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Hot Water Extract from *Saccharomyces cerevisiae* Scavenges DPPH and Reduces Senescence Associated β-Galactosidase (Sa-β-Gal) in Human Dermal Fibroblasts

(Ekstrak Air Panas daripada *Saccharomyces cerevisiae* Menghimpun DPPH dan Mengurangkan β-Galaktosidase (Sa-β-Gal) Berkait Senesens dalam Fibroblas Dermal Manusia)

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ABSTRACT

Extracts from *Saccharomyces cerevisiae* are incorporated in a lot of cosmetic products on the market, but the benefits of the extracts lack scientific reports. We tested the toxicity and anti-senescent activity of an extract from *S. cerevisiae* on an *in vitro* model, the human dermal fibroblast (HDF) cell culture. We chronicled the development of the extraction method and the subsequent biochemical assays. We used two extraction methods which were hot water extraction and rapid spin. The optimum duration and growth phase to harvest *S. cerevisiae* were determined by 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay, which also proved that the extracts exhibited antioxidant activity. Hot water extract showed a higher antioxidant activity, and not toxic to HDF. When subjected to senescence-associated β-galactosidase (SA-β-Gal) assay, the hot water extract significantly reduced the expression of SA-β-Gal in pre-senescent (passage 20, 30 < population doubling < 40) and senescent (passage 30, population doubling > 50) HDF. In conclusion, *S. cerevisiae* hot water extract possessed antioxidant activity by scavenging DPPH, and anti-senescent activity by reducing the expression of SA-β-Gal in pre-senescent and senescent HDF.

Keywords: Ageing; DPPH; *Saccharomyces cerevisiae*; senescence

ABSTRAK

Ekstrak daripada *Saccharomyces cerevisiae* digabungkan dalam banyak produk kosmetik di pasaran. Ia dikatakan mempunyai kesan yang baik pada kulit manusia, tetapi masih kurang laporan saintifik tentang keberkesanan ekstraknya. Kami telah menguji kesan ketoksikan dan aktiviti anti-penuaan bagi ekstrak mentah daripada *S. cerevisiae* pada model *in vitro*, kultur sel fibroblas dermal manusia (HDF). Kami melaporkan pembangunan kaedah pengekstrakan dan asai biokimia seterusnya. Kami menggunakan dua kaedah pengekstrakan iaitu pengekstrakan air panas dan putaran cepat. Tempoh dan fasa pertumbuhan optimum untuk menuai *S. cerevisiae* ditentukan dengan asai 2,2-difenil-1-pikrilhidrazil (DPPH), yang juga membuktikan bahawa ekstrak mentah menunjukkan aktiviti antioksidan. Ekstrak air panas menunjukkan aktiviti antioksidan yang lebih tinggi dan tidak toksik kepada HDF. Apabila dilakukan asai β-galaktosidase berkait penuaan (SA-β-Gal), ekstrak air panas mengurangkan ekspresi SA-β-Gal dalam HDF pra-senesens (pasaj 20, 30 < populasi berganda < 40) dan senesens (pasaj 30, populasi berganda > 50). Kesimpulannya, ekstrak air panas *S. cerevisiae* mempunyai aktiviti antioksidan dengan menghimpun DPPH dan aktiviti anti-senesens dengan mengurangkan ekspresi SA-β-Gal dalam HDF pra-senesens dan senesens.

Kata kunci: DPPH; penuaan; *Saccharomyces cerevisiae*; senesens

INTRODUCTION

Recently, *Saccharomyces cerevisiae* yeast extracts have become part of the ingredients in numerous cosmetic products, as they are claimed to have beneficial effects to the human skin. However, there is limited literature describing the mechanism of action of the extracts on the skin resulting in aesthetic effects. In a clinical study, products containing *S. cerevisiae* extract in combination with several vitamins had shown a beneficial effect on skin texture and appearance (Gaspar et al. 2008).

S. cerevisiae extract is rich in amino acids, which can improve skin moisture, as well as peptides, proteins and polysaccharides that can promote wound healing and cell renewal (Kim & Yun 2006; Péterszegi et al. 2003).

