Natural substances with therapeutic potential in wound healing

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Untreated wounds are a significant health problem that affects the whole world. The healing wounds capacity of different plants and animals extracts is due to their wide variety of bioactive compounds with epithelial cell regenerative effects on wounds. The present study aims to investigate the efficacy of 3 combined preparations (extract 1, extract 2 and extract 3) as potential regenerative products used for healing of skin wounds. The gel compositions were prepared by mixing the extracts of garden snail mucus H. aspersa and plants (leaf extract of Plantago major and/or flower extract of Calendula officinalis) in 5:1:1 ratio. Their wound healing activity was evaluated using the excision wound model on female Wistar rats. The percentage of wound contraction area was calculated precisely on the 3rd, 6th, 10th, 14th and 19th day. Our results demonstrated that extract 3- treated group (containing extract from P. major and snail mucus extract) exerted the best rate of wound closer in the period between 6th and 10th day post injury. The reduction of wound area was by 55% on the 6th day and by 21% on the 10th day after injury versus the control untreated group, respectively. Extract 1 was the best for wound closing in the period between 3rd and 6th day post injury. The wound area was reduced by extract 1 on the 3rd post injury day by 133% and by 61% on the 6th day versus the control. In conclusion the best wound healing effect of Gel 1 suggests a synergic mechanism of action among the three ingredients: mucus from H. aspersa, P. major leaf extract and extract of Calendula flowers.

Key words: snail Helix aspersa mucus extract; Calendula flower extract; Plantago major leaf extract; healing effects

INTRODUCTION

Untreated wounds are one of the most significant health problems in the world and most often lead to complications and limb amputation [1]. Wound healing is a complex process that goes through four partially overlapping stages: hemostasis, inflammation, proliferation and maturation or tissue modelling [1, 2]. The efficiency of healing depends on the synchrony of the four phases, and it can be influenced by many internal (endogenous) and external (exogenous) factors.

Although there are good clinical practices in the world that prevent delayed of chronic wounds/ulcers healing, their effectiveness is still unsatisfactory. In this regard, numerous studies have been conducted on folk methods of treatment as an alternative to modern clinical practices [3, 4]. A number of herbal preparations including extracts and/or purified biologically active compounds with plant origin have been used to treat skin lesions and are applied in the form of emulsions, creams and ointments [5-7].

Extracts of Plantago major (Plantaginaceae) and Calendula officinalis (Asteraceae) are ones of the most widely used natural products for the treatment of skin wounds. Plantago major leaves have been used as a wound healing herbal agent for many years in folk and traditional medicine [5]. Flowers of Calendula officinalis (calendula, marigold) is another medicinal plant which is also used in the modern world due to its pharmacological actions including wound healing and antioxidant. Some authors provide evidence that they increase the activity of white blood cells and accelerate tissue repair [8]. Stimulating angiogenesis, granulation, epithelialization and wound contraction effects were also proven [9, 10].

The combination of plant extracts with some animal products like a honey, propolis or snail extract significantly increases the effectiveness of treatment [7, 11, 12]. The positive wounds healing effects of different plants extracts is due to their
bioactive compounds richness like polysaccharides, lipids, caffeic acid derivatives, flavonoids, iridoid glycosides, terpenoids, phytosterols, essential oils, fatty acids, vitamins, etc., which determines their anti-inflammatory, antibacterial, antifungal and antioxidiant properties [11, 12].

The wound-healing effect of mucus from Helix aspersa has been related to its antioxidiant capacity, possibility to stimulate fibroblast proliferation and also migration and survival of keratinocytes [13, 14]. These results shed light on the molecular mechanisms underlying the regenerative properties of mucus, based on its promoting effect on skin cell migration, proliferation and survival [14].

Recently, Gubitosa et al. (2020) used snail mucus from garden snails "Helix aspersa Müller" conjugated with gold nanoparticles (AuNPs-SS) to investigate its anti-inflammatory activity and wound healing potential [15]. The in vitro test for AuNPs-SS safety in human keratinocytes demonstrated that AuNPs-SS accelerates wound closure, being associated with increased expression of the urokinase receptor (uPAR), which converts plasminogen to plasmin, degrades the extracellular matrix and directly controls cell adhesion, differentiation and proliferation [15]. The authors explain the observed effect by the presence of functional groups in the proteins, peptides and amino acids (and/or polyphenols) of snail mucus present on the surface of the nanoparticles [15].

The aim of our team was to search alternative pathways for wound healing with bioactive compounds from natural sources. Hence, the present study aims to investigate the efficacy of combined preparations obtained from garden snail mucus (Helix aspersa), and extracts from medicinal plants Calendula officinalis (flower extract) and Plantago major (leaf extract), as potential regenerative products used for healing of skin wounds.

