

RESEARCH ARTICLE

Extraction, Isolation, Characterisation and Pharmacological Investigations of Beta Escin from Horse Chest Nut Seed Extract

Poonam Chougule^{1,2*}, Kailasam Koumaravelou¹, Nilesh Chougule³

^{1*}Department of Centre for Research and Development, PRIST University, Vallam, Thanjavur, Tamil Nadu, India.

²Ashokrao Mane College of Pharmacy, Peth-Vadgaon, Kolhapur, Maharashtra, India.

³Ashokrao Mane Institute of Pharmacy, Ambap, Kolhapur, Maharashtra, India.

Received: 22th August, 2023; Revised: 27th September, 2023; Accepted: 05th October, 2023; Available Online: 25th December, 2023

ABSTRACT

Horse chestnut seeds (*Aesculus hippocastanum*) have long been recognized for their potential therapeutic properties, particularly due to the presence of bioactive compounds like beta-escin. This study aimed to comprehensively explore the extraction, isolation, characterization, and pharmacological investigations of beta escin from horse chestnut seed extract. Beta escin was extracted from horse chestnut seeds through a series of solvent extraction and purification steps. The isolated compound was then characterized using various analytical techniques, including UV, IR, DSC, X-RPD and GC/MS. Pharmacological studies were carried out to evaluate beta-escin's possible medicinal applications. In this anti-inflammatory investigation, it was found that the carrageenan-induced reduction in swelling in diclofenac gel begins after 30 minutes, with a value of 11.71%, whereas the effect of escin gel takes 60 minutes to manifest, with a value that is slightly lower at 4.55%. Pharmacological research demonstrated a variety of positive benefits, such as anti-inflammatory, antioxidant, and vascular protecting qualities, which may have prospective uses in the treatment of edema and chronic venous insufficiency.

Keywords: Horse chestnut seeds, Isolation, Beta Escin, Seed extract, Anti-inflammatory

International Journal of Pharmaceutical Quality Assurance (2023); DOI: 10.25258/ijpqa.14.4.25

How to cite this article: Chougule P, Koumaravelou K, Chougule N. Extraction, Isolation, Characterisation and Pharmacological Investigations of Beta Escin from Horse Chest Nut Seed Extract. International Journal of Pharmaceutical Quality Assurance. 2023;14(4):977-986.

Source of support: Nil.

Conflict of interest: None

INTRODUCTION

The resurgence of interest in herbal remedies can be attributed to a growing awareness of their potential benefits, coupled with a desire for more natural and holistic approaches to healthcare. In addition to their historical significance, many herbs and plant-based compounds are now being studied for their medicinal properties, opening up new avenues for scientific research and innovation in herbal medicine. This renewed focus on herbal remedies has led to the development of standardized herbal products that meet quality and safety standards, making them more accessible and reliable for modern consumers seeking alternative healthcare options.¹ Some of these involve preparing herbal substances by heating or steeping them in alcoholic beverages or honey. Finished herbal products refer to herbal mixtures crafted from one or multiple herbs. When multiple plants are combined, they are termed "mixture herbal products." These herbal remedies play a vital role in various traditional medical practices and therapies, including Chinese medicine, *Ayurveda*, Unani, Naturopathy, Osteopathy, and Homeopathy. India boasts a rich heritage in the field of herbal medicine, where these remedies were once

employed much like modern pharmaceuticals. Ayurveda, a traditional Indian healthcare system, places a strong emphasis on disease prevention and holistic treatment approaches.²

A. hippocastanum L., A plant belonging to the Hippocastanaceae family, native to the southern Balkan Peninsula in Southeast Europe, is known for its small-scale growth. The plant material from this species is primarily used in topical applications for addressing conditions such as edemas, minor bruises, localized skin and subcutaneous tissue inflammations, hemorrhoids, fragility of capillaries in the skin, and venous insufficiency. Escin, found in this plant, can be classified into two distinct categories, escin and escin, based on factors like their melting points, hemolytic properties, solubility in water, and specific rotations. It has been observed that Japanese horse chestnut seeds, partly owing to cyclooxygenase (COX) inhibition, possess certain anti-inflammatory properties. Apart from coumarins and saponins, there may be other chemical compounds within these seeds that have a COX-inhibiting effect. Furthermore, aside from its anti-inflammatory action, the escin component exhibits significant antioxidant and anti-edematous (anti-swelling) properties.^{3,4}

*Author for Correspondence: pnchougule1008@gmail.com

Recently, natural products are widely explored for their effectiveness in preventing circular diseases, more specifically varicose vein/chronic venous insufficiency. The natural phytochemicals obtained from plants and traditionally used as medicines can serve as substitutes to synthetic chemicals and antibiotics to treat varicose vein problems. Limited synthetic agents are available, for treating varicose veins they produce unwanted side effects.

'Escin' (hemolytic) and 'prosapogenin' (non-hemolytic) are two crystalline compounds that may be isolated from the saponin mixture found in *A. hippocastanum* seeds. Chestnut seeds have been utilized to extract bioflavonoids like quercetin, kaempferol, and their glycosylated derivatives, as well as antioxidants such as proanthocyanidin A2, and coumarins like esculin and fraxin. Extensive scientific research has confirmed that the anti-swelling, anti-exudative, and blood vessel-protective properties of extracts from the HCE are primarily attributed to aescin. Aescin is a combination of naturally occurring triterpene saponins and is known for its topical activity, whereas aescin is recognized for its oral effectiveness. Numerous herbal oral formulations containing a blend of aescin and other plant constituents are commercially available, renowned for their ability to alleviate swelling, reduce inflammation, and enhance venous tone.⁵ The reported safety of this extract intends to do further research for the separation of these two polymorphic forms. Out of which β aescin has topical action for treatment management for chronic venous Insufficiency and other circulatory disorders.

Therefore, looking out the importance of escin as an important phytoconstituent to be incorporated into formulations for the management of varicose vein, the present study attempts to extract, isolate, characterize and screen of pharmacological actions of beta escin, the principal active constituent of horse chestnut seed.⁶

MATERIALS AND METHODS

Horse chestnut seed procured from local market of Shimla. Escin (Pure form Isolated), egg albumin (95% Pure). Every other chemical used were of analytical grade.

Methodology

Sourcing and authentication of horse chest nut seeds

Fully matured seed of the Indian Horse Chestnut were procured from local market of Shimla. Seeds are authenticated from dept. of botany Padmabhushan Vasantdada Patil Science College Tasgaon, Dist- Sangli Maharashtra. The seed sample was meticulously cleaned to remove any dirt, dust, or other debris. To remove any moisture content, shade dried for two to three days. With the use of a grinder, the seeds were ground and stored in an airtight plastic container. Further analysis is done out by using this powder.