Previous studies utilised various methods of extraction to acquire certain active component in yeast. Compounds such as α-mannan is more soluble in alkaline condition (Li et al. 2019), while β-glucan is abundantly harvested by methanol extraction method (Gallo et al. 2014). Mannoproteins (Liu et al. 2008) and mannans (She et al. 2016) can be extracted by water; however, the isolation of active compounds is tedious, time-consuming, and requires a large amount of solvents, compared to a simple crude extraction. Presently, there is no available documentation of research on the effect of hot water extract from *S. cerevisiae* on human skin, much less that on human dermal fibroblast culture.

The active involvement of skin fibroblast in wound healing (Chowdhury et al. 2019; Michopoulou & Rousselle 2015) and synthesis of extracellular matrix (Tracy et al. 2016) makes skin fibroblast an essential model in the study of ageing and anti-ageing mechanism (Sorrell et al. 2007). Dermal fibroblasts are long-lived cell population (Rubin & Reisner 2014) capable of accumulating cellular damages (Starr & Starr 2014), whereby they undergo adaptive function which are often caused by extrinsic ageing. Impairment of dermal fibroblast and its complementary remodelling of the dermal extracellular matrix (ECM) causes skin wrinkles which is the most occurring phenotypic result in extrinsically aged skin (Starr & Starr 2014). These traits have enabled dermal fibroblast to become a preferred, established model in the study of extrinsic ageing processes at cellular level.

Senescent fibroblasts can be differentiated from the young based on the changes in morphology and biochemical content. Young fibroblasts are long and spindle shaped, but those that have senesced are large and flattened (Despres et al. 2019). Additionally, senescent cells express senescence-associated β-galactosidase (SA-β-gal), a known biomarker for cellular ageing that is almost undetectable in the young (Despres et al. 2019; Dimri et al. 1995; Momtaz et al. 2019). This differential trait is used to establish the age of cell cultures (Despres et al. 2019; Jaafar et al. 2018).

In this study, the extraction methods and duration of culture in harvesting *S. cerevisiae* were optimised. Then, the toxicity and anti-senescent activity of hot water extract on human dermal fibroblast (HDF) cell culture were tested.

Materials and Methods

S. cerevisiae culture

A single colony of *S. cerevisiae* (ATCC® 201390™, Genotype: MATa/MATalpha his3delta1/his3delta1 leu2delta0/leu2delta0 lys2delta0/+ met15delta0/+ ura3delta0/ura3delta0) grown on an agar plate was inoculated into 5 mL of yeast extract peptone dextrose (YPD) broth (BD, Massachusetts, USA). The cells were grown overnight (18 h) at 30 °C in an orbital shaking incubator (220 rpm) to an OD_{600} of 2.0 and diluted back to an OD_{600} of 0.2.

Extraction methods

Every 3 h during the 15-hour culture process, 2.55 × 10^6 cellsmL^{-1} of yeast culture was harvested via centrifugation at 1,722 xg for 10 min (Eppendorf® 5810, Hamburg, Germany). The cells collected in the pellet were recovered and suspended in 5 mL of sterile distilled water, where the cells were subjected to two extraction methods which were hot water extraction and rapid spin. Subsequently, the harvested cells were boiled at 100 °C for 20 min using reflux method, followed by centrifugation at 17,220 xg (Eppendorf® 5810, Hamburg,Germany) for 20 min (Yusof et al. 2010). The supernatant separated via centrifugation was kept as extract at -80 °C. Meanwhile, for rapid spin extraction, the harvested cultures were centrifuged twice at 400 xg at 4 °C for 10 min (Zarei et al. 2016). The supernatant (extract) was kept at -80 °C, and three biological replicates of S. c*erevisiae* were cultured to produce the extracts.

Antioxidant activity assay

An amount of 8 mg of 2,2-diphenyl-1-picrylhydrazyl (DPPH) was dissolved in 100 mL of ethanol and brought to 200 mL of total solution with sterile distilled water producing a 0.1 mM solution. DPPH solution (1.8 mL) was mixed with 0.2 mL of extract and incubated in the dark for 30 min. The absorbance of the samples was measured at 517 nm (UV mini 12s40 Shimadzu UV-Visible spectrophotometer). Sterile distilled water was used as the control, where the assay was conducted with the three different biological replicates of *S. cerevisiae* extracts. The antioxidant activity of each sample was calculated using the formula below (Gazi et al. 2004):

$$\text{Antioxidant activity (units mL}^{-1}) = ([A_{br}–A_{ar}]/S) \times 100$$

where A_{br} is the absorbance before reaction; A_{ar} is the absorbance after the reaction has taken place; and S is the volume of the sample (mL).

