

Effect of *Scutellariae herba* extracts in experimental model of skin burns: histological and immunohistochemical assessment

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Abstract

The skin burns are an issue of great interest and seriousness in the public health domain, by their destructive features. Natural medicinal products are extensively used from ancient times, in ethnopharmacology, for the treatment of skin injuries (burns, wounds, ulcerations) due to the local modulation of the cellular response, in terms of emollient, demulcent, astringent, anti-inflammatory, antimicrobial, epithelizing, wound-healing, immunomodulatory and antioxidant effects. **Aim:** Histological and immunohistochemical assessment of antiseptic, anti-inflammatory, astringent and cicatrizing effects of *Scutellariae* (*altissimae*, *galericulatae*, *hastifoliae*) *herba* extracts administered in the form of 20% topical preparations (cold-creams), in experimental model of third degree skin burns, at Wistar rats. Caffeic acid and chlorogenic acid derivatives were identified in all herbal samples. The most active was the cold-cream with 20% *Scutellariae hastifoliae herba* soft extract, promoting the neoangiogenesis vessels and granulation tissue. Flavonoids, tannins and polyphenol carboxylic acids are the main active principles responsible for antiseptic, anti-inflammatory, astringent and cicatrizing effects of herbal extracts. Beeswax, from the formulation of cold-creams, acts as emollient, epithelizing, cicatrizing and biostimulator.

Keywords: *Scutellariae herba*, extracts, experimental model, rats, skin burns.

Introduction

By their destructive features, skin burns are an issue of great interest and seriousness in the public health domain; annually, millions of burns cases are registered worldwide, some of them inflicting critical injuries and more than 250 000 deaths [1–4].

Often, skin burns require specialized emergency care because of their drastic complications due to impaired skin integrity, such as: microbial invasion [5, 6], depth and extent of the lesions at the level of epidermis, dermis, muscles tendons, bone tissue, kidneys, liver, lungs [1, 6], endocrine disequilibrium with severe psychological impact, stress and even mental disturbances [1, 2, 7, 8], unaesthetic scars [3, 9].

The mechanism of skin burns healing is very complex, involving intra- and extra-cellular signals and responses in reference to hemostasis, inflammation, proliferation and remodeling. Formation of new blood vessels, also known as angiogenesis, is crucial for the restoration of injured tissues due to the local supply with nutrients, vitamins, enzymes and growth factors [10–17].

Natural medicinal products are extensively used from ancient times, in ethnopharmacology, for the treatment of skin injuries (burns, wounds, ulcerations) due to the local modulation of the cellular response, in terms of emollient, demulcent, astringent, anti-inflammatory, antimicrobial, epithelizing, wound-healing, immunomodulatory and antioxidant effects [18–21].

In this respect, *Scutellaria* species (*Lamiaceae* family) highlighted useful pharmacological properties, due to their content of active principles (flavonoids, diterpenoids, essential oil, phenylethanoid glycosides, lignan glycosides, polysaccharides, carotenoids) [22, 23]: antioxidant [24, 25], antitumoral, anti-angiogenesis and immunomodulatory [26, 27], antimicrobial and antiviral, anticonvulsant, hepatoprotective [23, 28], anti-inflammatory, antipyretic, tonic [29], anti-feedant [22, 30].

The aim of our study is represented by the histological and immunohistochemical assessment of antiseptic, anti-inflammatory, astringent and cicatrizing (regenerative) effects of *Scutellariae* (*altissimae*, *galericulatae*, *hastifoliae*) *herba* extracts administered in the form of 20% topical preparations (cold-creams), in experimental model of third degree skin burns, at Wistar rats.

Materials and Methods

Plant material

The plant material (aerial part) was collected from *Scutellaria* spp. in blossom, from the South-West (Oltenia) Region of Romania: *S. altissima*, in June 2015 – from the surroundings of Radovan Village (Valea Rea zone), Dolj County; *S. galericulata*, in August 2015 – edges of the Lotru River, Brezoi Village (Vâlcea County);

S. hastifolia, in June 2015 – from the Bucovăț Forest (Dolj County). Considering the preparation of extracts, the plant material was preserved in optimum conditions of temperature, light and humidity. Voucher specimens are deposited in the Herbarium of the Vegetal & Animal Biology Department, Faculty of Pharmacy, University of Medicine and Pharmacy of Craiova, Romania.

Reagents and solvents

Analytical grade reagents and solvents were from Merck Millipore (Darmstadt, Germany) and from Sigma-Aldrich (Seelze, Germany).

Preparation of tinctures and cold-creams with herbal extracts

Starting from the vegetal material, according to the Romanian Pharmacopoeia Xth edition [31], 20% tinctures were prepared by percolation (70% ethanol as extraction solvent), and then physicochemically characterized: S1 – *Scutellariae altissimae herba*, S2 – *Scutellariae galericulatae herba*, and S3 – *Scutellariae hastifoliae herba*. After filtration, tinctures were stored in the refrigerator, in brown-glass bottles, until use. The preparation of cold-creams with 20% herbal soft extracts was accomplished according to our previously registered Patent (OSIM, 2012) [21, 32].

