https://doi.org/10.31925/farmacia.2025.2.21 **ORIGINAL ARTICLE**

MYRICETIN-FISETIN COMBINATORIAL TREATMENT AS A POTENTIAL STRATEGY FOR CUTANEOUS MELANOMA – AN *IN VITRO* STUDY

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Manuscript received: February 2025

Abstract

Cutaneous melanoma (CM) is a global health challenge with an increasing incidence risk. Conventional treatments include chemotherapy, specific surgeries, radiotherapy or immunotherapy, but in recent decades it has been observed that these treatment options display several limitations (*e.g.* toxic effects or treatment resistance). Botanical compounds and, in particular, the class of flavonoids are versatile agents with manifold biological activities recognised in the medico-pharmaceutical domain, among which myricetin and fisetin are acknowledged, two phytocompounds that have proven their potential in the treatment of CM through their antiproliferative and pro-apoptotic effects. Given these aspects, the present work aims to explore MYR and FIS and their combinatorial treatment in the CM cell line B164a5 and healthy JB6 Cl 41-5a cells. The results revealed that the two compounds possessed promising activity in inhibiting B164a5 cells, and the combination of MYR+FIS exerted superior effects compared to individual treatment of the agents by decreasing cell viability, inducing cell dysmorphology, reducing cell number, producing apoptotic-like nuclei and mitochondrial damage. Furthermore, the results suggested that the association between the two compounds was selective, avoiding damage to normal epidermal cells.

Rezumat

Melanomul cutanat (CM) reprezintă o provocare pentru sănătatea mondială, cu un risc de incidență în creștere. Tratamentele convenționale includ chimioterapia, operații chirurgicale specifice, radioterapia sau imunoterapia, dar în ultimele decenii s-a observat că aceste opțiuni de tratament prezintă mai multe limitări (de exemplu, efecte toxice sau rezistență la tratament). Compușii botanici și în special clasa flavonoidelor sunt agenți versatili, cu multiple activități biologice recunoscute în domeniul medico-farmaceutic, printre care sunt miricetina (MYR) și fisetina (FIS), doi fitocompuși care și-au dovedit potențialul în tratamentul CM prin efectele lor antiproliferative și pro-apoptotice. Având în vedere aceste aspecte, lucrarea de față își propune să exploreze MYR și FIS și tratamentul lor combinatorial în linia celulară de CM B164a5 și asupra celulelor sănătoase JB6 Cl 41-5a. Rezultatele au arătat că cei doi compuși posedă o activitate promițătoare în inhibarea celulelor B164a5, iar combinația MYR+FIS a exercitat efecte superioare comparativ cu tratamentul individual al agenților prin scăderea viabilității celulare, inducerea dismorfologiei celulare, reducerea numărului de celule, producerea de nuclee de tip apoptotic și deteriorarea mitocondrială. În plus, rezultatele au sugerat că asocierea dintre cei doi compuși a fost selectivă, evitând deteriorarea celulelor epidermiale normale.

Keywords: myricetin, fisetin, combinatorial treatment, cutaneous melanoma, epidermal cells

Introduction

Malignant melanomas are produced by malignant transformation of melanocytes, which are melanin pigment-producing cells originating from the neural crest. The activity and behaviour of melanocytes are governed by a multitude of factors, including ultraviolet radiation. Among the most popular types of melanomas is cutaneous melanoma (CM), which affects a large sector of the population and holds an alarming incidence and mortality in comparison to other types of cancers [29]. Due to the global spread in recent decades, it has been estimated that up to 331,722 new cases may be diagnosed annually. Furthermore, epidemiologic studies have shown impressive variations in the geographic and population incidence of cutaneous melanoma, and therefore, among the highest incidence rates have been found to be in Australia and New