Extraction and Purification

The Horse Chest Nut seed sample dried at 40°C was pulverized into powder, and a 100 g sample was weighed and ground. This powder from horse chestnut seeds was soaked in methanol for

4 days, resulting in a methanol extract. After filtration and evaporation, 97 g of dried material was obtained. The crude methanol extract was subjected to hexane and methanol (3:1) treatment, separating the hexane layer for oil extraction. The aqueous extract was mixed with ethyl acetate and methanol (3:1), yielding fractionated layers. The aqueous layer was combined with n-butanol and methanol extract (3:1). The fractionated n-butanol layer was evaporated under pressure at 60 to 70°C, concentrated, and lyophilized, resulting in a rich source of escin.⁷

Isolation

Here the purification of specific saponin components found in natural and edible seeds using a Shimadzu LC-2010A system with a preparative HPLC column (YMC-Pack ODS, 150 mm length, 10 mm inner diameter). To achieve this, here employed a mobile phase consisting of a mixture of methanol and 10 mM sodium phosphate buffer (pH 7) at a flow rate of 3 mL/min, with a ratio of 62:38 (v/v). We monitored the elution of saponins by measuring the absorbance at 230 nm.

For analytical purposes, we also utilized YMC-Pack ODS AM (150 mm length, 6 mm inner diameter) with the same mobile phase as previously mentioned, flowing at a rate of 0.8 mL/min. To convert escins into deacetylescins, for that subjected them to a 48 hour treatment at room temperature with a 5% potassium carbonate solution (pH 11.7). Afterward, we carried out chemical elution twice using 5 mL of 70% methanol through a C18 Maxiclean cartridge column. also adjusted the pH of the solutions to approximately 7 using 0.1 N HCl before this elution step and also performed two washes with 5 mL of 40% methanol. Then confirmed the success of this conversion by conducting HPLC analysis using the same YMC-Pack ODS AM analytical column (150 mm length, 6 mm inner diameter) as previously mentioned.⁸

Characterization Studies

Confirmatory tests for presence of flavonoids within extract

Shinoda Test: Mix 0.5 g of magnesium turnings with 5 mL of 95% ethanol, a few drops of strong hydrochloric acid, and a dry powder or extract. The colors orange, pink, red, and purple are seen.

Test with sulphuric acid: When sulphuric acid is added, flavones and flavanols dissolve and produce a dark yellow solution. Chalcones and aurones produce crimson or reddish-blue solutions. Orange and red colors come from flavones.

Physicochemical Characterisation Studies

Few physicochemical properties such as description, colour, odor and photo-microscopy were evaluated.

Melting Point

The melting point of beta escin was determined by using capillary method. Sample taken into the capillary tube which is enclosed by one side after that capillary was pass from flame and capillary was allowed to heat and the specific temperature was noted at which the sample was completely melted.

Solubility Studies

Solubility pattern of escin in different solvents such as water, Water, methanol, benzene, chloroform, acetone and carbon tetrachloride was determined by dispersing small amount of sample in glass test and vortexing for 5 minutes.

Measurement of UV Spectrum for Escin

Sample solution at a concentration 1mg/ml was prepared using purified water in 100 mL volumetric flask using isolated escin. From this stock, made a series of dilutions. Working standards were prepared of concentration 30, 40, 50, 60, 70, 80, 90, 100 µg/mL with addition of water in 10 mL volumetric flask. All prepared sample solutions were scanned between 200 to 400 nm. The absorption maxima from UV spectrum graph was observed and confirmed with literature data.⁹

FTIR Spectroscopy Analysis

Here IR spectroscopy to capture the spectrum of the isolated escin and subsequently compare it to reference spectra for the drug. The escin sample was positioned on the sample platform and compressed between the knob and the platform. Fourier-transform infrared (FTR) spectra of the escin samples were then obtained using a Perkin and Elmer FTI-Frontier Spectrometer.¹⁰

The measurements were carried out under normal room temperature conditions, spanning a spectrum ranging from 4000.00 to 400.00 cm⁻¹, and with a spectral resolution of 5 cm⁻¹.

Differential Scanning Calorimetry Analysis

Differential Scanning Calorimetry (DSC) thermograms were produced using the Shimadzu DSC instrument, known as the DSC-60. The analysis was carried out over a temperature range of 30.00 to 300°C, using approximately 3.00 grams of the sample enclosed in sealed aluminum pans. During the initial run, the scan was conducted at a heating rate of 10°C per minute, with a nitrogen gas flow rate of 100 mL per minute. Prior to the analysis, the instrument was calibrated using reference materials. The data acquired was then processed using the available software.¹¹

X-Ray Powder Diffraction Studies (X-RPD)

XRPD analysis was conducted using a D8 Focus X-ray Diffractometer manufactured by Bruker Scientific Germany. The instrument was configured with K α copper radiation (wavelength $\lambda = 1.5418$), employing a current of 40 mA and a voltage of 45 kV. A sample spinner equipped with an X Celerator detector at 2.122° was used. The analysis was carried out at room temperature, with a scanning range of 2 θ from 4° to 50°, utilizing a step size of 0.033° and a duration of 19.68 seconds per step. For stability studies, diffractograms were acquired using a different X-ray diffractometer, the D8 Phaser from Bruker, which utilized K α copper radiation. This instrument operated with a current of 10 mA and a voltage of 30 kV, and detection was performed using a one-dimensional scintillation counter detector.

Gas Chromatography

The analysis was conducted using a Waters Instrument at the Quadrant Laboratory in Sangli. The sample was dissolved in

DMSO, and a blank run was performed to confirm the DMSO peak. The study aimed to ascertain the quantity of residual sample within the extract, with particular attention to the solvent of major concern, methanol.

