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In vitro anti-melanoma effect of polyphenolic compounds

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ABSTRACT

Objective: To evaluate the effects of phenolic acids (caffeic, ferulic, and coumaric acids) and flavones (luteolin and apigenin) on the proliferation and melanogenesis in murine melanoma B16-F10 cells.

Methods: Cell proliferation was determined after 24 and 48 hours of incubation using MTT assay. The effects of these tested compounds on cell cycle progression were analyzed by flow cytometry. Moreover, the melanin content and tyrosinase activity were measured spectrophotometrically at 475 nm.

Results: Luteolin and apigenin exhibited significant anti-proliferative activity against B16-F10 cells, while caffeic, ferulic, and coumaric acids induced slight inhibition after 24 and 48 hours of incubation. The tested compounds disturbed cell cycle progression of B16-F10, by a subsequent decrease in G_1 and arrested cycle progression in either G_1/S or G_2/M phase. Furthermore, apigenin provoked an increase in melanin content of B16-F10 cells. In contrast, luteolin, caffeic, ferulic and coumaric acids induced a decrease in melanin content of B16-F10 cells by inhibiting tyrosinase activity.

Conclusions: These active polyphenols may be used as skin whitening agents or natural tanning agents to treat skin pigmentation disorders.

KEYWORDS: Polyphenols; Cytotoxic activity; Cell cycle; Melanin content; Tyrosinase activity; Melanoma

1. Introduction

Melanogenesis is a process that produces melanin by cells called melanocytes. It leads to pigmentation in the skin, eyes, hair, nasal cavity, and inner ear and protects the hypodermis, the layer under the skin, from damage by UV radiation[1]. Disorders of hyperpigmentation, including solar lentigines, infections, and, allergic reactions, occur widely and gain interest among scientists and dermatologists[2]. Otherwise, hypopigmentation leading to discoloration or complete lack of pigmentation of underlying tissue represents either a serious problem. Hence, regarding the nature of pigmentation disorders, people are usually looking forward to uniform skin color.

Several compounds that interfere with this biosynthetic pathway have been identified as pigmentation modulators for cosmetic purposes, such as skin whitening or pro-pigmenting agents[3]. However, few substances are used in commercial skin products due to their carcinogenic potential. Many side effects, such as dermatitis and irritation are caused by agents used to treat skin hyperpigmentation[4].

Significance

Our study demonstrated the potential use of tested compounds as antiproliferative agents against B16-F10 melanoma cells and as modulator agents of melanogenesis for cosmetic purposes. These compounds also showed a lower cytotoxic effect on normal primary human keratinocytes. Nevertheless, further studies are needed to verify the molecular mechanism of these compounds in modulation of skin pigmentation under clinical or physiological conditions.

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Tyrosinase is a crucial enzyme involved in melanin biosynthesis. Indeed, melanogenesis entails the hydroxylation of *L*-tyrosine to 3,4-dihydroxy-*L*-phenylalanine (*L*-DOPA)[5], which is then oxidized to dopaquinone by tyrosinase. In fact, the treatment of pigmentation disorders is based on the application of tyrosinase inhibitors[6] and should improve the efficacy of melanoma therapy. Melanoma is certainly an issue that cannot be ignored and on which our efforts need to be focused due to its aggressive metastatic potential[7].

Polyphenols, potent bio-actives, and low toxic substances are recently used to treat pigmentary disorders. Thus, previous studies reported that many natural compounds such as flavonoids and phenolic acids which represent one of the most omnipresent groups of plant phenolics inhibit or enhance melanin biosynthesis[8] and have multiple biological potentials like antiproliferative, anticancer, antioxidant, and antimelanogenic activities. They may play a part in chemotherapeutic treatments and prevent many human diseases[9].

In the present study, we investigated the effects of flavones (luteolin and apigenin) and phenolic acids (caffeic, coumaric, and ferulic acids) on cell proliferation and cell cycle progression in a murine sarcoma cell line (B16-F10). In addition, we evaluated the effect of each compound on tyrosinase activity and melanin production.

