https://doi.org/10.31925/farmacia.2021.4.18 **ORIGINAL ARTICLE**

CHITOSAN SUPPORTS CONTAINING *IMPATIENS NOLI-TANGERE* AND *SYMPHYTUM OFFICINALE* HYDROALCOHOLIC EXTRACTS IN BURNS TREATMENT: ANTIMICROBIAL AND HEALING EFFECTS

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Abstract

Burn injuries have a major impact on population health, an optimal burn wound dressing with both healing and antibacterial effect being needed. Our purpose was to assess the healing efficacy of chitosan supports containing different quantities of a hydroalcoholic extract obtained from *Impatiens noli-tangere* and *Symphytum officinale*, in a rat burn wound model. The antibacterial effect of the extract was also assessed. The efficacy of topical daily administration of the supports and of silver sulphadiazine was evaluated by measuring the thermal lesion area. In addition, tissular hydroxyproline content and IL-6, TNF-alpha plasmatic levels were measured. The chitosan support with the highest content of plant extract showed the most pronounced healing effect. The mixed extract was shown to possess a moderate antibacterial effect, the most sensitive microorganism being *S. aureus*.

Rezumat

Arsurile au un impact major asupra sănătății populației, pansamentul optim al arsurilor necesitând, atât efect de vindecare cât și antibacterian. Scopul studiului a fost de a evalua eficacitatea suporturilor de chitosan conținând cantități diferite de extract hidroalcoolic obținut din *Impatiens noli-tangere* și *Symphytum officinale*, într-un model de leziuni termice la șobolani. De asemenea, a fost evaluat efectul antibacterian al extractului. Eficacitatea administrării locale zilnice a suporturilor și a sulfadiazinei de argint a fost evaluată prin măsurarea ariei leziunii. În plus, au fost determinate conținutul de hidroxiprolină tisular și nivelele plasmatice IL-6, TNF-alfa. Suportul cu cel mai mare conținut de extract a prezentat cel mai mare efect de vindecare. Extractul mixt a demonstrat efect antibacterian moderat, microorganismul cel mai sensibil fiind *S. aureus*.

Keywords: chitosan, healing effect, burn wound model, *Impatiens noli-tangere*, *Symphytum officinale*

Introduction

Burns are pathologies associated with high morbidity and mortality. Worldwide, more than 6 million people suffer from severe burns, with a rate of death exceeding 300,000 persons *per* year [1]. The topical preparations available for their treatment contain mainly anti-bacterial substances, such as silver sulphadiazine, rather than substances promoting healing [2]. Several medicinal plants improve the healing process of burn wound, by promoting various phases of the wound healing process [3, 4].

The rat burn model was found to possess translational accuracy in wound healing studies [5]. The thermal lesion results in an inflammatory response, with macrophages being attracted to the wound site. They impede microbial wound infection and initiate wound closure. Secreted cytokines, such as tumoural necrosis factor alpha (TNF-α) and interleukin-6 (IL-6), activate fibroblasts to produce type III collagen and fibronectin and also to secrete further cytokines which in turn attract keratinocyte cells to the injury site [6]. Keratinocytes are essential in the re-epithelialisation process, thus restoring the barrier function of the epithelium [7].

Symphytum officinale L., comfrey, *Boraginaceae* family, has been used in traditional medicine for its anti-inflammatory, analgesic and anti-oedematous effects, attributed to polysaccharides and polyphenolic compounds in its composition [8, 9]. The content in allantoin, another main constituent of *S. officinale*, is positively correlated with cell division intensification, the growth of connective tissue, bone, cartilage and with wound healing enhancement [8, 9]. *Impatiens noli-tangere*, touch-me-not, *Balsaminaceae* family, is being used traditionally for its antioxidant [10-12], anti-inflammatory [11, 12] and anti-microbial effect [12], having a high content of organic acids, anthraquinones, flavonoids and phenolic acids.

