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Genistin inhibits UV light-induced plasmid DNA damage and cell growth in human melanoma cells

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Abstract

In recent years, genistein has received considerable attention because epidemiologic studies showed that consumption of soybean-containing diets was associated with a lower incidence of certain human cancers in Asian populations. In vitro studies further showed that such chemopreventive and antineoplastic effects were associated with the antioxidant activity of genistein and inhibitor activities on cell proliferation and angiogenesis. Genistein was shown to arrest the growth of malignant melanoma in vitro and to inhibit ultraviolet (UV) light-induced oxidative DNA damage. Recently, it has been demonstrated that genistin, as other flavonoid glycosides, is partly absorbed without previous cleavage and does not have to be hydrolyzed to be biologically active. Therefore, not only isoflavone aglycons, but also glycosides can be of physiological relevance. In the present study, we evaluated in cell-free systems the effect of genistin and daidzin on pBR322 DNA cleavage induced by hydroxyl radicals, generated from UV photolysis of hydrogen peroxide, and their superoxide anion scavenging capacity. In addition, we investigated the growth inhibitory activity of these isoflavones against human melanoma cell line (M14). Under our experimental conditions, genistin and daidzin showed a protective effect on DNA damage and exhibited a superoxide dismutase-like effect, but only genistin was able to reduce significantly the vitality of M14 cells, confirming the importance of the 5,7-dihydroxy structure in the A ring. These results suggest that also genistin, due to its antioxidant and anticarcinogenic properties, contributes to the overall biological activity of soy and could have promising applications in the field of dermatology.

Keywords: Genistin; pBR322 damage; UV; Superoxide anion; Cell growth; Melanoma cells

1. Introduction

The bioflavonoids are aromatic secondary plant metabolites belonging to the class of plant phenolics. They are highly diverse in both their chemical structure and proposed biological functions. Flavonoids are benzo-γ-pirone derivatives that can be grouped according to the presence of different substituents on the rings and to the degree of benzo-γ-pirone ring saturation. Flavonoids per se are compounds in which the benzenoid substitution is at the 2 position; compounds with substitution at the 3 position are properly termed isoflavonoids [1]. Genistein (4′,5,7-trihydroxy-isoflavone), daidzein (4′,7-dihydroxy-isoflavone), and their 7-glycosides, genistin, and daidzin are the major isoflavonoids of soybeans and soy products. Genistein and genistin are present at ~4.6–18.2 and 200.6–960 μg/g, respectively, in soybean, soybean nuts, and soy powder. In recent years, genistein has received considerable attention because epidemiologic studies showed that consumption of soybean-containing diets was associated with a lower incidence of certain human cancers in Asian populations [2]. In vitro studies further showed that such chemopreventive and antineoplastic effects were associated with the antioxidant activity of genistein and inhibitor activities on cell proliferation and angiogenesis [3–7]. Genistein was shown to arrest the growth and induce the differentiation of malignant melanoma in vitro [8]. Moreover, it has been demonstrated that this isoflavone significantly inhibits ultraviolet (UV) light-induced oxidative DNA damage [8]. Nevertheless, genistein, like daidzein, seems to have some disadvantages, which considerably limit its potential clinical utility; these include rapid in vivo metabolism and excretion, low serum level after oral

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administration, and poor solubility [9]. It has been demonstrated that genistin, the predominant form found in plants, like other flavonoid glycosides [10–12], is partly absorbed without previous cleavage and does not have to be hydrolyzed to be biologically active [13]. Therefore, not only isoflavone aglycons, but also glycosides can be of physiological relevance.

In the present study, we evaluated in cell-free systems the effect of genistin and daidzin on pBR322 DNA cleavage induced by hydroxyl radicals (·OH), generated from UV photolysis of hydrogen peroxide (H_2O_2) and their superoxide anion (O_2^-) scavenging capacity, using a method that excludes the Fenton-type reaction and the xanthine/xanthine oxidase system. In addition, we investigated the growth inhibitory activity of these isoflavone glycosides against human melanoma cell line (M14), testing several cellular parameters such as cell vitality, cytotoxicity, and DNA damage. In addition, because a substantial body of data suggests that reactive oxygen species are associated with tumor promotion, acting as second messengers for signal transduction pathways that regulate cell proliferation, intracellular oxidants were determined.

