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Development of biotechnological formulations containing *Viscum album* L. for topic and transdermal use

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I dedicate this work to my father Ivan, who always believes in me and does everything he can to support my dreams.

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"Do not be afraid of your difficulties. Do not wish you could be in other circumstances than you are. For when you have made the best of an adversity, it becomes the stepping stone to a splendid opportunity."

H.P. Blavatsky

ABSTRACT

BATISTA, João Vitor da Costa. **Development of biotechnological formulations containing *Viscum album* L. for topic and transdermal use**. Rio de Janeiro, 2019. Thesis (MSc in Plant Biotechnology), Federal University of Rio de Janeiro.

Viscum album L. (Santalaceae), also known as mistletoe, is a semi-parasitic plant that grows in different host trees. In complementary medicine, European mistletoe extracts have been widely used in cancer treatment for at least 100 years. However, the complex phytochemical composition of mistletoe extract, as well as the low water solubility of some of its main pharmacological components, have motivated the development of new delivery systems. From a pharmaceutical standpoint, new systems for drug release focus mainly on releasing new drugs at a gradual and controlled pace, improving bioavailability, reducing side effects, protecting the drug from any degradation, carrying different classes of hydrophilic and lipophilic substances, reducing therapeutic doses and intake, as well as increasing patients´ compliance by decreasing pain and avoiding hospitalization for delivery of treatment. Considering the efficacy of the antitumoral therapy of *V. album* and new systems of pharmaceutical release, the goal of the present work was to develop and evaluate the antitumoral potential of new formulations containing *V. album* extract prepared from plants collected from the host tree *Abies alba*, due to previous results demonstrating the higher *in vitro* activity of this subspecies against tumoral cells (Yoshida and Molt-4) The research involved several chemical analyses of the plant derived extract, the development of new systems of pharmaceutical application with a new adhesive patch and hydrogel, the study of the stability of these application systems and the evaluation of their antitumoral activity *in vitro*, comparing the formulations containing dry and aqueous extract The herbal drug (*V. album*) was prepared as a lyophilized dry extract and chemically characterised by thin layer chromatography, as well as by its total flavonoids content. The hydrogel of poloxamer 407 20% containing 5% of dry or aqueous extract presented stability in pH, total flavonoids content and microbiological analyses. The hydrogel was differentiated through rheology parameters and studies of *in vitro* permeability indicated that only the hydrogel showed a transdermal potential, after 8 hours of tests. Both formulations (hydrogel and patch) presented selectivity towards tumoral cells of sarcoma and leukaemia (Yoshida and Molt-4) when compared to normal human keratinocytes (HaCaT), with higher antitumoral activity from the dry extract, compared to the aqueous one. The current

project presents, for the very first time, the development of new topical formulations based on *V. album* and the promising antitumoral activity of the ethanolic lyophilized extract in comparison to the aqueous extract.

Key words: *Viscum album*, dry extract, pharmaceutical formulation, hydrogel, patch, topic, transdermal, rheology, *in vitro*, cytotoxicity.

RESUMO

BATISTA, João Vitor da Costa. **Desenvolvimento de formulações biotecnológicas contendo *Viscum album* L. para uso tópico e transdérmico**. Rio de Janeiro, 2019. Dissertação (Mestrado em Biotecnologia Vegetal), Universidade Federal do Rio de Janeiro.

Viscum album L. (Santalaceae), popularmente conhecido como visco, é uma planta semi-parasita que cresce em diferentes árvores hospedeiras. Na medicina complementar, os extratos do visco europeu têm sido utilizados no tratamento de câncer há mais de 100 anos. No entanto, a complexa composição fitoquímica dos extratos de visco associada à baixa solubilidade em água dos seus principais constituintes farmacológicos tem motivado o desenvolvimento de novos sistemas carreadores. Do ponto de vista farmacêutico, os novos sistemas de liberação de drogas visam fornecer uma liberação gradual e controlada, melhorar a biodisponibilidade, reduzir os efeitos colaterais, proteger o fármaco contra a degradação, carrear as diferentes classes de substâncias hidrofílicas e lipofílicas, reduzir a dose terapêutica, o número de administrações, assim como aumentar a adesão do paciente ao tratamento ao diminuir a dor e evitar a hospitalização para a administração da medicação. Considerando a eficácia da terapia antitumoral de *V. album* e os novos sistemas de liberação de fármacos, o objetivo do presente projeto foi o desenvolvimento e a avaliação do potencial antitumoral de novas formulações contendo extratos de *V. album* preparados a partir de plantas coletadas do hospedeiro *Abies alba*, devido a resultados prévios demonstrando uma maior atividade *in vitro* desta subespécie frente a linhagens tumorais (Yoshida e Molt-4) O plano de pesquisa envolveu as análises químicas do insumo ativo vegetal, o desenvolvimento dos sistemas de liberação tópica na forma de adesivo (patch) e hidrogel, a análise reológica do hidrogel, o estudo de estabilidade dos sistemas desenvolvidos e a avaliação da atividade antitumoral *in vitro*. O insumo ativo vegetal (*V. album*) foi preparado na forma de extrato seco liofilizado e caracterizado quimicamente por cromatografia em camada fina e teor de flavonoides totais. Os hidrogéis de polaxamer 407 a 20% contendo 5% do extrato seco ou aquoso apresentaram estabilidade quanto ao pH, ao teor de flavonoides totais e aos ensaios microbiológicos. Os hidrogeis foram diferenciados a partir dos parâmetros de reologia e os estudos de permeação *in vitro* indicaram o potencial transdérmico apenas do hidrogel, a partir de 8h de ensaio. Ambas as formulações (hidrogel e adesivo) apresentaram seletividade para as células tumorais de sarcoma e leucemia (linhagens Yoshida e Molt-4)

quando comparadas aos queratinócitos humanos normais (linhagem HaCaT), sendo observada maior atividade antitumoral do extrato seco em comparação ao aquoso. Este projeto apresenta, pela primeira vez, o desenvolvimento de novas formulações tópicas a base de *V. album* e a atividade antitumoral promissora do extrato etanólico liofilizado em comparação ao extrato aquoso.

Palavras-chave: *Viscum album*, extrato seco, formulação, hidrogel, adesivo, tópico, transdérmico, reologia, *in vitro*, citotoxicidade.

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ABBREVIATIONS

B16F10	murine melanoma cell line
CFU	colony-forming unit
DAD	diode array detector
DMSO	dimethyl sulfoxide
HaCat	immortalized human keratinocytes
HCT 116	human colorectal carcinoma cell
HIV	human immunodeficiency virus
HPLC	high performance liquid chromatography
HPLC-DAD-MS	high performance liquid chromatography with diode array detection – mass spectrometry
HPLC-MS	high performance liquid chromatography – mass spectrometry
HPLC-UV	high performance liquid chromatography with ultraviolet detector
IC-50	half maximal inhibitory concentration
K562	human chronic myeloid leukemia cell
Molt-4	human acute T lymphoblastic leukaemia
MTT	[3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide
NP/PEG	diphenylborinic acid aminoethylesther and polyethylene glycol
PBS	phosphate-buffered saline
PEG 400	polyethylene glycol 400
PEO	polyethylene oxide
pH	potential of hydrogen
PPO	polyphenylene oxide
PVA	polyvinyl alcohol
PVPK90	polyvinylpyrrolidone K90
Rf	retardation factor
TLC	thin layer chromatography
UHPLC	ultra high performance liquid chromatography
UV-Vis	Ultraviolet visible
WHO	world health organization
WST-1	colorimetric cell viability kit assay
Yoshida	Yoshida ascites sarcoma cell
3ITT	Three interval thixotropy test

CONTENTS

1. INTRODUCTION

The high incidence of cancer is considered a very important topic in public health. Significant efforts are being carried out by the scientific community to develop new therapeutic approaches against cancer, as well as new efficient drugs with less adverse effects. In that context, natural products are considered important sources of substances that act against cancer, being known that approximately 55% of antitumoral drugs approved between 1940 and 2014 came from natural resources (NEWMAN; CRAGG, 2016).

Aqueous extracts of *Viscum album* have a long history in complementary therapy against cancer, primarily in Central Europe (TRÖGER *et al.*, 2013). The most studied active substances in aqueous extracts of *V. album* are lectins and viscotoxins. These compounds induce macrophage cytotoxicity, stimulate phagocytosis by immune cells, increase cytokines secretion and stimulate cytotoxic effects in several cell lines *in vitro* (ESTKO *et al.*, 2015; TIMOSHENKO; CHERENKEVICH; GABIUS, 1995). The use of *V. album* did not show benefits for melanoma treatment when submitted to high level clinical trial (KLEEBERG *et al.*, 2004); however, a retrospective study observed a few benefits from the aqueous extracts of *V. album* (AUGUSTIN *et al.*, 2005).

Some studies have described the medicinal application of substances from *V. album* in hydro alcoholic tinctures, associated or not with chemotherapy. It was possible to correlate the antitumoral activity of these preparations with the presence of ethanolic antitumoral substances when Ehrlich carcinoma cells (STAN *et al.*, 2013), and HeLa cancer cells (SARPATAKI *et al.*, 2015) were used as models. Moreover, Ceboyic *et al.* (2008) demonstrated the efficacy of *V. album* non-polar extract, prepared by CO_2 supercritical fluid, in the cytotoxicity against Ehrlich carcinoma cells, confirming the importance of improving extractive methodologies. The study developed by Melo *et al.* (2018) showed the antitumoral activity of *V. album* tinctures against B16F10 and K562 cell lines by apoptosis triggering and cell cycle alterations. The presence of important substances, such as chlorogenic acid, caffeic acid, sakuranetin, naringenin 5-methyl ether, syringenin 4-*O*-glycoside (eleutheroside B), syringenin 4-O-apyoyl-glycoside (polygalactenoside E), alangilignoside C and ligalbumoside A, identified in these tinctures is probably involved with the antiproliferative and apoptotic effects,

emphasizing the importance of new studies with ethanolic soluble substances in *Viscum album* cancer pharmacotherapy.

V. album preparations, as most phytotherapic compounds, present therapeutic limitations attributed to low solubility and adsorption, along with chemical instability. Specially for phytotherapic formulations, the encapsulation in transdermal delivery systems improves absorption and bioavailability, decreases the therapeutic dose, because only a small amount of raw material is needed, and increases stability, since these delivery systems promote physical and chemical protection to the encapsulated raw materials (FALCÃO *et al.*, 2018; MENDES *et al.*, 2017). Besides, a very important benefit of this new route of administration would be seen in the paediatric population, as a non-invasive transdermal route would be an alternative to oral and intravenous routes (DELGADO-CHARRO; GUY, 2014). Currently, only one topic formulation is approved by the United States Food and Drug Administration (FDA) for cancer treatment of keratosis actinic and superficial basal cell carcinoma (PRINCE *et al.*, 2018).Therefore, the development of a transdermal delivery system containing hydrophilic and hydrophobic substances of *V. album* extracts can improve the antitumoral potential of this therapy as well as its local effect.

The present work focused on the development of topical formulations for transdermal delivery of *V. albu*m as a new route of administration with systemic effect. Stability studies of these formulations were carried out to verify chemical and rheological parameters. Also, *in vitro* cytotoxicity assays were performed using tumoral and non-tumoral cell lines.

2. LITERATURE REVIEW

2.1. CANCER

According to the World Health Organization (WHO) (2017), cancer is a generic term used to describe a large group of diseases characterized by abnormal cell growth beyond its usual limits and it is one of the main causes of death worldwide. Although 80% of cancer development is attributed to environmental factors, risk factors, intrinsic susceptibility and other elements are also associated with this chronic disease (INCA, 2019).

The main and predominant components of cancer include chronic inflammation, genomic instability and abnormal vascularization (HUREZ *et al.*, 2018). Amongst these, the immune system has already been recognised as the most dominant factor in cancer control, since weakness in the immunity parameters is related not only to the carcinogenesis process but also to cancer progression (GOPALAKRISHNAN *et al.*, 2018). It is known that the unbalance in the processes of cellular proliferation and cell death is one of the key factors for cancer development. Controlled mechanisms of apoptosis must be re-established, as a crucial part of this process involves signalling neighbouring cells with phagocytic capacity that the apoptotic cell is no longer required (LYONS; CLARKE, 1997; MARTIN; GREEN, 1995). Even though an efficient vascular growth was created, due to genetic mutations and an environment changing, if the apoptotic mechanism is not re-established, the support of phagocytic cells is not enough to control the proliferation of tumoral cells (LOIZZI *et al.*, 2017; ZIYAD; IRUELA-ARISPE, 2011).

In the last decade, there was a considerable progress in cancer treatment, with the development of several drugs focused on the molecular aspect of the disease. Although surgery, chemotherapy and radiotherapy persist as main treatment strategies, they present several limitations, like low probability of cure after cancer recurrence, adverse effects due to low selectivity of the drugs and high costs related to the use of high technology (HAMAGUCHI; WADA, 2017). Most chemotherapy and radiotherapy adverse effects are due to their non-selectivity for tumoral cells, as they act as antimitotic, stopping cell cycle completely instead of activating the apoptotic pathway (BAIG *et al.*, 2016; BASKAR *et al.*, 2014; VAN VUUREN *et al.*, 2015).

The limitations presented by the main therapies currently in use generate an interest in the development of drugs with higher selectivity and specificity of action against several types of tumours, proposing new therapeutic methodologies such as epigenomics, nanotechnology and natural metabolites (LEITE; DA CUNHA; COSTA, 2018).

The Plantae Kingdom presents a wide range of resources for research and development of new drugs, as can be observed throughout history in the use of plants` raw forms in teas, ointments and syrups, among others. Thus, this Kingdom is targeted in the need for discovery and development of new drugs with anticancer therapeutic activity (GALI-MUHTASIB *et al.*, 2015). Plants and their metabolites have already shown promising results against a lot of malignancies, in association to radio and chemotherapy (REJHOVÁ *et al.*, 2018; YING *et al.*, 2018). There are four classes of plant-derived anticancer drugs already in the market: the vinca alkaloids (vinblastine, vincristine and vindesine), the epipodophyllotoxins (etoposide and teniposide), the taxanes (paclitaxel and docetaxel) and the camptothecin derivates (camptotecin and irinitecan), isolated from *Catharanthus roseus* G. Don., *Podophyllum peltatum* L., *Taxus brevifolia* Nutt. and *Campototheca acuminata* Decne, respectively (CRAGG; PEZZUTO, 2016; DEMAIN; VAISHNAV, 2011; DESAI *et al.*, 2008). Amongst the most used and researched plant metabolites against cancer, alkaloids, terpenoids and sulphydryl compounds can be highlighted (GALI-MUHTASIB *et al.*, 2015) and have been found in the plant species *Viscum album* (BÜSSING, 2000).

2.2. *VISCUM ALBUM*

Viscum album is a semi parasit, evergreen, perennial, shrub native to Europe, from the Santalaceae family (VICAS; RUGINA; SOCACIU, 2012), whose main subspecies are ssp. *album*, ssp. *abietis* e ssp. *austriacum* (BÜSSING, 2000). Only *V. album*, that presents white berries from which its name is derived, is used in cancer therapy (Figure 1). For nutrition, development and fixation, *V. album* anchors itself to a host tree via a haustorium, which does not tend to be harmful to the tree due to its slow growth of only one pair of leaves a year (MAGANO, 2012). For at least 200 years, *V. album* has been used for health benefits, being indicated for menstrual disorders, epilepsy, high blood pressure, arthrosis, diabetes, neuralgia, bleeding and endometriosis,

among other conditions. In the beginning of the 20th century, it was introduced in the treatment of cancer, based on the anthroposophical indications of Rudolf Steiner and Ita Wegman (LEV; EPHRAIM; BEN-ARYE, 2011). Nowadays, it remains in use in the form of an aqueous injectable extract, as part of complementary therapy to standard cancer treatment, mainly in Europe (BAR-SELA, 2011).

Figure 1. *Viscum album* on a host tree (left) and its leaves and berries (right).

The aqueous extracts of *V. album* are widely studied and the main components described as responsible for the antitumoral activity are viscotoxins (JUNG *et al.*, 1990), viscolectins (DEBRAY *et al.*, 1992) and polysaccharides (MUELLER; HAMPRECHT; ANDERER, 1989). Viscolectins are glycoproteins, which have cytostatic effect and immunomodulatory activity (FRANZ, 1986). Viscotoxins are polypeptides with a cytotoxic activity that depends not only on its concentration but also on its composition (SCHALLER; URECH; GIANNATTASIO, 1996).

However, the cytotoxic and cytostatic activity of *V. album* cannot be explained only for the presence of viscotoxins and viscolectins (HAJTO, 1986; KOOPMAN *et al.*, 1990). Other substances, such as phenolic compounds, alkaloids, terpenes and oligosaccharides have already been identified in aqueous and ethanolic extracts of *V. album* (MELO *et al.*, 2018; ORHAN *et al.*, 2006, 2014; PANOSSIAN *et al.*, 1998; PIETRZAK; NOWAK; OLECH, 2014; POPOVA, 1991; SINGH *et al.*, 2016; VICAŞ *et al.*, 2011). This chemical variety is probably involved in the antitumoral activity of the hydro alcoholic extracts of *V. album* (ALPSOY *et al.*, 2010; MELO *et al.*, 2018; PARK;

HYUN; SHIN, 1999), considering that viscolectins and viscotoxins are found in higher amount in the aqueous extracts (PANOSSIAN *et al.*, 1998).

Previous studies evaluated the antitumoral potential of *V.album* extracts obtained from different host trees (a) *V. album* ssp. *album* from host trees *Malus domestica*, *Quercus* sp. e *Ulmus* sp., (b) *V. album* ssp. *austriacum* from host tree *Pinus sylvestris* and (c) *V. album* ssp. *abietis* from host tree *Abies alba*. It was observed that the extract obtained from the host tree *Abies alba* presented the highest antitumoral potential, then being of choice for the present study (BATISTA *et al.*, 2015; HOLANDINO *et al.*, 2018).

It is known that the principal substances associated to the antitumoral activity of *V. album* are degraded by oral intake (MUHEEM *et al.*, 2016). Thus, the search for new pharmaceutical forms for *V. album* administration is a field to be developed. Even though topical products have existed for centuries, their numbers have been increasing and they are becoming more common along with transdermal products. Despite the recent development, they have already been shown as a good alternative route to systemic administration (SHAH *et al.*, 1992). Also, the opportunity to deliver drugs locally creates the possibility of improving efficacy and safety of cancer chemotherapy, since local delivery minimizes harmful side effects (MOSES; BREM; LANGER, 2003).

2.3. SKIN

The skin (Figure 2), the biggest organ of the human body, acts as a physical barrier, besides also being involved in body temperature and blood pressure regulation and in the sensorial and endocrine functions. It is divided into three layers: epidermis, dermis and hypodermis (EDWARDS; MARKS, 1995).

The epidermis is composed of stratified squamous epithelium, acting as an interface between internal and external environments. The structural strength and the control of water evaporation are given by the keratinocytes, the flexibility by the lipids and the protection against radiation by the melanocytes and hairs. Composed mainly by dead keratinocytes, the *stratum corneum* is the most external layer of the skin. Several cell types, like fibroblasts, collagen, elastin, nervous tissues and blood vessels, compose the dermis and provide strength, resistance, elasticity and nourishment to the skin. At last, the hypodermis is composed essentially by a layer of adipose tissue, creating a

connection between the skin and the rest of the body and acting like a thermal insulation and shock absorber (CASEY, 2002; KUSUMA *et al.*, 2010).

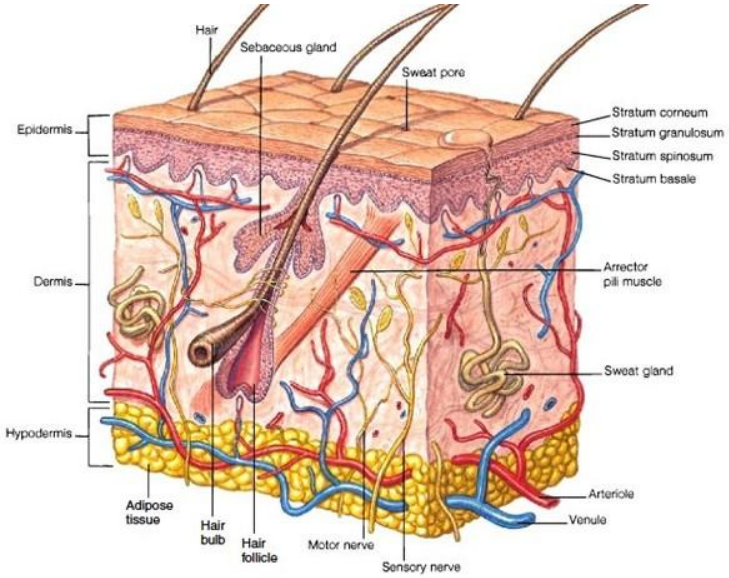

Figure 2 The schematic skin architecture with some of its components. (Source: https://medium.com/@cindymeza/layers-of-the-skin-fa974368418)

2.4. PHARMACEUTICAL FORMULATIONS FOR TOPICAL DELIVERY

In general, during the development of new therapeutic systems, as well as of new pharmaceutical formulations, five important factors have to be observed: (a) half-life of the drug; (b) excipients toxicity; (c) route of administration to minimize drug metabolism and increase biodisponibility; (d) use of the lowest concentration of the drug able to reach therapeutic effect and (e) frequency of drug administration (ZAFFARONI, 1991).

The main routes of drug administration for systemic effect are oral and parenteral. In regards to the oral route, the main challenges to bioavailability of the drug are variations in the gastrointestinal tract, pH, motility, drug degradation, food interaction and first pass effect. In addition, the high drug concentration needed to obtain the desirable therapeutic effect can cause undesirable side effects (PRAUSNITZ, 2004).

