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Article

Evaluation of Natural Extracts in Animal Models of Pain and Inflammation for a Potential Therapy of Hemorrhoidal Disease

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Abstract: The aim of this work was to assess the analgesic effect of three *Vitis vinifera* L. leaf extracts and the anti-inflammatory effect of three gels obtained from *Aesculus hippocastanum* L. seed extracts using animal models, as a preliminary study for the future development of topical preparations based on the combination of extracts with synergistic therapeutic effects on hemorrhoid disease. The analgesic effect was determined by means of the writhing test in mice. The anti-inflammatory effect was determined after administration of carrageenan or kaolin in the rat paw. Extraction using glycerol yielded the highest amounts of flavonoids for both *V. vinifera* leaves (37.27 ± 1.174 mg/L) and *A. hippocastanum* seeds (53.48 ± 0.212 mg/L). The highest total phenolic contents were registered for the *V. vinifera* 20% ethanolic extract (615.3 ± 34.44 mg/L) and for the *A. hippocastanum* glycerolic extract (247.8 ± 6.991 mg/L). The writhing test revealed that the *V. vinifera* ethanolic extract induced the most efficient analgesia (57.20%, $p < 0.01$), better than that induced by the positive control. In the carrageenan inflammation model, only the gel obtained from the *A. hippocastanum* glycerolic extract significantly reduced paw edema (17.27%, $p < 0.05$). An anti-inflammatory effect was also observed in the kaolin inflammation model but was not statistically significant (10.12%, $p > 0.05$). Our findings indicate that *V. vinifera* and *A. hippocastanum* extracts may have potential uses for the treatment of pain and inflammation associated with hemorrhoid disease.

Keywords: hemorrhoids; inflammation; pain; plant extracts; horse chestnut; grapevine

1. Introduction

Hemorrhoid disease is a prevalent symptomatic gastro-intestinal pathology. This ailment is defined as the enlargement and displacement of anal cushions, leading to the formation of vascular and connective tissue clusters. Approximately 10 million Americans are diagnosed annually with hemorrhoids [1,2]. Considering the quality of life is significantly affected for patients suffering from hemorrhoids, the management of this disease is of great importance. Typical symptoms include discomfort, itching, pain, inflammation, and bleeding [3].

Hemorrhoids can be treated using several surgical and nonsurgical approaches [1]. Surgical methods can include open and closed hemorrhoidectomy, Doppler-guided hemorrhoidal artery

ligation, and stapled hemorrhoidopexy [4]. Lifestyle and dietary modifications are required for an efficient management of hemorrhoids, involving fiber supplements, adequate hydration, and straining avoidance [5]. Drug therapy usually consists in the administration of topical formulations of corticosteroids, local anesthetics, antiseptics, or oral venotonics, such as flavonoids [6]. However, a downside of these therapies, that include long-term administration of topical corticosteroids, is represented by the risk of side effects caused by systemic absorption [7].

Botanical medicines or herbal remedies are natural products obtained from medicinal plants, which are a highly diverse source of bioactive compounds, namely, phytochemicals [8]. The application of natural products as an alternative therapeutic approach is in continuous growth worldwide [9], since recent studies showed that plant extracts are rich in phenolic and other compounds with good antioxidant, anti-inflammatory, anti-atherogenic, and antimicrobial activities [8,10]. Moreover, the concept of nutraceuticals has recently emerged. Nutraceuticals include phytocomplexes derived from vegetal food products and complexes of secondary metabolites of animal origin that are administered in a pharmaceutical form to alleviate certain conditions because of their beneficial pharmacological effects [11].

Previous reports indicated that medicinal plants can alleviate certain symptoms, such as pain, inflammation, and bleeding, in patients with hemorrhoids and are efficient as adjuvant therapies in both incipient and advanced hemorrhoid disease [12]. Many natural remedies have been widely used in traditional medicine for treating various conditions, including hemorrhoid disease, such as *Aesculus hippocastanum* L. (horse chestnut) and *Vitis vinifera* L. (grapevine) [13–15]. *A. hippocastanum* L. extracts, whose main active components are aescin and flavonoids, have various beneficial effects, including anti-inflammatory, venotonic, and vascular protective effects and antioxidant activity [16–19]. A horse chestnut seed extract was reported to possibly reduce capillary permeability and edema and inhibit enzymes involved in proteoglycan degradation, which warrants its use in hemorrhoid treatment [20]. *V. vinifera* L. can provide a wide variety of active chemical compounds, such as flavonoids, polyphenols, anthocyanins, and stilbene derivatives. Several pharmacological studies that evaluated grapevine extracts reported analgesic and antipyretic effects, antioxidant effects, cardioprotection, and hepatoprotection [19,21]. Moreover, the use of grapevine extracts in traditional medicine for their anti-hemorrhoidal properties and as a potential treatment for chronic venous insufficiency was previously reported [22,23].

Data provided by preclinical and clinical studies showed that horse chestnut seed extracts can induce weak genotoxicity on strains of *Salmonella typhimurium*, have greater toxicity after parenteral administration than after oral administration in laboratory animals, and may produce transient side effects in humans, such as itch, headaches, dizziness, and gastrointestinal disturbances [24]. Grapevine extracts were found to be non-toxic after acute oral administration and safe to use in cosmetic preparations [25,26].

Several studies showed that the parenteral administration of the main phytochemical constituent of horse chestnut seeds, i.e., aescin, showed a significant reduction of edema in animal models of inflammation and clinically significant beneficial effects on hemorrhoids [27,28]. Ethanolic grapevine leaf extracts showed antinociceptive activity in animal models of pain, and the authors suggested that the active constituents produce analgesia at a peripheral level [29]. In this research, we aimed to assess the analgesic effect of three *V. vinifera* leaf extracts and the anti-inflammatory effect of three gels obtained from *A. hippocastanum* seed extracts using animal models, as a preliminary study to identify the most efficient extraction method for the future development of topical preparations potentially useful for the treatment of symptomatic hemorrhoid disease.