2. Methods and materials

2.1. Chemicals

Daidzin and genistin (>99%) were obtained from the Extrasynthese (Geany Cedex, France); pBR322 plasmid DNA, 3(4,5-dimethylthiazol-2-yl)2,5-diphenyl-tetrazolium bromide (MTT), and 2′,7′-dichlorofluorescein diacetate (DCFH-DA) were obtained from Sigma Aldrich Co (St. Louis, USA); β-nicotinamide adenine dinucleotide (NADH) was obtained from Boehringer Mannheim (Germany). All other chemicals were purchased from GIBCO BRL Life Technologies (Grand Island, NY, USA).

2.2. Antioxidant activity in cell-free systems

2.2.1. DNA cleavage induced by hydrogen peroxide UV photolysis

The experiments were performed, as previously reported [14], in a volume of 20 μl containing 33 μM in bp of pBR322 plasmid DNA in 5 mM phosphate saline buffer (pH 7.4), and isoflavone glycosides at 100, 200, and 400 μM concentration. Immediately prior to irradiating the samples with UV light, H_2O_2 was added to a final concentration of 2.5 mM. The reaction volumes were held in caps of polyethylene microcentrifuge tubes, placed directly on the surface of a transilluminator (8000 μW cm^{-1}) at 300 nm. The samples were irradiated for 5 min at room temperature. After irradiation, 4.5 μl of a mixture, containing 0.25% bromophenol blue, 0.25% xylene cyanol FF, and 30% glycerol, were added to the irradiated solution. The samples were then analyzed by electrophoresis on a 1% agarose horizontal slab gel in Tris–borate buffer (45 mM Tris–borate, 1 mM EDTA). Untreated pBR322 plasmid was included as a control in each run of gel electrophoresis, conducted at 1.5 V/cm for 15 h. Gel was stained in ethidium bromide (1 μg/ml, 30 min) and photographed on Polaroid-Type 667 positive land film.

2.2.2. Scavenger effect on superoxide anion

Superoxide anion was generated in vitro as described by Paoletti et al. [15]. The assay mixture contained in a total volume of 1 ml 100 mM triethanolamine–diethanolamine buffer, pH 7.4, 3 mM NADH, 25 mM/12.5 mM EDTA/$MnCl_2$, and 10 mM β-mercapto-ethanol; some samples contained genistin and daidzin at different concentrations (25, 50, 100, and 200 μM). After 20 min of incubation at 25°C, the decrease in absorbance was measured at λ=340 nm.

2.3. Study on human tumor cell line

2.3.1. Cell culture and treatments

M14 human melanoma cells were grown in RPMI containing 10% fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 25 μg/ml fungizone. After 24 h of incubation at 37°C under a humidified 5% carbon dioxide to allow cell attachment, the cells were treated with different concentrations (12, 25, 50, and 100 μM) of genistin and daidzin, and incubated for 72 h under the same conditions.

Stock solutions of genistin and daidzin were prepared in DMSO. Control cultures received DMSO alone. The final concentration of this solvent was kept constant at 0.27%.

2.3.2. MTT bioassay

MTT assay was performed as described by Mosmann [16]. Briefly, the cells were set up 6×10^3 cells per well of a 96-well, flat-bottomed 200 μl of microplate. Cells were incubated at 37°C in a humidified 5% CO_2/95% air mixture and treated with isoflavone glycosides for 72 h. Four hours before the end of the treatment time, 20 μl of 0.5% MTT in phosphate-buffered saline (PBS) was added to each microwell. Cells were washed once before adding MTT. After 4 h of incubation at 37°C, the supernatant was removed and replaced with 100 μl of DMSO. The optical density of each well sample was measured with a microplate spectrophotometer reader (Digital and Analog Systems, Rome, Italy) at 550 nm.

