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Development and characterization of an emulgel based on a snail slime useful for dermatological applications

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ABSTRACT

Snail slime is an interesting material for effective dermatological use (e.g. wounds). Its properties are stricly connected to the origin. In this paper a snail slime, deriving from the species Helix aspersa Muller and obtained from a company, was deeply characterized and then properly formulated. The slime, obtained by Donatella Veroni method, was firstly submitted to NMR analysis in order to evaluate the chemical composition. The main molecules found are glycolate and allantoin, well known for their activities in wound healing promotion. In vitro experiments performed on keratinocytes, revealed the snail slime ability to promote cellular well-being. Moreover, the microbiological analysis showed high activity against many strains involved in wounds infections such as gram+ (e.g. *S. aureus*, *S. pyogenes*), gram- (e.g. *P. aeruginosa*, *E. coli*) and the yeast *C. albicans*. The effect on skin elasticity was evaluated as well by the instrument Cutometer® dualMPA580. The snail slime was then formulated as hydrophilic gel, using a combination of corn starch and sodium hyaluronate as polymers, then used as external water phase of an O/W emulgel. The formulation is physically stable and easily spreadable and demonstrated antimicrobial activity as observed for slime alone, suggesting its suitability to be used for wound treatment.

1. Introduction

Helix aspersa Muller (HAM) is the most prevalent snail specie in european countries, especially in mediterranean regions (Dinica et al., 2021). These terrestrial mollusks consist of a small body able of retracting into a spiral shell under danger conditions (Cilia et al., 2018). Helix aspersa Muller (HAM) possess four glands in the feet epithelium responsible for the production of a viscoelastic material called mucus or slime. The slime is very important for locomotion as it provides snails with both adhesive and lubricant properties. Moreover, the slime avoids the dehydration and protects the snail from possible predators (Cilia et al., 2018). HAM nutrition varies depending on the season and the areas in which snails live. Since many years snails was considered a succulent food both for humans and animals as rich in minerals, essential amino acids and polyunsaturated fatty acids (PUFAs). The interest in the secretions of this animal has also always been alive. The snail slime, known and used since ancient times, has also been highly appreciated in cosmetics and pharmaceuticals fields. The use of snail slime in medicine dates back to the ancient Greeks and Hippocrates suggested its use to treat skin inflammation (Cilia et al., 2018; Greistorfer et al., 2017; Ekin et al., 2018).

The suitability of a snail slime for skin application is connected to its chemical composition depending on the growth conditions such as environmental factors (temperature, humidity) and feeding. As reported by numerous scientific researches, allantoin and glycolic acid are the main molecules found in slime, responsible for interesting activities such as collagen synthesis stimulation in fibroblasts, cell turnover stimulation and water content retention in the extracellular matrix (Dinica et al., 2021; Laneri et al., 2019). In addition to, snail slime is rich in glycoproteins (e.g. achacin) (Cilia et al., 2018; Ehara et al., 2002) and organic acids (e.g. acetic, citric, lactic, tartaric) responsible for the antimicrobial activity (Hirshfield et al., 2003; Carpenter et al., 2009; Vassilev et al., 2020). The antioxidants superoxide dismutase and polyphenols are also present (Cilia et al., 2018; Onzo et al., 2021) as well as essential amino-

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acids (e.g. valine, leucine, isoleucine, tryptophan) which are able to form peptides secondary structure. Finally, vitamins (e.g. A,C,E) gives to the snail slime very important properties for skin health and are useful for the treatment of some skin diseases. Vitamin A is responsible for cellular differentiation, playing an important role in immune activity and epithelial barrier function. Vitamin C (water-soluble) and vitamin E (water insoluble) are valuable antioxidant molecules useful for free radical's neutralization. All these activities are important in preventing skin aging process. Based on this, snail slime is considered a precious material to be used for the development of products intended for skin use. Since the 18th century snail slime-based products have been purposed for cosmetic and dermatological use (Cilia et al., 2018). Nowadays, snail slime is largely employed in cosmetic products in which its anti-ageing activity is exploited. However, the main compounds found in snail slime could be also useful to treat many skin problems such as wounds. Each snail slime presents specific peculiarities connected to the specie as well as the growth conditions. Thus, it was considered interesting to evaluate the dermatological applications for wound treatment of a snail slime obtained from Helix aspersa Muller specie bred in Italy (hilly terrain in the Tuscany region) and furnished by the company "Natura Italiana della dott.ssa Serena Castrini". Currently this snail slime is used from the latter in cosmetic products available on the market but, its use in dermatological field it has not yet explored.

The aim of the present project was to perform a characterization of this specific snail slime aiming to evaluate its activities and its suitability for wound treatment and to formulate it in a semisolid formulation in order to purpose a product that can be easily employed in dermatological field for example for the treatment of damaged skin (wound).

2. Materials and methods

2.1. Materials

Helix aspersa Muller snail slime (HAMS) was gently supplied by "Natura Italiana della dott. ssa Serena Castrini" (Chiusi, Siena, Italy). The commercial slime was purchased through the appropriate commercial channels. DMSO was purchased from Thermo-Fisher Scientific (Waltham, MA, USA). Corn starch, glycerol, hyaluronic acid sodium salt (MW 1500–1800 Da), were purchased by A.C.E.F. s.p.a. (Fiorenzuola d'Arda (PC), Italy). Olifeel® TD O/W was purchased from ROELMI HPC (Origgio, Italy). Trypsin (EDTA), streptomycin, penicillin, glutamine, fetal bovine serum (FBS), Dulbecco's modified Eagle medium (DMEM), PBS (phosphate buffered saline) were purchased from Microtech srl (Pozzuoli, NA, Italy). Ultrapure water was obtained by reverse osmosis process in a MilliQ system Millipore (Rome, Italy). Other reagents and solvents were of analytical grade and used without further purification.

2.2. Snail slime extraction

HAMS was extracted during snails'growth in open fields in the middle of vegetation. Rapeseed, kale, proteor cabbage and chard were used as suitable feeding and white clover is used in order to protect the gastropods from predators and adverse weather conditions. The extraction was performed by Donatella Veroni method based on the use stimulating solution or sterilized and/or ozonated water using the extractor machine OzoSnail (Veroni and Franzoni, 2017).

2.3. Snail chemical characterization

The snail slime qualitative and quantitative composition was studied by NMR analysis using the Bruker 600 MHz NMR spectrometer and the ELISA microplate filter reader equipped with Gen5TM sw (Bio-Tek Instruments).

The protein content was indirectly quantified through the Kjeldahl method, according to ISO1871:2009 (Food and feed products. General guidelines for the determination of nitrogen by the Kjeldahl method). This method consists of a three-step approach: digestion, distillation, and titration. The first step was performed with the aid of a catalyst (Kjeltabs Se/3,5 – Foss Analytical a/s, Hillerod, Denmark) at high temperature in sulfuric acid (Sigma-Aldrich, MO, USA). The other two steps were carried on by the automated Kjeldahl analyzer Buchi AutoKjeldahl Unit K-370 (BUCHI Italia s.r.L Cornaredo Italy). The nitrogen content was quantified according to Eq. (1):

$$\frac{(V-B)xNx14x100}{Wx1000} \quad (1)$$

where: V is the volume of HCl standard solution used for the sample titration (mL); B is the mean value of the volume of HCl standard solution used for black's titration (mL); N is the normality of HCl standard solution (0.2), 14 is nitrogen molecular weight, W is the sample weight (g).

The protein content was then obtained multiplying the nitrogen content with the protein conversion coefficient (6.25).

2.4. Rheological analysis

The viscosity was measured by a Stresstech HR rheometer (Rheological Instruments, AB Milano, Italy) provided with cone-plate geometry, diameter 40 mm, angle 1°. The shear stress was set in the range 10 – 1000 Pa working at 25.0℃ ± 0.1 (room temperature, R.T.), n = 3 ± SD.

2.5. Effect on skin elasticity

The effect on skin elasticity was evaluated by the instrument Cutometer® dual MPA580, purchased from KELISEMA s.r.L. (Tavernerio (CO) Italy). The *Helix aspersa Muller* slime snail (hereinafter called HAMS), which was the subject of the study, was compared to a marketed product (hereinafter called commercial slime),

The study was performed on 27 people divided in four groups according to age and gender:

- women < 40 (average age 26.7 ± 4.9, n = 10),
- women > 40 (average age 55.0 ± 5.7, n = 6);
- men < 40 (average age 26.12 ± 4.0, n = 8);
- men > 40 (average age 59.0 ± 6.2, n = 3).

The number of people in each group is different and depends on their availability to participate. Allergic subjects as well as people undergoing to dermatological treatments were not included in the study. Every participant was provided for a sample of both HAMS and commercial slime, to apply once a day for 15 days. Commercial slime was applied on the left side of the face and HAMS on the right side. In order to avoid interference, during the period of use of the slimes the use of other cosmetics and dermatological products was prohibited.

According to the method reported by Kawalkiewicz et al. (2021) three time points were fixed as listed below:

- T0: measurement performed before slimes application,
- T1: measurement taken after one week of application,
- T2 measurement taken after two weeks of application.