2. Materials and Methods

2.1. Reagents

The solvents used for extraction and gel preparation were of commercial grade. Carbopol 940, sodium benzoate, and triethanolamine were acquired from Sigma-Aldrich (Sigma-Aldrich, Taufkirchen, Germany). The compounds administered to experimental animals were as follows: urethane (Sigma-Aldrich), kaolin (Sigma-Aldrich), carrageenan (Sigma-Aldrich), acetic acid (Sigma-Aldrich), metamizole sodium (Zentiva SA, Bucharest, Romania), and diclofenac sodium 5% gel (Hexal AG, Holzkirchen, Germany).

2.2. Plant Material and Extracts Preparation

A. hippocastanum seeds and *V. vinifera* leaves were provided by Hofigal SA (Romania). The plant materials identity was verified, and voucher specimens were stored at the Pharmaceutical Botany and Cell biology Department, University of Medicine and Pharmacy "Carol Davila", Bucharest. Both materials were grounded using a Swantech Sample Mill SJ 500 (France). Each material was extracted under reflux for 60 min with water, 20% ethanol, and 20% glycerol, using a plant material/solvent ratio of 1:10 (*w*/*v*). After filtration, the extractive solutions were stored at 4 °C. The solvents were selected so that the future extracts could be easily incorporated in a topical pharmaceutical form such as gel, because gel formulations use glycerin as an excipient and ethanol or other hydrophilic solvents.

Total polyphenol content (TPC) and total flavonoid content (TFC) were determined using the Folin–Ciocalteu method (λ = 750 nm) and the aluminum chloride method (λ = 429 nm) [30,31]. The assays were performed in triplicate using a UV–VIS spectrophotometer (Halo DB-20-220; Dynamica, Salzburg-Mayrwies, Austria). TPC and TFC were calculated using standard calibration curves and were expressed as means ± standard deviation (SD) using gallic acid equivalents (GAE) mg/L for TPC and mg/L quercetin equivalents (QE) for TFC.

2.3. Hydrogels Preparation

The *A. hippocastanum* extract gels were obtained by diluting a 2% carbopol 940 gel with the plant extracts in increasing concentrations under continuous stirring. The 2% carbopol base gel was obtained using carbopol 940, water, sodium benzoate (0.1%), triethanolamine, and glycerol (24%). The hydrogels were analyzed for their spreadability capacity and their pH values [32].

2.4. Animals

All experimental procedures were performed in accordance with bioethics norms proposed by the WMA Declaration of Helsinki and the NIH Guide for the Care and Use of Laboratory Animals. The experimental protocol was approved by the Bioethics Commission of the Faculty of Pharmacy, University of Medicine and Pharmacy Carol Davila, Bucharest.

Laboratory animals (NMRI mice and Wistar rats) were purchased from INCDMI Cantacuzino Institute, Bucharest, and were accustomed for two days to the new habitat. Food (grains for mice and rats (Cantacuzino Institute, Bucharest) and drinking water were made available to the animals ad libitum. The animals were housed on a light/dark cycle of 12 h, under constant humidity and temperature, monitored with a thermohigrometer. The recorded values were between 35% and 45% for humidity and 20 and 22 °C for temperature. After experimentation, the animals were euthanized according to the standard guidelines [33], by intraperitoneal injection of an overdose of thiopental sodium, 200 mg/kg b.w.

2.5. Antinociception

Animal models of peripheral pain induction are often based on chemical stimulation by intraperitoneal administration of an acetic acid solution or intraplantar injection of a formalin solution [34,35]. The analgesic effect of three *V. vinifera (folium)* extracts was evaluated using the writhing test, which detects the peripheral analgesic action mediated by endogenous substances such as prostaglandins and arachidonic acid metabolites via the cyclooxygenase pathway [36]. In this experiment, 40 male NMRI mice were divided into five groups and kept on a fasting period of 4 h before receiving the treatments presented in Figure 1.

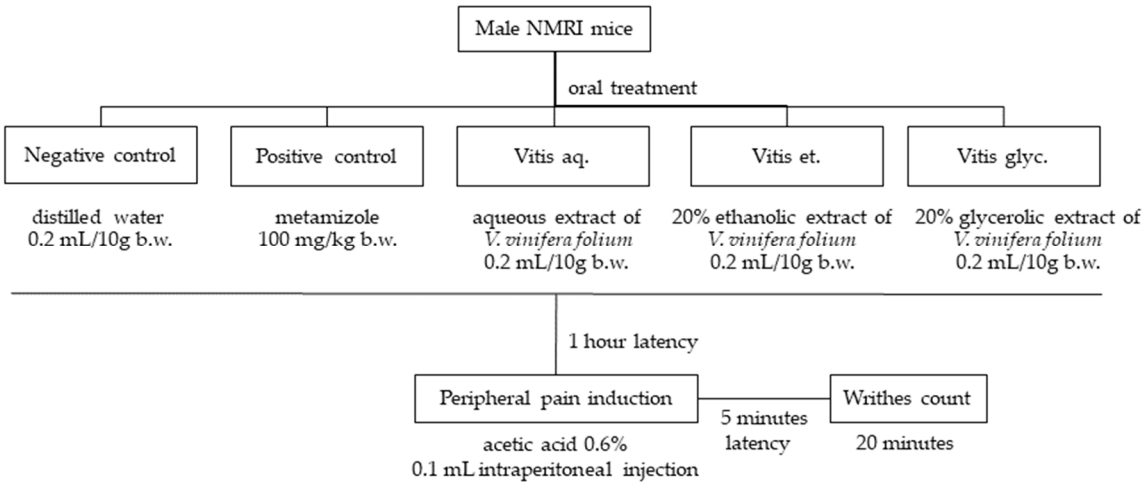

Figure 1. Experimental protocol for antinociception evaluation. Vitis aq.: *Vitis vinifera* aqueous extract; Vitis et.: *V. vinifiera* 20% ethanolic extract; Vitis glyc.: *V. vinifera* 20% glycerolic extract; b.w.: body weight.

