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NATURAL DRUGS

EFFECTS OF *PHALLUS IMPUDICUS* EXTRACT FOR ACCELERATING HARD-HEALING WOUNDS IN DIABETES-INDUCED RATS

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Abstract: The multifunctional characteristics of *Phallus impudicus* extract have encouraged research into its potential use in medical applications. Diabetes mellitus is a metabolic disease accompanied by chronic hyperglycemia caused by absolute or relative insulin deficiency. This study aimed to demonstrate the potential role of *P. impudicus* extract in accelerating wound healing in diabetic rats. For the induction of diabetes, streptozotocin (STZ, concentration 100 mg/mL) at a dose of 30 mg/kg b.w. in citrate buffer, pH 4.5 was used. Wounds were excised by cutting a 10-mm-diameter flap of full-thickness skin with a barb under inhalation anesthesia and 5% isoflurane under aseptic conditions. Wounds were treated with a standard preparation and with a prototype product containing an alcoholic extract of *P. impudicus*. Several metabolites that promote wound healing, including amino acids, sugars, organic acids, and other compounds, were identified in the ethanolic extract by GC-MS. In the MTT assay, the alcoholic extract of *P. impudicus* was nontoxic and stimulated fibroblast proliferation, which was also expressed as an increase in DNA and collagen synthesis. A hydrogel containing an extract of the fungus *P. impudicus* was shown to accelerate the period of wound desquamation in the experimental animals compared to the control groups. There was also a statistically significant ($p > 0.005$) reduction in SOD activity and TBARS compared to the control group, indicating an intensive healing process in which oxidative enzyme systems play a significant role.

Keywords: *Phallus impudicus* extract, wound healing, diabetes wounds, hard-healing wounds

The multifunctional characteristics of *Phallus impudicus* extract have encouraged research into its potential use in medical applications. Science is constantly seeking new evidence of the biological activity of extracts of natural origin.

Scientists are constantly seeking natural substances with beneficial adaptogenic, antioxidant, immunomodulatory, or antitumor properties. Thus, in medical practice, increasing attention is being paid to the development of medications based on natural raw materials. Natural-based drugs and medicines should not cause any side effects on the physiological functions of the human body. Research data suggests that *P. impudicus* extract may be a promising source of this type of safe drug [1, 2].

In non-traditional (alternative) medicine, water and alcoholic extracts from fresh or dry fungi are widely used. This may be related to a broad spectrum of action in the treatment of cardiovascular diseases, malignant tumors, and sexual weakness, and even during chemotherapy or radiation therapy to prevent metastases and relapse of oncological diseases [3-5].

The biologically active components of *P. impudicus* include polysaccharides, peptides, phospholipids, glucans, polyphenols, vitamins, unsaturated fatty acids, and chitin. Moreover, the presence of active compounds is characterized by pleiotropic biological activities and multitarget properties. Therefore, the development of safer, more effective, and controlled therapeutics based on the raw extract or active compounds of *P. impudicus* requires more detailed information on its potentially toxic effects in clinical practice [6, 7].

The stinkhorn (*Phallus impudicus* L. ex Pers.) of the family Phallaceae is a common and widely distributed fungus in Europe and North America. Its fetid smell and unmistakable appearance make it one of the most easily recognizable species of fungi. The mature fruiting body (an inedible white

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hollow stalk-like stipe, 10-–30 cm high, with a cap 2-–4 cm wide, covered with olive brown to dark brown slime-containing spores) evolved from an egg-shaped form (a whitish to yellowish round structure, up to 6 cm across, usually partly submerged in the ground). The "eggs,", which lack the unpleasant scent, are edible and regarded as delicacies in some countries.

According to the literature, natural products are rich sources of therapeutic agents and have inspired researchers to work on these natural products with advancements in synthetic methodologies and to try to synthesize or produce analogs of natural compounds with improved immunopharmacological properties [1, 2]. These natural products include fungi, which contain rich sources of novel organic compounds with interesting immunobiological properties [3, 4]. Identification of bioactive molecules from these fungi using high-throughput screening. For example, ergoflavin, isolated from an endophytic fungus, showed anti-inflammatory and anticancer activities [4].

Diabetes is one of the major unsolved problems in modern medicine. According to the World Health Organization (WHO), diabetes (Latin: diabetes mellitus, literally: honey leak) is a chronic metabolic disease characterized by high blood glucose levels known as hyperglycemia, which is the result of absolute or relative insulin deficiency. Epidemiological data from 2018 indicate that 5 million people die annually worldwide due to diabetes and its complications. It is projected that by 2040, 642 million people in the 20–79 age group will have diabetes, and a further 25% – 30% of adults with diabetes do not know they have the disease. Unfortunately, Poland is one of the leading countries in this infamous statistic. By 2015, three million adults were reported to have diabetes. By 2040, this number is expected to rise to over four million people.

The pharmacoeconomic aspect is worth noting here. It is projected that by 2040, the costs of diabetes treatment will increase by 19% globally, while the costs in Poland amounted to more than PLN nine billion in 2017. Diabetes is not only a disease that consumes a huge amount of financial resources but also causes social costs, the so-called non-medical costs due to employee absenteeism, reduced productivity, and consequently, the premature abandonment of professional activity (Report of the Institute of Health Protection, 2018).

The aim of this study was to assess the potential role of *P. impudicus* extract in accelerating wound healing in diabetic rats.

EXPERIMENTAL

Materials and Methods

Plant Material Collection

The fruiting bodies of the fungus are found from May to November in various types of forests, often in beech forests, as well as in parks and thickets, on fertile, humus-rich soils, in shady and moist places, singly or in small groups, quite common throughout Poland.

Mushroom fruiting bodies measuring 4-–5 cm × 3-–5 cm were collected in July 2018, selected, cleaned, dried, and subjected to qualitative analysis.