The following face sites were considered for slimes application corner eye (CE) for women and forehead (F) for men (Figure S1, Supplementary material).

The measurements were performed working in the following conditions: a negative pressure of 400 mbar was used for 2 s of suction and 2 s of release. Ten cycles of suction and release were performed for each measurement so that skin fatigue was also considered according to the conditions reported in literature (Ohshima et al., 2013). The measurements were performed in the temperature range of (20–22 °C) and in the relative humidity conditions of 40–60 % properly measured by

KELISEMA sensor for environmental conditions.

For each measurement three curves were registered and each datum represent the mean of three measurements ± SD. The measured parameters were: i) R0 (mm) = expression of skin compactness/elasticity, ii) R2 (%) = overall elasticity, iii) R5 (%) net elasticity, iv) R7 (%) = ratio between immediate recovery and total amplitude.

2.6. Statistical analysis

The relationship between the participants age and the measured parameters of each facial regions was studied using Pearson's correlation coefficient test (Ryu et al., 2008). P value was used to evaluate the statistical significance. f $p < 0.05$ was considered to be statistically significant (Ohshima et al., 2013).

2.7. Antimicrobial activity

The antimicrobial activity was evaluated against 10 strains, including Gram positive bacteria, Gram negative bacteria and yeasts. All the tested strains were bought from Microbiologics, St. Cloud, MN, USA. *Propionibacterium acnes* was bought from Mecconti S.A.R.L. Sp. z o.o., (Warsaw, Poland). The experiments were performed using agar well diffusion technique (Pagano et al., 2021). For each microorganism an initial suspension of 0.5 McFarland in 0.9 % sterile saline solution was prepared and 100 µl were distributed on Mueller-Hinton agar (MHA)/ Mueller-Hinton agar 5 % defibrinated sheep blood (MHAB) plates (Oxoid Limited, Basingstoke, UK) by a swab. Then, 50 µl of product, snail slime which was tested alone and diluted at 10 % (v/v), at 30 % (v/v) and at 50 % (v/v), were placed in a hole (diameter of 7 mm) previously made in the center of the plate that was incubated according to their specific growth conditions. Negative control was set up using sterile demineralized water, while antibiotic/antifungal discs (Oxoid Limited, Basingstoke, UK) were used as positive controls. After the incubation time, the presence of the inhibition halo was investigated and the diameter was measured by a gauge. The experiments were performed in triplicate and the mean of the inhibition zone and standard deviations were calculated. The data obtained were statistically analyzed by an analysis of variance (ANOVA) model, using the generalized linear model (GLM) procedure of SAS (SAS Institute Inc., Cary, NC, USA, 2001). A mixed model was used with treatments (HAMS as it is and diluted with distilled water: HAMS 10 % v/v, HAMS 30 % v/v, HAMS 50 % v/v and positive control) and microorganisms as fixed effects. The duplicate effect was determined to be insignificant and was deleted from the model. To explain significant mean differences ($p < 0.05$), Tukey's post-hoc analysis was performed.

2.8. Cytotoxicity, wound healing and morphological analysis

Cells viability was evaluated by MTT test. The experiments were performed on HaCat cells (human immortalized keratinocyte cell line) bought from I.Z.S.L.E.R. (Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna), chosen as representative of epidermidis. Cells were growth in monolayer cultures, in DMEM complete medium supplemented with 10 % heat- inactivated FBS, 2 mM of L-glutamine, and antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin) and incubated at 37 °C under 5 % CO_2 atmosphere. Cells were seeded onto 96-well plate (density of 1×10^4 cells/well). After 24 h fresh complete medium was replaced and cells were incubated with HAMS scalar dilutions (2 %, 5 %, 10 %, 30 % and 50 % v/v) in DMEM for 6 h, 12 h and 24 h. Then, MTT reagent dissolved in PBS 1X was added to the culture in order to obtain a final concentration of 0.5 mg/ml. The experiment was repeated twice using the snail samples in two different pH conditions: HAMS as it is (pH 2.52) and neutralized (pH 7.2) by using a sodium carbonate – sodium bicarbonate buffer (0.1 M) (Dawson et al., 1986). In all the experiments, untreated cells (CTR) were used as negative controls (Pagano et al., 2022). The supernatant was removed from each well after 3 h of incubation and the formed formazan salt crystals dissolved using DMSO (200 µl). After 30 min the results were acquired by an automatic microplate reader (Eliza MAT 2000, DRG Instruments, GmbH). The absorbance values (OD) were measured spectrophotometrically at $\lambda_{max} = 540$ nm. Cell viability calculation by MTT assay was performed by using Eq. (2) (n = 3 ± SD).

$$\%viability = \frac{Averageoftreatedcells - background}{Averageofcontrolcells - background} x100 \quad (2)$$

A one-way ANOVA test was performed using the Graphpad program (GraphPad Prism 9.2.0.332, GraphPad software, San Diego, CA, USA). The wound healing test (Pagano et al., 2022) was performed by Cyto-Select™ Wound Healing Assay Kit (Cell Biolabs, Inc., San Diego, USA) that allows to simulate in vitro a wound. It was used to study the effect of the snail slime on keratinocytes growth. This technique consists in the performing a linear thin scratch "wound" (creating a gap) in a confluent keratinocyte monolayer. The images of cells filling the gap are taken at regular time intervals. HaCat cell were seeded in a 24-well plate at the final concentration 5×10^4 cell/500 µl (1×10^5 /ml) and incubated overnight. Inserts were removed and cells were washed with PBS 1X to remove dead cells and debris. The cells were then incubated using two dilutions (10 % and 30 % v/v) of neutralized HAMS. After 6 and 12 h, the treatments were removed and cells were primarily washed with PBS 1X, then fixed with MeOH: EtOH ice-cold (1:1) and frozen for 5 min at −20 °C. All wells were rinsed gently with PBS 1X to remove fixatives and finally stained using methylene blue solubilized in PBS 1X (10 mg/ml). This solution was added to the cells and left in contact for 15 min at R.T. Then, the cells were washed two times with PBS 1X and finally with sterile water. The wound field was photographed by using a confocal microscope (Orma-Eurotek 620 with LCD) equipped with a Nikon D800 camera using a 4X magnification.

2.9. Morphological analysis

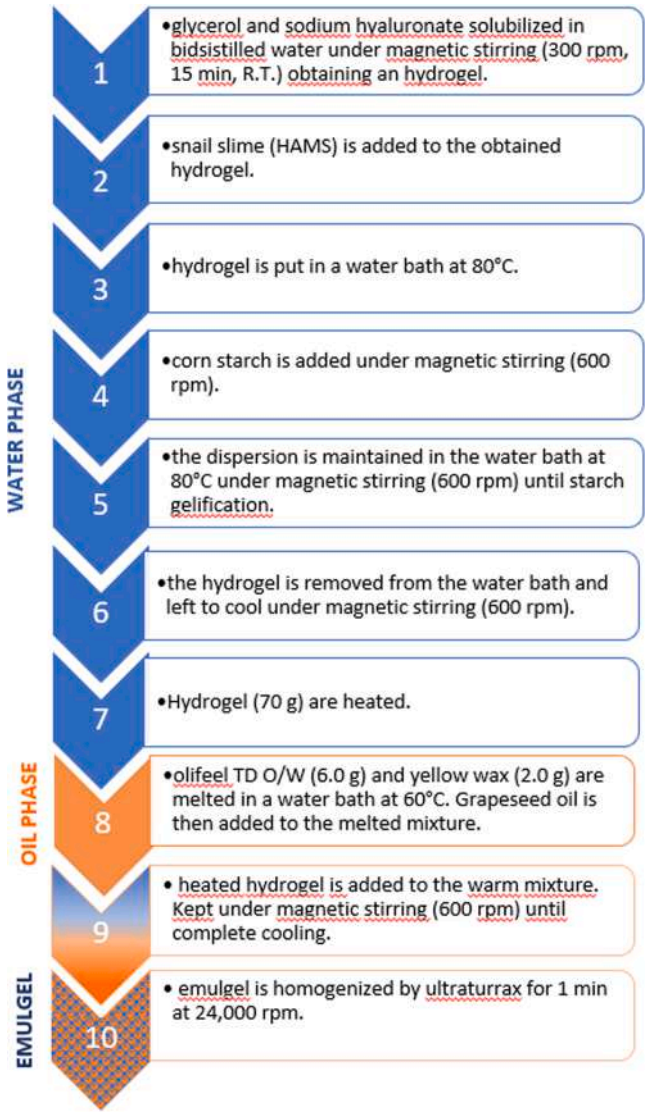
Morphological analysis was carried out by seeding the HaCat cells in a 6-well plate at the final concentration 2 $x10^5$ cell/well and incubated overnight. The cells were treated for 6 and 12 h with 10 % and 30 % neutralized HAMS. The cells were fixed and stained as previously described in 2.8 paragraph. Imaging was performed with 40X magnification (Orma-Eurotek 620 with LCD confocal microscope equipped with a Nikon D800 camera).

