Investigating the effect of phytoextracts on protecting *Saccharomyces cerevisiae* from oxidative stress

Koo Jun Yuan, Ryan Teoh, Zong Sheng Hao

1-26

ABSTRACT

Environmental stressors can lead to an increased generation of reactive oxygen species (ROS) which can lead to oxidative stress when ROS levels exceed detoxification mechanisms. Addition of antioxidant-rich phytoextracts to cells would be beneficial as the antioxidants would scavenge free radicals. In this study, UV radiation, hydrogen peroxide, and ethanol were used to induce oxidative stress in *Saccharomyces cerevisiae*, which resulted in a significant decrease (86%, 83%, and 95% respectively) in colony forming units (CFUs). However, addition of 10% (v/v) of aqueous extracts of *Zingiber officinale* and *Capsicum annuum* showed protective effects on yeast cells, restoring the CFU with only a loss of 17% and 4% for UV-treated cells; 11% and 37% for cells added with hydrogen peroxide; and 44% and 35% for cells treated with ethanol, respectively. The mixture of aqueous extracts was not found to exhibit synergistic effects in restoring survival of yeast cells treated with UV and hydrogen peroxide.

INTRODUCTION

Saccharomyces cerevisiae, also known as baker's yeast, has many industrial uses, such as brewing, winemaking, and baking bread. However, in these processes, *S. cerevisiae* also experiences oxidative stress resulting from environmental stresses. In the brewing process, for example, optimal oxygen levels are required for fermentation, and hence, for beer making to take place. Thus, yeast is constantly exposed to oxygen, not just in brewery propagation, but also when placed in fermentation vessels (Briggs, Boulton, Brookes, & Stevens, 2004; Gibson, Lawrence, Leclaire, Powell, & Smart, 2007). Serial repitching, a process whereby yeast cropped at the end of the fermentation is reused in subsequent fermentations and re-exposed to oxygen as before, can only be conducted a finite number of times before quality of yeast and hence fermentation performance both progressively worsen (Gibson et al., 2007). Due to the fact that reactive oxygen species (ROS) plays a direct role in cellular aging (Halliwell & Gutteridge, 1999) and replicative lifespan in yeast is correlated to the antioxidant potential of the cell (Barker, 1999; Van Zandycke, Sohier, & Smart, 2002), the amount of oxygen exposure of a certain batch of yeast and its ability to mitigate the effects of ROS generation might be used to determine the number of times serial repitching can be carried out (Gibson et al., 2007).

Similarly, in wine-making, yeast cells experience processes involving aeration, nutrient deprivation, and dehydration, which can all induce oxidative stress (Garre, Raginel, Palacios, Julien, & Matallana, 2009). Multiple studies conducted with laboratory yeast strains have shown ROS accumulation during dehydration resulting in protein denaturation, lipid peroxidation and damage to nucleic acids (Pereira, Panek, & Eleutherio, 2003; França, Panek, & Eleutherio, 2007). As for the process of bread-making, cells of *S. cerevisiae* are exposed to the multiple environmental stresses that have become associated with baking - these include freeze-thawing, high-sucrose, and air-drying. Due to the denaturation of antioxidant enzymes and damage done to the mitochondrial membrane caused by the said stress conditions, *S. cerevisiae* cells often experience oxidative stress during baking (Takagi & Shima, 2015).

As shown above, oxidative stress can lead to the deterioration of fermentation efficacy in *S. cerevisiae*. By exogenously adding antioxidants to *S. cerevisiae*, *S. cerevisiae* cells would be aided in the scavenging of free radicals, and could potentially become more resistant to environmental stresses present in the brewing, wine-making, and bread-making processes which lead to oxidative stress (Biradar et al., 2017).

By investigating whether phytoextracts of certain commonly-found plants in Singapore contain the necessary antioxidants to ameliorate the effects of oxidative stress on *S. cerevisiae* cells, they can potentially be developed to protect *S. cerevisiae* cells from environmental stresses which they are exposed to during said industrial processes, *S. cerevisiae* cells would possess increased replicative lifespan, fermentation capacity, viability and vitality, leading to heightened fermentation efficacy, reducing production costs while maximising profit for breweries, wineries, and bakeries alike.

OBJECTIVES AND HYPOTHESES

This study aims to investigate whether the aqueous extracts of *Zingiber officinale* (ginger) and *Capsicum annuum* (red capsicum) would be able to restore the survival of yeast cells exposed to UV radiation, hydrogen peroxide and ethanol, and whether a mixture of these two phytoextracts would exhibit synergistic effect in protecting yeast cells. We hypothesise that the extracts are able to protect yeast cells against UV radiation, hydrogen peroxide, and ethanol, and that a synergistic effect would be observed with a combination of extracts.

