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**HEALTH SCIENCES**

# Microencapsulation of DEET in Solid Lipid Microparticles: production, characterization and safety evaluation

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**Abstract:** DEET is considered the gold standard for insect repellent products. However, it behaves as a strong skin permeant. DEET was encapsulated in Solid Lipid Microparticles (SLM) and characterized in terms of morphology, particle size, cytotoxicity and *ex vivo* permeation. The particles exhibited micrometric size with a spherical shape. In addition, we developed and validated an analytical method for DEET quantification by high performance liquid chromatography (HPLC), which was selective, linear, precise, accurate and robust. The toxicity test in cell culture of keratinocytes, fibroblasts and macrophages showed that the formulation did not present cytotoxicity. The SLM were able to decrease the skin permeation of DEET in relation to the free active in ethanol with gain in the safe. Microparticles were able to increase the skin retention of DEET, which can contribute to extend the time of repellent action. The results showed that Solid Lipid Microparticles are safe and promising topical formulation to insect bite prevention.

**Key words:** DEET, Solid Lipid Microparticles, *Ex vivo* permeation, Cytotoxicity.

## INTRODUCTION

Arboviruses (from “viruses transmitted by arthropods”), are viruses that have part of their replication cycle in insects and can be transmitted to humans and animals by the sting of blood-sucking arthropods (Weaver & Reisen 2010). The diseases transmitted by these viruses, known as arboviruses like, Dengue, Zika, Chikungunya, Yellow fever, West Nile Virus, are still neglected diseases that need greater investments and research in ways of preventing, diagnosing and fighting transmitters. A method for arboviruses prevention is the use of insect repellent product, a topical substance that inhibit arthropods’ landing and bites in animals and humans (Nerio et al. 2010). Repellents have their application on the skin or over clothes and the belief is that the action is due the formation of a vapor barrier over the skin, which is unpleasant to the insects (Barradas et al. 2013).

Topical repellents can be natural (derived from essential oils) or synthetic. Natural repellents come from essential oils that consist of volatile organic compounds, mainly a mixture of terpenoids and aromatic compounds that are products of the secondary metabolism of plants (Maia & Moore 2011, da Silva & Ricci-Junior 2020). Among synthetic repellents, there are countless active ingredients available in the Brazilian market such as N, N-diethyl-meta-toluamide (DEET), ethyl Butylacetylaminopropionate (IR3535®) and Icaridin (Picaridin) (1-piperidine carboxylate 2- (2-hydroxyethyl) ) -1-methylpropylester). However, the most effective repellents are DEET and Permethrin. The latter, despite having its classification as an insecticide in the group of

pyrethroids, acts as a clothing repellent, more specifically in military clothing (Tavares et al. 2018). For over half a century, the use of DEET as an insect repellent remains and they discovered it after a research performed by the United States Department of Agriculture, in collaboration with the United States Military Department, on substances with potential repellent action. The goal was to develop a substance that, in addition to its repellent action, was also safe for human use along with a low irritating action (Legeay et al. 2018).

DEET has its authorized use for application on the skin and on the surface of clothes; it is present in several pharmaceutical presentations, such as aerosol, creams, gels, sprays and lotions, in multiple concentrations. Products with concentrations of 10-20% DEET have an average action time of 4-6 hours, respectively (Tavares et al. 2018).

DEET was encapsulated in Solid Lipid Microparticles (SLM) to reduce the active permeation. This carrier system grants product stability, biocompatible components use and less toxic to the user. The microencapsulation of DEET in SLM is simple, used materials and methods of low cost with absence of solvents in their production friendly to the environment (Bayón-Cordero et al. 2019, Karr et al. 2012, Silva et al. 2011).

Gasco (1993) patented the term “Solid Lipid Microparticles” (SLM) and used as an option to other colloidal systems such as liposomes. They are a composition of one or combined solid lipids at room temperature, with surfactants and co-surfactants (if necessary), the active compound of interest for encapsulation, preservatives and water (Bayón-Cordero et al. 2019, Souto & Müller 2006). The drug-lipid interaction, solubility of the drug in the lipid, the chemical structure of the lipid and its polymorphic state influences on active’s release profile (Jaspart et al. 2007, Das et al. 2013). Solid Lipid Microparticles have size ranging of 1 to 1000 µm (Kheradmandnia et al. 2010).

The objectives of this work were microencapsulation of DEET in SLM, characterization in relation to size, polydispersity index, morphology, content and finally safety evaluation by cytotoxicity assays in fibroblasts, keratinocytes and macrophages and *ex vivo* permeation using natural membrane.

## MATERIALS AND METHODS

### Material

The active pharmaceutical ingredient N, N-dimethyl-meta-toluamide (DEET) was from Welpharm CO®. The excipients used to develop the formulation: Stearic Acid - Pharma Nostra®, Polysorbate 80 and Methyl Paraben - Vetec®. The purified water used to prepare the mobile phase we obtained through the Sartorius® system. To develop and validate the HPLC analytical methodology, we used the following reagents: Methanol AG - Merck®, Hydrochloric Acid ACS and Phosphoric Acid - Sciavicco®, Sodium Hydroxide ACS - Neon®, Hydrogen Peroxide – Dynamic®, Copper Sulfate Pentahydrate - Siavicco®. The DEET and methyl paraben standard references were from the United States Pharmacopeia Reference Standard. We filtered the mobile phase and diluent through a vacuum filtration system composed of a 0.45 µm membrane. The samples, Active Pharmaceutical Ingredient (API), International Chemical Reference Substances (ICRS) and matrix were filtered through 0.45 µm filters (PTFE - Polytetrafluoroethylene - 25 mm - Analítica®) and 0.22 µm (PVDF - Polyvinylidene fluoride - 25 mm - FilterPro®). The chromatographic columns used were C18 125 mm x 4 mm x 5 µm, from ACE® and Merck®.

## METHODS

### Preparation of Solid Lipid Microparticles (SLM)

The technique used to prepare the SLM was hot homogenization, using Stearic Acid - Pharma Nostra®, as lipid, Polysorbate 80 - Vetec®, as surfactant, N, N-dimethyl-meta-toluamide (DEET) - Welpharm CO®, as the active, Methyl Paraben - Vetec®, as a preservative, and distilled water. For formulation development, we considered the evaluation of adequate proportions of lipid and surfactant, since they may interfere in particle size, viscosity and stability. Table I displays the proportions applied to the formulation.