Extract Preparation

A portion of the dried and homogenized mushroom fruit (10 g) of *P. impudicus* was placed in an orangish glass / dark glass bottle, and 30 g of 40% ethanol, obtained by diluting 96% ethanol (Honeywell, Germany) with deionized water (Millipore Direct-Q 3 UV), was added. It was mixed thoroughly and set aside in a dark, cool, dry place for incubation for 14 days.

During the 14-day incubation period, the mixture was mixed manually three times a day. After 14 days, the incubated mixture was filtered. The resulting extract was transferred to an orangish glass / dark glass bottle and stored in a refrigerator at 2-°C – 8°C.

Sample Preparation

The extract (0.5 mL) was evaporated at room temperature under a stream of compressed nitrogen (ABCHEM, N_2). After complete evaporation of the solution, the extract was derivatized for one hour at 80°C with 0.5 mL of N,Obis(trimethylsilyl)trifluoroacetamide (STFA).

GC-MS (Gas Chromatography – Mass Spectrometry) Analysis

Qualitative analysis of the extract was performed using a gas chromatograph (Agilent Technologies—GC 7820A) with a mass spectrometer (MS D5977B).

The experimental conditions of the GC-MS system were as follows: column Agilent HP-5ms Ultra neutral 30 m × 250 um × 0.25 um. The flow rate of the mobile phase (carrier gas: He) was set at 1.1 mL/min. The initial temperature (50°C) was raised to 200°C at 10°C intervals and then to 300°C at 5°C intervals, and the injection volume was 1 μL. Samples were run in the range of 33-650 m/z and the results were compared using the NIST 17 library search program.

Evaluation of Biological Activity in Vitro

Fibroblast Culture

Fibroblasts were obtained from ATCC (CRL-2072, ATCC, Manassas, VA, USA). The cells were maintained in DMEM supplemented with 5% fetal bovine serum (FBS), 50 IU/mL penicillin, and 50 µg/mL streptomycin (Gibco, Thermo Fisher Scientific, Waltham, Massachusetts, USA) at 37°C in a humidified atmosphere in the presence of 5% CO_2. Cells were counted using a hemocytometer and cultured at 1×10^5 cells per well in 2 mL of growth medium in six-well plates (Costar, Avantor, USA). After 90% confluence was achieved, the cells were passaged onto appropriate culture plates using phosphate buffer with 0.05 trypsin and 0.02% EDTA. Cells were counted in a hemocytometer and transferred to the appropriate plates at 1 × 105 cells suspended in 1 mL of culture medium. The culture medium was replaced with fresh medium every 48 h. Fibroblasts reached the contact inhibition state after six days of growth. Cells between eight and 14 passages were used in this study. Cells in the contact inhibition state were washed three times with 0.15 mol/L NaCl solution and then treated with the test agent.

Cell Viability Assay

The toxicity of the tested substances was determined using the method described by Carmichael (5) with some modifications. In living cells, the tetrazolium salt (MTT) is converted to purple formazan by mitochondrial dehydrogenases. In dead cells, the conversion process does not occur. Cells in the contact inhibition state were incubated for 24 hours at 37°C in a 5% CO_2 atmosphere in medium containing different concentrations of the test agent. The medium was discarded and the cells were rinsed three times with phosphate buffered saline (PBS). The cells were then incubated for 4 h in 1 mL of PBS with 25 µL of MTT (5 mg/mL, Sigma-Aldrich, USA). The medium was removed from the wells, and the cells were lysed with 1 mL of DMSO with 20 µL of Sorensen's buffer (0.1 mol/L glycine with 0.1 mol/L NaCl, pH 10.5, (Sigma-Aldrich, USA). Absorbance was measured at 570 nm. Cell viability of the treated cells was calculated as a percentage of the control cells.

DNA Biosynthesis Assay

Fibroblast proliferation was measured by the incorporation of [methyl-3 H]-thymidine (Hartman Analytic GmbH, Braunschweig, Germany) into DNA. Prior to the experiment, fibroblasts were cultured in a 24-well plate at 1×10^4 cells/well with 1 mL of growth medium. After 48 h, the cells reached confluence and were incubated in FBS-free DMEM (Gibco, Thermo Fisher Scientific, USA) with *Melittis melissophyllum* L. extracts under experimental conditions of wound healing assay or experimental inflammation induced by IL-1, for 24 h and next with 0.5 µCi/mL of [methyl-3 H]-thymidine for 4 h. PBS rinsed cells (three times with 2 mL) were solubilized with 1 mL of 0.1 mol/L sodium hydroxide containing 1% SDS and 5 mL of scintillation fluid Ultima Gold XR (PerkinElmer, Waltham, USA). Incorporation of the tracer into DNA was measured by Liquid Scintillation Analyzer Tri-Carb 2810 TR (PerkinElmer, Waltham, USA) and calculated using Quant Smart TM software (PerkinElmer, Waltham, USA).

Collagen Biosynthesis Assay

Collagen biosynthesis was measured using the method described by Peterkofsky et al.. Human skin fibroblast cells in a contact-inhibited state were incubated for 24 hours in a medium supplemented with 5[3H]-proline (5 µCi/mL, 28 Ci/mmol) and the test compound at different concentrations. After 24 hours, the cells were washed twice with 1 mL of DPBS, followed by the addition of 0.5 mL of DPBS with 10 mmol/L proline (115 mg/100 mL DPBS). The cell suspension was transferred from individual meshes to Eppendorf tubes and ultrasonicated thrice for 20 s at 0°C. Proteins in the homogenates were precipitated by adding 2 mL of 20% TCA with 20 mmol/l proline. After five minutes, the samples were centrifuged for 10 minutes at 1000 × g and 4°C. The supernatant was discarded and the precipitate was dissolved in 1 mL of 5% TCA with 10 mmol/L proline and centrifuged again. The resulting supernatant was discarded, and the precipitate was dissolved in 0.6 ml of 0.2 mol/L NaOH. Two samples (0.2 mL each) of the lysate were collected. Each sample was neutralized by adding 0.16 ml of 0.15 mol/L HCl and 0.1 mL of 1 mol/L Tris-HCl, pH 7.2. 20 µL of 62.5 mmol/L N-ethylmaleimide (NEM) and 10 µL of DPBS with collagenase (concentration 1 mg/mL) (test sample) or without collagenase (control sample) were then added to both samples. The samples were incubated for 90 minutes at 37°C. Next, 0.5 mL of 10% cold TCA was added and cooled for 5 minutes at 0°C. The samples were then centrifuged, and the collected supernatant was transferred to scintillation vials containing 4 mL of scintillation fluid. The radioactivity of the samples was measured, and the collagen content was expressed as dpm of

5[3H]-proline incorporated into bacterial collagenase-susceptible proteins per mg of protein contained in the cell homogenate extract and as relative values to the control.

