The antimicrobial effectiveness of *Tulbaghia violacea* and *Allium sativum* on the *in vitro* growth of *Candida albicans*

BY

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DECLARATION

I, Wesley Reddy, hereby declare that the work presented is entirely my own and not of any other person, unless explicitly acknowledged (including citation of published and unpublished sources). The work has not previously been submitted in any form to the Durban University of Technology or to any other institution for assessment or for any other purpose.

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ABSTRACT

Aim

This study sought to compare the antifungal potential of *Tulbaghia violacea* to that of *Allium sativum.* Mother tinctures and a respective selection of homoeopathic potencies were evaluated in terms of their inhibitory effect on the growth of *Candida albicans in vitro.*

Methodology

This experimental study employed microbiological assays to evaluate the antifungal potential of *Tulbaghia violacea* (wild garlic) while comparing this activity to *Allium sativum* (garlic) against *Candida albicans in vitro. Allium sativum* bulbs and fresh *Tulbaghia violacea* bulbs and leaves were processed into 1:2 ethanolic extracts. These were diluted into 1:10 mother tinctures in 8% ethanol and succussed to prepare respective 3CH, 5CH, 8CH, 13CH and 21CH homoeopathic potencies (CH-centesimal Hahnemannian – diluting substance by a factor of 100 followed by rigorous shaking before the next dilution).

The inhibitory effect of the respective preparations was investigated using the microtitre plate assay. The absorbance of *Candida albicans*, in Sabouraud dextrose broth, was measured at 630 nm with a Multiskan Go microplate spectrophotometer in the presence and absence of selected test substances. The absorbance readings of the test groups were compared to the negative control (*Candida albicans* left untreated). Additionally, disc- and well diffusion assays were used to further explore the antifungal effectiveness by measuring the zones of inhibition in Sabouraud dextrose agar plates.

Quantitative data analysis was conducted using the Statistical Package for the Social Sciences (SPSS) version 25. Descriptive statistics were used to analyse the sets of data produced from the microtitre assay. The differences between the Pre- and post-incubation absorbance was analysed.

Results

The microtitre assay produced data, which was used for the comparative analysis of the 1:10 mother tincture, 3CH, 5CH, 8CH, 13CH and 21CH test groups indicated that the 1:10 mother tinctures were the most statistically significant test group as $p = 0.006$. Additionally, the analysis confirmed that the inhibitory properties of *Allium sativum* were better than that of *Tulbaghia violacea*. The pairwise comparative analysis found that all tested groups were statistically significant when compared to the negative control (*Candida albicans* left untreated). Both the well and disc diffusion assay did not produce measurable zones of inhibition and therefore no comparative data were obtained from this investigation.

Conclusion

The study found that *Tulbaghia violacea* mother tinctures and homoeopathic potencies were effective in inhibiting the growth of *Candida albicans.* It can be concluded that *Tulbaghia violacea* does indeed contain antifungal properties, which can be further investigated by means of *in vivo* testing. According to the comparative analysis *Allium sativum* demonstrates better antifungal effectiveness than *Tulbaghia violacea*.

DEDICATION

This dissertation is dedicated to the memory of my late uncle, Navendran Reddy. Thank you for your support and for instilling into me the value of an education. I wish you were here to celebrate this achievement with me.

ACKNOWLEDGEMENTS

I would like to acknowledge and express my gratitude to the following individuals that have supported me in my research of this dissertation and have had a significant role in my life:

Professor Ashley Ross, my supervisor and mentor, thank you for your guidance, motivation, and support, not only throughout the research process, but also during my studies. Your contributions towards the research of my dissertation are highly appreciated.

Doctor Charlene Pillay, my co-supervisor, thank you for guiding me with the microbiological component of my research and for your knowledge, time and contributions to my dissertation.

My family, Sharon, Kriben and Wendall, thank you for the support and patience throughout this journey. To my mother, this would not be possible without you, thank you!

The **Ravidutt family**, Micaela, Hanesh and Devi, no words can express my gratitude for the love, support and kindness you have shown me. Thank you!

Doctor Shraddha Sirpal, my dear friend and laboratory technician at the department of homoeopathy. For assisting me with the manufacturing of the homoeopathic potencies investigated in this study, and for imparting to me your knowledge of homoeopathic pharmacy. You helped nurture and develop my passion for homoeopathic pharmacy, thank you!

The **Department of Homoeopathy**, for allowing me to make use of the laboratory and laminar flow room to manufacture the test substances used for this study.

The **Department of Biotechnology and Food Science and technical staff**, for allowing me to conduct my experiments within the post graduate laboratory.

Mr Deepak Singh, thank you for your time and contributions towards my research.

Professor Himansu Baijnath, for sourcing and verifying the plant material needed for this research.

W. Last cc, for preparing the crude ethanolic extracts of the two plants used in this research.

The **research and postgraduate support** at the Durban University of Technology for their financial support of this study.

A special thanks to **Doctor Madhu Maharaj**, for seeing potential in me and giving me an opportunity. For this I will always be grateful, Thank you!

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DEFINITION OF TERMS

Antibacterial: "A substance that kills bacteria or inhibits their growth" (Merriam-Webster Dictionary 2022b).

Antifungal: "A substance that kills fungi or inhibits their growth" (Merriam-Webster Dictionary 2022c).

Antimicrobial: A substance capable of destroying or inhibiting the growth of micro-organisms such as bacteria or fungi (Merriam-Webster Dictionary 2022d).

Anthelmintic: An agent that destroys or causes the expulsion of parasitic intestinal worms (Merriam-Webster Dictionary 2022a).

Centesimal Hahnemannian: Diluting substance by a factor of 100 followed by succussion at each stage (O'Reilly 1996).

Emetic: A substance that induces vomiting (Merriam-Webster Dictionary 2022e).

Hypertension: Abnormally high arterial blood pressure (Merriam-Webster Dictionary 2022f)

***In vitro*:** Scientific tests in the laboratory, often in test-tubes or petri plates (van Wyk, van Oudtshoorn and Nigel 1997).

Mother tincture: "The original tincture prepared with the aid of alcohol directly from the crude drug. It is the precursor for the preparation of different potencies and the starting point for the production of homoeopathic medicines" (Maiti *et al.* 2013).

Potency: A degree of potency of a medicine created from the process of potentization (O'Reilly 1996).

Potentisation: a process used to release the medicinal potential of a crude substance which involves trituration or dilution and succussion (O'Reilly 1996).

Succussion: the second step in the potentization of a liquid which involves a shaking with an impact, such as striking a vial on the palm of a hand (O'Reilly 1996).

Trituration: a dry method of potentization in which a non-soluble crude substance is rubbed, grinded and pounded with a proportion of lactose monohydrate in a mortar (O'Reilly 1996).

LIST OF ABBREVIATIONS

ATCC - American Type Culture Collection

CFU/ml - colony forming units per ml

CH – Centesimal Hahnemannian

CHAPTER 1: THE INTRODUCTION

1.1. CONTEXT OF THE STUDY

Candida albicans is an infectious yeast that causes candidiasis (thrush), a fungal infection that generally affects the cutaneous mucosa of the body, most commonly the oral cavity, genitalia, gastrointestinal or respiratory tract (Centers for Disease Control and Prevention 2022). *Candida albicans* is found naturally on the body and in the gastrointestinal mycobiota of healthy individuals (Centers for Disease Control and Prevention 2022). The fungus is a commensal species in a healthy human, however, it may display colonization and become pathogenic when host factors change due to predisposing factors (Neville, d'Enfert and Bougnoux 2015). Some common predisposing factors that lead to candidiasis are HIV/AIDS, tuberculosis, diabetes or cancer. Additionally, being pregnant, or taking oral antibiotics, contraceptives or certain drugs have been linked to *Candida albicans* overgrowth (Bvumbi *et al.* 2021).

According to the Centers for Disease Control and Prevention (2022), there has been a significant increase in antifungal resistance in response to existing treatments. Antifungal resistance has made fungal infections extremely difficult to treat. Therefore, alternative methods of treatment need to be explored.

South Africa is one of the most biodiverse countries in the world, home to more than 22 000 indigenous plant species, which accounts for 10% of the world's flowering species of plants (Prinsloo and Street 2012). From this abundant selection of flora, many plants have long been used for their therapeutic medicinal qualities, primarily in traditional medicine. Presently, there is still a large population of South Africans that use traditional medicine and medicinal plants for their primary health care needs (Saxena and Kukreti 2020).

Tulbaghia violacea (wild garlic) has been used by traditional healers for the treatment of several ailments including tuberculosis, gastrointestinal pathologies, fever and even cancers (Aremu and van Staden 2013). However, *Tulbaghia violacea* has no documented homoeopathic applications. Therefore, the purpose of this study was to compare domesticated *Allium sativum*, which has known herbal uses in the treatment

of Candidiasis, to *Tulbaghia violacea*. This will serve as a first step towards the homoeopathic appraisal of the plant as an alternative treatment for candidiasis.

1.2. AIM OF THE STUDY

The aim of this study was to compare the *in vitro* antifungal effectiveness of *Tulbaghia violacea* and *Allium sativum* mother tinctures and a respective selection of homoeopathic potencies on the growth of *Candida albicans.*

1.3. STATEMENT OF THE OBJECTIVES

1.3.1. Objective one:

To determine the antifungal effectiveness of *Tulbaghia violacea* mother tincture and 3CH, 5CH, 8CH, 13CH and 21CH potencies on the *in vitro* growth of *Candida albicans*.

1.3.2. Objective two:

To determine the antifungal effectiveness of *Allium sativum* mother tincture and 3CH, 5CH, 8CH, 13CH and 21CH potencies on the *in vitro* growth of *Candida albicans.*

1.3.3. Objective three:

To compare the relative *in vitro* antifungal effectiveness of respective *Tulbaghia violacea* and *Allium sativum* tinctures and potencies on the growth of *Candida albicans.*

1.4. HYPOTHESIS

1.4.1. Hypothesis one:

It was hypothesised that *Tulbaghia violacea* mother tincture and selected potencies would have a significant effect in inhibiting the *in vitro* growth of *Candida albican*s.

1.4.2. Hypothesis two:

It was hypothesised that *Allium sativum* mother tincture and selected potencies would have a significant effect in inhibiting the *in vitro* growth of *Candida albican*s.

1.4.3. Hypothesis three:

It was hypothesised that *Tulbaghia violacea* would display a greater inhibitory effect on the *in vitro* growth of *Candida albicans,* than *Allium sativum.*

1.5. DELIMITATIONS

- This study was limited to one strain of yeast, namely *Candida albicans.*
- This study was limited to two herbs, namely *Tulbaghia violacea* and *Allium sativum*.
- This was an *in vitro* study.
- The incubation temperature for growth was 37°C.
- This study was limited to two microbiological assays, namely the microtitre and the disc/well diffusion assay.

1.6 OUTLINE OF THE STUDY

Chapter two presents a review of the available literature and the gaps that exist currently. This supports the methodology employed in the study, which is explained in Chapter three. Chapter four aims to report on the findings and analysis of the data produced by the experimental study. The findings are then discussed in Chapter five and conclusions are reached in Chapter six with additional recommendations based on the findings and limitations of this study.

CHAPTER 2: LITERATURE REVIEW

2.1. ETHNOBOTANY

2.1.1. Definition

Ethnobotany is a field of research that investigates the botanical knowledge of human beings, and their use of the available plants in their surrounding environment, as foods and medicine (Balick and Cox 1996; Pei, Alan and Wang 2020).

2.1.2. Significance

Ethnobotany has a fundamental role in mankind as it is estimated that 80% of people living in developed countries make use of traditional medicine for their health care needs (Chug, Bali and Koul 2018; Saxena and Kukreti 2020). Medicinal plants form a large component of these traditional therapeutics (Lonkala and Reddy 2019).

The role of traditional medicinal plants can be seen in indigenous cultures, as the importance of medicinal plants is more observable than in developed societies, where the botanical origins of plants are often unknown or not understood by most individuals (Pei, Alan and Wang 2020). This is also due to the complex mechanisms of production in which synthetic alternatives are produced and therefore the botanical origins are often forgotten (Balick and Cox 1996). The integration of indigenous and scientific knowledge plays as significant role in maintenance of human health as it is estimated that 50% of prescription drugs are derived from plants or other natural sources by investigating the ethnomedical uses of these substances (Chug, Bali and Koul 2018; Pei, Alan and Wang 2020).

This integration can be seen in Western medicine, where the search for new drugs by screening, testing and refinement plants, has resulted in the discovery of many useful therapeutic substances that can be used to treat human diseases (Lonkala and Reddy 2019). One of these drugs is aspirin; the medicinal origins of aspirin date back as far as 3000 BC, where ancient civilisations such as the Sumerians, Egyptians and Greeks made use of different parts of the willow tree (*Salix alba*) and meadowsweet (*Spiraea ulmaria*). Based on the various ancient medical texts detailing the traditional uses of

these plants, scientists were able to identify potential therapeutic applications. After extensive scientific investigation, the substance, salicyclic acid was identified as the compound responsible for the therapeutic action of these plants. Scientists were then able to synthetically produce salicyclic acid and improve the compound by adding an acetyl group to the salicyclic acid to reduce the irritant properties. This was the birth of aspirin (acetyl salicylic acid) (Connelly 2014).

2.1.3. South African Ethnobotany

South Africa has a large and diverse botanical landscape that is home to approximately 22 755 plant species. From this large selection, many plants contain unique compounds that have the potential to become useful therapeutic agents. Currently, it is estimated that only 3000 of these plants are used as traditional medicines and many of which have been scientifically evaluated (Twilley, Rademan and Lall 2020; Oyedeji-Amusa, Sadgrove and van Wyk 2021). This accounts for less than 15% of the plants found in South Africa. The remaining 85% of South African plants remain untouched, however; intense and targeted study of these plants based on their traditional uses could potentially unlock new phytopharmaceuticals.

Prinsloo and Street (2012) listed some South African traditional medicinal plants that have been researched extensively and thereby gained international acclaim:

- *Agathosma betulina* (Buchu)
- *Aloe ferox* (Bitter Aloe)
- *Aspalathus linearis* (Rooibos)
- *Harpagophytum procumbens* (Devil's claw)
- *Hypoxis hemerocallidea* (African potato)
- *Pelargonium sidoides* (Geranium)
- *Sclerocarya birrea* (Marula)
- *Siphonochilus aethiopicus* (Wild ginger) and
- *Sutherlandia frutescens* (Cancer bush)

The widespread and diverse botanical landscape of South Africa is also home to rich cultural diversity, in which, African traditional medicine is practiced by herbalists from

indigenous communities such as the Khoi, Ndebele, Swazi and Zulu ethnic groups. This represents the main source of ethnobotany in South Africa (Che *et al.* 2017).

2.1.4. African Traditional Medicine in South Africa

According to Che *et al.* (2017), “traditional medicine refers to the knowledge, skills, and practices of various indigenous cultures.” The knowledge and practice of traditional medicine originates from the beliefs, theories, observations, and experiences of the indigenous South African cultures and is passed down from generation to generation to prevent disease and maintain health.

The interaction between the indigenous communities and the flora in the surrounding environment serves as the foundation of South African ethnobotany. African traditional herbalists have an extensive knowledge of various plants and how to use them as medicines, when faced with illness, they look to their surroundings for medicine. The knowledge of how to use plants as medicine is said to have come from their ancestors or is passed down from generation to generation from traditional healers to apprentice via word of mouth (Oyedeji-Amusa, Sadgrove and van Wyk 2021).

Studies of traditional knowledge of medicinal plants provides an insight into the traditional uses of the plants in treating numerous ailments. By applying scientific methodologies to determine the chemical profile and composition of traditional medicinal plants, potential therapeutic treatments can be developed (Chug, Bali and Koul 2018).

2.1.5. Phytopharmacological research as a link between Ethnobotany and medicine

There have been several local and international studies aimed at exploring the vast botanical resources found in South Africa. The intention of these initiatives was to link the indigenous and scientific understanding of medicinal plants by using microbiology as aid. The integration of the traditional and scientific paradigms allows for the preservation of traditional knowledge while determining a chemical profile of the

biologically active compounds present in indigenous medicinal plants (Prinsloo and Street 2012).

McGaw, Jäger and van Staden (2000) evaluated 46 indigenous plants based on their traditional uses for antibacterial, anthelmintic, and anti-amoebic properties using the disc diffusion and microdilution assays. *Tulbaghia violacea* was one of the investigated plants which did not display any antibacterial activity, however; it did have an anthelmintic effect against *Caenorhabditis elegans* (roundworm).

In a study conducted by Motsei *et al.* (2003), 24 traditional South African medicinal plants were used to find an alternative treatment for *Candida albicans* infections. Garlic and wild garlic had the best activity against the three *Candida albicans* strains used. The minimum inhibitory concentrations values (MIC) of the two aqueous bulb extracts were 0.56 and 3.25 mg/ml respectively. MIC values indicate the lowest drug concentration that prevents microbial growth. This indicated that garlic was more effective in inhibiting the growth of *Candida albicans*.

Based on the traditional uses, Bungu *et al.* (2006) investigated the anticancer properties of *Tulbaghia violacea* bulb and leaf methanol extracts. The results indicated that the extract was successful in inducing apoptosis in colon, cervical, breast and oesophageal cancer cell lines.

Eloff *et al.* (2008) investigated seven traditionally used South African plant species that had previously been screened for antifungal activity against *Candida albicans* and were tested for antifungal and antibacterial activity against several other species of fungi and bacteria. The results showed varying degrees of activity against fungi and bacteria. *Curtisia dentata* (Assegai) had the best antifungal and antibacterial activity against the other six species.

Mackenzie (2012) investigated the anticancer properties of both *Tulbaghia violacea* and *Allium sativum* bulb and leaf extracts to determine if these plants display anti-proliferative effects on Jurkat cells. The study found that *Tulbaghia violacea* can induce oxidative stress and therefore cell death by means of apoptosis. This was in

line with the findings of Bungu *et al.* (2006). The cytotoxic effect of *Tulbaghia violacea* on healthy cells was not investigated.

