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Article

Pomegranate oil-based nanocapsules enhance 3,3'-diindolylmethane action against melanoma cells

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Cutaneous melanoma, a highly aggressive skin cancer, is the leading cause of death among skin neoplasms. Hence, this study focused on developing a 3,3'-diindolylmethane (DIM)-loaded pomegranate oil (PO) nanocapsule suspensions and their potential effects on melanoma cells. Nanocapsule suspensions were prepared by the interfacial deposition method (Eudragit® RS100). Medium-chain triglyceride (MCT) nanocapsules were produced for comparison. The of nanocapsule suspensions showed nanometric size (<180 nm), low polydispersity index (<0.12) (as measured by dynamic light scattering), positive zeta potential (tested by capillary microelectrophoresis), pH below 5.0 (as measured by potentiometry), DIM content, and nearly 100% encapsulation efficiency (evaluated by HPLC). The formulations were considered non-irritating (according to HET-CAM) regardless of the core constitution. Notably, PO-based nanocapsules showed a superior performance compared to the MCT-containing formulation in delaying DIM light degradation (tested by UVC radiation), lowering genotoxicity (determined by *Allium cepa*), and increasing *in vitro* antioxidant action (measured by ABTS and FRAP). Additionally, the PO-based formulation demonstrated higher *in vitro* antitumoral activity in B16F10 than MCT nanocarriers (IC_{50} of 6.49 and >24 µg/mL, respectively), and antimetastatic capacity by reducing B16F10 cell adhesion, colonization, and migration. Based on these results, this study suggests that the nanoencapsulation of DIM into PO nanocapsules may be a promising approach in melanoma treatment.

Keywords: Indole derivatives. Skin cancer. Nanoparticles. Metastasis. Cell invasion.

INTRODUCTION

Cancer is a disease that has achieved significant morbidity and mortality rates worldwide. It is characterized by the rapid and disorganized hyperproliferation of cells. Cutaneous melanoma, a type of skin cancer that originates in melanocytes, is primarily caused by excessive exposure to solar radiation. Notably, it is considered the leading cause of fatalities among skin cancers due to its elevated metastatic rate and invasive potential (Iqbal *et al*., 2019). Therefore, due to melanoma's aggressiveness and the deficiency of effective and safe drugs for its management, it is vital to explore new approaches (Rigon *et al*., 2015). The potential of phytochemicals with antitumor properties has been among the areas explored (Kim *et al*., 2019).

Plant-derived active compounds have been extensively investigated as treatments for various disorders. As such, their utility and benefits to human

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health have drawn increasing scientific interest over the years (Kim *et al*., 2019; Marchiori *et al*., 2017a; Gomes *et al*., 2018). 3,3'-Diindolylmethane (DIM) is a derivative of indole-3-carbinol (I3C) oligomerization, a commonplace phytochemical found in cruciferous vegetables (*Brassica*). In terms of cancer treatment, numerous beneficial properties have been attributed to DIM, including antioxidant activity, the inhibition of neoplastic cell invasion and metastatic angiogenesis, and the induction of cellular apoptosis. Importantly, the potential of DIM to combat breast, stomach, and prostate cancer has been well demonstrated (Amare, 2020; Thomson *et al*., 2017; Hajra *et al*., 2017). Notably, DIM significantly reduces human melanoma cell viability through the expression modulation of genes involved in cell cycle arrest and pro-apoptotic genes (e.g., BAX and caspases 3 and 9). It also increases p53 and p38 MAPK gene expression, reactive oxygen species, and decreases the expression of erythroid nuclear factor 2 (Heo *et al*., 2018; Wang *et al*., 2020), indicating that this phytochemical could serve as a promising option for cancer management.

However, despite its potential, DIM is thermosensitive, photolabile, and highly lipophilic, thus presenting reduced solubility in biological fluids and oral bioavailability (Patel *et al*., 2012). These challenges can potentially be overcome by incorporating DIM into nanocapsules, a reservoir nanocarrier formed by an oil core surrounded by a polymeric shell. Nanocapsules excel in encapsulating highly lipophilic compounds due to their propensity to deposit in the oily core or remain adsorbed to the polymeric shell (Zielińska *et al*., 2020). Furthermore, this nanosystem has been widely employed to deliver drugs with antitumor potential for various neoplasms, including melanoma. Thus, studies have shown that drugs associated with polymeric nanocapsules have presented selective cytotoxicity *in vitro* against melanoma cells (Ferreira *et al*., 2018) and resulted in a reduction in tumor volume and the occurrence of metastasis in a murine melanoma model (Carletto *et al*., 2016).

Due to the polymer wall and nanometric size, the structural organization of nanocapsules is essential for protecting drugs against photodegradation (Osmari *et al*., 2023). Notably, oily core-based vegetable sources play a pivotal role in drug photostabilization due to the antioxidant compounds (e.g., polyphenols, flavonoids, and fatty acids) in their composition. Pomegranate oil (PO) has a rich constitution of polyphenols, flavonoids, and fatty acids (primarily punic acid), given its antioxidant, anti-inflammatory, and antitumor activities (Lydia *et al*., 2020; Zare, Shaverdi, Kalaee, 2021). This vegetable oil has been used as the oily component of nanocarriers (nanoemulsions and nanocapsules), promoting increased drug photostability, controlled drug release, and improved biological effects (Ferreira *et al*., 2015; Marchiori *et al*., 2017a). Similarly, evidence has shown that PO can induce cellular apoptosis, inhibiting angiogenesis and the proliferation of various tumor cells (e.g., breast, prostate, and glioma cells) (Ferreira *et al*., 2015; Lydia *et al*., 2020). Finally, although DIM-loaded PO nanocapsules appear promising for cancer treatment, their association has not been explored to date.

Given this background, a nanocapsule suspension containing DIM and PO was designed to investigate the antitumor potential of both compounds. The formulations were characterized and evaluated using *in vitro* approaches to assess cytotoxic and antimetastatic potential. The antioxidant properties and safety, using alternative models, were also examined.

MATERIAL AND METHODS

Reagents

DIM (99.1% purity) was procured from Fagron (Brazil). Span® 80 (sorbitan monooleate), 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), 3(4,5-dimethyl)-2,5diphenyltetrazolium bromide (MTT), Dulbecco's Modified Eagle's Medium (DMEM), penicillin/streptomycin, 0.25% trypsin/ EDTA solution, and fetal bovine serum (FBS) were obtained from Sigma-Aldrich Co. (USA). Medium-chain triglycerides (MCT) and Tween® 80 (polysorbate 80) were purchased from Delaware (Porto Alegre, Brazil). Pomegranate oil was supplied by Florien (Brazil). Dimethyl sulfoxide (DMSO) was acquired from NEON (Brazil). Eudragit® RS100 (EUD) was generously donated by Evonik (São Paulo, Brazil). All other chemicals and solvents were analytical grade and utilized as received.

Methods

Pre-formulation studies

Preliminary steps involved assessing the solubility of DIM in the oil core of the nanocapsules and the compatibility between each oil and the polymeric material. Additionally, for comparative purposes, nanocapsules composed of MCT were prepared. To determine the DIM solubility in PO and MCT, an excess of DIM powder was incorporated into 2 mL of oil, maintained under magnetic stirring for 12 h, and then centrifuged at 3,000 rpm for 10 min. The resulting supernatant was diluted with methanol and quantified by HPLC using a previously validated methodology (Mattiazzi *et al*., 2019). The chromatographic instruments and conditions were as follows: an LC-10A HPLC system (Shimadzu, Japan) equipped with a SIL-20A HT valve sample automatic injector, a UV-VIS SPD-M20A detector, an LC-20AT pump, CBM-20A system controller, a guard column, and a Kinetex C_{18} Phenomenex column (250 x 4.60 mm, 5 µm; 100 Å).

For the polymer dissolution/swelling estimate, EUD films were obtained from a polymeric dispersion (2 g) in acetone and the subsequent evaporation of the solvent at room temperature. The films were weighed (80 mg) and soaked in the PO for 60 days (n = 3). The films were extracted from the oil at predetermined intervals and carefully dried with absorbent paper. The variation in weight was determined using an analytical balance.

Nanocapsule suspension preparation and characterization

Formulations (n = 6) were prepared utilizing the interfacial deposition of the preformed polymer method (Fessi *et al*., 1989). An organic phase containing EUD (1.0 w/v%), Span 80® (0.77 w/v%), MCT or PO (3.0 w/v%), acetone (50 mL), and DIM (0.1 w/v%) was maintained under magnetic stirring at 40 °C until complete solubilization of the constituents. The organic phase was then incorporated into a Tween 80® (0.77 w/v%) aqueous dispersion (50 mL) with continued magnetic stirring. Finally, the solvent was eliminated through evaporation under reduced pressure to achieve a volume of 10 mL, which corresponds to 1.0 mg/mL of DIM (NC-MCT-D or NC-PO-D). Non-loaded formulations (NC-MCT or NC-PO) were prepared using the same procedures.

After preparation, the samples were evaluated relative to pH, particle size, polydispersity index, zeta potential, drug content, and encapsulation efficiency (n = 6). The pH was ascertained with a calibrated potentiometer (pH 21 model, Hanna Instruments). Particle size and polydispersity index (PDI) were examined through photon correlation spectroscopy, whereas zeta potential was evaluated via microelectrophoresis utilizing the Zetasizer® Nanoseries (Malvern Instruments). Samples were diluted (1:500) using ultrapure water or a 10 mM NaCl solution before analysis.

Quantification of DIM within nanocapsules was conducted via HPLC, as specified in section 2.2.1. For the total DIM content, an aliquot (100 µL) from each sample (NC-MCT-D and NC-PO-D) was diluted in 10 mL of methanol to extract the drug. Subsequently, the samples were filtered through a 0.45 µm nylon membrane and introduced into the HPLC system. The encapsulation efficiency (EE%) was determined using the ultrafiltration/centrifugation technique per the following sequence: an aliquot (300 µL) of each sample was transferred into a centrifugal filter device (Amicon® Ultra, 10,000 MW, Millipore), and the free drug was separated from the nanostructures at 2200 ×g for 10 minutes. Non-nano-encapsulated DIM was determined in the ultrafiltrate, while the entrapped active compound was calculated as dictated by Equation 1.

$$EE\% = \frac{Total\ DIM\ content - Free\ DIM\ content}{Total\ DIM\ content} x100 \quad (1)$$

Photostability evaluation

Aliquots (700 µL) of nanocapsule suspensions and the DIM methanolic solution (1 mg/mL) were placed in transparent plastic cuvettes and positioned equidistantly inside a mirrored chamber (1 m x 25 cm x 25 cm) containing a Phillips TUV lamp—UVC, 30 W, the source of ultraviolet C light (n = 3). At predetermined intervals (0, 1, 2, 3, 4, 8, 12, 24, and 48 h), an aliquot of each sample (NC-MCT-D and NC-PO-D) was withdrawn,

and the DIM content was determined using the HPLC method (section 2.2.1). Dark controls (cuvettes shielded from light) were assessed simultaneously.

