

Skin Wound Healing Promoting Effect of Polysaccharides Extracts from *Tremella fuciformis* and *Auricularia auricula* on the *ex-vivo* Porcine Skin Wound Healing Model

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Abstract. In this study we focused on the wound healing promoting effect of polysaccharides purified from *Tremella fuciformis* and *Auricularia auricula* by using the *ex-vivo* porcine skin wound healing model (PSWHM) as a tool for wound healing evaluation due to human ethics and animal right concerns, and more practical and high throughput experiment. Using previously reported protocol with modifications, purified polysaccharides from *A. auricula* and *T. fuciformis* were obtained at 0.84 and 2.0% yields (w/w), 86.60 and 91.22% purity, respectively, with small amounts of nucleic acid and protein contamination. The PSWHMs (3mm circular wound) were divided into five groups, each group (n=22) was treated with one of the following concentrations of polysaccharides extracts (1, 10 and 100µg/wound of *T. fuciformis* or *A. auricula*) or control solutions (10µl 10mM PBS), or 10µl 25ng/ml EGF (internal control). Then the treated PSWHMs were cultured at 37°C with 5% CO₂ for 48 hours before histological and microscopic evaluation. Epidermal or keratinocyte migration distances from the edges of each wound were measured, normalized with the PBS control group and expressed as mean%. The results clearly showed that both purified polysaccharides extracts promoted statistically significant wound healing effect (ANOVA, at *P*-values < 0.05) at the concentrations 100µg per wound for *A. auricula* (140.43%) and at 1µg per wound for *T. fuciformis* (125.38%) among three different concentrations tested for each extract.

Keywords: Wound healing, *ex-vivo* model, *Tremella fuciformis*, *Auricularia auricula*, Polysaccharides

1. Introduction

Wound healing *in-vivo* is a complex process comprising different phases: inflammation, granulation, tissue formation including re-epithelialisation, matrix formation and remodelling with the interplay of various cell types and cytokines^[1]. Epidermal regeneration is an important part of cutaneous wound healing, providing the closure of the wound and restoration of skin functions. It is a concerted process involving the proliferation and migration of keratinocytes at the wound margins, the differentiation of keratinocytes in the regenerated epidermis and a variety of interactions with components of the dermis^[1, 2]. Wounds cause discomfort and are more prone to infections. So, if any remedies can accelerate skin wound healing process, it would be benefit for clinical uses especially for diabetic patients.

Pig has been demonstrated as a human comparable *in-vivo* model because of more anatomical and physiological similarity to humans than other animals^[2]. Due to human ethics and animal right concerns, and more practical and high throughput experiments, we decided to use the *ex-vivo* porcine skin wound healing model (PSWHM) as an evaluation tool for wound healing bio-active compounds.

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Auricularia auricula (wood ear, Jews ear, jelly ear fungus, Auriculariaceae) is an edible black–brown mushroom with high content of carbohydrates (approximately 63% of dried fruit bodies), proteins and minerals (Ca, P, and Fe). The main monosaccharide composition of *A. auricula* polysaccharides is glucose (72%), mannose (8%), xylose (10%) and fucose (10%) [3]. The backbone chain of the polysaccharides is (1→3) β-D-glucans. In recent decades, *A. auricula* polysaccharide was found to have potential biological activities such as antioxidant [4], hypoglycemic, hypolipidemic, anti-inflammatory [3], anticoagulant activities [5], etc.

Tremella fuciformis (silver ear fungus, white jelly fungus, Tremellaceae) has been appreciated as an edible mushroom. The chemical structure of the polysaccharides consists of a linear backbone of (1→3) α-D-mannan with the side chains composed of glucuronic acid, xylose and fucose. The ratio of mannose, fucose, xylose, and glucuronic acid is 9:1:4:3 that makes glucuronic acid accounted for 17.6% of total polysaccharides [6, 7]. *T. fuciformis* polysaccharides also has been studied for its medicinal purposes such as anti-oxidant [6], hypoglycemic, memory improvement, suppressing fat accumulation, lowering plasma cholesterol, etc.

There are many pharmacological studies using both *in-vitro* and *in-vivo* models of polysaccharides isolated from fungi, but only several studies focused on the wound healing promoting effect of the fungal polysaccharides. Bae *et al.* (2005) demonstrated that polysaccharides isolated from *Phellinus gilvus* enhance dermal wound healing in streptozotocin-induced diabetic [8] and normal rats [9]. Kwon *et al.* (2009) also reported the positive effects of *Sparassis crispa* on wound healing in streptozotocin-induced diabetic rats [10]. Gao *et al.* (2004) reported that *Ganoderma lucidum* polysaccharide fractions accelerated healing of acetic acid-induced ulcers in rats [11]. In this study we focused on the wound healing promoting effect of polysaccharides purified from *T. fuciformis* and *A. auricula* by using PSWHM.

