect of consumption of phytoestrogens in oestrogen-positive breast cancer remains to be elucidated. In conclusion, our study demonstrates that various phytoestrogen-containing plant extracts and genistein are able to specifically modulate antioxidant properties and differentiation of studied cells.

Introduction

It has been well demonstrated that oestrogen loss at menopause is followed by skin atrophy, decreased collagen and water content, loss of elasticity (Brincat et al., 1985; Affinito et al., 1999; Calleja-Agius and Brincat,

2012), as well as poor wound healing (Brincat et al., 2005). In particular, the negative effect of menopause on the wound healing results from fragile skin that tears and bruises easily and is thus susceptible to trauma. Hormone replacement therapy (HRT) was therefore used to treat menopausal symptoms and to promote wound healing in the elderly (Ashcroft et al., 1997). However, HRT could also be linked with several negative side effects, for example, on endometrium or cardiovascular system (Pickar et al., 2017).

Breast cancer is the second most frequently diagnosed cancer and the leading cause of death among woman in developed countries (DeSantis et al., 2014). Data from human studies have shown great differences in the breast cancer incidence among women with Western lifestyles (e.g., United States and Western Europe) and women from Asia (e.g., Japan and China) (Xie et al., 2013), which may result from different soy consumption (Wu et al., 1996; Korde et al., 2009). Whereas in Asian countries the average daily intake of soy isoflavones represents 25–50 mg (Messina et al. 2006), in US and/or Europe it is less than 1 mg per day (Horn-Ross et al., 2001). So far, several epidemiologic studies evaluating the relation between soy intake and breast cancer incidence have been conducted worldwide (Wu et al., 2008, 2015; Liu et al., 2014). Although all of these studies had some limitations, it is generally accepted that high soy intake is associated with reduced risk of breast cancer in Asian women. Until now only a very few studies have been focused on red clover extracts in relationship to cancer development. Treatment with red clover is safe and well tolerated in healthy women and does not produce breast cancer-promoting effects (Powles et al., 2008).

During the last decades, plant extracts containing phytoestrogens have increasingly been used as an alternative to oestradiol HRT in the treatment of menopausal disorders (Huntley and Ernst, 2004; Boot et al., 2006). The phytoestrogens are a group of natural compounds that exert oestrogenic activities. Isoflavonoids such as daidzein, genistein, biochanin A and formononetin are the most studied natural compounds exerting oestrogenic activities (Cornwell et al., 2004). For example, red clover mainly contains glycosides of formononetin and biochanin A (Beck et al., 2003) that are hydrolysed and metabolized to daidzein and genistein, respectively (Fig. 1). On the other hand, genistein and daidzein, in the form of glycosides, are the main isoflavonoids present in soybeans and soy products (Wang and Murphy, 1996). Therefore, red clover and soy may be considered as efficient sources of isoflavonoids exerting oestrogenic activities that may be used as food supplements for women suffering menopausal disorders. Since phytoestrogens exert agonist/antagonistic effects, they may be classified as natural selective oestrogen receptor modulators (SERMs) (Brzezinski and Debi, 1999). The efficacy and safety of genistein aglycone in a low oestrogen environment may be a direct consequence of its greater affinity for the oestrogen receptor (ER) β than ER-α and

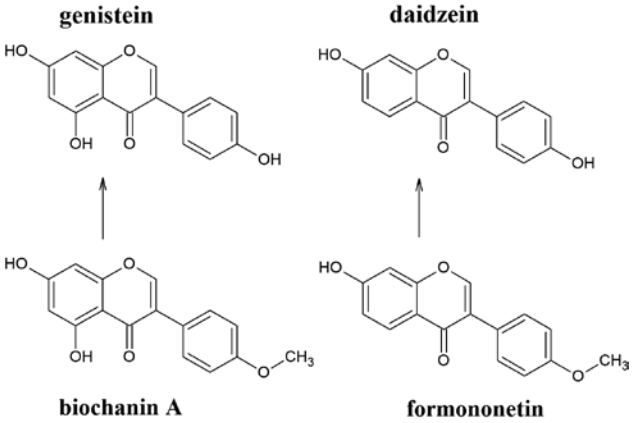

Fig. 1. Natural isoflavonoids: biochanin A and formononetin are metabolized to genistein and daidzein, respectively.

several times lower potency than oestradiol. Notably, ER-β is more widely distributed within the skin and skin structures (Thornton et al., 2003).

It is well known that differentiation and proliferation of cells as well as production of extracellular matrix (ECM) belong to crucial biological processes involved in tissue repair and tumour growth. Diet has been shown to influence breast cancer incidence, recurrence, and prognosis (Ziaei and Halaby, 2017) as well as skin physiology in post-menopausal women (Irrera et al., 2017). Hence, the aim of the present study was to compare the effects of different plant extracts (from red clover flowers and soybeans) containing phytoestrogens on NIH-3T3 (antioxidant properties, proliferation and ECM production), HaCaT and MCF-7 (antioxidant properties, proliferation and differentiation) cells. To complete the panel of experiments, we used isoflavonoid genistein for comparison.

Material and Methods

Reagents

The Dulbecco's Modified Eagle's Medium (DMEM), penicillin-streptomycin mixture, phosphate-buffered saline (PBS), foetal bovine serum (FBS), dimethyl sulphoxide (DMSO), genistein, daidzein, formononetin, biochanin A and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) were purchased from Sigma-Aldrich (Steinheim, Germany). 2',7'-Dichlorodihydrofluorescein diacetate (DCFH-DA) was purchased from Cayman Chemical (Ann Arbor, MI). Primary antibodies high-molecular-weight keratin, keratin 8 were purchased from DakoCytomation (Glostrup, Denmark), keratin 14 from Sigma-Aldrich (St. Louis, MO), keratin 19 from Dakopatts (Glostrup, Denmark), wide-spectrum keratin and Sox-2 from Abcam (Cambridge, UK). Secondary antibody goat anti-mouse was purchased from Sigma-Aldrich and swine anti-rabbit from Santa Cruz Biotechnology (Santa Cruz, CA). Concentrated hydrochloric acid (analytical-reagent grade) was from Centralchem (Bratislava, Slovak Republic).