To determine the degradation kinetics of DIM, the data were fitted to zero-order (Equation 2) and first-order (Equation 3) equations by plotting concentration (μg/mL) against time (h) and the natural logarithm of concentration (μg/mL) against time (h), respectively. The correlation coefficient (*r*) and degradation rate constant (*k*) were determined from the equations. Moreover, the half-life was calculated using Equations 4 and 5 for zero order and first order, respectively.

$$C = C_0 - k_0.t \quad (2)$$

$$C = C_0.e^{-k.t} \quad (3)$$

$$t_{\frac{1}{2}} = \frac{0.5.C_0}{k_0} \quad (4)$$

$$t_{\frac{1}{2}} = \frac{0.693}{k} \quad (5)$$

Where: C is the concentration at t time, C_0 is the initial concentration, k is degradation rate constant, and $t_{1/2}$ is the half-life.

Antioxidant capacity determination

The samples were assessed at 0.1, 0.5, 1.0, 2.0, 4.0, and 6.0 μg/mL concentrations. Pure DIM and MCT were solubilized in methanol, whereas PO was solubilized in DMSO. All formulations and isolated constituents were diluted in distilled water to attain the desired concentrations for testing. The ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) radical scavenging capacity of the formulations was evaluated as described by Re and colleagues (1999). The ABTS radical cation solution (0.3763 mM) was prepared by combining the ABTS stock solution (7 mM) with potassium persulfate (140 mM) 12 hours prior to the assay (final ABTS concentration 42.7 μM). The samples were incubated with the ABTS solution for 30 minutes under light protection (n = 3). The absorbance was measured using a UV/Vis spectrophotometer (Shimadzu, Japan) at 734 nm (ABTS assay). Pure ABTS and ascorbic acid solutions were negative and positive controls, respectively. The experiment was carried out in triplicate (n = 3), and the radical scavenging activity was indicated as a percentage of scavenging capacity, as follows (Equation 6).

$$SC\% = 100 - \frac{(ABS - ABB)\ X\ 100}{ABC} \quad (6)$$

where SC% is the scavenging capacity in percentage, ABS represents the absorbance of the incubated sample with ABTS, ABB stands for the blank sample absorbance, and ABC is the negative control absorbance. The ferric reducing antioxidant power (FRAP) assay was done using the protocol suggested by Benzie and Strain (1996), although there were some modifications. The samples were kept in a 290 μL of FRAP working solution, which contained 25 volumes of 300 mM acetate buffer pH 3.6, 2.5 volumes of 10 mM 2,4,6-tripyridyl-s-triazine, and 2.5 volumes of 20 mM $FeCl_3 \cdot 3H_2O$, at 37 °C for 60 minutes. At the end of the experiment, the product was measured under a spectrophotometer at 630 nm. Distilled water and ascorbic acid were used as the negative and positive controls. The assay was done in triplicate (n = 3), with the results expressed as μM Fe^{2+}/mL, utilizing a curve of $FeSO_4.7\ H_2O$ (20 mM) as the analytical standard.

Cell cytotoxicity

Murine fibroblast (L929) and melanoma (B16F10) cell lines were acquired from the Banco de Células do Rio de Janeiro (BCRJ). These cell lines were grown and maintained in a low-glucose Dulbecco's modified Eagle's (DMEM) medium, supplemented with 10% (v/v) fetal bovine serum (FBS) and penicillin/streptomycin (100 U/L). The cells were nurtured in a humidified atmosphere containing 5% CO_2 at 37 °C.

The cell viability was determined using the MTT assay (Mosmann, 1983). To do so, approximately 4 x 10^5 cells were seeded in 96-well plates. The plates were then incubated at 37 °C, within a humidified environment of 5% CO_2, for 24 hours and then earmarked for testing. Initially, concentrated DIM or PO stock solutions in

DMSO were prepared and subsequently diluted in DMEM to meet the desired treatment concentrations.

The formulations used for the exploration of cytotoxicity were prepared under aseptic conditions, making provisions to prevent contamination by microorganisms, as suggested in studies that discuss the sterilization possibilities for nanoformulations (Patel, Zode, Vansal, 2020; Bernal-Chávez *et al*., 2021). This process includes the initial sterilization of glassware, disinfection of equipment, and the preparation environment, as well as utilizing personal protective equipment such as caps, gloves, and masks to forestall the contamination of the formulations. All the formulations and isolated constituents were diluted in DMEM to attain the desired concentrations, corresponding to 1, 2, 4, 6, 12, 18, and 24 µg/mL of DIM.

The treatment and DMSO concentrations in DMEM (0.01%) were chosen based on previous studies (Mattiazzi *et al*., 2019; da Silva *et al*., 2022). DMEM (negative control) was also tested. The cells underwent the treatments outlined for 24 hours and were subsequently incubated with 20 µL of MTT for 4 hours at 37 °C. The formazan crystals formed were solubilized using 200 µL of DMSO and assessed as an absorbance at 570 nm. These assays were conducted in quintuplicate, and the cell viability of the treated groups was calculated relative to the control culture with DMEM (negative control), which symbolizes maximum viability (100%). IC_{50} values were calculated utilizing GraphPad Prism® software (version 6). The selectivity index (SI) was calculated by dividing the IC_{50} value obtained in a healthy cell by the IC_{50} value in a tumor cell (Widiandani *et al*., 2023).

Antimetastatic effect against murine melanoma cells

Based on B16F10 cytotoxicity data, a concentration of 4 µg/mL was selected to assess the antimetastatic effect of DIM (nanoencapsulated or not) on this cell line. This concentration allows us to observe the influence of treatments in modifying the malignancy of cells without inducing cell death (da Silva *et al*., 2022). In the cell adhesion assay, melanoma cells (B16F10) were seeded at 2 x 10^5 cells/well in 24-well plates and incubated with the samples for six hours at 37 °C. After the medium was discarded, the non-adhered cells were removed, and the remaining cells were stained with 500 µL of 0.5 % (m/v) crystal violet for 30 minutes at 37 °C. Then, the dye was discarded, and the cells were solubilized with 1% sodium dodecyl sulfate (m/v). Finally, optical density was measured at 570 nm using a microplate reader to analyze cell adhesion (da Silva *et al*., 2022). Data were collected in triplicate and expressed in terms of absorbance.

The capacity for colony development was assessed using a clonogenic assay. Melanoma cells (B16F10) were seeded in 6-well plates at 500 cells per well. The treatments were added and then incubated for five days. Subsequently, cells were stained with crystal violet and the colonies formed were quantified using ImageJ software. The assay was performed in triplicate, with the data expressed based on the number of colonies formed (da Silva *et al*., 2022).

The scratch wound assay evaluated cell migration, as outlined by Bürk (1973). Initially, 3 x 10^5 melanoma cells (B16F10) were seeded in 1 mL DMEM medium with 10% FBS in 24-well plates. Once a confluent layer was established, a straight wound was created by pressing the tip of a pipette tip, guided by a ruler in parallel along the diameter of the well. The suspended cells were gently aspirated, and the wells were washed with PBS. Then, the cells were treated with fresh DMEM containing the various treatments, without serum, to avoid proliferation interference. The wound's area was photographed before and after incubation for 20 hours and was determined using the ImageJ software. Cell migration was measured with reference to the negative control wound (DMEM) area, defined as 100% cell migration.

Cytotoxicity and genotoxicity evaluations

The cytotoxicity and genotoxicity of the formulations were evaluated using the *Allium cepa* test (Osmari *et al*., 2023). Twenty-four *A. cepa* bulbs were divided into eight groups, each consisting of three onions, for each treatment: (1) distilled water (negative control), (2) glyphosate 7.5 µg/mL (positive control), (3) vehicle (aqueous dispersion of polysorbate 80 at 5%), (4) non-nanoencapsulated DIM at 1 mg/mL in the vehicle, (5) NC-MCT, (6) NC-PO, (7) NC-MCT-D, and (8) NC-PO-D.

Each bulb, across all treatments, was rooted in distilled water for three days. After this, they were subjected to their respective treatment for 24 hours. The negative control group remained in distilled water. After a 24-hour treatment period, the bulbs were preserved in a 3:1 (v/v) ethanol: acetic acid solution for 24 hours. Subsequently, they were placed in 70% (v/v) ethanol and refrigerated at 4 ± 2 °C until the analysis. For microscopic examination, the meristematic region was cut and hydrolyzed with 0.2 M HCl for 5 minutes. After this, the excess HCl was removed, and the root tips were stained with 2% (w/v) acetic orcein under pressure from a glass stick. Two slides were prepared for each onion, using two root tips. These slides were analyzed using optical microscopy at 40x magnification, and the number of interphase, prophase, metaphase, anaphase, and telophase cells were observed. In addition, any chromosomal aberrations were assessed. For each group, 6,000 cells were analyzed (2,000 cells per onion). Using this data, the mitotic index (MI%) and chromosomal aberrations index (CA%) were calculated and expressed as percentages, according to Equations 7 and 8, respectively.

$$MI\% = \frac{Total\ of\ cells\ in\ mitosis}{Total\ of\ cells\ observed} x100 \quad (7)$$

$$CA\% = \frac{Number\ of\ anomalous\ cells}{Total\ of\ cells\ observed} x100 \quad (8)$$

Irritant potential evaluation

The Hen's Egg-Chorioallantoic Membrane test (HET-CAM) was conducted in accordance with the methodology recommended by the Interinstitutional Committee for Validation of Alternative Methods (ICCVAM, 2010). The experimental study was approved by the Research Ethics Committee of the Federal University of Santa Maria (Rio Grande do Sul State, southern Brazil) and was registered under CEUA No. 5428271020. Initially, the outermost shell and the white membrane of fertilized hen's eggs were removed after ten days of incubation (37 °C and 65% relative humidity). Three hundred microliters of each formulation were directly applied to the chorioallantoic membranes (CAM) (n = 4/formulation). After 20 seconds, the samples were rinsed with a 0.9% NaCl solution, and CAM reactions, such as vasoconstriction, hemorrhage, and coagulation, were monitored over 300 seconds. The samples (NC-MCT, NC-MCT-D, NC-PO, and NC-PO-D) were compared to non-nanoencapsulated materials (MCT, PO, and DIM in an aqueous solution with 10% of Tween® 80 and 10% of DMSO). The positive (0.1 M NaOH) and negative (0.9% NaCl solution) controls were also evaluated. The IS was calculated using Equation 9.

$$CA\% = \frac{Number\ of\ anomalous\ cells}{Total\ of\ cells\ observed} x100 \quad (9)$$

where h is hemorrhage time, v is vasoconstriction time, and c is coagulation time. From the IS values obtained, the lesions were classified as non-irritant (0–0.9), slightly (1–4.9), moderate (5–8.9), and severe irritant (9–21).

Statistical analysis

The results were expressed as the mean ± standard deviation (SD) or standard error of the mean (SEM). The data distribution was assessed using the D'Agostino Pearson normality test. Depending on the experimental design, the results were statistically analyzed through a one-way or two-way analysis of variance (ANOVA), followed by the Newman-Keuls post-hoc test. A p-value of less than 0.05 was considered statistically significant. All statistical analyses and the production of figures were conducted using the GraphPad Prism® version 6 statistical software program.

RESULTS

Pre-formulation studies

Phytochemical solubility in MCT and PO was 17.9 ± 0.8 and 6.0 ± 0.2 mg/mL, respectively ($p < 0.05$). Regarding the polymer dissolution/swelling, after 60 days, the weight of the polymer films was similar to their initial value (W_0: 83.6 ± 3.1 mg; W_{60}: 84.2 ± 3.3 mg) ($p > 0.05$).

