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Original Article

In vitro skin permeation of escin in the new gel formulation of *Aesculus hippocastanum* (Horse Chestnut)

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Abstract: *Aesculus hippocastanum* L. (Hippocastanaceae) is used in topical pharmaceutical and cosmetic formulations by industries. The aim of this work is to develop a new *A. hippocastanum* product having good stability and better skin permeation compared with the other products on the market. Two new gel formulations were prepared to evaluate escin *ex vivo* permeation through pig skin by using Franz-type diffusion and to analyse by HPLC. The first gel formulation has contained only *A. hippocastanum* extract (G1) and the second gel formulation has contained a mixture of *A. hippocastanum* and *M. chamomilla* extracts (G2), as active ingredients. The reference gel has escin as active ingredient and it was purchased from a pharmacy. G2 showed significantly better skin permeation ability with good stability compared to G1 and reference gel. Owing to its favourable penetration and stability characteristics the new gel formulation could be an alternative for the treatment of chronic venous insufficiency and inflammatory soft tissue inflammation.

Key words: *Aesculus hippocastanum*, gel, escin, HPLC, stability.

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Introduction

Medicinal plants have been widely used by pharmaceutical industries as therapeutic agents. Two of these plants, namely, *Aesculus hippocastanum* L. (Hippocastanaceae) and *Matricaria chamomilla* L. (Asteraceae), are used in topical pharmaceutical and cosmetic formulations for their anti-inflammatory and anti-oedema activity on the capillary permeability (Montenegro et al., 2007; De Almeida et al., 2013). These plants are cultivated around the world and they have several ethnopharmacological

and medicinal uses. *A. hippocastanum* is a member of Hippocastanaceae family and commonly known as horse chesnut. It grows in Balkan region of Eastern Europe but also widely cultivated in all temperate regions of North America, Europe and Asia (Baibado & Cheung, 2010). The seeds and the bark of young branches are used in medicine. Escin is the major active constituent isolated from the seed of A. *hippocastanum* and a complex mixture of triterpene saponins. Escin exists in two forms, α and β, and β-escin appears to be the active component of the mixture in pharmaceutical products (Sirtori 2001). These compounds have been clinically used as an anti-inflammatory agents and could be effective in the treatment of chronic venous insufficiency (CVI), hemorrhoids and post-operative oedema (Matsuda et al., 1997; Sirtori, 2001). According to Wilkinson and Brown, "The escin is capable of inhibiting hyaluronidase activity, however, inhibition of hyaluronidase has been determined to be insufficient to account for the powerful venotonic properties of *A. hippocastanum* extracts on the microvasculature, and it has been suggested that inhibition of other enzymes, including collagenase, elastase and β-glucuronidase, all of which are involved in defining the integrity of the extravascular matrix, might explain why extracts of *A. hippocastanum* were markedly more effective than escin alone" (Wilkinson & Brown, 1999). Therefore, we have preferred to use extracts in our formulations.

Matricaria chamomilla is a member of Asteraceae family and commonly known as German chamomile. The dried flowers of *M. chamomilla* contain terpenoids (α-bisabolol, α-bisabolol oxides A and B and matricin) and flavonoids that possess anti-inflammatory properties. These active compounds penetrate below the skin surface into the deeper skin layers. *M. chamomilla* is useful topical anti-inflammatory agent for this reason (Gupta et al., 2010; Srivastava et al., 2010). The aqueous extract of chamomile flowers is found to have anti-inflammatory activity, a wound-healing effect and a skin-soothing effect.

In addition to the extract is a natural preservative and potent antimicrobial activity against a wide spectrum microorganisms because of containing chamazulene, bisabolol and apigenin. It is safe for the body, producing neither toxicity nor irritation, and can be used to cosmetics and pharmaceuticals formulations (Park et al., 2007). There of, we have preferred to add aqueous extract of *M. chamomilla* to gel formulation to produce more stable product.

The aim of this study was to produce a gel having good stability and effective than reference gel contains escin as an active substance and sold in the market only for its anti-inflammatory effect. The experiments were performed on two formulations. The first formulation (G1) was prepared with an *A. hippocastanum* extract containing similar active matter with the reference gel. The second formulation (G2) was prepared with mixture of *A. hippocastanum* and *M. chamomilla* extracts to increase stability and activity.

The quantities of the escin in G1, G2 and reference gel, before and after the Franz-type diffusion test were determined by HPLC. The stability of the gels were tested for 1 month at 25°C ±2 °C/60 % RH and 40°C ±2 °C/75 % RH ±5 conditions.

Materials and methods

Plant material

The dried methanol extract of *A. hippocastanum* and the dried flowers of *M. chamomilla* were obtained from Martin Bauer Group (Germany).

