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THE EFFECT OF A TOPICAL TREATMENT BASED ON *SAMBUCI FLOS* EXTRACT IN EXPERIMENTAL THERMAL THIRD DEGREE SKIN BURNS

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ABSTRACT

In the European ethnopharmacology, elder flowers are used external in poultices for the treatment of burns and blisters. *Sambuci flos* tincture was prepared and physico-chemically analyzed according to Romanian Pharmacopoeia. The soft extract, obtained from tincture, was embedded in an ointment base of cold cream type, prepared according to United States Pharmacopoeia. The study was performed on three groups of adult male Wistar rats, each of ten animals, kept under standard conditions, both before and after the burns. Skin burns from the dorsal region were treated daily as follows: cold cream with 10% *Sambuci flos* extract (SFE) for the first group; 1% silver sulfadiazine cream (SDA) for the second group; the third group, considered as control, received the cold cream base (CCB). The favorable evolution of burnt skin wounds in the SFE group, compared to SDA and CCB groups, is mainly due to the astringent, anti-inflammatory, antiseptic and cicatrizing action of the natural product.
Keywords: *Sambuci flos*, extract, cold cream, topical treatment, third degree skin burns.

INTRODUCTION

Sambucus nigra L., elder, elderberry, European elder, *Caprifoliaceae* family, is a species native to most of Europe, northwest Africa and southwest Asia. It is a deciduous shrub or small tree of 4–6 m tall. It grows in a variety of conditions including both wet and dry fertile soils, primarily in sunny locations, forests, riversides (Atkinson and Atkinson, 2002; Ciocârlan, 2000).

From the phytochemical point of view, *S. nigra* species contain: cyanide glycosides (Ssambunigrin), flavonosides (rutin), anthocyanins (in fruits), saponins, catechic tannin, mucilages, volatile oil (in flowers), polyphenolic acids (caffeic acid and chlorogenic acid), sugars, organic acids, vitamins (ascorbic acid), lipids, amino acids, mineral salts. Sickening smell of fresh flowers is due to some aliphatic amines: ethylamine, *n*-propylamine, *i*propyl-amine, *n*-buthylamine, etc. Through enzymatic hydrolysis, sambunigrin released hydrocyanic acid, benzaldehyde and glucose (Bruneton, 2009; Kislichenko and Vel'ma, 2006; Lamaison *et al.*, 1991).

Elder flowers have the following pharmacological actions: diuretic and diaforetic (flavonosides, saponins), antitussive (sambunigrin), expectorant (saponins), softener and immunostimulatory (mucilages), slightly laxative, antirheumatic. It is used to treat influenza and other respiratory illnesses (virosis) accompanied by fever. Due to easy action laxatives and diuretics, the elder flowers (*Sambuci flos*) are used in diets. Elder flowers are given external as hot baths or poultices, for the treatment of furunculosis, abscesses, burns, blisters, rheumatism (Bojor and Popescu, 2009; Bruneton, 2009; Ciocoiu *et al.*, 2009; Roschek *et al.*, 2009; Wright *et al.*, 2007).

Around the world, every year millions of cases with skin burns are recorded, ranging from newborns to the third age. The suffering is more or less severe, depending on the extent and depth of the lesions. Severe burns have a catastrophic influence on the patient, both by the suffering and disabilities they cause, as well as the unaesthetic scars. Patients that survive burns on large areas heal slowly, with frequent infections and hormonal, liver, lung or kidney imbalances (Jeschke *et al.*, 2007).

The skin is a barrier structure that protects the body from external aggression, particularly against microbial attack. The complications of burns represent some of the most difficult pathologies. At the worldwide level, it is estimated that burns cause about 265 000 deaths per year. The mortality rate by burns depends on the severity of lesions, the patient's age, associated diseases, etc. (Forjuoh, 2006).

MATERIALS AND METHODS

Plant material

From the *S. nigra* species, the flowers were collected in May 2010, from the Botanical Garden of the University of Craiova, Dolj County, Romania. Voucher specimens are deposited in the Herbarium of the University of Medicine and Pharmacy of Craiova.

Reagents and solvents

All of the analytical grade solvents and reagents were purchased from Merck (Darmstadt, Germany).

