Biocide development for hard surfaces using extracts and compounds from *Callistemon citrinus*

Mvududu Sandra M.

(R115475Y)

Thesis submitted to the Department of Biochemistry, Faculty of Science, University of Zimbabwe, in partial fulfilment of the Requirements for the Degree of Master of Science in Biotechnology

Supervisor: Professor S. Mukanganyama

June 2019
DECLARATION

I, Sandra Mutsa Mvududu, hereby declare that the experimental work described in this report was carried out in the Biomolecular Interactions Analyses Laboratory (BIA), in the Department of Biochemistry, at the University of Zimbabwe from September 2018 to May 2019. This is my original work and has not been reproduced from someone else’s work. The work has not been submitted previously to qualify for any other academic award. Where use has been made of other people’s work, it has been referenced in the text.

…………………………………. …………………………………….

Signature    Date
DEDICATION

I dedicate this project to my daughter; she has been with me throughout my journey in obtaining my Master’s degree. My loving, caring family and my husband for their endless support.
ACKNOWLEDGEMENTS

I would like to express my special thanks to my Supervisor, Professor S. Mukanganyama and for his guidance and support throughout the project. It was not any easy journey but he was able to motivate me and provide for my project to be a success. Ms W. Mozirandi was also there to assist and guide me.

I would like to also thank the Department of Chemistry and the Faculty of Science innovation Centre (FASIC) for providing me with reagents for my project. My BIA colleagues were also very supportive with special mention to Lydia and Clarence.

Lastly I would like to thank my family for their support and patience. My husband for being supportive and understanding.
ABSTRACT

The global is now focusing on the use of herbal products and among them are herbal disinfectants. Plants contain a variety of secondary metabolites which include phenolic compounds, tannins, terpenoids, alkaloids and flavonoids. *C. citrinus* is a plant native to Australia which have been found to have antimicrobial properties. Herbal products can be environmentally safe as they are biodegradable and prevent environmental contamination. The aims of the study where to use the extracts from *C. citrinus* leaves to make a disinfectant to be used for cleaning hard surfaces. The test microorganisms to determine the antimicrobial activity of the *C. citrinus* leaf extract were *S. aureus*, *E. coli* and *C. albicans*. The leaves were dried and ground into a coarse powder which was used for the extraction process. Both total extraction and serial exhaustive extraction were used to obtain extracts from the leaves. The antimicrobial activities of the extracts were determined using the microbroth dilution method. The MTT assay was used to determine the viable cells after incubation of the test microbes and the extracts. The MIC was determined for the most potent extracts where total inhibition was observed. The hexane, DCM, ethyl acetate and acetone extracts of *C. citrinus* were effective against *S. aureus*. The ethanol extract had the highest inhibitory activity against *C. albicans* and *E. coli* was resistant to all the extracts tested against it. The MIC for the hexane extract was 50 µg/ml and for the DCM extract it was 25 µg/ml against *S. aureus*. The ethyl acetate extract had an MIC of 100 µg/ml against *S. aureus*. The DCM extract was used to formulate the disinfectant and formulation 1 was prepared using sulphonic acid and sodium hydroxide as the base. Formulation 2 was prepared using glycerine as the base. The agar disk diffusion method was used to test the effectiveness of the disinfectants formulated. Formulation 1 and 2 activity was tested against *S. aureus*, *C. albicans* and *E. coli*. *S. aureus* and *C. albicans* were susceptible to both formulations, the base for formulation 1 had antimicrobial activity as ZOI were observed around the disks containing the base as the negative control. *E. coli* was resistant to both formulations.

