Efficacy and Safety of Innovative Sunscreen (e)-4-(tert-butyl)-n’-((e)-3-(4-methoxyphenyl) allylidene) benzidrazida (TBMAB)

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Palavras-chave
Skin Cancer
Citotoxicity
UV Filters
Sunscreen

Abstract
The constant or repeated unprotected exposure to solar radiation can result in the onset of various harmful effects, such as inflammation, genetic mutation and hyperpigmentation, even than previous photoaging. The use of photoprotective formulations is one of the most effective tools to avoid this. However, most of the UV filters are unstable to UV radiation, can even permeate the skin and cause hepatotoxic effects. The aim of this study was to assess the efficacy and safety of an innovative sunscreen substance (E)-4-(tert-butyl)-N’-((E)-3-(4-methoxyphenyl)allylidene) benzhydrazide. Methods: The sunscreen formulation containing the innovative substance was subjected to various stress conditions: 45±2°C, 5±2°C and exposure to sunlight. These samples were evaluated in relation to its rheology, pH, viscosity, density and SPF to determine its stability. The assessment of TBMAB release from the formulation was performed in Microette equipment. Hepatoma cells (HepG2) were used to determine the TBMAB cytotoxicity. Results and Discussion: In the stability study, no significant changes were observed in the formulation. The maximum concentration of TBMAB released from the formulation was 3.63 µg.cm-2. Also, it was evident the cytotoxic effect on the concentrations well above 3.63 µg.cm-2, displaying the safety of this substance, considering HepG2 cells. Conclusion: These results demonstrate that TBMAB is a suitable innovative substance to be used in sunscreens, exhibiting stability, desired SPF value and safety, considering HepG2 cells. Thus, it could be a new alternative to prevent skin cancer.

Eficácia e segurança de filtros solares inovadores (E)-4-(TERT-BUTIL) -N’- ((E) -3- (4-METOXIFENIL) ALILIDENO) BENZIDRAZIDA (TBMAB)
A exposição constante e/ou repetida e desprotegida à radiação solar pode resultar no aparecimento de vários efeitos nocivos, como inflamação, mutação genética, hiperpigmentação e, até mesmo, o fotoenvelhecimento. O uso de formulações fotoprotetoras é uma das ferramentas mais eficazes para evitar estes efeitos deletérios. No entanto, a maioria dos filtros solares está instável à radiação UV, pode permear a pele e causar efeitos hepatotóxicos. O objetivo deste estudo foi avaliar a eficácia e a segurança de uma substância fotoprotetora inovadora (E)-4-(terc-butil)-N’-((E)-3-(4-metoxifênil)alilideno) benzidrazida. Métodos: A formulação de filtro solar contendo uma substância inovadora foi submetida a várias condições de estresse: 45 ± 2°C, 5 ± 2°C e exposição à luz solar. Essas amostras foram avaliadas em relação à sua reologia, pH, viscosidade, densidade e FPS para determinar sua estabilidade. A avaliação da liberação de TBMAB à partir da formulação foi realizada em equipamentos Microette. As células de hepatoma (HepG2) foram usadas para determinar a citotoxicidade do TBMAB. Resultados e Discussão: No estudo de estabilidade, não foram observadas alterações significativas na formulação. A concentração máxima de TBMAB liberada a partir da formulação foi de 3.63 µg.cm-2. Também ficou evidente o efeito citotóxico nas concentrações acima de 3.63 µg.cm-2, demonstrando a segurança dessa substância, considerando as células HepG2. Conclusão: Esses resultados demonstram que o TBMAB é uma substância inovadora adequada para ser usada na fotoproteção, exibindo estabilidade, valor desejado de SPF e segurança, considerando as células HepG2. Assim, poderia ser uma nova alternativa para prevenir o câncer de pele.

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**INTRODUCTION**

The constant and repeated unprotected exposure to sun radiation can result in the onset of various harmful effects, such as inflammation, genetic mutation, hyperpigmentation, photoaging and, inclusive, induction of malignant tumors (RASS; REICHRATH, 2008; REIS JUNIOR et al., 2016). The number of skin cancers increased 77% between 1992 and 2006, in the US, as a result of the constant exposure to sun radiation and destruction of the ozone layer (SIEGEL; MILLER; JEMAL, 2015).

Skin cancer is classified at melanoma and non-melanoma cancers. The melanoma is related with higher occurrence of genetic mutation and metastasis (ARMSTRONG; KRICKER, 2001; BOUKAMO, 1999; DENNIS; BEANE FREEMAN; VANBEEK, 2003) being dependent of the time and intensity of exposure to solar radiation (GRUIJL, 1999; GORDON, 2013). The non-melanoma reaches basal cells (is the most frequent) (SIEGEL; MILLER; JEMAL, 2015; SOEHNGE; OUHTIT; ANANTHASWAMY, 1997).

