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Anticarcinogenic potential of the Morin bioflavonoid against SK-MEL-5 human melanoma cells

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ABSTRACT

Melanoma is an aggressive skin cancer. Due to its metastatic potential, melanoma is the leading cause of death of all skin cancer types, and its incidence and mortality has increased dramatically over the last 30 years. The anti-cancer potential of several flavonoids against melanoma is widely studied, but not the action of the Morin flavonol on human melanoma SK-MEL-5 cells. In this study, SK-MEL-5 cells were exposed to the bioflavonoid Morin and to the chemotherapeutic cisplatin. Morin was able to reduce the viability of SK-MEL-5 cell lines depending on the concentration and the cultivation time, with significant values from 200 µM and reaching cytotoxicity greater than 80% at the dosage of 800 µM. The apoptotic effect exhibited dose and time dependence, with results higher than 70% in 24 hours of cultivation under a concentration of 800 µM of flavonol and 100% after 36 hours of exposure in 400 µM of Morin. The cisplatin positive control showed 70% of cytotoxicity at the lowest concentration. Morin, at a concentration of 400 µM, was able to reduce the mitochondrial membrane potential at more than 60% for 36 hours, suggesting the involvement of the intrinsic pathway of cell death by apoptosis. We also evaluated the activation of caspases-3 and -6 and confirmed the apoptotic activity of the extract. The results suggest that the Morin compounds affect cell viability in the tested cell line SK-MEL-5, inducing cell death by apoptosis.

The EC_{50} was 203.2±1.13 µM for Morin and 18.57±1.08 µM for cisplatin. Although cisplatin (cis-diamminedichloroplatinum-II) exhibited the best anti-proliferative effect on SK-MEL-5 cells when compared to Morin, this chemotherapeutic agent has limited clinical application because it generates severe and irreversible side effects resulting, mainly in neuropathies, renal and cardiac insufficiency. Therefore, our next strategic study will investigate the combined action of Morin and cisplatin (as well as other chemotherapeutic agents) with the objective of determining the lowest dose of the chemotherapeutic-bioflavonoid formulation that provides the best results in the anticarcinogenesis of human melanoma.

Keywords: *Melanoma, Morin, Cancer, Cell line SK-MEL-5, Apoptosis, Caspases.*

1. INTRODUCTION

Cancer is a major public health problem worldwide and is the second leading cause of death in the United States. Responsible for most deaths (75%) linked to skin cancers, melanoma is the fifth highest incidence in men and the sixth in women, and its incidence and mortality has increased dramatically over the last three decades and its incidence and mortality rate has increased dramatically over the last three decades. In 2017, more than 87,000 new cases of melanoma have been diagnosed with nearly 10,000 deaths in the United States [1-3].

Plant-derived bioactive compounds, especially polyphenolics, have been shown to reduce tumorigenesis, preventing metastasis and/or increasing chemotherapy and radiotherapy efficacy [4-8]. Flavonoids are polyphenolic compounds, a class of plant secondary metabolites exhibiting various pharmacological activities including anti-cancer action [6,9,10]. They have been reported to interfere in the initiation, promotion and progression of cancer by modulating different enzymes and receptors in signal transduction pathways related to cellular proliferation, differentiation, apoptosis, inflammation, angiogenesis, metastasis and reversal of multidrug resistance [11-14].

The effects of various flavonoids are known about signaling pathways associated with progression of countless cancer types, including melanoma [4,15-19], but not the action of morin on human tumorigenic melanoma cells. Morin (3, 5, 7, 2', 4'-pentahydroxyflavone) is a widely distributed flavone, originally isolated from members of the Moraceae family, being biologically active, occurring ubiquitously and is widely consumed [20,21]. This flavonoid has been the subject of multiple experimental studies investigating mechanisms of action for the control of cancer. Morin exhibits antioxidant properties, inhibition of lipid peroxidation, activation/inactivation of enzymes and transporter proteins, anti-proliferative action and induction of cell death by apoptosis [5,7,20,22]. This bioflavonoid has been known for its chemo-preventive activities by inducing mitochondrial mediated apoptotic events in mammary carcinogenesis [23], Leukemia cells [10,24,25], HCT-116 human colon cancer cells [26] and anti-metastatic effects on highly metastatic human breast cancer cells [27-29].