Data from different extraction methods were compared against the control (ddH_2O) using the Wilcoxon Rank Sum Test, followed by Greenhouse Geisser correction (epsilon < 0.75), using IBM SPSS version 21.

HUMAN DERMAL FIBROBLAST (HDF) CELL CULTURE

Human dermal fibroblast (HDF) that was first cultured from a previous research (Jaafar et al. 2018) was used in this study. The HDF was cultured in DMEM, 1.0 gL^{-1} of glucose with L-glutamine and sodium pyruvate, 10% FBS and 1% antibiotic-antimycotic mixed stock solution at 37 °C in 5% CO_2 humidified incubator. Upon reaching confluency at 80 to 90% rate in a T-25 flask (SPL Lifesciences Co. Ltd. Gyeonggi-do, South Korea), the cells were detached from the flask using cell dissociation reagent (Nacalai Tesque, Kyoto, Japan) for subculturing. Serial passaging of HDFs was carried out with 1:4 expansion degree ratio. Passaging of cells were monitored to determine population doubling (PD), which was used to represent the age of replicative-senescent cells as young (passage 4, PD < 12), pre-senescent (passage 20, 30 < PD < 40) and senescent (passage 30, PD > 50) (Makpol et al. 2012).

HDF VIABILITY ASSAY

The extracts acquired from both methods were assayed using the antioxidant activity assay to determine whether the extracts possess antioxidant ability. The results showed that hot water extraction method produced an extract with a higher antioxidant activity. Therefore, hot water extract was used for the remainder of the research. Cell viability assay was performed to establish the optimum dose of the hot water *S. cerevsiae* extract. HDF (2500 cells) were seeded into each well of 96-well plate in 100 µL of media and incubated for 24 h at 37 °C, with 5% CO_2. Upon reaching 70 to 80% confluence, the media was replaced with 100 µL of fresh media with 10% (v/v) of hot water yeast extract with various concentration ratio (yeast:HDF) and incubated further for 24 h. The concentration of yeast cells was varied according to yeast:HDF ratio of 1,000:1, 100:1, 10:1, 1:1, 1:10, 1:100, 1:1,000, 1:10,000, 1:100,000, 1:1,000,000, 1:10,000,000 and 1:100,000,000. Then, the treatment medium was replaced with fresh media and 10 µL of water-soluble tetrazolium salts-8 (WST-8) for 4 h, in accordance with the manufacturer's protocol (Nacalai Tesque, Kyoto, Japan). The absorbance was measured at 450 nm using Enspire® multimode plate reader (PerkinElmer, Massachusetts, USA). Unpaired *t*-test was used to compare the data between each age group to the control (untreated cells) using Prism 7 software (GraphPad Software, California, USA). Differences were considered significant when $p < 0.05$.

SENESCENCE-ASSOCIATED B-GALACTOSIDASE (SA-β-Gal) ASSAY

Human dermal fibroblast (HDF) was divided into three experimental groups consisting of young (passage 4, PD < 12), pre-senescent (passage 20, 30 < PD < 40) and senescent (passage 30, PD > 50). HDF was incubated for 24 h with the treatment media, which contained 10% (v/v) of hot water extract (yeast:HDF ratio of 10:1). SA-β-Gal assay was conducted as instructed by the manufacturer (Sigma, Darmstadt, Germany). A total of 5×10^4 HDF was seeded in a six-well plate and incubated with fixation buffer (2% formaldehyde/0.2% glutaraldehyde) for 6 to 7 min at room temperature. Next, the cells were rinsed three times with phosphate-buffered saline and incubated with 1 $mgmL^{-1}$ of X-Gal in a buffer containing 40 mM citric acid/phosphate (pH 6.0), 5 mM K_3FeCN_6, 5 mM K_4FeCN_6, 150 mM NaCl, and 2 mM $MgCl_2$ for 6 h at 37 °C in the absence of CO_2. The percentage of blue cells observed in 100 cells under a light microscope (Olympus CKX53 Cell Culture Microscope) was calculated. Repeated measures of ANOVA and Bonferroni adjustment was used to assess the significant differences between groups and differences were considered significant when $p < 0.05$.

