European Journal of Pharmaceutics and Biopharmaceutics 210 (2025) 114684

Contents lists available at ScienceDirect

European Journal of Pharmaceutics and Biopharmaceutics

journal homepage: www.elsevier.com/locate/ejpb

Research paper

Breaking the virus: Yeast glucans as an effective alternative to acyclovir in HSVI treatment

Diana Tavares-Valente [a,b], Helena Moreira [a,b], Pedro Sousa [a], Manuela Amorim [a], António Conde [c], Manuela Pintado [a], João Fernandes [a,b], João Azevedo-Silva [a,b,*]

[a] *Universidade Católica Portuguesa, CBQF—Centro de Biotecnologia e Química Fina—Laboratório Associado, Escola Superior de Biotecnologia, Rua Diogo Botelho 1327, 4169-005 Porto, Portugal*

[b] *Amyris Bio Products Portugal, Unipessoal Lda, Rua Diogo Botelho 1327, 4169-005 Porto, Portugal*

[c] *Hospital Lusíadas, Av. da Boavista 171, 4050-115 Porto, Portugal*

ARTICLE INFO

Keywords:
HSVI infection
Yeast Glucans
Immune stimulation
Skin *ex vivo* model
Circular economy

ABSTRACT

Glucans, structural polysaccharides in the yeast cell wall, are known for their biological and immunomodulatory capacities, helping in prevention and management of infections. Herpes simplex virus type 1 (HSVI) is a prevalent infection that causes great comorbidity and is challenging to treat due to the adverse effects of standard antiviral drugs like acyclovir. This study assessed the potential of yeast glucans extracted from two different origins – a steviol-glycoside producing strain and a wild-type strain- to circumvent HSVI infection, either *in vitro* and *ex vivo*. Treatment with glucans in keratinocytes and macrophages *in vitro* reduced cell infection similarly to acyclovir. However, unlike acyclovir, glucans demonstrated an immunostimulatory effect, increasing the production of IL-1β, TNF-α and IL-6. Additionally, both glucans were formulated with squalane for skin application. This formulation improved glucans penetration in the skin, restored skin structure and reduced the cytopathic effect of HSVI infection. In summary, this study highlights yeast glucans as a natural therapeutic alternative for HSVI treatment, offering an option with an excellent safety profile. Moreover, using glucans from industrial side-streams promotes a sustainable approach, contributing to the circular economy.

1. Introduction

Glucans are a heterogeneous group of polysaccharides, consisting of D-glucose monomers linked by glycosidic bonds, and are divided into α and β conformation. They are important structural elements of the cell wall or serve as energy storage in bacteria, fungi including yeast, algae, and plants, while they are absent in vertebrate and invertebrate organisms [1]. The structure of glucans diverges between different sources. The most reported consist of glucose polymers with a backbone of (1 → 3)-β –glycosidic bonds and different variation of side chains. Oat β-glucans for instance, have (1–4)-d-glucopyranose units, which are separated every 2–3 units by single β-glucans (1–3) glucose units. β-glucans derived from yeast and fungi typically have a (1 → 3)-β –backbone with a small number of (1 → 6)-β –linked side chains [2,3].

Despite the rich diversity of glucan structures, β-glucans are classified as biological response modulators, and are known to have immunogenic properties [4]. Beside this activity many other biological activities have been reported, including anti-inflammatory [5], anti-infection [6], radioprotective [7], and anti-tumor [8]. They are recognized by the human immune system as non-self molecules, named pathogen-associated molecular patterns (PAMPs) inducing both innate and adaptive immune responses [9]. They are recognized by pattern recognition receptors (PRRs), expressed by many different immune cells acting in upregulation of antigen presentation and costimulatory molecules, activation of complement pathway, chemotaxis and activation of pro-and anti-inflammatory signals [10,11]. The main PRRs involved are Dectin-1, complement receptor (CR3), scavenger receptors (SR), lactosylceramide (LacCer), and toll-like receptors (TLRs), e.g., TLR-2/6, and trigger responses mainly in macrophages, neutrophils, natural killer cells (NKs), and dendritic cells (DCs) *in vitro* [12].

More recently, it was also demonstrated that they have strong antioxidant properties related to the frequent exposition of these polysaccharides to oxidative burst and their competence to buffer against radicals [13] as well as promotion of skin health, protection against ultraviolet irradiation and wound healing ability [14,15].

The World Health Organization estimates that ~67 % of the world's

* Corresponding author at: Universidade Católica Portuguesa, CBQF—Centro de Biotecnologia e Química Fina—Laboratório Associado, Escola Superior de Biotecnologia, Rua Diogo Botelho 1327, 4169-005 Porto, Portugal.

https://doi.org/10.1016/j.ejpb.2025.114684
Received 22 November 2024; Received in revised form 26 February 2025; Accepted 28 February 2025
Available online 2 March 2025
0939-6411/© 2025 The Authors. Published by Elsevier B.V. This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/).

Table 1
Antibodies applied to the immunohistochemistry and immunofluorescence methods. (Ms: mouse; Rb: rabbit).

Antibody		Brand	Reference	Dilution	Host
Primary antibodies	Keratin 10	Abcam (UK)	Ab9026	1:100	Ms
	Keratin 14	Covance (USA)	PRB-160P	1:800	Rb
	HSVI	Dako		Ready to use	Rb
Secondary antibodies	Alexa Fluor 488	Invitrogen (USA)	A21206	1:500	Rb
	Alexa Fluor 594	Invitrogen (USA)	A21203	1:500	Ms

population is infected by HSVI, excluding asymptomatic carriers [16]. Symptoms of HSVI infection vary, from lesions in the oral-facial region ("cold sores"), to herpes keratitis, the leading cause of infectious blindness, to herpes encephalitis, which can be fatal. The viruses establish latent infections in the body, leading to recurrent episodes of symptoms due to stress, immunosuppression, or other stimuli [17]. Antiviral medications, such as acyclovir, valacyclovir, and famciclovir, are commonly used to treat HSV infections, working by inhibiting the replication of virus inhibiting DNA polymerase [18]. These medications can help reduce the severity and duration of symptoms. However, its chronic and indiscriminate use has triggered the emergence of virus resistance and systemic adverse effects, such as neurotoxicity with low therapeutic responses [16]. Therefore, there is much interest in developing new alternative therapies to treat HSVI infections. Compounds of natural origin, such as polysaccharides, where glucans are included, have been demonstrated as promising antiviral agents by presenting low cytotoxicity and high efficacy in their treatment of herpes simplex infections [19].

This work hypothesizes that yeast derived glucans, due to their immunostimulant and skincare properties, might present an efficient topical treatment against HSVI infection. For that, two glucans' extracts obtained from a wild-type yeast and an industrial steviol-glycoside producing yeast strain were evaluated *in vitro* and *ex vivo* for their capacity to counteract HSVI infections and promote skin health.

2. Materials and methods

2.1. Materials

Two glucan extracts obtained from *Saccharomyces cerevisiae* cell wall – a parental CEN.PK strain (WT-Glu) and a steviol-glycoside producing strain from industrial fermentation residue (SG-Glu) were used for this study. The extraction process and characterization are detailed in the Sousa *et al.* [20]. Briefly, SG-Glu has 92 % purity, a molecular weight of 451 KDa and is composed by glucose monomers with a β-(1,3)-linked backbone and β-(1,6)-linked side-chains. WT-Glu has 94 % purity, a molecular weight of 387 KDa and is composed by glucose monomers with a β-(1,3)-linked backbone and β-(1,6)-linked side-chains.