Plant material

Dried flowers from *Trifolium pratense* L. (Fabaceae family) were obtained from herbal company FYTOPHARMA a.s., Malacky, Slovak Republic (flowers were collected in Slovakia in 2016 and in Albania in 2015). Soy beans from *Glycine max* L. Merr. (Fabaceae family) were grown and obtained from agricultural cooperative Vysoká nad Uhom, Slovak Republic in 2016.

Extract preparation

The water infusions from the dried flowers of red clover were prepared based on the Czech-Slovak Pharmacopoeia, 4th ed. Briefly, 20 g of flowers were macerated in 200 ml of boiled water for 5 min and then cooled at room temperature for 45 min. The obtained infusions were filtered with cotton wool and lyophilized at –53 °C and 0.043 Pa in a SCANVAC CoolSafeTM freeze dryer (LaboGeneTM, Lynge, Denmark) according to the manufacturer's instructions. Subsequently, water extracts from TrSlW (Slovak red clover) and TrAlW (Albanian red clover) were obtained.

The ethanolic extracts of red clover were obtained by extracting dried flowers with 96% ethanol for three weeks with the help of occasional shaking. After evaporation of the solvent under reduced pressure, the dried ethanol TrSlEt (Slovak red clover) and TrAlEt (Albanian red clover) extracts were obtained.

The water decoction from the soybeans was prepared according to the Czech-Slovak Pharmacopoeia, 4th ed. Briefly, crushed beans (20 g) were macerated in 200 ml of boiled water for 30 min in a boiled water bath with occasional mixing. The obtained decoction was filtered with cotton wool and lyophilized (as above). Water extract from soybeans GlW was obtained.

The ethanolic extract of crushed soybeans (300 g) was obtained by extraction using 70% ethanol (450 ml) for two weeks. The extract was filtered through filter paper and then twice shaken in a splitting funnel with n-hexanol. After evaporation of the solvent under reduced pressure, dried ethanol extract from soybeans GlEt was obtained (Table 1).

Table 1. List of tested extracts in the experiments

Abbreviation	Extract
TrSlW	Water extract from the flowers of Slovak red clover
TrAlW	Water extract from the flowers of Albanian red clover
TrSlEt	Ethanol extract from the flowers of Slovak red clover
TrAlEt	Ethanol extract from the flowers of Albanian red clover
GlW	Water extract from soybeans
GlEt	Ethanol extract from soybeans

High-performance liquid chromatography (HPLC)

The Agilent 1200 HPLC system (Waldbronn, Germany) consisting of a binary pump, autosampler, column thermostat, DAD detector and software ChemStation was used. The separation was carried out using a C18 column Poroshell 120 EC-C18, 4.6 × 50.0 mm, 2.7 µm particle size (Agilent). The mobile phase (MP) consisted of methanol and water containing 0.1% formic acid (HCOOH) and 1 mM HCOONH4, filtered through a 0.2 µm filter. The gradient elution started from 10% methanol until 100% methanol at 18 min. The flow rate was 0.3 ml/min. Detection was performed by a DAD detector set at 254 nm. The column was maintained at 30 °C.

Sample preparation for HPLC

About 10 mg of lyophilised material was diluted in methanol in an ultrasonic bath. Samples were first diluted in 0.5 ml of DMSO and then filled to the mark by methanol. Five µl of samples were directly injected to the HPLC-DAD system.

Acid hydrolysis of samples: aliquot amounts of sample solvents were placed into vials, and to each the same volume (1 : 1) of hydrochloric acid (HCl) was added. Vials were sealed using aluminium crimp caps with rubber septum. Hydrolysis took place in an oven set at 100 °C for 60 minutes. After cooling to room temperature, aliquots of hydrolysed samples were transferred and analysed.

Identification and quantification of isoflavones

Identification and determination of the concentration of daidzein, genistein, formononetin and biochanin A was performed using authentic standards. The calibration curves were prepared for each sample from a mixed standard solution of isoflavones.

Cell cultures

NIH-3T3 (mouse embryonic fibroblast) cells, HaCaT cells (human keratinocyte cell line, Boukamp et al., 1998; Cell Lines Service, Eppelheim, Germany) and human cancer cell line MCF-7 (ER-positive human breast adenocarcinoma; ECACC, Porton Down, Salisbury, UK) were used in the experiments. Cells were grown at 37 °C in humidified atmosphere with 5% CO_2 in DMEM supplemented with 10 % FBS and 1 % antibiotics (100 IU/ml penicillin and 100 µg/ml streptomycin).

In vitro analysis of cytotoxicity and cell proliferation

The effect of the tested compounds on the activity of mitochondrial dehydrogenases of cells was assessed as reduction of tetrazolium salt MTT. Briefly, cells were seeded (9,000 cells/100 µl/well) in a 96-well plate in culture medium. Various concentrations of the tested water extracts contained in the medium or ethanol extracts and genistein contained in DMSO and medium

(the final concentrations of DMSO did not exceed 0.1 %) were added 24 h after seeding. After 24 h of incubation, the MTT salt (final concentration 0.45 mg/ml) was added and cells were incubated for another 3 h. After removing the medium with MTT, DMSO was added to lyse the cells, and the absorbance was measured at λ = 570 nm using an Infinite M200 spectrofluorometer (Tecan, Männedorf, Switzerland). The amount of the formed formazan (correlating to the number of viable and metabolically active cells) was calculated as a percentage of control cells.

