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DESENVOLVIMENTO E ESTABILIDADE DE UM GEL ANTI-IDADE COM EXTRACTO HIDROALCOÓLICO DE SALVIA SP.

DEVELOPMENT AND STABILITY OF AN ANTI-AGING GEL WITH HYDROALCOHOLIC EXTRACT FROM SALVIA SP.

DESARROLLO Y ESTABILIDAD DE UN GEL ANTIENVEJECIMIENTO CON EXTRACTO HIDROALCOHÓLICO DE SALVIA SP.

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RESUMO

Introdução: A busca incessante de produtos vindo da natureza para aplicar na procura do tratamento, cura ou até mesmo o bem-estar associado à própria estética, tem colaborado fortemente para aprofundar a área de estudo e conhecimento em cosméticos, para assim poder atender às exigências da população mundial.
Objetivos: Este trabalho visou o desenvolvimento e avaliação da estabilidade de formulações de dois géis (carbopol e metilcelulose) a partir da incorporação dos óleos essenciais das espécies *S. officinalis e S. elegans* e o extrato hidroalcoólico das mesmas sendo este último, utilizado como princípio ativo.
Métodos: Realizaram-se testes de estabilidade físico-química, organoléptica dos géis, teste de irritabilidade ocular (teste HET-CAM) e hidrodestilação em Clevenger para assim recolher o rendimento dos óleos e utilizá-los também como conservante natural nos géis.
Resultados: Como resultados significativos obtiveram-se: testes de ciclo de luz houve apenas a alteração da cor após 15 dias. Nos testes de congelamento/descongelamento houve mudança no gel de carbopol para *S. officinalis* no aspecto e para *S. elegans* no valor de pH e aspecto, no gel de metilcelulose em S. *elegans* houve apenas alteração no valor de pH. O teste de estabilidade acelerada houve alteração para os dois géis, nas amostras contendo o gel de carbopol houve mudança de coloração e desidratação parcial do gel, no de metilcelulose ocorreu a completa desidratação.
Conclusões: As amostras dos géis com extrato hidroalcoólico de *S. elegans* e *S. officinalis* nas concentrações de 5%, 2,5% e 1,25% obtiveram boa estabilidade de acordo com os testes a que foram submetidos.

Palavras-Chave: *Salvia officinalis*; *Salvia elegans*; gel; extrato hidroalcoólico; óleo essencial

RESUME

Introduction: The incessant search for products from nature to apply in the search for treatment, cure or even the well-being associated with the aesthetics itself, has strongly collaborated to deepen the area of study and knowledge, so as to meet the demands of the world population.
Objectives: This work aimed at the development and evaluation of the stability of two gels formulations (carbopol and methylcellulose) from the incorporation of the essential oils of the species *S. officinalis* and *S. elegans* and their hydroalcoholic extract.
Methods: Physicochemical stability, organoleptic gel tests, eye irritability test (HET-CAM test) and Clevenger hydrodistillation were performed to determine the oil yield and to use them as a natural preservative in the gels.
Results: As significant results we have: In light cycle tests there was only a color change after 15 days. In freeze / thaw tests there was a change in carbopol gel for *S. officinalis* in appearance and for *S. elegans* in pH and appearance, whereas in methylcellulose gel in *S. elegans* there was only change in pH. The accelerated stability test showed alteration for both gels, in the samples containing carbopol gel there was color change and evaporation of part of the gel, while in methylcellulose the complete dehydration.
Conclusions: The samples of the gels with hydroalcoholic extract of *S. elegans* and *S. officinalis* in concentrations of 5%, 2.5% and 1.25% obtained good stability according to the tests to which they were submit.