METHODS

Growth of yeast cells (preparation of yeast preculture)

S. cerevisiae cells were inoculated into 10 ml potato dextrose broth and grown overnight at 30°C in a shaking incubator. The number of cells were determined using a haemocytometer and standardised at approximately 1 x 10^{7} cells ml^{-1}.

Preparation of plant extracts

1 g of plant sample was ground in 10 ml of deionised water using a mortar and pestle. The entire mixture was then transferred to a 50-ml centrifuge tube and heated to 48°C for 20 min in a water bath. The mixture was centrifuged at 7000 rpm for 10 min, the supernatant was collected and was filter-sterilised through a microfilter.

Effect of plant extracts on yeast cells exposed to ultraviolet light

Four mixtures were prepared and left to stand at room temperature for 20 min. Control setups 1 and 2 each consisted of 3 ml of yeast preculture and an equal volume of sterile water. The mixture in control setup 2 was then transferred into a Petri dish for UV exposure for 30 s. This tested the effect of UV light on the growth of yeast cells in the absence of antioxidants. Control setup 3 consisted of 3 ml of yeast preculture and an equal volume of plant extract. This tested the effect of antioxidants on the growth of yeast cells. The test setup contained 3 ml of yeast preculture and an equal volume of plant extract. This mixture was then transferred into a Petri dish for UV exposure for 30 s to test if the antioxidants protected the cells from damage caused by free radicals from UV light. Five replicates of each setup were prepared. Mixtures from control setups 1 and 3 and test set-up were serially diluted to 10^{-4} with saline solution, while mixtures of control setup 2 were diluted to 10^{-2}. 0.1 ml of the diluted cultures were spread evenly with a sterile spreader on potato dextrose agar. The plates were incubated at 30°C and the number of yeast colony forming units was determined after 24 h.

Effect of plant extracts on yeast cells treated with hydrogen peroxide

Three setups were prepared. Control setup 1 contained 2 ml of yeast preculture with 3 ml of sterile water. Control setup 2 consisted of 2 ml of yeast preculture with 2 ml of sterile water and 1 ml of 2 mM hydrogen peroxide solution. This tested the effect of hydrogen peroxide on the growth of yeast cells in the absence of antioxidants. The test setup had 2 ml of yeast preculture added with 2 ml of plant extract and 1 ml of 2 mM hydrogen peroxide solution. This tested the effect of antioxidants on the growth of yeast cells treated with hydrogen peroxide

solution. Five replicates of each setup were prepared. The set-ups were incubated at 30°C for 60 min. Serial dilutions were then carried out with saline solution. 0.1 ml of the diluted cultures were spread evenly with a sterile spreader on potato dextrose agar. The plates were incubated at 30°C and the number of yeast colony forming units was determined after 24 h.

Effect of plant extracts on yeast cells treated with ethanol

Three setups were prepared. Control setup 1 contained 3 ml of yeast preculture with 3 ml of sterile water. Control setup 2 contained 3 ml of yeast preculture with 1.8 ml of sterile water and 1.2 ml of absolute ethanol to give a final concentration of 20% (v/v) ethanol. This tested the effect of ethanol on the growth of yeast cells in the absence of antioxidants. The test setup was made up of 2 ml of yeast preculture added with 1.8 ml of plant extract and 1.2 ml of absolute ethanol. This tested the effect of antioxidants on the growth of yeast cells treated with ethanol. Five replicates of each setup were prepared. Serial dilution, plating and incubation of plates were done according to that previously described.

DPPH antioxidant test

1,1-diphenyl-2-picryl-hydrazil (DPPH) is a free radical which produces a purple solution when dissolved in methanol. When it is reduced by antioxidants, a change in colouration from purple to yellow is observed. The negative control consisted of 1.0 ml of DPPH, 1.9 ml of methanol and 0.1 ml of sterile water. In the test set-up, plant extract replaced sterile water. Five replicates were prepared. For the respective blanks for each set-up, methanol was added instead of DPPH solution. The initial absorbance was measured at 517 nm against the respective blanks and the mixtures were then left to stand in the darkness for 20 min, before the final absorbance readings were measured. The radical scavenging activity (in %) was then calculated based on the following formula: $\frac{Final\ absorbance\ of\ control - Final\ absorbance\ of\ test}{Final\ absorbance\ of\ control} \times 100\%$

RESULTS

Effect of phytoextracts on yeast cells after UV exposure

Exposure of *S. cerevisiae* cells to UVC light for 30 s was found to decrease colony forming units by 86%, but addition of aqueous extract of *Zingiber officinale* showed significant ameliorative effects, reducing loss of CFUs to only 17% (Fig. 1). Addition of aqueous extract of *Capsicum annuum* showed even stronger ameliorative effects, restoring CFUs with only a loss of 4% (Fig. 2). However, mixture of both extracts did not restore CFUs at a significantly

higher rate than the individuals extract, thus it was not found to exhibit synergistic effect (Fig. 3). The Kruskal-Wallis test p value was 0.00547 for setups with ginger, 0.00896 for capsicum, and 0.0015 for the mixture, showing that the differences in number of colony forming units were statistically significant. The plates are shown in Fig. 4.

