Polysaccharides of Mushroom *Phallus impudicus* Mycelium: Immunomodulating and Wound Healing Properties

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Abstract: Mushroom *Phallus impudicus* is traditionally used in Asia and Northern Europe as a remedy of folk medicine. This review summarizes our own results on the artificial cultivation of the *P. impudicus* mycelium and some data on the study of the biological activity of polysaccharides isolated from this mycelium. The cultural-morphological and physiological-biochemical properties of the mycelium of the new fungal strain of *P. impudicus* were studied. The optimization of the nutrient medium composition and cultural conditions enabled to increase the biomass yield by 1.3-fold, and the contents of polysaccharides by 1.5 to 1.7-fold. The polysaccharides isolated from this mycelium possessed immunomodulating properties *in vitro* as well as *in vivo* in experimental rats with streptozotocin-induced diabetes. The ointment containing 10% *P. impudicus* polysaccharides enhanced cutaneous wound healing in experimental rats, accelerating epithelialization, contraction and growth of granulation tissue as compared to the ointment base. Taken together, polysaccharides of *P. impudicus* mycelium are promising raw material for the development of functional food as well as new therapeutic and prophylactic drugs.

Key words: polysaccharides; mycelium; *P. impudicus*; immunomodulation; wound healing

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白鬼笔蘑菇菌丝体多糖：免疫调节和伤口愈合特性

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摘要：白鬼笔蘑菇在亚洲和北欧是一种传统的民间医药。本文综述了我们人工培养白鬼笔菌丝体的研究成果，以及从白鬼笔菌丝体中分离出的多糖的生物活性数据。对白鬼笔新菌株菌丝体的培养形态特征和生理生化特性进行了研究。经过优化培养基的营养物构成和培养条件能够使菌体生物量提高 1.3 倍，多糖含量提高 1.5~1.7 倍。从该菌丝体中分离的多糖在体外和在体内（链脲佐菌素诱导的糖尿病大鼠）均具有免疫调节作用。与原始软膏相比，含有 10%白鬼笔多糖的软膏，能促进实验大鼠的皮肤创面愈合，促进肉芽组织的上皮形成、收缩和生长。综上所述，白鬼笔菌丝体多糖有望成为开发功能性食品以及新型治疗和预防药物的原材料。

关键词：多糖；菌丝体；白鬼笔；免疫调节；伤口愈合

In countries of Southeast Asia (China, Japan, Korea and others), Basidiomyceta fungi (higher fungi) are considered not only as the most important source of food and feed protein, but also as valuable raw materials for production of biologically active substances used in creation of various therapeutic and prophylactic agents of a wide spectrum of action[1].

The biological effect of most medicinal mushrooms is largely determined by polysaccharides. The oncostatic effect of polysaccharides isolated from fruit bodies of some basidiomycetes was established, which led to active study of these compounds, as well as search for their producers[2]. Mushroom polysaccharides also have hepatoprotective, antioxidant, chemo- and radioprotective, antimicrobial, antiviral, lipid-lowering and other effects. At the same time, higher basidiomycetes are not widely used as producers of polysaccharides although some of them have low toxicity, immunostimulating and antitumor activities, low toxicity and are used in countries

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of South-East Asia for treatment of certain oncological and chronic infectious diseases in immunodeficiencies. The most important commercially available polysaccharides are lentinan, isolated from *Lentinus edodes*, schizophillan from *Schizophillum commune*, PSK and PSP from *Tinea versicolor* and griffron-D from *Grifola frondosa*[3]. Polysaccharides with oncostatic effect, isolated from fungi, are either water-soluble β-D-glucans, β-D-glycans with heterosaccharide chains of xylose, mannose, galactose and uronic acid, or α-D-glucan-protein complexes-proteoglycans. Less commonly, oncostatic properties are found in glycans with the α-glycosidic bond. As a rule, protein-bound glucans have a higher immunostimulating effect than the corresponding glycans[3].

Most of immunomodulatory and antitumor polysaccharides are glucans having β-(1→3) -glycosidic bonds in the main chain and β-(1→6) in the branches. The difference in biological activity is associated with the size of molecules, solubility in water, the degree of branching, and the presence of a corresponding spatial structure (three-chain right-handed spiral). Fungi contain polysaccharide-protein complexes or glycoproteins which are represented by polypeptide chains or small proteins with which the polysaccharide chains of β-D-glucan are tightly bound[4].