Oyedeji-Amusa, Sadgrove and van Wyk (2021) reviewed the link between the ethnobotany and chemistry of the South African *Meliaceae* family of plants. Out of the twelve plants reviewed, ethnomedical records were found for eight plants, while chemical records were found for ten plants. Traditionally, 35% of plant material used comes from the roots of plants and 33% comes from the bark, which contributes to overharvesting and eventual destruction of the natural flora. Chemical analysis indicates that 42% of phytochemicals are found in the bark and 17% in seeds, this highlights the importance of investigating sustainable methods of using traditional medicinal plants.

Tulbaghia violacea and *Allium sativum* were identified for their unique chemical composition and their rich ethnobotanical usage for further investigation, with the aim to add to the available ethnobotanical knowledge and establish a link to homoeopathy. Below is a detailed review of the literature related to both *Tulbaghia violacea* and *Allium sativum*.

2.2. *Tulbaghia violacea*

2.2.1. Family

Tulbaghia violacea is a plant that is classified under the *Amaryllidaceae* family, subfamily *Allioideae* (van Wyk, van Oudtshoorn and Nigel 1997; Ranglová, Krejčová and Kubec 2015).

2.2.2. Common Names

Tulbaghia violacea is known by common names such *wild garlic (English), wilde knoffel (Afrikaans) and isihaqa (Zulu)*(van Wyk, van Oudtshoorn and Nigel 1997; Aremu and van Staden 2013).

2.2.3. Botanical Description

Wild garlic is a perennial plant with a bulb-like corm/rhizome that strongly resembles *Allium sativum*. Long narrow leaves arise from top of the corm which grows in clumps that reach a height of about 20-35 cm (Ranglová, Krejčová and Kubec 2015). The flowering stem produces ten or more pale purple flowers at the tip of the slender stalk. All parts of the plant have a strong smell of garlic when physically damaged (van Wyk, van Oudtshoorn and Nigel 1997).

2.2.4. Distribution

Tulbaghia violacea is named in honour of Ryk Tulbagh, Governor of the Cape, 1751–1771 as most of the species are found in the Eastern Cape (Aremu and van Staden 2013; Ranglová, Krejčová and Kubec 2015). *Tulbaghia violacea* is indigenous to Southern Africa as the plants grow naturally in the Southeast region of South Africa, in the area between Knysna and KwaZulu-Natal as indicated in ***Figure 2.1***. It may also be found as far north as Zimbabwe (van Wyk, van Oudtshoorn and Nigel 1997).

Figure 2.1: The orange are indicates the distribution of *Tulbaghia violacea* in South Africa (van Wyk, van Oudtshoorn and Nigel 1997).

2.2.5. History and Traditional Uses:

- The Early Cape colonists used *Tulbaghia violacea* as a remedy for pulmonary tuberculosis and as an anthelmintic (Hutchings 1996; Ranglová, Krejčová and Kubec 2015).
- The Zulus and Sothos use *Tulbaghia violacea* for treating ailments such as tuberculosis, constipation, and intestinal worms. Traditionally, infusions of the bulbs were used as love charms, as well as to treat other conditions such as rheumatism, paralysis, and high fevers. The leaves may be eaten to treat sinus headaches, or colic, flatulence, and restlessness in infants (Hutchings 1996).
- Rastafarians have been known to drink wild garlic extracts to treat coughs, colds, and influenza (Aremu and van Staden 2013).
- In the Transkei, the corms are rubbed all over the body as a protection against evil spirits. The plants are also cultivated near the homestead to keep snakes away (Hutchings 1996).
- Ranglová, Krejčová and Kubec (2015), reported wild garlic as being used traditionally to treat fevers, fits, paralysis, headaches, oral or ear infections, rheumatism, high blood pressure, heart, chest and stomach ailments.

2.2.6. Plant Sections Used

The fresh corms and leaves are used (van Wyk, van Oudtshoorn and Nigel 1997; Aremu and van Staden 2013).

2.2.7. Constituents of wild garlic

Chemical studies of *Tulbaghia violacea* have shown the presence of alkyl cysteine sulfoxide lyases, which suggest that the odour-production mechanisms may be similar to those produced by allinase-like enzymes in the genus *Allium* (Hutchings 1996). It was believed that the main active compound of *Tulbaghia violacea* was allicin. However, recent investigations have found that the main active compound found in *Tulbaghia violacea* is 2,4,5,7-tetrathiaoctane-4-oxide (thiosulphinate marasmicin) (Ranglová, Krejčová and Kubec 2015).

According to research conducted to investigate the chemical constituents present in the rhizomes, moderate radical-scavenging activities were determined on the essential oils of *Tulbaghia violacea* (Mackenzie 2012). Furthermore, the constituents of *Tulbaghia violacea* are: dimethyl trisulphide, dimethyl disulphide, methyl-(methylthio)-meth 2,4-dithiapentane, (methylthio)acetic acid, (methylthio)acetic acid, 2-(methylthiol) ethanol, propanitrile, 3-(methylthio)-2,4-dithiapentane, bis-(methylthio) disulphide (Afolayan, Bradley and Olorunnisola 2012).

2.2.7.1. Marasmicin - the phytochemical found in wild garlic

Thiosulphinate marasmicin is a Sulphur compound first identified by Thamburan *et al.* in 2006 and is responsible for the strong odour and antimicrobial activities of *Tulbaghia violacea* (Ranglová, Krejčová and Kubec 2015). Marasmicin is an enzyme-mediated product formed when there is mechanical trauma to the bulb or leaves of the plant as shown in ***Figure 2.2***. Marasmin S-(methylthiomethyl) cysteine-4-oxide) is a cysteine-derived amino acid that is found in the cytoplasm of *Tulbaghia violacea*. When tissue disruption occurs, C-S lyase, that is normally found in the cell vacuoles, is released and catalyzes the breakdown of marasmin into thiosulphinate marasmicin (2,4,5,7-tetrathiaoctane-4-oxide) (Ranglová, Krejčová and Kubec 2015). This process is similar to the formation of allicin from alliin in *Allium sativum.*

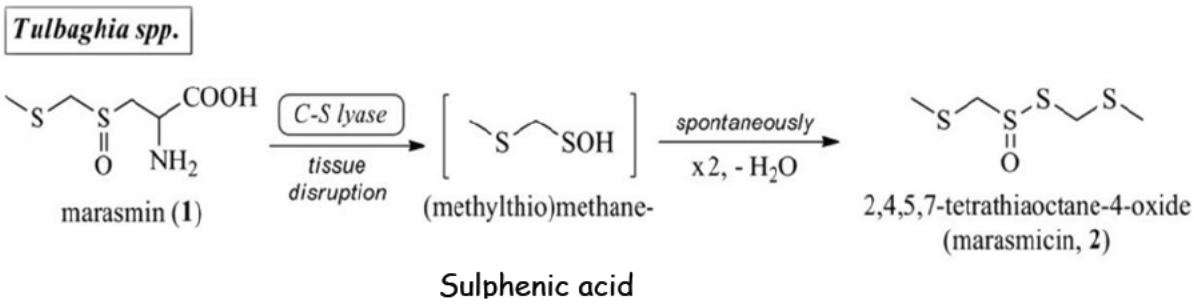

Figure 2.2: Formation of Marasmicin from Marasmin in *Tulbaghia violacea*
(Ranglová, Krejčová and Kubec 2015).

Marasmicin is a highly volatile compound due to the complex mechanism of formation. Ranglová, Krejčová and Kubec (2015) found that enzyme-mediated reaction by C-S lyase to produce the compound marasmicin is significantly reduced by the application of heat to the sample or the use of organic solvents as an extractant. Heat and organic solvents inactivate C-S lyase, which results in a decrease in the formation of marasmicin.

2.3. *Allium sativum*

2.3.1. Family

Allium sativum is a plant that is classified under the *Amaryllidaceae* family, subfamily *Allioideae* (Saxena and Kukreti 2020), although sometimes it is assigned to the *Liliaceae* family (Majewski 2014).

2.3.2. Common Names

Allium sativum is known by the common name, garlic (Davies 2000).

2.3.3. Botanical Description

Allium sativum is a commercially cultivated bulb. Each bulb can be divided into six to twelve segments, referred to as cloves. The leaves are tall and flat, and form at the base of the stem. An erect stem grows from the garlic bulb to form a cluster arrangement of flowers, which vary in colour from purple to white (Davies 2000).

2.3.4. Distribution

Cultivated varieties of garlic can be found all over the world (Davies 2000).

2.3.5. History and Traditional Uses

Allium sativum has recorded historic uses that date as far back as 5000 years. Some believe garlic originated in Western Siberia, while others think it came from Central Asia. However, the exact origins of garlic are unknown. Presently, *Allium sativum* is one of the most cultivated plants in the world and most well-known for its culinary uses (Saxena and Kukreti 2020). According to folklore, garlic is referred to as the "poor man's treacle" which translates to the "poor man's heal all" (Davies 2000).

- Archeologists have found drawings of garlic in Egyptian tombs, which date back to 3700 years BC (Bayan, Koulivand and Gorji 2013; Majewski 2014).
- Slaves building the Great Pyramids in Egypt were fed garlic as part of their daily food ration to keep their strength up (Davies 2000).
- Roman soldiers chewed garlic before fighting in a battle (Majewski 2014).
- The Greeks had over 62 medicinal uses of garlic, for conditions such as rabies, leprosy, scorpion bites, smallpox, and anthrax, among others (Davies 2000). Athletes were fed garlic to increase their stamina (Bayan, Koulivand and Gorji 2013).
- In Roman lore, garlic is one of the five healing foods (Davies 2000)
- The Slavs claimed it to protect against snakebites (Majewski 2014).
- During World War I and II, a mixture of garlic juice and sphagnum moss was used to control infection and treat wounds on the battlefield (Davies 2000).
- Fishermen in Africa use garlic to repel crocodiles (Majewski 2014).
- In Europe, "garlic is believed to ward off vampires, demons, evil spirits and have other magical properties" (Majewski 2014).
- According to Davies (2000) some of the documented traditional uses of garlic are bronchitis, pneumonia, emphysema, colds, asthma, coughs, tuberculosis, whooping cough, intestinal worms, diabetes, acne, high blood pressure, angina, hyperlipidemia, anaemia, cancer, candidiasis, salmonella infections, dysentery, constipation, hay fever and allergic rhinitis.
- Mackenzie (2012) found that *Allium sativum* has superior immune-stimulatory, antilipidaemic, antitumour, antineoplastic properties.
- Saxena and Kukreti (2020) stated that the regular use of an *Allium sativum* mouthwash may inhibit the formation of dental caries.

2.3.6. Plant Sections Used

The *Allium sativum* bulb is used medicinally (Majewski 2014).

2.3.7. Constituents of garlic

Garlic contains more than 2000 biologically active compounds. Some of which are volatile, water-soluble, and oil-soluble organic sulphur compounds such as: diallyl sulphide, diallyl disulphide, diallyl trisulphide (Majewski 2014). According to Barrett (2004), garlic has a sulphur content that is four times greater than that of any other high sulphur content vegetables. The organic Sulphur compounds of *Allium sativum* are alliin and scordinin A and B (Majewski 2014). Alliin is the naturally occurring cysteine sulphoxide that breaks down to form the thiosulphinates (allicin), ajoenes (E-ajoene, Z-ajoene), vinyldithiins (vinyl-1,3-dithiin, vinyl-1,2-dithiin) and sulphides (diallyl disulphide and diallyl trisulphide) (Majewski 2014).

2.3.7.1. Allicin - the phytochemical found in garlic

Allicin (diallyl thiosulphinate) is a highly volatile organic sulphur compound that acts as a plant defence mechanism. Allicin is responsible for the antimicrobial properties and odour of garlic (Barrett 2004). According to (Majewski 2014), allicin contains stronger antibiotic potential than penicillin or tetracycline. However, allicin is ignored in orthodox medicine due to the complex mechanism of production of allicin from the breakdown alliin [***Figure 2.3***], and the instability of the compound.

The sulphoxide alliin is released when the *Allium sativum* bulb undergoes any physical trauma such as being cut/ crushed or processed in any way. Alliin then interacts with the enzyme alliinase which then undergoes hydrolysis and condensation and is converted into allicin (diallyl thiosulphinate) [***Figure 2.3***] (Barrett 2004). The volatile compound allicin is highly unstable and undergoes rapid breakdown into other compounds. “Due to the reactive nature of the compound, there are no products on the market, which would contain a detectable amount of allicin” (Majewski 2014).

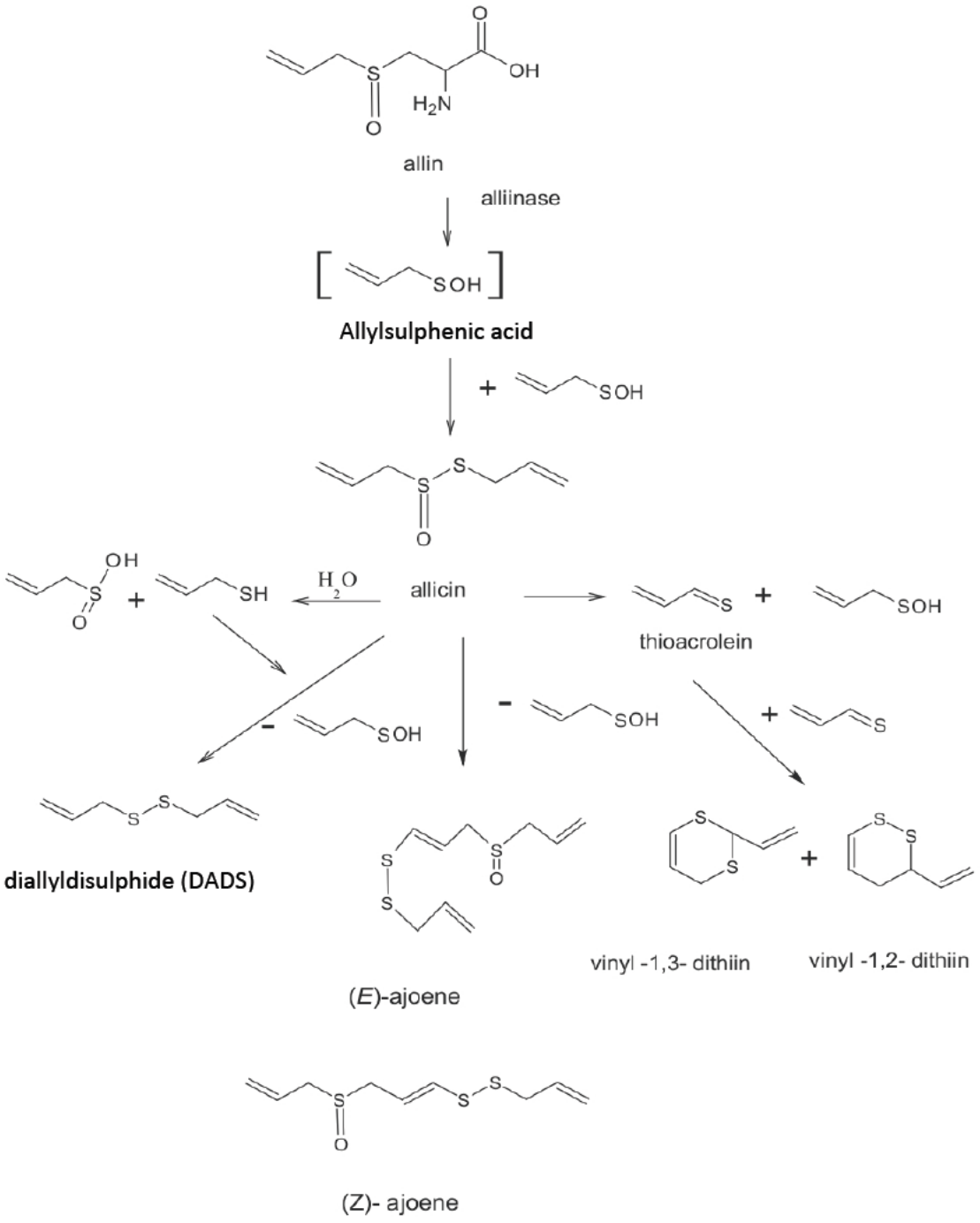

Figure 2.3: Schematic breakdown of alliin (Majewski 2014).

2.4. *Tulbaghia violacea* and *Allium sativum* antimicrobial research

Numerous research studies conducted on *Tulbaghia violacea* and *Allium sativum* have aimed to evaluate the antimicrobial properties of these plants:

- Hutchings (1996) noted a general bacteriostatic action of *Tulbaghia violacea*, whilst later studies by McGaw, Jäger and van Staden (2000) reported negative results in antimicrobial tests against *Escherichia coli, Klebsiella pneumoniae*, *Staphylococcus aureus* and *Bacillus subtilis*.

- Ncube *et al.* (2011) conducted an experiment comparing indoor and outdoor cultivated *Tulbaghia violacea.* Extracts of the two groups of plants were obtained in petroleum ether and dichloromethane. These were evaluated for antibacterial and antifungal activities against four species of bacteria and one species of fungi (*Candida albicans).* Spectrophotometry analyses was used to determine the saponins and phenolic compounds. The results indicated that the micro propagated *Tulbaghia* extract in petroleum ether displayed the best antibacterial activity and yielded the greatest number of phytochemical compounds.

- Afolayan, Bradley and Olorunnisola (2012) tested the cytotoxic activity of *Tulbaghia violacea* on the brine shrimp lethality bioassay. It was discovered that the toxicity was dependent on the concentration of the extract. Concentrations above 5.0 µg/ml were cytotoxic, which is due to the presence of polysulfides that are known to have potential anticancer, antimicrobial, and antifungal effects.

- Aremu and van Staden (2013) reviewed several research studies of *Tulbaghia violacea* and found discrepancies in the reported antimicrobial activity of the plant. Ranglová, Krejčová and Kubec (2015) set out to determine the reasons for such discrepancies and it was concluded that different methods of extraction resulted in varying levels of the antimicrobial compound marasmicin. It was concluded that freshly harvested rhizomes that were blended with water contained the most marasmicin.

- Lonkala and Reddy (2019) used the disc diffusion assay to investigate the antibacterial activity of *Carica papya* (papaya) and *Allium sativum* individually and in a combination against six strains of bacteria. The results obtained demonstrated that both plants are potentially effective in supressing the growth of selected bacteria. A combination of both plant extracts proved to be most effective against the six strains of bacteria tested.

