INVESTIGATING THE ANTICANCER EFFECTS OF LINALOOL AND PERILLYL ALCOHOL ON *SACCHAROMYCES CEREVISIAE*

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ABSTRACT

Cancer is a lethal disease that healthcare industries worldwide have been grappling with since time immemorial - and Singapore is no exception. According to the Singapore Cancer Society, 12,775 new cancer cases are diagnosed annually in Singapore, showing the severity of the problem. Existing cancer treatments like surgery, radiation and chemotherapy have their limitations, such as the inability to combat metastasis, undesirable side effects, and cost. Hence, there has been growing interest in using affordable, natural cures like lavender essential oil to treat cancer. Linalool and perillyl alcohol are 2 phytochemicals present in lavender essential oil which have demonstrated anti-tumoral effects in mice and humans. This study aimed to elucidate the pro-cell death and antiproliferative potential of linalool and POH on *Saccharomyces cerevisiae* (*S. cerevisiae*), which acts as a cancer model. Linalool and POH were added in varying doses to *S. cerevisiae* cultures. Cell death was analysed with the trypan blue and gel electrophoresis assay, inhibition of proliferation was analysed with the colony forming unit assay, and antioxidant activity was analysed with the 2,2-diphenyl-1-picrylhydrazyl assay. Both linalool and POH showed statistically significant pro-cell death and antiproliferative effects on *S. cerevisiae*, with the minimum concentration of 50% cell death being 10.069 mM and 658.762 μgmL^{-1} respectively. Both chemicals also likely acted through an apoptotic pathway. However, POH potentially induced an antioxidant response in *S. cerevisiae*, causing an increase in DPPH radical scavenging activity.

1. INTRODUCTION

Cancer is one of the most prevalent diseases in the world today, and mortality from cancer is increasing (Safarzadeh, Shotorbani & Baradaran, 2014). Cancer is currently the leading cause of death in Singapore, accounting for 29.7% of deaths in 2015 (Ministry Of Health Singapore, 2018). It was estimated that the lifetime risk for developing cancer in the Singapore

population is approximately 1 for every 4-5 people (Lee, Chew, Chow, Kuo & Ho, 2014), showing that the cancer problem is extremely pertinent in Singapore.

Common cancers such as breast, lung and prostate cancer are primarily treated via surgery, radiation therapy and chemotherapy (Mayo Clinic, 2019). However, in late stage cancers, secondary malignant tumours known as metastases are likely to develop and propagate deep throughout the circulatory or lymphatic system (American Society of Clinical Oncology, 2019), making the surgical removal of tumours extremely difficult, and at times unfeasible (WebMD LLC, 2018). Radiation and chemotherapy both have their disadvantages as well, because of undesirable side effects such as hearing loss (OncoLink, 2016). Furthermore, chemotherapy also entails multidrug resistance (Gautam, Mantha & Mittal, 2014) and a hefty medical bill. Chemotherapy was estimated to cost as high as $30,000 over an 8-week period, and a single cancer episode lasts around 3-4 months (Selby, 2018). In light of the downsides of the existing cancer treatment methods, a new type of therapy - herbal medicine - has taken the medical arena by storm. Herbal medicine contains antioxidant properties and therefore alleviates side effects that current cancer treatments have for less cost and possibly greater effectiveness. Thus many scientists have increasingly been trying to find natural "cures" for cancer, especially in developing nations (Safarzadeh, Shotorbani & Baradaran, 2014), like lavender essential oil.

Lavender essential oil has been known to reduce stress and relieve pain and has recently been said to be anti-tumoral. Its volatile constituents can easily pass through the blood-brain barrier, which is a common obstacle for drug delivery, because of their small size and lipophilicity (Gayathiri *et al.*, 2016). Lavender essential oil also contains two phytochemicals, linalool and perillyl alcohol (POH), which have been shown to trigger apoptosis (Gayathiri *et al.*, 2016) and exhibit antiproliferative effects in cancer cells.

In a 2016 study, linalool was administered to 16 mice xenografted with human cancer cells. Tap-water was administered as a control, and the linalool treatment groups were given a 100 μg/kg low-dose solution or 200 μg/kg high-dose linalool solution. It was found that the tumors and organs from the mice in the high-dose group showed a 55% reduction in mean tumor weight when compared to the mice in the control group. This study showed that linalool

exhibited an anticancer effect as well as minimal toxic effects on healthy cells, and that it has potential for application in cancer therapy (Iwasaki *et al.*, 2016).