Evaluation of in Vivo Wound-Healing Activity

Experimental Animals

Male Wistar rats (280-–300 g) were used for the experiment. All the animals were housed in group cages as appropriate in a room with a 12 h light/dark cycle and allowed access to tap water and standard rat food. Before the initiation of the experiment, the animals were acclimatized to the laboratory conditions for a period of five days. The care and handling of animals were in accordance with internationally accepted laws and the guidelines set forth in Directive 2010/63/EU of the European Parliament and of the Council of 22 October 2010 on the protection of animals used for scientific purposes. The study was approved by the Local Ethics Committee for animal experiments (No. 07/18 of 19 April 2018).

Experimental Model of Diabetes

For the first four weeks, the animals were fed a high-fat feed. After this time, the animals were weaned from the feed for 12 hours leaving only free access to water. The animals then received an intraperitoneal injection of 0.075 mL of streptozotocin solution (STZ, concentration 100 mg/mL) prepared immediately prior to administration at a dose of 30 mg/kg b.w. in citrate buffer, pH 4.5. One week after STZ administration, fasting blood glucose was measured, and animals with blood glucose levels above 145 mg/dL were qualified for further testing. In the next step, one week apart, the rats received another dose of STZ (intraperitoneally, at a dose of 30 mg/kg b.w., 0.075 mL). After another week, blood glucose was measured and animals with blood glucose levels above 200 mg/dL were qualified for the study. Rats in which diabetes was not induced based on veterinary advice were sacrificed for organ harvesting for the development of new test methods.

Experimental Model of Skin Damage

Based on the aim and objectives of the study, the animals were randomized into five groups that received the appropriate treatment:

1. negative control – vehicle/substrate;
2. positive control – a commonly used wound-healing agent available on the pharmaceutical market (Karnosil);
3. product prototype (hydrogel) containing 10% of a test extract of *P. impudicus*;
4. permeation enhancer e.g. DMSO + substrate;
5. permeation enhancer + product prototype.

According to the protocol for standard treatment of skin infections, a commonly used wound-healing agent available on the pharmaceutical market, was used as a comparison solution.

The experimental groups were randomized according to body weight as the leading trait. Each group included six animals. All procedures were performed under inhalation anesthesia with 5% isoflurane (1000 mg/g Isotek, VetAgro, Poland) under aseptic conditions. Hair was manually removed from the interscapular region of each animal.

The wounds were excised by cutting a 10-mm-diameter flap of full-thickness skin with a barb. The animals received the analgesic metamizol (40 mg/kg) intramuscularly into the quadriceps muscle of the thigh in a volume of 0.15 mL immediately after wounding. Analgesic treatment was continued for the following days with 200 mg/kg of metamizol per animal. The analgesic was administered until approximately day 10 of treatment. Immediately after wound debridement, 150 mg was applied to the skin at the site of injury. Treatment was continued until the wound healed completely. Treatment was repeated daily under aseptic conditions.

Daily Wound Care and Sampling

During daily dressing changes, the dynamics of the epidermis and wound surface contractions were observed until complete healing was achieved. Dressings were changed under light inhalation anesthesia with 5% isoflurane (1000 mg/g Isotek, VetAgro, Poland).

At each dressing change, the wound was washed with acetenisept.

Determination of Tissue Collagen Content

The following day, after complete healing, the animals were sacrificed with 5% isoflurane, and the reconstructed tissue was excised and stored at -20°C until assayed. Frozen tissue samples were crushed and homogenized in 4°C lysis buffer containing pepsin (Sigma P7012) in 0.5 M acetic acid. The residual unhomogenized tissue was removed by centrifugation at 12000 × g for 10 min. Acid-soluble total collagen (types I-IV) was measured spectrophotometrically in the resulting homogenates. Sirius red dye solutions were prepared by dissolving 1 g of Sirius red F3B in 100 mL of a saturated aqueous picric acid solution containing

0.05% Tween 20. The dye solution was added to the tissue homogenate, incubated for 45 minutes at room temperature with constant stirring, and then centrifuged (12000 × g, 10 minutes). The resulting precipitate was suspended in 0.5 M NaOH and shaken for 30 minutes. Absorbance of the bound dye was measured at 560 nm using a microplate spectrophotometer. Commercially available bovine type I collagen was used to generate the calibration curve.

Determination of Selected Antioxidant Enzyme Activity

Frozen tissue samples were homogenized in a chilled to 4°C solution of 0.15 M KCl containing 5 mM EDTA (1: 10 w/v) using a Potter-type homogenizer. Tissue residues were removed by centrifugation at 5000 g for 10 minutes. The resulting homogenates were measured for enzyme activity, reduced and oxidized glutathione levels, and concentration of lipid peroxidation end products (TBARS).

The total superoxide dismutase (SOD) concentration was measured using the modified Misr method of inhibition of auto-oxidation of adrenaline in sodium carbonate buffer at pH 10.2.

Catalase activity was measured using the Aebi method.

Reduced and oxidized glutathione levels were measured spectrophotometrically using an enzymatic recycling method with glutathione reductase, in the presence of NADPH.