RESULTS AND DISCUSSION

It is hypothesised that ageing occurs because of injurious assaults by free radicals to the cells (Harman 1956; Wang et al. 2021). The deleterious effects of the free radicals can be stalled by maintaining a high intracellular concentration of antioxidants (Xian et al. 2021). In the human skin, fibroblasts are actively involved in wound healing (Chowdhury et al. 2019) and the synthesis of extracellular matrix (Tracy et al. 2016). In ageing skin, impairment of skin or dermal fibroblasts and their complementary remodelling of the dermal extracellular matrix causes wrinkling, which is the most occurring phenotypic result (Starr & Starr 2014); this makes them an essential model in the study of ageing mechanism (Maarof et al. 2018; Tan et al. 2018).

DPPH SCAVENGING OF *S. cerevisiae* CRUDE EXTRACTS

Hot water extract from *S. cerevisiae* cultured for 3 h showed the highest antioxidant activity (59 $unitsmL^{-1}$)

(Figure 1), which was during the exponential phase of cell culture growth (Tajul Arifin et al. 2019). The effect could be owed to the fact that most of the abundant proteins are produced during the exponential phase (Boucherie 1985), as well as *de novo* amino acids (Mülleder et al. 2016). Alkaline amino acids, such as histidine and lysine, have antioxidant abilities (Dash & Ghosh 2017), which could contribute to the antioxidative performance of the extract. Furthermore, the antioxidant, D-erythroascorbic acid, synthesised by *S. cerevisiae* could have been included in the extract (Huh et al. 1998). The antioxidant activity showed a significant decreasing trend with increasing hours of culture ($p < 0.05$). Meanwhile, the rapid spin extract also possessed antioxidant activity but at a lower magnitude than that of the hot water extract. When the datasets from different extraction methods were compared, statistical analysis showed that there was no significant difference in the extraction methods ($p = 0.317$). Moreover, the preliminary study (unpublished data) of the authors of this work showed that yeast extracts exerted an increasing antioxidant capability (samples were taken at every 30 min), peaking at 3 h of culture. Therefore, to keep the method short for subsequent experiments, hot water extract was prepared from *S. cerevisiae* cultured for 3 h.

Yeast cell wall consists of polysaccharides, such as α-mannan and β-glucan, that are proven to have antioxidant capability (Machová & Bystrický 2013).

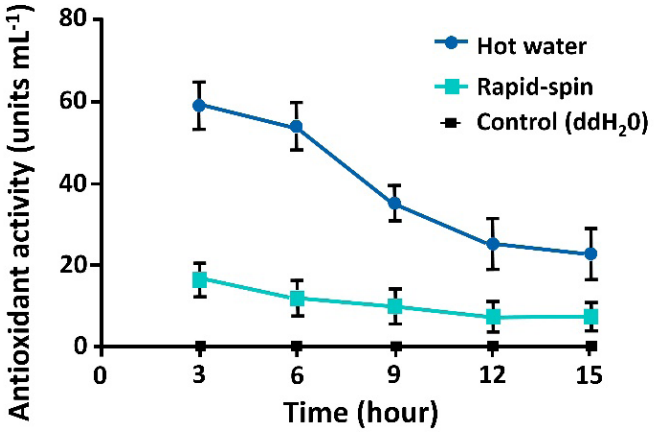

FIGURE 1. The antioxidant activity of *S. cerevisiae* extracts based on two different extraction methods which were hot water extraction and rapid spin. The extracts were prepared from *S. cerevisiae* cultured at different durations of 3, 6, 9, 12, and 15 h. All data were represented as mean + SD (n = 3). Wilcoxon Rank Sum Test was used to analyse the data between different extraction methods. There was a statistically significant decrement in the antioxidant activity over the hours ($p < 0.05$)

Comparative studies have proven that β-glucan has more potent radical scavenging capability than water-soluble antioxidant D-mannitol (Kogan et al. 2005). Furthermore, β-glucan exerts anti-ageing properties by stimulating the production of collagen (Wei et al. 2002) and promoting the migration and proliferation of human dermal fibroblast (Son et al. 2005). The antioxidant activities shown in this study could be due to the presence of such polysaccharides in the extract.