Pharmacological Investigations of Beta Escin

In-vitro anti-inflammatory study by protein denaturation method

• Sample preparation

A pure extract sample of escin was meticulously prepared using purified water as the solvent. To assess its anti-inflammatory and antioxidant properties, a 10% w/v concentration was meticulously formulated. This prepared sample solution underwent filtration through muslin cloth to eliminate any undesirable particles, ensuring a pristine sample. Subsequently, various strengths of the filtered escin solution, including 2, 4, and 10 mg/mL, were meticulously prepared to investigate its diverse biological activities.¹²

Procedure

The reaction mixture, comprising fresh hen's egg albumin, phosphate buffered saline (PBS) with a pH of 6.4, different concentrations of escin (Sample Code-VL04), and double-distilled water, had a total volume of 1-mL. After incubating the mixtures in an incubator at 37°C for 15 minutes, they were subsequently heated at 70°C for 5 minutes. Following cooling, their absorbance at 660 nm was measured, with a reference provided by a control solution. Ibuprofen was utilized as a reference medication and handled similarly at concentrations of 2 and 4 mg/mL to determine absorbance¹³ with the use of the following formula, the %inhibition of protein denaturation was determined.

$$\% \text{ inhibition} = \frac{\text{Abs of Control} - \text{Abs of Test}}{\text{Abs of test}} \times 100$$

Anti-Oxidant Study: DPPH Free Radical Scavenging Assay

We conducted an antioxidant study using the DPPH free radical scavenging assay, following the approach outlined by Olszewska *et al.* In this assay, water solutions of Escin Pure extracts were tested.

To prepare the DPPH radical solution, we dissolved 36 g of DPPH in methanol to create a concentration of 91 M, and this solution was stored at 4°C in the dark. The initial absorbance of DPPH in methanol (at a ratio of 2:1, v/v) was measured at 517 nm, resulting in a value of 0.700 ± 0.030.

For the DPPH measurements, analytical samples were prepared by mixing 2 mL of the equilibrated DPPH solution with 1-mL of escin methanol solution at three different concentrations (166.67–500.20 g/mL). After incubating for 60 minutes in the dark at room temperature, the absorbance at 517 nm was recorded to determine the reduction in the DPPH radical concentration.

We followed the same procedure to prepare the DPPH control, which did not contain any escin sample. To determine the concentration (EC50) required reducing the initial DPPH radical concentration by 50%, we plotted the three different

escin concentrations against the percentage of remaining DPPH radical. As a reference compound, vitamin E 1% solution in methanol was used.¹⁴ The escin pure extract's activity was then translated into VEAA, or vitamin E equivalent antioxidant activity.

In-vivo studies of Escin Extract

The *in-vivo* studies of escin extract were conducted at the Animal House within the Biocyte Institute of Research and Development, located at Plot No. 20, Shriram Residency, Kalanagar, Sangli, Maharashtra, India, with a postal code of 416416. The study was conducted in compliance with regulations, as evidenced by registration number 2114/PO/RC/S/20/CPCSEA and the date of registration, which was November 26, 2020.

Anti-Inflammatory Activity by using the Carrageenan Paw Induced Inflammation Model

To assess the anti-inflammatory effects of carrageenan, we conducted an experiment involving wistar rats, divided into three groups of three rats each, to establish an inflammation model.

In the first group, inflammation was induced by injecting carrageenan, and no treatment was administered to this group.

The second group, serving as the reference, and the third group, which received the test substance, were both treated before carrageenan injection. The treatment involved the topical application of Diclofam gel (2 mg/paw) to the second group and the test substance (2 mg/paw) to the third group. These treatments were administered at doses appropriate for the level of edema, effectively addressing the swelling.

To apply the test substance and diclofam, the plantar surface of the hind paw was gently massaged with the index finger, performing 50 strokes.

We used a digital calliper to measure the extent of edema in each treated group before and after the carrageenan injection. Edema was quantified as the proportional increase in paw volume resulting from the inflammatory injection. Consequently, the degree of edema was determined by calculating the volume difference between the initial measurement (0 hours) and subsequent time points, including 30 minutes, 60, 90, 120 and 150 hours following the carrageenan injection.

Percentile edema inhibition was calculated according to the following formula:

$$\% \text{ Inhibition} = [1 - (Vt - V0)] \times 100$$

Where,

VT = represents the edema volume in the drug treated group.

V0 represents the edema volume in the Carr group.

Anti-oedematous Potential by Mouse Writhing Test

To induce wheezing in rats, they will be administered 0.2 mL of intraperitoneal 0.6% acetic acid (volume/volume). For the mice, 30 minutes before the test, they will receive intraperitoneal injections of one of the following substances: water, Tris, L. beta escin (at doses of 8, 40, or 200 mg per kilogram of body weight) suspended in water, Dometacin

(at a dose of 10 mg per kilogram of body weight) diluted in Tris buffer (pH 8.2), or water. The observation of writhing behavior in the mice will commence 15 minutes after the intraperitoneal injections. Throughout the rat edema and writhing tests, standard laboratory conditions will be maintained, including a temperature range of 21 to 24°C, relative humidity between 40 to 60%, and a 12-hourlight–dark cycle.¹⁵

RESULTS AND DISCUSSION

The compound is soluble in water and methanol but insoluble in benzene, chloroform, acetone, and carbon tetrachloride. The melting point was found to be within the range of 224 to 228° by using the capillary method.

UV Spectrum of Escin

This analytical method was carried out by using UV spectrophotometer. For this current study, the absorbance maxima wavelength for escin was selected at 346 nm (Figure 1). A Calibration curve study was employed for escin (Figure 2)

FTIR Spectra

In Figure 3, we observe the FTIR spectrum of an extract derived from native horse chestnut. The presence of O-H vibrations is evidenced by the fact that the dominant region of escin aligns with the broad stretching vibration at 3400 cm^{-1} . The sharp band at 2929 cm^{-1} is a consequence of the -CH₂ stretching vibration within the saponin unit. At 2368 cm^{-1} , we detect the absorption peak linked to C-H stretching brought about by hydrogen atoms within methane rings. The peak at 1647 cm^{-1} corresponds to the bending vibration of