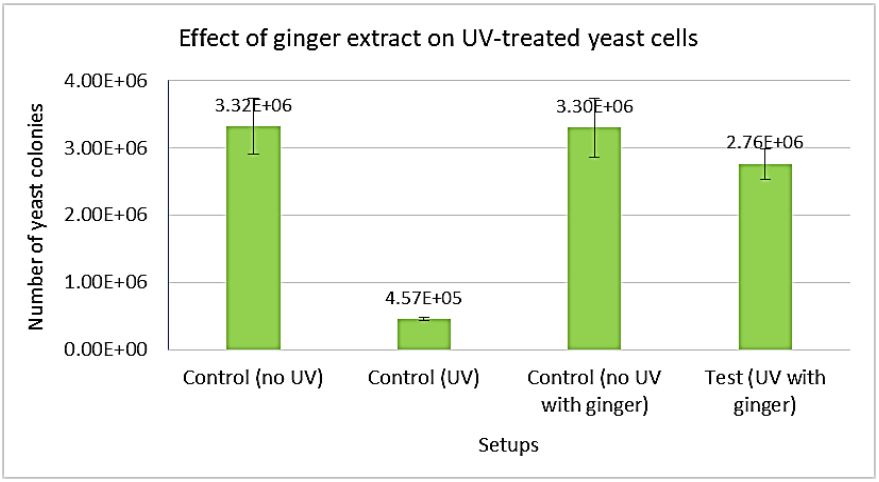

Fig. 1: Graph showing the effect of ginger extract on UV-treated yeast cells.

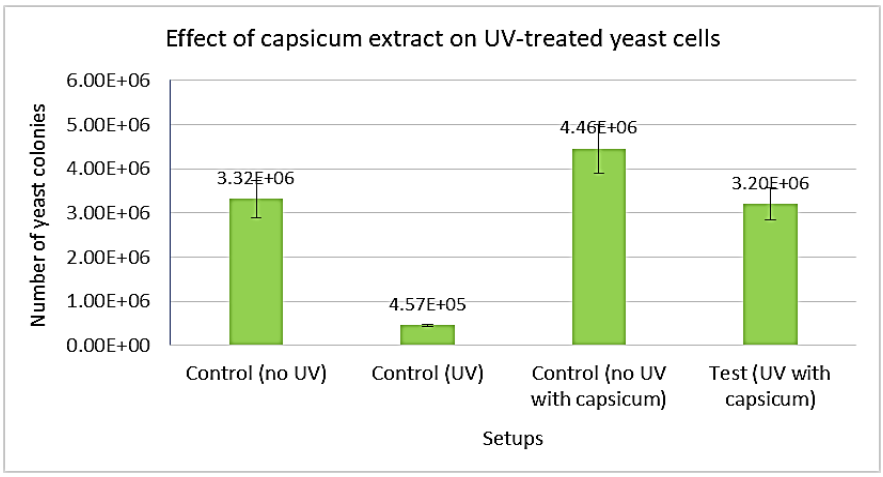

Fig. 2: Graph showing the effect of capsicum extract on UV-treated yeast cells.