Keywords: *Callistemon citrinus*, antimicrobial, biocide formulation, sulphonic acid, glycerine, *S. aureus*, *E. coli*, *C. albicans*
# Table of Contents

DECLARATION ........................................................................................................................................ ii
DEDICATION .......................................................................................................................................... iii
ACKNOWLEDGEMENTS ........................................................................................................................ iv
ABSTRACT ............................................................................................................................................... v
LIST OF FIGURES ................................................................................................................................... ix
LIST OF TABLES ..................................................................................................................................... xi
LIST OF APPENDICES .......................................................................................................................... xii
LIST OF ABBREVIATIONS .................................................................................................................... xiii

CHAPTER 1 ............................................................................................................................................... 1
  1 INTRODUCTION ................................................................................................................................ 1
    1.1 Background ..................................................................................................................................... 1
    1.2 Hard surface cleaning and disinfection ....................................................................................... 2
    1.3 Hospital acquired infections (HAIs) or nosocomial infections .................................................. 3
    1.4 Disinfectants and microbial resistance ....................................................................................... 4
    1.5 *Callistemon citrinus* (older name - *Callistemon lanceolatus*) .................................................. 5
      1.5.1 Classification ......................................................................................................................... 6
      1.5.2 *Callistemon citrinus* uses .................................................................................................... 6
      1.5.3 Pharmacological properties of *Callistemon citrinus* ........................................................... 7
    1.6 Biocides ....................................................................................................................................... 8
      1.6.1 Classification of biocides ......................................................................................................... 8
      1.6.2 Modes of action of disinfectants ............................................................................................ 9
      1.6.3 Factors affecting the effectiveness of a disinfectant ............................................................... 11
    1.7 Test microorganisms .................................................................................................................... 13
      1.7.1 *Staphylococcus aureus* ......................................................................................................... 13
      1.7.2 Virulence factors of *S. aureus* .............................................................................................. 14
    1.8 *Escherichia coli* 0157 .................................................................................................................... 15
      1.8.1 Epidemiology .......................................................................................................................... 17
    1.9 *Candida albicans* ........................................................................................................................ 18
      1.9.1 Epidemiology .......................................................................................................................... 19
    1.10 Plant extraction methods ............................................................................................................ 19
      1.10.1 Low or room temperature methods .................................................................................... 20
      1.10.2 High temperature extraction methods ................................................................................. 20
      1.10.3 Optional temperature methods ............................................................................................ 20
    1.11 Antimicrobial susceptibility tests ............................................................................................... 21
4.5 Biocide tests ..................................................................................................................50
  4.5.1 Formulation 1 .......................................................................................................51
  4.5.2 Formulation 2 .......................................................................................................53
  4.5.3 In- loco biocide tests .........................................................................................56

CHAPTER FIVE .....................................................................................................................58

5 Discussion ..........................................................................................................................58
  5.1 Plant extraction .........................................................................................................58
  5.2 Bioactivity of the extracts .......................................................................................60
  5.3 Biocide Formulations ..............................................................................................63
    5.3.1 Formulation 1 ......................................................................................................63
    5.3.2 Formulation 2 ......................................................................................................63
  5.4 Biocide tests: Agar disk diffusion method .................................................................64
    5.4.1 Formulation 1 ......................................................................................................64
    5.4.2 Formulation 2 ......................................................................................................65
    5.4.3 In loco biocide tests for Formulation 2 ...............................................................66

CHAPTER SIX ...................................................................................................................67

6 Conclusion .........................................................................................................................67
  6.2 Recommendations .....................................................................................................67

CHAPTER SEVEN ...............................................................................................................68

References .............................................................................................................................68

CHAPTER 8 ..........................................................................................................................75
**LIST OF FIGURES**