The activation of caspases (cysteine-aspartic proteases), a critical event in the induction of apoptosis and its mitochondria-associated signaling pathways, can be targeted as potential therapies against malignancies, including melanoma. Caspases-3, -8 and -9 are especially studied in melanoma [16,30-34], but there are no studies on the actions of Morin on caspase-6 activity, one of the targets of this study. This paper will present a pioneering study of the cytotoxicity of the Morin bioflavonoid and the chemotherapeutic cisplatin on SK-MEL-5 human melanoma cells.

2. EXPERIMENTAL SECTION

2.1. Cell lines and reagents. The SK-MEL-5 human melanoma cell lines (ATCC® HTB-70™) were obtained from the Recognizing Biology Laboratory of the Biosciences and Biotechnology Center, at the State University of North Fluminense, in Campos dos Goytacazes city, Brazil. The cells were cultured in D-MEMF12 (Gibco®-BRL, Invitrogen, Carlsbad, CA), supplemented with 20 µg/mL gentamycin (Gibco®) and 10% fetal bovine serum (Gibco®). The cultures were replicated every 2 days and kept in an incubator (Forma Scientific Inc., model 3159) at 37°C, with 5% of CO_2 and humidity control. Morin, 3-[4,5-dimethyl-2-thiazolyl]-2,5-diphenyl-2H-tetrazolium bromide (MTT, Sigma®, St. Louis, USA); cisplatin [*cis*-diamminedichloroPt(II)], acridine orange and ethidium bromide (Sigma-Aldrich Chemistry®, St. Louis, USA).

2.2. MTT and LDH assays. The S-KMEL-5 cell lines were plated into a 100 µL/well ($1x10^6$ cells/mL) in 96-well plates treated with the flavone Morin at final concentrations of 50, 100, 200, 400 and 800 µM. The cells were maintained at 37 °C, with 5% of CO_2 and humidity control. After 48 hours, cell viability was assessed by the colorimetric MTT assay [35]. Part of the culture supernatants were used for the quantification of the enzyme lactate dehydrogenase (LDH) performed using commercial diagnostic kit (Doles Reagents®, GO, Brazil). The determination of LDH levels is proportional to the number of dead cells by in vitro necrosis.

2.3. Assessment of apoptosis using fluorescence microscopy. The cells were incubated for 12, 24 and 36 hours with the active compound Morin, then stained with a solution of 10 µg/mL of acridine orange (Sigma®) and 10 µg/mL of ethidium bromide (Sigma®). We evaluated the rate of apoptosis and necrosis activity by fluorescence microscopy (Zeiss Axioplan®, Germany) [36].

2.4. Analysis of mitochondrial membrane potential (ΔΨm) by flow cytometry. The mitochondrial membrane potential was examined using the lipophilic cationic fluorescent dye JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethyl-benzimidazol-carbocyanine iodide) [37]. The tumor cell lines were treated with the Morin compound (400 µM) and incubated for 48 hours at 37 °C under 5% CO_2 (Horn Jr. et al., 2013). The results were obtained using FACSCallibur™ flow cytometer (BD Biosciences) and the data were analyzed using WinMDI software version 2.9.

2.5. Caspase activity assay. To prove if the compounds were able to induce apoptosis, a caspase colorimeter assay kit (Invitrogen®) was used containing substrates for caspases-3 and -6. The cultures containing $1x10^6$ cells/mL were incubated with a concentration of 400 µM of active flavonoid for 48 hours. Subsequently, the cells were centrifuged for 5 minutes at 1500 rpm and re-suspended in 50 µL lysis buffer. The samples were centrifuged for 1 min (10,000x g) and then added 50 µL of the substrate (4 µM). The samples were incubated at 37 °C for 2 hours, in the dark. The absorbance of cells was measured at 405 nm with the microplate spectrophotometer (Multiskan™ Thermo LabSystems Milford, MA, model 352). As negative controls, the cells were incubated with D-MEMF12 (Gibco®) and supplemented with 20 µg/mL of gentamycin (Gibco®) and 10% fetal bovine serum (Gibco®).

2.6. Statistical analysis. The results were expressed as a mean ± standard deviation, and analyzed independently. The assays were analyzed by ANOVA - One way - followed by Tukey using the Graph Pad Software 5.0 program. Significant differences were considered as $P<0.05$.