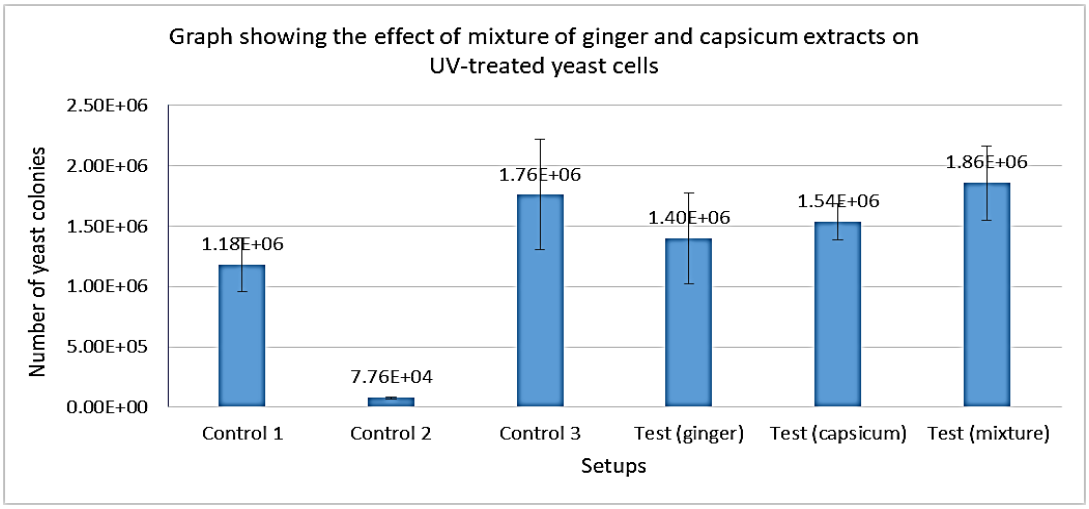

Fig. 3: Graph showing the effect of a mixture of ginger and capsicum extracts on UV-treated yeast cells. No synergistic effect was observed when extracts were used in combination.

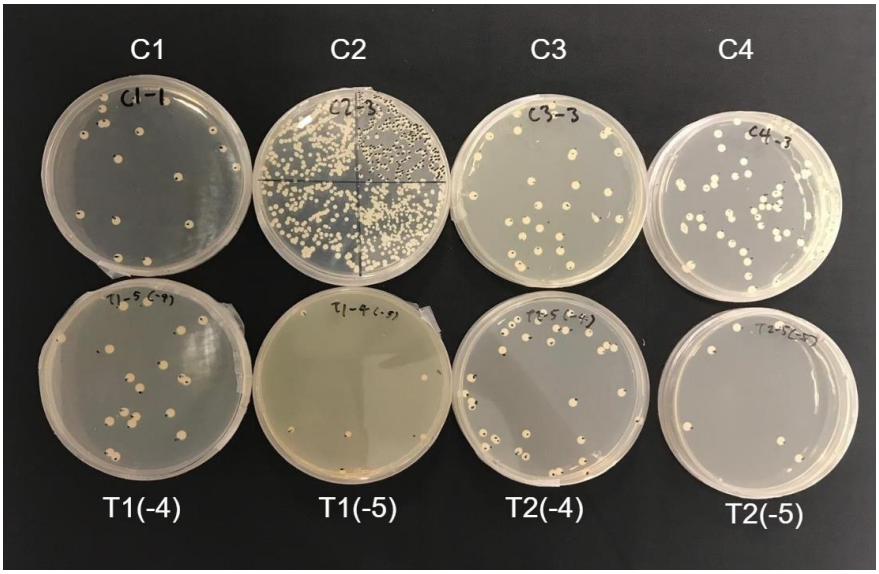

Fig. 4: Plates with yeast colonies. C1 and C2 represent Control 1 (no UV) and Control 2 (UV), respectively. C3 and C4 represent Control 3 (ginger extract) and Control 4 (capsicum extract), respectively. T1 and T2 represent Test 1 (ginger extract with UV) and Test 2 (capsicum extract with UV), respectively. Dilution factors for all setups were 10^{-4} or 10^{-5}, but 10^{-2} for Control 2.

Effect of phytoextracts on yeast cells treated with hydrogen peroxide

Treatment of *S. cerevisiae* cells with hydrogen peroxide decreased CFUs by 83%, but addition of aqueous extract of *Capsicum annuum* restored the CFUs with a loss of 37%, while the addition of aqueous extract of *Zingiber officinale* restored the CFUs with a loss of only 11% (Fig. 5). A mixture of extracts was not found to exhibit synergistic effect (Fig. 6). The p values in the Kruskal-Wallis test were 0.0062 and 0.0023 for individual extracts, and the mixture, respectively, showing statistical significance in the number of yeast colonies among setups.

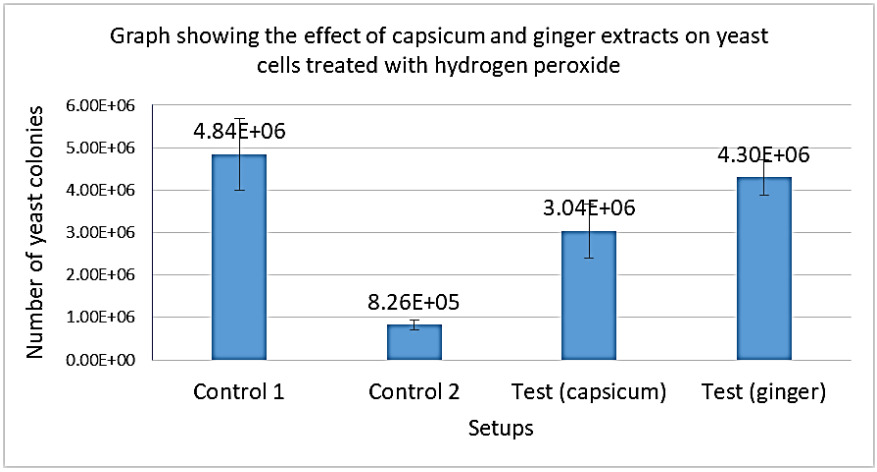

Fig. 5: Graph showing the effect of capsicum and ginger extracts on yeast cells treated with hydrogen peroxide.