Hot water extract showed greater antioxidant capability than that of the rapid spin method. The hypothesis is that hot water extracts have many chemical constituents, such as phenols (Kim et al. 2011) and polysaccharides (Wu et al. 2021; Xu et al. 2018), that exhibit antioxidant activities. The various recovery methods for polysaccharide produce different yield for different combination methods, but the most consistent and better yields were produced at high temperature (Gu & Pan 2014; Zhang et al. 2013). In fact, higher extraction temperature yielded higher amount of polysaccharides in other organisms (Arasi et al. 2016; Gu & Pan 2014). This is because high temperature could maximise the diffusion coefficient and solubility rate of polysaccharide in hot water solvent (Lee et al. 2017). Hence, the heating process during hot water extraction could justify the distinction result in antioxidant activity assay. Further analysis and characterisation of the hot water yeast extract by metabolomics must be performed to ascertain the constituents that contributed to the antioxidant effect.

HOT WATER EXTRACT FROM *S. cerevisiae* IS NON-TOXIC TO HDF

When compared to the untreated cells, almost all concentrations of the hot water extract significantly increased ($p < 0.05$) the viability of young and pre-senescent HDF to more than a 100% (Figure 2), except for 1:10 000 in the young group (Figure 2(a)), and 1:1000, 1:10,000 and 1:100,000 in the pre-senescent group (Figure 2(b)). The number of senescent cells were significantly increased ($p < 0.05$) when compared to the untreated cells, when treated with 1,000:1, 100:1, 10:1, 1:1 and 1:10 concentrations (Figure 2(c)). This result

