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Dosage of Bioactive Molecules in the Nutricosmeceutical *Helix aspersa* Muller Mucus and Formulation of New Cosmetic Cream with Moisturizing Effect

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Abstract

The present study was carried out to provide the allantoin and glycolic acid contents in the *Helix aspersa* Muller mucus of common Campania land (Italy) by using chromatographic method. The study continued with the formulation of a snail mucus cosmetic cream, whose ability to hydrate the skin was evaluated comparing the skin hydration and trans-epidermal water loss (TEWL) effects of a stable cosmetic preparation. The skin TEWL and skin hydration effects were measured by TEWAMETER and corneometer probe, respectively, at the beginning, after 1 hour, and 24 hours.

Keywords

Snail mucus, allantoin, glycolic acid, corneometry, cosmetologic application, nutricosmeceutical

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The term "nutricosmeceutical" is a relatively new term obtained by combining "nutraceuticals" and "cosmeceuticals".[1] It is used by the industry, although not recognized by the FDA and EU legislation, to describe supplements, functional foods, and beverages that contain active ingredients able to improve human beauty and health. The favorable economic circumstances and the growth of elderly population are having a highly positive impact on the nutricosmetics market. The worldwide market for nutricosmetics will grow by 5.0% compound annual growth rate (CAGR) from 2017 to 2025 to become US$7.93 bn worth by 2025.[2] The nutricosmetics market is segmented by the type of ingredient, application (skin care, hair care, nail care), and region. It is expected that in Europe cosmeceuticals for the elderly will be sold more, as the average age has increased, while in the United States the herbal supplements will be sold which is identified as the highest impact rendering driver for this region. Skin care dominates the market with a growth expectation of 4.5% (CAGR) to reach $194 961 million in 2024.[3] Demand for natural cosmetics has increased the global market for snail beauty products (snail extracts, snail mucus, snail oil, snail serum, or snail filtrate). Snails are common food consumed by millions of people worldwide[4,5] and are a source of secondary metabolites used in cosmetics and medicine. Snail meat (escargot) is rich in protein and mineral sources although low in lipid content. However, n6/n3 and PUFA/SFA ratios were found in good range according to HMSO.[6] The pharmacologic activities of helix slime components are broadly studied and recognized.[7] Snail mucus is used in cosmetic, to stimulate the formation of collagen, elastin, and dermal components that repair the signs of photoaging and to reduce the damage generated by free radicals.[8] The aim of this work was to determine for the first time allontoin and glycolic acid concentrations in snail mucus (a nutricosmeceutical product) of *Helix aspersa* Muller mucus (*Helix Complex*) from Campania to assess its potential as a source of useful molecules for the nutricosmeceutical industry and to formulate a moisturizer cosmetic cream able to have high moisturizing properties. Moisturizers are topical products designed to prevent dry skin and enhance and preserve the skin barrier function. The newest generation of moisturizers contains barrier repair ingredients in addition to traditional moisturizer components. Snail mucus is a mixture of active substances commonly thought to have healthy properties for the treatment of skin disorders such as allantoin, collagen, elastin, and glycolic acid together with glycoproteins (mucin, achacin) and mucopolysaccharides.[9] Allantoin, or 5-ureidohydantoin, is one from several uric acid oxidation products.[10,11] According

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Table 1. Linearity Data (Line Equation with a Standard Calibration Curve) in Spiked Samples of Snail Mucus (N = 5 for Each Concentration Level).

Component	Line equation	R^2	LLOD (mg/mL)	LLOQ (mg/mL)
Allantoin	$Y = 28.114x + 0.65$	0.9972	0.004	0.0125
Glycolic acid	$Y = 70.482x + 9.2$	0.9926	0.130	0.2500

to US Food and Drug Administration (FDA) and EU regulation on cosmetic products, allantoin is a safe and effective active compound for skin protection.[12] It is helpful to fight skin damage since it has desquamating action and helps cell proliferation and wound healing. Moreover, allantoin increases the water content of the extracellular matrix and forms complexes with irritant and sensitizing substances.[13] For these reasons, it is used in cosmetics and personal care products such as shampoos, lotions, creams, lipsticks and in topical pharmaceutical agents for treatment of skin diseases including antiacne product. Glycolic acid, or alpha-hydroxyacetic acid, has an excellent capability to penetrate skin, to increase collagen synthesis by fibroblasts, to modulate matrix degradation and collagen synthesis through keratinocyte-released cytokines,[13-15] to accelerate epidermal turnover, to prevent melanin formation in melanocytes by directly inhibiting tyrosinase activity,[16] and to increase UV-induced pigmentation.[17] It is used at concentrations ≤10%, and at final pH ≥ 3.5 in cosmetic and pharmaceutical products for the treatment of skin diseases including actinic keratosis, hyperkeratosis, and seborrheic eczema.[18-22]