2. Materials and methods

2.1. Chemicals and reagents

Flavones and phenolic acids were purchased from Extrasynthese (Genay, France). Trypsin, penicillin, streptomycin, vitamins, sodium pyruvate, RPMI-1640 medium, non-essential amino acids, and fetal bovine serum were purchased from Sigma Cell Culture (Courtaboeuf, France). Moreover, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) was purchased from Euromedex (Mundolsheim, France). Triton X-100 was purchased from Biomatik Corporation (Cambridge, UK), *L*-DOPA and ribonuclease A (RNase) were purchased from Sigma Aldrich (St. Louis, USA) and dimethyl sulfoxide was procured from Sigma-Aldrich (St. Quentin Fallavier, France). Ethylenediaminetetraacetic acid (EDTA; Honeywell Burdick and Jackson, Germany), tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl; Biobasic, Canada), phosphate-buffered saline (PBS; Gibco by Life Technology, France) and sodium hydroxide (NaOH; Applichem, Germany) were bought. Propidium iodide was purchased from Sigma-Aldrich (Steinheim, Germany). Kojic acid was obtained from Fluka.

2.2. Culture of B16–F10 and primary human keratinocyte (PHK) cells

B16-F10 murine melanoma cells were cultured in RPMI supplemented with 10% fetal bovine serum, 1% non-essential amino acids (100×), 1% *L*-glutamine (200 mM), 1% vitamins (100×), 1% penicillin (10 000 U/mL), streptomycin (10 000 μg/mL) and 1% sodium pyruvate (100 mM).

PHK cells were cultured in a CnT-07BM basal medium. Cells were incubated at 37 ℃ in a humidified atmosphere with 5% CO_2 and the medium was renewed every day.

2.3. Antiproliferative assay of B16–F10 and PHK cells

The antiproliferative activity of flavones and phenolic acids on B16-F10 melanoma and PHK cells was evaluated by MTT assay, which is used to measure cellular metabolic activity as an indicator of cell viability. Cells (5×10^3 cells/well) were seeded in 96-well plates and incubated overnight at 37 ℃. Then, 100 μL of various concentrations of the tested compound were added to cells. After incubation for 24 h and 48 h, cells were washed and treated with MTT (2 mg/mL). Two hours later, the formazan blue formed in the cells was dissolved by adding dimethyl sulfoxide. The absorbance was measured at 570 nm on a microplate reader (Thermo Scientific). The concentration of 50% cell inhibition (IC_{50}) was calculated from the graph of inhibition percentage against different molecule concentrations.

2.4. Cell cycle progression of B16–F10 cells

Murine B16-F10 cells (5×10^5 cells) were incubated for 24 h and then treated with different concentrations of compounds for 48 h. After incubation for 30 min at room temperature with ribonuclease A (10 mg/mL) and staining for 10 min with 50 mL propidium iodide (1 mg/mL), cell cycle analysis was conducted using a flow cytometry system (Beckman Coulter, Switzerland). Percentages of cells in each phase of the cell cycle were determined.

2.5. Melanin content assay of B16–F10 cells

Melanin content was determined, as described by Lee *et al*[10]. Briefly, B16-F10 cells (10^5 cells/well) were incubated for 24 h at 37 ℃ and 5% CO_2. Then, different concentrations of flavones (10, 25, and 50 μM) and phenolic acids (500, 800, and 1 000 μM) were added to cells for 48 h. After treatment, cells were trypsinized and solubilized in tubes containing 1 mL of Triton X100 (0.1%). The intracellular melanin content was measured by spectrophotometric absorbance at 475 nm.

2.6. Tyrosinase assay of B16–F10 cells

The tyrosinase activity was assessed by measuring the rate of *L*-DOPA oxidation[11]. B16-F10 cells were treated with flavones (50 μM) and phenolic acids (1 000 μM) for 48 h, and 10^6 cells were solubilized with Triton X100 (0.1%). After centrifugation at 12 000 rpm for 15 min at 4 ℃, the supernatant was mixed with *L*-DOPA (0.15%). The tyrosinase activity was determined spectrophotometrically at 475 nm, every minute for 10 min, after the

addition of *L*-DOPA. Kojic acid (1 000 μM) was used as a positive control.

2.7. Statistical analysis

The results are presented as mean±standard deviation (SD) and all experiments were carried out in triplicate. Statistical comparisons among groups were analyzed using one-way and two-way analysis of variance (ANOVA), followed by Dunnett's test, using GraphPrism software 6.01. Statistical significance was considered at *P*-value<0.05.