A 2014 study also investigated the antiproliferative effects of POH on human glioblastoma multiforme (GBM). Since patients with GBM inevitably develop resistance to the standard temozolomide used during chemotherapy, the study explored the cytotoxic activity of the novel agent NEO212, a conjugate of temozolomide to POH, on three human temozolomide-resistant glioma cell lines. For in vivo evaluation of NEO212, temozolomide-resistant glioma cells were implanted into immune-incompetent mice, and NEO212 was administered. The study showed that NEO212, at equimolar concentrations of temozolomide, was more cytotoxic for temozolomide-resistant cells than temozolomide and not toxic to normal cells. It resulted in DNA strand breaks, endoplasmic reticulum stress and inhibited autophagy, thus causing subsequent death of tumor cells. The study concluded that POH had minimal organ and bone marrow toxicity and effectively entered the tumor (Cho *et al.*, 2014).

A significant problem that scientists studying cancer face is the high cost of many of the novel drugs and therapeutic agents (Cagan & Meyer, 2017). Given the anti-carcinoma properties of the phytochemicals linalool and POH, lavender essential oil is a potential suitable herbal treatment with its low cost and availability in many countries (Safarzadeh, Shotorbani & Baradaran, 2014).

This study examined the effects of using linalool and POH on *Saccharomyces cerevisiae* (*S. cerevisiae*) and elucidate its antiproliferative and pro-cell death potential. *S. cerevisiae* can be exploited as a suitable cancer model as it mimics 3 cancer properties, namely: the autonomous drive to proliferate, tissue invasion and metastasis, and the evasion of apoptosis (LaMorte, 2016). Firstly, upregulation of Ras occurs when *S. cerevisiae* is exposed to glucose, facilitating G_1 to S phase transition (Cazzanelli *et al*., 2018), showing how *S. cerevisiae* proliferates on its own similarly to how cancer grows by itself. Secondly, enzymes involved in lipid metabolism which play crucial roles in metastasis are highly conserved in *S. cerevisiae* (Cazzanelli *et al.*, 2018). Lastly, *S. cerevisiae* undergoes an apoptotic process similar to mammalian apoptosis in areas like radical oxygen species (ROS) production, protease activity and a major role played by

mitochondria (Guaragnella *et al.*, 2014), showing that although *S. cerevisiae* does not model the evasion of apoptosis directly, its similarities with mammalian cancer's apoptotic mechanism still make it invaluable as a simple eukaryote to study cancer.

The findings of this study was combined with results from the Academies of Loudoun, which will be researching on the gliosarcoma 9L/lacZ cell line to determine the feasibility of using lavender essential oil extracts as an effective treatment for cancer.

2. OBJECTIVES AND HYPOTHESIS

This study aimed to examine the pro-cell death and antiproliferative effects of linalool and POH on *S. cerevisiae*. Cell death was examined with trypan blue and gel electrophoresis assay, inhibition of proliferation was measured with the colony forming unit assay and oxidative stress was analysed with the DPPH assay. The lowest concentration of linalool and POH at which 50% cell death occurred (LC50) was then determined.

It was hypothesized that the addition of linalool and POH will significantly decrease the proliferation and survival of *S. cerevisiae*.

3. MATERIALS AND METHODS

3.1 Quantification of linalool and POH in lavender essential oil

Gas chromatography (GC) and high performance liquid chromatography (HPLC) was performed on a sample of *Lavender angustifolia* essential oil (61718, Sigma-Aldrich) to confirm the presence of linalool (51782, Sigma-Aldrich) and POH (77311, Sigma-Aldrich) in lavender essential oil. For HPLC, the solvent system was acetonitrile and ultrapure water in a ratio of 70:30, and reading wavelength was 205 nm.

3.2 Cells and reagents

S. cerevisiae (156250, Carolina Biological Supply) was inoculated into 50 mL sterile Potato Dextrose Broth (PDB) (Oxoid) and incubated overnight at 35°C. Cell concentration was standardized to optical density 0.1AU with a uv-vis spectrophotometer set to 600 nm, and cell density was counted with a hemocytometer. Pure DMSO (Sigma-Aldrich) was used to dissolve linalool to achieve final concentrations of 4, 8, 12.443, 16 and 20.738 mM, and POH to achieve

final concentrations of 175, 350, 590, 700 and 800 μgmL^{-1}. The cell culture was mixed with PDB and either linalool or POH, and seeded in 12mL centrifuge tubes. The final concentration of DMSO was maintained below 0.5%. The centrifuge tubes were incubated at room temperature on a rocker set to 25 rocks/min for 1 day. Various assays were then performed to elucidate the pro-cell death and antiproliferative properties of linalool and POH.