2.3.3. Lactic dehydrogenase release

Lactic dehydrogenase (LDH) activity was spectrophotometrically measured in the culture medium and in the cellular lysates at 340 nm by analyzing NADH reduction during the pyruvate–lactate transformation, as previously reported [17]. Cells were lysed with 50 mM Tris–HCl+20 mM EDTA, pH 7.4+0.5% sodium dodecyl sulfate, further disrupted by sonication and centrifuged at 13,000*g* for 15 min. The assay mixture (1 ml final volume) for the enzymatic analysis contained 33 μl of sample (5–10 μg of protein) in 48 mM

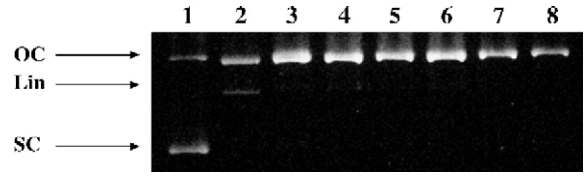

Fig. 1. Effect of genistin and daidzin on the protection of supercoiled DNA against ·OH generated by the photolysis of H_2O_2. Lane 1, untreated DNA; lane 2, 2.5 mM H_2O_2; lane 3, genistin (100 μm)+2.5 mM H_2O_2; lane 4, daidzin (100 μM)+2.5 mM H_2O_2; lane 5, genistin (200 μM)+2.5 mM H_2O_2; lane 6, daidzin (200 μM)+2.5 mM H_2O_2; lane 7, genistin (400 μM)+2.5 mM H_2O_2; lane 8, daidzin (400 μM)+2.5 mM H_2O_2. SC, supercoiled circular DNA; LIN, linear form; OC, open circular form.

PBS, pH 7.5, plus 1 mM pyruvate and 0.2 mM NADH. The percentage of LDH released was calculated as percentage of the total amount, considered as the sum of the enzymatic activity present in the cellular lysate and that in the culture medium. A Hitachi U-2000 spectrophotometer (Hitachi, Tokyo, Japan) was used.

2.3.4. Reactive oxygen species assay

Reactive species determination was performed by using a fluorescent probe DCFH-DA, as previously described [17]. DCFH-DA diffuses through the cell membrane; it is enzymatically hydrolyzed by intracellular esterases and oxidized to the fluorescent 2′,7′-dichlorofluorescein in the presence of ROS. The intensity of fluorescence is proportional to the levels of intracellular oxidant species. One hundred microliters of 100 μM DCFH-DA dissolved in 100% methanol was added to the cellular medium where the acetate group is not hydrolyzed, and the cells were incubated at 37°C for 30 min. After incubation, cells were lysated and centrifuged at 10,000*g* for 10 min. The fluorescence (corresponding to the radical species-oxidized 2′,7′-dichlorofluorescein) was monitored spectrofluorometrically using a Hitachi F-2000 spectrofluorimeter (Hitachi): excitation, 488 nm; emission, 525 nm. The total protein content, measured according to Bradford [18], was evaluated for each sample, and the results are reported as fluorescence intensity per milligram protein and compared to relative control.

2.3.5. DNA analysis by COMET assay

The presence of DNA fragmentation was examined by single cell gel electrophoresis (COMET assay), according to Singh et al. [19]. Briefly, $0.8–1\times10^5$ cells were mixed with 75 μl of 0.5% low melting agarose and spotted on slides. The "minigels" were maintained in lysis solution (1% *N*-laurosil-sarcosine, 2.5 M NaCl, 100 mM Na_2EDTA, 1% Triton X-100, 10% DMSO, pH 10) for 1 h at 4°C, and then denatured in a high pH buffer (300 mM NaOH, 1 mM Na_2EDTA, pH 13) for 20 min, and finally electrophoresed in the same buffer at 18 V for 45 min. At the end of the run, the minigels were neutralized in 0.4 M Tris–HCl, pH 7.5, stained with 100 μl of ethidium bromide (2 μg/ml) for 10 min, and scored using a fluorescence microscope (Leica, Wetzlar, Germany) interfaced with a computer. Software (Leica-QWIN) allowed us to analyze and quantify DNA damage by measuring (a) tail length, intensity, and area; (b) head length, intensity, and area. These parameters are employed by the software to determine the level of DNA damage as (a) the percentage of the fragmented DNA (TDNA) and (b) tail moment (TMOM) expressed as the product of TD (distance between head and tail) and TDNA.

2.4. Statistical analysis

Results were analyzed using one-way ANOVA followed by Dunnett's post hoc test for multiple comparisons with control. All statistical analyses were performed using the statistical software package SYSTAT, version 9 (Systat, Evanston, IL, USA).

