

EVALUATION OF *ALLIUM AMPELOPRASUN* VAR. *PORRUM* EXTRACT GELS IN A WOUND HEALING ANIMAL MODEL

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Abstract. Hemorrhoids are one of the most common conditions affecting humans, more than 50 % of the population over 50 years of age having experienced hemorrhoid problems. Treatment options mainly depend on the location, grade and severity of hemorrhoids and range from lifestyle modifications, oral and topical medication, nonsurgical and surgical treatment. In this research we aimed to assess the wound healing effect of three gels obtained from *Allium ampeloprasun* var. *porrum* bulb, stem and leaves on an animal model, as a preliminary study for future development of topical preparations with potential use in symptomatic hemorrhoid disease. Three hydrophilic *A. ampeloprasun* gels were prepared using *A. ampeloprasun* aqueous, 20 % ethanolic and 20 % glycerolic extracts. Total polyphenol content (TPC) and total flavonoid content (TFC) were determined for each extract using the Folin-Ciocalteu method and the aluminum chloride methods. The effect on healing rate was assessed using an animal model of wound healing. The highest TPC and TFC values were obtained with water extraction. *A. ampeloprasun* 20 % ethanolic extract gel induced the highest wound contraction rate and showed the lowest TPC and TFC contents, thus the effect on wound healing could not be correlated with these classes of phytochemicals. The beneficial effect of the hydrophilic gel formulated with *A. ampeloprasun* var. *porrum* 20 % ethanolic extract on the rat model of wound healing could warrant its potential use in the topical treatment of hemorrhoid disease

Key words: Wound healing, hemorrhoid, plant extracts, leek.

INTRODUCTION

Wound healing is an essential physiological process responsible for restoring a lesion induced by a local aggression. Regeneration and repair of the tissue are linear processes that consist in cell and biochemical events divided into three stages: inflammatory reaction, proliferation and remodeling [5]. For instance, wound healing is crucial for patients suffering from hemorrhoids. Hemorrhoidectomy often results in postoperative pain, due to the spasm of internal sphincter and wound

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healing processes. Therefore, local treatments for pain relief and wound healing improvement are of high importance for patients that went through surgical removal of hemorrhoids [16].

Hemorrhoids are one of the most common conditions affecting humans with more than 10 million patients experiencing hemorrhoidal symptoms in USA. It is estimated that more than 50 % of the population over 50 years of age have experienced hemorrhoid problems [19]. Hemorrhoids are defined as symptomatic enlargement and abnormally downward displacement of anal cushions [10]. The main symptoms include bleeding, anal swelling, pain, pruritus and evacuation difficulties [18].

Treatment options mainly depend on the location, grade and severity of hemorrhoids and range from lifestyle modifications, like increasing the intake of dietary fiber and fluids in the diet, oral and topical medication, nonsurgical treatment like rubber-band ligation and sclerotherapy, to surgical treatment [9].

Topical treatments (suppositories, creams, ointments) usually combine, locally applied corticosteroids, anaesthetics, lubricants, protectors and venotonics. Studies suggest that, in the short term, topical treatments improve localized symptoms [7].

There is evidence that herbal preparations are also effective in relieving hemorrhoids symptoms especially in the early stages of disease. Usually botanical extracts with anti-inflammatory, analgesics, wound healing, antibacterial, and astringent properties are used [11, 15]. Also, application of an *Aloe vera* herbal wound healing cream on the surgical site after open hemorrhoidectomy was effective in reducing postoperative pain on resting and during defecation and healing time, in the patients compared with the placebo group [3].

Allium ampeloprasum var. *porrum* L. (leek) is a perennial plant, cultivated and used as food. The bulbs are used in Brazil, in respiratory and digestive disorders, for its anti-inflammatory and antitussive properties, respectively spasmolytic and stomachic [1]. Moreover, a saponine isolated from *A. ampeloprasum* var. *porrum* L., demonstrated antiinflammatory and gastroprotective properties using murine *in vivo* models [1].

Other *Allium* species such as *A. cepa* and *A. sativa* are known for their wound healing properties. It was previously shown that topical administration of *Allium* extracts reduced the inflammatory stage of healing, increased the wound contraction rate and decreased the epithelization period [4, 20]. Moreover, phytoconstituents such as tanins and flavonoids were considered to play a key role in the wound healing activity, due to antibacterial and radical scavenging properties [20]. In addition, an *A. ampeloprasum* subsp. *iranicum* cream was tested in a double-blind randomized placebo control trial that showed significantly better outcomes for the leek group compared to the standard antihemorrhoidal cream and placebo groups for bleeding severity and overall subjective improvement [13].