- Enejiyon *et al.* (2020) determined the antibacterial activity of *Allium sativum* and *Allium cepa* against *Salmonella typhi*, *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Klebsiella pneumoniae* and *Escherichia coli.* Water, ethanol and acetone extracts of both plants were tested using the well diffusion method. The results of the study indicated that both plants contain varying levels of inhibition and therefore it was concluded that the Allium species of plants display antibacterial activities against Gram-positive and Gram-negative bacteria.

- Magryś (2021) found that using *Allium sativum* in combination with conventional antibiotics may be beneficial in inhibiting the growth of multidrug-resistant bacteria.

2.5. *Candida albicans*

2.5.1. Classification

Candida albicans is an opportunistic infectious yeast that belongs to the genus *Candida*, family *Saccharomycetaceae* (Barlows *et al.* 1991).

2.5.2. Candidiasis

Different species of *Candida* are normally found in the gastrointestinal microflora. However, due to the opportunistic nature of the fungi, it becomes pathogenic in immunocompromised individuals (Centers for Disease Control and Prevention 2022). *Candida albicans* causes candidiasis (thrush), which affects the muco-cutaneous parts of the body, most commonly the oral cavity, genitalia, gastrointestinal tract or respiratory tract. Candidiasis can also occur on cutaneous sites, such as nails, nail folds, hair follicles, among other sites. In addition to superficial and mucosal lesions, it also has the potential to disseminate and produce bloodstream candidiasis (Bondaryk, Kurzątkowski and Staniszewska 2013).

2.5.3. HIV/AIDS and Candidiasis

HIV/AIDS, diabetes or pregnancy can make an individual more susceptible to secondary infections (Budree 2004; Bvumbi *et al.* 2021). Candidiasis can also occur as an unintended consequence of medical treatments, such as making use of antibiotics or oral contraceptives (Bondaryk, Kurzątkowski and Staniszewska 2013). South Africa has the highest prevalence of HIV/AIDS internationally (Allinder and Fleischman 2019). Additionally, every two out of three HIV/AIDS patients suffer from candidiasis as a result of being immunocompromised (Eloff *et al.* 2008; Saxena and Kukreti 2020). The presence of Oropharyngeal candidiasis is now regarded as the earliest sign of HIV.

2.5.4. Drug Resistance

Although *Candida albicans* is part of the normal microbiota, it is now emerging as a significant pathogen due to increasing resistance to available drugs (Bungu *et al.* 2006). The excessive use of antibiotics such as antibacterial and antifungal agents has resulted in multiple drug resistance (MDR) of both pathogenic bacteria and fungi (Saxena and Kukreti 2020). According to Bondaryk, Kurzątkowski and Staniszewska (2013), *Candida albicans* is known to be the leading opportunistic fungal pathogen causing infections in immunocompromised individuals, who are already heavily medicated due to their chronic medical conditions.

Fluconazole is regarded as the standard treatment for candidiasis. However, there are reports of reduced effectiveness after prolonged use of fluconazole for the treatment and prevention of *Candida albicans* infections. There are many other azole antifungals available such as posaconazole, voriconazole, ketoconazole and itraconazole, all of which produce similar side effects such as skin rashes, nausea, vomiting, diarrhoea, headaches and hepatitis (Thamburan *et al.* 2006).

A common clinical presentation of *Candida albicans* is oral candidiasis, this is particularly evident in HIV positive persons. Oral candidiasis can be diagnosed by identifying the creamy white curd-like patches in the mouth, which causes a sore throat, difficulty in chewing and swallowing. Systemic symptoms include diarrhoea, weight loss and blood infections. Superficial candidiasis is rarely fatal; however, it does lower the quality of life of an infected person (Bondaryk, Kurzątkowski and Staniszewska 2013).

2.6. HOMOEOPATHY

Homoeopathy is a complementary system of medicine that treats disease by using substances that produce ‘similar’ symptoms to the disease, when ingested by healthy individuals. Homoeopathic remedies/potencies are produced by a process of potentization/dynamization, which involves serial dilution and vigorous shaking (succussion) after each dilution. The process of potentization is claimed to make a substance less toxic and more therapeutic (O'Reilly 1996).

Homoeopathy is holistic in nature and makes use of highly diluted potencies to provide a remedial effect. The most used potencies in homoeopathy are: 6CH ($1:10^{12}$), 12CH ($1:10^{24}$), 30CH ($1:10^{60}$), 200CH ($1:10^{400}$), 1M ($1:10^{2000}$), and 10M ($1:10^{20000}$). Due to the high dilutions used and the lack of side effects, finding a homoeopathic treatment for candidiasis will be highly beneficial as most infected individuals are already on multiple medication for pre-existing medical conditions with many side effects.

2.6.1. The Fibonacci Series and Homoeopathy

According to Ghose (2022), The Fibonacci series is a universal law that can be seen occurring in nature and consists of a mathematical sequence of numbers created by adding the sum of the preceding two numbers to form the series:

- 0 + 1 = 1
- 1 + 1 = 2
- 2 + 1 = 3
- 3 + 2 = 5
- 5 + 3 = 8
- 8 + 5 = 13
- 13 + 8 = 21
- 21 + 13 = 34 and so on

The Fibonacci series was rediscovered by an Italian mathematician named Leonardo Bonacci (1170-1250) from ancient Hindu-Arabic texts written in Sanskrit 200 B.C. Some examples of the Fibonacci series occurring naturally can be found as the mathematical proportion of the structure of leaves, seeds and flowers, which allow for optimal sunlight absorption, plant growth, pollination or seed compaction (Tőkés and Tőkés 2014). The shell of Chambered Nautilus follows the golden ratio and allows for optimal growth without changing the shape of the animal. The Fibonacci series can even be observed in the inner and external ear of human beings, which helps with sound conduction (Ghose 2022).

The Fibonacci series was relatively recently introduced into Homoeopathy by Rozencwajg, as a framework for choosing homoeopathic potencies in greater alignment to patterns occurring in nature, rather than the base-6 and base-10 patterns more routinely employed (i.e. 6-12-30 or 30-200-1000) (Tőkés and Tőkés 2014). For this research project, the potencies 3CH, 5CH, 8CH, 13CH, and 21CH were tested for antifungal activity against *Candida albicans*.

2.5.2. Research linking ethnobotany with homoeopathy

To date, the only homoeopathic research done on *Tulbaghia violacea* at the Durban University of Technology was conducted by Invernizzi (2002). *Tulbaghia violacea* was tested on Gram negative bacteria using the disc diffusion assay. None of the samples tested showed any positive antimicrobial results. Some of the suggestions made were to:

- Replace filter paper discs with blank susceptibility discs.
- Use Vernier calipers for more accurate measurements.
- Manufacture the experimental herbal tinctures instead of purchasing them as this allows for greater control over the type of sample used, extraction methodology and final concentration of the samples.

In a study conducted by Dummer (2003) the antimicrobial activities of an indigenous plant, *Withania somnifera* (Ashwaganda), was investigated *in vitro*. Using the disc diffusion assay, 1X and 6X homoeopathic potencies were tested against *Bacillus cereus*, *Pseudomonas aeruginosa*, *Klebsiella pneumonia*, *Escherichia coli* and *Staphylococcus aureus*. The study found both 1X and 6X ineffective at inhibiting the growth of the selected group of bacteria.

Budree (2004) tested ethanolic extracts of *Commiphora molmol, Hydrastis canadensis* and *Warburgia salutaris* against *Candida albicans* by making aid of the disc diffusion assay. The results indicated that only *Hydrastis canadensis* was effective in inhibiting the growth of *Candida albicans.*

A double-blind study was conducted by Maharaj (2006) to evaluate the effectiveness of a topical complex containing *Allium sativum* and *Hydrastis canadensis* mother tinctures combined with *Apis mellifica* and *Urtica urens* in D3 homoeopathic potencies, as an alternative treatment for *Tinea pedis.* However, it was concluded that the complex was not effective in the treatment of *Tinea pedis.*

Shaffique *et al.* (2018) reviewed the ethnobotanical, phytochemical and homoeopathic uses of *Echinacea angustifolia* (purple cone flower) to determine if there is a link between the three paradigms. The review concluded that the traditional use in the

treatment of bacterial and fungal infections has been verified by *in vitro* studies and is also used for septic conditions in homoeopathy.

2.7 CONCLUSION

After an extensive review of the available literature, the importance of ethnobotany in identifying potential therapeutic substances was highlighted. The rich traditional medicine background combined with a diverse assortment of indigenous flora found in South Africa, resulted in *Tulbaghia violacea* being identified for its unique phytochemical: marasmicin. A plant that shares a similar phytochemical is *Allium sativum*, which has documented historic uses that date back to 5000 years. The similarities in these plants have resulted in numerous investigations of their antimicrobial properties, however to date there has only been one homoeopathic research study conducted on *Tulbaghia violacea*. Thrush was identified as a problematic opportunistic fungal infection which has an urgent need for intervention that does not produce side effects and cannot result in antifungal resistance. After a comprehensive review of the available literature, a gap in the existing knowledge was identified and a study was designed to investigate the antifungal properties of *Tulbaghia violacea,* which is outlined in the following chapter.

CHAPTER 3: METHODOLOGY

3.1. RESEARCH DESIGN AND SETTING

This study sought to investigate the antifungal potential of an indigenous plant: *Tulbaghia violacea* in homoeopathic potencies and as a mother tincture. The antifungal activity of *Tulbaghia violacea* was also compared to a parallel set of homoeopathic potencies and a mother tincture, prepared from *Allium sativum.* This study consisted of two *in vitro* experimental investigations:

1. Determining the absorbance (optical density/growth) of *Candida albicans* before and after incubation with and without administrating a homoeopathic potency or mother tincture in a microtitre plate.
2. Using the disc/well diffusion method to determine the minimum inhibitory concentration, by measuring the zones of inhibition produced by the respective mother tinctures and homoeopathic potencies.

The research study was an *in vitro,* quantitative, experimental microbiological study that was carried out in the Department of Biotechnology and Food Science at the Durban University of Technology (DUT), Steve Biko campus.

3.2. MATERIALS

3.2.1. Preparation of the *Tulbaghia violacea* and *Allium sativum* ethanolic extracts, mother tinctures and homoeopathic potencies

The samples of *Tulbaghia violacea* (roots, rhizome, and leaves) and *Allium sativum* (bulb only) were sourced and verified by Professor Himansu Baijnath, an experienced botanist from the University of KwaZulu-Natal (Westville campus). *Tulbaghia violacea* was sourced from a natural environment as the plant grows naturally in Durban, whereas *Allium sativum* was purchased from a retail store as the plant is not indigenous to Southern Africa.

The procedure for the manufacture of the mother tinctures and homoeopathic potencies were as follows:

- The mass of the fresh plant material (1300 g *Allium sativum* and 780 g *Tulbaghia violacea*) was determined by using a mass scale and placed into zip lock bags at the Homoeopathic pharmacy laboratory on the DUT Ritson campus. The air was removed from the zip lock bags by submerging the bags in a tub of water. The bags were then sealed and packed into a cardboard box. The box was then sent via overnight courier to W. Last.cc in Mulbarton, Johannesburg.
- At W. Last cc, 1:2 (1-part ethanol: 2 parts plant material) ethanolic extracts of *Allium sativum* and *Tulbaghia violacea* were prepared according to a modified method 2A of the German Homoeopathic Pharmacopoeia [***Appendix 3***]. A 1:2 dilution was used for the extraction process for the ethanolic extracts as this allowed the extracts to be further diluted into 1:10 mother tinctures with a final alcohol concentration below 10%.
- Special care was taken when transporting the plants from Durban to Johannesburg, by sealing the plants in zip lock bags and removing the air from the bags, the zip lock bags were then wrapped with bubble wrap, to prevent any physical damage, boxed and then sealed.
- Once the 1:2 ethanolic extracts were returned to the department of Homoeopathy via courier services, the samples were diluted with deionized water, (obtained from a 4 step reverse osmosis filter) from the homoeopathy clinic dispensary, into 1:10 mother tinctures [***Appendix 4***].
- After 25 ml volumes of the 1:10 mother tinctures were prepared, respective 25 ml volumes of 3CH, 5CH, 8CH, 13CH and 21CH homoeopathic potencies were prepared with 8% ethanol [***Appendix 4***].
- All final alcohol percentages were 8% to minimise the possible antifungal effect of the ethanol used in preparation of the potencies.

3.2.2. Source of the *Candida albicans* sample

The *Candida albicans* standard American Type Culture Collection (ATCC10231) samples were obtained from the Department of Biotechnology and Food Science at the Durban University of Technology (DUT). Five colonies of a diameter >1 mm were sub-cultured onto a fresh Sabouraud agar plate and allowed to grow overnight at 30°C. This was regarded as the **starting culture** and was used for subsequent inoculations.

3.3. METHODS
3.3.1. THE MICROTITRE PLATE ASSAY:

3.3.1.1. Preparation of the growth medium for the microtitre plates

The medium of choice for this assay was Sabouraud dextrose broth which is commonly used to cultivate and isolate dermatophytes, such as *Candida albicans* (Sagar 2019). One litre of Sabouraud dextrose broth was prepared according to the manufacturer's (Merck KGaA, batch number: 1023936) specifications as follows:

- Using a mass scale, 30 g of Sabouraud dextrose broth powder was massed out and added to 1 litre of distilled water in a 2000 ml screw top flask.
- In a different screw top flask, 250 ml of 0.85% saline solution was prepared by mixing 2.125 g of sodium chloride (Manufacturer: Sigma Aldrich, Batch number: SZBFO350V) chemical standard with 250 ml distilled water.
- The two flasks were autoclaved at 121ºC for 20 minutes and thereafter left to cool to room temperature before further use.

3.3.1.2. Preparation of the inoculum for the microtitre plates

- A laminar flow unit (Bioflow-II labotech) was sterilized by wiping the surface with 96% ethanol and clean tissue paper.
- The inoculum was prepared by suspending 10 ml of the prepared Sabouraud broth with five >1 mm diameter colonies of the *Candida albicans* starting culture which was then allowed to incubate for 24 hours at 30°C.
- A volume of 4 ml of sterile 0.85% saline was added to 400 µl of the 24-hour old *Candida* cultures to form the **stock culture**.

- The absorbance of the stock culture was read at 530 nm in a UV-visible spectrophotometer (Thermo Scientific Genesys 150).
- The stock culture was adjusted with the 0.85% saline solution to match that of 0.5 McFarland standard solution (A 0.5 McFarland standard is prepared by mixing 0.05 ml of 1% Barium chloride and 9.95 ml of 1% Sulphuric acid to form a solution with a turbidity to match the approximate cell density of $1.5x10^8$ colony forming units per ml (CFU/ml) (Tankeshwar 2021). The turbidity of the solution was verified by testing the absorbance in a spectrophotometer at 530 nm.
- The **culture broth** was prepared by adding 0.01 ml from the prepared stock cultures to 10 ml of the prepared Sabouraud dextrose broth.

3.3.1.3. The controls

Two types of controls were used in this study: blank controls, and negative controls:

1. Broth sterility/ blank control: wells 1 A-C and 1 E-G contained 200 µl of broth only. The purpose of the blank controls was to determine if the broth only was sterile enough to not produce a positive absorbance after incubation, to have a reference point for comparison of the experiment absorbance readings, and to further validate the methodology of the assay.
2. Negative controls: wells 2 A-C and 2 E-G contained the broth culture only and was not subjected to any test substances, the purpose of the negative control was to indicate the growth abundance of *Candida albicans* when left untreated, to provide a reference point for comparison of the experimental data (treatment groups), and to illustrate the functionality of the experimental design.

3.3.1.4. Preparation of the microtitre plates

The 96 well microtitre plate used in this research consists of 8 vertical columns labelled A-H, and 12 horizontal rows numbered 1-12. ***Table 3.1*** represents the microtitre plate map of the following:

- Two hundred microlitres of **broth only** were added to wells numbered 1A, 1B, 1C, 1E, 1F, and 1G.

- Two hundred microlitres of the culture broth (broth + *Candida albicans* inoculum) were added to wells numbered 2A, 2B, 2C, 2E, 2F and 2G.
- One hundred microliters of the culture broth were added to wells numbered 3-8 A–C and E-G.
- Rows D and H: 1-8 was left blank to physically isolate *Tulbaghia violacea* (A-C) and *Allium sativum* (E-G) samples.
- One hundred microliters of mother tincture or homoeopathic potency were then added to each well as follows:
    1. 3A, 3B, 3C - *Tulbaghia violacea* 1:10 Mother tincture
       4A, 4B, 4C - *Tulbaghia violacea* 3CH
       5A, 5B, 5C - *Tulbaghia violacea* 5CH
       6A, 6B, 6C - *Tulbaghia violacea* 8CH
       7A, 7B, 7C - *Tulbaghia violacea* 13CH
       8A, 8B, 8C - *Tulbaghia violacea* 21CH [***Table 3.1***]
    2. 3E, 3F, 3G - *Allium sativum* 1:10 Mother tincture
       4E, 4F, 4G - *Allium sativum* 3CH
       5E, 5F, 5G - *Allium sativum* 5CH
       6E, 6F, 6G - *Allium sativum* 8CH
       7E, 7F, 7G - *Allium sativum* 13CH
       8E, 8F, 8G - *Allium sativum* 21CH [***Table 3.1***]
- Each potency and mother tincture was tested three times in a single microtitre plate [***Figure 3.1***], representing one cycle. Each cycle was repeated two times.