In vitro migration (wound healing) assay

The migration activity of cells following treatment was assessed using a wound healing assay. Briefly, cells were cultured in a 24-well plate in the medium until confluent. A 2 mm pipette tip was used to wound the monolayer of cells. Afterwards, the medium was removed, the cells were washed twice with PBS, and fresh medium containing the studied extracts or genistein at different concentrations was added. The wounded area was photographed immediately after wounding (t = 0 h) and following 18 h at the same place. The migration distance (gap size) was determined using image analysis software QuickPHOTO MICRO 2.2 (Promicra, Czech Republic) as a percentage of the original area measured at time 0.

Detection of intracellular oxidative stress

Generation of reactive oxygen species (ROS) was assessed using a DCFH-DA probe (Cayman Chemical). Cells were seeded in the black plate in the amount of 25,000 cells/100 µl/well with serum-free medium. After a 24 h incubation period, DCFH-DA dissolved in serum-free medium (10 µM final concentration) was added. Following 15 min, samples (extracts or genistein) at various concentrations were added, and after an additional 60 min, the intracellular fluorescence was measured (λ (excitation/emission) = 480/530, Infinite M200 spectrofluorometer (Tecan, Switzerland) .

Immunocytochemistry

The NIH-3T3, HaCaT and MCF-7 cell lines were seeded on a cover glass at a density of 2,000 cells/cm^2 and cultured for 24 h. The tested samples were then added to the medium at final concentrations of 12.5 µg/ml for extracts and 12.5 µM for genistein. Cells were then cultured for another six days. During this period, the medium with the samples was changed once. After that, cells were washed with PBS and fixed in 2% paraformaldehyde (pH = 7.2). Non-specific binding of secondary antibody was blocked by pre-incubation with normal swine serum (DAKO, Glostrup, Denmark) diluted in PBS for 30 min. Antibodies used in the present investigation are described in Table 2. Control of specificity was performed by replacement of the first step antibody by irrelevant antibody of the same isotype directed against antigen not occurring in the cells. The nuclei of cells were counterstained by DAPI (Sigma-Aldrich), specifically recognizing DNA. The specimens were mounted to Vectashield (Vector Laboratories, Burlingame, CA, USA) and inspected by an Eclipse 90i microscope (Nikon, Tokyo, Japan) equipped with NIS-Elements AR.40.00 for data storage and analysis and with a ProgRes MF CCD camera (Jenoptik Optical Systems GmBH, Jena, Germany)

Statistical analysis

Analyses were performed using GraphPad Prism 7.0 software. Data are presented as mean ± standard deviation. Comparisons between groups were made using the Student's *t*-test with equal variance.

Results

Identification and quantification of isoflavones

Three water extracts and three ethanol extracts from red clover flowers or soybeans were prepared and stored under appropriate conditions. The amounts of aglycones and glycosides of isoflavones daidzein, genistein, formononetin and biochanin A were quantified by the HPLC method (Fig. 2).

All extracts contain aglycones and glycosides of the isoflavones, and we thus found reasonable to quantify the isoflavones after the hydrolysis as well. As previously demonstrated (Luthria et al., 2007), our data also confirmed that the content of isoflavones was found higher when extracted with ethanol. In the red clover extracts, biochanin A dominated, whereas soybean extracts showed dominating genistein and daidzein among the isoflavones, which is in agreement with previously published studies (Wang et Murphy, 1996; Beck et al., 2003). The highest isoflavone content of 26 µg/g dried

Table 2. Reagents used for immunocytochemistry

	Abbreviation	Host	Secondary antibody	Channel
High-molecular-weight keratin	HMWK	mouse monoclonal	goat anti-mouse	TRITC-red
Keratin 8	K10	mouse monoclonal	goat anti-mouse	TRITC-red
Keratin 14	K14	mouse monoclonal	goat anti-mouse	TRITC-red
Keratin 19	K19	mouse monoclonal	goat anti-mouse	TRITC-red
Wide-spectrum keratin	WSK	rabbit polyclonal	swine anti-rabbit	FITC-green
Sox-2	SOX-2	rabbit polyclonal	swine anti-rabbit	FITC-green

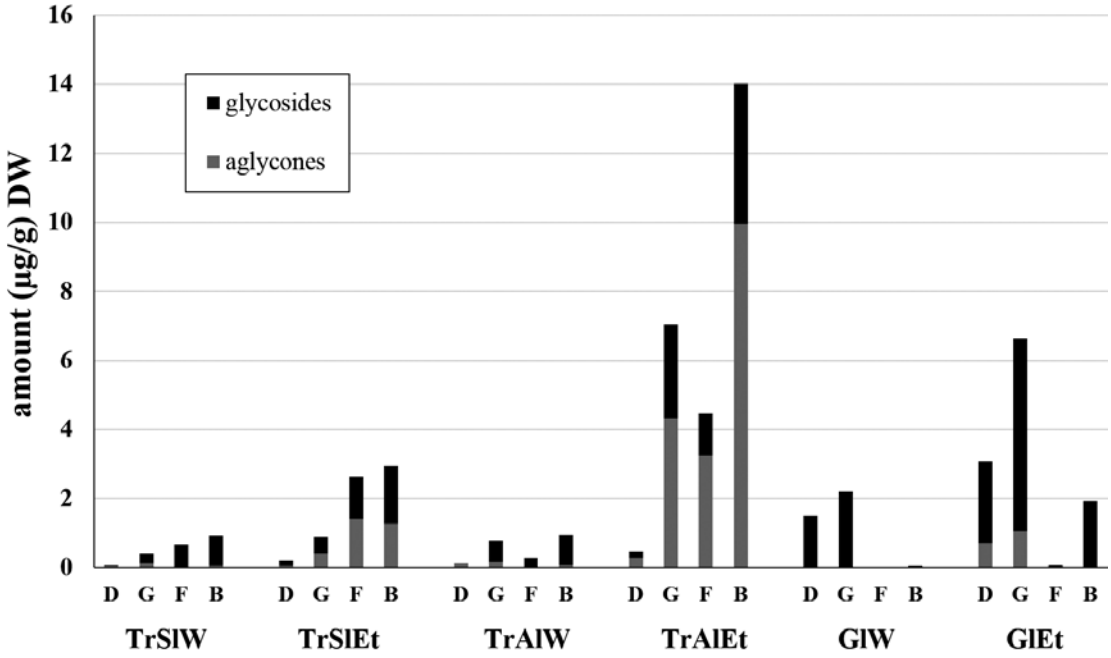

Fig. 2. Identification and quantification of isoflavones from six tested extracts
The amounts of aglycones (grey columns) and glycosides (black columns) of isoflavones daidzein ("D"), genistein ("G"), formononetin ("F") and biochanin A ("B") are expressed as µg/g dried weights of the extracts.

