Investigating antifungal properties of crop plant extracts

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Digital Object Identifier (DOI): https://doi.org/10.82406/xsdw-w963

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Abstract

The cumulative estimate for the number of people affected by Fungal pathogens annually is over a billion (Brown et al., 2024). They pose an immediate threat to human health by causing life-threatening infections and destroying crops. Fungal pathogens significantly impact agriculture due to limited options for antifungal agents, creating a strong selection pressure for antifungal resistance (Fisher et al., 2024; Oliver and Hewitt, 2014). If left unchecked, antifungal resistance can cause major crop losses, which could be disastrous in developing countries where crops can provide up to 50% of an individual's caloric intake (Brauer et al, 2019; FAO, 2024). To combat this, novel fungicides are necessary. One option to develop and or discover novel fungicides is to utilise naturally produced defences, e.g. plant secretions. To screen some of these natural defences, extracts of 14 plant species were made up and tested against *Candida albicans* and *Saccharomyces cerevisiae*. The four best performing extracts were, Pak choi, garlic, tomato, and strawberry. For these four extracts further analysis into the potential bioactive compounds could be used as starting points for developing novel fungicides.

Keywords: Mycology; Fungicides; Drug discovery; Antifungal resistance; Agrochemicals

Introduction

This project was completed as part of the University of Lincoln Undergraduate Research Opportunity Scheme (UROS), which allows students to work alongside academics to develop employability and research skills. UROS applies Student as

Producer principles and Student as Researcher approaches to projects, allowing students to their develop knowledge about the work surrounding their discipline. This project follows this model through continuous support of the student in the form of both assistance in the lab, as well as meetings to discuss project progress in the research and writing stages. This project specifically examines the use of plant extracts as a stepping stone to develop novel antifungal agents to overcome the threat of antifungal resistance.

Project Background

While the threat of Fungal pathogens (FP) has historically been neglected in public health, their impact is of an ever-growing global concern, which is encouraged by climate change (Case et al., 2025; Brown et al., 2024). FP pose a significant risk to human health directly and indirectly. Figures from a literature review by Denning (2024) suggest that approximately 6.55 million people are impacted by a life-threatening FP annually, with 2.55 million deaths caused directly.

Additionally, FPs are detrimental to food security globally, where 15% of crops are lost pre-harvest, and a further 15% are lost post-harvest (Fisher et al., 2012; Savary et al., 2019). As of 2019, the most common crops were cereals, such as coarse grains, rice, and wheat. In developing countries, cereals can make up to 50% of a person’s daily caloric intake (Brauer et al., 2019; FAO, 2024). FPs such as *F. graminearum* and *B. graminis* commonly infect cereals, potentially reducing crop yields by up to 70% (Singh et al., 2016). Whilst currently under control, *de novo* development of resistance could be catastrophic, with over 40% of cereals being sprayed with just one compound (Jørgensen and Heick, 2021).

To combat antifungal resistance, novel compounds are needed with different target sites and mechanisms of action. Many approaches are used to discover new fungicides, including using existing natural compounds within living species (Steinberg and Gurr, 2020). As fungi can infect plants, the plant may produce a fungicide as a defence mechanism; this has been previously shown in alliums (Khounganian et al., 2023). This research aims to assess the efficacy of antifungal properties of other crop species to determine the best starting point for developing novel antifungal compounds.

Literature Review

This review has identified multiple studies using plant extracts to find bioactive compounds for use against pathogenic species, though most of this work focuses on bacteria. This has meant that most of the samples chosen for this project have prior research found in the literature. Figure 1 shows a phylogenetic tree of all samples.

