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Biotechnology

Physico-Chemical Study of the Pseudo-Protein and Bioactive Plant Substance Melilot

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Abstract. Solid Ointment Melilot (SOM) was created by combining biodegradable, biocompatible, and bioactive biomimetic polymer – pseudo-protein used as a matrix and a mixture of bioactive plant substances Melilot. The initial form of the ointment is an aerosol (spray), which is convenient to apply after plastic surgery and cosmetic procedures on the skin area of any topology, for the prevention of scars, stains, and other complications. SOM after plastic surgery is used in the proliferative and differential phases. Biologically active composition of Melilot – small molecules of secondary metabolites overcome the lipid barrier and contribute to the activity of biologically active substances: cytokine mediators, proteases, differentiation of monocytes into macrophages. A technological scheme for obtaining the biologically active component Melilot has been developed. It is obtained from a substance isolated from five plants (*Melilotus officinalis* L., *Juglans regia* L., *Trifolium praténse* L., *Chelidonium majus* L., *Maclura pomifera* (Raf.) C.K.Schneid). The antimicrobial activity of the SOM and Melilot against different microorganisms was studied by the ager disc-diffusion method. The following parameters were defined in Melilot: total phenolic compounds, total sugars, organic acids, chromium trace element, % dry matter, antioxidant activity, pH, vitamin C. The acute toxicity and local irritation of the SOM were studied as well. The SOM (the initial form – spray) was prepared. After applying a liquid spray, the solvent (alcohol) quickly evaporates, and the solid ointment formed which represents a pseudo-protein film impregnated with Melilot tightly adheres to the surface providing normal wound healing and a prolonged action of a plant remedy Melilot.

Keywords: solid ointment, pseudo-protein, bioactive plant remedy, spray

Introduction

In some cases, various complications accompany plastic surgery, the main cause of which is the subsequent inflammation after violating the skin integrity and, eventually, the connective tissue is formed in this place. Various types of spots and scars are often formed in the injured tissue. The same complications are sometimes observed after cosmetic procedures such as mechanical, chemical, enzymatic, and physical peeling.

We used a pseudo-protein (PP) made of amino acid L-leucine as a matrix, and a mixture of plant

substances called Melilot for obtaining a biocomposite preparation [1] we called solid ointment.

Especially promising as biomaterials are PPs composed of amino acid L-leucine [2] which is an essential branched-chain amino acid showing a wide spectrum of physiological activity [3,4]. L-leucine (Leu) can regulate several cellular processes such as protein synthesis, tissue regeneration, and metabolism, it may help in healing skin and muscle tissue, etc. Owing to unique biological properties the PPs composed of Leu have a huge potential for applications as bioresorbable and bioactive biomaterials.

For preparing the said biocomposite preparation we called solid ointment (initial form - spray), to select L-leucine based PP as a matrix. Preference was given to a copolymer poly(ester urea-*co*-ester amide) **(1L6)$_{0.7n}$-(8L6)$_{0.3n}$** [5,6] which showed an optimal cell supporting properties among the PPs studied.

The used plant substances "Melilot" was made from the local flora: *Melilotus officinalis* L., *Juglans regia* L., *Trifolium praténse* L., *Chelidonium majus* L., *Maclura pomifera* (Raf.) C.K.Schneid.

We called "the solid ointment" a remedy that is applied to the problem area after plastic surgery. The initial form of the solid ointment is a spray that is convenient to apply to surfaces of any topology and difficult-to-reach places. Being sprayed and followed by rapid evaporation of the solvent (alcohol) the remedy forms an elastic and thin (0.05-0.1 μm) film on the skin's surface. The film is air and water vapor permeable ("breathable"), protects the problem area from the penetration of bacteria.