TBARS was determined according to the method of Buege and August with the modifications of Esterbauer and Cheeseman. This method is based on the reaction of the formation of a colored complex of lipid peroxidation products with thiobarbituric acid (TBA) in an acidic environment at 100°C. The main lipid peroxidation product reacting with thiobarbituric acid is MDA; so for simplicity, the level of all substances reacting with TBA is presented as the MDA concentration. A reaction mixture (4.5 mL) consisting of 0.375% thiobarbituric acid (TBA), 15% trichloroacetic acid (TCA) in 0.25 N HCl was added to 0.5 mL of blood or plasma hemolysate. The samples were incubated in a water bath for 20 minutes at 100°C to optimize the reaction conditions of MDA with TBA. The samples were then cooled and centrifuged at +4°C for 15 minutes at 2000 × g. After centrifugation, the supernatant was collected and the extinction was measured at $\lambda = 532$ nm against the reaction mixture incubated under the same conditions using a Cary 60 UV-Vis spectrophotometer from Agilent Technologies. The colored product, formed from the combination of dialdehydes with thiobarbituric acid, was evaluated spectrophotometrically at 532 nm. TBARS concentrations were calculated based on an extinction coefficient of 1.56 × 105 M-1 cm-1.

Statistics

The obtained results were processed using Graph Pad Prism v. 6.0. Data in the tables are presented as mean ± standard error of the mean. The differences between the control and study groups were determined using one-way ANOVA. Values of $p < 0.05$ were considered statistically significant.

RESULTS

Gas Chromatography-Mass Spectrometry (GC-MS) Analysis of P. Impudicus Extracts

Several metabolites, including amino acids, sugars, organic acids, and other compounds, were identified in the ethanolic extract by GC-MS (Figure 1). Among them, chemical structures were found to possess wound healing accelerating properties according to the literature. The dominant wound healing component in the studied extract was D-mannose (94.69%). Among the organic acids that promote wound healing, malic acid (92.54%) has been found (Table 1), as well as significant amounts of furoic acid (91.31%), which has preservative properties and also significant amounts of butanedioic acid (96.88%) which is known as "natural" antibiotic because of its relative acidic nature (Table 1). It is also worth mentioning that numerous amino acids, such as glycine (93.26%), L-valine (92.47%), and L-isoleucine (93.81%), have a beneficial effect on reducing the wound surface area by accelerating the granulation process and reducing inflammation, which in turn has a positive effect on reducing wound healing time.

Determination of Cell Viability (MTT Test)

Evaluation of cytotoxicity is an important step in the study of wound healing aids. The effect of ethanolic extract of *P. impudicus* on fibroblast cell viability was shown. The ethanolic extract increased the test index starting at a concentration of 0.05%, and at 0.1% and 0.2% the MTT values were higher than those of the aqueous extract. When the content of *P. impudicus* in the ethanol extract increased, a decrease in the index was observed, approaching the values of the control group.

The obtained data indicated the complete non-toxicity of ethanolic extracts at the tested

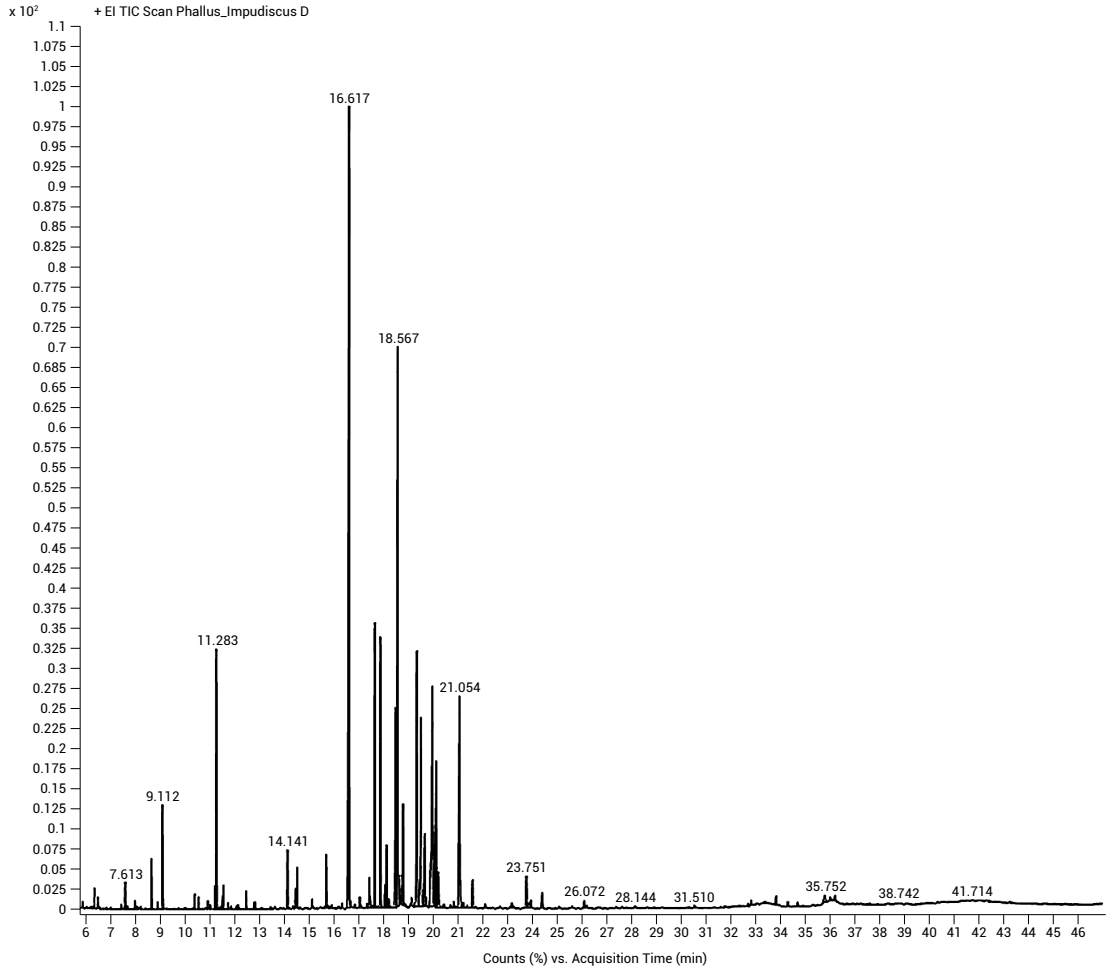

Figure 1. GC-MS chromatogram of the ethanolic extract of *P. impudicus*.

