Quality evaluation of a Brazilian marketed herbal medicine made from Schinus terebinthifolius Raddi: changes in the legislation for registration throughout 20 years

Avaliação da qualidade de um medicamento à base Schinus terebinthifolius Raddi (aroeira) comercializado no Brasil: adequação à legislação ao longo de 20 ano

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ABSTRACT
Considering the importance of the evaluation of quality and performance attributes of herbal medicines, this work evaluated a gynecological gel containing aroeira (*Schinus terebinthifolius* Raddi), which was previously registered in 2001, and is still marketed in Brazil. Using the updated guides and legislation as a basis, the product was evaluated by checking the pH, spreadability, rheological behavior, *in vitro* release test, *ex vivo* permeation and retention study using porcine vaginal mucosa in Franz diffusion cells, and histological evaluation of the porcine vaginal mucosa before and after application of the product. After the permeation study, it was verified that gallic acid (chemical marker quantified) does not cross the mucosa, therefore, it is safe for local use. In addition, the pH of the formulation is within the range considered normal for use in healthy women. Finally, the histological evaluation of the mucosa showed changes compatible with the application of tannins (major compounds of the plant), and without changes indicative of loss of tissue integrity.

Keywords: Phytotherapeutic Drugs, In Vitro Techniques, Mucous Membrane, Vaginal Creams, Foam and Jelly, Anacardiaceae.

RESUMO
Considerando a importância da avaliação dos atributos de qualidade e desempenho dos medicamentos fitoterápicos, este trabalho avaliou um gel ginecológico contendo aroeira (Schinus terebinthifolius Raddi), que foi registrado anteriormente em 2001, e ainda é comercializado no Brasil. Utilizando os guias e legislação atualizados como base, o produto foi avaliado através da verificação do pH, espalhabilidade, comportamento reológico, teste de liberação in vitro, estudo de permeação e retenção ex vivo usando a mucosa vaginal suína nas células de difusão de Franz, e avaliação histológica da mucosa vaginal suína antes e depois da aplicação do produto. Após o estudo de permeação, foi verificado que o ácido gálico (marcador químico quantificado) não atravessa a mucosa, portanto, é seguro para uso local. Além disso, o pH da formulação está dentro da faixa considerada normal para uso em mulheres saudáveis. Finalmente, a avaliação histológica da mucosa mostrou alterações compatíveis com a aplicação de taninos (principais compostos da planta), e sem alterações indicativas de perda de integridade do tecido.

Palavras-chave: Fitoterápicos, Técnicas In Vitro, Membrana Mucosa, Cremes Vaginais, Espuma e Geléia, Anacardiaceae.

1 INTRODUCTION

The use of medicinal plants in the population for therapeutic purposes is an ancient practice, with records dating back thousands of years before Christ. However, only in the nineteenth century did researchers start looking for active components present in medicinal plants, leading to the concept of the first active substance with the characteristics currently available. Over time and based on knowledge of folk medicine, different ways of using medicinal plants and herbal medicines have been used (Dutra, 2016).

According to ANVISA (National Health Surveillance Agency), herbal medicines are those obtained with the exclusive use of active plant raw materials, whose safety and efficacy are based on clinical evidence and characterized by the consistency of their quality. Interest in herbal medicines has been increasing significantly worldwide, representing about 3% of the global drug market, with a turnover of around $30 billion annually (Marques, 2013; Dutra, 2016; Silva, 2017).

The standardization of the herbal medicines registry in Brazil began in 1967 with the publication of Ordinance no. 22, which required only information about the plant, including its botanical identification, plant drug characterization, organoleptic, physicochemical and phytochemical characteristics, as well as references or scientific research to substantiate therapeutic use, absence of toxic effect and pharmacological experimentation in animals. Despite being a rising market and having a fundamental role for the population, there was no specific regulation for herbal medicines until the mid-1990s. This allowed the disordered growth of this market because until then they were considered low risk medicines, and there were no concrete studies about their use (Oshiro, 2016).