Table 3.1: The microtitre plate map

	1 *(Broth)*	**2** *(B.C)*		**3** *(1:10)*	**4** *(3CH)*	**5** *(5CH)*	**6** *(8CH)*	**7** *(13CH)*	**8** *(21CH)*
A	Broth only	Broth culture	*Tulbaghia violaceae* (T.V)	B.C+ T.V *(1:10)*	B.C+ T.V 3CH	B.C+ T.V 5CH	B.C+ T.V 8CH	B.C+ T.V 13CH	B.C+ T.V 21CH
B	Broth only	Broth culture		B.C+ T.V 1:10	B.C+ T.V 3CH	B.C+ T.V 5CH	B.C+ T.V 8CH	B.C+ T.V 13CH	B.C+ T.V 21CH
C	Broth only	Broth culture		B.C+ T.V 1:10	B.C+ T.V 3CH	B.C+ T.V 5CH	B.C+ T.V 8CH	B.C+ T.V 13CH	B.C+ T.V 21CH

D	Blank row								
E	Broth only	Broth culture	*Allium sativum* (A.S)	B.C+ A.S 1:10	B.C+ A.S 3CH	B.C+ A.S 5CH	B.C+ A.S 8CH	B.C+ A.S 13CH	B.C+ A.S 21CH
F	Broth only	Broth culture		B.C+ A.S 1:10	B.C+ A.S 3CH	B.C+ A.S 5CH	B.C+ A.S 8CH	B.C+ A.S 13CH	B.C+ A.S 21CH
G	Broth only	Broth culture		B.C+ A.S 1:10	B.C+ A.S 3CH	B.C+ A.S 5CH	B.C+ A.S 8CH	B.C+ A.S 13CH	B.C+ A.S 21CH
H	Blank row								

KEY: T.V – *Tulbaghia violacea*, A.S – *Allium sativum*, B.C – Broth culture

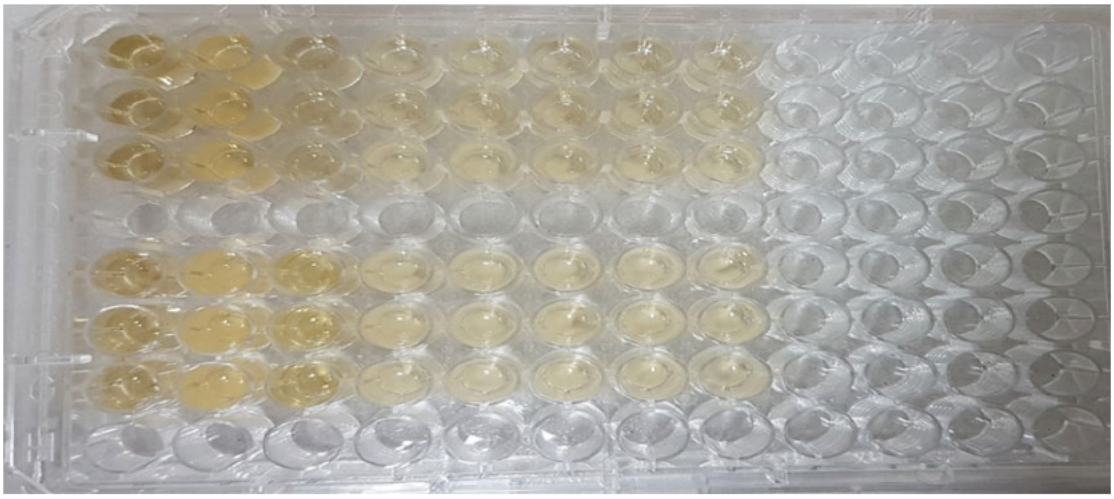

Figure 3.1: The microtitre plate

3.3.1.5. The absorbance measurements of the microtitre plates

Once the microtitre plate was set up as illustrated in ***Table 3.1***, the plate was immediately read at 630 nm using a Multiskan Go microplate spectrophotometer. The absorbance values were recorded in ***Appendix* 5A and *5B*** as before incubation. Thereafter, the plate was covered with a lid, labelled with a marker, wrapped with parafilm, and placed in an incubator at 33°C for 24 hours.

After incubation, the microtitre plate was read at 630 nm in the microplate spectrophotometer. The results were recorded in ***Appendix 5A*** **and** ***5B*** as after incubation. This process was repeated twice, and the results of trial two were recorded in ***Appendix 5C*** **and** ***5D*****.**

3.3.2. THE WELL AND DISC DIFFUSION ASSAY: SUSCEPTIBILITY TEST

The antifungal activity of the two plants was also determined by measuring the zones of inhibition using the well diffusion method.

3.3.2.1. Preparation of the growth medium for the petri plates

The medium of choice for this Assay was Sabouraud dextrose agar which is commonly used to cultivate and isolate dermatophytes such as *Candida albicans* (Sagar 2019). A total of 1 litre of Sabouraud dextrose agar was prepared according to the manufacturer (Merck KGaA: 1019903) specification as follows:

- Using a mass scale, 65 g of Sabouraud agar powder was weighed out and added to 1 litre of distilled water in a Schott bottle.
- The flask was heated and agitated until the medium was completely dissolved and thereafter autoclaved at 121ºC for 20 minutes. The flask was removed from the autoclave and left to cool at room temperature.
- Under a sterile laminar flow unit, approximately 40 ml of agar was poured aseptically into twelve sterile petri plates and allowed to solidify.

Twelve petri were labelled as follows:
Tulbaghia violacea - 1:10, 3CH, 5CH, 8CH 13CH, 21CH.
Allium sativum - 1:10, 3CH, 5CH, 8CH 13CH, 21C [***Table 3.2***]

- Each petri plate was demarcated into four sections to allow for the triplicate testing of one test substance and one blank control [***Figure 3.2***].

3.3.2.2. Preparation of the inoculum for the petri plates

Using the methodology described by Thamburan *et al.* (2006), under a sterilized laminar flow unit, a **saline culture** was prepared by suspending five individual colonies from the starting culture in 10 ml of sterile saline solution (0.85%) and adjusted to the 0.5 McFarland Standard.

3.3.2.3. The controls

Negative controls: one out of the four wells/disc contained the saline culture only and was not subjected to any test substances. The purpose of the negative control was to indicate how much growth of *Candida albicans* occurred when left untreated, to provide a reference point to compare the experimental data (treatment groups) to and finally to illustrate that the functionality of the experimental design of the assay.

3.3.2.4. Preparation of the well diffusion assay

- Once the Sabouraud agar had solidified, four wells were punched into the agar using the back end of a sterile P1000 micropipette tip.
- One hundred microlitres of the prepared saline culture was then added to each plate and spread evenly with a glass spreader.
- The plates were allowed to dry for two minutes with the lids in place.
- This process was repeated for all the agar plates.
- Ten microlitres of the various concentrations were added to each well according to ***Table 3.2***.

Table 3.2: The petri plate labels for the well diffusion assay

Plate number	*Tulbaghia violacea*	*Allium sativum*
1	1:10	-
2	3CH	-
3	5CH	-
4	8CH	-
5	13CH	-
6	21CH	-
7	-	1:10

8	-	3CH
9	-	5CH
10	-	8CH
11	-	13CH
12	-	21CH

The plates were incubated at 37°C for 24 hours. Thereafter, the zones of inhibition were observed and measured.

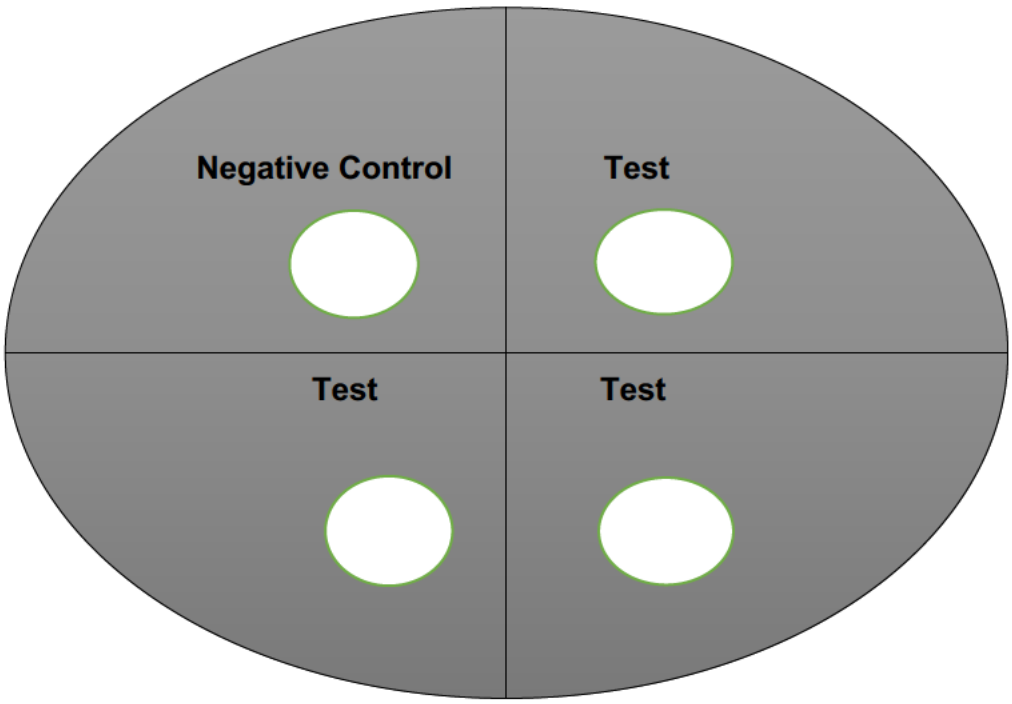

Figure 3.2: The petri plate map

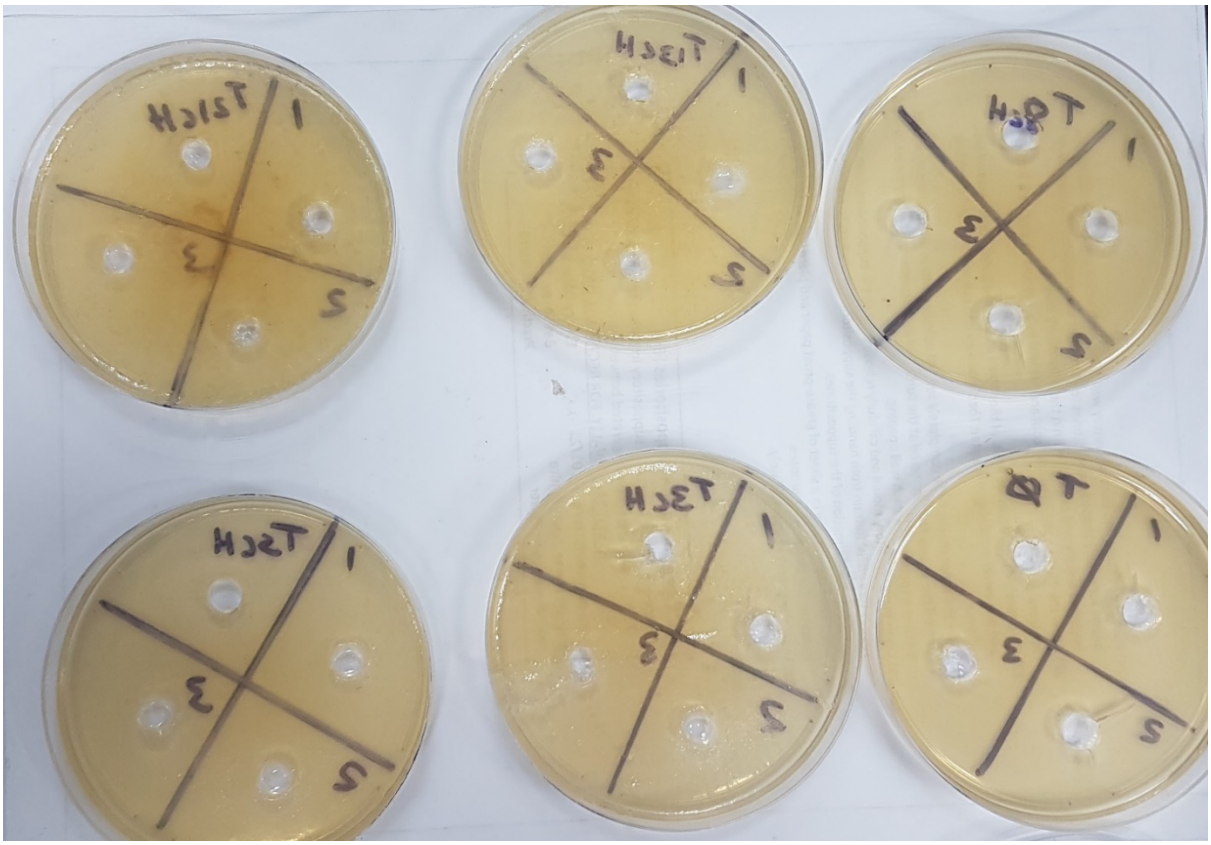

Figure 3.3: The petri plates of *Tulbaghia violacea* using the well method

3.3.2.5. Preparation of the disc diffusion assay

After six unsuccessful attempts to elicit zones of inhibition using the well diffusion method, it was decided that the disc diffusion methodology would be used instead:

- Twelve Sabourad agar plates were prepared, no holes were punched into the agar.
- Under a sterilized laminar flow unit, the culture was prepared once again by suspending five individual colonies from the starting culture in 10 ml of sterile 0.85% saline solution and adjusted to the 0.5 McFarland Standard (Tankeshwar 2021).
- The culture (10 µl) was added to each plate and spread evenly with a glass spreader.
- The plates were allowed to dry for two minutes with the lids in place.
- This process was repeated for all agar plates.

- Number 4 Whatman filter paper was cut into 5 mm discs, placed into a 100 ml Schott bottle and autoclaved at 121ºC with a pressure of 103 kPa for 20 minutes.
- Once cooled, three discs per plate were placed evenly in twelve petri plates using sterile forceps. The petri plates were then closed and labelled according to the remedy they would receive. The fourth quadrant was unmarked to indicate the blank control.
- The three test discs placed on the agar plates were saturated with 10 µl of the respective test substance before being sealed and incubated for 24 hours at 33°C.
- Three of each impregnated discs were placed on the respective petri plate according to ***Table 3.3***.

Table 3.3: The petri plate labels for the disc diffusion assay

Plate number	*Tulbaghia violacea*	*Allium sativum*
1	1:10	-
2	3CH	-
3	5CH	-
4	8CH	-
5	13CH	-
6	21CH	-
7	-	1:10
8	-	3CH
9	-	5CH
10	-	8CH
11	-	13CH
12	-	21CH

3.3.2.6. Measurements of the zones of inhibition

The petri plates were sealed with parafilm, inverted, and incubated at 37°C for 24 hours. Thereafter, the plates were removed and observed. Zones of inhibition were measured by using a vernier calliper.

3.4. STATISTICAL METHODS FOR DATA ANALYSIS

The statistical package used for quantitative data analysis was the Statistical Package for the Social Sciences (SPSS) Version 25. Using SPSS, descriptive statistics were applied to analyse the data. Non-parametric tests were conducted to analyse the differences in the antifungal effectiveness of the different homoeopathic potencies and mother tinctures. The One-Sample Kolmogorov-Smirnov Test was used to determine the distribution of the data.

The Mann-Whitney U Test is used to compare differences between two independent sets of data that are not normally distributed (Laerd statistics 2023b). The Mann-Whitney U Test was used to compare the differences of the test groups of each plant before and after incubation.

The Kruskal-Wallis test is a non-parametric test that is an extension of the Mann-Whitney U Test and is used to determine statistically significant differences between two independent data sets (Laerd statistics 2023a). The Kruskal-Wallis test was used to compare the pairwise effect of each test group. The statistical analysis presented in Chapter Four represents the findings of the research methodology employed in this Chapter.

CHAPTER 4: THE RESULTS

4.1. INTRODUCTION

The Microtitre plate assay described in Chapter Three produced the primary experimental data. The well- and disc diffusion assay did not produce measurable zones of inhibition. This will be fully explored and discussed in the next Chapter. The quantitative data produced from the Microtitre plate assay was analysed using descriptive and inferential statistics to determine the relationship of the test substances on the growth of *Candida albicans in vitro.* This Chapter provides a descriptive and visual representation of the statistical analysis of the data.

The One-Sample Kolmogorov-Smirnov Test for normality indicated that the data does not follow a normal distribution. The median and interquartile range was used to describe central measure and variability, and non-parametric tests were conducted. The Mann-Whitney U Test was used to compare the differences of the test groups of each plant pre- and post-incubation and the independent-samples Kruskal-Wallis test with analysis of variance was used to compare the pairwise effect of each test group.

4.2. DESCRIPTIVE RESULTS FOR WILD GARLIC

The data analysis in ***Table 4.1*** shows the descriptive results before and after incubation of the controls, respective *Tulbaghia violacea* potencies and the 1:10 mother tincture. The result suggests that the negative control with broth and *Candida albicans* had the highest median absorbance value after 24 hours of incubation (**1.247**) while the lowest was observed for the 1:10 mother tincture (**0.070**). This indicates that there was rapid growth of *Candida albicans* after 24 hours of incubation when left untreated and that the 1:10 mother tincture was the most inhibitory of the growth of *Candida albicans.* The homoeopathic potencies did display general inhibition of the growth of *Candida albicans* as the median values ranged from **0.137** to **0.194**. No significant difference was reported in the samples before incubation.