Therefore, in this research we aimed to assess the wound healing effect of three gels obtained from *A. ampeloprasun* var. *porrum* bulb, stem and leaves extracts on an animal model, as a preliminary study for future development of topical preparations with potential use in symptomatic hemorrhoid disease.

MATERIALS AND METHODS

CHEMICALS AND REAGENTS

Carbopol 940, sodium benzoate, triethanolamine, thiopental sodium and nefopam were purchased from Sigma-Aldrich (Sigma-Aldrich, Taufkirchen, Germany). A commercially available ointment (Cicatrizin®, Tis Pharmaceutical, SA) containing *Symphytum officinale*, *Hypericum perforatum*, *Matricaria recutita* and *Calendula officinalis* extracts was used as the reference treatment (positive control) in the animal wound healing model [12]. Solvents used for extraction and gel preparation were of commercial grade.

PLANT EXTRACTS PREPARATION

A. ampeloprasun var. *porrum* bulb, stem and leaves were provided by Hofigal SA, Romania. The identity of plant materials was confirmed and voucher specimens were stored at the Pharmaceutical Botany and Cell biology Department, University of Medicine and Pharmacy “Carol Davila”, Bucharest. All three materials were grounded with a Swantech Sample Mill SJ 500, France. Each material was extracted under reflux for 60 min at 100 °C with water, 20 % ethanol and 20 % glycerol, respectively, using a material:solvent ratio of 1:10 (w:v). The extractive solutions were stored at 4 °C after filtration. The selection of extraction solvents was done taking into consideration the feasibility of further incorporation into a topical pharmaceutical formulation, such as gels, since both glycerol and ethanol are used as common excipients for such forms.

TOTAL POLYPHENOL AND TOTAL FLAVONOID CONTENTS

The determination of total polyphenol content (TPC) and total flavonoid content (TFC) were performed using the Folin-Ciocalteu method ($\lambda = 750$ nm) and the aluminum chloride method ($\lambda = 429$ nm), respectively [14, 17]. Both assays were performed in triplicate and the absorbances were measured with a UV-VIS spectrophotometer (Halo DB-20-220; Dynamica, Salzburg-Mayrwies, Austria). Standard calibration curves were used for calculating TPC and TFC, which were

expressed as the means \pm standard deviation (SD) of gallic acid equivalents (GAE) mg/L for *TPC*, and as mg/L quercetin equivalents (QE) for *TFC*.

HYDROGELS PREPARATION

The *A. ampeloprasun* extract gels were obtained as follows: 2 % carbopol 940 gels were diluted with each leek extract in increasing concentrations under continuous stirring, whereas the 2 % carbopol base gel was obtained with carbopol 940, water, sodium benzoate (0.1 %), triethanolamine, and glycerol (24 %). The spreadability and pH values were determined for all three hydrogels [8].

EXPERIMENTAL PHARMACOLOGY

All experimental procedures have been performed in accordance with bioethics norms in animal research for scientific purposes, under Law 43/2014 on the protection of animals used for scientific purposes and Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes. The scientific protocol has been approved by the Bioethics Commission of Carol Davila University of Medicine and Pharmacy, Bucharest.

Male Wistar rats weighting between 200–300 g (8–12 weeks old) have been purchased from INCDMI Cantacuzino, Bucharest. Food (grains for mice and rats, Cantacuzino Institute, Bucharest) and drinking water were available to animals *ad libitum*. Animals were housed under constant humidity and temperature, monitored with a thermohygrometer, recorder values ranging between 35–45 % for humidity and 21 ± 1 °C for temperature, respectively. At the end of the experiment, rats were euthanized according to the standard protocols, by administration of an intraperitoneal overdose with 200 mg/kg b.w. thiopental sodium.

The effect on wound healing was evaluated using an experimental model based on the induction of burn injuries on rodents and the investigation of their healing rate [2, 6]. Thirty male Wistar rats were divided into 5 equal groups ($n = 6$) and the dorsal fur was shaved 24 hours prior to experimentation.

During the first experiment day, general anesthesia was induced by intraperitoneal administration of 60 mg/kg b.w. thiopental sodium. Moreover, animals received intraperitoneal injections of nefopam 20 mg/kg b.w. for analgesia. After disinfection with alcoholic solution for surgical use, burns were created by placing on shaved dorsal skin cylindrical metallic bodies with a mass of 100 g and a diameter of 2 cm, heated in physiological saline at 100 °C. The hot bodies were left on the animal skins for 10 seconds. After 5 min, animals were topically given on the burnt surface 0.2 mL of the preparations shown in Table 1. The topical preparations were administered once daily for 14 days. The aspect of the wounds was recorded with a digital camera after burn induction (day 0) and after 1, 3, 5, 7, 10 and 14 days.