Characterization of nanocapsule suspensions

All formulations exhibited a white/bluish opalescent appearance without visible precipitation. The results of the physicochemical characterization are outlined in Table I. The suspensions demonstrated pH values within the acidic range (4.7–4.8) and a positive zeta potential (+7.0 – +8.1 mV). The structures featured an unimodal particle distribution (PDI > 0.12) and size within the nanometric range (<80 nm). PO-based formulations were larger than those prepared with MCT ($p < 0.05$). Both DIM-containing formulations boasted high encapsulation efficiency (98.5%) and phytochemical content nearing the theoretical value (1 mg/mL).

TABLE I - Physicochemical characterization of the nanocapsule suspensions

	Formulations			
	NC-MCT	**NC-PO**	**NC-MCT-D**	**NC-PO-D**
pH	4.8 ± 0.1	4.7 ± 0.1	4.7 ± 0.1	4.7 ± 0.1
Mean diameter (nm)	165 ± 2	176 ± 3***	162 ± 3	175 ± 3###
Polydispersity index	0.11 ± 0.01	0.12 ± 0.03	0.10 ± 0.01	0.11 ± 0.02
Zeta potential (mV)	+7.4 ± 1.4	+7.0 ± 1.4	+8.1 ± 1.0	+7.0 ± 1.0
Drug content (mg/mL)	-	-	0.94 ± 0.01	0.97 ± 0.02
Encapsulation efficiency (%)	-	-	98.5 ± 0.9	98.5 ± 1.2

Mean ± standard deviation ($n = 6$). One-way ANOVA followed by Newman-Keuls. (***) $p < 0.001$: significant difference between NC-MCT and NC-PO. (###) $p < 0.001$: significant difference between NC-MCT-D and NC-PO-D. **Abbreviations:** NC-MCT: medium-chain triglyceride nanocapsules; NC-PO: pomegranate oil nanocapsules; NC-MCT-D: medium-chain triglyceride nanocapsules containing 3,3'-diindolylmethane; NC-PO-D: pomegranate oil nanocapsules containing 3,3'-diindolylmethane.

Photostability study

After an 8-hour exposure to UVC radiation, the methanolic solution displayed less than 10% of the residual DIM concentration (6.6 ± 4.4%). Simultaneously, the nanocapsules exhibited values approximately 12-fold higher ($p < 0.05$), revealing 77.6 ± 3.6% and 82.3 ± 4.9% for NC-MCT-D and NC-PO-D of DIM remaining content, respectively (Figure 1). Because of these higher DIM remaining contents in nanocapsules, the experiment was prolonged for up to 48 hours, which indicated similar residual DIM content for NC-MCT-D (35.1 ± 6.7%) and NC-PO-D (42.9 ± 1.2%) ($p > 0.05$).

Based on the correlation coefficient (r), the DIM methanolic solution adhered to zero-order degradation kinetics, and the formulations were more compatible with first-order degradation kinetics. The obtained half-life values demonstrated that the non-nanoencapsulated DIM had a half-life of 4.28 ± 0.10 hours, while the nanocapsules exhibited values roughly 8.5 and 9.5-fold higher ($p < 0.001$) for NC-MCT-D and NC-PO-D, respectively. Furthermore, the PO core resulted in a longer half-life for NC-PO-D (40.88 ± 1.42 hours) when compared to NC-MCT-D = 36.49 ± 3.33 hours ($p < 0.05$) (Table II).

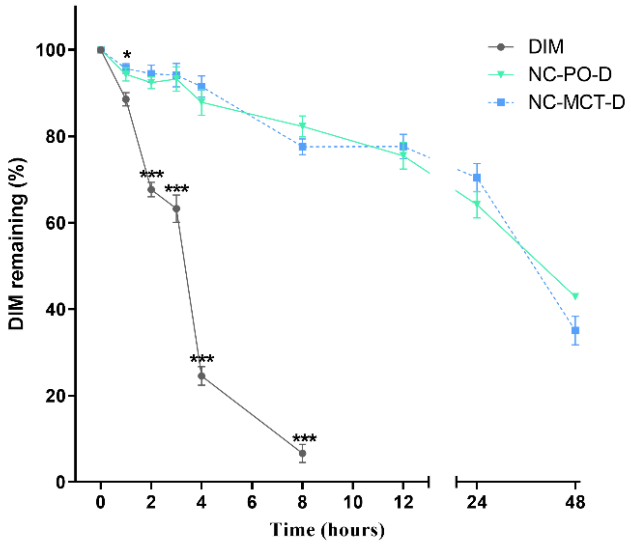

FIGURE 1 - Remaining concentration of DIM from nanocapsules and methanolic solution after UVC radiation exposure. Mean ± standard deviation (n = 3). Two-way ANOVA followed by the Newman-Keuls. (*) $p < 0.05$, (***) $p < 0.001$: significant difference between non-nanoencapsulated DIM and nanocapsule suspensions (NC-MCT-D and NC-PO-D).

TABLE II - Parameters obtained in the UV-C degradation kinetics study

	Correlation coefficient (r)	
	Zero-order	**First-order**
DIM	**0.9846 ± 0.0021**	0.9532 ± 0.002
NC-MCT-D	0.9334 ± 0.0111	**0.9613 ± 0.0149**
NC-PO-D	0.9339 ± 0.0144	**0.9739 ± 0.0078**
	Degradation constant (k)	**Half-life ($t_{½}$)**
DIM	166.787 ± 2.687 µg/mL/h	4.28 ± 0.10 h***
NC-MCT-D	0.019 ± 0.001 h^{-1}	36.49 ± 3.33 h
NC-PO-D	0.017 ± 0.001 h^{-1}	40.88 ± 1.42 h #

Mean ± standard deviation (n = 3). One-way ANOVA followed by Newman-Keuls. (***) $p < 0.001$: significant difference between methanolic solution DIM and nanocapsules. (#) $p < 0.05$: significant difference between NC-MCT-D and NC-PO-D. Abbreviations: DIM: 3,3'-diindolylmethane; NC-MCT-D: medium-chain triglyceride nanocapsules containing 3,3'-diindolylmethane; NC-PO-D: pomegranate oil nanocapsules containing 3,3'-diindolylmethane.

Antioxidant capacity determination

The pure and both DIM formulations exhibited the highest radical scavenging properties compared to other samples tested (Figure 2A). Generally, nanocapsules demonstrated superior or equal performance in neutralizing ABTS radicals compared to non-nanoencapsulated DIM ($p < 0.05$). At intermediate concentrations (1 and 2 µg/mL), NC-PO-D (56.1 and 87.2%) displayed greater scavenging activity than NC-MCT-D (45.3 and 79.0%) and pure DIM

(45.2 and 77.7%) ($p < 0.05$). The oils and non-loaded formulations indicated that nanoencapsulation enhanced radical scavenging capacity for both MCT and PO ($p < 0.05$). The NC-PO demonstrated more substantial activity than pure PO (2.8 and 4.6%) ($p < 0.001$) and NC-MCT (9.8 and 15.7%) ($p < 0.01$).

Regarding the FRAP assay, DIM nanoencapsulation improved its antioxidant potential. Notably, while non-nanoencapsulated DIM showed slight FRAP potential, NC-PO-D and NC-MCT-D presented superior performance in the 1–6 µg/mL interval ($p < 0.001$; Figure 2-B). Significantly, the formulation composed by PO (3529 ± 129 µM/mL) demonstrated superior performance in comparison to the NC-MCT-D (3105 ± 258 µM/mL) ($p < 0.05$). Pure oils and unloaded formulations presented no or discrete antioxidant potential ($p > 0.05$).

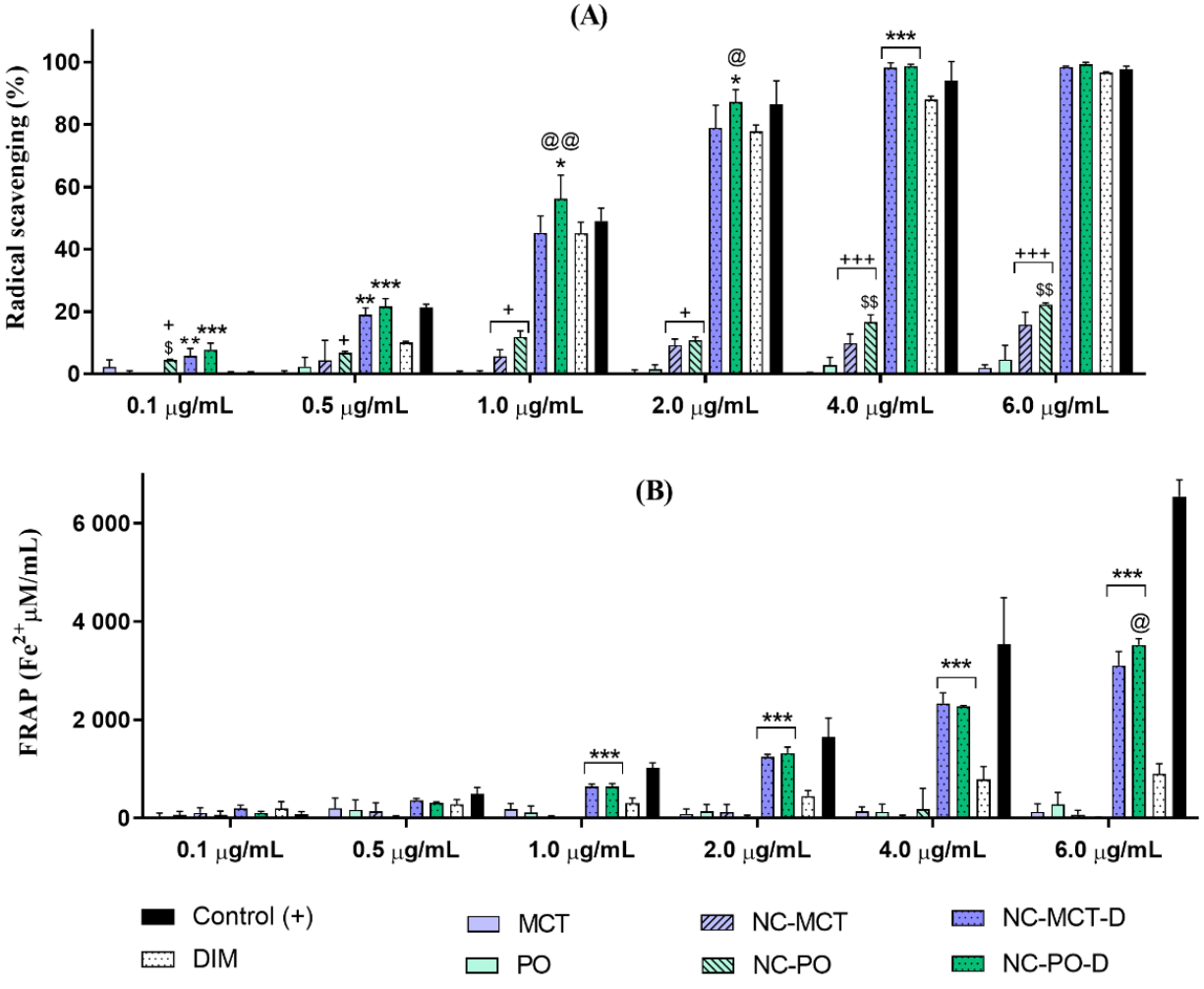

FIGURE 2 - Antioxidant potential of non-nanoencapsulated DIM, pure oils, DIM-loaded nanocapsules, and non-loaded nanocapsules by ABTS (A) and FRAP (B). Mean ± standard deviation (n = 3). One-way ANOVA followed by Newman-Keuls. (*) $p < 0.05$, (**) $p < 0.01$, (***) $p < 0.001$: significant difference between non-nanoencapsulated DIM and DIM-loaded nanocapsules. (@) $p < 0.05$, (@@) $p < 0.01$: significant difference between NC-MCT-D and NC-PO-D. ($) $p < 0.05$, ($$) $p < 0.01$: significant difference between NC-MCT and NC-PO. (+) $p < 0.05$, (+++) $p < 0.001$: significant difference between non-nanoencapsulated oils and non-loaded nanocapsules.