One hour after administration, the mice received 0.1 mL of 0.6% acetic acid via intraperitoneal injection for peripheral pain induction. The experimental animals were placed in individual plexiglass cages and, after a latency period of 5 min, an observer counted the abdominal writhes of each mouse for 20 min. A writhe is defined as the elongation of the body followed by torsion and extension of the hind limbs [37].

2.6. Anti-Inflammatory Effect

The anti-inflammatory activity of three *A. hippocastanum* (semen) extracts was evaluated using the plethysmometric method, consisting in the intraplantar administration of inflammatory agents such as kaolin or carrageenan [38]. The pharmacological model of induction of edema by carrageenan is mostly used in research for assaying the anti-inflammatory effects of natural products [39,40].

Inflammation was induced in 70 male Wistar rats, distributed into 10 equal experimental groups. The animals were anesthetized intraperitoneally with a 13% urethane solution, 1300 mg/kg b.w. After general anesthesia induction, the initial hind paw volume was determined with the plethysmometer apparatus (Ugo Basile, Gemonio, Italy). Thereafter, 0.2 g of each treatment gel was applied to the surface of the right paw and massaged gently 50 times with the index finger. The topical gels that were used as treatments are specified in Figure 2. Inflammation was induced by intraplantar administration of inflammatory agents as follows: 0.1 mL of a 1% carrageenan suspension to five groups and 0.2 mL of a 10% kaolin suspension to the remaining five groups. The evolution of the induced edema was observed for 3 h following the intraplantar injections.

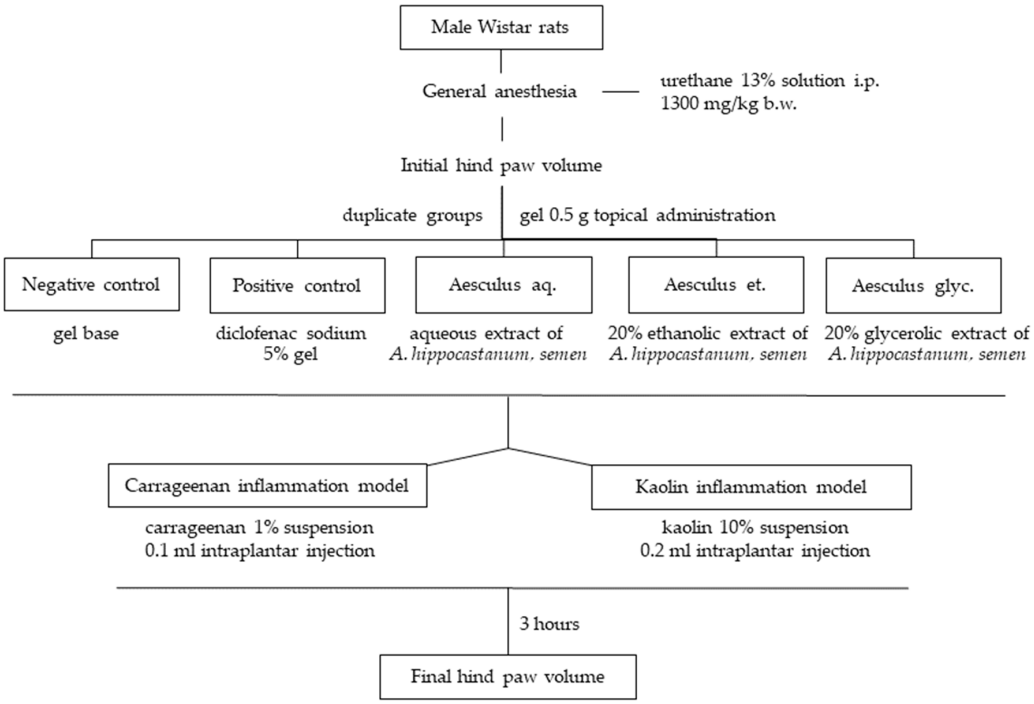

Figure 2. Experimental protocol for anti-inflammatory effect evaluation. Aesculus aq.: *Aesculus hippocastanum* aqueous extract; Aesculus et.: *A. hippocastanum* 20% ethanolic extract; Aesculus glyc.: *A. hippocastanum* 20% glycerolic extract; b.w.: body weight; i.p.: intraperitoneal injection.

2.7. Statistical Analysis

The evaluation of the obtained experimental data was performed using the GraphPad Prism v.5.0 software (GraphPad Software Inc., San Diego, CA, USA). The distribution of the biological responses was tested using the Kolmogorov–Smirnov normality test. Parametric and non-parametric statistical tests were applied using the predefined 95% confidence interval, and the statistical significance threshold was set at 0.05. The experimental results are expressed as arithmetic mean ± standard deviation (mean ± SD).

3. Results

3.1. Total Polyphenolic and Flavonoid Contents

The results obtained from TPC and TFC assays are presented in Table 1. The highest TFC value was registered in the 20% glycerol extracts. Of the three solvents, TPC values were higher in the 20% glycerol extract from *A. hippocastanum* and in the 20% ethanol extract from *V. vinifera.*

Table 1. Total flavonoid content (TFC) and total polyphenol content (TPC) of *A. hippocastanum* and *V. vinifera* extracts.