Macroscopic wound analysis was performed using the ImageJ v.1.52 software (National Institutes of Health, Bethesda, MD, USA) by measuring the wound area (cm²). The healing rate was determined by quantification of wound contraction rates using formula (1).

$$\text{Wound contraction rate (\%)} = \frac{\text{Initial wound area} - \text{Measuring day area}}{\text{Initial wound area}} \times 100 \quad (1)$$

Table 1

Experimental groups and treatments

Group	Name	Treatment
1	CON	gel base (control treatment)
2	REF	Cicatrizin® (reference treatment)
3	APA	aqueous extract gel of <i>Allium ampeloprasum</i>
4	APE	20 % ethanolic extract gel of <i>Allium ampeloprasum</i>
5	APG	20 % glycerolic extract gel of <i>Allium ampeloprasum</i>

STATISTICAL ANALYSIS

Statistical analysis of obtained experimental data has been performed using GraphPad Prism v.5.0 software (GraphPad Software Inc., San Diego, CA, USA). The distribution of biological data was determined using Kolmogorov-Smirnov normality test. Parametric and non-parametric statistical tests were applied with 95 % confidence intervals (CI95%) and statistical significance threshold was set as $\alpha = 0.05$. Results were expressed as mean \pm standard deviation (mean \pm SD) and mean \pm standard error of the mean (mean \pm SEM).

RESULTS

AND TOTAL PHENOLIC CONTENT

Total flavonoids Experimental data obtained from *TFC* and *TPC* assays are shown in Table 2. *TFC* was obtained in large amounts by extraction with water and 20 % glycerol, while *A. ampeloprasum* aqueous extract showed the highest *TPC* content.

Table 2.
TFC and TPC of *Allium ampeloprasum* extracts

Sample	TFC (mg/L)		TPC (mg/L)	
	Mean \pm SD	CI95%	Mean \pm SD	CI95%
<i>Allium ampeloprasum</i> aqueous extract	16.40 \pm 0.056	15.89 – 16.91	206.6 \pm 6.888	189.5 – 223.7
<i>Allium ampeloprasum</i> 20 % ethanolic extract	13.09 \pm 1.096	3.23 – 22.93	190.8 \pm 6.535	174.6 – 207.1
<i>Allium ampeloprasum</i> 20 % glycerolic extract	16.91 \pm 0.756	10.11 – 23.70	203.7 \pm 6.599	187.3 – 220.0

SD – standard deviation ($n = 3$); CI95% – 95 % confidence interval of mean.

HYDROGELS PREPARATION

Gel formulations using carbopol were successfully prepared, were characterized by a translucent, clear, red-brown aspect, and had a pH of 7.3 ± 0.3 (Hanna HI-98127 pH Tester). Macroscopic inspection showed that all gel formulations containing *A. ampeloprasum* extracts were homogeneous and presented a good spreadability.

EFFECT ON WOUND HEALING

A wound healing animal model was implemented for evaluating the efficacy of *A. ampeloprasum* extract gels on injuries created by burn induction. The burn duration of 10 seconds yielded wounds with a mean size of 6.06 ± 0.84 cm², the minimum burn area being 5.1610 cm² and maximum 8.2580 cm². The lowest wound area was observed for the reference group, while the highest mean area was recorder for the group assigned to *A. ampeloprasum* 20 % ethanolic extract treatment (Fig. 1).

The wounds created in experimental animals were round and pale, with distinctive margins showing signs of erythema. The white aspect of the injuries reveal the induction of severe edema. Moreover, no blisters were noticed in wounded rats (Fig. 2). After 1 and 3 days of topical treatment applications, the edema persisted and small signs of crust formation were observed. During 5th and 7th days of wound monitoring, darker, brown crusts were clearly formed in all groups, but the healing

signs were more obvious for groups receiving Cicatrizin ointment and *A. ampeloprasum* 20 % ethanolic and 20 % glycerolic extract gels. After 10 days, signs of wound shrinkage were more obvious for the reference treatment group. Furthermore, during the final day of the experiment, crust detachment and epithelization could be noticed, although both processes were incomplete.