3.3 Trypan blue cell death assay

Cell samples were diluted with PDB until countable with a hemocytometer. 0.4% trypan blue (Sigma-Aldrich) was added to a final concentration of 50% (v/v). Viable and non-viable *S. cerevisiae* were then counted using a hemocytometer. Trypan blue traverses the phospholipid bilayer of non-viable cells, causing them to stain dark blue.

3.4 Colony forming unit assay

Potato Dextrose Agar (Oxoid) plates were prepared in advance. Based on the cell density obtained from the trypan blue assay, cell samples were serially diluted by factors of 10 with and spread on agar plates. The agar plates were then incubated for one day at room temperature before colonies were counted with a colony counter.

3.5 Gel electrophoresis assay

A 1% (w/v) agarose (Bio-Rad) and TAE buffer solution (Invitrogen) was used to cast the agarose gel. *S. cerevisiae* DNA was then extracted using the Yeast DNA Extraction Kit (78870, Thermo Scientific). DNA samples were loaded with bromophenol blue (Invitrogen) as the loading dye and SYBR Green (Invitrogen) as the stain alongside a 1kb ladder (Promega). Electrophoresis was run for 1 hour at 80V, after which DNA bands were visualized with a UV transilluminator.

3.5 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay

DPPH powder (Sigma-Aldrich) was dissolved in methanol (Sigma-Aldrich). 1mL of DPPH solution was added to 2mL methanol to prepare a DPPH control. 1mL of DPPH solution was added to 1.9mL methanol and 0.1mL cell sample to prepare DPPH test groups. Test groups were incubated in the dark at room temperature for 0min before absorbance was read at 517nm. In the presence of antioxidants, purple DPPH will form colourless DPPH-H, thus antioxidant activity

can be measured by spectrophotometric analysis. The DPPH radical scavenging activity (RSA) increases with antioxidant activity, and it is calculated by the formula:

$$\%RSA = \frac{A_0 - A_1}{A_0} \times 100$$

where A_0 is the absorbance of the DPPH control

and A_1 is the absorbance of the DPPH test group

4. RESULTS AND DISCUSSION

4.1 Quantification of $C_{linalool}$ and C_{POH} in lavender essential oil

GC and HPLC results showed that the concentration of linalool (hereby abbreviated as $C_{linalool}$) and concentration of POH (hereby abbreviated as C_{POH}) in *Lavender angustifolia* essential oil was 295 µM and 123 µM respectively. The image output can be found in Appendix A3 and A4 respectively.

4.2 Effect of $C_{linalool}$ and C_{POH} on cell viability

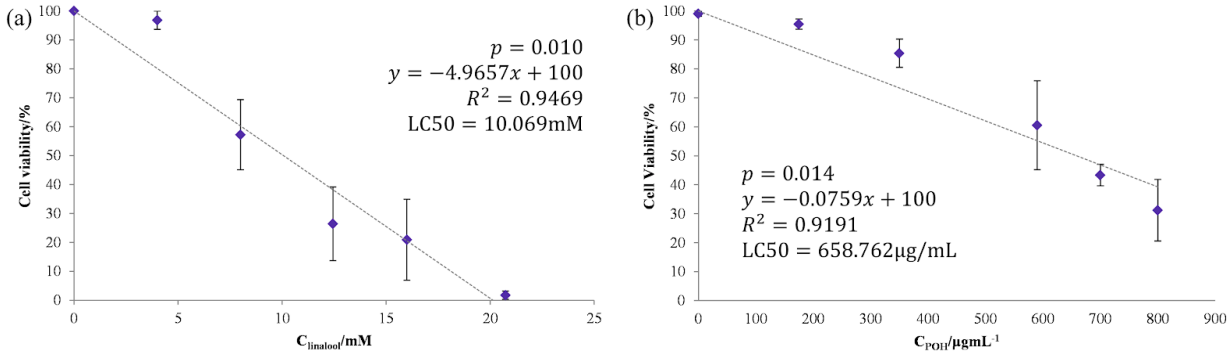

Fig. 4.2.1: Linalool and POH significantly decrease cell viability. *S. cerevisiae* culture was mixed (n=3) with linalool ranging from 0 to 20.738 mM (**a**) or POH ranging from 0 to 800 µgmL^{-1} (**b**) and incubated for 24 h. Cell viability was analysed with the trypan blue assay (n=3). LC50 was determined by substituting y = 50 into the trendline equation and solving for x.

As shown in Fig. 4.2.1, as $C_{linalool}$ increases from 0 to 20.738 mM (a) and C_{POH} increases from 0 to 800 µgmL^{-1} (b), there is a significant decrease in cell viability from 100% to 1.77% (a) and 100% to 31.2% (b) respectively.