**EXPERIMENTAL**

**Extract preparation**

Extracts from medicinal plants C. officinalis and P. major, and from garden snail (H. aspersa) were prepared, as follows:

*C. officinalis* extract was prepared using dry flowers, extracted by maceration with aqueous ethanol solution (45% v/v ethanol), in a ratio of solvent to raw material 4:1. The maceration process takes place in a closed vessel in the dark for 12 hours, at a temperature of 45 °C, with continuous shaking. The extraction process ends with ultrasonic extraction at room temperature for 5 minutes at temperature 25 °C (frequency: 40 kHz). After a series of operations, such as: decantation, pressing, centrifugation, vacuum filtration, concentration on a vacuum rotary evaporator, the resulting extract was lyophilized. An extract containing 15% ethanol and 35% glycerol was prepared, with concentration 100 mg/ml dry extract of calendula.

The extraction of the biologically active substances from *P. Major* was made from 300 g of deciduous plantain leaves purchased commercially. Maceration was performed in the dark with an aqueous-alcoholic solution containing 50% ethanol for 24 hours at room temperature (3: 1 solvent to raw material ratio). The extraction process was completed in an ultrasonic bath, in order to better extract the active substances. Ultrasonic extraction was performed for 5 minutes at 25 °C, using an ultrasonic cleaning bath (frequency: 40 kHz). After a series of operations, such as: decantation, pressing, centrifugation, vacuum filtration, concentration on a vacuum rotary evaporator, the resulting extract was lyophilized and finally 3.0 g of dry extract was obtained. Prepared aqueous extract contained 150 mg/ml dry extract of *P. major*.

*Snail extract preparation.* The mucus was collected from snails *H. aspersa*, grown in Bulgarian eco-farms using patented technology, so the snails survived without disturbing their biological functions [16]. The resulting crude mucus extract was homogenized and centrifuged to remove coarse impurities. After several steps of filtration (also object of patented technology), the native mucus extract was obtained. The protein concentration in the native mucus extract was determined by Bradford assay [17]. The three analysed compositions with potential regenerating effect (CERE) were prepared by mixing the extracts of garden snail, plantain and/or calendula in 5:1:1 ratio.

**Mass spectometric analysis of molecular mass of peptides**

Peptides with MW below 10 kDa were analyzed by MALDI-TOF-TOF mass spectrometry on an AutoFlexTM III. High Performance MALDI-TOF&TOF System (Bruker Daltonics). 1.0 μl of the mixture of 2.0 μl of matrix solution (7 mg/ml of α-cyano-4-hydroxycinnamic acid in 50% ACN containing 0.1% TFA) and 1.0 μl of the sample
were mixed and spotted on a stainless steel 192-well target plate. The mixture of angiotensin I, Glu-1-fibrinopeptide B, ACTH (1–17), and ACTH was used for calibration of mass spectrometer. The MS/MS spectra were carried out in reflector mode and the amino acid sequences of peptides were identified by precursor ion fragmentation using MALDI-MS/MS analysis.

**SDS-PAGE electrophoresis**

The native fresh extract from mucus was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 5% stacking gel and 12% resolving gel according to Laemmli method with modifications [18]. 20 μg of the sample was loaded on the gel. Protein standard mixture ranging from 10 kDa to 250 kDa (Precision Plus Protein™ Standard All Blue, Bio-Rad Laboratories, Germany) was used as molecular marker. Coomassie Brilliant Blue G-250 staining was used for the visualization.

**In vivo studies on the regenerating effect on wounds of the three combined extracts**

**Laboratory animals:** Female adult Wistar rats (190-220 g) were adapted under standard conditions of the local vivarium for 7 days before the experiment. Rats were divided in 4 groups and received food and water ad libitum.

**Experimental model of excision wounds:** The rats were anaesthetized by Chloral hydrate (400 mg/kg intraperitoneally (i.p.). All animals were depilated at dorsal thoracic region and full thickness of skin was cut off from a pre-determined area. The wound area was measured immediately by tracing it on transparent paper and calculated in square centimeters [19]. For measuring wound contraction, the same protocol was followed on the 3rd, 6th, 10th, 14th and 19th day and the percentage of wound contraction area was calculated.

The raw area at the time of wounding was considered as 100%, and the wounding day was considered as day zero.

**Treatment:** All wounds were initially disinfected with 3% of H2O2 and further daily treated with different CERE according to the groups. Control rats did not receive any treatment. Rats were divided in single cages to protect animals from additional hurts and infections. Each experimental group contained of 6 animals. The wound healing activity of the three snail gels (CERE 1, CERE 2 and CERE 3) was evaluated by monitoring wound’s contraction percentage in comparison to the controls using the excision wound model.

**Statistical analysis**

The statistical analysis of the experimental data was performed according to Student Fisher’s t-test and the results were considered significant at \( P < 0.05 \).