Sample	TFC (mg/L)		TPC (mg/L)	
	Mean ± SD	CI 95%	Mean ± SD	CI 95%
A. hippocastanum aqueous extract	31.01 ± 1.386	18.56–43.46	154.3 ± 0.176	152.7–155.8
A. hippocastanum 20% ethanolic extract	34.92 ± 8.085	14.84–55.01	160.2 ± 3.971	150.4–170.1
A. hippocastanum 20% glycerolic extract	53.48 ± 0.212	51.57–55.39	247.8 ± 6.991	239.1–256.5
V. vinifera aqueous extract	18.26 ± 1.824	13.73–22.79	266.2 ± 17.57	238.2–294.1
V. vinifera 20% ethanolic extract	18.47 ± 1.442	5.51–31.43	615.3 ± 34.44	305.8–924.8
V. vinifera 20% glycerolic extract	37.27 ± 1.174	26.72–47.82	505.3 ± 34.68	419.2–591.5

SD: standard deviation (n = 3); CI 95%: 95% confidence interval of the mean.

3.2. Hydrogels Preparation

Carbopol gel formulations were successfully prepared. They were clear, translucent, red-brown in color, and had a pH of 7.3 ± 0.3 (Hanna HI-98127 pH Tester). A visual inspection revealed that all formulations were homogeneous and presented a good spreadability.

3.3. Analgesia

Experimental results showing the mean number of writhes performed by the animals in the experimental groups after acetic acid administration, the analgesic effect of the tested *V. vinifera* extracts compared to those of the negative control, and the statistical significance of the obtained data are presented in Figure 3.

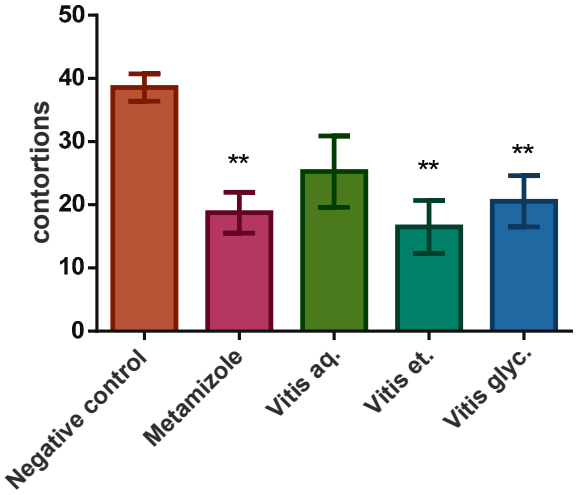

Figure 3. Mean number of writhes for each group after treatment and acetic acid injection (mean ± SD). The *V. vinifera* ethanolic extract was associated with the most potent decrease in the number of writhes (** $p < 0.01$). Metamizole and the glycerolic extracts showed a slightly lower analgesic effect (** $p < 0.01$). The *V. vinifera* aqueous extract reduced the number of writhes, but the difference with respect to the control was not statistically significant ($p > 0.05$).

Both the positive control and the tested *V. vinifera* extracts showed an obvious analgesic effect, as highlighted by the decrease in the average number of writhes, compared to the negative control group. The most potent analgesic effect was observed in the group that received the ethanolic extract (57.20%, $p < 0.01$). The group treated with metamizole and the one receiving the glycerolic extract showed a slightly lower, but statistically significant, analgesic effect ($p < 0.01$).

The mean difference between the number of writhes observed in the negative control and that determined in animals treated with the *V. vinifera* aqueous extract was not statistically significant (34.51%, $p > 0.05$). Although the ethanolic extract exhibited a slightly more potent analgesic effect than metamizole, the difference between these groups was statistically insignificant.

3.4. Anti-Inflamatory Effect

The anti-inflammatory activity of the gels obtained from *A. hippocastanum* extracts was evaluated using carrageenan and kaolin inflammation models. The experimental data obtained using carrageenan, relative to the baseline values prior to the induction of edema, are shown in Table 2.

Table 2. Hind paw volume (cm^3) of rats receiving intraplantar carrageenan and topical gels.

Parameter	Treatment Groups				
	Negative Control	Positive Control	Aesculus aq.	Aesculus et.	Aesculus glyc.
Initial paw volume (Mean ± SD)	1.73 ± 0.19	1.70 ± 0.06	1.65 ± 0.11	1.65 ± 0.13	1.81 ± 0.17
Final paw volume (Mean ± SD)	2.21 ± 0.36	2.01 ± 0.18	1.97 ± 0.15	2.11 ± 0.29	2.00 ± 0.18
Paw volume increase (%)	28.10	18.04	19.01	28.30	10.83
Paired *t*-test (*p*)	* (0.0102)	** (0.0039)	** (0.0028)	*** (0.0008)	** (0.0021)

*** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$.

Carrageenan injection led to an increase in paw volume in all groups. The volume increase of the rat paws was statistically significant in all groups, in comparison with the baseline value. Three hours after carrageenan administration, the largest edema was recorded in the group treated with *A. hippocastanum* ethanolic extract (28.30%, $p < 0.0001$), followed by the negative control group (28.10%, $p < 0.05$), the positive control group (19.01%, $p < 0.01$), and the *Aesculus* aq. group (18.04%, $p < 0.01$). The lowest inflammatory response was recorded in the group treated with the glycerolic extract (10.83%, $p < 0.0001$).

In comparison with the negative control group, the most potent anti-inflammatory effect was observed for the group receiving the *A. hippocastanum* glycerolic extract (17.27% lower mean difference of paw volume). Diclofenac and the aqueous extract showed a similar anti-inflammatory effect, with a decrease of mean differences of paw volumes by 10.06% and 9.08%, respectively (Figure 4). The ethanolic extract of *A. hippocastanum* had no observable anti-inflammatory effect. The edema reduction by treatment with diclofenac gel was not statistically significant, whereas the glycerolic extract induced a statistically significant decrease of the inflammatory response (Mann–Whitney, $p < 0.05$).