Human herpesvirus 1 (HSVI) (VR-1493) was purchased from American Type Culture Collection (ATCC). Immortalized human keratinocyte cells (HaCaT) were obtained from Cytion (300493). The human leukemia monocytic cell line THP-1 (TIB-202) was purchased from ATCC. Dulbecco's Modified Eagle Medium (DMEM), RPMI 1640, Fetal Bovine Serum (FBS), Newborn Calf Serum (NCBS), 199 V, antibiotic–antimycotic (penicillin–streptomycin–amphotericin B), Gill's hematoxylin and Pierce BCA assay kit were obtained from Thermo Fisher Scientific (Waltham, MA, USA). TC insert, for 12-well plates, PET, transparent, 1 μm were obtained from Sarstedt. Phorbol-12-myristate-13-acetate (PMA), Fluoromount media and Acyclovir were acquired from Sigma-Aldrich (St. Louis, MO, USA). ELISA assay kits were purchased from Biolegend (San Diego, CA, USA). Vectastain Elite ABC-HRP kit and peroxidase substrate DAB were acquired from VectorLabs (Newark, USA). All the other reagents used were analytic grade.

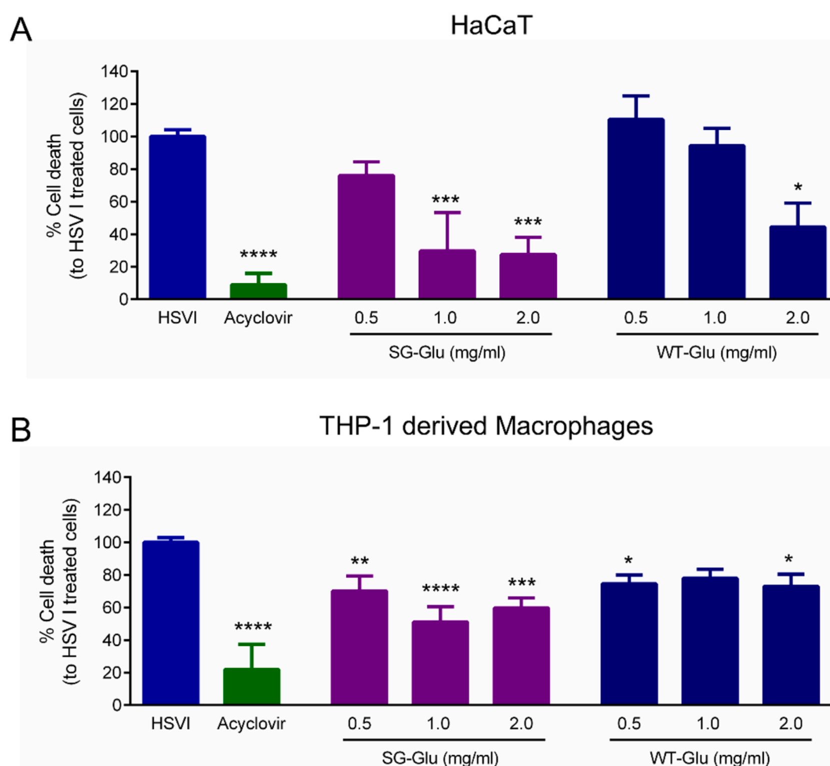


Fig. 1. Metabolic inhibition of HaCaT cells (A) and mTHP-1 cells (B) treated with glucans (SG-Glu and WT-Glu) and acyclovir (0.1 mg/mL). Data are represented as mean ± SD from at least three replicates for each sample. Significant differences between samples and control are indicated as $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$, $^{****}p \leq 0.0001$.

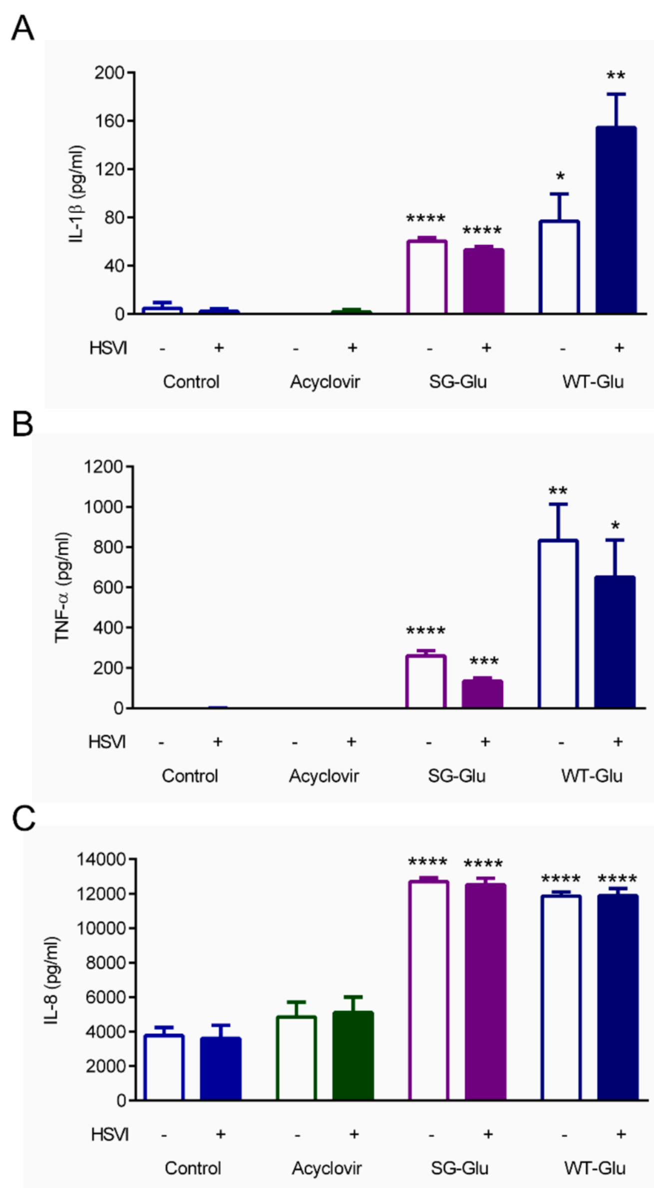


Fig. 2. Cytokine expression upon HSVI infection and treatment with glucans and acyclovir. IL-1β (A), TNF-α (B) and IL-8 (C) production of mTHP-1 cells upon exposure to native glucans (SG-Glu and WT-Glu) and acyclovir. Data are represented as mean ± SD and significant differences between samples and control are indicated as $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$, $^{****}p \leq 0.0001$.

2.2. Cell culture

HaCaT cells were cultured in DMEM supplemented with FBS (10 % v/v) and antibiotic–antimycotic (1 % v/v). THP-1 cells were cultured in RPMI 1640 medium supplemented with 10 % (v/v) FBS, 1 % (v/v) antibiotics and 2-Mercaptoethanol at 0.05 mM. All cultures were maintained in a humified atmosphere at 37 °C with 5 % CO_2. THP-1 cells were used in its differentiated macrophage-like form (mTHP-1). For this, PMA at a final concentration of 50 nM was added immediately before the seeding and cells were incubated for 48 h at 37 °C with 5 % CO_2, to allow differentiation before exposing the cells to the compounds.