Zealand [22]. Surgeries, chemotherapy and immunotherapy are all procedures used to treat patients, and new approaches have progressed in recent years. However, researchers are looking for new therapeutic options that offer promising results and prolong the lives of oncologic patients. In this regard, natural plant-derived compounds have become an opportunity due to their properties, developing into a significant source of oncological drugs and offering novel potential anticancer candidates [20]. In addition, the need for new therapies is also driven by resistance to treatment and the side effects associated with conventional treatments [6]. Flavonoids are botanical components that have acquired recognition for their versatile pharmacological properties, including anticancer, antioxidant, anti-inflammatory, antiviral, neuroprotective and cardioprotective properties, and they are also commonly used in skin care and cosmetics [34]. Myricetin (MYR) and fisetin (FIS) originate from the flavonol subclass and are acknowledged for their antitumor potential, even in CM [2, 26, 30]. MYR is a compound found in many natural plants, such as bayberry, and exerts many biological functions that underpin the development of future perspectives on its use. The therapeutic effects of MYR have been observed in various tumours, atherosclerosis, inflammatory diseases, diabetes, cerebral ischemia or microbial pathogenic infections [31]. FIS is a natural compound found in numerous fruits and vegetables (*e.g.* cucumbers, onions, apples). It possesses impressive biological properties (antioxidant, antiproliferative, pro-apoptotic) that outline its potential in the medical-pharmaceutical sphere. It has also been observed that FIS inhibits the MAPK and NF-kB signalling pathways in various cancer cells [26]. Additionally, an important attribute of FIS is its photoprotective potential, one study demonstrating that FIS 5 - 25 μM inhibited the expression of cyclooxygenase-2 (COX-2) and matrix metalloproteinase (MMP)-1, MMP-3, MMP-9 caused by ultraviolet B (UVB) irradiation in human skin fibroblasts and, moreover, FIS suppressed UVB-induced collagen degradation [5]. MYR has also been highlighted as a potent candidate for the prevention of UV-induced skin aging, and recently it was reported that this natural compound suppresses UVB-induced skin cancer by targeting Fyn [17].

The present work aims to explore *in vitro* 2 natural compounds belonging to the flavonoid class (MYR and FIS) and their combinatorial treatment as potential candidates for the alternative treatment of CM using the 2D cell line B164a5 as an experimental model, as well as their influence on normal epidermal cells using JB6 Cl 41-5a cells.

Materials and Methods

Reagents and instruments

Myricetin, fisetin, the phosphate-buffered saline (PBS) and the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) viability kit were provided by Sigma-Aldrich, Merck KGaA (Darmstadt, Germany). The Dulbecco's Modified Eagle Medium (DMEM), Eagle Minimum Essential Medium (EMEM) and dimethyl sulfoxide – DMSO (solvent) were obtained from PAN-Biotech GmbH, Aidenbach, Germany; foetal bovine serum (FBS- 30-2020™), the penicillin/streptomycin mixture and trypsin-EDTA solution were purchased from American Type Culture Collection (ATCC) Manassas, VA, USA; while Hoechst 33342 dye and MitoTracker™ Red CMXRos from Thermo Fisher Scientific (Waltham, MA, USA). The devices used, Cytation 5 (plate reader) and Lionheart FX (automated microscope), were provided by BioTek Instruments Inc. (Winooski, VT, USA).

Cell Culture Protocol

The experiments were performed using two different cell lines: B16 melanoma 4A5 (CVCL_4612; CLS Cell Lines Service) up to passage 10 and JB6 Cl 41-5a neonatal BALB/c epidermal cells (CRL-2010™; ATCC, Manassas, VA, USA) up to passage 17. Both cell lines were cultured in the specific growth medium (DMEM for B16 melanoma 4A5 cells and EMEM for JB6 Cl 41-5a cells) supplemented with 10% FBS (for DMEM), 5% FBS (for EMEM) and 1% antibiotic mixture (100 U/mL penicillin/100 μg/mL streptomycin). The cells were maintained in a humidified incubator, under standard conditions of 37°C and 5% CO_2. The cell morphology was also microscopically examined every day during the experiments. Myricetin and fisetin were dissolved to form the stock solution in DMSO. During the experiments, the concentration of DMSO did not exceed 0.5%.

Cell viability assay via MTT method

The cell viability was analysed using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) method to evaluate the impact of MYR, FIS and the association between MYR and FIS (20, 30 and 40 μM) on B164a5 and JB6 Cl 41-5a cell lines for a period of 24 h. The cells were cultured in 96-well plates at 1×10^4 cells/well density and treated with the compounds of interest when the desired confluence was reached. At the end of the treatment (24 h), 100 μL of fresh medium and 10 μL of MTT were added to each well, and the plates were introduced into the incubator. Next, after 3 hours of incubation, 100 μL of MTT solubilising solution was added and the plates were maintained for 30 minutes at room temperature. The absorbance was read at 570 and 630 nm wavelengths as a final step using the Cytation 5 device.

Bright-field cell morphology evaluation

For this analysis, the cells were cultured in 96-well plates at 1×10^4 cells/well density and treated with the compounds of interest when the desired confluence was reached. The impact of the test samples MYR, FIS and the association between MYR and FIS (20,

30 and 40 μM) on B164a5 and JB6 Cl 41-5a cell morphologies was investigated by taking representative photos of the control and treated cells under brightfield illumination (at magnification 20×) using the Lionheart FX automated microscope. Next, the obtained pictures were processed in the Gen5™ Microplate Data Collection and Analysis Software (Version 3.14) from BioTek Instruments Inc. (Winooski, VT, USA).