weight (0.0026 %) was included in the TrAlEt extract. However, the content of isoflavones and other phytochemicals may vary depending on the conditions of plant cultivation (Tsukamoto et al., 1995), which may represent a limitation of the present analysis.

MTT assay

We performed the MTT cytotoxicity tests in NIH-3T3 fibroblasts, HaCaT keratinocytes and human cancer cell line MCF-7 with the studied extracts and genistein. Generation of formazan correlates directly with the number of viable cells containing active mitochondrial reductases. The determined inhibitory concentrations (IC_{50}) are listed in Table 3.

In detail, genistein showed preferred cytotoxicity in cancer cell line MCF-7 when compared to 3T3 and HaCaT cells. The water extracts of *T. pratense* and soy beans were generally less cytotoxic than ethanol extracts for each tested cell line. Moreover, both extracts from Slovak and Albanian red clovers acted in a similar fashion. However, red clover extracts were found more cytotoxic than soybean extracts, which is probably not related to the content of isoflavones.

Migration (wound healing) assay

To further examine whether treatment by non-toxic concentrations of the samples affects cell migration, "wound healing assay" was performed. However, results from the present experiments (Figs. 3–5) revealed poor concentration dependency of the tested extracts on cell migration in all tested cell lines.

In detail, the effects of the tested samples on migration of NIH-3T3 fibroblasts (Fig. 3) were not significant, except for significant positive effects of TrAlW, GlEt

Table 3. Inhibition of viability of three cell types by the tested extracts and genistein after 24-h incubation expressed as IC_{50}

	IC_{50}		
	NIH-3T3	HaCaT	MCF-7
TrSlW (µg/ml)	>> 800	242.80 ± 0.09	> 800
TrSlEt (µg/ml)	> 400	130.70 ± 0.17	> 400
TrAlW (µg/ml)	> 800	> 800	> 800
TrAlEt (µg/ml)	290.20 ± 0.58	133.90 ± 0.22	369.1 ± 0.6
GlW (µg/ml)	>> 800	>> 800	>> 800
GlEt (µg/ml)	>> 800	>> 800	> 800
Gen (µM)	681.80 ± 0.19	228.60 ± 0.35	140.3 ± 0.3

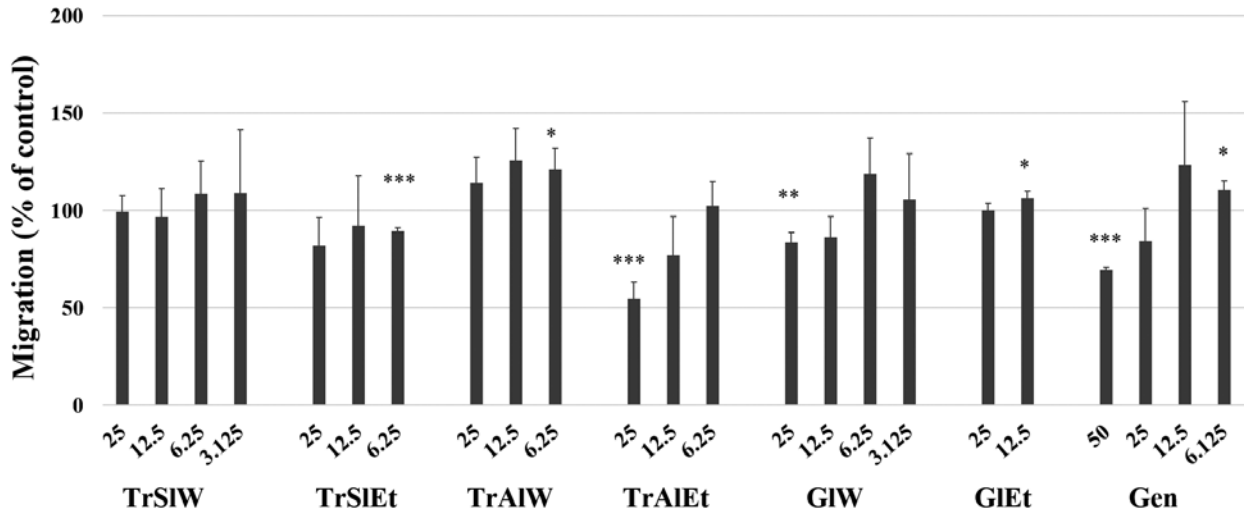

Fig. 3. Effect of the tested extracts or genistein on migration of NIH-3T3 cells expressed as the percentage of the control (100 %)

The values are the means ± SD of three independent experiments (* indicates $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ vs. untreated control).

and genistein at selected concentrations. In contrast, significant inhibitory effects of TrSlEt, TrAlEt, GlW and genistein at higher tested concentrations were recorded.

Similarly, the migratory potential of HaCaT keratinocytes (Fig. 4) was significantly inhibited by the soybean ethanol extract and genistein. On the other hand, red clover water extracts improved cell migration at certain concentrations.

Finally, the tested samples positively affected migration of MCF-7 cancer cells, TrAlEt at 6.25 µg/ml and GlW at 6.25 µg/ml, while TrSlW at 3.125 µg/ml was found inhibitory. Other tested concentrations showed no significant effects on the cell migration.