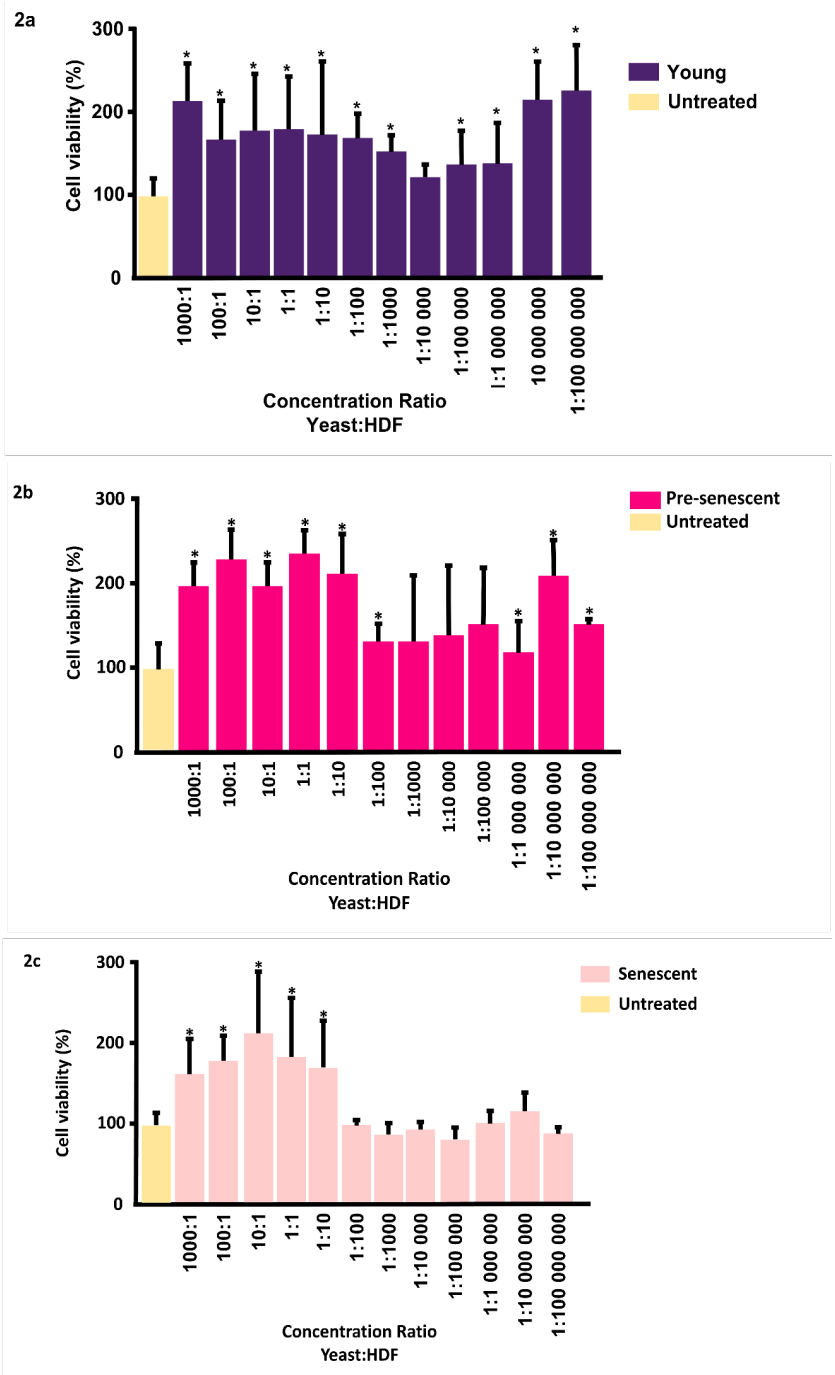

FIGURE 2. The percentage of viable HDF following treatment with various concentration of hot water extract from *S. cerevisiae*. Different number of *S. cerevisiae* cells per HDF ratio (yeast:HDF) were harvested for extract preparation, and used to treat HDF. In each age group the data from each concentration was compared to the control (untreated) by unpaired *t*-test. Data were represented as mean ± SD (n = 3). 2a) Young HDF + hot water extract 2b) Pre-senescent HDF + hot water extract 2c) senescent HDF + hot water extract. *Significantly different ($p < 0.05$) compared to the control (untreated) group

suggested that the senescent cells thrived better in the presence of the yeast extract. The yeast:HDF ratio of 10:1 was chosen as the treatment dose, to ensure that the HDF receive sufficient amount of yeast extract.

HOT WATER EXTRACT FROM *S. cerevisiae* REDUCED SA-β-Gal IN HDF

SA-β-Galactosidase is an established biomarker for cellular senescence in both *in vitro* and *in vivo* studies. The level of this biomarker can be used to differentiate senescent cells between quiescent and terminally differentiated cells (Dimri et al. 1995). It is a pH-dependent staining process, where the biomarker is only detectable at pH 6.0 (Dimri et al. 1995). There was a small number of blue-stained cells observed in the young (PD < 12) HDF (Figures 3(A) & 4). As the cells senesced further, the number of blue-stained cells in the pre-senescent (30 < PD < 40) group (Figures 3(B) & 4) significantly increased, compared with the young cells ($p < 0.001$). The highest number of blue-stained cells can be observed in the senescent (PD > 50) group (Figures 3(C) & 4), which was significantly higher than that in the young group ($p < 0.001$).

The number of blue-stained cells were significantly reduced in all treated groups, when compared with their untreated counterparts: $p < 0.01$ for the young, $p < 0.05$ for the pre-senescent and $p < 0.001$ for the senescent (Figures 3(a), 3(b), 3(c) & 4).

As opposed to epithelial cells, fibroblasts have more spread out structural proteins like fibronectin, actin and microtubules that give rise to the spindle shape of fibroblasts (Marceau et al. 1980). Young fibroblasts appear smaller than the pre-senescent and senescent counterparts (Figure 3), while the pre-