3. Results

Fig. 1 shows the electrophoretic pattern of DNA after UV photolysis of H_2O_2 (2.5 mM) in the absence and presence of genistin and daidzin (100, 200, and 400 μM). DNA derived from pBR322 plasmid showed two bands on agarose gel electrophoresis (lane 1); the faster moving band corresponded to the native form of supercoiled circular DNA (scDNA) and the slower moving band was the open circular form (ocDNA). The UV irradiation of DNA in the presence of H_2O_2 (lane 2) resulted in the cleavage of scDNA to ocDNA and linear form (linDNA), indicating that ·OH generated from UV photolysis of H_2O_2 produced DNA strand scission. The addition of isoflavone glycosides (lanes 3–8) to the reaction mixture of H_2O_2 suppressed the formation of linDNA. The action of these natural compounds was comparable to that of trolox and ascorbic acid as previously reported [20]. The treatment of plasmid DNA with isoflavonoids alone did not change the migration pattern (data not shown).

The superoxide anion scavenging capacity of these isoflavones was tested using the method of Paoletti et al. [15], which excludes the Fenton-type reaction and the xanthine/xanthine oxidase system. Also, in this assay,

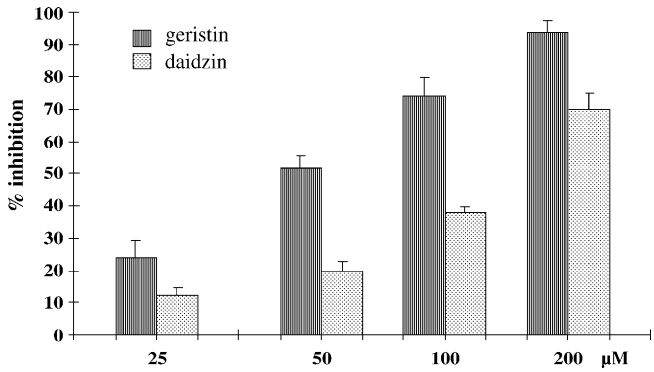

Fig. 2. Scavenger effect of genistin and daidzin at different concentrations (25, 50, 100, and 200 μM) on O_2^- expressed as percentage of inhibition of NADH oxidation; rate of O_2^- production was 4 nmol/min. Each value represents the mean±S.D. of four experiments performed in duplicate.

Table 1
Cell viability in M14 cells after the treatment with genistin and daidzin for 72 h (12, 25, 50, and 100 μM)

Treatment	MTT	% LDH released
Control	100	10.0±2.5
Genistin		
12 μM	81.6±2.6*	10.7±2.7
25 μM	78.1±2.9*	12.5±1.8
50 μM	64.2±1.6*	12.1±4.7
100 μM	59.1±4.6**	9.1±3.8
Daidzin		
12 μM	95.9±2.6	10.8±4.7
25 μM	96.7±2.9	11.1±2.8
50 μM	95.6±1.6	12.6±4.3
100 μM	97.1±2.6	13.1±2.8

Each value represents the mean±S.D. of the three experiments performed in duplicate.
* Significant vs. control untreated cells ($P<.05$).
** Significant vs. control untreated cells ($P<.01$).

genistin and daidzin showed a dose-dependent superoxide scavenging effect (Fig. 2). Genistin exhibited the major effect and, under our experimental conditions, at 200 μM, corresponded in activity to 0.08 U/mg protein superoxide dismutase (SOD) [20].

The extract was tested in vitro for its potential human tumor cell growth inhibitory effect on M14 human melanoma cell line using MTT assay, a nonradioactive, fast, and economical assay widely used to quantify cell growth. MTT is a yellow water-soluble tetrazolium salt. Metabolically active cells are able to convert the dye to its water-insoluble dark blue formazan by reductive cleavage of the tetrazolium ring. The results, summarized in Table 1, show that only genistin exhibited a significantly ($P<.01$) and a dose-dependent inhibitory effect on this human cancer cell examined, and at higher concentration (100 μM), the cell viability was 59%.

Lactic dehydrogenase release was also measured to evaluate the presence of cell necrosis as a result of cell disruption subsequent to membrane rupture (Table 1). Under our experimental conditions, treatment of cell cultures with genistin and daidzin did not result in a significant increase in LDH release.