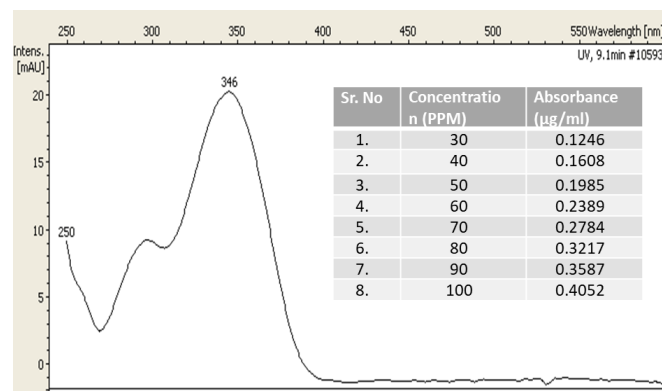


Figure 1: Absorption maxima of escin

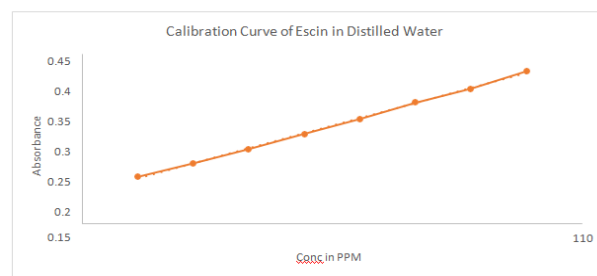


Fig. Calibration Curve of Escin

Figure 2: Calibration curve of escin

H-O-H, which is a characteristic feature commonly found in the spectra of escin Ia and escin IIb. Saponin molecules possess hydroxyl groups, leading to water absorption. The bands at 1081 and 1156 cm^{-1} are attributed to the stretching, bending, and asymmetric stretching of the C-O-C glycosidic bond, respectively. Meanwhile, the 1365 cm^{-1} band can be traced back to the bending modes of O-C-H, C-C-H, and CO-H. In the range between 1365 and 1413 cm^{-1} , we observe absorption bands resulting from C-H bending vibrations. The absorption peak at 1160 cm^{-1} is likely due to C-O bending. Within the 800 to 1300 cm^{-1} range of the infrared (IR) spectrum, we can use this information to analyze the saponin's short-range order, as the spectra of isoescin I and IIb are particularly sensitive to structural changes at the molecular level, particularly with respect to short-range order.

DSC Analysis

In order to determine the purity and melting point of the crude horse chest nut extract and escin, DSC measurements were made. Using a Perkin Elmer model and Pyris 6 software, differential scanning calorimetry was used to evaluate the thermal behavior of isolated escin. A sample weighing around 5.0 mg was put into an aluminum pan, hermetically sealed under nitrogen, and run over a temperature range of 30 to 300°C at a scanning rate of 10°C per minute in a dynamic nitrogen atmosphere. As a guide, a sealed aluminum pan that was empty was employed. Similar thermal behavior was seen with crude Horse chestnut extract. The observed results are shown in Table 1 and Figure 4.

XRD Analysis

Figure depicts the native horse breast nut extract's X-ray diffraction pattern. According to the XRD pattern, isoesin and escin are the two main active ingredients in horse chestnut extract. distinct polymeric versions of escin, escin and isoesin, with distinct arrangements of hydroxyl double helices. The escin type is a saponin-specific diffraction peak with prominent peaks at around 15° and 23° and an unresolved doublet at approximately 17° and 18°. Beta saponin, which demonstrates isoescin type, exhibits the greatest diffraction peak at around 17° 2, a few minor peaks at approximately 15, 20, 22, and 24°, and a distinctive peak at approximately 5.6°. Escin polymorph primarily constitutes the crystalline phase. The similar escin-type pattern in smooth European horse chestnut with B-type allomorph at the centre and escin type

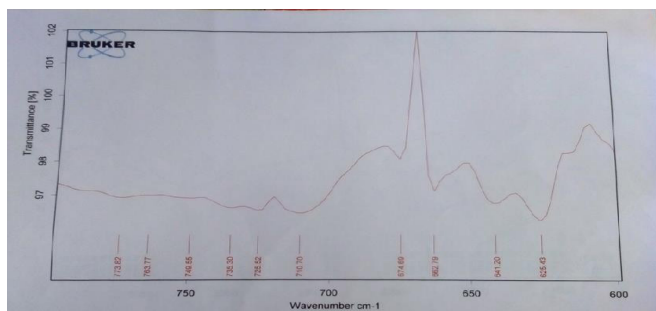


Figure 3: FTIR spectra of Horse Chest Nut Extract

Table 1: DSC analysis

Sample	Peak maxima(°C)
Escin Isolated	221
Crude Horse Chest Nut Extract	105

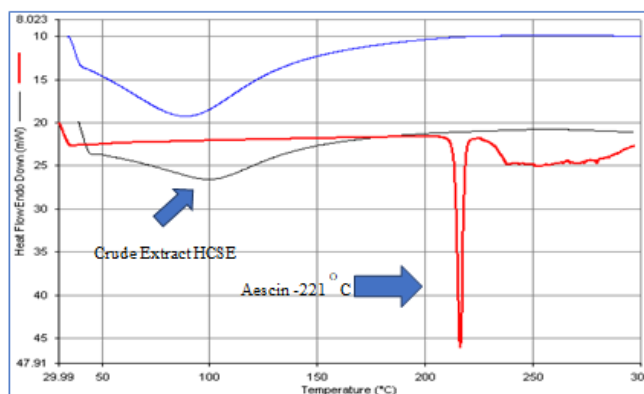


Figure 4: DSC Thermogram of Escin samples

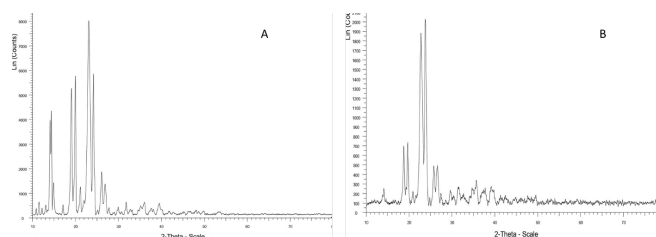


Figure 5: X-ray Diffraction of Escin sample A (A) Escin sample B (B)

located towards the outside of the granule.¹⁶ The XRD results revealed that Horse chestnut extract showed typical saponin-type pattern with characteristic peaks at 5.70, 15.00, 17.30, 18.87, 23.30 and 26.39°. The escin was determined to have a crystallinity percentage of 32.78% (as shown in Figures 5A and 5B). This crystallinity in escin arises from the organized arrangement of double helices formed by clusters of short-DP chains containing CH-OH3 groups.

GC Analysis

By the GC Chromatogram, the absence of solvent –methanol confirms the solvent that we have used for isolation is within the limit (Figure 6).