Only 28 years after the publication of Ordinance no. 22, there was the first update for the registration of herbal medicines, which introduced, for proof of efficacy and safety, the presentation of preclinical and clinical toxicology and pharmacology studies through Ordinance no. 6 of January 31, 1995. Since then, some changes have been occurring in the legislation, with the aim of standardizing the registration of herbal medicine, in order to ensure greater efficacy and safety of these medicines (Tappin, 2013).

A survey conducted by Carvalho (2015) *et al*. showed that in 2008 there were 512 herbal medicines registered in Brazil, while in 2011 and 2015 only 382 and 359 were found respectively. There has been, therefore, a 31% drop in herbal medicines registered in the country, either due to renewal of registration being denied due to the lack of

compliance with health requirements or the lack of interest of industries in adapting to new legislation (Perfeito, 2012 Oak, 2018).

The current legislation for the registration of herbal medicines in Brazil is the RDC no. 26 of May 13, 2014, which differentiates traditional herbal product from herbal medicine with the latter being required to prove its safety and efficacy through clinical studies. (ANVISA, 2014; OSHIRO, 2016). Despite successive updates in the laws regulating the registration of herbal medicines, the change in the regulatory scenario of this product group is considered recent.

In the specific case of topical herbal medicines, there is no guide to evaluate their performance. However, it is noteworthy that in Brazil, the "Guide on Quality Requirements for Topical and Transdermal Product Registration" was recently published by the Brazilian Health Regulatory Agency (ANVISA) in 2019. This guide describes tests for quality evaluation such as description, pH, content and rheology, and tests for performance evaluation, which can be done through in vitro release test (IVRT) or in vitro permeation assay (IVPT).

Given the previous information, the aim of this work was to assess the quality and safety of an herbal medicine of vaginal use marketed in Brazil for about 20 years, through quality and performance assays described in literature.

2 MATERIALS AND METHODS

Analytical method validation

The analytical method was developed by the industry responsible for the production of Kronel (INFAN), and was validated following the guidelines of RDC No. 27/2012 of the National Health Regulatory Agency (Brasil, 2012) for bioanalytical methods, with modifications related to the mobile phase and column brand and type. The parameters evaluated were selectivity, residual effect, linearity, precision and accuracy, and recovery.

Materials

Kronel® (gel of *Schinus terebinthifolius* Raddi) samples as well as the placebos were kindly donated by Hebron LTDA and used as the subject of this evaluation. Two different lots of Kronel® with the following numbers were used: 1807018 and 1906019. The standard for Gallic acid was purchased from Sigma-Aldrich lot 1371, drug content 100%. All the solvents and reagents used in the analyses were HPLC grade

Quality evaluation tests

Organoleptic characteristics such as appearance, odor and color of the products were evaluated. The pH was analyzed by Hanna® pH21 pH meter, previously calibrated with buffer solutions pH 7.0 and pH 4.0 at room temperature, using 3 replicates.

The spreadability evaluation was conducted using the method adapted from Borghetti & Knorst (2006), in three replicates, in which 0.5 g of the formulation was applied to a glass plate placed on graph paper, and a glass plate of known weight was placed on the sample. After one minute of spreading, the diameter was measured in opposite directions and the mean diameter was calculated. This procedure was repeated up to a total of 5 plates. The results were expressed as sample spreadability as a function of the applied weight.

The viscosity and rheological properties of the formulations were determined at room temperature using the Rheology International Digital Viscometer. The apparent viscosity was determined using spindle 6 with rotation of 30 rpm.

The content was evaluated using gallic acid as a standard for being the chemical marker of the plant. The gallic acid content of the formulations was quantified after an amount of the gel was added to 60 mL of a diluent solution (a solution containing water and hydrochloric acid 1% and methanol) followed by an ultrasonic bath and HPLC analysis, using a previous validated methodology.