ROS generation

After 60 min incubation of the studied cell lines with the tested samples, direct fast anti/pro-oxidant effects were confirmed by specific fluorogenic probe DCFH-DA (Figs. 6–8). The reduction/production of ROS by the samples could shed some light on the partial molecular mechanisms of cytotoxicity. After passing through the plasma membrane, DCFH-DA, a lipophilic and non-fluorescent compound, is de-esterified to hydrophilic phenol H_2DCF (dihydrodichlorofuorescein), which may be oxidized to fluorescent DCF (2',7'-dichlorofluorescein) by a process usually considered to involve ROS.

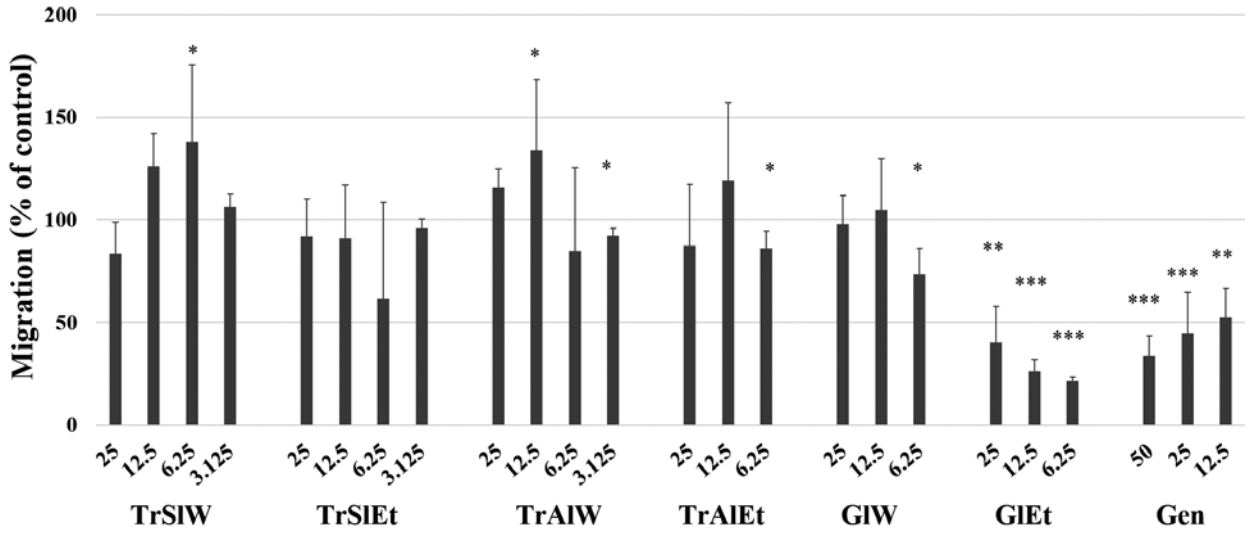

Fig. 4. Effect of the tested extracts or genistein on migration of HaCaT cells expressed as the percentage of the control (100 %)

The values are the means ± SD of three independent experiments (* indicates $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ vs. untreated control).

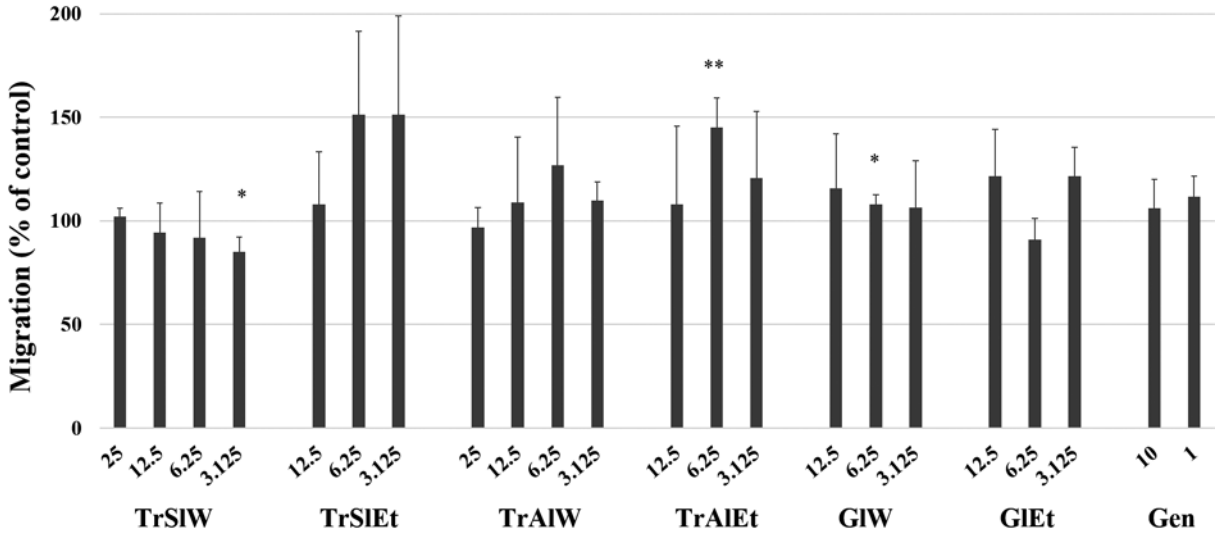

Fig. 5. Effect of the tested extracts or genistein on migration of MCF-7 cells expressed as the percentage of the control (100 %)
The values are the means ± SD of three independent experiments (* indicates $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ vs. untreated control).