Table 1. Qualitative chromatographic analysis of *P. impudicus* fruiting body extract.

Lp.	RT	Name	Probability of occurrence [%]
1	5.933	glycine, TMS derivative	64.01
2	6.127	L-alanine, TMS derivative	90.91
3	8.092	lactic acid, 2TMS derivative	84.86
4	8.738	L-alanine, 2TMS derivative	92.82
5	8.986	glycine, di-TMS	93.26
6	9.216	2-furoic acid, TMS derivative	91.31
7	9.494	L-leucine, TMS derivative	79.8
8	9.808	L-isoleucine, TMS derivative	87.22
9	10.046	Beta-alanine, 2TMS derivative	90.02
10	10.257	L-norvaline, 2TMS derivative	73.53
11	10.455	L-valine, 2TMS derivative	92.47
12	10.845	succinic acid, monoethyl ester-, (TMS)	94.28
13	11.024	L-serine, 2TMS derivative	89.59
14	11.269	L-leucine, 2TMS derivative	89.85

Table 1. Qualitative chromatographic analysis of *P. impudicus* fruiting body extract (cont.).

Lp.	RT	Name	Probability of occurrence [%]
15	11.583	L-isoleucine, 2TMS derivative	93.81
16	11.616	L-proline, 2TMS derivative	81.31
17	11.802	butanedioic acid, 2TMS derivative	96.88
18	11.904	picolinic acid, TMS derivative	95.65
19	12.12	glyceric acid, 3TMS derivative	96.9
20	12.182	uracil, 2TMS derivative	94.79
21	12.226	2-butenedioic acid, (E)-, 2TMS derivative	96.4
22	12.357	2-ketobutyric acid, TMS derivative	78.92
23	12.522	serine, 3TMS derivative	93.74
24	12.639	alanine, 2TMS derivative	71.53
25	12.836	homoserine, 3TMS derivative	73.83
26	12.887	L-threonine, 3TMS derivative	97.76
27	13.117	glycerol, 3TMS derivative	81.24
28	13.267	sebacic acid, 2TBDMS derivative	65.02
29	13.438	malic acid 1-ethyl ester, 2TMS	84.97
30	13.625	malic acid, 4-ethyl ester, 2TMS	87.99
31	13.785	citrulline, 4TMS derivative	65,37
32	13.946	2-Aminomalonic acid, N,O,O,-TMS	80.92
33	14.081	L-Aspartic acid, 3TMS derivative	73.83
34	14.187	malic acid, 3TMS derivative	92.54
35	14.497	erythritol, 4TMS derivative	90.02
36	14.567	L-5-oxoproline,, 2TMS derivative	81.45
37	14.684	5-methylcytosine, 2TMS derivative	62.31
38	14.907	L-norleucine, 2TMS derivative	65.47
39	14.987	L-cysteine, 3TMS derivative	70.42
40	15.425	L-asparagine, 2TMS derivative	87.29
41	15.673	L-ornithine, 3TMS derivative	73.24
42	15.743	L-glutamic acid, 3TMS derivative	90.56
43	15.882	tyramine, 2TMS derivative	79.1
44	16.218	D-arabinopyranose, 4TMS derivative (isomer 2)	87.14
45	16.247	homocysteine, 3TMS derivative	79.53
46	16.276	Beta-D-(+)-xylopyranose, 4TMS derivative	76.31
47	16.382	asparagine, 3TMS derivative	89.76
48	16.671	3-hydroxypicolinic acid, 2TMS derivative	70.31
49	16.722	L-lysine, 3TMS derivative	80.5
50	17.039	ribitol, 5TMS derivative	84.15
51	17.094	arabinitol, 5TMS derivative	88.3
52	17.375	D-(+)-ribono-1,4-lactone (R,S,R)-, 3TMS derivative	73.22
53	17.591	Meso-erythritol, 4TMS derivative	72.75
54	18.201	L-(-)-sorbofuranose, pentakis(trimethylsilyl) ether	74.76
55	20.414	D-glucitol, 6TMS derivative	94.35
56	20.611	D-xylopyranose, 4TMS derivative	82.9

Table 1. Qualitative chromatographic analysis of *P. impudicus* fruiting body extract (cont.).

Lp.	RT	Name	Probability of occurrence [%]
57	21.276	D-mannose, 5TMS derivative	94.69
58	21.451	D-xylose, 4TMS derivative	82.31
59	21.546	D-lyxose, 4TMS derivative	88.03
60	22.112	D-ribose, 4TMS derivative	78.62
61	23.716	Scyllo-inositol, 6TMS derivative	81.89
62	26.177	cystathionine, 4TMS derivative	78.63
63	29.625	oleamide, TMS derivative	79.06
64	30.103	D-(+)-glucosamine, 4TMS derivative	71.7
65	30.585	uridine, 3TMS derivative	77.31
66	33.569	levoglucosan, 3TMS derivative	74.58
67	33.879	adenosine, 4TMS derivative	82.26
68	36.462	D-(-)-fructopyranose, 5TMS derivative (isomer 2)	75.38
69	38.547	D-(+)-cellobiose, (isomer 1), 8TMS derivative	90.4
70	40.198	D-psicopyranose, 5TMS derivative (isomer 1)	72.3

concentrations. As the MTT test correlates well with cell proliferation, it can be concluded that the ethanolic extract of *P. impudicus* stimulates fibroblast proliferation. However, as its concentration increased, there was significant inhibition of this process.