2.3. Multiplicity of infection (MOI) determination

HaCaT and mTHP-1 cells were cultured and exposed to different concentrations of HSVI determined according to the following formula (1):

$$\text{MOI} \times (2 \times 10^{7}) \times (1/\text{titer in pfu/ml}) = \text{volume (ml) virus stock needed} \quad (1)$$

Solutions for viral infection were prepared with 199 V medium which contains less serum than cell maintenance medium and is used solely when inoculating cells with virus. Cells were incubated with virus inoculum for 2 h at 37 °C to allow virus absorption. After incubating for 48 h, cell viability was evaluated using the PrestoBlue cell viability reagent (Thermo Fisher Scientific, Waltham, MA, USA).

2.4. HSVI cytopathic effect

HSVI cytopathic effect and the effect of glucans against infection was evaluated using the PrestoBlue cell viability reagent. Briefly, 96-well microplates were seeded with 100 μL of a cell suspension at a concentration of 1.0 x 10^5 cells/mL for HaCaT and mTHP-1 and incubated for 24 h. After incubation, cells were incubated with HSVI at correspondent MOI for 2 h (HaCaT MOI = 0.2 and mTHP-1 MOI = 5). Then, the media with virus was replaced by fresh medium with glucans at different concentrations or acyclovir for 48 h. After incubating for 48 h hours, 10 μL of PrestoBlue was added to each well and allowed to react for 2 h. The cell viability was then measured by fluorescence spectrometry, with an emission and excitation wavelength of 560 nm and 590 nm, respectively.

2.5. Inflammation and cytokine analysis

Briefly, HaCaT and mTHP-1 cells were cultured in a 12-well plate with 1 mL of a cell suspension of (2.5 x 10^5 cells/mL) and exposed to glucans (2 mg/mL), and acyclovir (0.1 mg/mL) after inflammatory stimulus, the HSVI infection. Cytokine quantification was done through enzyme-linked immunosorbent assay (ELISA) kits, according to the manufacturer's instructions. Protein cell content was calculated through Pierce BCA assay kit. All results were converted as pg of IL-1β, TNF-α and IL-8 per μg of protein.

2.6. Crystal violet assay

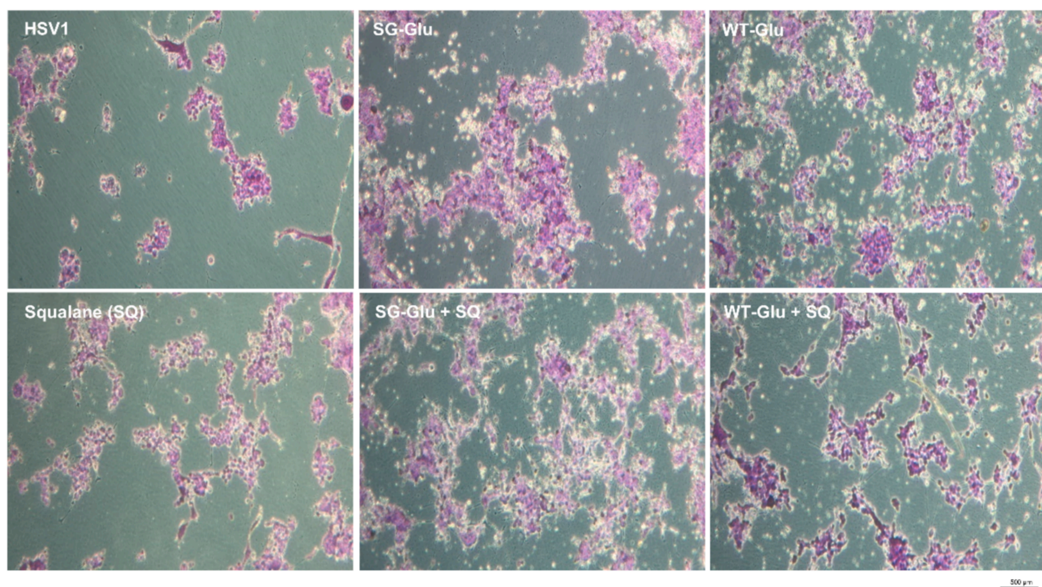
For crystal violet assays, 1.25 x 10^5 cells/mL cells were seeded in 24-well plates and treated with 2 mg/mL of glucans and 0.1 mg/mL of acyclovir, after HSVI infection for 48 h. Untreated cells were used as control. After incubation, the medium containing the compounds was removed, cells were washed twice with PBS and formed colonies were fixed for 5 min with 3.7 % (w/v) paraformaldehyde in PBS and stained for 20 min with 0.05 % (w/v) crystal violet in distilled water. Then, the plates were washed 4 times in a stream of tap water. After air-dry the plates at room temperature, images were taken using ZEISS (Jena, Germany) Optical Microscope Axiocam 208 Color Camera.

2.7. Human skin explants preparation

The studies conducted using human skin explants were approved by the Ethical Committee of Hospital Lusíadas (Porto, Portugal) and Universidade Católica Portuguesa (UCP, Porto, Portugal) with the approval code EO_E0121. The studies followed the WHO Guiding Principles on Human Cell, Tissue and Organ Transplantation. For all the *ex vivo* assays, healthy skin was obtained from abdominoplasty surgeries. Written informed consent from all participants was obtained under the established protocol. All the skin tissues were used within hours after surgery. Skin was maintained in sterile containers with PBS and Penicillin-Streptomycin at 10 % (v/v).

In sterile conditions, skin was placed epidermis down and the adipose layer was completely removed with scissors, remaining only the epidermal and dermal skin layer. For *ex vivo* HSVI model, a 12 mm

A



B

Formulation	Non-absorbed (%)	Skin-absorbed (%)
SG-Glu + SQ	41.1 ± 3.9	58.9 ± 3.9
WT-Glu + SQ	50.3 ± 7.2	49.7 ± 7.2

Fig. 3. Evaluation of glucans formulations in squalane. (A) Microscope images of HaCaT cells stained with crystal violet after HSV-1 infection and exposure to different formulations. The scale bar is set at 500 μm. (B) Skin permeation quantification after exposing skin explants to exposure to squalane formulation with SG-Glu and WT-Glu.

biopsy punch (3 cm^2) was pierced through the skin explants, which were then placed inside transwell inserts (with a diameter of 1.2 mm), epidermis side up, previously positioned in a 12-well plate with 600 μL of supplemented DMEM with FBS (10 % v/v) and Penicillin-Streptomycin (1 % v/v), and allowed to stabilize overnight at 37 °C in 5 % CO_2.

2.8. Formulations for skin applications

Glucans were incorporated into an emulsion with squalane (SQ, Amyris Inc.) and water to enhance skin penetration. The emulsion was prepared at a proportion of 5 % (w/v) SQ, 5 % (w/v) glucans, and 90 % water. Acyclovir was used as a positive control, and previously dissolved in 1 M HCl and added to the vehicle (water and squalane emulsion, serving as the negative control) at a concentration of 0.01 %. All mixtures were homogenized using an ultrasonic homogenizer probe (CY-500 sonicator, Optic Ivymen System, Barcelona, Spain) at amplitude of 70 % for 30 s to ensure stable emulsion formation.