3. RESULTS SECTION

3.1. Viability of SK-MEL-5 cells treated with the Morin and Cisplatin compouds. Cells were exposed to different concentrations of the tested compounds Morin and Cisplatin over a 48 hour period, and cell viability was measured by the MTT colorimetric assay (Fig. 1). Morin was able to reduce the viability of SK-MEL-5 cell lines in a concentration-dependent manner with significant values from 200 µM and reaching cytotoxicity higher than 80% in the dose of 800 µM. The cisplatin positive control exhibited 70% cytotoxicity at the lowest concentration (50 µM). The EC_{50} was 203.2 ± 1.13 µM for Morin and 18.57 ± 1.08 µM for cisplatin.

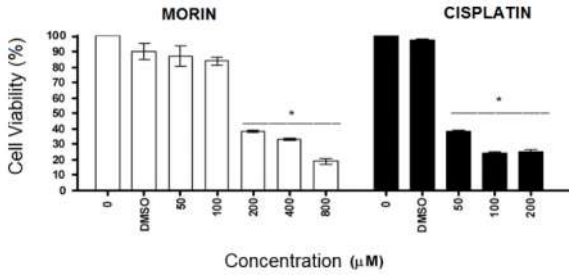

Fig. 1. Cytotoxic effects of Morin and Cisplatin (positive control) in the SK-MEL-5 human melanoma cells, after 48 hours of incubation, evaluated through the colorimetric MTT assay (n=3). The DMSO concentration was 1%. *P<0.05; statistically significant values compared to the negative control (zero, cell culture medium).

3.2. Assessment of cell viability by the release of lactate dehydrogenase (LDH). The LDH enzyme released by SK-ME-5 cells was measured by spectrophotometry, after 48 hours of treatment with the Morin flavonoid at different concentrations up to 800 µM. The dose-dependent increase in LDH release in treatments (Fig. 2) confirm cell necrosis.

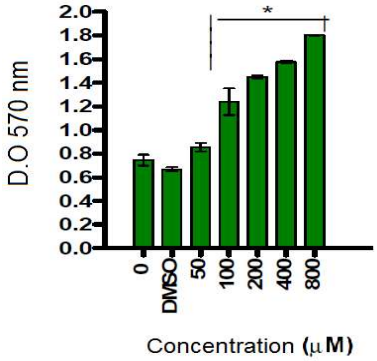

Figure 2. Evaluation of the release of LDH (lactate dehydrogenase). The SK-MEL-5 cells were treated with different concentrations of the Morin

compound for 48 hours. The concentration of DMSO was 1%. *P<0.05; statistically significant values compared to the negative control (zero, cell culture medium).

3.3. Evaluation of the cell death mechanism (fluorescence microscopy). The percentage of apoptosis induced by the compounds, Morin and cisplatin, in the SK-MEL-5 human melanoma cell line was evaluated (Fig. 3 and Fig. 4). The apoptotic effect exhibited dose and time dependence, with results higher than 70% in the first 24 hours of cultivation reaching 100% after 36 hours of exposure to 400 µM of the flavonol (Fig. 3). The chemotherapeutic cisplatin already showed significant results from 25 µM (Fig. 4).

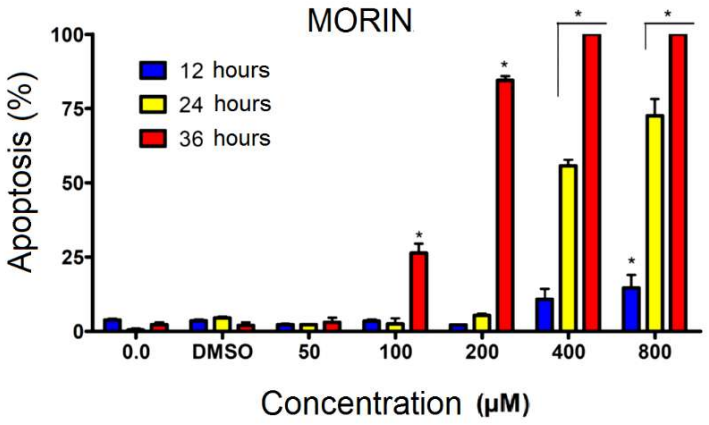

Figure 3. The percentage of apoptosis induced by Morin in neoplastic SK-MEL-5 line determined by fluorescence microscopy at 12, 24 and 36 hours. The DMSO concentration was 0.2%. *P<0.05; statistically significant values compared to the negative control (zero, cell culture medium).