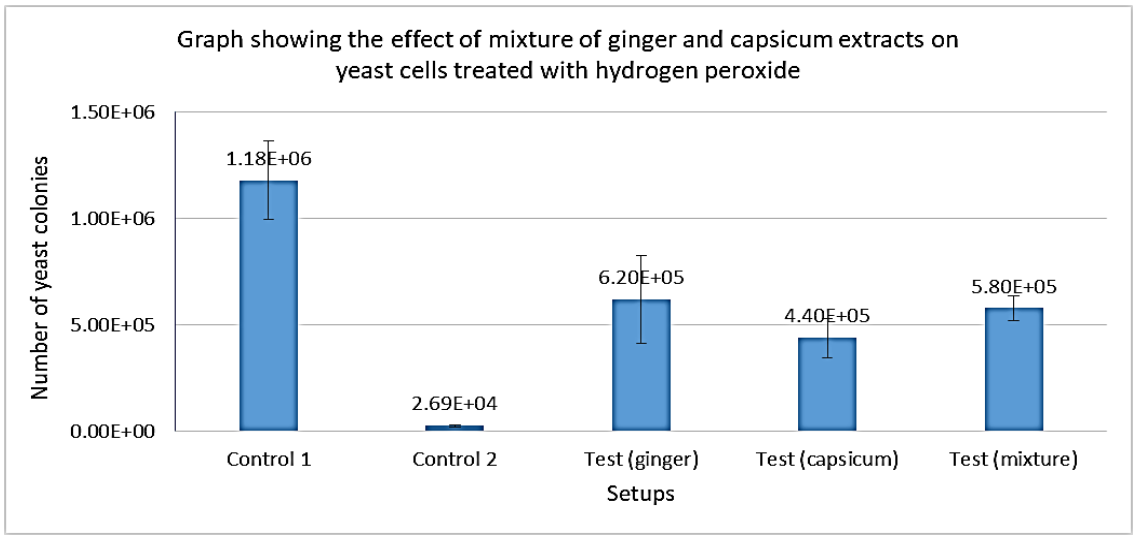

Fig. 6: Graph showing the effect of mixture of ginger and capsicum extracts on yeast cells treated with hydrogen peroxide.

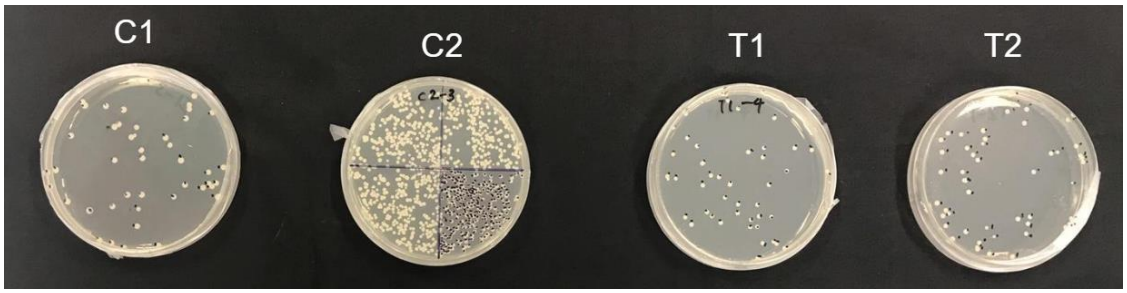

Fig. 7: Plates with yeast colonies. C1 and C2 represent Control 1 (no hydrogen peroxide) and Control 2 (hydrogen peroxide), respectively. T1 and T2 represent Test 1 (ginger extract with hydrogen peroxide) and Test 2 (capsicum extract with hydrogen peroxide), respectively. Dilution factors for all setups were 10^{-4}, but 10^{-2} for Control 2.

Effect of phytoextracts on yeast cells treated with ethanol

Treatment of *S. cerevisiae* cells with ethanol decreased CFUs by 95%, but addition of aqueous extract of *Zingiber officinale* restored the CFUs with a loss of 44%, while the addition of aqueous extract of *Capsicum annuum* restored the CFUs with a loss of only 35% (Fig. 8). The Kruskal-Wallis test p value was 0.00151, indicating that the difference in the mean number of yeast colonies in setups was statistically significant.

