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In vivo comparative assessment of incised wound healing in rats after application of hydrogel/organogel formulation containing St. John's wort methanol extract

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Over the years, St. John's wort (*Hypericum perforatum* L.) has been shown to contain important bioactive ingredients with substantial physiological and pharmacological activity. The present study aims to evaluate the healing intensity of incised wounds on rats treated with a semi-solid hydrogel/organogel (bigel) formulation containing a hyperforin-rich extract from St. John's wort. Three methods to obtain hyperforin-rich methanol extract from St. John's wort were applied and evaluated for effectiveness – percolation method, ultrasonic extraction, and Soxhlet extraction. The extracted amount of hyperforin was determined by reverse-phase HPLC analysis. The Soxhlet extraction technique was most appropriate for this study's purposes (3.552 mg/mL). Hyperforin-rich methanol extract was included in a bigel as a semi-solid formulation. The therapeutic potential of the developed formulation was evaluated for healing intensity and compared with a commercial product. Both were applied for 10 days on incised wounds (50 mm) inflicted on rats. The efficacy parameter is defined as the tensile strength applied on already healed wounds through a particular experimental setup. An *in vivo* experiment was performed with 21 male Wistar rats, divided into three groups at random. Group A was not treated with therapeutic products. Groups B and C were treated with a commercial product, and with bigel containing an extract of St. John's wort, respectively. The tensile strength registered for group B ((3.7±0.2) N) was lower than that stated for group C ((6.4±0.7) N). The obtained differences are statistically significant ($p<0.05$). As a result of the study accelerated and most effective wound healing was found in the experimental group treated with bigel containing St. John's wort extract rich in hyperforin.

Keywords: bigel, *Hypericum perforatum* L., hyperforin, tensile strength.

INTRODUCTION

Plants have long been used as wound healing agents, being a good source of diverse phytocomponents that, compared to synthetic molecules, are easily absorbed by humans and animals and are therefore an alternative model for drug development.

St. John's wort (SJW) has a rich historical background, one of the oldest used in traditional medicine and, therefore, the most extensively investigated medicinal herbs [1]. The medicinal properties of this species were known even back in Hippocrates' time due to its wound/burn healing and anti-inflammatory action [2]. Modern methods of qualitative and quantitative analysis prove the content of a rich palette of biologically active substances (BAS), which are used in the healing of wounds and various skin disorders, as well as in the treatment of diseases of the gastrointestinal, respiratory, and nervous systems (depression) [3].

SJW is the main source of active pharmaceutical ingredients (APIs) such as hyperforins (phloroglucinols) and hypericins (naphtho-dianthrones), and many other BAS, as a broad range of flavonoids (rutin, quercetin, miquelianin, quercitrin, amentoflavone, hyperoside). It has been found that the major BAS in SJW is hyperforin (HPF). The highest HPF concentrations were found in leaves, flowers, and fruits [4].

HPF belongs to the polycyclic polyprenylated acylphloroglucinols family and has a unique architecture. HPF is a mixture of bicyclic interconverting tautomers derived from SJW. The structure contains asymmetric vicinal quaternary centers and a densely functionalized tetracarbonyl

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array (Fig. 1) [5]. HPF is poorly stable when exposed to light and oxygen [6]. However, it is quite stable in protic solvents such as methanol and in *in vivo* systems, as well as at low temperatures, which to some extent explains its broad therapeutic potential [7, 8]. HPF exhibits good protective and potent antioxidant and anti-inflammatory activity in topical applications.

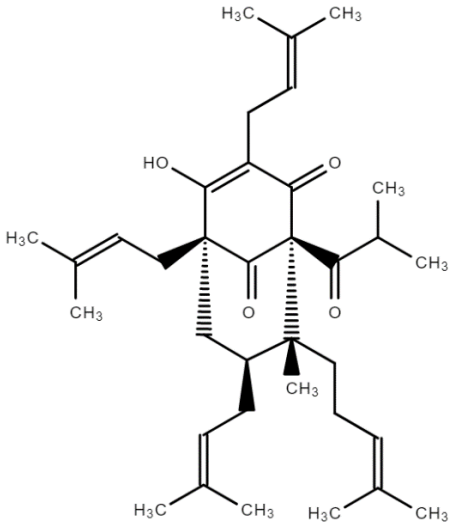

Figure 1. Chemical structure of hyperforin.

