The Association Between Fisetin and Rutin Triggers an Enhanced Cytotoxicity in A431 and A375 Skin Cancer Cells

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Abstract

Background: Skin cancer is a public health problem and conventional treatments lead to multiple limitations that affect patients' well-being. Fisetin and rutin are two phytocompounds that have demonstrated versatile biological properties, including anticancer effects. Methods: A431 and A375 cells were used to evaluate the two compounds and their associative treatment. For investigation, after 24 hours of treatment, cell viability was assessed by MTT technique and cell morphology was analyzed. Results: The results indicated that the associative treatment is more potent compared to the individual treatment of the agents, producing a dose-dependent decrease in cell viability and enhancing cell dimorphologies with increasing the concentration. Conclusion: Associative treatment represents a promising approach as an alternative for skin cancer, exerting a superior effect compared to individual treatment. However, future research directions should be directed towards elucidating the mechanism of action of the FIS+RUT treatment, assessing the impact in 3D experimental models, and verifying the efficacy by incorporation into different nanoformulations.

Keywords: fisetin, rutin, skin cancer, melanoma, in vitro

INTRODUCTION

Skin cancer is among the most common cancers in the United States and can be broadly categorized into melanoma or nonmelanoma skin cancer. Recent studies have shown an alarming increase in cases in recent decades, but nevertheless the exact incidence is difficult to detect due the under-reporting. The treatment strategies are varied and include surgical excision, cryotherapy, chemotherapy or immunotherapy, but the current options are considered a global health problem which affects the well-being of cancer patients due to a number of disadvantages [1,2]. In these aspects, botanical compounds have shown to be promising agents for the prevention and alternative treatment of skin cancers and melanomas, possessing versatile biological activities that make them the subject of numerous research studies [3,4]. Furthermore, it was noted that phytocompounds offer the advantage of a non-toxic profile and a pleiotropic mechanism of action [5]. Medicinal phytochemical compounds are found in a multitude of sources (e.g. plant, marine, microbial) and have been found to include chemo-preventive abilities that have been suggested to be closely related to antioxidant properties. Since it has been realized that natural products may have potential in the symptomatic treatment of cancers and in addition may reduce the adverse effects produced by anticancer therapy, there is an increasing rate of self-medication represented by the oncology patient population who choose botanical agents due to their safety and efficacy [6].

(FIS) and rutin (RUT) are two natural compounds belonging to flavonoid class [7]. FIS is a phytoestrogenic flavonoid distributed in various fruits and vegetables and with numerous biological properties including anti-inflammatory, antioxidant and anticancer effects through anti- invasion, anti-metastatic and apoptosis promoting activities by increasing pro-apoptotic Bax and caspase 3/8. Additionally, it was also noted that FIS modulates different signaling pathways and increases the efficacy of chemotherapeutic agents [5]. RUT presents a diverse spectrum of pharmacological properties, including anti-inflammatory, antioxidant, antibacterial and antimutagenic. Besides, RUT has been shown to minimize photoaging of the skin by enhancing skin density and elasticity through the regulation of extracellular matrix enzymes [8–10]. An important fact is that the natural compound RUT demonstrated its ability in a panel of cancers, including cutaneous melanoma [11]. The skin is the largest organ of the human body which has the role of protection against external factors such as radiation, chemicals, allergens or infections. Alongside the aforementioned abilities in skin cancers and melanoma, flavonoids have also revealed their therapeutic potential for a number of other skin-related conditions (e.g. atopic dermatitis, psoriasis, vitiligo) [12].

Aim and objectives

The aim of the current work was the in vitro assessment of two natural compounds belonging to the flavonoid class, namely FIS and RUT and their association (FIS + RUT), regarding their impact on two distinct 2D cancer cell lines, i.e. the A431 (epidermoid carcinoma) and the A375 (malignant melanoma) cell lines.

MATERIAL AND METHODS

Reagents and Instruments

The tested samples fisetin and rutin and the MTT (3-(4,5-dimethylthiazol2-yl)-2,5-diphenyltetrazolium bromide) kit were obtained from Sigma-Aldrich, Merck KGaA (Darmstadt, Germany). The Dulbecco's Modified Eagle Medium (DMEM) and dimethyl

sulfoxide – DMSO (solvent) were obtained from PAN-Biotech GmbH, Aidenbach, Germany; fetal bovine serum (FBS- 30-2020 ™), the penicillin/streptomycin mixture, and trypsin-EDTA solution were purchased from American Type Culture Collection (ATCC) Manassas, VA, USA. The devices used, Cytation 5 (plate reader) and Lionheart FX (automated microscope) were from BioTek Instruments Inc. (Winooski, VT, USA).