The antitumor effect of polysaccharides is based on increasing immunity. Pharmacologically they are classified as biological response modifiers. The mechanism of the cancerostatic action is explained by activation of the immune system:β-D-glucan binds to the surface layer of a lymphocyte or a specific serum protein that activates macrophages, T-, NK-cells and other effector cells. This leads to an increase in the production of antibodies, interleukins (IL-1 and IL-2) and interferon[5]. The activation of the immune system by β-1,3-glucan is non-specific, which allows the use of β-glucan both as a preventive measure and as an auxiliary drug for various diseases accompanied by a general decrease in immunity.

One of the most interesting and little-studied mushrooms is a common stinkhorn (*P. impudicus*) traditionally used in folk medicine in Asia and Northern Europe. The literature contains few data on the biochemical composition of *P. impudicus*. The carbohydrate composition of the mycelium has been studied, with the main component being glucose (47.0%)[6]. Significant amounts of mannose (24.0%) and galactose (18.4%) have also been found. *P. impudicus* has been shown to form polysaccharide Pl-2 (glucomannan)[3]. Fifty-nine different compounds were identified among which predominated dimethyl trisulfide, cis- and trans-β-ocimenes, 2-phenyl acetaldehyde and 2-phenyl ethanol[7].

P. impudicus has antitumor, antioxidant and immunostimulating properties[8-11]. Some studies indicate the active ingredient polysaccharide nature of *P. impudicus*, others evaluate the pharmacologic activity of its extracts. The administration of *P. impudicus* extract inhibited the rate of tumor growth, reduced tumor volumes and increased the efficacy of cyclophosphan -containing cytostatic therapy in mice with Ehrlich adenocarcinoma[8]. Additionally, these authors suggested that this extract having antioxidant properties[9] significantly increased the survival rate and improved the general condition of mice subjected to total single γ-irradiation at a dose of 7 Gy. The extract of *P. impudicus* inhibited intravascular platelet aggregation as well as antiaggregative and anticoagulative activities of the vascular wall in breast cancer patients after chemotherapy and hormonal treatment[10]. Some data suggested immunomodulating activity of this mushroom. An aqueous extract of *P. impudicus* showed an impaired immune response and caused overexpression of IL-4 and TNFα in splenocyte cell culture[11].

P. impudicus is rarely found in nature and production of mycelium biomass of this mushroom using biotechnological methods is relevant. Investigation of the physiological and biochemical characteristics of *P. impudicus* under artificial cultivation will allow further development of biotechnology for production of biomass and polysaccharides to create new therapeutic and prophylactic drugs.

1 Studies of *Phallus impudicus* growth under varying cultivation conditions

Since the resourses of fungus *P. impudicus* are limited, the relevant objective was to obtain mycelium biomass using biotechnology techniques. We carried out research into growth of new strains of the mashroom *P. impudicus* under varying cultivation conditions[12,13]. We used strains of *P. impudicus* isolated from fruit bodies gathered in forests of Grodno and Minsk regions (Belarus). The culture and morphological properties of the mushroom strains were studied using agar-containing media. The surface mycelium was obtained when the mushroom was grown in liquid nutritional media in Erlenmeyer flasks under steady-state conditions. The deep mycelium was grown under conditions of mixing using a shake-flask propagator (120 r/min). In agar media, the strains produced white colonies with concentric circles. The edge of the colonies was uneven, ascending and fimbriate. The airy mycelium was high, cottony and fluffy. The colony reversum was uncolored. The mycelium had a weak mushroom smell. Despite the colonies being high and dense, their growing up to the brim of a Petri dish was observed as late as after 50 days of cultivation. The linear growth rate was 0.46~1.2 mm/day and the growth coefficient (GK) was 6.0~10.8. The colonies of the *P. impudicus* strains studied grew faster in glucose peptone agar compared to beer wort.