In-vitro Studies

Venotonic Properties of Escin

Escin is the main bioactive compound found in horse chestnut seed extract. Its ability to inhibit hyaluronidase activity has led to the proposal that assessing the inhibition of other enzymes like collagenase, elastase, and glucuronidase could help us understand the maintenance of the integrity of the matrix outside of blood vessels. It has been shown that hyaluronidase inhibition is insufficient to explain the potent venotonic effects of horse chestnut seed extracts on the microvasculature. Horse chestnut seed extract's primary active ingredient demonstrates anti-inflammatory, anti-edematous, and

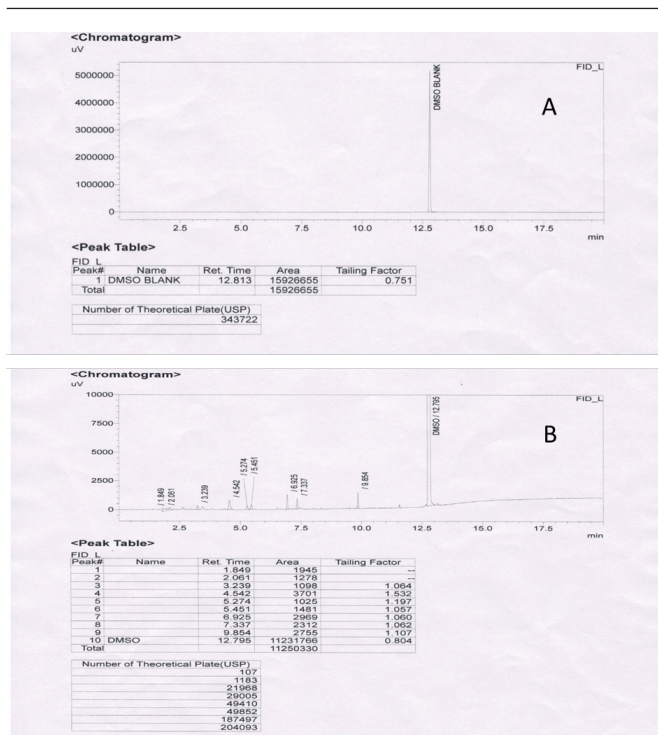


Figure 6: GC chromatogram of blank- DMSO (A) Aescin isolated sample (B)

venotonic characteristics that may be due to decreased vascular permeability. Escin’s anti-inflammatory characteristics have been demonstrated in animal models, and they suggest that lowering leukocyte activation and adhesiveness, it can affect the problem of inflammatory intermediates. In a study published in 2016 by one of the scientists, it was found that escin had anti-inflammatory effects in a rat model of pleurisy.^{17,18} Where the application of escin was found to reduce the leukocyte relocation into plural activity and prevented the proclamation of inflammatory intermediaries. In the human study, the patients who was suffering from chronic venous insufficiency exhibited that the 5mg escin given intravenously daily two times for a week which resulted in 33% decrease of leukocyte density, 50% decrease in macrophage numbers.

The venotonic property of escin is determined by the use of animal investigations and in vitro experiments. The ability to increase prostaglandin F2 production was the venotonic trait. This prostaglandin increases venous contractility and prevents the breakdown of mucopolysaccharides in venous tissue. When dogs were given 25 to 50 mg of escin, the venous pressure increased by about 21%, and the ultimate maximum pressure increased by 30% relative to baseline. The pure escin increased venous tone in the *in-vitro* research by 10 to 20% at low dosages.

Anti-inflammatory Potential of Escin

Here UV spectroscopy as an analytical technique to investigate the therapeutic effects of pure escin extract in water on protein denaturation, specifically using egg albumin derived from fresh hen’s eggs. In this study, ibuprofen served as the standard against which we measured the percentage inhibition of protein

denaturation. The reaction mixture, with a total volume of 2 mL, consisted of 0.1 mL of 5% egg albumin (from fresh hen’s eggs), 1.8 mL of phosphate-buffered saline (PBS) at pH 6.4, and 0.1 mL of the escin sample (at concentrations of 2, 4, and 10 mg/mL). Double-distilled water was used as a control in the same volume. After incubating the mixtures at 37°C in an incubator for 15 minutes, they were further heated to 57°C for a duration of 30 minutes. This allowed us to assess the impact of escin on protein denaturation in a dose-dependent manner,^{19, 20} their absorbance at 660 nm was measured after cooling, using a vehicle as a reference.

From below Table 2, it is inferred that the lower concentration of water extract of escin has very little anti-inflammatory effect. As the concentration is increased around 10 mg/mL the mean %inhibition is 88.52% (Table 2) which is quite promising. Escin, a plant flavonoid molecule, is a powerful antioxidant and free radical scavenger. Consequently, there is a strong link between the total phenolic content of plant/herb extracts and their antioxidant properties. Similar relationships were seen in the current investigation as well. Water-based extracts of escin show moderate anti-inflammatory effects when compared to Standard ibuprofen solution at the highest dose (10 mg/mL).^{21, 22}

Anti-Oxidant Potential of Escin

Aescin has demonstrated its effectiveness in preventing the onset of edema in various inflammation models, including paw edema induced by irritants such as ovalbumin, dextran, cotton pellet, carrageenin, and bradykinin. Additionally, it

Table 2: *In-vitro* anti-inflammatory activity of VL-O4 by protein denaturation method

Sample Code	Concentration in mg/mL	Anti-Inflammatory activity	
		Absorbance at 660 nm	%inhibition
Blank	-	1.45	
Ibuprofen	2	0.78	46.20
	4	0.13	91.03
	10	0.18	96.15
VL-04/01	2	1.04	28.27
VL-04/02	4	0.62	57.24
VL-04/03	10	0.11	88.52

Table 3: *In-vitro* anti-oxidant activity of VL-O4 by DPPH assay method

Sample Code	Concentration in mg/mL	DPPH	
		EC 50 mcg/mL	VEAA (per gram fraction)
Blank	-	0.00	0.00
Vitamin E	2	23.8 ± 0.5	96.99
Vitamin E	4	40.3 ± 1.2	125.11
Vitamin E	10	90.4 ± 0.9	725.21
VL-04/01	2	71.3 ± 2.5	243.44
VL-04/02	4	121.7 ± 4.5	540.52
VL-04/03	10	171.4 ± 5.6	650.60

has been effective in inhibiting serous peritonitis induced by formalin injection in rats and carrageenin-induced peritonitis in mice.²¹⁻²³ On the flip side, aescin does not exhibit the ability to alleviate edema in inflammation models that simulate the later proliferative phase, including pocket-granuloma and formalin-induced paw edema in rats. Furthermore, it proves ineffective in experimental models of polyarthritis. Two plant-derived flavonoid compounds, proanthocyanidin and escin, possess remarkable antioxidant capabilities and are highly efficient at scavenging free radicals. Consequently, there exists a significant correlation between the overall phenolic content found in plant and herb extracts and their antioxidant properties^{24,25} (Table 3 and Figure 7).