Cell Culture Conditions

The experiments were performed using two different 2D cell lines: A-431 CRL-1555 ™ (epidermoid carcinoma) and A-375 CRL-1619 ™ (malignant melanoma) obtained from ATCC, Manassas, VA, USA. Both cell lines were cultured in the specific growth medium DMEM supplemented with 10% FBS and 1% antibiotic mixture (100 U/mL penicillin/100 µg/mL streptomycin). The cells were maintained in a humidified incubator, at 37°C and 5% CO2. Fisetin and rutin were dissolved to form the stock solution in DMSO. The concentration of DMSO did not exceed 0.5%.

Cell Viability – The MTT Test

The cell viability was evaluated via the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) method to observe the impact of FIS, RUT and FIS+RUT (30, 40 and 50 µM) on A431 and A375 cells. After 24 hours, 100 µL of fresh medium and 10 µL of MTT were inserted to each well and the plates were introduced in the incubator. After 3 hours of incubation, 100 µL of MTT solubilizing solution were added and the plates were maintained for 30 minites at room temperature. The absorbance was read at 570 and 630 nm wavelengths at Cytation 5.

Bright-Field Cell Morphology Evaluation

The impact of the test samples FIS, RUT and FIS+RUT (30, 40 and 50 µM) on A431 and A375 cell morphologies was evaluated by taking representative photos of the control group (untreated cells) and treated cells under brightfield illumination (magnification 20×) using the Lionheart FX automated microscope. The obtained pictures were processed in the Gen5™ Microplate Data Collection and Analysis Software (Version 3.14) - BioTek Instruments Inc. (Winooski, VT, USA).

RESULTS

The impact of FIS, RUT and FIS+RUT Treatment on Cell Viability

In the case of treatment of A431 cells with FIS (Figure 1A), the results obtained indicated that cell viability decreased in a concentration-dependent manner, i.e. treatment with 30 µM FIS reduced cell viability to 40%and the highest concentration tested of 50 µM produced a decline in cell viability to a threshold of 31%. Treatment with RUT in A431 cells (Figure 1B) exhibited a dose dependent reduction in cell viability, reaching at the highest concentration tested (50 µM) a percentage of 64%. The combinatorial treatment between RUT and FIS (Figure 1C) resulted in a decreasing cell viability under the same dose-dependent trend, with the percentages gradually diminishing with increasing dose from 36% (at the lowest concentration of 30 µM) to 26% (at 50 µM).

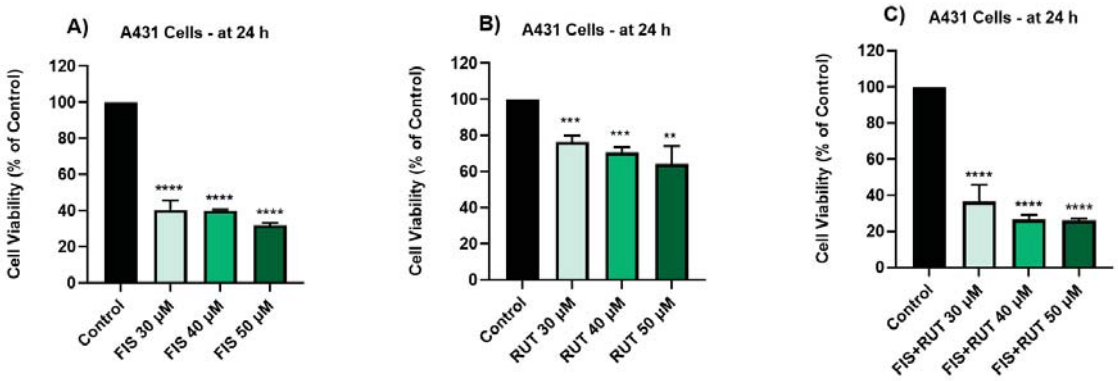

Figure 1. Graphical representation of cell viability percentages 24 hours after treatment of A431 cells with (A) FIS (30, 40, 50 μM), (B) RUT (30, 40, 50 μM), (C) and the combinations between FIS and RUT (30, 40, 50 μM). The results are presented as percentages (%) normalized to control (untreated cells). All data are expressed as mean values ± SD from three independent experiments done in triplicate. For analyzing the statistical differences between the control group - untreated cells and treated groups, the One-way ANOVA test was conducted, followed by the Dunnet's multiple comparison post-test. "*" marks statistical significance (** $p<0.01$; *** $p \leq 0.001$; **** $p \leq 0.0001$).