Cell cytotoxicity

Non-nanoencapsulated DIM significantly diminished fibroblast viability (L929) in a concentration-dependent manner compared to the control group ($p < 0.05$; Figure 3). The cytotoxic effect was diminished by DIM nanoencapsulation into NC-MCT-D ($p < 0.05$), but not by NC-PO-D ($p > 0.05$), findings that were confirmed through IC_{50} values (6.17, >24, and 6.49 µg/mL, respectively; Table III). At the highest concentration level (24 µg/mL), all samples induced statistically significant cytotoxic effects on L929 cells ($p < 0.05$).

The evaluation of cytotoxicity against B16F10 cells showed that non-nanoencapsulated DIM exhibited significant cytotoxicity only at the highest concentrations (18 and 24 µg/mL). However, DIM-containing nanocapsules induced a cytotoxic effect at lower concentrations ($p < 0.05$; Figure 4). Remarkably, NC-MCT-D exhibited significant cytotoxicity at 12 µg/mL ($p < 0.05$). Simultaneously, NC-PO-D demonstrated a cytotoxic effect starting from half of the active concentration of its counterpart formulation (6 µg/mL) ($p < 0.05$). Calculated IC_{50} values supported these findings, indicating that nanoencapsulation enhances DIM's antitumoral action against B16F10 cells (Table III). Values of 0.34, 1.03, and 0.74 were observed for SI for free DIM, NC-PO, and NC-PO-D, respectively. This suggests that the nanoencapsulation of DIM in nanocapsules composed of PO resulted in a slight increase in selective toxicity for tumor cells.

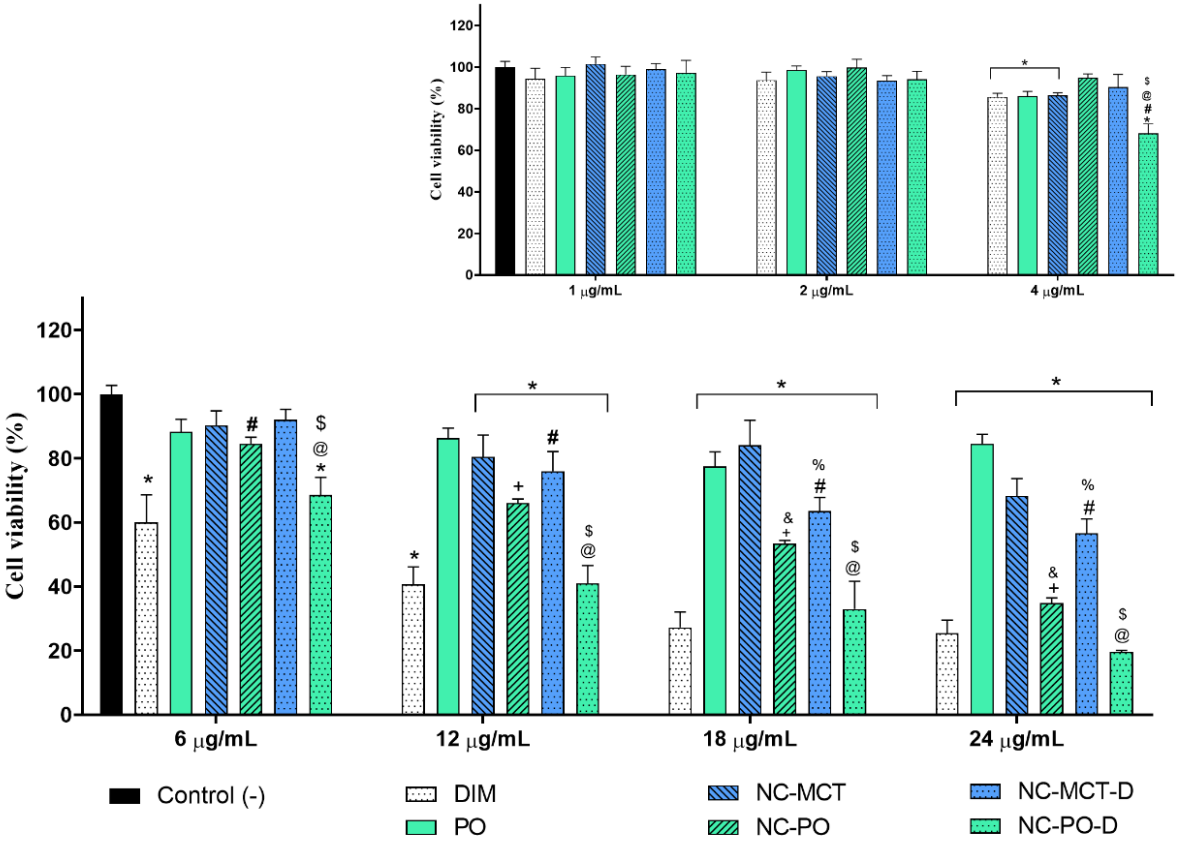

FIGURE 3 - The cytotoxic effect of non-nanoencapsulated DIM, PO, DIM-loaded nanocapsules, and non-loaded nanocapsules against murine fibroblast cells (L929) was studied. The results are presented as the mean ± standard deviation ($n = 5$). A one-way ANOVA test followed by Newman-Keuls post hoc test was used for comparison. (*) $p < 0.05$: significant difference between control and the other groups. (+) $p < 0.05$: significant difference between PO and NC-PO. (#) $p < 0.05$: significant difference between non-nanoencapsulated DIM and DIM-loaded nanocapsules. (&) $p < 0.05$: significant difference between NC-MCT and NC-PO. (%) $p < 0.05$: significant difference between NC-MCT and NC-MCT-D. ($) $p < 0.05$: significant difference between NC-PO and NC-PO-D. (@) $p < 0.05$: significant difference between NC-MCT-D and NC-PO-D.

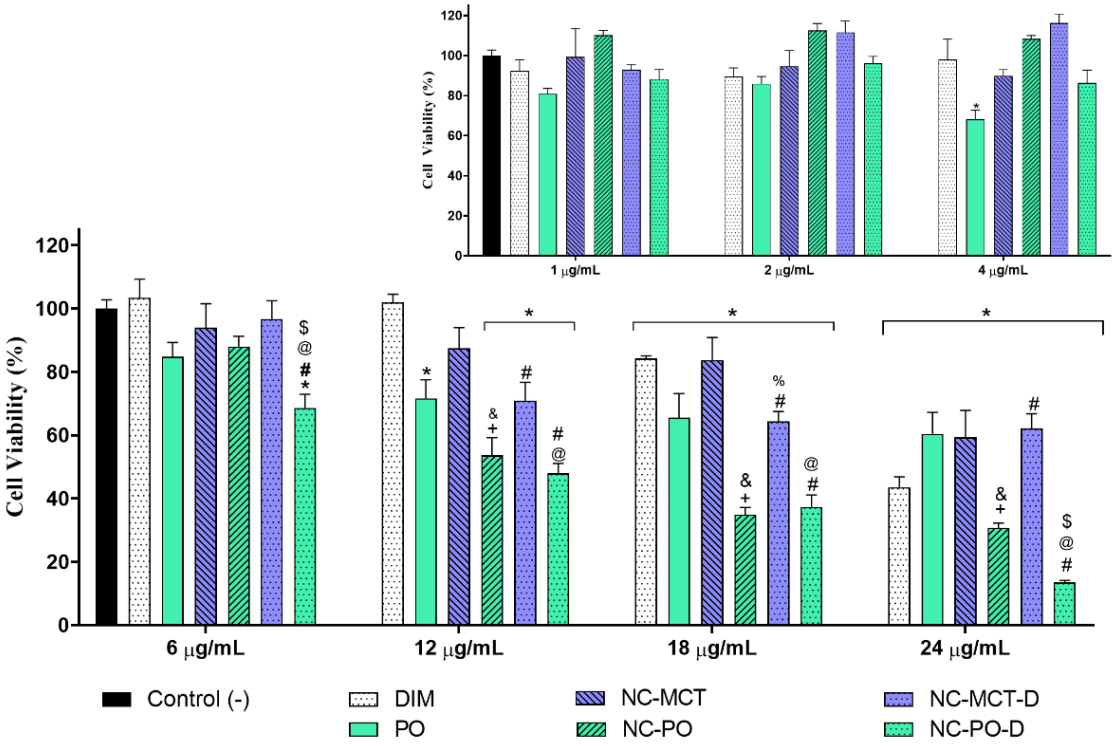

FIGURE 4 - The cytotoxic effect of non-nanoencapsulated DIM, PO, DIM-loaded nanocapsules, and non-loaded nanocapsules against murine melanoma cells (B16F10) was tested. The data are represented as mean ± standard deviation (n = 5). The statistical analysis was carried out using one-way ANOVA followed by the Newman-Keuls post hoc test. (*) $p < 0.05$: significant difference between the control and the remaining groups. (+) $p < 0.05$: significant difference between PO and NC-PO. (#) $p < 0.05$: significant difference between non-nanoencapsulated DIM and DIM-loaded nanocapsules. (&) $p < 0.05$: significant difference between NC-MCT and NC-PO. (%) $p < 0.05$: significant difference between NC-MCT and NC-MCT-D. ($) $p < 0.05$: significant difference between NC-PO and NC-PO-D. (@) $p < 0.05$: significant difference between NC-MCT-D and NC-PO-D.

TABLE III - IC_{50} values obtained after different treatments for 24 h in different cell lines

	IC_{50} (μg/mL)	
	L929	**B16F10**
DIM	6.17	18.41
PO	> 24.00	> 24.00
NC-MCT	> 24.00	> 24.00
NC-PO	10.85	8.32
NC-MCT-D	> 24.00	> 24.00
NC-PO-D	6.49	8.71

Abbreviations: PO - pomegranate oil; DIM: 3,3'-diindolylmethane; NC-MCT: medium-chain triglyceride nanocapsules; NC-PO: pomegranate oil nanocapsules; NC-MCT-D: medium-chain triglyceride nanocapsules containing 3,3'-diindolylmethane; NC-PO-D: pomegranate oil nanocapsules containing 3,3'-diindolylmethane.

Antimetastatic effect against murine melanoma cells

The antimetastatic potential was investigated through adhesion, migration, and colony formation tests. The NC-PO, NC-MCT-D, and NC-PO-D diminished cell adhesion ($p < 0.05$; Figure 5). In relation to the colony formation assay, although all treatments decreased the incidence of cell colonies, non-nanoencapsulated DIM exhibited superior performance in this test ($p < 0.05$; Figure 6). A lower number of colonies were observed for NC-PO-D than NC-MCT-D ($p < 0.05$). The cell migration test revealed that only the DIM-loaded PO nanocapsules reduced cell mobility compared to the control group ($p < 0.05$; Figure 7).

