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Research article

EXPLORING THE THERAPEUTIC POTENTIAL OF BEE VENOM COMPONENTS IN WOUND HEALING: A COMPREHENSIVE EVALUATION OF MORPHOMETRIC, BIOCHEMICAL, AND HISTOPATHOLOGICAL MARKERS

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Bee venom (BV) and its components, secretory phospholipase A2 (sPLA2) and *Apis cerana* secapin-1 (AcSecapin-1), have potential effects on wound healing. This study aims to evaluate impact of BV, sPLA2, and AcSecapin-1 on full-thickness wound healing in male Wistar Albino rats over a 7-day period. Various morphometric (body weight, wound contraction), biochemical (hydroxyproline, oleic acid, IL-8, TGF-β1, redox parameters), and histopathological markers (reepithelialization, inflammatory cells, angiogenesis, fibroblast activation, and collagen density) were assessed. Treatment with sPLA2 and AcSecapin-1 increased oleic acid levels. IL-8 levels increased with sPLA2 treatment, and TGF-β1 levels increased with AcSecapin-1 treatment. BV and its components led to a decrease in FRAP levels. Additionally, BV treatment resulted in reduced angiogenesis, and both BV and sPLA2 treatments reduced inflammatory cells. All groups exhibited wound contraction without delay or regression. sPLA2 and AcSecapin-1 induced alterations in the wound healing milieu, without systemic changes. The treatment groups, except for the AcSecapin-1 group, showed an anti-inflammatory effect, identified by reduced inflammatory cell accumulation. Only the BV treatment suppressed angiogenesis. In conclusion, BV, sPLA2, and AcSecapin-1 demonstrate distinct effects on wound healing, with BV showing notable anti-inflammatory and anti-angiogenic properties, while sPLA2 and AcSecapin-1 influenced cytokine and oleic acid levels.

Keywords: Angiogenesis, Honeybee, Oleic acid, Phospholipase A2, Secapin, Wound healing

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INTRODUCTION

Wound healing is a complex and dynamic process involving various factors contributing to the restoration of tissue integrity [1]. The healing process begins immediately after injury with the release of various growth factors, platelet factors, cytokines, and low molecular weight compounds [2]. Typically, the wound healing cycle comprises hemostasis (a few minutes), inflammation (0-5 days), proliferation (5-21 days), and remodeling (21 days-2 years or longer) stages. However, any disruption caused by external or internal factors can prolong each stage and lead to unsatisfactory healing outcomes, even resulting in chronic wound conditions [3].

Cytokines, chemokines, and growth factors released during inflammatory processes in the skin play a significant role in wound healing [4]. The wound healing processes have been identified in studies conducted on healthy Wistar rats. These processes can vary depending on the characteristics of the wound and the overall health status of the rat [5,6].

The treatment of wounds poses a significant burden on both patients, causing emotional and functional trauma, and healthcare professionals. Despite technological and scientific advancements, the treatment of chronic wounds remains a major challenge. Various factors triggering wound formation, such as inappropriate bed rest, chemical exposures, and diabetes, can delay wound healing. Among the current treatment methods, medications can cause some side effects and negatively affect wound healing [7]. Natural product ingredients are considered economically advantageous over pharmaceuticals due to the latter's associated high expenses, so nowadays the therapeutic use of natural substances obtained from bee products is increasing in the medical field [8]. Bee products are particularly popular due to their potential in wound healing. These include honey, propolis, and bee venom (BV) [9–13]. Each of them contains unique active ingredients and is also used for skin health maintenance [6]. Particularly, BV contains peptides, biological amines, and enzymes known to have positive effects on wound healing. Bee venom exhibits anti-inflammatory, antibacterial, and analgesic effects and enhances immune responses [14]. However, limited information is available on the specific effects of BV or its components on wound healing. This study aims to evaluate the individual effects of BV components such as *Apis mellifera* secretory phospholipase A2 (sPLA2) and *Apis cerana* secapin-1 (AcSecapin-1), aiming to understand possible synergistic effects and provide a more comprehensive understanding of the mechanisms of action and potential therapeutic use of these components in wound healing. The study utilized Wistar albino rats as subjects to measure morphometric, biochemical, and histopathological markers, thereby contributing to the body of knowledge on the medicinal properties of bee venom components.

MATERIALS AND METHODS

Bee venom, sPLA2, and AcSecapin-1

The BV was sourced from a local beekeeper engaged in beekeeping activities in the Izmir/Foça region (38.6704° N, 26.7579° E). In mid-April 2021, BV was collected from ten hives in the same apiary using a BV collector (Beewhisper 5.0, IGK Electronics Ltd.) according to the protocol provided by the manufacturer (www.beewhisper.com). The BV was subsequently stored in raw form at – 80°C for later use. To remove potential impurities, the collected BV samples were reconstituted in phosphate-buffered saline solution (PBS, pH 7.4). The solution was then centrifuged at 15,000 RPM for 30 minutes at +4°C. The supernatant was carefully collected with a syringe and subjected to coarse filtration using filter paper, followed by fine filtration with a polytetrafluoroethylene filter with a pore size of 0.22 μm. Subsequently, the samples were transferred to sterile amber vials and used immediately. *Apis mellifera* phospholipase A2 (sPLA2) was procured in powdered form (5mg) from Sigma Aldrich (Product code P9279). *Apis cerana* secapin-1 (AcSecapin-1), was produced by Elabscience in powdered form (5mg) with >95% HPLC purity, consistent with the peptide amino acid sequence (QLITVPVRCPPNYDFIKGRCREKIP) [15].

