Journal of
Clinical and Experimental Pharmacology

Bettega et al., Clin Exp Pharmacol 2016, 6:1
DOI: 10.4172/2161-1459.1000201

Research Article Open Access

Experimental Confirmation of the Utility of *Nasturtium officinale* Used Empirically as Mouth Lesion Repairing Promotor

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Abstract

Nasturtium officinale R.Br. (Brassicaceae), "watercress", is an herbal plant widely consumed as food by many cultures. Along with this, it is used in folk medicine to help in treating asthma, bronchitis, hypertension, as well as other diseases. Rural Italian communities have used *N. officinale* as an adjuvant to cure *bovine stomatitis* without any scientific evidence that supports its use for such end. This study evaluated the efficacy of *N. officinale* as an adjuvant on the healing of traumatic ulcers on rat tongue dorsum.

Method: In four control-experiment group-pairs (eight animals per group) there were punch induced 0.5 mm lesions on tongue dorsum, to mimic traumatic ulcers. Rats from control groups received saline solution onto lesions daily. Animals from experimental groups received oral solution containing 15% *N. officinale* ethanolic extract onto lesions. Animals were euthanized at 2, 7, 14, and 21 days after lesion induction. Lesions were qualitatively evaluated by hematoxilin-eosine stain, and quantitatively, by picrosirius red stain under polarized light.

Results: There were observed higher deposits of total, immature and mature collagen in experimental groups than its respective control groups after 14 and 21 days.

Conclusion: Results revealed that the solution containing 15% *N. officinale* extract promoted wound healing on rats tongue dorsum with higher collagen deposits.

Introduction

The most common mouth cavity lesions are the traumatic ulcers. The loss of the epithelium and the exposition of the underlying conjunctive tissue characterizes the lesions, causing injury and discomfort [1,2]. Empirically, different population groups use plant extracts as auxiliaries on the traumatic ulcers healing process [3-5].

Searching for cost-effective and easily accessible herbal remedies, which might induce or improve the repairing process, our group realized that *Nasturtium officinale* (common name "water cress") has been applied for long by Italian rural communities for veterinarian purpose in stomatitis treatment [6,7]. Besides that, *N. officinale* showed therapeutic potential to heal ulcer on chemical or heat-induced rabbit skin [8].

N. officinale is a perennial medicinal plant, belong to the family of Cruciferae (Brassicaceae), it can be found in many countries and be traded as a fresh plant. It is eaten raw in salads, or can be cooked in soups. Moreover, it is also used in popular medicine in several countries as expectorant, hypoglycemic, stomach stimulating, and anti-hypertensive [9,10]. It has also some beneficial effects in diabetes, bronchitis, tuberculosis, asthma and scurvy [11].

In despite of its ethno botany and ethno veterinary importance, and how easy it is to obtain *N. officinale*, there are no studies have been published yet that proof its efficiency to treat traumatic ulcer, which is very common in dentistry.

The objective of this study was to evaluate *N. officinale* as an adjuvant in the healing process on rat tongues.

Materials and Method

Extract of *N. officinale* and oral solution

The vegetable material was collected in December, 2005, identified by Dr. Gert Hatschbach and registered at the Curitiba Botanical Museum (Brazil), under the number 248503. The material was pre-established in environmental conditions and established with forced ventilation to 45°C . The granulometric regulation was made in a knife grinder, with a sieve of 8 mm.

A cotton blanket of 3 cm tick was adapted over a porous Teflon plate, inside the Soxhlet extractor [12]. 500 grams of vegetal material was added in the extractor, 1500 ml of 85% ethanol over the vegetal material, to wetting, for 30 minutes. It was added 1500 ml of 85% ethanol in a 3000 ml balloon attached to the extractor. After having connected the condenser on the extractor, it started the heating process to continuing extraction. After 5 hours of extraction, the balloon extract was concentrated to approximately 800 ml. The extract was transferred to the volumetric balloon and the volume was completed with 85% ethanol.