Preparation of tincture

The tincture from *Sambuci flos* was obtained by percolation, according to the Romanian Pharmacopoeia

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Xth edition, in a ratio of vegetal product/extraction solvent (70^{c} ethanol) 1:5. The 20% tincture was filtered and then stored in dark bottles in the refrigerator, until use (F.R. X, 1993).

Physico-chemical analysis

Tincture is a clear liquid, colorful. A slightly sediment may form on standing and that is acceptable as long as the composition is not changed significantly. Determination of relative density of the tincture was performed using a pycnometer, on the fourth decimal precision. Refractive index determination was made with Abbé refractometer. The quality conditions were established according to Romanian Pharmacopoeia Xth edition: iron (up to 0.001%), heavy metals (up to 0.001%), alcohol concentration, and evaporation residue (F.R. X, 1993).

Identification of flavonosides

Thin-layer chromatography (TLC) can separate and identify, using appropriate standards, a series of flavonosides and their aglycones (Wagner and Bladt, 1996):

- Stationary phase: silica gel Merck, 10×10 cm plates;
- Mobile phase: ethyl acetate–water–formic acid–acetic acid (72:14:7:7);
- Sample: 20% tincture of *Sambuci flos*;
- Standards (s): rutoside, hyperoside, apigenol-7-neohesperidoside, quercitrin, luteol-7-glucoside, apigenol-7-glucoside (0.1% methanolic solutions);
- The amount applied onto the starting line: 20 µL of sample solution, 10 µl of standards' solutions – spots applied are linear (bands) of 1 cm width;
- Migration distance: 12 cm;
- Revelator: NEU/PEG reagent, followed by examination of plates in UV light (λ 365 nm).

Quantitative analysis of flavonosides

The quantitative determination of flavonosides was made using spectrophotometric method, through the reaction with aluminum chloride, according to the Romanian Pharmacopoeia Xth edition. The standard curve was obtained using appropriate extinction values of rutoside solutions (F.R. X, 1993).

Choice of the cold cream type formulation

Cold creams, also called aqueous waxes or soft creams, are ointment-emulsions H/L-type bases, which are made from waxes, liquid paraffin, sodium tetraborate and water. By rubbing the skin, through dissolution of emulsion, the water evaporates from the composition of the cream. The process is endothermic and causes a local cooling effect. Ointment-emulsions H/L-type bases are recommended in the treatment of subcutaneous and chronic inflammations of the skin. Because of easily application, patients often prefer cold creams instead of greasy ointments (USP 34–NF 29, 2011).

Preparation of a cold cream with 10% vegetal extract

Sambuci flos tincture was evaporated slowly until the consistency of a soft extract, at 50–60^{0}C, under reduced pressure, using a Heidolph rotary evaporator. The soft extract was weighed and then embedded in a cold-cream H/L-type ointment base, prepared according to United States Pharmacopoeia. The vegetal extract was dissolved in a small amount of diluted alcohol (solvent used for extraction) and then emulsified in the ointment base, at room temperature, by continuous grinding (USP 34–NF 29, 2011).

Experimental model

The study was performed on three groups of adult male Wistar rats, each of ten animals, weighing between 290 and 350 g. The rats were kept under standard conditions of light, temperature, food and water (*ad libitum*), both before and after the burns, in the animal facility of the University of Medicine and Pharmacy of Craiova.

The Ethics Committee of the University of Medicine and Pharmacy of Craiova, in accordance with the European Council Directive No. 86/609/EEC, the European Convention on the Protection of Vertebrate Animals (2005), and the Government Ordinance No. 37/2.02.2002, approved the experimental protocol.

General anesthesia was induced by intramuscular injection of 85 mg/kg ketamine hydrochloride (Ketalar®, Parke-Davis) and 6 mg/kg xylazine hydrochloride (Rompun®, Bayer). The hair was removed from the higher dorsal region of the rats. The third degree burns were inflicted on an area of approx. 1.5 cm^2. Burns were generated using a special stainless steel device, cone-shaped, weighing 350 g, with 1 cm diameter, and equipped with a control thermometer. The metallic device, heated in boiling water, to 100^{0}C, was applied to each animal locally for five seconds (Busuioc CJ *et al.*, 2011; Popescu FC *et al.*, 2011).

The creams were applied daily under the form of thin films. Skin burns from the dorsal region were treated as follows: cold cream with 10% *Sambuci flos* extract (SFE) for the first group; 1% silver sulfadiazine cream (SDA) for the second group; the third group, considered as control, received the cold cream base (CCB).

