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Flavones and Phenolic Acids as Potential Inhibitor of B16F10 Melanoma Cells Growth and Modulator of Melanogenesis

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Keywords:

Flavones; Phenolic acids; Antiproliferative activity; Melanogenesis; Cell cycle arrest

1. Abstract

Pigmentation disorders leading to discoloration or hyperpigmentary of underlying tissue occur widely and gain interest. This study is within the framework of strategies under-developing safe compounds to modulate melanognonesis pathway. Phenolic acids and flavones were tested for their effects, on the viability in murine melanoma (B16-F10) and in primary human keratinocyte (PHK). Flavones, luteolin and apigenin exhibited significant anti-proliferative activity against cell skin cancer B16-F10, while phenolic acids, caffeic, ferulic and coumaric acids, induced slight inhibition. Tested compounds were found to disturb cell cycle progression of B16-F10, by a subsequent decrease of post-mitotic phase G1 and arrest cycle progression at either G_1/S or G_2/M. We further examined and evaluated the effects of flavonoids and phenolic acids on melanogenesis in the melanogenic cells model, murine B16F10 cells. While, apigenin and ferulic acid revealed an ability to enhance melanogenesis melanoma cells; luteolin, caffeic and coumaric acids decreased the melanin contents of the cells. To understand the mechanism underlying the melanogenesis modulation, we further evaluated the tyrosinase activity using L-DOPA as tyrosinase substrate. This study underlines the potential use of tested compounds as therapeutic agents in the treatments of human melanoma and as modulator of melnogenesis for cosmetic purpose.

2. Introduction

Melanogenesis is a crucial biosynthetic pathway responsible for determining eye, hair, and skin color and protecting hence, the body from serious damage caused by ultraviolet (UV) radiation [1]. Hyperpigmentary disorders, including solar *lentigines*, melasma and, postinflammatory hyperpigmentation, 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. Some agents used to treat skin hyperpigmentation, like hydroquinone, can cause side effects such as dermatitis and skin irritation (Maeda and Fukuda 1996).

Tyrosinase is a crucial enzyme involved in melanin biosynthesis. Indeed, the melanogenesis entail the hydroxylation of L-tyrosine to L-3,4-dihydroxyphenylalanine (L-DOPA) [4], then the oxidation of L-DOPA to L-DOPA quinine through the enzymatic reaction of tyrosinase. Tyrosinase inhibitors are considered as options for

the treatment of some dermatological issues associated with pigmentation disorders [5]. The inhibition of melanogenesis should obviously 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 its aggressive metastatic potential [6].

Polyphenols, potent bio-actives and low toxic substances, are recently used to treat pigmentary disorders. Many inhibitors and enhancers of melanin biosynthesis have been described. Kojic acid, for example, is a well-known tyrosinase inhibitor produced by *Aspergillus* and *Penicillium fungi* [7]. Some polyphenols, nevertheless, have been described as melanin inducer.

Flavonoids and phenolic acids represent one of the most pervasive groups of plants phenolics. Acting as reducing agents, free radical scavengers, and quenchers of singlet oxygen formation, flavonoids and phenolic acids components may play important roles in chemotherapeutic treatments and other human diseases [8].

In the present study, we investigated whether flavones, luteolin and apigenin, and phenolic acids, caffeic, coumaric and ferulic acids, were able to inhibit cell proliferation and cell cycle progression in murine sarcoma cell line (B16F10), without compromising the viability of primary human keratinocytes (PHK) cells. In the continuing search for effective melanogenesis modulators from natural sources, we evaluated the effect of each of compounds on melanin content and tyrosinase activity. In addition, effects of all tested compounds on progression through the cell cycle were analyzed by flowcytometry.

3. Material and Methods

3.1. 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). 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), 3,4-dihydroxy-L-phenylalanine (L-DOPA) and ribonuclease A (RNase) were purchased from Sigma Aldrich (St. Louis, USA) and dimethyl sulfoxide (DMSO) 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). Propidium iodide (PI) was purchased from Sigma-Aldrich (Steinheim, Germany).