Results

Detection of Bioactive Compounds

The allantoin and glycolic acid content in the crude extract from different batches of Helix Complex was chemically analyzed by HPLC using the method proposed by El Mubarak et al [8] with some modifications. According to European medicines agency guidelines, validation, linearity, LLOD, LLOQ, precision, and accuracy are considered. Direct dilution of mucus in water or buffer was not possible and led to poor recoveries. Good separation and recovery levels were achieved dissolving mucus in buffer solution (1 mL snail mucus +4 mL phosphate buffer pH 2.7; 10 mM) with the aid of ultrasonic bath for 15 minutes.[8] Three different flow rate values for the mobile phase (0.5, 0.7, and 1.0 mL/min) were tested. The value of 1 mL/min determined a good separation. Detection of allantoin and glycolic acid was done at 200 nm (maximum absorbance) after spectra monitoring with UV detector. In these conditions, glycolic acid (2.82 ± 0.07 minutes) elutes earlier than allantoin (3.9 ± 0.06 minutes). The linearity, LLOD, and LLOQ of analytical procedure are summarized in Table 1.

Intra- and interday repeatabilities are reported in Table 2.

Results on method stability agree with data reported by El Mubarak et al.[8] Allantoin and glycolic acid were analyzed in three different lots of snail mucus (at a concentration of 1 mL mucus/10 mL buffer solution). The results are shown in Table 3.

Measurement of Skin Hydration

Skin hydration was evaluated comparing the skin hydration and trans-epidermal water loss (TEWL) effects of a stable cosmetic preparation. TEWL is an in vivo measurement method for testing stratum corneum barrier function of the human skin. Twenty females aged between 20 and 65 years were enrolled in this randomized study. Four areas were identified

Table 2. RSD (%) and Recoveries for 3 Consecutive Days in Spiked Samples of Snail Mucus (N = 5 at Each Concentration Level).

Component	Theoretical concentrations (mg/mL)		Recovery (%)	RSD (%)
Allantoin	1.0	Day 1	105.26	2.54
		Day 2	100	3.0
		Day 3	105.26	2.63
		Total	103.5	2.58
	0.5	Day 1	99.2	2.16
		Day 2	98.3	1.90
		Day 3	99.1	0.44
		Total	98.9	1.36
	0.25	Day 1	100	0.87
		Day 2	113.1	3.80
		Day 3	113.2	2.08
		Total	108.7	1.58
Glycolic acid	1.00	Day 1	98.4	1.7
		Day 2	95.24	2.49
		Day 3	99.0	2.40
		Total	97.5	1.01
	0.50	Day 1	86.9	2.13
		Day 2	90.59	1.54
		Day 3	91.24	2.12
		Total	89.6	3.5
	0.25	Day 1	100	2.12
		Day 2	109	4.21
		Day 3	113	3.56
		Total	107.3	1.10

Table 3. Values of Allantoin and Glycolic Acid in 3 Different Lots of Snail Mucus (Concentration Was Expressed in g/L).

Products	Allantoin	Glycolic acid
Snail mucus (g/L) lot 1	0.37 ± 0.60	3.29 ± 125
Snail mucus (g/L) lot 2	0.4 ± 0.3	3.42 ± 423
Snail mucus (g/L) lot 3	0.45 ± 0.52	3.35 ± 443
Media	0.41	3.35

on the cleaned and dried flying part of the forearm. Each area was of 4 cm^2 and was applied 0.5 ± 0.10 g of each product and spreader until its adsorption. On the same area TEWL and corneometry were measured before the cream application (T0), after 1 hour by its application, and after 24 hours (T24h) with the maximum reproducibility. All participants completed the study and no adverse event was observed. The results are reported in Table 4.

Corneometry determining the capacitance of the stratum corneum and reflecting the relative stratum corneum moisture was measured with a corneometer CM 820. The results are reported in Table 5.