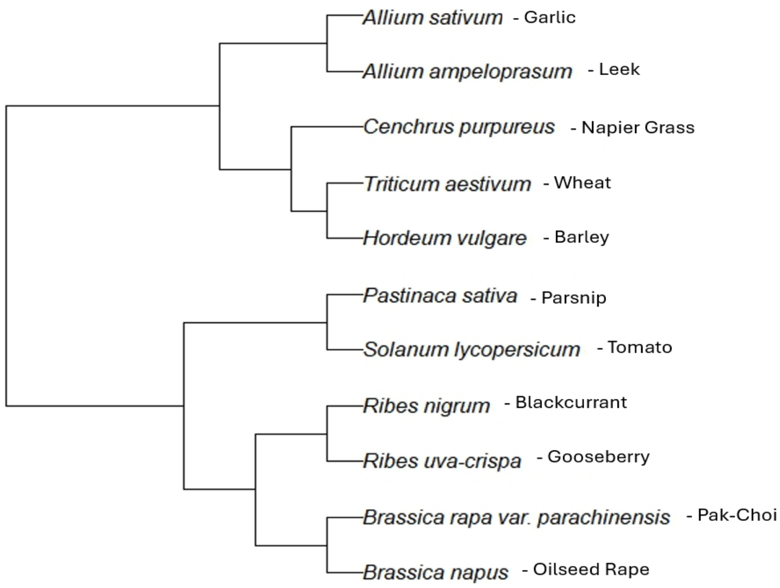

Figure 1 Phylogenetic tree of samples used to show both the species and common names. The phylogenetic tree was made in R using libraries connected to the interactive tree of life to determine how the species are ordered. Strawberry is not shown here as it does not have a taxononical classififcation due to how recenctly we as a species created it.

For example, Garlic extracts were investigated by Khounganian et al., identifying garlic had antifungal properties against *C albicans* but did not test against *S cerevisiae*. Leek extracts have antibacterial properties, being effective against both Gram-positive and negative bacteria (*Staphylococcus aureus* and *Escherichia coli* respectively) (Xie et al., 2023). Extracts of the grass genus *perpureum* (common name of Napier grasses) were tested by Chuah and Sudau, 2022 finding activity against anthracnose in mangoes, a fungal disease which causes fruit rot. Wheat and barley were shown to contain compounds which possess antifungal properties but the efficacy of those compounds in extract was not assessed (Patzke et al., 2017; Bohlmann et al., 1988; Hejgaard et al., 1992). Similarly, this can be seen in the leaves of parsnips which contain polyphenols and xanthotoxin which both have antifungal properties (Kenari et al., 2021). Tomato extracts of the fruit leaf and stem all show antifungal properties (Tam et al., 2021). Extracts of blackcurrant were shown to be active against a variety of bacterial species (Ejaz et al., 2023). Extracts of gooseberry were assessed for anticandidal properties finding that those with higher polyphenol content showed greater levels of growth inhibition (Krisch et al., 2009).

Three different varieties of Pak-choi were shown to inhibit the growth of *Candida albicans* (Park et al., 2025). Several varieties of strawberry show antimicrobial activity (Seleshe et al., 2017). Oilseed rape was the only extract which did not have any credible prior literature surrounding its antimicrobial properties.

A major gap in the literature, however, is that most of the work surrounding plant extracts exclusively investigates the fruits so this work aims to evaluate the properties using leaves to form extracts as fungi commonly infect both leaves and fruits.

Additionally, there is no work which directly compares the efficacy of the extracts to one another.

Methodology

An overview of the methodology used to determine the best starting point for antifungal compound development is provided in figure 2:

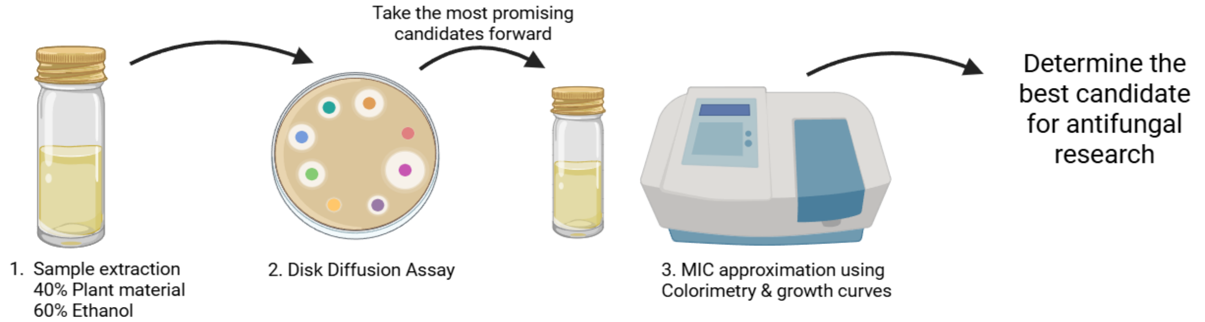

Figure 2 An outline of the methodology used to determine the best performing extracts.