2.10. HAMS formulation

HAMS was formulated as O/W emulgel (scheme 1) in which the external water phase is represented by a hydrogel prepared using both sodium hyaluronate (NaHy) and corn starch (CS) as polymers. The optimized hydrogel has the following composition:

CS	4.0 w/w%
NaHy	1.5 w/w%
glycerol (Gly)	3.0 w/w%
neutralized HAMS	30.0 w/w%
Bidistilled water	61.5 w/w%

Briefly, Gly and NaHy were solubilized in bidistilled water under magnetic stirring (300 rpm) at R.T. for 15 min. Then, HAMS was added to the obtained hydrogel and the mixture kept under magnetic stirring (500 rpm) in a water bath thermostated at 80 °C (starch gelification temperature). Lastly, CS was added and, once starch gelification occurred, the formed hydrogel was allowed to cool under constant magnetic stirring (600 rpm) at R.T. The hydrogel was prepared in a hermetically closed jar to prevent water loss during heating. This hydrogel (70 % w/w) was then used as water phase (W) of an O/W emulgel (30:70 w/w O/W ratio) reaching HAMS amount of 21 % w/w in the final formulation. The oil phase (O) has the following composition: liquid lipophilic phase



Scheme 1. Description of the procedure followed for emulgel preparation.

(grapeseed oil) 22 % w/w, emulsifying agent (Olifeel TD O/W) 6.0 % w/w solid lipophilic phase (yellow wax) 2.0 % w/w.

The emulgel was prepared as follows; the solid components of the oil phase (O) and the emulsifier were melted in a water bath at 60 °C. Then, the liquid lipophilic phase was added to the obtained molten mass. The hydrogel, used as water phase (W), was heated and then added to the prepared oil (O) phase and the obtained emulgel was kept under magnetic stirring (400 rpm) until complete cooling. Then, in order to better promote the O phase dispersion, the emulgel was homogenized by ultraturrax T25 IKA LABORTECHNIK for 1 min at 24,000 rpm.

2.11. Formulation characterization

The morphology of the oil droplets of the emulgel was observed by a Nikon Eclipse 80i optical microscope (Melville, LA, USA).

The spreadability was evaluated according to Arvouet-Grand et al. (1995). One gram of formulation was pressed between two glass plates 20 cm square and its diameter (spread diameter) was measured after one min. Under these experimental conditions, they were applied the following terms: Semi-stiff creams to samples with a spread diameter $\leq$ 50 mm and semifluid creams to those with a spread diameter $>$ 50 mm but $<$ 70 mm (Garg et al., 2002).

The physical stability was evaluated in different conditions:

1. storage at R.T. for three months protected from light.
2. centrifugation (Hettich zentrifugen, Universal 32 R) at 2500 rpm for 20 min at R.T.
3. accelerated stability testing. The test was performed for 3 months, during which all formulations are submitted to 4 cooling-heating cycles. Specifically, during each cycle the sample is stored for 7 days in a refrigerator at 4 °C, 7 days at R.T. and 7 days in oven at 40 °C. The aim of this assay is to evaluate emulgel modifications, due to fast temperature changes, such as discontinuity, separation, change in colour and gas production.

2.12. Emulgel antimicrobial activity

The antimicrobial activity of the prepared emulgel was evaluated by the same procedure followed for the not formulated HAMS and reported in par. 2.7.

3. Results and discussion

3.1. Chemical characterization

By NMR analysis it was possible to know the main molecules contained in the HAMS of interest. The obtained results (Table 1) show that benzoate, citrate and glycolate are the most abundant molecules found. Benzoate presence is justified as it is the main component of the product used from the producer as snail preservative. Glycolate is an interesting molecule able to stimulate collagen synthesis as well as to enhance the cellular turnover by promoting the exfoliation process, avoiding pores obstruction (Okano et al., 2003). Citrate is useful as preservative as prevents bacterial and microbial growth. Another molecule widely present in HAMS is allantoin and this is very important for a material to be applied to the skin, because of its multiple beneficial activities on the skin. This molecule in fact is responsible for fibroblasts proliferation and synthesis of both collagen and elastin (Araújo et al., 2010). It has a desquamating action and participates to wound healing processes inhibiting the migration of inflammatory cells to the wounds. Furthermore, thanks to its ability to increase the water content in the extracellular matrix, allantoin has a moisturizing action.

In addition to, essential amino acids such as threonine, leucine, valine, and isoleucine and others (e.g. alanine, proline, valine) were identified. Their presence is very important as it is well known that

Table 1
Metabolites and relative concentrations obtained by NMR analysis performed on HAMS sample.

HAMS Metabolites	Concentrations (μM)
Glycolate	390062.7
Citrate	99993.6
Benzoate	6088.8
Allantoin	583.5
Acetate	235.5
Tartrate	103.8
Betaine	100.8
Lactate	61.5
Acetoacetate	12.3
Acetoin	15.3
Alanine	22.2
Proline	24.0
Malonate	21.6
Glycine	15.9
Threonine	14.1
Leucine	13.5
Mannose	7.8
2-Hydroxyisovalerate	6.0
Valine	5.4
Ornithine	4.5
Isobutyrate	3.6
Isoleucine	3.0
Choline	1.5

amino acids participate to the synthesis of skin proteins such as keratins, collagen and elastin. This could represent a valuable support for damaged skin repair, acid-base balance and water retention in cellular layers as well as in the maintenance of skin microbiome (Joanna, 2020).

The analyzes carried out also revealed that the free amino acids hydroxyproline and glutamic acid were not present (detection level μM).

The protein content was determined through the Kjeldahl method that revealed a content of 0.08 g/100 g and 0.14 g/100 g for HAMS and commercial slime respectively.

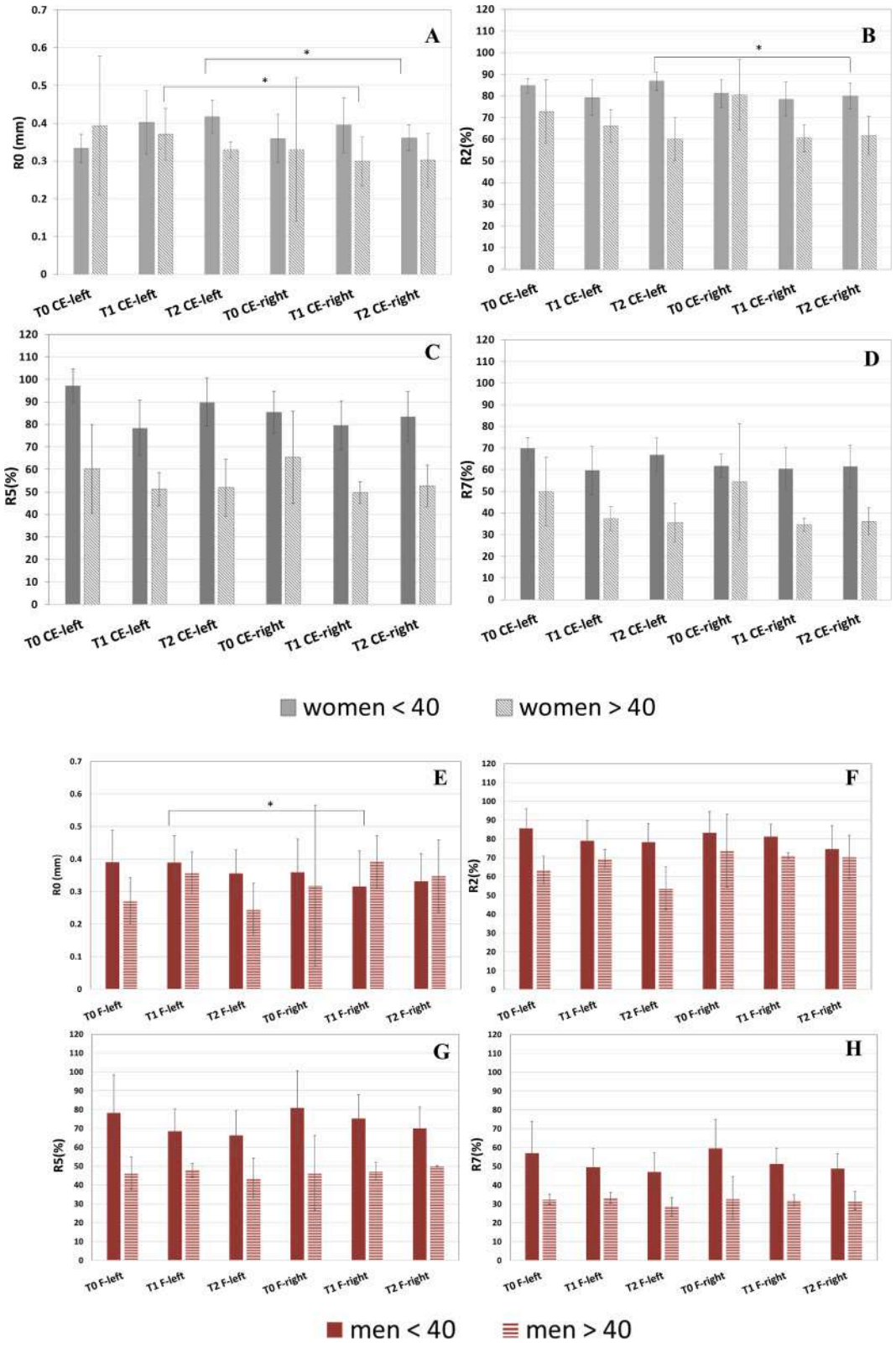


Fig. 1. Values of parameters **A**) R0; **B**) R2; **C**) R5; **D**) R7 for women < 40 and women > 40; **E**) R0; **F**) R2; **G**) R5; **H**) R7 for men < 40 and men > 40 (*$p < 0.05$).