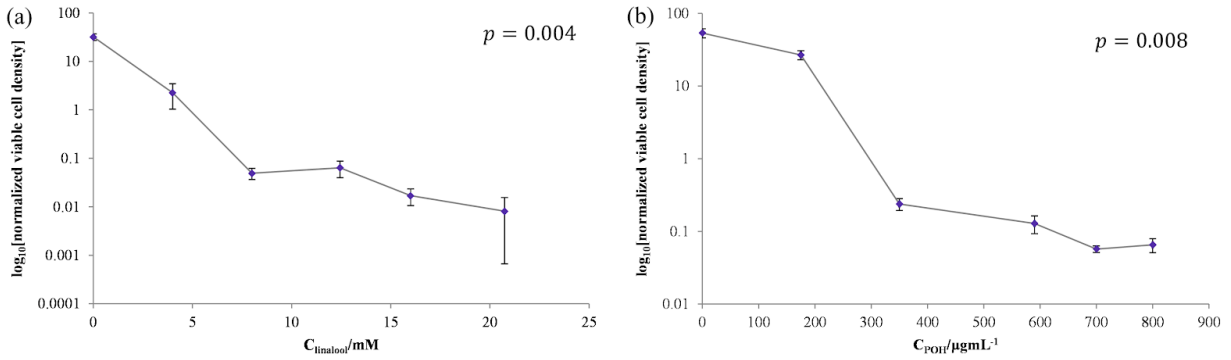

Fig. 4.2.2: Linalool and POH significantly decrease normalized viable cell density. *S. cerevisiae* culture was mixed (n=3) with linalool ranging from 0 to 20.738 mM (**a**) or POH ranging from 0 to 800 µgmL^{-1} (**b**) and incubated for 24 h. Normalized viable cell density was determined with the trypan blue assay (n=3) by dividing viable cell density by initial cell density to reconcile batch differences.

As shown in Fig 4.2.2, as $C_{linalool}$ increases from 0 to 20.738 mM (a) and C_{POH} increases from 0 to 800 µgmL^{-1} (b), there is a significant decrease in $\log_{10}$[normalized viable cell density] from 32.0 to 0.00810 (a) and 53.6 to 0.0652 (b) respectively. This is because linalool stimulates the secretion of TNF-α, IFN-γ and IL-2, which are all proteins related to cell death. This led to the activation of JNK, caspase 3 and other related proteins and enzymes to achieve an apoptotic cytotoxicity effect (Shen, Wang, Chen & Chang, 2013). On the other hand, POH downregulates RAD52, a gene known to play a role in homologous recombination, genomic stability and DNA repair (Ansari, Fatima & Hameed, 2017), causing prolonged DNA damage and apoptosis.

4.3 Effect of $C_{linalool}$ and C_{POH} on cell proliferation

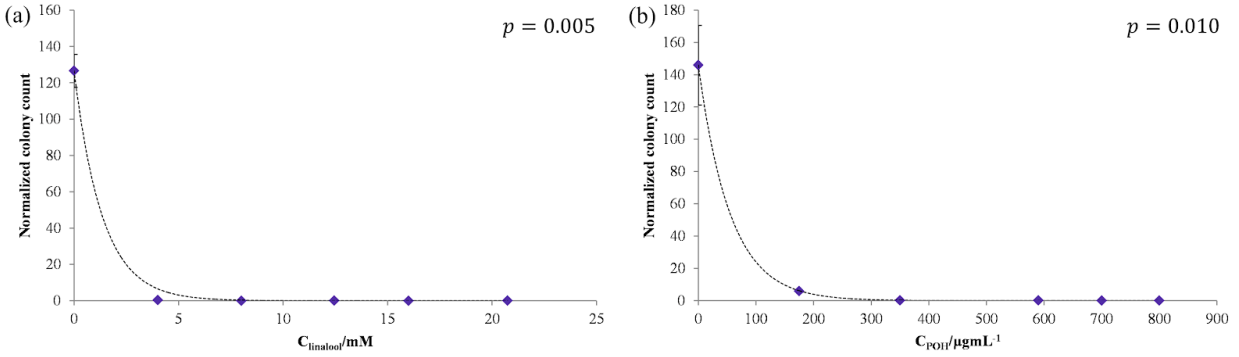

Fig. 4.3.1: Linalool and POH significantly inhibit cell proliferation. *S. cerevisiae* culture was mixed (n=3) with linalool ranging from 0 to 20.738 mM (**a**) or POH ranging from 0 to 800 µgmL^{-1} (**b**) and incubated for 24 h. Normalized colony count was determined with the colony forming unit assay (n=3) by dividing colony count by initial cell density to reconcile batch differences.