Sample Collection

Sample collection was done throughout the project, with initial collection creating the library of plants, and subsequent collection was done to recoup sample stocks.

Fungal species

The fungal species used were two yeasts, *Candida albicans* and *Saccharomyces cerevisiae* and one filamentous fungi *Rhizopus stolonifera*

Media preparation & plate inoculation

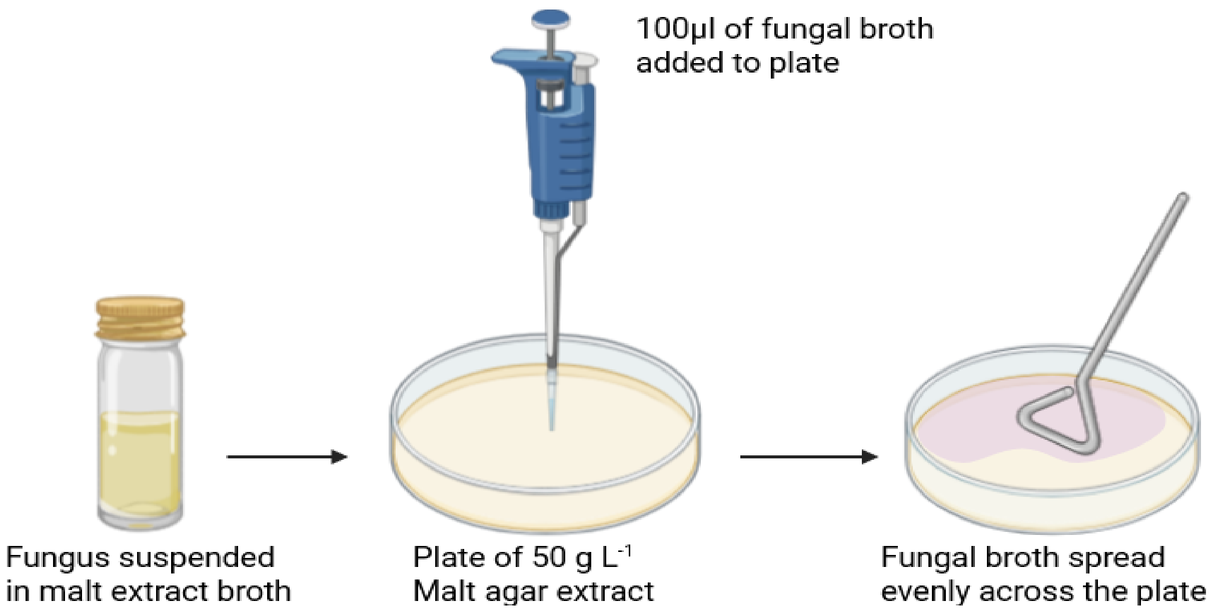

Figure 3 Method for creating plates, using a yeast

Fungal growth media were prepared using malt extract agar (MEA) at a concentration of 50 g L^{-1}. When inoculating the agar plates with a yeast, 100μL of the fungal broth was pipetted onto the centre of the plate and spread using a plastic spreader to coat the surface of the agar (figure 3).

When inoculating the agar with filamentous fungi a piece of agar was taken from an existing plate and placed in the centre of a new agar plate and left to incubate for 24 hours before its use in any assays (figure 4).

Sample preparation & extraction

Samples were prepared for extraction by mechanical lysis, cutting the plant material into small pieces, then using a mortar and pestle to further break it down into a paste. When using smaller masses of plant material, a pipette tip was used to break down the material. Results of testing concluded the best solvent was ethanol. Ethanol was then added to form a solution equating to a W/V 40% plant extract. Once broken-down extracts were placed in a 55°C incubator overnight to further break down cellular components. They were then stored at 5°C.