Up till now, a number of publications have claimed that Escin extract has antioxidant qualities. Pure extract Escin obtained from *Aesculus hippocastanum*, whose phytochemical makeup is well known, demonstrated either greater radical-scavenging capacities in the DPPH test when compared with standard Vitamin E samples ($EC_{50} = 90.4$ g/mL for 10 mg dose), while 10 mg/mL escin dose ($EC_{50} = 174.4$ g/mL), lower concentrations such as 2 mg/mL showed $EC_{50} = 71.3$ g/mL and 4 mg/mL showed $EC_{50} = 121.7$ g/mL. Additionally, the antioxidant activity of vitamin E, which was used as a benchmark, was maximally inhibited by the DPPH radical scavenging experiment in a dose-dependent manner.²⁶⁻²⁸

All samples of vitamin E and escin water-based extract showed an antioxidant potential in the DPPH free radical scavenging assay with 10 mg/mL having the highest Vit E equivalent antioxidant activity (VEAA = 650.60/g dw), followed by 4 and 2 mg/mL strengths (VEAA = 540.52 and 243.44/g, respectively). Similar variations in reducing capability, as determined by the Vitamin E strengths (2–10 mg/mL), were found between the fractions, as indicated in Table 6. In comparison to escin, which has a similar strength of 650.60 mg, the VEAA score for the 10 mg strength was much higher at 725.21. Lower strengths nevertheless have dose-dependent VEAA values that are straightforwardly associated with escin levels. These findings show that the antioxidant activity of escin water-based extract at all strengths is substantially dosage dependent and rises with increasing phenol concentration.^{29,30} There are strong positive correlations ($r = 0.989$, $p < 0.0001$) between the EC_{50} values and the estimates of antioxidant activity obtained in the DPPH. This

linear relationship between the two variables is statistically significant, which shows that phenolic chemicals have a substantial role in the antioxidant activity of the investigated escin pure product. The results show that the flavonoids such as escin and other major phenolic components of horse chestnut extract have good polarity. As a result, they are mostly soluble and easily extractable with water, a universal solvent. Here, we can draw the conclusion that additional research is being done to determine whether escin is the only active component of horse chestnut seeds, which may be principally responsible for their antioxidant activity.³¹

In-vivo Studies of Escin

Escin demonstrates a minimum of three distinct pharmacological effects:

- Affects swell and inflammation;
- Has an impact on venous tone; and
- Protection against hypoxia-induced endothelial damage.³²⁻³⁴

In conditions like chronic venous insufficiency (CVI), such as in cases of blood stasis, we can envision the inflammatory process as a sequential series of events. It starts with a reduction in ATP levels within endothelial cells. This decrease triggers the release of inflammatory agents like prostaglandins and platelet-activating factors, accompanied by a rise in cellular calcium concentrations. Consequently, polymorph nuclear neutrophils are attracted to the site, becoming activated and adhering to the area. Additionally, during an inflammatory response, histamine and serotonin are released, both of which contribute to an increase in capillary permeability.³⁵ The migration of leukocytes from blood vessels to surrounding tissues has significant consequences, including the worsening of inflammation, the development of edema, and pathological alterations in veins.

Numerous preclinical studies conducted over many years have consistently supported the beneficial effects of escin on inflammation and edema (as shown in Figure 8 and detailed in Table 4). Escin's anti-inflammatory properties are notably observed and can be reliably evaluated using a rat paw model. It has been found that escin possesses specific anti-inflammatory properties, reducing vascular permeability in inflamed tissues and thereby preventing edema formation. Additionally, scientific literature suggests potential antioxidative effects associated with escin. When applied in the form of a topical gel, escin has the potential to reduce both inflammation and edema in rat paw edema models by suppressing the release of inflammatory agents.³⁶

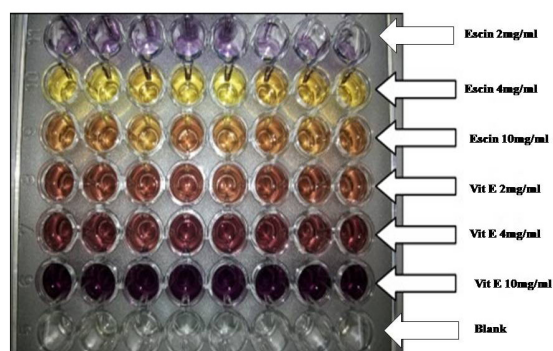


Figure 7: DPPH Assay of Escin Pure Extract

Table 4: In-vivo anti-inflammatory activity

Time Interval (minutes)	Standard (%)	Test Preparation (Escin Gel) (%)
30	11.71	-8.64
60	10.20	4.55
90	21.27	12.24
120	33.40	21.55
150	41.92	32.00

Researchers have explored the hypothesis that escin can mitigate the harmful cellular responses triggered by hypoxia and have examined the underlying mechanisms using various *in-vitro* and *ex-vivo* models. One noteworthy finding is related to PECAM-1, a molecule that appears to translate mechanical stresses, such as shear stress, into biochemical signals. PECAM-1 plays a vital role in maintaining the integrity of adherent junctions at points where endothelial cells are

Table 5: Details of the treatment group

Control group	NAD NAD
Disease group	Diffuse parakeratosis (+++) with hyperkeratosis (++) and focal area of cellular debris Diffuse parakeratosis (+++) with hyperkeratosis(++) Diffuse parakeratosis (+++) with hyperkeratosis(++) Diffuse parakeratosis (+++) with hyperkeratosis(++)
Escin formulation (Sample-1)	Focal parakeratosis (++) with hyperkeratosis (+) Focal parakeratosis (++) with hyperkeratosis (+) Multifocal parakeratosis (+++) with hyperkeratosis (++) with focal epidermoid cyst (+)
Escin formulation (Sample-2)	NAD focal parakeratosis (+++) with hyperkeratosis (++) with focal epidermoid cyst (+) NAD
Diclofenac ointment Market sample	NAD Multifocal parakeratosis (+) with hyperkeratosis (+)

