ACTA BIOLOGICA CRACOVIENSIA Series Zoologia 53: 47–61, 2011

Analgesic, Antiedematous and Wound Healing Properties of AX/09 in the Treatment of Swelling and Poorly Healing Skin Defects in Rodents

Tadeusz Librowski[1]*, Karolina Pytka*, Kinga Sałat*, Anna Rapacz*, Janusz Kurek** and Andriej Waleriewicz Pantiuchin***

** Department of Pharmacodynamics, Jagiellonian University, Medical College, Medyczna 9, 30-688 Cracow, Poland, ** Nes Pharma, Ostrogskich 5, 33-100 Tarnów, Poland, *** Dept. of Pharmaceutical Technology and Biotechnology, Saratov State Medical University, Bolshaya Kazachia st., 112, 410012 Saratov, Russia*

Accepted September 12, 2011

In the present paper the therapeutic effect of AX/09 preparation containing a mixture of terpene natural oils and AVILIN, a thick viscous liquid with anti-inflammatory, antibacterial and tissue-regenerating properties is studied. The antinociceptive and antiedematous activities of AX/09 were investigated in the Randall-Selitto test and carrageenan-induced hind paw edema model in rats. The burn wound-healing properties of AX/09 in chemical and thermal burn models were studied in mice and rats, respectively. To determine the stage of the wound repair process a few methods were used. The wound surface area was measured planimetrically. The development of the granulation tissue and its transformation into young skin, the presence of infiltrates and exudates within the damaged skin were measured micrometrically by skinfold thickness.

Planimetric observations of the wound surface area and micrometric measurements of effusions and infiltrates showed that the healing process of the chemically-induced burn wound was more balanced and much faster after treatment with AX/09. In this model, in animals treated with AX/09 the whole granulation tissue was transformed into young epidermis after 17 days, while in the group treated with AVILIN and in the control group a similar effect was observed after 20 and 23 days, respectively. Much less convincing results were observed in animals treated with AX/09 in thermally-induced burn models. In animals treated with AX/09 complete wound closure by the newly formed epidermis was observed after 21 days. In the group treated with *vehiculum* a similar effect was observed after 22 days and in the control group after 24 days. There were no toxic skin reactions after the first contact with the skin, as well as after multiple applications of AX/09. AX/09 did not cause a delayed-type allergic reaction. The efficacy of AX/09 and its influence on the burn wound healing process, especially in chemical models, seems to be of special value for the clinical, especially dermatological, practice.

Key words: Burn wound, polivinylbutylic ether, terpene natural oils, carrageenan-induced paw edema, contact allergy, skin defects, chemical burn, thermal burn

[1] mflibrow@cyf-kr.edu.pl

PL ISSN 0001-530X

INTRODUCTION

The most relevant role of the skin is the protection of inner parts of the body from pathogens and damage coming from the external environment. Apart from this protective role against mechanical, physical and chemical factors it also regulates the heat, secretory and resorbing activities and immune processes (Adam et al., 1968).

The skin aging process inhibits the synthesis of collagen and proteoglycans and leads to the impairment of angiogenesis (Adam et al., 1968) which plays an important role in embryogenesis and is essential for such processes as the hair cycle and wound healing (Adam et al., 1968). The mechanism of skin cell damage is difficult to determine but it is usually connected with the damage to one of four intracellular systems: aerobic respiration, cell membrane, synthesis of enzymes and structural proteins, and the genetic apparatus repair systems. There is evidence that the onset and acceleration of the skin aging process is associated with the action of oxygen free radicals produced in the skin. Free oxygen radicals can react with many components of skin cells causing peroxidation of the genetic material and lipids, which results in the damage to the cell membranes, inhibition of prostacyclin synthesis and inactivation of enzymes. Under physiological conditions there is a large variety of free radical-scavenging mechanisms the human organism is supplied with. One of them is the superoxide dismutase which catalyzes decomposition of peroxide radicals.

Burn not only disturbs the function of the skin but it also exposes the whole body to many dangerous factors from the environment. The extension and depth of burn wounds depend on the temperature, duration of heating and cooling of the tissue. There are numerous causes of burns and therefore they are classified as thermal, chemical, electrical and radiant burns (Potocki, 1987). Thermal burns are caused by high temperatures, and their severity depends on the temperature and time of contact with the skin. Skin damage caused by high temperatures is a consequence of coagulation necrosis with changes within the capillaries. This leads to increased vascular permeability and loss of both plasma and electrolytes. Chemical burns occur as a result of exposure of the skin or the mucous membranes to caustic liquids, such as concentrated acids, alkalis or salts of some heavy metals. This leads to necrosis of the affected surface. The depth of chemical burns depends on the concentration of acids or alkali and the time of their action (Mountaineer, 1992). Burn leads to coagulative necrosis which does not undergo tissue autolysis but is rejected by demarcation. Insusceptibility of necrotic collagen to skin collagenase causes delayed regeneration, characteristic of burn wounds in comparison with incised wounds, (Kopec-Szlęzak, 1985). Additionally, changes in electrolyte levels at the site of the burn wound and even in the blood serum are also important. The most prominent one is the escape of potassium ions; they become replaced by sodium ions, which results in excessive swelling, enhances inflammatory processes and delays the regeneration. It is a very well-known fact that severe, extensive and deep burns may be the cause of death. Therefore, the consequences of burns causing clinical and metabolic disorders are called “burn disease” (Potocki, 1987).

Proper skin tissue regeneration comprises vascular regeneration, connective regeneration and epithelial regeneration. Regeneration of epidermis after deep burns depends not only on the degree and extent of its destruction but also on the degree of skin damage (Potocki, 1987). Conditions in which vascular regeneration takes place are not fully understood yet. However, the mechanisms of connective and epithelial regeneration are relatively well-known. Two antagonistic hormonal systems: somatropin (STH) with androgen and deoxycorticosterone and corticotropin (ACTH) with glucocorticoids and foliculin play a significant role in it. In addition to the endocrine system, some vitamins are involved in the regeneration process: vitamin B_{12} which supports the action of STH and mineralocorticoids, vitamins A, C, PP, B_2 which facilitate the tissue regeneration processes through their direct effect on collagen, and vitamin E which supports directly the effect of ACTH and glucocorticoids (Dioufa et al., 2010).