Effects on DNA Synthesis

Increased DNA synthesis by fibroblasts and activation of their proliferation are prerequisites for successful wound healing. DNA synthesis was rapidly activated in fibroblast cells already incubated with a minimal amount of ethanol extract of *P. impudicus* (0.05%), increased with the next dose (0.1%), and was then rapidly inhibited when incubated with the 0.2% dose. Nevertheless, these changes, despite a clear trend, were not statistically significant.

Effects on Collagen Synthesis

Changes in collagen biosynthesis were reflected by differences in the amount of isotopically labelled proline incorporated into the collagen fibers. The results showed that the addition of ethanol extracts at a concentration of 0.1% increased collagen biosynthesis, while the use of an ethanol extract at a concentration of 0.01% impaired collagen biosynthesis.

Effects of P. Impudicus Ethanolic Extract on Mechanical Wound Healing

Animals with blood glucose levels above 200 mg/dL were eligible for the experiment. The overall parameters are presented in Table 2. In all study groups, animal weight was statistically significantly lower ($p > 0.01$) than in the control group, while glucose levels were statistically significantly higher ($p > 0.01$) than in the control group, ranging from 296 ± 26 to 307 ± 19 mg/dL (Table 2). Karnosil gel was used as a positive control. It is an active medicinal cosmeceutical that accelerates skin regeneration. Thanks to its precise, topical application, the hydrogel forms an occlusive layer that inhibits water evaporation evaporation and reduces water loss through the epidermis. Karnosil is excellent for the

Table 2. General parameters

	Animal numbers [n]	**Body weight** [g]	**Glucose** [mg/dL]
Negative control	12	398 ± 16	89 ± 7
Positive control (Karnosil)	12	312 ± 25**	307 ± 19***
Product prototype (hydrogel) *P. impudicus*	10	304 ± 36**	301 ± 24***
DMSO + substrate	8	289 ± 26**	298 ± 17***
DMSO + product prototype	7	315 ± 32**	296 ± 26***

**$p < 0.01$, ***$p < 0.001$ vs negative control

care, protection and maintenance of irritated skin exposed to: abrasions, wounds, cuts, burns, scalds (including sunburn), bedsores.

Karnosil's active formula effectively promotes skin regeneration thanks to silver ions and carnosine. Thanks to its occlusive layer, it protects the skin from the adverse effects of external factors as well as factors of bacterial and fungal origin. The d-panthenol and allantoin contained in Karnosil soothe irritations and redness, reduce itching, provide soothing relief, and leave the skin in good condition. The innovative hydrogel formula stimulates rapid skin regeneration. Karnosil is also recommended for diabetics, for use on skin at risk of damage. Topical application of Karnosil is ideal for the care of affected areas in people who frequently develop sores and bedsores.

Its indications include care for, protection from, and maintenance of the good condition of irritated skin exposed to abrasions, wounds, cuts, burns, scalds (including sunburn), and bedsores.

A hydrogel containing an extract of the fungus *P. impudicus* was shown to accelerate the period of wound desquamation in the experimental animals compared to the control groups. The onset of scab rejection in animals receiving hydrogel + DMSO occurred on day nine, and in animals in the control groups (control, Karnosil) on days 11 and 9-–10, respectively. Complete rejection of the scab in the animals receiving hydrogel + DMSO occurred on day 16, and on days 17-–20 in the control group animals. In animals receiving hydrogel + DMSO, full wound healing occurred at approximately the same time, that is, on day 18, while in animals of the control groups, full wound healing occurred on days 18–20 (Table 3).

A hydrogel containing an extract of the fungus *P. impudicus* was shown to reduce wound size during healing in a statistically significant manner in experimental animals compared to the control groups. The wound size of animals in the control group was 80.2 ± 55.1 mm after 10 days and decreased to 34.4 ± 10.5 mm after 20 days. While in animals treated with hydrogel containing an extract of the fungus *P. impudicus*, the wound size was already nearly 30% smaller after 10 days of healing compared to the control group, and after a further 10 days, it decreased by a further 28% and on the 20th day of healing and was 16.0 ± 4.3 mm (Table 4).

Collagen is one of the main proteins found in humans and animals, accounting for approximately 80% – 85% of the extracellular matrix of the dermis. The physical properties of collagen provide the skin with the tensile strength, integrity, and structure of

Table 3. Effects of a hydrogel containing an extract of the fungus *P. impudicus* on wound healing parameters.

	Negative control	**Positive control (Karnosil)**	**Product prototype (hydrogel) *P. impudicus***	**DMSO + substrate**	**DMSO + product prototype**
Beginning of epidermis, days	5.7 ± 0.4	5.1 ± 0.3	5.0 ± 0.4	5.3 ± 0.4	4.7 ± 0.2
Onset of scab rejection, days	10.9 ± 0.4	9.5 ± 0.3*	10.0 ± 0.4	10.5 ± 0.7	9.0 ± 0.7*
Complete rejection of scab, days	20.3 ± 1.1	17.3 ± 0.7	18.2 ± 1.0	18.6 ± 1.1	16.7 ± 0.5*
Complete healing, days	22.6 ± 1.2	18.9 ± 0.7	20.3 ± 1.3	20.9 ± 1.3	18.5 ± 0.6

*$p < 0.05$ compared to the negative control

Table 4. Effects of a hydrogel containing an extract of the fungus *P. impudicus* on changes in relative wound size (wound shrinkage).

Group	**Time after injury, days**	
	10	**20**
Negative control	80.2 ± 5.1	34.4 ± 10.5
Positive control (Karnosil)	59.6 ± 7.8[ab]	16.5 ± 3.8[ab]
Product prototype (hydrogel) *P. impudicus*	66.0 ± 3.6[ab]	19.0 ± 6.5[ab]
DMSO + substrate	73.3 ± 4.8	33.6 ± 8.3
DMSO + product prototype	57.2 ± 4.2 [ab]	16.0 ± 4.3[ab]

[a]$p < 0.05$ compared to the negative control;

[b]$p < 0.05$ compared to the DMSO+substrate.

normal and remodeled skin, such that accurate measurements of collagen levels can be used to assess the effectiveness of the wound healing process.