Discussion

Detection of Bioactive Compounds

Allantoin and glycolic acid were analyzed in 3 different lots of snail mucus (at a concentration of 1 mL mucus/10 mL buffer solution). Both compounds are highly polar molecules able to interact with endcapping (as it happens with Vision HT C18 High Load column silica column) mainly via H-bonding. The effect of mobile phase pH was studied in association to the retention time, peak height, and shape. Buffer solutions of different pH values were prepared by adding phosphoric acid 0.1 M to 10 mM NaH_2PO_4. According to El Mubarak et al[8] the optimal mobile phase pH was found to be 2.7, comparing the curves in the resulting pH/time plots. Increasing the concentration of the mobile phase to 20 mM NaH_2PO_4 did not determine change in peak shape and analytes' retention time. Three different flow rate values for the mobile phase (0.5, 0.7, and 1.0 mL/min) were tested. The value of 1 mL/min determined a good separation. To validate the analytic procedure, method linearity was detected. The linearity of an analytical procedure consists in finding results directly proportional to the concentration of analyte in the sample. For the construction of calibration curves the method of constant addition was used. The correlation coefficient of the linear regression line is considered linear in the range studied, because it is approximately 1 (R^2 glycolic acid = 0.9926; R^2 allantoin = 0.9972). Successively, was found the lowest amount of analyte in sample (relative standard deviation < 15%), the accuracy (relative error < 20%), and the method precision under the same operating conditions over a short interval of time. Method validation showed high recovery, precision, and sensitivity.

Measurement of Skin Hydration

The increase in demand for natural, herbal, and biological products has focused the attention of cosmetic companies on the snail slime which contain molecules able to stimulate skin cells and keep skin hydrated. Immediately after the snail creams application, the elevated TEWL values reached levels close to normal. After applications of snail cream 5% and snail cream 10%, TEWL values increased. In low concentration glycolic acid is believed to facilitate progressive weakening of cohesion of the stratum corneum intercellular material, resulting in uniform exfoliation of its outermost layers[23] and evaporation of the intercorneocyte water. Instead, we note a decrease in

Table 4. TEWL Measurements Following a Single Topical Application of Slime Cream Over Time (Hours).

Time (h)	Cream formulation	Number of volunteers in which a variation of TEWL is measured	TEWL	Improvement of samples compared to control (%)	*P*
0	Placebo	20	14 ± 3.21		
	Snail cream 2%	20	11.08 ± 1.42		
	Snail cream 5%	20	12.59 ± 2.41		
	Snail cream 10%	20	12.44 ± 2.50		
1	Placebo	20	13.85 ± 1.92	+2.54	0.018*
	Snail cream 2%	20	10.79 ± 1.30	-1.17	0.050*
	Snail cream 5%	20	12.85 ± 1.50	+4.13	0.038*
	Snail cream 10%	20	12.98 ± 1.96	+6.1	0.044*
24	Placebo	20	13.88 ± 2.00	+0.86	0.049*
	Snail cream 2%	20	10.12 ± 1.96	-7.81	0.082
	Snail cream 5%	20	11.17 ± 1.94	-10.1	0.047*
	Snail cream 10%	20	10.55 ± 2.24	-14.9	0.016*

TEWL, transepidermal water loss.
*$P < 0.001$ vs $T = 0$. Results are presented as mean ± SEM.

Table 5. Corneometry Measurements Following a Single Topical Application of Slime Cream Over Time (Hours).

Time (h)	Cream formulation	Number of volunteers in which a variation of TEWL is measured	Corneometry test	Improvement of samples compared to control (%)	*P*
0	Placebo	20	35.74 ± 6.78		
	Snail cream 2%	20	32.92 ± 4.55		
	Snail cream 5%	20	31.68 ± 5.82		
	Snail cream 10%	20	32.25 ± 6.77		
1	Placebo	20	37.17 ± 7.62	+5.8	0.050*
	Snail cream 2%	20	36.21 ± 4.34	+11.10	0.025*
	Snail cream 5%	20	35.49 ± 5.22	+15.26	0.035*
	Snail cream 10%	20	36.45 ± 7.93	+15.16	0.049*
24	Placebo	20	35.71 ± 6.94	+1.8	0.031*
	Snail cream 2%	20	37.9 ± 5.18	+15.78	0.0026*
	Snail cream 5%	20	35.25 ± 5.44	+12.8	0.050*
	Snail cream 10%	20	38.2 ± 8.31	+20.3	0.017*

Notes. *$P < 0.001$ vs $T = 0$. Results are presented as mean ± SEM.

TEWL values, immediately after 2% snail mucus cream application. After 24 hours, the water is regenerated and accumulated in the intercorneocyte spaces creating a more compact hydrolipidic film as shown by the significant decrease of the TEWL for all 3 formulations (at 2%, 5%, 10%). Regarding the results of corneometry, all formulations (2%, 5%, 10%), with increasing concentration of snail mucus in the cream, immediately after the application of the moisturizing cream, determine highly statistically significant ($P = 0.0026$-0.050) improvement in hydration. This result may be due to allantoin in the snail mucus, an extremely moisturizing substance. In addition, snail mucus by itself has a moisturizing action capable of supporting the effect of allantoin.