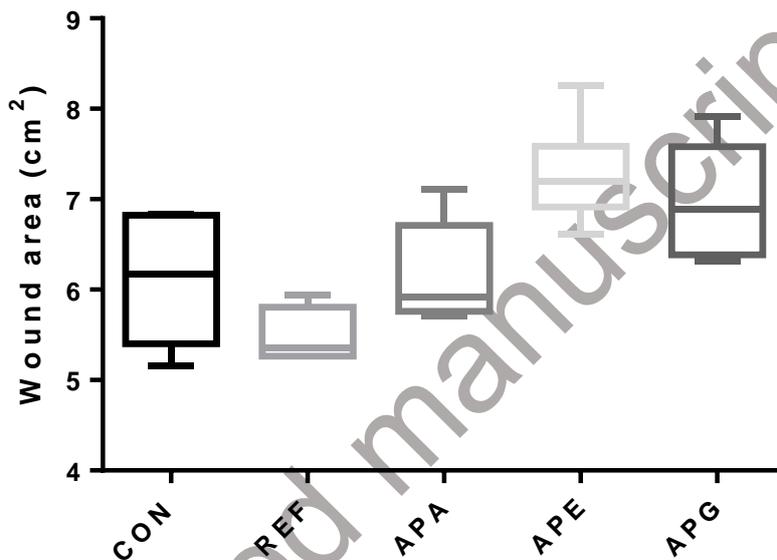


Fig. 1. Box plots of wound areas (cm²) induced by burns with metallic cylindrical bodies.

The evolution of wound healing effects is depicted in Fig. 3, expressed as wound contraction rates. The recorded healing rates were non-linear during the 2 weeks experiment. In day 1, the highest contraction rates were observed for REF and APG rat groups, while for APA group there was an increase in burnt area. Interestingly, contraction rates increased from day 1 to day 3 for all groups, except for REF, which registered a seemingly plateau effect until 5th day, and a slight increase in wound size from day 5 to day 7. Another increase in healing rates was observed for the rest of the groups during measurements from 5th day of the experiment. During day 7, an increase of mean contraction rate was observed only for APA group, whilst all the remaining treatment groups showed slight decrease in wound size. From day 7 to day 10, an abrupt decrease of wound area was noticed for CON and REF groups, an intermediate healing rate for APA and APG groups and a plateau effect for APE treatment. Between 10th and 14th days, the highest increase

in contraction rates was recorded for APE group. After 2 weeks of topical treatment, the highest wound healing rates were observed for groups treated with CON, REF and APE, while APA and APG treatments produced a lower recovery rate than the base gel or reference ointment.

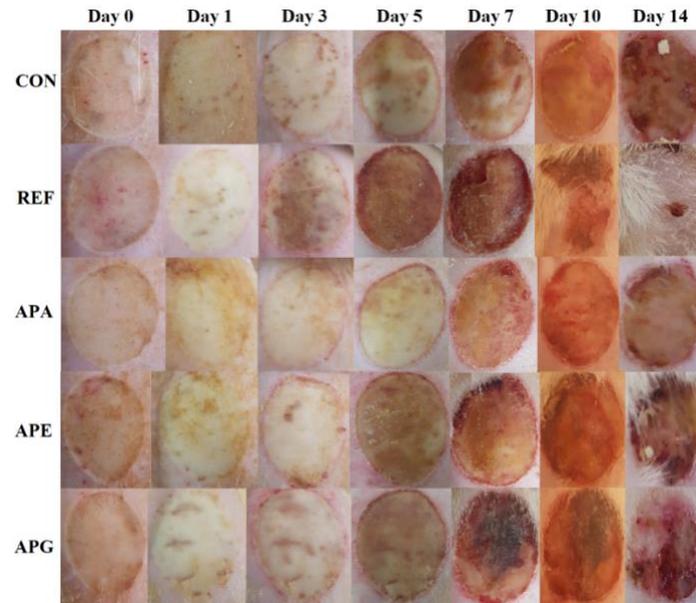


Fig. 2. Over time macroscopic changes of burn wounds in Wistar rats during 2 weeks treatment.