3.2. Effect on skin elasticity

Skin elasticity is a factor that influences some processes, for example skin healing capability in certain conditions such as wounds. It has been observed that a skin showing limited elasticity, such as aged skin, shows reduced healing capacity (Potekaev et al., 2021; Ariffin Khalid et al., 2022). Based on this, the use of a product able to increase skin elasticity could improve its intrinsic wound healing capacity. Literature data documents snail slime capacity to improve skin elasticity due to the stimulation of collagen and elastin fibres production (Rashad et al., 2023).

In order to perform a preliminary evaluation, HAMS effect on skin elasticity was studied by a Cutometer® dual MPA580. The study aimed to compare the influence on skin elasticity, after 15 days of application, of both HAMS and a commercial slime used for comparison. With this aim 27 volunteers, without skin pathologies or pre-existing allergy problems, were enrolled. The parameters R0 (skin pliability/firmness, low values suggest high skin firmness, Darren, 2022), R2 (express skin ability to recover its original structure, high values suggest good skin elastic properties), R5 (net elasticity, an increase of this value means an improvement of skin elasticity) and R7 (skin ability to return to its original position after deformation) were considered as they are the main related to skin elasticity.

For women groups the most interesting results were obtained for R0 parameter. For women < 40, a statistically significant difference was detected between the two slimes at T2 (Table 1S, Supplementary material). In particular R0 was lower for the right side compared to the left one, suggesting a better skin firmness for the side treated with HAMS (Fig. 1A). For the left side an increase of R0 value from T0 to T2 was detected (Fig. 1A) suggesting a worsening of skin firmness by using commercial slime. For the women > 40 the statistically significant difference (Table 2S, Supplementary material) was detected at T1 with the R0 lowest value (0.29) measured for the right side, treated with HAMS, suggesting that the latter could slightly improve this parameter (Fig. 1A). In the case of R2 parameter a statistically significant difference was observed at T2, observing a value slightly higher for the side treated with commercial slime. For the other two parameters no-statistically significant differences were detected both for women (Fig. 1C and Fig. 1D) and men groups (Fig. 1E-H, Table 3S and 4S, Supplementary material). In general, the preliminary evaluation performed suggest that HAMS shows a positive influence on woman skin elasticity. However, for a better understanding of the effect on skin elasticity will be useful to perform the study for a prolonged time.

3.3. Antimicrobial activity

The next step was to evaluate HAMS potential antimicrobial activity on the strains reported in Table 2. HAMS was employed as it is (not diluted) and diluted with sterile water to obtain the following concentrations: 10 % (v/v), 30 % (v/v) and 50 % (v/v) (Figure S2, Supplementary material). As HAMS was microbiologically stabilized from the producer by the addition of a preservative, the experiment was performed on the latter used as control (alone) and diluted with water at the same concentrations in which it is present in the slime. After incubation, the inhibition halos (diameter expressed in mm) were measured. For the preservative non-inhibition halos were obtained (Figure S3, Supplementary material) while HAMS demonstrated activity against all the tested strains as testified by the inhibition halos measured (Table 2). The diameters decrease as HAMS amount decreases (not diluted and diluted: 50 %, 30 % and 10 %) suggesting a concentration dependent activity.

However, it must be underlined that the agar diffusion method, used to perform the preliminary evaluation of the antimicrobial activity, does not allow to detect inhibition halos for low amount of sample assayed. It can be hypothesized that in the snail slime sample 10 % v/v, the concentration of active molecules responsible for the antimicrobial activity is very low thus, the effect on microorganism growth cannot be

Table 2
Inhibition halos (mm) measured for different amounts of HAMS and for the emulgel. Values are expressed as means ± standard deviation. Different letters in the same row (A,B,C) indicate differences between mean values within each microorganism ($p < 0.05$); different letters in the same column (a,b,c,d) indicate differences between mean values for different micro-organisms within the extracts concentration ($p < 0.05$).CIP-ciprofloxacin, CN-gentamicin, KCA-ketoconazole, LZ-linezolid, P-penicillin G, TE-tetracycline.

	strain	growth conditions	HAMS	HAMS 50 %	HAMS 30 %	HAMS 10 %	Positive control	Emulgel
Gram positive bacteria	*E. faecalis WDCM 00087*	37 °C for 24 h ± 2	23.42 ± 0.68^{Ce}	14.96 ± 0.44^{Bbc}	8.89 ± 0.11^{Aa}	–	TE30μg/disc 22.16 ± 0.82	11.16
	S. epidermidis WDCM 00,036	37 °C for 24 h ± 2	24.79 ± 0.21^{Bf}	14.65 ± 0.47^{Aabc}	14.45 ± 0.49^{Ae}	–	CN10μg/disc 28.64 ± 0.36	12.95
	S. aureus WDCM 00034	37 °C for 24 h ± 2	25.04 ± 0.50^{Cf}	17.63 ± 0.53^{Be}	11.67 ± 0.63^{Ac}	–	TE30μg/disc 21.42 ± 0.54	11.28
	S. aureus MR BAA1708	37 °C for 24 h ± 2	19.58 ± 0.37^{Cb}	14.71 ± 0.53^{Bbc}	11.79 ± 0.26^{Acd}	–	LZ30μg/disc 23.57 ± 0.45	10.32
	S. pyogenes ATCC 19615	37 °C for 24–48 h ± 2	23.85 ± 0.18^{Cef}	20.20 ± 0.42^{Bf}	16.70 ± 0.99^{Af}	–	TE30μg/disc 28.31 ± 0.29	12.76
	B. subtilis WDCM 00003	30 °C for 24 h ± 2	22.05 ± 0.28^{Dd}	16.15 ± 0.77^{Cd}	13.44 ± 0.23^{Bde}	11.32 ± 0.67^{Ac}	P10UI/disc 29.88 ± 0.21	11.78
	B. cereus WDCM 00001	30 °C for 24 h ± 2	19.88 ± 0.20^{Dc}	14.38 ± 0.55^{Cabc}	12.77 ± 0.36^{Bd}	9.97 ± 0.31^{Ab}	P10UI/disc 16.36 ± 0.75	–
	C. perfringens WDCM 00,007	37 °C for 24–48 h ± 2 in anaerobiosis	21.35 ± 0.33^{Cd}	16.83 ± 0.28^{Bde}	12.98 ± 0.13^{Ad}	–	P10UI/disc 31.15 ± 0.15	–
Gram negative bacteria	*P. aeruginosa WDCM 00025*	37 °C for 24–48 h ± 2	20.31 ± 0.53^{Cc}	14.88 ± 0.21^{Bc}	10.17 ± 0.74^{Ab}	–	CN10μg/disc 18.35 ± 0.79	9.91
	E. cloacae WDCM 00,083	37 °C for 24 h ± 2	18.60 ± 0.96^{Cb}	13.69 ± 0.44^{Bab}	9.03 ± 0.16^{Aa}	–	CIP5μg/disc 31.91 ± 0.66	10.51
	E. coli WDCM 00,013	37 °C for 24 h ± 2	19.85 ± 0.20^{Dc}	14.66 ± 0.40^{Cbc}	12.26 ± 0.54^{Bcd}	7.88 ± 0.81^{Aa}	CIP5μg/disc 16.82 ± 0.36	11.50
	K. pneumonia WDCM 00,097	37 °C for 24 h ± 2	18.57 ± 0.37^{Cb}	13.87 ± 0.24^{Babc}	10.43 ± 0.19^{Ab}	–	CIP5μg/disc 29.73 ± 0.22	9.45
	P. mirabilis WDCM 00,023	37 °C for 24 h ± 2	19.36 ± 0.29^{Dbc}	16.27 ± 0.31^{Cd}	14.92 ± 0.11^{Be}	12.67 ± 0.37^{Ad}	CIP5μg/disc 35.12 ± 0.14	11.81
Yeast	*C. albicans WDCM 00,054*	37 °C for 24 h ± 2	15.44 ± 0.45^{a}	–	–	–	KCA10μg/disc 22.60 ± 0.29	–

Table 3
Compositions of the prepared hydrogels.