Determination of gallic acid content in the formulations

An amount of 1.00 g of the Kronel® Gel product was weighed, and added to 60 mL of a diluent solution. The sample was extracted in an ultrasonic bath for 30 minutes. The contents of the beaker were transferred to a 100 mL volumetric flask. The volume was completed with q.s.p. diluent and homogenized solution. Then filtered to a vial using a 0.45µm x 13mm diameter filter, or equivalent. The samples were injected in triplicate on HPLC under chromatographic conditions.

Performance evaluation tests

In vitro release test (IVRT)

The in vitro release test was performed using artificial hydrophilic membranes from Millipore® made of PVDF, which were previously hidrated in receptor solution (phosphate buffer 4.5), for 12 hours.

A single dose (300 mg) of the formulation and the placebo was applied to the membranes (1,77 cm^2 area exposed) mounted in a Franz cell (Vision® Microette). The

donor compartments were kept closed and the receptor solution (6mL), which was kept under constant stirring by a magnetic bar. Twelve replicates were evaluated and after 7 hours study maintained at 37°C, samples of 1 mL from the receptor solution were collected and quantified by High Performance liquid chromatography.

In vitro permeation test (IVPT)

The porcine vaginal mucosa was sourced from a local abattoir at Paulista-Brazil. Using scalpels and scissors, the vaginal mucosa was excised and packed in saline until the permeation experiment was performed (no longer than 2 hours). A single dose (300 mg) of the formulation and the placebo was applied to the porcine vaginal mucosa (1,77 cm^2 area exposed) mounted in a Franz cell (Vision® Microette). The donor compartments were kept closed and the receptor solution (6mL) consisted of sodium phosphate buffer (pH 4.5), which was kept under constant stirring by a magnetic bar. Twelve replicates were evaluated and after 7 hours study maintained at 37°C, samples of 1 mL from the receptor solution were collected. At the end of the permeation study, the mucous membranes were removed from the equipment for the residual formulation collection procedure and cleaning. Then the retention procedure was performed. Residual formulation was removed from the vaginal mucosa by a cleaning procedure using swabs (Lights Medical Manufacture – China) containing isopropyl alcohol. After this procedure, gallic acid was extracted from the swabs, using the retention procedure, the amount of gallic acid extracted from the residual formulation was analyzed by HPLC and expressed as quantity ± standard deviation.

Histological evaluation of the porcine vaginal mucosa

The tests were performed according to the protocol established by Almeida et al (2016). Histological analysis was conducted using fresh porcine vaginal mucosa (white), and porcine vaginal mucosa after 6 hours of exposure to Kronel® gel and placebo. The collected material was cleaved and dipped in a 10% neutral buffered formaldehyde (NBF) solution, remaining for 48 hours. After this procedure, the fragments were dehydrated in ethyl alcohol in increasing concentrations, diaphanized by xylol, impregnated and included in paraffin. The blocks were cut in a microtome and adjusted to 5 μm. Thus, the obtained cuts were placed on slides smeared with albumin and kept in a regulated oven at a temperature of 37 ºC, for 24 hours for drying. Hematoxylin-Eosin (H.E.) staining technique was used, and the sections were analyzed under a light microscope, under fixed