Animal experiments

A total of 44 male Wistar Albino rats were utilized in the study, with approval obtained from the Afyon Kocatepe University Experimental Animals Local Ethics Committee (Approval No: 49533702/50). The study spanned a duration of 7 days. Throughout the study, strict adherence to ethical principles in animal experimentation and compliance with ARRIVE guidelines were maintained. Rats were randomly assigned to five groups, with 8 animals allocated per group: Wound Control Group (C), Wound + Contractubex® Treatment Group (Cx), Wound + Bee Venom Group (V), Wound + sPLA2 Group (P), and Wound + AcSecapin-1 Group (S) and housed separately in cages. Standard rat pellet feed and water were provided ad libitum to all groups. The weights of the rat groups were measured on days 0, 3, 5, and 7. On the 7th day, the measurements were taken immediately before anesthesia and blood sampling. Additionally, it is important to note that the welfare unit of the experimental animals' facility monitored the weight parameter. This unit ensured that the animals were kept in suitable, stress-free conditions throughout the study. At the end of the experiment, animals were sacrificed under isoflurane anesthesia, and blood samples were collected via exsanguination into heparinized tubes and sent to the laboratory. Blood samples were kept at 4°C for 2 hours before centrifugation, followed by separation of plasma and storage at – 20°C. Additionally, samples were collected from the wound area for ELISA, histopathological examination, hydroxyproline, oleic acid, and free radical analysis, and stored at – 18°C.

Full thickness wound model

To create wounds, rats were anesthetized with isoflurane, and the dorsal area of each rat was shaved and cleaned with 70% ethanol. A template measuring 15 mm x 15 mm square was placed over the area where the wound would be created, and the perimeter was marked with a skin marking pen (Figure 1). Subsequently, full-thickness excision of the skin was performed [16]. Due to the flexible nature of the excision areas, wound formation occurred with measurements that did not fully adhere to the template in some animals. To mitigate the initial inequality in wound area measurements, the area of the excised regions at the beginning of the study was designated as the baseline measurement (Area 0), and this difference was accounted for in statistical calculations by using Area 0 as a covariate.

Injection and dosage method

Wound control group rats were administered 200 µl of PBS solution (pH: 7.4), while treatment group rats were administered BV, sPLA2, and AcSecapin-1 at a dose of 200 µg/kg [16]. Throughout the duration of the study, Contractubex® was applied topically to cover the area where the wound was created. Bee venom, sPLA2, and AcSecapin-1 were dissolved in PBS. Injections were administered daily to the four edges of the wound areas from central points, totaling 200 µl in volume with 50 µl at each injection site (Figure 1).

Monitoring wound healing

The wound area was photographed on days 0, 3, 5, and 7, each time using a sterile ruler 10 cm long. After calibration with the ruler using ImageJ software, the wound areas were measured in square centimeters (cm^2) using the software's area calculation function.

Preprocessing of skin samples

Skin samples were thawed from – 18°C to 4°C for dissolution. These samples were thoroughly washed with ice-cold PBS to remove any remaining blood on the sample. After removing the wound crusts, the remaining sample was divided into two halves, one for the measurement of oleic acid (OA), interleukin-8 (IL-8), and transforming growth factor-β1 (TGF-β1), and the other half for the measurement of hydroxyproline. The samples were weighed and placed into separate sample containers accordingly.

For the samples intended for the measurement of OA, IL-8, and TGF-β1, each sample was placed into a 50 mL beaker. Ice-cold PBS solution (pH 7.4) was added at a ratio of 1:10 (w/v). The sample underwent mechanical homogenization for 4 minutes using an IKA-T18 Ultra Turrax homogenizer and was thoroughly mixed. The resulting suspension underwent a second homogenization using an ultrasonic homogenizer (Bandelin Sonopuls; 20 kHz power) at 50% power, repeated 5 times

for 10 seconds each, with 10-second intervals of immersion and resting in iced water between each cycle.

A 0.5 mL portion of this suspension was transferred to a screw-capped glass test tube for the measurement of OA. The remaining suspension was stored at – 18°C for later use for IL-8 and TGF-β1 measurements.

For the measurement of hydroxyproline, the samples were further processed using a scalpel to divide them into approximately 2 mm-sized small pieces for later use.

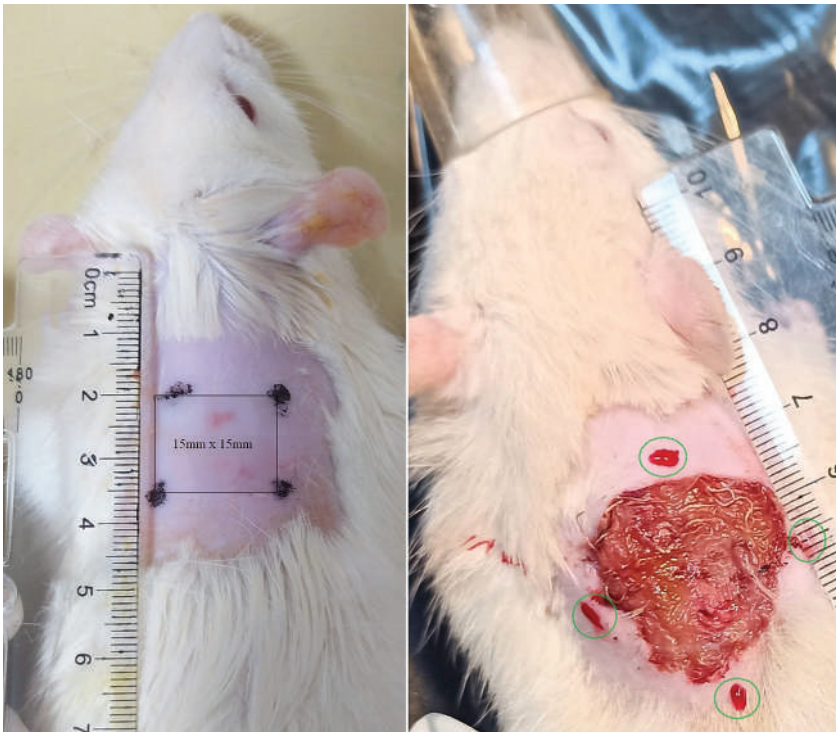

Figure 1. Transparent acetate template measuring 15mm x 15mm placed on the dorsal region of the rat (**left**). Daily injection sites with green circles (**right**).