Materials and Methods

Helix aspersa Muller Mucus (Helix Complex) Collection and Sterilization

Snail mucus (secretions) was kindly provided by *Terra Fertilis*, a farm of natural breeding of Italian snails; *Helix aspersa* Muller sample lots: C 121701; D 151701; E 191701.

Microbiological Characterization

To test microbiological contamination, 100 µL of Helix Complex mucus was plated on culture dishes containing culture media tryptic soy agar (Biomerieux, Italy). The number of colonies was evaluated after 24 to 48 hours at 37°C and expressed as colony forming unit (CFU). The identification of contaminating bacteria was performed by Gram staining (Liofilchem, Italy). The presence of contaminating fungi was evaluated by plating Helix Complex on Sabouraud medium plates (Biomerieux, Italy). The serum presented a total bacterial amount less than 100 CFU/g as well as for molds and yeasts and total absence of pathogens.

Chemical Characterization of Allantoin and Glycolic Acid

The allantoin and glycolic acid content in the crude extract from different batches of Helix Complex was chemically analyzed by HPLC using the method proposed by El Mubarak et al. [8] with some modifications.

Mucus snail treatment. Mucus (1 mL) is dissolved in 4 ml potassium phosphate buffer (pH 2.7; 10 mM). After stirring using a Vortex device for about 15 minutes, 1 mL is dissolved in 4 mL natrium phosphate buffer (pH 2.7; 10 mM). Finally, the sample is filtered by Corning filter (0.8 µm).

Reagent and chemicals for chemicals characterization. High-quality standard allantoin was supplied from FLUKA (St Louis, MO, USA) with purity > 98.0% and glycolic acid from Sigma-Aldrich (St Louis, MO, USA) with purity of 99.0%. HPLC-grade acetonitrile (CH_3CN) and methanol (CH_3OH) were purchased from Honeywell Burdick & Jackson (Seelze, Germany); mono-natrium phosphate (NaH_2PO_4), phosphoric acid (H_3PO_4), and natrium hydroxide (NaOH) from Merck KgaA (Darmstadt, Germany); and *n*-hexane ($CH_3(CH_2)_4CH_3$) from Fisher Scientific (Hampton, UK). Ultra-pure water from a MilliQ® instrument (Millipore, Billerica, MA, USA) was used. All solvents were filtered through 0.22 µm filters (Titan Membrane, Millipore).

Standards preparation. Allantoins and glycolic acid standards were prepared at 3 concentration levels: 1, 0.5, 0.25 mg/mL in potassium phosphate buffer (pH 2.7; 10 mM).

Apparatus used for chemical characterization. The chromatographic system consisted of an HPLC Jasco LC-Net II/ADC (JASCO International Co., Ltd. Tokyo, Japan) with a 20 µL Rheodyne 8125 injector (Rheodyne, Ronhert Park, CA, USA), an UV Detector UV "Jasco MD-2010 plus" (JASCO

Table 6. Ingredients of Moisturizer Creams (100 g).

Ingredient class	*Helix aspersa* Muller mucus cream
Phase O	
Emollient	Cetyl alcohol 2.5 g
Emulsifier	*Prunus amygdalus* dulcis oil 2.5 g
	Polyglyceryl-3methylglucose distearate 5.0 g
	Triticum vulgare germ oil 7.5 g
Antioxidant/emulsifier	*Triticum vulgare* germ oil 7.5 g
Phase W	
Base	Aqua
Antioxidant	Tocopheryl acetate 1.0 g
	Parfum 0.1 g
Barrier repair ingredients	Snail mucus (0% (placebo), 2%, 5%, and 10%)
Preservants	Sodium benzoate, potassium sorbate phenoxyethanol (0.5%)

International Co., Ltd. Tokyo, Japan) (λ 200 nm), and reversed phase Vision HT C18 High Load 5 μm (250 mm × 4.60 mm, 4 μm) column (Grace, Munich, Germany).

Allantoin and glycolic acid determination method. *Helix aspersa* Muller mucus samples were chromatographed on a Vision HT C18 High Load column 250 mm × 4.6 mm ID, 5 μm particle size, using the following eluting solution: pre-run: 100% CH_3CN, 1 to 8 minutes, 100% NaH_2PO_4; 9 to 26 minutes (30% NaH_2PO_4 + 70% CH_3CN); 27 to 30 minutes, 100% CH_3CN.The flow rate of the mobile phase was 1 mL/min. Elution was monitored at 200 nm.