Extract preparation

10 g dried flowers of *M. chamomilla* were extracted with 400 mL hot water (90°C) for 20 minutes. This procedure was repeated three times. The infusion was filtered, than lyophilizated and stored at -80 °C.

Quantitative determination of escin in *Aesculus hippocastanum* extract gels by HPLC Chromatographic HPLC Conditions

The methanol extract and gels have been analyzed by HPLC-DAD. The HPLC system consisted of a Shimadzu 10A model (Shimadzu Analytical and Measuring Instruments, Kyoto, Japan), a pump (LC-10AD), a diode-array detector (DAD) (SPD-M10A) and an autosampler (SIL-10AD).

Separation was accomplished with an ACE C18 column, 250 x 4.6mm i.d., 5 µm (Advanced Chromatography Technologies, Aberdeen, Scotland). The elution conditions were as follows: flow rate: 1.5 mL/min; column temperature: 40 °C; injection volume: 10 µl; run time: 15 min; detection: 205 nm.

The isocratic solvent system (solvent A: 0.05% triflouroacetic acid in water (v/v)) and solvent B: acetonitril (70:30, v/v) was used. All solvents were filtered through a 0.45 µm filter prior to use and degassed in an ultrasonic bath.

The system control and the data analysis procedure were performed with Shimadzu LC Solutions software.

Chemicals

Escin (E1378) was obtained from Sigma-Aldrich (Taufkirchen, Germany). Milli-Q ultrapure water was obtained from Millipore (Billerica, MA), HPLC grade acetonitrile (1.00030) was obtained from Merck (Darmstadt, Germany) and trifluoroacetic acid (411564) was obtained from Carlo Erba Reagent (Val de Reuil, France). Reparil Gel (Madaus GmbH) was used as a reference product.

Standard Preparation

The five point calibration curve for escin was prepared with external standard solution within the concentration range of 250 – 15.625 ng/mL, in mixture of acetic acid/mobile phase (1:6, v/v). The experiment was conducted for three times providing the same conditions. The calibration curve was constructed by using average of peak area.

Sample Preparation

The dried methanol extract and gels were dissolved in acetic acid/mobile phase (1:6, v/v). All samples were filtered through a 0.45 µm filter into a vial for HPLC analysis. Each sample was prepared and injected for three times.

Gel preparation

The weighted amount of Carbopol 940 was added in to the sufficient quantity of water with gentle mixing. The mixture was left at room temperature overnight to complete swelling of the polymer. After the formation of clear blank gel, preweighted amounts of Propylene glycol, herbal extract(s), Tween 20 and Triethanolamine were added respectively into the gel. The pH value of the gel mixture was adjusted to 7.4. The weight of gels were completed to 100 g by gently adding purified water.

The composition of the gel formulations (G1 and G2) are shown in Tables 1 and 2.

Table 1. Compositions of *A. hippocastanum* extract gel formulation (G1) % (w/w)

Ingredients	Amounts (g)
A. hippocastanum extract (standardized)	4.65
Carbopol 940	1.8
Propylene glycol	10.6
Triethanolamine	0.6
Tween 20	1.0
Purified water to	100.0

Table 2. Compositions of *A. hippocastanum* and *M. chamomilla* extract gels formulation (G2) % (w/w)

Ingredients	Amounts (g)
A. hippocastanum extract (standardized)	4.65
M. chamomilla extract (standardized)	0.4
Carbopol 940	1.8
Propylene glycol	10.6
Triethanolamine	0.6
Tween 20	1.0
Purified water to	100.0

Reparil gel contains, Escin, Diethylamine salicylate, Lavender oil, Neroli oil, Carbopol 980, Softigen 767, Disodium EDTA, Trometamol, Isopropyl alcohol and purified water.

Stability test of the formulations

Stability studies on the gel formulations were performed by keeping at the temperature of 25ºC±2ºC and 40ºC±2ºC. Three samples of optimized formulation (G1, G2 and Reference Gel) were taken in glass vials and were kept at accelerated temperature of 25ºC and 40ºC at ambient humidity for the period of a month. Thereafter to measure the escin quantity of the samples were analyzed by HPLC.

Skin permeation studies of gel formulations

Ex vivo permeation studies were performed through pig skin by using vertical Franz diffusion cells. A piece of skin was placed between donor and receiver compartments with temperature maintained at 37°C±1°C using a water circulator. The receptor phase was 12.0 mL of isotonic Phosphate buffer (pH 7.4) at 37ºC (±0.5°C) and stirred continuously at 800 rpm. At predetermined time intervals (2h, 4h, 6h and 24h) 0.3 mL samples were collected from receptor phase and replaced by an equal volume of fresh medium to keep the volume constant.

The samples were analyzed by using HPLC and cumulative amount of escin was calculated.