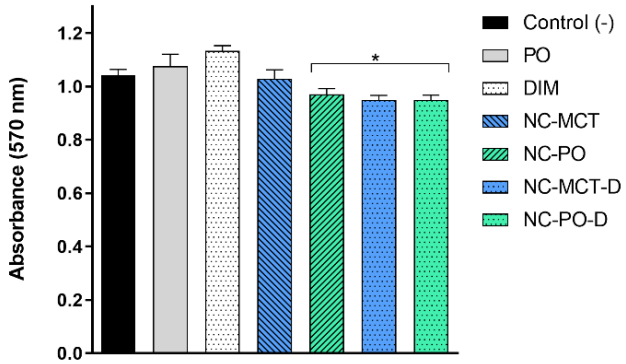

FIGURE 5 - Evaluation of adhesion potential in murine melanoma cells (B16F10): mean ± standard deviation (*n* = 3). One-way ANOVA followed by Newman-Keuls. (*) $p < 0.05$: significant difference between the control and the other groups.

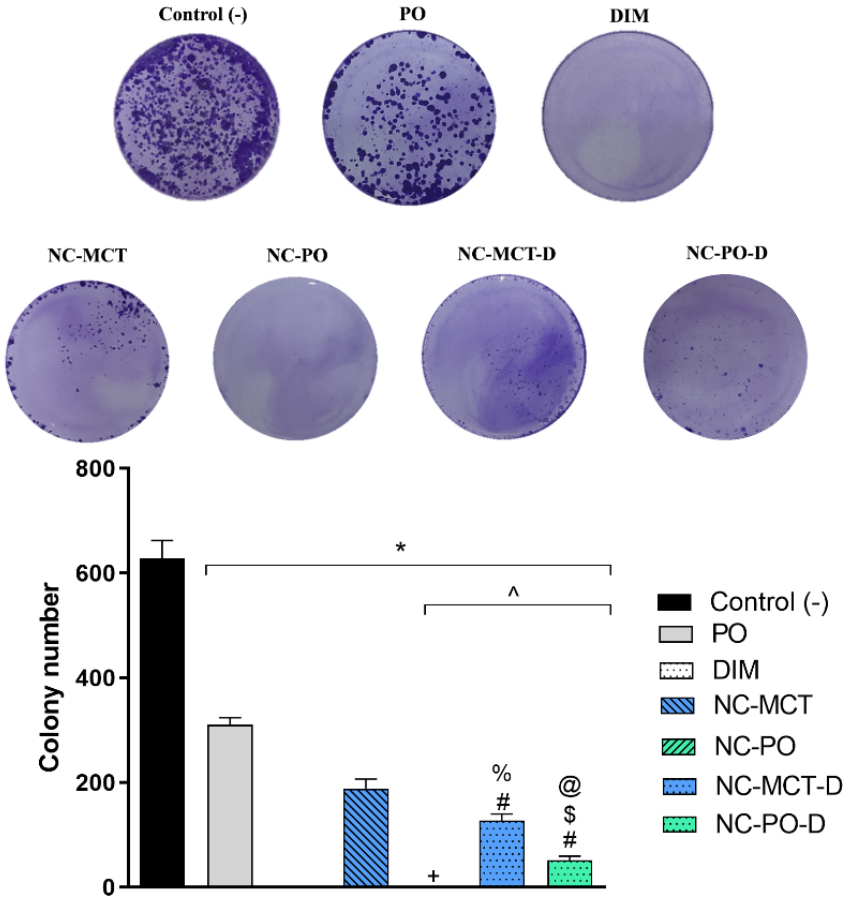

FIGURE 6 - Evaluation of colony formation in murine melanoma cells (B16F10). Mean ± standard deviation (*n* = 3). One-way ANOVA followed by Newman-Keuls multiple comparisons test was used. (*) $p < 0.05$: significant difference between the control group and the other groups. (+) $p < 0.05$: significant difference between PO and NC-PO. (^) $p < 0.05$: significant difference between NC-MCT and the other nanocapsules. (#) $p < 0.05$: significant difference between non-nanoencapsulated DIM and DIM-loaded nanocapsules. (%) $p < 0.05$: significant difference between NC-MCT and NC-MCT-D. ($) $p < 0.05$: significant difference between NC-PO and NC-PO-D. (@) $p < 0.05$: significant difference between NC-MCT-D and NC-PO-D.

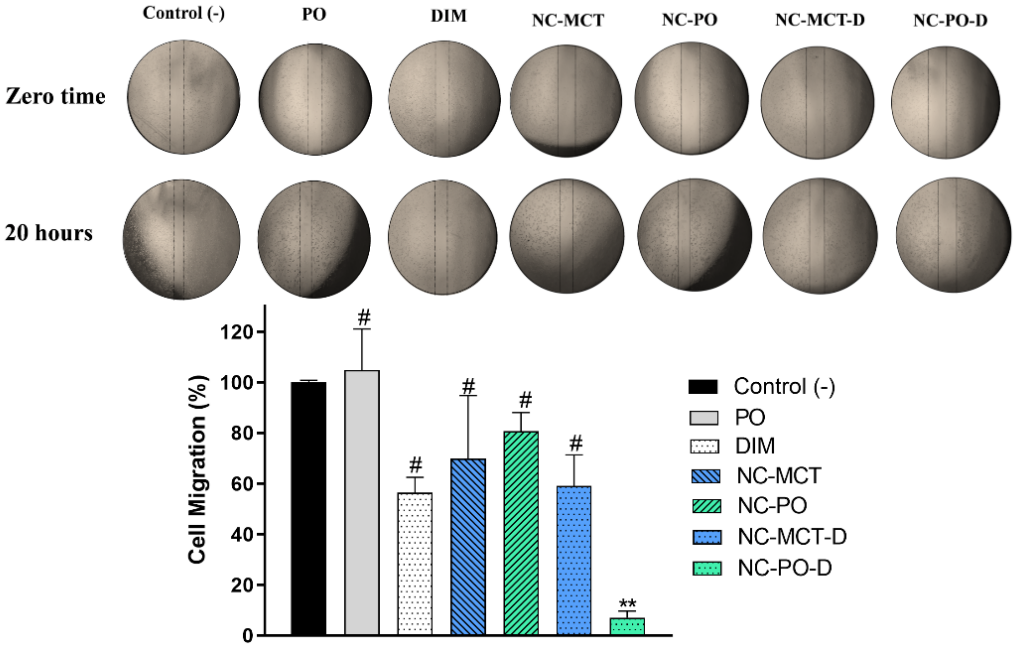

FIGURE 7 - Evaluation of migration in murine melanoma cells (B16F10). Mean ± standard deviation (n = 3). One-way ANOVA followed by Newman-Keuls. (**) $p < 0.01$: significant difference between control and the other groups. (#) $p < 0.05$: significant difference between NC-PO-D and the other groups.

Cytotoxicity and Genotoxicity by *Allium cepa* assay

Excepting the NC-MCT, which caused an increase in MI% (8.6 ± 0.7%; $p < 0.05$), the treatments presented MI% similar to that of the negative control group ($p > 0.05$). As anticipated, the glyphosate group (positive control) experienced a significant reduction in MI% (2.0 ± 0.2%) (Figure 8A; $p < 0.001$). With regard to genotoxicity, both non-nanoencapsulated DIM and NC-MCT-D led to a significant increase in CA% compared to the negative control (Figure 8B; $p > 0.01$). Neither NC-PO-D nor non-loaded nanocapsules showed this effect ($p > 0.05$). The positive control group exhibited a CA% higher than that of the negative control and all other treatments ($p < 0.001$). In accordance with these findings, Table IV presents the types and frequency of chromosomal aberrations observed for each group, with metaphase being the most frequent event in DIM, NC-TCM-D, and positive control groups. Figure 9 illustrates the various phases of mitosis and images of the chromosomal aberrations found in this study.

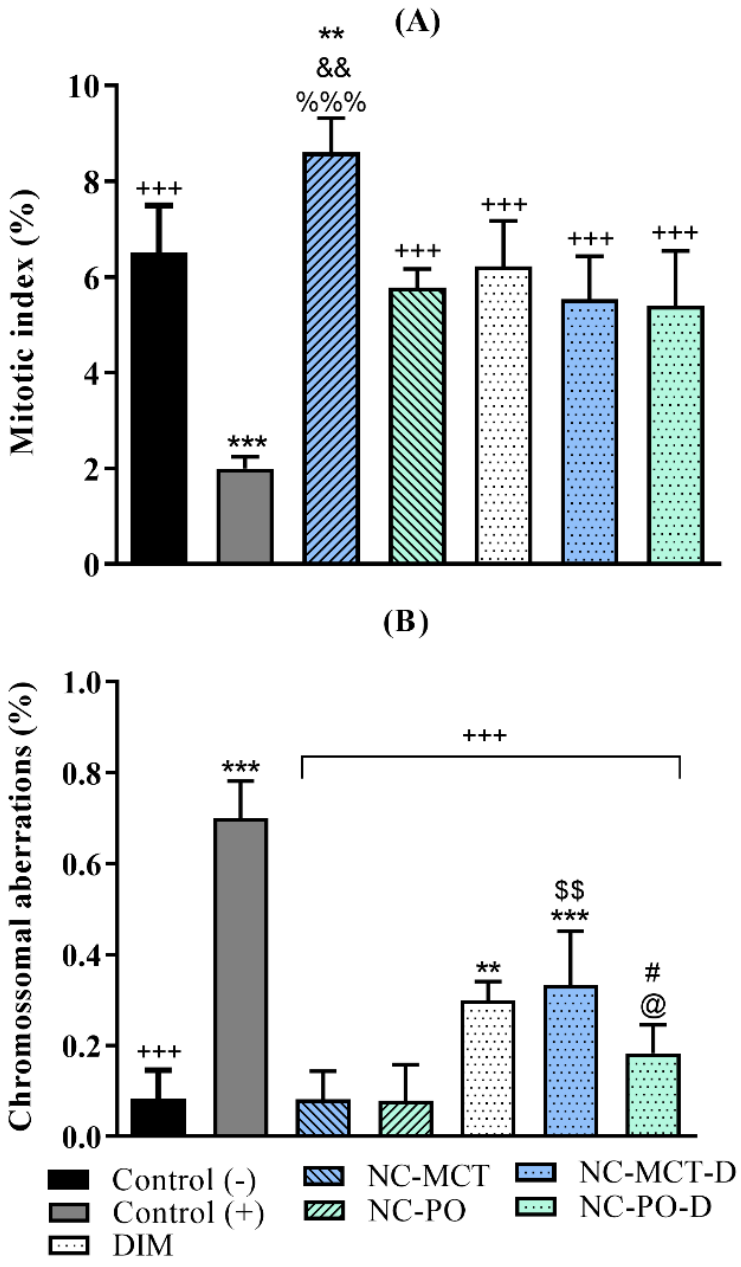

FIGURE 8 - Mitotic index (A) and chromosomal aberrations index (B) obtained for the different treatments by the *Allium cepa* assay. Mean ± standard deviation (n = 3). One-way ANOVA followed by Newman-Keuls. (**) $p < 0.01$ (***) $p < 0.001$: significant difference between negative control and the other groups. (+++) $p < 0.001$: significant difference between positive control and the other groups. (%%%) $p < 0.001$: significant difference between NC-MCT and the other nanocapsules. (&&) $p < 0.01$: significant difference between NC-MCT and non-nanoencapsulated DIM. ($$) $p < 0.01$: significant difference between NC-MCT-D and NC-MCT. (#) $p < 0.05$: significant difference between non-nanoencapsulated DIM and DIM-loaded nanocapsules. (@) $p < 0.05$: significant difference between NC-MCT-D and NC-PO-D.