The use of sunscreens is the most efficient tool of skin cancer prevention (BOUKAMP, 1999; WRIGHT; SPENCER; FLOWERS, 2006). However, most of the UV filters are unstable to UV radiation, can even permeate skin and cause adverse effects such as contact dermatites, allergic reaction, mutation and toxicity (REIS JUNIOR et al., 2016). Avobenzone is one of the filters with the highest photoprotection against UVA radiation however, this compound has adverse effects such as: photoinstability and incompatibility with other sunscreens. Ethylhexyl p-methoxycinnamate is one of the filters with the highest photoprotection against UVB radiation however, this compound presents photostability (BERNEBURG; PLETTENBERG; KRUTMANN, 2000; WRIGHT; SPENCER; FLOWERS, 2006; SIEGEL; MILLER; JEMAL, 2015).

In previous experiments performed in the Laboratory of Pharmaceutical Chemistry of UNESP, were synthesized 8 active compounds with protection capacity against UVA and UVB radiation. The compound (E)-4-(tert-butyl)-N’-((E)-3-(4-methoxyphenyl)allylidene) benzidrazida - TBMAB was obtained by molecular hybridization of the pharmacophoric groups of Avobenzone with octyl methoxycinnamate and showed higher activity photoprotective in relation to the other synthesized compounds (REIS JUNIOR et al., 2016). Thus, the aim of this study was to assess the efficacy and safety of an innovative sunscreen (E)-4-(tert-butyl)-N’-((E)-3-(4-methoxyphenyl)allylidene) benzidrazida – TBMAB.

**METHODS**

**SYNTHESIS OF TBMAB**

The TBMAB synthesis (Figure 1) was performed according to the methods described by Reis (REIS et al., 2014).
Development of sunscreen formulation

Table 1 shows the components of the sunscreen formulation. A sunscreen formulation should be stable at different temperatures and conditions without the occurrence of degradation of formulation components, mainly the active compound.

Table 1 - Composition of the sunscreen formulation.

<table>
<thead>
<tr>
<th>INCI name</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Helianthus Annuus (Sunflower) Seed Oil (and) Sodium Polyacrylate (and)</td>
<td>4</td>
</tr>
<tr>
<td>Xylitol (and) Caprylic Acid (and) Glyceryl Stearate</td>
<td></td>
</tr>
<tr>
<td>Dibutyl Adipate</td>
<td>2</td>
</tr>
<tr>
<td>Alkyl Benzoate C_{12-15}</td>
<td>5</td>
</tr>
<tr>
<td>Caprylic/Capric Triglyceride</td>
<td>4</td>
</tr>
<tr>
<td>(E) -4- (tert-butyl) -N' - ((E) -3- (4-methoxyphenyl) allylidene) benzhydrazide – TBMAB</td>
<td>2</td>
</tr>
<tr>
<td>PPG-5-Ceteth-20</td>
<td>2</td>
</tr>
<tr>
<td>Green coffee oil</td>
<td>2</td>
</tr>
<tr>
<td>Aqua</td>
<td>qsp 100</td>
</tr>
</tbody>
</table>

Assessment of the formulation stability

The sunscreen formulation was subjected to various stress conditions: 45±2°C, 5±2°C and exposure to sunlight. During 90 days, SPF, PFUVA, pH and rheology was measured periodically (ISAAC et al., 2008).

SPF and PFUVA in vitro (Optometrics) assessment

The sunscreen formulation (0.11 g) was spread on a Transpore® tape. Measurements were performed in the Optometrics SPF 290 equipment, a spectrophotometer with detection via integrating sphere. The SPF (sun protection factor) and PFUVA (UVA protection factor) values were determined. Readings were made in the range of 290 nm to 400 nm in triplicate. The control was prepared using the emulsion without TBMAB (DUTRA et al., 2004).

pH assessment

The sunscreen formulation was dispersed in distilled water (10% w/w) for pH determination. The pH values need to be between 5.5 and 6.5, which are compatible with the skin pH (ISAAC et al., 2008).

Rheology measurements

The rheology measurements were performed in a Brookfield rheometer using the cone-plate type sensor (C40 / 2° Ti). The data were analyzed by the software Rheowin 3. All assays were performed in triplicate, at a temperature of 32 ± 1°C.

The viscosity and flow properties were determined by application of a shear rate, from 0 – 100 s-1 for 120 seconds to ascending curve followed by a shear rate of 100 – 0 s-1 for 120 seconds to descending curve (ISAAC et al., 2008; GALLEGOS; FRANCO, 2011).