Cell number evaluation

Initially, the cells were cultured in 96-well plates at 1 x 10^4 cells/well density and stimulated when they achieved the desired confluence. To evaluate the influence of MYR, FIS and the combinatorial treatment between MYR and FIS (20, 30 and 40 μM) on the cell number of the B164a5 cell line, the cells were captured after 24 hours of treatment at 4x magnification using the Lionheart FX automated microscope. After this, the images were analysed using the Cell Analysis Tool included in the Gen5™ Microplate Data Collection and Analysis Software (Version 3.14) using the corresponding protocol indicated by the manufacturer.

Nuclear immunofluorescence staining using Hoechst 33342

Hoechst 33342 staining was performed to visualize the modifications induced by MYR, FIS and the association between the two (MYR+FIS) on the nuclear shape of B164a5 cells. In short, the cells were cultured in 12-well plates (1 x 10^5 cells/well) and allowed to attach. When the desired confluence was reached, they were treated with the samples of interest at the highest concentration tested, *i.e.* 40 μM of MYR, FIS and the combinations between MYR and FIS. Following the applied 24 h treatment, Hoechst staining solution (1:2000 dilution in PBS) was prepared, the culture medium was removed from the plates, the prepared staining solution was added, and the plates were then incubated in the dark for 5 - 10 min. Subsequently, the staining solution was removed, and the cells were washed three times with PBS. Image processing and analysis were conducted using the Lionheart FX automated microscope (at magnification 20x) and Gen5™ Microplate Data Collection and Analysis Software (version 3.14) from BioTek Instruments Inc. (Winooski, VT, USA).

Mitochondrial immunofluorescence staining using MitoTracker™ Red CMXRos

The aspect of mitochondria in B164a5 CM cells was examined through the immunofluorescence staining via MitoTracker™ Red CMXRos. In short, the cells were cultured in 12-well plates (1 x 10^5 cells/well) and allowed to attach, and when the desired confluence was reached, they were treated with the samples of interest at the highest concentration tested, *i.e.* 40 μM of MYR, FIS and the combination of MYR and FIS. MitoTracker™ Red CMXRos was dissolved in DMSO to a final concentration of 1 mM and then diluted in the specific culture media (DMEM) to a working concentration of 300 nM. Live B164a5 CM cells were incubated at 37ºC and 5% CO_2 with the prepared solution for 30 - 45 minutes and rinsed with PBS (3 times 1000 μl/ well). Image processing and analysis were conducted using the Lionheart FX automated microscope (at magnification 20x) and Gen5™ Microplate Data Collection and Analysis Software (version 3.14) from BioTek Instruments Inc. (Winooski, VT, USA).

Statistical analysis

The results were statistically analysed using GraphPad Prism software (GraphPad Software, San Diego, CA, USA, www.graphpad.com), Version 9.3.1. The differences between the MYR, FIS, MYR+FIS treated groups and control were determined using One-way ANOVA and Dunnett's multiple comparison tests. All statistically significant results were marked using "*", as follows: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

Results and Discussion

CM is a malignancy arising from melanocytes of the skin, with the incidence growing in recent decades. Managing CM in advanced stages is complex, especially for patients who have developed brain metastases. For these reasons, multidisciplinary support is crucial for the well-being of patients in these stages [21]. Currently, treatment approaches include surgical resection, chemotherapies, radiotherapy and immunotherapies, depending on the tumour's stage, location and genetics. However, the present treatment options have an emerging decreasing success rate due to treatment resistance, toxic effects on the organism and the lack of survival benefit. There are also limitations for targeted therapy, which can produce satisfactory therapeutic effects in a short period of time. Still, in the same context, tumours can develop resistance to treatment rapidly (in about a few months) [3]. Recently, findings revealed that flavonoids are promising candidates in the fight against CM, reducing invasion, inhibiting proliferation and inducing cell apoptosis [19]. It has been noticed that flavonoids interact with several molecular targets involved in melanoma pathogenesis (*e.g.* p53, Bcl-2, MAPK pathway or caspases 3 and 9). In addition, a study demonstrated that flavonoids blocked the two-pore channel 2 (TPC2) found in the melanosome membrane and consequently boosted melanin production and minimised melanoma cell proliferation, migration and invasion [7]. Toward these aspects, the purpose of the present study was to evaluate the potential of two flavonoids, namely MYR and FIS and the association between MYR and FIS as promising candidates in the treatment of CM in the B164a5 cell line and also conducting a preliminary examination of the cytotoxic impact in a healthy epidermal cell line, namely JB6 Cl 41-5a to observe the selectivity capacity of the compounds. Flavonoids MYR and FIS have been chosen based on the reported beneficial effects