As shown in Fig. 4.3.1, as $C_{linalool}$ increases from 0 to 20.738 mM (a) and C_{POH} increases from 0 to 800µgmL^{-1} (b), there is a significant decrease in normalized colony count from 127 to 0.0216 (a) and 146 to 0.00001 (b) respectively. Since the colony forming unit assay assumes that each colony arises from a single cell, a decrease in colony count implies a drop in the number of cells that can proliferate. Hence both linalool and POH exhibit antiproliferative properties against *S. cerevisiae*. This is because linalool induces G_0 and G_1 cell cycle arrest by downregulating Cdk4 and cyclin A and upregulating p21 and p27 (Rodenak-Kladniew *et al.*, 2018), while POH downregulates S-phase specific cyclins such as CLB5 and CLB6 (Ansari, Fatima & Hameed, 2017), causing *S. cerevisiae* to be arrested at S-phase and hence inhibiting its proliferation.

4.4 Effect of linalool and POH on DNA health

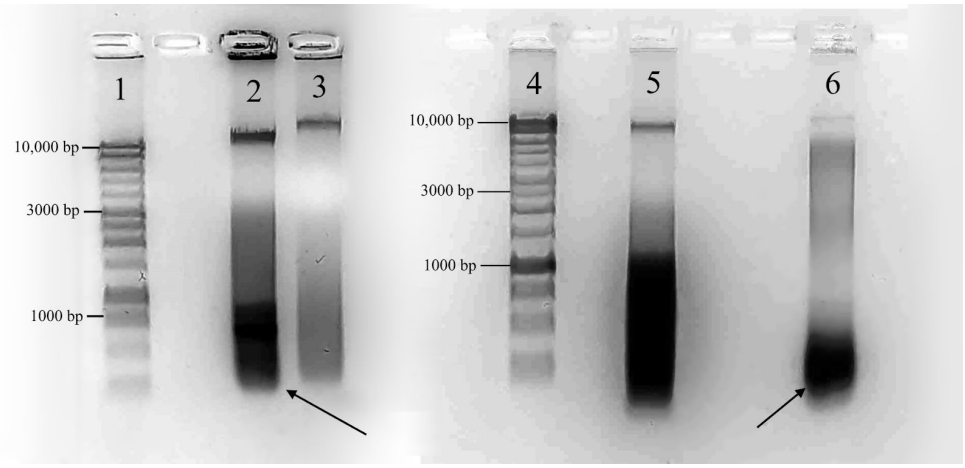

Fig. 4.4: Agarose gel electrophoresis of *S. cerevisiae* DNA samples treated with linalool and POH. DNA from *S. cerevisiae* samples were extracted with Yeast DNA Extraction Kit (78870, Thermo Scientific). Gel electrophoresis was run at 80V for 60min with bromophenol blue as the loading dye and SYBR Green as the stain. Lanes: 1, 4 - 1kb DNA ladder. 3, 5 - Negative control containing only 0.1% DMSO. 2 - 4 mM Linalool. 6 - 175 µgmL^{-1} POH.

As shown by the black arrows in Fig. 4.4, more small DNA fragments less than 1000 bp in size are present in the experimental groups (Lane 2 and 6), suggesting that both chemicals induced DNA fragmentation and possibly act through an apoptotic pathway. Berić *et al.* (2007) performed the alkaline comet assay on *S. cerevisiae 3A* and found that linalool induces DNA strand breaks as a result of pro-oxidative activity (Labieniec, Gabryelac, & Falcioni, 2003), while POH activates the IRE1α pathway in endoplasmic reticulum (ER) stress, releasing C/EBP homologous protein (CHOP) and causing subsequent DNA fragmentation (Cho *et al.*, 2012).