The SOM (The solid ointment containing plant substances "Melilot") does not contain synthetic dyes and fragrance additives. All components in it bear a functional load and retain activity (see below) The polymer film prevents the expulsion of water out of the cells, protects the skin from drying, keeps its elasticity, and stimulates the physiological activity of cells [5,6]. Taking into account the obtained previously positive biological characteristics of both the PP matrix and herbal "Melilot" we suppose that the new SOM will help to create an invisible "cosmetic" scar after plastic surgery and protect the body from the formation of normotrophic, atrophic, hypertrophic, keloid scars, brown, red spots and itching during the healing process. Therefore, we decided to prepare SOM and study some of its most important biological properties.

Materials and Methods

The PP – poly(ester urea-*co*-ester amide) **(1L6)$_{0.7n}$-(8L6)$_{0.3n}$** was synthesized as reported previously [6].

The selected plant raw materials for the preparation of "Melilot" were collected in the field. Aboveground part during flowering of: *Melilotus offfiicinalis* L. *Trifolium praténse* L. *Chelidonium majus* L. Unripe fruits *Juglans regia* L. and immature fruits (green) *Maclura pomifera* (Raf.) C.K.Schneid.

Each plant was processed separately, within a maximum of 72 hours after collection (refrigerated before processing). The plants were washed and dried, then homogenized with 45-50°C conditionally alkaline mineral water (pH 7,1). 1 kg plants – 50 ml of mineral alkaline water.

The homogenate was boiled for 5-8 min and filtered. 96% ethyl alcohol was added (1:1) to the filtrate. The filtrate was kept 12 h at 5°C. The filtrate was subjected to centrifugation at 4000 rpm for 10 min. The supernatant was vacuum filtrated (a pore size of 5-8 μm).

The selected substances are mixed in the following proportion: *Maclura pomifera*; *Chelidonium majus* L., *Trifolium praténse* L., *Melilotus offfiicinalis* L., *Juglans regia* L. (30%: 30%: 15%: 10%) and the Melilot was prepared.

The SOM (the initial form – spray) containing a 5% solution of the PP in 96% ethyl alcohol and 3% dry Melilot (relative to the mass of the PP) was prepared. The PP **(1L6)$_{0.7n}$-(8L6)$_{0.3n}$** was purchased from Polymer Solutions, LLC (Tbilisi, Georgia).

The antimicrobial activity of the SOM and Melilot against different microorganisms was studied by the ager disc-diffusion method [7,8]. Reference cultures (bacterial strains) have been selected as test objects grown on agar slant tubes from the G. Eliava Institute of Bacteriophages, Microbiology and Virology.

Gram-positive bacteria: *Staphylococcus aureus* ATCC 25923; *Staphylococcus epidermidis* 28384 clinical; *Bacillus cereus* ATCC 10876;

Gram-negative bacteria: *Pseudomonas aeruginosa* ATCC 27853; *Esherichia coli* ATCC 25923;

We studied the antimicrobial activity of the SOM.

After 24 hours of incubation, the test microorganisms were transferred from test tubes to Petri dishes with TSA (Tryptic Soy Agar). Then holes, so called wells, were cut in agar with a sterile borer (12 mm in diameter). 200 µl of SOM (composed of a 5% solution of the PP in 96% ethyl alcohol along with 3% (per mass of the PP) of dry Melilot was poured into each well, and Petri dishes were placed in a thermostat at 37°C for 24 hours. The 96% ethyl alcohol was served as the control. In parallel the bactericidal activity of the Melilot solution in 45% ethyl alcohol was carried out in the same way. 200 µl of Melilot were poured into the well. The 45% ethyl alcohol was served as the control. The antimicrobial activity of the research objects was evaluated by the diameter of the inhibition zone of the test organisms. The experiment was repeated three times for each test-organism.

Melilot's chemical analyses were conducted using conventional analytical methods.

The following parameters were defined: total phenolic compounds – OIV-MA-AS2-10, total sugars – GOST 8756.13-87, organic acids – OIV-MA-AS313-04, chromium trace element – GOST 30178-96, % dry matter – GOST 28562-90, antioxidant activity – FRAP-Method, pH – GOST 26188-2016, vitamin C – GOST 24556-89.