demonstrated on different CM cell lines, evidencing antiproliferative activities [1, 33] and thus standing out as valuable alternative strategies for CM. The concentrations to be tested (20 - 40 μM) for MYR and FIS were selected according to the specialised literature where the 2 compounds have demonstrated anticancer potential on other CM cell lines [1]. As an initial step in examining the impact of the 2 compounds and their association, cell viability was assessed 24 h after treatment for both B164a5 cells and healthy epidermal cells - JB6 Cl 41-5a by MTT technique. The MTT method represents a test based on the conversion of MTT into formazan crystals by living cells, which is very popular for the evaluation of cytotoxicity [24]. For the treatment of B164a5 cells (Figure 1A), the results showed a dose-dependent decrease for all compounds; thus, for MYR at the highest concentration of 40 μM, the percentage of viable cells reached about 66%, while for FIS at the same concentration of 40 μM, the percentage achieved 63%. For the association of the two compounds at 20 μM, the cell viability was approximately 53%, and at 30 μM, it reached 50%. In contrast, for the combination of the two, at the highest concentration of 40 μM, the percentage of viable cells reached the threshold of approximately 49%. In the case of the treatment of healthy epidermal JB6 Cl 41-5a cells (Figure 1B), MYR at concentrations of 20 and 30 μM showed a cell stimulation. In contrast, the highest tested concentration of 40 μM slightly decreased the viability to about 96%. For FIS, the lowest tested concentration (20 μM) decreased the viability up to 82%, while the highest tested concentration (40 μM) reduced the viability of the cells up to about 68%. When the two compounds MYR and FIS were combined, the decrease in cell viability was also dose-dependent, with the lowest percentage occurring at 40 μM, *i.e.* 89%.

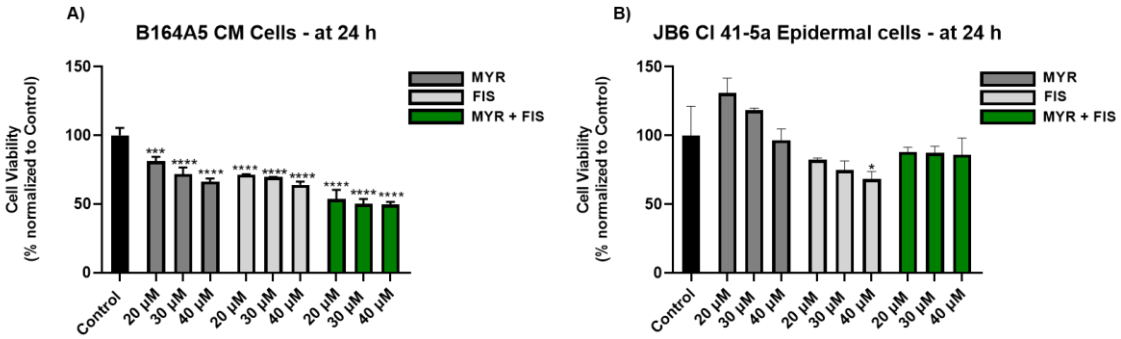

Figure 1.

Graphical representation of cell viability percentages 24 h after treatment of B164a5 cells (A) and JB6 Cl 41-5a cells (B) with MYR (20, 30, 40 μM), FIS (20, 30, 40 μM) and the combinations between MYR+FIS (20, 30, 40 μM)

The results are presented as percentages (%) normalised to control (untreated cells). All data are expressed as mean values ± SD from three independent experiments done in triplicate. The One-way ANOVA test was conducted to analyse the statistical differences between the control and treated groups, followed by Dunnett's multiple comparison post-test. "*" marks statistical significance (* $p < 0.05$; *** $p \leq 0.001$; **** $p \leq 0.0001$)

The CM cells were found to be strongly affected by the combinatorial treatment of the two flavonoids, demonstrating a superior action compared to the administration of each compound individually. Similarly, MYR (0 - 100 μM) was also shown to produce a dose-dependent decrease in cell viability on A431 cells and in the same dose and time range, affecting the healthy FR2 cells less, according to MTT analysis [32]. In another study performed on the A375 CM cell line, MYR demonstrated that upon treatment for 48 hours in the concentration range of 10 - 100 μM, cell viability decreases dose-dependently, with the percentage of viability being below 60% starting from the concentration of 40 μM [1]. Moreover, it was observed that MYR potently inhibited tumour promoter-induced transformation of neoplastic cells by directly inhibiting MEK, JAK1, Akt and MKK4 kinase activity, thus being considered a promising agent for skin cancer chemoprevention. In a mouse skin model, MYR attenuated ultraviolet B (UVB)-induced COX-2 expression and skin tumour formation by upregulating Fyn [18], and a study by Jheng-Hua Huang and co-workers showed that MYR produced a protective effect for healthy keratinocyte cells against UVB radiation [13]. Iftode *et al.* revealed that FIS in the 5 - 25 μM concentration range decreases the viability of A375 cells in a dose-dependent manner over 72 h, and, in the same study, it was observed that the 5 - 25 μM concentration range of FIS does not affect the viability of HaCaT cells [14]. FIS has been shown to inhibit the MAPK and NF-kB signalling pathways and has been recognised for its anti-proliferative and antioxidant properties [26]. Similarly, another paper indicated that FIS in the dose range 20 - 80 μM reduces the viability of CM cells dose-dependently and time-dependently, with the lowest percent viability achieved at the 80 μM dose after 48 h [33]. Hosseini *et al.* have suggested that the association