Keywords: *Salvia officinalis*; *Salvia elegans*; gel; hydroalcoholic extract; essential oil

RESUMEN

Introducción: La búsqueda incesante de productos de la naturaleza para aplicar en la búsqueda de tratamiento, cura o incluso el bienestar asociado con la estética en sí, ha colaborado fuertemente para profundizar el área de estudio y conocimiento, a fin de satisfacer las demandas de la población mundial.
Objetivos: Este trabajo tuvo como objetivo el desarrollo y evaluación de la estabilidad de dos formulaciones de geles (carbopol y metilcelulosa) a partir de la incorporación de los aceites esenciales de las especies *S. officinalis* y *S. elegans* y su extracto hidroalcohólico.
Métodos: La estabilidad fisicoquímica, las pruebas de gel organoléptico, la prueba de irritabilidad ocular (prueba HET-CAM) y la hidrodestilación de Clevenger se realizaron para recolectar el rendimiento de aceite y también para usarlos como conservantes naturales en los geles.
Resultados: Como resultados significativos tenemos: En las pruebas de ciclo de luz solo hubo un cambio de color después de 15 días. En las pruebas de congelación / descongelación hubo un cambio en la apariencia del gel de carbopol para *S. officinalis* y para *S. elegans* en pH y apariencia, mientras que en el gel de metilcelulosa en *S. elegans* solo hubo cambio en el pH. La prueba de estabilidad acelerada mostró alteración para ambos geles, en las muestras que contenían gel de carbopol hubo cambio de color y evaporación de parte del gel, mientras que en la metilcelulosa la deshidratación completa.
Conclusiones: Las muestras de los geles con extracto hidroalcohólico de S. elegans y S. officinalis en concentraciones de 5%, 2.5% y 1.25% obtuvieron buena estabilidad de acuerdo con las pruebas a las que fueron sometidos.

Palabras clave: *Salvia officinalis*; *Salvia elegans*; gel; extracto hidroalcohólico; aceite esencial

INTRODUCTION

The mixing and handling of plant, animal and mineral substances started the first formulations based on natural products produced by man. Ancient civilizations had this first contact, realizing that some plants had a "curative" potential. With the beginning of the isolation of active ingredients, medicinal plants started to be used based on their known activities (Viegas Jr, Bolzani, & Barreiro, 2006). With the demand for current technologies, the development of drugs and cosmetics, using natural products, has grown increasingly in order to meet the requirements and the growing demand of the world market. To be satisfactory, they need to be in compliance considering their quality, safety and effectiveness, according to their physical-chemical characteristics (Silva et al., 2015).

Due to the immense diversity of metabolites, mainly the secondary ones, plants are a major source of compounds with therapeutic interest, which have the capacity to originate new formulations that use diverse chemical, physical, cosmetological, pharmacological, botanical, toxicological and agronomic knowledge (Brandão, David, Couto, & Nascimento, 2010).

Salvia officinalis is a species from the Middle East and the Mediterranean that belongs to the Lamiaceae family and it is considered an aromatic plant with medicinal properties (Baricevic & Bartol et al., 2000 quoted by Povh & Ono, 2008). Used in folk medicine for the treatment of different types of conditions, highlighting its emenagogue, diaphoretic, germicidal, anti-inflammatory, antioxidant, astringent and insecticidal characteristics (Evans, 1991; Hertwig, 1991; Costa, 1994 como citados em Povh & Ono, 2008). They present in their composition several active substances, among them hydroxycinnamic acids (chlorogenic acid), triterpenes, (ursolic acid), diterpenes (carnosolic acid), volatile oils (including thujone and camphor) and phenols in particular flavonoids (apigenin and luteolin-7-glucosides) (Draelos, & Thaman, 2006.

Salvia elegans is from Mexico and Guatemala, also belonging to the Lamiaceae family, known for being widely used as a natural preservative or flavoring and used also, in several diseases linked to the central nervous system, like as an antidepressant (González-Cortazar et al., 2013). Previous studies demonstrate the great antioxidant capacity, associated with its richness in caffeic acid (Pereira et al., 2018).

The essential oils present in aromatic species are compounds derived from the secondary metabolism of plants that have a particular odor, changing the organoleptic characteristics of plants, acting as chemical messengers between plant and environment (Araújo, 2009 quote in Silva, et al., 2015). For the two plants studied, the following constituents can be obtained for *S. elegans* and *S. officinalis* respectively: borneol (17.4%), β-eudesmol (10.4%), bornyl acetate (5%) guaiol (4.8%); and α-thujene (25.8%), viridiflorol (20.4%), β-thujene (5.7%) camphor (6.4%) (Ali, et al., 2015).