The creep and relaxation assay was obtained using a shear stress of 1 Pa for 300 seconds and, after, accompanying the recovery for over 300 seconds, ceasing the applied tension (ISAAC et al., 2008; GALLEGOS; FRANCO, 2011).
**TBMAB release**

The release of TBMAB from the sunscreen formulation was studied in a Microette equipment. Modified Franz cells were used, with 1.77 cm² of diffusion area, and cellulose membrane (Sigma-Aldrich) (FRANZ, 1975). The Modified Franz cell compartment was filled with 7.0 mL of 0.1 M phosphate buffer (pH 7.4) and ethoxylated sorbitan monooleate surfactant 20OE (3%) at a temperature of 32 ± 5 °C and stirring at 300 rpm (LEHMAN; RANEY; FRANZ, 2011). Samples of 295 mg of the sunscreen formulation were placed on the cellulose membrane (FRANZ, 1975). The amount of active released was quantified by UV spectrophotometry after 2, 4, 6, 8 and 12 hours of testing (ANVISA, 2003).

**Cytotoxicity assays**

The degree of cytotoxicity was determined by MTT assay. In this assay, living cells are able to reduce 3- (4,5-dimethyl-2-thiazolyl) -2,5-diphenyl-2H-tetrazolium bromide (MTT), forming violet formazan crystals (MOSMANN, 1983). The hepatoma cells (HepG2) were cultured in DMEM and treated with different concentrations of TBMAB. The cell density for the cytotoxicity test (MTT) was 1 x 10⁵ cells. mL⁻¹. After treatment, spectrophotometric reading was performed in microplate reader in a wavelength of 595 nm (ABE; MATSUKI, 2000). As positive control, DMSO 10% were employed and the negative control was DMEM.

**Statistical analysis**

All results were subjected to statistical analysis of variance test using the ANOVA and Tukey test for multiple comparisons of average. The resulting values equal to or less than 5% was considered statistically significant (STHLE; WOLD, 1989).

**Results and discussion**

**SPF and PFUVA in vitro (Optometrics) assessment**

The sun protection factor (SPF) and PFUVA assessment help in the determination of the activity and of the possible degradation of the sunscreen formulation constituents. Through Tukey test, was evidenced no significant difference, both in the SPF (Figure 2) and PFUVA (Figure 3) between the control sunscreen formulation (5 ± 2 °C) and the formulations subjected to physical stress (45 ± 2 °C and exposure to sunlight). These results indicate that the sunscreen active is stable under increased temperature and/or exposure to sunlight (SPRINGSTEEN et al., 1999).

![Figure 2 - SPF values for 90 days.](image-url)
A higher SPF compared to PFUVA may be related to the caffeine's ability to increase the SPF of some chemical filters, justified by its UVB absorption capacity (SHEHATA; RIZK; REND, 2016). This synergistic capacity of the green coffee oil is due to the possibility of intermolecular hydrogen bonds, which can occur between a pair of electrons free of caffeine and the functional clusters of the tested solar filter (LEONE, 2018). The same comparison was done in relation to the PFUVA of the formulations, and no differences were verified between the sample maintained at 5 ºC and the formulations subjected to physical stress (45 ± 2 ºC and exposure to sunlight).

**Figure 3 - Assay PFUVA.**

<table>
<thead>
<tr>
<th>PFUVA</th>
<th>Time (day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.4</td>
<td>10</td>
</tr>
<tr>
<td>0.8</td>
<td>20</td>
</tr>
<tr>
<td>1.2</td>
<td>30</td>
</tr>
<tr>
<td>1.6</td>
<td>40</td>
</tr>
<tr>
<td>2.0</td>
<td>50</td>
</tr>
</tbody>
</table>

**PH Assessment**

When pH reduces to values below 5.5 they can be related to the degradation of the constituents of the formulation. Also, it can cause dermal irritation. Thus, during the stability studies, the maintenance of pH values between 5.5 and 6.5 was observed. Furthermore, no significant differences in the pH values were evidenced (Figure 4) between the control sunscreen formulation (5 ± 2 ºC) and the samples exposed to physical stress (45 ± 2 ºC and exposure to sunlight). These results, once again, indicate that the sunscreen formulation is stable under increased temperature and/or exposure to sunlight (ANVISA, 2004; CORRÊA, 2012).

**Figure 4 - pH values measured during 90 days.**

<table>
<thead>
<tr>
<th>pH</th>
<th>Time (day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>10</td>
</tr>
<tr>
<td>8</td>
<td>20</td>
</tr>
<tr>
<td>10</td>
<td>30</td>
</tr>
</tbody>
</table>

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RHEOLOGY MEASUREMENTS

FLOW PROPERTIES

The rheogram showed on Figure 5 demonstrated that does not exist linear relationship between shear stress and shear rate values, specifying the non-Newtonian behavior of the sunscreen formulations. This type of non-Newtonian behavior is fitted by the Herschel-Bulkley model (GALLEGOS; FRANCO, 1999; ALVES, 2004; BARRY; WARBURTON, 1968), where the flow occurs only from a specific shear stress depending of a yield stress (T₀) that should be exceeded.