The evolution of the skin burns was monitored daily, for three weeks. Taking into consideration the macroscopic appearance of the lesions, the rats in all groups had initially third degree skin burns: necrosis of the epithelial and underneath connective tissue, until the muscle layer; about 4 mm perilesional edema; intense erythema.

Histological study

From each group of animals, under general anesthesia, burnt skin was sampled at intervals of 7, 14 and 21 days, with approx. 3 mm of perilesional area, in order to dynamically track the evolution of the healing process. Immediately after sampling, the burnt skin fragments were fixed in 10% buffered neutral formalin for 72 hours, at room temperature, and included in paraffin. For the histological study, 4 μm thick serial sections were cut using a Microm HM350 rotary microtome equipped with a waterfall based section transfer system (STS, Microm). Sections were routinely stained with Hematoxylin–Eosin and then assessed under the light microscope.

Microscopy and image acquisition

The sections were photographed with a Nikon Eclipse 90i microscope (Apidrag, Romania) equipped with a QImaging Rolera cooled CCD camera. Images were captured and archived using the Image ProPlus 7 AMS software (Media Cybernetics Inc, Buckinghamshire, UK).

Statistical analysis

Statistical evaluation of the experimental results was performed using Student's *t*-test. For $p<0.05$, differences were considered statistically significant.

RESULTS AND DISCUSSION

Physico-chemical analysis of *Sambuci flos* tincture

The results of the physico-chemical analysis of *Sambuci flos* tincture are shown below (Tables 1 and 2, Fig. 1).

Table 1. Physico-chemical analysis of *Sambuci flos* tincture

Physico-chemical characteristics	*Sambuci flos* tincture
Aspect	clear liquid
Color	yellow-orange, with green shades
Smell	typically, aromatic
Taste	slightly bitter, burning, aromatic
Relative density	0.9551
Refractive index	1.3680
Iron [%]	–
Heavy metals [%]	–
Alcohol content [%]	68.5
Evaporation residue [%]	4.25
Qualitative analysis (TLC)	flavonosides
Quantitative spectrophotometric analysis [mg rutoside / 100 mL tincture]	7.68

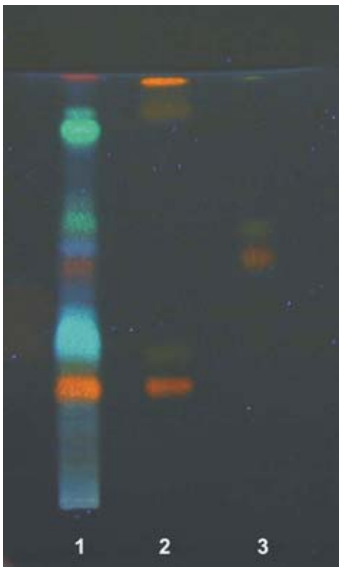

Fig. 1. TLC chromatogram of *Sambuci flos* tincture (1). Standards, bottom-up: (2) rutoside, hyperoside, apigenol-7-neohesperidoside, quercitrin; (3) luteol-7-glucoside, apigenol-7-glucoside.

Table 2. Results of TLC analysis of *Sambuci flos* tincture

Sample No.	R_f	Color (VIS)	Fluorescence (UV)	Observations
1.	0.36	yellow	yellow-orange	rutoside
	0.61	yellow	yellow-orange	probably luteol-7-glucoside
2.	0.36	yellow	yellow-orange	rutoside (s)
	0.43	yellow	yellow-green	hyperoside (s)
	0.58	yellow	yellow-orange	apigenol-7-neohesperidoside (s)
	0.98	yellow	yellow-orange	quercitrin (s)
3.	0.61	yellow	yellow-orange	luteol-7-glucoside (s)
	0.69	yellow	yellow-green	apigenol-7-glucoside (s)

TLC analysis performed for *Sambuci flos* tincture has revealed the presence of rutoside and possibly of luteol-7-glucoside. Some blue fluorescent bands could be due to polyphenol carboxylic acids and coumarins, often found in flavonosidic extracts.