Two systems were assembled for each formulation: the first formed by stearic acid, polysorbate 80 and DEET, kept at 75°C in a water bath – the oily phase - and the second formed by 0.1% methyl paraben in distilled water – the aqueous phase - also at 75°C in a water bath sufficient for 20 mL. After the homogenization of both systems, the mixture of stearic acid/ polysorbate 80/ DEET, at 75°C was slowly added to the distilled water with methyl paraben heated at same temperature. We homogenized the entire system in water bath (75 °C) for 4 minutes. The homogenization was performed in an ultrasonic processor (UP100H, Hielscher) for 2 min (Cycle 0.5 / Amplitude 40%). The temperature of the dispersion was reduced to 5 °C using an ice bath for precipitation of the Solid Lipid Microparticles. Subsequently, we stirred the system again using a magnetic stirrer, for 20 minutes.

### Determination of particle size and polydispersity index

After formulation preparation, formulations had particle size and polydispersity index (Span) checked using the Mastersizer 2000 Malvern Panalytcal® device, by setting equipment to perform the analysis at 25°C.

### Optical microscopy

To evaluate SLM morphology, we used Quimis® optical microscope with a 10-x objective to characterize the particles produced in the formulation.

### Determining the pH value

The pH was checked at room temperature (25°C), by inserting Micronal® potentiometer electrode into the formulations, previously calibrated with pH 4.0 and 7.0 buffer solutions.

### Development and validation of analytical methodology for DEET quantification

The ICRS, the API and sample were prepared in the concentration of 0.2 mg/mL diluted in methanol. For DEET quantification in the repellent formulation, we checked the adequate parameters described in the USP (United States Pharmacopeia 2018), except for the diluent and the chromatographic column. The limit established by USP is from 92 to 108%. A degradation study complemented method validation. We used this formulation with DEET 10% to validate the analytical methodology. The equipment used for DEET quantification was the Shimadzu® Ultra-Fast Liquid Chromo graph, composed by the LC Solution® software.

**Table I.** Formulation of Solid Lipid Microparticles containing DEET.

| Formulation | Estearic acid | DEET | Polysorbate 80 | Methylparaben | Water (EQF) |
|---|---|---|---|---|---|
| 1 | 10% | 10% | 5% | 0.1% | 20 mL |

DEET - N,N-dimetil-meta-toluamida; EQF - enough quantity for.

The chromatographic column used was C18 (Octadecyl) 125 mm x 4 mm x 5 μm from ACE®. For analytical methodology development, the system used methanol and purified water acidified with phosphoric acid in proportion of (45:55) as mobile phase and methanol as diluent. The defined flow was 1.0 mL / min. The temperature was 25°C. The wavelength of the UV-Vis detector was 235 nm. The injection volume was 10 μL.

The analytical methodology validation was according to the Guide for Analytical Methods Validation (National Health Surveillance Agency 2017), which determines the quantitative tests for determining the active principle in pharmaceutical products and raw materials, in addition to identification. In this category, the recommended validation parameters are:

**Selectivity:** Dilution of the ICRS of DEET in methanol at 0.2 mg/mL and compared to a sample at the same concentration. To evaluate possible interferences present in the formulation, the ICRS was compared to the active prepared at 0.2 mg/mL, diluent and matrix.

**Linearity:** Linearity was assessed by checking the proportionality between the concentration and the response obtained from a chromatographic signal. The acceptable correlation coefficient must be greater than 0.990. Thus, three analytical curves were constructed in 5 (five) concentrations of the ICRS at 0.160, 0.180, 0.200, 0.220, 0.240 mg/mL from the stock solution in the concentration of 2 mg/mL diluted in methanol, as diluent. In addition, we obtained the correlation coefficient, determination, equation of the regression line from y to x, estimated by the least squares method and residual dispersion graph. The level of significance used for the statistical tests is 5%. Residues analysis allowed homoscedasticity assessment. Statistical calculations performance using Microsoft Office Excel 2007 software.

*Accuracy (repeatability and intermediate precision):* The repeatability test was performed with 6 (six) solutions in 100% concentrations - 0.2 mg/mL - prepared individually. The intermediate precision performance occurred with the same aforementioned concentrated solutions, on different days, with different analysts under the same conditions proposed by the analytical method. Precision has its demonstration by calculating the relative standard deviation (RSD) of the samples and RSD between the runs of the first and second days. The formula for calculating the standard deviation is below:

$$RSD = (SD / DMC) \times 100$$

Where, RSD is the relative standard deviation, SD is the standard deviation and DMC is the determined mean concentration.

**Accuracy**: The accuracy assessed from 3 (three) sample concentrations of 80% (0.160 mg / mL), 100% (0.200 mg/mL) and 120% (0.240 mg/mL), which included method linear range. It`s expressed by the relationship between the average concentration determined experimentally and the corresponding theoretical concentration (National Health Surveillance Agency 2017). For accuracy, 9 (nine) samples were prepared with 3 (three) replicates, accuracy calculation is:

$$A = (AEC / TC) \times 100$$

Where, A is the accuracy, AEC is the average experimental concentration and TC is the theoretical concentration.

**Robustness**: Robustness assessed whether the method was capable to resist to small variations in the parameters determined by National Health Surveillance Agency (2017). The samples and ICRS were prepared at 0.2 mg/mL in methanol. In this work, as a chromatographic method was used, the aforementioned legislation determined acceptable variations in

the pH of the mobile phase (if applicable), in the composition of the mobile phase, different lots or manufacturers of columns, temperature and flow of the mobile phase. The conditions evaluated in the robustness test: variation in mobile phase composition (methanol: solution A (45:55)/ (50:50)/ (40:60), columns from different manufacturers, temperature (25±2°C), flow (1±0.2 mL), filters (PTFE - Polytetrafluoroethylene - 25 mm and 0.22 μm PVDF - Polyvinylidene fluoride - 25 mm); reference chemical solution from DEET and sample stability. All materials used are in sub item 2.1.

**Forced Degradation Test**: The test should promote degradation greater than 10% and less than what would lead to complete sample degradation (National Health Surveillance Agency 2015). The sample, active and matrix were prepared at 0.2 mg/mL and subjected to forced conditions of acid degradation, basicity, oxidative stress, ion metal exposure, thermal analysis and photo-stability for degradation study. That is complementary to the selectivity test to assess possible interferences in the active signal. After exposure, the samples were analyzed by HPLC. Parameters: acid degradation (HCl 6 M/ 80°C water bath for 1h), basicity (NaOH 6M / 80°C water bath for 1h), oxidative stress ($H_2O_2$ 10% / 80°C water bath for 1h), ion metal exposure (CuSO4 0.5 M /24 h at room temperature), thermal analysis (60°C/5 h) and photo-stability (sunlight/ 24 h).