3. Results

3.1. Cell viability assay

Since the main purpose of our study was to explore natural and safe pigmentation modulators, the cytotoxicity effect of the studied compounds against PHK cells was tested. Furthermore, the cytotoxic effect on B16-F10, a well-known human melanoma cell line, was determined. As shown in Figure 1, the proliferation of B16-F10 cells was inhibited by flavones and phenolic acids in a time- and

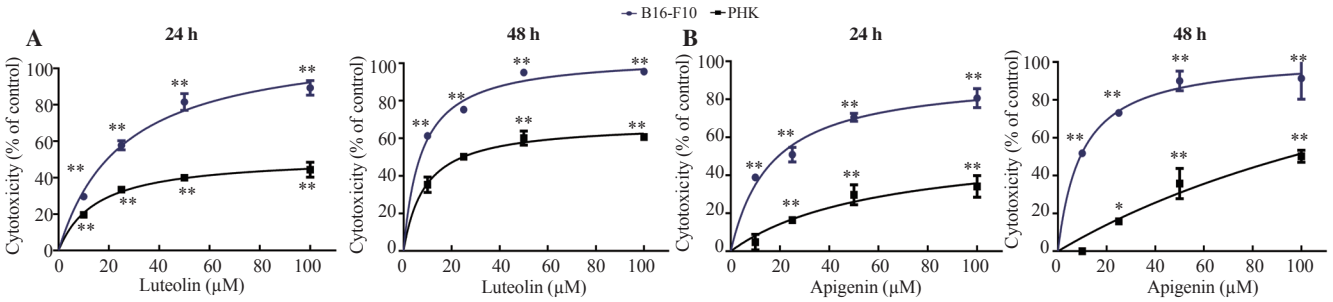

Figure 1. Inhibitory effect of luteolin (A) and apigenin (B) on B16-F10 and primary human keratinocyte (PHK) cells after 24 h and 48 h of incubation. The cell viability percentage was assessed by MTT assay. Results are expressed as mean±SD of three independent experiments and analyzed by Dunnett's test. $^{*}P<0.05$ and $^{**}P<0.01$ compared with the control group.

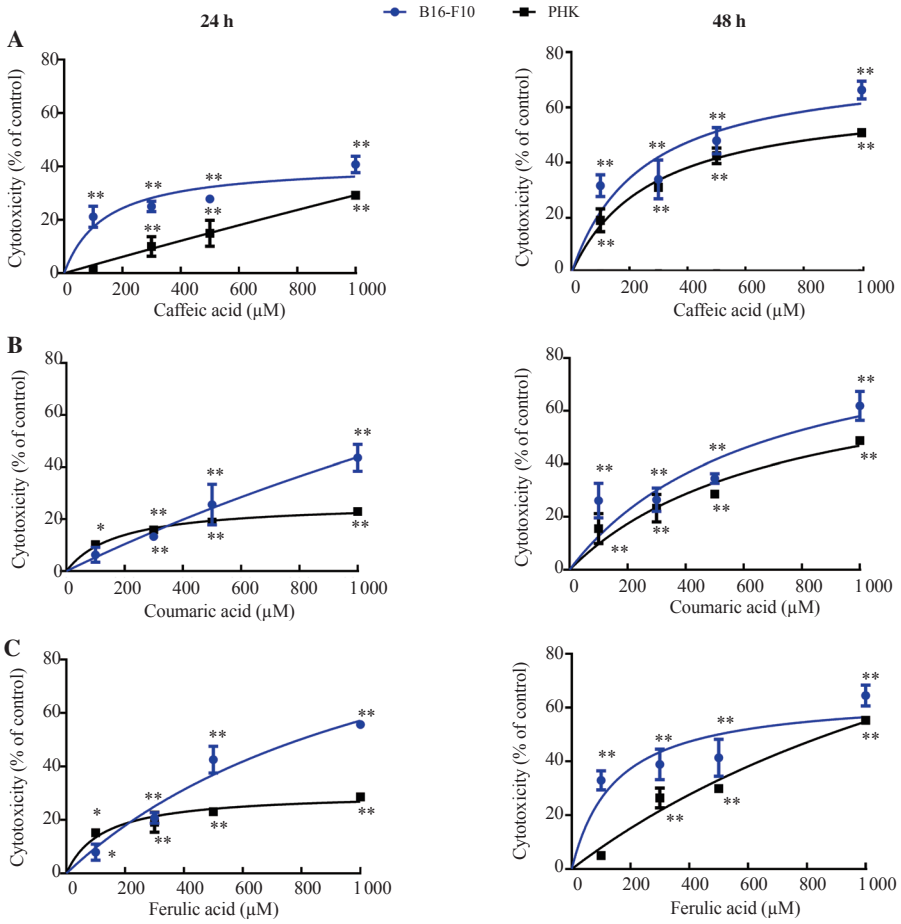

Figure 2. Inhibitory effect of caffeic acid (A), coumaric acid (B), and ferulic acid (C) on B16-F10 and PHK cells after 24 h and 48 h of incubation. Growth inhibition was assessed by MTT assay. Results are expressed as mean±SD of three independent experiments and analyzed by Dunnett's test. $^{*}P<0.05$ and $^{**}P<0.01$ compared with the control group.