Skin Hydration Determination

Reagent and chemicals for skin hydration determination. Cetyl alcohol, prunus amygdalus dulcis oil, polyglyceryl-3-methylglucose distearate, *Triticum vulgare* germ oil, and tocopheryl acetate were purchased from ACEF (Fiorenzuola D'Arda, Italy) and Parfum by Farotti essenze (Rimini, Italy).

Formulation development. The formulation used is an O/W emulsion containing ingredients listed in Table 6. For the qualitative evaluation, 4 snail slime creams were used at different snail mucus concentrations: 0% (placebo), 2%, 5%, and 10%. The oil phase (O) is prepared and placed on a heating plate so that a liquid oily component is formed. At the same time the water is heated up to the same temperature of the oil phase. Subsequently the aqueous phase is poured flush into the oil phase, shaking vigorously; it starts to cool the emulsion in an ice bath and, once cold, the remaining components are added. The pH of emulsions was calculated with a pHmeter Crison GPL20 and was 5.0 to 5.5; viscosities 26 570 to 29 460 mPa s (L4, 20 rpm) were calculated with a rheometer Visco Basic Plus, Fungilab.

Apparatus used for skin hydration determination. Probe tewameter® dual mpa 580 (Courage + Khazaka electronic GmbH, Köln, Germany), Probe Corneometer DUAL MPA 580 (Courage + Khazaka electronic GmbH, Köln, Germany), and Software CK-MPA-Multi-Probe-AdapterFB (Courage + Khazaka electronic GmbH, Köln, Germany).

Volunteer selection. The experiments were carried out on 20 healthy female Caucasian subjects aged between 20 and 65. All volunteers signed an informed consent sheet, according to the Helsinki Declaration of ethical principles for medical research and were subjected to a thorough case history and a careful clinical examination.

The inclusion criteria were the following:

- healthy female Caucasian subjects aged between 20 and 65;
- during the study and 24 hours before the study, other topical or systemic treatments were excluded, except for the application of the moisturizers in the study;
- all the subjects were carefully selected, cooperative, discerning, and able to follow instructions and comply with the study requirements;
- all the subjects should not expose themselves to UV before the study.

The exclusion criteria considered were the following:

- pregnant or breastfeeding women;
- subjects with a history of skin hyper-reactivity or intolerance reactions to cosmetic products/ingredients;
- subjects with diseases in the period immediately preceding the current study;
- subjects during topical or systemic treatment with any drug that may affect the outcome of the test or suffering from skin diseases (eczema, psoriasis, lesions);
- -subjects treated with topical retinoids in the previous 6 months before the start of the study and of systemic retinoids in the previous 12 months;

- subjects who performed treatments with topical products based on alpha- and beta-hydroxy acids in 45 days before the start of the study.

Application of cream and skin hydration determination. About 0.5 ± 0.10 g of snail cream (placebo, 2%, 5%, and 10% snail cream) was spread on 4 areas of the volunteers' forearm volar area (area ~ 12 cm^2). Skin hydration was carried out using TEWL and corneometry probes for skin measurements, under standard conditions of temperature and humidity (T = 25°C, humidity 50% ± 5% RH) after an acclimation period of about 30 minutes. Measurements were made before cream application and after 1 and 24 hours from the treatment. Results are presented as mean ± SD for a Gaussian distribution and as the median and 25th to 75th percentile values for a non-Gaussian distribution and counts.

Statistical Tests

We use the Mann-Whitney *U*-test or unpaired Student *t*-test if data were continuous and independent and for paired data we use the paired Student *t*-test. The Pearson's correlation coefficient was used to assess correlation between the continuous variable. For all tests, significance was achieved at $P < 0.05$. Statistical tests were performed using SPSS software (version 15).

Conclusion

A snail mucus cosmetic cream with moisturizing properties was formulated. The amount of mucus to be added was evaluated according to allantoin and glycolic acid content in *H. aspersa* Muller mucus of common Campania land (Italy) and skin hydration measured by TEWL and corneometry. An HPLC method, developed and validated according to the European Commission Decision 2002/657/EC, allowed to quantify glycolic acid (3.35 g/L) and allantoin (0.41 g/L) in the mucus. A cream containing mucus (2%) gives a desirable moisturizing action.

Declaration of Conflicting Interests

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