AVILIN (Vinylinum, polyvinylbutylic ether) is a light yellow, thick viscous liquid, characterized by specific odour and minimally soluble in water. It has been shown to have anti-inflammatory, antibacterial and tissue-regenerating properties. AVILIN is used in the treatment of wounds and ulcers. It affects epithelization and tissue regeneration. Vinylinum is also used in abscesses, boils, ulcers of digestive tract, abcessed

wounds, inflammatory states of the mammary gland, frostbites and inflammatory diseases, to treat injuries, wounds and burns of various degree (Adam et al., 1968; Bazhenov, 1950; Tikhonova et al., 1970). Internally AVILIN can be used in the treatment of peptic ulcers in the stomach and duodenum, catarrh and colic (Benes, 1960; Kolesov, 1966; Potocki, 1987). The lotion acts protectively on erosions, accelerates regeneration of the mucosa and has some anesthetic and anti-inflammatory properties. It is completely safe after oral administration as its particles are not absorbed from the gastrointestinal tract (Balon, 1977; Vitalyeva et al., 1967).

Essential oils have been widely used in medicine and in production of perfumes and cosmetics for centuries. They are important both in the treatment of different types of wounds and in revitalizing the skin (Lans, 2007; Shetty et al., 2006; Shim et al., 2007; Shukla et al., 1999; Stevenson et al., 2002; Upadhyay et al., 2009a; Upadhyay et al., 2009b; Zhuang et al., 2009). They have the ability to stimulate cellular metabolism and improve the skin structure as they accelerate regenerative processes of the cells (Kamath et al., 2003; Kolesov, 1966). Essential oils improve blood supply to the skin, which makes the skin more flexible. By regulating the secretion of fatty substances they restore skin equilibrium. It has also been demonstrated that essential oils have local anaesthetic and anti-inflammatory properties, which may be very valuable in medicinal practice (Harish et al., 2008; Lans, 2007; Liu et al., 2008).

In the present paper the therapeutic effect of AX/09 containing a mixture of terpene natural oils and AVILIN as *vehiculum* is studied. The antinociceptive, antiedematous and burn wound-healing properties of AX/09 in chemically- or thermally-induced burn models are investigated.

MATERIALS AND METHODS

Chemicals

The reference drugs used – indomethacin and ibuprofen – were provided by Sigma Aldrich (Germany). Polyvinylbutylic ether (AVILIN) was supplied by NES Pharma (Poland); thiopentone sodium was purchased from Werner W. (Germany); 40% hydrobromic acid solution (Hydrobromic acid 40% pure) and 0.9% NaCl solution were provided by Polskie Odczynniki Chemiczne (Polish Chemical Reagents, Poland).

Test equipment

The development of carrageenan-induced paw edema was measured with the use of plethysmometer (Ugo Basile Plethysmometer, Type 7140). In the Randall-Selitto test the pain threshold was measured by means of analgesiameter (Ugo Basile Analgesy Meter, Type 37215).

Animals

The experiments were carried out at the Department of Pharmacodynamics, Jagiellonian University, Medical College in Cracow and at the Department of Pharmaceutical Technology and Biotechnology, Saratov State Medical University, Russia. Adult male Albino Swiss mice weighing 18-30 g, male Albino Wistar rats (180-250 g) and guinea pigs (250-300 g) were used in the experiments. The animals were kept in groups of 15 mice or 3 rats in cages at a room temperature of 22±2° C, under light/dark (12:12) cycle and had free access to food and water before the experiments. Each experimental group consisted of 6-12 animals/dose and each animal was used only once. Before the experimentation the animals were habituated to the vivarium for a minimum of 72 h. The experiments were performed between 8 a.m. and 3 p.m. The procedures were approved by the Local Ethics Committees of the Jagiellonian University in Cracow and Saratov State Medical University, Russia.

Experiments

Anti-inflammatory activity of AX/09 in the carrageenan-induced hind paw edema model

Male albino Wistar rats used in the hind paw edema test were divided into four groups, one of which was the control. To induce inflammation, 0.1 ml of 1% carrageenan solution in water was injected into the subplantar tissue of the hind paw. Prior to this, paw diameters were measu-

red. The development of paw edema was measured plethysmographically. After carrageenan injections, the investigated AX/09 ointment was applied at a constant volume of 0.2 ml by smearing the exact volume of the formulation over the entire surface of the swollen feet, 1, 2, 3 and 23 h after the carrageenan injection. Indomethacin (gel and ointment -Metindol)) and ibuprofen (gel) were used as the reference drugs and they were applied to the swollen rat paw in a way similar to that used for AX/09. Measurements were made six times: for the first time before the injection of carrageenan, at the peak point of swelling, after the third postinjection hour, three times at one-hour intervals from the peak inflammation point, and after 24 h. The degree of inhibition of rat paw edema accumulation, expressed as %, was calculated by the formula:

$$\% = \frac{K - d}{K} \cdot 100$$

K = average increase in the volume of the rat paw in the control group at the appointed hour, d = average increase in the volume of the rat paw after the administration of the investigated compound at the appointed hour (Winter et al., 1962).

Analgesic activity in the Randall-Sellito test

To induce inflammation 0.1 ml of a carrageenan solution in saline was injected subcutaneously into the plantar surface of the left hind paw of the rat. After carrageenan injection, the investigated AX/09 ointment preparation was applied at a constant volume of 0.2 ml by smearing the exact volume of the formulation over the entire surface of the swollen feet, 1, 2, 3 and 23 h after injection of carrageenan. Indomethacin (gel and ointment – Metindol)) and ibuprofen (gel) were used as the reference drugs and they were applied to the swollen rat paw in a way similar to that used for AX/09. After the third hour (i.e. at the top point of swelling), using an analgesia meter, pressure was applied through the tip to the plantar surface of the rat's foot, at a constant rate (expressed in g), to the point at which the animal struggled, squealed or attempted to bite. The increase in the pain threshold (in %) was calculated by subtracting the force applied to the control group from the force applied to the drug group, and dividing the difference by the force applied to the control group. The percentage of analgesia was calculated according to the following formula:

$$\% \text{ analgesia} = (100 \times B)/A - 100$$

Where: A = mean pressure (in g) for the control group; B = mean pressure (in g) for the test group (Randall and Selitto, 1957).