Each milliliter of the oral solution to be used in the study had 0,15 ml of extract of *N. officinale*, 1,080 mg of feniletilglucosinolato (PEITC),

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Received: November 17, 2015; **Accepted:** January 05, 2016; **Published:** January 11, 2016

Citation: Bettega PVC, Johann ACBR, Alanis LRA, Bazei IF, Miguel OG, et al .(2016) Experimental Confirmation of the Utility of *Nasturtium officinale* Used Empirically as Mouth Lesion Repairing Promotor. Clin Exp Pharmacol 5: 201. doi:10.4172/2161-1459.1000201

0,846 mg de flavonoids (in rutin) e 0,256 mg of phenylpropanoid (in chlorogenic acid). The extract of *N. officinale* was regulated within these three elements which are the oral solution markers.

Animals

To accomplish this study, the project was evaluated and approved by the Animal Use Ethnical Committee (CEUA-PUCPR–445/2009). For that end, it was used 64 male Wistar rats with age of 45-50 days and corporal mass of ca.200 g. They were fed with pelletized ration Nuvilab® CR-1 (Nuvital Nutrientes Inc.) and water ad libitum, with clear-dark cycles of 12 hours and environmental temperature (25 ± 1°C).

Inducing the ulcers

The rats were anesthetized with sodium thiopental (30 mg/kg) via intraperitoneal before the mechanical inducing in the very middle of the top of the tongue, through a punch of 0.5 mm. The technique consists in rotating and pressing movements with the tool over the area to be removed, it has to be deepening until one realizes some resistance [13]. During the following three days of inducing ulcers, the animals were treated with mepiridina (2 mg/kg i.p.) to reduce pain in order to facilitate feeding.

Experimental treatment

Soon after the mechanical induction of ulcer, rats were organized in eight groups of eight species. Four groups acted like controls (Gctrl2, Gctrl7, Gctrl14, Gctrl21) and received topical application of sterilized saline solution only once a day, always at the same time. The other four groups (Gexp2, Gexp7, Gexp14 e Gexp21) received daily aliquot of oral solution (50 µL) of *N. officinale* on the ulcers for 2, 7, 14, and 21 days, respectively. The animals in each pair of experimental-control-groups were euthenized by overdose of sodium thiopental (120 mg/kg) via intraperitonial and intracardiac injections, after the mentioned periods.

Processing and histological analysis

After the death of each pair of experimental-control-groups, the tongues were removed with a blade of scalpel 12 and conditioned in universal collectors with buffered formalin 10%. The tongues placed on formalin were organized in blocks with paraffin and the areas with ulcers were divided up to 5 µm thick, according to standard technique. The cuts were diaphanized and by hematoxilina-eosina (HE) and picrosirius red (SR) [13].

The cuts by HE were evaluated qualitatively in optical microscope Olympus® Medusa BX-40 with magnification of 400X, by two independent observers. The variable intensity of inflammation, content of collagen and angioneogênese were all evaluated.

The cuts colored by SR were evaluated in microscope Olympus® BX-50 with polarized lens Olympus® U-Pot and micro camera Dinolite® with magnification of 400X, by only one observer, to determine the presence of mature and immature collagen on the area of lesion. The mature lesion presents an orange-red color, while the immature is yellow-green [14]. To aim analysis and statistics, the images of four areas were randomly obtained. The images were analyzed in the program of morphometry Image Proplus™ 4.5 (Media Cybernetics, Silver Spring, MD), which supplied the mature and immature collagen area, expressed in squared micrometers. The final values were determined from the average of the four areas.

Statistical analysis

It was used the statistical package SPSS® 18.0 (SPSS Inc., Chicago, IL.), the significant level adopted was 5% ($p<0,05$). The normality Kolmogorov-Smirnov test was done, in which it was realized that only two of the eight treatments did not show normal distribution, two-way ANOVA was opted. The variance homogeneity test of Levene revealed that the variance was heterogeneous, being opted by the parametrical multiple comparison test of Games-Howell.