3.2. Cells and Culture Conditions

B16-F10 mouse melanoma cells were cultured in RPMI supplemented with 10% heat-inactivated fetal bovine serum, 1% non-essential amino acids (100×), 1% L-glutamine (200 mM), 1% vitamins (100×), 1% penicillin (10,000 Uml^{-1}), streptomycin (10,000 μgml^{-1}) and 1% sodium pyruvate (100 mM). Cells were grown at 37°C in a humidified atmosphere containing 5% CO_2.

Primary human keratinocytes (PHK) were isolated as primary cells from the human foreskin. Keratinocytes were isolated from the epidermis using overnight 4 °C dispase/trypsin digestion (Joly-Tonetti et al., 2013). Isolated keratinocytes were grown in a CnT-07BM.1 basal medium (CELL N TEC). Incubation was performed at 37°C in a humidified atmosphere with 5% CO_2. The medium was renewed every day. For all experiments, cells were seeded at passage numbers 1–3 and treated upon reaching 60–80% confluence.

3.3. Cell Viability Assay

The effect of flavones and phenolic acids (Figure 1) on the viability of B16-F10 melanoma and PHK cells was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyltetrazoliumbromide (MTT) assay, which is based on the cleavage of a tetrazolium salt by mitochondrial dehydrogenase in viable cells. Cells were seeded in 96-well microtitration plates at a concentration of 5×10^3 cells/well, and incubated overnight at 37°C. Twenty-four hours after seeding, cells were treated with 100 µl of various concentrations of the tested compound, before incubating the plates for 24 h and 48 h at 37 °C. Cells were washed once before adding 50 µl of 2 mg/ml MTT. After 2 h of incubation at 37 °C, the medium was discarded, and the formazan blue formed in the cells was dissolved by adding 100 µl DMSO. Negative control without the tested compound but with DMSO was prepared in the same manner. Optical density (OD) was measured at 570 nm on a microplate reader (Thermo Scientific). The IC_{50} value (the concentration of 50% cell inhibition) was calculated from the graph of inhibition percentage against different molecules concentrations.

3.4. Determination of Melanin Content

Melanin released by cells was measured, as described previously [9]. Briefly, B16-F10 cells (10^5 cells/ well) were seeded into a 25 cm^2 culture dish with 5 ml of culture medium, and incubated for 24 h at 37 °C, 5% CO_2. Then, cells were treated with flavones (10, 25 and 50 µM) and phenolic acids (500, 800 and 1000 µM) for 48 h. After treatment, melanogenesis activity (closely related to the amount of produced melanin) was estimated from the amount of melanin retained in cells (intracellular melanin). Adherent cells were detached by incubation in trypsin–EDTA 0.05%; 10^6 cells were solubilized in tubes containing 1 ml of Triton X100 (0.1%). Spectrophotometric absorbance of intracellular melanin content was measured at 475 nm. Absorbance was compared against a standard curve of known concentrations of synthetic melanin, and amounts of melanin were estimated.

3.5. Tyrosinase Activity

Tyrosinase enzyme activity was estimated by measuring the rate of 3, 4-dihydroxy-L-phenylalanine (L-DOPA) oxidation, as de-

scribed previously by [10], with slight modifications. Briefly, B16-F10 cells were treated with flavones (50 µM) and phenolic acids (1000 µM) for 48 h, and 10^6 of viable cells were then solubilized in phosphate buffer (0.1 M; pH 6.8) containing 0.1% Triton X100. Lysate was clarified by centrifugation at 12,000 rpm for 20 min at 4°C; 400 µl of supernatant was mixed with 400 µl of L-DOPA (0.15%), and absorbance was measured spectrophotometrically at 475 nm, every minute for 10 min, after the addition of the substrate (L-DOPA).

Fig 1

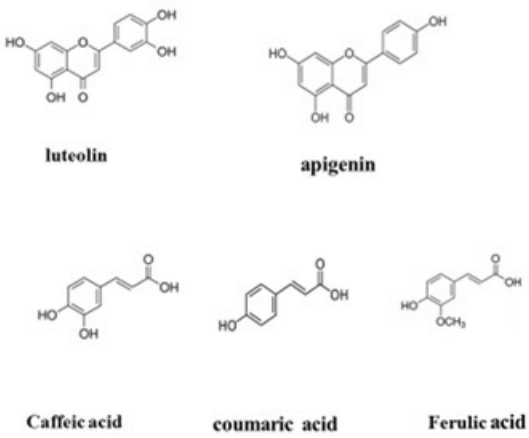

Figure 1: The chemical structure of luteolin, apigenin, caffeic acid, p-coumaric acid and ferulic acid.