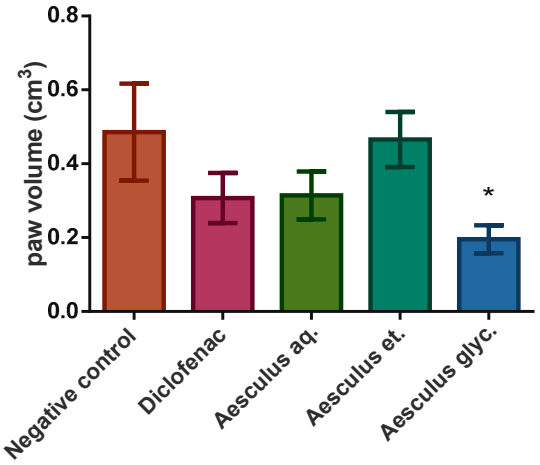

Figure 4. Difference of paw volume between initial and final measurements in the carrageenan inflammation model (mean ± SD). The *A. hippocastanum* glycerolic extract induced a statistically significant decrease of the inflammatory response (* $p < 0.05$). The *A. hippocastanum* ethanolic extract showed no observable anti-inflammatory effect ($p > 0.05$). The edema reduction by treatment with diclofenac gel was not statistically significant and was comparable to that observed after treatment with the *A. hippocastanum* aqueous extact ($p > 0.05$).

Following kaolin intraplantar injection, all treatment groups showed increased paw volumes. The differences in paw volumes between initial and final measurements were statistically significant in all groups (Table 3).

Table 3. Hind paw volume (cm^3) of rats receiving intraplantar kaolin and topical gels.

Parameter	Treatment Groups				
	Negative Control	Positive Control	Aesculus aq.	Aesculus et.	Aesculus glyc.
Initial paw volume (Mean ± SD)	1.69 ± 0.13	1.67 ± 0.18	1.63 ± 0.10	1.67 ± 0.08	1.75 ± 0.12
Final paw volume (Mean ± SD)	2.28 ± 0.20	2.14 ± 0.13	2.06 ± 0.22	2.24 ± 0.10	2.18 ± 0.18
Paw volume increase (%)	35.00	27.89	26.49	34.00	24.88
Paired *t*-test (*p*)	*** (<0.0001)	*** (0.0002)	** (0.0016)	*** (<0.0001)	*** (<0.0001)

*** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$.

Three hours after kaolin administration, the greatest increase of hind paw volume was observed in the negative control group (35.00%, $p < 0.0001$), followed by the *Aesculus* et. group (34.00%, $p < 0.001$), the positive control (27.89%, $p < 0.01$), and the *Aesculus* aq. group (26.47%, $p < 0.01$). The lowest increase was recorded in the group receiving the glycerolic extract (24.88%, $p < 0.0001$).

Compared to the control group, the most potent anti-inflammatory effect was observed in the group receiving the *A. hippocastanum* glycerolic extract (10.12% less edema development). Diclofenac and the aqueous extract showed a comparable anti-inflammatory effect, leading to a decrease of edema by 7.11% and 8.5%, respectively, when compared to the control group (Figure 5). *Aesculus* et. showed no anti-inflammatory effect. Surprisingly, the anti-inflammatory effect of diclofenac was not statistically significant. Although the glycerolic extract showed the highest efficacy in preventing edema induction, the experimental data analysis showed no statistically significant results, although close to the significance threshold ($p = 0.0517$).

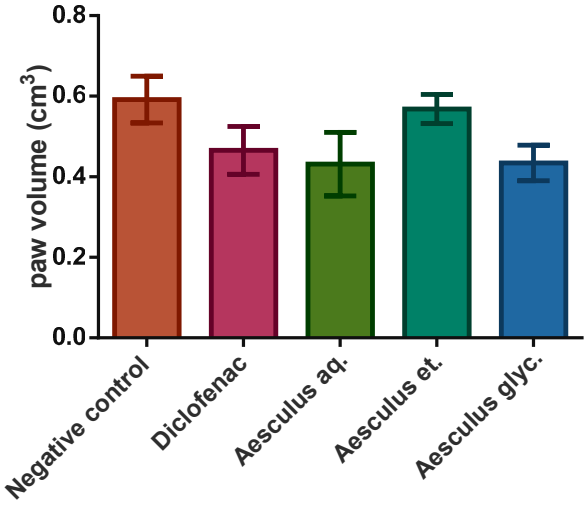

Figure 5. Difference of paw volume between initial and final measurements in the kaolin inflammation model (mean ± SD). The *A. hippocastanum* ethanolic extract showed no significant anti-inflammatory effect ($p > 0.05$). Diclofenac sodium and the *A. hippocastanum* aqueous extract reduced paw edema, but the differences were not statistically significant ($p > 0.05$). The *A. hippocastanum* glycerolic extract showed a statistically insignificant anti-inflammatory effect ($p = 0.0517$).

4. Discussion

Hemorrhoid disease is a discomforting gastro-intestinal pathology, whose main symptoms are pain and inflammation [1–3]. The use of botanical remedies for the traditional treatment of hemorrhoids has been previously documented [13–15]. Available data indicate that products obtained from *A. hippocastanum* L. seeds (horse chestnut) and *V. vinifera* L. leaves (grapevine) have clinically relevant beneficial effects on the main symptoms of hemorrhoid disease [20,22]. Thus, the aim of this study was to investigate the effects of several extracts obtained from horse chestnut seeds and grapevine leaves in pain and inflammation animal models, in order to establish the best extraction method for further preparation of a topical formulation with potential use as a hemorrhoids symptomatic treatment.