For A375 cells, the treatment with FIS (Figure 2A) slightly reduced the cell viability concentration-dependently up to 87%, which was observed at the highest concentration examined, i.e. 50 μM; and according to Figure 2B, RUT decreased cell viability up to 91%.The associative treatment of FIS + RUT (Figure 2C) provided for the 30 μM treatment a cell viability of 83%, at the 40 μM treatment the cell viability decreased to 80%, while at the highest concentration of 50 μM the threshold of about 74% was reached.

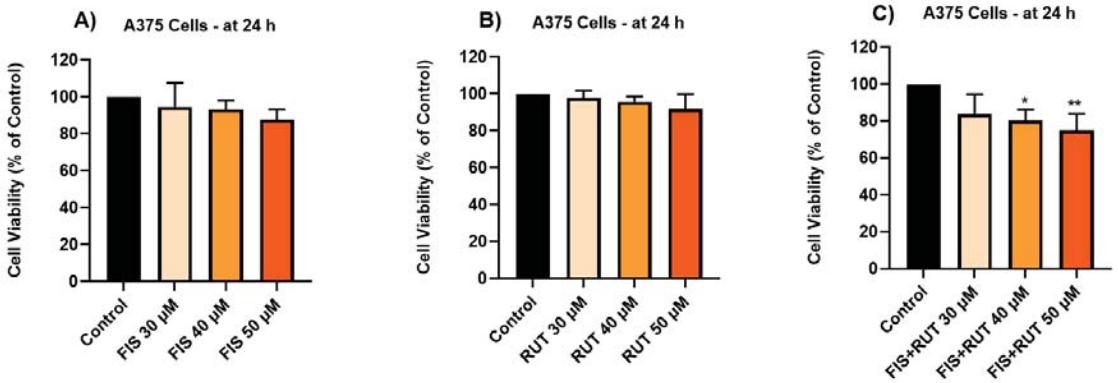

Figure 2. Graphical representation of cell viability percentages 24 hours after treatment of A375 cells with (A) FIS (30, 40, 50 μM), (B) RUT (30, 40, 50 μM), (C) and the combinations between FIS and RUT (30, 40, 50 μM). The results are presented as percentages (%) normalized to control (untreated cells). All data are expressed as mean values ± SD from three independent experiments done in triplicate. For analyzing the statistical differences between the control group - untreated cells and treated groups, the One-way ANOVA test was conducted, followed by the Dunnet's multiple comparison post-test. "*" marks statistical significance (* $p < 0.05$ and ** $p<0.01$).

Analysis of Cellular Morphology

The next step of the investigation of FIS, RUT and FIS+RUT combinatorial treatment after 24 hours of treatment on A431 and A375 cells was to analyze the cell morphology. In the case of A431 cells, as shown in Figure 3, FIS treatment produced a dose-dependent decrease in cell confluency, with the highest concentration of 50 μM showing a massive rounding of the shape and a decrease in size. Application of RUT treatment decreased cell confluence gradually and produced cell rounding at 50 μM, while the combinatorial treatment between

FIS and RUT showed dysmorphologies from the lowest concentration of 30 µM that were accentuated in a dose-dependent manner up to 50 µM where a massive cell rounding, reduced confluence and signs of detachment of cells from the plate were noticed.