A hydrogel containing an extract of the fungus *P. impudicus* was shown to increase the concentration of collagen in the remodeled tissue of a healing wound. In the control group, the collagen concentration was 21.30 ± 1.38 mg/g tissue, whereas in the group treated with hydrogel containing *P. impusicus* fungus extract, the collagen concentration in the healing, remodeled tissue was significantly higher ($p > 0.05$) at 28.40 ± 1.57 mg/g tissue (Table 5).

Considered one of the most important antioxidant enzymes, catalase helps remove the toxic by-products of reactive oxygen species. In animals treated with a hydrogel containing an extract of the fungus *P. impudicus*, a slight reduction in catalase activity from 28.4 ± 2.1 units/mg protein to 25.0 ± 0.71 units/mg protein was observed, which may indicate its consumption in the process of healing and tissue remodeling. In contrast, for the other enzymes, SOD, GSH, and GSSG, the changes in enzyme activity were not significant (Table 6). The lack of changes in SOD activity was also evident in the absence of changes in the concentration of lipid peroxidation end products (TBARS) in the remodeling tissue compared to the control group (Table 7).

Table 5. Effects of a hydrogel containing an extract of the fungus *P. impudicus* on the collagen content of the remodeled skin at the wound site, mg/g tissue.

Group	**Collagen content**
Negative control	21.30 ± 1.38
DMSO + substrate	23.82 ± 1.03
Product prototype (hydrogel) *Phallus impudicus*	25.71 ± 1.48[a]
DMSO + product prototype	28.40 ± 1.57[ab]

[a]$p < 0.05$ compared to the negative control;

[b]$p < 0.05$ compared to the DMSO + substrate.

Table 6. Effects of a hydrogel containing an extract of the fungus *P. impudicus* on selected biochemical parameters in remodeled skin.

Group	**SOD** unit./mg protein	**Catalase** unit./mg protein	**GSH** nmol /mg protein	**GSSG** nmol /mg protein
Negative control	13.0 ± 0.9	28.4 ± 2.1	8.8 ± 0.6	1.2 ± 0.2
Positive control (Karnosil)	17.8 ± 0.6[a]	22.5 ± 0.5[a]	6.0 ± 0.4[a]	1.4 ± 0.3
DMSO + substrate	13.7 ± 0.7[b]	23.5 ± 0.8[a]	6.1 ± 0.3[a]	1.0 ± 0.3
Product prototype (hydrogel) *P. impudicus*	12.7 ± 0.5[b]	23.3 ± 1.0[a]	7.6 ± 0.7	0.9 ± 0.3
DMSO + product prototype	13.1 ± 0.5[b]	25.0 ± 0.71	7.8 ± 0.8	0.9 ± 0.2

[a]$p < 0.05$ compared to the negative control;

[b]$p < 0.05$ compared to the positive control.

Table 7. Effects of a hydrogel containing an extract of the fungus *P. impudicus* on the content of lipid peroxidation end products (TBARS) in remodeled skin.

Group	**TBARS** nmol/mg protein
Negative control	0.54 ± 0.04
Positive control (Karnosil)	1.00 ± 0.07[ac]
DMSO + substrate	0.80 ± 0.08[ab]
Product prototype (hydrogel) *P. impudicus*	0.58 ± 0.04[bc]
DMSO + product prototype	0.54 ± 0.02[bc]

[a]$p < 0.05$ compared to the negative control;

[b]$p < 0.05$ compared to the positive control);

[c]$p < 0.05$ compared to the DMSO + product prototype.

DISCUSSION

Skin wound healing is a multiphase process involving the regeneration of damaged tissues. The proliferative phase, which includes the processes of epithelization, granulation, and contraction, is traditionally chosen to study the properties of wound healing with pharmacological preparations in the experiment. These processes require the involvement of several cell types, of which fibroblasts play a leading role. Fibroblasts are involved in the key processes of normal wound healing by participating in the formation and contraction of new extracellular matrix, mainly in the form of collagen structures. Fibroblasts condition the contraction process by producing and releasing an extracellular matrix that contracts to bring the wound edges closer together. In an in vitro study, we demonstrated that the extract of *Phallus impudicus* is non-toxic to fibroblast cultures and stimulates their proliferation and DNA synthesis.

In a full-thickness skin wound model, *P. impudicus* showed high pharmacological potential, as evidenced by the reduction in contraction and epithelization time, as well as the initiation of scab formation and rejection. In this case, the therapeutic efficacy of the plant extract was superior to that of the classical extract, which has clear antimicrobial properties and is widely used in the clinic for wound care [33]. In recommending standard use ointment for wound surface treatment, it should be emphasized that this antimicrobial agent at bactericidal concentrations can be unchanged, and in some cases enhance fibroblast proliferation and stimulate fibroblast proliferation and DNA synthesis [33]. It should be noted that the wound-healing activity of pharmacological substances is influenced by many factors, including the ability to inhibit microbial infection and inhibit oxygen radical production, as well as immunomodulatory and anti-inflammatory properties. In the current literature, there are indications of the biological activity of *P. impudicus* and its use to treat wound and skin infections.

The healing activity of plant extracts is attributed to the synergistic action of chemical compounds and their metabolites contained in them. Gas chromatographic-mass spectrometric analysis (GC-MS) performed in the present study revealed the presence of a significant amount of compounds with wound-healing activity in the extract of *Phallus impudicus*. The predominant compound in the extract is D-mannose, which arrests the inflammatory response and reduces granulation tissue formation in an experimental wound model in rats [14], and citric acid, whose wound-healing promoting activity is due to its ability to bind active oxygen radicals on the wound surface [30].

Glucan is a potent macrophage stimulant that occurs in *P. impudicus* extract, similar to levoglucosan. There is some evidence that glucans mediate effects through activation of macrophages, neutrophils, natural killer (NK) cells, and lymphocytes. Moreover, it has been reported that β-glucans have immunostimulatory activity and enhance wound healing by increasing macrophage infiltration into injury sites and stimulating tissue regeneration [34]. Moreover, β-glucans directly increase the synthesis of types I and III collagen [35, 36], stimulating collagen regeneration and facilitating wound healing. We also found that the collagen content of the remodeled skin increased when patches containing *P. impudicus* extracts were used.