Furthermore, HPF does not induce phototoxicity. In dermal application, HPF is used for the treatment of skin diseases, such as neurodermatitis. The treatment with hyperforin-rich extract dermal products leads to improvement of the stratum corneum moisture, subjective skin parameters and reduces skin surface dryness, indicating stabilization and improvement of the barrier [9].

For effective administration of hyperforin, alone or in combination, it is indispensable that an optimal topical formulation has to be used. Furthermore, to ensure the dermal carrier's compliance, compatibility, and stability, it is desirable to consider the lipophilic characteristics of hyperforin and the necessary conditions for skin hydration [10]. In this relation, in the interest of the study, an inherently innovative hybrid dermal dosage form was chosen, optimally meeting the listed criteria.

In the family of semi-solid formulations, bigels are innovative structurally hybridized systems of two different phases – hydrogel and organogel. The symbiosis between these two types of gels allows overcoming some of the main disadvantages of the initial semi-solid systems, namely the limited ability of the hydrogel to penetrate the skin's lipophilic barriers and the low patient compliance of organogel due to its stickiness and oily residues [11]. At the same time, they expressly combine the advantages of the two precursor forms: 1) the ability to include both hydrophilic and lipophilic drug substances; 2) enrichment of hydration of stratum corneum; 3) provision of opportunities for local, transdermal, and modified drug delivery; and 4) improved patient compliance [12].

The present study evaluates the healing intensity of incised wounds on rats treated with a bigel formulation containing a hyperforin-rich extract from SJW. The article describes extracts preparation from SJW (flowers and leaves), determination of HPF, obtaining a bigel for topical application, and wound modeling and assessment.

MATERIALS AND METHODS

Materials

All materials used in the study including SJW dried flowers and leaves powdered to approx. 2 mm (Bilec Company, Bulgaria); methanol (≥99.9%, analytical grade, Fisher Chemical); hyperforin standard (≥ 85%, HPLC grade, Merck, Germany); acetonitrile (> 99.8%, HPLC grade, Fisher Chemicals UK), phosphoric acid (HPLC grade, Acros Organics Germany); double-distilled water (Gesellschaft für Labortechnik mbH, Germany), borago oil (Alteya Organics Bulgaria), poloxamer 407 (Sigma Life Science, USA), sorbitan monostearate (Span® 60, Thermo Fisher Kandel GmbH, Germany), ketamine 5% (Bremer Pharma GmbH, Germany), xylazine 2% (Alfasan Int., Netherlands), jodseptadon 10% (Chemax Pharma Ltd., Bulgaria), sodium chloride 0.9% solution (B. Braun Melsungen AG, Germany) were of pharmaceutical grade.

Methods

Preparation of the experimental extracts. Three different methods for SJW extracts preparation were used – percolation, sonication, and hot Soxhlet extraction, as all of them were performed with methanol in the absence of light.

Extract 1 **(E1)** was obtained by a percolation method in a closed vessel for 24 hours. First, 1 g of SJW was extracted at room temperature with 100 mL of MeOH. Next, the solvent was removed, and the solid residue from the extraction process was compressed to optimize yield. Finally, the liquid obtained after the compression and the solvent removed earlier were mixed and concentrated to a final volume of 50 mL.

Extract 2 **(E2)** was obtained by a sonication method. First, 1 g of dried SJW material was extracted with 100 mL of MeOH for 30 minutes at 40°C in an ultrasonic bath. Then, the reagent used was removed, and a new extraction was performed with 100 mL of MeOH for 30 min at 40°C. Finally,

the two extracts were combined and concentrated to a final volume of 50 mL.

Extract 3 **(E3)** was obtained by the Soxhlet extraction method**.** The plant material of SJW (5 g) was extracted with MeOH (500 mL) for 5 h (approximately nine cycles) in the Soxhlet apparatus [8].

After the condensation, as a general procedure, 1 mL sample of each of the extracts was taken for HPLC analysis, whereafter extracts obtained were shielded from light in dark bottles and stored in a refrigerator at -18°C until subsequent use.