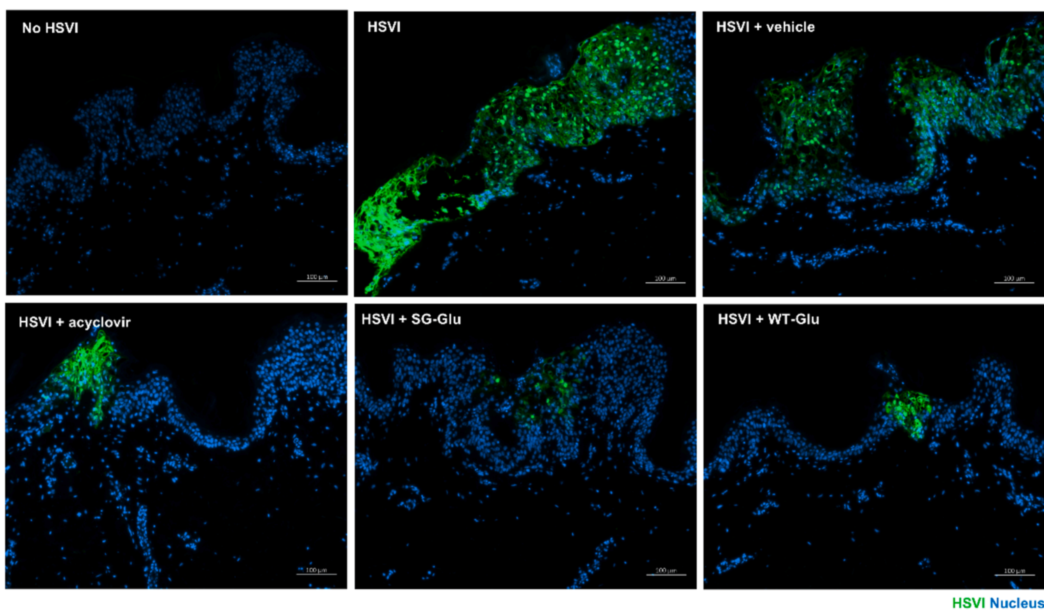
2.9. Glucans skin permeation

Skin explants with 20 mm-diameter (after adipose layer remotion) were placed in each cell of FRANZ cells. At the beginning of the permeation experiment, 0.1 mL of glucans formulations were applied in duplicate, onto the *stratum corneum* of each epidermis sample in the donor compartment of each cell. Receptor compartments were filled with PBS (phosphate buffer) pH 7.4. The system was kept at 32 °C by a circulating water bath and the membrane surface temperature was maintained at this temperature throughout the 24 h of the experiment. At the end, the skin was removed from the Franz diffusion cell and each side was gently treated with 1 mL of DMSO with LiCl (0.25 M) to wash out the unabsorbed water insoluble glucans. A standard curve was done with various concentrations of glucans and its corresponding fluorescence with calcofluor stain, to quantify the non-absorbed fraction. Fluorescent intensity was determined using a microplate reader (Synergy H1, BioTek) with excitation at λex 360 nm and emission at λem 430 nm. Results are expressed as percentage of non-absorbed formulation.

2.10. Preparation of an ex vivo human skin HSVI infection model

The *ex vivo* model for HSVI infection was developed based on [21]. Skin explants were treated with a derma-roller/microneedles (1,200 needles of 1.5 mm in length) 20 times in 4 directions (SR150; Skin Radiance, London, UK) to allow virus penetration through the epidermis into the dermis. 9×10^6 HSVI copies in 100 μl were placed onto the top of microneedled pre-treated skin explants and fresh culture media was added on the bottom of the transwell. The virus inoculum was kept at 37 °C for 48 h and after that, the media was replaced by fresh media every day. After 3 days of culture, skin explants were exposed to glucans or acyclovir emulsions for 4 days. Afterwards, they were harvested for paraffin embedding or for quantitative PCR virus DNA detection.

A



B

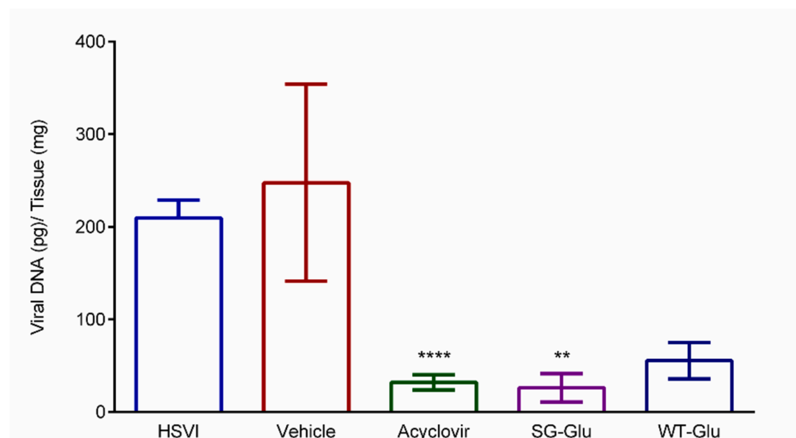


Fig. 4. Treatment with glucans formulation decreases the viral load present in the skin upon HSVI infection. (A) Immunofluorescence staining of HSVI (green) upon HVS1 infection and after exposure to formulations. The scale bar is set at 100 µm. Nuclei (blue) were counterstained with DAPI. (B) Quantification of viral DNA on skin by qPCR upon HVS1 infection and after exposure to formulations. Results are normalized by tissue weight. The data is represented as mean ± SEM from three replicates for each sample. Significant differences between samples and HSVI control are indicated as $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$, $^{****}p \leq 0.0001$.

2.11. Immunohistochemistry

Skin explants previously embedded in paraffin were cut with a thickness of 5 µm. Heat-induced antigen retrieval was performed in deparaffinized and rehydrated slides using Tris-EDTA buffer pH 9 or Sodium citrate buffer pH 6, depending on the antibody used. When using markers based on the enzyme peroxidase, endogenous peroxidases were blocked with 3 % (v/v) H_2O_2, for 15 min. Sections were then permeabilized with 0.2 % (v/v) Triton X-100 for 15 min at room temperature. Universal horse serum (HS) at 2.5 % (v/v) was added for 1 h to block non-specific binding and then primary antibodies diluted in HS 1 % (v/v) (Table 1), were added to the skin sections and let incubate overnight at 4 °C. For detection, both chromogenic and fluorescent probes were used. In the case of the chromogenic, the Vectastain Elite ABC-HRP kit and the respective peroxidase substrate DAB were followed according to the manufacturers' protocol. Nuclei were stained with Gill's hematoxylin. In case of fluorescence, after primary antibody incubation, the sections were incubated with the secondary antibody (Alexa Fluor 488 anti-rabbit or 594 anti-mouse) (Table 1) diluted at 1:500 in HS 1 % (v/v) and incubated for 1 h. DAPI (0.02 mg/mL) was used for nuclear staining. Slides were mounted with aqueous Fluoromount media. All the sections were analysed under a Zeiss Axio M2. Imager (Zeiss, Germany) microscope and images were acquired using the ZEN 2012 software (Zeiss, Germany).

2.12. Hematoxylin & eosin staining

Once the slides were deparaffinized, the Hematoxylin and eosin Y (H&E) staining was performed following standard protocols to observe the overall skin structure. Slides were cleared with xylene and mounted using non-aqueous Entellan media. All the sections were analysed under a Zeiss Axio M2.Imager microscope and images were acquired using the ZEN 2012 software.