Results

Qualitative analysis

Two days after the mechanical induction of ulcer, the control group as well as the experimental group, showed the same characteristics of intense polymorph nuclear inflamed filtered, similar amount of collagens fibers and few newly formed blood vessels (Figures 1A and 1B).

Seven days have passed after the induction of ulcers and the inflamed filtered polymorph nuclear and mononuclear showed itself invariable in the experimental-control pair. The filtered was moderate or intense in both groups. However, in Gexp7 a larger number of fibroblasts and higher concentration of collagen fibers were observed (Figures 1C and 1D). On the other hand, Gctrl7 showed larger angiogenesis.

As time went by, the mononuclear inflamed filtered showed a tendency of reduction, varying from intense to discreet, with similar proportion of blood vessels, in both Gctrl14 and Gexp14. Similar to the previous one, it was realized a larger amount of fibroblasts and of collagen fibers in Gexp14 than Gctrl14 (Figures 1E and 1F).

The healing process evolved to consolidation and, at the end of period of 21 days post ulceration, the inflamed infiltered showed discreet or inexistent with similar proportion of blood vases in Gctrl21 and Gexp21. As expected, a visible increase in fibroblasts, deposition of more organized collagen fibers, and remodeling of the tissue was observed in Gexp21 (Figures 1G and 1H).

Quantitative analysis of the collagen content

The parametrical multiple comparison test of Games-Howell revealed a larger amount of total collagen in the experimental group than in the control group, in the 14 days groups (1220,66 ± 413,05 μm^2 versus 218,83 ± 77,25 μm^2, $p=0,00296$), as well as in the 21 days groups (979,79 ± 411,22 μm^2 versus 143,41 ± 26,58 μm^2 , $p=0,01835$) (Figure 2).

A greater amount of immature collagen was verified comparing Gexp14 with Gctrl14 (304,36 ±110,87 μm^2 versus 72,88 ± 23,03 μm^2, $p=0,00534$). Similarity of behavior was also observed after 21 days post ulceration (205,07 ± 59,54 μm^2 versus 59,53 ± 14,86 μm^2, $p=0,09321$) (Figure 2).

Larger amount of mature collagens were found in the experimental groups Gexp14 (916,30 ± 308,35 μm^2 versus 145,95 ± 55,04 μm^2, $p=0,0343$) and Gexp21 (774,72 ± 354,40 μm^2 versus 83,88 ± 14,30 μm^2, $p=0,01298$) in relation to their respective control (Figure 2).

Discussion

N. officinale has been used in different therapeutic and dietary purposes by several ethnical groups. However, to the best of our knowledge, *N. officinale* has been applied empirically bysome Italian rural communities as a repairing promoter in stomatitis that shows