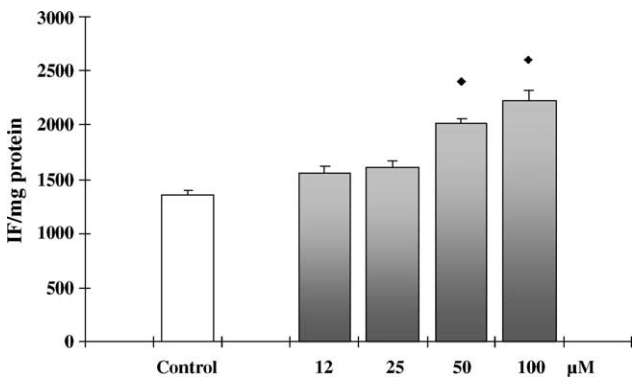

Fig. 3. Intracellular oxidants in M14 human tumor cell line untreated and treated with genistin at different concentration (12, 25, 50 and 100 μM). Each value represents the mean±S.D. of three experiments performed in duplicate. ◆ Significant versus control untreated cells ($P<.01$).

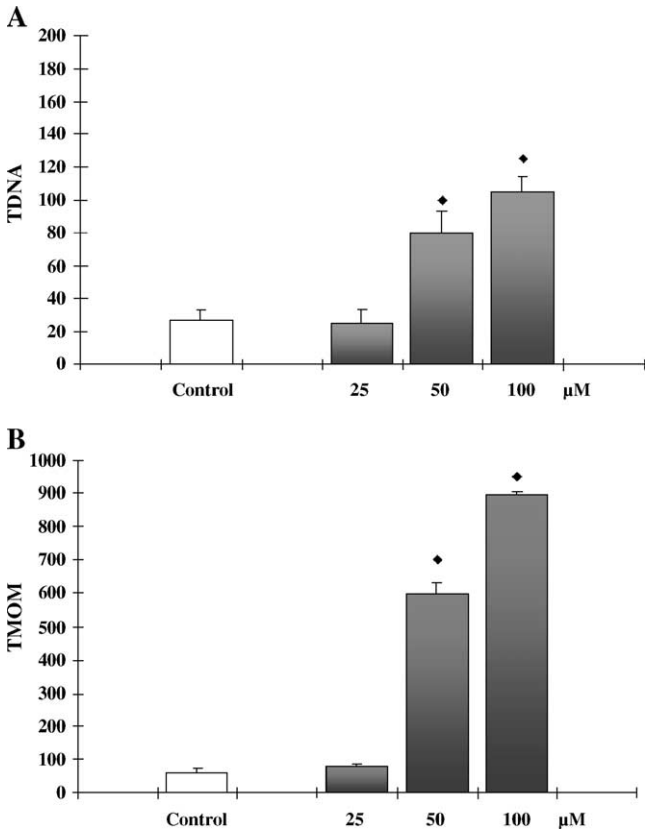

Fig. 4. COMET assay of genomic DNA of human M14 cells, untreated and treated with genistin for 72 h. (A) TDNA values, (B) TMOM values. The values are the mean±S.D. of three experiments performed in duplicate. ◆ Significant versus control untreated cells ($P<.01$).

The results of ROS formation are presented in Fig. 3. Genistin induced a significant and dose-dependent increase in ROS formation when compared with the untreated control.

DNA damage was analyzed using COMET assay, a sensitive method for detecting DNA strand breaks in a single cell and a versatile tool that is highly efficacious in human biomonitoring of natural compounds [21]. The results of TDNA and TMOM (Fig. 4), representing the percentage of the fragmented DNA and the product of TD (distance between head and tail) and TDNA, respectively, clearly evidence a dose dependent DNA damage to cells exposed to genistin for 72 h, in particular, at the highest concentration.

4. Discussion

The incidence of human skin cancers has significantly increased in the past 20 years and continues to increase at an alarming annual rate of 4% [8]. Therefore, the development of safe and effective preventive agents against photocarcinogenesis has become an important subject in dermatological research. Melanoma and nonmelanoma skin cancers are among the most prevalent cancers in the human population. Skin provides a protective barrier against environmental