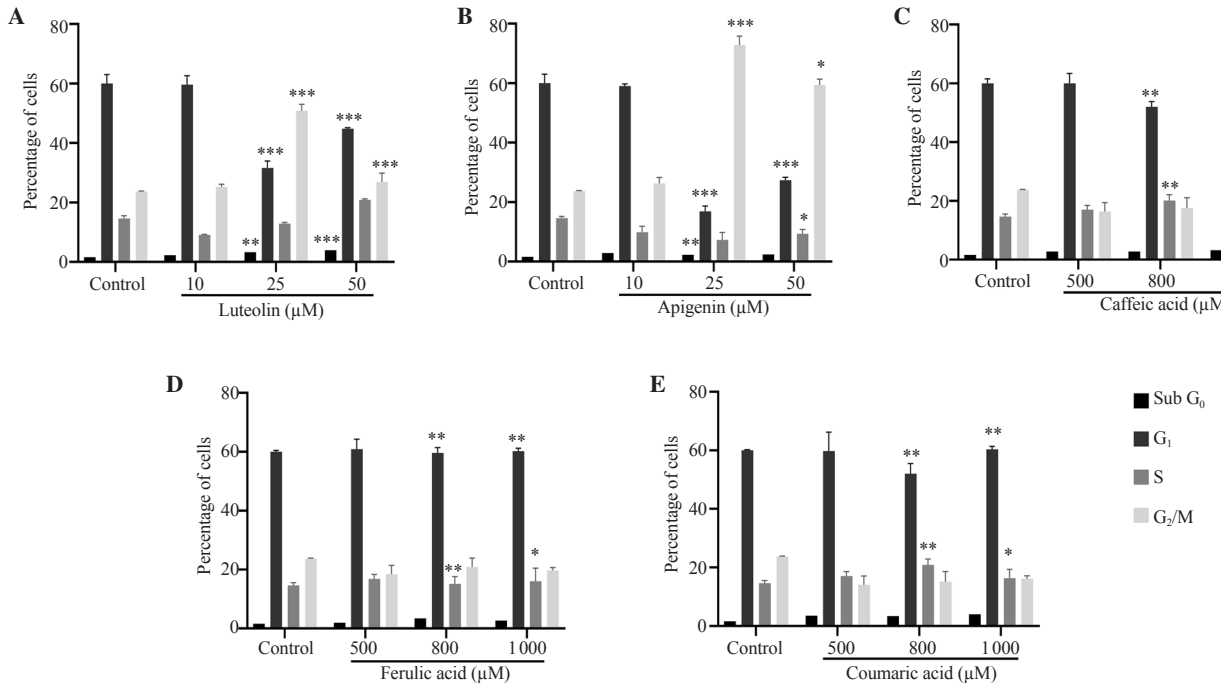

Figure 3. Cell cycle progression of B16-F10 cells after 48 h incubation with flavones (A: luteolin; B: apigenin) and phenolic acids (C: caffeic acid; D: ferulic acid; E: coumaric acid). Values represent mean±SD of three independent experiments and analyzed by Dunnett's test. $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$ compared with the control group.