When dealing with the parenteral route, the main problems are related to low patient compliance due to pain attributed to the invasive administration process and also to the drug toxic effects when injected at high concentrations (PRAUSNITZ, 2004). Due to these challenges, topical route is an alternative to parenteral drug administration (HENRY *et al.*, 1998).

Topical delivery can be carried out through ocular, rectal, vaginal and skin routes, wherein the latter is the organ with the fastest and easiest access, being considered the main route for topical delivery (MONICA; GAUTAMI, 2014). Systems of topical delivery are usually applied onto the skin to treat dermatological problems and the goal is to create a reservoir of the drug in the skin (TOUITOU, 2002). In general, semisolid formulations are selected for having the highest retention time in the skin, liquid formulations for having the fastest absorption rate and shortest retention time in the skin and transdermal patches for generating a continuous and controlled release (WILLIAMS, 2003). Once the formulations reach the goal regarding penetration through the skin, optimization can be carried out (FLATEN *et al.*, 2015).

2.4.1. Transdermal delivery

Due to the problems with systemic drug administration cited above (like enzymatic degradation, low intestinal absorption, first pass effect, pain and inconvenience of injection, among others), transdermal route becomes an attractive option for administration of new drugs and of drugs that already exist (PRAUSNITZ; LANGER, 2008; PRAUSNITZ; MITRAGOTRI; LANGER, 2004).

As the skin evolved to prevent the inflow of toxins to the organism and to avoid water loss, it naturally developed low permeability to external molecules, with the *stratum corneum* being the first barrier for transdermal absorption. Another point to consider is the nature of the product to be transported. Hydrophilic products present low permeability and are generally used with permeation enhancers, favouring the passive transportation through the follicles and glandular ducts (PRAUSNITZ; MITRAGOTRI; LANGER, 2004). Then, the absorption can be verified by the drug level in the blood, the drug and its metabolites in the urine and the patient's clinical response (SHINGADE *et al.*, 2012).

Transdermal drug delivery presents some advantages: elimination of first pass effect; decrease in drug blood level with reduction of side effects; decrease in fluctuation of drug blood level; easy elimination in cases of intoxication; decrease of drug intake, increase in patient's compliance to treatment; increase in the therapeutic potential of the drug when avoiding problems associated with gastrointestinal absorption; and therapeutic

effect reached with lower daily dose (RASTOGI; YADAV, 2012; SHINGADE *et al.*, 2012; TANNER; MARKS, 2008).

Disadvantages of transdermal drug deliveries are: the need of presenting physical-chemical characteristics compatible with *stratum corneum* penetration; possibility of skin irritation; variation in the absorption according to the application site within the same person, from person to person and related to the patient age; drug with low molecular mass (<500 Da) and intermediate partition coefficient (NAIK; KALIA; GUY, 2000; RASTOGI; YADAV, 2012; SHINGADE *et al.*, 2012). These problems can be bypassed with the use of vesiculated systems, like liposomes, micro emulsions, nanoparticles and others. Additional strategies, like chemical enhancers (solvents and surfactants) and the use of specific techniques (iontophoresis, electroporation, ultrasound) also permit the increase in efficacy with transdermal drugs use (PRAUSNITZ; LANGER, 2008).

2.4.2. Hydrogels

Currently established as pharmaceutical formulations, hydrogels have become increasingly more popular as a result of its high content of water, soft consistency, flexibility and biocompatibility (LAFFLEUR, 2017). The first hydrogel described dates from the 1960s and hydrogel use began to rise in the 1970s for biomedical applications (CHIRANI *et al.*, 2015). They are defined as three-dimensional polymeric matrices and are hydrophilic, able to absorb high amounts of water and biological fluids and, due to the high content of water, porosity and consistency, capable of simulating natural alive tissues (CALÓ; KHUTORYANSKIY, 2015). Hydrogels present ionic and non-ionic characters and their networks can be composed of homopolymers or copolymers, becoming insoluble due to the presence of chemical or physical reticulation. Besides, the thermodynamic compatibility allows for the hydrogel swelling process in an aqueous environment (GIOFFREDI *et al.*, 2016; PEPPAS *et al.*, 2000).

Hydrogels can reach a sustained and targeted release of drugs, increasing the drug effects and lowering the side effects at the same time (CHIRANI *et al.*, 2015). Compared to other formulations, hydrogels offer easier application and use, appropriate adhesiveness and consistency, ease of manufacturing and good elasticity and flexibility (KATHE; KATHPALIA, 2017). A lot of developed hydrogels are used by the

pharmaceutical marketing for different applications, such as: wound dressing, contact lenses, drug delivery systems, hygiene products and tissue engineering (CALÓ; KHUTORYANSKIY, 2015; CHIRANI *et al.*, 2015).

Pinto *et al.* (2017) developed nanostructured hydrogels with repellent properties, which had decreased permeability through the skin when compared to a commercial formulation. Garala *et al.* (2013) developed a temperature-sensitive *in situ* gel for periodontal disease, obtaining a sustained drug release for a period of 6 h and satisfactory physical-chemical properties. For ocular delivery, Desai & Blanchard (1998) developed a pilocarpine system with sustained release, which they found to be better than the conventional eye drops. In a review about polymeric hydrogels, the authors concluded that hydrogel dressings are convenient for any wound and burn types regardless of wound shape (KAMOUN; KENAWY; CHEN, 2017).

Regarding cancer, some papers have also described the use of hydrogels. The application of hydrogels in melanoma skin cancer has several advantages over other drug delivery systems and conventional therapies, like easier accessibility and lower cost (VISHNUBHAKTHULA; ELUPULA; DURÁN-LARA, 2017). Balakrishnan *et al.* (2019) demonstrated the potential of a hydrogel sustained release doxorubicin *in situ* in drug resistant cancers, stimulating new studies for injectable hydrogels for cancer treatment. A review about injectable hydrogels for local cancer therapy concluded that this pharmaceutical formulation reduced toxicity in normal tissues, provided localized and sustained delivery of the drugs to the tumour and was more efficient in cell apoptosis and tumour growth inhibition (NOROUZI; NAZARI; MILLER, 2016).

2.4.3. Patches

Patch, or transdermal drug delivery systems, is a device loaded with drug and usually applied onto the skin to deliver a specific dose through the skin to the blood (SHINGADE *et al.*, 2012; TANNER; MARKS, 2008). There are three generations of patches: (a) first generation consists of a system able to deliver small molecules, which is already used by oral or parenteral routes; (b) second generation has permeation enhancers, which is used mainly in creams and ointments for topical use and in a few transdermal system of delivery of small molecules, like iontophoresis and ultrasound; lastly, (c) the third generation, which has permeation and biochemical enhancers, makes

it even easier to reach the desirable target, like *stratum corneum* disturbers in micro and nano scales (micro needles, thermal ablation and micro dermabrasion) (PRAUSNITZ; LANGER, 2008).

Over the years, transdermal patches have proven significant clinical benefits over other dosage forms. The first transdermal patch was approved in the 1980s to relieve symptoms of motion sickness, vomiting and nausea (TANNER; MARKS, 2008). Patch delivery systems offer a non-invasive procedure, which allows continuous intervention, with a slow, controlled and sustained release (NAIK; KALIA; GUY, 2000). They improve patient acceptance and compliance, have direct access to target or disease site and provide an alternative when oral dose is not possible, like in unconscious or nauseated patients (BROWN *et al.*, 2006). Besides the drug characteristics to skin penetration, an important characteristic of patches is adhesiveness, which is critical to efficacy, safety and quality of the product (WOKOVICH *et al.*, 2006).

Another challenge for patch development is regarding stability, creating the need for proper packing and then adding an extra step during the manufacturing process, making it more expensive when compared to tablets and capsules. Skin irritation is also an important factor to be taken into account, as with constant application it can lead to irritant contact dermatitis and decrease patients' compliance. The main difficulty, though, is still the absorption, which is affected by physicochemical nature of the drug, the site of application, thickness and integrity of the epidermis, blood flow and skin hydration and thickness. (PAUDEL *et al.*, 2010; SANTOS *et al.*, 2018; STEVENS *et al.*, 2015). All of those together make the pre formulation study development of the patch a challenge to make an efficient product.

Banerjee *et al.* (2014) developed a transdermal patch for prophylaxis against a nicotinic cholinergic receptor agonist, which had a drug release profile of up to 72 h. A methadone transdermal patch was developed to be used as detoxification for heroin addicts, being obtained a characterized patch with an adequate permeation of methadone (MUÑOZ *et al.*, 2017). A transdermal patch composed of simvastatin was developed and evaluated for its stability, composition and permeation, which resulted in a successful adhesive (PARHI; PADILAM, 2018). Ramadan *et al.* (2018) obtained a transdermal patch containing lamivudine for human immunodeficiency virus (HIV) treatment capable of reaching blood circulation for about 6 days, which could decrease viral resistance and toxicity. A randomized, multicentric trial investigated the analgesic efficacy and safety of a transdermal patch containing fentanyl compared to standard opioid treatment in cancer

patients. This study showed that the topical formulation containing a lower opioid drug load was safe and not inferior to the standard treatment (KRESS *et al.*, 2008).

The literature also describes the use of patches for cancer patients. A pilot study done with patients with neuropathic pain showed the efficacy of lidocaine patch in the management of cancer chronic pain (CHEVILLE *et al.*, 2009). A highly aggressive cancer, pancreatic cancer, was evaluated with a 3D-printed biodegradable patch, which exhibited flexibility, prolonged release and significant suppressive effect on the growth of a subcutaneous pancreatic cancer xenograft with minimized side effects (YI *et al.*, 2016). An interesting strategy with a vaccine patch containing B16F10 tumour lysates cells combined with melanin delivery was tested as an intradermal microneedle patch for gradual release into skin melanoma model. The results showed that this vaccine induced tumour regression and was also able to create innate and adaptive immune response when mice were inoculated with B16F10 melanoma cells (YE *et al.*, 2017).

2.4.4. Polymers

Polymers are the most important constituents for transdermal drug delivery and, therefore, need to present biocompatibility, consistency, efficiency on drug release to the skin and chemical compatibility with the system's components. It controls the release of the drug from the formulation and should allow the drug to diffuse properly (GEETHU *et al.*, 2013; SAHU *et al.*, 2017). According to George & Suchithra (2019), a polymer can be defined as a macromolecular structure made, partly or entirely, from a large number of similar units bonded together, containing large chains with the same or different functional group. The classification of polymers is broadly divided into natural or synthetic polymers (BRADY *et al.*, 2017; GEORGE; SUCHITHRA, 2019; GROWNEY KALAF *et al.*, 2017; GYLES *et al.*, 2017; VILLANOVA; ORÉFICE; CUNHA, 2010).

Natural polymers have some disadvantages, like microbial contamination, batch-to-batch variation, uncontrolled rate of hydration and reduced viscosity. The synthetic polymeric system has some disadvantages as well, such as high cost, toxicity, environmental pollution during synthesis, non-renewable sources, higher risk of side effects and poorer patient compliance (GEORGE; SUCHITHRA, 2019). Natural polymers advantages are biocompatibility, biodegradability and non-toxicity, whereas

synthetic polymers are hydrophobic and possessing of strong covalent bonds within their matrix, which allows for higher durability and mechanical strength (GYLES *et al.*, 2017).

The most used natural polymers for transdermal delivery systems are: cellulose derivatives (gelatine, starch, chitosan, etc); while for synthetic polymers, some important examples are: polyvinyl alcohol, polybutadiene, polysiloxane silicone rubber, butyl rubber, etc). (RASTOGI; YADAV, 2012; SINGH, S.; SAROHA; HIMANSHI, 2016; SUGIBAYASHI; MORIMOTO, 1994; VILLANOVA; ORÉFICE; CUNHA, 2010).

Polyvinyl alcohol (PVA) (Figure 3a) is a water-soluble, non-ionic biocompatible, biodegradable and non-toxic synthetic polymer, characterized by being an odourless, flavourless and translucency granulated powder. Its physical characteristics depend on the polymerization and hydrolysis content, being the latter what classifies the PVA as hydro soluble or partially hydrolysable, which presents distinct solubility. Due to its crosslinking hydrogen bonds, it possesses mechanical strength, chemical and thermal stability. (BRADY *et al.*, 2017; DEMERLIS; SCHONEKER, 2003; GYLES *et al.*, 2017; KATHE; KATHPALIA, 2017; KAZA; PITCHAIMANI, 2007; THONG; TEO; NG, 2016; YADAV; KANDASUBRAMANIAN, 2013).

PVA has already been used for contact lens, articular cartilage, vascular cell culturing, fibbers and food application, but its most described use is for transdermal drug delivery (BHUNIA *et al.*, 2013; DEMERLIS; SCHONEKER, 2003). Fully hydrolysed grade types, which are soluble in hot water, have some advantages as topical formulations, such as tissue-like elasticity, high water content, ease fabrication and ease sterilization, presenting good film forming characteristics and good adhesive properties (NUTTELMAN *et al.*, 2001; STAUFFER; PEPPAS, 1992; THONG; TEO; NG, 2016).

Another polymer with interesting properties is Poloxamer 407 (Figure 3b), a non-ionic triblock copolymer of poly ethylene oxide (PEO)- polypropylene oxide (PPO)-polyethylene oxide (PEO), mainly available as the brand name Pluronic®. The letter in the notation stands for liquid (L), paste (P), or flakes (F), whereas the first two numbers, multiplied by 300, indicate the molecular weight of the PPO block and the last number, multiplied by 10, the percentage weight fraction of the PEO block. The commonly used poloxamer in hydrogels is Pluronic® F127, which has a weight percentage of 70% PEO and a molecular weight of PPO around 4000 (DUMORTIER *et al.*, 2006; GARALA *et al.*, 2013; GROWNEY KALAF *et al.*, 2017).

Poloxamers are generally recognized as safe excipients, having been used in pharmaceutical formulations and various applications, such as injectable, oral, rectal,

ophthalmic, cutaneous, nasal and vaginal systems (BODRATTI; ALEXANDRIDIS, 2018; GIULIANO *et al.*, 2018; MATANOVIĆ; KRISTL; GRABNAR, 2014; TATINI *et al.*, 2015). Amongst the different types of poloxamers, poloxamer 407 has probably been the most used in drug delivery studies (MATANOVIĆ; KRISTL; GRABNAR, 2014; WANG, W. Y. *et al.*, 2016).

Aqueous solution of poloxamer 407 has thermo sensible properties that make it undergo sol-gel transition with increasing temperature. When the copolymer molecules aggregate in micelles by dehydration in oxide propylene hydrophobic block, it creates a thermo reversible gel for topical drug delivery. Poloxamer hydrogels have been described as cell and drug carriers due to their low toxicity, good drug solubilizing and loading capacity, reversal thermal gelation, ability to gel in physiological conditions, good drug release characteristics and achieving improved compliance and bioavailability (BAN *et al.*, 2017; DUMORTIER *et al.*, 2006; GIOFFREDI *et al.*, 2016; GIULIANO *et al.*, 2018; GROWNEY KALAF *et al.*, 2017).

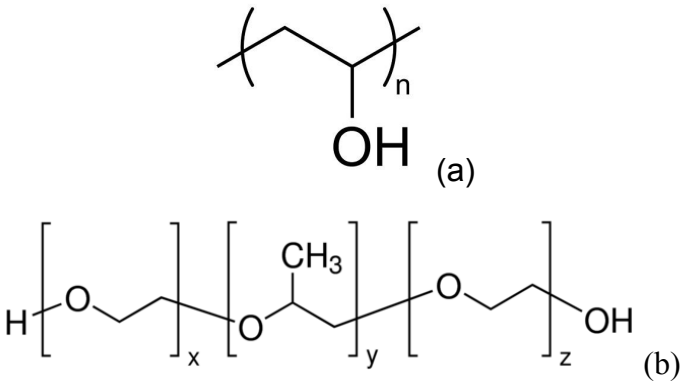

Figure 3. Molecular structure of PVA (a) and Poloxamer 407 (b). (Source: Sigma-aldrich)

Natural and vegetable sources became a big field of research for the development of new products for cancer treatment. Based on the known antitumoral use of *V. album*, this project aimed to develop a new route of administration as well as new formulations containing this plant extract for cancer treatment.

3. OBJECTIVES

3.1. GENERAL

Develop a topical transdermal formulation containing *Viscum album* and evaluate its antitumoral activity *in vitro*.

3.2. SPECIFICS

- Determine the main chemical constituents in the *V. album* dry extract;
- Develop a hydrogel containing dry and aqueous extract;
- Develop a patch containing dry and aqueous extract;
- Evaluate the *in vitro* cutaneous permeation of the formulations containing *Viscum album* dry extract;
- Determine the hydrogel rheological characteristics;
- Evaluate the hydrogel stability;
- Evaluate the *in vitro* cytotoxicity assays of *Viscum album* formulations in tumoral and non-tumoral cell lines.

4. MATERIAL AND METHODS

4.1. *VISCUM ALBUM* SAMPLES

4.1.1. Dry extract

The berries, leaves and stems from the semi parasite *V. album* were collected in January 2018 and 2019 before 11:30h from the host tree *Abies alba* (A), in St. Pantaleon, Switzerland. The botanic identification was previously done by Prof Dr. Marcelo Guerra Santes, from Universidade Estadual do Rio de Janeiro, and a voucher (C.H. Quaresma 18.328) was deposited at the Herbarium of the Faculdade de Formação de Professores, Universidade Estadual do Rio de Janeiro, Brazil.

The *Viscum album* mother tinctures were prepared from fresh herbs following the Brazilian Homeopathic Pharmacopeia, in which the use of maceration process is required (BRASIL, 2011). Briefly, the grinded fresh herbs were kept in contact with the extracting liquid in a glass vessel, protected from light and heat. The samples were stirred daily, for a total period of 21 days. Finally, the samples were filtered and the mother tinctures, at a final ethanol 45% v/v concentration, were obtained (Table 1).

Table 1. Mother tincture and dry extract data

Year	Fresh Herb (g)	Volume (mL)	Dry extract (g)
2018	603.82	2,374	59.45
2019	275.40	1,300	28.0

The mother tinctures were taken to rotatory evaporator (Büchi, Vacuum Pump V-700) to eliminate the maximum of its hydro alcoholic content. The balloon was kept at 40 ºC under vacuum (Büchi 461 Water Bath).

Lastly, these residues were frozen in a freezer at -80 ºC and then added to a lyophilizer (Christ Beta 2-8 LD) for a period of 24 h (-43 ºC/0.09 mbar), obtaining the dry extract of *V. album*.

4.1.2. Aqueous extract

The aqueous extract of *V. album*, named Iscador®, was donated by the pharmaceutical company Iscador AG (Arlesheim, Switzerland). It was an aqueous solution 1:5 from *V. album spp abietus* (Lot 1804/8141), the same subspecies used to prepare the dry extract, prepared from fresh herbs in a concentration of 200 $mg.mL^{-1}$ and it contained a standard amount of viscotoxins of 421 $\mu g.mL^{-1}$.

4.2. CHEMICAL MARKERS IDENTIFICATION IN THE DRY EXTRACT

The dry extract was analysed for its chemical components, identifying the major markers by thin layer chromatography (TLC) and high performance liquid chromatography coupled to mass spectroscopy (HPLC-MS), and quantified by HPLC with UV detection (HPLC-UV-Vis). This was carried out with an adapted version of the methodology described in the French Homeopathic Pharmacopoeia (ANSM, 2010).

4.2.1. Thin layer chromatography

TLC analysis was performed according to the monograph of *V. album* in the French Homeopathic Pharmacopeia, using a silica gel plate 60 F254 (0.22 μm, SiliCycle, Quebec, Canada). A solution of distilled water, methanol (Grupo Química), glacial acetic acid (Merck) and dichloromethane (Vetec) (2:3:8:15 v/v/v/v) was used as mobile phase. As standard solution, 2 mg of chlorogenic acid (Tedia) and 2 mg of caffeic acid were solubilized in 20 mL of methanol. 10 mg of dry extract was solubilized in 1 mL of ethanol 45% v/v. The detection was made by spraying a NP/PEG (10 $g.L^{-1}$ of diphenylborinic acid aminoethylester in methanol, followed by a solution of macrogol 400 50 $g.L^{-1}$ in methanol). The plate was analysed under ultraviolet light (365 nm) before and after the detection solution was sprayed. The sample was characterized by its retention factor (Rf) compared to the standard solution (ANSM, 2010).

4.2.2. Total flavonoids content

Determination of the total flavonoid content in the dry extract was carried out according to the method developed by Rolim *et al.* (2005). The concentration of total flavonoids in equivalents of rutin was evaluated by ultraviolet-visible spectrophotometer (UV-Vis) (Thermo Fischer, Genesys 10S) at the wavelength 361 nm. To plot a calibration curve, six different concentrations (5.0; 10.0; 15.0; 20.0; 25.0 and 30.0 $\mu g.mL^{-1}$) of a standard solution of rutin were used. A mixture of ethanol 96 ºGL and acetic acid 0.02 M (99:1) was used as solvent (Solvent 1) to prepare the solutions. The linear equation was obtained by linear regression in Excel®.

4.2.3. High performance liquid chromatography with ultraviolet detection – HPLC-UV

The analyses were conducted in HPLC Dionex Ultimate 3000 equipped with ultraviolet detection (Thermo Fisher Scientific, USA). The sample was prepared as follows: a solution of acidified acetonitrile (Tedia) and acidified water with 0.1% v/v of formic acid (Tedia) each was prepared in a proportion of 1:9 (v/v) (solution T) respectively. Then, 50 mg of the dry extract was solubilized in 1 mL of ethanol 45% v/v and added in a 5 mL volumetric balloon, filling it up with solution T. At the end, the sample was filtered in a 0.45 µm membrane filter (ANSM, 2010; MELO *et al.*, 2018).