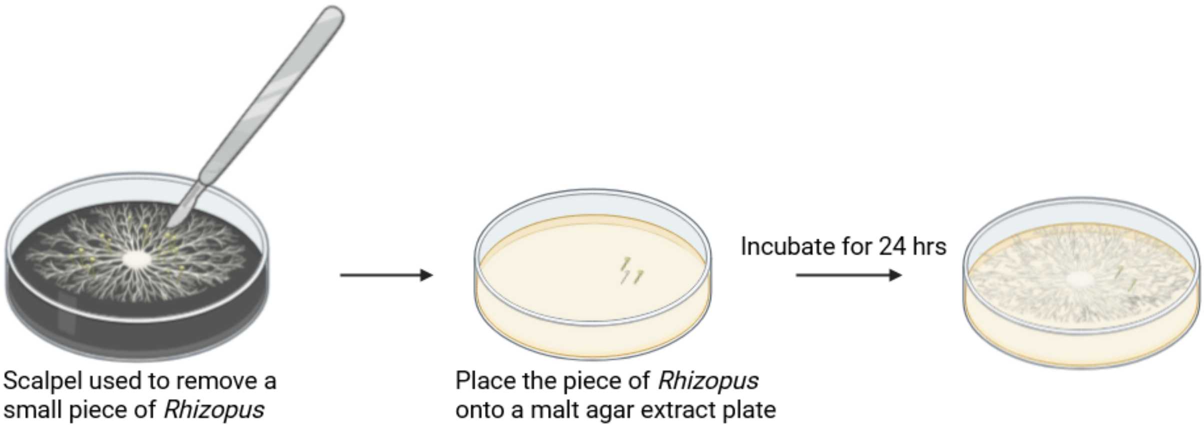

Figure 4 Method for creating plates when working with a filamentous fungus

Assays

Shown in figure 5, the primary assay used was based on the Kirby-Bauer Disk Diffusion Susceptibility test detailed by Hudzicki (2009).

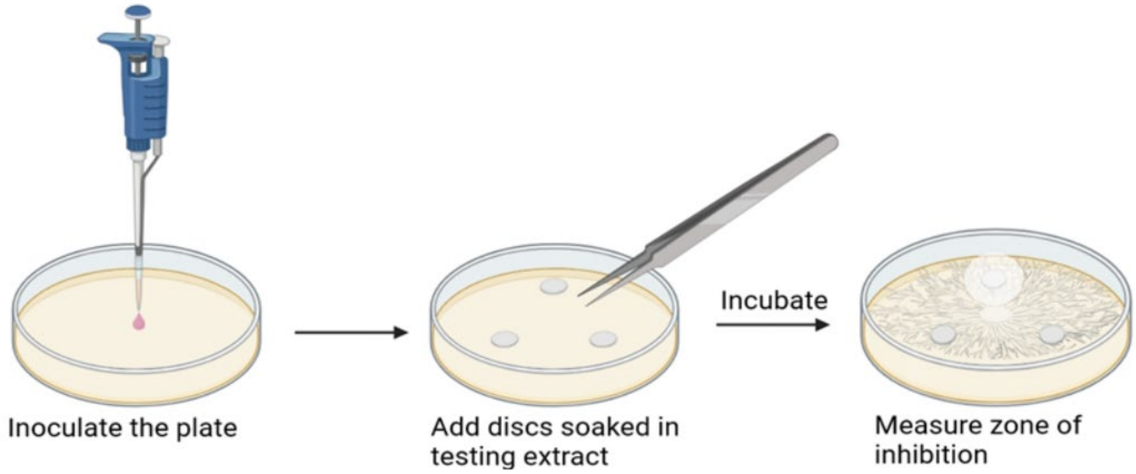

Figure 5 Disc diffusion assay methodology

Ratios were used to approximate the minimum inhibitory concentration (MIC), as illustrated in figure 6. This is because the bioactive compound is of an unknown concentration within the plant, meaning µg/ml is an inappropriate unit.