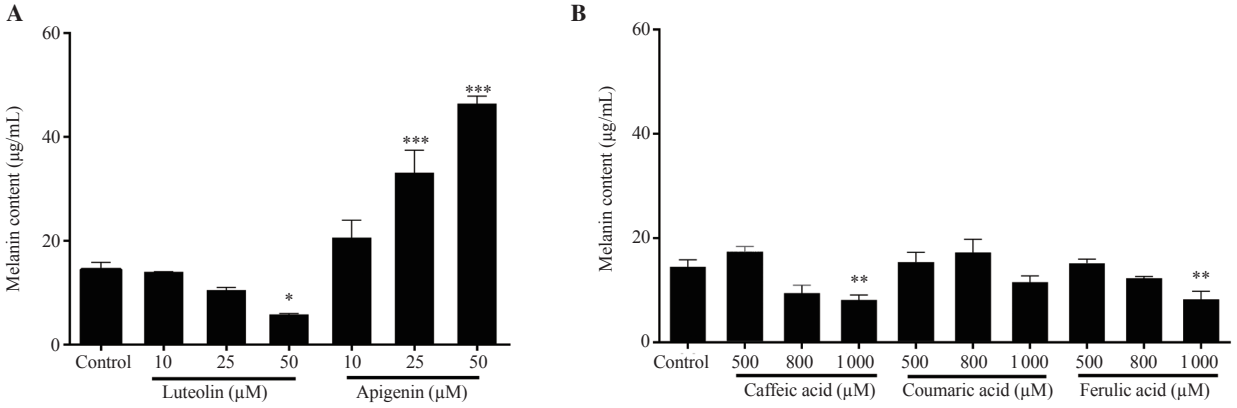

Figure 4. Effect of flavones (A) and phenolic acids (B) on melanin content in B16-F10 cells after 48 h of incubation. Values represent mean±SD of three independent experiments and analyzed by Dunnett's test. $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$ compared with the control group.

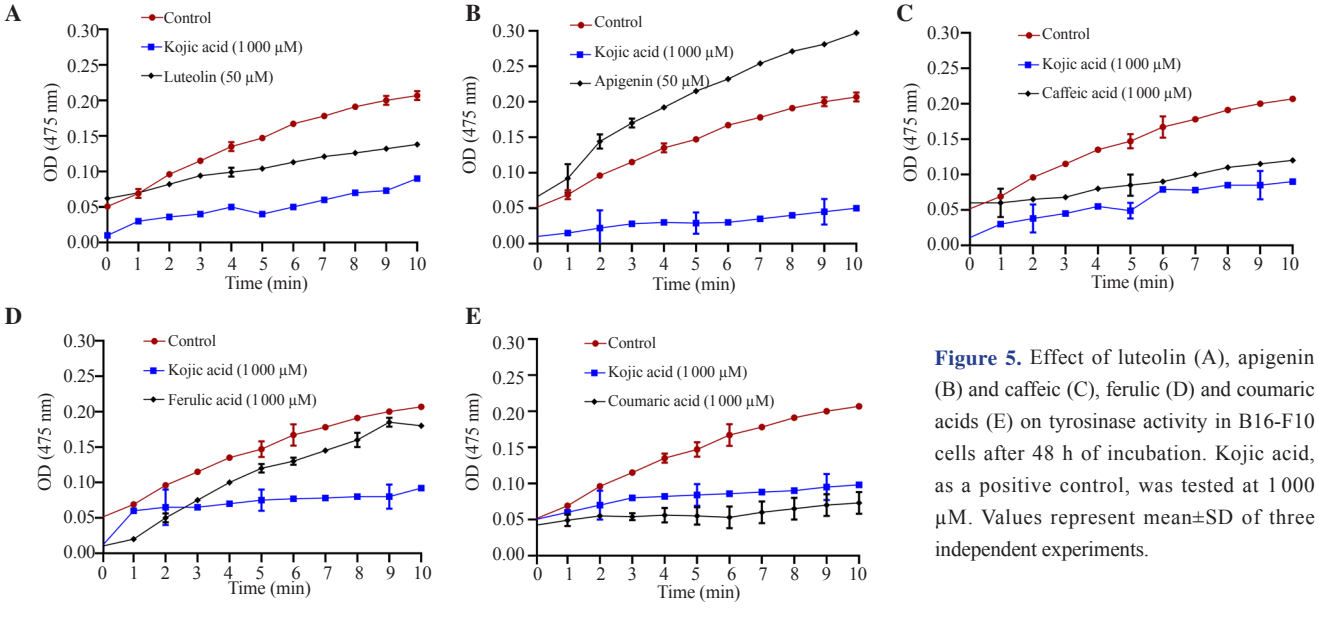

Figure 5. Effect of luteolin (A), apigenin (B) and caffeic (C), ferulic (D) and coumaric acids (E) on tyrosinase activity in B16-F10 cells after 48 h of incubation. Kojic acid, as a positive control, was tested at 1 000 μM. Values represent mean±SD of three independent experiments.

concentration-dependent manner. The IC_{50} values for B16-F10 cells receiving luteolin and apigenin treatment for 24 h were 22 μM and 25 μM, respectively. After 48 h of treatment, the IC_{50} values were 4 μM and 5 μM, respectively.