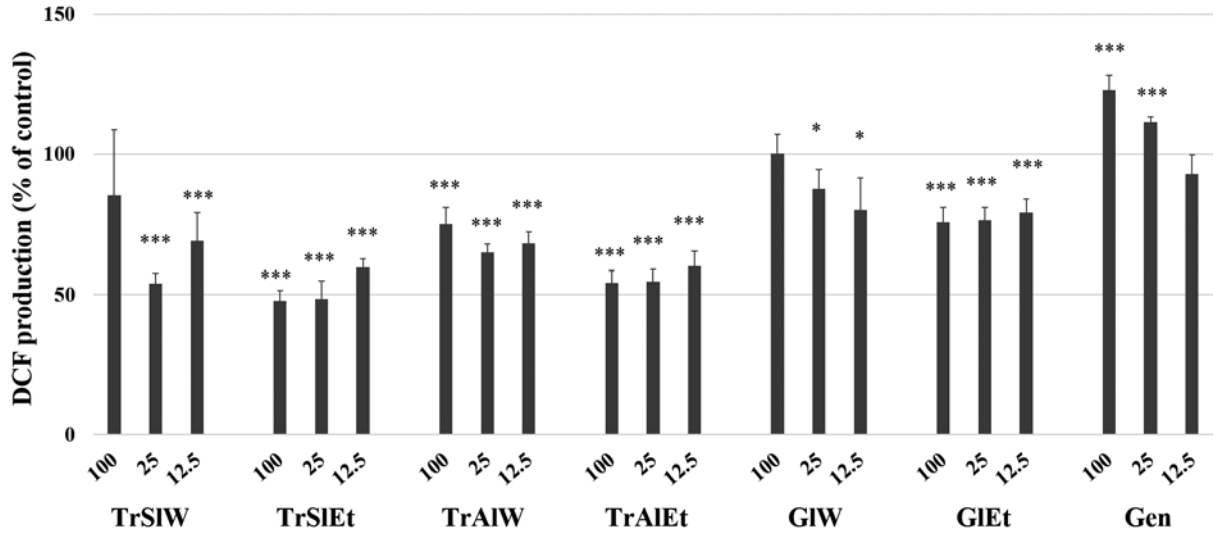

Fig. 6. Effects of the tested extracts or genistein on the ROS level in NIH-3T3 cells after 60 min incubation
The values are expressed as percentage of control (100 %) and are the means ± SD of three independent experiments (* indicates $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ vs. untreated control).

The extracts in the same concentrations caused similar effects on the fluorescence of DCF in all tested cell lines (Figs. 6–8). In general, the four red clover extracts significantly reduced ROS in the cells compared to the control in the tested concentrations. On the other hand, the same concentrations of soybean extracts exerted no significant effects on the ROS levels or acted as mild antioxidant or pro-oxidant. A pronounced pro-oxidant effect induced by GlW was observed in MCF-7 cells (Fig. 8).

Immunocytochemistry of cells

We examined whether the tested extracts modulate formation of the fibronectin-rich extracellular matrix in NIH-3T3 fibroblasts. However, the differences were not significant in any of the tested extracts (Fig. 9).

To determine whether the studied extracts can also modulate the differentiation pattern of epithelial cells, we next stained a panel of selected keratins (Fig. 9). Interestingly, the expression of luminal marker keratin-8 in MCF-7 was significantly increased following treatment with TrSlW, TrAlW and genistein. However, in comparison to control experiments, the tested extracts did not induce/reduce either the expression of keratin-19 or the expression of keratin-14 both in HaCaT and MCF-7 cells.

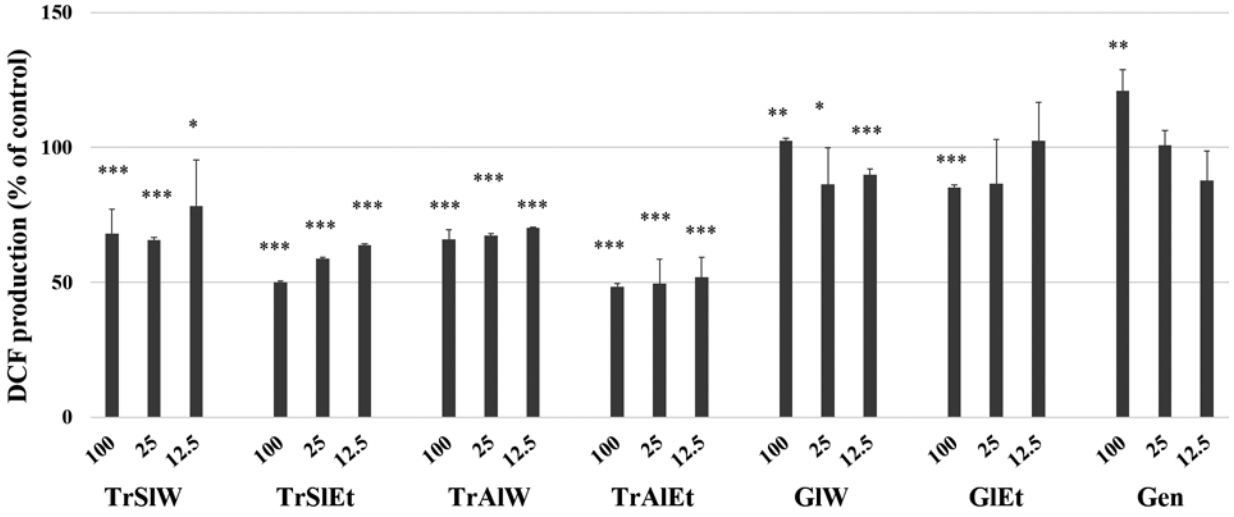

Fig. 7. Effects of the tested extracts or genistein on the ROS level in HaCaT cells after 60 min incubation
The values are expressed as percentage of control (100 %) and are the means ± SD of three independent experiments (* indicates $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ vs. untreated control).