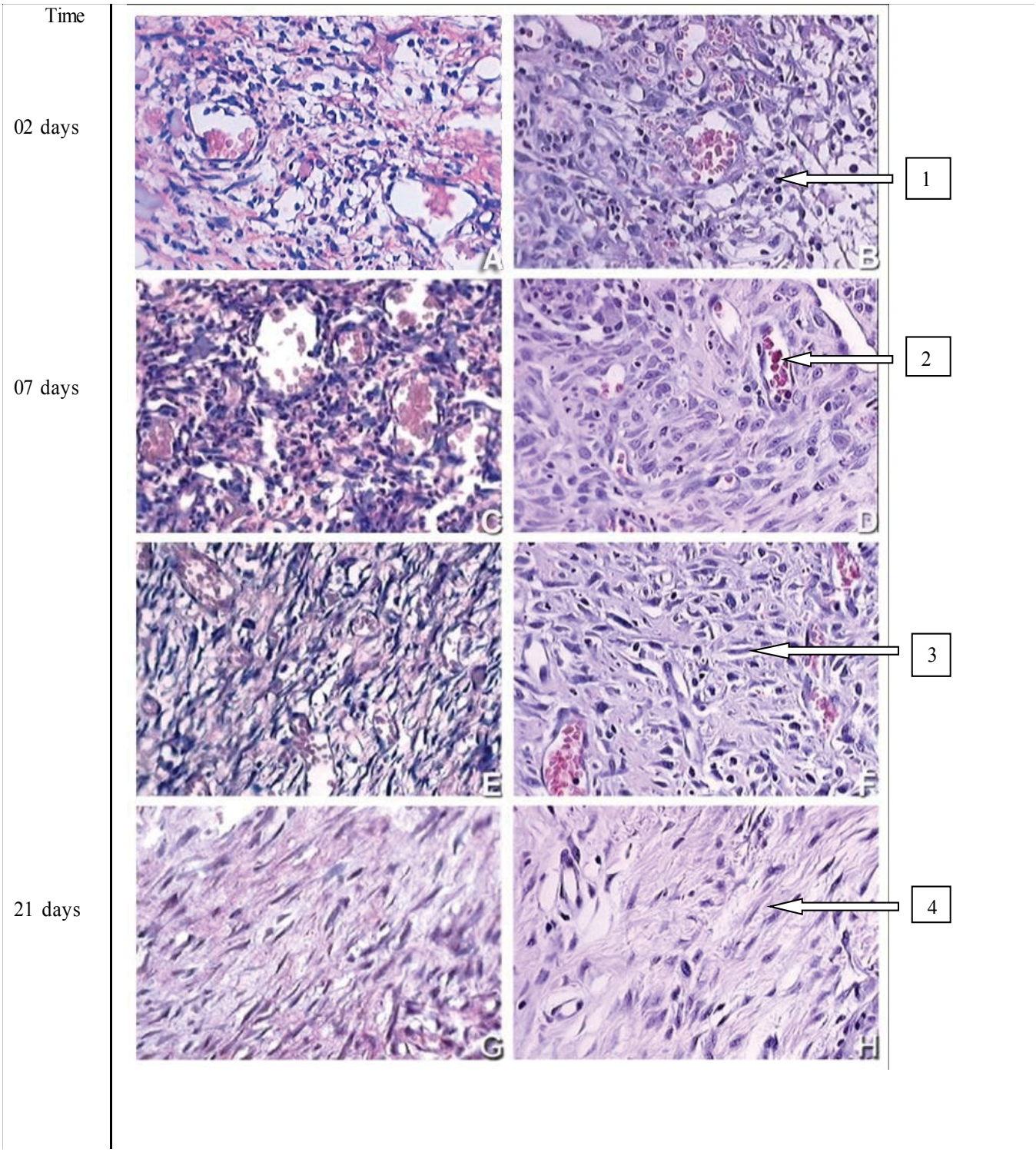

Figure 1: Lesion qualitative evaluation. Photomicrography of the injured area of experimental and control groups in the periods of 2, 7, 14 and 21 days (magnification 400X, HE), showing evolution of the process along the period.

Label:

1 Inflammatory cells
2 Blood vessel
3 Fibroblasts
4 Collagen fibers

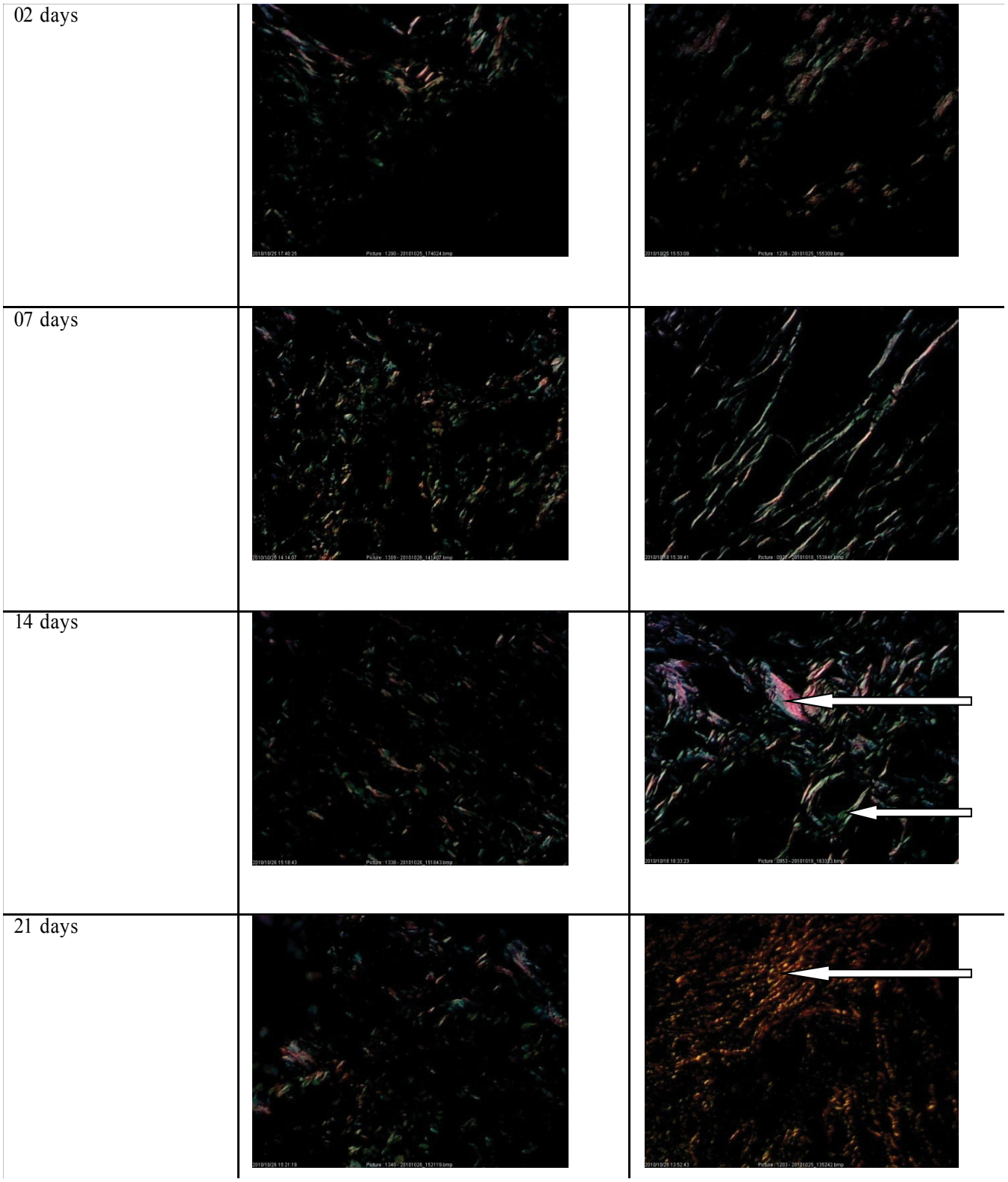

Figure 2: Collagen quantity evaluation by picrosirius red technique.
Photomicrography of injury on rat tongues from control and experimental groups in the periods of 2, 7, 14 and 21 days (magnification 400X, SR).
Label: Mature collagen (5); Immature collagen (6)

ulcer type characteristics [6]. As *N. officinale* is a plant that is adapted to different climates and can be grown and maintained easily. Importantly, this plant has a potential of therapeutic applications and healing which paved the way for evaluating such applicability in a detailed way.

The mouth traumatic ulcer's healing process is very complex, being characterized by taking out the conned tissue and replaced by a conjunctive tissue. The standard healing process can be divided in (1) inflamed stage; (2) proliferative stage of fibroblasts and blood vessel; and (3) maturing stage and collagen remodeling [15-17]. Any medicinal strategy that promotes or accelerates the remodeling to the physiological condition is certainly desirable, because it would collaborate to reduce the discomfort and the risk of later infection.

It was observed that the solution of *N. officinale* promoted an increase in fibroblasts with a consequent lift of deposition of collagen before the seventh day of post ulceration, and it remained constant throughout the experimental interval. Still, the phytochemical accelerated the substitution rate of immature collagen (rich in fibers, type III thin) to mature collagen (rich in fiber, type I thick) [18,19].