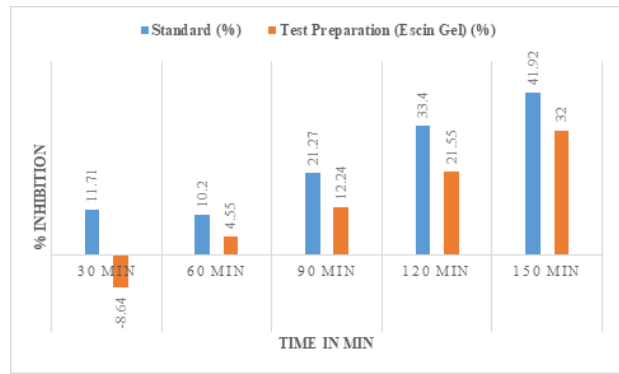


Figure 8: %inhibition of the anti-inflammatory activity

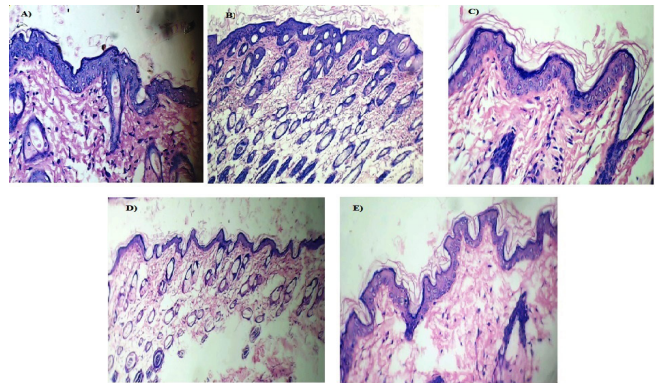


Figure 9: Histopathological study of the anti-inflammatory activity

Table 6: Histopathological details of the *in-vivo* study

Lesions/group	Control	Disease	Escin formulation (Sample-1)	Escin formulation (Sample-2)	Diclofenac ointment Market sample
No. of animals	02	04	03	03	02
Parakeratosis focal (+)	00	00	00	00	00
(++)	00	00	00	00	00
(+++)	00	00	01	01	00
Multifocal (+)	00	00	00	00	01
(++)	00	00	00	00	00
(+++)	00	00	01	00	00
Diffuse(+)	00	00	00	00	00
(++)	00	00	00	00	00
(+++)	00	04	00	00	00
Hyperkeratosis Focal (+)	00	00	01	00	00
(++)	00	00	00	00	00
(+++)	00	00	00	01	00
Multifocal (+)	00	00	00	00	01
(++)	00	00	01	00	00
(+++)	00	00	00	00	00
Diffuse(+)	00	00	00	00	00
(++)	00	04	00	00	00
(+++)	00	00	00	00	00
Other Lesions Cellular debris	00	01	00	00	00
Epidermoid cyst	00	00	01	01	00

connected. Additionally, PECAM-1 serves as a crucial regulator in scenarios involving the migration of neutrophils through the basement membrane during conditions like inflammation, ischemia-reperfusion injury, and oxidative stress.³⁷

According to the findings on escin gel's anti-inflammatory activity, the activity was carried out by paw edema caused by carrageenan. Rubbing the paw allowed for the evaluation of the inflammation's decrease. The example provided displayed good activity.

Histopathological Changes

Figure 9 Photomicrographs Histopathological changes after treatment. Normal skin (A); Inflamed skin (B); skin treated with Escin gel Sample-1 (C), skin treated with Escin gel Sample-2, (D) and skin treated with reference Diclofenac Ointment (D). The detailed treatment group and their observation are depicted in Tables 5 and 6.

CONCLUSION

In summary, this study on horse chestnut seed extract, following USP 29 Monograph standards, has provided valuable insights and promising results. The isolation method efficiently separated beta-aescin, validated through multiple structural identification techniques. *In-vivo* studies demonstrated escin's anti-inflammatory potential, with results comparable to diclofenac gel. The acetic acid-induced writhing test confirmed escin's anti-odematous potential akin to Indomethacin. Histopathological findings supported skin compatibility with escin gel, suggesting its therapeutic promise. Enhancing oral solubility and topical permeability could further optimize its anti-inflammatory and anti-odematous effectiveness.