Thin-layer chromatography (TLC) analysis

TLC analysis of polyphenols from the aboveground parts of the three *Scutellaria* spp. has been achieved by using a CAMAG (Muttentz, Switzerland) system, according to the specialty literature [33–35]: TLC silica gel 60 F₂₅₄ 20×10 precoated glass plates as stationary phase (Merck, Darmstadt, Germany); chloroform–methanol (1:1, v/v) mixture for pre-washing; chloroform–ethyl acetate–toluene–formic acid–methanol (15:20:10:10:1, in volumes) as mobile phase, in a vapor-equilibrated chromatographic tank (CAMAG 20×10 cm twin trough chamber); herbal samples – 20% methanolic extracts from *Scutellariae* (*altissimae*, *galericulatae*, *hastifoliae*) *herba*; standards (Merck) – 0.05% methanolic solutions of caffeic acid, chlorogenic acid, quercetin and rutin; migration distance 80 mm; application of samples (5 µL) and standards (1–3 µL) using CAMAG Linomat 5 semiautomatic system (spray gas – nitrogen, dosage speed – 150 nL/s, band length – 8 mm); detection with CAMAG TLC Scanner 3 photodensitometer (at UV 254 nm, without derivatization, with deuterium–wolfram lamp, 20 mm/s scanning speed, 100 µm/step resolution, absorption as measurement mode); winCATS ver. 1.4.3 software package applied for spectra acquisition, processing and quantification analysis.

Experimental model of thermal skin burns in rats

Animals

The study was performed on five groups of common adult Wistar rats, each of 10 animals, weighing between 300 and 350 g. At the Animal Facility of the University of Medicine and Pharmacy of Craiova, animals were kept under standard conditions of light, temperature, humidity, food and water (*ad libitum*), both before and after the

experiment. The Ethics Committee of the University of Medicine and Pharmacy of Craiova approved the experimental protocol, according with the European Council Directive No. 86/609/November 24, 1986 (86/609/EEC), the European Convention on the Protection of Vertebrate Animals used for experimental and other scientific purposes (December 2, 2005), the Romanian Government Ordinance No. 37/February 2, 2002 and the Romanian Parliament Law No. 43 (April 11, 2014) on the protection of animals used for scientific purposes [13–17, 20, 21].

Procedures

General anesthesia was induced using intramuscular injection of 85 mg/kg body-weight (b.w.) Ketamine hydrochloride (Ketalar[®], Parke-Davis) and 6 mg/kg b.w. Xylazine hydrochloride (Rompun[®], Bayer). The hair was removed on an area of cca. 5 cm², on the higher dorsal region of the rats. A special cone-shaped stainless steel device (1 cm diameter, 350 g weight), equipped with a control thermometer was used for the infliction of third-degree burns, on an area of 1.5 cm². Device was applied locally, after the heating in boiling water (100°C), on the dorsal region of each rat for five seconds [13–17, 20, 21].

For the wound healing of each experimental group, thin films of five topical preparations were applied daily: cold-creams with 20% herbal soft extracts (S1, S2 and S3), 1% silver sulfadiazine (SDA) cream for reference group, and cold-cream (CC) base, as control.

Evolution of third-degree skin burns and the welfare of animals were daily monitored, for three weeks. No animal died during the experiment.

Histological study

For the dynamically assessment of the angiogenesis process, the granulation tissue on the burn wound, with cca. 3 mm of perilesional area, was collected from each group of rats, under general anesthesia, at three, seven, 14 and 21 days from the skin burns infliction, and the remaining wound was surgically sutured. After sampling, the burnt-skin pieces were fixed in 10% buffered neutral formalin, for 72 hours, at room temperature, and then processed for histological paraffin inclusion technique. Using a Microm HM350 rotary microtome (MICROM International GmbH, Walldorf, Germany), equipped with a waterfall-based section transfer system (STS, Microm), 4 µm thick serial cross-sections were cut for the histological study. Hematoxylin–Eosin (HE) classical staining was used for the light microscopy [20].

Immunohistochemical study

Immunohistochemical (IHC) study started from 3 µm thick cross-sections added on poly-L-lysine coated slides (Sigma-Aldrich, Munich, Germany) and stored one day, at 37°C, in the thermostat. Then, after dewaxing and hydration of the cross-sections, the histological material was incubated in 1% hydrogen peroxide solution for 30 minutes. Next, cross-sections were washed in tap water and boiled in citrate buffer solution (pH 6) for 20 minutes, for antigen unmasking. After the boiling, cross-sections were cooled for 15 minutes and then washed in phosphate-buffered saline (PBS). Endogenous peroxidase blocking was made with 2% skimmed milk for 30 minutes. Next,

cross-sections were incubated with primary antibody, overnight, at 4°C. The signal was amplified using peroxidase secondary antibody on polymer support (EnVision, Dako) for 30 minutes. 3,3'-Diaminobenzidine (DAB, Dako) was used for the signal detection. After the contrasting with Mayer's Hematoxylin, the slides were covered with DPX (Fluka). For the evaluation of the angiogenesis process, we used anti- α -smooth muscle actin (α -SMA) antibody (monoclonal mouse anti-human muscle actin, clone HHF35, 1:100 dilution, Dako) [13–15, 20].