Determination of irritant action of AX/09

Studies were carried on white male guinea pigs. The animals received a standard diet and drinking water *ad libitum*. Experiments were carried out in two groups of animals: the test group (15 AX/09-treated animals) and the control group (10 animals treated with *vehiculum*). Every day for 10 days 0.5 g of AX/09 or *vehiculum* was applied by smearing (not rubbing) it on guinea pig's right side of the body. The reaction was induced on the surface area of 5 x 5 cm under a loose bandage with sterile gauze and cellophane. Skinfold thickness was measured with a caliper (accuracy of 0.05 mm) before and on the last day of the experiment. The results were checked daily for 10 days. The development of erythema and an increase in skinfold thickness in 50% of the tested animals was regarded as a positive result.

Evaluation of allergenic properties of AX/09 – ability to cause delayed contact allergy

The aim of the test was to determine whether the tested preparation can be an antigen that causes a delayed hypersensitivity reaction in normal guinea pigs and to assess whether the substrate preparation causes delayed skin allergy. The study was carried out on white male guinea pigs. The animals were divided into two groups: the control group (10 animals treated with *vehiculum*) and the test group (15 animals treated with AX/09). Every day for 8 days approximately 0.1 g of the investigated AX/09 or *vehiculum* was applied by smearing it on the shaved and degreased with alcohol and ether right side of the guinea pig's skin (surface area 5 x 5 cm) (the induction phase). If there was no response,

then on the 15th day of the experiment, on the left side of the guinea pig's skin, 0.01 g of the evoking dose of the investigated AX/09 or *vehiculum* diluted 10 times was applied (the expression phase). The results which caused a rash, skin changes and an increase in skinfold thickness measured with a caliper 24, 48 and 72 h after applying the evoking dose, were considered as positive.

Chemical burn induced by 40% hydrobromic acid

Contact burns were induced by the use of a stamp pad moistened with 40% hydrobromic acid solution and placed for 2 min on the mouse body being under general anesthesia (thiopentone sodium – 75 mg/kg; intraperitoneal administration). Then, the burnt surface was washed with plenty of 0.9% NaCl and dried with sterile gauze. After the burn had been induced, the animals were divided into three groups:

Group I – animals without any treatment

Group II – animals treated with *vehiculum* (AVILIN)

Group III – animals treated with AX/09

The investigated preparation (AX/09) or *vehiculum* (0.1 g) was applied to the wound once a day until the complete healing was achieved. AX/09 and *vehiculum* were used for the first time 24 h after the burn and then every 24 h for 20 days after washing the wounds with 0.9% NaCl and drying with sterile gauze. The surface area of the burn wound was thoroughly evaluated every 24 h. Furthermore, the burn wound surface area was measured planimetrically on the 4^{th}, 7^{th}, 9^{th}, 12^{th}, 16^{th}, 19^{th} and 22^{nd} day after the burn. Skinfold thickness was measured before the burn and 4, 7, 9, 12, 16, 19 and 22 days after the burn. At the same time the appearance of the burn wound, i.e. the size and appearance of the scab, infiltration, swelling or redness were observed. The wound was considered healed when the defect was completely covered by the newly formed epidermis.

Thermal burns

Contact burns of 2 cm^2 were made in rats under general anaesthesia (thiopentone sodium; 75 mg/kg, intraperitoneal administration) using a heated to 100°C copper tip for 12 s. The animals were divided into 3 groups:

Group I – animals without any treatment (control group)

Group II – animals treated with *vehiculum*

Group III – animals treated with AX/09

0.2 g of AX/09 or *vehiculum* was applied to the wound once a day until the complete healing of the wound was achieved. The surface area of the burn wound was measured planimetrically 5, 7, 9, 13, 15, 19, 21 and 24 days after the burn. Skinfold thickness was measured before and after the burn (i.e. after 5, 7, 9, 13, 15, 19, 21 and 24 days). At the same time the appearance of the burn wound (i.e. the size and appearance of the scab, infiltration, swelling and redness) was observed. The wound was considered healed when the defect was completely covered by the epidermis.

Statistical analysis

Student's t-test was used to determine the significance of differences between the mean values obtained for the control and treatment groups, to evaluate statistically the results concerning the burn wound and skinfold thickness. Differences were considered significant when $p<0.05$.

RESULTS

Analgesic and antiedematous effects in the carrageenan-induced hind paw edema

Polyvinylbutylic ether, AX/09 as well as the reference drugs did not show the antinociceptive effect in the Randall-Selitto test. However, in the carrageenan-induced paw edema test, in the second and third hour of observation, both polyvinylbutylic ether (AVILIN) and AX/09 showed a greater antiedematous effect than indomethacin or ibuprofen. AVILIN showed the strongest antiedematous effect as compared with the reference compounds (Fig. 1).

Evaluation of the irritant effect

The investigated preparation applied topically every 24 h for 10 days did not lead to inflammatory changes in experimental animals. Toxicodermal