3.6. Cell Cycle Analysis Using Flow Cytometry

Mouse melanoma cells (B16F10 5 × 10^5 cells) were seeded into a 50 cm^2 culture dish and incubated for 24 h. Cells were treated with different concentrations of compounds for 48 h, trypsinized and washed twice in PBS (pH = 7.4). Cells were harvested and incubated for 15 min at room temperature and washed twice in cold PBS (pH = 7.4). After treatment with Ribonuclease A (10 mg ml^{-1}) for 30 min at room temperature and staining with 50 ml propidium iodide (1 mg ml^{-1}) for 10 min, cell cycle analysis was conducted using FACS system (Beckman Coulter, Switzerland). Percentages of cells in each phase of the cell cycle were calculated.

3.7.Statistical Analysis

All tests were carried out in triplicate and results were presented as mean ± SD (Standard deviation). Statistical comparisons among groups were analyzed using one-way and two-way analysis of variance (ANOVA), followed by Tukey's multiple comparison test, using GraphPrism software. Statistical significance was considered for p-value < 0.05

4. Results

4.1. Cell Viability Assay

Since the main purpose of the present study was to investigate natural and safe pigmentation modulators, the cytotoxicity effect of studied molecules was tested against primary human keratinocytes, PHK. Furthermore, we have evaluated their cytotoxic effects on a well-known human melanoma cell line, B16F10. As revealed by cytotoxic curves, the proliferation of B16F10 cells was inhibited by flavones and phenolic acid in a time- and concentration-dependent manner. Indeed, the inhibitory concentration of 50 percent of tumoral cells (IC_{50}) was 22 µM for luteolin and 25 µM for apigenin, respectively (Figure 2 and 3). Very interestingly, the same treatments carried out on primary human keratinocytes cells show a slight cytotoxic effect.

Fig 2

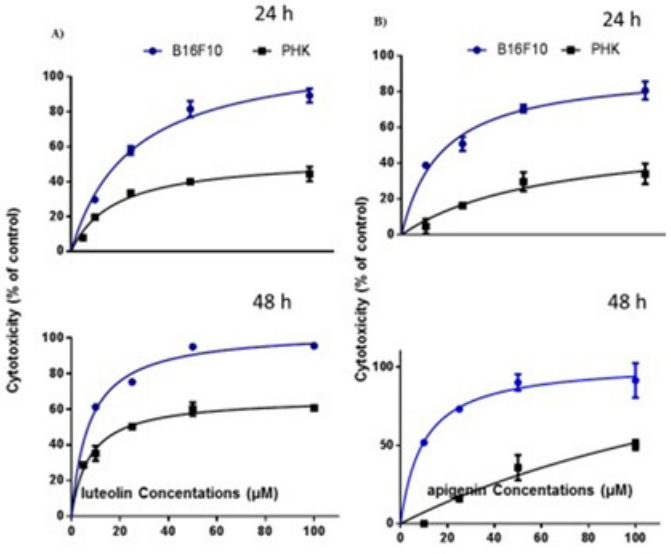

Figure 2: Inhibitory effect of flavones on B16-F10 melanoma cells and primary human keratinocytes (PHK). After treatment of B16-F10 and PHK with increasing concentrations (0-100 µg/mL) for 24 and 48 h of luteolin **(A)** and apigenin **(B)**, the percentage of cell viability was determined by MTT assay. Results are expressed as mean percentage of control growth ± SD of three independent experiments

Phenolic acid seemed to be less effective against melanoma cells. Indeed, cells exposed with higher concentrations ranging from 100 to 1000 µM of caffeic, coumaric and ferulic acid showed higher viability rate. Interestingly, treatment with the same doses induced no significant toxicity on PHK cells, except for the highest tested concentration of 1000 µM and after 48 hour of direct exposure to the agents (Figure 3).