### DEET quantification in the repellent formulations

DEET concentration was measurement in the formulations. DEET was extracted from the Solid Lipid Microparticles with methanol at hot. An aliquot of the formulation was added to a bottle. The extractor solvent (70 °C) was added to dissolve the microparticles and extract DEET. The solution was filtered and injected on the HPLC (filter with pore of 0.45 μm) to quantify DEET. Analytical curve was also obtained in order to assess the amount of DEET extracted from the repellent formulation.

### Cell Culture and Viability

The cytotoxicity studies were carried out in culture of cells that make up the skin: human dermal fibroblasts (ATCC® PCS-201-012 ™), human dermal keratinocytes (HaCaT, BCRJ 0341) and murine macrophages (RAW 264.7) (ATCC® TIB-71™). The formulation used is an aqueous suspension containing DEET encapsulated in SLM (SLM-DEET). Formulation was sterilized for UV-light and was diluted with culture medium. For the assays, we placed the three cell lines in 96-well culture plates at a concentration of $2.5x10^5$ cells/well. After 24h of incubation, we added a sample of the test formulation (SLM-DEET 10%) to the wells in different concentrations of DEET (5; 10; 25; 50 and 100 μg/mL). Concentrations of 5 to 100 μg/mL were established according to previous studies of our research group for drugs in formulations of cutaneous administration (Marques et al. 2018, Pinto et al. 2017). After this procedure, we incubated the plates again for 24 hours. Cell viability was determined using the colorimetric assay by reducing tetrazolium salts, such as 3- (4, 5-dimethyl-2-thiazolyl) -2, 5-diphenyl-2H-tetrazolium bromide (MTT). At the end of the treatment period, we added 20 μL of a MTT solution (5 mg/mL) to each well. Then, we incubated the cultures at 37 °C, protected from light, until the formazan crystals observation. To solubilize formazan crystals, we added 100 μL of dimethyl sulfoxide (DMSO) to each well and performed the spectrophotometric reading of the absorbance, at a wavelength of 570 nm, in an ELISA plate reader. The percentage of cell viability expresses the cytotoxicity of the formulation, by calculation of the ratio between the number of cells treated and the control.

The control (cells treated with culture medium) represented 100% cell viability (Marques et al. 2018, Pinto et al. 2017).

The mean and standard deviation of the experiments were calculated using GraphPad Prisma program (Version 5.1). In cytotoxicity studies, the mean value of cell viability (%) of the control was compared with the mean value of cell viability (%) of concentrations of 5, 10, 25, 50 and 100 µg/ml. Statistical analysis was performed in the GraphPad Prisma program (Version 5.1) using t-tests (and nonparametric tests) with a significance level of $p < 0.05$.

### *Ex vivo* skin permeation study

In the *ex vivo* permeation studies, the pig ear skin was used as a natural membrane to separate the donor compartment from the recipient of the diffusion cell. Swine skin was used due to its similarity to human skin. Ears were obtained from a slaughterhouse in Rio de Janeiro (Brazil). The pieces of total skin were removed from the outer region of the ear with a scalpel. Subsequently, the adipose tissue was removed with scissors and tweezers. The skin pieces were washed with distilled water and stored in poly (vinyl chloride) films at -3 °C. On the day of the *ex vivo* permeation study, the natural membranes were defrosted at room temperature (25 °C) (Vieira et al. 2020).

Franz diffusion cells were used for *ex vivo* permeation studies. The receptor compartment of the diffusion cell was filled with 8 mL of PBS (phosphate-buffered saline) pH 7.4 containing 5% ethanol to follow the sink condition. The receiving medium was maintained at 32 °C and constantly homogenized by a magnetic bar. Pig ear skin was placed in the diffusion cell with the dermis directed to the receptor solution and the epidermis facing the donor compartment. Amount of 300 mg of formulation (SLM containing 10% DEET, 30 mg of repellent) or 300 mg of ethanolic solution containing 10% DEET (30 mg of free repellent) were added to the donor compartment of the diffusion cell. The system was occluded with a Teflon disc and 0.5 mL of the receiving solution was collected at 1, 2, 4 and 6 hours after administration of formulation and replaced with clean receptor solution. The diffusion area of the cell is 1.77 $cm^2$. The solutions were filtered (filter with pore of 0.45 µm) and the amount of DEET that permeated the skin was measured by HPLC. The skins were removed from the diffusion cells after 6 hours of experiment. The excess formulation was removed from the skin surface by cotton moistened with purified water. The treated area of the skin with the formulation was removed with a scalpel. The extracted tissue was cut in small pieces and added to a tube containing 5 mL of methanol to extract DEET. The tubes were shaken for 5 minutes and after 24 hours of extraction, the solutions were filtered and the DEET retained on the skin was quantified by HPLC. The experiments were performed with n= 5 determinations for each sample.

The samples were analyzed by HPLC by the previously validated method. The analytical curve was linear in the range of 30 to 300 µg/mL with $R^2$=0.9992.

The mean and standard deviation of the experiments were calculated using GraphPad Prisma program (Version 5.1). In the *ex vivo* permeation studies, the averages values of free DEET permeated were compared with the averages values of DEET encapsulated in SLM for each time in hours. In the skin retention studies, the averages values of free DEET were compared with the averages values of the active encapsulated in SLM. Statistical analysis was performed in the GraphPad Prisma program (Version 5.1) using t-tests (and nonparametric tests) with a significance level of $p < 0.05$.

# RESULTS AND DISCUSSION

## Characterization of Solid Lipid Microparticles (SLM) containing DEET

After SLM production, we evaluated the pH, organoleptic characteristics, particle size and polydispersity index (Span). Organoleptic characteristics are very important acceptance requirements for a new product, as parameters such as appearance, color, odor and sensory are evaluated, which are essential for product stability assessment.

The macroscopic aspect evaluation of the repellent formulation presented milky, odorless and homogeneous white color. The microscopic evaluation occurred by means of the optical microscopy technique used to characterize the morphology of the SLM produced in this work (Figures 1a and 1b), obtained with a Quimis® optical microscope and Motic Images Plus 2.0® software using a 10x lens.