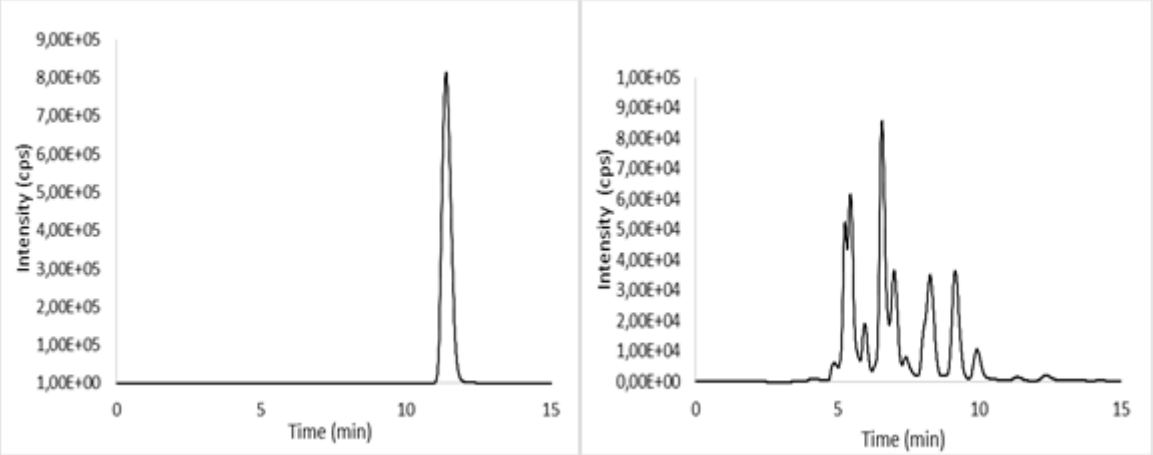
focus and field clarity, using 40x and 100x magnifications. A descriptive analysis of the results obtained was performed.

3 RESULTS AND DISCUSSION

Assay

The evaluated method demonstrated necessary chromatographic selectivity, without endogenous matrix interference in the analyte retention times, as shown in the chromatograms bellow (Figure 1).

Figure 1. (A) Chromatogram of gallic acid in methanolic solution (LoQ) (B) Chromatogram white sample of the extracted vaginal mucosa



Necessary linearity was obtained for the samples, with an average determination coefficient of 0.9999. The verification of precision and accuracy, intracurrent and intercurrent, was performed through the analysis of 5 (five) replicates of each of the samples LoQ, LQC (low quality control), MQC (medium quality control) and HQC (high quality control) in 3 (three) runs on different days. The concentrations of injections at each point were obtained and from these the RSD was calculated to demonstrate precision and the RSE to demonstrate accuracy. The recovery of gallic acid from the mucosa was 31.16%.

Quality evaluation tests

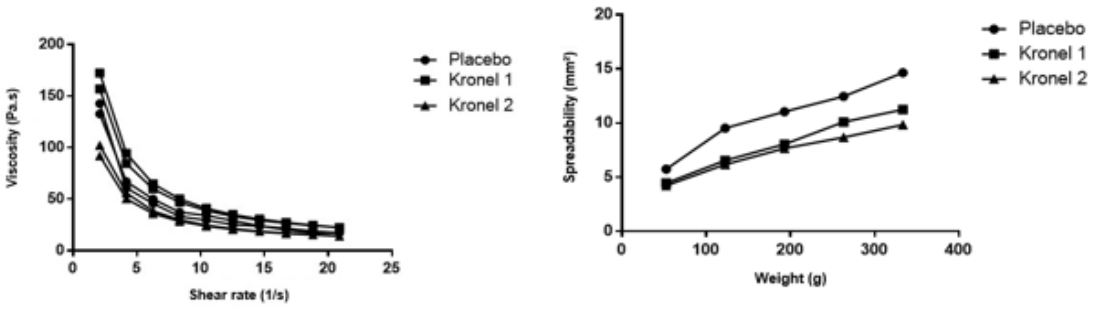
The formulations were analysed macroscopically regarding organoleptic characteristics in order to identify any instability such as color modification, odor and precipitates. Both lots presented an intense red color with light odor and homogeneity. The pH values of lots 1807018 and 1606019 were 4,47±0,02 and 4,44±0,01 respectively, which demonstrates that both are within the parameters of vaginal health, defining the

formulation as compatible with the route of administration (O'HANLON; MOENCH; CONE, 2013).