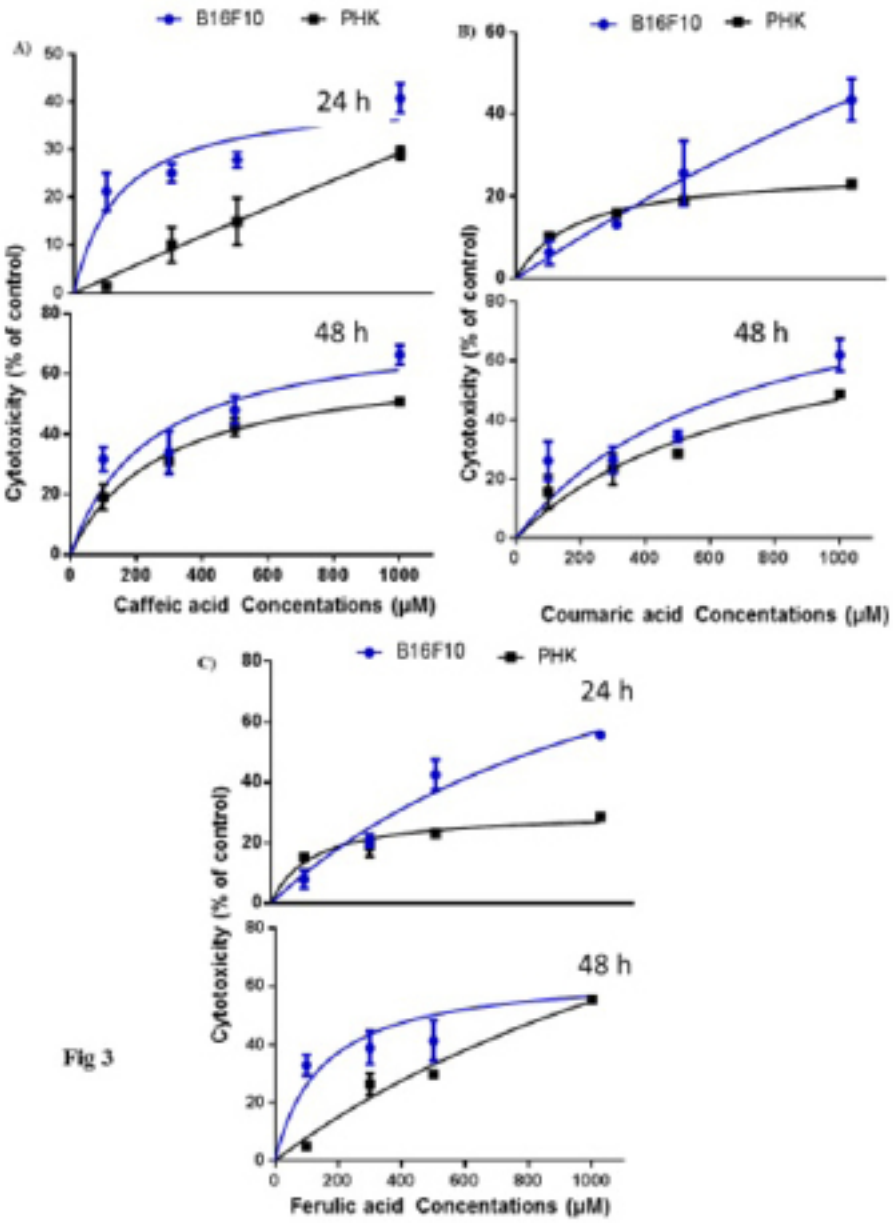

Fig 3

Figure 3: Inhibitory effect of phenolic acids on B16-F10 melanoma cells and primary human keratinocytes (PHK). Growth inhibition of B16-F10 and PHK cells were determined by crystal violet assay after 24 and 48 h of exposure to increasing concentrations (0-1000 µg/mL) of caffeic acid **(A)**, coumaric acid **(B)** and ferulic acid **(C).** Results are expressed as mean percentage of control growth ± SD of three independent experiments