**RESULTS AND DISCUSSION**

A number of natural extracts have been used in the care of skin wounds for many years due to their therapeutic activities, including anti-inflammatory, antimicrobial and cell stimulating properties. The native combined extracts of fresh mucus from garden snail (*H. aspersa*) as well as of some medicinal plants were known by folk medicine. Even though they were used for ages, new interest towards their skin regeneration properties had rapidly increased in the last few years [8, 14, 19]. Recent data discovered the variety of active components of the natural extracts, responsible for their biological activities. However, interdisciplinary studies are necessary for development of efficient standardized products for use in the medical practice.

Our team reported recently new data about antimicrobial activity of several peptide and protein fractions isolated from garden snail *H. aspersa* [16, 20, 21]. These data are in agreement with established antimicrobial properties of the mucus from *H. aspersa*, and *A. fulica* [22]. In the region above 100 kDa, proteins might correspond to glycoproteins and mucines, which have been detected in the mucus from *H. aspersa* and *H. pomatia* [23]. Additionally, the mucus from the garden snail has been found to contain allantoin (2,5-Dioxo-4-imidazolidinyl urea), glycolic acid, glutathione (GSH) and peptides with antibacterial and antioxidant properties [24, 25].

In fact, the proteins, peptides and amino acids (and/or polyphenols) in the mucus play a key role in cell regeneration and growth, preventing the effects of inflammatory disease [15, 26]. Recently, Gubitosa and co-workers explained the observed effect by the presence of functional groups in the proteins, peptides and amino acids (and/or polyphenols) of snail mucus present on the surface of AuNP [15]. Therefore, new studies revealed the regenerative properties of the secretion of *H. aspersa*. *In vitro* tests showed that the snail mucus promotes proliferation, migration and survival of keratinocytes and dermal fibroblasts [14]. On the
basis of this knowledge as well as having in mind the results from our previous studies, we prepared the combined extracts with potential regenerating effect (CERE) on open wounds. The complex mixture of natural extracts, contains garden snail mucus, C. officinalis and P. major. We expect that their accelerating effects on wound healing processes will be due to stimulation of the regeneration process and prevention of the wound infection.

The content of the prepared snail extract was analyzed by 12% SDS-PAGE. Fig. 1 shows that the mucus is a complex mixture of substances with different molecular weight. Several protein bands in wide range 25 - 35 kDa, 38 - 40 kDa, 45-50 kDa; 80-90 kDa and above 250 kDa were detected from SDS-PAGE (Fig. 1). Moreover, the mucus peptides with a MW below 3 kDa were determined by MALDI-MS analyzes (Fig. 2).

The amino acid sequence of different peptides from mucus of C. aspersa clearly demonstrates presence of many important amino acids such as glycine, proline and tryptophan [16], associated with established antibacterial activities in peptides.

The extracts of C. officinalis and P. major were prepared as it was described under Experimental section. Both extracts contain 15% ethanol and 35% glycerol, 100 mg dry Calendula extract and/or 150 mg dry P. major extract in 1 ml. The garden snail extract was obtained as it was described previously [16]. We combined the extracts after homogenization of aqueous mucus (with a concentration of 0.3 mg/ml protein determined by Bradford analysis) [17] and/or an extract of P. major and/or an extract of calendula. The different CERE formulations tested in this study are shown in Table 1.

The wound healing potential of three tested combined extracts CERE 1, CERE 2 and CERE 3 was evaluated by using an excision wound model. Our results showed that local application of CERE 1 and CERE 3 accelerated the progression of wound healing as compared to control untreated animals. The positive effect on wound healing of CERE 1 and CERE 3 gels was different both in terms of strength and in terms of the stage of wound healing processes in which it occurs (Fig. 3). The healing effect of CERE 2 on wounds was not significant.

![Fig. 1. SDS-PAGE of H. aspersa mucus (1), MW marker (2).](image1)

![Fig. 2. Mass spectrometric spectrum of peptide fraction with Mw < 3 kDa by AutoflexTM III, High Performance MALDI-TOF&TOF/TOF Systems (Bruker Daltonics, Bremen, Germany).](image2)
Table 1. Composition of the studied Combined Extracts with potential Regenerating Effect (CERE).