2.13. Tissue DNA virus isolation and qPCR

Tissue samples of 25 mg of were cut into small pieces and processed using NZY tissue gDNA isolation kit, NZYTECH (Lisbon, Portugal), according to the manufacturer's instructions. Then, we proceeded with qPCR (thermocycler QTower, AnalyticJena, Germany). The primers of

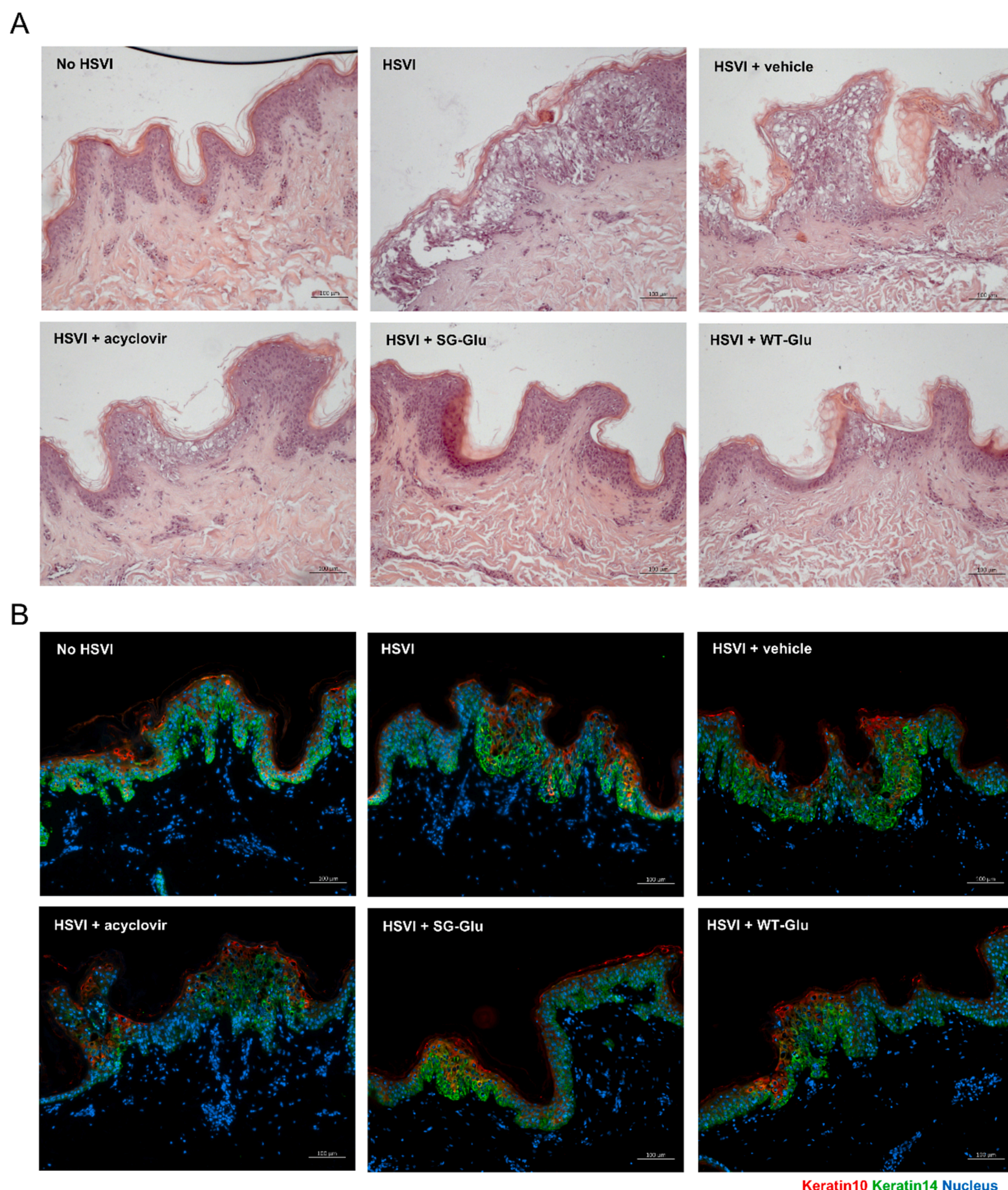


Fig. 5. Treatment with glucans formulations restore skin structure upon HSVI infection. (A) Representative images of H&E and (B) immunofluorescence staining of keratin-10 (red) and keratin-14 (green) upon HVS1 infection and after exposure to glucans and acyclovir formulations. The scale bar is set at 100 μm. Nuclei (blue) were counterstained with DAPI.

HSVI were purchased to Eurofins with the following sequences:

***HSVI fwd*: TCAAGGCCACCATGTACTACAAAGACGT**

***HSVI rev*: GCCGTAAAACGGGGACATGTACACAAAGT**

The results of qPCR were normalized to a HSVI concentration, presented as viral DNA concentration (pg) per mg of tissue.

2.14. Statistical analysis

All the experiments were run at least in triplicate. All the statistical analysis was performed using Statistica software, version 14.0, USA. Data analysis and comparisons between groups were calculated as follows: for cytophatic effect of HSVI was performed a one-way ANOVA with Dunnett's multiple comparison test; Cytokine expression profile was analyzed using Student's *t*-test and the viral DNA quantification was performed through a non-parametric Wilcoxon matched pairs test was performed, as the normality assumption was not met for any dataset according to the Shapiro–Wilk test. Graphics were generated using GraphPad Prism v9.4.0 (La Jolla, USA).

3. Results

3.1. Glucans protect against HSVI cytopathic effect in vitro

The impact of yeast glucans on the cytopathic effect of HSVI was evaluated in HaCaT cells and macrophages derived from THP-1 cells. For this, glucans were previously extracted from two different sources of yeast: an industrial steviol glycoside producer spent strain (SG-Glu) and the parental wild-type strain (WT-Glu). Both glucans' extracts were previously characterized as described by Sousa P., *et al.* [20]. Acyclovir was used as a positive control to counteract the HSVI infection. For the keratinocyte cell line HaCaT it was observed that both SG-Glu and WT-Glu were able to protect cells from the death induced by HSVI infection

(Fig. 1A). This protective effect was especially evident with higher glucans concentration. For SG-Glu at 1.0 and 2.0 mg/mL and for WT-Glu at 2 mg/mL, a significant decrease of cell death was attained. In macrophages the protective effect against HSVI infection of both glucans was lower than in keratinocytes (Fig. 1B). Nevertheless, a significant reduction in HSVI-induced metabolic inhibition was observed, with cell viability increasing by approximately 30 % in macrophages treated with glucans. However, this protective effect did not show a clear dose–response relationship.

3.2. The immunostimulant properties of glucans are independent of HSVI infection

Cytokines are crucial modulators between immune cells and non-immune cells, and as such they play a vital role in all functions of the immune system. It is well documented that in primary HSVI infection the antiviral capacity of immune cells is characterized by macrophages activation and some export of cytokines in skin cells [22]. Unfortunately, studies are still lacking concerning comprehensive study of cytokines during HSVI infection. It is known that IL-1β and TNF-α protected from HSVI effects, however, about IL-8 little information was reported [23].