The secondary metabolites present in both plants have antioxidant characteristics, as they have phenolic compounds, mainly flavonoids and terpenoids, that can act against free radicals, which are one of the factors responsible for cell aging (Sousa, et al., 2007).

The search for cosmetics with potential to delay cellular aging has increased among the population, requiring the market to develop and research new formulations with active principles capable to prevent the action of free radicals, being therefore one of the important characteristics for an anti-aging formulation its antioxidant capacity (Mariotti, & Frasson, 2011).

Gel formulations allow the speed of a treatment that would, otherwise, be more prolonged or more expensive. This type of formulation is a vehicle for water-soluble and fat-soluble active ingredients (Melo, Domingues, & Lima, 2018), allowing easy spreading and excellent absorption.

According to Directive 76/768 / EEC, the cosmetic product must be safe, in other words, not cause any damage to the human health when used in topical application (Chorilli, Scarpa, Leonardi, & Franco, 2007), for the purpose of cleaning and protection. According to Oriqui, Mori and Wongtschowski (2013), stability tests expose cosmetics to numerous variables, such as temperature, luminosity, vibration, among others, with the aim of analyzing the product's behavior against these factors. These tests are important to establish the shelf life, as well as stipulate adequate storage conditions and data on the degree of confidence and safety of cosmetic products.

The objective of this project is to formulate anti-aging gels in order to combat the undesirable effects of aging, associated with skin oxidations based on carbopol and methylcellulose, using the hydroalcoholic extract of *Salvia officinalis* and *Salvia elegans* in three different concentrations (1.25%, 2.5% and 5%) as an active ingredient and the essential oil as a natural preservative to check its stability and safety with centrifugation, mechanical vibration, light and dark cycles, freezing tests / defrosting, stability accelerated, pH and HET-CAM. In addition, an in vitro culture method was developed for the same species for further development of the active principle with total independence from the alternation of seasons, harvest or pests that may alter the yield.

1. METHODS

The study deals with a quantitative experimental research, which "consists in determining an object of study, selecting the variables that would be able to influence it, defining the forms of control and observation of the effects that the variable produces on the object" (Gil , 2002, p.47), with the researcher as an active agent in the process.

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1.1 Plant Material and Obtaining Hydroalcoholic Extract

The samples of *S. officinalis* and *S. elegans* were collected at the Polytechnic Institute of Bragança - Portugal, from October/2018 to October/2019 for the later stages in the production of gels and *in vitro* culture development of the two species of Salvia. After harvesting, 5 g of plant were weighed and hydroalcoholic extraction was performed. The extraction was started by placing the cut leaves and stems in 150 mL of 80% ethanol at room temperature under magnetic stirring for 30 minutes. The mixture was filtered and the procedure was performed two more times, and the obtained extract was stored at -20 °C.

1.2 Chemical analysis of the hydroalcoholic extract

Ultra-high Performance Liquid Chromatography coupled to Diode Array Detector and an Electrospray Mass Spectrometer (UHPLC-DAD-ESI/MSn) analyses of phenolic profiles from the *S. officinalis* and *S. elegans* extracts were carried out with a mass spectrometer Thermo Xcalibur Qual Browser (Thermo Scientific, San Jose, CA, USA) using the conditions previously described by Afonso et al (2017).

1.3 Preparation of Carbopol and Methylcellulose gel

The carbopol® gel was prepared using the formula with 15% absolute ethanol, 85% purified water and 2 grams of carbopol® for each 100 ml of formulated gel. For this, the absolute ethanol was mixed with the purified water, in the proportions specified previously, and with the aid of a tamise the carbopol was sprinkled over the hydroalcoholic mixture, leaving it to stand for 24 hours. Triethanolamine was used to correct the pH to a value of 7, using approximately 1 mL of this for each gram of carbopol contained in the gel. As for the preparation of the methylcellulose gel, the same proportions of absolute ethanol and water mentioned above were use, however, 2 ml of glycerin was added for each 100 mL of manipulated gel. The manipulated gel is made having absolute ethanol with water mixed and, methylcellulose was sprinkled over the glycerin. Finally, the hydroalcoholic mixture was added, mixing quickly and with the help of a magnetic stirrer the gel was homogenized. After the preparation of the two types of gels, were added hydroalcoholic extracts of *S. officinalis and S. elegans* in three different concentrations (1.25%, 2.5% and 5%) to be subjected to stability tests.