The characterization of the SLM started with the verification of the particle size and polydispersity index (Span) of the formulation containing DEET, with the aid of the particle analyzer by laser diffraction (Mastersizer 2000 Malvern Panalytical®). The result of the analysis was in Figure 1b.

With the aid of a potentiometer previously calibrated with pH 4.0 and 7.0 buffers at 25°C, the pH verification of the formulations obtained the results shown in Table II.

With the results obtained, it appears that the formulations presented the pH within the normal range of the skin's pH (4.8-5.6); therefore, they have less chance of causing irritation, besides not altering the bactericidal / fungicidal function and skin protection (Leonardi et al. 2002). SLM projects a promising scenario due to its topical application to cosmetics and other pharmaceutical products, in addition to the use

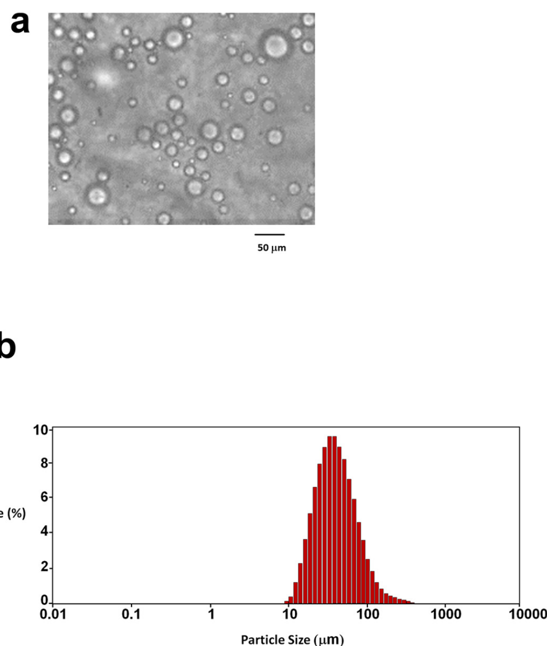



**Figure 1. Image of Solid Lipid Microparticles containing DEET (a). Image obtained using a Quimis® Optical Microscope with 10x objective, Motic Images Plus 2.0® software. Polydispersion (Span) and Size of Solid Lipid Microparticles containing DEET (b) obtained using Mastersizer 2000 Malvern Panalytcal®.**

of components that are biocompatible (Tursilli et al. 2007).

The permeation of drugs and other substances through the skin occurs by transdermal or transfollicular route. Moreover, the routes allow the passage of particles with different size range. In general, particles larger than 10 µm remain on the skin surface, particles between 3 and 10 µm can enter in the hair follicles, and when smaller than 3 µm, they can penetrate the follicles and the stratum corneum (Lademann et al. 2001). The results obtained from the analysis of the formulations in the equipment by laser diffraction are in Table II. The mean diameter was 62.53±1.75 µm. Microparticles reduces the absorption capacity through the skin, due to its size greater than 10 µm, therefore maximizing the maintenance of the repellent product developed on the skin surface (Tavares et al. 2018, Tursilli et al. 2007).

**Table II.** Results of polydispersion (Span) and Size of Solid Lipid Microparticles.

| Formulation | DEET | pH | Span | Size ( µm) |
|---|---|---|---|---|
| 1 | 10% | 5.72±0121 | 5.04±0.38 | 62.53±1.75 |

**DEET - N, N-dimethyl-meta-toluamide. Results are mean ± SD of n=3 determinations.**

Span value is a statistical parameter to evaluate the particle size distribution. Particles have a monomodal size distribution with low Span value (Teeranachaideekul et al. 2007). Span value ranges of zero to infinity, so a value close to zero means that the size distribution is close to monomodal. The Span value obtained was 5.04±0.38 with size distribution characteristic of microparticles. This parameter can be influenced by the type of lipid used, drug concentration and technique used for production (Albertini et al. 2008).

### Development and validation of the analytical methodology

The analytical methodology for DEET quantification in the repellent formulation of this work was from a method described in United States Pharmacopoeia (2018), which included topical solutions containing ethyl or isopropyl alcohol. However, due to the repellent formulation made up of SLM containing 10% DEET, this type of solutions present at United States Pharmacopoeia (2018), did not fit into the possible suitability of the method and subsequent validation compliant to the requirements of the Validation guide for analytical methods (National Health Surveillance Agency 2017).

Among the modifications in the analytical methodology, we used a solely methanol diluent, since, the one recommended by United States Pharmacopoeia did not solubilize the formulation containing 10% DEET. The second was in relation to the characteristics of the chromatographic column used in this work. The column indicated for DEET analysis at USP 41 NF 36 (United States Pharmacopoeia 2018), was Octadecylsilane (C18) 15 cm long, 4.6 mm in diameter and 3.5 µm in particle size. However, we used a chromatographic column with similar characteristics, with the stationary phase also composed of C18, but with 12.5 cm in length, 4.0 mm in diameter and 5 µm in particle size.

The changes in these parameters were tested and evaluated for compliance with the requirements present in Table III, which were satisfactory. Therefore, the validation step begun after these tests.

The results of the analytical method developed are in Table IV.

The DEET retention time was 11.2 min. Through comparative analysis of the ICRS, API, sample and matrix chromatograms, we noticed that there were no interferences present in the formulation that could elute at the same time as DEET retention. This confirmed result by the chromatograph coupled to a diode array detector (DAD), known by the acronym from the Photo Diode Array (PDA), compared the peak obtained from the sample spectrum with that of the ICRS. Thus, indicating the level of purity greater than or equal to 0.9999 determined

**Table III.** Chromatographic evaluation criteria.

| Solution | Rating criteria |
|---|---|
| Calibration ICRS | DPR (%) of the replicate areas must be ≤ 1.0% |
| Resolution (R) | Minimum 1.5 |
| Theoretical dishes (N) | Minimum 2000 |
| Tail factor | Maximum 2.0 |

**USP 41 NF 36 (USP 2018).**

in the ultraviolet region with a wavelength of 235 nm. Therefore, the method is considered selective according to the established by the National Health Surveillance Agency (2017), due to the absence of matrix or diluent peaks that can interfere in the standard time of the analyte signal.

Five (5) concentrations of the ICRS were used to obtain the analytical curve within the range established by the method (80 to 120%) prepared in triplicate from the same mother solution. From the results, we constructed three analytical curve as in Figure 2a, 2b and 2c. In this work, the values of ($R^2$) of the calibration curves of Figures 2a, 2b and 2c were 0.9971, 0.9999 and 0.9978, respectively, very close to this line.