Each solution was made up to 10 ml, where a 50% extract solution required 5 ml of malt extract broth (MEB) and 5 ml of plant extract, and a 10% extract solution required 9 ml of MEB and 1 ml of plant extract. 100 µl of inoculant was then added. At intervals of 1,6-, and 24-hours absorbance measurements were taken using colourimetry to determine the level of cellular growth where greater absorbance values equate to greater levels of cellular growth. Cell cultures were placed on a shaker at 28°C to promote cell growth.

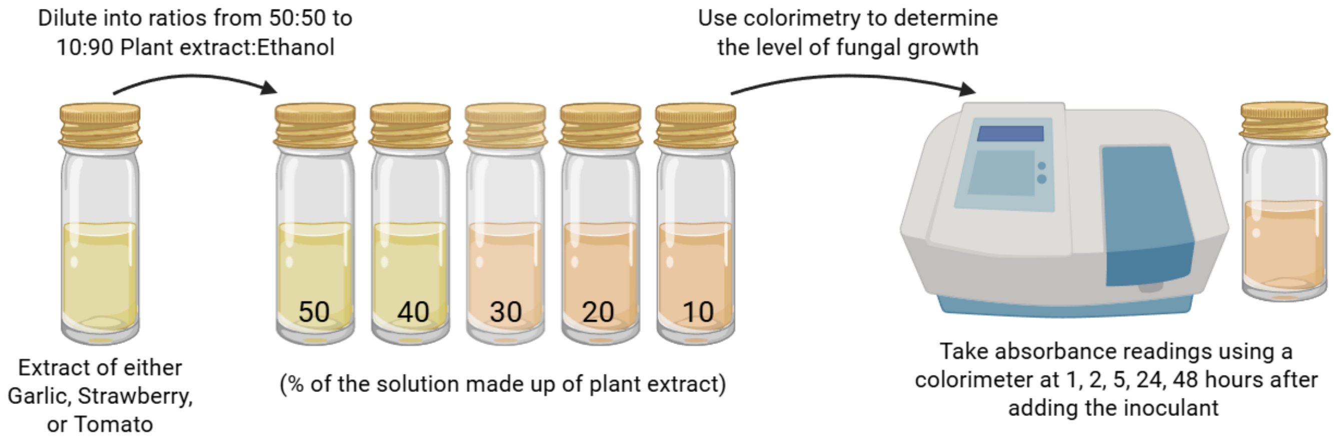

Figure 6 The methodology for approximating the minimum inhibitory concentration of an extract.

Results

Using a box and whisker plot (figure 7) of the data allowed immediate comparison between the crop extracts and resulted in selecting the four best extracts. This was done based on the median Parsnip and tomato have similar medians but the variance of tomato data trends upwards).