between flavonoids (*e.g.* quercetin and FIS) has a synergistic effect, and the anticancer effect is more potent in different cancer cell lines [12]. In the same way, FIS was also combined with aspirin for treating CM, and cell viability results indicated that the anti-cancer effect is stronger with the combination of the two compounds compared to individual treatment in A375 cells, without affecting healthy cells under treatment [14].

In general, according to ISO Standard 10993-5:2009, a sample causes cytotoxicity if the viability of the cells is reduced more than 30% [8]; therefore, in the present situation, FIS at the highest concentration of 40 μM is the only compound that decreases the viability of healthy cells below 70%, reaching 68%. Besides, the combinatorial treatment displayed a protective effect on normal JB6 Cl 41-5a cells even at the highest dose, attenuating the reduction in viability observed following FIS 40 μM treatment.

In continuation, the effect of MYR, FIS (20, 30 and 40 μM) and the combination of MYR and FIS (20, 30 and 40 μM) was evaluated at the morphological level after 24 h of treatment. The results for B164a5 cells (Figure 2A) revealed that a dose-dependent decrease in cell confluency was obtained in the case of MYR treatment. For FIS treatment, in the same manner, cell confluency decreases in a concentration-dependent manner, and dysmorphologies such as cell elongation and cell shrinkage appear at both 30 μM and 40 μM concentrations. For the association between the 2 compounds, a prominent decline in confluency was noticed from the lowest concentrations (20 and 30 μM) compared to untreated cells, the reduction being dose-dependent. Furthermore, for MYR+FIS, frequent dysmorphologies such as cell elongation, cell rounding and cell shrinkage can be observed starting at the lowest concentrations, and traces of cell debris can be observed at the highest concentration tested (40 μM). Regarding healthy JB6 Cl 41-5a cells, according to Figure 2B, MYR did not induce any morphological changes, maintaining the specific appearance of the cells throughout the treatment. For FIS-treated cells, a slight decrease in confluency and signs of debris can be observed starting at the mid-concentration of 30 μM. When the 2 compounds were combined, a slight decline in confluency was observed only at 40 μM.