Spreadability is one of the essential features of pharmaceutical forms for topical administration and is closely related to their administration in the action site. Spreadability values obtained for both lots of Kronel® and placebo are presented in figure 2A, and there are no statistically significant differences among them. The spreadability data of the formulations are very similar to a study perfomed by Kalita in 2017 with metronidazole microsphere-loaded bioadhesive vaginal gel. The study shows that as the viscosity of the formulation increases, the spreadability of the formulations decreases.

After rheological studies, it was shown that all formulations presented non-Newtonian pseudoplastic behavior , justified by the decrease of the viscosity as the shear rate increases. The study of the rhelogical properties is very relevant for the pharmaceutical industry, since the products consistence must be reproducible lot to lot, and thus ensuring the technological quality of the final product. (Aulton, 2005; Lahoud, 2010). Rheological behavior of the formulations is shown in figure 2

Figure 2. (A) Spreadability of the formulations (B) Rheological behavior of the formulations (different lots of Kronel® and placebo).



Determination of gallic acid content in the formulations

According to the manufacturer's specifications, it establishes that the values of gallic acid in Kronel vaginal gel formulations are between 0.27 mg and 0.33 mg per gram of the gel. After performing the test to determine the gallic acid content in the two Kronel batches, it was possible to observe that both are within the specified, as shown in table 1 below.

Table 1. Average values of gallic acid content in formulations.

	Kronel® lot 1	Kronel® lot 2
Content ±SD	0,29± 0,02	0,31±0,15

Perfomance evaluation tests

IVRT

The release of drugs defined in a simplified way as the process by which a drug is released from its pharmaceutical form e becomes available to be absorbed by the skin (Chowdary; Rajyalakshmi, 1987). For the pharmaceutical industry, the IVRT are of great relevance in quality control of drug at different stages of life cycle, as such characteristics must remain constant during the shelf life of the product (Praça, 2010). Furthermore, the evaluation of drug release can be an applicable tool to assess the reproducibility batch to batch of the final product and to control changes during the life cycle of the product (Anvisa, 2019).

After conducting the in vitro release tests, the different lot studied were compared statistically according to the SUPAC-SS 1724 guide from FDA that provides recommendations about post-registration changes to non-sterile semi-solids dosage forms. For lots to be considered equivalents, the confidence interval should be between 75 and 133, 33%. The comparative statistical evaluation of the lots showed that both are considered similar, with a confidence interval of 83,84% - 104,86%. Therefore, it is possible to guarantee the reproducibility of the lots following the Guide no 20 from ANVISA.