TABLE IV - Total aberrant cells in *Allium cepa* exposed to different treatments (6000 cells observed for each group)

	Chromosome aberrations				
	PD	**MD**	**AB**	**SA**	**BN**
Negative control	0	3	0	0	2
Positive control	1	25	4	12	0
Vehicle	0	6	0	0	0
DIM	0	14	1	3	0
NC-MCT	1	5	0	0	0
NC-PO	0	8	1	0	0
NC-MCT-D	0	12	0	2	6
NC-PO-D	0	7	0	0	7

Abbreviations: PD: disturbed prophase; MD: disturbed metaphase; AB: anaphase bridge; AS: sticky anaphase; BN: Binucleation; DIM: 3,3'-diindolylmethane; NC-MCT: medium-chain triglyceride nanocapsules; NC-PO: pomegranate oil nanocapsules; NC-MCT-D: medium-chain triglyceride nanocapsules containing 3,3'-diindolylmethane; NC-PO-D: pomegranate oil nanocapsules containing 3,3'-diindolylmethane.

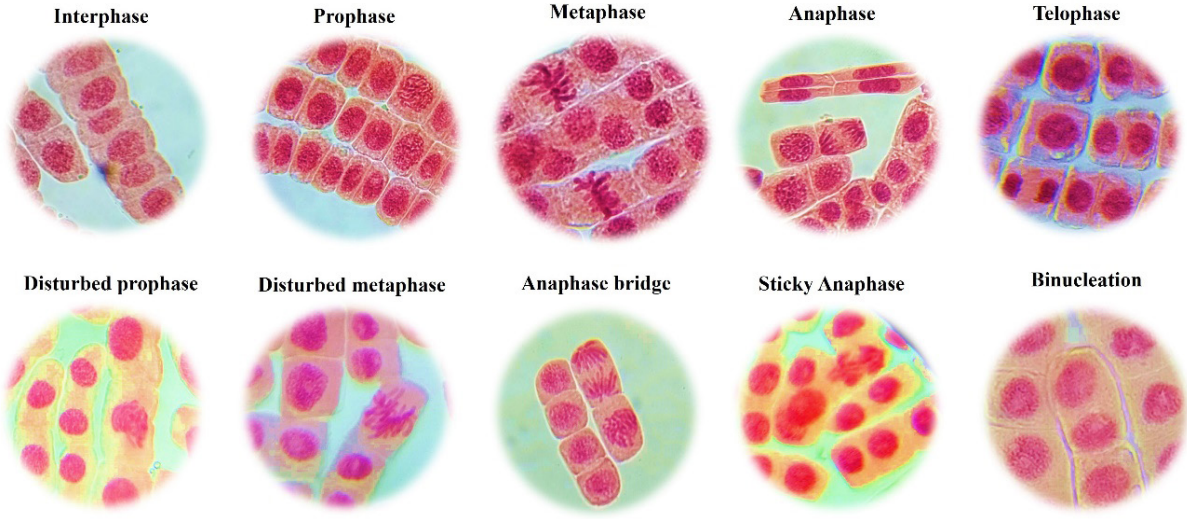

FIGURE 9 - Representative image of cells in different phases of mitosis and chromosomal aberrations observed by the *Allium cepa* assay.

Irritant potential assessment of the formulations by HET-CAM assay

The test results indicated that neither the formulations nor their constituents caused any vasoconstriction, bleeding, or coagulation following a 300-second CAM exposure (Figure 10). The positive control used for this test, 0.1 M NaOH, resulted in the coagulation of the vessels and registered an SI of 8.0 ± 0.1, categorizing it as a moderate irritant.

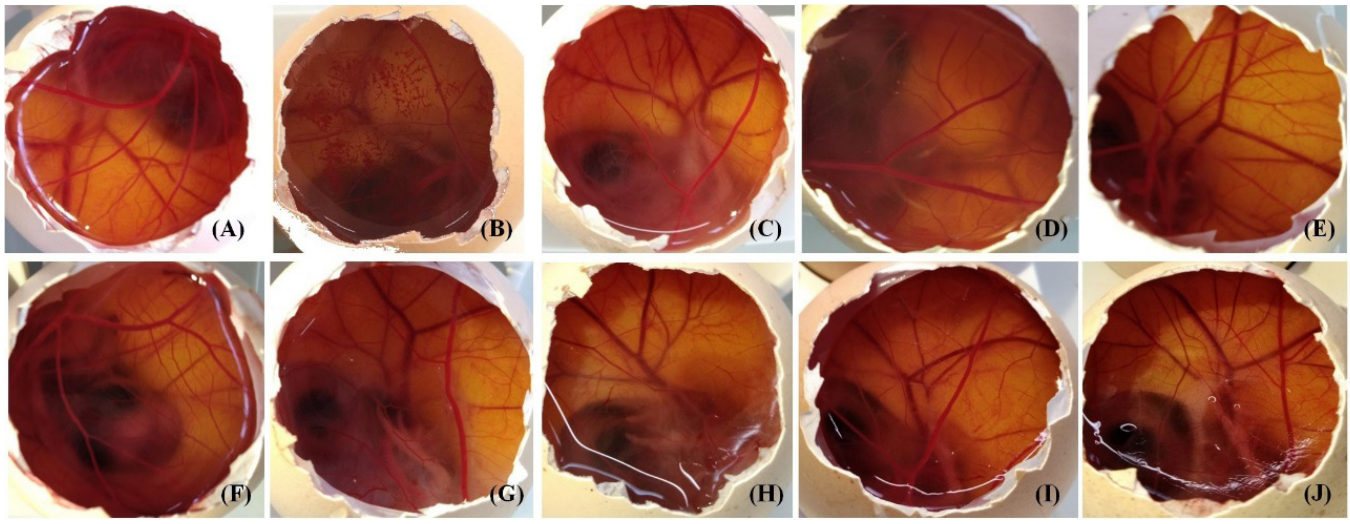

FIGURE 10 - Images of the chorioallantoic membrane (CAM) after the application of saline solution - negative control (A), 0.1 M NaOH: positive control (B), vehicle: aqueous dispersion containing 10% polysorbate 80 and 10% DMSO (C), MCT (D), PO (E), non-nanoencapsulated DIM (F), NC-MCT (G), NC-MCT-D (H), NC-PO (I) and NC-PO-D (J).

DISCUSSION

Compounds of natural origin have been extensively investigated for their therapeutic potential in treating various diseases (Kim *et al*., 2019; Marchiori *et al*., 2017a; Gomes *et al*., 2018). DIM, a phytochemical, has exhibited various therapeutic benefits against several types of cancer. However, its use in conventional dosage forms is limited due to its unfavorable physicochemical characteristics (Patel *et al*., 2012). Our data collectively suggest that the association of DIM with PO-based nanocapsules enhances its photostability and scavenger properties, mitigates its genotoxicity, and significantly potentiates its antitumor action. Remarkably, the results also suggest that using PO as the oily core positively influences the general physicochemical and biological properties of formulations.

Pre-formulation studies assist in estimating the compatibility among the constituents of nano-based formulations, ensuring the proper formation of the core-shell structure. In this context, EUD and PO are compatible materials for composing a nanocarrier system, and DIM's solubility in PO underscores the optimal design of formulations. After 60 days, no significant change in the weight of the EUD polymeric films was observed under PO contact, corroborating previous studies regarding EUD's compatibility with synthetic or vegetable oils (Contri *et al*., 2013; Gomes *et al*., 2018). The drug content and the high EE% can be attributed to DIM's affinity for the oil core, which is supported by its solubility in both PO and MCT. Due to its core's lipophilic character, nanocapsules can appropriately encapsulate low water-soluble drugs like DIM (Osmari *et al*., 2023).

Moreover, the zeta potential displayed the positive surface of the particles, a characteristic of the EUD polymer due to the presence of quaternary ammonium groups (Osmari *et al*., 2023). Thus, these formulations facilitate better interaction with the skin's surface, extending the residence time at the application site. They also improve permeation and absorption by the targeted tissue, proving beneficial for therapeutic purposes in melanoma treatment (Contri *et al*., 2016). The use of MCT or PO as the nanocarrier core and the presence or absence of DIM did not alter the pH or zeta potential values, reinforcing that the polymer encapsulates these constituents at the interface of the particles.

Solid tumors such as melanoma often have fenestrations (200–2000 nm) in blood vessels and poor lymphatic drainage, resulting in passive targeting of nanosystems to tumor tissue through enhanced permeation and retention (EPR). Therefore, nanocapsules smaller than 200 nm, like those developed in this study,

are promising for melanoma therapy since they can deposit in tumor tissue, leading to increased antitumor activity (Carletto *et al*., 2016). Previous studies have shown that formulations prepared with EUD display a size in the nanometric range and a narrow polydispersion index (Osmari *et al*., 2023). Unlike other parameters, the results indicated a slight increase in size when PO was used in the formulations. Vegetable oils typically have a lower density and higher viscosity than MCT, resulting in larger nanocapsules (Contri *et al*., 2013).

DIM is reported as a photolabile compound, and nanocapsules are well-known reservoir systems recognized for their ability to increase drug photostability (Luo *et al*., 2013; Mattiazzi *et al*., 2019). Our results show the enhanced DIM photostability of nanoencapsulation under an accelerated condition (UVC radiation). This finding is significant, particularly when considering future applications in melanoma treatment through the incorporation of the suspension into hydrogels and polymeric films for cutaneous application. Such formulations must be photostable, given that they can be applied in body regions exposed to radiation (Marchiori *et al*., 2017a; Giuliani *et al*., 2022). The oil type influenced the photodegradation rate, as NC-PO-D exhibited a longer half-life than NC-MCT-D. This finding underscores the importance of the PO constitution in enhancing DIM photostability. Besides the particles' ability to reflect and scatter UV radiation, the antioxidant components in PO can absorb UV radiation, resulting in less degradation of the bioactive (Lydia *et al*., 2020). This assertion is reinforced by the higher antioxidant power observed for PO nanocapsules containing DIM in the ABTS and FRAP assays.

In addition to enhancing photostability, antioxidant activity is closely tied to the ability to reduce DNA damage and inflammatory and carcinogenic processes (Dalcin *et al*., 2019). The ABTS test assesses the compounds' ability to eliminate free radicals by donating electrons or hydrogen atoms, thus neutralizing the $ABTS^{\cdot+}$ cation. Conversely, the FRAP assay considers the compounds' ability to donate electrons, leading to the reduction of ferric ions (Fe^{3+}) to ferrous ions (Fe^{2+}). Indole compounds, such as DIM, have been identified as antioxidants due to two secondary amines in their structure capable of donating a hydrogen atom to neutralize radicals (Mattiazzi *et al*., 2019). In addition, the pyrrole ring in these compounds plays a crucial role in reducing Fe^{3+} (Kamnev *et al*., 2001). The antioxidant power of PO, rich in carotenoids, tocopherols, and fatty acids, has been recognized (Lydia *et al*., 2020). The combination of DIM and PO into nanocapsules may have strengthened the known antioxidant activity of these constituents. This enhancement might be attributable to an increase in the contact surface on the nanometer scale, allowing closer contact between molecules (Marchiori *et al*., 2017a; Mattiazzi *et al*., 2019).