Figure 5 - Flow properties measurements.

The hysteresis area is defined as the area between the ascendent (0-100s⁻¹) and the descendent (100-0s⁻¹) flow curves. Thus, we could consider that how much higher is the difference between the hysteresis areas of the formulations subjected to different stress conditions, higher is the instability of the sunscreen formulation. Through Tukey test, was evidenced no significant difference of the hysteresis area between the control formulation (5 ± 2 °C) and the formulations under physical stress (45 ± 2 °C, exposure to sunlight). These results indicate that the sunscreen formulation is stable under increased temperature and / or exposure to sunlight (GALLEGOS; FRANCO, 1999; ALVES, 2004; BARRY; WARBURTON, 1968).

Figure 6 - Assay viscosity.

VISCOSITY

According to the results (Figure 6), all samples showed a decrease in viscosity with increasing shear rate.
This rheological behavior is related deformation, random orientation and/or disaggregation of molecules for to facilitate flow. There was no significant difference between the viscosity of sunscreen formulations under different physical stresses (45 ± 2°C or exposure sunlight) and the control sample (5 ± 2°C) (GALLEGOS; FRANCO, 1999; ALVES, 2004; BARRY; WARBURTON, 1968).

**Creep and Relaxation**

During the application of shear stress (0-300 sec), the elastic properties (creep) of the sunscreen formulation is observed. After ceasing the shear stress (300-600 sec), the viscous behavior of the material (relaxation) can be observed (Figure 7) (GALLEGOS; FRANCO, 1999; ALVES, 2004; BARRY; WARBURTON, 1968).

![Figure 7 - Creep and relaxation assay.](image)

Thus, the sunscreen formulations subjected to various physical stress conditions (5 ± 2°C, 45 ± 2°C and exposure to sunlight), had not presented significant differences, indicating that the sunscreen formulation is stable under different temperatures (GALLEGOS; FRANCO, 1999; ALVES, 2004; BARRY; WARBURTON, 1968).

**Scan analysis by spectrophotometry UV**

The phosphate buffer (0.1 M - pH 7.4) with sorbitan monooleate surfactant ethoxylated EO 20 (3%) was used as solvent for determination the maximum absorption wavelength of TBMAB. According to the results, the maximum absorption wavelength is λ=338 nm (ANVISA, 2003).

**TBMAB release**

The TBMAB release was quantified by means of UV spectrophotometry after 2, 4, 6, 8 and 12 hours of testing. According to the results (Figure 8), the maximum concentration of TBMAB released occurred after 12 hours (4.05 µg/cm2). There was no significant difference (p<0.05) of sunscreen active released after 8 and 12 hours (Tukey test) (FRANZ, 1975).
Efficacy and safety of innovation sunscreen (e) -4- (tert-butyl)-n...

Figure 8 - TBMAB released after 2, 4, 6, 8 and 12 hours.

Cytotoxicity assays
In this essay (Figure 9), it was evidenced that the active sunscreen in a concentration of 0.00022321 g.mL-1, increased cell viability (16.17%) compared to control group. However, it was evident cytotoxic effect in concentrations higher than 0.00022321 g.mL-1 (MOSMANN, 1983; ABE; MATSUKI, 2000). The use of the HepG2 strain is functional to assess the xenobiotic potential if the studied compound crosses the skin barrier. The liver is an important xenobiotic metabolizer, so analyzing the cytotoxic potential for this organ is extremely important (ATES et al., 2014; ATES et al., 2017; VINHAL et al., 2020).

Figure 9 - Cytotoxicity assay of TBMAB.
**Conclusion**

In the stability study, no significant changes were observed in the formulation. The average SPF of the sunscreen formulation was 3.5. The maximum concentration of active released was 3.63 µg.cm\(^{-2}\). In the assay of hepatotoxicity, was observed that the active sunscreen increased cell viability by 16.17% at a concentration of 0.223 g.mL\(^{-1}\) on comparison to control group; however, it was evident cytotoxic effect on the concentrations well above 0.223 g.mL\(^{-1}\).

These results demonstrate that this sunscreen formulation is safe and effective and endorse the possibility of this asset be promising prototype of a new class of sunscreens to prevent skin cancer.

**Acknowledgement**

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**Reference**


SPRINGSTEEN, A., YUREK, R., FRAZIER, M., CARR, K.F. *In vitro* measurement of sun protection