Macroscopic findings

Evolution of burnt skin wounds, after the application of creams, was variable, as follows (Fig. 2):

- animals of SFE group, treated with cold cream containing 10% *Sambuci flos* extract, had a favorable evolution, with a very good epithelization, the wound healing being almost complete for 21 days (90.66%);
- the second group (SDA), treated with 1% silver sulfadiazine cream, and the control group (CCB), treated with cold cream base, showed a delay of wound healing, the epithelization at 21 days being incomplete (76.66% and 68%, respectively), compared with SFE group.

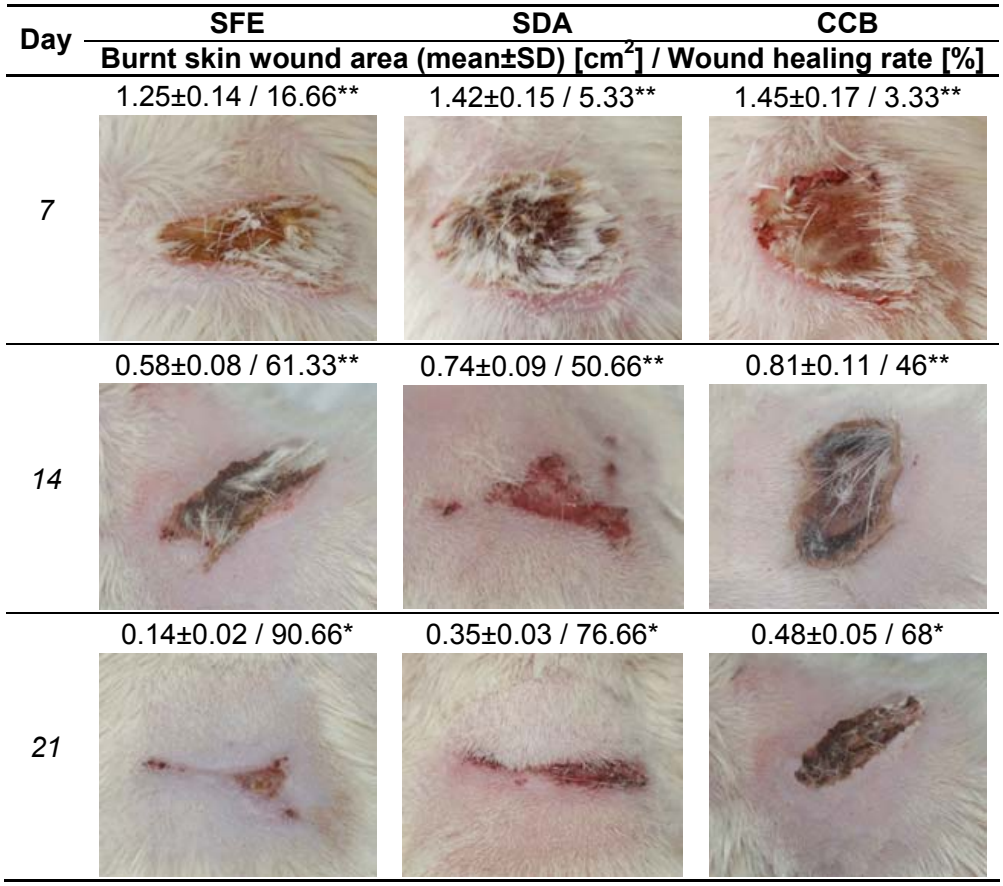

Fig. 2. Evolution of burnt skin wounds, after the application of creams: SFE – cold cream with 10% *Sambuci flos* extract; SDA – 1% silver sulfadiazine cream; CCB – cold cream base (control group); SD – standard deviation; * – $p<0.01$; ** – $p<0.05$.

Microscopic findings

The application of a metallic device heated to 100^0C for five seconds on the skin of the Wistar rats resulted in an immediately noticeable white-gray area of coagulation necrosis. The macroscopic appearance of the wound changed in the following days, becoming light brown-blackish, with raised edges surrounded by a reddish zone of hyperemia and edema.