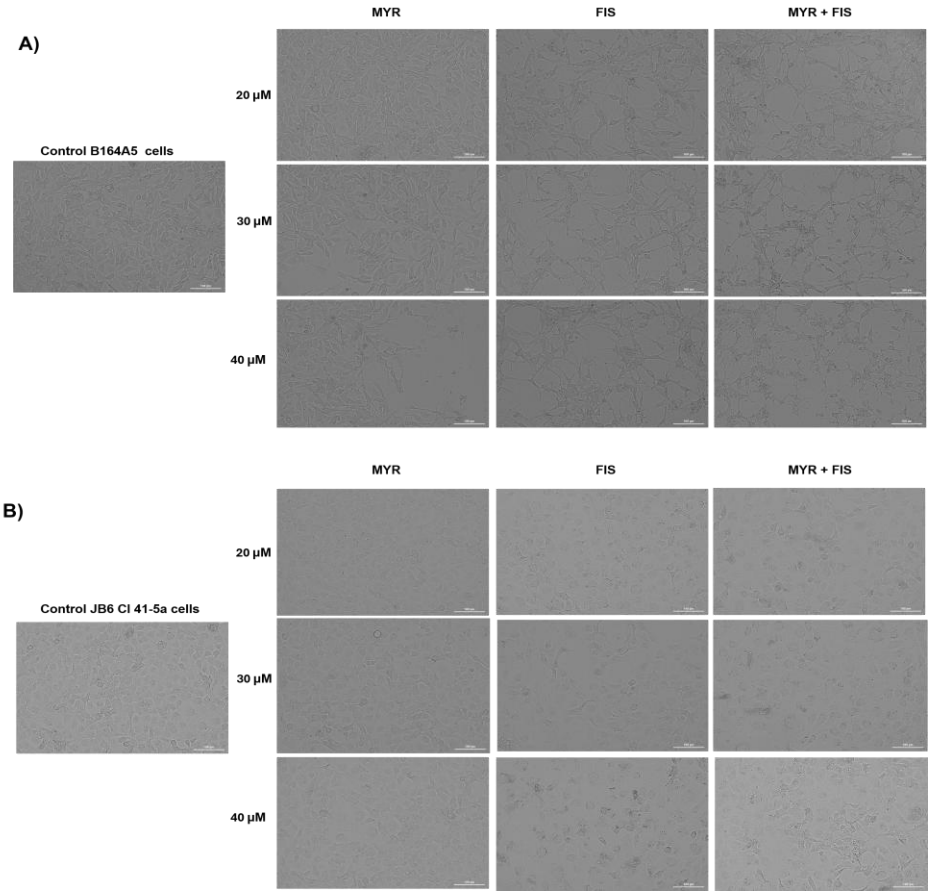

Figure 2.

Representative images illustrating the morphological changes observed at 24 h of treatment of B164a5 cells (A) and JB6 Cl 41-5a cells (B) with MYR (20, 30 and 40 μM), FIS (20, 30 and 40 μM) and the combinations between MYR+FIS (20, 30 and 40 μM)

The pictures were taken at a magnification of 20×, and the scale bar indicates 100 μm

Cell morphology examination represents a key tool method that shows shape alterations occurring in cell lines following different treatments, thus playing an essential role in evaluating cytotoxicity [8]. In this sense, the impact of MYR treatment on cell morphology has been investigated in other cell lines. Thus, in the SK-BR-3 cell line (breast cancer), MYR (10 and 20 μM) induced the appearance of autophagic vacuoles after 24 h of treatment [10]. FIS was morphologically analysed by Khalid Imtiyaz and his associates in A375 (cutaneous melanoma) and A431 (human epidermoid carcinoma) cell lines in the dose range 4 - 128 μM. It was observed that both cell lines are affected by the natural compound, causing a concentration-dependent decrease in confluency, cell size reduction and cell shrinkage [15]. Also, another study showed that FIS 20 μM produced cell shrinkage after 72 h of treatment in A375 cells [14]. Regarding the impact on healthy epidermal cells, the two compounds and their association did not show signs of cytotoxicity, the results of the morphological analysis being in concordance with those obtained from the cell viability analysis by the MTT method. Furthermore, it has been noted that natural compounds benefit from a particular selectivity, affecting cancer cells and avoiding toxicity in healthy tissues [11]. The main target of chemotherapy is to kill cancer cells, but in many cases, it also destroys the host's healthy or immune cells, ultimately affecting the endogenous anti-tumour response. Under these aspects, there is a considerable interest in studying repercussions of the cytotoxic effects of chemotherapeutic drugs, considering that the damage or killing of immune cells may promote tumour growth [28].

Observing that the two natural compounds tested, as well as the combinatorial treatment between the two have favourable effects in killing cancer cells and possess the ability to be selective, the next step of the exploration was to investigate the impact of MYR, FIS and MYR+FIS on the number of B164A5 cells at 24 h of treatment. According to Figure 3, MYR dose-dependently decreased cell number, thus at the highest concentration of 40 μM it reduced the cell number up to about 55%; FIS showed a dose-dependent reduction reaching a percentage of 40% and the association between the two compounds MYR+FIS at the highest concentration of 40 μM caused a percentage of cell number reaching about 36%.

In another paper, it was observed that MYR has the ability to reduce the invasion and motility of A431 cells; thus, at a concentration of 20 μM, MYR inhibited cell invasion up to 66% [32]. *In vivo,* oral administration of FIS (45 mg/kg body weight and 90 mg/kg body weight, 5 times/week) was demonstrated to reduce tumour size in a transgenic genetically modified mouse model of metastatic melanoma [25]. Concerning combinatorial treatments with phyto-compounds as alternative strategies for CM, Chen *et al.* demonstrated that the association of 2 other natural compounds (*i.e.* borneol and curcumin) produced more favourable effects on A375 CM cells compared to individual treatment of the parent compounds, the results being in all cases dose-dependent and moreover, the association of botanical compounds displayed a synergistic effect which induced apoptosis [4]. In the same way, the evaluation of cell number highlighted that the combinatorial treatment of MYR and FIS resulted in a much stronger effect.