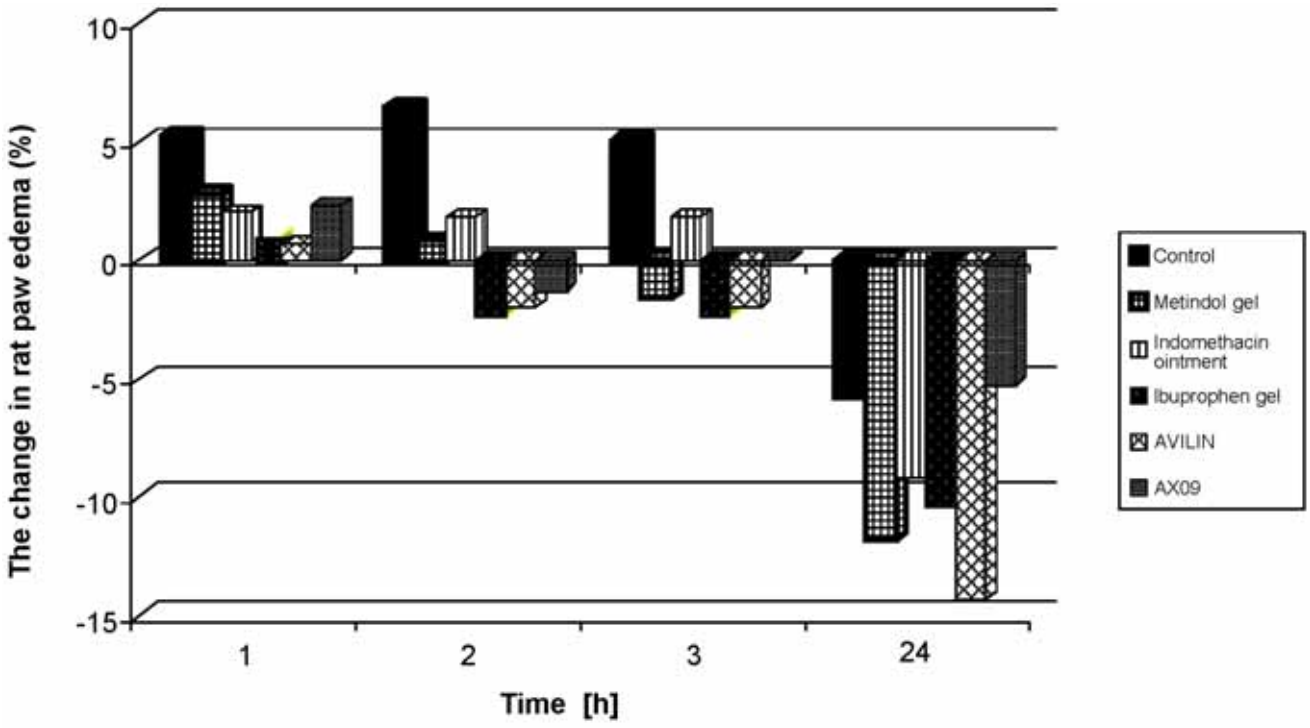

Fig. 1. Antiedematous effects of AX09, AVILIN and the reference drugs on carrageenan-induced paw edema

reactions (erythema, increase in skinfold thickness) after treatment with AX/09 also did not occur after the first application to the skin or after 10 days. The *vehiculum* did not show an irritating effect either.

Evaluation of allergenic properties of AX/09 (ability to cause delayed contact allergy)

In order to determine the ability of AX/09 to induce symptoms of the late-type immune response, the influence on the formation and spreading of erythema and the formation of exudate and swelling of the skin was evaluated. Neither AX/09 nor *vehiculum* demonstrated positive reactions (erythema or swelling) in the animals tested. The control animals also did not react positively.

Chemical burn in mice

In this study the surface area of the burn wound was evaluated and the skinfold thickness was measured planimetrically before and after the burn. Four days after the burn had been induced, the surface area of the wound was: 136.62 mm^2 (control mice), 103.20 mm^2 (*vehiculum*-treated animals), and 80.50 mm^2 (AX/09-treated animals). The results were statistically significant (for AX/09 at $p < 0.01$). After 9 days the wound size in the control group was 86.62 mm^2, in the *vehiculum*-treated group – 46.20 mm^2 and in the mice treated with AX/09 – 32.70 mm^2. After 16 days the wound surface area was: 15.87 mm^2, 5.80 mm^2 and 2.5 mm^2, respectively (Table 1). Comparing the surface area of the wound after 16 days with that after 12 days, the wound size was 4 times smaller in the group treated with AX/09 and approximately 3 times smaller in the *vehiculum*- and control- treated mice (Table 1). Comparing in turn the surface area of the wound after 16 days with that after 4 days, the wound size was 30 times smaller in the mice treated with AX/09, nearly 18 times smaller in the animals treated with *vehiculum* and nearly 9 times smaller in the control group. The increase in skinfold thickness measured 4 days after the burn was 1.98

TABLE 1. The influence of AX/09 and *vehiculum* (AVILIN) on the average surface area (in mm^2) of chemically-induced burn wounds in mice

Day of measurement	CONTROL	average	AVILIN	average	AX/09	average
4	179 133 135 160 126 152 102 106	**136.625**	145 75 105 79 50 140 110 83 155 90	**103.2**[a]	80 75 73 67 110 81 72 66 75 106	**80.5**[c]
7	153 110 125 150 113 104 82 85	**115.25**	105 50 55 55 50 140 80 65 92 50	**74.2**[b]	30 55 50 25 75 40 42 30 60 100	**50.7**[c]
9	143 76 107 97 90 81 58 41	**86.625**	67 25 25 44 40 90 45 50 45 31	**46.2**[c]	15 25 25 12 50 25 35 25 40 75	**32.7**[c]
12	88 50 59 42 50 29 25 17	**45.0**	20 15 0 20 20 40 7 15 18 10	**16.5**[c]	0 15 7 0 15 10 0 0 10 41	**9.8**[c]
16	65 25 13 0 15 0 0 9	**15.875**	0 0 0 0 23 35 0 0 0	**5.8**[c]	0 0 0 0 0 0 0 0 25	**2.5**[c]
19	35 13 0 0 10 0 0	**7.25**	0 0 0 0 0 20 0	**2.0**[c]	0 0 0 0 0 0 0	**0**[c]
22	0 0 0 0 0 0 0	**0**	0 0 0 0 0 0 0	**0**	0 0 0 0 0 0 0	**0**

The burn wound induced by the use of 40% hydrobromic acid solution
Significant difference (Student's t-test) compared with the vehicle-treated group: [a]$p < 0.05$;
[b]$p < 0.01$; [c]$p < 0.001$

mm in the control group, 1.5 mm in the vehicle-treated group, and 1.33 mm in the group treated with AX/09. The results were statistically significant ($p < 0.01$). After 9 days, in the case of mice, the increase in skinfold thickness was: in the control animals – 1.75 mm; in the vehicle-treated ones – 1.06 mm, and the in AX/09-treated mice – 0.86 mm. These results were not statistically significant. After 16 days the increase in skinfold thickness was: in the control group – 0.962 mm, in the vehicle-treated group – 0.67 mm, and in the group treated with AX/09 – 0.32 mm (Tables 2, 2A, 2B).

After 4 days in most control animals the wounds were open, oozing, only in a few cases covered with a dry scab. The same effect was observed in the vehicle-treated group. In contrast, in animals treated with AX/09, wounds were mostly dry and covered with thin scabs over the entire surface. In this group an early stage of the granulation process could be observed and it was demonstrated by the presence of the "epidermal seam" (i.e. a red stripe at the border of the skin and the granulation tissue). On the 10th day in the control group the wounds were mostly covered with dark, semi-protruding scabs. In the group treated with the vehicle, in half of the animals the scabs fell off. In the vast majority of animals treated with AX/09 no scabs were observed, moreover, the early skin formation process had begun. On the 17th day the wounds were completely healed and covered by the newly formed epidermis in all the mice treated with AX/09, in 80% of the animals in the vehicle- treated group and in 50% of the animals in the control group (Table 3). The complete healing of the wound in 100% of the control animals was found on the 23rd day of observation.