Table 4.1: Descriptive results for *Tulbaghia violacea*

		Time of incubation	Mean (median) and interquartile range of *Tulbaghia violacea* absorbances						
			Number of tests	Mean	Standard Deviation	Median	Percentile 25	Percentile 75	95.0% Lower CL for Mean
Experiment	Broth only	Before	6	0.068	0.001	0.068	0.067	0.069	0.066
		After	6	0.069	0.006	0.069	0.064	0.071	0.063
	B & C	Before	6	0.065	0.002	0.066	0.065	0.067	0.063
		After	6	0.992	0.472	**1.247**	0.545	1.317	0.496
	Tincture 1:10	Before	6	0.070	0.001	0.071	0.070	0.071	0.069
		After	6	0.071	0.002	**0.070**	0.069	0.072	0.068
	Potency 3CH	Before	6	0.053	0.002	0.053	0.052	0.054	0.051
		After	6	0.221	0.149	0.194	0.110	0.311	0.064
	Potency 5CH	Before	6	0.052	0.002	0.052	0.051	0.054	0.050
		After	6	0.140	0.081	0.137	0.054	0.213	0.055
	Potency 8CH	Before	6	0.053	0.003	0.052	0.051	0.054	0.050
		After	6	0.161	0.106	0.150	0.079	0.221	0.050
	Potency 13CH	Before	6	0.054	0.004	0.053	0.051	0.055	0.050
		After	6	0.181	0.141	0.149	0.053	0.294	0.033
	Potency 21CH	Before	6	0.052	0.002	0.052	0.051	0.053	0.050
		After	6	0.181	0.105	0.184	0.069	0.276	0.071

Figure 4.1 provides visual evidence of the difference before and after incubation. The analysis indicates that the 1:10 mother tincture produced lowest median value and therefore the most inhibition (least growth) The untreated control group (sample containing broth and *Candida* only) produced the most growth of *Candida albicans.*

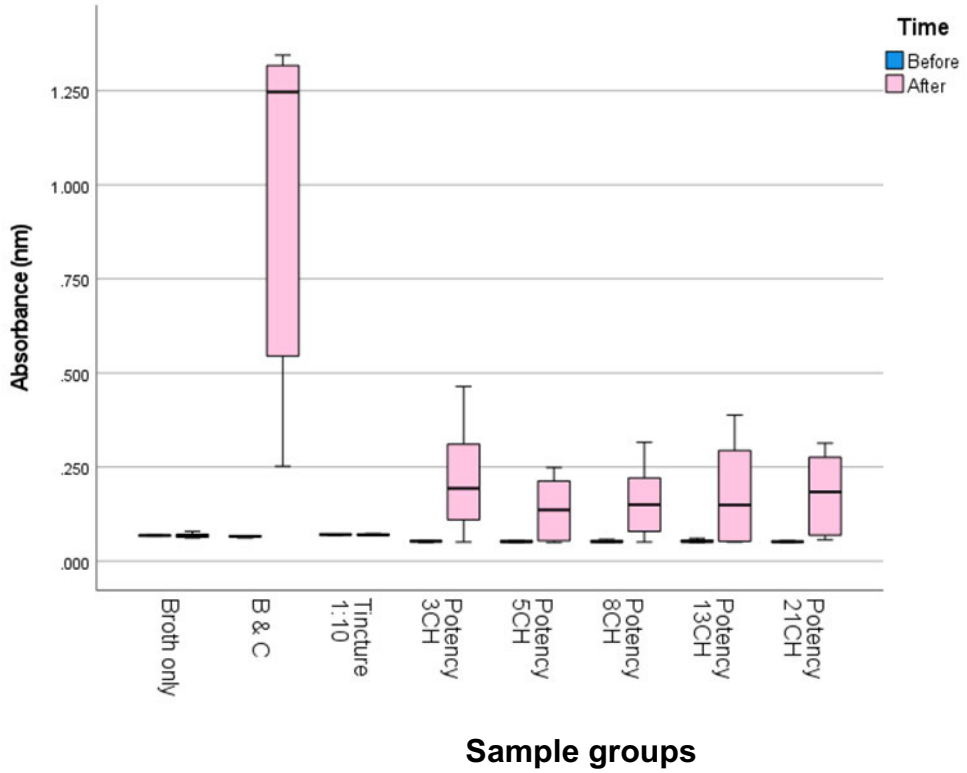

Figure 4.1: **Box plots comparing before and after incubation for *Tulbaghia violacea*.**

4.3. DESCRIPTIVE RESULTS FOR GARLIC

The data analysis in ***Table 4.2*** show the descriptive results before and after incubation of the controls, respective *Allium sativum* potencies and the 1:10 mother tincture. The result suggests that the sample with broth and *Candida albicans* (negative control) had the highest median absorbance value after 24 hours of incubation (**1.073**) while the lowest was observed for the 3CH potency (**0.094**). This indicates that there was a rapid growth of *Candida albicans* after 24 hours of incubation when left untreated and that the 3CH potency was the most inhibitory to the growth of *Candida albicans.* The mother tincture and other potencies did display an inhibitory effect on the growth of *Candida albicans* as the median values ranged from **0.112** to **0.230**. No significant difference was reported in the samples before incubation.

Table 4.2: Descriptive results for *Allium sativum*

		Time of incubation	Mean (median) and interquartile range of *Allium sativum* absorbances						
			Number of tests	Mean	Standard Deviation	Median	Percentile 25	Percentile 75	95.0% Lower CL for Mean
Experiment	Broth only	Before	6	0.063	0.002	0.063	0.062	0.065	0.061
		After	6	0.060	0.003	0.062	0.059	0.062	0.056
	B & C	Before	6	0.065	0.003	0.065	0.061	0.066	0.061
		After	6	0.938	0.479	**1.073**	0.461	1.347	0.435
	Tincture 1:10	Before	6	0.100	0.001	0.100	0.099	0.101	0.098
		After	6	0.127	0.025	0.136	0.114	0.142	0.100
	Potency 3CH	Before	6	0.054	0.000	0.054	0.054	0.054	0.054
		After	6	0.139	0.078	**0.094**	0.079	0.221	0.042
	Potency 5CH	Before	6	0.052	0.001	0.051	0.051	0.053	0.050
		After	6	0.195	0.112	0.139	0.103	0.291	0.055
	Potency 8CH	Before	6	0.051	0.001	0.051	0.051	0.052	0.050
		After	6	0.189	0.142	0.112	0.106	0.237	0.012
	Potency 13CH	Before	6	0.053	0.002	0.054	0.052	0.054	0.051
		After	6	0.278	0.255	0.186	0.089	0.425	0.010
	Potency 21CH	Before	6	0.054	0.002	0.054	0.052	0.055	0.052
		After	6	0.329	0.329	0.230	0.051	0.593	-0.016

Figure 4.2 further provides visual evidence of the differences before and after incubation. The analysis suggests that the 3CH potency produced lowest median result and therefore the most inhibition (least growth). The untreated control group (sample containing broth and candida only) produced the most growth of *Candida albicans*.

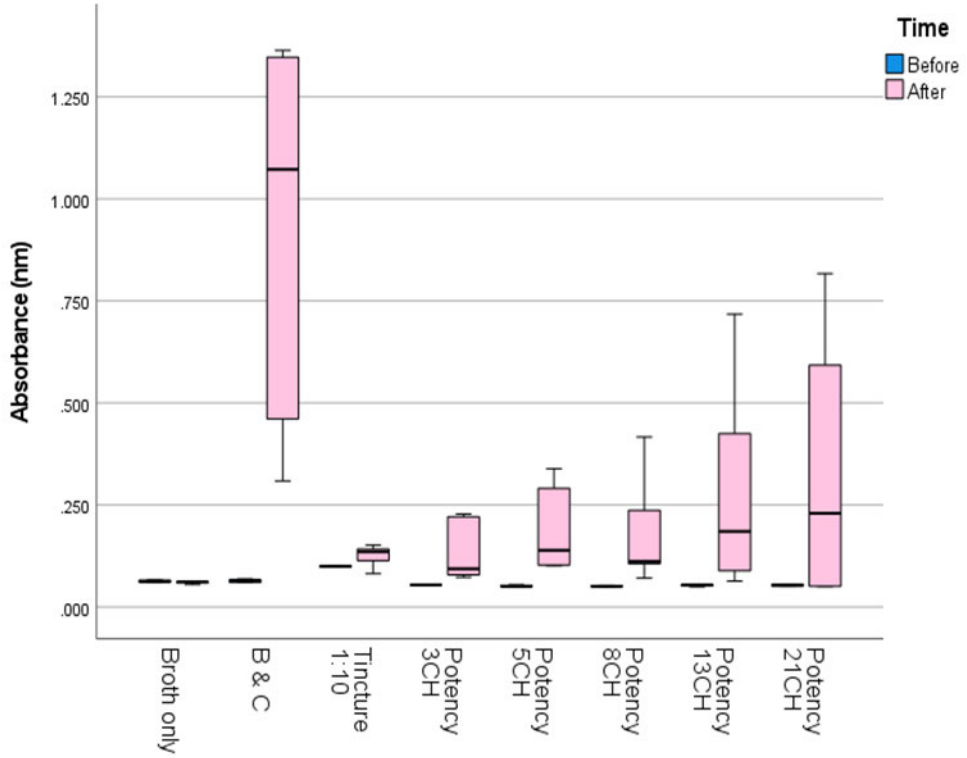

Figure 4.2: **Box plots comparing before and after incubation for *Allium sativum*.**

4.4. COMPARATIVE ANALYSIS OF THE INHIBITORY PROPERTIES OF THE SAMPLES

Research objective number three of this study was to compare the differences in the inhibitory properties of *Tulbaghia violacea* (test group) versus *Allium sativum* as a mother tincture and at different potencies. Non-parametric tests were performed to compare the differences between the two groups.

4.4.1. Descriptive analysis

The Mann-Whitney U Test was used to compare the differences between the plants at each potency and each time interval. The data analysis in ***Table 4.3*** shows the results of the Asymptotic Significance (2-tailed) p-values. The 1:10 mother tinctures produced statistical significance at $p < 0.05$.

Table 4.3: **Differences between the test substances at each time interval**

Potency	Time	p-value
Broth	Before	**0.025**
	After	**0.012**
B + C	Before	0.404
	After	0.631
1:10	Before	**0.012**
	After	**0.006**
3CH	Before	0.124
	After	0.465
5CH	Before	0.827
	After	0.465
8CH	Before	0.291
	After	0.584
13CH	Before	0.926
	After	0.337
21CH	Before	0.139
	After	0.873

The comparative analysis indicates that the 1:10 mother tinctures produced a statistically significant difference between both plants after incubation as $p = 0.006$. A comparison of the means (medians) indicates that *Tulbaghia violacea* had a mean (median) of 0.071 (0.070) and *Allium sativum* was 0.127 (0.136).

The Mann-Whitney Test was also used to compare the differences between each plant test group before and after incubation. The data analysis in ***Table 4.4*** shows the results of the Asymptotic Significance (2-tailed) p-values.

Table 4.4: Comparing before and after separately at each test substance

	Group	p-value
Broth only	*Tulbaghia violacea*	0.795
	Allium sativum	0.085
B & C	*Tulbaghia violacea*	**0.006**
	Allium sativum	**0.004**
1:10	*Tulbaghia violacea*	1.000
	Allium sativum	0.087
3CH	*Tulbaghia violacea*	**0.044**
	Allium sativum	**0.022**
5CH	*Tulbaghia violacea*	0.079
	Allium sativum	**0.008**
8CH	*Tulbaghia violacea*	**0.028**
	Allium sativum	**0.009**
13CH	*Tulbaghia violacea*	0.090
	Allium sativum	**0.006**
21CH	*Tulbaghia violacea*	**0.006**
	Allium sativum	0.335

The analysis indicated a statistically significant incubation for *Tulbaghia violacea* (3CH, 8CH and 21CH) and *Allium sativum* (3CH, 5CH, 8CH and 13CH), as the mean (median) difference between pre- and post-incubation were as follows:

Tulbaghia violacea:

- 3CH - pre-incubation 0.053 (0.053), post-incubation 0.221 (0.194)
- 8CH - pre-incubation 0.053 (0.052), post-incubation 0.161 (0.150)

- 21CH - pre-incubation 0.052 (0.052), post-incubation 0.181 (0.184)

Allium sativum

- 3CH - pre-incubation 0.054 (0.054), post-incubation 0.139 (0.094)
- 5CH - pre-incubation 0.052 (0.051), post-incubation 0.195 (0.139)
- 8CH - pre-incubation 0.051 (0.051), post-incubation 0.189 (0.112)
- 13CH - pre-incubation 0.053 (0.054), post-incubation 0.278 (0.186)

Figure 4.3 further illustrates the absorbance differences in the result measured for the antifungal formulations against *Candida albicans*. The image visibly confirmed that the inhibitory properties of *Allium sativum* were better than that of *Tulbaghia violacea.*

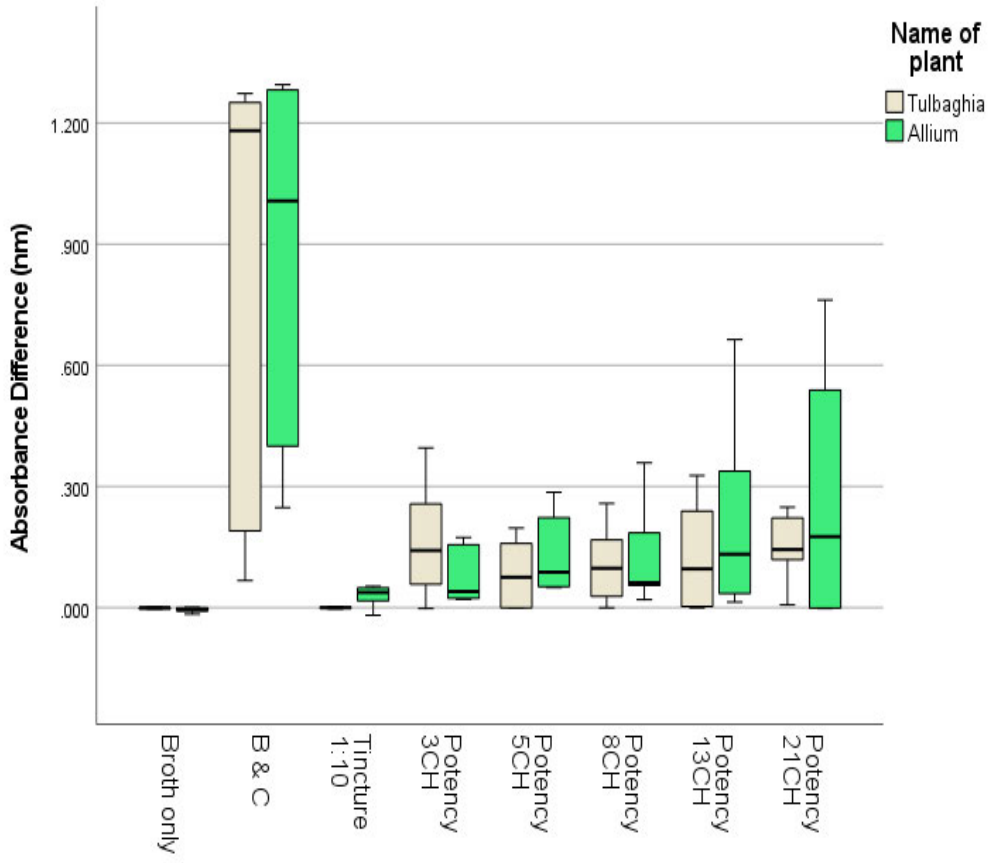

Figure 4.3: Mean (median) differences between each plant test group.

4.4.2. Pairwise comparisons between each test substance

The Independent-Samples Kruskal-Wallis Test was used to determine if there are any statistically significant pairwise differences between each test group. In the series of tables and figures that follow, the pairwise comparisons of *Allium sativum* and *Tulbaghia violacea* pre- and post-incubation are presented.

In ***Tables 4.5*** and ***4.6*** the pre-incubation and post-incubation pairwise comparisons of *Tulbaghia violacea* are displayed. ***Figures 4.4*** and ***4.5*** provide a visual representation of the comparisons.

Table 4.5: Pairwise comparison of *Tulbaghia violacea* before incubation

Pairwise Comparisons of Experiment				
Sample 1-Sample 2	Test Statistic	Std. Error	Std. Test Statistic	Sig.
Potency 21CH -Potency 5CH	1.100	7.354	0.150	0.881
Potency 21CH -Potency 8CH	2.433	7.041	0.346	0.730
Potency 21CH -Potency 3CH	2.800	7.354	0.381	0.703
Potency 21CH -Potency 13CH	3.767	7.041	0.535	0.593
Potency 21CH -B & C	18.300	7.354	2.488	**0.013**
Potency 21CH -Broth only	21.933	8.492	2.583	**0.010**
Potency 21CH -Tincture 1:10	26.000	7.354	3.535	**0.000**
Potency 5CH -Potency 8CH	-1.333	7.041	-0.189	0.850
Potency 5CH -Potency 3CH	1.700	7.354	0.231	0.817
Potency 5CH -Potency 13CH	-2.667	7.041	-0.379	0.705
Potency 5CH -B & C	17.200	7.354	2.339	**0.019**
Potency 5CH -Broth only	20.833	8.492	2.453	**0.014**
Potency 5CH -Tincture 1:10	24.900	7.354	3.386	**0.001**
Potency 8CH -Potency 3CH	0.367	7.041	0.052	0.958
Potency 8CH -Potency 13CH	-1.333	6.714	-0.199	0.843
Potency 8CH -B & C	15.867	7.041	2.253	**0.024**
Potency 8CH -Broth only	19.500	8.222	2.372	**0.018**

Potency 8CH -Tincture 1:10	23.567	7.041	3.347	**0.001**
Potency 3CH -Potency 13CH	-0.967	7.041	-0.137	0.891
Potency 3CH -B & C	15.500	7.354	2.108	**0.035**
Potency 3CH -Broth only	19.133	8.492	2.253	**0.024**
Potency 3CH -Tincture 1:10	23.200	7.354	3.155	**0.002**
Potency 13CH -B & C	14.533	7.041	2.064	**0.039**
Potency 13CH -Broth only	18.167	8.222	2.209	**0.027**
Potency 13CH -Tincture 1:10	22.233	7.041	3.158	**0.002**
B & C -Broth only	3.633	8.492	0.428	0.669
B & C -Tincture 1:10	-7.700	7.354	-1.047	0.295
Broth only -Tincture 1:10	-4.067	8.492	-0.479	0.632

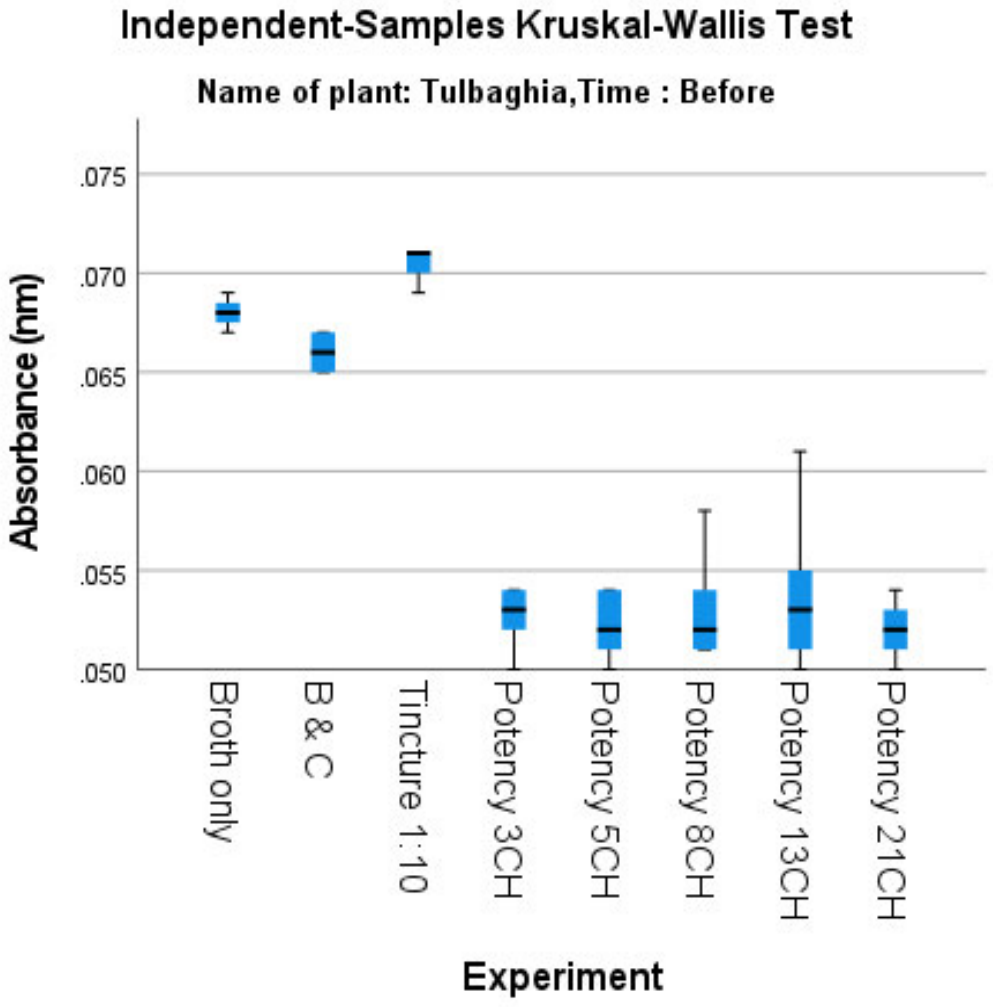

Figure 4.4: The pairwise comparison of *Tulbaghia violacea* before incubation.