HPF HPLC determination

Chromatographic conditions. The HPLC-UV analysis of HPF was performed with a Thermo Scientific UltiMate 3000 Analytical LC System, equipped with a variable wavelength vibration detector (Dionex UltiMate 3000 VWD) and a diode array detector (Dionex UltiMate 3000 DAD-3000 Diode Array Detector) (Thermo Scientific, USA). Thermo Scientific HYPERSIL GOLD AQ C_{18} (150 mm × 4.6 mm, 5 µm) analytical column, protected by an HYPERSIL GOLD AQ C_{18} (10 mm × 4.6 mm, 5 µm) guard-column, was used. The elution was in isocratic mode with a mobile phase consisting of 0.3 % phosphoric acid and acetonitrile (10/90, v/v). The flow rate was set at 0.8 mL/min, and the temperature of the columns and the autosampler was maintained at 25°C and 10°C, respectively. The wavelength of the UV detector was set at 273 nm, and the injection volume was 20.0 µL. Thermo Scientific™ Chromeleon™ 7.2 Chromatography Data System software™ was used for the systemic control and data analysis.

The qualitative and the quantitative determination of HPF were made according to the retention time and UV spectrum of the substance in the standard samples. In addition, quantitative analysis was performed using the method of external standardization.

Sample preparation

Standard solutions – A standard stock solution of HPF with concentration of 50.0 µg/mL was prepared in methanol. During the development of the method it was found that isocratic elution with acetonitrile and 0.3% aqueous solution of phosphoric acid is most suitable and provides sharp and symmetrical peaks of HPF. After serial dilutions working standard solutions with concentration of 50.0, 40.0, 30.0, 20.0, 10.0 and 1.0 µg/mL were obtained. They were used for calibration curve construction (x = concentration of the standard solutions [µg/mL] and y = peak area [mAU*min]) and method validation.

For method validation, the parameters proposed by the International Conference on Harmonisation (ICH) were evaluated [13].

Test solutions – samples of herbal extracts were filtered with syringe filters Minisart® NY25 (0.2 µm, d = 25.0 mm, Sartorius™, Germany). The so-obtained samples were serially diluted with methanol to obtain specimens with a final concentration of 5.0 µg/mL. After that, six aliquots of each extract were injected into the HPLC system.

Bigel preparation

The two phases (hydrophilic and lipophilic) required for the bigel composition were prepared separately. The hydrogel contains Poloxamer 407 25.0% and purified water 75.0 % (w/w). The organogel contains borago oil 85.0 % (w/w) and Span® 60 15.0 % (w/w). The weighed poloxamer 407 was dispersed in the purified water (25°C, 400 rpm). A stable hydrogel was formed. Span® 60 and E3 (1 mL) were dissolved in borago oil (60°C, 100 rpm). A bigel was obtained at hydrogel/organogel ratio of 70:30. The heated organogel was added step by step to the hydrogel under continuous stirring (500 rpm) to obtain a homogeneous mixture and cool to ambient temperature. Color, homogeneity, consistency, and phase separation of the bigel were inspected visually [14].

Experimental animals

The study was performed on 21 male Wistar rats weighing between 200 and 250g each, provided by the Medical University of Varna, Bulgaria. The animals were situated in plastic cages in a well-ventilated room at a temperature of 22 ± 1°C, relative humidity about 55%, and a 12/12 light/dark cycle was maintained. They were subjected to a standard pellet diet and water *ad libitum* throughout the experiment in the Vivarium of MU-Varna.

The experimental protocol was implemented with the approval of the Commission for Ethical Treatment of Animals at the Bulgarian Food Safety Agency (permit number: 265/02.06.2020). Furthermore, all empirical procedures were conducted according to the relevant institutional and national rules and regulations following the international guidelines (EU Directive, 2010/63/EU for animal experiments), the Basel Declaration [15], and the International Council for Laboratory Animal Science ethical guideline for researchers [16].

Wound modeling by incision

The experimental animals were anesthetized by intramuscular application of 5% ketamine (35.0 mg/kg) and 2% xylazine (5.0 mg/kg). The skin

of the dorsum (thoracolumbar region) of each rat was prepared for aseptic surgery by shaving and sterilization with povidone-iodine (10% cutaneous solution) [17]. Two paravertebral incisions, 5 cm long, located 1.5 cm from the midline on both sides of the rat, were made through the entire thickness of the skin. The wounds were closed with three interrupted surgical sutures and cleaned daily with 0.9% saline [18].