Acquisition of images and the microscopic assessment

Areas of maximum vascular density (by "hot spot" method) were chosen for the assessment of angiogenesis vessel density and five microscopic images were captured. Then, the "manual tagging" features from ImageProPlus software package were used for the vessel count. For automatic calculation of vascular densities, the acquired data were exported to an Excel spreadsheet. Microscopic evaluation was achieved by grabbing 40 \times images under the Nikon Eclipse 55i microscope equipped with a 5 Mp CCD (charge-coupled device) color sensor (Apidrag,

Romania). Image ProPlus 7 AMS package (Media Cybernetics, Inc., Marlow, Buckinghamshire, UK) was used for capture, storing and analyzing of images [13–15, 20].

Statistical analysis

Student's *t*-test was applied for the statistical analysis of the physicochemical experimental data. For $p < 0.05$, the differences were considered statistically significant. ANOVA (analysis of variance) test was used for the assessment of the differences between the effects of topical preparations, taking into account the means and standard errors for each period of time.

Results

TLC fingerprint of the polyphenols from *Scutellariae herba* extracts is highlighted in Figure 1. Caffeic acid ($R_f \sim 0.92$) and chlorogenic acid ($R_f \sim 0.25$) derivatives have been identified in all herbal samples, as follows: 234 mg% caffeic acid derivative (*Scutellariae hastifoliae herba*), 116 mg% caffeic acid derivative (*Scutellariae galericulatae herba*) and 92 mg% chlorogenic acid derivative (*Scutellariae altissimae herba*).

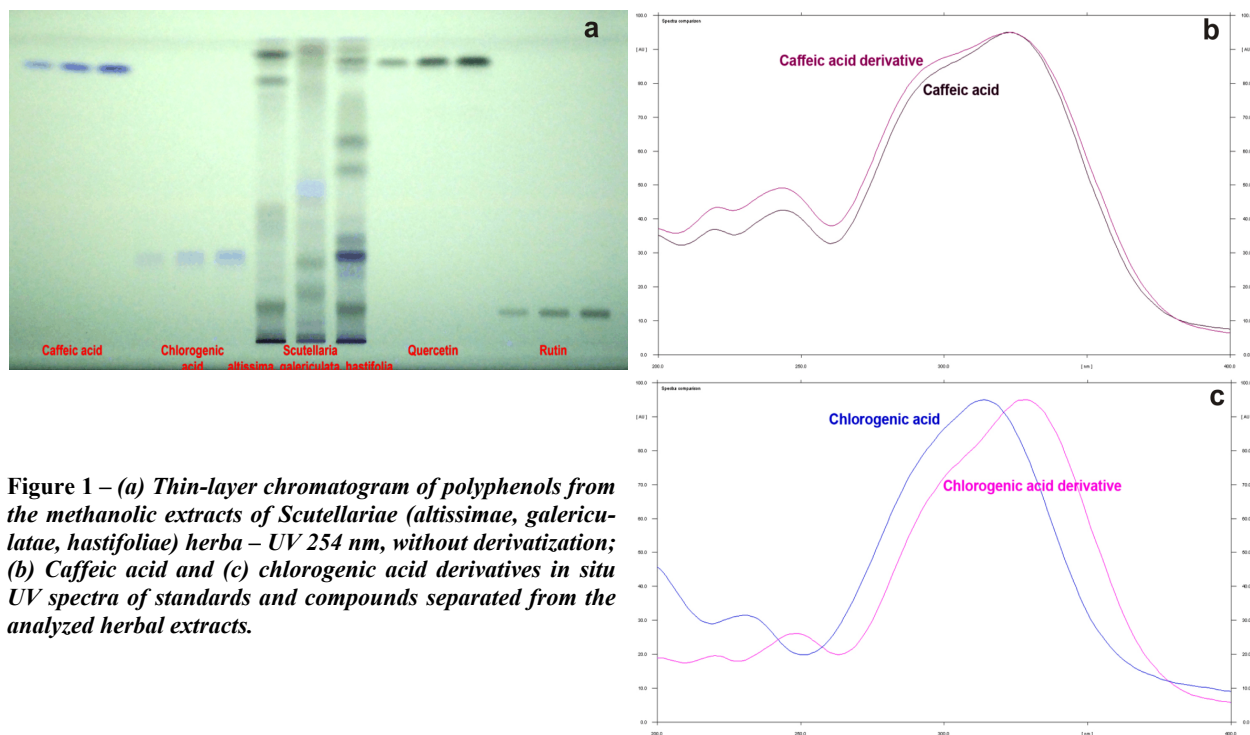


Figure 1 – (a) Thin-layer chromatogram of polyphenols from the methanolic extracts of *Scutellariae (altissimae, galericulatae, hastifoliae) herba* – UV 254 nm, without derivatization; (b) Caffeic acid and (c) chlorogenic acid derivatives in situ UV spectra of standards and compounds separated from the analyzed herbal extracts.

Immediately after the application of metallic device heated to 100°C, for five seconds, a white-gray area of coagulation necrosis of epidermis, dermis and superficial muscles occurs on the rat skin together with alteration of the vascular network, hyperemia and edema.

The evolution of burnt skin wounds was variable, following the use of topical preparations (Figures 2–5): cold-cream groups ($S3 > S1 > S2$) exhibited a good epithelization, a favorable evolution and an almost complete wound healing at 21 days; a delay of wound healing with an incomplete epithelization after 21 days was highlighted for SDA (reference) and CC (control) groups, compared with S1–S3 groups.