2. Materials and Methods

2.1. Polysaccharides Extraction and Purification

The polysaccharides of *T. fuciformis* and *A. auricula* were isolated by the method suggested by Yoon *et al.* (2003) with some modifications until purified polysaccharides obtained. Briefly, *A. auricula* and *T. fuciformis* were initially dried at 70°C and ground using mortar. Dried powders were suspended and refluxed in methanol. The pellets were collected, suspended and refluxed in sterile water. Clear supernatants were collected after low speed centrifugation. The polysaccharides in the concentrated supernatant were precipitated with absolute ethanol, subsequently re-suspended in sterile water, and dialyzed by using 10kDa cut off membrane. The purified solutions were finally lyophilized [5]. Protein contamination in polysaccharides extracts were analyzed by bicinchoninic acid (BCA) protein assay and SDS-PAGE. Nucleic acid contamination was measured by UV absorbance at 260nm. The content of polysaccharides in the extracts was determined by Phenol-Sulfuric acid method [12].

2.2. Wound Healing Efficacy of *A. auricula* and *T. fuciformis* Polysaccharides on PSWHM

Porcine ears were obtained from a local slaughterhouse, were washed and disinfected with PBS and 70% ethanol. Biopsy punch (diameter 6mm) was used to obtain circular porcine skins from the areas between the ridges of the ear. Subsequently, small circular wound (diameter 3mm) was generated on each excised skins by removing epidermis and upper dermis from the center using 3mm biopsy punch under sterile condition. The PSWHMs were divided into five groups for each purified mushroom polysaccharides extract, each group (n=22) was treated with 10μl of one of the following extracts or control solutions; 0.1, 1 and 10mg/ml *A. auricula* or *T. fuciformis* polysaccharides extracts, 10mM PBS (as control), or 25ng/ml EGF (as an internal control). The wound healing models were then incubated with 5% CO₂ at 37°C for 48 hours. After incubation, all wound models were collected, snap-frozen, and stored at -80°C. The wounds were cryo-sectioned at 8μm thick (until the middle of the wound was reached). Next, the sections were stained with hematoxylin/eosin by standard method. Afterwards, all sections were histologically evaluated by microscopy. Epidermal or keratinocyte migration distances from the edges of each wound were measured. The migration was normalized with the PBS control group and expressed as mean% ±SE. Statistical analysis was

performed using One-way analysis of variance (ANOVA) followed by Tukey's multiple range test, *P*-values < 0.05 are considered to be statistically significant. Analysis was done with SPSS 16.0.

3. Results and Discussion

3.1. Polysaccharides Extraction and Purification

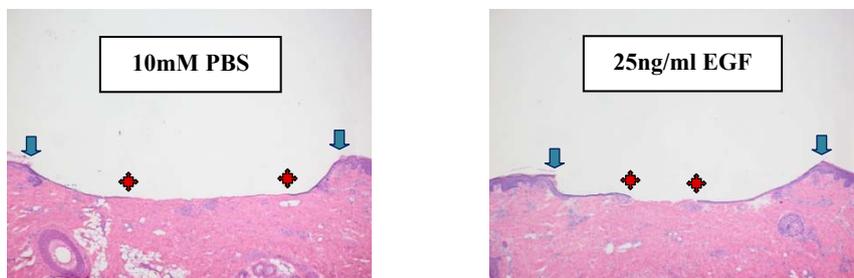
Table 1: Characteristics of the purified polysaccharides extracts from *A. auricula* and *T. fuciformis*

Characteristics	<i>A. auricula</i>	<i>T. fuciformis</i>
Appearance	White sponge	White sponge
%yield (w/w, dry weight)	0.84%	2.0%
Protein contamination		
- BCA protein assay	1.98%	3.02%
- SDS-PAGE	< 0.25%	< 0.25%
Nucleic acid contamination	0.79%	3.02%
Polysaccharides determination	86.60%	91.22%