Extraction using 20% glycerol yielded the highest total content of flavonoids for both *V. vinifera* leaves and *A. hippocastanum* seeds. The total phenolic content was higher in the 20% ethanolic extract of grapevine leaves and the 20% glycerolic extract of horse chestnut seeds. The results indicated that the anti-inflammatory and analgesic effects of the two extracts may be induced by other phytochemicals beside polyphenols and flavonoids, such as coumarins, saponins, and catechins. Numerous studies have shown that horse chestnut seeds contain several escins which dose-dependently inhibited the increase of vascular permeability and showed good anti-inflammatory effects [19,41,42]. The grapevine extracts are rich in polyphenols such as flavonoids, catechins, and stilbenes [43]. Among them, caffeic acid, quercetin, kaempferol glycosides, and resveratrol were identified as the major compounds of extracts with anti-inflammatory and analgesic activity [29,44]. Future research is needed to further determine the chemical profile of the extracts in order to advance their production for clinical studies.

Both the ethanolic and the glycerolic extracts obtained from *V. vinifera* leaves showed a statistically significant pain alleviation in mice. The chemical stimulation test revealed that the ethanolic extract induced the most efficient analgesia, followed by the glycerolic extract. Moreover, the grapevine ethanolic extract showed a more potent analgesic effect than the reference substance, metamizole sodium. Thus, we suspect that extraction with water may not provide a sufficient amount of phytochemicals with analgesic effect. Our findings are similar to previously reported data which showed that grapevine leaves ethanolic extracts alleviate peripheral pain through supposedly non-central mechanisms [29]. The toxic profile of the extracts was not tested, because the plants are used in traditional medicine without reported toxic effects. Future research is planned to evaluate the final pharmacological product tolerability.

Two inflammation and edema models were used to test the anti-inflammatory potential of *A. hippocastanum* seed extracts. Extraction with two solvents, water and 20% glycerol, produced a decrease in hind paw edema in the carrageenan inflammation model. However, only the gel obtained from the 20% glycerolic extract of *A. hippocastanum* seeds induced a statistically significant reduction of hind paw edema. Although this effect was also observed in the kaolin inflammation model, the anti-inflammatory effect was not statistically significant in this case. This finding suggests that the glycerolic extract might be rich in active constituents able to interfere with the proinflammatory mechanism of carrageenan, which produces an increase in TNF-α, IL-1, nitric oxide, and PGE-2 levels [45]. Moreover, previous published studies highlighted that horse chestnut ethanolic extracts inhibit elastase and hyaluronidase, thus diminishing capillary permeability and edema [20]. However, our experimental results showed that the ethanolic extract had no significant anti-inflammatory activity, probably because of the low concentration of ethanol used for extraction.

5. Conclusions

The experimental results obtained in animal models of pain and inflammation further support the traditional use of *V. vinifera* L. and *A. hippocastanum* L. in treating symptoms of hemorrhoid disease, such as pain and inflammation. *V. vinifera* L. ethanolic and glycerolic leaf extracts showed a significant effect on chemically induced algesia, while an *A. hippocastanum* L. glycerolic seed extract exhibited a good anti-inflammatory effect on carrageenan-induced edema. These findings are only partially related to the total polyphenolic and flavonoid contents, thus, the pharmacological effects could also be credited to other phytochemical classes, such as coumarins, saponins, and catechins. A limitation of this study is linked to the lack of a specific and accessible animal model of hemorrhoid disease, thus, our research focused on the two main symptoms of the discussed condition. Further studies are required to establish the efficacy of a topical medication combining the two botanical products.

6. Patents

The present work resulted in the submission of Patent Application No. A/01033/2018 to the Romanian State Office for Inventions and Trademarks.

Author Contributions: Conceptualization, O.C.S., G.M.N., and O.T.O.; Data curation, D.P.M., G.N., and G.M.N.; Funding acquisition, M.I., G.I., and O.T.O.; Investigation, D.P.M., O.C.S., G.N., M.I., A.-S.S., S.N., G.I., G.M.N., and O.T.O.; Methodology, D.P.M., O.C.S., G.N., M.I., A.-S.S., S.N., G.I., G.M.N., and O.T.O.; Project administration, M.I. and O.T.O.; Writing—original draft, D.P.M., O.C.S., and G.N.; Writing—review & editing, G.M.N. and O.T.O.

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Conflicts of Interest: The authors declare no conflict of interest.