The most active growth was shown by strains 4 and 5, with the maximum linear growth rate being 0.46~1.2 mm/day, the growth coefficient was 10.8. The microscopic examination of the vegetative mycelium of the *P. impudicus* strains showed septate, branched, interwoven in different directions hyphae as well as merging of the hyphae occurring with the help of anastomoses. There were also seen large swollen cells that were often of irregular shape and contained spheric crystals. The mycelium had buckles corresponding to the small arc-shaped disjunctors located opposite the lateral septum of the hypha. Under solid-phase cultivation, complete encrustation of the grain substrate by the vegetative mycelium of P. impudicus was observed during 6~8 weeks. After 8~10 weeks, formation of primordia was observed. Studies of the effect of cultivation temperature showed that the optimum temperature for growth of the *P. impudicus* strains studied was 22~24 ℃. The low cultivation temperature (18~20 ℃) turned out to be more preferable for these mushrooms than the higher one (26~28 ℃). At a temperature of 4±1 ℃, the vegetative mycelium grew very poorly. At 30 ℃ and higher, mycelium growth essentially stopped. To select strains-producers of polysaccharides, we studied growth of the strains of the mushroom *P. impudicus* in beer wort 8ºB in Erlenmeyer flasks at 23~25 ℃. The biomass contained 5.5%~9.4% of endopolysaccharides. During deep cultivation of *P. impudicus*, accumulation of lipopolysaccharides in culture liquid was observed. The amount of the extracellular polysaccharides formed was 1.4~3.0 g/L. The most actively growing strains of *P. impudicus* produced 4.8~5.0 g/L of biomass containing 8.6~9.4 g/L of endopolysaccharides. The culture liquid contained 2.8~3.0 g/L of exopolysaccharides. Research was done into growth of the *P. impudicus* strains in liquid glucose peptone medium during surface (under steady-state conditions) and deep (using a shake-flask popagator) cultivations. During the surface cultivation in flasks under steady-state conditions, the mycelium grew as individual downy pieces, then a uniform thick film was formed and the culture liquid adjacent to the film became jelly-like due to exopolysaccharides. Sixty cultivation days was required to obtain 10~15 g/L of biomass. The culture liquid filtrate contained 3.4~5.3 g/L of exopolysaccharides. During deep cultivation in flasks using a shake-flask propagator and mixing, the mycelium of *P. impudicus* grew as pellets of varying diameters (1~5 mm). Growth as mycelium clots was sometimes observed. To obtain 5.0~5.6 g/L of biomass, the duration of cultivation amounted to 30 days. The surface mycelium contained 65%~66% of total carbohydrates, 14.9%~17.7% of endopolysaccharides, 6.2%~9.7% of true protein and 1.8%~2.0% of lipids. The high content of carbohydrate compounds in the surface mycelium may be explained by attachment of exopolysaccharides to the surface layer. The deep mycelium contained 52-55% of total carbohydrates, 8.5%~9.0 % of endopolysaccharides, 9.5%~10.2% of true protein and 2.3%~2.5% of lipids. The cultural liquid contained up to 3.0 g/L of extracellular polysaccharides.

It is shown that *P. impudicus* can be grown on media containing rye, wheat, soy flour, and whey, which opens up the prospect for the development of a complex nutrient media. The slightly acidic pH of the medium (4.0~5.0) was more favorable for growth of the biomass. A decrease in pH to 3.5~3.7 was observed during mycelium growth. The optimal temperature for deep cultivation of *P. impudicus* mycelium was 22~24 ℃. When growing the mushroom at a temperature above 26 ℃, a significant slowdown in its growth was observed. The optimization of the nutrient medium composition and cultivation conditions increased the biomass yield by 1.3-fold and the content of the total polysaccharides - by 1.5 to 1.7-fold.

Comparative study of the chemical composition of the fruit bodies and cultivated mycelium of *P. impudicus* was carried out. The content of polysaccharides in the samples studied was not different (Table 1).

Table 1 Chemical composition of body fruit and mycelium of *P. impudicus*, % (Means ± standard error)

	Body fruit	Surface mycelium	Deep mycelium
Total carbohydrates	50.8±3.0	54.1±3.3	50.6±2.5
Endopolysaccharides	13.5±0.6	14.6±0.8	13.2±0.4
Exopolysaccharides	None	2.0±0.5	4.0±1.0
Chitin	12.5±0.5	7.0±0.3[a]	11.2±0.6
Crude protein	25.2±0.4	20.0±0.8	21.0±0.6[a]
True protein	13.5±1.1	12.2±0.3	13.4±0.2
Lipids	1.4±0.1	2.0±0.1[a]	2.3±0.2[a]
Total phenolic compounds	0.64±0.018	0.82±0.014	0.46±0.016

Note: a: $p<0.05$ as compared to the Body fruit group.