The cytotoxicity of the formulations against murine fibroblast cells (L929) was evaluated to determine their biocompatibility. By encapsulating DIM in PO nanocapsules, the cytotoxicity of the bioactive was maintained, aligning with the study by Baccarin et al. (2015), which showed the cytotoxicity of PO nanoemulsions on murine fibroblasts (3T3). This outcome may be attributed to the oil's composition, potentially containing polyphenols capable of reacting with transition metals to form free radicals in a biological medium, potentially leading to harmful cellular effects (Marchiori *et al*., 2017a). PO, a vegetable oil, holds several bioactive molecules providing numerous pharmacological properties, including anti-inflammatory, antinociceptive, and antitumoral effects (Amri *et al*., 2017; Ferreira *et al*., 2016; Marchiori *et al*., 2017b; Lydia *et al*., 2020). Interestingly, due to its wealth of antioxidant compounds, PO may exhibit a dual profile, acting as a pro-oxidant at higher concentrations, resulting in cytotoxic and associated effects. A study by Yuan *et al.* (2009) showed pro-oxidant activity after administering 3 g/day of punicic acid to 15 healthy young individuals for 28 days. In rats, a 0.6 g/kg dose of punicic acid demonstrated antioxidant effects, while higher doses of 1.2 g/kg were linked to pro-oxidant activity (Mukherjee *et al*., 2002). Hence, these findings are in harmony with our results, indicating that PO is a biologically active molecule whose concentration must be carefully determined to avoid toxic effects.

The antitumor activity of DIM is well-documented across various types of cancer, with recent cytotoxicity studies demonstrating its efficacy against certain melanoma cell lines (Amare, 2020; Heo *et al*.,

2018; Wang *et al*., 2020). This study reveals that the nanoencapsulation of DIM enhances its *in vitro* antitumor activity, yielding significantly higher cytotoxicity even at reduced concentrations. Furthermore, the *in vitro* antitumor effect was enhanced when DIM was carried in PO nanocapsules. This result may be explained by considering the beneficial properties of PO and when the oil associated with other bioactive nanostructures improved the *in vitro* antitumor effects (Ferreira *et al*., 2015; Lydia *et al*., 2020). Likewise, melding DIM with polymeric nanocapsules containing a primula oil core has improved antitumor activity *in vitro* (Mattiazzi *et al*., 2019). The therapeutic benefits conferred by pomegranate oil are intrinsically linked to its composition, which is replete with flavonoids, terpenes, and fatty acids, particularly punic acid. These constituents are believed to contribute to an antitumor effect against various forms of cancer by inducing cellular apoptosis, DNA damage, and cell cycle arrest (Zare, Shaverdi, Kalaee, 2021; Sharma *et al*., 2022; Cairone *et al*., 2023).In tumor development in aggressive neoplasms such as melanoma, metastases formation is highly recurrent. Considering cutaneous melanoma, when it has already reached metastasis, the patient receives the worst prognosis, and the treatment is often only palliative (Iqbal *et al*., 2019). Thus, the search for therapeutic alternatives with the potential to reduce the ability to form metastasis is highly relevant. Cell adhesion is one of the steps that make up metastasis development, allowing tumor cells to adhere to a new site for proliferation (Guan, 2015). Thus, nanocapsules composed of PO or DIM and nanocapsules consisting of such an association have shown promising results since they reduce cell fixation, which may indicate that the formulations have the potential to reduce cell adhesion in new tumor sites (Li *et al*., 2015).

Colonization, wherein tumor cells establish colonies in invaded tissue or organs to initiate secondary tumor development, represents another crucial phase in metastasis. The current findings indicate that all treatments managed to curb colony formation, thereby reducing the potential for new tumor colonization (Ye *et al*., 2020). The greater number of colonies observed with nanoencapsulated DIM, compared to free DIM, may be correlated with the controlled release of active substances facilitated by polymeric nanocapsules, a factor potentially contributing to attenuating *in vitro* biological effect*s* (Mattiazzi *et al*., 2019). Regardless, incorporating DIM within PO nanocapsules was associated with a reduced colonization effect compared to MCT nanocapsules, underscoring the enhanced antimetastatic potential engendered by combining vegetable oil and bioactive substances in nanocapsules.

The subject of migration pertains to the capacity of tumor cells to migrate from the primary tumor region, infiltrating the blood vessels and other organs (Zare, Shaverdi, Kalaee, 2021). Within this context, the results propose that the combination of DIM and PO in polymeric nanocapsules is critical in decreasing the migration ability of tumor cells. Accordingly, these findings substantiate the hypothesis that the nanoencapsulation of DIM within PO nanocapsules holds promise in minimizing events associated with metastatic potential in melanoma cells.

Indeed, prior studies have revealed the potential of DIM in reducing the formation and migration of diverse tumor cells (Ye *et al*., 2020). Similarly, both PO and its main component (punic acid) have demonstrated significant inhibition of migration, adherence, and cell colony formation (Zare, Shaverdi, Kalaee, 2021). Furthermore, in line with other studies in which migration and colony assays were conducted on B16F10 cells, it has been noted that PO nanocapsules containing DIM possess an antimetastatic potential that is equal to or possibly surpasses that of chemotherapeutic drugs (Chang *et al*., 2023).

The toxicological evaluation of nanomaterials is a critical aspect of creating new formulations. Alternative models such as HET-CAM and *A. cepa* have been employed for this purpose (Giuliani *et al*., 2022; Osmari *et al*., 2023). The toxicity measured by the Allium cepa test can be influenced by various characteristics of nanoparticles, such as composition, size, shape, and surface charge. Previous research has shown that this model is sensitive to chemotherapy drugs such as 5-fluorouracil and cyclophosphamide, leading to MI% reduction at concentrations up to 0.5 mg/mL (Gupta *et al*., 2020). In contrast, PO nanocapsules containing DIM (1 mg/mL) did not alter the number of cells in mitosis, denoting an absence of cytotoxicity against the model.

Among the tested samples, only NC-MCT led to an increase in MI% compared to the negative control. This observation suggests that including DIM or PO in the nanocapsules might counteract the effect observed for NC-MCT, potentially due to their antioxidant capacities, which mitigate the potential damage caused by the cationic polymer. Evidence of nanoencapsulation of an antioxidant bioactive into EUD nanocapsules reversing the cytotoxic and genotoxic effects of unloaded nanocapsules has been demonstrated in a previous study (Dalcin *et al*., 2019).

Traditional alkylating chemotherapy drugs, such as dacarbazine, have been shown in prior studies to induce genotoxic effects in healthy cells, potentially leading to secondary cancers (Etebari *et al*., 2015). In the present study, DIM displayed mild genotoxicity, less than the positive control. Some researchers have investigated the genotoxicity of metabolites from cruciferous vegetables and reported minor genotoxic potential. While it is known that the nanoencapsulation of bioactive compounds can diminish genotoxicity (Marchiori *et al*., 2017a), only PO nanocapsules were able to reduce DNA damage, demonstrating the protective effect of this vegetable oil. This finding aligns with prior studies that demonstrated the *in vivo* antigenotoxic potential of PO (Aly, Salman, Hassan, 2014). Given the potential use of these formulations in antitumor therapy and existing treatment limitations, the results from the *A. cepa* assay indicate that PO nanocapsules containing DIM may prove beneficial as an antitumor treatment, considering the lack of cytotoxicity and diminished genotoxicity.

Conventional antitumor therapies available for melanoma often have adverse effects, such as photosensitivity, erythema, and severe skin irritation (Leite *et al*., 2021). Moreover, drug candidates like imiquimod can result in similar reactions as topical melanoma therapy, such as vitiligo, erythema, and local irritation. In this study, the HET-CAM test was used to preliminarily investigate the irritant potential of the formulations. Exposure of the CAM to the samples showed no signs of irritation from either the isolated constituents or the nanocapsule suspensions (loaded and unloaded), indicating the safety of the developed nanostructured systems. Previous research has supported the non-irritating nature of nanocapsules with similar compositions using the same methodology (Osmari *et al*., 2023). This outcome suggests that these nanocapsule suspensions may hold promise as a stepping-stone in developing formulations intended for cutaneous use, such as gels and films. These formulations could potentially offer a topical therapeutic alternative for cutaneous melanoma, with a lower risk of adverse events, such as skin irritation.

After a thorough analysis of the data, we have identified the potential of a PSO-based formulation for treating melanoma. In order to optimize this potential, a suitable formulation that can be applied topically will be created, ensuring effective delivery of DIM, a promising treatment for melanoma. To achieve this, a cutaneous-compatible formulation will be developed and characterized, ensuring it fulfills all the necessary requirements for cutaneous delivery. The rheological properties will be measured to determine consistency, flow behavior, and the DIM permeation profile within the skin layers. Recognizing the importance of safety in this formulation, we will scrutinize its safety profile to guarantee its safe use for topical application. Our primary objective is to create an effective and safe treatment for melanoma that is easily applied to the skin.

CONCLUSION

The pomegranate oil nanocapsules containing DIM showed promise as drug carriers with antitumor activity, as these formulations had characteristics suitable for colloidal systems. They also demonstrated greater photostability of DIM, reduced genotoxic potential, and improved antioxidant effects *in vitro*. In terms of antitumor effects, the PO nanocapsules containing DIM were effective against melanoma cells without increasing cytotoxicity in healthy cells. Additionally, the combination of DIM and vegetable oil in polymeric nanocapsules reduced events associated with the metastatic process, suggesting that they could be used to inhibit tumor progression. Furthermore, nanocapsule suspensions have the potential to be used as a platform for combining PO and DIM to enhance the anti-melanoma effect and enable the delivery of bioactive and vegetable

oil in final formulations for cutaneous applications, such as gels and polymeric films. Therefore, this study encourages the scientific community to invest in research to better understand these nanosystems for future use in anti-melanoma therapy.

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DECLARATION OF COMPETING INTEREST

The authors declare no conflicts of interests in this manuscript.

REFERENCES

Aly FAE, Asmaa SS, Zeinab MH. Evaluation of the antimutagenic effect of pomegranate seed oil against genotoxicity induced by nitrobenzene in mice. Glob J Pharmacol. 2014;8(2):189-195.

Amare DE. Anti-Cancer and Other Biological Effects of a Dietary Compound 3,3′-Diindolylmethane Supplementation: A Systematic Review of Human Clinical Trials. Nutr Diet Suppl. 2020;12:123-137.

Amri Z, Lazreg-Aref H, Mekni M, El-Gharbi S, Dabbaghi O, Mechri B, et al. Oil characterization and lipids class composition of pomegranate seeds. BioMed Res Int. 2017:1-8.

Baccarin T, Mitjans M, Ramos D, Lemos-Senna E, Vinardell MP. Photoprotection by Punica granatum seed oil nanoemulsion entrapping polyphenol-rich ethyl acetate fraction against UVB-induced DNA damage in human keratinocyte (HaCaT) cell line. J Photochem Photobiol B Biol. 2015;153:127-136.

Benzie IFF, Strain JJ. The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": The FRAP assay. Anal Biochem. 1996;239(1):70–76.

Bernal-Chávez S, Del Prado-Audelo ML, Caballero-Florán IH, Giraldo-Gomez DM, Figueroa-Gonzalez G, Reyes-Hernandez OD, et al. Insights into terminal sterilization processes of nanoparticles for biomedical applications. Molecules. 2021;26(7):2068.

Bürk RR. A factor from a transformed cell line that affects cell migration. Proc Natl Acad Sci USA. 1973;70(2):369–372.