Table 4.6: **Pairwise comparison of *Tulbaghia violacea* after incubation**

Pairwise Comparisons of Experiment				
Sample 1-Sample 2	Test Statistic	Std. Error	Std. Test Statistic	Sig.
Broth only -Tincture 1:10	-2.500	8.299	-0.301	0.763
Broth only -Potency 5CH	-7.500	7.913	-0.948	0.343
Broth only -Potency 13CH	-10.667	7.913	-1.348	0.178
Broth only -Potency 8CH	-12.250	7.913	-1.548	0.122
Broth only -Potency 21CH	-12.250	7.913	-1.548	0.122
Broth only -Potency 3CH	-14.500	7.913	-1.832	**0.067**
Broth only -B & C	-30.833	7.913	-3.897	**0.000**
Tincture 1:10 -Potency 5CH	-5.000	8.299	-0.602	0.547
Tincture 1:10 -Potency 13CH	-8.167	8.299	-0.984	0.325
Tincture 1:10 -Potency 8CH	-9.750	8.299	-1.175	0.240
Tincture 1:10 -Potency 21CH	-9.750	8.299	-1.175	0.240
Tincture 1:10 -Potency 3CH	-12.000	8.299	-1.446	0.148
Tincture 1:10 -B & C	28.333	8.299	3.414	**0.001**
Potency 5CH -Potency 13CH	-3.167	7.913	-0.400	0.689
Potency 5CH -Potency 8CH	-4.750	7.913	-0.600	0.548
Potency 5CH -Potency 21CH	-4.750	7.913	-0.600	0.548
Potency 5CH -Potency 3CH	7.000	7.913	0.885	0.376
Potency 5CH -B & C	23.333	7.913	2.949	**0.003**
Potency 13CH -Potency 8CH	1.583	7.913	0.200	0.841
Potency 13CH -Potency 21CH	-1.583	7.913	-0.200	0.841
Potency 13CH -Potency 3CH	3.833	7.913	0.484	0.628
Potency 13CH -B & C	20.167	7.913	2.549	**0.011**
Potency 8CH -B & C	18.583	7.913	2.349	**0.019**
Potency 21CH -B & C	18.583	7.913	2.349	**0.019**
Potency 8CH -Potency 3CH	2.250	7.913	0.284	0.776
Potency 21CH -Potency 3CH	2.250	7.913	0.284	0.776
Potency 8CH -Potency 21CH	0.000	7.913	0.000	1.000
Potency 3CH -B & C	16.333	7.913	2.064	**0.039**

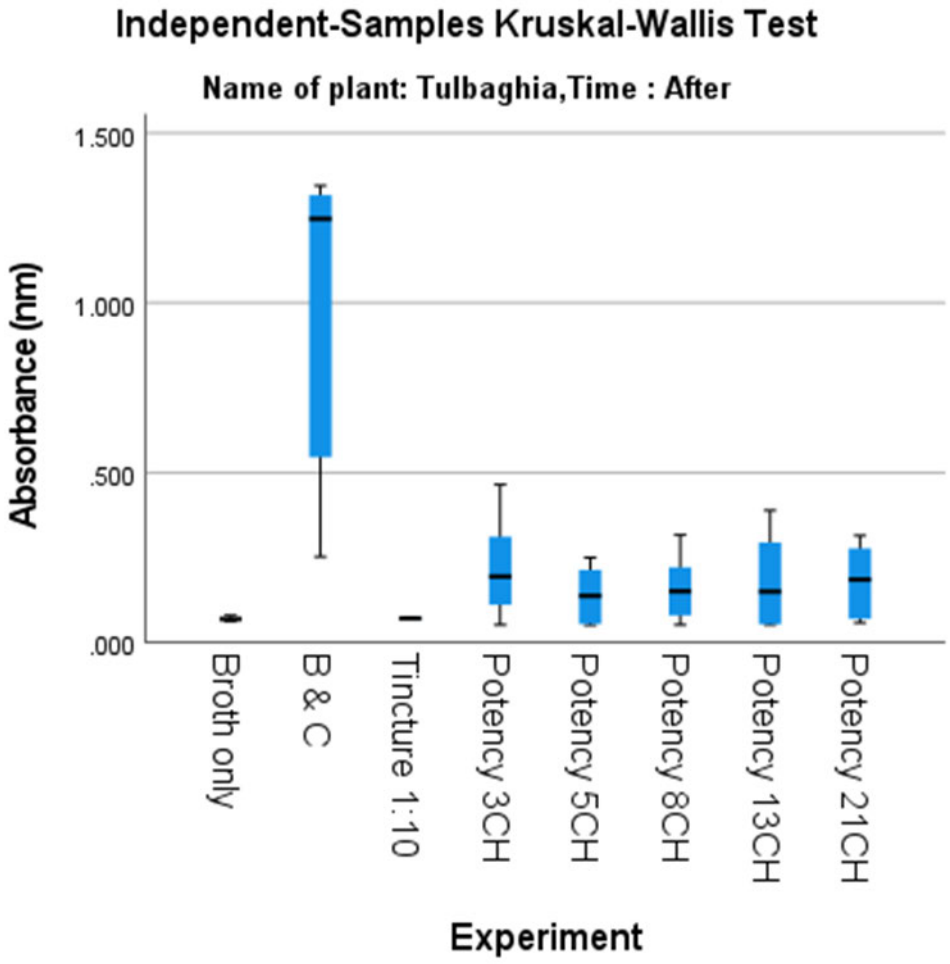

Figure 4.5: **The pairwise comparison of *Tulbaghia violacea* after incubation.**

After incubation the: 1:10 mother tincture (0,001), 3CH (0,039), 5CH (0,003), 8CH (0,019) 13CH (0,011) and 21CH (0,019) were statistically significant when compared to the broth and Candida (negative control).

Tables 4.7 and ***4.8*** show the pairwise comparison of *Allium sativum* before and after incubation. ***Figures 4.6*** and ***4.7*** are visual representations of the data analysis.

Table 4.7: Pairwise comparison of *Allium sativum* before incubation

Pairwise Comparisons of Experiment				
Sample 1-Sample 2	Test Statistic	Std. Error	Std. Test Statistic	Sig.
Potency 8CH -Potency 5CH	2.500	7.164	0.349	0.727
Potency 8CH -Potency 13CH	-6.500	7.164	-0.907	0.364
Potency 8CH -Potency 21CH	-9.450	6.859	-1.378	0.168
Potency 8CH -Potency 3CH	11.700	8.272	1.414	0.157
Potency 8CH -Broth only	22.700	7.164	3.169	**0.002**
Potency 8CH -B & C	23.617	6.859	3.443	**0.001**
Potency 8CH -Tincture 1:10	30.700	7.598	4.040	**0.000**
Potency 5CH -Potency 13CH	-4.000	7.164	-0.558	0.577
Potency 5CH -Potency 21CH	-6.950	6.859	-1.013	0.311
Potency 5CH -Potency 3CH	9.200	8.272	1.112	0.266
Potency 5CH -Broth only	20.200	7.164	2.820	**0.005**
Potency 5CH -B & C	21.117	6.859	3.079	**0.002**
Potency 5CH -Tincture 1:10	28.200	7.598	3.711	**0.000**
Potency 13CH -Potency 21CH	-2.950	6.859	-0.430	0.667
Potency 13CH -Potency 3CH	5.200	8.272	0.629	0.530
Potency 13CH -Broth only	16.200	7.164	2.261	**0.024**
Potency 13CH -B & C	17.117	6.859	2.496	**0.013**
Potency 13CH -Tincture 1:10	24.200	7.598	3.185	**0.001**
Potency 21CH -Potency 3CH	2.250	8.009	0.281	0.779
Potency 21CH -Broth only	13.250	6.859	1.932	0.053
Potency 21CH -B & C	14.167	6.540	2.166	**0.030**
Potency 21CH -Tincture 1:10	21.250	7.312	2.906	**0.004**
Potency 3CH -Broth only	11.000	8.272	1.330	0.184
Potency 3CH -B & C	11.917	8.009	1.488	0.137
Potency 3CH -Tincture 1:10	19.000	8.651	2.196	**0.028**
Broth only -B & C	-0.917	6.859	-0.134	0.894

Broth only -Tincture 1:10	-8.000	7.598	-1.053	0.292
B & C -Tincture 1:10	-7.083	7.312	-0.969	0.333

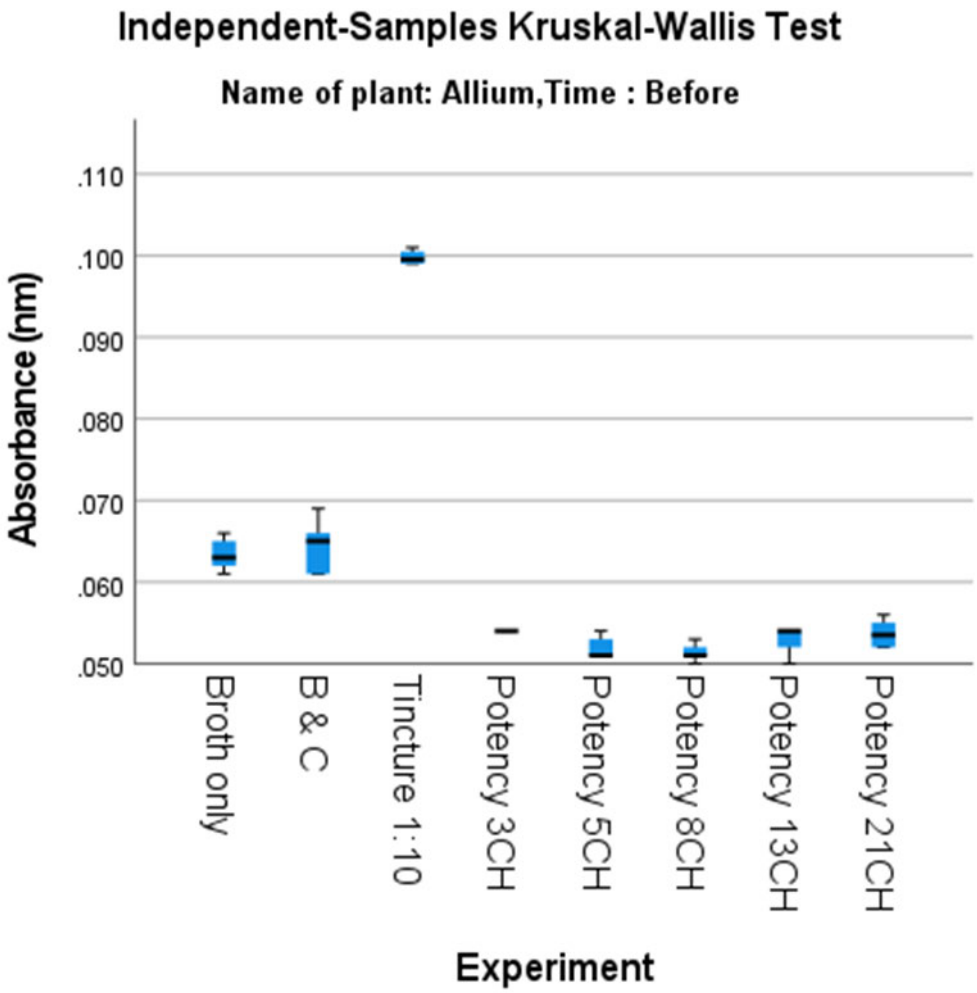

Figure 4.6: **Pairwise comparison of *Allium sativum* before incubation.**

Table 4.8: Pairwise comparison of *Allium sativum* after incubation

Pairwise Comparisons of Experiment				
Sample 1-Sample 2	Test Statistic	Std. Error	Std. Test Statistic	Sig.
Broth only -Potency 3CH	-12.900	8.122	-1.588	0.112
Broth only -Potency 21CH	-14.383	7.776	-1.850	0.064
Broth only -Tincture 1:10	-16.050	7.776	-2.064	**0.039**
Broth only -Potency 8CH	-16.700	8.122	-2.056	**0.040**
Broth only -Potency 5CH	-18.400	8.122	-2.265	**0.023**
Broth only -Potency 13CH	-18.800	7.776	-2.418	**0.016**
Broth only -B & C	-33.967	7.776	-4.368	**0.000**
Potency 3CH -Potency 21CH	-1.483	7.776	-0.191	0.849
Potency 3CH -Tincture 1:10	3.150	7.776	0.405	0.685
Potency 3CH -Potency 8CH	-3.800	8.122	-0.468	0.640
Potency 3CH -Potency 5CH	-5.500	8.122	-0.677	0.498
Potency 3CH -Potency 13CH	-5.900	7.776	-0.759	0.448
Potency 3CH -B & C	21.067	7.776	2.709	**0.007**
Potency 21CH -Tincture 1:10	1.667	7.414	0.225	0.822
Potency 21CH -Potency 8CH	2.317	7.776	0.298	0.766
Potency 21CH -Potency 5CH	4.017	7.776	0.517	0.605
Potency 21CH -Potency 13CH	4.417	7.414	0.596	0.551
Potency 21CH -B & C	19.583	7.414	2.641	**0.008**
Tincture 1:10 -Potency 8CH	-0.650	7.776	-0.084	0.933
Tincture 1:10 -Potency 5CH	-2.350	7.776	-0.302	0.762
Tincture 1:10 -Potency 13CH	-2.750	7.414	-0.371	0.711
Tincture 1:10 -B & C	17.917	7.414	2.416	**0.016**
Potency 8CH -Potency 5CH	1.700	8.122	0.209	0.834
Potency 8CH -Potency 13CH	-2.100	7.776	-0.270	0.787
Potency 8CH -B & C	17.267	7.776	2.220	**0.026**
Potency 5CH -Potency 13CH	-0.400	7.776	-0.051	0.959
Potency 5CH -B & C	15.567	7.776	2.002	**0.045**
Potency 13CH -B & C	15.167	7.414	2.046	**0.041**

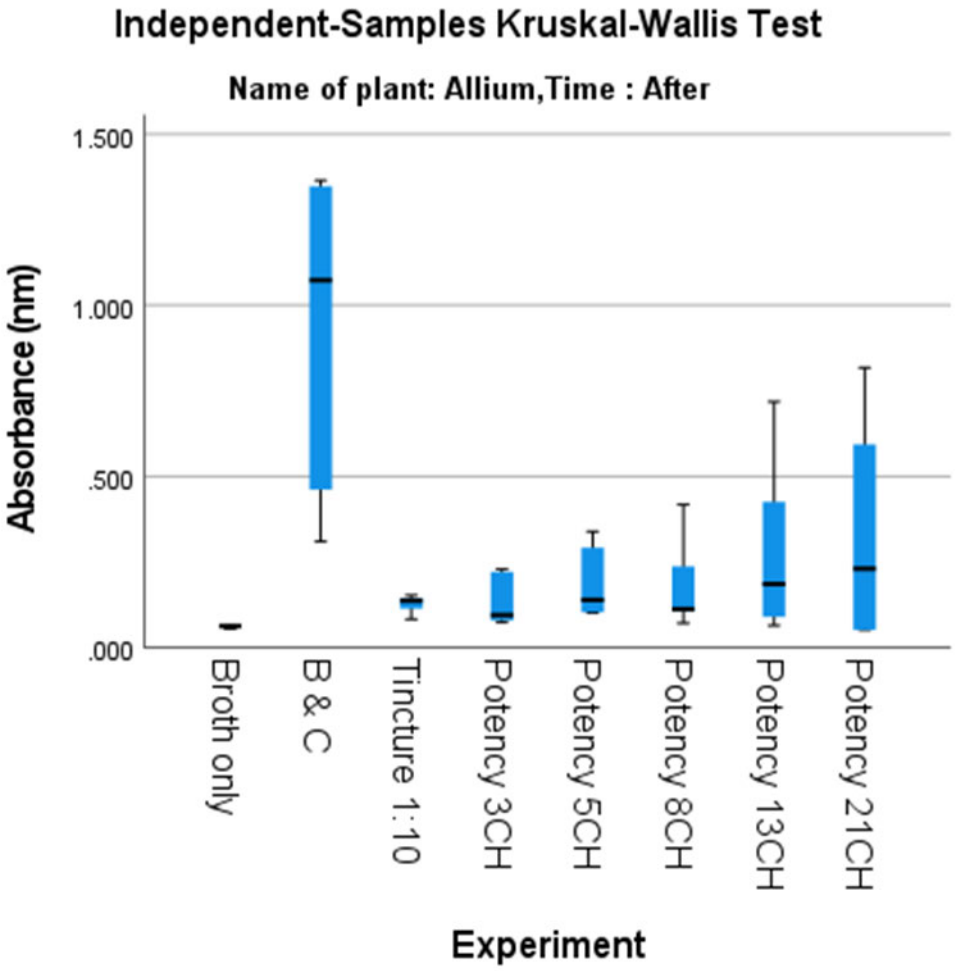

Figure 4.7: The pairwise comparison of *Allium sativum* after incubation.