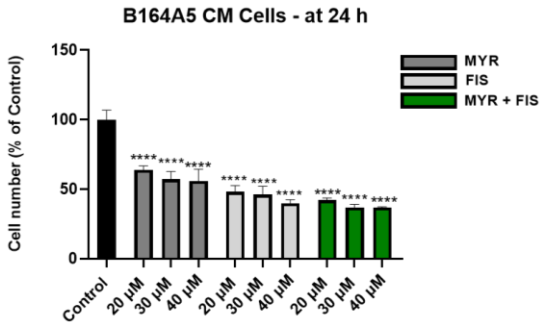

Figure 3.

Graphical representation of cell number percentages 24 h after treatment of B164a5 cells with MYR (20, 30 and 40 μM), FIS (20, 30 and 40 μM) and the combinations between MYR+FIS (20, 30 and 40 μM) The results are presented as percentages (%) normalised to control (untreated cells). All data are expressed as mean values ± SD from three independent experiments done in triplicate. For analysing the statistical differences between the control group and treated groups, the One-way ANOVA test was conducted, followed by Dunnett's multiple comparison post-test. "*" marks statistical significance (**** $p \leq 0.0001$)

Starting from the premise that the two compounds are promising options in the treatment of CM cells, and that the association between MYR and FIS yielded superior results against cancer cells, the subsequent step of the study explored the impact of the compounds in more detail, investigating the influence of MYR, FIS and MYR+FIS at the nuclear and membrane levels. The Hoechst (2'-[4-ethoxyphenyl]-5-[4-methyl-1-piperazinyl]-2,5'-bi-1H-benzimidazole trichloride trihydrate) method is widely used in various fields such as cell biology, molecular biology and pathology. It aims to study nuclear structure and behaviour, while having economic advantages [27]. Other advantages are its specificity for DNA by emitting a blue fluorescence and its minimal cytotoxicity, which makes this approach ideal for experimental studies [27]. The nuclear impact on B164a5 cells was assessed after 24 h of treatment with MYR, FIS and the combination of the two compounds at the highest concentration previously tested, *i.e.* 40 μM. Figure 4 shows MYR exhibited no significant nuclear changes, with the cells retaining their nuclear shape similar to those shown in control cells. FIS decreased nuclear

confluency and induced dysmorphologies characterized by condensed nuclei with reduced size and shape (white arrows). The combination of MYR+FIS produced major dysmorphologies including massive chromatin condensation, nuclear shrinkage, shape irregularities and a significant reduction in nuclei (white arrows).

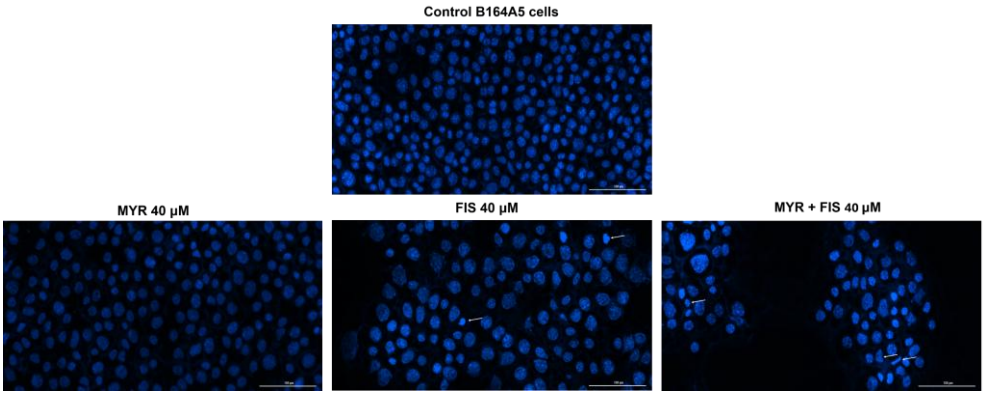

Figure 4.

Representative images showing the changes in nuclear morphology at 24 h of treatment of B164a5 CM cells with MYR (40 µM), FIS (40 µM) and the combinations between MYR+FIS (40 µM)

The pictures were taken at a magnification of 20×, and the scale bar indicates 100 µm. The white arrows indicate nuclear modifications

Apoptosis is also known as programmed cell death. It is characterised by specific morphological changes or energy-dependent biochemical mechanisms and is considered a vital element for a multitude of processes (*e.g.*, functioning of the immune system) [9]. The structure of the nucleus is a marker that indicates the type of cell death, hence in terms of apoptosis the nuclear changes that can occur are chromatin condensation or nuclear contraction [27], changes that also seem to occur following treatment with FIS (40 µM) or the combination of MYR and FIS (40 µM) in B164a5 cells. Wei Sun *et al.* showed that MYR induced dose-dependent nuclear changes in A431 cells, with the 40 µM concentration inducing the most evident nuclear dysmorphologies [32]. FIS (5-20 µM) after 72 h of treatment on A375 cells showed that it produced nuclear apoptotic changes in a dose-dependent manner, with the number of nuclei being reduced with increasing concentration, and also in the same study, it was observed that the association between FIS and aspirin can produce apoptotic-like cell death effects in a higher percentage compared to individual FIS treatment [14].