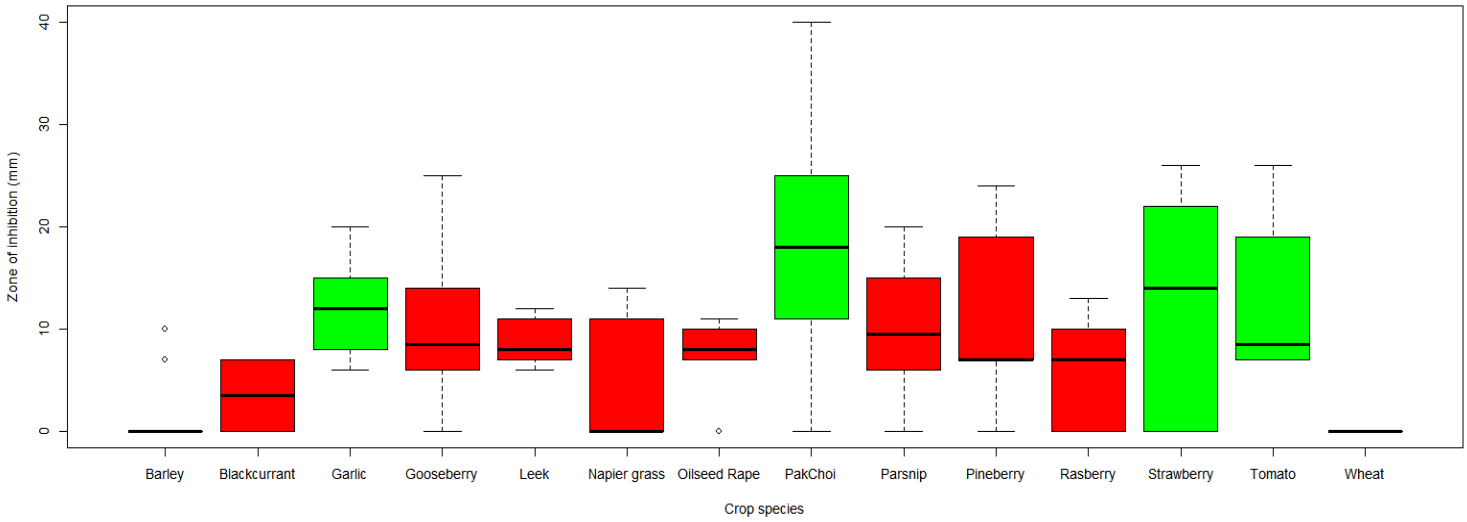

Figure 7 Box and whisker plots of the zones of inhibition created by each crop species. Highlighted in green are the four species chosen for further analysis.

After selecting the four extracts to move forward with further analysis was conducted, such as looking at the differences between the zone of inhibition when the FP was changed. This included *Rhizopus stolonifera,* but no extract showed any activity against it, as shown in figure 8. Due to time constraints Pak-Choi was not used in any further assays but was a part of statistical testing.

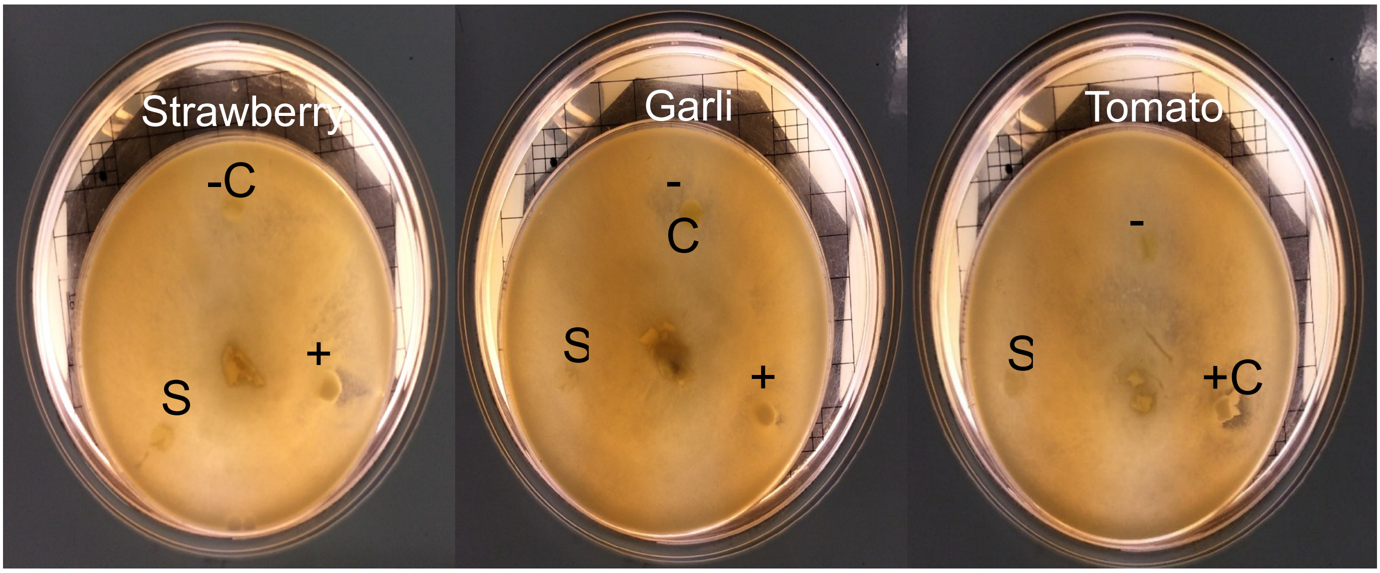

Figure 8 Plates from disc diffusion activity assays against R stolonifera. -C refers to a negative control soluition comprised of 50% ethanol. +C refers to a positive control cream made from 2% w/v coltrimazole. S refers to the 50% plant extract solution