<table>
<thead>
<tr>
<th>Combined extract 1 (CERE 1)</th>
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<tbody>
<tr>
<td>Native water mucus extract</td>
<td>25 ml</td>
</tr>
<tr>
<td>Extract from <em>Plantago major</em></td>
<td>5 ml</td>
</tr>
<tr>
<td>Extract from <em>Calendula officinalis</em></td>
<td>5 ml</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Combined extract 2 (CERE 2)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Native water mucus extract</td>
<td>25 ml</td>
</tr>
<tr>
<td>Extract from <em>Calendula officinalis</em></td>
<td>5 ml</td>
</tr>
<tr>
<td>Distilled water</td>
<td>5 ml</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Combined extract 3 (CERE 3)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Native water mucus extract</td>
<td>25 ml</td>
</tr>
<tr>
<td>Extract from <em>Plantago major</em></td>
<td>5 ml</td>
</tr>
<tr>
<td>Distilled water</td>
<td>5 ml</td>
</tr>
</tbody>
</table>

The complex process of wound-healing includes the following stages: coagulation, inflammation, collagenase, wound contraction and epithelialization [1, 2]. It is well known that approximately after 3 days from the initial wound, the proliferative phase centres around fibroblasts and the production of both collagen and ground substance forms the basis for the tissue scaffold of the wound area. This is a stage of enhanced macrophage secretion of growth factors and cytokines that promotes tissue proliferation and cell migration. Meanwhile, endothelial cells enter in a rapid growth phase and angiogenesis occurs within the granulation tissue, creating a rich vascular network supplying this very active area of healing [27], and the wound tissue matures and restores [8].

Our results demonstrated that wounds treated with CERE 1 (containing extract from *P. major*, *C. officinalis* and mucus extract) exerted the best rate of wound closer in the period between 3th and 6th day post injury (Figs. 3, 4, 5 and 6). The wound area was reduced by CERE 1 on the 3rd day post injury by 133% (*P < 0.01*) and by 61% (*P < 0.05*) on the 6th day versus the control. From the 10th days after injury until the end of our experiment the rate of wound healing in CERE 1 and control group was commensurable.

The best wound healing effect of CERE 1 suggests a synergic mechanism of action among the three ingredients, namely the native snail mucus, the extract of *P. major* and the extract of Calendula. The obtained results are in accordance with the known literature data, proving the effectiveness of the main components of the used extracts in the treatment of wounds of different origin. Wound-healing positive effect of mucus from *Helix aspersa* was already reported and it is due to the antioxidant capacity and possibility for stimulation of fibroblast proliferation [13]. Methanol and aqueous extracts from the leaves of *Plantago major* show regenerating and stimulating effect on wounds caused by burns [28], processes connected with enhanced cell proliferation and migration [29]. On the other hand, wound healing by enhancing fibroblast proliferation, collagen bundle synthesis and revascularization in skin injuries treated by combination of *Plantago major* with *Aloe vera* was also reported [30]. Ethanol extract of *Calendula blossom* significantly improves wound healing in experimentally induced thermal burns in rats due to the increase in collagen hydroxyproline and hexosamine content [31]. Antimicrobial, antioxidant properties, improved recovery from the inflammation phase and increased production of granulation tissue were also reported [32-34].

![Graph showing effect of combined extracts on wound contraction](image-url)

*Fig. 3. Effect of combined extracts CERE 1, CERE 2 and CERE 3 on excision wound expressed as percentage of wound contraction.*

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M. Lazarova et al.: Natural substances with therapeutic potential in wound healing
Fig. 4. Control rats untreated with CERE. Measurement of the size of the wound on: (a) 3rd, (b) 6th, (c) 10th, (d) 14th and (e) 19th day.

Fig. 5. Treated rats with CERE 1 and measurement of the size of the wound on: (a) 3rd, (b) 6th, (c) 10th, (d) 14th and (e) 19th day.

Fig. 6. Treated rats with CERE 3 and measurement of the size of the wound on: (a) 3rd, (b) 6th, (c) 10th, (d) 14th and (e) 19th day.

The herbal agents from leaves of *P. major* and *Calendula* extracts also have shown beneficial effects of the complex product in the treatment of wounds. Samuelsen et al. reported that the topical treatment with *P. major* extract eradicated the infections and healed the wounds [35]. However, our data showed that application of only the extract of *Calendula* with mucus extract (CERE 2) is the less efficient combination. The obtained experimental results clearly demonstrated positive effects of preparations CERE 1 and CERE 3 on wound healing process. Due to the richness of biologically active compounds in the three extracts, CERE 1 was found to be with the highest wound-healing potential. It is known that plant extracts are rich in vitamins C, A and K, glycosides, alantoin, tannins terpenoids, flavonoids, saponins, coumarines, quinones, volatile oil, carotenoids and amino acids [11]. These phytoconstituents have wide applicability as antioxidant, antimicrobial, anti-inflammatory, anti-ulcer, anti-proliferative, antiparasitic, hypoglycemic, hypolipidemic and wound healing potential in experimental and clinical trials [7, 32].

CONCLUSION

On experimental rat’s excised wound models we observed significant acceleration of healing process
of the wounds treated with CERE 1 and CERE 3 in comparison to untreated controls. The best wound healing effect showed mixture from mucus from H. aspersa, P. major leaf extract and extract of Calendula flowers (CERE 1). Further research shall clarify the individual role of biologically active ingredients as well as their interactions.

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REFERENCES