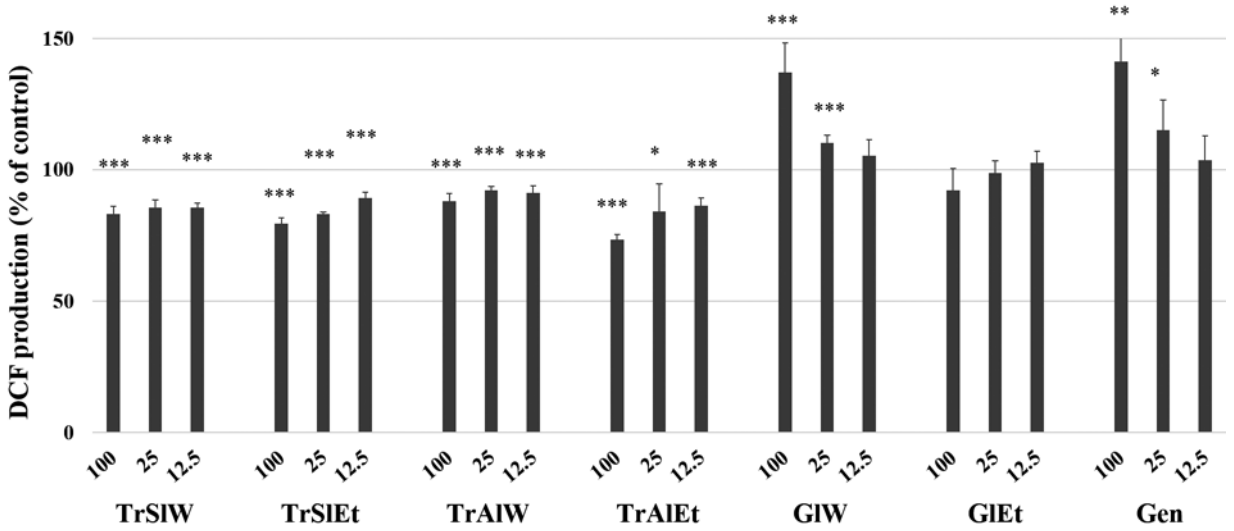

Fig. 8. Effects of the tested extracts or genistein on the ROS level in MCF-7 cells after 60 min incubation
The values are expressed as percentage of control (100 %) and are the means ± SD of three independent experiments (* indicates $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ vs. untreated control).

Discussion

In the immunocytochemical part of our study, we revealed the possibility to determine whether the studied extracts could modulate the differentiation pattern of epithelial cells. We next stained a panel of selected keratins in HaCaT keratinocytes and MCF-7 cancer cells. Most importantly, the expression of luminal marker keratin-8 (Iyer et al. 2013) in MCF-7 was significantly increased following treatment with TrSlW, TrAlW and genistein, but not when treated with GlW. The expression of keratin-8 may be associated with poor prognosis (Dvořánková et al., 2012; Bonin et al., 2015). However, cells exposed to isoflavones *in vitro* may respond differently than their cellular counterparts in animal models (Ziaei et Halaby, 2017). From this point of view, the effect of consumption of phytoestrogens in oestrogen-positive breast cancer remains to be elucidated. The only difference between the studied extracts was observed in the GlW sample in the absence of formononetin and biochanin A. It is well known that formonentin and biochanin A are metabolized into genistein and daidzein *in vivo*, and thus it may be hypothesized that the content of these two compounds in the GlW extract was too low to induce keratin-8 expression *in vitro*. Therefore, it has to be answered whether this mechanism would also be present at the *in vivo* level. In addition, in comparison to control experiments, the tested extracts