Thermal burn in rats

After 5 days the burn wound surface area in control rats was 153.4 mm^2, in the vehicle-treated group – 155.4 mm^2, and in the rats treated with AX/09 – 154.8 mm^2 (Table 4). After 13 days the burn wound surface area in the control group was 58.8 mm^2, and in the vehicle-treated group – 52.4 mm^2. In the group treated with AX/09 the burn wound size was the same as that in the vehicle-treated group (Table 4). Nineteen days after the burn the wound surface area in the control, vehicle-treated and AX/09-treated groups was 9.2 mm^2, 3.4 mm^2 and 2.0 mm^2, respectively (Table 4). Comparing the surface area of the wound 19 days after the burn with that after 5 days, it was approximately 16 times smaller in the control group, approximately 45 times smaller in the *vehiculum*-treated group and almost 80 times smaller in the group treated with AX/09 (Table 4). Skinfold thickness measured 5 days after the burn was 5.26 mm in the control group, 5.88 mm in the vehicle-treated group, and 5.06 mm in the group treated with AX/09 (Table 5, 5A, 5B). After 13 days the skinfold thickness in the control-, vehicle- and AX/09- treated groups was 4.06 mm, 3.04 mm and 2.74 mm, respectively. After 19 days, the skinfold thickness was 2.2 mm in the control group, 1.36 mm in the vehicle-treated group and 1.26 mm in the AX/09–treated group (Table 5, 5A, 5B). Five days after the burn the wounds in the majority of animals in all the groups were dry, but in some cases they were open and oozing. On the 9th day of observation the wounds in the control group were dry and sealed, covered with dark scabs. In the animals treated with the *vehiculum* and AX/09 the wounds were dry and the scabs partly fell off. Moreover, there was clearly visible red granulation tissue. After 15 days the wounds in the control group were covered with thin scabs. The same effect was observed in the *vehiculum*-treated group. In this group the newly formed epidermis was noticeable. In animals treated with AX/09 an advanced stage or even the completion of the granulation process was observed. On the 17th day after the burn the wounds in the control group and in the *vehiculum*-treated group were still covered with small scabs. In some animals there was epidermis, which was demonstrated by the presence of the "epidermal seam". In animals treated with AX/09 the wounds were without scabs. The process of wound healing had begun. After 21 days the wounds in the control group and the *vehiculum*-treated group were covered by epidermis and in 50% of the animals the healing process could be observed. In animals treated with AX/09 the wounds were completely covered by epidermis and the process of reparation was already finished. In the *vehiculum*-treated group and the control group the reparation process was completed after 22 and after 24 days, respectively (Table 6).

TABLE 2. The increase in skinfold thickness (Δl) in control mice (Chemical burn - HBr)

	l_0	l_1	Δl_1	l_4	Δl_4	l_7	Δl_7	l_9	Δl_9	l_{12}	Δl_{12}	l_{16}	Δl_{16}	l_{19}	Δl_{19}	l_{22}	Δl_{22}
1.	**5**	12	**7**	25	**20**	23	**18**	23	**18**	21	**16**	19	**14**	13	**8**	8	3
2.	**4**	12	**8**	35	**31**	28	**24**	28	**24**	20	**16**	17	**13**	12	**8**	6	**2**
3.	**4**	12	**8**	23	**19**	23	**19**	23	**19**	21	**17**	19	**15**	10	**6**	6	**2**
4.	**6**	13	**7**	23	**17**	24	**18**	22	**16**	20	**14**	15	**11**	10	**4**	7	**1**
5.	**4**	10	**6**	25	**21**	24	**20**	23	**19**	20	**16**	14	**10**	10	**6**	6	**2**
6.	**7**	13	**6**	25	**18**	25	**18**	22	**15**	17	**10**	10	**3**	10	**3**	9	**2**
7.	**6**	13	**7**	21	**15**	20	**14**	20	**14**	15	**9**	10	**4**	8	**2**	7	**1**
8.	**5**	11	**6**	22	**17**	22	**17**	20	**15**	17	**12**	12	**7**	8	**3**	5	**0**
Average			**6.875**		**19.75**		**18.5**		**17.5**		**13.75**		**9.62**		**5.0**		**1.625**
Average in mm			**0.6875**		**1.975**		**1.85**		**1.75**		**1.375**		**0.962**		**0.5**		**0.162**

TABLE 2A. The increase in skinfold thickness (Δl) in the *vehiculum*-treated mice (Chemical burn – HBr)

	l_0	l_1	Δl_1	l_4	Δl_4	l_7	Δl_7	l_9	Δl_9	l_{12}	Δl_{12}	l_{16}	Δl_{16}	l_{19}	Δl_{19}	l_{22}	Δl_{22}
1.	**5**	12	**7**	20	**15**	18	**13**	18	**13**	15	**10**	13	**8**	9	**4**	7	**2**
2.	**4**	10	**6**	23	**19**	21	**17**	20	**16**	13	**9**	10	**6**	8	**4**	6	**2**
3.	**3**	12	**9**	14	**11**	14	**11**	12	**9**	11	**8**	9	**6**	6	**3**	4	**1**
4.	**4**	11	**7**	18	**14**	20	**16**	18	**14**	16	**12**	10	**6**	6	**2**	4	**0**
5.	**5**	12	**7**	21	**16**	19	**14**	17	**12**	16	**11**	13	**8**	7	**2**	6	**1**
6.	**4**	8	**4**	22	**18**	22	**18**	22	**18**	19	**15**	15	**11**	12	**8**	6	**2**
7.	**4**	12	**8**	17	**13**	16	**12**	16	**12**	14	**10**	10	**6**	7	**3**	4	**0**
8.	**4**	11	**7**	19	**15**	17	**13**	11	**7**	10	**6**	8	**4**	6	**2**	4	**0**
9.	**5**	14	**9**	17	**12**	18	**13**	17	**12**	15	**10**	10	**5**	7	**2**	6	**1**
10.	**3**	9	**6**	20	**17**	19	**16**	19	**16**	15	**12**	10	**7**	5	**2**	5	**2**
Average			**7.0**		**15**		**14.3**		**10.6**		**10.3**		**6.7**		**3.2**		**1.1**
Average in mm			**0.7**		**1.5**[a]		**1.4**[a]		**1.1**[a]		**1.0**		**0.7**		**0.3**[a]		**0.11**

Significant difference (Student's t-test) compared with the vehicle-treated group: [a]$p < 0.05$