References

1. Sun, Z.; Migaly, J. Review of Hemorrhoid Disease: Presentation and Management. *Clin. Colon Rectal Surg.* **2016**, *29*, 22–29. [PubMed]
2. Lohsiriwat, V. Hemorrhoids: From basic pathophysiology to clinical management. *World J. Gastroenterol.* **2012**, *18*, 2009–2017. [CrossRef] [PubMed]
3. Mott, T.; Latimer, K.; Edwards, C. Hemorrhoids: Diagnosis and Treatment Options. *Am. Fam. Physician* **2018**, *97*, 172–179.
4. Acheson, A.G.; Scholefield, J.H. Management of haemorrhoids. *BMJ* **2008**, *336*, 380–383. [CrossRef] [PubMed]
5. Lohsiriwat, V. Treatment of hemorrhoids: A coloproctologist's view. *World J. Gastroenterol.* **2015**, *21*, 9245–9252. [CrossRef] [PubMed]
6. Brown, S.R. Haemorrhoids: An update on management. *Ther. Adv. Chronic Dis.* **2017**, *8*, 141–147. [CrossRef]
7. Coondoo, A.; Phiske, M.; Verma, S.; Lahiri, K. Side-effects of topical steroids: A long overdue revisit. *Indian Dermatol. Online J.* **2014**, *5*, 416–425. [CrossRef] [PubMed]
8. Durazzo, A.; D'Addezio, L.; Camilli, E.; Piccinelli, R.; Turrini, A.; Marletta, L.; Marconi, S.; Lucarini, M.; Lisciani, S.; Gabrielli, P.; et al. From Plant Compounds to Botanicals and Back: A Current Snapshot. *Molecules* **2018**, *23*, 1844. [CrossRef] [PubMed]
9. Kumar, A.H.S. Rediscovering the Drug Discovery with Natural Products as Therapeutic Tools. *J. Nat. Sci. Biol. Med.* **2018**, *9*, 1. [CrossRef] [PubMed]
10. Altemimi, A.; Lakhssassi, N.; Baharlouei, A.; Watson, D.; Lightfoot, D. Phytochemicals: Extraction, Isolation, and Identification of Bioactive Compounds from Plant Extracts. *Plants* **2017**, *6*, 42. [CrossRef] [PubMed]
11. Santini, A.; Novellino, E. Nutraceuticals—Shedding light on the grey area between pharmaceuticals and food. *Expert Rev. Clin. Pharmacol.* **2018**, *11*, 545–547. [CrossRef] [PubMed]
12. Hashempur, M.H.; Khademi, F.; Rahmanifard, M.; Zarshenas, M.M. An Evidence-Based Study on Medicinal Plants for Hemorrhoids in Medieval Persia. *J. Evid.-Based Complement. Altern. Med.* **2017**, *22*, 969. [CrossRef] [PubMed]
13. Karatoprak, G.Ş. Horse Chestnut. *Nonvitam. Nonminer. Nutr. Suppl.* **2019**, 295–299. [CrossRef]
14. Foca, G.; Ulrici, A.; Cocchi, M.; Durante, C.; Vigni, M.L.; Marchetti, A.; Sighinolfi, S.; Tassi, L. Seeds of Horse Chestnut (*Aesculus hippocastanum* L.) and Their Possible Utilization for Human Consumption. In *Nuts and Seeds in Health and Disease Prevention*; Academic Press: Cambridge, MA, USA, 2011; pp. 653–661.
15. Deliorman Orhan, D.; Orhan, N.; Özçelik, B.; Ergun, F. Biological activities of *Vitis vinifera* L. leaves. *Turk. J. Biol.* **2009**, *33*, 341–348.
16. Margină, D.; Olaru, O.T.; Ilie, M.; Grădinaru, D.; GuȚu, C.; Voicu, S.; Dinischiotu, A.; Spandidos, D.A.; Tsatsakis, A.M. Assessment of the potential health benefits of certain total extracts from *Vitis vinifera*, Aesculus hyppocastanum and Curcuma longa. *Exp. Ther. Med.* **2015**, *10*, 1681–1688. [CrossRef] [PubMed]
17. Küçükkurt, I.; Ince, S.; Keleş, H.; Küpeli Akkol, E.; Avcı, G.; Yeşilada, E.; Bacak, E. Beneficial effects of *Aesculus hippocastanum* L. seed extract on the body's own antioxidant defense system on subacute administration. *J. Ethnopharmacol.* **2010**, *129*, 18–22. [CrossRef] [PubMed]
18. Senatore, F.; Mścisz, A.; Mrugasiewicz, K.; Gorecki, P. Steroidal constituents and anti-inflammatory activity of the horse chestnut (*Aesculus hippocastanum* L.) bark. *Boll. Soc. Ital. Biol. Sper.* **1989**, *65*, 137–141.
19. Wilkinson, J.A.; Brown, A.M.G. Horse Chestnut—Aesculus Hippocastanum: Potential Applications in Cosmetic Skin-care Products. *Int. J. Cosmet. Sci.* **1999**, *21*, 437–447. [CrossRef]
20. MacKay, D. Hemorrhoids and varicose veins: A review of treatment options. *Altern. Med. Rev.* **2001**, *6*, 126–140.