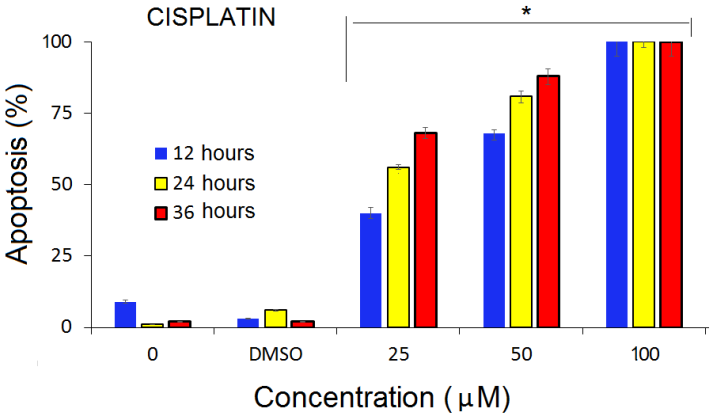

Figure 4. The percentage of apoptosis induced by Cisplastin (positive control) in the neoplastic SK-MEL-5 line determined by fluorescence microscopy at 12, 24 and 36 hours. The DMSO concentration was 0.2%. *P<0.05; statistically significant values compared to the negative control (zero, cell culture medium).

3.4. Alteration of mitochondrial membrane potential (ΔΨm). It can be seen in the control group, for the evaluated cell lines, that the cell population is predominantly found in quadrant 'C' of the graph corresponding to the region containing living cells with viable mitochondria (Figure 5). For Morin and cisplatin (positive control) treatments, a migration in the cell population from quadrant 'C' to 'D' was observed, representing cells with dissipation of the mitochondrial membrane potential (FL1), at concentrations starting from 200 µM mainly at 36 hours of incubation. Morin at a concentration of 400 µM was able to reduce the mitochondrial membrane potential at more than 60% for 36 hours.

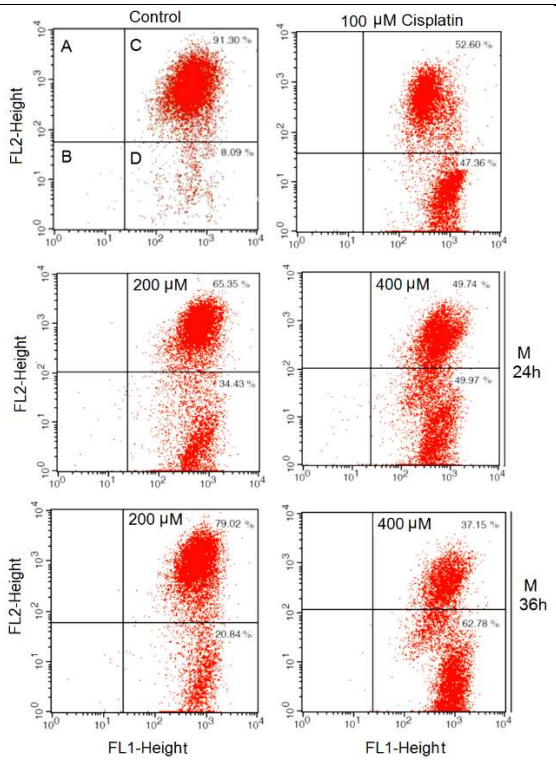

Figure 5. Analysis of mitochondrial membrane potential (ΔΨm) determined by flow cytometry of SK-MEL-5 cells treated with the Morin flavonoid (M) over a period of 24 and 36 hours of incubation. The proportion was expressed as a percentage. Quadrant 'C' consists of normal cells containing viable mitochondria and quadrant 'D' is composed of cells containing mitochondria with a loss of membrane potential.

3.5. Evaluation of the activity of caspases-3 and -6. When exposed to the Morin compound SK-MEL-5 human melanomic cells exhibited significantly higher values in the activity of caspase-3 and -6 (Fig. 6), important enzymes in triggering cell death by apoptosis.

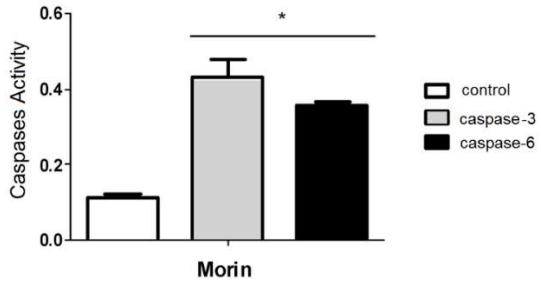

Figure 6. Activity of caspases-3 and -6 exposed to the Morin compound (400 µM) in the SK-MEL-5 human cell line after 48 hours of incubation. The DMSO concentration was 0.2%. *P<0.05; statistically significant values compared to the negative control (zero, cell culture medium).