Sample	CS (w/w %)	Gly (w/w %)	NaHy (w/w %)	HAMS (w/w %)	Bidistilled water (w/w %)	Aspect	Observations
CS_NaHy_1	10	10	0.5	30	49.5		Discontinuous, the solid part (starch) tends to separate from liquid part. Sticky, poor spreadable, residues on the skin after application.No gas developed.
CS_NaHy_2	10	5.0	0.5	30	54.5		Discontinuous, the solid part (starch) tends to separate from liquid part. Sticky, poor spreadable, residues on the skin after application.No gas developed.
CS_NaHy_3	10	3.0	0.5	30	56.5		Discontinuous, the solid part (starch) tends to separate from liquid part. Sticky, poor spreadable, residues on the skin after application.No gas developed.
CS_NaHy_4	5.0	5.0	0.5	30	59.5		Discontinuous, the solid part (starch) tends to separate from liquid part. Sticky, well spreadable, residues on the skin after application.No gas developed.
CS_NaHy_5	5.0	3.0	0.5	30	61.5		Discontinuous, the solid part (starch) tends to separate from liquid part. Not sticky, well spreadable, few residues on the skin after application.No gas developed.
CS_NaHy_6	4.0	3.0	0.5	30	62.5		Continuous, the solid part (starch) tends to separate from liquid part. Not sticky, well spreadable, few residues on the skin after application.No gas developed.
CS_NaHy_7	4.0	3.0	1.0	30	62.0		Continuous, the solid part (starch) tends to separate poorly from liquid part. Not sticky, well spreadable, few residues on the skin after application.No gas developed.

(*continued on next page*)

Table 3 (*continued*)

Sample	CS (w/w %)	Gly (w/w %)	NaHy (w/w %)	HAMS (w/w %)	Bidistilled water (w/w %)	Aspect	Observations
CS_NaHy_8	4.0	3.0	1.3	30	61.7		Continuous, the solid part (starch) tends to separate poorly from liquid part. Not sticky, well spreadable, few residues on the skin after application.No gas developed.
CS_NaHy_9	4.0	3.0	1.5	30	61.5		Continuous, no separations were observed. Not sticky, well spreadable.No gas developed.

perceived. Probably the lack of inhibition halo is just the result of the limited assay sensitivity.

For the yeast *C. albicans* the activity was observed for not diluted HAMS. This result is very interesting as studies reported in literature performed on different slimes, did not show activities against this yeast (Pitt et al., 2015) while a certain activity was observed from soft tissue extract metabolites (Azeem et al., 2022). This is very important because in the latter case it was necessary to take soft tissue from the snails with risks of animal suffering. Thus, the antifungal ability of the studied slime suggests an interesting application of this material without snails suffering.

The antimicrobial activity, observed especially against the strains reported in Table 2 (Figure S2, Supplementary material), namely *E. faecalis*, *S. epidermidis*, *S. aureus*, *S. aureus MR*, *S. pyogenes*, *P. aeruginosa*, *K. pneumoniae* notoriously involved in wounds infection, suggests that HAMS can find interesting applications in products intended for wounds treatment. This remarkable aspect deserves to be explored further as there is no clear medical data in scientific literature. In one of the few studies on this subject, Pitt *et al.* observed that the slime of the species *H. aspersa* showed antimicrobial activity mainly attributable to proteins with molecular weights between 30 and 100 kDa (Pitt et al., 2015).

3.4. Cytotoxicity wound healing and morphological analysis

Considering the possibility to formulate HAMS in a topical product for wound treatment, the effect on cells viability was evaluated using keratinocytes as cell line representative of epidermidis.

The experiment was performed on stabilized HAMS in the perspective to perform an evaluation on the slime that realistically can be used as raw material in a dermatological formulation.

Before to perform the experiment, HAMS pH value was measured resulting very low (2.52), probably attributable to the presence of benzoic acid as preservative. Considering that, two different MTT assays were done. In the first one HAMS pH was maintained unmodified. The obtained results after 6 (Fig. 2A), 12 (Fig. 2B) and 24 h (Fig. 2C) of exposure show that cell viability is maintained > 60 %, after 6 and 12 h, for all the dilutions except for 50 % v/v. Prolonging the exposure time to 24 h the safest higher concentration is 5 % v/v. It was hypothesized that the observed cytotoxicity could be due to the culture medium acidification by HAMS. Thus, it was considered useful to solve this problem adjusting HAMS pH to neutral values (pH 7.2) using a sodium carbonate – sodium bicarbonate buffer (0.1 M). It should be considered in fact, that the studied slime is already used in commercially available products, such as serum (containing high slime percentages), which pH is neutral. Thus, the second MTT assay was performed on neutralized HAMS diluted samples. The obtained results show that cell viability resulted $>$ 70 % also for the highest HAMS dilution (50 % v/v) for an exposure time of 6 h (Fig. 2A). Increasing the exposure time to 12 h (Fig. 2B) and 24 h (Fig. 2C), the highest safe concentration resulted 30 % v/v measuring a viability value > 80 %.

Considering that the optimal results were obtained using neutralized HAMS, two safe concentrations (10 % and 30 % v/v) were assayed in vitro in order to evaluate the healing activity. Generally, the average residence time on the skin of a dermatological product is about 12 h considering that the product is removed after 8–12 h by cleansing. The obtained results (Fig. 3) show that both concentrations are not able to promote the wound field closure within 12 h. However, the presence of cell aggregates is detectable in the wound field of cells treated with both concentrations. This suggests that HAMS possess a certain ability to stimulate keratinocytes growth. We don't forget that wound healing process is elaborate due for high cellular diversity, complexity, and plasticity and in vitro models of wound healing despite their limitations, provide an unprecedented opportunity to further explore the molecular and cellular features of wound repair. In order to better investigate this aspect, the morphological analysis was performed. From the observation of keratinocytes, the morphology, confluence and growth indicate good biocompatibility of neutralized HAMS (Fig. 4).

3.5. Snail slime formulation

The preliminary characterization performed suggested that the studied HAMS could be a useful material for dermatological products intended for wound treatment. In this perspective, it was considered interesting to use HAMS as ingredient of a semisolid formulation. In order to purpose an effective, but at the same time a sustainable formulation, natural raw materials were chosen for the preparation. Firstly, HAMS was formulated as hydrophilic gel using the recipe of "starch glycerolate", reported in the Farmacopea Ufficiale Italiana XII Ed. (F.U.XII Ed.), as starting point. It consists of: corn starch (CS) 10 % w/w; glycerol (Gly) 70 % w/w and water 20 % w/w. Starch is a biodegradable and biocompatible polysaccharide, able to form a gel under specific temperature conditions (Pan et al., 2021), used since many years in topical products due to its soothing capacity and thus useful for damaged skin (e.g. wounds). Together to this, it was considered interesting to evaluate CS combination to the biopolymer sodium hyaluronate (NaHy), a glycosaminoglycan known for its wound healing capacity (Voinchet et al., 2006; Colella et al., 2012).