IVPT

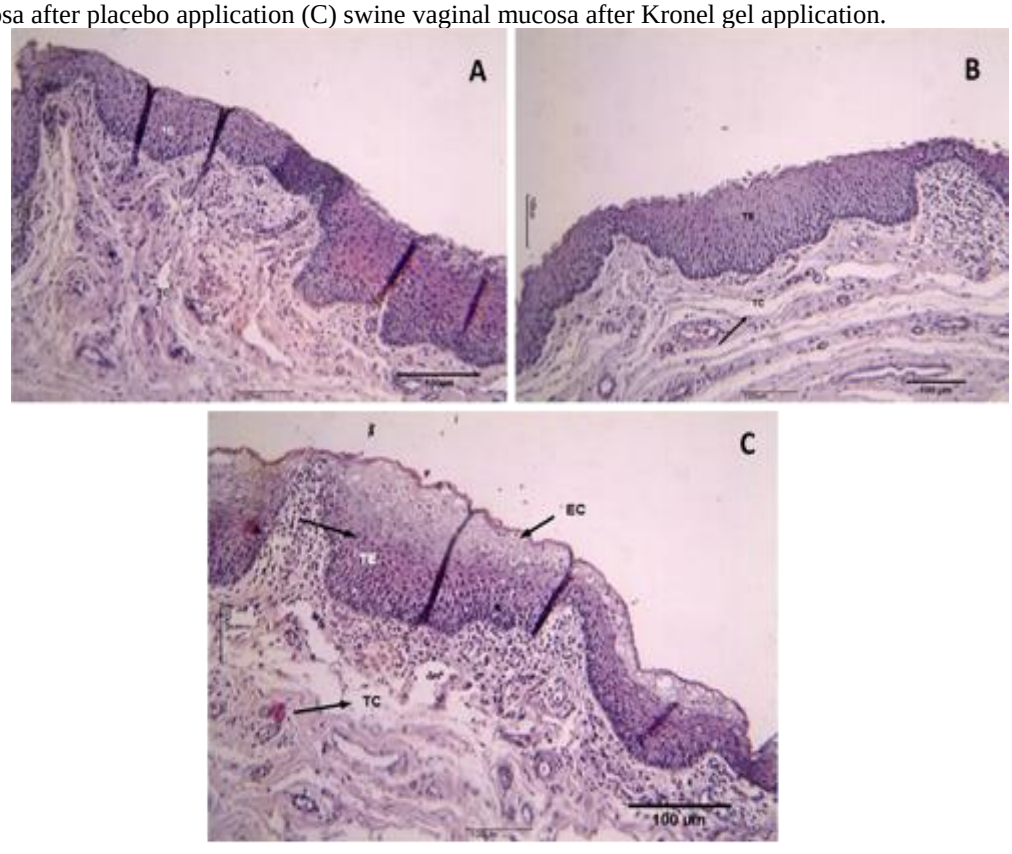
Considering a topically administrated product, it is desirable to have retention on the vaginal surface with a low degree of absorption (MACHADO, 2015). In this way, IVPT were performed with the aim of quantifying gallic acid, after 7 hours of contact with the porcine vaginal mucosa. The time of contact was based on the product's recommendation, added the average sleep time for healthy individuals (18 – 25 years old), that is between 7 and 9 hours (HIRSHKOWITZ, 2015). Vaginal porcine mucosa was used in this study due to its morphological and composition similarity with the human vagina. (Van Eyk, 2005; Squier, 2008).

The results showed that it was not possible to detect an amount equal to or greater than 1ug/mL of gallic acid in the receptor solution, demonstrating the safety of topical use of the product. Approximately 86,56% of gallic acid was recovered, demonstrating that the used for quantification was appropriate, since the FDA recommends the recovery be between 85 and 115% (FDA, 2015). Of this total, approximately 18% was found in the porcine vaginal mucosa, while 68, 56% remained in the residual formulation after the conclusion of the test.

Histological evaluation of the porcine vaginal mucosa

The microscopic evaluation of the histological sections was performed in order to verify possible changes in the porcine mucosa in details. When comparing the epithelial tissue (ET) of the Kronel® group to the same tissue of the white and placebo groups it was possible to verify that the first one is thicker, as shown in figure 3 below. The changes observed in the porcine vaginal mucosa are tipical of changes caused by tannins and do not indicate evidence of loss of mucosal integrity after the administration of the product (Valotto et al., 2011).

Figure 3. Histological sections at 100x magnification: (A) fresh porcine vaginal mucosa (B) porcine vaginal mucosa after placebo application (C) swine vaginal mucosa after Kronel gel application.



4 CONCLUSIONS

When comparing the laws that regulated the registration of herbal medicines over 20 years, it was possible to observe an increase in the rigor of the requirements added to each one of them, thus forcing manufacturers to adapt the standards, in order to guarantee a greater security for the final consumer.

Technology proved to be an ally in meeting these requirements, since some assessments started to be carried out in a more practical and quick way, however, requiring more specific equipment, tools and training.

After evaluating the quality and performance requirements of Kronel® vaginal gel, it was possible to conclude that, according to the requirements required for a formulation of local action, Kronel® was considered a product with pharmaceutical quality and safe for its intended use. It was reproducible batch by batch, gallic acid did not permeate the mucosa, and the formulation showed characteristics compatible with the route of administration, without causing significant changes in the evaluated mucosa.

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