4.2. Cell Cycle Analysis

Studies have previously shown that various natural compounds (i.e. resveratrol, xanthohumol) affect cell viability by disturbing cell cycle progression. Melanoma cells were exposed for 48 h to the different compounds. The results reveal that the percentage of cells in the post-mitotic phase G1 increased after exposure to different concentrations of caffeic, coumaric and ferulic acids; preventing cells from enterings phase (Figure 4). Indeed, we observed a marked increase of cells percentage in the S and G2/M phases after exposure to different concentrations of flavones and phenolic acids. flavones were found more efficient than phenolic acids, to cause repercussions on the distribution of melanoma cells in G1-the different phases of the cell cycle (Figure 4)

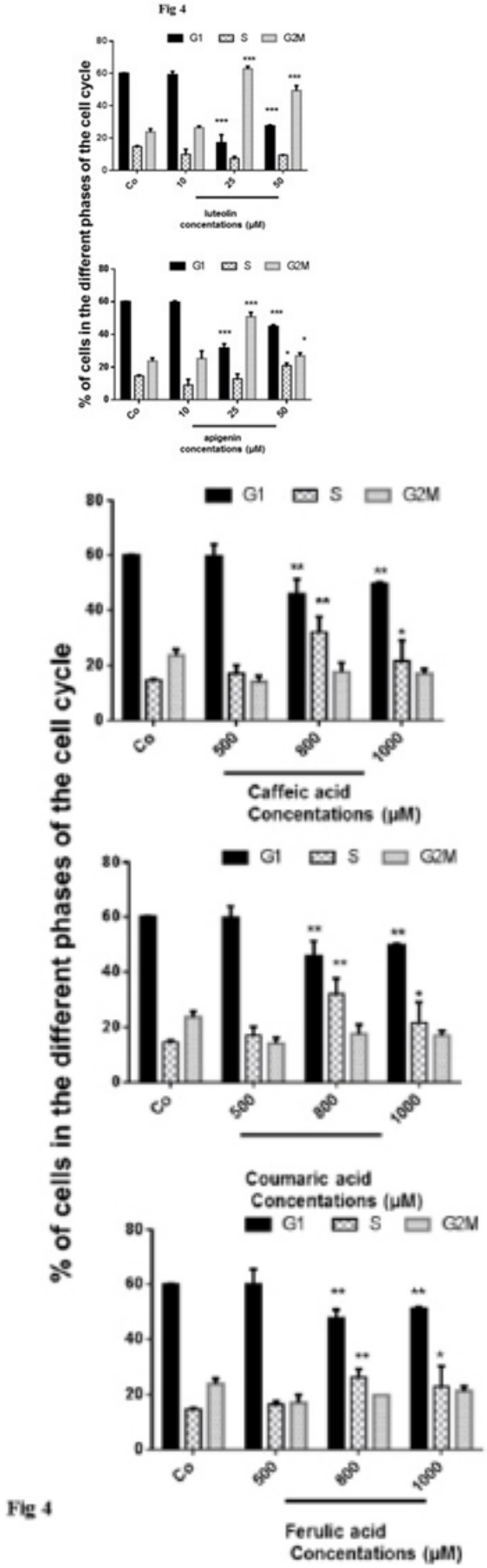

Figure 4: Cell cycle distribution of B16F10 cells after treatment with luteolin, apigenin caffeic acid, ferulic acid, and coumric acid. Values represent the mean ± SD of three separate experiments

4.3. Effect of Flavones and Phenolic Acids on Melanin Synthesis and Tyrosinase Activity

To investigate the effect of phenolic compounds on melanin synthesis, B16-F10 melanoma cells were exposed to different concentrations of the molecules for 48 h and then melanin contents were measured (Figure 5). Our experiment indicates that luteolin, caffeic and coumaric acids and ferulic acids significantly decreased the production of intracellular melanin in the compared to the rate of melanin synthesis in untreated cells. Whereas apigenin and ferulic acid stimulated significantly the production of intracellular melanin in a dose dependent manner when compared to the untreated cells.

As far as melanin synthesis pathway involved a rate-limiting regulatory melanogenic enzyme (Hearing, 1999), which is the tyrosinase, we attempted the assessing of the aforementioned protein in cells incubated with different doses of the tested molecules. Since the finding of melanogenic activity showed a doses depending relation activity- dose, we chose to test the highest concentrations (50 µM for flavones and 1000 µM for phenolic acids) to examine tyrosinase activity. Our results revealed that apigenin increased tyrosinase activity in a dose and time dependent manner, whereas luteolin, caffeic, coumaric and ferulic acids decreased tyrosinase activity (Figure 6).