Oleic acid analysis

A modified method [17–20] was developed for measuring oleic acid (OA), verified with a commercial standard (olive oil). For measurement, 0.5 mL of suspension was mixed with 7.5 mL ethanol:cyclohexane (1:2, v/v) and centrifuged at 4100 RCF for 10 minutes at 4°C. The supernatant was discarded, and the remaining organic layer was transferred to a beaker and heated at 60°C to separate the solvent. To measure free fatty acid content, 50 μL of the oily residue was mixed with 2 mL ethanol-diethyl ether (1:2, v/v) and titrated with 0.1 M KOH containing 1% phenolphthalein until a

pale pink color persisted for 30 seconds. The volume of titration solution consumed was recorded, and the percentage of OA content was calculated according to previous studies [18,20].

Hydroxyproline analysis

Collagen measurements were performed according to the method described previously, which quantifies the high concentration of hydroxyproline imino acid in the collagen content. The amount of hydroxyproline was calculated in accordance with the previous study [21].

IL-8 and TGF-β1 analysis

For the measurement of IL-8 and TGF-β1, ELISA kits specifically designed for rats (ELK Biotechnology Rat IL-8 and TGF-β1 ELISA Kit) were utilized. The separated skin sample suspensions were centrifuged (4°C, 4100 RCF, 10 min), while the previously separated plasmas were brought to room temperature and immediately used for analysis. The required sample wells, chemicals, and samples were brought to room temperature. The analyses were conducted according to the kit user manual.

Analysis of oxidative stress parameters

For the measurement of malondialdehyde (MDA) in blood, the double heating method was used, where lipid peroxides react with thiobarbituric acid [22]. The measurement of reduced glutathione (GSH) was conducted using Ellman's procedure [23]. The activity of the enzyme superoxide dismutase (SOD) was determined by the reaction of superoxides with nitroblue tetrazolium and the inhibition of this reaction by SOD [24]. The ferric reducing antioxidant power (FRAP) analysis was also used to assess non-enzymatic antioxidant capacity [25].

Histopathological evaluation

Tissue samples were fixed in 10% formalin solution, embedded in paraffin blocks, and cut into 4 μm thick sections. The sections were stained with hematoxylin and eosin (H&E) [26] as well as Mallory's triple stain [27] Subsequently, they were evaluated using a Nikon Eclipse 80i light microscope at 4x magnification. Semi-quantitative scoring (0-none, 1-very mild, 2-mild, 3-moderate, 4-severe) was performed for reepithelialization, inflammatory cells, angiogenesis, fibroblast activation, and collagen density during histopathological examination [28].

Statistical analysis

IBM SPSS package program (v20) was utilized for the statistical analysis of the data. Firstly, the assumption of normality was examined. In this regard, Shapiro-Wilk and Kolmogorov-Smirnov tests were applied to assess the normal distribution of the data

[29]. Additionally, Skewness-Kurtosis measurements were evaluated to determine if they fell within the range of – 1.5 to +1.5 [30]. For data showing normal distribution ($p>0.05$), the zeroth day area (Area 0) was considered as a covariate, and covariance analysis (ANCOVA) was performed. For data not meeting the normal distribution criterion ($p<0.05$), a non-parametric covariance analysis (Quade nonparametric ANCOVA) was employed [31].

RESULTS

Group weights

The mean weight of all groups has decreased relative to their initial weights. However, it was observed that the differences in weights measured on the same measurement day across groups and over time within the same group were statistically insignificant ($p>0.05$) (Figure 2).

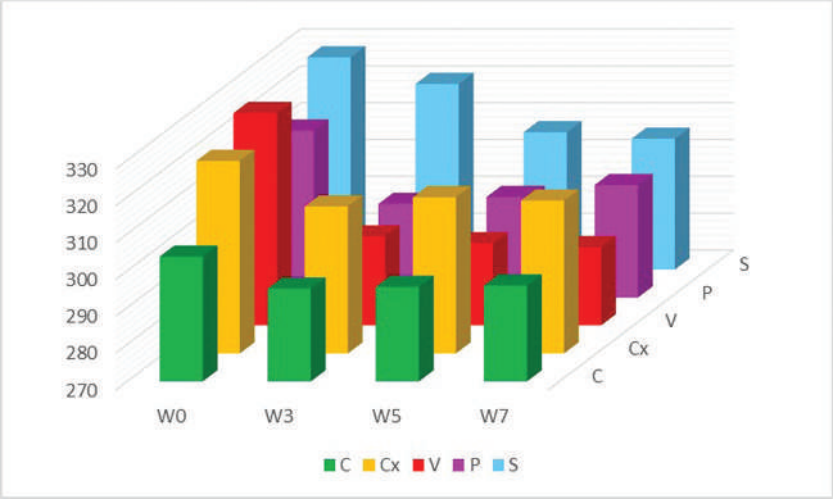

Figure 2. Changes in group weights over days. Abbreviations: **C**, wound control group; **Cx**, Contractubex® group; **V**, wound + bee venom group; **P**, wound + sPLA2 group; and **S**, wound + AcSecapin-1 group; **W0, W3, W5, W7**, mean group weights measured on days 0 to 7.

Wound areas

Based on wound area calculations, no statistically significant differences ($p<0.05$) were observed among the groups in terms of wound closure on days 0, 5, and 7. However, on the 3rd day, wound contraction in the Cx group occurred faster compared to the P and S groups. All groups exhibited contraction of wound areas relative to the initial wound area on the first day, which continued to show statistical significance until the final day of the experiment (Figure 3).