The acute toxicity and local irritation of the SOM were studied as well. The study was conducted on 12 adult male Wister albino rats with a body weight of 200-250 grams. The animal care protocol followed the recommendations of the National Research Council Committee Update of the Guide for the Care and Use of Laboratory Animals (USA), to minimize animal pain and/or discomfort, both during manipulation and animal sacrifice after the experiment [9].

Results and Discussions

We decided that for preparing the said biocomposite preparation we called solid ointment (initial form – spray), to select L-leucine based PP as a matrix. Preference was given to a copolymer poly(ester urea-co-ester amide) (1L6)0.7n-(8L6)0.3n [4,5] which showed an optimal cell supporting properties among the PPs studied.

The solid ointment containing plant substances Melilot (hereafter labeled as SOM) was prepared by mixing of ethanol solution of PP (1L6)0.7n-(8L6)0.3n with a solution of Melilot. After spraying on the wound surface and ethanol evaporation the formed film tightly adheres the wound surface and provides sustained release of the bioactive substances of plant origin deposited in it, similar to artificial skin "PhagoBioDerm" impregnated with bacteriophages and other medications [5,6] providing a prolonged action of bioactive compounds.

The obtained results show no activity of Melilot against *Escherichia coli* and *Staphylococcus aureus*, whereas SOM is active against the latter as well (Table 1). It is important that with other three bacteria (*Staphylococcus epidermidis, Bacillus cereus, Pseudomonas aeruginosa*) SOM revealed by far higher activity as compared with pure Melilot. Based on this data, it can be concluded that PP enhances the bactericidal activity of the Melilot components. The 96% (used for preparing SOM) and 45% (used for preparing the Melilot) ethyl alcohol were served as the control showing no inhibition effect with the examined bacteria (zero zone of inhibition).

Table 1. The antimicrobial activity

№	Test-cultures	Zone of inhibition, diameter (mm)	
		SOM (spray)	"Melilot"
1	*Staphylococcus aureus* ATCC 25923	8 ± 0.7	0
2	*Staphylococcus epidermidis* 28384 clinical	18 ± 0.7	8 ± 0.5
3	*Bacillus cereus* ATCC 10876	16 ± 0.7	4 ± 0.5
4	*Pseudomonas aeruginosa* ATCC 27853	11 ± 0.7	6 ± 0.5
5	*Escherichia coli* ATCC 25923	0	0

Table 2. Chemical analysis of Melilot

	Parameter	Result
1	Total phenolic compounds, mg/L; gallic acid equivalent (GAE)	632.31
2	Total sugars, g/l	12.0
3	Organic acids, HPLC g/l	
	Tartaric acid	9.2
	Lactic acid	2.16
	Malic acid	0.3
4	Trace element chromium mg/l	0.056
5	% dry matter, refractometry	2.84
6	pH	6.34
7	Antioxidant activity, mg/l; spectrophotometry	45.34
8	Vitamin C, mg/100 ml	33.0

Antioxidant activity and chemical composition of Melilot was studied (Table 2). According to these characteristics, a comparison of Melilot with various objects showed that its content of polyphenolic compounds (632.31 mg/l; gallic acid equivalent) is 2.5-3 times higher than that of objects rich in these compounds, like European type wines made of Chinese, Tsolikouri, Manavi green and other varieties of grapes (212.2 mg/l, 214.9 mg/l, 250.2 mg/l; respectively) [10].

The antioxidant activity of Melilot (45.34 mg/l) actually exceeds the antioxidant potential of Tsitska grape samples grown in the Adjara and Imereti zones (30.1-44.5 mg/l) of Georgia [11]. The content of vitamin C in "Melilot" (33 mg/100 ml) is only 2 times lower than that of freshly squeezed lemon juice (62 mg), which is known for this vitamin and 1.5-3 times higher than the fruits of blackthorn, hawthorn, cranberry, and viburnum [12].

The assessment of acute toxicity and local irritation of the SOM showed no histological or cytological pathology of the organs after applying the preparation confirming non-toxicity of the SOM. The tested sample also does not cause local skin irritation. All these speaks for a high safety of the SOM.