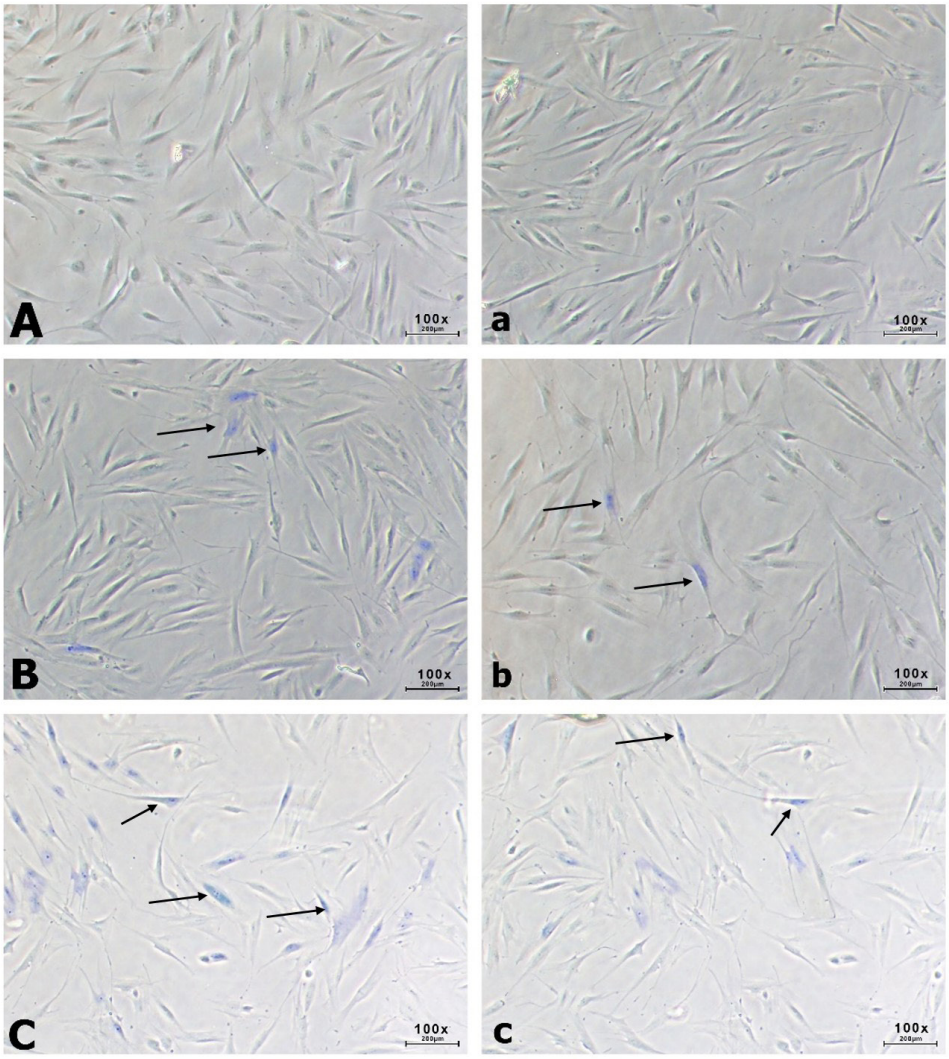

FIGURE 3. SA-β-Gal staining in treated (yeast:HDF ratio of 10:1) and untreated HDFs. (A) Young HDF and (a) Young HDF + hot water extract (B) Pre-senescent HDF and (b) pre-senescent HDF + hot water extract (C) senescent HDF and (c) senescent HDF + hot water extract. Positive blue stain of SA-β-Gal appeared in pre-senescent and senescent HDF, as indicated by arrows

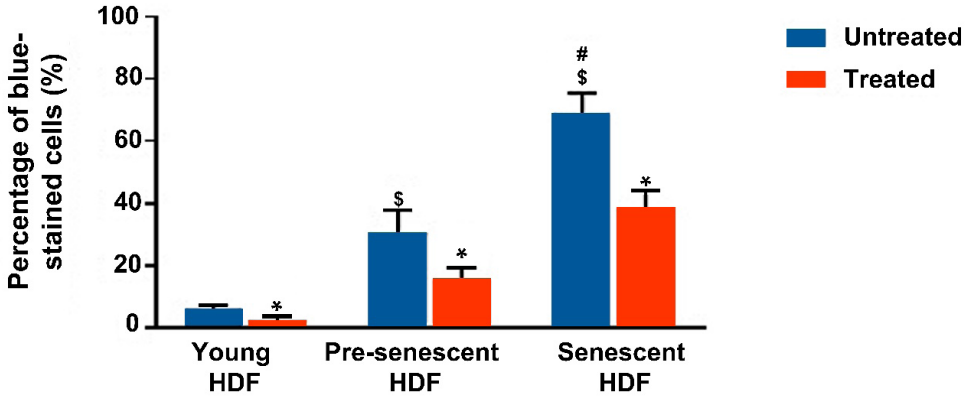

FIGURE 4. The percentage of blue-stained HDF cells detected by SA-β-Gal assay. The data were analysed by unpaired *t*-test (n = 3). All the treated groups showed a significant reduction in the number of blue-stained cells when compared with their respective control group. The percentage of blue-stained cells in the untreated senescent and pre-senescent groups were significantly higher than the untreated young group. *Significant difference between treated and untreated cells within the same age group. $Significant difference between untreated group with the untreated young cells. #Significant difference between untreated senescent and untreated pre-senescent cells. $*^{\#\$}p < 0.05$.

senescent fibroblasts appeared longer, and have enlarged cellular body. The same outcome was observed in the morphology of senescent cells, where they appeared the longest and the flattest, as also observed in previous studies (Makpol et al. 2013; Tan et al. 2018). The larger flattened cytoplasm was attributed to an increase in the number and size of lysosomes (Lipetz & Cristofalo 1972), enlarged cisternae in the Golgi complex (Despres et al. 2019), accumulation in autophagic vacuoles (Gerland et al. 2003) and in less regular occurrence, the rise in the number of glycogen particles (Hariton et al. 2018).