Separation was performed in a reverse phase column (C-18, 250 mm x 4,6 mm x 5,0 µm; Kromasil, Akzo Nobel) using water-formic acid 0.1% v/v (A) and acetonitrile-formic acid 0.1% v/v (B) as mobile phases: (i) 0-20 min, 10% B, (ii) 20-25 min, 10-15% B, (iii) 25-45 min, 15% B, (iv) 45-50 min, 15-100% B, (v) 50-55 min 100% B, (vi) 55-57 min 100-10%, and (vii) 57-65 min 10% B, as described in the monograph of *V. album* (ANSM, 2010). The flow rate used was 1.0 $mL.min^{-1}$ and the injected volume of 100 µL. The absorption spectrum in the UV-Vis was obtained at 325 nm.

4.2.4. HPLC-DAD-MS

The analyses were conducted in UHPLC Dionex Ultimate 3000 coupled with diode array detector (DAD) (Thermo Fisher Scientific, USA) and tandem mass

spectrometry with electrospray ionization (LCQ Fleet Ion Trap-Thermo Fisher Scientific, USA). The sample was prepared as described above in the HPLC-UV methodology.

The flow rate used was 1.0 $mL.min^{-1}$ and the injected volume of 20 µL. The absorption spectrum in the UV-Vis was obtained at a range from 200 to 400 nm. Mass spectra were performed in negative ion mode (ANSM, 2010; MELO *et al.*, 2018).

4.3. PHARMACEUTICAL FORMULATIONS DEVELOPMENT

4.3.1. Dry extract solubility in pharmaceutical solvents

V. album dry extract solubility was determined in different pharmaceutical excipients by adding an excess of the extract (10 mg) in 2 mL of each excipient. The vial was kept at room temperature, stirring, for 72 h until equilibrium. The mixtures were removed from the stirrer and centrifuged at 3,000 rpm for 15 min. The supernatant was removed and filtered in a 0.45 µm membrane filter and the chemical analysis was performed by UV-Vis (λ=361nm) to quantify the extract solubility in each excipient (AHMAD *et al.*, 2017).

4.3.2. Hydrogel

The hydrogel formulation was prepared with 20% w/w of Poloxamer 407 dispersed in distilled water and kept under refrigeration for at least 24 h for complete hydration, dispersion and dissolution of the polymer. After that, other components of the formulation – 5% w/w of propylene glycol, 5% w/w of Transcutol® (diethylene glycol monoethyl ether), 5% w/w of the dry extract or the aqueous extract – were added and homogenized in an ice bath, with a magnetic stirrer, until the dry extract was completely soluble. At long last, the formulation was weighed in an airless dispenser (Anwander, Switzerland) and kept at room temperature to acquire consistency.

4.3.2.1. Stability studies

The hydrogels stability was evaluated at 7, 15, 30, 60, 90 and 180 days after their manufacturing. They were kept in an acclimatized chamber for accelerated stability evaluation, with controlled temperature (40 ± 5 ºC) and humidity (75 ± 5%) (BRASIL, 2005).

4.3.2.1.1. Batches definition

Each batch was named with 3 distinct numbers. The first number represents the production batch (1, 2 or 3). The second number is related to the pool of each batch that would be sent to different analyses: for the dry extract, 2 pools were produced (physical-chemical and microbiological analyses) and for the aqueous extract, 3 pools (physical-chemical, microbiological and drug content analyses) were produced. The third number designates the time of analysis (1 to 6).

In total, 36 hydrogels with dry extract and 54 hydrogels with aqueous extract were produced. The batches were differentiated by the following colors: batch 1, yellow color; batch 2, green color; and batch 3, blue color (Figure 4).

Figure 4. Hydrogel batch label

4.3.2.1.2. pH

The electrode from the portable pH meter (model 922, Bante Instrument®) previously calibrated was inserted directed into the hydrogel in the primary pack, at room

temperature. After a constant value was obtained, the pH for each sample was recorded and calculated for its mean and standard deviation, in Excel®.

4.3.2.1.3. Drug content

For the hydrogel with dry extract, the drug content measurement was given by its total flavonoid quantification, according to the method developed by Rolim *et al.* (2005), as previously described (item 4.2.2).

200mg of the hydrogel was added to a 25mL volumetric flask and it was filled up with solvent 1 (blank solution). Solvent 1 was used to set the spectrophotometer and the 3 different batches were measured. Data was plotted in Excel® and its mean and standard deviation were calculated from the line equation.

The content of chlorogenic acid was also evaluated after 1 and 6 months of the hydrogels manufacturing. In a volumetric flask of 10 mL, 2.0 g of the hydrogel were dissolved in solution T (1:9 acetonitrile and water, with 0.1% v/v of formic acid, respectively) and analysed by HPLC-DAD. The separation was performed in a reverse phase column (C-18, 250 mm x 4,6 mm x 5,0 μm; Kromasil, Akzo Nobel) in an HPLC equipped with: ultimate 3000 pump LPG; auto sampler WPS-3000 TSL; columns compartment TCC-3000 SD and diode array detector DAD (Thermo Fisher, São Paulo, Brazil). Water-formic acid 0.1% v/v (A) and acetonitrile-formic acid 0.1% v/v (B) were used as mobile phases in the gradient mode: (i) 0-20 min, 10% B; (ii) 21-26 min, 100% B; (iii) 27-37 min, 10% B. The flow rate used was 1.0 $mL.min^{-1}$ and the injected volume of 70 μL. The detection was performed at 325 nm.

4.3.2.1.4. Rheology

The rheological analyses of the hydrogels were carried out at Federal Institute of Rio de Janeiro, under the supervision of PhD Catarina Amorim Oliveira.

Rheometer Anton Paar MCR 302 (Figure 5) with Peltier system and thermal bath Tecnal TE 2015 for temperature control were used for rheological measurements. Results were obtained by Rheoplus/32 software. A type CP-40-2 (40 mm diameter and angle of 2 º) geometry and a temperature of 32ºC were used during the rotational and oscillatory analyses.

Figure 5. Rheometer Anton Paar MCR 302 (a). Before the analyses: (b) hydrogel dry extract; (c) hydrogel aqueous extract; (d) During the analysis.

A temperature sweep between 50 to -10ºC was carried out to determine the viscosity as a function of temperature. Hydrogels' viscosity curve was obtained applying a shear rate in the range from 1 to 100s^{-1}.

Three-interval thixotropic test (3ITT) was carried out to evaluate the thixotropy by means of percentage values of structure recovery for each sample. First, a low shear rate of 1s^{-1} was applied and then a high shear rate of 100s^{-1} was applied. Lastly, the recovery was at the low shear rate of 1s^{-1}. Amplitude sweeps (constant frequency of 10 rad.s^{-1}) and frequency (deformational amplitude constant of 0.1%) tests were also carried out to obtain the structural characteristics of the samples, like the flow and strength of the hydrogels.

Rheological characteristics were also carried out to evaluate the stability of the hydrogels in the same stage as the other analyses, at 7, 15, 30, 60, 90 days after their preparation. They were kept in a chamber with controlled temperature (40 ± 5 ºC) and humidity (75 ± 5%).

4.3.2.1.5. Microbiological analyses

The microbiological analyses were carried out at Laboratório de Controle de Qualidade de Medicamentos, Alimentos e Cosméticos (LACMAC) from the School of Pharmacy – UFRJ. The internal methodology 0001 – Microbiological analysis of

pharmaceutical products and non-sterile raw materials was used, as preconized by the Brazilian Pharmacopeia 5th edition (BRASIL, 2010). The limits for the analysis were: (i) total microbiological charge – maximum 10^2CFU.mL^{-1}; (ii) molds and yeasts – maximum 10^2CFU.mL^{-1}; (iii) *Staphylococcus aureus* – absent; (iv) *Pseudomonas aeruginosa* – absent and (v) *Escherichia coli* – absent.

Transfer 10 g of the sample to a flask containing 90 mL of casein-soy broth (Casoy broth) and mix until complete dissolution of the sample.

Total Count of Aerobic Microorganisms

With sterile pipette, transfer 1 mL aliquots of 1:10 dilution to four Petri dishes. Carefully add about 20 mL of casein soy agar (TSA - bacterial counting medium) to two plates and, to the other two, add about 20 mL of potato dextrose agar (BDA - mold and yeast counting medium) and 0.3 ml of 10% tartaric acid. The temperature of the culture media should be at a maximum of 45 °C. Homogenize the contents of the plates by performing gentle circular and eight-shaped movements. After agar solidification, invert the plates and incubate in an oven under the following conditions: - Casein Soy Agar (TSA): 32,5 °C ± 2,5 °C for 24 hours and up to 48 hours. - Potato dextrose agar (BDA): 22,5 °C ± 2,5 °C for 5 to 7 days.

Reading results

After the incubation period, with the help of the colony counter, examine the plates for microbial development and count the number of colony forming units. For each test (bacteria and fungi), calculate the arithmetic mean between the counts found on the two plates and multiply the result by the dilution factor. Express the result as CFU/g of product. If there is no microbial growth in either plate, express the result as <dilution factor/g or mL. Examples: Dilution 1:10 - Dilution Factor 10: <10 CFU/g.

Search for specific microorganisms

Search for *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Escherichia coli*

Transfer 10 mL of 1:10 dilution (previous item) to flask containing 90 mL of casein-soy broth (Casoy Broth). Homogenize and incubate at 32.5 °C ± 2.5 °C for 18 to 24 hours.

1. Research on *Staphylococcus aureus*

After the incubation period, transfer an aliquot of the casein-soy broth using a sterile inoculation loop onto the surface of salty mannitol agar or Baird-Parker agar. Invert the plate and incubate at 32.5 °C ± 2.5 °C for 18 to 48 hours.

After the incubation period, growth of black colonies surrounded by translucent halo indicates probable presence of *S. aureus*. If these colonies are present, proceed to further identification through complementary biochemical tests.

For isolation and purification of cultures, remove a small portion of the central part of the colony with sterile inoculation loop or needle and transfer it to a plate containing non-selective solid culture medium (casein-soy agar) by stripping the inoculum. Incubate the plate at 32.5 °C ± 2.5 °C for 18 to 24 h.

After incubation period, perform Gram stain on suspected colonies. If Gram-positive cocci are observed, perform the catalase test.

Catalase Test

For the test, remove a portion of the culture under study with an inoculation loop and pass it to a slide. Add one drop of 3% v / v hydrogen peroxide solution over the smear. The test is considered positive when there is bubbling due to the release of oxygen. According to the catalase test result, classify the microorganism as catalase positive or catalase negative. Verifying that the microorganism is catalase positive, perform the coagulase test.

Coagulase Test

The coagulase test must follow the instructions on the manufacturer's kit.

Incubate the isolated colony in BHI (Brain and Heart Infusion) broth for 24 hours at 32.5 °C ± 2.5 °C. From the culture isolated in the BHI broth proceed with the coagulase test. Perform coagulase testing using coagulase kit as per manufacturer's instructions. Transfer 0.3 ml of the culture into sterile tubes and add 0.5 ml of reconstituted lyophilised plasma. Incubate at 32,5 °C ± 2,5 °C. Observe the presence of clot in the first 4 hours and up to 18 to 24 hours. The presence of any degree of coagulation indicates a positive reaction to the coagulase test. According to the result of the coagulase test, classify the microorganism as coagulase positive or coagulase negative. The positivity of the test indicates the probable presence of *S. aureus* in the product.

Express the search result for *S. aureus* as present or absent.

2. Research on *Pseudomonas aeruginosa*

After the incubation period, transfer an aliquot of the casein-soy broth (previous item) using a sterile inoculation loop onto the cetrimide agar surface. Invert the plate and incubate at 32.5 °C ± 2.5 °C for 18 to 48 hours.

After incubation, the growth of greenish-yellow and fluorescent colonies under UltraViolet light indicates a probable presence of *P. aeruginosa*. If these colonies are present, proceed to further identification through complementary biochemical tests.

For isolation and purification of cultures, remove a small portion of the central part of the colony with a sterile inoculation loop and put it in a plate containing a non-selective solid culture medium (eg casein-soy agar) by stripping the inoculum. Incubate the plate at 32.5 °C ± 2.5 °C for 18 to 24 h.

After the incubation period, perform Gram stain on suspected colonies. If Gram-negative rods are observed, test for growth at 42 °C.

Growth test at 42 °C

Transfer pure cultures to tube containing BHI broth. Incubate the tube at 42 °C for 48 h. The positivity of this test is indicated by the presence of turbidity in the tube. P. aeruginosa strains grow at 42 ° C. Express the result of *P. aeruginosa* research as present or absent.

3. Research on *Escherichia coli*

After the incubation period (previous item), transfer 1 mL of casein-soy broth into a flask containing 100 mL of MacConkey broth. Homogenize and incubate the flask at 42 °C to 44 °C for 24 to 48 hours.

After this, transfer an aliquot of MacConkey broth using a sterile inoculation loop onto the surface of MacConkey agar. Invert the plate and incubate at 32.5 °C ± 2.5 °C for 18 to 48 hours.

After the incubation period, the growth of pink to reddish pink colonies, whether or not surrounded by a bile precipitate zone indicates a probable presence of *E. coli*. If these colonies are present, proceed to further identification through complementary biochemical tests.

For isolation and purification of cultures, remove a small portion of the central part of the colony with a sterile inoculation loop and put it in a plate containing a non-selective solid culture medium (eg casein-soy agar) by stripping the inoculum. Incubate the plate at 32.5 °C ± 2.5 °C for 24 to 48 h.

After incubation period, perform Gram stain on suspected colonies. If Gram-negative rods are observed, transfer a loop of the pure culture to EMB agar. Incubate the plate at 32.5 °C ± 2.5 °C for 24 hours. Observation of dark metallic-bright colonies indicates the likely presence of *E. coli* in the medium. Proceed to the next tests to confirm the result.

Transfer a pure culture loop to tubes containing *E. coli* (EC) broth and Brilliant Green Bile (BGB) broth, respectively, and Durham tubes. Incubate EC broth at 44 ° C for 24 h and BGB broth at 32,5 ° C ± 2,5 ° C for 24 h.

After the incubation period, observation of turbidity and gas formation in the tubes indicates likely presence of *E. coli* in the medium. Express the *E. coli* search result as present or absent.

4.3.2.2. Evaluation of the occlusive factor

To evaluate the hydrogels occlusive effect, small glasses of 40 mL and 4.6 cm in diameter were filled with 30g of distilled water and covered with cellulose filter paper (90mm, Whatman number 6 and cutting size of 3µm). The hydrogel was homogeneously spread onto the filters (13.3mg.cm^{-2}) and kept in a chamber at 40 ºC for 72h. This was carried out in triplicate. The water mass was evaluated on an analytical balance after 6h, 24h, 48h and 72h. Three glasses were covered with cellulose paper without the hydrogel and were used as controls. The occlusion factor (F) from the gel was calculated from the equation:

$$F = \left(\frac{A-B}{A}\right) x100 \qquad \text{Equation 1}$$

Where A represents the water loss from the glass without the hydrogel (control) and B, the water loss from the glass with the hydrogel (TEERANACHAIDEEKUL *et al.*, 2007; WISSING; MÜLLER, 2002).

4.3.2.3. Spreadability

Spreadability quantitative determination from the hydrogel was performed as follows: two diagonals were marked on a glass blade to obtain their intersection as a central point. On this point, it was applied around 25 mg of the formulation on the opposite side of the mark. Another blade, with its mass known, was carefully applied over the formulation, coinciding to the first blade. After one minute, it was recorded the diameter of the hydrogel circle on the intersection of the diagonals. The same was carried out using an additional 2g weight, with measurement always at an interval of one minute (Figure 6). A second 2g weight was added and finally a 5g weight was used, as shown in Table 2. From the radius obtained, the areas from the correspondent surface were calculated, applying the circle area formula ($A = \pi.r^2$, $\pi = 3.14$, in which r = radius obtained from the formulation spreadability) (FERNANDEZ-MONTES, 2005).

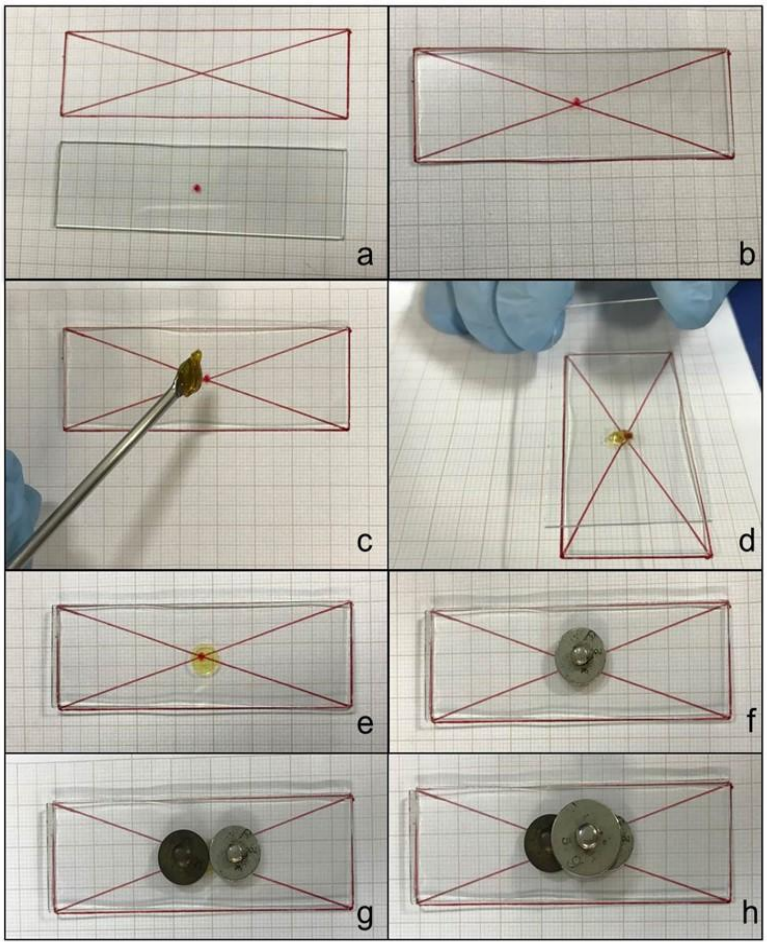

Figure 6. Spreadability test: (a) diagonals on millimetric paper; (b) slide placement on the diagonal; (c) approximately 25 g of hydrogel was put onto the slide; (d) addition of slide with known weight onto the hydrogel; (e) spread caused by the superior slide; (f) 2 g weight added onto the 2nd slide; (g) another 2g weight added onto the 2nd slide; (h) a 5 g weight added onto the two 2 g weights.

Table 2. Spreadability determination and weights used

Analysis	Weights
1st	Glass blade (known weight)
2nd	Glass blade + 2 g weight
3rd	Glass blade + 2 g weight + 2 g weight
4th	Glass blade + 2 g weight + 2 g weight + 5 g weight

4.3.3. Patch

The development of the PVA patch was based on an adapted Patch-non-Patch® technique (PADULA *et al.*, 2007).

First, it was prepared a solution of 20% w/w of PVA in distilled water under magnetic stirring for a period of 24 h. This solution was heated up to 90 ºC until complete dissolution and solubilisation of the polymer. An adhesive solution was prepared by adding 21.3% w/w of polyvinylpyrrolidone K90 (PVPK90) and 12% w/w of polyethylene glycol 400 (PEG 400) in 66.7% w/w of distilled water. Furthermore, they were mixed as follows (Table 3): 5% w/w of the dry extract or of the aqueous extract was added to the PVA solution at 57% w/w; then 4% w/w of sorbitol solution at 70% was added; the adhesive solution was added at 27% w/w and lastly 7% w/w of propylene glycol.

Table 3. Patch composition

Component	Concentration (% w/w)
PVA 20%	57.00%
Adhesive	27.00%
Sorbitol solution 70%	4.00%
Propylene glycol	7.00%
V. album extract	5.00%
Adhesive	
PVPK90	21.30%
PEG 400	12.00%
Water	66.70

This system was left resting to eliminate air bubbles created during magnetic stirring. Then, it was weighted on a silicone paper and carried to an oven at 60 ºC for 30 min. After this, the patch was weighted for calculation of the water loss and final mass.

4.3.3.1. Drug content

For the patch with dry extract, the drug content measurement was given by its total flavonoid quantification, according to the method developed by Rolim *et al.* (2005), described on item 4.2.2.

150 mg of the patch was added to a volumetric flask of 25 mL and it was filled up with solvent 1 (blank solution). Solvent 1 was used to set the spectrophotometer and the patch was measured. Data was plotted in Excel® and its mean and standard deviation were calculated from the line equation.

The content of chlorogenic acid was also evaluated. After 5 months of manufacturing, 145 mg of the patch was dissolved in solution T (1:9 acetonitrile and water with 0.1% v/v of formic acid) in a volumetric flask of 10 mL and analysed by HPLC-DAD, as described on item 4.3.2.1.3.

4.3.3.2. Evaluation of the occlusive factor

To evaluate the occlusive effect from the patch, small glasses of 40 mL with 4.6 cm in diameter were filled with 30 g of distilled water, covered with the patch (0.55 mg) and kept in a chamber at 40 ºC for 72 h. This procedure was carried out in triplicate. The water mass was evaluated on an analytical balance after 6, 24, 48 and 72 h. Three glasses were covered with cellulose paper (90 mm, Whatman nº 6 and cut size of 3 μm) and were used as control. The occlusion factor (F) from the patch was calculated from the equation 1 shown in item 4.3.2.2.