Experimental rats were randomly divided into three experimental groups with 7 animals per group. Group A was defined as a control group without treatment. Group B was defined as a reference group where rats were treated with a medicinal product – multifunctional cream, a blend of herbal extracts from *Aloe Vera*, *Prunus amygdalus*, *Vitex negundo*, and *Rubia cordifolia*. Group C was defined as an experimental group, and animals were treated with a bigel contained SJW extract. The test formulations were administered topically, once daily for a research period of ten days. The sutures were eliminated on the 9th day. On the 10th day, the wound healing strength was determined by measuring the force required to disrupt its integrity. By tensiometer was measured the tensile strength of the repaired skin after simulated surgical wounds with an experimental setup [19], which was constructed in our laboratory (Fig. 2).

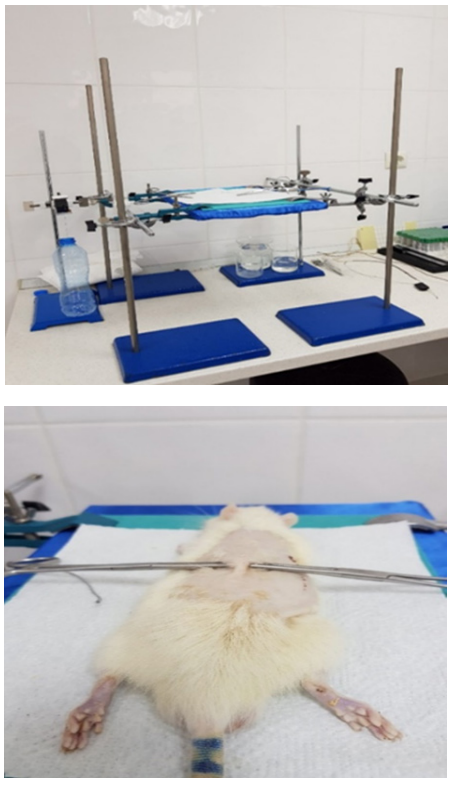

Figure 2. Experimental setup for determination of the force of rupture of the wound.

Determination of the force of rupture of the wound

The tensile strength of the treated restored skin is considered a parameter for skin repairing. A force gauge Halda Haldex AB 150 Switzerland, was used to measure the rupture force of the wounds. A calibration model was created to establish the range of values measured with the force meter *($y=0.2086x$, $R^2=0.9991$)*.

Statistical analysis

All measurements during the *in vivo* experiment were made seven times. The results shown in the tables are averaged. The standard deviation is calculated. The experimental data were approximated by linear dependences, and the coefficient of determination was obtained. ANOVA: single factor analysis was performed. The rate for statistical significance is defined as the accepted significance level $p < 0.05$.

RESULTS AND DISCUSSION

Three types of extracts were obtained by the described methods and then concentrated to a final volume of 50 mL. Their color and odor were assessed for compliance with the investigated herb. A validated HPLC method was used to quantify HPF in the extracts samples.

Results from the HPLC-UV analysis

Linearity in the range of 1.0 - 50.0 µg/mL was assessed (sixfold analysis) by the straight-line equation (*$y = 0.3028x - 0.0053$*) and the correlation coefficient ($R^2 > 0.999$).

The LOQ of HPF was found to be 1.0 µg/mL referring to a signal-to-noise ratio ($S/N \geq 10$). In addition, the comparison of series blank and standard samples showed the ability of the system to detect the target analyte unequivocally (Fig. 3).

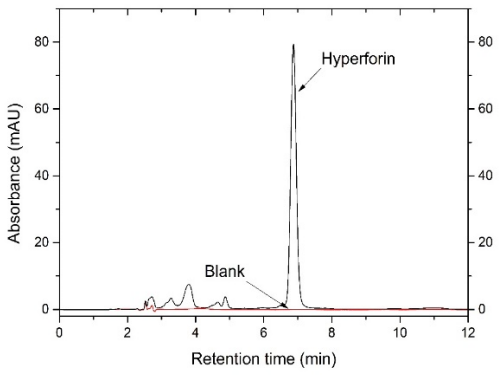

Figure 3. Comparison between a standard sample of HPF (black) and a blank sample (red).