Phenolic acids were less effective against melanoma cells. Indeed, B16-F10 cells exposed to higher concentrations ranging from 100 to 1 000 μM of compounds after 24 h of treatment showed a higher viability rate compared to flavones. The IC_{50} value was 800 μM for ferulic acid and exceeded 1 000 μM in the presence of caffeic and coumaric acids. Nevertheless, the IC_{50} values were around 500 μM, 780 μM, and 550 μM for caffeic, coumaric and ferulic acids, respectively after 48 h of treatment. As expected, the tested compound showed lower cytotoxic effects on PHK cells compared to B16-F10 cells (Figure 2).

3.2. Cell cycle analysis

The percentage of cells in the G_1 phase markedly decreased after exposure to 25 and 50 μM of luteolin and apigenin (Figure 3). Moreover, we observed a marked increase in the G_2/M phase after exposure to different concentrations of flavones (25 μM and 50 μM) (P<0.05). However, caffeic, ferulic, and coumaric acids induced G_2/M phase arrest. Flavones were found more efficient than phenolic acids to regulate the distribution of melanoma cells in different phases of the cell cycle (Figure 3).

3.3. Effect of flavones and phenolic acids on melanogenesis

To explore the effect of phenolic compounds on melanin production, B16-F10 cells were treated with different concentrations of the compounds for 48 h, and then melanin contents were determined (Figure 4). A significant decrease was found in the production of intracellular melanin when cells were treated with the highest concentration of luteolin, caffeic, and ferulic acids compared to the untreated cells (P<0.05). In contrast, apigenin stimulated significantly melanin production in a dose-dependent manner (P<0.05).

The tyrosinase activity was also measured in cells treated with the tested compounds. Since the melanogenic activity showed a dose-dependent change, we chose to examine the effect of the compounds at the highest concentrations (50 μM for flavones and 1 000 μM for phenolic acids) on tyrosinase activity. According to the results, apigenin increased tyrosinase activity while luteolin and caffeic, coumaric, and ferulic acids decreased tyrosinase activity (Figure 5). Interestingly, the inhibitory effect of coumaric acid on tyrosinase activity was more significant than that of the positive control kojic acid.

4. Discussion

All organisms exist in different colors which arise from the unique distribution of pigments throughout the body. Pigmentation is regulated by genetic, environmental, endocrine factors, and natural plant products that modulate the amount, type, and distribution of melanins in the skin, hair, and eyes[11,12]. Therefore, in response to the growing interest in natural compounds, new approaches have been under improvement for several years to search for safe compounds, thus enabling the skin to better withstand treatment[13].

Despite various therapeutic studies on flavonoids and phenolic acids (chemotherapeutic efficacy, free radical scavengers, *etc*)[14], there is scarce information about their effects on the melanogenesis process. The present study highlights the ability of flavones and phenolic acids to act as anticancer agents against a melanoma cell line and as modulatory agents of melanogenesis.

Before the investigation of melanogenesis, herein, we tested the cytotoxicity of flavonoids and phenolic acids against PHK cells. In comparison with B16-F10, significantly lower toxicity was observed in PHK cells suggesting that natural compound selectively targets tumor cells rather than normal cells. The higher inhibitory effect affecting cytotoxic and/or antiproliferative activities obtained with luteolin and apigenin may be attributed to the different factors involving the saturation and the position of the C_2-C_3 bond as well as the number and substitution of hydroxyl groups in the A and B rings[15,16]. Indeed, significant variations in biological tested activity may be attributed to the modifications of their chemical structures.

Programmed cell death is a highly regulated process used to eliminate damaged and cancerous cells. Dysregulation of cell cycle progression is one of the triggering aspects of apoptosis[17]. Substances that can disturb cell-cycle progression, leading to cell cycle arrest may represent a good option to prevent and treat cancer[18]. Thus, considerable interest has been attributed to the effect of polyphenols on arresting cell cycle progression[19].

In the present study, we assessed the ability of both flavones and three phenolic acids to disturb the cell cycle in melanoma cells. Natural products can disturb cell cycle progression by arresting cell division at either G_1/S or G_2/M checkpoints in a dose-dependent manner.