Hydroalcoholic extracts of these two Romanian indigenous plants were embedded in chitosan dressings,

and their healing effects were assessed using a model of rat thermal lesion. Chitosan is a biopolymer with high biocompatibility and widely used as a topical dressing in wound management as it possess intrinsic antimicrobial properties, and ensures an optimal delivery of extrinsic antimicrobial and healing agents to wounds and burns [13, 14].

Materials and Methods

Plant material

Extraction procedure - ultrasound-assisted extraction

Ground plant material was extracted with ethanol/ water (50% v/v for *I. noli tangere* and 70% v/v for *S. officinale*, 100 g ground material *per* litre of solvent) in an ultrasonic apparatus (Elma Transsonic 460/H, frequency 35 kHz) for 1 h. The extract was filtered under vacuum (No.1 Whatman filter paper), processed by microfiltration (MF) (Millipore filters with 45 µm), followed by ultrafiltration (UF) (Millipore membrane with cut-off 1000 Da) using a KMS Laboratory Cell CF-1.

Preparation support with bioactive compounds

Chitosan supports were prepared by evaporation method. 2.1, 4.2 respectively 6.3 mL of each extract were entrapped in the polymeric solution by dispersion, obtaining supports with 6 cm diameter.

Estimation of total polyphenols content

The phenolic total content was determined by the Folin-Ciocâlteu method, as previously described [15]. Total polyphenols content was expressed as gallic acid equivalents (GAE) in mg/L of extract.

Estimation of total flavonoid content

The total flavonoid content was assessed using the adapted version of aluminium chloride colorimetric method [16]. The results were expressed as mg rutin equivalents (RE)/L of extract.

HPLC-MS analyses of phenolic compounds

The polyphenol content was determined using a previously described HPLC method [3], using a HPLC system, equipped with a C18 Nucleosil 3.5, 4.6 mm x 50 mm, Zorbax column, and coupled to a MS detector (Shimadzu LCMS-2010 EV, Shimadzu Europe). A mix of formic acid in water (pH = 3.0)/formic acid in acetonitrile (pH = 3.0) was employed as mobile phase. The polyphenolic compounds separation was performed using binary gradient elution. Gallic acid, chlorogenic acid, ellagic acid, caffeic acid, rutin, rosmarinic acid, luteolin, quercetin, quercetin 3-β-D-glucoside, apigenin, umbelliferone and kaempferol were used as reference standard.

Antibacterial effect of the plant extract

The hydroalcoholic plant extract was tested *in vitro* against 5 bacterial species and strains: *Bacillus subtilis* ATCC 6623, *Proteus mirabilis* ATCC 29245, *S. aureus* ATCC 25923, *S. epidermidis* ATCC 12228 and *Escherichia coli* ATCC 8739, using the disk diffusion method [17]. Sterilized Petri dishes (9 cm diameter) were inoculated with 0.01 mL of bacterial inoculums (10^5 - 10^6 bacteria *per* mL), in Muller-Hinton agar. Sterile filter paper discs (Whatman, 6 mm in diameter) loaded with 6.3 mL mixed plant extract, were placed on the top of Mueller-Hilton agar plates. Gentamycin, ciprofloxacin and ampicillin were used as positive control. Ethanol (50%) was used as negative control. The Petri dishes were placed at 4°C for 1 - 2 h and then incubated at 35 ± 0.1°C for 18 - 24 h. At the end of the period, the inhibition zones formed on the media were measured with a transparent ruler in millimetres.

Animals

Wistar adult male rats (n = 35; 197 ± 48 g), purchased from "Carol Davila" University Biobase, Bucharest, were left for five days to accommodate, before starting experimental procedures. They were housed in a ventilated cage system, with a bedding of wood sawdust, under controlled light/dark cycle conditions (12 h light/12 h dark; lights on at 6:00 AM), with free access to water and food pellets. The temperature ranged between 20 and 22°C and the relative humidity was maintained at 35 - 45%. All procedures were carried out according to EU Directive 2010/63/UE and with the approval of the Institutional Animal Care and Use Committee.