Conclusion

The novel biocomposite-solid ointment Melilot (SOM) has been elaborated on the bases of L-leucine-base biocompatible and bioactive polymer – pseudo-protein (matrix) and plant substances Melilot (active ingredient). The initial form of the remedy is spray. After being sprayed at the wound surface the solvent (ethanol) is evaporated forming a thin and transparent, bioactive film ("solid ointment") tightly adhered to the wound surface. The new remedy was developed to reduce/alleviate the complications after plastic surgery or cosmetic procedures, to protect the skin from undesirable consequences after plastic surgery and cosmetic procedures, and to avoid or reduce scar formation. The antibacterial activity and safety of the new remedy were assessed. A more detailed study of the SOM including case studies with volunteers is in progress.

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ნაშრომში განხილულია ბიოდეგრადირებადი, ბიოთავსებადი და ბიოაქტიური ბიომიმეტური პოლიმერის – ფსევდოპროტეინისა (გამოყენებული მატრიცად) და ბიოაქტიური მცენარეული სუბსტანცია მელილოტის შერწყმით მიღებული მალამო აეროზოლის ფორმით. იგი გამოიყენება პლასტიკური ქირურგიისა და კოსმეტიკური პროცედურების შემდეგ ნებისმიერი ტოპოლოგიის კანის უბნებზე, ნაწიბურების, ლაქების და სხვა გართულებების პროფილაქტიკისთვის. პლასტიკური ოპერაციის შემდეგ მალამო გამოიყენება პროლიფერაციულ და დიფერენციალურ ფაზაში. მელილოტის ბიოლოგიურად აქტიური შემადგენლობა – მეორეული მეტაბოლიტების მცირე მოლეკულები ლახავს ლიპიდურ ბარიერს და ხელს უწყობს ბიოლოგიურად აქტიური ნივთიერებების მედიატორების ციტოკინების, პროტეაზების აქტივობას, მონოციტების დიფერენცირებას მაკროფაგებად. შემუშავდა და დაიხვეწა მალამოს ბიოაქტიური კომპონენტის, მელილოტის მიღების ტექნოლოგიური სქემა. იგი მიიღება ხუთი მცენარიდან გამოყოფილი სუბსტანციებისგან (***Melilotus officinalis*** L., ***Juglans regia*** L., ***Trifolium praténse*** L., ***Chelidonium majus*** L., ***Maclura pomifera*** (Raf.) C.K.Schneid). შევისწავლეთ ბიოდეგრადირებადი ამინომჟავური პოლიმერის – აფსკწარმომქმნელი ფსევდოპროტეინების, მელილოტის და მყარი მალამოს ანტიმიკრობული აქტიურობა სხვადასხვა მიკროორგანიზმის მიმართ აგარ-დიფუზიური მეთოდით. მელილოტში განისაზღვრა: საერთო ფენოლური ნაერთები, საერთო შაქრები, ორგანული მჟავები, მიკრო ელემენტი ქრომი, მშრალი ნივთიერება %, ანტიოქსიდანტური აქტივობა, pH, ვიტამინი C. მყარ მალამოში შესწავლილ იქნა მწვავე ტოქსიკურობა და ადგილობრივი გამღიზიანებლობა. შემუშავდა მყარი მალამოს მიღების ტექნოლოგია და დამზადდა აღნიშნული მალამო. თხევადი სპრეის გამოყენების შემდეგ, გამხსნელი (ალკოჰოლი) სწრაფად აორთქლდება და შედეგად მიღებული მყარი მალამო, რომელიც არის მელილოტით გაჟღენთილი ფსევდოპროტეინული ფირი, მჭიდროდ ეკვრის ზედაპირს, რაც უზრუნველყოფს ჭრილობის ნორმალურ შეხორცებას და მცენარეული სუბსტანცია მელილოტის მოქმედების ხანგრძლივ ეფექტს.

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