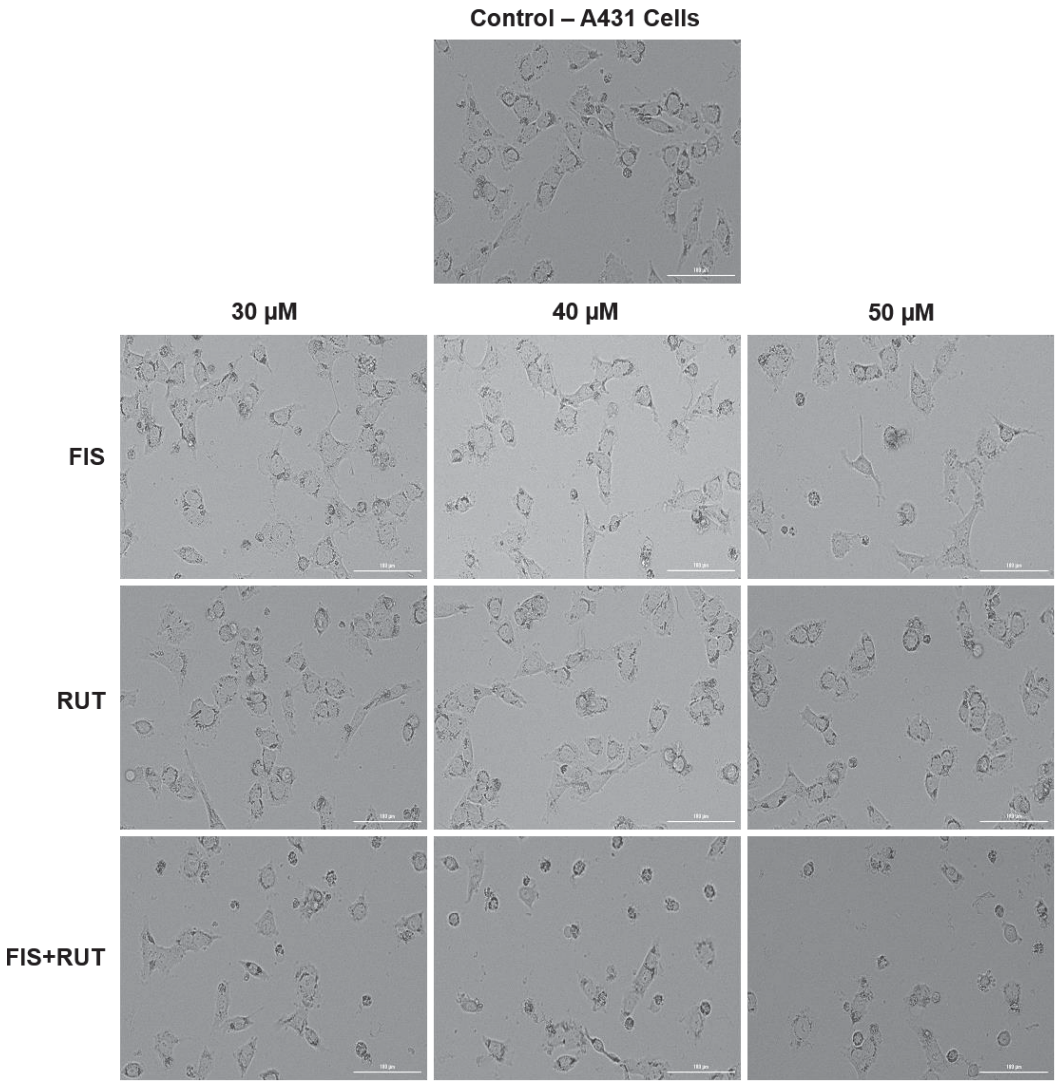

Figure 3. Representative images illustrating the morphological changes observed at 24 h of treatment of A431 cells with FIS (30, 40, 50 µM), RUT (30, 40, 50 µM) combinations between FIS + RUT (30, 40, 50 µM), The pictures were taken at a magnification of 20×, and the scale bar indicates 100 µm

For A375 cells (Figure 4), the 24 h treatment with FIS and RUT did not induce significant changes in cell morphology. In contrast, upon application of the combinatorial treatment between FIS and RUT, at the highest tested concentration of 50 µM, slight signs of detachment of the cells from the plate and a reduction in confluency could be observed.