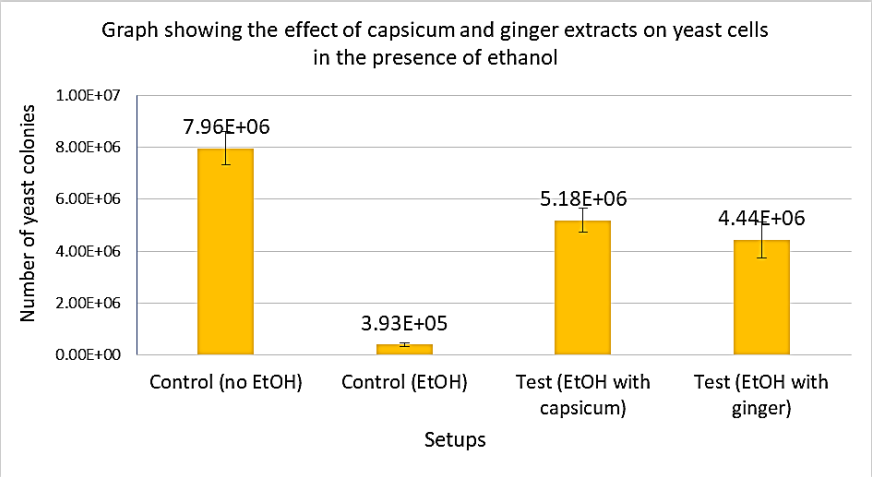

Fig. 8: Graph showing the effect of capsicum and ginger extracts on yeast cells treated with ethanol.

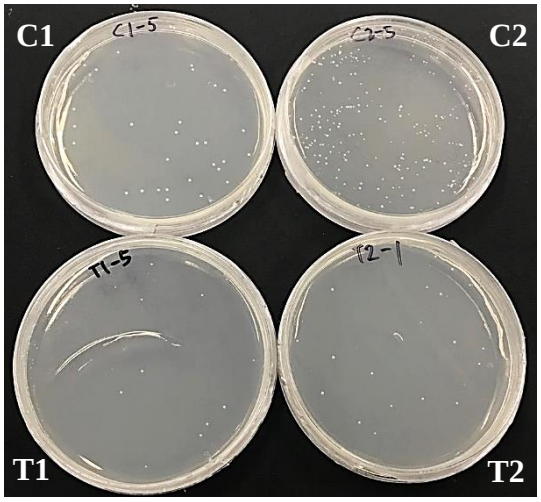

Fig. 9: Plates with yeast colonies. C1 and C2 represent Control 1 (no ethanol) and Control 2 (ethanol), respectively. T1 and T2 represent Test 1 (ginger extract with ethanol) and Test 2 (capsicum extract with ethanol), respectively. Dilution factors for all setups were 10^{-4}, but 10^{-2} for Control 2.

DPPH antioxidant assay

DPPH antioxidant assay was carried out to quantify the radical scavenging activities of both extracts. The radical scavenging activity of *Zingiber officinale* was determined to be 60.33% whereas that of *Capsicum annuum* was 65.45% (Fig. 10). The Mann-Whitney U test gave a pvalue of 0.01208, showing that the differences in final absorbance of the control and test were statistically significant.

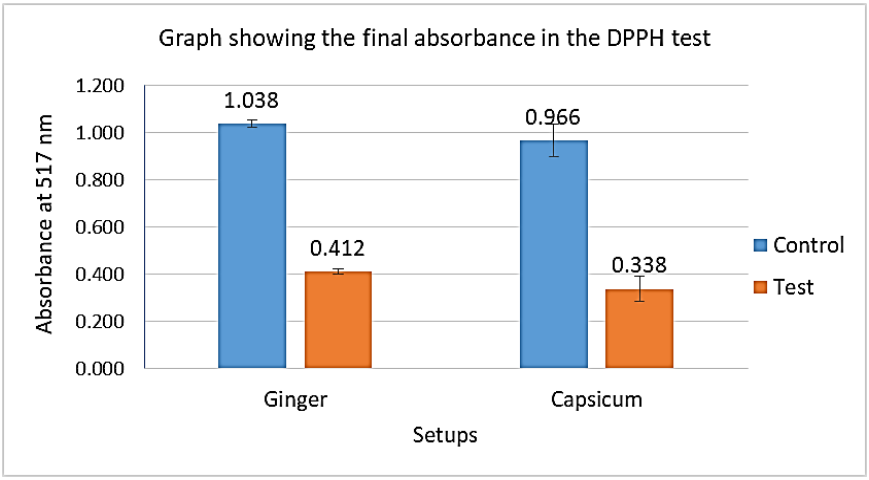

Fig. 10: Graph showing final absorbance in the DPPH test.