To evaluate this parameter and prove the directly proportional relationship between concentration (variable x) versus response (variable y), the mathematical model called the calibration curve represented by the simple linear regression equation $y = ax + b$ and its dispersion diagram (Ribeiro et al. 2008). In addition to the coefficients a (slope) and b (linear coefficient) it is also possible to calculate the correlation coefficient (r) that varies between -1 and +1, where r the closer to 1, the greater the response due to the increase in concentration and the smaller the error in y, resulting in a positive correlation (Ribeiro et al. 2008). From the calculations performed, the correlation coefficient of the resulting calibration curves in Figures 2a, 2b and 2c were, respectively, 0.9985, 0.9999 and 0.9989, therefore, they are in accordance with the requirements of the National Health Surveillance Agency (2017) regarding compliance to consider a linear parameter.

To detect deviations from linearity, presence of atypical samples, heteroscedasticity and suitability of the regression model employed we performed the analysis of residual graph (Ribeiro et al. 2008). According to the residual graph, as in Figure 2d, it appears that the data are homogeneously dispersed and with constant variance due to the random distribution around zero that are confirmed by the Cochran test, comparing the highest variance with the others, where:

**Table IV. Results of the validation of the analytical method.**

| Parameters | DEET (Content / RSD) | Acceptance criteria |
|---|---|---|
| Selectivity | No interference | No interference |
| Linearity | 0.998 | r > 0.990 |
| Precision[a] | 105.63 ± 0.66 % | < 5% |
| Precision[b] | 106.09 ± 0.38 % | < 5% |
| Accuracy (80%)[c] | 95.75 ± 0.43 % | < 5% |
| Accuracy (100%)[d] | 97.26 ± 0.65 % | < 5% |
| Accuracy (120%)[e] | 104.01 ± 0.24 % | < 5% |
| QL (µg/mL) | 20 | - |
| DL (µg/mL) | 9.5 | - |

**r = correlation coeficiente , RSD - Relative standard deviation. [a]Precision (RSD) (%) Intraday with n = 6 determinations. [b]Precision (RSD) (%) Interday with n = 6 determinations in 2 days (n = 12 determinations). Accuracy (RSD) (%) based on 3 concentration levels: low (80%) (0.160 mg/mL)[c], medium (100%) (0.200 mg/mL)[d] and high (120%) (0.240 mg/mL)[e] and n = 3 determinations for each concentration level. DL= Detection Limit (DL=(SDx3.3)/S); QL= Quantification limit (QL=(SDx10)/S); SD-standard deviation of the y intercept of three curves; S is the mean of the slope of the three curves.**

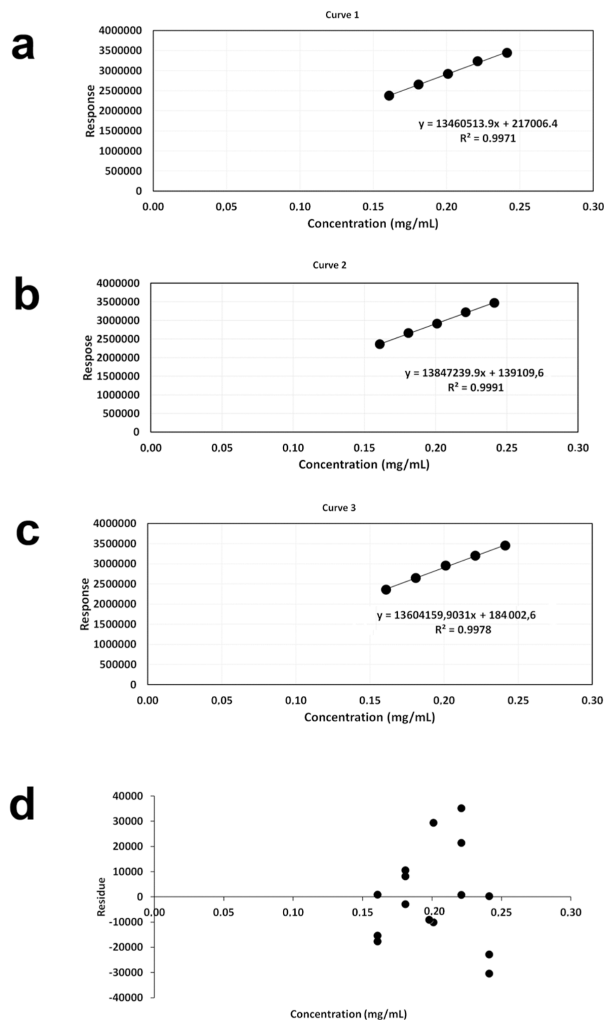



**Figure 2. Analytical curves prepared with DEET ICRS diluted in methanol: Curve 1 (AaCurve 2 (b), Curve 3 (c) and Residual analysis of analytical curves (d).**

### *Cochran Test*

Tabulated C = 0.6838 (Table C for a significance level of α = 5%). This value considers the number of points analyzed, which in this case are 5 (five), 80, 90, 100, 110 and 120% and the number of replicates of each curve, which are 3 (three). The calculated C = 0.4016 for the values of each curve is less than the tabulated C, which means that the variances are homogeneous in the three curves. The results obtained from the precision test (repeatability and intermediate precision) displayed RSD values of 0.66 and 0.38, respectively. It appears that the DPR is within the specified, that is, below 5% as required by National Health Surveillance Agency (2017). Regarding the percentage recovered and DPR for the accuracy test, it was obtained for the levels of 80%, 100% and 120%, respectively, 95.75±0.43%, 97.26±0.65% and 104.01±0.24%, which are in accordance with the legislation.

The robustness results are in Table V. Robustness is a validation parameter analyzed in order to verify the sensitivity to small variations imposed in the proposed analytical method (National Health Surveillance Agency 2017, Ribani 2004). The conditions evaluated for robustness were: the relative standard deviations between the areas of all the conditions mentioned above are below 5%, therefore, one can consider the satisfactory repeatability of the analyzed parameters (flow, temperature, filter, mobile phase, column and stability of the reference chemical substance and sample).

The tail factor calculated by all parameters was less than 2.0, which means that the peak shape had a Gaussian aspect, that is, sharp and symmetrical, thus not affecting the resolution of the chromatograms. A chromatographic signal with a large tail may interfere with an adjacent signal, affecting the chromatographic resolution and, therefore, the separation of the substances present and, consequently, the purity of the signal (Neto 2009).