In line with the present finding, several authors demonstrated that flavones-mediated inhibitory effects on cell proliferation were in part attributed to cell cycle arrest at the G_2/M phase[20]. Moreover, Zhao *et al*.[20] showed that apigenin suppressed melanoma cells (A375 and C8161 cells) by arresting cells in the G_2/M phase and inducing apoptosis.

Furthermore, George *et al*.[21] demonstrated that luteolin inhibits the proliferation of HaCaT and human melanoma A375 cells by disturbing cell cycle and promoting apoptosis. Kilinc *et al*.[22] demonstrated that ferulic and *p*-coumaric acids reduced the percentages of G_1-phase cells and increased the percentages of S and G_2/M phases in Caco-2 cells.

Moreover, our results on cell cycle progression of B16-F10 cells showed a modulatory effect of caffeic acid on cell cycle distribution. In fact, Pelinson *et al*.[23] reported that this compound altered the cell cycle of human melanoma SK-Mel-28 cells.

A recent study showed that *Origanum vulgare* L. hydroalcoholic extract inhibited melanogenesis and melanoma cell proliferation. The oregano extract obtained by chromatographic methods confirmed the presence of caffeic acid, *p*-coumaric acid, and apigenin[24]. Moreover, another study revealed that rosemary extract enhanced the cytotoxic effect on ovarian cancer cell lines (RA2780) by disturbing the cell cycle[25].

Our finding follows a previous study that phenolic substances having the hydroxyl group, methoxy, or polyphenols with ortho- or para-dihydroxyl groups, or phenols containing condensed rings have a significant apoptotic effect and they also reported the order of effectiveness of caffeic > ferulic > *p*-coumaric acids[26,27].

Epidermal melanocytes are involved in skin pigmentation by the regulation of melanin synthesis and ensuing transfer of the pigment to keratinocytes. The murine B16-F10 cells are used as a melanogenic cell model to evaluate the effect of tested flavones and phenolic acids on melanogenesis.

In B16-F10 cells, luteolin repressed the synthesis of melanin in a dose-dependent manner. However, cells exposed to the highest concentration of apigenin produced an amount of melanin nearly three times greater than control (untreated cells). Comparing the structures of apigenin and luteolin, the only extra hydroxyl group in luteolin may be responsible for the observed inhibition of melanin synthesis. Similar to others[28], an increase in melanogenesis activity in B16 cells was detected in cells treated with plant extracts from mango-steen, *Erica multiflora*, *Pyrostegia venusta*, and *Daphne gnidium*.

The assessment of melanin amount in B16-F10 exposed to different phenolic acids revealed that caffeic, coumaric, and ferulic acids inhibit melanogenesis at the highest concentration. However, conflicting data are found in the literature in regard to the effect of ferulic acid on melanogenesis. Some works revealed a stimulatory effect while others exhibited an inhibitory effect[29].

To understand the mechanism underlying the melanogenesis modulation by flavones and phenolic acids, we further evaluated the tyrosinase activity. In this study, we observed that luteolin, caffeic, coumaric, and ferulic acids inhibited tyrosinase activity in B16-F10 cells, as revealed by the enzyme kinetic curve. In this respect, many works reported that methoxylation in ferulic acid replaced with hydroxylation in caffeic acid is a substantially more effective activity. However, ferulic acid is estimated to be more active than *p*-coumaric acid, since the electron-donating methoxy group led to increased stabilization of the aryloxy radical through electron delocalization after hydrogen donation by the hydroxyl group[27].

Recent studies showed that caffeic and *p*-coumaric acids reduced the mushroom tyrosinase activity more effectively than kojic acid[30,31]. Besides, many reports found that the level of tyrosinase protein in cells decreased when they were treated with *Pistacia atlantica* subsp. *mutica* extract, which contains quercetin, luteolin, isoquercetin, rutin, and other polyphenols[32].

Based on the results, it can be concluded that flavones and phenolic acids exert a significant cytotoxic effect against melanoma cells with significant induction of cell cycle arrest. Interestingly, luteolin, and caffeic, coumaric, and ferulic acids could increase tyrosinase inhibitory activity and could be a potential therapeutic agent for skin disease treatment such as skin cancers. However, further studies are needed to elucidate the mechanisms of action of the tested compounds at the molecular and cellular levels and to explore the *in vivo* anti-melanoma potential.

Conflict of interest statement

The authors declare that they have no conflict of interest.

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Authors' contributions

FS performed the experiments and analyzed the data; MM and IMB helped in flow cytometry analysis; LCG and SKJ supervised the overall project.

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