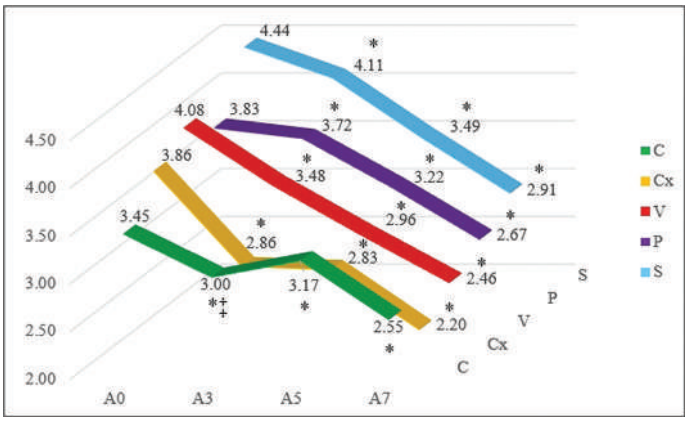

Figure 3. Changes in wound areas over days. *****, Wound contraction on subsequent days is statistically significant compared to the first day ($p<0.05$). **‡**, Contraction in the Cx group is statistically significant compared to the P and S groups ($p<0.05$). Abbreviations: **C**, wound control group; **Cx**, Contractubex® group; **V**, wound + bee venom group; **P**, wound + sPLA2 group; and **S**, wound + AcSecapin-1 group; **A0, A3, A5, A7**, mean wound area measurements (cm^2) on days 0-7.

Hydroxyproline and OA levels

No statistically significant difference ($p<0.05$) was observed among the groups in hydroxyproline measurements, indicating similar levels across the groups (Figure 4). Statistically significant differences ($p<0.001$) were observed among the groups in OA measurements. An increase in OA levels was observed in groups Cx, P, and S compared to the control group. Group Cx had higher OA levels than group V, but lower levels compared to groups P and S. Group V had lower OA levels compared to groups P and S. Group P had the highest OA levels compared to all other groups (Figure 4).

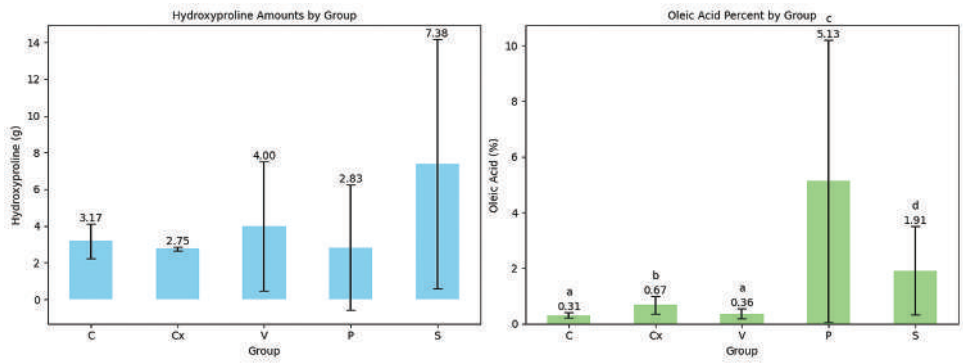

Figure 4. The values of hydroxyproline and OA in samples taken from wound areas are presented. Abbreviations: **C**, wound control group; **Cx**, Contractubex® group; **V**, wound + bee venom group; **P**, wound + sPLA2 group; and **S**, wound + AcSecapin-1 group. Superscript letters denote statistical differences among the groups ($p<0.001$).

IL-8 and TGF-β1 levels

IL-8 levels were determined from skin samples by plotting a standard curve (R^2=0.987), and IL-8 concentrations (pg/ml) were determined accordingly. A statistically significant difference ($p<0.005$) was observed among the groups. An increase in IL-8 levels was observed in groups Cx, P, and S compared to the control group. Additionally, IL-8 levels in groups Cx, P, and S were higher than those in the V group (Figure 5).

For TGF-β1 measurements from skin samples, a standard curve was plotted (R^2=0.999), and TGF-β1 concentrations (pg/ml) were determined accordingly. A statistically significant difference ($p<0.05$) was observed among the groups. A dramatic increase in TGF-β1 levels was observed in the S group compared to all other groups (Figure 5).

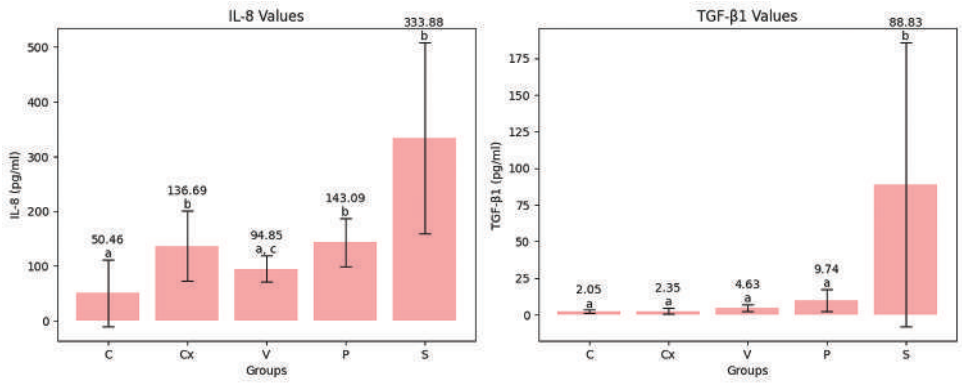

Figure 5. IL-8 and **TGF-β1** values were obtained from samples taken from the wound areas. Abbreviations: **C**, wound control group; **Cx**, Contractubex® group; **V**, wound + bee venom group; **P**, wound + sPLA2 group; and **S**, wound + AcSecapin-1 group. Superscript letters denote statistical differences among the groups ($p<0.005$ for IL-8; $p<0.05$ for TGF-β1).