Table 2. Quantitative analysis of HPF in samples.

№	Name of the sample	Peak area [mAU*min]	Sample concentration [µg/mL]	Concentration of plant extract [mg/mL]
1	E1	3.33	11.01	2.20
2	E2	1.93	6.38	1.28
3	**E3**	**5.37**	**17.76**	**3.55**

The developed HPLC method was used to evaluate the analytical yield of the extraction procedures.

The presented results are an argument for the choice of extract E3 obtained by the Soxhlet extraction method, as it provides the highest extraction yield of HPF (Fig. 4 and Table 2).

Bigel preparation

A stable biphasic semi-solid formulation (bigel) for topical use as a vehicle of hyperforin-rich extract of SJW was obtained. A tube inversion test confirmed the gel formation. The bigel formulation had a white color with a creamy appearance and a pleasant scent of borage oil.

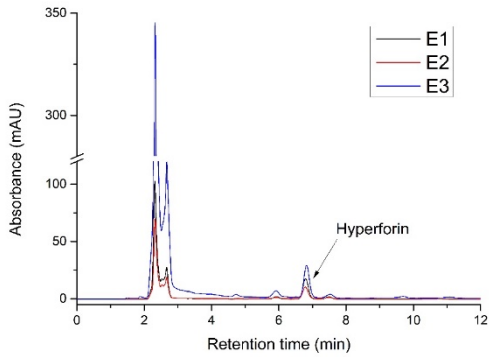

Figure 4. Overlapped chromatograms of test samples (E1 - black; E2 – red; E3 – blue).

Results from in vivo experiment

The effects of the bigel containing E3 extract of SJW were tested over 10 days in an experimental group of 7 rats. The average mass (m, g) required to tear the wounds of rats and the average values for calculated tensile forces (P, N) by groups and their standard deviations are shown in Table 3.

Table 3. Average of tensile forces by groups

Group	m, g	P, N	SD, N
A	397.55	3.95	0.20
B	382.26	3.75	0.23
C	666.67	6.41	0.70

Similar experimental results were found between control group A (without treatment) and reference group B (treatment with the commercial product). The average values of tensile forces for these groups are similar, because they lie in the same confidence interval. The experimental group C, composed of rats treated with bigel containing SJW extract, showed a significant difference and greater forces required to tear the wounds compared to the other 2 groups.

The results obtained from one-way analysis of variance (ANOVA) are presented in Tables 4, 5 and 6.

A statistical difference was found between control and experimental groups as well as between reference and experimental groups. A comparison was made between groups A and B. There is not a statistically significant difference between groups A and B ($F_{critical} > F$). The results demonstrate that the hyperforin-rich SJW extract containing bigel significantly increases the rupture resistance of the healed wound more than in the control and reference groups. These results complement the trends identified in previous studies and confirm the role of hyperforin as a potent anti-inflammatory and antioxidant agent [20, 21]. Undoubtedly, the included borage oil, which has been proven to have broad therapeutic potential, also contributes to these results [22].

Table 4. ANOVA analysis between control group A and reference group B

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups A and B	0.06	1	0.06	0.33	0.58	4.97
Within Groups A and B	1.85	10	0.19			
Total	1.91	11				

Table 5. ANOVA analysis between control group A and experimental group C

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups A and C	21.12	1	21.12	19.91	0.0012	4.96
Within Groups A and C	10.60	10	1.06			
Total	31.73	11				

Table 6. ANOVA analysis between reference group B and experimental group C

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups B and C	23.44	1	23.44	19.81	0.0012	4.96
Within Groups B and C	11.83	10	1.18			
Total	35.27	11				

CONCLUSIONS

The lack of specific wound healing products other than antibiotics, anti-inflammatory, and analgesic medicinal products used in allopathic treatment is one of the main reasons for the continued search for effective alternative resources for this purpose.

This study found accelerated and most effective wound healing in the experimental group treated with bigel containing SJW extract rich in HPF. Further studies of SJW and hyperforin, respectively, for its wound healing properties, will help determine its efficacy and range of application.

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