DISCUSSION

Hydrogen peroxide is a reactive oxygen species itself, so the loss in CFUs after treatment with hydrogen peroxide can be attributed to the action of ROS which causes oxidative stress, directly leading to cell death. UV light induces oxidative stress by releasing inflammatory cytokines which results in the generation of ROS (Goswami, Sharma, & Haldar, 2013). As for ethanol, the process of oxidative stress is much more complicated as it is induced via multiple pathways such as damage to the cell membrane leading to increased cell membrane permeability, as well as depletion of antioxidant enzymes and release of inflammatory cytokines (Wu & Cederbaum, 2004). The reduction in loss of CFUs can be attributed to the plant antioxidant compounds found in the phytoextracts which scavenge free radicals, therefore protecting yeast cells from oxidative stress. Such ameliorative effects have been found for several plant extracts, such as one study where apple polyphenols ameliorated ROS-mediated aging in yeast cells (Stirpe et al., 2017), and another where antioxidants from *Centella asiatica*, *Phyllanthus emblica*, *Asparagus racemosus*, *Tinospora cordifolia* restored protected *S. cerevisiae* cells from oxidative stress induced by malachite green (Biradar et al., 2017).

Both capsicum and ginger extracts showed strong ameliorative effects, which also corresponds with the high antioxidant levels found in these extracts. Our results match those of scientific literature. One *in vivo* study reported that ginger essential oil increased antioxidant marker enzymes like catalase and glutathione peroxidase, and hence the cells' viability of hydrogen peroxide-induced oxidative stress (Höferl et al., 2015). One *in vitro* study on mice skin cells reported that 6-paradol and its derivative 6-dehydroparadol, which are both strong antioxidant compounds found in ginger, attenuated oxidation of DNA bases induced by hydrogen peroxide and UV (Chung, Jung, Surh, Lee, & Park, 2001). Administration of extract rich in 6-shogaol (another antioxidant in ginger) to mice has also been shown to restore protein expression of antioxidant enzymes such as superoxide dismutase, glutathione peroxidase and catalase (Bak, Ok, Jun, & Jeong, 2012).

As for capsicum, compounds like ascorbate, as well as gallic acid, which are both found in high amounts in *Capsicum annuum*, could have been the main factor behind the restoration of yeast CFUs. One study by Wu et al. (2011), showed that ascorbate and gallic acid scavenged hydrogen peroxide by mitigating the oxidant-induced growth arrest of the yeast cells. Ascorbic acid was also able to influence Cr(VI) toxicity by scavenging free radicals formed during Cr(VI) to Cr(III) reduction (Poljšak et al., 2005). Another antioxidant in the capsicum could be

melatonin. Treatment with melatonin increased viability for yeast cells after hydrogen peroxide stress up to 35% more than for the untreated controls, while stress amelioration reached 40% for UVC light (Bisquert, Muñiz-Calvo, & Guillamón, 2018).

CONCLUSIONS

In the present study, oxidative stress was induced on *Saccharomyces cerevisiae* via exposure to UV light, hydrogen peroxide, and ethanol, resulting in significant loss of colony forming units (CFUs). However, loss of CFUs was greatly restored with the addition of aqueous extract of ginger (*Zingiber officinale*) and red capsicum or red bell pepper (*Capsicum annuum*), which can be attributed to the antioxidant compounds present in both plants. This is supported by the results from the DPPH assay showing the strong free-radical scavenging properties of both extracts. However, no synergistic effect was found for the ginger-capsicum mixture.

A limitation was the difficulty to standardise the antioxidant content in the extracts of ginger and capsicum each time a fresh batch of extract was prepared, thus leading to varying concentrations of antioxidants and varying ability to protect yeast cells against agents of oxidative stress.

Further research could be conducted using other inducers of oxidative stress apart from those used in the present study, and also investigate whether other solvents could be used to effectively extract antioxidants from the plants.

REFERENCES

Bak, M., Ok, S., Jun, M., & Jeong, W. (2012). 6-Shogaol-rich extract from ginger up-regulates the antioxidant defense systems in cells and mice. *Molecules*, 17(7), 8037-8055. doi:10.3390/molecules17078037

Barker, M. (1999). Effect of Cu, Zn superoxide dismutase disruption mutation on replicative senescence in *Saccharomyces cerevisiae*. *FEMS Microbiology Letters*, 177(2), 199-204. doi:10.1016/s0378-1097(99)00308-0

Biradar, S., Tamboli, A., Patil, T., Khandare, R., Govindwar, S., & Pandwar, P. (2017). Phytoextracts protect *Saccharomyces cerevisiae* from oxidative stress with simultaneous enhancement in bioremediation efficacy. *Indian Journal of Experimental Biology*, 55, 469-478.