1.4 Physical-chemical stability tests

Centrifugation

Centrifugation tests were performe by adding 0.3 grams of each gel in eppendorf tubes. These tubes were introduce in a centrifuge for 30 minutes at 24 ° C and 3000 r.p.m (Agência Nacional de Vigilância Sanitária [ANVISA], 2004). The test was performe in triplicate for each concentration of hydroalcoholic extract of two species of sage and for gels without an active ingredient.

Mechanical Vibration

Mechanical vibration tests were performed by adding 0.3 grams of each gel in eppendorf tubes and submitting each tube to vibration in a vortex for 15 seconds. It was carried out in triplicate, for each concentration of hydroalcoholic extract, and for gels without an active ingredient.

1.5 Light and Dark Cycles

The light cycles were performed exposing and the samples to a photoperiod of 16 hours in light and 8 hours in the dark, for two weeks, to evaluate the stability of the gels in relation to color, odor and appearance. All tests were performed in triplicate for gels with no active ingredient and for gels with active ingredient in concentrations of 5%, 2,5% e 1,25% hydroalcoholic extract of *S. elegans* and *S. officinalis.*

According Marx (2004, p. 5) “The lighting used in the tests should simulate the intensity to which the cosmetic would probably be exposed.”

1.6 Temperature at 25 ° C

The samples were submitte in triplicate, for two weeks, at an ambient temperature of 25 ° C controlled in an oven, to evaluate their stability.

1.7 Freeze-thaw cycles

Samples of 5 grams of each gel were submitte, in triplicate, to alternating temperatures of 45 ° C and -20 ° C at regular intervals of 24 hours, for two weeks, in order to assess the stability with regard to color, odor, pH and appearance. Second Marx (2004) some changes can be identified through this test, such as suspension problems occurring crystallization or cloudiness of the samples as well as instabilities in the formulations.

1.8 Accelerated Stability

Samples containing 3g of each prepared gel were subjected to a temperature of 40 ° C ± 2 ° C at 75% ± 5% relative humidity (H.R) for two weeks. Stability was verified by evaluating color, odor, pH and appearance (Bouranen, 2017).

1.9 Determination of pH
The pH was determined by means of pH tapes, obtaining the values through the reference colorimetry.

1.10 Testes organolépticos
The appearance, color and odor were evaluated through visual and olfactory examination in order to analyze the integrity of the gels for two weeks regarding all physical-chemical tests performed. Organoleptic assessment was classified according to the following criteria: 1- No visible changes; 2- Moderate change; 3- Changed.

1.11 Eye irritation assessment using the HET-CAM test
Twenty chicken eggs, from the transmontana autochthonous breed, were use per test, which were place in an incubator for 10 days. A flashlight was use determine the presence of the embrio and inshure that the eggs were fertilized, as needed by the validated protocol. These eggs were test for possible bleeding and irritabilities, witch the formulations could cause, when in contact with the chorea-allantoic membrane (HET-CAM). A negative control (0.9% NaCl) and a positive control (1% NaOH) and 3 replicates of the samples were use. 3 grams of each formulation were place in contact with the choro-allantoic membrane and parameters of irritability, hemorrhage or coagulation were evaluate. The procedure was performe according to the guidelines of the Interagency Coordinating Committee on Advancing Alternatives the Validation of Alternative Methods (2010).

1.12 In vitro culture of Salvia elegans and Salvia officinalis
For the in vitro culture of *Salvia elegans* and *Salvia officinalis* the MS culture medium was use, supplemented with the kinetin phytoregulators (1 mg) and IBA (0.5 mg) and 20 g of sucrose, per liter of prepared culture medium. To prepare the plant material for inoculation, it was necessary an initial disinfection, which was carried out for 7 minutes, with agitation, in a 5% chlorine solution, plus 10 drops of tween 80 per 100 mL of sterile solution. After washing in sterile water, the explants were move to a 70% ethanol solution for 5 minutes. The explants were wash in sterile water and, together with the previously prepared culture medium, the in vitro inoculation was carried out, in a laminar flow chamber. Inoculation was do, using tubes and flasks, which were placed in an *in vitro* culture chamber for vegetative multiplication, with controlled light system, and temperature (16h of light / 8 hours of darkness with lamps dayligth at 24°C ± 2°C).