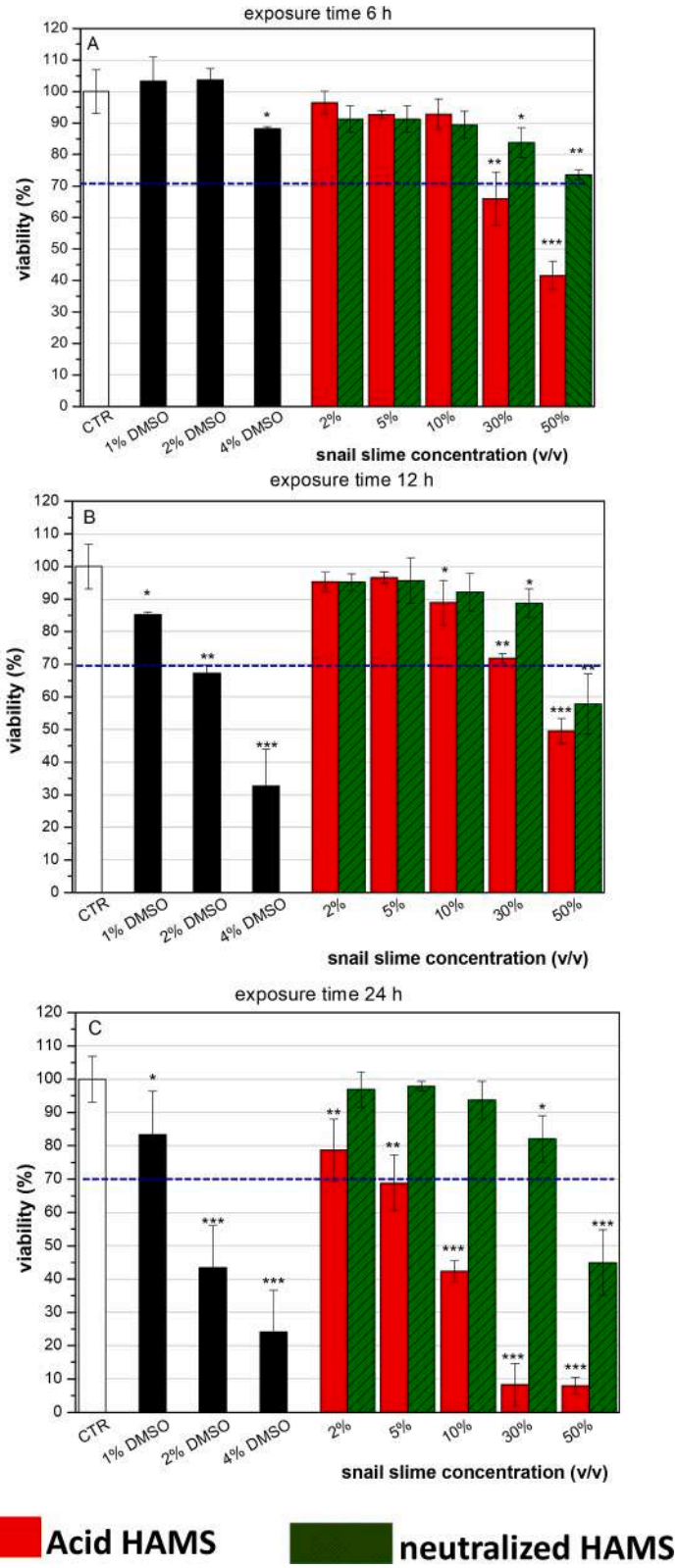


Fig. 2. **A**) HaCaT cells viability incubated using dilutions of acidic (pH 2.52) and neutralized HAMS (pH 7.2) after an exposure time of **A**) 6 h, **B**) 12 h and **C**) 24 h. The negative control (CTR) is represented by untreated cells in DMEM (white bar), the positive control by cells treated with DMSO in three different concentrations (1 %, 2 %, 4 %, black bars). The percentage of viable cells in comparison to the control was reported as the mean ± SD of five independent experiments. Dotted lines indicate 70 % cells viability. *$p < 0.01$, **$p < 0.001$ and ***$p < 0.0001$, treatments versus CTR (one-way ANOVA test).

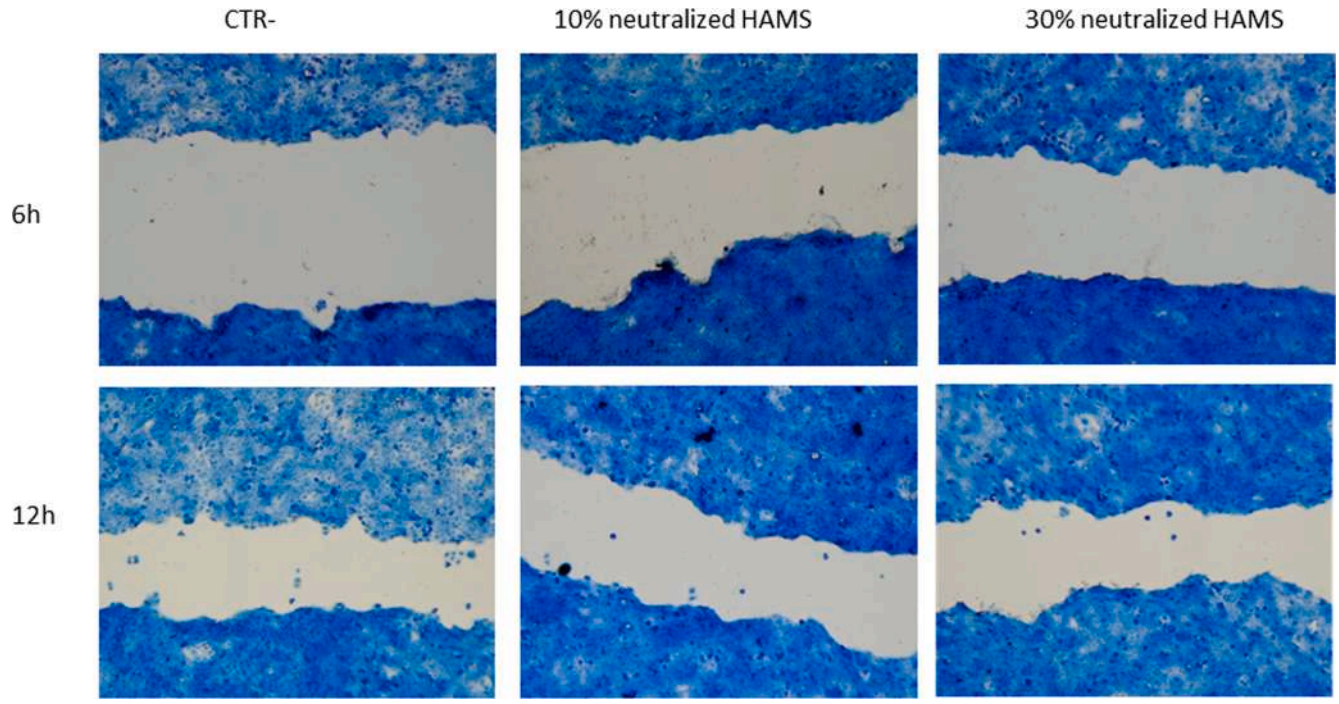


Fig. 3. Wound field observed at 6, 12 and 24 h for untreated cells (CTR) and for cells treated with two different neutralized HAMS concentrations (10 % v/v and 30 % v/v), magnification 4X.

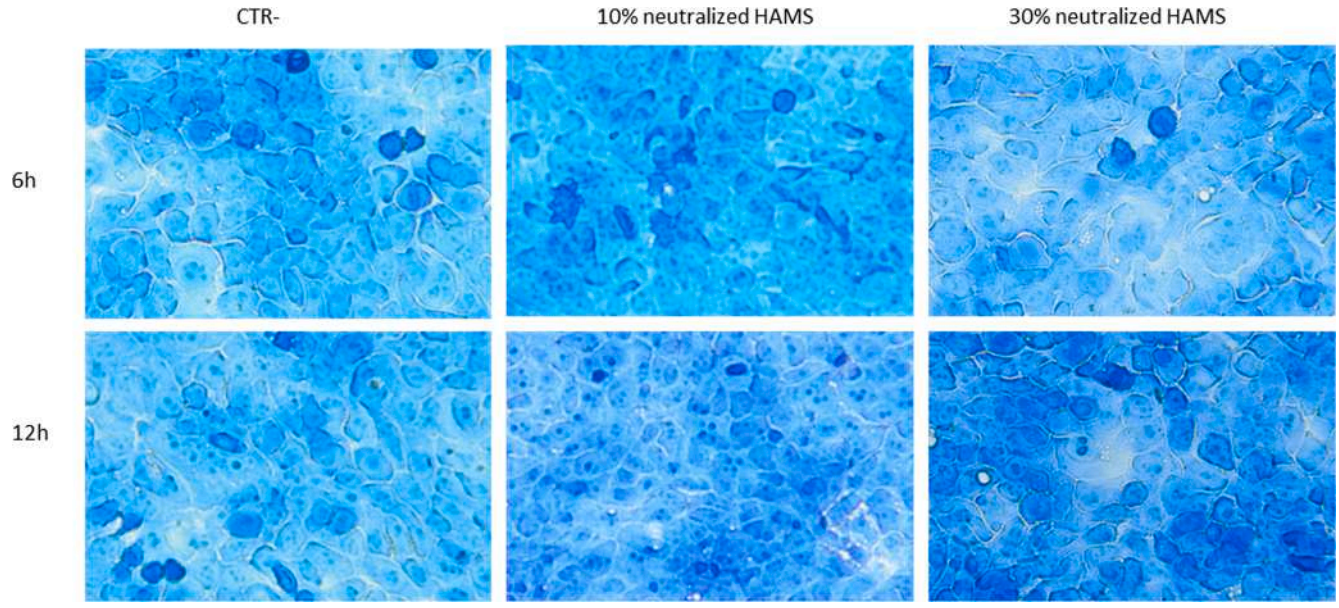


Fig. 4. Optical microscopy images of HaCat cell line after 6 h and 12 h of exposure time of neutralized HAMS dilutions (10 % v/v and 30 % v/v), magnification 40X.

Considering the results obtained from both the antimicrobial activity and cytotoxicity assays it was decided to use the HAMS (neutralized) dilution 30 % v/v, as provided with good activity and at the same time safety for cells (Fig. 2). Many attempts were made in order to find the most suitable % of CS, Gly and NaHy to obtain a stable, uniform and spreadable gel. CS was assayed in the range between 4–10 % w/w (for percentages below 4 % w/w the gel is too fluid while for values > 10 % w/w the gel is very thick and viscous) while Gly amount was used in the range 3–10 % w/w (Table 3). In regard to NaHy, it was decided to investigate a range 0.5–1.5 % w/w (Table 3) considering the amount generally used in the commercially available products. The best results were obtained for the gel containing 4 % w/w of CS and 1.5 % w/w of NaHy (CS_NaHy_9). The latter was then used as external water phase of an O/W emulgel and the composition of a previously optimized cream (Pagano et al., 2021) was used as reference. As the O/W ratio used was 30/70 w/w, the final HAMS concentration in the emulgel was 21 % w/w. The emulgel final composition turned out to be as follows: i) oil phase (O): grapeseed oil (22 g), Olifeel TD O/W (6.0 g), yellow wax (2.0 g); ii) water phase (W): CS_NaHy_9 (70 g).