Starting with third day until 21st day, the mean number

of angiogenesis vessels gradually decreased for all five groups of tested creams. S3 group recorded the largest mean number of angiogenesis vessels comparing with S1, S2, CC and SDA groups (Figure 6).

Three and seven days after the burns infliction, at the surface of skin wound, a thick coagulation necrotic area containing deformed collagen fibers with variable tinctoriality and remnants of pilosebaceous follicles with marked signs of degeneration were highlighted. The collagen fibers from the viable area are separated by an inflammatory infiltrate zone of polymorphonuclear neutrophils. The postcombustion edema and inflammatory reaction were much reduced for S1–S3 cold-cream groups. The inflammatory reaction was much stronger and the

presence of edema vacuoles between necrotic area and the muscular layer predominated into the microscopic lesions for SDA and CC experimental groups (Figures 2 and 3).

Fourteen days after the burned injury, at the necrosis site and into the underlying viable conjunctive tissue, for S1–S3 cold-cream groups, the skin wounds exhibited a thinned coagulation necrotic area with immune system cells. A thin band of polymorphonuclear neutrophils and lymphocytes delimitates the boundary between necrotic

area and viable tissue. Into the wound depth, a rich angiogenesis vascular network was highlighted. A small mean number of neoformation vessels but with large area and perimeter was observed for all experimental groups. For SDA and CC groups, coagulation necrotic area remained more abundant with extensive inflammatory infiltrate and edema vacuoles. Also, a thick belt of polymorphonuclear neutrophils and lymphocytes mark the limit of necrotic area and viable conjunctive tissue (Figure 4).