This research shows that the cultivated mycelium *P. impudicus* can be used as a source of the corresponding polysaccharides.

2 Immunostimulating effect of *Phallus impudicus* polysaccharides *in vitro*

Many polysaccharides isolated from Basidiomycetes mushrooms have a wide range of pharmacologic activities. Most important among these effects of mushroom polysaccharides have antitumor and immunomodulating properties[14].

At present, a number of polysaccharides with immunomodulating properties have been isolated from mushrooms. Such substances as schizophyllan, krestin, and lentinan, have been used in oriental medicine as immunomodulators[15]. Lentinan isolated from the fruit body or mycelium of shiitake mushroom (*L. edodes)* was the most promising immunomodulator among the polysaccharides[16, 17].

Very limited data in the current literature suggest an immunomodulating effect of polysaccharides isolated from *P. impudicus*[11]. In an *in vitro* experiment, we evaluated the effect of polysaccharides isolated from mycelium of *P. impudicus* on the immunologic parameters using venous blood of healthy volunteers and a lymphocyte population isolated from this blood as an object of study. We evaluated the effects of the polysaccharides on the phagocytic activity of neutrophyls, the relative content of T-lymphocytes and the expression of CD25+ antigens by T- and B-lymphocytes. We used polysaccharides from *L. edodes* with the known immunomodulating properties[18] and immunosuppressor mofetil mycophenolate[19] as reference substances.

Both the tested polysaccharides enhanced the expression of CD25+ (Table 2) which is the α-subunit of the interleukin-2 (IL-2) receptor actively engaged in a wide range of immune responses, including regulation of cell phagocytic activity[20]. IL-2 plays a pivotal role in regulating the adaptive immune system by controlling immune response, survival and proliferation of regulatory T cells, which are required for the maintenance of immune tolerance[21]. A number of autoimmune and immunodeficiency diseases are associated with disordered mechanisms of IL-2 receptor expression because IL-2 was originally identified as a growth factor critical for T cell proliferation *in vitro*[22]. In accordance with the above results, polysaccharides of *L. edodes* and *P. impudicus* similarly increased the amount of T lymphocytes evaluated as T-active rosetting in human blood**.**

Phagocytosis is one of the key mechanisms of adaptive immune response to pathogens. In the process of phagocytosis, macrophages process and present antigens to lymphocytes and then ingest and destroy pathogens, which in turn elevates the innate immune response[23]. Our results demonstrated that all the investigated polysaccharides increased the phagocytotic uptake of macrophages (Table 2), whereas the effect of *P. impudicus* polysaccharides on phagocytic activity was significantly higher than that of *L. edodes***.** In addition, both the polysaccharides significantly increased the number of latex particles absorbed by a single cell while this indicator was also significantly higher in samples with addition of polysaccharides of *P. impudicus* as compared to *L. edodes* polysaccharides.

Table 2 Effect of polysaccharides from *P. impudicus, L. edodes* and mofetil mycophenolate on immunologic parameters in human blood *in vitro* (Means ± standard error)

	Control	Polysaccharides from *L. edodes*, 0.1 mg/mL	Polysaccharides from *P. impudicus*, 0.1 mg/mL	Mofetil mycophenolate 0.1 mg/mL
CD25+ expression/%	27.1±0.5	36.4±0.5[a]	36.0±1.1[a]	17.0±1.4[abc]
T- lymphocytes/%	21.5±0.8	39.9±2.5[a]	45.0±1.1[a]	11.8±0.8[abc]
Phagocytic index/%	52.5±1.2	65/5±1.2[a]	77.8±1.6[ab]	31.5±0.9[abc]
Number of phagocytized particles per 1 cell	5.6±0.1	6.8±0.1[a]	7.5±0.2[ab]	3.7±0.2[abc]

Note: a: $p<0.05$ as compared to the control group; b: $p<0.05$ as compared to the *L. edodes* group; c: $p<0.05$ as compared to the *P. impudicus* group.