Grapeseed oil was chosen as lipophilic liquid phase, selected for its proven antioxidant activity and healing capacity (Rekik et al., 2016). Yellow wax was used as solid lipophilic phase while Olifeel TD O/W (cetearyl olivate), deriving from olives non-edible fraction, was chosen as emulsifying agent.

In order to perform a better dispersion of the oil droplets, the prepared emulgel was homogenized by ultraturrax. As testified by the images acquired by the optical microscope in fact, the oil droplets of the untreated emulgel are dimensionally non–uniform (Fig. 5A) while after ultraturrax treatment an improvement of the homogeneity was obtained (Fig. 5B). This also gave greater homogeneity to the entire formulation. The rheological analysis shows (Fig. 6) a shear thinning behavior, important property contributing to an easy and pain-free spreading necessary for the application on damaged skin as a wound. Emulgel spreadability was also evaluated according to the method described by Arvouet-Grand et al. (1995). The obtained results showed a spread diameter < 50 mm (Fig. 5 insert) that classify the emulgel as semistiff, right compromise between a good viscosity, necessary to ensure the stability, and good spreadability on skin.

Lastly, the physical stability was evaluated. For biphasic formulations in fact, as emulgels, the evaluation of physical stability (no separation phenomena) during the storage period is an aspect that must be carefully evaluated to calculate their shelf-life. By the accelerated stability tests it is possible to simulate, in a short time period, the conditions responsible for product modification, generally occurring during a long period. In this study the following tests were performed:

1. storage at R.T. protected from light for 3 months,
2. centrifuge test,
3. accelerated tests with variable temperature cycles, performing three weekly cycles (one week in a refrigerator at 4 °C, one week at R.T., one week in oven at 40 °C). At the end of the application of these stresses, the formulation was observed to evaluate whether separation phenomena occurred. The obtained results (Figure S4, Supplementary material) showed that it was homogeneous, without phase separation and therefore stable in the investigated period.

Emulgel antimicrobial activity was finally evaluated by agar diffusion method. The obtained results (Table 2) show that HAMS activity is maintained also after its formulation. In particular the emulgel resulted active against all the strains tested for HAMS except for *C. albicans*, *B. cereus* and *C. perfringens*. These results are very interesting as the strains sensitive to the emulgel are often involved in wounds infections (Tomic-Canic et al., 2020) suggesting that the developed formulation can be used for wound treatment as suitable alternative to formulations containing conventional antimicrobials as antibiotics whose use is not recommended due to microbial resistance problem.

4. Conclusion

The aim of this work was to develop a new semisolid formulation for wound treatment using a snail slime. Firstly, this raw material was deeply characterized and then used to realize a dermatological formulation.

The NMR analysis revealed the most abundant molecules such as organic acids, including glycolic acid, allantoin and vitamins, substances useful in the support of the wound healing process. This slime showed antibacterial activity against many microbes involved in wound infection such as *S. aureus*, *S. aureus MR*, *P. aeruginosa*. In vitro experiments on keratinocytes revealed evident cellular well-being suggesting its potential benefits on skin.

Based on these findings, an O/W emulgel was successfully realized by combining HAMS with corn starch and sodium hyaluronate (external water phase) in order to obtain a synergism in wound healing promotion. The formulation resulted stable, easily spreadable and active against the bacteria involved in wound infections as slime alone. These characteristics suggest the possible application in the wound treatment representing a suitable alternative to semisolid formulations containing conventional antibiotics which use is not recommended due to microbial resistance problems.

In addition to these technical and healthy properties, this formulation is also appreciable because of its biosustainability. It was realized by using ingredients from natural sources in the perspective to plan a product respectful for both human and environment health.

CRediT authorship contribution statement

Cinzia Pagano: Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Project administration, Investigation, Formal analysis, Data curation, Conceptualization. **Maria Rachele Ceccarini:** Writing – review & editing, Validation, Methodology, Formal analysis, Data curation. **Alessia Marinelli:** Writing – original draft, Validation, Methodology, Formal analysis, Data curation. **Anna Imbriano:** Writing – review & editing. **Tommaso Beccari:** Writing – review & editing, Resources, Investigation, Funding acquisition. **Sara Primavilla:** Writing – review & editing, Methodology, Formal analysis, Data curation. **Andrea Valiani:** Resources, Investigation, Funding acquisition. **Maurizio Ricci:** Writing – review & editing, Resources, Project administration, Investigation, Funding acquisition. **Luana Perioli:** Writing – review & editing, Writing – original draft,

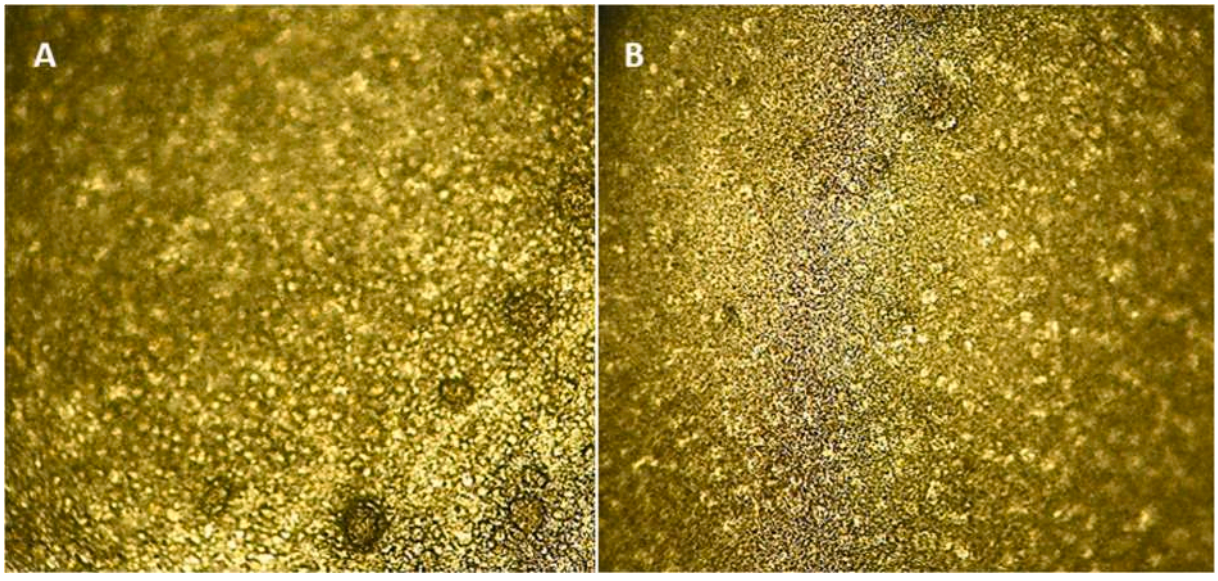


Fig. 5. Optical microscope images of emulgel **A**) after preparation; **B**) after homogeneization by ultraturrax.

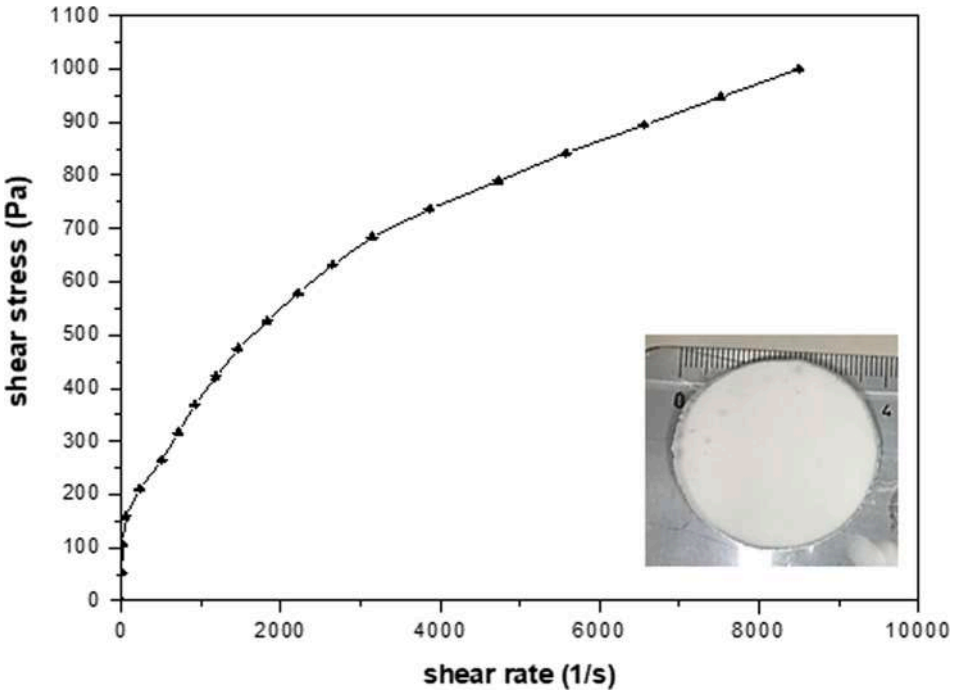


Fig 6. Rheological profiles obtained at 25 °C of the emulgel. Insert: spread diameter measured for the emulgel.