Burn wound model

Rats were anesthetized with thiopental and dorsum was shaved. The shaved area was disinfected with 3 x 3 cm sterile gauze soaked in alcohol. A 100 g cylindrical stainless-steel rod (1 cm diameter) was heated to 100°C in boiling water. Temperature was monitored using a thermocouple. Burn infliction was limited to the loin. The rod rested on its own weight for 10 seconds at two different sites on each rat. The size of the wounds was measured with a micrometre. Animals received analgesia during the postoperative period.

Study design

After burn infliction, the following topical treatments were applied daily: group 1 – no treatment (control group); group 2 – 1% silver sulphadiazine 1 mL *per* application; group 3 – chitosan support impregnated with 2.1 mL of mixed extract, prepared from equal quantities of each individual plant extract (S1); group 4 – chitosan support impregnated with 4.2 mL of mixed extract, prepared from equal quantities of each individual plant extract (S2); group 5 – support impregnated with 6.3 mL mixed extract, prepared from equal quantities of each individual plant extract (S3). The wounds were covered with a sterile gauze with adhesive margins and absorbent body, 7.2 x 5 cm (Cosmopor, Hartmann). The general appearance and degree of wound healing of the burn wound (the lesion area) were assessed on days 1 (after 10 hours after burn infliction), 2 (after acute administration), 4 and 7 (after subacute treatment). The area of the

lesion was calculated using the following formula: $A = \pi R_m^2$, (R_m = average radius).

Assessment of hydroxyproline content

On day 8, the animals of each group were euthanized by decapitation. The tissue of the lesion was excised, weighed, dried at 600°C for 12 hours and the weight after drying was determined. After samples processing, the absorbance was measured at 500 nm and the hydroxyproline content was calculated using a standard curve of pure L-hydroxyproline [18].

Assessment of cytokines concentration

Blood was taken from the tail at day eight and centrifuged. It was determined the concentration of tumour necrosis factor alpha (TNF-α) and interleukin 6 (IL-6) using an ELISA kit according to the manufacturer's instructions (Enzo Life Sciences, USA). The limits of detection for TNF-α and IL-6 were 5, respectively 20 pg/mL.

Statistical analysis

Statistical analysis was performed using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego California USA). D'Agostino & Pearson test was used for establishing the distribution of the response. Data are reported as means ± standard error of the mean (SEM). Parametrical Student's t-Test was employed. A confidence interval (CI) of 95% was employed and p values of 0.05 or less were considered to be significant.

Results and Discussion

Extracts characterization

The total polyphenols and total flavonoids content measured in the extracts are presented in Table I.

The HPLC-MS analysis was used to identify the individual polyphenols present in the examined extracts. The results are displayed in Table II.

Table I

The total polyphenols and total flavonoid content of assessed extracts

Extract	Total polyphenols content, mg GAE/L	Total flavonoids content, mg QE/L
***Impatiens noli-tangere* hydroalcoholic extract**		
MF extract	590.2	184.8
UF concentrate extract	983.7	449.5
***Symphytum officinale* hydroalcoholic extract**		
MF extract	999.4	83.1
UF concentrate extract	1303.1	114.1

MF = microfiltration, UF = ultrafiltration

Table II

HPLC-MS polyphenolic profile of extracts

	Concentration of polyphenolic compounds (µg/mL)	
Compound [M/z]	***I. noli-tangere***	***S. officinale***
Ursolic acid [455.4]	5.56 ± 0.4	4.05
Chlorogenic acid [353]	4.37 ± 0.2	0.25
Caffeic acid [179]	-	3.08
Rosmarinic acid [359]	49.86 ± 2.8	224.93
Umbelliferone [161]	-	2.95 ± 0.1
Quercetin [301]	10.33 ± 0.8	3.79 ± 0.1
Luteolin [285]	0.14 ± 0.01	0.17 ± 0.01
Apigenin [269]	0.09 ± 0.01	-
Rutin [609]	44.93 ± 3.0	4.91 ± 0.1
Ellagic acid [301]	1.58 ± 0.1	0.38 ± 0.02
Quercetin 3-β-D-glucoside [463]	56.70 ± 4.2	8.89 ± 0.2

Antimicrobial activity

The susceptibility of the bacterial strains to the extract as well as that of the positive controls, can be found in Table III.