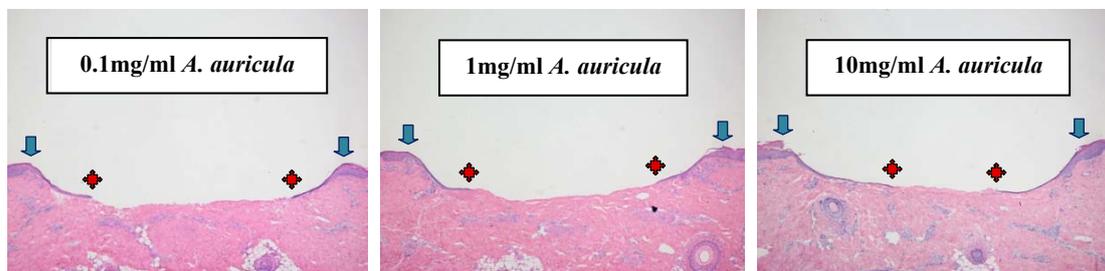
By using the protocol mentioned above, the purified polysaccharides white sponge-like powders from *A. auricula* and *T. fuciformis* were obtained with the yields 0.84 and 2.0%, respectively (Table 1). The polysaccharides content in purified extracts of *A. auricula* was 86.60% and in *T. fuciformis* extract was 91.22%. The protein contamination in both polysaccharides extracts was less than 0.25% by SDS-PAGE analysis. Notably, using BCA protein analysis method resulted in a higher amount of protein content. The reducing ability of some polysaccharides in the purified extracts may cause this interference. The nucleic acid contamination was 0.79% in purified extracts of *A. auricula* and 3.02% in *T. fuciformis* extracts.

3.2. Wound Healing Efficacy of *A. Auricula* and *T. fuciformis* Polysaccharides Extracts on PSWH

Control group



Polysaccharides from *A. auricula* extract (Treatment groups)



Polysaccharides from *T. fuciformis* extract (Treatment groups)

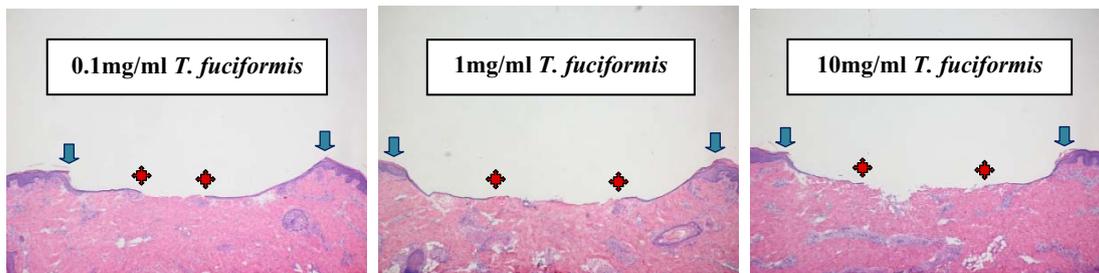


Fig. 1: Histology of PSWHMs (hematoxylin&eosin, 4X). Effect of three different doses (0.1, 1 and 10 mg/ml, 10 μ l per wound) of *A. auricula* and *T. fuciformis* purified polysaccharides extracts on epidermal layer migration of PSWHM after 48 hours exposure. 10mM PBS was used as control and 25ng/ml EGF was used as positive internal control. Arrows indicate the edges of the wounds, and stars mark the tip of epidermal layer migration.

Histological study in Fig. 1, all wounds treated in control and various treatment groups were sound, with no sign of apoptosis, necrosis or bacterial contamination indicating the validity of PSWHM model and no toxicity of the purified extracts at tested concentrations. Morphology of wound margins, epidermis and dermis layers were normal. The fibroblast cells set firmly and normal. Keratinocyte or epidermal layer migration in each side of the wound was measured from the edge of the wound (shown as arrow) to the migration tip (shown as star). The efficacy of wound healing promoting effect of *A. auricula* and *T. fuciformis* purified polysaccharides extracts was expressed as normalized epidermal migration (%means \pm SE) against PBS control group (Fig. 2 and 3).

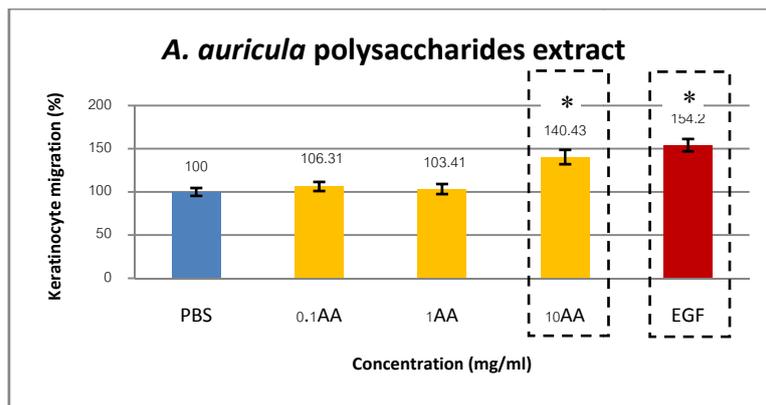


Fig. 2: Histograms and ANOVA analysis. Effect of three different doses (0.1, 1 and 10 mg/ml, 10 μ l per wound) of *A. auricula* purified polysaccharides extracts (AA) on epidermal layer migration of PSWHM after 48 hours exposure. 10mM PBS was used as control and 25ng/ml EGF was used as positive internal control. Values shown on histograms represent the normalized %means \pm SE. (n=22; * $P < 0.05$).