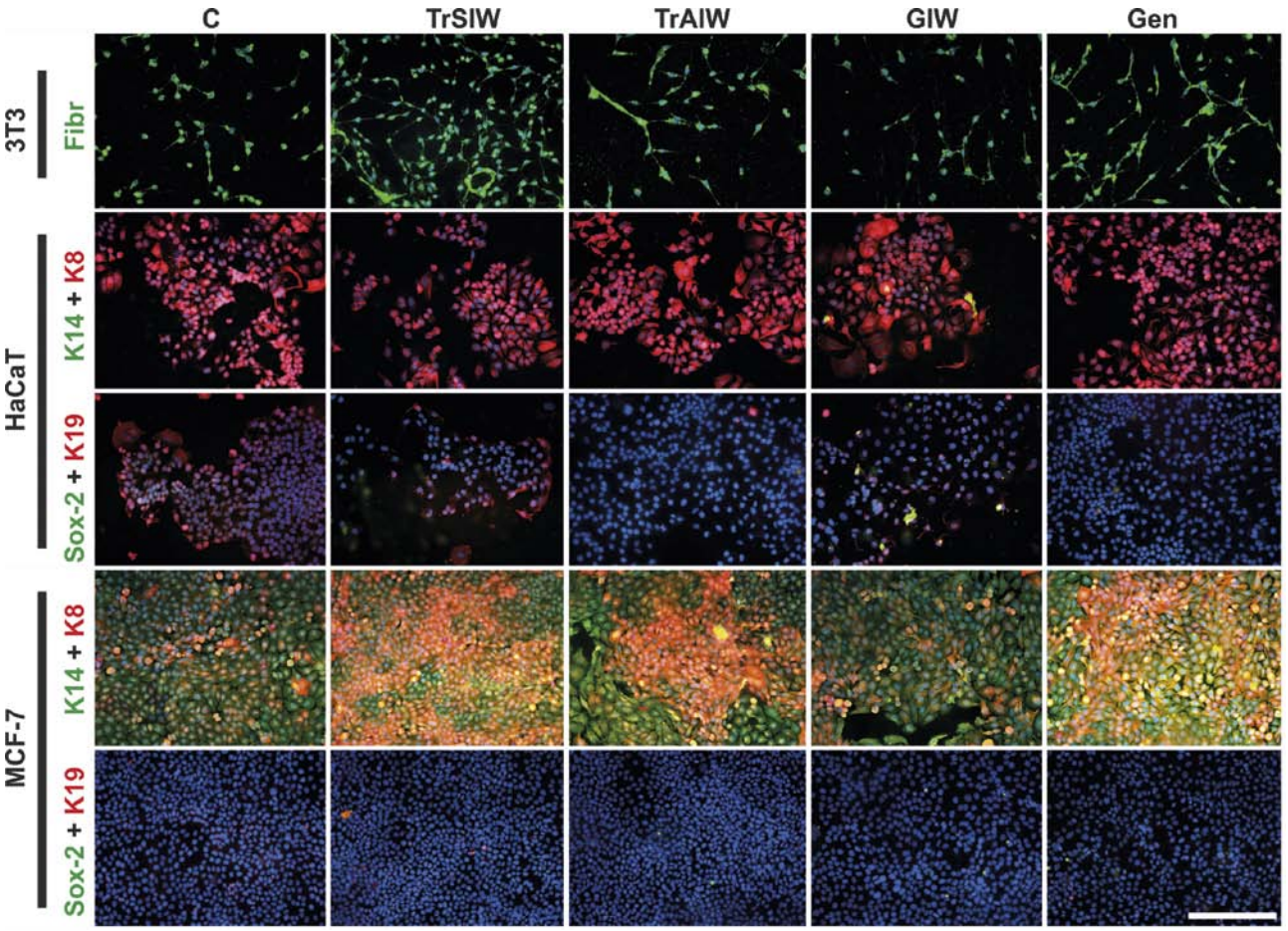

Fig. 9. Expression profile of NIH-3T3 fibroblasts, HaCaT keratinocytes and MCF-7 breast cancer cells
In particular, the expression of keratin-8 in MCF-7 cells changed following treatment with TrSlW, TrAlW and genistein, but not after GlW (all extracts at 12.5 µg/ml/genistein at 12.5 µM final concentration). Other parameters remained stable (scale bar 50 µm).

did not modulate the expression of keratin-19 (Dvořánková et al., 2005), one of the putative markers of poor differentiation, nor did they alter expression of the basal/myoepithelial marker keratin-14 (Wetzels et al., 1989).

The MTT assay is a frequently used protocol to evaluate the cytotoxicity/proliferation *in vitro* (Mosmann, 1983; Zaczyńska et al., 2018). Our study was the first to examine the cytotoxic effect of genistein in HaCaT keratinocytes. Moreover, our data regarding the cytotoxicity of genistein in MCF-7 cells agree with a previously published study (Liu et al., 2013), but assessment of NIH-3T3 cell viability showed less cytotoxic effects in our study than previously seen (Rucinska et al., 2008).

The *in vitro* scratch wound healing assays are beneficial for early basic studies of the wound healing capacity of the tested samples (Kuzan et al., 2017). In a highly coordinated biological process of dermal wound healing, skin fibroblasts interact with surrounding cells such as keratinocytes, fat and endothelial cells, as well as inflammatory and immune cells (Gál et al., 2017). Fibroblasts produce ECM, glycoproteins, adhesive molecules and various cytokines (Le Pillouer-Prost, 2003). Therefore, we used keratinocytes and fibroblasts for the *in vitro* experiments. As previously shown, human keratinocyte cell line HaCaT and MCF-7 cancer cells express both ER-α and ER-β (Pawlak et Wiebe, 2007; Peržeľová et al., 2016) and their expression may also be expected in mouse embryonic fibroblasts NIH-3T3 (Walker et Korach, 2004). The tendency to increase cell migration was seen in non-cancer cells treated with water extracts, while the ethanol extracts rather decreased migration. Interestingly, both extracts rather increased migration of cancer cells, which presents an aspect with crucial significance for clinical practice.

Flavonoids and isoflavonoids have also been shown to influence the intracellular redox status and to have antioxidant properties (Guo et al., 2002; Williams et al., 2004). In this context, we examined how genistein and the studied extracts affect ROS generation *in vitro*. Our study revealed that lower concentrations of genistein had no effects on the generation of ROS, whereas higher concentrations exerted significant pro-oxidant effects in all tested cell lines. Similarly, genistein at nanomolar concentrations showed almost no effects on the production/reduction of ROS using a similar model of endothe-

lial cells, but it increased SOD activity following peroxide-induced ROS generation (Fáber et al., 2018). It has also been shown that genistein acted as an antioxidant at lower tested concentration (5 μM) and as a pro-oxidant at higher tested concentrations (200 μM) after 48 h of incubation (Chen et al., 2014). Similarly, daidzein and genistein at lower tested concentrations (less than 20 μM; 24 h of incubation) protected cells against oxidative damage especially with respect to DNA (Foti et al., 2005), whereas genistein at 20 μM acted as a pro-oxidant and caused apoptosis of leukaemic cells (Kim et al., 2014). From this point of view, not only the concentrations, but also the time of treatment represents a significant factor influencing the modulatory effects of phytoestrogens on the oxidative stress. Regarding possible underlying mechanisms of the tested extracts, it has been shown that the antioxidant properties of *T. pratense* were attributed to the high content of flavonoids, especially glycosides of kaempferol, quercetin, luteolin and apigenin (Kaurinovic et al., 2012; Tundis et al., 2015). Furthermore, the soybean isoflavone extract also exerted antioxidant properties *in vitro* and elevated the levels of CAD and SOD enzymes *in vivo* (Liu et al., 2005). Phenolic acids, including chlorogenic, caffeic, ferulic, p-coumaric, syringic and others as well as tannins, flavonoids and proanthocyanidins are present in soybeans and possess antioxidant activity (Malenčić, et al., 2007). On the other hand, the fatty acids present in soybeans could act as pro-oxidants (Waraho et al., 2009).

In conclusion, our results showed only weak correlations between direct anti/pro-oxidant effects and cytotoxicity of the tested samples, which may be caused by the composition of the tested extracts and presence of ballast, but with no relationship to the content of genistein. However, pure genistein showed a direct correlation between significant pro-oxidative effects at cytotoxic concentrations and almost no pro-oxidative effects at non-cytotoxic concentrations. In particular, the modulatory effect on MCF-7 cell differentiation may be found potentially interesting for further experimental work, also using oestrogen receptor antagonists to explore whether these effects are ER-mediated or not.

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