Table 3 Effects of *P. impudicus* polysaccharides (PIP) on immunologic parameters in rats with streptozotocin-induced diabetes (Means ± standard error)

	Control	Diabetes	Diabetes + PIP, 10 mg/kg	Diabetes + PIP, 30 mg/kg
Complement activity,CH50	63.7±2.71	51.0±3.80[a]	81.0±3.31[ab]	68.5±3.69[b]
Circulating immune complexes, a.u.	17.8±4.30	53.3±11.85[a]	39.4±6.43 [a]	21.3±6.38 [b]
Phagocytic index, %	80.7±3.60	57.4±2.07 [a]	65.0±2.32[ab]	71.1±5.22[b]

Note: a: $p<0.05$ as compared to the Control group; b: $p<0.05$ as compared to the Diabetes group;

The second reference substance, mofetil mycophenolate, an immunosuppressant which is capable of inhibiting proliferation of T and B lymphocytes and producing antibodies[19], exerted the opposite effect on the parameters studied. As compared to all the groups studied, mofetil mycophenolate significantly up-regulated the expression of CD25+ and decreased the number of T lymphocytes and phagocytosis-characterizing parameters (Table 2).

Thus, the presented data demonstrated immunomodulatory activity of *P. impudicus* polysaccharides which exceeded similar activity of the known immunostimulant, polysaccharides of *L. edodes*.

3 Effect on immunologic parameters in rats with streptozotocin-induced diabetes

Type 1 diabetes is a chronic autoimmune disease that is characterized by specific destruction of insulin-secreting is let *β*-cells, resulting in insulin deficiency, hyperglycemia as well as diabetic complications[24]. Development of diabetes involves disturbances in both the innate and adaptive immune systems. Innate immune stimulation may be beneficial in prevention or treatment of type 1 diabetes[25]. Given increasing evidence for the role of innate immunity in type 1 diabetes, we therefore investigated therapeutic effects of *P. impudicus* polysaccharides on innate immunity in rats with streptozotocin-induced diabetes.

Significant disorders in the immune system: A decrease in the activity of the complement system and nonspecific resistance assessed by the intensity of phagocytosis as well as a dramatic increase in the production of circulating immune complexes were observed in rats with type 1 diabetes mellitus (Table 3). These changes in cellular and humoral immunity factors contribute to progression of the diabetic process and precede the development of infectious and vascular complications.

The treatment of diabetic rats with polysaccharides of *P. impudicus* showed immunomodulating properties of this substance. The polysaccharides administration caused activation of the complement system, dose-dependently decreased the value for circulating immune complexes and significantly enhanced the phagocytic activity of blood cells. Therefore, we can conclude that *P. impudicus* polysaccharides can be considered as a suitable candidate for correction of immune disorders in type 1 diabetes.

4 Study of wound-healing effect of *Phallus impudicus* polysaccharides in rat experiment

Earlier we found a significant therapeutic effect of *P.*

impudicus extract from fruit bodies of the mushroom on healing of burn wounds[26]. It was shown that the ointment containing this extract had a strong reparative effect and accelerated the burns wound healing in these rats.