A comparison of the four best extracts is shown by box and whisker plots in figure 9. The choice of crop species in the ethanol extract had a statistically significant difference on the diameter of the zone of inhibition (X^2= 11.795, df = 3, P = 0.0081). A Bonferroni correction was applied, and so effects are reported at a 0.0083 level of significance. The median (interquartile range) diameters are, 12 mm (8.25-14.75) for garlic, 18 mm (11.75-24.75) for Pak Choi, 14 mm (0-22) for strawberry 8.5 mm (7-18.25) for tomato. To find the statistically significant differences a Dunn test was conducted. The results of the Dunn test showed that Pak choi had a significantly greater zone of inhibition when compared to the other extracts, (garlic – p=0.0079, strawberry – p= 0.0217, tomato – p= 0.0125)

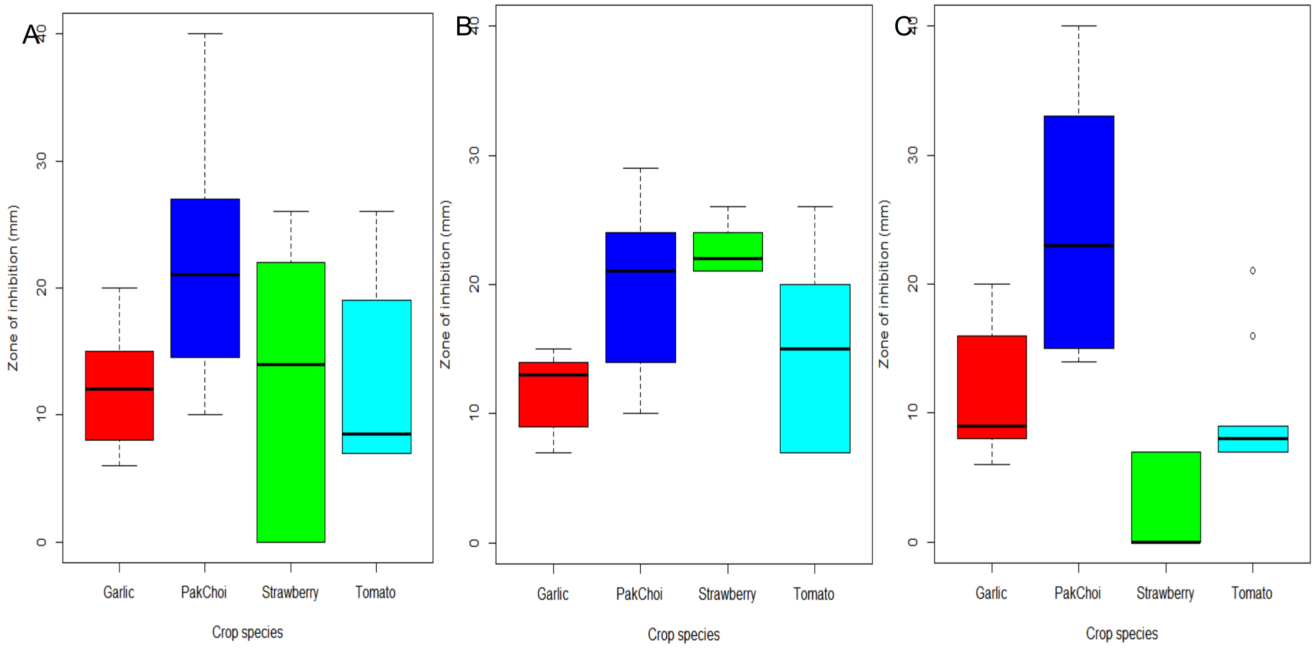

Figure 9 A boxplot of the zones of inhibitions for the top four candidates. Graph A shows both species of fungus. Graph B shows Candida albicans. Graph C shows Saccharomyces cerevisiae

As a part of further analysis, a colourimetry-based growth curve was attempted using the garlic extract however the data generated proved inconsistent and resulted in figure 10 where a correctly produced growth curve is shown for comparison.