The levels of cytokines IL-1β, TNF-α and IL-8 produced by macrophages treated with glucans and under HSVI infection were evaluated (Fig. 2A–C, respectively). The results indicate that SG-Glu and WT-Glu trigger the production of IL-1β, TNF-α and IL-8 in macrophages, with both glucans presenting significant increases in all cytokines when compared to untreated cells (control). The infection of HSVI did not significatively alter this pattern of cytokine production, as infected cells treated with glucans presented similar expression levels as non-infected cells exposed to glucans. Additionally, acyclovir slightly increases only IL-8 levels in macrophages, in cells infected or not infected with HSVI although not significative.

3.3. Glucans formulated with squalane retain their HSVI protective properties

Large molecules such as glucans have limited skin permeation. Thus, to explore the potential of glucans as an antiherpetic agent in the skin, it is important to prepare a formulation which enhances the permeation of glucans. In this work, we have explored the combination of glucans with squalane (SQ), a saturated oil used in skin care products as a moisturizer, recognized by its hydration and anti-inflammatory properties. Also, and although SQ is primarily used as an emollient, some reports have point out the SQ permeation enhancement capacity [24].

The antiherpetic activity of formulations was qualitatively evaluated in HaCaT cells to understand if SQ interferes with glucans activity (Fig. 3A). We can observe that both glucans + SQ increase the cell surface area when compared with squalane alone, thus indicating that these formulations retain the glucans antiherpetic capacity. Furthermore, the permeation studies of glucans in these formulations were performed in Franz Cells using human skin explants treated for 24 h and evaluated by calcofluor staining (Fig. 3B). It was observed that SQ formulations with SG-Glu and WT-Glu result in epidermal absorption of 58.9 % and 49.7 %, respectively.

3.4. Validation of glucans HSVI protective properties in an ex vivo model

To further evaluate the potential of glucans application in the treatment of HSVI infections in skin (*herpes labialis*) an *ex vivo* skin model mimicking HSVI infection was used. This model was adapted from the one described by Tajpara *et al.* [21]. Skin explants exposed to HSVI particles were incubated for 4 days to let the infection spread. Afterwards, treatment with SG-Glu, WT-Glu and acyclovir formulated with SQ was performed for 3 days, twice a day. A vehicle formulation containing the same backbone, without any active ingredient, was used as a control.

The viral infection on the skin was confirmed through immunofluorescence for HSVI (Fig. 4A). The presence of HSVI was extensively detected in skin infected with HSVI when compared to non-infected skin (No HSVI). Upon treatment with both glucans' formulations, the virus load drastically decreases. While the same trend was observed for the infected skin treated with acyclovir, the vehicle alone did not affect the infection. These results were further confirmed by the quantification of specific HSVI viral DNA present in the tissue (Fig. 4B). Indeed, a significant decrease in HSVI infection in skin treated with glucans was attained. This decrease is like the one observed for acyclovir treated skin. It is important to note that the acyclovir formulation used in this work is in the same range of values of the most common topical application creams used as first line of treatment, acyclovir (20–40 pg/mg).

Furthermore, we evaluated the skin structure after HSVI infection and treatment with glucans. From the H&E staining and observing the overall skin structure, it was possible to perceive the skin damage (cytopathic effect) induced by the HSVI infection in the epidermis when compared to the compared to non-infected skin (No HSVI) with no alterations at the dermal level (Fig. 5A). However, it seems that the epidermal damage is drastically reduced upon treatment with acyclovir and both SG-Glu and WT-Glu formulations. Additionally, key skin matrix proteins responsible for epidermal structural integrity, namely keratin 10 and 14 (K-10 and K-14, respectively) were assessed. During skin HSVI infection, it was observed a great disorganization between both keratins, with apparent scattered expression of K-14 contrary to predominantly localization at the *stratum basale*. Notwithstanding, while it seems that both keratins expression were restored in presence of acyclovir and glucans formulations (Fig. 5B), a higher degree of restored epidermal integrity was attained with the latter.

4. Discussion

Glucans are well-known for their immunostimulatory properties, interacting with PAMP receptors on immune cells, particularly dectin-1 and TLR4 [25]. The recognition of glucans by these receptors triggers an inflammatory response which may present different levels, considering glucans structure, length and origin [26]. Glucans from yeast present a structure (1 → 3)-β with side branches (1 → 6)-β which have been reported to present an increased immunostimulatory activity when compared to glucans of other origin [27]. Associated to this, previous studies have shown that glucans can have antiviral activity due to their activation of the innate immune cells, reprogramming host cells to a trained immunity that regulates the antiviral response [28–30].

Taking the immunostimulant properties of glucans together with their antiviral activity, this work focused on the potential of yeast glucans as a novel treatment option for herpes viral infection. HSVI skin infection, commonly referred as *herpes labialis,* is a highly contagious viral infection that primarily causes oral herpes, characterized by cold sores or fever blisters that appear around the mouth or on the face [31]. Treatment for HSVI infection includes antiviral medications, which can help reduce the severity of symptoms and shorten the duration of the infection [32], but its use has led to acyclovir-resistant HSVI infections often associated with systemic adverse effects [33].

The capacity of glucans to counteract HSVI infection was proven using an *ex vivo* skin model for HSVI infection that was optimized based on the work reported by Tajpara, *et al* [21]. Herein, we have observed that, just like acyclovir, the gold-standard treatment of HSVI infection, glucans had the capacity to decrease the levels of HSVI infection in a model of higher complexity. While the mechanisms of action of acyclovir are well described and are based on the inhibition of virus replication machinery by direct inhibition of DNA polymerase, which is responsible for viral genome replication [34], glucans mechanisms of action are still unknown. Notwithstanding, we found that this effect might be related to two glucans properties. In one hand, our glucans protected both keratinocytes and macrophages differentiated from THP-

1 cells from the cytopathic effect of HSVI in a dose-dependent manner. In the other, glucans stimulated the production of the inflammatory cytokines IL-1β, TNF-α and IL-8 in macrophages *per se* and independent of any other stimuli. These results were in accordance with what was expected due to the fact that glucans from yeast present a structure (1 → 3)-β with side branches (1 → 6)-β which have been reported to present an increased immunostimulatory activity when compared to glucans of other origin [27], and those particularly with a (1 → 3)-β-d-glucan backbone, have been shown to stimulate nitric oxide generation and cytokine production in immune cells [4]. This has an effect in the recruitment and activation of effector cells, such as natural killer cells and cytotoxic T lymphocytes, which play a crucial role in clearing virus-infected cells [4]. For instance, recruited effector $CD8^+$ T cells clear the infected lesions *via* direct killing or interferon-γ (IFN-γ)-mediated control of HSV-infected cells that is secreted by infiltrating $CD4^+$ T cells [28]. Moreover, other studies have also showed that glucans can stimulate the production of IFN-β, which is associated with enhanced ubiquitination of TBK1, a key molecule in the antiviral immune pathway [30]. All this taken together, indicate that our active ingredients may be triggering an immune response in macrophages in our optimized *ex vivo* HSVI skin model, which may promote the activation of the innate and trained immune mechanisms of cells and plays a critical role in orchestrating the mechanisms to counteract the viral infection.