Fig 5

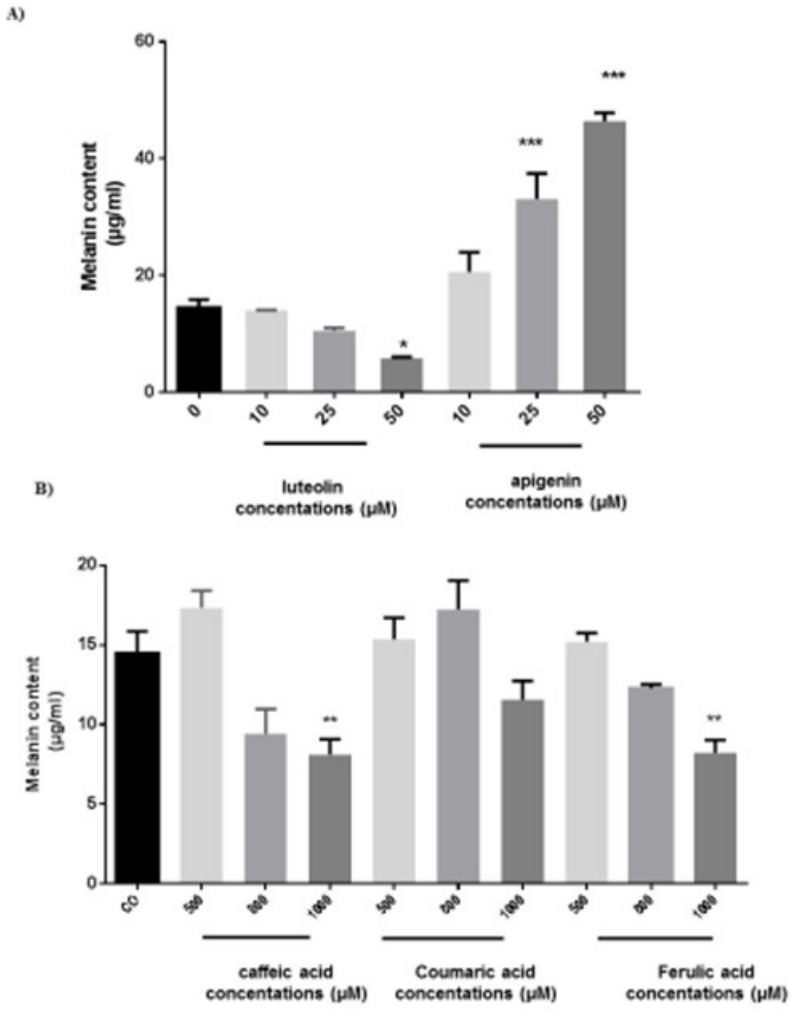

Figure 5: Effect of flavones **(A)** and phenolic acids **(B)** on melanin content in B16F10 cells after 48 h incubation. Values represent the mean ± SD of three separate experiments. Data are expressed as mean ± SEM of three independent experiments. *p* values were determined by a student est. $*p < 0.05$ and $***p < 0.001$.