4.5 Effect of C_{POH} on DPPH RSA

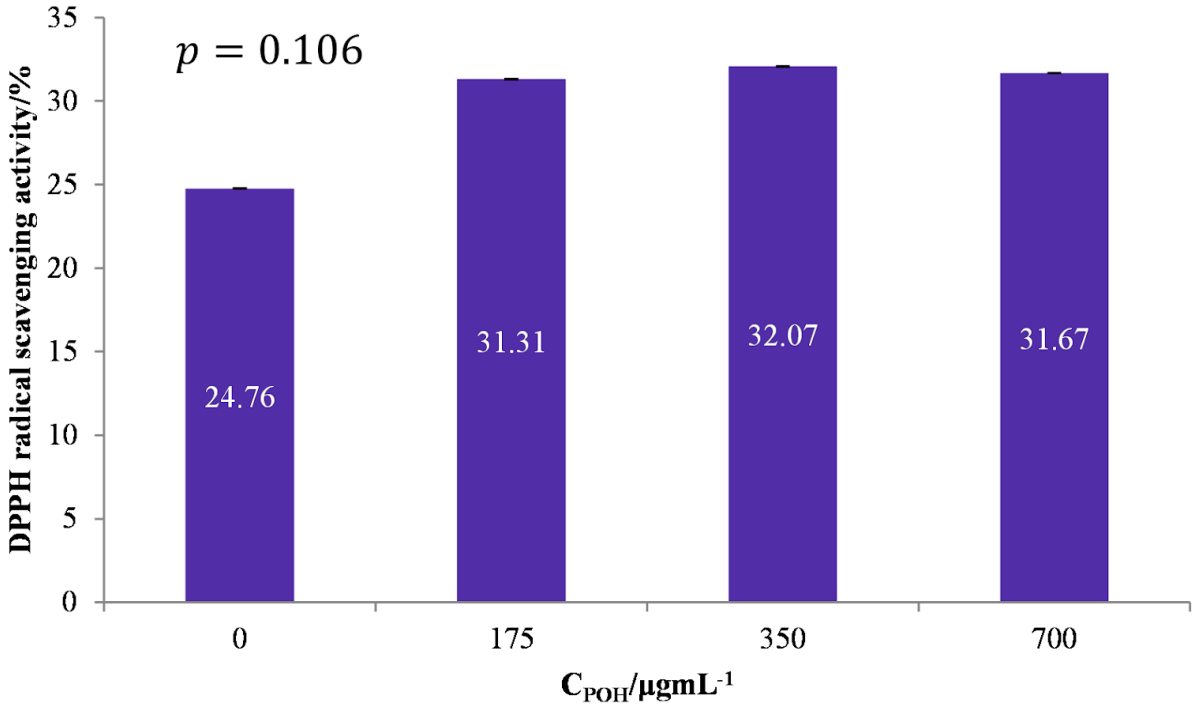

Fig. 4.5: POH has no significant effect on DPPH RSA. *S. cerevisiae* culture was mixed (n=3) with POH ranging from 0 to 700 µgmL^{-1} and incubated for 24 h before the DPPH assay was used to determine the RSA.

It was expected that POH would induce oxidative stress as it upregulates CHOP (as shown in Section 4.4) and CHOP has been shown to contribute to oxidative stress (Cao & Kaufman, 2014). However, as shown in Fig. 4.5, the DPPH RSA of 175, 350 and 700 µgmL^{-1} POH is greater than that of the control, even though the difference is statistically insignificant. One reason to explain this behaviour would be POH's tendency to induce an antioxidant response in cells. Khan, Nafees & Sultana (2011) demonstrated that POH protected against ethanol induced acute liver injury in rats by inhibiting oxidative stress and ameliorating the activity of hepatic antioxidant enzymes. This shows that POH is able to induce cell death in *S. cerevisiae* even without inducing oxidative stress. As for linalool, no clear nor explainable trend was observed for the effect of $C_{linalool}$/mM on DPPH RSA/%. Results for the effects of $C_{linalool}$/µgmL^{-1} on DPPH RSA/% can be found under Appendix A5.

5. CONCLUSION AND FUTURE WORK

Within the limits of this experiment, both linalool and POH exhibit statistically significant antiproliferative and pro-cell death effects on *S. cerevisiae*, with their LC50 being 10.069 mM and 658.762 μgmL^{-1} respectively. Both chemicals are likely to act through inducing apoptosis in *S. cerevisiae* as they both induce DNA fragmentation. An increase in C_{POH} also causes an increase in DPPH RSA, possibly because POH induces an antioxidant response in *S. cerevisiae*. In the future, *Lavender angustifolia* essential oil and combinations of linalool and POH will be used on *S. cerevisiae* to investigate if there is a synergistic effect between the two compounds. The comet assay will be used to characterize DNA damage instead of gel electrophoresis to confirm if both chemicals act through apoptosis. Additionally, late stage gliosarcoma cell lines can be used in replacement of *S. cerevisiae* as an even better cancer model to elucidate the antiproliferative and pro-cell death effects of linalool and POH.