The final step in assessing the two compounds and their combinatorial treatment was the analysis at mitochondrial levels using cell-permeable MitoTracker probes, which stain active mitochondria in living cells [23]. According to Figure 5, MYR 40 µM did not produce significant effects on mitochondria compared to untreated cells. In contrast, FIS 40 µM and the combinatorial treatment (at 40 µM) produced a massive mitochondrial condensation (white arrows), mitochondrial damage being more frequent and specific in the case of associative treatment (MYR+FIS).

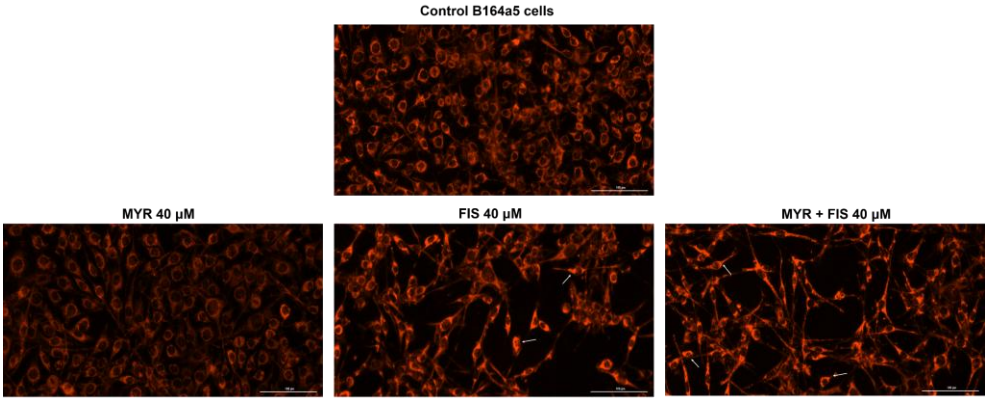

Figure 5.

Representative images showing the changes in mitochondrial morphology at 24 h of treatment of B164a5 CM cells with MYR (40 µM), FIS (40 µM) and the combinations between MYR+FIS (40 µM)

The pictures were taken at a magnification of 20×, and the scale bar indicates 100 µm. The white arrows indicate mitochondrial modifications

Mitochondria play an essential function in energy metabolism, controlling the stress response and several biosynthetic processes, as well as being crucial signal mediators for the propagation of various cellular outcomes, being complex organelles. Furthermore, mitochondria constitute the principal source of intracellular reactive oxygen species generation. They are implicated in cellular Ca^{2+} homeostasis, contain a self-destructive set of apoptogenic factors that can be released to promote cell death, thereby presenting a common scaffold for metabolism and apoptosis (programmed cell death) [35]. About MYR, it has been found to downregulate Bcl-2 and translocate Bax into mitochondria, leading to the release of cytochrome c from the mitochondrial membrane [16]. FIS caused the damage of the mitochondrial transmembrane potential as reported in a study carried out by Kang Wang *et al.* on human uveal melanoma cells and in addition boosted the levels of the pro-apoptotic proteins Bcl-2 and cytochrome c [36].

The present work proved that the combinatorial treatment of MYR and FIS induces superior effects on CM cells compared to individual treatment of the flavonoids and, at the same time, reveals a selective behaviour avoiding toxicity in healthy cells. Future research directions should be oriented towards investigations on the mechanism of action realised by the combinatorial treatment. However, the effect may be attributed to the fact that both compounds can target or modulate similar signalling pathways or expressions (NF-kB, MAPK, MMP-9). Furthermore, both MYR and FIS are recognised as phytocompounds with antitumor action [5, 10, 16, 17, 26]. Other areas of research on the topic could be complemented by evaluation in 3D experimental models and the exploration of combinatorial treatment incorporated in targeted delivery systems or nanoformulations, which could produce advanced effects.

Conclusions

The new findings suggested that the combinatorial treatment between MYR and FIS displayed a superior effect compared to individual treatment on CM cells, producing a dose-dependent decrease in cell viability, cell dysmorphologies, and diminished confluency, reducing cell number, causing apoptotic-like cell death signs, and inducing mitochondrial condensation. In addition, the combinatorial treatment strategy presented selectivity on B164a5 cells, avoiding cytotoxic effects in normal epidermal JB6 Cl 41-5a cells.

Conflict of interest

The authors declare no conflict of interest.

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