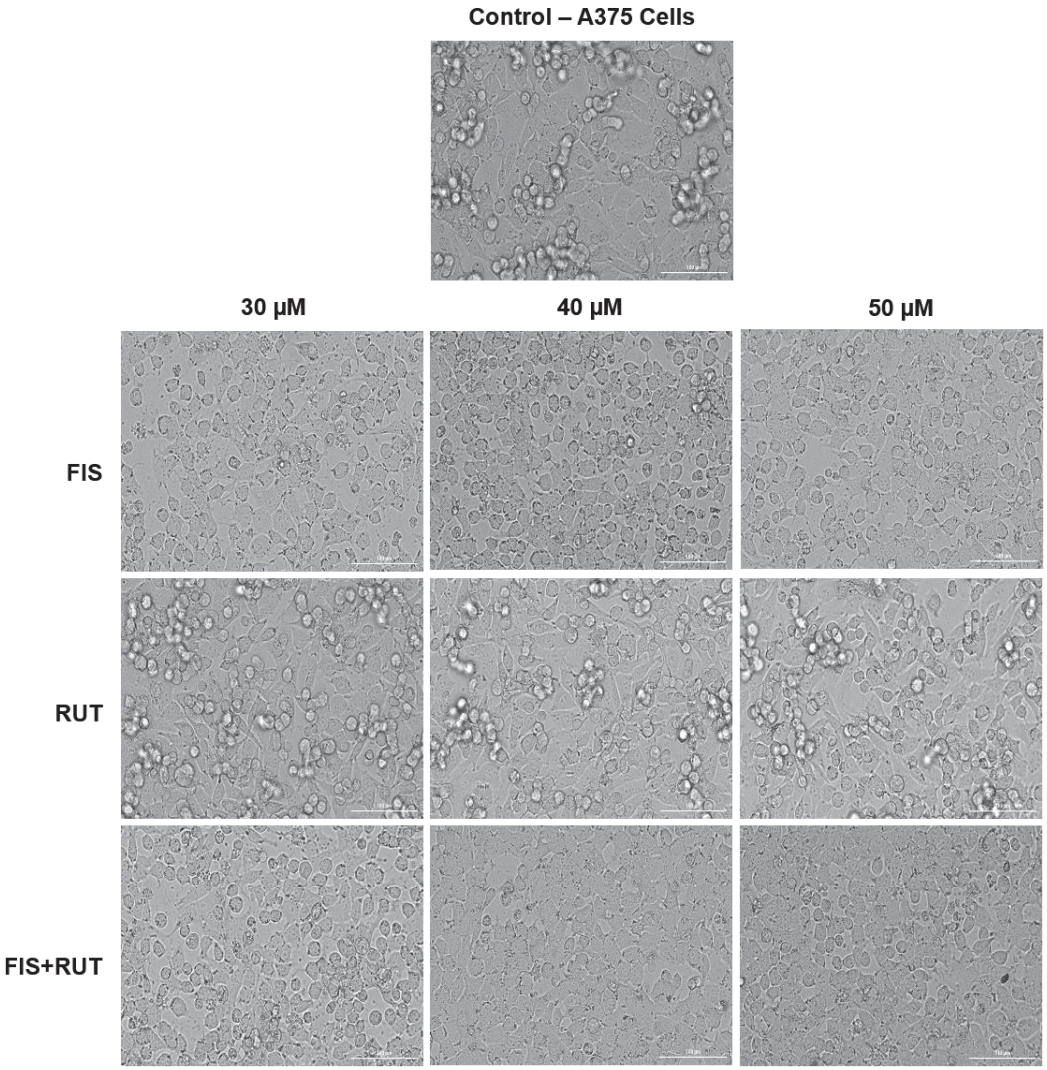

Figure 4. Representative images illustrating the morphological changes observed at 24 h of treatment of A431 cells with FIS (30, 40, 50 µM), RUT (30, 40, 50 µM) combinations between FIS + RUT (30, 40, 50 µM), The pictures were taken at a magnification of 20×, and the scale bar indicates 100 µm

DISCUSSIONS

Skin cancer is one of the most aggressive forms of cancer and is formed by the non-repair of deoxyribonucleic acid in skin cells which leads to mutations and genetic defects. There are certain parameters such as symmetry, color, shape or size that are used to identify skin cancer and to differentiate melanoma from benign skin cancer [13]. Considering the current treatments, there are several limitations among which the most recognized include toxic effects, high costs and resistance to treatment [2] . Phytochemicals are biologically active products that are derived from plants, and in recent decades have been found to possess multiple anti-cancer properties that are cost-effective and tolerated. Also, the anticancer activities exerted by herbal compounds are the result of their antioxidant, anti-inflammatory, anti-proliferative and anti-angiogenic properties [14]. Based on these reasons, researchers have turned their attention to finding new approaches or therapeutic alternatives for the management of skin cancers.