4.4. CYTOTOXICITY EVALUATION *IN VITRO*

Both formulations, hydrogel and patch, were evaluated to verify their *in vitro* cytotoxicity using WST-1 colorimetric assay, incubating the cells with different

concentrations of the formulation varying from 2 to 200 mg.mL^{-1}, for a period of 24 h (HOLANDINO *et al.*, 2018). Three different cell lines were used: Molt-4 (human T lymphoblast; acute lymphoblastic leukaemia, non-adherent), Yoshida (ascites sarcoma cell, adherent) and HaCat (immortal HaCat keratinocytes, non-tumoral, adherent). They were grown in RPMI 1640 (Sigma) supplemented with 10% foetal bovine serum, 2 mM L-glutamine and 1% penicillin-streptomycin, in a humid environment with 5% CO_2 at 37 ºC. Cell lines were kept in those conditions for a period of 24 h to reach at least 90% of confluent cells and the monolayer cell lines (Yoshida and HaCat) were harvested with trypsin/EDTA. 90 µL of the cell suspension were seeded (1x10^4 cell.ml^{-1}) in a 96 cell well plate for 24 h. Then, 90 µL of the formulations, previously weighed and solubilized in medium, were added to the wells in different concentrations (2, 5, 10, 15 e 20 mg.mL^{-1}). The rate of viability was evaluated after 24 h of incubation by adding 20 µL of WST-1 (Roche, Germany) in each well. The absorbance at 450 and 690 nm was measured in the plate reader after 1:30 h of incubation at 37 ºC in the dark. The viability percentage was calculated in comparison to the negative control (cells treated with hydrogel and patch without the extract) using the mean of 3 independent experiments in triplicate.

4.5. SKIN PERMEATION STUDY *IN VITRO*

4.5.1. Skin preparation

Pig ear skin was chosen for experiments because it presents a close similarity to human skin (FLATEN *et al.*, 2015; TODO, 2017). The pig ears were obtained from a local slaughterhouse and kept under refrigeration for transportation to the laboratory, where they were cleaned with water and the skin was separated using a scalpel. The subcutaneous tissue – hypodermis and blood vessels – was removed (SANTIS *et al.*, 2013). The skins were packed in plastic paper, covered with aluminium paper and kept in the freezer at –20 ºC until their use, for up to a maximum of 60 days.

4.5.1.1. Chlorogenic acid extraction and recovery from epidermis and dermis

Pig ear (1 cm^2) was added with 20 µL of dry extract solution in three different concentrations (10, 50 and 100 mg.mL^{-1}) in triplicate. 1 cm^2 of pig ear without the extract

was also analyzed to evaluate the interference of the skin in the chromatography results. After 1 hour, each skin was added to an eppendorf and submitted to the extraction method, adding 1.5 mL of the mobile phase (water 0.1 % formic acid + acetonitrile 0.1% formic acid – 9:1 v/v, respectively) and shaken for 90 s. Then it was centrifuged for 10 min at 6,400 rpm, filtered through a 0.45 µm pore size filters (Whatman) and analysed by HPLC-UV, as described on item 4.3.2.1.3.

Analytical curve was built with a standard of chlorogenic acid in six different concentrations (1, 5, 10, 15, 20 and 30 $\mu g.mL^{-1}$).

Dry extract concentration is standardized to its concentration of chlorogenic acid as follows:

10 $mg.mL^{-1}$: in equivalents of chlorogenic acid is 188.80 $\mu g\ mL^{-1}$

50 $mg.mL^{-1}$: in equivalents of chlorogenic acid is 944.00 $\mu g\ mL^{-1}$

100 $mg.mL^{-1}$: in equivalents of chlorogenic acid is 1,888.00 $\mu g\ mL^{-1}$

4.5.2. Receptor medium selection

The selection of the receptor medium was based on the solubility of dry extract in phosphate buffer (PBS) (pH 7.4) in the absence and presence of different surfactants: ethanol, tween 20 and propylene glycol (0.5, 1.0 and 2.0% v/v). Experiments were performed using an excess of the dry extract added to the receptor medium and kept under moderate agitation for 24 h in a water bath maintained at 37 ± 0.5 ºC. Subsequently, the samples were centrifuged (6,400 rpm, 10 min) and the aqueous phase filtered through 0.45 µm pore size filters and analysed by HPLC, as described on item 4.3.2.1.3.

4.5.3. Skin permeation and retention assay

The *in vitro* skin permeation study of *V. album* dry extract formulations was carried out in a Franz type vertical cell diffusion system (Figure 7), composed of a donor compartment with diffusional area of 1.54 cm^2 and a receptor compartment with a maximum volume of 7 mL. The methodology followed the technique described by Pinto *et al*. (2017).

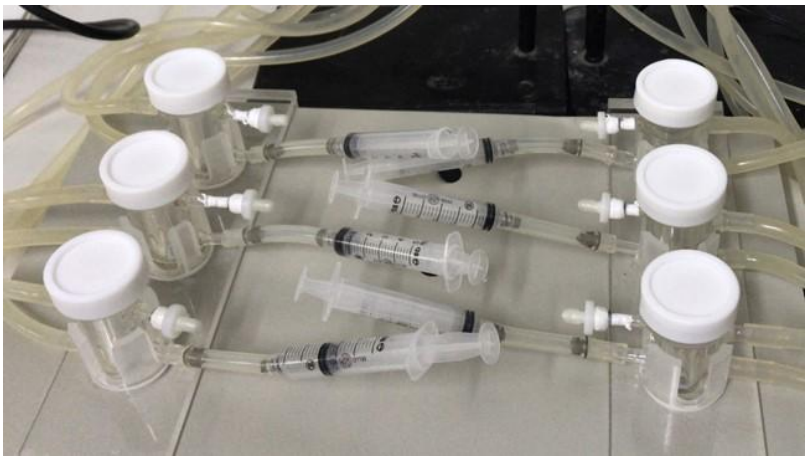

Figure 7. Franz type vertical cell diffusion system

Pig skin samples were put with the epidermis turned up to the donor compartment. The receptor medium was PBS pH 7.4 (USP, 2012). Experiments were carried out at 37 ºC under constant stirring (500 rpm) for 24 hours. 300 mg of each formulation was applied onto the skin of each cell. 1 mL of the receptor medium was collected after 1, 2, 4, 8 and 24 h with refilling with 1 mL of fresh buffer solution. The aliquots were filtered through a 0.45 μm pore size filters disc (Whatman) and analysed by HPLC-UV as described on item 4.3.2.1.3.

After 24 h, the permeation study was finished. The pig ear skins were removed from the Franz type diffusion cell and the formulation excess was removed with cotton dipped in distilled water. In order to evaluate the drug retained in the epidermis and the dermis, these layers were separated using a scalpel to collect the epidermis, following a method standardized by our group (Figure 8). Thereafter, the epidermis and the dermis (cut into small pieces) were inserted in eppendorf containing 1.5 mL of mobile phase (water 0.1% formic acid + acetonitrile 0.1% formic acid – 9:1 v/v, respectively) for chlorogenic acid extraction. The eppendorfs were shaken for 90 seconds and centrifuged for 10 minutes at 6,400 rpm. Then, all samples were filtered through a 0.45 μm pore size filters disc (Whatman) and analysed by HPLC-UV, as described on item 4.3.2.1.3. The results were expressed as mean and standard deviation of one experiment in sextuplicate.

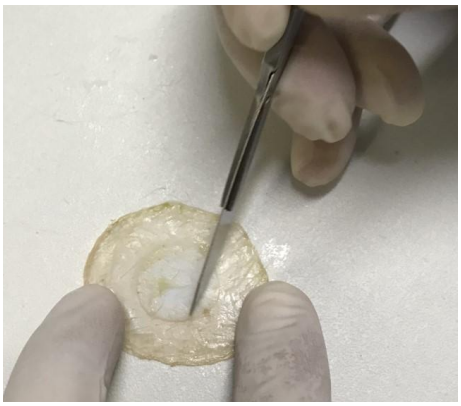

Figure 8. Separation of epidermis and dermis with scalpel for the extraction process.

4.6. STATISTICAL ANALYSIS

All experiments were analysed using GraphPad 8 (California, USA), significant differences between the samples were determined by one-way ANOVA and $p<0.05$ was considered to be statistically significant. Tukey post-test was used for the cytotoxicity *in vitro* experiments.

5. RESULTS AND DISCUSSION

5.1. CHEMICAL ANALYSES

5.1.1. Thin layer chromatography

Thin layer chromatography analysis was done using two chemical standard solutions and the solubilized *V. album* dry extract. The standards presented two characteristic bands with Rf of 0.7 and 0.95, which were correspondent to chlorogenic and caffeic acids, respectively (Figure 9). The sample presented only one band at 0.7 related to the chlorogenic acid but yellow-reddish bands were also detected in the plate, typical of phenolic acids (WAGNER; BLADT, 1996).

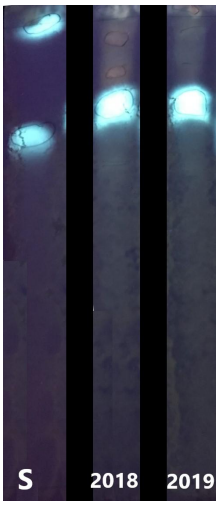

Figure 9. Thin layer chromatography. S - standards (chlorogenic [lower band] and caffeic [upper band] acids); 2018 and 2019 - solubilized dry extract of *V. album* of each year.

5.1.2. Total flavonoid content

The total flavonoid content was determined by the spectrophotometer method using rutin as the standard. The absorbance from a series of concentrations of rutin was plotted to generate a calibration curve (y=0.0283x + 0.0004) with R^2 of 0.99466. In this analysis, the total flavonoid content in 2018 and 2019 *V. album* dry extract from the host tree *Abies alba*, in equivalents of rutin, was 31.38 $mg.g^{-1}$ and 28.39 $mg.g^{-1}$ respectively (Table 4).

Table 4. Total flavonoids content (mg.g^{-1}) in the *V. album* ssp *abietis* dry extract.

Year	Total flavonoids (mg.g^{-1})
2018	31.3790 ± 1.3474
2019	28.3899 ± 0.8610

The content of total flavonoids in *Viscum album* spp. *abietis* was previously analysed and varied from 3.200 mg.g^{-1} to 9.955 mg.g^{-1} when extracts produced by different solvents and extraction methods were analysed (PIETRZAK; NOWAK; OLECH, 2014). However, the flavonoid content in berries of *V. album* from different subspecies (*ssp album* and *ssp austriacum*) and host trees, varied from 0.270 to 0.428 mg.g^{-1} (PIETRZAK *et al.*, 2017). Tahirovic & Basic (2017) evaluated the total flavonoids from the leaves and the stems of *V. album ssp album* from different host trees, finding a variation range from 2.29 to 5.05 mg.g^{-1}. Kang (2015) evaluated the total flavonoid content in a different subspecies of *V. album* (host tree *Quercus acutissima*) and found a concentration of 36.38 mg.g^{-1}. Comparing the result of this study to the previous ones described in the literature, it was possible to emphasize the higher flavonoid content detected in *Viscum album* dry extract. Considering the different extraction methods, analytics and plant parts used as well as the subspecies analyzed, the result showed a similar average amount of total flavonoid content to other subspecies (KANG, 2015), suggesting that the method used in the present work could be used as a quality parameter for the analysis of flavonoid content in *V. album* extracts. However, more studies are necessary to evaluate changes with harvesting season, subspecies, plant parts analyzed, solvents and extract methodology used.

5.1.3. HPLC-DAD-MS

The dry extract was analysed by HPLC-DAD-MS to identify its content of chlorogenic acid, since this phenolic acid is used as a *V. album* chemical marker according to the French Pharmacopeia (ANSM, 2010). It was possible to extract the mass ion for the chlorogenic acid *m/z* 353, as shown in Figure 10.

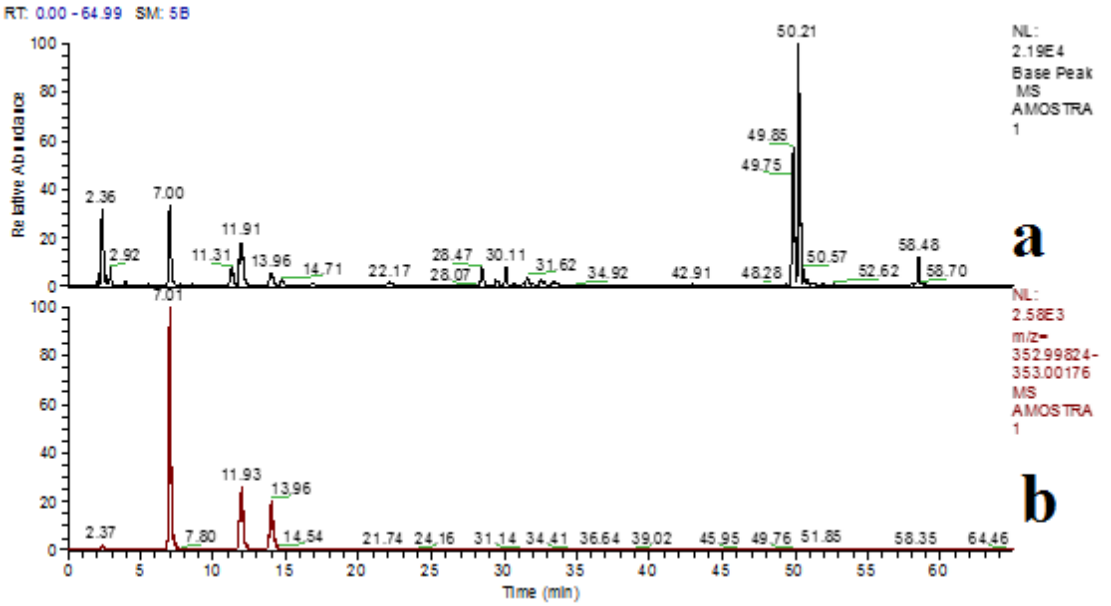

Figure 10. Base peak ion-chromatogram of *V. album* dry extract 2019. a – base peak ion-chromatogram of MS spectra. b – extracted ion-chromatogram of MS/MS spectra of chlorogenic acid within the *m/z* range 352.990-353.001.

Three peaks were identified for the mass of 353. These peaks at 7.01, 11.93 and 13.98 min suggest the presence of more than one isomer of chlorogenic acid. Mocan *et al.* (2016) described the first peak as 3-O-caffeoylquinic acid, the second as 5-O-caffeoylquinic acid and the third as 4-O-caffeoylquinic acid. Meinhart *et al.* (2017) also demonstrated the identification of different isomers of chlorogenic acid and they appear in the chromatogram in the same order described above. Chlorogenic acids are a group of molecules synthesized between hydroxycinnamic acids and quinic acid. Although their biosynthesis is still unclear, except for the 5-O-caffeoylquinic acid, it is suggested that isomerases cause the migration from the main 5- position to positions 3- or 4- (CAMPA *et al.*, 2008). Popova (1991), Luczkiewicz *et al.* (2001) and Melo *et al.* (2018) have already identified the presence of more than one isomer of chlorogenic acid in *V. album* extracts.

5.1.4. HPLC-UV

The quantification of chlorogenic acid, the chemical *Viscum album* marker according to the French Pharmacopeia, was also done by HPLC-UV. A standard of chlorogenic acid was used to create a calibration curve (y = 0.2391x – 0.2659) with

R^2=0.99919. In figure 11 and 12 it's possible to see the chromatogram of the 2018 and 2019 dry extract, respectively.

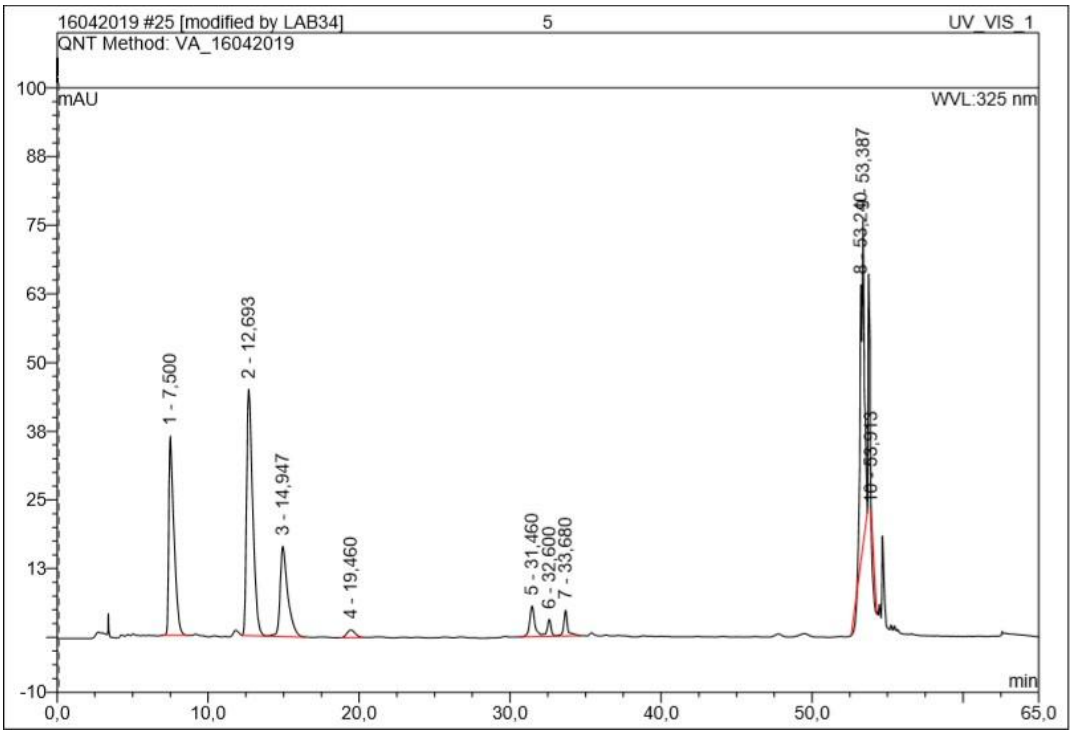

Figure 11. HPLC-UV chromatogram of *V. album* 2018 dry extract. Peaks 1, 2 and 3 represents the three isomers of chlorogenic acid (λ=325 nm).

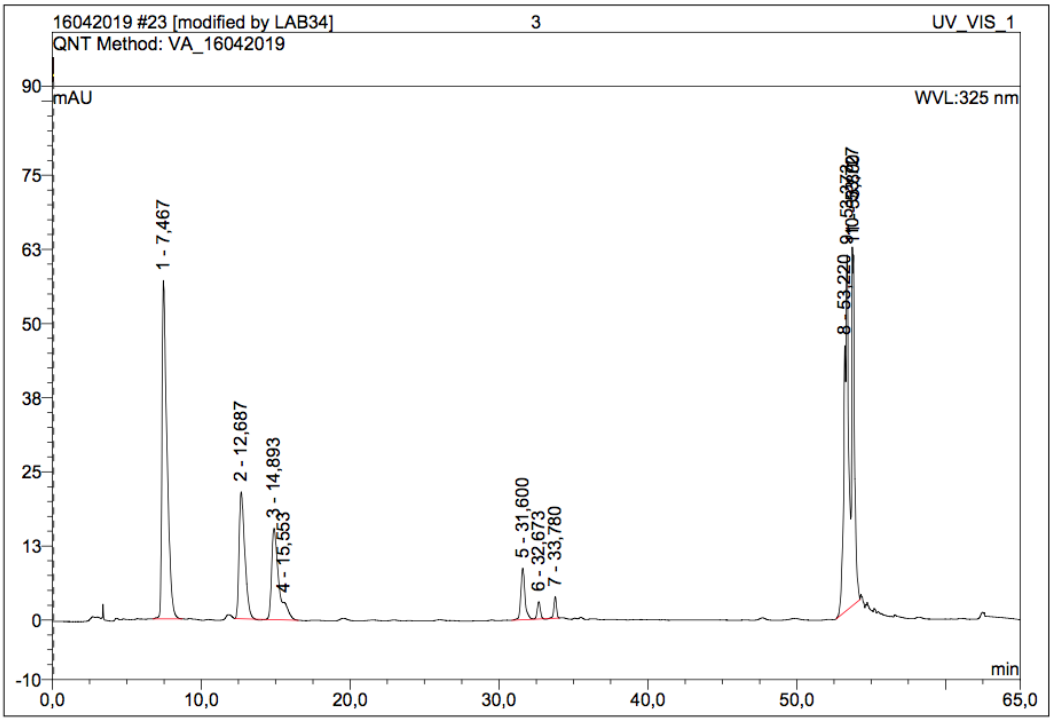

Figure 12. HPLC-UV chromatogram of *V. album* 2019 dry extract. Peaks 1, 2 and 3 represents the three isomers of chlorogenic acid (λ=325 nm).

It was possible to identify the chlorogenic acid at the retention time of 7.5, 12.6 and 14.8 min. According to the calibration curve created from the chemical standard analysis, the amount of chlorogenic acid present in both dry extracts was calculated as a sum of the three peaks, resulting in 18.88 (2018, winter extract) and 16.75 $mg.g^{-1}$ (2019, winter extract). Although *V. album* harvests were done in different years (2018 and 2019) it was possible to quantify similar amounts of chlorogenic acid, emphasizing the importance to use this chemical marker as a quality parameter for *V. album* preparations.

Luczkiewicz *et al.* (2001) quantified the total amount of chlorogenic acid in different subspecies of *V. album.* The amounts detected were 19 mg% (host tree *Acer platanoides* L.) and 13.5 mg% (host tree *Sorbus aucuparia* L.), which are similar to the *V. album* (host tree *Abies alba*) of the present study.

Coffee is reported as the main source of chlorogenic acids, ranging from 4.0 to 11.0 $mg.g^{-1}$ (MEINHART *et al.*, 2017). When comparing this data to the *V. album* chlorogenic acids content registered in the present work (18.88 and 16.75 $mg.g^{-1}$), it can be said that these dry extracts are rich in chlorogenic acids and this presented enough reproducibility even when harvests from different years are used. However, it is also important to emphasize the period of the year used in the harvest. Urech and Baumgartner (2015) showed important differences in the content of viscolectin and viscotoxin when different months of the year were compared. Because of this, it is important to standardize the period and the season of the year used for the harvest, in order to guarantee the quality parameters of the dry extract. In the present work, the period was January 2018 and January 2019 and the season was winter for both collections.