Glucans are also known for their deeply nourishing and hydrating properties, which help boost the skin's barrier, smooth skin, and protect it from daily environmental stressors. Their properties have also been associated with calming effects on irritated skin – helping to reduce redness and irritation –, and with anti-ageing properties – (collagen promotion) – [35], which shows their benefic potential in the skin care industry [20]. This is in concordance with our results where we show that emulsions containing yeast glucans decrease the overexpression of the K-14 expression resulting in a repair of the tissue integrity. Therefore, our glucans have the unique capacity to restore the skin homeostasis and tissue structure while, at the same time, are reducing the levels of HSVI infection.

Being a natural product with a GRAS status, glucans may be seen as an excellent alternative for the management of herpes labialis outbursts. In this work, yeast glucans extracted from two different sources were used: one derived from a wild-type strain grown under controlled production conditions, and the other from an industrial fermentation residue of an industrial strain used for steviol-glycoside production. Although presenting different origins both showed similar performances. Due to their structure, glucans are large molecules presenting skin permeation limitations. They are known to have the capacity to reach dermis and epidermis when applied as a cream formulation or employing a specific vehicle (e.g., hyaluronic acid), by entering through the intercellular space rather than through cells [14]. Moreover, SQ properties have been related to their permeation enhancement capacity [24]. Therefore, in the *ex vivo* assays, a formulation containing SQ was used. We have demonstrated that while SQ improves glucans permeation in the skin, it preserves the activity of our active molecules without interfering with their ability to counteract the HSVI cytopathic effect. Interestingly, both glucans demonstrated similar performance in HSVI infection treatment. This is an important aspect in a context of circular economy, where sustainable approaches and the valorization of industrial wastes are important issues. The results herein obtained might pave the way for the application of glucans industrial side-streams in high-value application.

5. Conclusion

In this work the antiherpetic capacity of glucans extracted from different origins was evaluated *in vitro* and *ex vivo*. Glucans are well-known for their immunomodulatory and skin-repairing properties. In this study, we demonstrated their potential in counteracting HSVI infection and promoting skin restoration, supporting their further exploration in topical formulations. The results demonstrated that glucans present an anti-herpetic capacity either *in vitro* or *ex vivo*, contributing to the restoration of skin natural structure. The *ex vivo* HSVI infection of human skin model has provided valuable insights on impairment of epidermal barriers, the susceptibility of the epidermis and dermis to HSVI, and the role of skin barriers in preventing infection. This work brings a new insight into how yeast glucans can enhance our skin health, reduce virus infection and be used for skin barrier preservation. This study also highlights the potential of yeast-derived glucans from industrial by-products, aligning with sustainable approaches by repurposing fermentation residues for high-value applications.

CRediT authorship contribution statement

Diana Tavares-Valente: Writing – original draft, Methodology, Investigation. **Helena Moreira:** Writing – review & editing, Methodology, Investigation. **Pedro Sousa:** Methodology, Investigation. **Manuela Amorim:** Writing – review & editing, Methodology, Investigation, Conceptualization. **António Conde:** Methodology, Conceptualization. **Manuela Pintado:** Writing – review & editing, Methodology, Investigation, Conceptualization. **João Fernandes:** Writing – review & editing, Supervision, Methodology, Investigation, Conceptualization. **João Azevedo-Silva:** Writing – review & editing, Supervision, Methodology, Investigation, Conceptualization.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: DTV, PS, MA, MEP, JCF and JAS are inventors on a patent application covering the use of glucans for HSVI treatment (EP 4 201 397 A1). All other authors declare no competing interests.

Acknowledgements

This research was funded by National Funds from FCT Fundação para a Ciência e a Tecnologia through project UID/Multi/50016/2019 and from ANI Agência Nacional de Inovação through project Alchemy (POCI-01-0247-FEDER-027578).

Data availability

Data will be made available on request.

References

[1] C. Caseiro, J.N.R. Dias, C.M.G. de Andrade Fontes, P. Bule, From cancer therapy to winemaking: the molecular structure and applications of β-glucans and β-1, 3-glucanases, Int. J. Mol. Sci. 23 (6) (2022) 3156, https://doi.org/10.3390/ijms23063156.

[2] G.D. Brown, S. Gordon, Fungal β-glucans and mammalian immunity, Immunity 19 (3) (2003) 311–315, https://doi.org/10.1016/S1074-7613(03)00233-4.

[3] A. Estrada, C. Yun, A. Van Kessel, B. Li, S. Hauta, B. Laarveld, Immunomodulatory activities of oat β-glucan in vitro and in vivo, Microbiol. Immunol. 41 (12) (1997) 991–998, https://doi.org/10.1111/j.1348-0421.1997.tb01959.x.

[4] I. Urbancikova, D. Hudackova, J. Majtan, Z. Rennerova, P. Banovcin, M. Jesenak, Efficacy of pleuran (β -glucan from pleurotus ostreatus) in the management of herpes simplex virus type 1 infection, Evid. Based Complement. Alternat. Med. 2020 (2020) 1–8, https://doi.org/10.1155/2020/8562309.

[5] Seong SK, Kim HW. Potentiation of Innate Immunity by β-Glucans. Mycobiology. 2010 Jun;38(2):144-8. doi: 10.4489/MYCO.2010.38.2.144. Epub 2010 Jun 30. PMID: 23956643; PMCID: PMC3741566.

[6] G. Camilli, G. Tabouret, J. Quintin, The complexity of fungal β-glucan in health and disease: effects on the mononuclear phagocyte system, Front. Immunol. 9 (2018), https://doi.org/10.3389/fimmu.2018.00673.

[7] Y. Gu, Y. Takagi, T. Nakamura, T. Hasegawa, I. Suzuki, M. Oshima, H. Tawaraya, Y. Niwano, Enhancement of radioprotection and anti-tumor immunity by yeast-derived β-glucan in mice, J. Med. Food 8 (2) (2005) 154–158, https://doi.org/10.1089/jmf.2005.8.154.

[8] L. Wu, J. Zhao, X. Zhang, S. Liu, C. Zhao, Antitumor effect of soluble β-glucan as an immune stimulant, Int. J. Biol. Macromol. 179 (2021) 116–124, https://doi.org/10.1016/j.ijbiomac.2021.02.207.

[9] B. Mallard, D.N. Leach, H. Wohlmuth, J. Tiralongo, Synergistic immunomodulatory activity in human macrophages of a medicinal mushroom formulation consisting of Reishi, Shiitake and Maitake, PLOS ONE 14 (11) (2019) e0224740, https://doi.org/10.1371/journal.pone.0224740.

[10] S. Awate, L.A. Babiuk, G. Mutwiri, Mechanisms of action of adjuvants, Front. Immunol. 4 (2013), https://doi.org/10.3389/fimmu.2013.00114.

[11] N. Petrovsky, P.D. Cooper, Carbohydrate-based immune adjuvants, Expert Rev. Vaccines 10 (4) (2011) 523–537, https://doi.org/10.1586/erv.11.30.

[12] R.P. Singh, A. Bhardwaj, β-glucans: a potential source for maintaining gut microbiota and the immune system, Front. Nutr. 10 (2023), https://doi.org/10.3389/fnut.2023.1143682.

[13] E. Machová, S. Bystrický, Antioxidant capacities of mannans and glucans are related to their susceptibility of free radical degradation, Int. J. Biol. Macromol. 61 (2013) 308–311, https://doi.org/10.1016/j.ijbiomac.2013.07.016.