2. RESULTS AND DISCUSSION

The obtaining of essential oil by hydrodistillation at Clevenger generated a yield of 0.45% for *S. elegans.* It was not possible to perform the extraction for the plant *S. officinalis* due to the lack of plant material. According to Moraes (2009), seasonality can influence the chemical compounds of plants and this can reflect on the yield of essential oil content according to the seasons. The harvest of S. elegans was carried out in the late summer period and throughout the autumn period, which was a possible reason for a low yield of essential oil, since in periods of low light and temperature it tends to decrease the production of essential oil for the species under study (Viecelli, & Cruz-Silva, 2009). For this reason, it was not possible to incorporate essential oil as a preservative in the gels using only hydroalcoholic extract.
The hydroalcoholic extracts of *S. officinalis* and *S. elegans* analysed by LC-DAD-ESI/MSn have shown similar phenolic profiles to the previous described for decoctions (Pereira et al, 2018). In more detail, *S. officinalis* was mainly composed by rosmarinic acid, apigenin-O-glucuronide, scutellarein-O-glucuronide and luteolin-7-O-glucuronide while rosmarinic acid, salvianolic acid K, luteolin-7-O-glucuronide and caffeic acid are the major constituents of *S. elegans* extract.
Concerning the color evaluating of the gels under study, it was noticed that carbopol® samples without the active ingredient were translucent. After the addition of hydroalcoholic extracts of *S. elegans* and *S.officinalis* at concentrations of 5%, 2.5% and 1.25%, which were of an intense green color, resulted in gels with a yellowish / greenish tint that intensified according to the increase in concentrations of extract in the gels (Figure 1). The samples of methylcellulose gels without addition of the active ingredient had a yellowish color, and the same with the incorporation of hydroalcoholic extracts with concentrations of 5%, 2.5% and 1.25% resulted in a yellowish / greenish hue that intensified according to the addition of the extract in the above values.

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Figure 1 - Carbopol gels with *S. elegans* hydroalcoholic extract in concentrations of 5%, 2.5%, 1.25% and without active ingredient in decreasing order.
Source: Elaborated by the authors, 2019.

Analyzing the stability tests, it was found that during centrifugation, none of the samples underwent any visible change. The centrifugation test makes it possible to identify phase separations, precipitations, among other visually perceptible changes, since it "produces stress in the sample, simulating an increase in the force of gravity, increasing the mobility of the particles and anticipating possible instabilities" (ANVISA, 2004, p. 35).

After the mechanical vibration test, the samples remained unchanged in relation to the organoleptic (aroma, color and general aspects) and physicochemical parameters. This type of test aims to evaluate the behavior of formulations subject to vibrations associated with means of transport, which can alter the characteristics of the samples (ANVISA, 2004).

According to the exposure of these samples to the light and dark cycle, color changes were detect in all those that had extracts from the two plants under study, which completely lost the shade presented before exposure to the test. Gels with no active ingredient, however, maintained their characteristic color, translucent to the carbopol gel and yellow to the methylcellulose gel.

During the exposure of the samples to an ambient temperature of 25 ° C, changes in color, odor and appearance were not observe, only small pH variations for *S. elegans* in the methylcellulose gel, where the pH varied from 6.1 to 5.6.

Exposure to extreme temperatures during the freezing and thawing test modified the appearance of the samples with carbopol® gels and with extract of *S. officinalis* and *S. elegans* (Figure 2), making them granular. The pH was changed in the samples of the carbopol® (3.6-4.1) and methylcellulose gels with *S. elegans* (5.6-6.1) represented in (Figure 3). In this type of trial "the occurrence of physical-chemical changes is frequent and even expected" requiring careful evaluation of the results (ANVISA, 2004, p. 16).