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APPENDICES

A1 Calculations for graph plotting

Calculations for Fig. 4.2.1:

$$\text{Cell Viability (\%)} = \frac{\text{Number of live cells}}{\text{Total cells}}$$

Calculations for Fig. 4.2.2:

$$\text{Viable Cell Density (cells/mL)} = \text{Average Viable Cells} \times 10^4 \times \text{Dilution Factor}$$

$$\text{Normalized Viable Cell Density (no units)} = \frac{\text{Viable Cell Density (cells/mL)}}{\text{Initial Cell Density (cells/mL)}}$$

Calculations for Fig. 4.3.1:

$$\text{Colony Count of original solution (colonies/mL)} = \text{Colony Count from agar plate} \times 10 \times \frac{1}{\text{Dilution Factor}}$$

$$\text{Normalized Colony Count (no units)} = \frac{\text{Colony Count}}{\text{Initial Cell Density}}$$

A2 Protocol for yeast DNA extraction

1. Pellet a 10mL S. cerevisiae culture grown overnight, resuspend the cells and transfer entire suspension to a 1.5mL microcentrifuge tube. Pellet cells by centrifugation at 3000-5000 × g for 5 minutes at room temperature. Discard the supernatant.

2. Suspend cells in Y-PER Reagent and scale accordingly, maintaining ratio of 8μL/1mg pellet. Mix by gently vortexing or inverting the tube or pipetting up and down until the mixture is homogeneous and incubate at 65°C for 10 minutes.

3. Centrifuge at 13,000 × g for 5 minutes, discard supernatant, add 400μL of DNA Releasing Reagent A, and 400μL of DNA Releasing Reagent B to the pellet for a total volume that should equal approximately 800μL. Mix to produce a homogenous mixture and incubate at 65°C for 10 minutes.

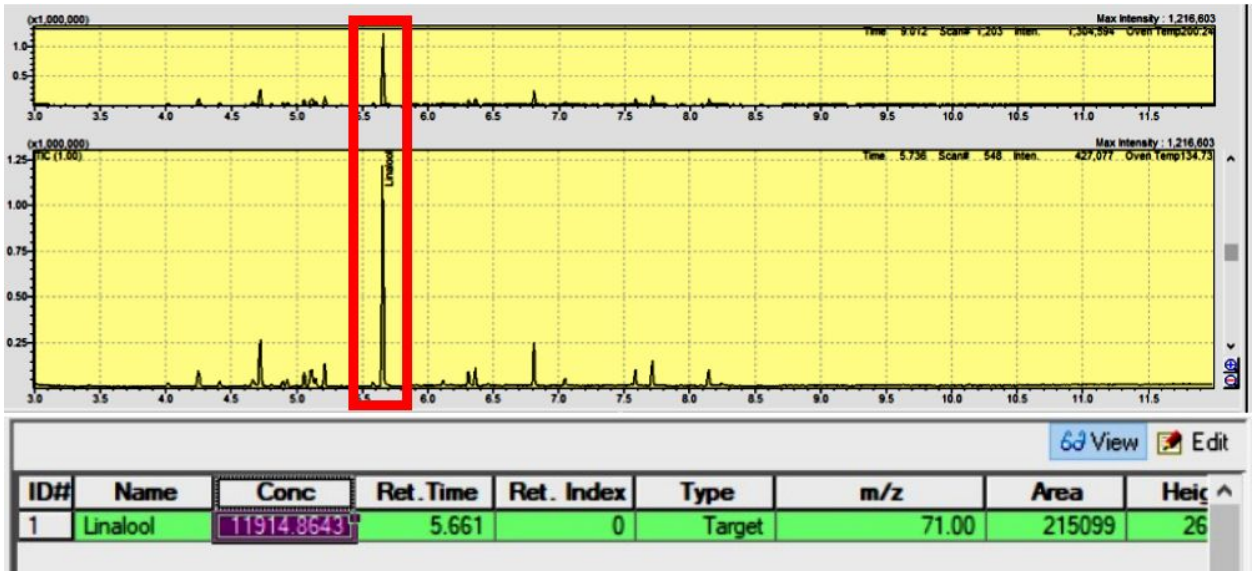
4. Add 200μL of Protein Removal Reagent to mixture and invert several times. Centrifuge at least 13,000 × g for 5 minutes and transfer supernatant to a new 1.5mL centrifuge tube.

5. Add 600μL isopropyl alcohol to fill tube. Mix gently by inversion. Precipitate genomic DNA by centrifuging the mixture at 13,000 × g for 10 minutes.

6. Remove supernatant and add 1.5mL of 70% ethanol to the pellet, invert several times and centrifuge at 13,000 × g for 1 minute to wash off any residual salts or cellular debris clinging to the DNA or tube.

7. Resuspend in 50μL TE buffer and flick the bottom of the tube before wash the sides of the tubes until all genomic DNA is in solution.