The objective of the present study was the evaluation of two natural compounds FIS and RUT, and the associative treatment of FIS+RUT on two distinct cell lines A431 and A375. RUT and FIS were selected on the basis of the specialized literature, that proved that the two flavanoids compounds exhibit potential in the alternative treatment of skin cancers and cutaneous melanomas, expressing anti-proliferative and anti-invasive effects on cancer cells. The concentration range chosen for the investigation was also considered taking into account the results indicated in the scientific literature, in which the two botanical compounds have been reported to display therapeutic effects on cancer cells [5,11]. The results suggested that for both compounds and for the associative treatment, the reduction in cell viability was dose-dependent in A431 cells (which were more strongly impacted by the treatment) and in A375 cells. FIS has been shown to inhibit growth and colony formation in A431 cells. Thus, another study showed that following MTT analysis, FIS (5- 80 μM) acts in a time- and dose-dependent manner and complementarily induces cell apoptosis and G2/M arrest [15]. FIS was also tested for 72 h in A375 cells where at 20 μM it decreased cell viability up to 86.40% [5]. Furthermore, FIS was also explored in combination with other compounds, i.e. aspirin, to check the combinatorial potential in the treatment of A375 cells and the results obtained showed promising results, the therapeutic association between the natural compound and aspirin being more potent compared to individual treatment of the compounds on cancer cells for a period of 72 hours [5]. About RUT was demonstrated to have the capacity to attenuate superoxide production, decrease adhesion and migration of human cancer cells and induce in vitro cytotoxicity in cancer cells [10] In addition, the bioflavonoid RUT modulates multiple signaling pathways among which can be listed NF-κB, PI3K/Akt/mTOR, Nrf2, ERK, JNK, or p38 MAPK [9]. Similarly, one study showed that RUT (1-50 μM) decreases the viability of RMPI-7951 and SK-MEL-28 malignant melanoma cells in a concentration-dependent manner after 24 hours of treatment, reaching at the highest concentration a cell viability of 60% for RPMI-7951 cells and 51.48% for SK-MEL-28 cells [11]. In order to investigate the cytotoxicity, microscopic analysis of cell morphology are crucial tools, providing information about the mechanism of action of the targeted samples and about the type of cell death involved [2]. The results of the cell morphology investigation suggested that the two compounds exert a more pronounced action when combined for both cell lines used in the study. In A431 cells, Pal et. al showed that treatment of cells with FIS significantly decreases the number of colonies compared to untreated cells. In the same study, using Annexin V/PI staining, FIS was observed to induce apoptosis of skin cancer cells [15] . Also, Iftode and colleagues noticed that at the morphologic level, treatment with FIS 20 μM for 72 hours caused a confluency reduction of A375 cells [5]. On the A375 cell line, RUT was further assayed in the dose range 1-75 μM for 24 hours and the data yielded that with increasing dose the cell confluency decreases, and at the highest concentrations cells become rounded and lose adhesion [8].

Moreover, an important aspect to investigate concerning the compounds with potential in anticancer therapy is their behavior on healthy cells. In this respect, both FIS and RUT have been evaluated individually in different healthy cell lines (such as HaCaT keratinocyte cells) and the data obtained indicated that the two compounds do not induce cytotoxicity [8]. This selectivity behavior of the two compounds represents an additional advantage, because among the primary limitations of chemotherapy is the lack of specificity on the target, often affecting healthy cells and consequently causing adverse effects to patients by compromising the immune system, chemotherapy having the ability to destroy even immune cells [16]. Combinatorial treatments between two naturally derived agents constitute a potentially effective approach for the treatment of different skin cancers, thus several studies with the objective of evaluating an associative treatment between two phytocompounds presented promising results [17].

The findings of the present work showed that RUT+FIS associative treatment exerted superior effects compared to individual administration of the compounds in skin cancer cells. In view of these aspects, future directions should focus on investigating the mechanism of action underlying the anticancer activity of the two compounds, as well as the association between the two substances; assessing the combinatorial treatment on 3D experimental models; performing a detailed screening of the associative treatment in healthy tissues; and studying the association of FIS+RUT in nanoformulations.

CONCLUSIONS

The results framed that FIS and RUT compounds are promising candidates as an alternative for skin cancer. Additionally, the combinatorial treatment of FIS and RUT demonstrated a stronger effect than individual treatment on A431 and A375 cells which was described by a dose-dependent decrease in cell viability and the induction of cellular dysmorphologies that were intestified with increasing the concentration.

Conflicts of Interest

The authors declare no conflict of interest.

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