Cairone F, Salviti C, Lazzetti A, Fabrizi G, Troiani A, Pepi F, et al. In-Depth Chemical Characterization of Punica granatum L. Seed Oil. Foods. 2023;12(8): 1592.

Carletto B, Berton J, Ferreira TN, Dalmolin LF, Paludo KS, Mainardes RM, et al. Resveratrol-loaded nanocapsules inhibit murine melanoma tumor growth. Colloids Surf B Biointerfaces. 2016;144:65-72.

Chang KF, Lai HC, Lee SC, Huang XF, Huang YC, Chou TE, et al. The effects of patchouli alcohol and combination with cisplatin on proliferation, apoptosis and migration in B16F10 melanoma cells. J Cell Mol Med. 2023(April):1-13.

Contri RV, Ribeiro KL, Fiel LA, Pohlmann AR, Guterres SS. Vegetable oils as core of cationic polymeric nanocapsules: influence on the physicochemical properties. J Exp Nanosci. 2013;8(7-8):913-924.

Contri RV, Fiel LA, Alnasif N, Pohlmann AR, Guterres SS, Schäfer-Korting M. Skin penetration and dermal tolerability of acrylic nanocapsules: Influence of the surface charge and a chitosan gel used as vehicle. Int J Pharm. 2016;507(1-2):12-20.

da Silva JLG, Viana AR, Passos DF, Krause LMF, Miron VV, Schetinger MRC., et al. Istradefylline modulates purinergic enzymes and reduces malignancy-associated factors in B16F10 melanoma cells. Purinerg Signal. 2022:1-18.

Dalcin AJF, Vizzotto BS, Bochi GV, Guarda NS, Nascimento K, Sagrillo MR, et al. Nanoencapsulation of the flavonoid dihydromyricetin protects against the genotoxicity and cytotoxicity induced by cationic nanocapsules. Colloids Surf B Biointerfaces. 2019;173:798-805.

Etebari M, Jafarian-Dehkordi A, Lame V. Evaluation of protective effect of amifostine on dacarbazine induced genotoxicity. Res Pharm Sci. 2015;10(1):73–79.

Ferreira LM, Cervi VF, Sari MHM, Barbieri AV, Ramos AP, Copetti PM, et al. Diphenyl diselenide loaded poly (ε-caprolactone) nanocapsules with selective antimelanoma activity: Development and cytotoxic evaluation. Mater Sci Eng C. 2018;91:1-9.

Ferreira LM, Sari MHM, Cervi VF, Gehrcke M, Barbieri AV, Zborowski VA, et al. Pomegranate seed oil nanoemulsions improve the photostability and *in vivo* antinociceptive effect of a non-steroidal anti-inflammatory drug. Colloids Surf B Biointerfaces. 2016;144:214-221.

Ferreira LM, Cervi VF, Gehrcke M, da Silveira EF, Azambuja JH, Braganhol E, et al. Ketoprofen-loaded pomegranate seed oil nanoemulsion stabilized by pullulan: selective antiglioma formulation for intravenous administration. Colloids Surf B Biointerfaces. 2015;130:272-277.

Fessi HPFD, Puisieux F, Devissaguet JP, Ammoury N, Benita S. Nanocapsule formation by interfacial polymer deposition following solvent displacement. Int J Pharm. 1989;55(1):1–4.

Giuliani LG, Pegoraro NS, Camponogara C, Osmari BF, Brum BT, Reolon JB, et al. Locust bean gum based hydrogel containing nanocapsules for 3,3 ′ -diindolylmethane delivery in skin inflammatory conditions. J Drug Deliv Sci. Technol. 2022;78(April):103960.

Gomes GS, Maciel TR, Piegas EM, Michels LR, Colomé LM, Freddo RJ, et al. Optimization of Curcuma Oil/ Quinine-Loaded Nanocapsules for Malaria Treatment. AAPS PharmSciTech. 2018;19(2):551–564.

Guan X. Cancer metastases: Challenges and opportunities. Acta Pharm Sin B. 2015;5(5):402–418.

Gupta S, Das D, Mitra PK, Halder S, Datta AK. Assessment of Cytotoxicity Induced by Hazardous Chemotherapeutic Drugs Cyclophosphamide and 5-Fluorouracil in Allium cepa Assay for Ecological Safety. Cytologia. 2020;85(2):151–155.

Hajra S, Basu A, Singha RS, Patra AR, Bhattacharya S. Attenuation of doxorubicin-induced cardiotoxicity and genotoxicity by an indole-based natural compound 3,3′-diindolylmethane (DIM) through activation of Nrf2/ ARE signaling pathways and inhibiting apoptosis. Free Radic Res. 2017;51(9-10):812–827.

Heo JR, Lee GA, Kim GS, Hwang KA, Choi KC. Phytochemical-induced reactive oxygen species and endoplasmic reticulum stress-mediated apoptosis and differentiation in malignant melanoma cells. Phytomed. 2018;39(December 2017):100–110.

Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM). ICCVAM-Recommended Test Method Protocol: Hen's Egg Test—Chorioallantoic Membrane (HET-CAM) Test Method. 2010;13(1):B30-B38.

Iqbal J, Abbasi BA, Ahmad R, Batool R, Mahmood T, Ali B, et al. Potential phytochemicals in the fight against skin cancer: Current landscape and future perspectives. Biomed Pharmacother. 2019;109(October 2018):1381–1393.

Kamnev AA, Shchelochkov AG, Perfiliev YD, Tarantilis PA, Polissiou MG. Spectroscopic investigation of indole-3-acetic acid interaction with iron (III). J Mol Struct. 2001;563:565-572.

Kim EK, Jang M, Song MJ, Kim D, Kim Y, Jang HH. Redox-mediated mechanism of chemoresistance in cancer cells. Antioxidants. 2019;8(10):1–18.

Leite MN, Viegas JSR, Praça FSG, Paula NA, Ramalho LNZ, Bentley MV, et al. Ex vivo model of human skin (hOSEC) for assessing the dermatokinetics of the anti-melanoma drug Dacarbazine. Eur J Pharm Sci. 2021;160(February):105769.

Li WX, Chen LP, Sun MY, Li JT, Liu HZ, Zhu W. 3'3-Diindolylmethane inhibits migration, invasion and metastasis of hepatocellular carcinoma by suppressing FAK signaling. Oncotarget. 2015;6(27):23776–23792.

Luo Y, Wang TT, Teng Z, Chen P, Sun J, Wang Q. Encapsulation of indole-3-carbinol and 3,3′-diindolylmethane in zein/ carboxymethyl chitosan nanoparticles with controlled release property and improved stability. Food Chem. 2013;139(1-4):224–230.

Lydia DE, Khusro A, Immanuel P, Esmail GA, Al-Dhabi NA, Arasu MV. Photo-activated synthesis and characterization of gold nanoparticles from Punica granatum L. seed oil: An assessment on antioxidant and anticancer properties for functional yoghurt nutraceuticals. J Photochem Photobiol B Biol. 2020;206(January):111868.

Marchiori MCL, Rigon C, Copetti PM, Sagrillo MR, Cruz L. Nanoencapsulation Improves Scavenging Capacity and Decreases Cytotoxicity of Silibinin and Pomegranate Oil Association. AAPS PharmSciTech. 2017a;18(8):3236–3246.

Marchiori MCL, Rigon C, Camponogara C, Oliveira SM, Cruz L. Hydrogel containing silibinin-loaded pomegranate oil based nanocapsules exhibits anti-inflammatory effects on skin damage UVB radiation-induced in mice. J Photochem Photobiol B Biol. 2017b;170:25-32.

Mattiazzi J, Sari MHM, Lautenchleger R, Dal Prá M, Braganhol E, Cruz L. Incorporation of 3,3′-Diindolylmethane into Nanocapsules Improves Its Photostability, Radical Scavenging Capacity, and Cytotoxicity Against Glioma Cells. AAPS PharmSciTech. 2019;20(2):1–11.

Mosmann T. Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. J Immunol Methods. 1983;65(1-2):55–63.

Mukherjee C, Bhattacharyya S, Ghosh S, Bhattacharyya DK. Dietary effects of punicic acid on the composition and peroxidation of rat plasma lipid. J Oleo Sci. 2002;51(8):513-522.

Osmari BF, Medeiros GA, Reolon JB, Prado VC, Brucker N, Cruz L. Cationic nanocapsule suspension as an alternative to the sublingual delivery of nifedipine nifedipine. Pharm Dev Technol. 2023;28(5):1–11.

Patel AR, Spencer SD, Chougule MB, Safe S, Singh M. Pharmacokinetic evaluation and *In Vitro*-In Vivo Correlation (IVIVC) of novel methylene-substituted 3,3′ diindolylmethane (DIM). Eur J Pharm Sci. 2012;46(1-2):8–16.

Patel D, Zode SS, Vansal AK. Formulation aspects of intravenous nanosuspensions. Int J Pharm. 2020;586:119555.

Re R, Pellegrini N, Proteggente A, Pannala A, Yang M, Rice-Evans C. Antioxidant activity applying an improved

ABTS radical cation decolorization assay. Free Radic Biol Med. 1999;26(9-10):1231-1237.

Rigon RB, Oyafuso MH, Fujimura AT, Gonçalvez ML, Prado AHD, Gremião MP, et al. Nanotechnology-based drug delivery systems for melanoma antitumoral therapy: A review. Biomed Res Int. 2015;2015:16–20.

Sharma K, Kesharwani P, Prajapati SK, Jain A, Jain D, Moby N, et al. An insight into anticancer bioactives from Punica granatum (Pomegranate). Anti-Cancer Agents in Medicinal Chemistry (Formerly Current Medicinal Chemistry-Anti-Cancer Agents). 2022;22(4):694-702.

Thomson CA, Chow HS, Wertheim BC, Roe DJ, Stopeck A, Maskarinec G, et al. A randomized, placebo-controlled trial of diindolylmethane for breast cancer biomarker modulation in patients taking tamoxifen. Breast Cancer Res Treat. 2017;165(1):97-107.

Wang X, Zhao Y, Yu M, Xu Y. PTEN/Akt signaling-mediated activation of the mitochondrial pathway contributes to the 3, 3′-diindolylmethane-mediated antitumor effect in malignant melanoma cells. J Med Food. 2020;23(12):1248-1258.

Widiandani T, Tandian T, Zufar BD, Suryadi A, Purwanto BT, Hardjono S, et al. *In vitro* study of pinostrobin propionate and pinostrobin butyrate: Cytotoxic activity against breast cancer cell T47D and its selectivity index. J Public Health Afr. 2023;14(1):2516.

Ye F, Li X, Sun K, Xu W, Shi H, Bian J, et al. Inhibition of endogenous hydrogen sulfide biosynthesis enhances the anti-cancer effect of 3, 3′-diindolylmethane in human gastric cancer cells. Life Sci. 2020;(261):118348.

Yuan G, Sinclair AJ, Xu C, Li D. Incorporation and metabolism of punicic acid in healthy young humans. Mole Nutr Food Res. 2009;53(10):1336-1342.

Zare M, Shaverdi H, Kalaee SEV. Anti-cancer effects of pomegranate seed oil on esophageal cancer cell line (KYSE-30). Gen Cell Tissue. 2021;8(1):17-21.

Zielińska A, Carreiró F, Oliveira AM, Neves A, Pires B, Venkatesh DN, et al. Polymeric nanoparticles: production, characterization, toxicology and ecotoxicology. Molecules. 2020;25(16):3731.

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