The retention time varied in methanol mobile phase: solution A (40:60) with an average time of 19.293 min, in the variation of Merck® column, with an average time of 16.259 min and flow variation of 0, 8 mL resulting in an average time of 14.909 min. With flow of 1.2 mL obtaining an average time of 9.855 min. Therefore, as recommended by National Health Surveillance Agency (2017), when the method is susceptible to variations due to different retention times, in this case, they must be controlled and the

**Table V.** Robustness results.

| Parameters | Tail factor | Retention time (min) | Area | Content (%) | RSD |
|---|---|---|---|---|---|
| Flow (mL) | | | | | |
| 0.8 | 1.423 | 14.909 | 3386206 | 102 | 0.07 |
| 1.0* | 1.386 | 11.861 | 2621918 | 102 | 0.45 |
| 1.2 | 1.430 | 9.855 | 2221871 | 102 | 0.10 |
| Temperature (°C) | | | | | |
| 23 | 1.285 | 11.807 | 2701555 | 96 | 0.16 |
| 25* | 1.462 | 11.545 | 2897039 | 106 | 0.10 |
| 27 | 1.349 | 11.216 | 2706143 | 92 | 0.05 |
| Filter (µm) | | | | | |
| 0.22 | 1.124 | 11.906 | 3204803 | 104 | 0.11 |
| Without filtering* | 1.118 | 11.893 | 2857437 | 98 | 0.09 |
| 0.45 | 1.124 | 11.906 | 2860110 | 98 | 0.63 |
| Column | | | | | |
| ACE®* | 1.628 | 11.761 | 2170480 | 100 | 0.52 |
| Merck® | 1.170 | 16.259 | 2830736 | 97 | 0.13 |
| Mobile phase | | | | | |
| MP 1 | 1.328 | 7.605 | 3123513 | 106 | 0.46 |
| MP* | 1.158 | 10.903 | 3076827 | 105 | 0.35 |
| MP 2 | 1.153 | 19.293 | 3362162 | 105 | 0.46 |
| Stability ICRS | | | | | |
| Recent preparation | 1.092 | 11.157 | 2959298 | 98 | 0.11 |
| After 24 h | 1.141 | 11.779 | 2977677 | 104 | 0.21 |
| Sample stability | | | | | |
| Recent preparation | 1.076 | 11.173 | 3030089 | 105 | 0.28 |
| After 24 h | 1.123 | 11.694 | 3027842 | 104 | 0.27 |

**RSD - Relative standard deviation, *Control. Mobile control phase (methanol: solution A) (45:55) Solution A - 0.1% phosphoric acid in purified water. Mobile control phase (methanol: solution A) (50:50) Solution A - 0.1% phosphoric acid in purified water. Mobile control phase (methanol: solution A) (40:60) Solution A - 0.1% phosphoric acid in purified water.**

precautions in the method informed. These changes in the retention time can result in the coelution of the API with other formulation components and therefore, reduce its purity and, consequently its quantification.

In relation to the percentage recovered from the analysis, only with the use of methanol mobile phase: water (40:60) we obtained a value outside the specified. It is important to highlight this variation in the method.

The solution of the reference solution and sample had its stability time analyzed, to guarantee that the results obtained were reproducible and reliable. The stability of the analytical solutions included in the robustness tests, therefore, the reference solution and the freshly prepared sample were prepared and injected, and they were stored at room temperature. After 24 hours, they were prepared, injected and compared with a new standard. The reference chemical and the sample showed

24-hour stability, as they showed recovery values within the established range (92 to 108%) and RSD below 5%. The other chromatographic evaluation criteria are within the specified, therefore, the reference sample and chemical substance can be considered stable in this period.

## Forced Degradation Test

The degradation tests that are complementary to the selectivity parameter. They include tests with matrix, API and sample in a wide pH range (acid and basic hydrolysis), oxidation, ion metal, heat and light. The tests did not present any impurities that would interfere in DEET signal attainment, as verified in Table VI, ensuring that the signal displayed is exclusively from the API. Summing up, DEET is a compound resistant to stress conditions presenting resistance to hydrolysis (Costanzo et al. 2007, United States Agency for Toxic Substances and Disease Registry 2015). Resistance to oxidative degradation was also observed through the results of the comparative analysis between the DEET control solution, active solution and the sample solution with oxidizing agent ($H_2O_2$ 10%). The latter presented a high level of purity guaranteed by DAD, or that is, the absence of degradants or impurities that may impair the quantification of the API in the product. The forced degradation by thermolysis is necessary to complement the selectivity test to evaluate the heat resistance and the appearance of possible degradation products that impair the product's quality and effectiveness. For degradation studies by thermolysis, ICH Q1A guide (European Medicines Agency 2003), recommends to carry out the test with an increase of 10°C above the accelerated stability study. The accelerated stability study is performed at a temperature of 40°C +/- 2°C (National Health Surveillance Agency 2019) and in the thermolysis test of this work the matrix, API and sample were kept in an oven at 60 °C for 4 h, that is, temperature above that recommended by the aforementioned resolution. This test, even with stress conditions higher than recommended, demonstrates that no product degradation formation interfered with the API signal, as by the results of Table VI. Hence, the conclusion that the microencapsulated insect repellent containing DEET is resistant to temperature. The recovered percentage showed a value above the determined working range, 92 to 108%, due to a possible evaporation of the aqueous phase that makes up the formulation due to the assigned heat resulting in a higher concentration of the API. There are two types of photolytic degradation study: confirmatory tests and forced degradation study. Confirmatory tests assess photosensitivity of the sample, active and placebo exposed to light totaling no less than 1.2 million lux and an integrated 200 watt h $m^2$ ultraviolet as recommended by ICH Q1B (European Medicines Agency 1996). The forced degradation tests, on the other hand, aim

**Table VI.** Results of forced degradation.

| Degradation condition | Content (%) | Peak purity |
|---|---|---|
| | **API** | |
| Acid | 108 | 0.99999 |
| Basic | 108 | 1.0000 |
| Oxidizing | 105 | 0.99999 |
| Metal Ion | 108 | 0.99999 |
| Temperature | 98.18 | 1.0000 |
| Photostability | 104.47 | 0.99999 |
| | **Sample** | |
| Acid | 98 | 0.99999 |
| Basic | 99 | 0.99999 |
| Oxidizing | 100 | 0.99999 |
| Metal Ion | 95 | 0.99999 |
| Temperature | 147.90 | 1.0000 |
| Photostability | 128.32 | 1.0000 |

API - active pharmaceutical ingredient.

to assist in the development of the analytical methodology in order to verify possible degradation products that may arise in the long-term and accelerated stability tests and impair the method selectivity (National Health Surveillance Agency 2015). In this work, the light intensity used was 3 (three) times higher than in the confirmatory test. Through the results, not observed product degradation that interfered with the retention time of the API. The sign of the same showed a high level of purity confirmed by the DAD. In this way, declared DEET resistance even using forced degradation conditions. The literature confirms the result of the photolytic resistance of DEET (European Chemicals Agency 2017).