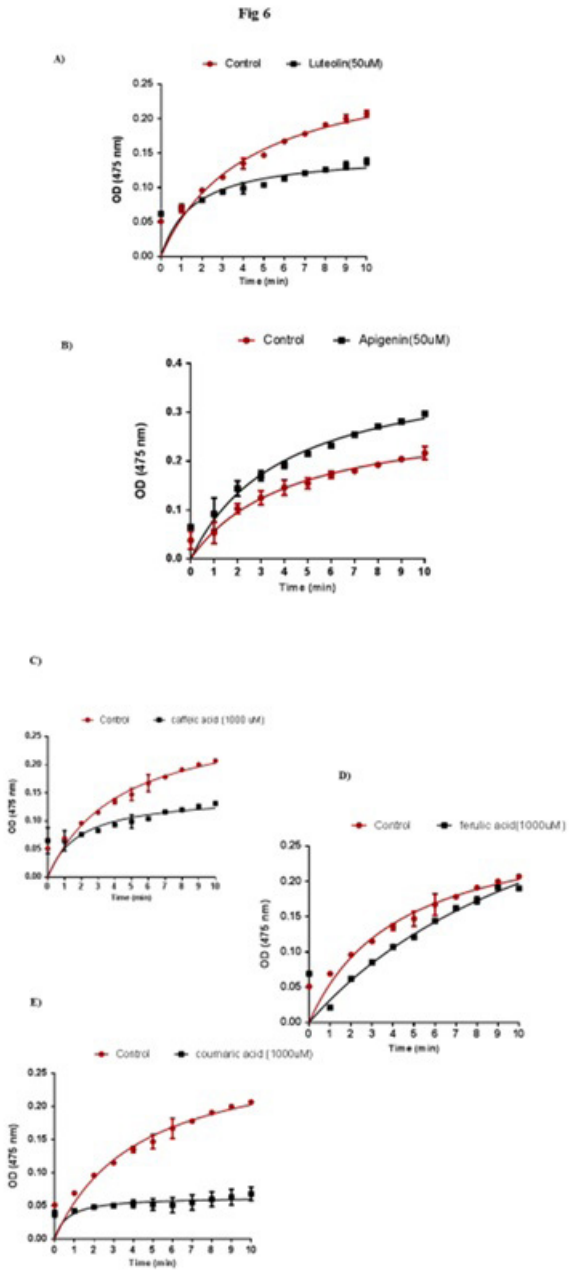

Figure 6: Effect of luteolin **(A)** and apigenin **(B)** caffeic acid **(C)**, ferulic acid **(D)**, and coumric acid **(E)** on tyrosinase activity in B16F10 cells after 48 h of incubation. Values represent the mean ± SD of three separate experiments

5. Discussion

Although the main function of melanogenesis is to protect against UV radiation, melanin pigment can also interfere with epidermal homeostasis and may entail a melanoma [11]. Numerous options have been shown as potent modulators of melanogenesis, however, their use in the cosmetics industry has been hampered by its toxicity and its side effects [12].

Therefore, in response to the growing interest in natural compounds, new strategies have been under development for several years aimed at searching safe compounds, thus enabling the skin to better withstand treatment [13].

In spite of various therapeutic approaches of flavonoids and phenolic acids (chemotherapeutic efficacy, free radical scavengers...) [14], there are a few information about its effects on melanogenesis process. The present study highlights the ability of flavones, luteolin and apigenin, and phenolic acids including caffeic, coumaric, and ferulic acids, to act as a chemopreventive against a melanoma cell line and as modulators of melanogenesis.

Prior to the investigation of melanogenesis, herein, we tested for the first time the cytoxic of above-mentioned molecules against primary human keratinocytes PHK. In comparison with B16F10, a significantly lower toxicity rate was observed in primary cells suggesting that natural compound selectively targets tumor cells rather than normal cells. The higher inhibitory effect obtained with luteolin and apigenin, may be attributed to the number of hydroxyl groups in the A and B rings [15].

Different factors affecting cytotoxic and/or antiproliferative activities of polyphenols involve 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 [16]. Indeed, any modifications in a molecule chemical structure can be responsible for significant variations in their activity.

One of the major incidents required for any cancer development known as a hallmark of malignant cells is deregulation of the cell cycle [17]. Substance that can disturb cell-cycle progression and lead to cell-growth arrest may represent a good option for cancer prevention and therapy strategies [17]. Thus, a considerable attention has been paid to the ability of polyphenols to inhibit cell-cycle progression [18].

In the current study, we evaluated the ability of both flavones and phenolic acids to disturb cell cycle in melanoma cells. Natural compounds have been found to arrest cell-cycle progression at either G_1/S or G_2/M boundaries in a dose dependent manner.

In line with the present finding, several authors demonstrated that flavonoids-mediated inhibitory effects of cell proliferation were accompanied by cell cycle arrest at G2/M phase

the ability of flavones to cause cell cycle arrest at G_2/M phase [19]. In fact, this study showed that treatment of breast cancer cells with flavonoids resulted in increase in percentage of cells at G2/M phase and decrease in percentage of cells at G1 and S phase in a dose-dependent manner. Moreover, another study conducted by Zhao et al., 2017 showed that apigenin suppressed of melanoma cells (A375and C8161 cells) by inducing G2/M phase arrest and apopotosis.