5.2. PHARMACEUTICAL DEVELOPMENT

5.2.1. Dry extract solubility

The solubility of drugs or extracts in solvents is important because it influences not only the choice of the components in the formulation but also their concentration, and it must be based on the solvent characteristics. Amongst the evaluated excipients, a solution containing poloxamer 407 and PVA presented the highest concentration of solubilized extract, followed by propylene glycol and transcutol® (Figure 13). PVA shows interesting characteristics, such as complete biodegradability and resistance,

originating formulations that are biocompatible, permeable and biodegradable (KIM *et al.*, 2003). Poloxamer 407 is considered as an inactive ingredient. Besides being thermo reversible, its solutions facilitate the solubilisation of more hydrophobic molecules, promote desirable strength and consistency of formulations and generate a non-occlusive gel (DUMORTIER *et al.*, 2006). Propylene glycol is widely used by the cosmetic and pharmaceutical industries, with the properties to potentiate cutaneous penetration, to act as humectant and to decrease viscosity (ASCENCIO *et al.*, 2016; GENIN *et al.*, 2015). Lastly, transcutol® was also chosen as a component of the formulation due to its biocompatibility, non-toxicity and its potential to promote cutaneous permeation (CENSI *et al.*, 2012), even though it presented a low solubility of the extract. The above-mentioned excipients were chosen for the hydrogel manufacturing and PVA was chosen as the main component for the patch development.

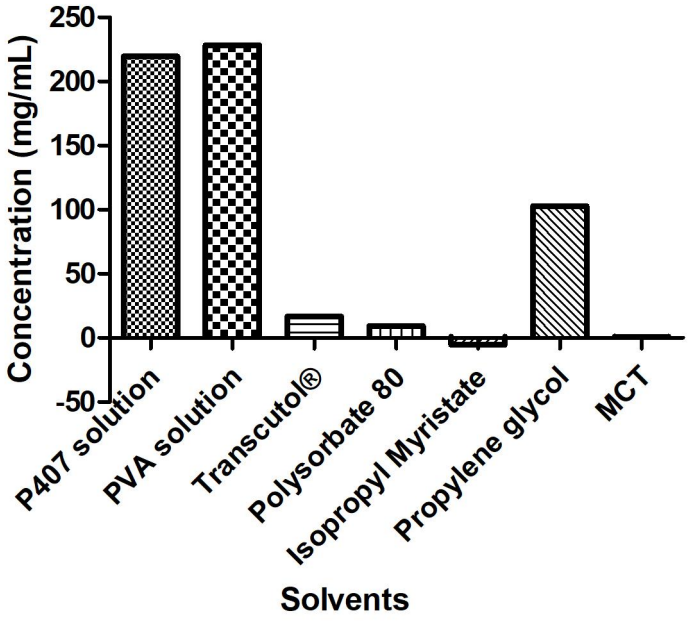

Figure 13. Total flavonoid concentration of the solubilised dry extract in different pharmaceutical solvents. P407 solution (poloxamer 407 15 % w/w); PVA solution (polyvinyl alcohol 20 % w/w); MCT (medium-chain triglycerides).

5.2.2. Hydrogel

The stability evaluation is used to provide evidence about the quality of the formulations developed when submitted to the influence of temperature and humidity in the primary packaging. The hydrogels were prepared and divided according to the

analysis in three different batches for each sample of *V. album*: dry extract and aqueous extract, labelled in different colours. They were packaged in 15 mL airless dispensers (Figure 14) and kept inside a chamber at 40 ºC with 75% of humidity, for the accelerated stability evaluation according to the Brazilian Guide for Stability Studies (BRASIL, 2005).

Figure 14. Hydrogel appearance. On the left with aqueous extract and on the right with the dry extract.

5.2.2.1. pH

The pH analyses were carried out using a benchtop pH meter for determined periods of time and presented stable values for both samples (Figure 15). For the aqueous extract the pH was 1 unit lower than for the dry extract, with an average of 4.5 and 5.5, respectively.

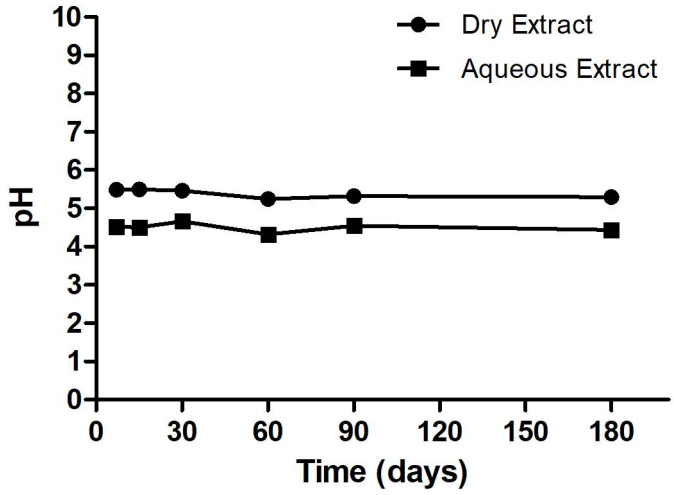

Figure 15. pH control (mean ± SD). Hydrogel containing 5% (w/w) of dry extract or 5% (w/w) aqueous extract. (n=3)

For cosmetic and topical formulations, compatibility with the skin is important, and maintenance of the skin pH is an important factor to preserve the cutaneous barrier and an adequate hydration of the skin (DUARTE *et al.*, 2017). Lambers *et al.* (2006) carried out an experiment to evaluate the skin surface pH. They demonstrated an average pH of about 4.7 and emphasized that pH variation is dependent on several factors, such as age, sex, race, diseases etc. The authors also highlighted that an acid skin pH is associated with a better ability to preserve the skin flora and its integrity. The acceptable pH range for topic formulations vary from 4.5 to 7.5 (DAUDT *et al.*, 2015). Our results are compatible with the pH variation desirable to topic formulations and both hydrogels presented promising results with pH stability while in storage.

5.2.2.2. Drug content

The evaluation of the hydrogels stability on their total content of flavonoids in equivalents of rutin, presented as a percentage, is shown below (Figure 16), with maintenance of the content for each batch during the period evaluated.

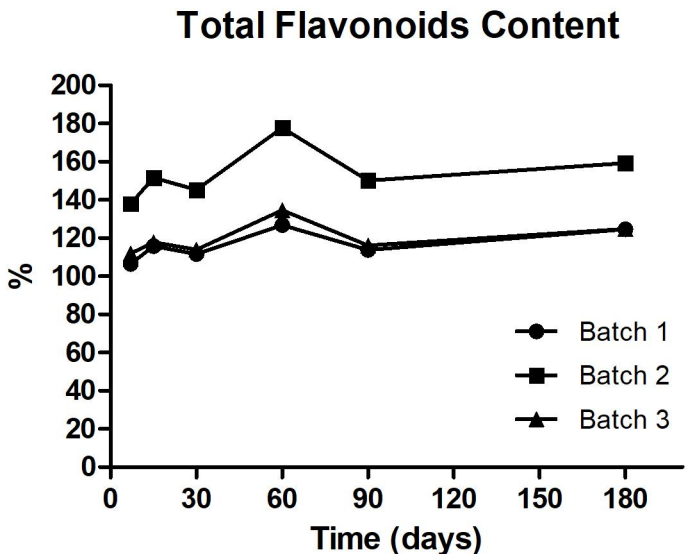

Figure 16. Stability of drug content on total flavonoids content in equivalents of rutin. Hydrogel containing 5% (w/w) of dry extract

Flavonoids are secondary metabolites in the polyphenolic group. They have a lot of therapeutic properties, such as antioxidant, antibacterial, antiviral and anticancer, among others. Rutin is a flavonol commonly used as a standard to quantify total

flavonoids present in plant extract and pharmaceutical and cosmetic products (ROLIM *et al.*, 2005). This direct method presented good experimental results and allows measurement of flavonoids in equivalents of rutin. Besides, it is a simple, quick and convenient technique, and, consequently, it is an important protocol to evaluate the stability of pharmaceutical formulations analysis.

It was shown that all three batches presented a higher and comparable content of total flavonoids in relation to the expected results. According to the Brazilian guide for phytotherapic drugs, the range for the analytic content can vary ± 20% when releasing the batch and ± 10 % during the stability studies (ANVISA, 2014). In the batch release, batch 1 presented 106%, batch 2 137% and batch 3 111%. Batch 2 was an outlier and presented values not similar to batches 1 and 3 since the first day of analysis. This difference could be attributed to experimental artefacts, such as a mistake in the weighting procedure or in the hydrogel homogenization. Besides, among the five aliquots removed from each specific time chosen for the stability studies, only the analysis done after 60 days extrapolated the maximum content allowed, which is 10%. New stability studies will be conducted in order to confirm these hypotheses.

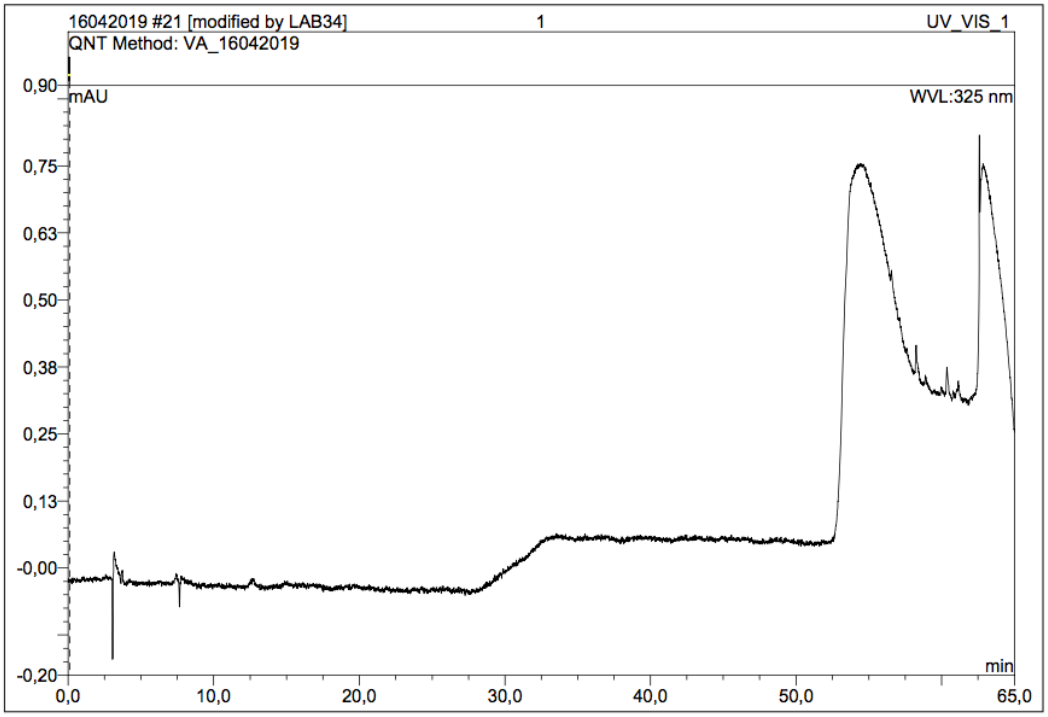

Figure 17. HPLC-UV Chromatogram from the hydrogel at 325 nm.

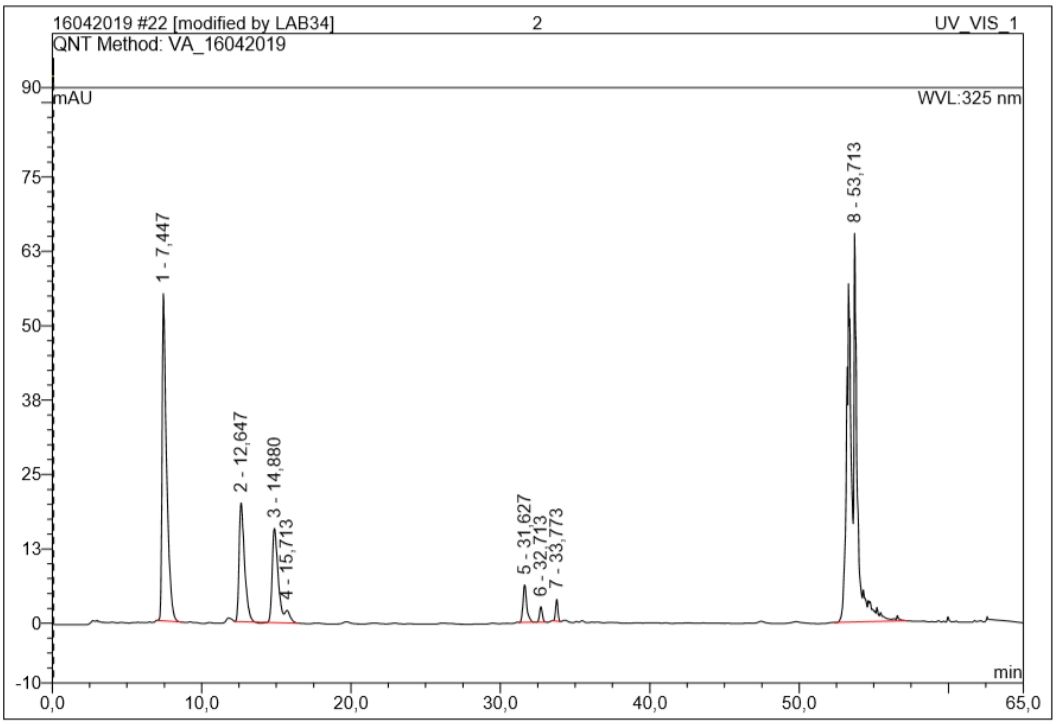

Figure 18. HPLC-UV chromatogram of hydrogel 5 % w/w dry extract 2019. Peaks 1, 2 and 3 represents the three isomers of chlorogenic acid (λ=325 nm).

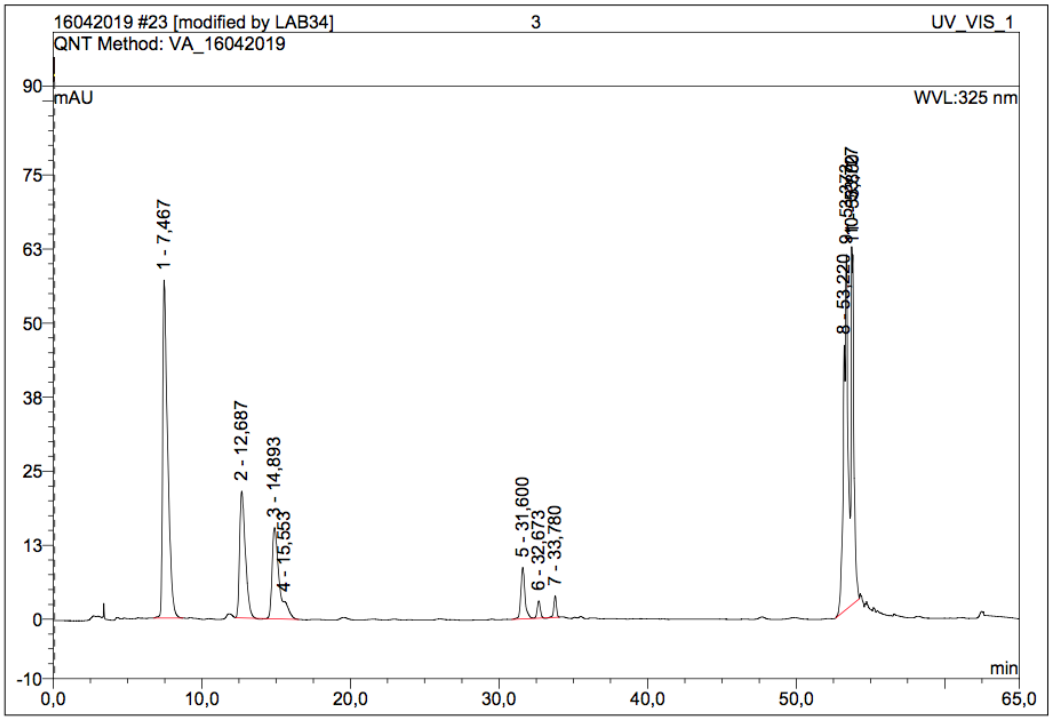

Figure 19. HPLC-UV chromatogram of *V. album* dry extract 2019. Peaks 1, 2 and 3 represents the three isomers of chlorogenic acid (λ=325 nm).

The content of chlorogenic acid after one month of manufacturing was evaluated at 90.17 %.

According to the Brazilian guide for phytotherapic drugs (ANVISA, 2014), the content can vary ± 10%, hence the chlorogenic content in the samples is within the allowed variation. Compared to the higher values found in the UV-Vis, the HPLC evaluation could be a better option for the stability analyses of drug content in *V. album* extracts as well as their content in formulations.

5.2.2.3. Rheology

Rheological analyses were made due to its importance in the development and the stability parameters related to semisolids formulations since they are considered as non-Newtonian fluids, in other words, complex systems with viscoelastic characteristics (QWIST *et al.*, 2019; WAGNER; MOUNT; GILES, 2014; ZIGNANI; TABATABAY; GURNY, 1995).

The samples' flow curve presented a non-linear relation, characteristic of non-Newtonian fluids. Correspondent viscosity curves are presented in Figure 20. All samples presented a substantial decrease of viscosity in a small range of shear. The initial (shear rate of 1 s^{-1}) and final (shear rate of 100 s^{-1}) viscosity for the hydrogel aqueous extract were 331.0 ± 2.8 Pa s and 2.8 ± 0.2 Pa s, respectively, and for the hydrogel dry extract were 372.5 ± 7.8 Pa s and 3.1 ± 0.3 Pa s, respectively.

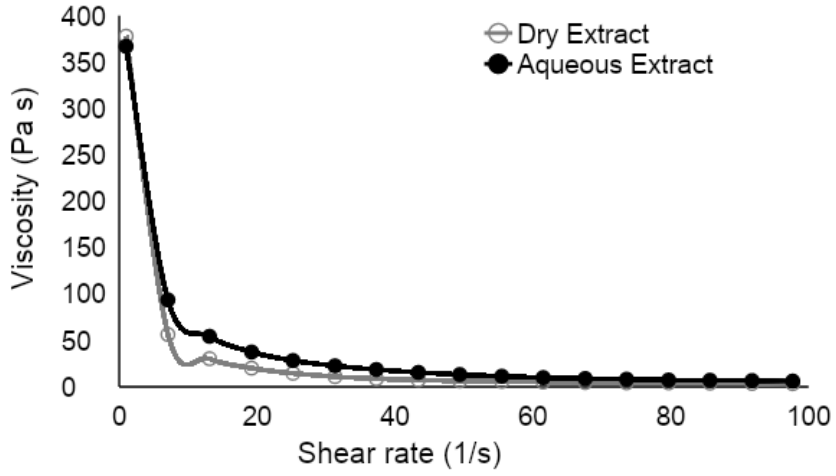

Figure 20. Viscosity curve of the hydrogel containing 5 % w/w of *V. album* dry extract and aqueous extract.

It is worth mentioning that rheology is closely related to the transportation phenomena in Engineering, creating a challenge when it comes to the comprehension of behaviour and characterization of non-Newtonian fluids. Based on this information, a temperature screening was carried out to follow the samples' viscosity properties. It is very well known that temperature influences rheological characteristics of poloxamer 407 gels (DESAI, S. D.; BLANCHARD, 1998; DUMORTIER *et al.*, 2006; GIOFFREDI *et al.*, 2016; GIULIANO *et al.*, 2018). In higher temperatures, viscosity is higher, while in lower temperatures (under refrigeration), the polymer solution behaves as a Newtonian fluid, the opposite of most materials, which present higher viscosity in lower temperatures. Values for the hydrogel aqueous extract were lower because it lowered the concentration of the polymer in the final formulation, when compared to the dry extract formulation. The results are represented in the Figure 21.

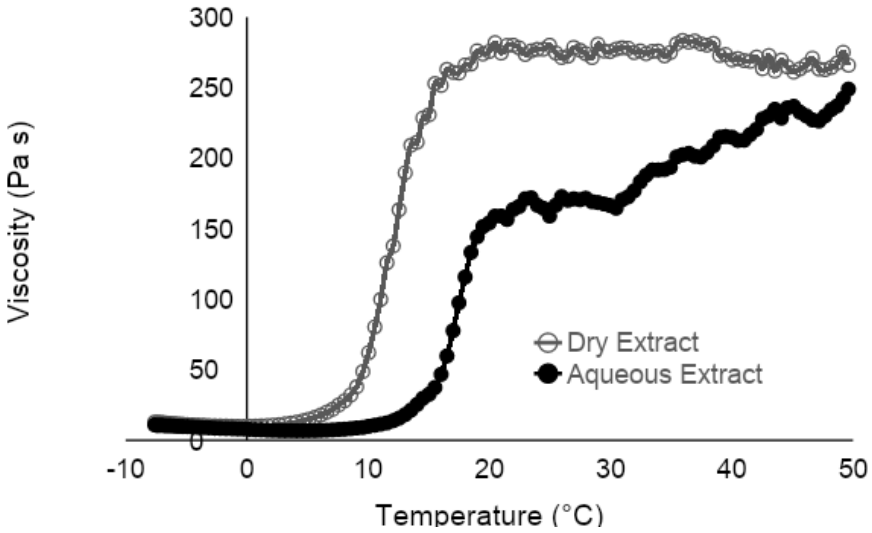

Figure 21. Temperature sweep of the hydrogel containing 5 % w/w of *V. album* dry extract and aqueous extract

In 1923, H. Freundlich observed that some gels fluidized by agitation process giving rise to liquid solutions. He proposed a new model for the deviations from Newtonian behaviour and named it as thixotropy (thixis: stirring, shaking; trepo: turning or changing), which is defined as the continuous decrease of viscosity with time when flow is applied to a sample that has been previously at rest and the subsequent recovery of viscosity in time when this flow is discontinued. Three elements are essential when considering thixotropy: viscosity, reversibility and decrease of viscosity induced by flow (MEWIS; WAGNER, 2009).