TABLE 2B. The increase in skinfold thickness (Δl) in mice treated with AX/09 (Chemical burn – HBr)

	l_0	l_1	Δl_1	l_4	Δl_4	l_7	Δl_7	l_9	Δl_9	l_{12}	Δl_{12}	l_{16}	Δl_{16}	l_{19}	Δl_{19}	l_{22}	Δl_{22}
1.	**5**	10	**5**	15	**10**	11	**6**	8	**3**	7	**2**	7	**2**	6	**1**	5	**0**
2.	**3**	10	**7**	18	**15**	16	**13**	14	**11**	11	**8**	5	**2**	6	**3**	4	**1**
3.	**6**	12	**6**	16	**10**	14	**12**	11	**5**	8	**2**	7	**1**	8	**2**	8	**2**
4.	**4**	10	**6**	18	**14**	15	**11**	12	**8**	8	**4**	6	**2**	6	**2**	5	**1**
5.	**3**	13	**10**	29	**26**	25	**22**	20	**17**	16	**13**	10	**7**	6	**3**	4	**1**
6.	**3**	9	**6**	14	**11**	14	**11**	11	**8**	10	**7**	7	**4**	4	**1**	3	**0**
7.	**4**	10	**6**	13	**9**	13	**9**	13	**9**	8	**4**	7	**3**	6	**2**	5	**1**
8.	**6**	12	**6**	20	**14**	18	**12**	10	**4**	9	**3**	8	**2**	7	**1**	6	**0**
9.	**5**	13	**8**	16	**11**	14	**11**	13	**8**	11	**6**	7	**2**	7	**2**	6	**1**
10.	**4**	12	**8**	17	**13**	16	**12**	17	**13**	16	**12**	11	**7**	6	**2**	4	**0**
Average			**6.8**		**13.3**		**11.9**		**8.6**		**6.1**		**3.2**		**1.9**		**0.7**
Average in mm			**0.68**		**1.33**[a]		**1.19**[b]		**0.86**[a]		**0.61**[c]		**0.32**[c]		**0.19**[c]		**0.07**[c]

Significant difference (Student's t-test) compared with the vehicle-treated group: [a]p< 0.05; [b]p< 0.01; [c]p< 0.001

TABLE 3. The influence of AX/09 and AVILIN on the time needed for burn wounds to heal completely as compared with the control group (Chemical burn – HBr)

	10 days	11 days	12 days	13 days	14 days	15 days	16 days	17 days	18 days	19 days	20 days	21 days	22 days	23 days
CONTROL	0/8	0/8	0/8	0/8	1/8	1/8	3/8	4/8	4/8	5/8	6/8	6/8	7/8	**8/8**
AVILIN	0/10	0/10	1/10	4/10	6/10	6/10	8/10	8/10	8/10	9/10	**10/10**			
AX/09	1/10	2/10	4/10	5/10	8/10	8/10	9/10	**10/10**						

x/y – the number of animals with the completed healing process per number of all animals in the group

TABLE 4. The influence of AX/09 and *vehiculum* (AVILIN) on the average surface area (in mm^2) of temperature-induced burn wounds in rats as compared with the control group

Day of measurement	Control	average	AVILIN	average	AX/09	average
5	180 187 137 146 117	**153.4**	137 167 159 156 158	**155.4**	157 167 134 159 157	**154.8**
7	132 125 117 132 94	**120.0**	137 127 113 121 123	**124.2**	125 105 128 110 121	**117.8**
9	100 105 98 125 88	**103.2**	118 108 98 102 108	**106.8**	98 95 118 93 115	**103.8**
13	50 51 52 97 44	**58.8**	51 58 54 48 51	**52.4**	45 61 55 40 61	**52.4**
15	30 41 51 57 19	**39.6**	21 18 34 13 19	**21.0**[a]	10 6 13 8 20	**11.4**[c]
19	11 7 11 11 6	**9.2**	5 0 0 7 5	**3.4**[c]	0 0 0 10 0	**2.0**[c]
21	6 0 0 8	**2.8**	3 0 0 5	**1.6**[a]	0 0 0 0	**0**[c]
24	0 0 0 0	**0**	0 0 0 0	**0**	0 0 0 0	**0**

Significant difference (Student's t-test) compared with the vehicle-treated group:
[a]$p < 0.05$; [c]$p < 0.001$

TABLE 5. The increase in skinfold thickness (Δl) in the control rats (Thermal burn)

	l_0	Day 5	Δl_5	Day 7	Δl_7	Day 9	Δl_9	Day 13	Δl_{13}	Day 15	Δl_{15}	Day 19	Δl_{19}	Day 21	Δl_{21}	Day 24	Δl_{24}
1.	**11**	65	**54**	82	**71**	74	**63**	40	**29**	34	**23**	29	**18**	20	**9**	16	**5**
2.	**11**	62	**51**	90	**79**	69	**58**	50	**39**	47	**36**	30	**19**	23	**12**	14	**3**
3.	**13**	68	**55**	72	**57**	80	**67**	64	**51**	61	**48**	40	**27**	25	**12**	20	**7**
4.	**12**	67	**55**	67	**55**	72	**60**	68	**56**	57	**45**	40	**28**	30	**18**	18	**6**
5.	12	60	**48**	68	**56**	74	**62**	40	**28**	35	**23**	30	**18**	22	**10**	17	**5**
Average			**52.6**		**63.6**		**62.0**		**40.6**		**35.0**		**22.0**		**12.2**		**5.2**
Average in mm			**5.26**		**6.36**		**6.2**		**4.06**		**3.5**		**2.2**		**1.22**		**0.52**

TABLE 5A. The increase in skinfold thickness (Δl) in rats treated with *vehiculum* (Thermal burn)

	l_0	Day 5	Δl_5	Day 7	Δl_7	Day 9	Δl_9	Day 13	Δl_{13}	Day 15	Δl_{15}	Day 19	Δl_{19}	Day 21	Δl_{21}	Day 24	Δl_{24}
1.	**9**	67	**58**	80	**71**	67	**58**	45	**36**	34	**25**	30	**21**	28	**19**	15	**6**
2.	**10**	75	**65**	68	**59**	52	**42**	36	**26**	28	**18**	24	**14**	20	**10**	14	**4**
3.	**11**	69	**58**	74	**63**	64	**53**	40	**29**	34	**23**	20	**9**	17	**6**	14	**3**
4.	**10**	66	**56**	50	**40**	57	**47**	40	**30**	27	**17**	24	**14**	20	**10**	15	**5**
5.	**10**	67	**27**	59	**49**	58	**48**	41	**31**	31	**21**	20	**10**	18	**8**	13	**3**
Average			**58.8**		**56.4**		**49.6**		**30.4**		**20.8**		**13.6**		**10.6**		**4.2**
Average in mm			**5.88**		**5.64**		**4.96**[a]		**3.04**		**2.08**[a]		**1.36**		**1.06**		**0.42**