Concluding, the degradation test did not show formation of products that interfere in DEET signal attainment, thus guaranteeing exclusivity.

### *DEET quantification in the repellent formulations*

The recovery of the SLM repellent was satisfactory and the amount of DEET in formulation was 9.95±0.22%. The initial concentration of DEET was 10% and the recovery was 99.5% being within the range of 98 to 102%.

### *In vitro* cytotoxicity test

Before using an *in vivo* formulation, an appropriate *in vitro* cytotoxicity study must be performed to ensure safety. Studies with human dermal fibroblast culture, human epidermis keratinocytes and blood murine macrophages were chosen because they are sensitive skin cells, since the formulation is designed to cutaneous administration.

In Figure 3 are the graphs referring to the cytotoxicity test performed with cell cultures and in them it is possible to observe how the formulation behaved in the concentrations of

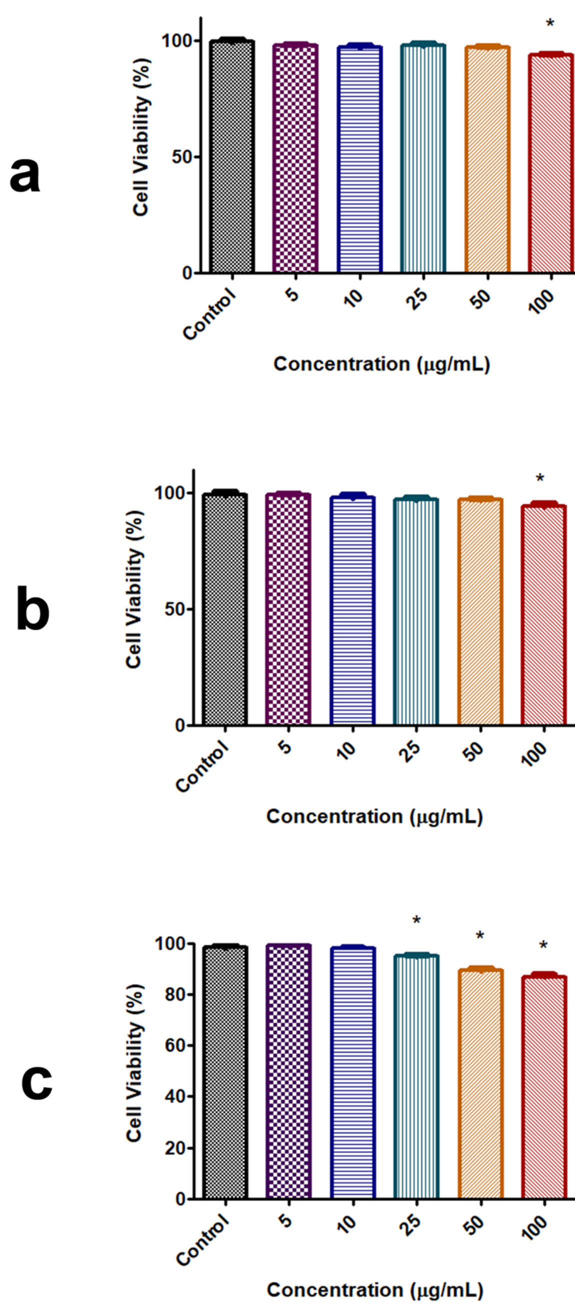



**Figure 3. Percentage of viable cells assessed by the MTT assay: (a) Keratinocytes (HaCat), (b) Fibroblasts (ATCC® PCS-201-012 ™) and (c) Macrophages (RAW264.7). Results are Mean Standard deviation of n = 5 determinations. * Mean value is significantly different between Control and test with $p < 0.05$.**

5, 10, 25, 50 and 100 µg/mL of DEET. Primarily, the formulation demonstrated absence of cytotoxicity to keratinocytes (Figure 3a) and fibroblasts (Figure 3b) at concentrations of 5 to 50 µg/mL. At the concentration of 100 µg/mL, we observed a small reduction in cell viability for keratinocytes (Figure 3a) and fibroblasts (Figure 3b) of the order of 93.6 and 94.2% with statistically significant differences compared to the control. In relation to the small reduction in cell viability, the formulation can be considered safe because cell viability remained above 93% for keratinocytes (Figure 3a) and fibroblasts (Figure 3b).

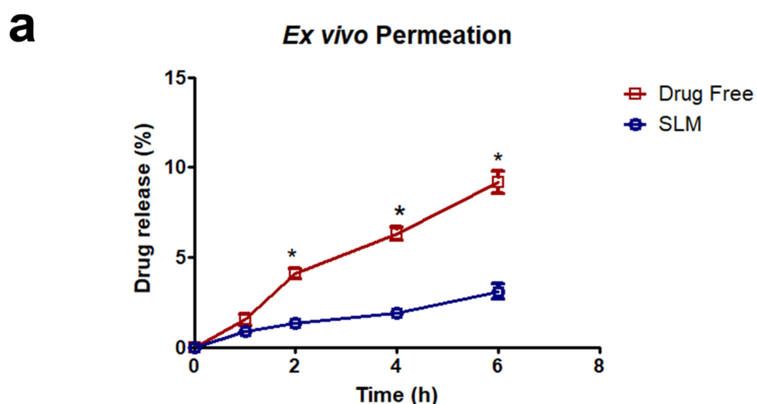
The formulation demonstrated absence of cytotoxicity to macrophages (Figure 3c) at concentrations of 5 to 10 µg/mL. At the concentration of 25 to 100 µg/mL, we observed a small reduction in cell viability for macrophages in the order of 95.1 to 86.6%, with a significant difference compared to the control. We can conclude that the formulation can be considered safe because cell viability remained above 86% for macrophages (Figure 3c). Macrophages are cells involved with the defense system and because they perform phagocytosis, they can be more sensitive to formulation. In general, the formulation with SLM containing DEET demonstrates absence of cytotoxicity. *In vivo* dermal irritability studies performance is required to support *in vitro* cytotoxicity studies to ensure the safety of the formulation.