The 3ITT is recommended to evaluate the thixotropic behaviour of a sample (PATEL *et al.*, 2015). This measurement is separated into three different sections to examine the time dependent structural degradation and recovery. The first section simulates the quiescent state before a sample is processed; therefore a low shear rate is applied. The second section simulates the shear during the application and, therefore, a high shear rate is applied. The third and last section describes the recovery after the application. It is expected, then, that the hydrogel developed presents a good recovery of its structure after skin application, keeping a good adherence and homogeneity. According to Figure 22, the dry and aqueous extracts hydrogels presented a good recovery, up to 50% after 10 seconds, with the dry extract hydrogel showing a slightly higher recovery in function of time (Table 5).

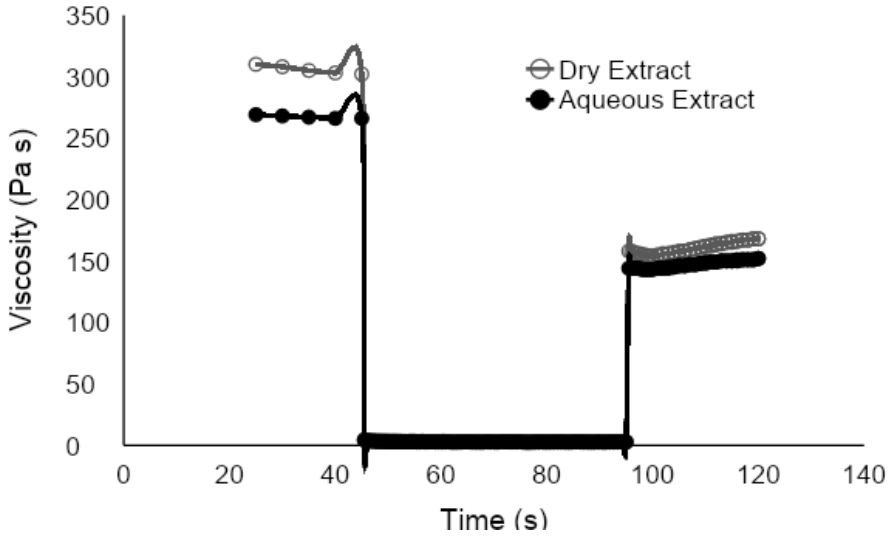

Figure 22. Three-interval thixotropic test of hydrogel containing 5 % w/w of *V. album* dry extract and aqueous extract

Table 5. Flow recovery of three-interval thixotropic test with different shear rate

Formulation	Recovery %	
	After 10 s	After 60 s
Hydrogel Aqueous Extract	54.9%	56.1%
Hydrogel Dry Extract	52.3%	55.3%

The elastic (G') and viscous (G'') modules in function of shear rate at a frequency of 10 rad.s^{-1} were plotted in Figure 23. At low deformation, G' (storage modulus) and G'' (loss modulus) are constant because the sample's structure is not

disturbed. This is called linear viscoelastic (LVE) region, in which both modules are parallels. As soon as the modules start to diminish, the structure is disturbed. Initially, G' value was higher than G'' value, inferring predominance of the elastic effects in both evaluated hydrogels. With the amplitude screening test, it can also be determined the limit of this elasticity, since the relation between such portions defines the strength of the internal network of the hydrogel, which affects the long-term stability.

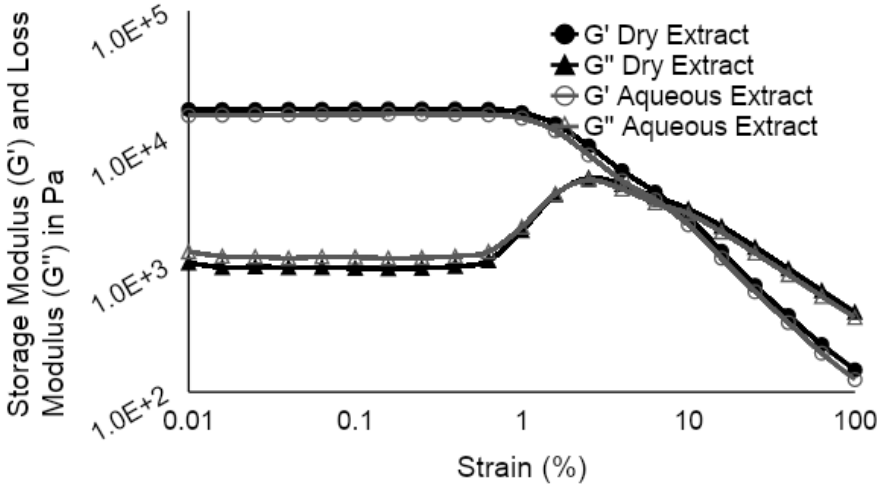

Figure 23. Strain sweep of both dry and aqueous hydrogels. (G' – elastic module; G" – viscous module).

In Table 6, the intersection value of G' and G'' is indicated, after which the viscous forces starts to prevail over the elastic ones. This intersection point would be the shear range in which occurs the break of the material structure, making it leak and behave predominantly as a liquid (G'>G''). In addition, this study can be employed to determine the hydrogel strength (BARRADAS *et al.*, 2018), in which the delta tangent (tan δ) is used, where δ = G''/G', and values inversely proportional to the hydrogel strength are obtained.

Table 6. Hydrogel cross over and tan δ

Formulation	Cross over	G"/G'
Hydrogel Aqueous Extract	2.461 e^{2}	8.47 e^{-2}
Hydrogel Dry Extract	2.723 e^{2}	6.23 e^{-2}

The loss tangent or the internal friction or softening (tan δ) is the ratio between the lost energy by cycle and the stored energy during the cycle. This relation is very

useful in the characterization of the hydrogel strength. According to Douglas (2018), fully developed or strong gel has G'>G", with more rigid materials presenting smaller tan δ values and more flexible materials presenting higher tan δ values. The hydrogels presented viscoelastic behaviour with tan δ values lower than 1, which indicates that exists a predominance of elastic properties and that it could form gel networks to exhibit gel like behaviour (HAO *et al.*, 2018). The gelation behaviour can be explained as a de-solvation and swelling process of the copolymer to form cross-linked aggregates when the temperature is raised and the hydrogen bonds are broken, favouring the hydrophobic interactions of the PPO domains defined as a spontaneous micellization process (BODRATTI; ALEXANDRIDIS, 2018; CHO; SHIN; OH, 1997). Such behaviour was verified in the studied hydrogels, with tan δ lower than 1, being the hydrogel with the dry extract the stronger one.

The stability study (Figure 24) indicates the viscosity values evaluated over the duration of the study (7, 15, 30, 60, 90 days). It can be seen that there was no statistically significant differences during the period analysed for each hydrogel. However, the mean viscosity for the hydrogel with dry extract and aqueous extract is 318.2 and 369.6, respectively, which are statistically different (Welch's t test; $p<0.001$). Different studies have shown the temperature and concentration influence over the viscosity of pharmaceutical preparations (BAN *et al.*, 2017; CHO; SHIN; OH, 1997; DESAI, S. D.; BLANCHARD, 1998) and by considering that the aqueous extract adds a higher content of liquid in the formulation, it is possible to understand its lower viscosity when compared to the dry extract hydrogel, which adds only a solid dry extract.

Similar profile was previous registered in which the addition of components, such as gellan gum and tween 80, also increased the gels viscosity (DEWAN *et al.*, 2017; YEO *et al.*, 2013). Gioffredi *et al.* (2016) observed the viscosity of poloxamer 407 20% w/v in PBS (244 Pa s) and DMEM (240 Pa s) as lower than the viscosity found in the hydrogels in the present work, which range between 300 and 400 Pa s. This higher viscosity could be explained by the presence of other components (propylene glycol and transcutol®) in the formulations.

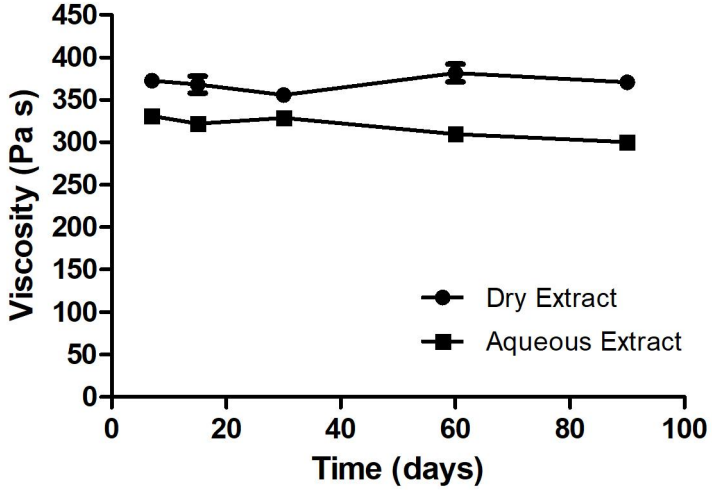

Figure 24. Hydrogels viscosity stability (mean ± SD) (n=2).

5.2.2.4. Microbiological analyses

The presence of pathogenic microorganisms always affects the shelf time of pharmaceutical products. It can lead to physicochemical disturbance and also alter its sterility (DAO *et al.*, 2018).

Tables 7 and 8 show the microbiological results of both hydrogels developed. None of the samples evaluated presented *S. aureus* or *P. aeruginosa*. Only one batch of the dry extract and one of the aqueous extract presented mold and yeasts. Two samples of the dry extract and four samples of the aqueous extract showed higher values for total microbiological charge. In some analyses, the products (Hydrogel dry extract Batch 1.1.3; Hydrogel aqueous extract Batch 1.3.1/4) would not be approved due to the higher microbiological count than the one preconized by the Brazilian Pharmacopeia (2010). In spite of that, as the analyses were evaluated for each batch in different periods of time and the contamination was not maintained, it could be inferred that: i) it might have been caused by package contamination, or ii) it was due to some experimental mistake.

In general, the gels developed presented very good microbiological stability, which could be explained by the presence of propylene glycol at the concentration of 5.0%, which was described by De Spiegeleer *et al.* (2006) as a good preservative, similar to parabens. Also, phenolic compounds, such as the ones present in *V. album,* have antimicrobial properties (AL JITAN; ALKHOORI; YOUSEF, 2018; EL GHARRAS, 2009) and that could also explain the microbiological stability of the formulations, which have already shown antimicrobial properties (HUSSAIN; KHAN; HUSSAIN, 2011).

Personal care products, such as cosmetics, pharmaceutical and food, are used daily and synthetic substances are largely used in them to contain microbial growth and prevent them from degradation (JIMÉNEZ-DÍAZ *et al.*, 2016). Amongst these synthetic products, parabens are extensively used as a preservative in cosmetics and pharmaceutical formulations due to their low cost and wide activity against microorganisms (CRINNION, 2010; TADE *et al.*, 2018). However, recently, paraben use in cosmetic and topical formulations has gained some attention, as some studies associated its use with health effects, such as hepato and dermal toxicity, skin irritation and higher cancer risk (CRINNION, 2010; KIZHEDATH; WILKINSON; GLASSEY, 2019; SONI; CARABIN; BURDOCK, 2005). This can be explained because parabens are not completely metabolised and are retained in the skin, generating cellular and mitochondrial disruption and estrogenic activity (DARBRE *et al.*, 2004; ISHIWATARI *et al.*, 2006). Due to those factors, the formulations of this work were developed without parabens or other artificial preservative ingredient.

Table 7. Hydrogel dry extract microbiological stability

Hydrogel Dry Extract																		
Batch	**1.1.x**						**2.1.x**						**3.1.x**					
Time (days)	**7**	**15**	**30**	**60**	**90**	**180**	**7**	**15**	**30**	**60**	**90**	**180**	**7**	**15**	**30**	**60**	**90**	**180**
Total Microbiological charge ($CFU.g^{-1}$)	<10	<10	**110**	<10	<10	<10	<10	<10	<10	<10	<10	<10	10	<10	<10	<10	<10	<10
Mold and yeasts ($CFU.g^{-1}$)	<10	<10	<10	<10	<10	**20**	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	10
Staphylococcus aureus	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
Pseudomonas aeruginosa	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
Escherichia coli	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A

Table 8. Hydrogel aqueous extract microbiological stability

Hydrogel Aqueous Extract																		
Batch	**1.3.x**						**2.3.x**						**3.3.x**					
Time (days)	**7**	**15**	**30**	**60**	**90**	**180**	**7**	**15**	**30**	**60**	**90**	**180**	**7**	**15**	**30**	**60**	**90**	**180**
Total Microbiological charge ($CFU.g^{-1}$)	20	<10	10	**235**	<10	<10	<10	<10	10	<10	<10	<10	<10	<10	100	<10	<10	<10
Mold and yeasts ($CFU.g^{-1}$)	**65**	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	**160**	<10	<10	<10	<10	<10	10
Staphylococcus aureus	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
Pseudomonas aeruginosa	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
Escherichia coli	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A

CFU – colony-forming unit; A – absence

5.2.2.5. Evaluation of the occlusive factor

The *stratum corneum,* which is composed of protein (mainly keratin), lipids and water, is an important barrier for skin penetration. The water content of the *stratum corneum* is variable, depending on the environment and the body site. The water content needs to be preserved, otherwise the skin starts to get chapped. If the surface is occluded, the *stratum corneum* can hydrate up to five times its dry weight and when fully hydrated, it has a higher permeability than in normal state (MICHAELS; CHANDRASEKARAN; SHAW, 1975; WISSING; LIPPACHER; MÜLLER, 2001).

Occlusive formulations lead to a decrease in skin water loss and the occlusion factor is a parameter that supports the evaluation of the formulations' ability to keep the skin hydrated (WISSING; LIPPACHER; MÜLLER, 2001). As the hydrogel is a formulation that needs to stay on the skin for a period of time, the sensorial aspects and the skin's hydration are important factors for consumers' adhesion (GARG *et al.*, 2002).

Firstly, it was determined the occlusive factor of the developed hydrogels containing the aqueous and dry extracts and the plain hydrogel, without any extract. During the spread process it was possible to observe that all the hydrogels were able to produce a homogeneous layer on the paper filter, covering the entire area. After 72 h of assay, the plain hydrogel, the one with the dry extract and the one with the aqueous extract of *V. album* presented 27.91%, 30.34% and 24.12% occlusive factors, respectively. Considering that zero represents a non-occlusive effect and 100 represents the maximum occlusion, the formulations presented around 25-30% of occlusive effect, with the hydrogel with the dry extract presenting a higher occlusive effect. This can be correlated to its higher content of solid composition compared to the other two developed hydrogels. In general, the poloxamer gel originates non-occlusive hydrogels, as previously described in the literature (DUMORTIER *et al.*, 2006).

5.2.2.6. Spreadability

Semisolid pharmaceutical formulations need to be on the skin for a period of time and should present high power of spreadability to provide a soft touch to the skin. Consumers evaluate formulations' characteristics, such as texture, odour, appearance and

spreadability properties, with the last parameter being specially relevant for the therapy's efficacy because affects drug delivery (GARG *et al.*, 2002).

Spreadability can be defined as the increase in area of a semisolid formulation on a surface after a certain time and it is important because topical formulations should be easy to apply on the skin (FERNANDEZ-MONTES, 2005).

Table 9. Hydrogel spreadability from the hydrogel; hydrogel 5% (w/w) dry extract; hydrogel 5% (w/w) aqueous extract.

Weight (g)	Hydrogel Ø(mm)	Area (mm^2)	Hydrogel Dry Extract Ø(mm)	Area (mm^2)	Hydrogel Aqueous Extract Ø(mm)	Area (mm^2)
5.1	6	28.27	6	28.27	6	28.27
7.1	9	63.62	7	38.48	9	63.62
9.1	9	63.62	8	50.26	9	63.62
14.1	9	63.62	9	63.62	9	63.62

Ø – diameter

In table 9, it can be seen that both plain hydrogel and hydrogel with aqueous extract spread to their maximum after adding the first 2 g weight and the hydrogel with dry extract needed to be under a greater weight to spread, reaching the same area than the others. Therefore, the hydrogel with dry extract was considered as having the same spreadability as the others, but in a longer period of time.

This result is in accordance to the rheology analyses, which gave us the behaviour of the two developed hydrogels, with a higher consistency and strength of the hydrogel containing dry extract when compared to the one containing aqueous extract, which lead to a lower spreadability of the former. In complement, a lower spreadability suggests a better site application, which could increase permeability and a more focused application. This is an important analysis as the therapeutic efficacy also depends on the hydrogels' capacity to spread (DANTAS *et al.*, 2016).

5.2.3. Patch

The development of the patches followed the Patch-non-patch® technique. However, this technique had to be adapted due to the lack of a shaping knife, used to

control the thickness of the patch. The manufacturing of the patches was standardized based on their weights.

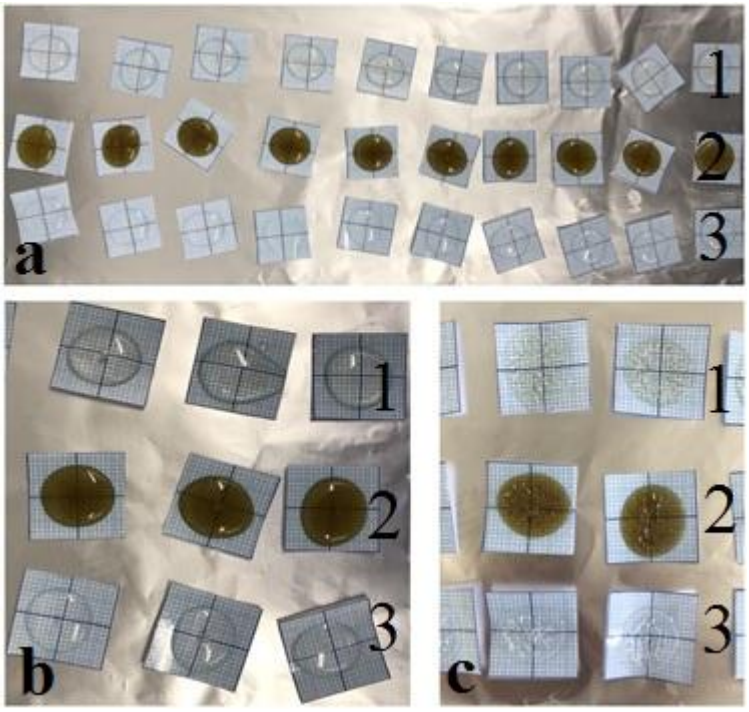

Figure 25. Patch development. A and B: patch before being taken to the oven. C: patch after 30 min in oven at 60 ºC. 1 – aqueous extract patch; 2 – dry extract patch; 3 – plain patch.

A standard plastic paper was cut into pieces with a known area and mass, and on it was weighted the patch. Then, it was taken to the oven for 30 min at 60 ºC (Figure 25). After that, they were weighed and the final mass of the patch and the water loss were calculated.

Our results showed that this procedure presented enough reproducibility, however it can be further improved. The plain patch and the patch with the aqueous extract had the same final mass and water loss (Figure 26). The patch with the dry extract presented a hydrophobic behaviour, which was reflected in the increase of final patch mass in comparison with the water loss. It can be inferred that due to the same amount of water content in the plain patch and in the patch with aqueous extract, as it was added at 5% w/w of an aqueous extract, the water present in the aqueous extract replaced the water in the plain patch mixture. When it comes to the dry extract patch, 5% (w/w) of a dry extract is added to the patch mixture, which makes it less aqueous. Conversely, the hydration process detected in the patch containing dry extract was expected, since the lyophilised extracts are highly hygroscopic (ABASCAL; GANORA; YARNELL, 2005).

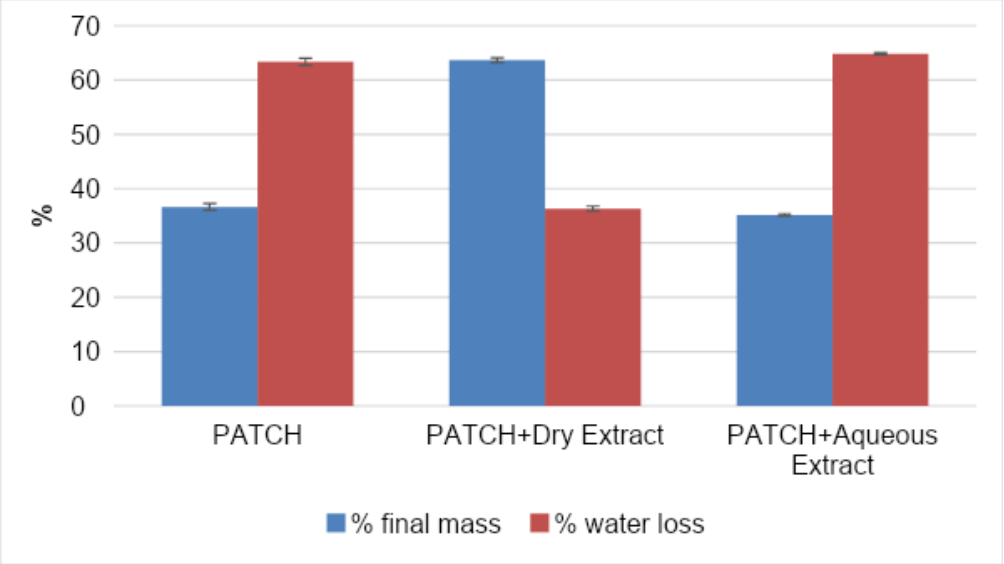

Figure 26. Percentage of final mass and water loss of each patch (mean ± SD) (n=10).