After incubation the: 1:10 mother tincture (0,016), 3CH (0,007), 5CH (0,045), 8CH (0,026) 13CH (0,041) and 21CH (0,008) were statistically significant when compared to the broth and Candida (negative control).

In ***Tables 4.9*** and ***4.10*** the pairwise comparison of the difference pre-incubation and post-incubation of both *Allium sativum* and *Tulbaghia violacea* are displayed. ***Figures 4.8*** and ***4.9*** are visual representations of the analysis.

Table 4.9: Pairwise comparison of the difference of *Tulbaghia violacea* before and after incubation

Pairwise Comparisons of Experiment				
Sample 1-Sample 2	Test Statistic	Std. Error	Std. Test Statistic	Sig.
Broth only -Tincture 1:10	-1.267	8.116	-0.156	0.876
Broth only -Potency 5CH	-13.350	8.116	-1.645	0.100
Broth only -Potency 8CH	-17.933	8.116	-2.210	**0.027**
Broth only -Potency 13CH	-18.183	8.116	-2.240	**0.025**
Broth only -Potency 3CH	-19.683	8.116	-2.425	**0.015**
Broth only -Potency 21CH	-21.300	8.477	-2.513	**0.012**
Broth only -B & C	-31.433	8.116	-3.873	**0.000**
Tincture 1:10 -Potency 5CH	-12.083	7.739	-1.561	0.118
Tincture 1:10 -Potency 8CH	-16.667	7.739	-2.154	**0.031**
Tincture 1:10 -Potency 13CH	-16.917	7.739	-2.186	**0.029**
Tincture 1:10 -Potency 3CH	-18.417	7.739	-2.380	**0.017**
Tincture 1:10 -Potency 21CH	-20.033	8.116	-2.468	**0.014**
Tincture 1:10 -B & C	30.167	7.739	3.898	**0.000**
Potency 5CH -Potency 8CH	-4.583	7.739	-0.592	0.554
Potency 5CH -Potency 13CH	-4.833	7.739	-0.625	0.532
Potency 5CH -Potency 3CH	6.333	7.739	0.818	0.413
Potency 5CH -Potency 21CH	-7.950	8.116	-0.980	0.327
Potency 5CH -B & C	18.083	7.739	2.337	**0.019**
Potency 8CH -Potency 13CH	-0.250	7.739	-0.032	0.974
Potency 8CH -Potency 3CH	1.750	7.739	0.226	0.821
Potency 8CH -Potency 21CH	-3.367	8.116	-0.415	0,678
Potency 8CH -B & C	13.500	7.739	1.745	0.081
Potency 13CH -Potency 3CH	1.500	7.739	0.194	0.846
Potency 13CH -Potency 21CH	-3.117	8.116	-0.384	0.701
Potency 13CH -B & C	13.250	7.739	1.712	0.087
Potency 3CH -Potency 21CH	-1.617	8.116	-0.199	0.842
Potency 3CH -B & C	11.750	7.739	1.518	0.129
Potency 21CH -B & C	10.133	8.116	1.249	0.212

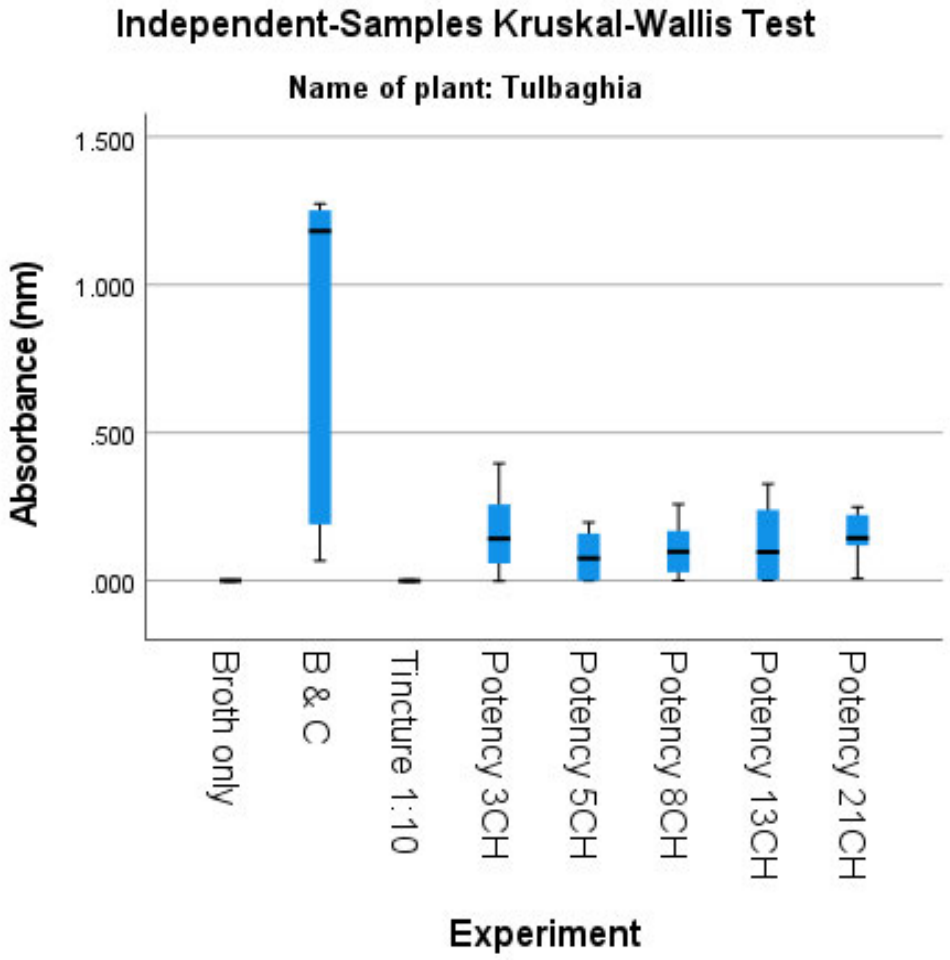

Figure 4.8: Pairwise comparison for the difference of *Tulbaghia violacea* before and after incubation

According to the comparison of the differences before and after incubation of *Tulbaghia violacea* in ***Table 4.9*** and ***Figure 4.8***, the 1:10 mother tincture (0.000) and 5CH (0.019) was statistically significant when compared to the control (broth and *Candida*). The inhibitory effect of the 1:10 mother tincture was statistically significant when compared to 3CH (0.017), 8CH (0.031), 13CH (0.029) and 21CH (0.014) at $p < 0.05$ This indicates that *Tulbaghia violacea* is more inhibitory in crude form.

Table 4.10: Pairwise comparison of the difference of *Allium sativum* before and after incubation

Pairwise Comparisons of Experiment				
Sample 1-Sample 2	Test Statistic	Std. Error	Std. Test Statistic	Sig.
Broth only -Tincture 1:10	-10.000	7.583	-1.319	0.187
Broth only -Potency 3CH	-15.633	7.953	-1.966	**0.049**
Broth only -Potency 21CH	-18.167	7.583	-2.396	**0.017**
Broth only -Potency 8CH	-20.833	7.953	-2.620	**0.009**
Broth only -Potency 13CH	-21.333	7.583	-2.813	**0.005**
Broth only -Potency 5CH	-22.033	7.953	-2.770	**0.006**
Broth only -B & C	-35.500	7.583	-4.682	**0.000**
Tincture 1:10 -Potency 3CH	-5.633	7.953	-0.708	0.479
Tincture 1:10 -Potency 21CH	-8.167	7.583	-1.077	0.281
Tincture 1:10 -Potency 8CH	-10.833	7.953	-1.362	0.173
Tincture 1:10 -Potency 13CH	-11.333	7.583	-1.495	0.135
Tincture 1:10 -Potency 5CH	-12.033	7.953	-1.513	0.130
Tincture 1:10 -B & C	25.500	7.583	3.363	**0.001**
Potency 3CH -Potency 21CH	-2.533	7.953	-0.319	0.750
Potency 3CH -Potency 8CH	-5.200	8.307	-0.626	0.531
Potency 3CH -Potency 13CH	-5.700	7.953	-0.717	0.474
Potency 3CH -Potency 5CH	-6.400	8.307	-0.770	0.441
Potency 3CH -B & C	19.867	7.953	2.498	**0.012**
Potency 21CH -Potency 8CH	2.667	7.953	0.335	0.737
Potency 21CH -Potency 13CH	3.167	7.583	0.418	0.676
Potency 21CH -Potency 5CH	3.867	7.953	0.486	0.627
Potency 21CH -B & C	17.333	7.583	2.286	**0.022**
Potency 8CH -Potency 13CH	-0.500	7.953	-0.063	0.950
Potency 8CH -Potency 5CH	1.200	8.307	0.144	0.885
Potency 8CH -B & C	14.667	7.953	1.844	0.065
Potency 13CH -Potency 5CH	0.700	7.953	0.088	0.930
Potency 13CH -B & C	14.167	7.583	1.868	0.062
Potency 5CH -B & C	13.467	7.953	1.693	0.090

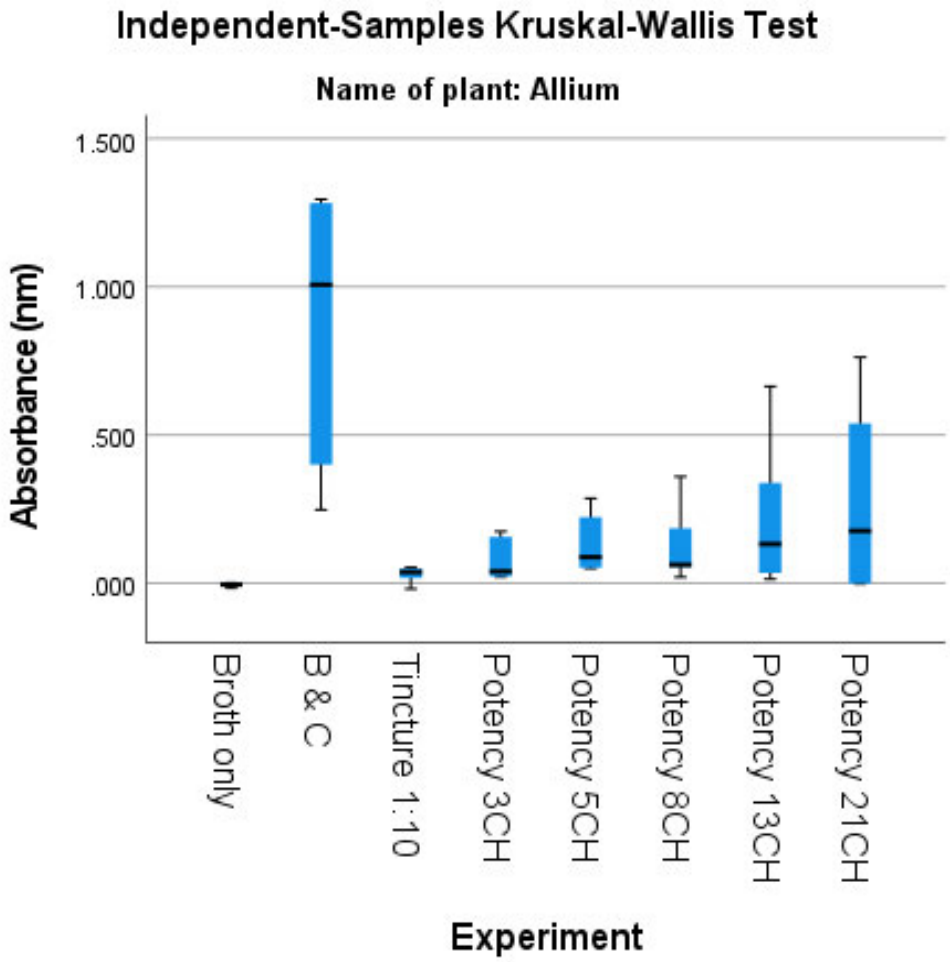

Figure 4.9: Pairwise comparison for the difference of *Allium sativum* before and after incubation.

According to the comparison of the differences before and after incubation of *Allium sativum* in ***Table 4.10*** and ***Figure 4.9***, the 1:10 mother tincture (0.001), 3CH (0.012) and 21CH (0.022) were statistically significant when compared to the control (broth and *Candida*) at $p < 0.05$.

CHAPTER 5: DISCUSSION

5.1. INTRODUCTION

The purpose of this study was to investigate the antifungal properties of *Tulbaghia violacea* on *Candida albicans* and compare this to the antifungal activity of *Allium sativum*. This study also aimed to investigate an indigenous plant that is extensively used in African traditional medicine in a homoeopathic context. This Chapter aims to present a discussion of the findings presented in Chapter Four, as related to the review of literature presented in Chapter Two, and a critical analysis of the microbiological assays used in the methodology as a mode of scientific investigation of the two medicinal plants.

5.2. DATA ANALYSIS OF THE MICROTITRE ASSAY

The data presented in Chapter Four reflects the findings the microtitre assay which was used as an experiment to determine the antifungal potential of *Tulbaghia violacea*. A respective selection of *Allium sativum* mother tincture and homoeopathic potencies were used as a point of reference for comparison. The results of this study, as shown in Chapter Four, demonstrated that *Tulbaghia violacea* and *Allium sativum* 1:10 mother tincture and homoeopathic potencies all contain varying degrees of antifungal inhibition when compared to the negative control.

The samples representing the negative controls (containing the broth culture only and no test substance) had the highest mean (median) absorbance at 0.992 (1.247) – *Tulbaghia violacea* and 0.938 (1.073) – *Allium sativum*, This provides an insight of the growth rate of *Candida albicans* when left untreated. Which indicates that the research methodology employed in this study provides suitable conditions for antifungal growth and provided uninhibited data. Based on the mean (median) differences presented in ***Tables 4.1*** for *Tulbaghia violacea* and ***4.2*** for *Allium sativum*, it can be deduced that the *Candida albicans* growth rate was inhibited by both test groups. ***Figures 4.1*** and ***4.2*** are visual representations of the data analysis presented in ***Tables 4.1*** and ***4.2.***

5.3. THE DISC DIFFUSION AND WELL DIFFUSION ASSAYS

The well diffusion assay was conducted as a secondary method of evaluation of the antifungal potential of *Allium sativum and Tulbaghia violacea*. However, the well diffusion assay did not produce significant zones of inhibition, therefore the disc diffusion assay was also conducted. Unfortunately, this methodology also did not produce any clear zones of inhibition. McGaw, Jäger and van Staden (2000), Dummer (2003) and Invernizzi (2002) also reported no antimicrobial activity when using the disc diffusion assay.

5.3.1. Modifications to the methodology

Three repetitions of the disc and well diffusion assay was conducted with slight modifications to variables that could have prevented clear zones of inhibition from forming:

- Manufacturing fresh agar and using the freshly solidified agar on the same day of manufacture, it was suspected that preparing agar and storing it in a fridge before usage could limit the formation of zones of inhibition.
- Incubation period, a longer incubation period of 36 hours was also investigated.
- Optimum incubation temperature was modified, 33°C, 35°C, 37°C was investigated.
- Drying time for the disc method was modified, 3, 16 and 24 hours was investigated.
- Not allowing the discs to dry.
- Multiple impregnations of the discs, as this allowed for more test substance to penetrate the discs, as suggested by Dummer (2003).
- A new batch of starting culture was produced on the day of the investigation.

Possible reasons as to why the well and disc diffusion assay did not produce any significant zones of inhibition:

- The disc and well diffusion assay may not have been an optimal methodology to evaluate the antifungal potential of the homoeopathic potencies. Homoeopathic potencies work on an energetic level rather than a physical. The absence of antimicrobial activity using the well and disc diffusion assay was also reported by Invernizzi (2002) and Dummer (2003).
- The test substances were too insoluble to diffuse out of the well.
- The ethanolic extraction method may not have allowed for adequate extraction of allicin and marasmicin as ethanol may not have stabilised the volatile compounds. Ranglová, Krejčová and Kubec (2015) reported that *Tulbaghia violacea* homogenized in water at low temperatures retains antimicrobial activity when compared to ethanol.
- The filter paper was not able adequately absorb the test substances in the disc diffusion assay, as noted by Invernizzi (2002).
- The active volatile compounds were not present in a high enough concentration to produce antifungal inhibition. Similarly, Dummer (2003), investigated a plant that contains volatile and reported no inhibition in the disc diffusion assay. A recommendation from the study is to test the essential oils of plants that contain volatile oils, which could not be done in this study.
- The content of the active compounds may have decreased significantly from the time of conducting the Microtitre assay. Ranglová, Krejčová and Kubec (2015) investigated if the storage and processing of *Tulbaghia violacea* influences the antimicrobial activity of the plant. The study concluded that heat, the use of organic solvents and storage would significantly inhibit the formation of marasmicin due to the inactivation of the enzyme C-S lyase.
- *In vitro* methodologies may not be appropriate to evaluate the antimicrobial potential of mother tinctures and homoeopathic potencies. Invernizzi (2002) and Dummer (2003) conducted similar studies and reported similar findings and recommended *in vivo* testing.
- The active compounds, allicin and marasmicin, could have possibly dissipated before the mother tinctures were potentized and diluted into the homoeopathic

potencies. During this study the ethanolic extracts were unable to be released from W. Last for roughly three months due to the payment protocol of DUT. The content of the active compounds may have decreased significantly from the time of production of the mother tinctures and remedies to the point at which the experiment was conducted. Ranglová, Krejčová and Kubec (2015) found that storage and high temperatures significantly decreases volatile oil content.