Furthermore, George et al., 2013 demonstrated that luteolin inhibits the proliferation of HACAT and human melanoma cells A375 and promotes cell cycle arrest and apoptosis with possible involvement of programmed cell death.

Besides [20] demonstrated that ferulic and *p*-coumaric acid decreased the proportion of cells in the G_1 phase and increased the

proportion of cells in the S and G_2 phases in Caco-2 cells. They concluded that *p*-coumaric and ferulic acids inhibited cell proliferation by presumably affecting different cell cycle phases.

Furthermore, our results on cell cycle progression of B16F10 cells are in agreement with recent studies showing cell cycle modulation of caffeic acid in human melanoma SK-Mel-28. In fact, treatment with caffeic acid increased the population of cells at G0/G1 phase, reduced the population of cells at S phase also reduced G2/M phase in these cells (Pelinson et al., 2019)

Our finding is in accordance with previous studies on the effects of hydroxycinnamates on the induction period of autoxidizing fats [21]. They also demonstrated the order of effectiveness caffeic > ferulic > p-coumaric acid. Phenolic substances are the most effective antioxidants al hydroxyl group, usual methoxy, or polyphenols with ortho- or para-dihydroxylic groups, or phenols containing condensed rings, for example, anthocyanins [16].

Epidermal melanocytes are involved in skin pigmentation by the regulation of melanin synthesis and ensuing transfer of the pigment to keratinocytes. The murine *B16F10* melanoma cells are used as melanogenic cells *model to evaluate the effect of tested flavones and phenolic acids on* melanogenesis. Of importance is that although some flavones have similar structures, they show opposite effects on melanogenisis regulation. Luteolin markedly inhibited melanin synthesis in B16F10 cells in a dose-dependent. However, cells exposed to the highest dose of apigenin produced an amount of melanin nearly three times greater than control (untreated cells). In comparing the structures of apigenin and luteolin (Figure 1), there is only one extra hydroxyl group in luteolin. It is postulated that this extra hydroxyl group in luteolin palyed an important role in determing some specificities of this molecule.

The assessment of melanin amount in B16 F10 exposed to different phenolic acids showed that caffeic acid, p-coumaric acid and ferulic acid inhibits the melanogenesisis in a dose dependent manner. *Conflicting data* are found in literature in regards to the effect of ferulic acid on melanogenesis. Some studies showed stimulatory effect [22] and others showed an inhibitory effect [23].

Tyrosinase plays a critical regulatory role in the biosynthetic pathway of melanin pigments, since it catalyzes the oxidation of monophenols, o-diphenols, and o-quinones [24]. To understand the mechanism underlying the melanogenesis modulation by flavonoid and phenolic acids, we further evaluated the tyrosinase activity using L-DOPA as tyrosinase substrate. We demonstrated that luteolin, caffeic acid, coumaric acid and ferulic acid decreased tyrosinase activity in B16F10 cells, as revealed by the enzyme kinetic curve. Similarly, in correlation with the anti-melanogenesis activity, apigenin were found to increase tyrosinase acivity.

This result may be explained that the concentrations of flavonoids required for the inhibition of tyrosinase enzyme reaction *in vitro* were too high, compared with those inhibiting melanogenesis in cells [25].

Regarding phenolic acids, methoxylation of the hydroxyl group in the ortho position of the diphenolics, as in ferulic acid, results in a decrease in the scavenging reaction, for instance, hydroxylation as in caffeic acid in place of methoxylation is substantially more effective. Ferulic acid is, indeed, expected to be more effective than p-coumaric acid, since the electron-donating methoxy group let to increased stabilization of the aryloxyl radical through electron delocalization after hydrogen donation by the hydroxyl group [26].

6. Conclusion

On the basis of the findings, Flavones and phenolic acids have a potential to be used as potent therapeutic agents in the treatments of human melanoma and as modulator of melanogenesis for cosmetic purpose. We also reported that phenolic acids, caffeic, ferulic and coumaric acids exerts a significant cytotoxic effect against melanoma cells with significant induction of cell cycle arrest. However same doses of same agents tested on normal skin cells showed no or low toxicity level.

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