The 5 bacterial strains used for the microbiological testing are frequently isolated in burn wounds [19]. Our results indicate that the hydroalcoholic extract possess moderate antibacterial activity, in accordance to similar studies [20, 21]. *S. aureus*, the bacteria which have been found to be one of the most common species able to readily infect burn wounds, seems to be the most sensitive microorganism, possibly as a result of specific phenolic compounds [20, 21].

Table III

Antimicrobial activities of the tested hydroalcoholic extract

Substance	**Inhibition area (mm)** ± SEM				
	S. aureus ATCC 25923	*S. epidermidis* ATCC 12228	*B. cereus* ATCC 11778	*P. mirabilis* ATCC 29245	*E. coli* ATCC 8739
Hydroalcoholic extract	21 ± 0.2	17 ± 0.4	4 ± 0.3	7 ± 0.3	6 ± 0.2
Ethanol	ND	ND	ND	ND	ND
Ampicilin 10 µg/disc	31 ± 0.7	21 ± 0.7	11 ± 0.5	19 ± 0.6	9 ± 0.3
Ciprofloxacin 10 µg/disc	30 ± 1.1	29 ± 0.8	33 ± 1.4	38 ± 1.2	36 ± 1.1
Gentamicin 10 µg/disc	28 ± 0.6	18 ± 0.2	29 ± 0.5	29 ± 0.8	26 ± 1

Values are the mean of 3 replicates. ND: Not determined

Rate of wound contraction

The wound evolution based on the values of the average surface/group of the thermal lesion areas before treatment (day 1) and following acute (1 administration) and subacute treatment (4 and 7 days of treatment) are presented in Figure 1.

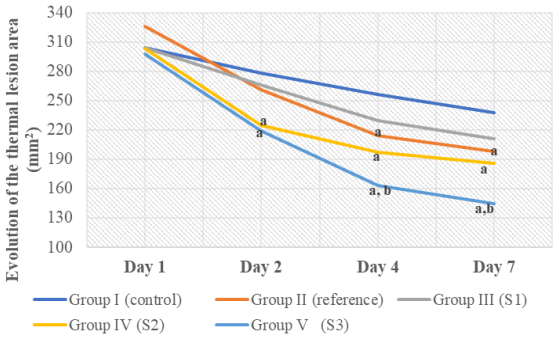

Figure 1.

Evolution of the thermal lesion area (mm^2)

a – change *vs*. control ($p < 0.05$, t Student, CI 95%); b – change *vs*. group II (reference) ($p < 0.05$, t Student, CI 95%)

A better healing pattern characterized through a higher wound contraction rate was observed in all treated groups when compared to control. Support 3 showed greater healing properties when compared with silver sulphadiazine, the results suggesting a significant enhancement of the cicatrisation process.

Biochemical analysis

The results of the assessment of the hydroxyproline content in granular tissue are given in Figure 2

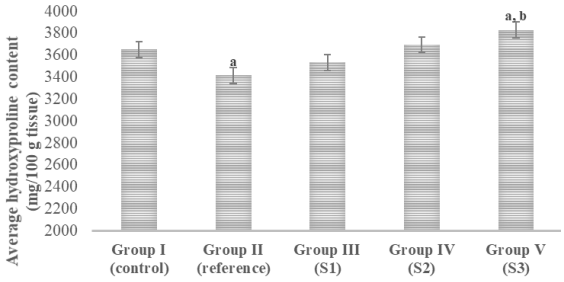

Figure 2.

Average content of hydroxyproline in granular tissue

a – change *vs*. control ($p < 0.05$, t Student, CI 95%); b – change *vs*. group II (reference) ($p < 0.05$, t Student, CI 95%)

Collagen ensures the integrity and the strength of the extracellular matrix and is essential for healing [22]. Hydroxyproline, a major constituent of collagen, is used as an index of collagen turnover [3, 4, 23].