Studies have demonstrated the cytotoxic potential of various plant extracts and natural compounds, especially flavonoids, against different types of tumor cells including melanoma [12,38]. Morin is a natural polyphenol with beneficial effects against several human diseases [20]. This study determined, for the first time, the cytotoxic effect of the Morin flavonol and its possible capability of inducing apoptosis in SK-MEL-5 melanoma cells.

The regulation in the expression of proteins such as cyclin D1 and c-Myc and suppression of the signal transducer and activator of transcription (STAT3) pathway are likely correlated to Morin's ability to inhibit cell proliferation by inducing blockage of sub-G1 cell cycle arrest [39,40]. In order to confirm death caused

by apoptosis promoted by the active compound Morin, quantitative and qualitative analyses were performed taking into consideration the recommendations of the Nomenclature of Cell Death Committee [41]. Nuclear morphology (perinuclear chromatin condensation, nuclear collapse and fragmentation) allowed us to distinguish apoptotic cells from necrotic and viable cells. Additionally, Morin promoted a high percentage of apoptosis in the melanoma line (Fig. 3 and Fig. 5).

Morin-induced apoptosis could be associated with promotion of reactive oxygen species (ROS) and derangement of mitochondrial membrane potential ($\Delta\Psi m$), promoting activation of caspases-3, -8 and -9 and triggering apoptosis (DNA fragmentation and sub-G1 phase of the cell cycle). Morin regulates the release of cytochrome c and increases activation of caspases-3, -8 and -9 [39], suggesting the enhancement of apoptosis through both extrinsic and intrinsic pathways. An analysis of caspases-3 and -6 in our results (Fig. 6) confirms an increase in its activity in SK-MEL-5 cells after 48 hours of treatment with the mitochondrial apoptotic pathway in a time and concentration dependent manner of the tested compounds. Action of these caspases have been seen in modulation of cellular autophagy and necroptosis [42,43]. Cisplatin is also known to interfere with mitochondrial activity, caspase activation, DNA damage, and cellular apoptosis [44,45].

The chemotherapeutic cisplatin exhibited the best anti-proliferative effect on SK-MEL-5 cells when compared to morine. The strong apoptotic and cytotoxic effect exhibited by cisplastin in this study may be mainly due to its action on the DNA and mitochondria of human melanoma cells [45,46]. Although widely used in the chemotherapeutic treatment of various types of cancer, cisplatin (cis-diamminedichloroplatinum-II) exhibits limited clinical application because it generates severe side effects resulting mainly in neuropathy, renal and cardiac insufficiency [44-47]. At a lower dose (0.1 or 1 μM), cisplatin induced non-reversible damage in primary animal hippocampal neurons. Both doses produced severe mitochondrial respiratory deficits and significant ROS production. When administered to the animal model (2-3 mg/kg), cisplatin induces mitochondrial damage and hippocampal neurotoxicity, as well as cognitive deficits [48,49].

Thus, a strategic study of the combined action of Morin and cisplatin would be promising (as well as other chemotherapeutic agents) with the objective of determining the lowest dose of the chemotherapeutic-bioflavonoid formulation that provides the best results in anticarcinogenesis of human melanoma. Morin has *in vivo* and *in vitro* selective action [27,39]. It had no toxic effect in rats at daily doses of 10 or 50 mg/kg in one week, exhibiting anti-cancer effects in metastatic breast tumors in animals [25,27]. This flavonoid is promising in the search for new antineoplastic agents and more *in vivo* and *in vitro* tests should be performed to elucidate the specific pathways of apoptosis induction promoted by this biocompound in these neoplastic lineages. Furthermore, the lowest effective concentration of *in vitro* Morin was found in our results, even though it is perhaps a constraint in studies for a long period of time, it could be tested and combined with chemotherapy to investigate synergistic effects.

4. CONCLUSIONS

The anti-cancer potential of several flavonoids in melanoma is widely studied, but not the action of the morphine flavonol on human melanoma SK-MEL-5 cells. For the first time, the flavonoid Morin showed, cytotoxic activity against SK-MEL-5 human Melanoma cell lines, significantly decreasing cell viability and inducing cell death by apoptosis in a metabolic pathway, dependent on caspases in a time and concentration-dependent manner. The anti-cancer property of this natural agent in human tumor cells may have promising therapeutic implications on melanogenesis.

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6. ACKNOWLEDGEMENTS

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