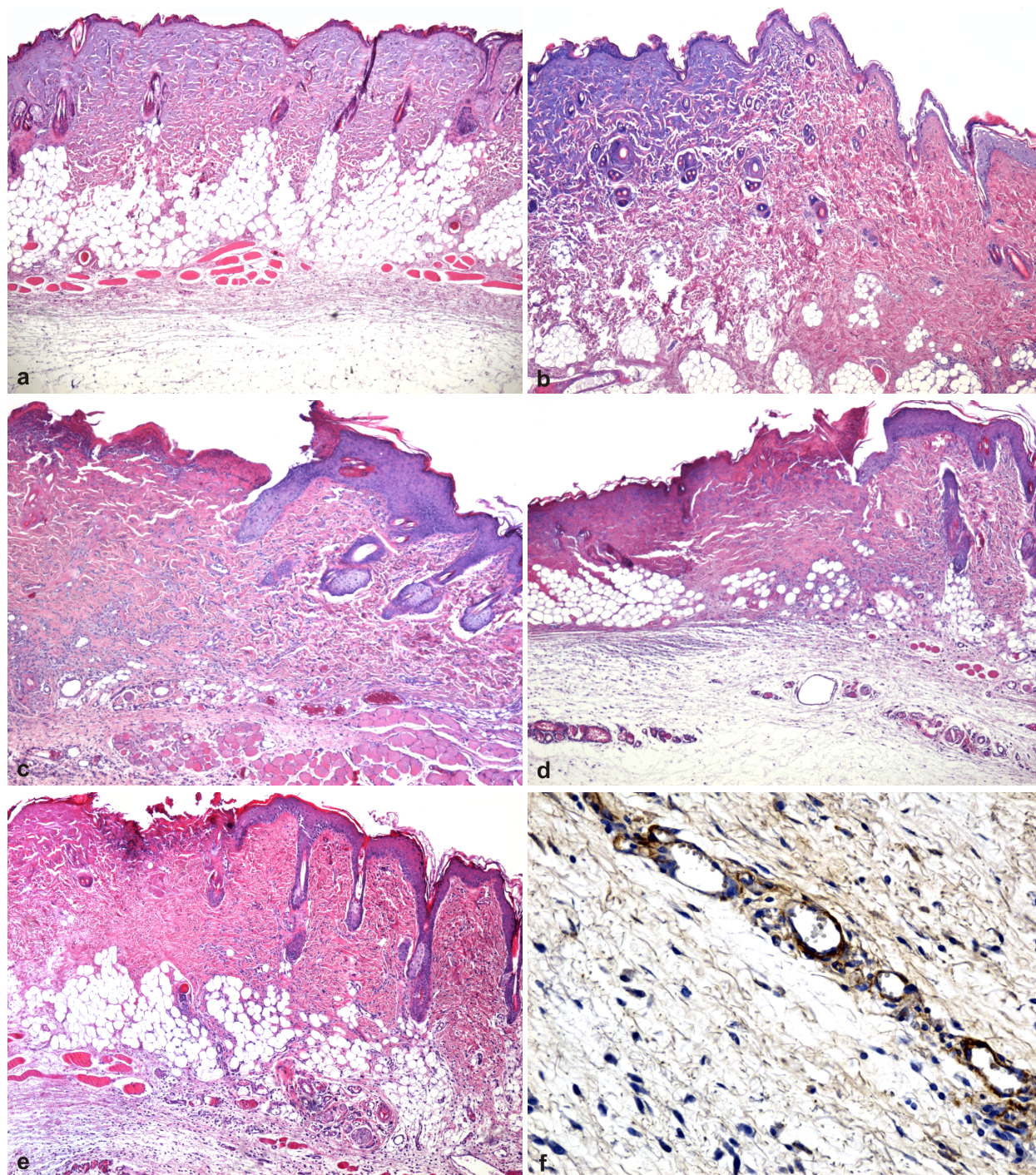


Figure 2 – Histological and immunohistochemical aspects on the evolution of burn wounds, three days after the application of creams: (a) Cold-cream containing 20% *Scutellariae altissima* herba extract (S1); (b) Cold-cream containing 20% *Scutellariae galericulatae* herba extract (S2); (c) Cold-cream containing 20% *Scutellariae hastifoliae* herba extract (S3); (d) 1% Silver sulfadiazine cream (SDA – reference group); (e) Cold-cream base (CC – control group); (f) Immunohistochemical aspects of angiogenesis vessels with positive immunostaining for α -SMA, after the application of S3 cold-cream. HE staining: (a–e) $\times 40$. Anti- α -SMA antibody immunostaining: (f) $\times 200$.

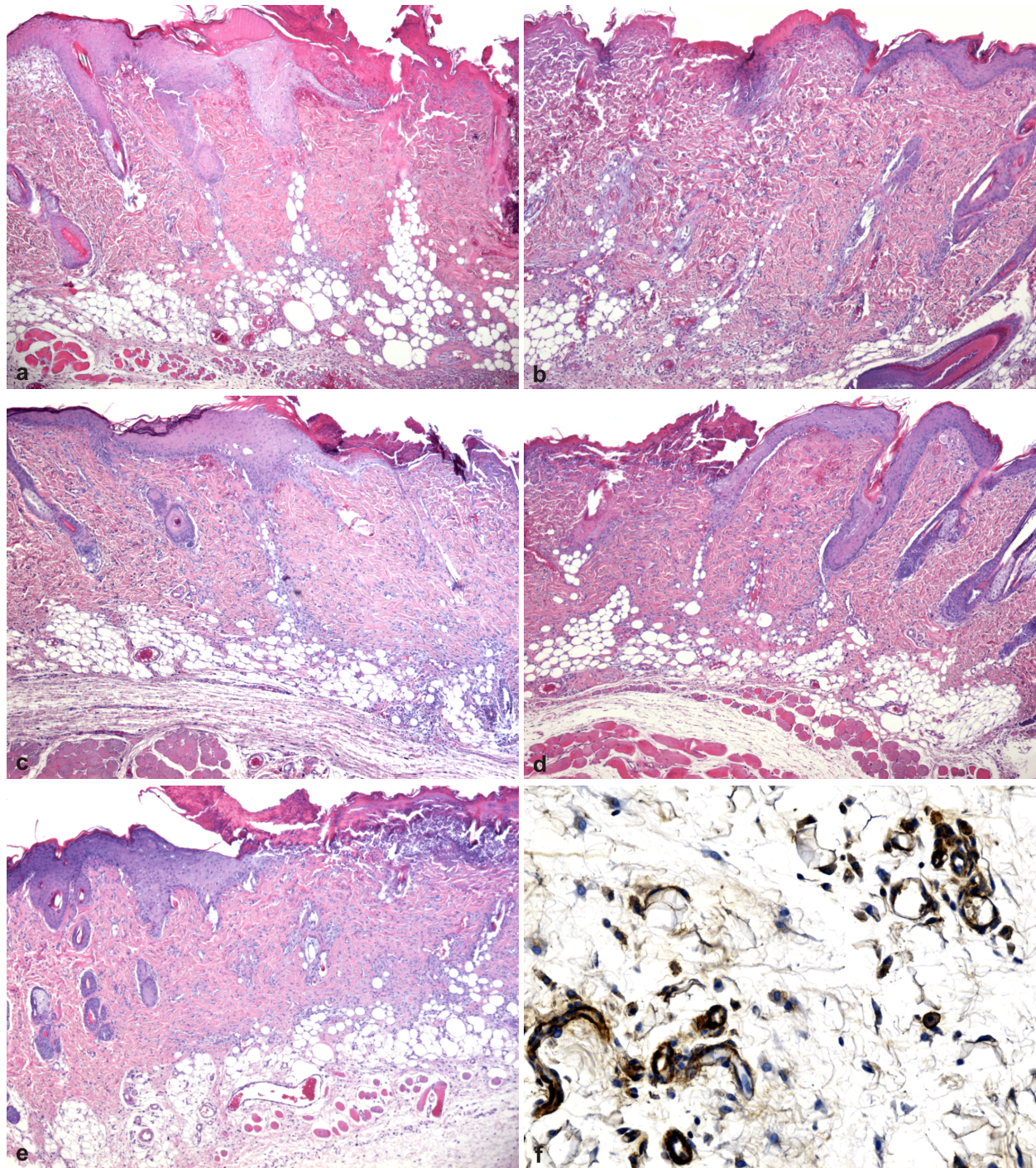


Figure 3 – Histological and immunohistochemical aspects on the evolution of burn wounds, seven days after the application of creams: (a) Cold-cream containing 20% *Scutellariae altissima* herba extract (S1); (b) Cold-cream containing 20% *Scutellariae galericulatae* herba extract (S2); (c) Cold-cream containing 20% *Scutellariae hastifoliae* herba extract (S3); (d) 1% Silver sulfadiazine cream (SDA – reference group); (e) Cold-cream base (CC – control group); (f) Immunohistochemical aspects of angiogenesis vessels with positive immunostaining for α -SMA, after the application of S1 cold-cream. HE staining: (a–e) $\times 40$. Anti- α -SMA antibody immunostaining: (f) $\times 200$.