5.2.3.1. Drug content

The content of chlorogenic acid evaluated was 61.37% and 66.12% before and after the patches were taken to the oven, respectively (Figure 27). Considering the Brazilian Guide for Phytotherapics (BRASIL, 2014), this would not be approved as the variation can only be up to ± 10% and it reached almost 40 %.

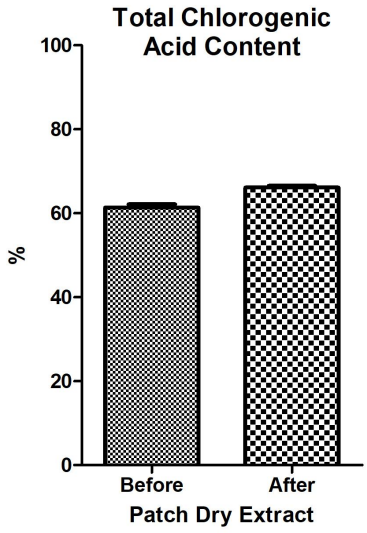

Figure 27. Total chlorogenic acid content in the patch before and after the heating process in the oven (mean ± SD) (n=3).

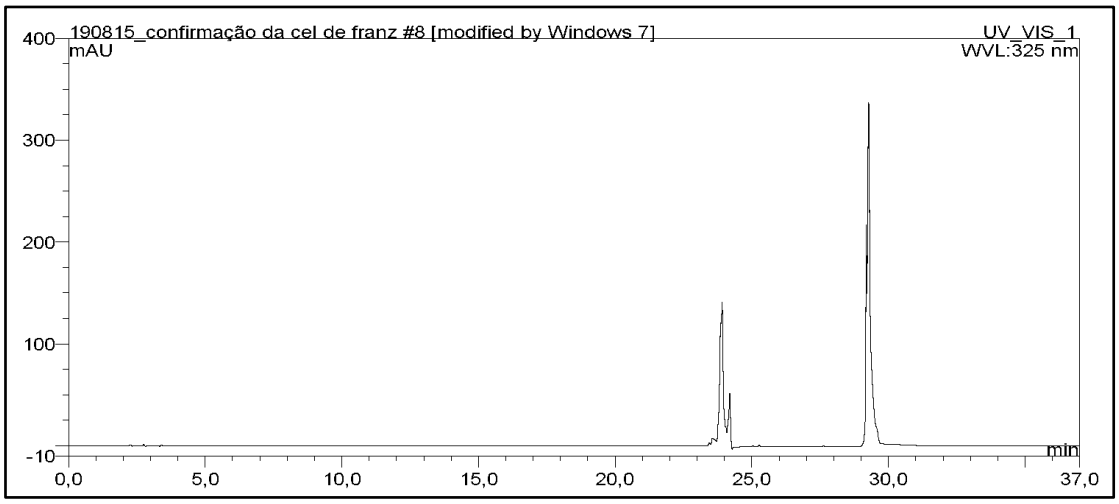

Figure 28. HPLC-UV patch chromatogram done at 325 nm.

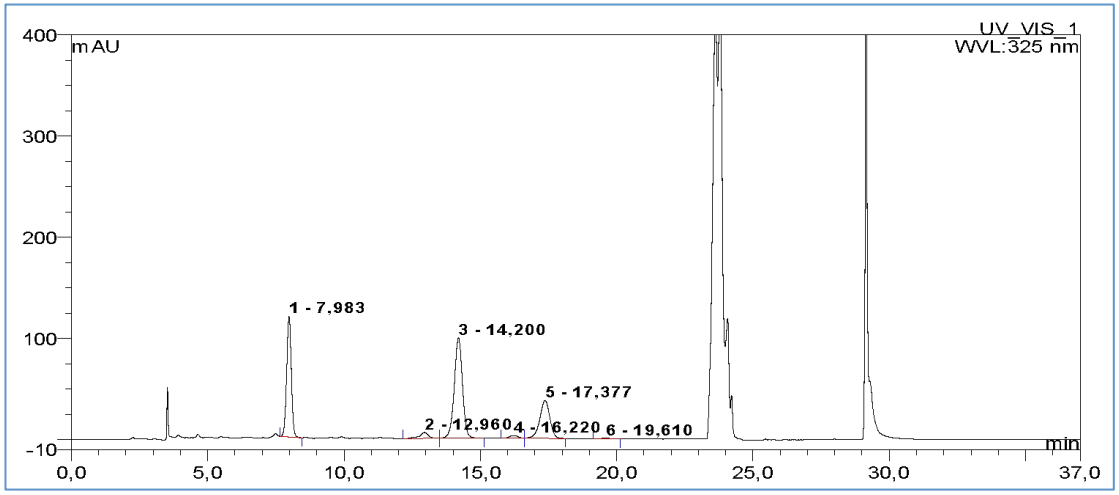

Figure 29. HPLC-UV chromatogram of the patch 5% w/w dry extract before being taken to the oven. Peaks 1, 3 and 5 represents the three isomers of chlorogenic acid (λ=325 nm).

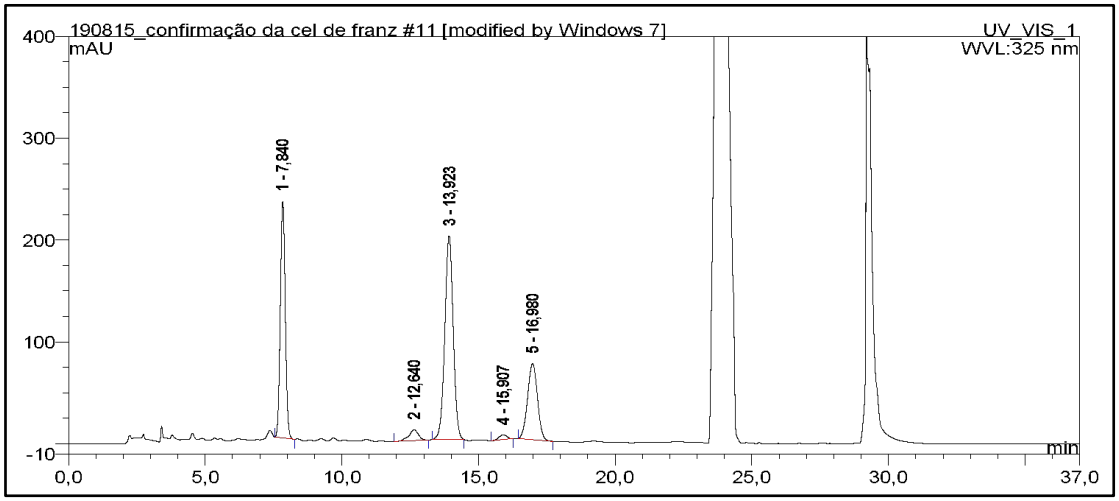

Figure 30. HPLC-UV chromatogram of the patch 5% w/w dry extract after being taken to the oven. Peaks 1, 3 and 5 represents the three isomers of chlorogenic acid (λ=325 nm).

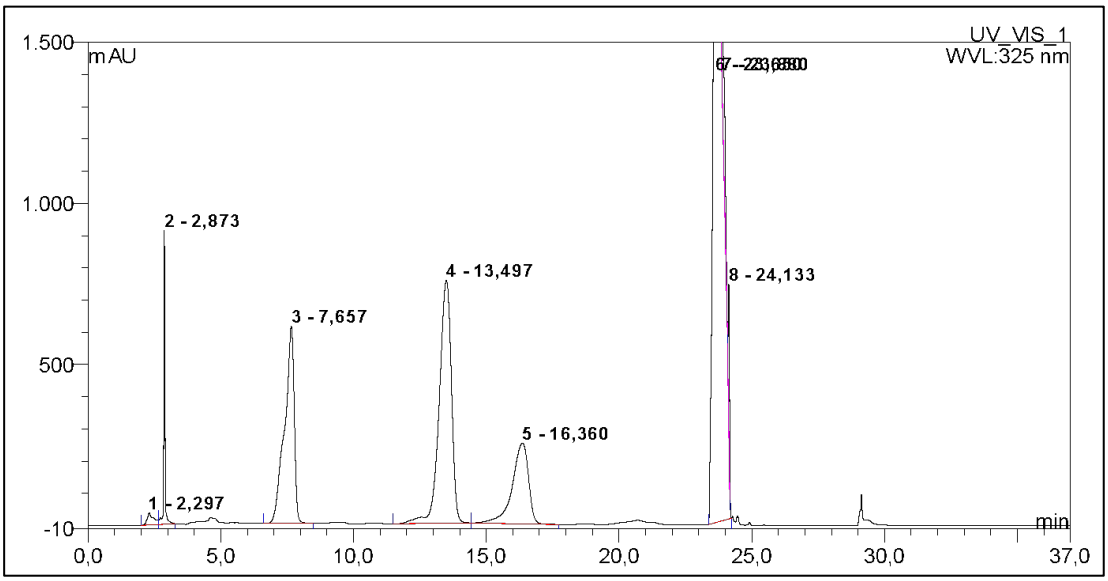

Figure 31. HPLC-UV chromatogram of V. album dry extract 2018. Peaks 3, 4 and 5 represents the three isomers of chlorogenic acid (λ=325 nm).

5.2.3.2. Evaluation of the occlusive factor

Firstly, the occlusive factors of the developed patch containing the aqueous and dry extracts and the plain patch, without any extract, were determined. Differently from the hydrogel analysis, paper was not used to cover the glass; the patch was put directly on the glass and evaluated (Figure 32).

Figure 32. Occlusion assay with patch containing dry extract

After 72 h of assay, the plain patch, the one with the dry extract and the one with the aqueous extract of *V. album* presented an average F of 35.64%, 23.39% and 20.29%, respectively. Considering that zero represents a non-occlusive effect and 100 represents the maximum occlusion, the formulations presented around 25% of occlusion effects. The patch-non-patch technique produces a non-occlusive patch (PADULA *et al.*, 2007), which can be seen with the low F value detected in our experiments.

5.3. PERMEATION STUDIES

5.3.1.1. Chlorogenic acid extraction and recovery from epidermis and dermis

As the formulations had the purpose to be transdermal, it was necessary to determine the amount of the chemical marker of the extract in the different layers of the skin as well as the amount that was able to cross the skin, in a way to discover the real potential of the formulations as transdermal or not. As so, in the present study the cutaneous retention and permeation evaluation of the developed formulations was carried out by the *in vitro* Franz cell type methodology, using pig ear skin as the biological membrane.

According to the extraction method, the solvent used for the chlorogenic acid extraction of the epidermis and dermis was acetonitrile:water (1:9) with 0.1% formic acid shaking for 90 s. Table 10 presents the validation data for this extraction procedure.

Table 10. Extraction method data

Dry extract mass (mg)	Chlorogenic acid mass (µg)	Recovery from total skin (%)	
		Mean	SD
10	188.8	65.22	24.16
50	944.0	62.02	9.51
100	1,888.0	75.95	4.93

5.3.2. Receptor medium selection

The receptor medium chosen for the *in vitro* permeation and retention experiments was PBS (pH 7.4) with 2% v/v of tween 20. This medium maintained the sink condition due to its adequate ability to solubilize the dry extract and, when compared to the other mediums tested, it was able to solubilize a higher amount of chlorogenic acid, as shown in the following table (Table 11).

Table 11. Chlorogenic acid quantification in different receptor mediums for permeation studies.

Chlorogenic acid concentration (µg.mL^{-1})				
		Surfactant		
Concentration	PBS	Ethanol	Propylene glycol	Tween 20
-	859.59	-	-	-
0.5 %	-	850.47	861.16	876.59
1.0 %	-	858.48	862.03	881.97
2.0 %	-	851.87	866.14	875.95

5.3.3. Permeation and retention assays

The hydrogel and patch permeation and retention experiments were performed in a Franz cell diffusion type, using pig ear skin as the biological membrane for a period of 24 hours. The amount of chlorogenic acid in each fraction collected (receptor medium, epidermis and dermis) was determined from the calibration curve developed from the standard in the mobile phase. Then, it was used to calculate the percentage of the chemical marker in each fraction – epidermis, dermis and receptor medium.

5.3.3.1. Hydrogel

A pilot assay was performed using 4 cells containing 300 mg of the hydrogel dry extract and 2 cells with the same components, except by the polymer being replaced by DMSO, as a control for permeation (Control).

In Figure 33, it is possible to observe the permeation of chlorogenic acid throughout the skin from 4 h to 24h.

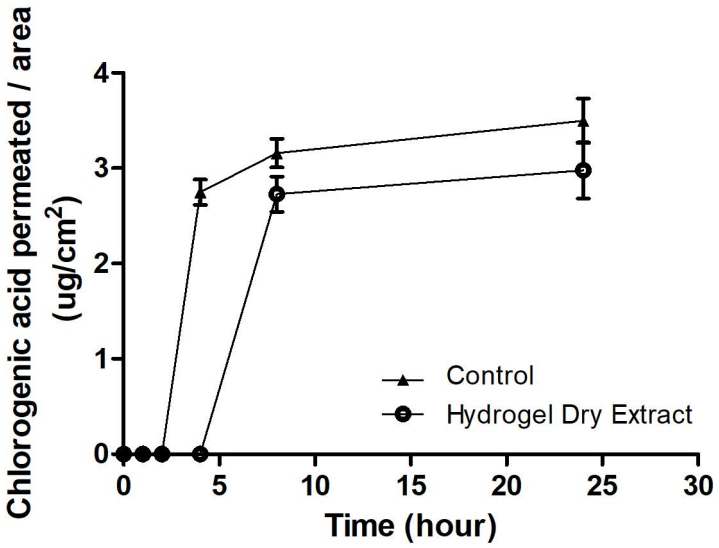

Figure 33. Hydrogel with 5% w/w dry extract permeation assay (mean ± SD).

The amount of chlorogenic acid permeated from the hydrogel was higher than 2 $\mu g.mL^{-1}$ after 8 hours, reaching almost 3 $\mu g.mL^{-1}$ after 24 h, almost the same as control, which presented 3 $\mu g.mL^{-1}$ already at 4 h. The lower flow of chlorogenic acid permeated in the hydrogel could be related to the poloxamer, because its viscosity retards the diffusional release of the drug (WANG *et al.*, 2016). An *in vitro* and *in vivo* transdermal study with different poloxamer 407 formulations and enhancers verified that the highest concentration was reached after 6 h of permeation (CHI; TAN; CHUN, 1996), which was also found in the present study.

After an initial lag time, permeation profile was linear with time, allowing the flow calculation in the stationary state (J_{ss}) (Table 12). The flow of each cell was calculated by linear regression, from 60 min.

Table 12. Chlorogenic acid flow from the hydrogel through the skin.

Cell	Flow (J_{ss}) ($\mu g.cm^{-2}.h^{-1}$)
1	5.964
2	7.842
3	7.614
4	7.896
Mean ± SD	7.33 ± 0.92

In addition to the amount of chlorogenic acid permeated, the amount retained in the epidermis and dermis after 24 h of the experiment was evaluated.

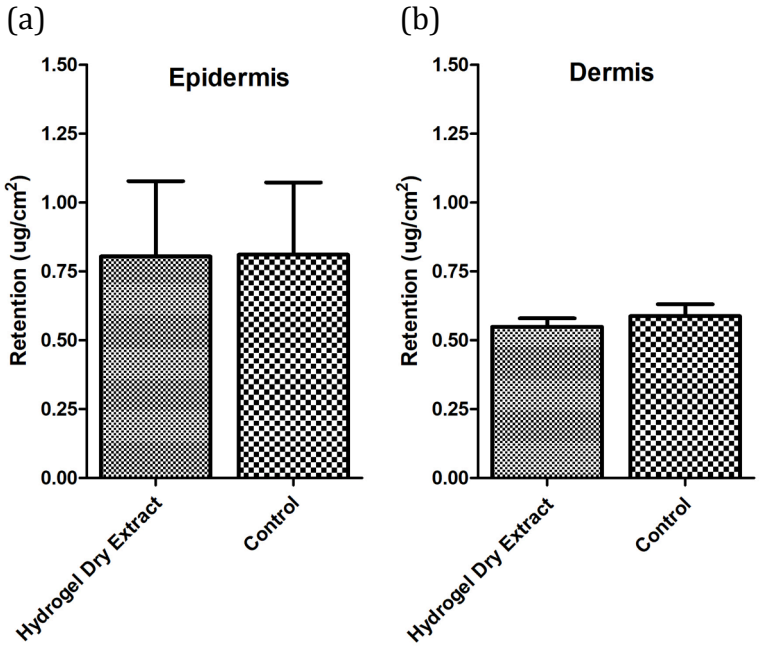

Figure 34. Amount of chlorogenic acid retained in (a) epidermis and (b) dermis after 24 hours of in vitro permeation and retention study (mean ± SD).

The hydrogel showed some amount of chlorogenic acid being retained both in the epidermis and dermis (Figure 34). However, the epidermis presented more chlorogenic acid than the dermis, which could be explained by the *stratum corneum* layer present in the epidermis, which is a major barrier to drug permeation (MICHAELS; CHANDRASEKARAN; SHAW, 1975; TODO, 2017). It could be observed that there was no difference between hydrogel and control, both presenting retention higher than 0.75 $\mu g.cm^{-2}$ in the epidermis and higher than 0.50 $\mu g.cm^{-2}$ in the dermis.

The amount of permeated chlorogenic acid was higher than the one retained, which is in accordance with the literature, due to the components of the studied hydrogel. A study showed that a combination of enhancers improve drug permeability throughout the skin (SHIN; CHO; OH, 2001), with the use of transcutol® increasing permeation by absorbing water due to its hygroscopic nature and the use of propylene glycol acting as a humectant, hydrating the skin and also improving the transdermal potential of the formulation (ESCOBAR-CHÁVEZ; QUINTANAR-GUERRERO; GANEM-QUINTANAR, 2005; GANEM-QUINTANAR *et al.*, 1997). Both enhancers were used in

the developed hydrogels and are probably involved in the permeation of chlorogenic acid from the dry extract into the formulations. It is also worth mentioning that there is no described study in the literature regarding transdermal evaluation of mistletoe extracts.

The hydrogel with the aqueous extract was not evaluated as the main active component, viscotoxin, is a polypeptide with a molecular mass of 5 kDa (SCHALLER; URECH; GIANNATTASIO, 1996; SINGH *et al.*, 2016) and it is already known that it is necessary a molecular weight lower than 500 Da for skin penetration (BROWN *et al.*, 2006; NAIK; KALIA; GUY, 2000; RASTOGI; YADAV, 2012). However, a future experiment could be created to evaluate this, as a study showed that insulin, which has a 3-7kDa size, can be delivered transdermal with a poloxamer 407 gel. This suggests that big size drugs could permeate the skin with chemical enhancers, iontophoresis, terpenes and fatty acids, as well as poloxamers can prevent denaturation of proteins and retain their biological activity (PILLAI; PANCHAGNULA, 2003).

5.3.3.2. Patch

4 cells with the patch and 2 cells with the formulation of the patch were tested without being taken to the oven, in a non-consistent form, as a highly viscous solution, considered as a control.

It was possible to verify that there was no permeation, neither for the patch nor the control. When evaluating the retention of chlorogenic acid in skin layers, it was not possible to quantify it on the dermis but there was a difference in retention between the control and the patch in the epidermis (Figure 35).

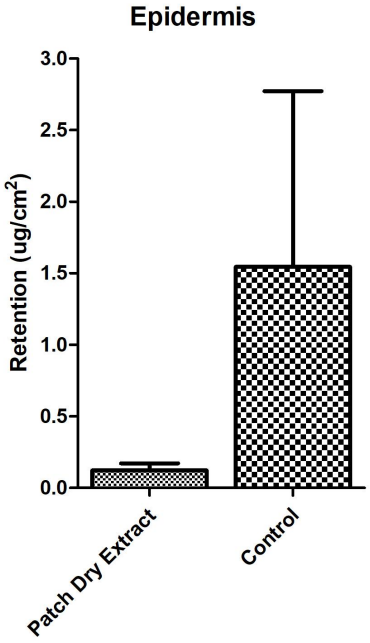

Figure 35. Amount of chlorogenic acid retained on epidermis after 24 hours of in vitro permeation and retention study (mean ± SD).

According to the obtained results, we can suggest that the patch had difficulties in delivering the extract, since there was no penetration and only a small amount retained onto the skin. The control, a viscous solution of the patch, resulted in a higher amount of chlorogenic acid content on the skin, even with a high standard deviation. This high standard deviation can be due to the variation during cleaning of the skin, since the patch solution is viscous and sticky.

5.4. CYTOTOXICITY EVALUATION *IN VITRO*

The cytotoxicity *in vitro* evaluation of the developed formulations was carried out in three different cell lines, two of them tumoral (Yoshida and Molt 4) and one non-tumoral (HaCat). In addition, those cell lines were either adherent (Yoshida and HaCat) or non-adherent (Molt 4). This was done in order to explore and study several possibilities.

The control cell line without any treatment was evaluated and considered as 100% viability. The formulations without *V. album* were also evaluated to verify if they were inactive and were considered as negative controls. The concentration for the negative controls was the same as the highest concentration used to test with *V. album*

(1000 µg extract = 20 mg.mL^{-1} of formulation). When analysing controls versus the formulations (negative controls), it was possible to verify that the hydrogel did not show statistical difference, but the patch did increase cell viability for the adherent cell group (HaCat and Yoshida) (Figure 36). Therefore, for the statistical analyses, all results were compared to the negative control (hydrogel and patch) and not to the control.