Significant difference (Student's t-test) compared with the vehicle-treated group: [a]$p < 0.05$

TABLE 5B. The increase in skinfold thickness (Δl) in rats treated with AX/09 (Thermal burn)

	l_0	Day 5	Δl_5	Day 7	Δl_7	Day 9	Δl_9	Day 13	Δl_{13}	Day 15	Δl_{15}	Day 19	Δl_{19}	Day 21	Δl_{21}	Day 24	Δl_{24}
1.	**9**	46	**37**	54	**45**	52	**43**	35	**26**	27	**18**	22	**13**	17	**8**	12	**3**
2.	**12**	66	**54**	52	**40**	50	**38**	41	**29**	34	**22**	23	**11**	21	**9**	16	**4**
3.	**12**	59	**47**	62	**50**	60	**48**	38	**26**	25	**13**	20	**8**	19	**7**	17	**5**
4.	**10**	66	**56**	59	**49**	52	**42**	41	**31**	32	**22**	27	**17**	20	**10**	16	**6**
5.	**10**	69	**59**	64	**54**	38	**28**	35	**25**	27	**17**	24	**14**	19	**9**	13	**3**
Average			**50.6**		**47.6**		**39.8**		**27.4**		**18.4**		**12.6**		**8.6**		**4.2**
Average in mm			**5.06**		**4.76**[a]		**3.98**[a]		**2.74**[c]		**1.84**[c]		**1.26**[c]		**0.86**[c]		**0.42**

Significant difference (Student's t-test) compared with the vehicle-treated group: [a]$p < 0.05$; [c]$p < 0.001$

TABLE 6. The influence of AX/09 and *vehiculum* (AVILIN) on the time needed for burn wounds to heal completely as compared with the control rats (Thermal burn)

	Day 17	Day 18	Day 19	Day 20	Day 21	Day 22	Day 23	Day 24
CONTROL	0/5	0/5	0/5	1/5	3/5	3/5	4/5	**5/5**
AVILIN	0/5	1/5	2/5	3/5	3/5	**5/5**		
AX/09	1/5	3/5	4/5	4/5	**5/5**			

x/y – the number of animals with the completed healing process per number of all animals in the group

DISCUSSION

The aim of this study was to analyze the burn wound healing process and the influence of AX/09 preparation on it. The comparison of pharmacological properties of AX/09 and *vehiculum* (AVILIN) would provide data to clarify the therapeutic value of AX/09. The present study demonstrated that the investigated AX/09 administered locally showed high antiedematous activity in the carrageenan test (Table 1). Anti-inflammatory and analgesic activities of the investigated AVILIN and AX/09 were more satisfactory than those of the reference compounds. To determine the stage of the wound repair process a few methods were used. The wound surface was measured planimetrically. The development of the granulation tissue and its transformation into young skin, the presence of infiltrates and exudates within the damaged skin were measured micrometrically by skinfold thickness. Planimetric observations of the surface area of experimental animal wounds and micrometric measurements of effusions and infiltrates showed that the healing process of the chemically-induced burn wound was more balanced and much faster after treatment with AX/09. The healing process in the control- and *vehiculum*-treated groups was much slower as compared with the AX/09-treated group, with a clear delay in the development of the granulation tissue and formation of young epidermis. The process of granulation and epithelialization progressing from the edges of the wound was more advanced in the skin of animals treated with AX/09, with highly significant regression of wound inflammation manifested by faint effusion. Elimination of the necrotic tissue (scabs), especially in chemical burns, was a result of the proliferation of the granulation tissue and its transformation into epidermis, and it was most intense in animals treated with AX/09. It should be emphasized that in chemically-induced burn wounds in animals treated with AX/09 the whole granulation tissue was transformed into young epidermis after 17 days. In contrast, in the control group and in the group treated with *vehiculum* such epithelial cells occurred rarely or in small numbers. Presumably, the reasons for the above effects are better conditions for wound healing caused by interaction of the wound with the AX/09 preparation. A similar effect, i.e. fully healed chemical wounds, in the control group and the group treated with AVILIN was observed after 23 and 20 days, respectively.

Much less convincing results were observed in the case of animals treated with AX/09 in thermally-induced burn models. It concerns mainly the first days after the burn during which AX/09 did not show any therapeutic effect. Therapeutic effects – rapidly progressing granulation and epithelialization with disappearing effusion could be observed after 17 days of treatment. However, after 21 days in animals treated with AX/09 complete wound closure by the newly formed epidermis was observed; simultaneously, dermis reconstruction in the burn area took place. In the group treated with *vehiculum* a similar effect was observed after 22 days, and in the control group after 24 days.

In clinical practice, for drugs used topically it is important that they have no toxicodermal or allergenic effects, especially anaphylactic or contact type effects. Experiments conducted on guinea pigs showed that AX/09, as well as the *vehiculum*, did not cause irritation or inflammation of the skin. There were no toxic skin reactions after the first contact with the skin, as well as after multiple applications of AX/09. Taking into consideration the fact that the guinea pig skin is commonly used for the determination of toxic or allergenic proper-

ties, the investigated AX/09 can be regarded as safe for the skin. As the reactivity of human skin is similar to the reactivity of guinea pig skin, it can be assumed that the investigated preparation will not cause toxic, irritant or allergenic reactions in humans. AX/09 did not cause a delayed-type allergic reaction which, contrary to the toxic effects, can occur after some time and at a distant site, even far from the site of antigen application. 2,4-dinitrochlorobenzen (DNCB), used for comparison purposes, caused severe erythema, edema and effusion characteristic of contact hypersensitivity.

CONCLUSIONS

In the present experiment it was demonstrated that AX/09 modifies and normalizes the process of burn wound healing. There were no toxicodermal reactions, neither after the first contact with the skin nor after 10 days of treatment with AX/09. The *vehiculum* that was used did not show any irritating effects either. Moreover, the skin test with *vehiculum* and AX/09 did not reveal any propensity to cause contact allergy in any of the animals tested.