Seven days after the injury, at the surface of burnt skin wound a thick coagulation necrosis area was observed. This zone is composed mainly of deformed collagen fibers, with variable staining capacity. The collagen fibers are separated from the viable area by an inflammatory infiltrate band made of polymorphonuclear neutrophils. We also found remnants of pilosebaceous follicles, with marked signs of degeneration. At the SFE group (cold cream with 10% *Sambuci flos* extract), the inflammatory reaction was much lower and the post-combustion edema was much reduced (Fig. 3a). At the SDA group (1% silver sulfadiazine cream), the microscopic lesions were dominated by the presence of vacuoles of edema, placed between necrosis area and the muscular plan (Fig. 3b). At the CCB group (cold cream base – control group), compared to SFE or SDA groups, the inflammatory reaction was much stronger both in width and concerning the number of polymorphonuclear cells (Fig. 3c).

At **14 days** after burn, the skin injuries treated with *Sambuci flos* extract cold cream showed a thinned coagulation necrosis area, with multiple cellular elements of the immune system, both at the site of necrosis and into the underlying viable conjunctive tissue. A thin band of polymorphonuclear neutrophils and lymphocytes marked the limit of necrosis area and viable tissue. Into the depth of the wound is remarkable the presence of angiogenesis rich vascular network (Fig. 4a). For SDA group, the coagulation necrosis area remained fairly thick. The vacuoles of edema are visible at the level of coagulation zone and into the underlying conjunctive tissue. A thick band of polymorphonuclear neutrophils and lymphocytes enclosed the limit of necrosis area and viable underlying conjunctive tissue (Fig. 4b). The CCB group shows the same zone of coagulation necrosis like SDA group, except the fact that the inflammatory infiltrate was more abundant and larger in area. Neoformation vessels were also observed, but in small number comparing to SFE group (Fig. 4c).

At **21 days**, for SFE group, the granulation tissue was relatively well-shuffled, meaning that the inflammatory infiltrate was much reduced. The neoformation vessels showed a remodeling process, in that they appeared mature-type blood vessels and the re-epithelization process was well expressed (Fig. 5a). For SDA group, at the wound surface persisted the same coagulation necrosis area, under which abundant inflammatory infiltrate was observed. The granulation tissue retained the character of a young tissue. Although present, the re-epithelization process of the burnt wound was reduced comparing to SFE cold cream (Fig. 5b). In the case of CCB group, was noted the persistence of coagulation necrosis area and of abundant inflammatory infiltrate with difficult re-epithelization: thin, discontinuous, and with variable thickness epithelium (Fig. 5c).

The favorable evolution of burnt skin wounds is due mainly of flavonoids and tannins content of cold cream with 10% *Sambuci flos* extract. Bioflavonoids have some useful properties: antioxidant, anti-inflammatory, epithelizing (healing) in burns, atonic and superficial wounds, capillaroprotective and vasculotropic – decrease the permeability and increase the resistance of the capillaries (Abu-Zinnadah, 2008; Becić *et al.*, 2005; Han *et al.*, 2005; Süntar *et al.*, 2010).

Pharmacological action and therapeutic effects of tannins based on their ability to precipitate proteins (Becić *et al.*, 2005; Kumar *et al.*, 2008):

- astringent action, underlying haemostatic effect in bleeding of small extent: in contact with tannin, the lesion forms a film or membrane of precipitation and/or coagulation, which tends to tighten;
- antiseptic action: in contact with tannin, the microorganisms from burnt wound are directly embedded into the precipitation and/or coagulation membrane;
- anti-inflammatory action, due to the local protection of precipitation and/or coagulation membrane, reducing the inflammatory process to extinction;
- epithelizing action, favorable in the treatment of small area burns, because of precipitation and/or coagulation protective membrane; in addition, tannin had local antitoxic effect through inactivation of degradation products which results in the catabolism of proteins.

In addition, the bees wax from the composition of cold cream has emollient, cicatrizing and biostimulative action.