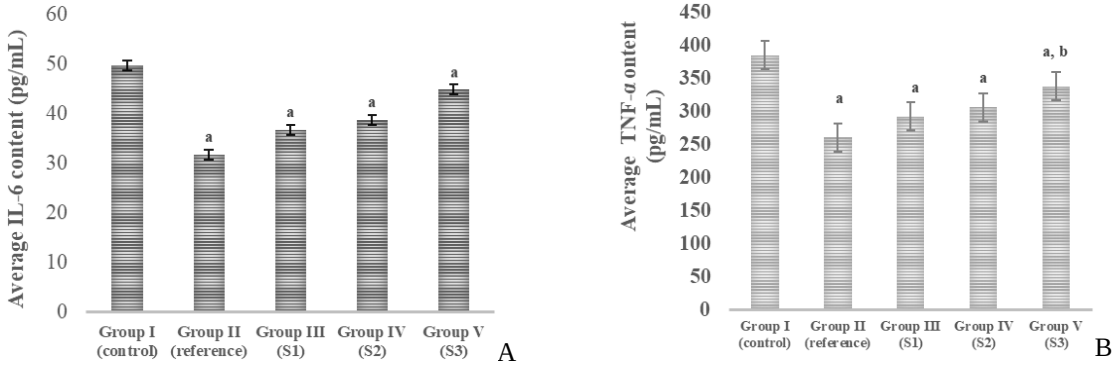

Figure 3.

Average blood concentration of proinflammatory cytokines

A – mean blood concentration of IL-6. B – mean blood concentration of TNF-α.

a – Change *vs*. control, $p < 0.05$, t Student, CI 95%; b – Change *vs*. group II (reference), $p < 0.05$, t Student, CI 95%

Support 3 significantly increases hydroxyproline content in the granular tissue, when compared to both control and silver sulphadiazine groups, indicating an increased collagen synthesis and thereby an increased

cellular proliferation. Along with increasing collagen content, all treated groups present a lower serum level of IL-6 and TNF-α when compared with the control group (Figures 3A, 3B).

Silver sulphadiazine determines a significant decrease in the serum concentrations of IL-6 and TNF-α *vs*. control. These results are in accordance with those of several authors [22, 24]. They also associate the alteration of the cytokines profile with a low healing effect of silver sulphadiazine [18-20]. The macrophages infiltrated at the site of injury secrete proinflammatory cytokines, such as IL-6 and TNF-α [22-26]. TNF-α determines the cascade release of secondary cytokines and humoral factors can induce the production of fibroblast growth factor-7 (FGF-7), suggesting that it can indirectly promote reepithelialisation [22, 25], while Il-6 has a mitogenic and proliferative [25] effect on keratinocytes [22]. Both cytokines seem to be necessary for the healing process, hypothesis which is in accordance with our data, support 3, the one that reduces non-significantly the cytokine profile when compared to control, demonstrating the greatest healing effect.

This decrease of cytokines level could be partly due to the presence of polyphenols and allantoin in the hydro-alcoholic extracts [26-30]. Furthermore, allantoin was shown to possess a complex healing effect, with topical application of allantoin leading to reduced chemotaxis of inflammatory cells into rat wounds and increased fibroblast proliferation and collagen synthesis [27-31], supporting thus the synthesis of extracellular matrix during wound healing.

In vitro assays demonstrated that caffeic acid and chlorogenic acid accelerated the proliferative response of fibroblasts, thus enhancing wound healing [29]. Polyphenolic compounds present in hydro-alcoholic extracts were shown to possess antioxidant and free radical scavenging properties, preventing the release of reactive species responsible for the oxidative stress and tissue damage in burns [10, 11].

Conclusions

Chitosan supports containing *Impatiens noli-tangere* and *Symphytum officinale* hydroalcoholic extracts possess a significant healing effect. The underlying mechanism is complex, including reduced chemotaxis of inflammatory cells into rat wounds and increased fibroblast proliferation and collagen synthesis.

Furthermore, the supports possess moderate anti-bacterial effect against *S. aureus*, one of the most common pathogens isolated in infected burn wounds, possibly due to specific phenolic compounds. These results suggest that the above-mentioned supports could be valuable tools for the treatment of burn wounds.

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Conflict of interest

The authors declare no conflict of interest.

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