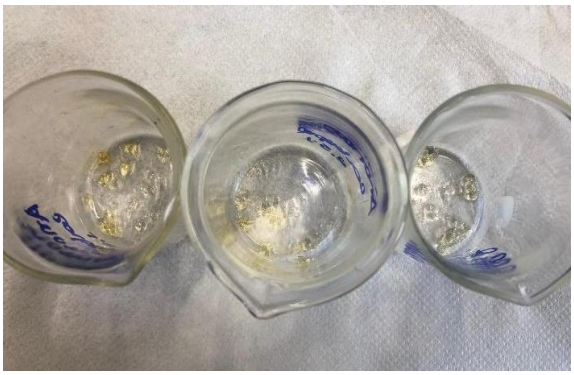

Figure 2 - Accelerated stability test for carbopol gel with *S. elegans* and *S. officinalis* in the 3 concentrations (1.25%, 2.5% and 5%).
Source: Elaborated by the authors, 2019.

Figure 3 - Accelerated stability test for methyl cellulose gel with *S. elegans* and *S.officinalis* in the 3 concentrations (1.25%, 2.5% and 5%).
Source: Elaborated by the authors, 2019.

In the accelerated stability test, after two weeks at 40 ° C ± 2 ° C at 75% ± 5% relative humidity (HR), the results for the carbopol gel, with the two Salvia species, were that, part of the gel water has evaporated. In the remain gel, there was a color change from translucent to light green and in relation to the methylcellulose gel there was complete dehydration.
The evaluation of eye irritability using the HET-CAM test did not show irritation, hyperemia or hemorrhage in the choro-allantoic membrane of the eggs used, being the same compared with the negative (0.9% NaCl) and positive (1% NaOH) controls, highlighting the possibility of not being irritating to the skin and eyes. All gels had an alcoholic odor before and after all tests. Possibly this would be not notice if the gels have the essential oils added, since the essential oils have fragrances more intense than the alcohol.
In vitro culture of *S. elegans* and *S. officinalis* with MS medium (Murashige & Skoog, 1962, as quoted in Pinto, Arello, Pinto, & Barbosa, 1996) and kinetin (1 mg / L) and IBA (0.5 mg / L) as phytoregulators, gave very positive results (Figure 4). After, the first week of inoculation, there was already the development of leaves and new meristems, with a multiplication rate of 50% after 1 month and after 28 days, root growth of the plants in the bottle was already observed, as can be seen in figure 4. Altogether, 50 explants were used to biomass development, with the aim of future works with this material, as these plants have no seasonal constraints.

Figure 4 - In vitro culture of *S. elegans* in Medium MS with phytoregulators Cinetina (1 mg) and IBA (0.5 mg) per liter, period of two months.
Source: Elaborated by the authors, 2019.

CONCLUSIONS

The samples of the gels with hydroalcoholic extract of *S. elegans* and *S. officinalis* in concentrations of 5%, 2.5% and 1.25% obtained good stability according to the tests to which they were submit.
The samples analyzed with the light and dark test lost their initial color completely. The appearance of carbopol gels with extract from both species was modified when subjected to the freezing and thawing test of the samples, becoming granular. There were variations in the pH of the carbopol and methylcellulose gels for the assay with *S. elegans*. The pH not changed for any of the two gels for *S. officinalis*. Despite the pH changes in the different tests, they never exceeded the ideal limits of the skin. According to

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Gonçalves, Brianezi, and Miot (2017), the skin has a pH between 4.6 and 5.8, that is, slightly acidic. This characteristic contributes to the smooth functioning of one of its main functions, which is to act as a protective barrier against foreign microorganisms.
The presence of phenolic compounds in the two plants, *S. elegans* and *S. officinalis*, validates the anti-aging activity of the gel, since according to Ali et al. (2015) these compounds are important antioxidant agents.
Regarding the *in vitro* culture of the two species, the result was promising. Plant development, was observed, with a meristematic multiplication rate close to the values in the literature (MIŠIĆ et al, 2006; Gostin, 2008; Pistelli et al, 2013), although using indole-3-butyric acid (IBA) (in the literature in general, α-naphthaleneacetic acid (NAA) is considered more efficient in obtaining complete plants with root system developed for acclimatization).
The continuity of studies is important, in order to complement and obtain more information on the subject.

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