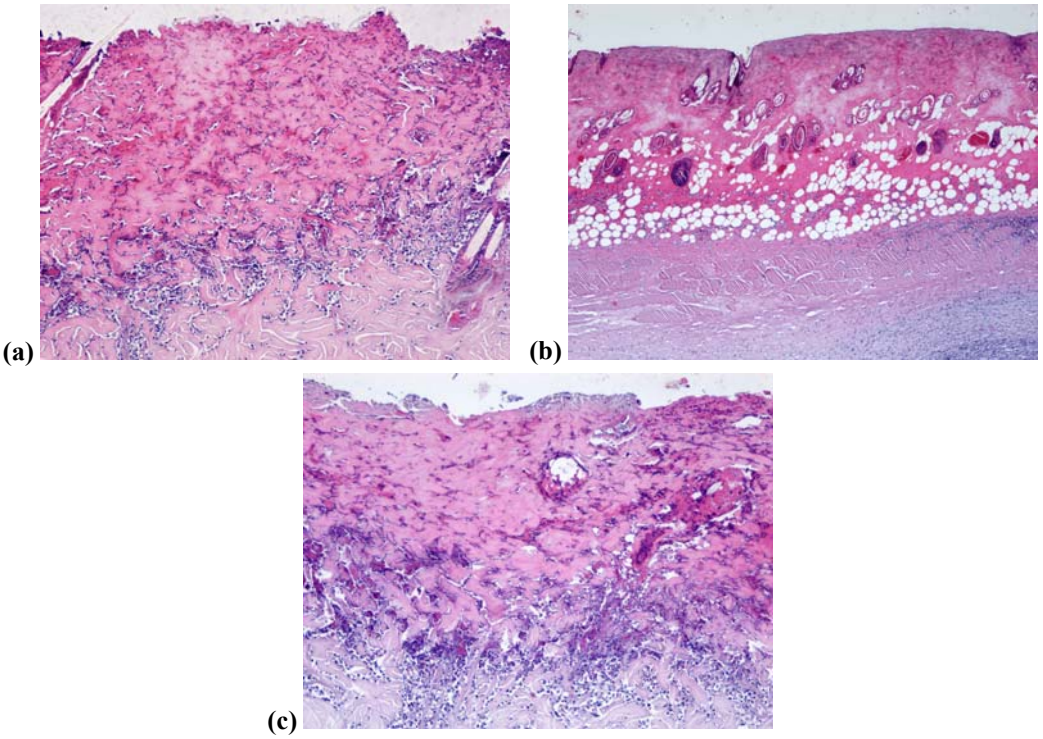

Fig. 3. Microscopic evolution of burnt skin wounds, at seven days after the application of creams: (a) cold cream with 10% *Sambuci flos* extract (HE stain, ×100); (b) 1% silver sulfadiazine cream (HE stain, ×40); (c) cold cream base – control group (HE stain, ×100).