Results

Quantitative determination of escin in *Aesculus hippocastanum* gels by HPLC

The identification of the escin peak in the samples was achieved by comparing retention time and UV spectra with commercial standards. The absorption maxima of the escin standard was determined at the wavelength of 205 nm.

It was observed that there exists linear relation between the escin peak areas and concentrations. Linear regression analysis of the data yielded a correlation coefficient (R^2) of 0.998 for escin. Calibration equation was shown on Figure 1.

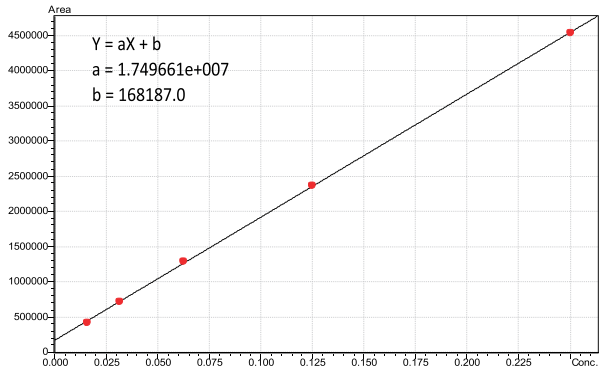

Figure1: Calibration curve of escin standard

Quantity of escin in Gel 1, Gel 2 and reference gel analyzed by HPLC-DAD, were found to be 1.03 mg/mL, 1.01 mg/mL and 1.03 mg/mL, respectively (Table 3). The results of the cutaneous absorption of the gels were shown on Table 4.

Table 3: Quantity of escin in the gels (n=3)

Gels	Quantity of escin (mg/mL)	%RSD
G1	1.03	9.32
G2	1.01	8.60
Reference Gel	1.03	6.41

Table 4: Quantity of escin in the gels after skin permeation tests

Gels	Time (hour)	Quantity of escin (mcg/mL)
G1	2	730.0
	4	850.0
	6	850.0
	24	850.0
G2	2	760.0
	4	850.0
	6	890.0
	24	890.0
Reference Gel	2	810.0
	4	810.0
	6	820.0
	24	840.0

Discussion

Chronic venous insufficiency (CVI) is a common health problem, particularly patients immobilized for prolonged periods. Signs and symptoms of CVI may include leg discomfort, heaviness, cramps, pain, oedema and skin changes (Angoules 2015; Aziz et al. 2015). Therefore, edema protective phytopharmaceutical preparations can be applied topically for the treatment of CVI. The seed extract of *A. hippocastanum*

has traditionally been used to treat patients with CVI (Suter, Bommer, and Rechner 2006). The German Commission E has approved the use of *A. hippocastanum* preparations for the treatment of CVI. *A. hippocastanum* seed extract is widely used in Europe and is becoming increasingly popular in North America as well, for the treatment of CVI with strong supportive scientific evidence. External preparations containing escin can be applied topically for the treatment of CVI.

Some clinical trials shows that the seed extract of *A. hippocastanum* is a safe and efficient shortterm treatment for CVI. Effects of escin in the gel form on skin perfusion in CVI treatment were evaluated by Ruffini et al., 2004. According to the results, topical treatment of venous microangiopathy with the gel was very effective in improving skin perfusion and nutrition (Ruffini et al. 2004). Efficacy of escin in topical gel on microcirculation in patients with chronic venous hypertension (CVH), and venous microangiopathy was determined in the other clinical study. The result showed that treatment with topical applications of the gel in areas of venous microangiopathy was beneficial (Belcaro et al., 2004).

This paper presents a new *A. hippocastanum* gel formulation having good stability and better skin permeation compared with the other products on the market. Any studies could be found on the literature research on this subject directly. There are prepared two gel formulation, namely G1 and G2 having wide temperature stability and skin permeation ability were compared with reference gel purchased from pharmacy. Stability test results showed that the new gel formulations have similar stability compared to the reference at 25 °C and 40 °C.

Franz-type diffusion test result showed that G2 has linear and maximum permeation during the 24 hours. 890 mcg/mL escin passed into the pig skin at the end of the 24 hours (Table 4). According to the results, G2 has good temperature stability with significantly better skin permeation ability compared with G1 and reference gel.

As a result, the new gel containing the mixture of *A. hippocastanum* and *M. chamomilla* extracts has good stability and skin permeation ability compared to other gels containing only escin or *A. hippocastanum* extract.

The most important advantages of the topical drugs to treatment diseases like CVI are easy application and less probability regarding the side effects

and drug interactions. Therefore, this preliminary work is expected to be a pioneer study in developing more effective topical medication. A patent application related to this study has been filled.

Owing to its favourable penetration and stability characteristics of the improved gel formulation (G2) in this study, the phytochemical product containing escin is an alternative to treatment of chronic venous insufficiency and inflammatory soft tissue conditions.

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