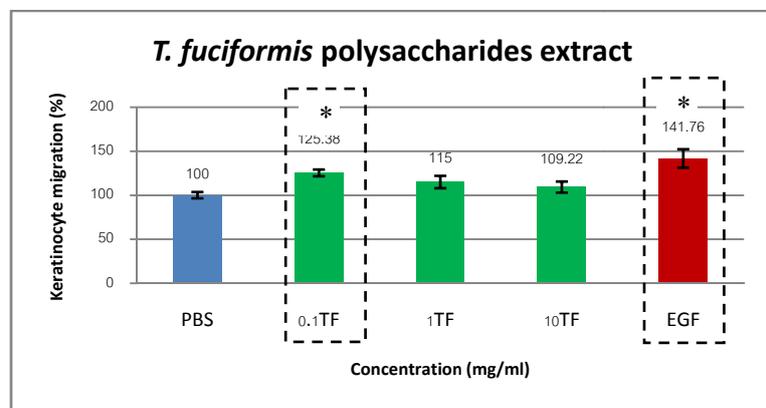


Fig. 3: Histograms and ANOVA analysis. Effect of three different doses (0.1, 1 and 10 mg/ml, 10 μ l per wound) of *T. fuciformis* purified polysaccharides extracts (TF) on epidermal layer migration of PSWHM after 48 hours exposure. 10mM PBS was used as control and 25ng/ml EGF was used as positive internal control. Values shown on histograms

represent the normalized %means \pm SE. (n=22; * $P < 0.05$).

The results in Fig. 1 and ANOVA analysis in Fig. 2, showed that only the wounds treated with 10 μ l of 10mg/ml of polysaccharides extract from *A. auricula* (10AA group) presented the highest and significant efficacy among the three concentrations of the extract. The results indicated that the purified polysaccharides extract from *A. auricula* could increase epidermal migration up to 40% when compared to 10mM PBS (140.43%, n=22, $P < 0.05$) comparable to the wounds treated with 10 μ l of 25ng/ml EGF (154.20%, n=22), which was used as a positive internal control.

Fig. 1 and ANOVA analysis in Fig. 3, the wounds treated with 10 μ l of 0.1mg/ml of polysaccharides extract from *T. fuciformis* (0.1TF group) showed the highest and significant efficacy among three concentrations of the extract. The result demonstrated that the purified polysaccharides extract from *T. fuciformis* could increase epidermal migration up to 25% when compared to 10mM PBS (125.38%, n=22, $P < 0.05$) whereas the wounds treated with 10 μ l of 25ng/ml EGF, which was used as a positive internal control, could increase the effect up to 42% (141.76%, n=22). Interestingly, the epidermal migration efficacy of purified extract of *T. fuciformis* in lowest concentration group was higher than the other two more concentrated groups. One possible explanation is that the purified extract might contain certain substances or other different polysaccharides that could neutralize wound healing effect, but not cause significant harm to the wound since at higher concentrations of the purified extracts still had some activity (15% and 9%).

4. Conclusion

PSWHM, the *ex-vivo* wound healing model developed in our laboratory, used in this study, demonstrated satisfactory results for wound healing bioactive compound screening. The method for polysaccharides purification from *A. auricula* and *T. fuciformis* was developed successfully. The purified polysaccharide extracts from *A. auricula* and *T. fuciformis* were approximately achieved at the yields of 0.84 and 2.0% w/w of raw dried mushrooms, and with the purity of polysaccharides at 86.60 and 91.22% respectively with small amount of nucleic acid and protein contamination. Both purified polysaccharides extracts showed statistically significant promotion of wound healing effect at 100 μ g per wound (10 μ l of 10mg/ml) for *A. auricula* and at 1 μ g per wound (10 μ l of 0.1mg/ml) for *T. fuciformis* among three different concentrations (1, 10, 100 μ g per wound) tested in each extract. These inconsistent results may due to the different amounts and types of polysaccharides in both extracts (glucans and mannans, side chains, monosaccharide composition) and may also due to other ingredients or different polysaccharides that may contain negative wound healing property. Therefore, further in-depth investigation for their quantity and quality of polysaccharides and their clinical use is necessary.

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