Cytotoxicity studies were done with the formulation because it is the focus of our study. DEET cytotoxicity (Free DEET) was evaluated by Tisch et al. (2002) in primary human nasal mucosal cells. The authors reported that no significant cytotoxic effect was observed after the incubation of DEET with human cells in the concentration range of 0.5-1.0 mM (95.56-191.3 µg/mL) (Tisch et al. 2002). The cytotoxicity study of Microparticles containing DEET was performed in the range of 5 to 100 µg/mL below the concentration of 191.3 µg/mL reported as non-cytotoxic by Tisch et al. (2002).

DEET is an efficient and safe insect repellent when used as recommended. From 5% of the DEET in the formulation, repellent effect is perceived (Stefani 2009). Thus, adults can use concentrations of 10% with a repellent effect of 4 hours as reported on registered products and available on the market (Tavares et al. 2018, Koren et al. 2003).

### *Ex vivo* skin permeation study

The results of the *ex vivo* permeation studies are shown in Figure 4. SLM provided slow and sustained release of the repellent. The free repellent in ethanolic solution exhibited higher permeation than the formulation containing DEET encapsulated in SLM (Figure 4a). The statistical analysis of the *ex vivo* permeation studies shows that after 2 hours there are statistically significant differences between the percentage of DEET released from the ethanolic solution and the active release from the SLM. Ethanol is a co-solvent in pharmaceutical formulations containing DEET (Tavares et al. 2018, Karr et al. 2012, Iscan et al. 2006). Ethanol is able to act as a permeation promoter of lipophilic drugs such as DEET by solubilizing the lipids of the epidermis concomitantly promoting a cutaneous dehydration with formation of pores, holes and fissures that enhance the permeation of DEET. Our results corroborate the results of Barradas et al. (2013) that encapsulated DEET in micellar system and performed *ex vivo* permeation studies using mouse skin. The authors observed that the free DEET in ethanolic solution exhibited higher permeation than the repellent in micellar system (Barradas et al. 2013). Thus, the results of *ex vivo* permeation for the free repellent have not been satisfactory because DEET must remain on the skin surface where it acts as an insect



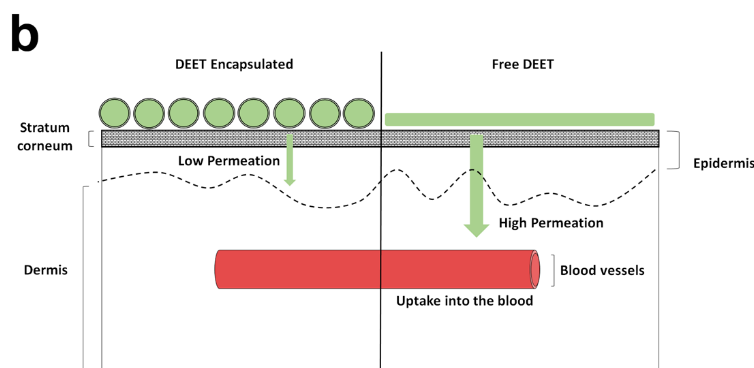




**Figure 4.** *Ex vivo* permeation studies: amount of DEET permeated in (%) (a) and illustration of skin permeation of free drug and encapsulated (b). Results are Mean Standard deviation of n = 5 determinations. * Mean value is significantly different with $p < 0.05$ between free DEET and encapsulated in Microparticles.

repellent. The skin permeation to the receptor solution of the *ex vivo* permeation study shows that this free DEET may eventually reach the bloodstream (Figure 4a). In this context, SLM reduced the permeation of DEET compared to the control in 6 hours. We can concluded that the formulation containing DEET encapsulated in SLM is safer than formulations containing free DEET in ethanolic solution.

After 6 hours of *ex vivo* permeation, the amount of DEET retained on the skin was determined and the results exhibited in Figure 5. The SLM provided higher retention of DEET in the skin than the free repellent and the difference was statistically significant with $p < 0.05$. The SLN remain on the skin surface probably adhered to the stratum corneum as a reservoir sustaining the repellent release, as we can see in the Figure 4b. Free DEET in ethanolic solution (Control) provided cutaneous permeation of the drug through the layers of the skin (epidermis and dermis) to the receptor solution where the permeant accumulates.

## CONCLUSIONS

Outlining, the analytical methodology for quantifying DEET is considered selective, linear, precise, accurate and robust for the evaluated parameters. Notably, the formulation is advantageous due to the use of few excipients, in addition to the active ingredient for its production. The toxicity test in cell culture of keratinocytes, fibroblasts and macrophages showed that the formulation did not present cytotoxicity; however, *in vivo* studies of irritability is required to ensure the safety of the formulation. The SLM were able to decrease

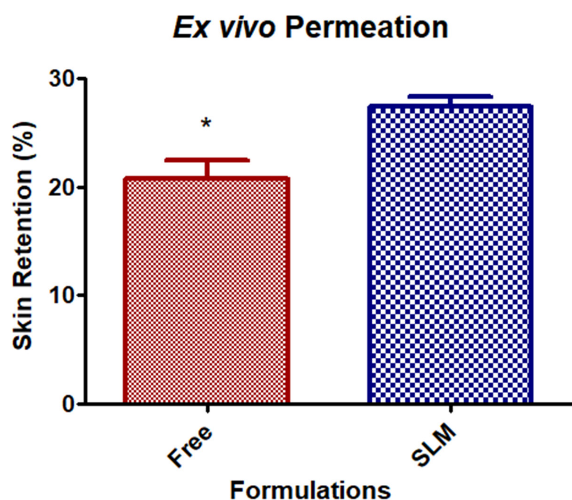



**Figure 5. Amount of DEET retained in the skin after *ex vivo* permeation studies: black bar shows the result of cutaneous retention of free repellent and white bar shows the result of the retention of microencapsulated DEET in SLM. Results are Mean Standard deviation of n = 5 determinations. * Mean value is significantly different with $p < 0.05$.**

the skin permeation of DEET in relation to the free active in ethanol, with security gain. The formulation components are biodegradable and biocompatible with the skin, thus being safe and with less risk of toxicity. The results showed that Solid Lipid Microparticles are safe and promising topical formulation to insect bite prevention.

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All authors contributed extensively to the work presented in this paper and helped to draft the manuscript. All authors discussed the results and contributed to the writing of the manuscript.