The greater deposition and acceleration in the transition of types of collagen contribute in the healing process of the ulcer [20]. In the context, the results obtained show that the *N. officinale* can be useful as an efficient therapeutic adjuvant in the formation of conjunctive tissue which sustains the repair of injury.

Further studies are warranted to examine the mechanism of action of *N. officinale* solution in promoting collagen maturation and healing process. Based on the proposal made by these authors [3,21], our group hold the theory that flavonoids (expressed in rutin) should inhibit the activities of metalloproteinase of matrix-1 (MMP-1) and/or reduce the expression of gene MMP-1, that would be involved in increasing the total collagen rate on the ulcer area.

Another possible explanation stems about the possibility that the PEITC found in *N. officinale* presents antimicrobial and antioxidant activities that would lead to a cleaner wound and favor the healing process [8]. Although such hypothesis may not be the most appropriate to explain the phenomenon. The retention of solution on the ulcer area and posology applied do not favor the contact of PEITC on the point of application for sufficient time intervals, to obtain the time-kill effect against a full microbial population.

It is noteworthy that the solution of *N. officinale* did not properly improve the inflammatory events, because the experimental and control pair showed a similar degree of inflammation along the experiment. Such findings show that *N. officinale* might not have intrinsic anti-inflammatory activities which also support the fact that its use for anti-inflammatory purposes is inconsiderable.

Of note, he angioneogenesis, an ordinary phenomenon during inflammatory events, was less pronounced in Gexp7. It is possible that components of *N. officinale* had promoted a more rapid secretion of collagen in such way that it implicated the rising of new vessels. That dynamic of events, in normal conditions (i.e., in the controls), was expected to occur after about the 14th day [22]. Still, *N. officinale* might have accelerated the reduction of vessels to be around the 7th day. Once more, it seems that flavonoids (expressed in rutin) might be responsible for relative collagen increment, with a consequent reduction in the blood vessels' density.

Although a fibrinopurulent membrane was noticed in equal densities in the control and treatment groups (data not showed), the presence of necrotic cells or other poisonous evidence were not detected. These findings suggest that the formulation used here was safe and did not imply any undesirable local effects.

The experimental therapeutic posology in this study consists of a daily administration of *N. officinale* solution. It is possible that a dosage paradigm that involves more frequent daily applications might contribute to the healing process and decrease the time needed for a full recovery. Moreover, the pharmaceutical formulation used (oral solution) might not have maximized the contact of *N. officinale* solution with the ulcerated area. Interestingly, the ethno veterinary practice of Italian rural communities have successfully treated the ulcerated areas in cattle through the use of raw leaves of *N. officinale* as a co-adjuvant which can increase the contact time between the ulcer and the raw leaves [6]. Based on the premise that the availability of the active principles in the leaves in nature might be smaller than the one in the extract applied here, it is reasonable to assume that the ulcerated areas were exposed to ta higher concentration of these active principles in the formulation used here compared to applying the raw leaves.

Maybe formulations in orabase increment the substantivity of phytochemical with a longer retention of the same and consequent increase of repairing activity. By narrowing the action area of the phytochemical and hamper its dispersion, an orabase presentation, besides raising the punctual repairing potential, it could also minimize undesirable effects on other tissues and organs, as well as supplant therapeutic contraindications as stomachic and intestinal ulcers [23] and pregnancy [24]. Such assumptions should be evaluated in following studies which cover dosage pharmacotechnic variations.

Conclusion

The watercress has been used by Italian rural communities in managing ulcers in cattle. However, this study, for the first time, provides an evidence of the healing potential of *N. officinale* as an adjuvant in treating mouth ulcer and its acceleration of repairing process. Watercress extract may offer a better therapeutic alternative for patients suffering from mouth ulcers. However, this study was conducted on rats and further studies are warranted to verify its applicability in humans.

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