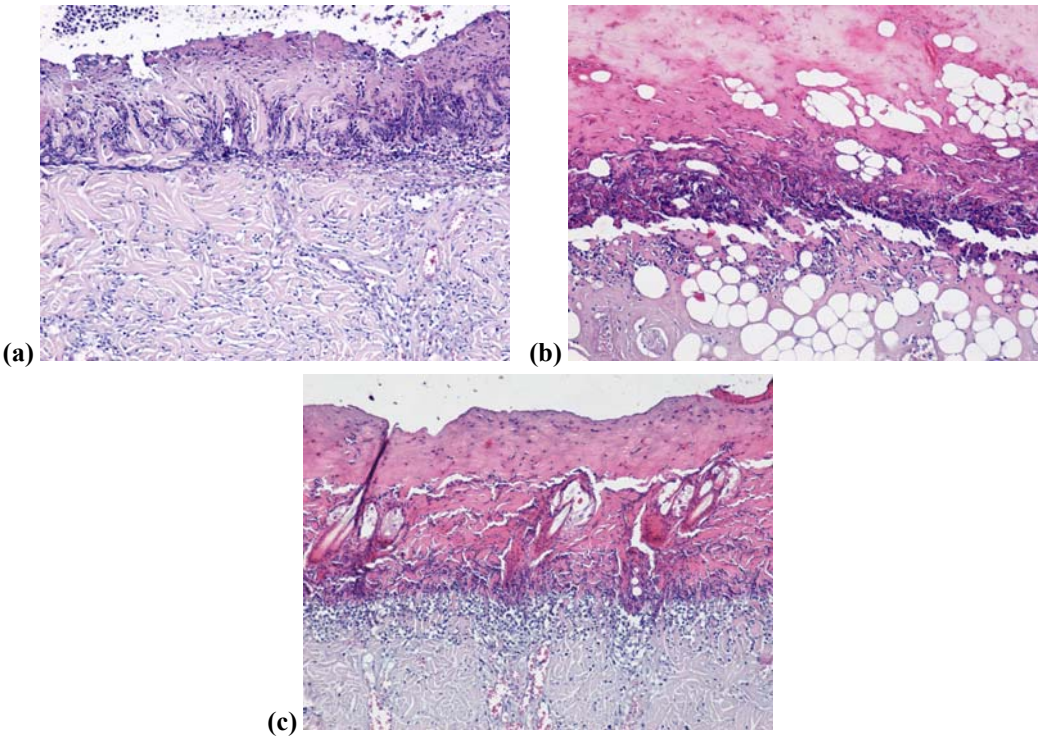

Fig. 4. Microscopic evolution of burnt skin wounds, at 14 days after the application of creams (HE stain, ×100): (a) cold cream with 10% *Sambuci flos* extract; (b) 1% silver sulfadiazine cream; (c) cold cream base (control group).

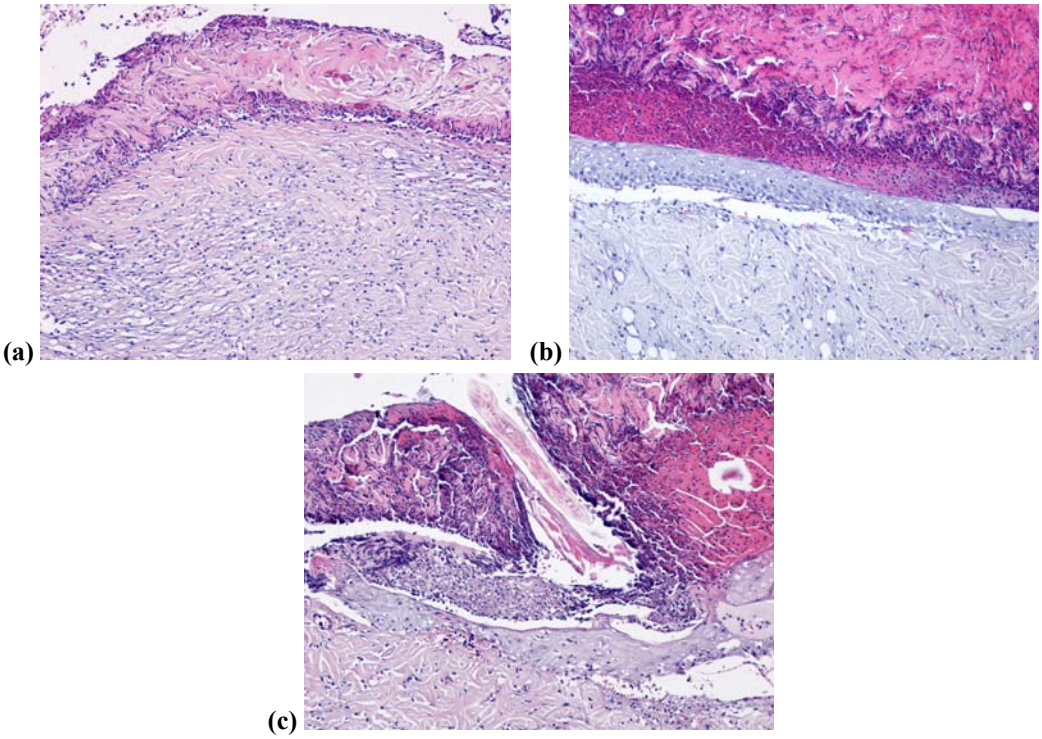

Fig. 5. Microscopic evolution of burnt skin wounds, at 21 days after the application of creams (HE stain, ×100): (a) cold cream with 10% *Sambuci flos* extract; (b) 1% silver sulfadiazine cream; (c) cold cream base (control group).

CONCLUSIONS

Studies on the preparation and physico-chemical characterization of *Sambuci flos* tincture, according to Romanian Pharmacopoeia, are based on the chemical composition and therapeutic uses of the natural product. The soft extract, obtained by evaporation of the tincture, was embedded in an ointment base of cold cream type, prepared according to United States Pharmacopoeia.

The favorable evolution of burnt skin wounds treated with 10% *Sambuci flos* extract cold cream, compared to the groups treated with 1% sulfadiazine cream and cold cream base (control) respectively, is mainly due to the astringent, anti-inflammatory, antiseptic and cicatrizing action of the phytocomplex. In addition to reducing the inflammatory reaction, the cold cream with vegetal extract favored the appearance of neoangiogenesis capillaries.

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