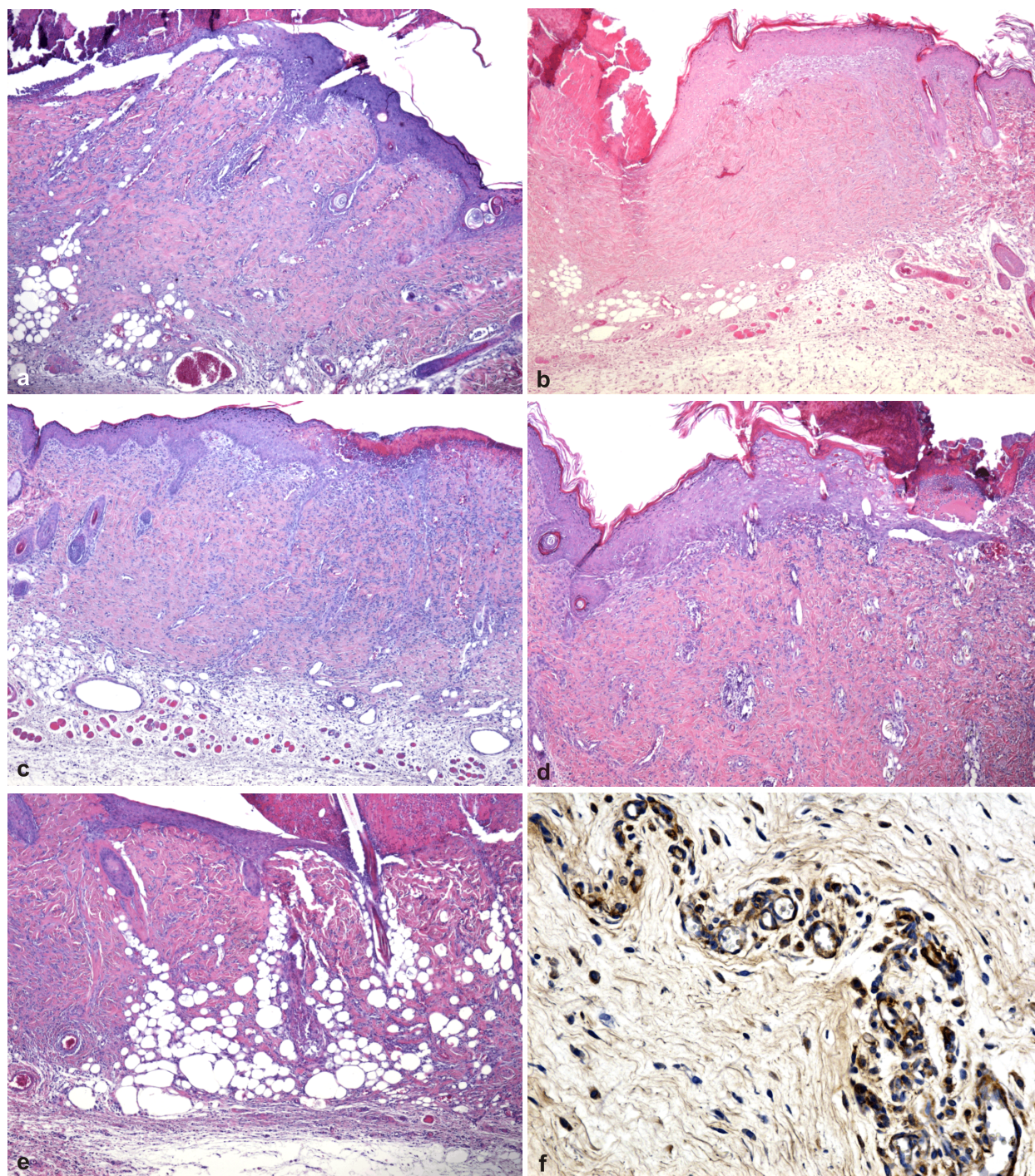


Figure 4 – Histological and immunohistochemical aspects on the evolution of burn wounds, 14 days after the application of creams: (a) Cold-cream containing 20% *Scutellariae altissima* herba extract (S1); (b) Cold-cream containing 20% *Scutellariae galericulatae* herba extract (S2); (c) Cold-cream containing 20% *Scutellariae hastifoliae* herba extract (S3); (d) 1% Silver sulfadiazine cream (SDA – reference group); (e) Cold-cream base (CC – control group); (f) Immunohistochemical aspects of angiogenesis vessels with positive immunostaining for α -SMA, after the application of S2 cold-cream. HE staining: (a–e) $\times 40$. Anti- α -SMA antibody immunostaining: (f) $\times 200$.