Cellular ageing or senescence is an irreversible process (Blagosklonny 2014). The enzyme β-Galactosidase is exclusively expressed by senescent cells like fibroblasts (Makpol et al. 2013) and myoblasts (Khor et al. 2017), but undetectable in the pre-senescent and young, giving rise to its moniker senescence-associated β-Galactosidase (SA-β-Gal) (Dimri et al. 1995). This unique characteristic allows the senescent cells to be distinguishable from quiescent and immortal cells (Dimri et al. 1995), propelling the detection method into a standard of senescence assay in fibroblast studies (Moon et al. 2019). However, this study observed some blue-stained young cells (Figure 4), positive of SA-β-Gal, which could be observed in previous studies (Gerland et al. 2003; Severino et al. 2000). Previous study (Gerland et al. 2003) reported a strong expression of SA-β-Gal in young cells (PD < 20, human diploid fibroblast MRC5), detected by transmission electron microscopy. However, this does not mean that the young group contains senescent cells but instead the staining was influenced by cell density (Severino et al. 2000). This study showed that the senescent cells highly expressed SA-β-Gal, but the expression was reduced when treated with the yeast extract.

The anti-senescence exhibited by the yeast extract could be due to its antioxidant effect, which reduced the oxidative stress in the cells. There are other studies that supported this interpretation, where antioxidant extracts simultaneously showed anti-ageing effects on cells. One study found that a leaf extract from *Pourthiaea villosa* had anti-ageing activity, by reducing the production of reactive oxygen species (ROS) in hydrogen peroxide induced senescent HDF. The leaf extract also had DPPH-scavenging effect (Choi et al. 2019). Another study found that the compound licochalcone D from licorice reduced the expression of SA-β-Gal in oxidative stress-induced senescent human bone marrow-mesenchymal stem cells (Maharajan et al. 2021). This was achieved via activation of adenosine 5' monophosphate-activated protein kinase (AMPK) pathway, in both their *in vitro* and *in vivo* models. In a rat model, researchers found that traditional Chinese medicine Pinggan-Qianyang decoction reduced SA-β-Gal expression, whilst reducing

superoxide anion level and increasing the level of antioxidant enzyme superoxide dismutase (Cui et al. 2021). In this study, prior to the SA-β-Gal assay, the cells already reached senescence based on the PD value, but the yeast extract reduced the number of cells that expressed SA-β-Gal. From this discovery, it was postulated that this extract might be able to reverse ageing or at least replicative senescence, phenotypically. To support this postulation, future research shall include studying the effect of this extract on telomere length (a marker of cellular ageing) and telomerase activity (Tsoukalas et al. 2019) in HDF. Telomere length is shortened as the cell ages (Ju et al. 2021). High glucose-induced senescent in human glomerular mesangial cells contained significantly shorter telomeres and higher level of SA-β-Gal than normal cells, but treatment of the senescent cells with tea polyphenols increased the length of telomeres and reduced the expression of SA-β-Gal (Cao et al. 2019). Although the results showed a promising application of this extract, there was lack of funding for clinical trial that limited the current study. Eventually, a clinical trial must be performed to observe the effect of this extract on the skin phenotype, such as the elasticity and firmness (Zhong et al. 2019), with the help of future funding.

Conclusion

This study demonstrated that hot water yeast extract can exhibit both anti-senescent and antioxidant. However, subsequent studies must be done to further support and verify the results of this work, as well as to characterise the biochemical pathways involved. Future studies will include determining the constituent of the extract via liquid chromatography mass spectrometry (LCMS), assaying proteins that are involved in skin ageing such as pro-collagen, matrix metalloproteinases and many others, determining the effect of the extract on HDF cell cycle and RNA microarray.

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