21. Nassiri-Asl, M.; Hosseinzadeh, H. Review of the Pharmacological Effects of *Vitis vinifera* (Grape) and its Bioactive Compounds. *Phyther. Res.* **2009**, *23*, 1197–1204. [CrossRef]
22. Dehdari, S.; Hajimehdipoor, H.; Esmaeili, S.; Choopani, R.; Mortazavi, S.A. Traditional and modern aspects of hemorrhoid treatment in Iran: A review. *J. Integr. Med.* **2018**, *16*, 90–98. [CrossRef] [PubMed]
23. Schaefer, E.; Peil, H.; Ambrosetti, L.; Petrini, O. Oedema Protective Properties of the Red Vine Leaf Extract AS 195 (Folia vitis viniferae) in the Treatment of Chronic Venous Insufficiency. *Arzneimittelforschung* **2011**, *53*, 243–246. [CrossRef] [PubMed]
24. Dudek-Makuch, M.; Nska-Sroka, S. Horse chestnut—Efficacy and safety in chronic venous insufficiency: An overview. *Rev. Bras. Farmacogn.* **2015**, *25*, 533–541. [CrossRef]
25. Geetha, S.; Devaraj, A. Acute oral toxicity studies of vitis vinifera (grapes) produced by microbial fertigation and foliar spray of panchagavya. *J. Pharm. Sci. Innov.* **2014**, *3*, 245–248.
26. Fiume, M.M.; Bergfeld, W.F.; Belsito, D.V.; Hill, R.A.; Klaassen, C.D.; Liebler, D.C.; Marks, J.G.; Shank, R.C.; Slaga, T.J.; Snyder, P.W.; et al. Safety Assessment of *Vitis vinifera* (Grape)-Derived Ingredients as Used in Cosmetics. *Int. J. Toxicol.* **2014**, *33*, 48S–83S. [CrossRef] [PubMed]
27. Sirtori, C.R. Aescin: Pharmacology, pharmacokinetics and therapeutic profile. *Pharmacol. Res.* **2001**, *44*, 183–193. [CrossRef]
28. Wang, H.; Zhang, L.; Jiang, N.; Wang, Z.; Chong, Y.; Fu, F. Anti-inflammatory effects of escin are correlated with the glucocorticoid receptor/NF-κB signaling pathway, but not the COX/PGF2α signaling pathway. *Exp. Ther. Med.* **2013**, *6*, 419–422. [CrossRef]
29. Aouey, B.; Samet, A.M.; Fetoui, H.; Simmonds, M.S.J.; Bouaziz, M. Anti-oxidant, anti-inflammatory, analgesic and antipyretic activities of grapevine leaf extract (*Vitis vinifera*) in mice and identification of its active constituents by LC–MS/MS analyses. *Biomed. Pharmacother.* **2016**, *84*, 1088–1098. [CrossRef]
30. Radulescu, C.; Stihi, C.; Ilie, M.; Lazurcă, D.; Gruia, R.; Olaru, O.T.; Bute, O.C.; Dulama, I.D.; Stirbescu, R.M.; Teodorescu, S.; et al. Characterization of Phenolics in *Lavandula angustifolia*. *Anal. Lett.* **2017**, *50*, 2839–2850. [CrossRef]
31. Olaru, O.T.; Venables, L.; Van De Venter, M.; Nitulescu, G.M.; Margina, D.; Spandidos, D.A.; Tsatsakis, A.M. Anticancer potential of selected Fallopia Adans species. *Oncol. Lett.* **2015**, *10*, 1323–1332. [CrossRef]
32. Jantarat, C.; Sirathanarun, P.; Chuchue, T.; Konpian, A.; Sukkua, G.; Wongprasert, P. In vitro antimicrobial activity of gel containing the herbal ball extract against propionibacterium acnes. *Sci. Pharm.* **2018**, *86*, 8. [CrossRef] [PubMed]
33. European Union. Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes Text with EEA relevance. *Off. J. Eur. Union* **2010**, *28*, 82–128.
34. Zanfirescu, A.; Cristea, A.; Nitulescu, G.; Velescu, B.; Gradinaru, D.; Zanfirescu, A.; Cristea, A.N.; Nitulescu, G.M.; Velescu, B.S.; Gradinaru, D. Chronic Monosodium Glutamate Administration Induced Hyperalgesia in Mice. *Nutrients* **2017**, *10*, 1. [CrossRef] [PubMed]
35. Weggel, L.; Pandya, A.; Weggel, L.A.; Pandya, A.A. Acute Administration of Desformylflustrabromine Relieves Chemically Induced Pain in CD-1 Mice. *Molecules* **2019**, *24*, 944. [CrossRef] [PubMed]
36. Gawade, S.P. Acetic acid induced painful endogenous infliction in writhing test on mice. *J. Pharmacol. Pharmacother.* **2012**, *3*, 348. [CrossRef] [PubMed]
37. Marzoli, F.; Marianecci, C.; Rinaldi, F.; Passeri, D.; Rossi, M.; Minosi, P.; Carafa, M.; Pieretti, S.; Marzoli, F.; Marianecci, C.; et al. Long-Lasting, Antinociceptive Effects of pH-Sensitive Niosomes Loaded with Ibuprofen in Acute and Chronic Models of Pain. *Pharmaceutics* **2019**, *11*, 62. [CrossRef]
38. Pashmforosh, M.; Rajabi Vardanjani, H.; Rajabi Vardanjani, H.; Pashmforosh, M.; Khodayar, M.; Pashmforosh, M.; Rajabi Vardanjani, H.; Rajabi Vardanjani, H.; Pashmforosh, M.; Khodayar, M.J. Topical Anti-Inflammatory and Analgesic Activities of Citrullus colocynthis Extract Cream in Rats. *Medicina* **2018**, *54*, 51. [CrossRef]
39. Hovaneţ, M.-V.; Oprea, E.; Ancuceanu, R.V.; Duţu, L.E.; Budura, E.A.; Şeremet, O.; Ancu, I.; Moroşan, E. Wound Healing Properties of Ziziphus jujuba Mill. leaves. *Rom. Biotechnol. Lett.* **2016**, *21*, 11842–11849.
40. Regina Nonato, F.; Adelita Almeida Barros, T.; Maria Lucchese, A.; Eduardo Cordeiro Oliveira, C.; Ribeiro dos Santos, R.; Botelho Pereira Soares, M.; Flora Villarreal, C. Antiinflammatory and antinociceptive activities of *Blechnum occidentale* L. extract. *J. Ethnopharmacol.* **2009**, *125*, 102–107. [CrossRef]

41. Matsuda, H.; Li, Y.; Murakami, T.; Ninomiya, K.; Araki, N.; Yoshikawa, M.; Yamahara, J. Antiinflammatory effects of escins Ia, Ib, IIa, and IIb from horse chestnut, the seeds of *Aesculus hippocastanum* L. *Bioorg. Med. Chem. Lett.* **1997**, *7*, 1611–1616. [CrossRef]
42. Özkan, E.E.; Mesut, B.; Polat, A.; Tan, N. In vitro skin permeation of escin in the new gel formulation of Aesculus hippocastanum (Horse Chestnut). *İstanbul Üniversitesi Eczac. Fakültesi Derg.* **2016**, *46*, 79–88.
43. Nadia, Z.; Aicha, M.; Sihem, H.; Abdelmalik, B. In vivo analgesic activities and safety assessment of *Vitis vinifera* L. and *Punica granatum* L. fruits extracts. *Trop. J. Pharm. Res.* **2017**, *16*, 553. [CrossRef]
44. Koşar, M.; Küpeli, E.; Malyer, H.; Uylaşer, V.; Türkben, C.; Başer, K.H.C. Effect of Brining on Biological Activity of Leaves of *Vitis vinifera* L. (Cv. Sultani Çekirdeksiz) from Turkey. *J. Agric. Food Chem.* **2007**, *55*, 4596–4603. [CrossRef] [PubMed]
45. Mansouri, M.T.; Hemmati, A.A.; Naghizadeh, B.; Mard, S.A.; Rezaie, A.; Ghorbanzadeh, B. A study of the mechanisms underlying the anti-inflammatory effect of ellagic acid in carrageenan-induced paw edema in rats. *Indian J. Pharmacol.* **2015**, *47*, 292. [CrossRef] [PubMed]