Twenty-one days after the burn, for S1–S3 groups, inflammatory infiltrate was much reduced with largely well recovered granulation tissue and a well expressed re-epithelization. Mature-type blood vessels exhibited a remodeling process with neoformation vascular network. Comparing with the third day, a small number of angiogenesis vessels but with the largest area and perimeter

was evidenced for all experimental groups. Persistence of coagulation necrosis area, an abundant inflammatory infiltrate and insufficiently developed granulation tissue, for SDA and CC groups, make the re-epithelization process to be difficult (Figure 5).

Comparing with SDA and CC groups (delayed and almost spontaneously wound healing), S1–S3 cold-creams

activate the development of angiogenesis capillaries and granulation tissue. The most active was S3 cold-cream with 20% *Scutellariae hastifoliae herba* soft extract.

For all tested creams, progressively decreasing in the mean number of angiogenesis vessels is directly correlated

with the maturation of granulation tissue (extracellular matrix – collagen fibers) and the reduction of inflammatory response, and inversely correlated with the lumen (diameter), area and perimeter of capillaries.

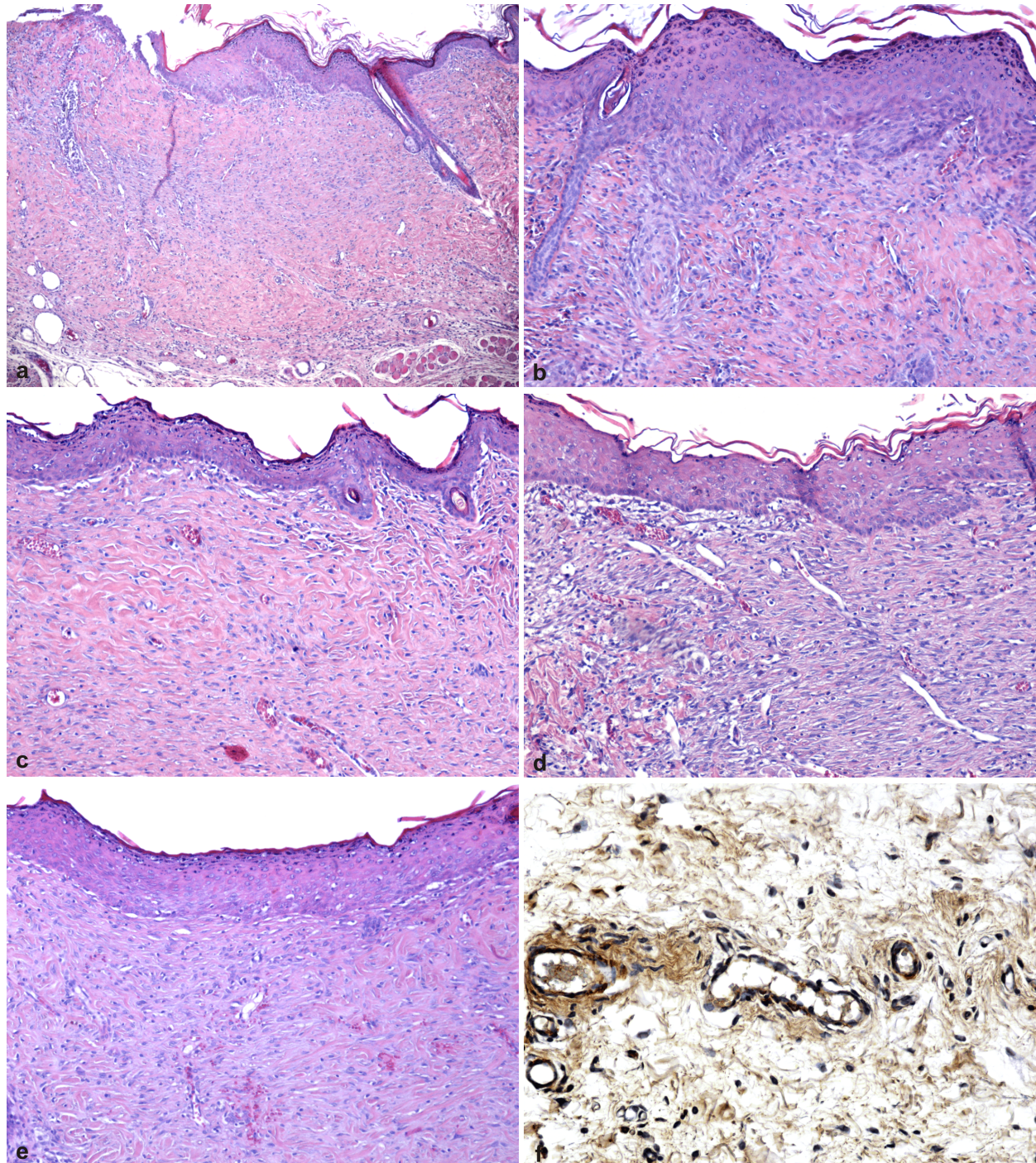


Figure 5 – Histological and immunohistochemical aspects on the evolution of burn wounds, 21 days after the application of creams: (a) Cold-cream containing 20% *Scutellariae altissima* herba extract (S1); (b) Cold-cream containing 20% *Scutellariae galericulatae* herba extract (S2); (c) Cold-cream containing 20% *Scutellariae hastifoliae* herba extract (S3); (d) 1% Silver sulfadiazine cream (SDA – reference group); (e) Cold-cream base (CC – control group); (f) Immunohistochemical aspects of angiogenesis vessels with positive immunostaining for α -SMA, after the application of CC base. HE staining: (a) $\times 40$; (b–e) $\times 100$. Anti- α -SMA antibody immunostaining: (f) $\times 200$.