5.4. CLINICAL SIGNIFICANCE OF THE STUDY

Traditional medicine plants have come under intense focus as there is an ever growing need to find new or complementary treatments for the numerous diseases that face mankind. Research conducted by McGaw, Jäger and van Staden (2000), Motsei *et al.* (2003), Eloff *et al.* (2008), Oyedeji-Amusa, Sadgrove and van Wyk (2021) highlighted the importance of investigating traditional medicinal plants. This study aimed to investigate an alternative and natural treatment for *Candida albicans* infections.

The findings of the study indicated that *Tulbaghia violacea* does demonstrate antifungal activity *in vivo*, this is in line with other studies that aimed to investigate the application of *Tulbaghia violacea* on opportunistic pathogenic fungal infections (Thamburan *et al.* 2006; Ncube *et al.* 2011; Somai and Belewa 2011).

In an indigenous herbal or homoeopathic context, *Tulbaghia violacea* (1:10) mother tincture could be prescribed to patients that suffer from candidiasis. The tincture may be used for topical applications in the form of a cream or as a mouth wash in the treatment of oral candidiasis. *Tulbaghia violacea* 3CH displayed the most antifungal inhibition from the homoeopathic potencies, and therefore may be used as an adjunct when treating candidiasis. Higher potencies are not recommended as they work on a biophysical rather than physical level.

This study also verified the antifungal activity of *Allium sativum* which is in line with the findings of Lonkala and Reddy (2019), Enejiyon *et al.* (2020) and Magryś (2021). This provides justification for the use of *Allium sativum* in an herbal and homoeopathic

context. A combination of garlic and wild garlic may prove to be the most effective against candidiasis.

From a conservation perspective Aremu and van Staden (2013) and Oyedeji-Amusa, Sadgrove and van Wyk (2021) highlighted the importance of finding sustainable alternatives to reduce overharvesting of natural resources. Manufacturing ethanolic extracts from traditional medicinal plants provides a sustainable alternative to reduce the issue of overharvesting of medicinal plants, as large volume of extracts can be produced from a small amount of raw material. Herbalists often use water in the production of the traditional medicine, which has a short shelf life and would require harvesting of new raw material for each patient. The use of ethanolic extracts, which are preserved for several years, would decrease the strain on natural resources.

5.5. CONCLUSION

Candida albicans is regarded as one of the most prevalent pathogenic fungal infection that occurs in humans. Thrush has a significant negative impact on the quality of life as it generally occurs secondary to other pre-existing conditions such as diabetes and HIV/AIDS. The findings of this study show that there is a potential for *Tulbaghia violacea* to be used as a natural treatment for thrush infections in humans in crude dosage and in low homoeopathic potency. Methods of processing the plant to ensure the main biologically active compounds are effectively utilized need to be investigated.

CHAPTER 6: CONCLUSIONS AND RECOMMENDATIONS

6.1. CONCLUSIONS

While conducting this research project two important questions became apparent:

1. What role do appreciable concentrations of phytochemicals play in the production/ extraction of plant mother tinctures and subsequent potencies of homoeopathic medicines?
2. What proportion of the indigenous flora of Southern Africa is underutilized for medicinal purposes because of their complex phytochemistry, which are either not understood or unable to be successfully extracted and utilized?

In the case of *Allium sativum* and *Tulbaghia violacea*, the main active phytochemicals that are responsible for the medicinal properties are volatile oils. These volatile oils give the plants their medicinal properties in crude form and can only be extracted by using hydro distillation. In this research, ethanolic mother tinctures were manufactured from fresh plants, in the process of making these tinctures, the plant material was chopped, this resulted in the enzymatic reaction that is responsible for the formation of volatile oils, allicin and marasmicin. However, these oils are not stable in ethanol, which means that there were little/ none of these volatile oils present in the final mother tincture and homoeopathic potencies.

An outcome of this study was to establish uniform results between two microbiological methodologies to establish consistency, as this further validates the use of these medicinal plants scientifically. In this study the microtitre assay produced visual and statistically significant results, while the disc/well diffusion did not, this inconsistency can be explained by the volatile nature of the biologically active compounds contained within these plants. One batch of test substances was produced and used on both assays, the microtitre assay was conducted first and the disc/well assay was conducted several weeks later. The samples may have degraded in the delay between each assay.

Although homoeopathic remedies are purported to work on a biophysical ('energetic') level, the consequences of these volatile oils being absent from base substances due to inadequate methods of extraction, even before the distinctly homoeopathic pharmaceutical processes, are unknown. In-depth research of such biochemicals and their effect on subsequently produced homoeopathic remedies is urgently required.

6.2. RECOMMENDATIONS

Upon conclusion of the study, and after due reflection upon the aim and objectives of the study, the results achieved and described in the preceding Chapters, and the limitations identified through implementation of the study, the following recommendations are made for future research in this and similar microbiological homoeopathic studies:

- Request a homoeopathic pharmaceutical company to produce the ethanolic extracts and homoeopathic potencies. In this study the ethanolic extracts were manufactured by W. Last cc, and the homoeopathic potencies by the researcher. A time delay of roughly three months between production of the ethanolic extracts and the homoeopathic potencies was experienced. This may be eliminated by manufacturing both the ethanolic extracts and potencies by a company or researcher. Additionally, it is highly recommended that any time delays be avoided when working with plants that contain volatile oils.
- Test a wider range of microbes, a limitation of this study was that antifungal potential would be established on only one type of fungi. However, studies have shown that *Tulbaghia violacea* displays inhibitory effects on a wide range of fungi.
- Use Yeast Peptone Dextrose as the medium of growth. In this study, sabouraud dextrose broth and agar were used as growth media. Using different growth media provides an insight into the growth rate of *Candida albicans* under different nutritional conditions.
- Test ethanolic extracts and homoeopathic potencies to determine the presence or absence of volatile compounds by means of high-pressure liquid chromatography.

- Investigate the effect of *Tulbaghia violacea in vivo* as this study was limited to *in vitro* applications only. *In vivo* testing is more complex and addresses safety, toxicity, and efficacy in a living organism and are considered more reliable.
- The plants should be grown from seed. This allows for a greater degree of control over factors that could not be accommodated in this study, such as; age of the plants, environmental conditions, sunlight, soil and nutrient conditions, the use of pesticides, herbicides or growth regulators (in the case of garlic which was commercially grown) and type of seed. These factors play a role in the development of active phytochemicals that are responsible for the medicinal properties of these plants.
- When conducting multiple experiments/ assays; produce one large batch of test substances and decant this into multiple smaller batches. One batch should be used per experiment or assay as this prevents cross contamination or degradation of the test samples. In this study, one batch of test substances were produced to prevent batch variation, however, the same bottles of test substances were used for all experimentation.
- Investigate synthetic methods of producing allicin and marasmicin, this will greatly reduce the demand for raw materials in the form of harvesting wild plants, which eventually loads to the destruction of the indigenous plants.
- The inclusion of a positive antifungal control, such as Fluconazole or Nystatin, would further validate the methodology employed and produce positive data to compare the test data to.
- The use of the alcohol used to manufacture the test substances and as control is high recommended, this will determine if the alcohol produced any inhibition. In this study 8% was used, to prevent the inhibitory effect of the alcohol. However, on retrospective review of the study, it is currently unknown if the alcohol did contribute to the inhibitory effect of the test samples.

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APPENDICES

Appendix 1: Permission application letter to use the laminar flow room

2 November 2019

Dr Maharaj

Head of Department: Homoeopathy

Request for Permission to Conduct Research

Dear Dr Maharaj,

My name is Wesley Reddy, a Master of health sciences student at the Durban University of Technology. The research I wish to conduct for my Masters dissertation involves The anti-microbial effectiveness of *Tulbaghia violacea* and *Allium sativum* on the *in vitro* growth of *Candida albicans*.

I am hereby seeking your consent to make use of the Laminar flow room to prepare the remedies I require.
I have provided you with a copy of my proposal which includes copies of the data collection tools and consent and/ or assent forms to be used in the research process.

If you require any further information, please do not hesitate to contact me: 074 568 5328, rwesley395@gmail.com.

Thank you for your time and consideration in this matter.

Yours sincerely,

Wesley Reddy
Durban University of Technology

Appendix 2: Permission application letter to use Microbiology laboratory

2 November 2019

Prof. FM Swalaha

Head of Department: Biotechnology

Request for Permission to Conduct Research

Dear Professor, Swalaha,

My name is Wesley Reddy, a Master of health sciences student at the Durban University of Technology. The research I wish to conduct for my Masters dissertation involves: The anti-microbial effectiveness of *Tulbaghia violacea* and *Allium sativum* on the *in vitro* growth of *Candida albicans.*

I am hereby seeking your consent to make use of the Microbiology laboratory at the Department of Biotechnology and Food Technology and its resources to conduct my research.

I have provided you with a copy of my proposal which includes copies of the data collection tools and consent

If you require any further information, please do not hesitate to contact me: 074 568 5328, rwesley395@gmail.com.

Thank you for your time and consideration in this matter.

Yours sincerely,

Wesley Reddy

Durban University of Technology

Appendix 3: Methodology used by W. Last to prepare the ethanolic plant extracts using the German Homoeopathic Pharmacopoeia Method 2A (modified)

The ethanolic extracts of *Tulbaghia violacea* and *Allium sativum* was prepared by W. Last according to method 2A of the German Homoeopathic Pharmacopoeia. Method 2A is used on plants that contain volatile oils and ensures that the final extract had a concentration of 1:2 (Benyunes 2005).

The procedure to manufacture 1300 g of *Tulbaghia violacea* 1:2 was as follows:

- The fresh plant material was washed and cleaned to remove any dirt or impurities. The plants were then finely chopped to less than 1 cm.
- A mass of 780 g *Tulbaghia violacea* was weighed out.
- For every 3 parts of plant material, 2 parts of 86% ethanol was added, 520 g of ethanol was added to *Tulbaghia violacea* and mixed well.
- A deviation from the German Homoeopathic Pharmacopeia was used to boost the extraction process. A 1000 W Ultrasonic transducer with a submersible sonotrope was placed in the extract.
- The extract was left to stand for eight days in a sealed bottle in a dark cupboard. Once every day the bottle was agitated to ensure even extraction.
- After eight days, the mixture was pressed through a mutton cloth to remove the solid plant material. The filtrate was then filtered again with 150 mm sterile Whatman filter paper at 5 µm (paper grade 597).
- Due to the loss of volume from the filtration process; the extract was measured after the filtration process and topped up with 86% ethanol to yield final volume of 1300 g *Tulbaghia violacea.*

The procedure to manufacture 2000 g of *Allium sativum* 1:2 was as follows:

- The fresh plant material was washed and cleaned to remove any dirt or impurities. The plants were then finely chopped to less than 1 cm.
- A mass of 1200 g *Allium sativum* was weighed out.
- For every 3 parts of plant material, 2 parts of 86% ethanol was added, 800 g of ethanol was added to *Allium sativum* and mixed well.
- A deviation from the German Homoeopathic Pharmacopeia was used to boost the extraction process. A 1000 W Ultrasonic transducer with a submersible sonotrope was placed in the extract.
- The extract was left to stand for eight days in a sealed bottle in a dark cupboard. Once every day the bottle was agitated to ensure even extraction.
- After eight days, the mixture was pressed through a mutton cloth to remove the solid plant material. The filtrate was then filtered again with 150 mm sterile Whatman filter paper at 5 µm (paper grade 597).
- Due to the loss of volume from the filtration process; the extract was measured after the filtration process and topped up with 86% ethanol to yield a final volume of 2000 g of *Allium sativum*.

Appendix 4: Liquid preparations made from ethanolic plant extracts using the German Homoeopathic Pharmacopoeia method 1A

Preparations made by method 1A are liquid preparations produced from the ethanolic extracts made by Method 2A. A modified version of method 1A was used to produce a 1:10 mother tincture which was then used to manufacture the Homoeopathic potencies of *Allium sativum* and *Tulbaghia violacea* respectively (Benyunes 2005).

To prepare 30 ml *Tulbaghia violacea* and *Allium sativum* mother tinctures (1:10) from the ethanolic extracts (1:2):
By means of a 10 ml pipette, 2 parts (6 ml) of the *Tulbaghia violacea* or *Allium sativum* was added to 8 parts (24 ml) of deionized water. No succussions.

To prepare 3 ml of *Tulbaghia violacea / Allium sativum* 1CH by means of dilution and succussion:
By means of micropipette 1 part (0.03 ml) *Tulbaghia violacea / Allium sativum* mother tincture was added to 99 parts (2.97 ml) of 8% ethanol in a 5 ml bottle. Succuss 100 times by hand and labelled as 1CH.

To prepare 3 ml of *Tulbaghia violacea / Allium sativum* 2CH by means of dilution and succussion:
By means of micropipette, 1 part (0.03 ml) of 1CH was added to 99 parts (2.97 ml) of 8% ethanol in a 5 ml bottle. Succuss 100 times and label as 2CH.

To prepare 25 ml of *Tulbaghia violacea / Allium sativum* 3CH by means of dilution and succussion
By means of micropipette,1 part (0.25 ml) of 2CH was added to 99 parts (24.75 ml) of 8% ethanol in a 30 ml bottle. Succuss 100 times and label as 2CH.

Subsequent dilutions were made using the steps above until the desired potencies were reached. 3CH, 5CH, 8CH, 13CH and 21CH were prepared in 25 ml volumes and all intermediate potencies were prepared in 3 ml volumes.

Appendix 5: Raw data

Appendix 5A: The absorbance readings of *Tulbaghia violacea* after trial one

***Tulbaghia violacea* absorbance before incubation (nm)**							
Broth only	B.C	1:10	3CH	5CH	8CH	13CH	21CH
0.069	0.066	0.071	0.054	0.052	0.053	0.053	0.052
0.074	0.067	0.071	0.054	0.054	0.054	0.055	0.054
0.093	0.072	0.089	0.068	0.072	0.058	0.061	0.065
***Tulbaghia violacea* absorbance after 24 hours of incubation (nm)**							
Broth only	B.C	1:10	3CH	5CH	8CH	13CH	21CH
0.069	1.317	0.072	0.311	0.249	0.221	0.215	0.196
0.071	1.311	0.070	0.175	0.213	0.219	0.294	0.276
0.079	1.345	0.086	0.464	0.128	0.316	0.388	0.314
***Tulbaghia violacea* absorbance difference**							
Broth only	B.C	1:10	3CH	5CH	8CH	13CH	21CH
0	1.251	0.001	0.257	0.197	0.168	0.162	0.144
0.003	1.244	0.001	0.121	0.159	0.165	0.239	0.222
0.014	1.273	0.003	0.396	0.056	0.258	0.327	0.249

Appendix 5B: The absorbance readings of *Allium sativum* after trial one

***Allium sativum* absorbance before incubation (nm)**							
Broth only	B.C	1:10	3CH	5CH	8CH	13CH	21CH
0.085	0.066	0.090	0.065	0.068	0.058	0.087	0.055
0.066	0.065	0.100	0.054	0.053	0.052	0.054	0.056
0.063	0.069	0.097	0.054	0.054	0.053	0.054	0.054
***Allium sativum* absorbance after 24 hours of incubation (nm)**							
Broth only	B.C	1:10	3CH	5CH	8CH	13CH	21CH
0.069	1.337	0.139	0.221	0.291	0.417	0.425	0.817
0.059	1.347	0.142	0.228	0.339	0.237	0.260	0.401
0.055	1.364	0.114	1.074	0.914	0.891	0.718	0.593
***Allium sativum* absorbance differences**							
Broth only	B.C	1:10	3CH	5CH	8CH	13CH	21CH
0.016	1.271	0.049	0.156	0.223	0.359	0.338	0.762
0.007	1.282	0.042	0.174	0.286	0.185	0.206	0.345
0.008	1.295	0.017	1.02	0.86	0.838	0.664	0.539

Appendix 5C: The absorbance readings of *Tulbaghia violacea* after trial two

***Tulbaghia violacea* absorbance before incubation (nm)**							
Broth only	B.C	1:10	3CH	5CH	8CH	13CH	21CH
0.068	0.065	0.071	0.050	0.051	0.051	0.051	0.050
0.061	0.067	0.070	0.053	0.054	0.051	0.050	0.051
0.067	0.062	0.069	0.052	0.050	0.051	0.053	0.053
***Tulbaghia violacea* absorbance after 24 hours of incubation (nm)**							
Broth only	B.C	1:10	3CH	5CH	8CH	13CH	21CH
0.068	1.183	0.073	0.212	0.050	0.081	0.051	0.057
0.062	0.545	0.069	0.051	0.054	0.051	0.053	0.069
0.064	0.252	0.069	0.110	0.145	0.079	0.083	0.172
***Tulbaghia violacea* absorbance differences**							
Broth only	B.C	1:10	3CH	5CH	8CH	13CH	21CH
0	1.118	0.002	0.162	0.001	0.03	0	0.007
0.001	0.067	0.001	0.002	0	0	0.003	0.018
0.003	0.19	0	0.058	0.095	0.028	0.03	0.119

Appendix 5D: The absorbance readings of *Allium sativum* after trial two

***Allium sativum* absorbance before incubation (nm)**							
Broth only	B.C	1:10	3CH	5CH	8CH	13CH	21CH
0.061	0.061	0.099	0.052	0.051	0.050	0.050	0.052
0.065	0.061	0.099	0.056	0.051	0.051	0.052	0.052
0.062	0.065	0.101	0.054	0.051	0.051	0.054	0.053
***Allium sativum* absorbance after 24 hours of incubation (nm)**							
Broth only	B.C	1:10	3CH	5CH	8CH	13CH	21CH
0.062	0.309	0.152	0.073	0.103	0.112	0.064	0.051
0.062	0.461	0.133	0.079	0.139	0.071	0.111	0.059
0.062	0.808	0.082	0.094	0.101	0.106	0.089	0.051
***Allium sativum* absorbance differences**							
Broth only	B.C	1:10	3CH	5CH	8CH	13CH	21CH
0.001	0.248	0.053	0.021	0.052	0.062	0.014	0.001
0.003	0.4	0.034	0.023	0.088	0.02	0.059	0.007
0	0.743	0.019	0.04	0.05	0.055	0.035	0.002