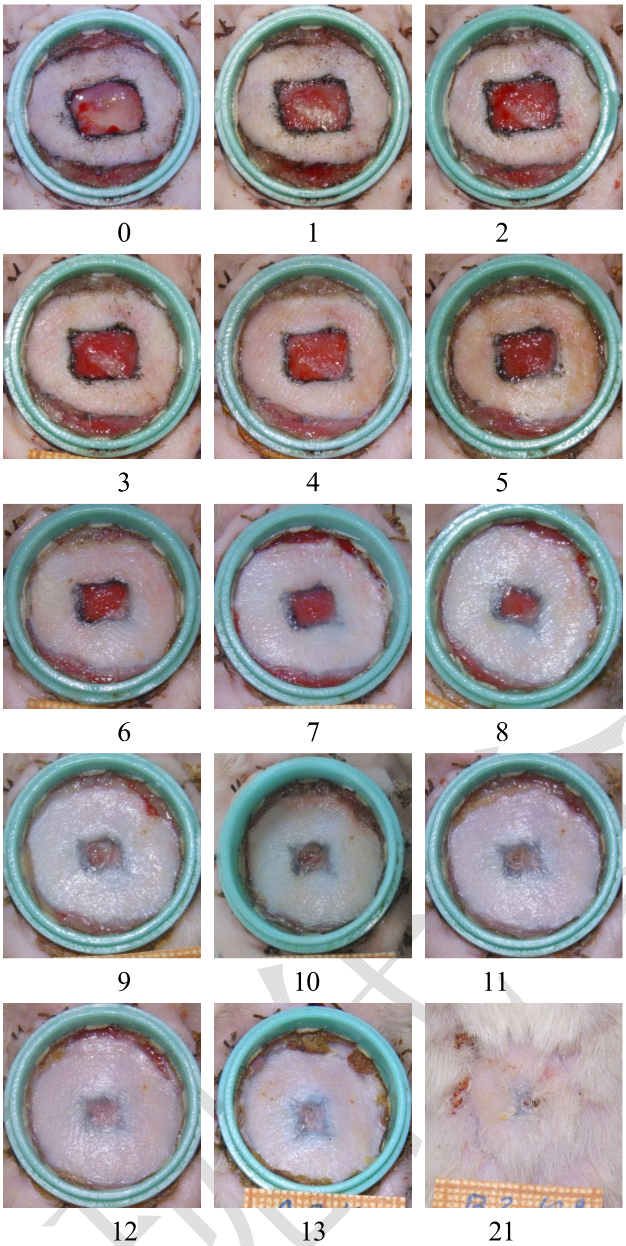

Fig.1 Dynamics of healing (days after wounding) of a full-thickness cutaneous wound in control rats treated with ointment base

Recently we studied the wound-healing effect of *P. impudicus* polysaccharides in a model of excision of a full-thickness skin flap in Wistar rats. The wound was closed by a protective chamber that was fixed to the body of the animal. The rats were divided into 2 groups: The 1st group consisted of control animals treated with the ointment base applied to the wound surface and the 2nd group was experimental one treated with the ointment containing 10% *P. impudicus* polysaccharides.