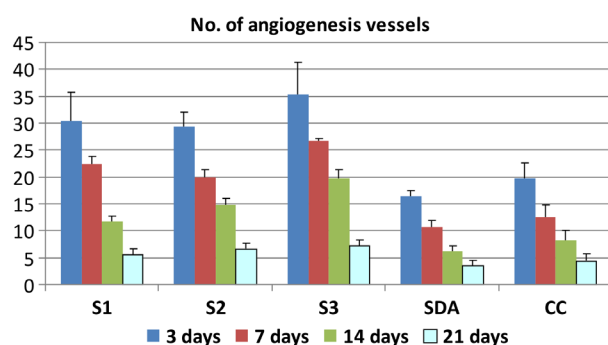


Figure 6 – Evolution of the number of angiogenesis vessels after the application of creams at three, seven, 14 and 21 days, respectively. S1: Cold-cream with 20% *Scutellariae altissima* herba extract; (b) S2: Cold-cream with 20% *Scutellariae galericulatae* herba extract; (c) S3: Cold-cream with 20% *Scutellariae hastifoliae* herba extract; (d) SDA: 1% Silver sulfadiazine cream (reference group); (e) CC: Cold-cream base (control group).

Discussion

With a high capacity of autoregeneration, the skin is a vital organ for the body homeostasis and a mechanical barrier against many external aggressive factors (UV irradiation, microorganisms, toxins, etc.); its functioning and capacity of regeneration is severely limited by wide area and high degree burns [11, 36].

At the skin level, some decisive cellular and molecular cascade events developed because of burn aggression: inflammation, proliferation and remodeling; for tissue repair and restoration of homeostasis, monocytes/macrophages and fibroblasts acting at the level of damaged area; monocyte adhesion at the injury site and biosynthesis of inflammatory markers, matrix remodeling proteins, and growth factors [13–17, 37–39]; migration of blood cells from the wound depth; stimulation of local inflammation; removing of the cellular debris/pathogens; proliferation of the connective tissue and extracellular matrix; angiogenesis – formation of a new network of vessels; granulation tissue for remodeling process [10, 37, 38, 40].

For the skin regeneration, angiogenesis exhibited a major role, closely related to necrosis area removing, activation of the resistance against the microorganisms attack and speeding the appearance of scarring [7].

Various cells, such as M2 macrophages, very important for the stimulation of different wound repair stages and for angiogenesis, release the proangiogenic factors, starting from bone marrow-derived circulating endothelial precursors [41–46].

Due to their easily application, for subcutaneous and chronic inflammations of the skin, patients often prefer cold-creams instead of greasy ointments. Cold-creams induce a local cooling effect, useful in the treatment of skin burns and wounds, by their peculiar composition, including waxes, liquid paraffin, sodium tetraborate and water. This category of topical preparations is much effective at dermal level when herbal extracts are added in the composition of cold-cream base [19–21, 32].

An ideal wound dressing exhibited three conditions,

such as: protecting of the damaged skin from infections; reducing inflammation; and activating cell proliferation for cellular destructions recovery. Natural products rich in some active principles like heteroglycans, flavonoids, tannins, anthracene-derivatives, essential oils, vitamins, minerals stimulate the wound-healing process, acting as emollient, anti-inflammatory, astringent, antiseptic, antioxidant, epithelizing and cicatrizing remedies [18–21, 47].

By their polyphenolic content (flavonoids, coumarins, tannins, polyphenol carboxylic acids and related compounds – some of them detected through TLC–densitometric analysis), the cold-creams with 20% *Scutellariae herba* soft extracts exhibited anti-inflammatory, astringent, antiseptic, epithelizing and cicatrizing effects in experimental model of third-degree skin burns. Also, all tested herbal extracts activate the angiogenesis process at the level of skin wound.

The role of bioflavonoids and tannins for a proper evolution of burnt skin wounds is undeniable. Capillaro-protective and vasculotropic, decreasing the permeability and increasing the resistance of the capillaries, antioxidant, anti-inflammatory, epithelizing and wound healing properties are widely recognized for flavonoids. Moreover, tannins exhibited astringent, antiseptic, hemostatic, anti-inflammatory and epithelizing actions. Beeswax, from the formulation of cold-creams, acts as emollient, epithelizing, cicatrizing and biostimulator [19–21, 32, 48, 49].

Conclusions

The effects of *Scutellariae (altissima, galericulatae, hastifoliae)* herba extracts administered as 20% topical preparations were assessed from the histological and immunohistochemical point of view, in experimental model of third degree skin burns, at Wistar rats. The most active was the cold-cream with 20% *Scutellariae hastifoliae* herba soft extract, promoting the neoangiogenesis vessels and granulation tissue. Flavonoids, tannins and polyphenol carboxylic acids are the main active principles responsible for antiseptic, anti-inflammatory, astringent and cicatrizing effects of herbal extracts.

Conflict of interests

The authors declare that they have no conflict of interests.

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