Blood levels of IL-8, TGF-β1, and redox markers

There was no statistically significant variance ($p<0.05$) noted across the groups in the measurements of IL-8 and TGF-β1 from blood samples. For the measurement of MDA levels in the blood, a standard curve was constructed (R^2=0.996), and MDA concentrations (μmol/ml) were determined accordingly. GSH measurements were expressed in mg/ml, while SOD measurements were expressed as % inhibition. There was no statistically significant difference observed among the groups in blood MDA, GSH, and SOD levels (Table 1). However, FRAP levels revealed a statistically significant difference among the groups ($p<0.001$). FRAP values of the YZ, YP, and YS groups remained lower compared to the control and C groups (Figure 6).

Table 1. Values and statistical analysis ($p<0.05$) of measurements from blood samples.

Groups	IL-8 (pg/ml)	TGF-β1 (pg/ml)	MDA (µmol/ml)	GSH (mg/ml)	SOD (% inhibition)
C	66.82±5.54	1.21±0.61	4.93±2.18	51.05±8.23	59.76±1.85
Cx	68.61±5.02	0.76±0.33	5.63±1.13	46.57±3.39	52.79±10.34
V	67.45±2.44	0.81±0.22	5.56±2.46	47.00±5.20	49.39±5.94
P	67.57±5.50	0.91±0.47	5.56±1.88	52.71±7.37	52.11±6.66
S	67.06±10.17	1.10±0.65	5.52±1.92	43.29±6.42	53.68±5.85
p	0.840	0.549	0.961	0.213	0.054

Note: C, wound control group; **Cx**, Contractubex® group; **V**, wound + bee venom group; **P**, wound + sPLA2 group; **S**, wound + AcSecapin-1 group; **IL-8**, interleukin-8; **TGF-β1**, transforming growth factor-β1; **MDA**, malondialdehyde; **GSH**, reduced glutathione; **SOD**, superoxide dismutase.

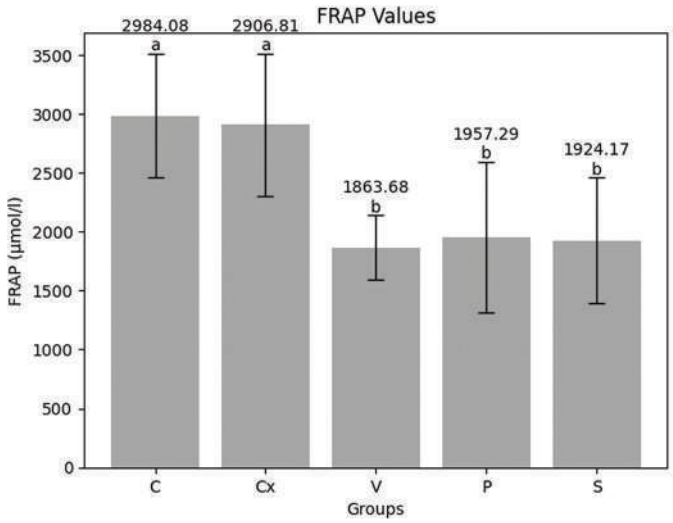

Figure 6. Values of FRAP measurements obtained from blood samples. Abbreviations: **C**, wound control group; **Cx**, Contractubex® group; **V**, wound + bee venom group; **P**, wound + sPLA2 group; **S**, wound + AcSecapin-1 group. The superscripted letters indicate statistical differences among the groups ($p<0.001$).

Histopathology

Histopathological evaluations of skin samples taken from the wound area revealed a decrease in inflammatory cells in groups Cx, V, and P compared to the control group ($p<0.05$). Additionally, inflammatory cell counts were higher in groups Cx, P, and S compared to group V (Figure 7, Figure 8). Angiogenesis was lower in group V compared to the control, P, and S groups ($p<0.05$). No statistically significant changes were observed in reepithelialisation, fibroblast activation, and collagen density (Table 2).