REFERENCES

1. El-Dakhly SM, Salama AA, Hassanin SO, Yassen NN, Hamza AA, Amin A. Aescin and diosmin each alone or in low dose-combination ameliorate liver damage induced by carbon tetrachloride in rats. *BMC Research Notes*. 2020 Dec;13(1):1-7.
2. Gallelli L. Escin: a review of its anti-edematous, anti-inflammatory, and venotonic properties. *Drug design, development and therapy*. 2019 Sep 27;3425-37.
3. Patlolla JM, Rao CV. Anti-inflammatory and Anti-cancer Properties of β -Escin, a Triterpene Saponin. *Current Pharmacology Reports*. 2015 Jun;1:170-8.
4. Pittler MH, Ernst E. Horse-chestnut seed extract for chronic venous insufficiency: a criteria-based systematic review. *Archives of Dermatology*. 1998 Nov 1;134(11):1356-60.
5. Hippocastanum HH. *Aesculus Hippocastanum (Aescin, Horse Chestnut) in the Management of Hemorrhoidal Disease*. *Turk J Colorectal Dis*. 2018;28:54-7.
6. Gandhi SV, Nilgar NM, Bhalekar MR. Formulation and evaluation of phytoconstituents emulgel for the treatment of varicose veins. *European Journal of Pharmaceutical and Medical Research*. 2018;5(7):432-9.
7. Mor D, Dande P. Varicose veins: an overview of current and herbal treatments. *International Journal of Pharmaceutical Sciences and Research*. 2017 May 1;8(5):1959.
8. Singh H, Sidhu S, Chopra K, Khan MU. The novel role of β -aescin in attenuating CC14-induced hepatotoxicity in rats. *Pharmaceutical biology*. 2017 Jan 1;55(1):749-57.
9. Jacobs BN, Andraska EA, Obi AT, Wakefield TW. Pathophysiology of varicose veins. *Journal of Vascular Surgery: Venous and Lymphatic Disorders*. 2017 May 1;5(3):460-7.
10. Nyavanandi D, Mandati P, Narala S, Alzahrani A, Kolimi P, Pradhan A, Bandari S, Repka MA. Feasibility of high melting point hydrochlorothiazide processing via cocrystal formation by hot melt extrusion paired fused filament fabrication as a 3D-printed cocrystal tablet. *International Journal of Pharmaceutics*. 2022 Nov 25;628:122283.
11. Butreddy A, Sarabu S, Almutairi M, Ajjarapu S, Kolimi P, Bandari S, Repka MA. Hot-melt extruded hydroxypropyl methylcellulose acetate succinate based amorphous solid dispersions: Impact of polymeric combinations on supersaturation kinetics and dissolution performance. *International Journal of Pharmaceutics*. 2022 Mar 5;615:121471.
12. Özkan EE, Mesut B, Polat A, Nur TA. In vitro skin permeation of escin in the new gel formulation of *Aesculus hippocastanum* (Horse Chestnut). *Journal of Faculty of Pharmacy of Istanbul University*. 2016 Dec 1;46(2):79-88.
13. Shareef NM, Abdul-Jalil TZ. Iraqi *Hyacinthus orientalis* L. Flowers as the Source of Bioactive Compounds Especially Stigmasterol: Identification, Isolation and Characterization. *International Journal of Drug Delivery Technology*. 2023;13(3):792-796.
14. Domanski D, Zegrocka-Stendel O, Perzanowska A, Dutkiewicz M, Kowalewska M, Grabowska I, Maciejko D, Fogtman A, Dadlez M, Koziak K. Molecular mechanism for cellular response to β -escin and its therapeutic implications. *PloS one*. 2016 Oct 11;11(10):e0164365.
15. Belcaro G, Dugall M, Maramaldi G, Togni S, Giacomelli L. Evaluation of the efficacy of a new escin-based, multi-component formulation in reducing eyelid edema. *Minerva Oftalmol*. 2016 Dec;58:65-9.
16. Li M, Lu C, Zhang L, Zhang J, Du Y, Duan S, Wang T, Fu F. Oral administration of escin inhibits acute inflammation and reduces intestinal mucosal injury in animal models. *Evidence-Based Complementary and Alternative Medicine*. 2015 Jan 1;2015.
17. Zelbieni E, Draksienė G, Savickas A, Kopustinskiene D, Masteikova R, Bernatoniene J. In vitro permeation studies of phenolics from horse chestnut seed gels prepared with different polyacrylic acid polymer derivatives. *Acta pharmaceutica*. 2015 Jun 30;65(2):181-9.
18. Belczak SQ, Sincos IR, Campos W, Beserra J, Nering G, Aun R. Venous-active drugs for chronic venous disease: A randomized, double-blind, placebo-controlled parallel-design trial. *Phlebology*. 2014 Aug;29(7):454-60.
19. Huang Y, Zheng SL, Zhu HY, Xu ZS, Xu RA. Effects of aescin on cytochrome P450 enzymes in rats. *Journal of Ethnopharmacology*. 2014 Jan 10;151(1):583-90.
20. Vujic Z, Novovic D, Arsic I, Antic D. Optimization of the extraction process of escin from dried seeds of *Aesculus hippocastanum* L. by Derringer's desirability function. *J. Anim. Plant Sci*. 2013;17(1):2514-21.
21. Mishra SK, Dash SL, Mohapatra R. In-vitro Anticancer Activity of Various Plant Extracts. *International Journal of Pharmaceutical Quality Assurance*. 2023;14(1):61-65.
22. Bartzoka ED, Lange H, Mosesso P, Crestini C. Synthesis of nano- and microstructures from proanthocyanidins, tannic acid and epigallocatechin-3-O-gallate for active delivery. *Green Chemistry*. 2017;19(21):5074-91.
23. Belcaro G, Dugall M, Maramaldi G, Togni S, Giacomelli L. Evaluation of the efficacy of a new escin-based, multi-component formulation in reducing eyelid edema. *Minerva Oftalmol*. 2016 Dec;58:65-9.

24. Wang XH, Xu B, Liu JT, Cui JR. Effect of β -escin sodium on endothelial cells proliferation, migration and apoptosis. *Vascular pharmacology*. 2008 Oct 1;49(4-6):158-65.
25. Singh H, Sidhu S, Chopra K, Khan MU. The novel role of β -aescin in attenuating CC14-induced hepatotoxicity in rats. *Pharmaceutical biology*. 2017 Jan 1;55(1):749-57.
26. Jang MH, Piao XL, Kim JM, Kwon SW, Park JH. Inhibition of cholinesterase and amyloid- β aggregation by resveratrol oligomers from *Vitis amurensis*. *Phytotherapy Research: An International Journal Devoted to Pharmacological and Toxicological Evaluation of Natural Product Derivatives*. 2008 Apr;22(4):544-9.
27. Belcaro G, Cesarone MR, Errichi BM, Ledda A, Di Renzo A, Stuard S, Dugall M, Pellegrini L, Rohdewald P, Ippolito E, Ricci A. Venous ulcers: microcirculatory improvement and faster healing with local use of Pycnogenol®. *Angiology*. 2005 Nov;56(6):699-705.
28. Allegra C, Carlizza A. Variants of functional venous disease. *Phlebology*. 2003;289-92.
29. Insull Jr W. Clinical utility of bile acid sequestrants in the treatment of dyslipidemia: a scientific review. *Southern medical journal*. 2006 Mar 1;99(3):257-74.
30. Fine AM. Oligomeric proanthocyanidin (OPCs). *Alternative Medicine Review*. 2003;8:442-50.
31. Belcaro G, Nicolaides AN, Geroulakos G, Cesarone MR, Incandela L, Sanctis MD. Essaven gel-local treatment of venous and microcirculatory diseases review of efficacy and new controlled, randomized studies-Essaven Gel--Review of Experimental and Clinical Data. *Angiology*. 2001;52(3):S1.
32. Hippocastanum HH. *Aesculus Hippocastanum (Aescin, Horse Chestnut) in the Management of Hemorrhoidal Disease*. *Turk J Colorectal Dis*. 2018;28:54-7.
33. Zhang H, Yang B. Successful treatment of pachydermoperiostosis patients with etoricoxib, aescin, and arthroscopic synovectomy: Two case reports. *Medicine*. 2017 Nov;96(47).
34. Feng JL, Wang ZW, Zhang J, Wang ZN, Liu F. Study on food-grade vitamin E microemulsions based on nonionic emulsifiers. *Colloids and Surfaces A: Physicochemical and Engineering Aspects*. 2009 May 1;339(1-3):1-6.
35. Sirtori CR. Aescin: pharmacology, pharmacokinetics and therapeutic profile. *Pharmacological Research*. 2001 Sep 1;44(3):183-93.
36. Bielanski TE. Horse-chestnut seed extract for chronic venous insufficiency. *Journal of family practice*. 1999 Mar 1;48(3):171
37. Winter CA, Risley EA, Nuss GW. Carrageenin-induced edema in hind paw of the rat as an assay for antiinflammatory drugs. *Proceedings of the society for experimental biology and medicine*. 1962 Dec;111(3):544-7.