insults and is the primary target for UV radiation effects [22]. Ultraviolet radiation is considered the major etiological factor in skin cancer. It has been suggested that the depletion of stratospheric ozone and human recreational behavior resulting in intensive sun exposure may further increase these rates. Solar UV consists of UVC (200–280 nm), UVB (280–320 nm), and UVA (320–400 nm). Recent evidence has identified the involvement of hydrogen peroxide in the response to physiological doses of UVB [22]. The antioxidant system of the skin battles reactive oxygen species and helps prevent UV-induced oxidative skin damage. Cutaneous damage, premature aging of the skin, and skin cancer ensue when UV exposure exceeds the protective capacity of the antioxidant system [23]. Supporting this cutaneous defense system with topical or oral antioxidants may provide a successful strategy for the prevention and treatment of skin cancer. Chemoprevention by means of phytochemicals has been the focus of many studies within the last decade. AICR and the World Cancer Research Fund advise that five or more servings of fruit and vegetables be consumed daily to reduce the risk of certain cancers [24]. The beneficial effects of fruits and vegetables for both healthy people and cancer survivors have sometimes been associated with the presence of various antioxidant micronutrients. Comprehensive reviews have detailed the findings supporting the role of green tea polyphenolic antioxidants as chemopreventive agents [25,26]. In mice, topical vitamin E has been shown to confer photoprotection by the inhibition of UV-induced thymine dimer formation, absorption of UVB radiation, and prevention of UV-induced immunosuppression [27–29]. Studies indicate that genistein potently inhibits UVB-induced skin carcinogenesis and photodamage in animals [8]. From our studies in vitro, it is possible to hypothesize that genistin could also be used in preventing skin damage; in fact, this isoflavone exhibits protection against DNA strand scission, induced by ·OH radicals, generated from UV photolysis of H_2O_2, suppressing the formation of linDNA. In addition, both genistin and daidzin exhibited a SOD-like effect, inhibiting the superoxide anion formation in a dose-dependent manner.

The study on human tumor cells clearly demonstrates that genistin was able to reduce significantly the vitality of M14 cells after 3 days of exposure, without an increase in cellular membrane breakage, as evaluated by percentage of LDH release, even at the highest dosage of 100 μM. On the contrary, daidzin, the chemical structure of which differs by only one hydroxyl group, was ineffective, confirming the importance of the 5,7-dihydroxy structure in the A ring of isoflavones. In fact, several data evidence that the number of hydroxyl groups strongly affects the biological activities of isoflavones. Genistein and daidzein inhibit tumor cell growth via different mechanisms. For example, genistein arrested cells in G2, while daidzein induced an accumulation of cells in G1 and exhibited inhibitory effect on cell proliferation at higher concentration [30]. It has been demonstrated in vitro that daidzein, due to its lipophilicity related to the lacking of the hydroxyl group in position 5, interacts with membrane phospholipids, and this could be in part the cause of a higher concentration of daidzein next to the membrane surface [31].

Higher amounts of ROS are produced in some cancer cells [32] that overactivate the mitogen-activated protein kinase (MAPK) signaling pathway resulting in constant activation of redox-sensitive transcription factors including nuclear factor-kappa B (NF-kB) and AP-1. Studies suggest that phenolic phytochemicals can scavenge the constitutively high amounts of ROS in cancer cells, thereby blocking MAPK signaling, activation of NF-kB and AP-1, and ultimately, the expression of responsive genes that stimulate cancer cell proliferation, thereby depriving them of an essential molecule needed for their existence [33]. On the other hand, phenolic phytochemicals can, paradoxically, induce the formation of ROS to achieve an intolerable level of high oxidative stress in cancer cells [33]. Epigallocatechin gallate, quercetin, and gallic acid each generated H_2O_2 in a time- and concentration-dependent manner when added to cell culture media [34]. We found similar results in M14 cells after genistin treatment. In fact, this isoflavone determined a significant increase in the intracellular oxidants and DNA strand breaks, as clearly evidenced by the values of TDNA and TMOM. On the other hand, a well-documented feature of human melanoma cells is that their antioxidant capacity is altered when compared with normal melanocytes; in fact, melanoma cells show decreased catalase activity [35]. In addition, it was shown that melanocytes and melanoma cells respond differentially to exogenous peroxides: melanoma cells were unable to suppress a peroxide stress and generated a prooxidant response [36].

5. Conclusion

In conclusion, this study, which can be considered as the starting point for further investigation, demonstrates that as reported for other glycoside flavonoids [10–12], genistin in its intact form may play a key role in the beneficial effects of soy. Our results suggest that due to its antioxidant and anticarcinogenic properties, the isoflavone genistin, like genistein, could have promising applications in the field of dermatology.

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