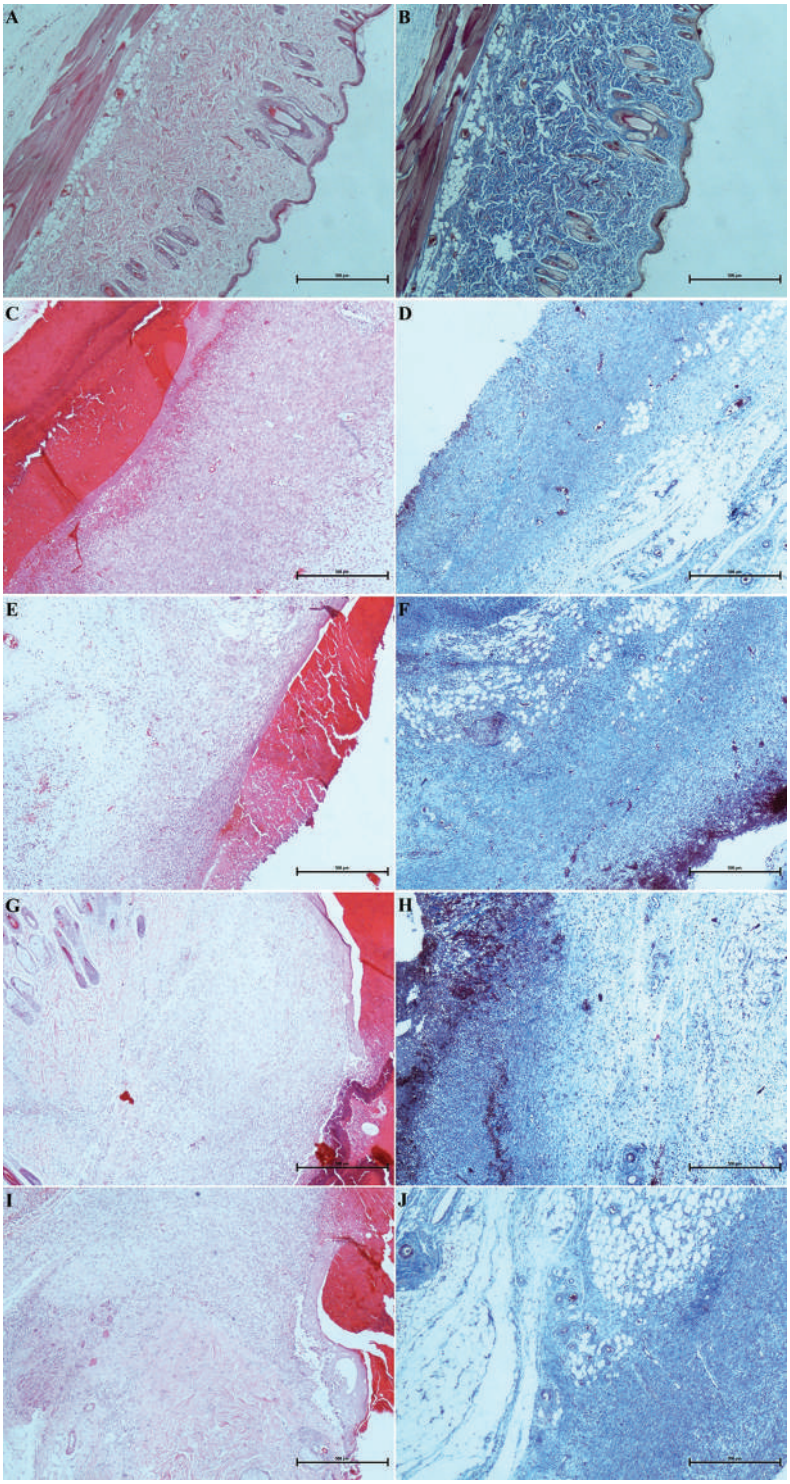
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Figure 7. Histopathological examination of skin samples. Preparations stained with H&E (**left panel-A, C, E, G,** and **I**) and Mallory's triple stain (**right panel-B, D, F, H,** and **J**) are presented. Microscopic images from top to bottom correspond to: wound control group (**A, B**); Contractubex® group (**C, D**); wound + bee venom group (**E, F**); wound + sPLA2 group (**G, H**); wound + AcSecapin-1 group (**I, J**).

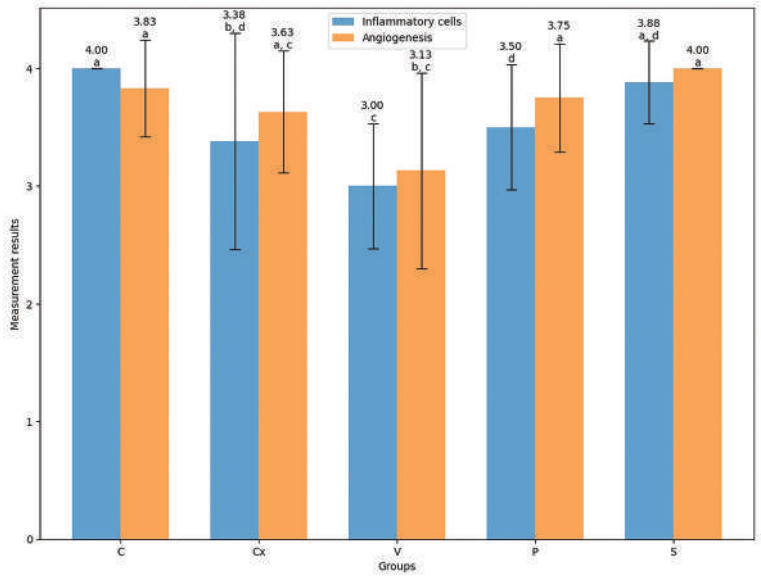

Figure 8. Inflammatory cells and angiogenesis values from samples taken from wound sites. Abbreviations: **C**, wound control group; **Cx**, Contractubex® group; **V**, wound + bee venom group; **P**, wound + sPLA2 group; **S**, wound + AcSecapin-1 group. The superscript letters indicate the statistical difference between groups ($p<0.001$).

Table 2: Values and statistical analysis of histopathological measurements obtained from wound area samples ($p<0.05$).

Groups	Reepithelialization	Fibroblast (H&E)	Fibroblast (Triple)	Collagen	Total Score
C	1.33±0.82	3.50±0.55	3.17±0.41	2.83±0.75	18.67±1.51
Cx	1.63±1.06	3.88±0.35	3.88±0.35	3.88±0.35	20.25±2.71
V	1.75±1.04	3.50±0.53	3.50±0.53	3.50±0.53	18.38±2.00
P	1.38±1.19	3.50±0.53	3.25±0.71	3.25±0.71	18.63±2.72
S	1.50±0.76	3.63±0.52	3.75±0.46	3.63±0.74	20.38±2.39
p	0.913	0.526	0.092	0.090	0.229

Note: C, wound control group; **Cx**, Contractubex® group; **V**, wound + bee venom group; **P**, wound + sPLA2 group; **S**, wound + AcSecapin-1 group; H&E, hematoxylin & eosin staining; Triple, Mallory's trichrome staining.