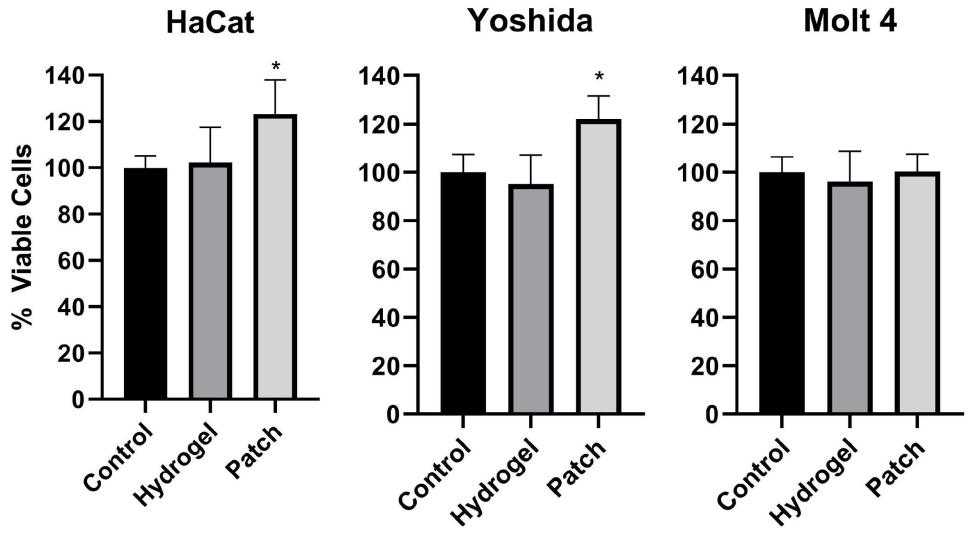

Figure 36. Cytotoxicity comparison between control and the formulations controls (plain hydrogel and plain patch) tested in the concentration of 20 mg.mL^{-1}. Cell growth was assessed with WST-1 assay, after 1:30h of incubation. Results are presented as mean SD from at least three independent experiments in relation to control. *p<0.0001, obtained with one way ANOVA with Tukey post-test.

The amount of the extracts present in each concentration of formulation tested in the cells is expressed in the following table (Table 13).

Table 13. Formulations concentration data for cell *in vitro* experiments

Formulation	***Viscum album ssp abietis***	
Gel/Patch (mg)	**Dry extract (ug)**	**Aqueous extract (ug)**
20	1000	1000
15	750	750
10	500	500
5	250	250
2	100	100

As the patch increased the cell viability, a comparison between the control patch and the patch with the extracts was performed to evaluate the influence of the formulations with extracts on cell viability in the same concentration (20 $mg.mL^{-1}$). Figure 37 shows that the addition of *V. album* extracts statistically decreased cell viability in relation to the patch, confirming the activity of the extracts on the tested cells. It is worth mentioning that the same pattern of cytotoxicity was observed for the three cell lines, being the dry extract the most cytotoxic.

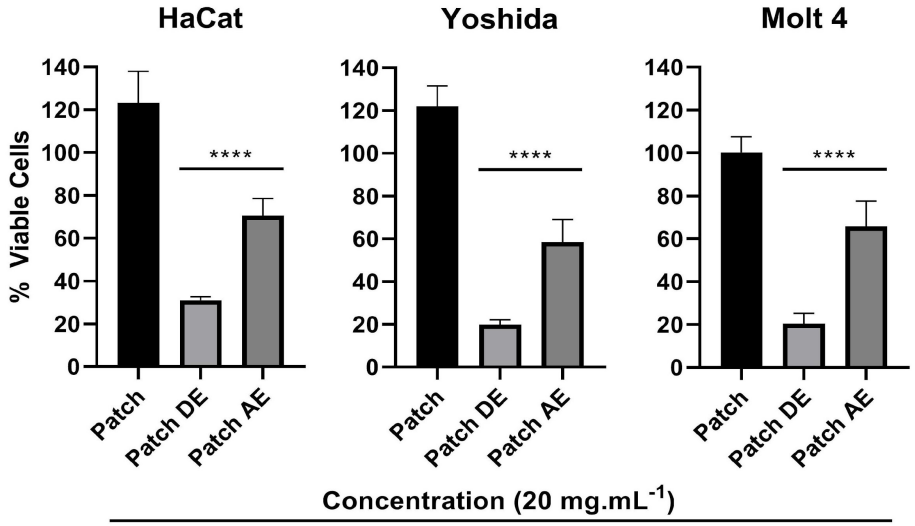

Figure 37. Comparison on cell viability when treated with the same concentration of the patch with and without *V. album* extracts.

When analysing the results for the HaCat cell line, we could verify that the plain hydrogel did not promote an increase in the cell viability, but the plain patch did (Figure 38). Even though, from 10 to 20 $mg.mL^{-1}$, all formulations were statistically able to decrease cell viability, the formulations with the dry extract were more potent than the ones with the aqueous extract. Hydrogel with aqueous extract at 2 $mg.mL^{-1}$ was not statistically different from the control.

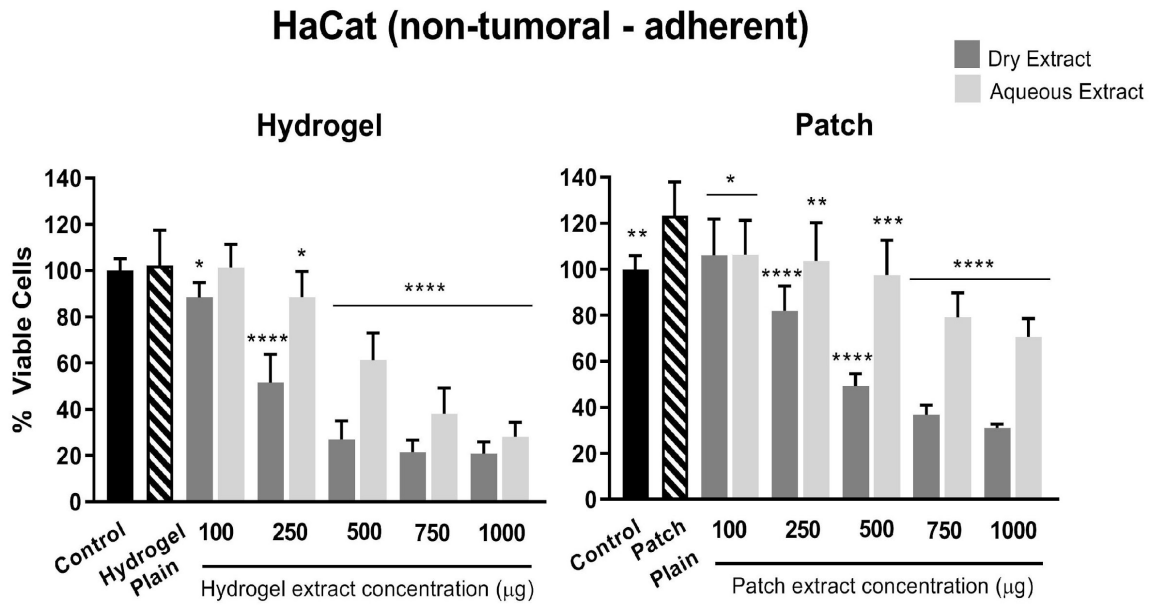

Figure 38. Dose response effect of the Hydrogel and Patch containing *V. album* dry and aqueous extract (5 % w/w) on the proliferation of HaCat cell line after 24 hours. The concentrations varied between 2 and 20 mg. Hydrogel and Patch were used as control at a concentration of 20 mg. Cell growth was assessed with WST-1 assay, after 1:30h of incubation. Results are presented as mean±SD from at least three independent experiments in comparison to hydrogel and patch controls. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001, obtained with one way ANOVA with Tukey post-test.

Yoshida cell line also had an increase in viability when tested with the plain patch but not with the plain hydrogel. An interesting result was observed with the hydrogel with the aqueous extract at 2 $mg.mL^{-1}$, which was also able to increase cell viability (Figure 39). The same pattern found in the HaCat cell line was observed when comparing the formulations with the aqueous and the dry extract, with the formulations with the dry extract being the most active.

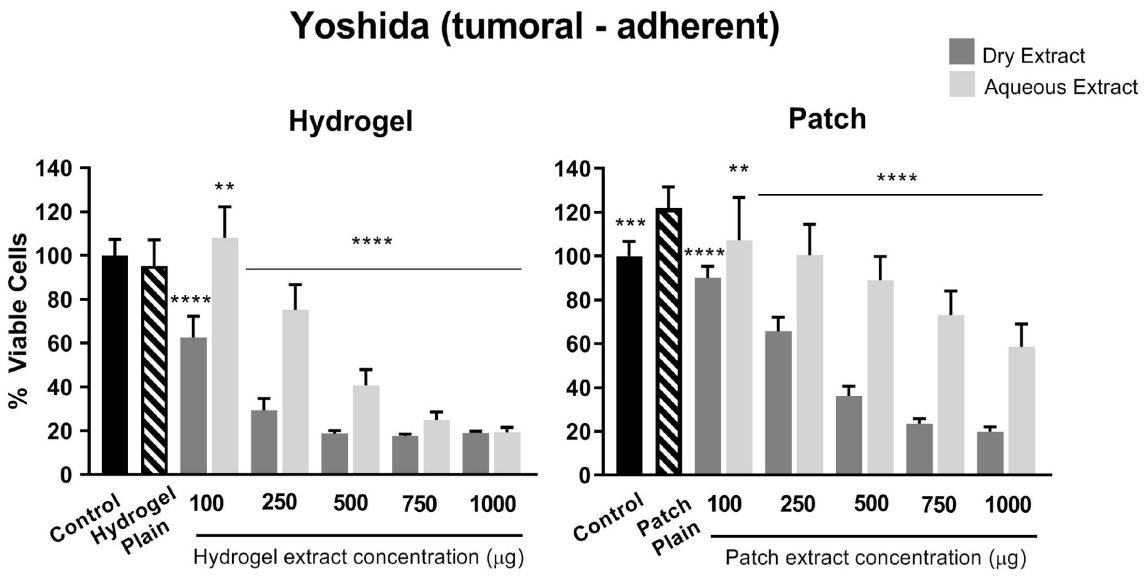

Figure 39. Dose response effect of the Hydrogel and Patch containing *V. album* dry and aqueous extract (5 % w/w) on the proliferation of Yoshida cell line after 24 hours. The concentrations varied between 2 and 20 mg. Hydrogel and Patch were used as control at a concentration of 20 mg. Cell growth was assessed with WST-1 assay, after 1:30h of incubation. Results are presented as mean±SD from at least three independent experiments in comparison to hydrogel and patch controls. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001, obtained with one way ANOVA with Tukey post-test.

The non-adherent cell line, Molt 4, did not have an increase in viability when tested with the plain hydrogel and the plain patch, a different result when compared to HaCat and Yoshida. Hydrogel with the aqueous extract at 2 and 5 $mg.mL^{-1}$ were not statistically different from the control and neither was the 2 $mg.mL^{-1}$ of the hydrogel with the dry extract (Figure 40). Formulations with the dry extract were more potent than the ones with the aqueous extract, following the same pattern observed for the other two cell lines.

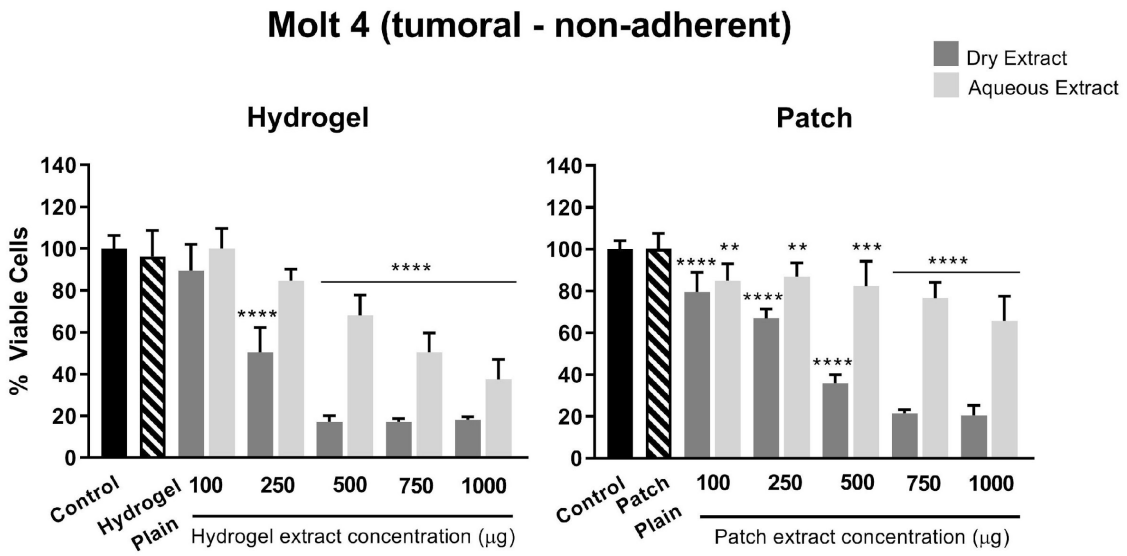

Figure 40. Dose response effect of the Hydrogel and Patch containing *V. album* dry and aqueous extract (5 % w/w) on the proliferation of Molt-4 cell line after 24 hours. The concentrations varied between 2 and 20 mg. Hydrogel and Patch were used as control at a concentration of 20 mg. Cell growth was assessed with WST-1 assay, after 1:30h of incubation. Results are presented as mean±SD from at least three independent experiments in comparison to hydrogel and patch controls. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001, obtained with one way ANOVA with Tukey post-test.

Adherent cell lines, HaCat (non-tumoral) and Yoshida (tumoral), increased their viability when tested with the plain patch, while the non-adherent Molt 4 (tumoral) did not. Lower concentrations were evaluated for the hydrogel and patch in HaCat cell line (Figure 41) to verify if it could present a hormesis response, which is defined by a low-dose stimulation and a high dose inhibition (CALABRESE, 2008). It is worth mentioning that the plain patch and hydrogel concentration is the same as the highest concentration tested with the extracts, so it was tested at 2 $mg.mL^{-1}$ instead of 20 $mg.mL^{-1}$.

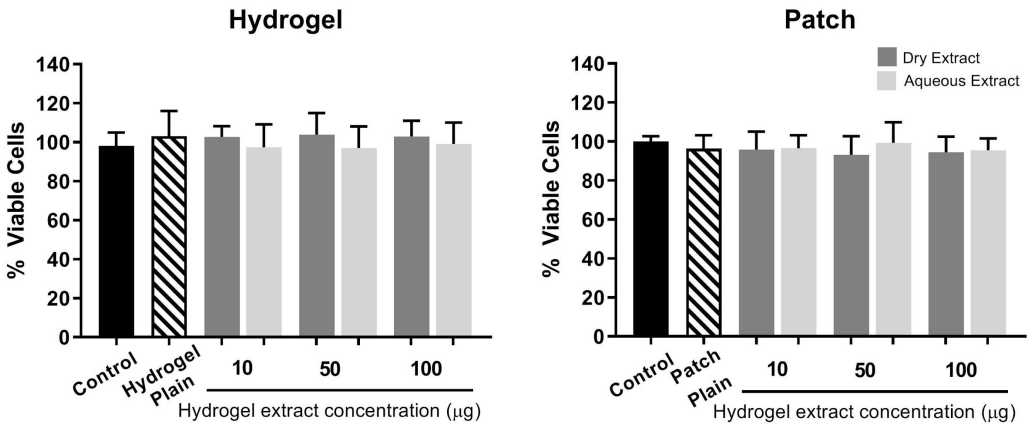

Figure 41. Dose response effect of the Hydrogel and Patch containing *V. album* dry and aqueous extract (5 % w/w) on the proliferation of HaCat cell line after 24 hours. The formulations concentration varied between 0.2 and 2 mg. Hydrogel and Patch were used as control at a concentration of 2 mg. Cell growth was assessed with WST-1 assay, after 1:30h of incubation. Results are presented as mean±SD from at least three independent experiments in relation to hydrogel and patch controls.

Trying to understand the increase in viability of the adherent cells, it was carried out a literature search about each component of the patch that could be increasing cell viability or proliferation. Seo *et al.* (2009) evaluated *in vitro* cytotoxicity of PVA hydrogels in L-929 fibroblasts and found that they increased the cell density when compared to the control and that cell growth inhibition was also not significant.

Wang *et al.* (2015) studied the effects of nanofibers made of PVPK90 loaded with curcumin as anticancer, evaluated the cytotoxicity of the nanofibers (with and without curcumin) and verified a slight increase in cell viability at 100 $\mu g.mL^{-1}$. Lasfargues *et al.* (1973) analysed a serum substitute that can support the continuous growth of mammary tumour cells and found out that 3,5 % of PVPK90 could replace hyaluronate, a critical compound needed for the growth of human breast carcinoma cells *in vitro*. Bioadhesive polymeric films based on usnic acid for burn wound treatment were analysed for cytotoxicity and each component of the films was evaluated. PVPK90, from 0.009 to 0.089 $\mu g.mL^{-1}$, was considered safe and did not increase HaCat viability *in vitro* (PAGANO *et al.*, 2019).

Silva *et al.* (2015) evaluated keratinocytes exposed to hyperosmotic stress and challenged HaCat cells with different concentrations of sorbitol (0.2-2.0 M in serum medium). MTT assay was carried out and it was verified that sorbitol presented a dose dependent response in lowering cell viability. Lu *et al.* (2014) verified that sorbitol induces a decrease in cell viability of human colorectal cancer cells (HCT116) in a dose

dependent manner. According to the results found in the literature, the increasing in the adherent cells viability is barely related to the sorbitol present in the patch.

As though PVA and PVPK90 are considered safe and biocompatible, more studies need to be carried out with these polymers for adherent cells to elucidate their influence in cell behaviour.

The low dose tested in the HaCat experiments evidenced that no hormesis is present at lower concentration of *V. album* formulations and gave an important result to the effect of the plain patch in cell viability since it did not increase cell viability at 2 $mg.mL^{-1}$ like it increased at 20 $mg.mL^{-1}$. It is important to observe that the influence of the PVA or PVPK90, the main components believed to increase cell viability in adherent cell lines, works in a dose dependent manner.

When comparing the results for cell response according to the extract, it was calculated the IC50 (Table 14). IC50 for hydrogel is lower with the dry extract when compared to the aqueous extract, for all cell lines tested. This means that the dry extract formulations are more potent than the aqueous extract formulations. When comparing the adherent cell lines, the IC50 for the tumoral (Yoshida) is half the one needed for the non-tumoral (HaCat), inferring the selectivity in cytotoxic activity for tumoral cells. The non-adherent tumoral cell line (Molt-4) is more resistant than the tumoral adherent one (Yoshida), presenting similar IC50 values than the adherent non-tumoral (HaCat).

Table 14. IC50 of the Hydrogel containing Dry extract and Aqueous extract

	Hydrogel IC50	
Cell line	**Dry extract**	**Aqueous extract**
HaCat	5.813	12.40
Yoshida	2.786	8.856
Molt 4	5.265	15.04

The same analysis was carried out with the patch (Table 15). Similar results were found, but it was possible to observe a lower IC50 for the tumoral non-adherent group (Molt-4) when compared to the adherent ones. IC50 for the tumoral adherent cell line (Yoshida) was lower than for the non-tumoral adherent one (HaCaT), confirming the selectivity of the dry extract formulations activity for adherent tumoral cells.

Both patches, with dry and aqueous extract, showed higher values of IC50 when compared to the hydrogel, which resulted in a better cytotoxic activity of the hydrogel compared to the patch.

Table 15. IC50 of the Patch containing Dry extract and Aqueous extract.

	Patch IC50	
Cell line	**Dry extract**	**Aqueous extract**
HaCat	11.14	26.46
Yoshida	7.383	22.59
Molt 4	6.881	86.10

.

6. CONCLUSION AND PERSPECTIVES

The dry extract of *V. album* was obtained and chemically analysed according to its flavonoids content, which was used as a chemical marker for the herbal drug. Also, it was possible to verify that the flavonoid content was stable in batches from two different years of harvesting and production.

Two pharmaceutical formulations – hydrogel and patch – were developed, using the aqueous and dry extracts of *V. album ssp abietis*, and several analyses were carried out to elucidate their properties.

The hydrogels were evaluated for stability during a period of 6 months and presented compatible pH for skin application, drug content and microbiological stability after 180 days of storage.

Rheological assays showed the differences between the hydrogel with aqueous extract and the hydrogel with dry extract, demonstrating their non-Newtonian behaviour and a higher viscosity in the dry extract hydrogel.

The skin permeation *in vitro* assay by Franz cell type showed the transdermal delivery ability of the hydrogel containing the dry extract, one of the main objectives of this work, for the *V. album* injection replacement or adjuvant use in clinical practice.

The patch did not present any cutaneous permeation in the pig ear skin assay, which could be evaluated in a different system for topic application.

According to the cytotoxicity assays, the formulations showed higher activity against tumoral cell lines, suggesting a promising selectivity effect. Also, the dry extract formulations presented a higher activity compared to the aqueous ones.

Other possibilities can be explored for the developed patch in this study, such as another route of application. Additionally, the stability of the patches is being evaluated.

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Paper Submission

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Phytochemical profile and *in vitro* antiproliferative activity of *Viscum album* ethanolic extracts

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Patent Deposit

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DECLARAÇÃO

Declaramos para os devidos fins que a pesquisa a qual resultou no trabalho de dissertação do aluno **João Vitor da Costa Batista - DRE nº 117338740**, título: ***"Development of biotechnological formulations containing Viscum album L. for topic and transdermal use"*** está em processo de depósito de pedido de patente nesta Agência.

Rio de Janeiro, 22 de outubro de 2019.

Flávia Lima do Carmo
Coordenadora da Agência UFRJ de Inovação

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