Bisquert, R., Muñiz-Calvo, S., & Guillamón, J. M. (2018). Protective role of intracellular melatonin against oxidative stress and UV radiation in *Saccharomyces cerevisiae*. *Frontiers in Microbiology*, 9, 318. doi:10.3389/fmicb.2018.00318

Briggs, D.E., Boulton, C.A., Brookes, P.A., & Stevens, R. (2004). Brewing: science and practice. *Journal of the Science of Food and Culture*, 86(1). doi:10.1533/9781855739062

Chung, W., Jung, Y., Surh, Y., Lee, S., & Park, K. (2001). Antioxidative and antitumor promoting effects of [6]-paradol and its homologs. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis*, 496(1-2), 199-206. doi:10.1016/s1383-5718(01)00221-2

França, M., Panek, A., & Eleutherio, E. (2007). Oxidative stress and its effects during dehydration. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology*, 146(4), 621-631. doi:10.1016/j.cbpa.2006.02.030

Garre, E., Raginel, F., Palacios, A., Julien, A., & Matallana, E. (2010). Oxidative stress responses and lipid peroxidation damage are induced during dehydration in the production of dry active wine yeasts. *International Journal of Food Microbiology*, 136(3), 295-303. doi:10.1016/j.ijfoodmicro.2009.10.018

Gibson, B.R., Lawrence, S.J., Leclaire, J.P., Powell, C.D., & Smart, K.A. (2007). Yeast responses to stresses associated with industrial brewery handling. *FEMS Microbiology Reviews*, 31(5), 535-569. doi:10.1111/j.1574-6976.2007.00076.x

Goswami, S., Sharma, S., & Haldar, C. (2013). The oxidative damages caused by ultraviolet radiation type C (UVC) to a tropical rodent *Funambulus pennanti*: Role of melatonin. *Journal of Photochemistry and Photobiology B: Biology*, 125, 19-25. doi:10.1016/j.jphotobiol.2013.04.008

Halliwell, B. & Gutteridge, J.M.C. (1999). Free radicals in biology and medicine. Oxford: Oxford University Press.

Höferl, M., Stoilova, I., Wanner, J., Schmidt, E., Jirovetz, L., Trifonova, D., . . . Krastanov, A. (2015). Composition and comprehensive antioxidant activity of ginger (*Zingiber officinale*) essential oil from Ecuador. *Natural Product Communications*, 10(6), 1085-1090. doi:10.1177/1934578x1501000672

Pereira, E.D., Panek, A.D., & Eleutherio, E.C. (2003). Protection against oxidation during dehydration of yeast. *Cell Stress & Chaperones*, 8(2), 120. doi: 10.1379/1466-1268(2003)0082.0.co;2

Poljšak, B., Gazdag, Z., Jenko-Brinovec, Š, Fujs, Š, Pesti, M., Bélagyi, J., & Raspor, P. (2005). Pro-oxidative vs antioxidative properties of ascorbic acid in chromium (VI)-induced damage: an *in vivo* and *in vitro* approach. *Journal of Applied Toxicology*, 25(6), 535-548. doi:10.1002/jat.1093

Stirpe, M., Palermo, V., Bianchi, M.M., Silvestri, R., Falcone, C., Tenore, G., Novellino, E., & Mazzoni, C. (2017). Annurca apple (M. pumila Miller cv Annurca) extracts act against stress and ageing in *S. cerevisiae* yeast cells. *BMC Complementary and Alternative Medicine*, 17(1), 200. doi: 10.1186/s12906-017-1666-7

Takagi, H. & Shima, J. (2015). Stress tolerance of baker's yeast during bread-making processes. *Stress Biology of Yeasts and Fungi,* 23-42. doi:10.1007/978-4-431-55248-2_2

Van Zandycke, S.M., Sohier, P.J., & Smart, K.A. (2002). The impact of catalase expression on the replicative lifespan of *Saccharomyces cerevisiae*. *Mechanisms of Ageing and Development,* 123(4), 365-373. doi:10.1016/s0047-6374(01)00382-7

Wu, D., & Cederbaum, A.I. (2003). Alcohol, oxidative stress, and free radical damage. *Alcohol Research & Health*, 27, 277-284.

Wu, M. J., Odoherty, P. J., Fernandez, H. R., Lyons, V., Rogers, P. J., Dawes, I. W., & Higgins, V. J. (2011). An antioxidant screening assay based on oxidant-induced growth arrest in *Saccharomyces cerevisiae*. *FEMS Yeast Research*, 11(4), 379-387. doi:10.1111/j.1567-1364.2011.00726.x