In many studies, the wound healing properties of mushrooms have been associated with their rich polysaccharide content. Polysaccharides from many different species of mushrooms are potent immune-modulating agents [37]. Polysaccharides isolated from *P. gilvus* reduced wound contraction and enhanced the re-epithelialization of 6-mm circular wounds in streptozotocin (STZ)-induced diabetic rats by topical application twice a day for five days [38]. It was also shown that the polysaccharide fraction of *P. impudicus* is involved in immunomodulatory activity, since it proved to be effective in enhancing phagocytosis and is useful in the treatment of immunodeficiency accompanied by different diseases. Their immunomodulatory effects are mediated by various immune cells, such as macrophages and T lymphocytes [39]. It is worth noting that the local use of *P. impudicus* polysaccharides on full-thickness cutaneous wounds could enhance healing with regard to epithelialization, contraction, and growth of granulation tissue. In our study, the *P. impudicus* extract showed the same properties. If we take into account the fact that the D-ribose present in the extract—a five-carbon simple sugar, a structural element of ribonucleosides, ribonucleotides, some coenzymes and vitamins, which has a moisturizing effect due to its ability to bind water—determines the correct and faster wound healing process in a moist environment according to Winter's theory [40].

Proteins can effectively accelerate wound healing and improve the nutritional status of an individual. The potential mechanism may be related to the acceleration of the entire process of wound healing, including the inflammation, proliferation, and remodeling phases. Wada et al. found that administering peripheral parenteral nutrition (PPN) solutions

containing amino acids immediately after surgery was effective for wound healing [41].

The extract of *P. impudicus* also contains amino acids that can affect wound healing processes at every stage of healing.

L-proline is an endogenous amino acid. It has an unusual cyclic structure, which means that the amino group is attached to the side chain, making it a secondary amine, thus creating a ring structure. Proline plays an important role in shaping the protein structure. It is a key component of collagen production. An interesting property of l-proline is its ability to bind water.

L-lysine is an organic chemical compound and an essential amino acid that is not synthesized in the human body. Lysine has been shown to participate in cross-linking between the three polypeptides in collagen, which results in its stability and tensile strength. It has a nourishing effect and enhances the activity of antioxidants.

Glycine is the simplest of the 20 standard protein amino acids. Due to its structure, glycine, unlike other amino acids, is not optically active. Glycine constitutes nearly one-third of all the amino acids that form collagen. Glycine is also a component of keratin in the hair. It is highly valued in modern cosmetics because it prevents the aging process by stimulating natural repair processes. Glycine is one of the most effective builders of collagen; therefore, it has a rejuvenating effect on the skin.

L-cysteine is an organic chemical compound derived from a group of endogenous amino acids. It forms a group of sulfur amino acids. Cysteine has the ability to stimulate collagen and elastin production. It accelerates wound healing and the cell renewal process.

β-alanine is a naturally occurring amino acid in which the amino group is attached to the β carbon. It has the ability to maintain and retain moisture in the skin.

α-Glutamic acid is a chemical compound derived from a group of amino acids with an acidic character. It is one of the most important neurotransmitters. It has the ability to bind moisture from the environment. It also possesses antioxidant properties.

Some of these compounds are antioxidants that interact with a wide range of oxygen and nitroxyl free radicals, thus capturing them and interrupting the pathological chain of oxidative stress and preserving cell life. In addition, plant polyphenols can inhibit the production of these radicals and enhance antioxidant defense activity. It is known that under normal physiological conditions, the wound healing process is directly influenced by the levels of oxygen free radicals, and reducing their levels and increasing their antioxidant activity promotes accelerated wound healing. Moreover, most of these compounds exhibit antioxidant and anti-inflammatory properties by effectively inhibiting the expression of anti-inflammatory cytokines and the activity of enzymes involved in eicosanoid production, which may contribute to shortening the inflammatory phase of wound healing. Some of them, such as amino acids, can improve the main building blocks for tissue growth, cell renewal, and repair throughout the wound healing process. Proteins also significantly affect the entire process of wound healing through their roles in RNA and DNA synthesis, collagen and elastic tissue formation, immune system function, epidermal growth, and keratinization. Thus, the results obtained in the present study indicate the high pharmacological potential of the topical application of *P. impudicus* extract, contributing to an increase in the rate of skin contraction and epithelization period in an experimental model of total mechanical wounds in rats. The positive effect of the studied extract on the proliferative phase of the wound healing process was realized through its antioxidant, antimicrobial, and immunomodulatory properties.

CONCLUSIONS

1. The study confirms that the extract of this mushroom may be useful in the treatment of diabetic wounds, which opens new avenues for the development of natural therapeutic agents.
2. The preparation containing *P. impudicus* extract demonstrated the ability to accelerate wound healing and desquamation in diabetic rats compared to the control groups.
3. Metabolites such as amino acids, sugars, and organic acids were identified in the ethanolic extract that promote healing processes, which confirms the biological activity of the extract.
4. The statistically significant reduction in SOD activity indicates that the extract may help reduce oxidative stress, which is an important aspect in the healing process.
5. Additional studies are needed to evaluate the safety and efficacy of *P. impudicus* extract in clinical practice and to understand its mechanisms of action.

These findings may serve as a basis for further studies and discussions on the medical applications of *P. impudicus* extract, especially in the context of the treatment of diabetic wounds and other related diseases.

Conflict of Interest

The authors declare no conflicts of interest.

Author's Contribution

A – Research concept and design: A.Z.;
B – Collection and/or assembly of data: A.Z., N.K., P.K.;
C – Data analysis and interpretation: A.Z., N.K.;
D – Writing the article: A.Z., N.K.;
E – Critical revision of the article: A.Z., P.K.;
F – Final approval of the article: A.Z.

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