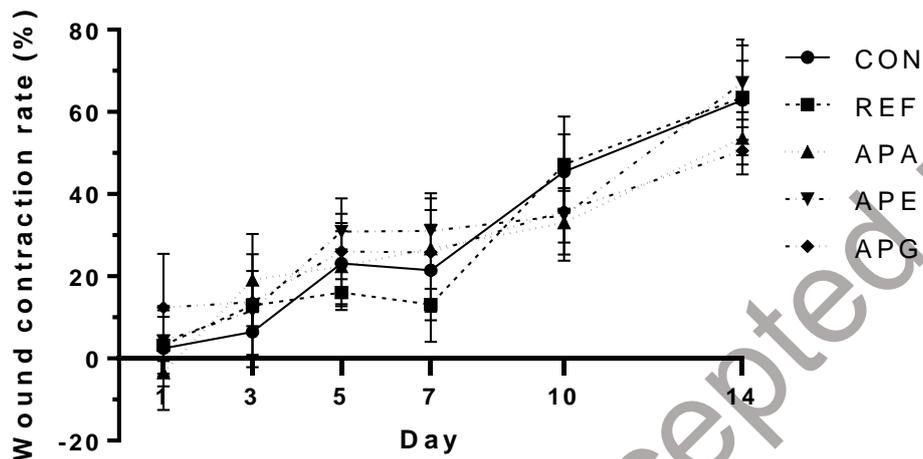


Fig. 3. Mean wound contraction rates (%) over 2 weeks.

Most notably, 3 days after injury induction, reference treatment lead to a higher statistically significant wound contraction of 12.86 % (t Student, $p < 0.05$), the effect on wound healing being 99.74 % higher than the control group (6.44 %). Moreover, treatment with the aqueous extract gel induced a healing rate of 19.10 %, which was significantly higher than the control by 196.68 % (t Student, $p < 0.05$). Both 20 % ethanolic and 20 % glycerolic extracts induced higher wound contraction rates than control, but the effects on wound healing were statistically insignificant. In the 5th experiment day, treatment with gel prepared from *A. ampeloprasum* 20 % ethanolic extract showed a mean contraction rate of 30.90 % and the effect was 33.57 % higher than the base gel (23.13 %), although the observed difference was close to the established statistical significance threshold (t Student, $p = 0.0599$). After 2 weeks of gel application, mean contraction rates of both reference (63.56 %) and 20 % glycerolic extract gels (67.13 %) were slightly higher than the rate of control group (62.85 %), showing stronger effects on wound healing with 1.13 % and 6.82 %, respectively. The recorded effects were not statistically significant (Fig. 4).

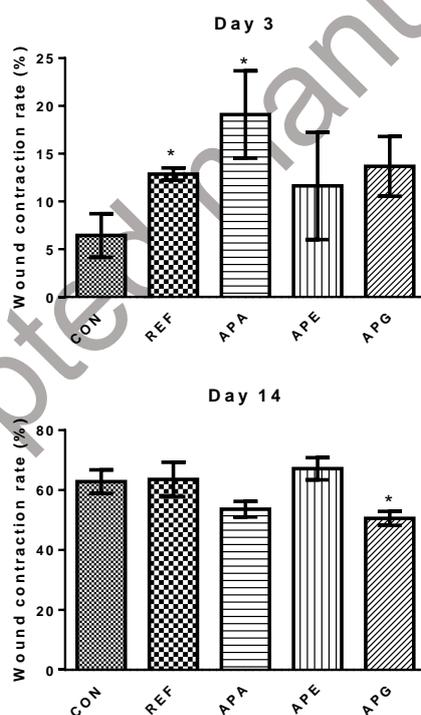


Fig. 4. Mean \pm SEM wound contraction rates measured after 3, 5 and 14 days of topical treatment: * – $p < 0.05$; # – $p = 0.0599$.

Since the *A. ampeloprasum* 20 % ethanolic extract showed the lowest total flavonoids and total phenolic content, the effect on wound healing could not be correlated with these classes of phytochemicals. Therefore, our findings suggest that the effects on wound healing of *A. ampeloprasum* var. *porrum* could be attributed to other phytochemical complexes. Surprisingly, wound contraction measurements revealed that both alcoholic and 20 % glycerolic extracts showed positive effects on wound healing after 1 week of topical treatment, but manifested a negative impact after 2 weeks. On the other hand, the 20 % ethanolic extract gel produced only slight differences in contraction rates between 5th and 10th day of application, but pronounced positive effects between days 1–5 and 10–14.

CONCLUSIONS

The beneficial effect of the hydrophilic gel formulated with *A. ampeloprasum* var. *porrum* 20 % ethanolic extract on the rat model of wound healing could warrant its potential use in the topical treatment of hemorrhoid disease. Further studies are needed to assess the effects of *A. ampeloprasum* extracts on histopathological modifications after burn injuries and to investigate the negative effects of hydroalcoholic and hydroglycerolic extracts on wound healing after 1 week of topical application.

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