In chemical burns AX/09 caused termination of the wound healing process, i.e. the complete closure of the wound by the newly formed epidermis, after 17 days. In the control group the same effect was observed after 23 days. In thermal burns AX/09 enhanced the granulation and new skin formation on the surface of the wound as compared with the control group. AX/09 considerably reduced inflammation and effusion within the wound, which was manifested by a faster decline in skinfold thickness measured micrometrically. The efficacy of AX/09 and its influence on the burn wound healing process, particularly in chemical models, seems to be of special value for the clinical, especially dermatological, practice.

REFERENCES

Adam, E., I. Mezei, and O. Butsich. 1968. Treatment of trophic ulcers of the leg with Shostakovskii's balm. *Vestn. Dermatol. Venerol.* 42: 69-70.

Atiyeh, B. S., S. A. Dibo, and S. N. Hayek. 2009. Wound cleansing, topical antiseptics and wound healing. *Int. Wound J.* 6(6): 420-430.

Balon, L.R. 1977. Use of Mixtures Shostakovskii's lotion in the treatment of several pathologic states of the soft tissues of the oral cavity. *Dentistry* (Mosk) 56: 32–34.

Bazhenov, A. P. 1950. Treatment of wounds with Shostakovskii's synthetic balsam (vinilin). *Khirurgiia* (Mosk) 6: 11–16.

Benes, I. 1960. Shostakovskii's Lotion – a new wide-spectrum drug. *Cas Lek Ceský* 99: 25–27.

Dioufa, N., A. V. Schally, I. Chatzistamou, E. Moustou, N. L. Block, G. K. Owens, A. G. Papavassiliou, and H. Kiaris. 2010. Acceleration of wound healing by growth hormone-releasing hormone and its agonists. *Proc. Natl. Acad. Sci.*107(43): 18611-18615.

Harish, B. G., V. Krishna, H. S. Santosh-Kumar, B. M. Khadeer-Ahamed, R. Sharath, and H. M. Kumara Swamy. 2008. Wound healing activity and docking of glycogen-synthase-kinase-3-beta-protein with isolated triterpenoid lupeol in rats. *Phytomedicine* 15: 763-767.

Jablonska, S., and T. Chorzelski. 1997. *Diseases of the skin.* PZWL, ed. IV.

Kamath, J. V., A. C. Rana, and A. R. Chowdhury. 2003. Pro-healing effect of Cinnamomum zeylanicum bark. *Phytother. Res.* 17: 970-972.

Kolesov, A. P. 1966. Treatment of cervical erosion of trichomonal etiology with Shostakovskii's balm. *Sov. Med.* 29: 123–125.

Kopec-Szlęzak, J. 1985. Regeneration of epidermis after third-degree burns in Wistar rats. Publisher SGGW-AR.

Lans, C. 2007. Comparison of plants used for skin and stomach problems in Trinidad and Tobago with Asian ethnomedicine. *J. Ethnobiol. Ethnomed.* 3: 1-12.

Liu, M., Y. Dai, Y. Li, Y. Luo, F. Huang, Z. Gong, and Q. Meng. 2008. Madecassoside isolated from Centella asiatica herbs facilitates burn wound healing in mice. *Planta Med.* 74: 809–815.

Mountaineer, D. 1992. *Outline of surgery*. PZWL, ed. II.

Potocki, J. L. 1987. *Nursing burns*. PZWL.

Randal, L. O., and J. J. Selitto. 1957. A method for measurement of analgesic activity on inflamed tissue. *Arch. Int. Pharmacodyn. Ther.* 111: 409-419.

Shetty, S., S. L. Udupa, A. L. Udupa, and V. R. Vollala. 2006. Wound healing activities of bark extract of Jatropha curcas Linn in albino rats. *Saudi Med. J.* 27: 1473–1476.

Shim, K. M., S. H. Choi, M. J. Jeong, and S. S. Kang. 2007. Effects of aucubin on the healing of oral wounds. *In Vivo* 21: 1037–1041.

Shukla, A., A. M. Rasika, G. K. Jain, R. Shankar, D. K. Kulshrestha, and B. N. Dhawan. 1999. *In vitro and in vivo* wound healing activity of asiaticoside isolated from Centella asiatica. *J. Ethnopharmacol.* 65: 1-11.

Stevenson, P. C., M. S. Simmonds, J. Sampson, P. J. Houghton, and P. Grice. 2002. Wound healing activity of acylated iridoid glycosides from Scrophularia nodosa. *Phytother. Res.* 16: 33–35.

Tikhonova, L. N., V. N. Tikhonova, and A. F. Kirsanov. 1970. Treatment of peptic ulcer in children with Shostakovskii's lotion and catamnesis. *Vopr. Okh.r Materin. Det.* 15: 91-96.

Upadhyay, N. K., R. Kumar, S. K. Mandotra, R. N. Meena, M. S. Siddiqui, R. C. Sawhney, and A. Gupta. 2009a. Safety and efficacy of healing Sea buckthorn (Hippophae rhamnoides L.) seed oil on burn wounds in rats. *Food Chem. Toxicol.* 47: 1146– 1153.

Upadhyay, N. K., R. Kumar, M. S Siddiqui, and A. Gupta. 2009b. Mechanism of wound-healing activity of Hippophae rhamnoides leaf extract in experimental burns. *Evid. Based Complement Alternate Med.*

Winter, C. A., E. A. Risley, and G. W. Nuss. 1962. Carrageenin-induced edema in the hind paw of the rat as an assay for anti-inflammatory drugs. *Proc. Soc. Exp. Biol. Med.* 11 1: 544-547.

Vitalyeva, G. M., R. I. Brodskii, V. L. Zelenskaia, and E. G. Peshkova. 1967. Complex treatment of periodontosis with the use of biomycin paste with Shostakovskii's balm. *Dentistry* (Mosk) 46: 89–90.

Zhuang, S. R., S. L. Chen, J. H. Tsai, C. C. Huang, T. C. Wu, W. A. S. Liu, H. C. Tseng, H. S. Lee, M. C. Huang, G. T. Shane, C. H. Yang, Y. C. Shen, Y. Y. Yan, and C. K.. 2009. Effect of citronellol and the Chinese medical herb complex on cellular immunity of cancer patients receiving chemotherapy/radiotherapy. *Phytother. Res.* 23: 785–790.