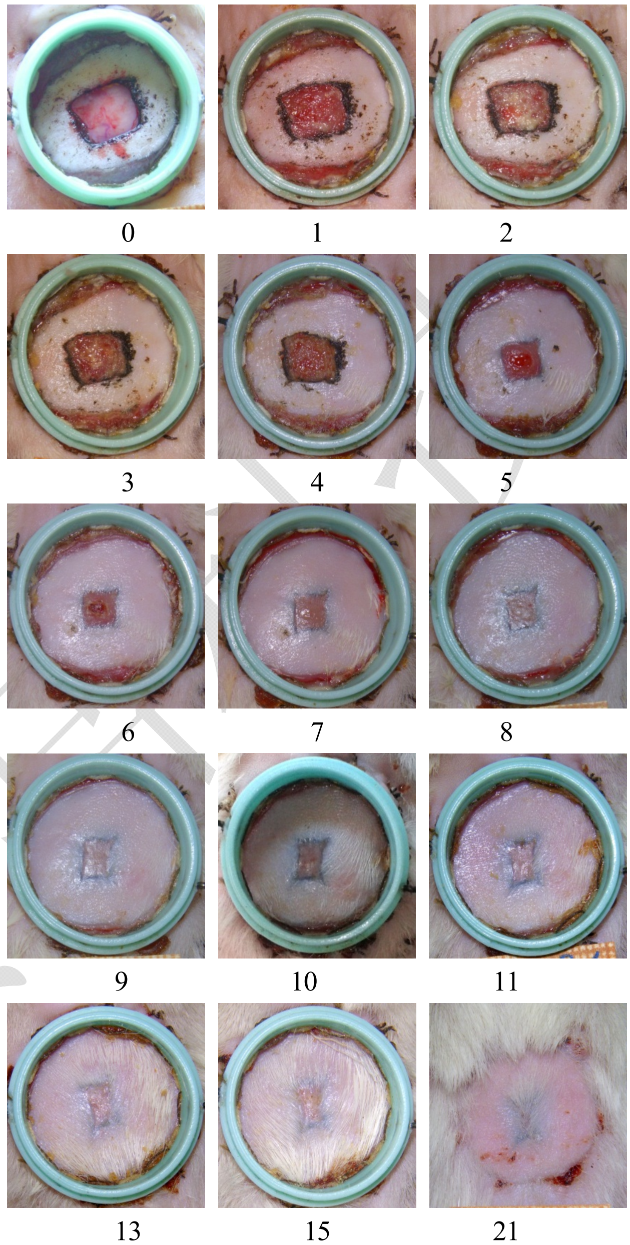

Fig.2 Dynamics of healing (days after wounding) of a full-thickness cutaneous wound in rats treated with ointment containing 10% polysaccharides of *P impudicus*

On the second day after removal of the skin flap, experimental animals treated with the ointment containing 10% *P. impudicus* polysaccharides locally applied to the wound surface showed mild swelling of the dermis and the adjacent subcutaneous tissue, perivascular hemorrhages per diapedesis and focal hemorrhages, intensive growth of granules and fibrin deposition that were observed along with venous plethora. On the third day, respectively, the area of the wound surface demonstrated fibrin deposits (under which granulation tissue was growing rapidly) that covered the entire wound surface with a dense layer. Along the edges of the wound, the regenerating epithelium was creeping onto the maturing granulation tissue (regeneration phase). On the 5th and the 6th days (phase of epithelialization and reorganization of the scar), there was observed intensive growth of epithelial tissue around the perimeter and a considerable contraction of the wound which had been nearly completely cleared of the fibrin plaque (Figures 1, 2). Complete epithelization of the wound surface and primary tension healing occurred on days 7~8. After the final healing, the surfaces of the wounds passed into tissue of the dermis without sharp boundaries, had an irregular rectangular shape without signs of hair growth and formed a subtle scar that was elastic, soft by touch and readily folding.

In contrast, on the second and the third days after the surgery, animals of the control group treated with the ointment base applied to the wound surface exhibited wound surface bleeding. The gauze bandages were abundantly saturated with serous hemorrhagic discharge, and the edema of the dermis and adjacent subcutaneous tissue remained. At this period, the formation of granulations was insufficient and a scant fragmented fibrin deposition was noticed (Figures 1, 2).

On the 5th~7th days, the wounds retracted. The surfaces of the majority of them were covered with a thin layer of fibrin, the granulation tissue was at the stage of formation, and the marginal epithelialization reached 1~2 mm along the entire perimeter of the wounds. By days 12~15 of the treatment with ointment base applications, there occurred, along with continuing contraction, a full epithelialization and primary tension healing of the wounds with the formation of small scars of an irregular rectangular shape that were dense by touch, soldered to the underlying tissues and had no signs of hair growth.

The study of the therapeutic effect of 10% *P. impudicus* polysaccharides ointment in a model of a full-thickness cutaneous wound in rats showed a pronounced wound-healing effect compared to the use of ointment base in control animals, which made it possible to shorten the periods of complete wound surface healing by 1.8 times. Thus, in the model of an acute full-thickness wound in rats, the ointment containing P. *impudicus* polysaccharides had a high therapeutic activity throughout all the phases of the wound process and accelerated the repair processes of the wound surface, causing faster maturation and remodeling of the granulation tissue, and also accelerated the recovery of the dermis, epithelial skin and its derivatives and shortened the periods required for complete healing of the wounds.

5 Conclusion

3.1 Taken as a whole, from the results presented here we can conclude that polysaccharides isolated from P. impudicus, are involved in immunomodulating activity, since they proved to be effective in enhancement of phagocytosis and useful in treatment of immunodeficiency accompanied by different diseases. Their immunomodulatory effect was mediated by various immune cells such as macrophages and T lymphocytes.

3.2 The present study also demonstrated that the local use of *P. impudicus* polysaccharides on full-thickness cutaneous wounds could enhance healing with regards to their epithelialization, contraction and growth of granulation tissue. There were significant differences in surface area and the period of wound healing between the groups, particularly when using the ointment containing polysaccharides of *P. impudicus*.

3.3 In conclusion, our results support the belief that polysaccharides of *P. impudicus* can improve the immune state and accelerate wound healing. Further pharmacological investigations should be carried out to evaluate the precise mechanism of these effects.

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