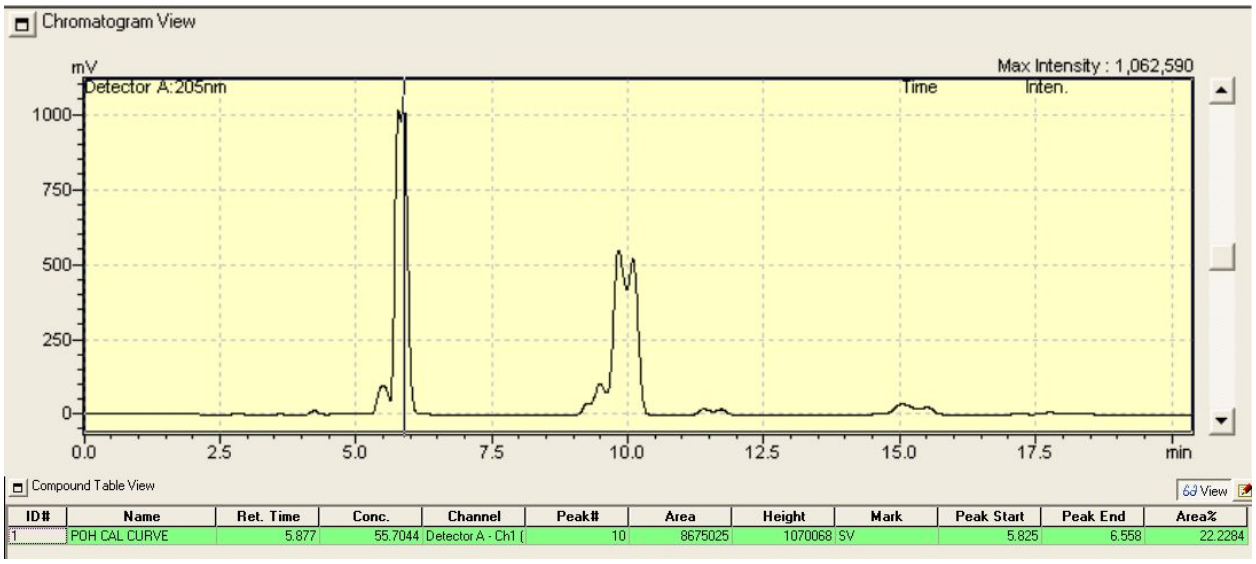
A3 GC result for linalool



ID#	Name	Conc	Ret.Time	Ret. Index	Type	m/z	Area	Heig
1	Linalool	11914.8643	5.661	0	Target	71.00	215099	26

$$\begin{aligned}
\text{Density of lavender oil} &= 0.879\ \text{mgL}^{-1} \\
&= 8.79 \times 10^{11}\ \text{ngL}^{-1} \\
C_{\text{linalool}}\ \text{in 200ppt lavender oil} &= 11.914\ \text{ppm} \\
C_{\text{linalool}}\ \text{in}\ 8.79 \times 10^{11}\ \text{ngL}^{-1}\ \text{lavender oil} &= \frac{8.79 \times 10^{11}}{200} \times 11.914 \\
&= 5.236203 \times 10^{10}\ \text{ppm} \\
C_{\text{linalool}}\ \text{in terms of}\ \mu\text{M} &= \frac{0.00005236203\ \text{mL}}{1\ \text{mL}} \times \frac{1}{154.25\ \text{gmol}^{-1}} \times \frac{0.87\ \text{g}}{1\ \text{mL}} \\
&= 295\ \mu\text{M (3 s. f.)}
\end{aligned}$$

A4 HPLC result for POH



ID#	Name	Ret. Time	Conc.	Channel	Peak#	Area	Height	Mark	Peak Start	Peak End	Area%
1	POH CAL CURVE	5.877	55.7044	Detector A - Ch1 [	10	8675025	1070068	SV	5.825	6.558	22.2284

$$\begin{aligned}
\text{Density of lavender oil} &= 0.879\ \text{gmL}^{-1} \\
&= 879000\ \text{mgL}^{-1} \\
&= 879000\ \text{ppm} \\
C_{\text{POH}}\ \text{in 2500 ppm lavender oil} &= 55.7044\ \text{ppm} \\
C_{\text{POH}}\ \text{in 879000 ppm lavender oil} &= \frac{879000}{2500} \times 55.7044 \\
&= 19585.667404\ \text{ppm} \\
&= 19585\ \text{ppm (to nearest whole number)}
\end{aligned}$$

$$C_{POH} \text{ in terms of } \mu M = \frac{0.000019585 \text{ mL}}{1 \text{ mL}} \times \frac{1}{152.23 \text{ gmol}^{-1}} \times \frac{0.959 \text{ g}}{1 \text{ mL}}$$
$$= 123.379 \ \mu M$$
$$= 123 \ \mu M \text{ (to 3 s. f.)}$$

A5 DPPH RSA data for Linalool