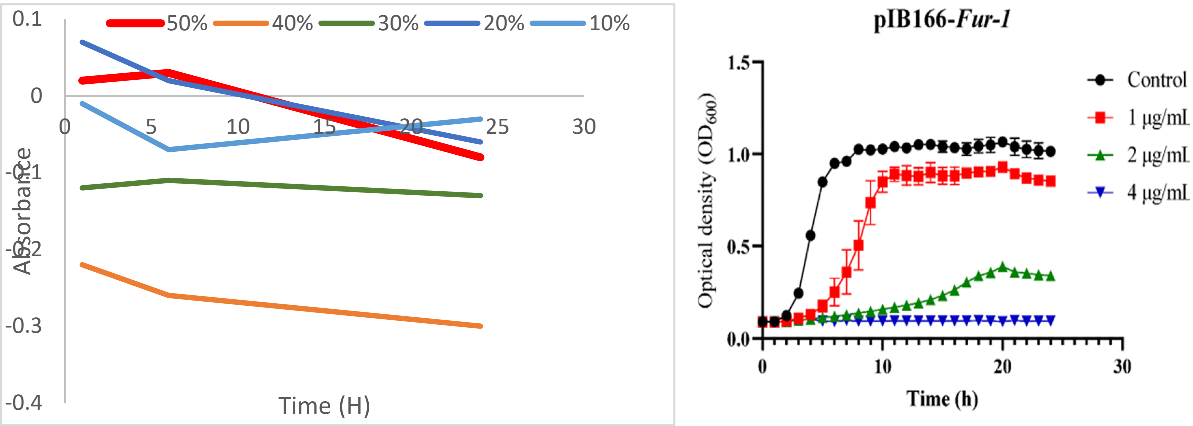

Figure 10 Left: A growth curve based on the data generated using garlic extracts. Right: A growth curve from Zewen et al., 2022 as a comparison to show what a growth curve is supposed to look like.

UROS Experience

By conducting this project, the UROS scheme has provided me with the opportunity to develop both technical and practical skills, along with written and IT based skills. Working alongside my supervisor gave me insight into the structure of research across multiple disciplines within the life sciences, something which is not an option for biochemists. Working outside of biochemistry has developed cross-disciplinary skills, which will benefit me when writing and conducting research for my dissertation. It also will support me when I graduate, as it will set me apart from other graduates when applying for my next steps.

As a part of the project, I learned lab etiquette and now understand how to conduct myself and experiments using a variety of equipment and techniques, such as the use of a laminar flow hood. Outside of the lab I was able to develop my writing skills as the report and poster are written under very different requirements when compared to coursework. I also developed IT skills, being able to learn BioRender as well as further develop my statistical abilities using a mix of R studio and Excel.
I believe that the UROS project is something that should be considered by every student regardless of their discipline as it offers an opportunity to further develop skills employers are looking for.

Conclusion

Pak Choi outperforms the other extracts; however, extracts of wheat and barley show no inhibition of growth. With cereals and grains making up a large bulk of the human diet the lack of growth inhibition by both wheat and barley is cause for concern (FAO 2019). Over 40% of cereals are sprayed using solely azoles (Jørgensen and Heick, 2021). Where the samples were taken from, it is likely that they were sprayed using azoles. These were expected to affect the growth of both *C albicans* and *S cerevisiae* as they are ethanol soluble and should have been in found in solution (Jørgensen and Heick, 2021; Kovács et al., 2009). The lack of innate resistance in these cereal crops implies a de novo development of resistance to azoles would be destructive to developing countries as they may lack the necessary infrastructure to cope (Case et al., 2025).

It must be considered that the data for Pak choi requires further repeats to determine whether the results are correctly representative, this is for two reasons. The pak choi was used as the initial testing so the techniques used were still being developed and it is possible that errors were made in the methods. Additionally, the environment used to test the pak choi extracts was not replicable as the external weather conditions changed. Given more time both technical and biological repeats should be completed to provide a more accurate result.

Overall Pak choi appears a good candidate for further chemical analysis to determine the bioactive compound and develop further understanding of the underlying chemical mechanism of inhibition.

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