Visualization, Supervision, Resources, Project administration, Investigation, Funding acquisition, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijpharm.2024.124337.

References

Araújo, L.U., Grabe-Guimarães, A., Mosqueira, V.C.F., Carneiro, C.M., Silva-Barcellos, N. M., 2010. Profile of wound healing process induced by allantoin. Acta Cir. Bras. 25, 460–466.

Ariffin Khalid, K., Faris Mohd Nawi, A., Zulkifli, N., Barkat, A., Hadi, H. Aging and wound healing of the skin: a review of clinical and pathophysiological hallmarks. Life 2022, 12(12), 2142.

Arvouet-Grand, A., Vennat, B., Lejeune, B., Pourrat, A., 1995. Formulation of Propolis Extract Emulsions. I. O/W creams based on non-ionic surfactants and various consistency agents pages. Drug Dev. Ind. Pharm. 21, 1907–1915.

Azeem, H.H.A.E., Osman, G.Y., El-Seedim, H.R., Fallatahm, A.M., Khalifam, S.A.M., Gharib, M.M., 2022. Antifungal Activity of Soft Tissue Extract from the Garden Snail Helix aspersa (Gastropoda, Mollusca). Molecules 27 (10), 3170.

Carpenter, C.E., Broadbent, J.R., 2009. External Concentration of Organic Acid Anions and pH: Key Independent Variables for Studying How Organic Acids Inhibit Growth of Bacteria in Mildly Acidic Foods. J. Food Sci. 74, 1–3.

Cilia, G., Fratini, F., 2018. Antimicrobial properties of terrestrial snail and slug mucus. J. Complement. Integr. Med. 15, 1–2.

Colella, G., Vicidomini, A., Soro, V., Lanza, A., Cirillo, N., 2012. Molecular insights into the effects of sodium hyaluronate preparations in keratinocytes. Clin. Exp. Dermatol. 37 (5), 516–520.

Darren, B.A., Lavin, V.C., Fahym, E.J., Griffinm, M., Guardinom, N., Kingm, M., Chenm, K., Lorenzm, P.H., Gurtnerm, G.C., Longakerm, M.T., Momenim, A., Wanm, D.C., 2022. Standardizing Dimensionless Cutometer Parameters to Determine In Vivo Elasticity of Human Skin. Adv Wound Care 11 (6), 297–310.

Dawson, R., Elliot, D., Elliot, W., Jones, K.M., 1986. Data for Biochemical Research, 3rd ed. Oxford Science Publ.

Dinica, R.M., Sandu, C., Botezatu, A.V.D., Busuioc, A.C., Balanescu, F., Mihaila, M.D.I., Dumitru, C.N., Furdui, B., Iancu, A.V., 2021. Allantoin from valuable romanian animal and plant sources with promising anti-inflammatory activity as a nutricosmetic ingredient. Sustainability 13 (18), 10170.

Ehara, T., Kitajima, S., Kanzawa, N., Tamiya, T., Tsuchiya, T., 2002. Antimicrobial action of achacin is mediated by L-amino acid oxidase activity. FEBS 531 (3), 509–512.

Ekin, I., Sesen, R., 2018. First record of the pest and parasitic intermediate host snail Cochlicella barbara (Linnaeus, 1758) in the south-eastern Anatolia. Middle East. J. Sci. 4 (1), 46–47.

Garg, A., Aggarwal, D., Garg, S., Singla, A.K., 2002. Spreading of semisolid formulations an update. Pharm. Technol. 26, 84–105.

Greistorfer, S., Klepalm, W., Cyran, N., Gugumuck, A., Rudoll, L., Suppan, J., von Byern, J., 2017. Snail mucus – glandular origin and composition in Helix pomatia. Zoology 122, 126–138.

Hirshfield, N., Terzullim, S., O'Byrne, C., 2003. Weak organic acids: a panoply of effects on bacteria. Sci. Prog. 86 (Pt 4), 245–269.

Joanna, B., 2020. Amino Acids and Short Peptides as Anti-Aging "Superfood". Int. J. Nutr. Sci. 5 (1), 1039.

Kawałkiewicz, W., Matthews-Kozaneckam, M., Janus-Kubiakm, M., Kubiszm, L., Hojan-Jezierskam, D., 2021. Instrumental diagnosis of facial skin—A necessity or a

pretreatment recommendation in esthetic medicine. J. Cosm. Dermatol. 20 (3), 875–883.
Laneri, S., Di Lorenzo, R., Sacchim, A., Dini, I., 2019. Dosage of bioactive molecules in the nutricosmeceutical Helix aspersa muller mucus and formulation of new cosmetic cream with moisturizing effect. Nat. Prod. Comm. 14 (8), 1–7.
Ohshima, H., Kinoshitam, S., Oyobikawa, M., Futagawam, M., Takiwakim, H., Ishikom, A., Kanto, H., 2013. Use of Cutometer area parameters in evaluating age-related changes in the skin elasticity of the cheek. Skin Res. Technol. 19 (1), e238–e242.
Okano, Y., Abem, Y., Masaki, H., Santhanam, U., Ichihashim, M., Funasakam, Y., 2003. Biological effects of glycolic acid on dermal matrix metabolism mediated by dermal fibroblasts and epidermal keratinocytes. Exp. Dermatol. 12 (2), 57–63.
Onzo, A., Pascalem, R., Acquavia, M.A., Cosmam, P., Gubitosam, J., Gaetam, C., Iannece, P., Tsybin, Y., Rizzim, V., Guerrierim, A., Ciriellom, R., 2021. Untargeted analysis of pure snail slime and snail slime-induced Au nanoparticles metabolome with MALDI FT-ICR MS. J. Mass Spectrom. 56 (5), 3–4.
Pagano, C., Baiocchim, C., Beccarim, T., Blasim, F., Cossignanim, L., Ceccarinim, M.R., Orabona, C., Orecchini, E., Di Raimo, E., Primavilla, S., Salvini, L., Di Michele, A., Perioli, L., Ricci, M., 2021. Emulgel loaded with flaxseed extracts as new therapeutic approach in wound treatment. Pharmaceutics 13 (8), 1107.
Pagano, C., Ceccarini, M.R., Faieta, M., Di Michele, A., Blasi, F., Cossignani, L., Beccari, T., Oliva, E., Pittia, P., Sergi, M., Primavilla, S., Serafini, D., Benedetti, L., Ricci, M., Perioli, L., 2022. Starch-based sustainable hydrogel loaded with Crocus sativus petals extract: A new product for wound care. Int. J. Pharm. 625, 122067.
Pan, B., Tao, J., Bao, X., Xiao, J., Liu, H., Zhao, X., Zeng, D., 2021. Quantitative study of starch swelling capacity during gelatinization with an efficient automatic segmentation methodology. Carbohydr. Polym. 255, 117372.
Pitt, S.J., Graham, M.A., Dedim, C.G., Taylor-Harrism, P.M., Gunn, A., 2015. Antimicrobial properties of mucus from the brown garden snail Helix aspersa. Br J Biomed Sci. 72, 174–181.
Potekaev, N.N., Borzykh, O.B., Medvedev, G.V., Pushkin, D.V., Petrova, M.M., Petrov, A. V., Dmitrenko, D.V., Karpova, E.I., Demina, O.M., Shnayder, N.A., 2021. The Role of Extracellular Matrix in Skin Wound Healing. J. Clin. Med. 10, 5947.
Rashad, M., Sampò, S., Cataldi, A., Zara, S., 2023. Biological activities of gastropods secretions: snail and slug slime. Nat. Prod. Bioprospect. 13, 42.
Rekik, D.M., Ben Khedir, S., Ksouda Moalla, K., Grati Kammoun, N., Rebai, T., Sahnoun, Z., 2016. Evaluation of Wound Healing Properties of Grape Seed, Sesame, and Fenugreek Oils. Evid Based Complement Alternat Med. 2016, 7965689.
Ryu, H.S., Joo, Y.H., Kim, S.O., Parkm, K.C., Younm, S.W., 2008. Influence of age and regional differences on skin elasticity as measured by the Cutometer®. Skin Res Technol. 14 (3), 354–358.
Tomic-Canic, M., Burgess, J.L., O'Neill, K.E., Strbo, N., Pastar, I., 2020. Skin Microbiota and its Interplay with Wound Healing. Am. J. Clin. Dermatol. 21 (Suppl 1), S36–S43.
Vassilev, N.G., Simovam, S.D., Dangalov, M., Velkova, L., Atanasov, V., Dolashkim, A., Dolashka, P., 2020. An 1H NMR- and MS-Based Study of Metabolites Profiling of Garden Snail Helix aspersa Mucus. Metabolites 10 (9), 360.
Veroni, D., Franzoni, G.M., 2017. EP3135107A2 Use of an ozonization device for the extraction of snail slime and related device, stimulating solution and process for the extraction of snail slime.
Voinchet, V., Vasseur, P., Kern, J., 2006. Efficacy and Safety of Hyaluronic Acid in the Management of Acute Wounds. Am. J. Clin. Dermatol. 7 (6), 353–357.