Correlation analysis

According to the Spearman correlation analysis results, there is a moderate positive correlation between IL-8 and TGF-β1 (rho=0.473, $p<0.01$), indicating a tendency for IL-8 levels to move together with TGF-β1 levels. There is a moderate positive correlation between OA and IL-8 (rho=0.504, $p<0.01$), indicating a tendency for OA levels to move together with IL-8 levels. There is a moderate negative correlation between blood IL-8 and SOD (rho=-0.342, $p<0.05$), indicating a tendency for blood IL-8 levels to move inversely with enzymatic antioxidant capacity (SOD). There is a strong positive correlation between inflammatory cells and angiogenesis (rho=0.478, $p<0.01$), indicating a tendency for the inflammatory cell score to move together with the angiogenesis score. There is a low-level positive correlation between OA and TGF-β1 (rho=0.383, $p<0.05$). There is a strong negative correlation between TGF-β1 and SOD (rho=-0.627, $p<0.001$). There is a moderate negative correlation between TGF-β1 and FRAP (rho=-0.453, $p<0.005$). There is a negative correlation between IL-8 levels and SOD activity (rho= – 0.378, $p<0.05$).

DISCUSSION

In this study, pain and discomfort induced by the treatments (bee venom, sPLA2, and AcSecapin-1 injections) may have led to a decreased appetite, resulting in a reduction in the intake of antioxidants that need to be consumed with food, that resulted in a decrease in animal feeding and subsequently weight loss.

Comparable levels of wound healing were observed among the groups on specific days. Treatment with Contractubex® may suggest prominent effects in rats in this group until the end of the third day. Additionally, significant improvement or contraction tendencies can be noted in all treatment groups.

Hydroxyproline plays a crucial role in wound healing, particularly in collagen synthesis and tissue repair [32]. We observed that Cx did not cause a significant change in the levels of hydroxyproline (Figure 4). Similarly, a previous study reported that a mixture of Contractubex® components, including allantoin, extractum cepae, and heparin, did not induce changes in hydroxyproline levels. Conversely, it has been stated that when these compounds are used individually, a decrease in hydroxyproline levels occurs [33].

Oleic acid has been studied for its pro-inflammatory effect, which speeds up the wound healing process [34]. In our study, variations in OA levels were detected among the groups, suggesting that treatments with Contractubex®, sPLA2, and AcSecapin-1 may be effective in OA production or accumulation. These intergroup differences in OA levels provide important clues for understanding molecular-level biochemical changes in the treatment groups. The observed increase in OA levels in the Cx and S groups has not been reported in the literature so far, highlighting the need for further in-depth research. Although the presence of sPLA2 in bee venom might not result in a measurable rise in OA levels, our study demonstrated that sPLA2, when used

in the P group, increased OA levels in the wound area, which aligns with previous findings [35,36]. The ability of sPLA2 to hydrolyze membrane phospholipids may have contributed to the increased presence of OA in the wound area.

Variations in IL-8 levels were observed in skin samples obtained from the wound area among different groups. The increase in IL-8 in the Cx, P, and S groups may indicate keratinocyte activation. Previous studies have reported similar results, showing that bee venom and sPLA2 can increase IL-8 levels. This suggests that the involvement of epidermal TLR receptors in the keratinocyte activation process enhances the inflammatory response [4]. Conversely, it has also been reported that bee venom and melittin can reduce IL-8 expression [6,37]. In this study, no change in IL-8 levels was observed in the V group, which may be attributed to the dosage of BV used. Furthermore, additional research is needed to fully understand the effects of Contractubex® and secapin on IL-8 levels.

AcSecapin-1 is one of the least studied peptides of BV, and therefore, limited information is available about its biological effects. Therefore, the dramatic increase in TGF-β1 levels due to AcSecapin-1 has been discussed based on the available data. We observed that AcSecapin-1 increased the production of the pro-inflammatory cytokine IL-8, and therefore, TGF-β1 may have increased in the wound tissue during the inflammation process. As reported previously, TGF-β1 plays a complex and critical role in wound healing by influencing cell growth, extracellular matrix accumulation, and tissue remodeling, while also regulating immune responses [6,38]. Additionally, the increase in OA levels may indicate cellular damage due to the influence of the inflammatory response which is not surprising since the previous finding in which *Apis mellifera* secapin induces inflammation [39].

The levels of IL-8, TGF-β1, MDA, GSH, and SOD in the blood samples were similar among the groups, indicating no significant changes in these parameters in the blood sampled on the 7th day of wound healing (Table 1). MDA is a marker of oxidative stress and lipid peroxidation, often elevated in response to tissue damage. GSH is a key antioxidant that helps in reducing oxidative stress and detoxifying harmful substances. SOD is an enzyme that plays a crucial role in protecting cells from damage by catalyzing the dismutation of superoxide radicals into oxygen and hydrogen peroxide [40]. Based on the results of the standard curve for measuring blood MDA levels, MDA concentrations were determined, yielding a high R^2 value indicating the reliability of these measurements (R^2=0.996). Despite the similarity in redox parameters, the difference in FRAP levels reflects a specific variation in antioxidant capacities among the groups (Figure 6). The FRAP test is commonly used to measure the non-enzymatic components of antioxidant capacity. In our study, this feature should be distinguished from markers reflecting enzymatic antioxidant capacity such as SOD and GSH. One limitation of this analysis is its inability to react with materials that act via hydrogen donation to reduce radicals (e.g., thiols and proteins) [41]. Therefore, the observed decreases in FRAP levels may indicate increased oxidative stress, uncontrolled accumulation of free radicals, and decreased appetite.

Contractubex®, a known medication used for scar management due to its bioactive ingredients, reduces inflammation and fibrosis [33]. Bee venom possesses anti-inflammatory, antinociceptive, antioxidant, and anti-apoptotic properties. The anti-inflammatory properties of bee venom are associated with melittin and PLA2 [42]. Our study revealed that interventions in the Cx, V, and P groups had a reducing effect on inflammatory cells. This suggests that the applications in these groups may have anti-inflammatory efficacy. Thus, the inflammatory phase, which is the initial stage of wound healing, appears to be influenced by Contractubex®, bee venom, and sPLA2.