[14] B. Du, Z. Bian, B. Xu, Skin health promotion effects of natural beta-glucan derived from cereals and microorganisms: a review, Phytother. Res. 28 (2) (2014) 159–166, https://doi.org/10.1002/ptr.4963.

[15] C. Nissola, M.L.K. Marchioro, E.V. de Souza Leite Mello, A.C. Guidi, D.C. de Medeiros, C.G. da Silva, J.C.P. de Mello, E.A. Pereira, A.M. Barbosa-Dekker, R.F. H. Dekker, M.A.A. Cunha, Hydrogel containing (1 → 6)-β-D-glucan (lasiodiplodan) effectively promotes dermal wound healing, Int. J. Biol. Macromol. 183 (2021) 316–330, https://doi.org/10.1016/j.ijbiomac.2021.04.169.

[16] J. Wouk, G.G. Celestino, B.C.D. Rodrigues, C.R.M. Malfatti, M.A.A. Cunha, A. Orsato, A.M. Barbosa-Dekker, R.F.H. Dekker, A.A.S.G. Lonni, E. Reis Tavares, L. C. Faccin-Galhardi, Sulfonated (1 → 6)-β- <scp>d</scp> -glucan (lasiodiplodan): a promising candidate against the acyclovir-resistant herpes simplex virus type 1 (HSV-1) strain, Biomacromolecules 23 (10) (2022) 4041–4052, https://doi.org/10.1021/acs.biomac.2c00156.

[17] G.A. Karasneh, D. Shukla, Herpes simplex virus infects most cell types in vitro: clues to its success, Virol. J. 8 (1) (2011) 481, https://doi.org/10.1186/1743-422X-8-481.

[18] D.M. Álvarez, E. Castillo, L.F. Duarte, J. Arriagada, N. Corrales, M.A. Farías, A. Henríquez, C. Agurto-Muñoz, P.A. González, Current antivirals and novel botanical molecules interfering with herpes simplex virus infection, Front. Microbiol. 11 (2020), https://doi.org/10.3389/fmicb.2020.00139.

[19] L. Chen, G. Huang, The antiviral activity of polysaccharides and their derivatives, Int. J. Biol. Macromol. 115 (2018) 77–82, https://doi.org/10.1016/j.ijbiomac.2018.04.056.

[20] P. Sousa, D. Tavares-Valente, C.F. Pereira, I. Pinto-Ribeiro, J. Azevedo-Silva, R. Madureira, Ó.L. Ramos, M. Pintado, J. Fernandes, M. Amorim, Circular economyeast: Saccharomyces cerevisiae as a sustainable source of glucans and its safety for skincare application, Int. J. Biol. Macromol. 265 (2024) 130933, https://doi.org/10.1016/j.ijbiomac.2024.130933.

[21] P. Tajpara, M. Mildner, R. Schmidt, M. Vierhapper, J. Matiasek, T. Popow-Kraupp, C. Schuster, A. Elbe-Bürger, A preclinical model for studying herpes simplex virus infection, J. Invest. Dermatol. 139 (3) (2019) 673–682, https://doi.org/10.1016/j.jid.2018.08.034.

[22] A. Mukhopadhyay, S. Indra, J. Ghosh, S. Biswas, P. Palit, D. Chattopadhyay, Herpes simplex virus-mediated skin infections: cytokines and its interplay, Explor. Immunol. (2024) 394–413, https://doi.org/10.37349/ei.2024.00148.

[23] J.B. Smith, J.J. Herbert, N.R. Truong, A.L. Cunningham, Cytokines and chemokines: The vital role they play in herpes simplex virus mucosal immunology, Front. Immunol. 13 (2022), https://doi.org/10.3389/fimmu.2022.936235.

[24] A.L.S. Oliveira, D. Valente, H.R. Moreira, M. Pintado, P. Costa, Effect of squalane-based emulsion on polyphenols skin penetration: Ex vivo skin study, Colloids Surf. B Biointerfaces 218 (2022) 112779, https://doi.org/10.1016/j.colsurfb.2022.112779.

[25] V. Vetvicka, T.V. Teplyakova, A.B. Shintyapina, T.A. Korolenko, Effects of medicinal fungi-derived β-glucan on tumor progression, J. Fungi 7 (4) (2021) 250, https://doi.org/10.3390/jof7040250.

[26] H.S. Goodridge, A.J. Wolf, D.M. Underhill, β-glucan recognition by the innate immune system, Immunol. Rev. 230 (1) (2009) 38–50, https://doi.org/10.1111/j.1600-065X.2009.00793.x.

[27] X. Zhong, G. Wang, F. Li, S. Fang, S. Zhou, A. Ishiwata, A.G. Tonevitsky, M. Shkurnikov, H. Cai, F. Ding, Immunomodulatory effect and biological significance of β-glucans, Pharmaceutics 15 (6) (2023) 1615, https://doi.org/10.3390/pharmaceutics15061615.

[28] A. Córdova-Martínez, A. Caballero-García, E. Roche, D.C. Noriega, β-Glucans could be adjuvants for SARS-CoV-2 virus vaccines (COVID-19), Int. J. Environ. Res. Public Health 18 (23) (2021) 12636, https://doi.org/10.3390/ijerph182312636.

[29] V. Vetvicka, P. Sima, L. Vannucci, Trained immunity as an adaptive branch of innate immunity, Int. J. Mol. Sci. 22 (19) (2021) 10684, https://doi.org/10.3390/ijms221910684.

[30] G. Wang, Z. Li, M. Tian, X. Cui, J. Ma, S. Liu, C. Ye, L. Yuan, M.S. Qudus, U. Afaq, K. Wu, X. Liu, C. Zhu, β-glucan induces training immunity to promote antiviral activity by activating TBK1, Viruses 15 (5) (2023) 1204, https://doi.org/10.3390/v15051204.

[31] D. Sharma, S. Sharma, N. Akojwar, A. Dondulkar, N. Yenorkar, D. Pandita, S. K. Prasad, M. Dhobi, An insight into current treatment strategies, their limitations, and ongoing developments in vaccine technologies against herpes simplex infections, Vaccines 11 (2) (2023) 206, https://doi.org/10.3390/vaccines11020206.

[32] D. Gopinath, K.H. Koe, M.K. Maharajan, S. Panda, A comprehensive overview of epidemiology, pathogenesis and the management of herpes labialis, Viruses 15 (1) (2023) 225, https://doi.org/10.3390/v15010225.

[33] K. Kłysik, A. Pietraszek, A. Karewicz, M. Nowakowska, Acyclovir in the treatment of herpes viruses – a review, Curr. Med. Chem. 27 (24) (2020) 4118–4137, https://doi.org/10.2174/0929867325666180309105519.

[34] S. Kausar, F. Said Khan, M. Ishaq Mujeeb Ur Rehman, M. Akram, M. Riaz, G. Rasool, A. Hamid Khan, I. Saleem, S. Shamim, A. Malik, A review: mechanism of action of antiviral drugs, Int. J. Immunopath. Pharmacol. 35 (2021) 205873842110026, https://doi.org/10.1177/20587384211002621.

[35] S. Gautier, E. Xhauflaire-Uhoda, P. Gonry, G.E. Piérard, Chitin–glucan, a natural cell scaffold for skin moisturization and rejuvenation, Int. J. Cosmet. Sci. 30 (6) (2008) 459–469, https://doi.org/10.1111/j.1468-2494.2008.00470.x.