We observed a decrease in angiogenesis in the V group; however, sPLA2 and AcSecapin-1 components did not exhibit such an effect. Similarly, a previous study has suggested that BV inhibits angiogenesis in wound healing [14]. Nevertheless, other studies have reported that bee venom increases TGF-β and VEGF expression, promoting angiogenesis in diabetic mice. Additionally, it has been shown to reduce caspase 3, – 8, and – 9 activities and stimulate endothelial progenitor cells in tissues due to this inhibition. Furthermore, bee venom limited inflammation by reducing ATF-3 and iNOS expression in diabetic wounds, thereby promoting neovascularization. Additionally, it activated angiogenesis by improving Ang-1/Tie-2 signaling [6,16,43]. The inconsistency between our study and these literature findings, despite using the same dose of BV, may be due to differences in bee species and the shorter duration of our study (7 days) compared to mentioned studies (15-20 days).

In the correlation analysis, the directions and strengths of the parameters moving together were compared, and it can be interpreted that IL-8, TGF-β1, and OA parameters showing low to moderate positive correlation with each other may serve as indicators of the inflammatory environment in the wound tissue. The moderate to strong negative correlation of TGF-β1 with FRAP and SOD, as well as both wound site IL-8 and blood IL-8 showing moderate negative correlation with SOD, suggests that the enzymatic antioxidant SOD is consumed/suppressed in both the wound site and systemically detectable pro-inflammatory responses. However, the lack of variation in SOD among the groups excludes this possibility. A robust positive correlation between inflammatory cell counts and angiogenesis parameters was noted during the 5th to 7th days of the wound healing process. This observation suggests that the peak of inflammation and angiogenesis phases coincides with this timeframe.

CONCLUSION

In conclusion, our study assessed the effects of BV, sPLA2, and AcSecapin-1 treatments on wound healing. We observed significant anti-inflammatory effects with Contractubex®, BV, and sPLA2 treatments, as evidenced by their impact on inflammatory cells and angiogenesis. Notably, BV treatment demonstrated inhibition of angiogenesis, contrasting with the effects of sPLA2 and AcSecapin-1. However, further investigation is warranted to fully understand the mechanisms underlying these effects and their potential clinical applications.

The observed variations in OA, IL-8, and TGF-β1 levels suggest complex interactions between components and mediators in wound healing, with Contractubex® and AcSecapin-1 inducing OA release, while sPLA2, Contractubex®, and AcSecapin-1 influence IL-8 and TGF-β1 levels, indicating their significant effects on keratinocyte activation and inflammation. Moreover, Contractubex®, BV, and sPLA2 exhibit anti-inflammatory effects, with BV specifically inhibiting angiogenesis, highlighting the role of IL-8 and TGF-β1 in inflammation-angiogenesis during wound healing.

These findings contribute to our understanding of the therapeutic potential of bee venom and its components in wound healing. Moving forward, additional comprehensive studies are needed to elucidate their precise mechanisms of action and to explore novel treatment strategies for wound healing in clinical settings.

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Authors' contributions

BDenk secured the study funding, designed the experimental research, performed statistical and data analysis, planned and carried out laboratory experiments, wrote the manuscript, and drafted figures. VY created full-thickness wounds in rats and contributed to manuscript drafting. BDayi and AS assisted in the experimental procedures, conducted histopathological evaluations, and wrote the histopathology findings. JS, UG, and ZS drafted, edited, and reviewed the final version of the manuscript. All authors read and approved the final version of the manuscript.

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ISTRAŽIVANJE TERAPIJSKOG POTENCIJALA KOMPONENTI PČELINJEG OTROVA U ZACELJENJU RANA: SVEOBUHVATNA PROCENA MORFOMETRIJSKIH, BIOHEMIJSKIH I HISTOPATOLOŠKIH MARKERA

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Pčelinji otrov (BV) i njegove komponente, sekretorna fosfolipaza A2 (sPLA2) i *Apis cerana* sekapin-1 (AcSecapin-1), imaju potencijalnu efikasnost u zarastanju rana. Cilj ovog rada je procena uticaja BV, sPLA2 i AcSecapin-1 na zarastanje rana pune debljine kod mužjaka Vistar Albino pacova tokom perioda od 7 dana. Procenjivani su različiti

paramerti, morfometrijski (telesna masa, kontrakcija rane), biohemijski (hidroksiprolin, oleinska kiselina, IL-8, TGF-β1, redoks parametri) i histopatološki markeri (reepitelizacija, inflamatorne ćelije, angiogeneza, aktivacija fibroblasta i gustina kolagena). Tretmani komponentama sPLA2 i AcSecapin-1 izazvali su povećanje nivoa oleinske kiseline. Povećanje nivoa IL-8s izazvao je PLA2 tretman, a povećanje nivoa TGF-β1 tretman sa AcSecapin-1. BV i njegove komponente dovele su do smanjenja nivoa FRAP. Pored toga, BV tretman je doveo do smanjenja angiogeneze, a tretmani BV i sPLA2 do smanjenja brojnosti inflamatornih ćelija. U svim grupama zabeležena je kontrakcija rane bez odlaganja ili regresije. sPLA2 i AcSecapin-1 izazvali su promene u okruženju zaceljivanja rana, bez sistemskih promena. Tretman grupe, osim AcSecapin-1 grupe, pokazale su antiinflamatorni efekat, identifikovan na osnovu smanjenja akumulacije inflamatornih ćelija. Samo je BV tretman sprečio angiogenezu. U zaključku, BV, sPLA2 i AcSecapin-1 pokazuju različite efekte na zarastanje rana, pri čemu BV pokazuje značajna antiinflamatorna i antiangiogena svojstva, dok sPLA2 i AcSecapin-1 utiču na nivoe citokina i oleinske kiseline.