<table>
<thead>
<tr>
<th>Figure 1.1</th>
<th>Gram positive <em>S. aureus</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 1.2</td>
<td><em>E. coli</em> bacterium</td>
</tr>
<tr>
<td>Figure 1.3</td>
<td><em>Candida albicans</em></td>
</tr>
<tr>
<td>Figure 2.1</td>
<td>A well labelled microtiter plate</td>
</tr>
<tr>
<td>Figure 4.1</td>
<td>Dried <em>Callistemon citrinus</em> leaves</td>
</tr>
<tr>
<td>Figure 3.2</td>
<td>Extracts of <em>Callistemon citrinus</em> against <em>Staphylococcus aureus</em> NCTC 6571</td>
</tr>
<tr>
<td>Figure 4.3</td>
<td>Activity of the different extracts used to screen <em>S. aureus</em>.</td>
</tr>
<tr>
<td>Figure 4.4</td>
<td>The activity of the crude extracts against <em>E. coli 0157</em></td>
</tr>
<tr>
<td>Figure 4.5</td>
<td>The percentage inhibitions of the extracts used to screen <em>E. coli 0157</em>.</td>
</tr>
<tr>
<td>Figure 4.6</td>
<td>The bioactivity of the 9 extracts with miconazole as the standard drug</td>
</tr>
<tr>
<td>Figure 4.7</td>
<td>Activity of the crude extracts at 100 µg/ml.</td>
</tr>
<tr>
<td>Figure 4.8</td>
<td>MIC for the ethyl acetate extract against <em>S. aureus</em></td>
</tr>
<tr>
<td>Figure 4.9</td>
<td>Cultured agar plate from the microtitre plate used for MIC determination</td>
</tr>
<tr>
<td>Figure 4.10</td>
<td>MIC for the DCM extract against <em>S. aureus</em></td>
</tr>
<tr>
<td>Figure 4.11</td>
<td>The agar plates used to determine the MBC of the DCM extract against <em>S. aureus</em></td>
</tr>
<tr>
<td>Figure 4.12</td>
<td>Disinfectant base containing sulphonic acid and sodium hydroxide</td>
</tr>
<tr>
<td>Figure 4.13</td>
<td>Glycerine based disinfectant</td>
</tr>
</tbody>
</table>
Figure 4.14  Antimicrobial activity of Formulation 1

Figure 4.15  The Zone of inhibition of formulation 2 against *S. aureus*

Figure 4.16  Average Zone of inhibition of Formulation 2 against *C. albicans*

Figure 4.17  Antimicrobial activity of formulation 2

Figure 4.18  Average Zone of inhibition of Glycerine based disinfectant against *S. aureus*

Figure 4.19  Average Zone of inhibition of Glycerine based disinfectant against *C. albicans*

Figure 4.20  Agar plates cultured before and after application of the disinfectant
LIST OF TABLES

Table 1.1   Classification of *C. citrinus*

Table 1.2   Disinfectants and cell structures they affect

Table 1.3   Cell surface factors of *S. aureus*

Table 1.4   Cytolytic toxins

Table 4.1   The mass and percentage yield after the different extraction processes
LIST OF APPENDICES

Appendix 1: Effect of the standard drug on *E. coli* 0157

Appendix 2: Effect of standard drug on *S. aureus*

Appendix 3: Effect of the standard drug on *C. albicans*
# LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Acronym</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>AIDS</td>
<td>Acquired Immune Deficiency Syndrome</td>
</tr>
<tr>
<td>C. citrinus</td>
<td><em>Callistemon citrinus</em></td>
</tr>
<tr>
<td>C. albicans</td>
<td><em>Candida albicans</em></td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethylsulfoxide</td>
</tr>
<tr>
<td>E. coli</td>
<td><em>Escherichia coli</em></td>
</tr>
<tr>
<td>EPEC</td>
<td>Enteropathogenic <em>E. coli</em></td>
</tr>
<tr>
<td>EIEC</td>
<td>Entero invasive <em>E. coli</em></td>
</tr>
<tr>
<td>ETEC</td>
<td>Enterotoxigenic <em>E. coli</em></td>
</tr>
<tr>
<td>EHEC</td>
<td>Enterohemorrhagic <em>E. coli</em></td>
</tr>
<tr>
<td>HAI</td>
<td>Hospital acquired infection</td>
</tr>
<tr>
<td>HUS</td>
<td>Haemolytic uraemic syndrome</td>
</tr>
<tr>
<td>MRSA</td>
<td>Methicillin-resistant <em>Staphylococcus aureus</em></td>
</tr>
<tr>
<td>NB</td>
<td>Nutrient broth</td>
</tr>
<tr>
<td>SDB</td>
<td>Sabouraud dextrose broth</td>
</tr>
<tr>
<td>S. aureus</td>
<td><em>Staphylococcus aureus</em></td>
</tr>
<tr>
<td>ZOI</td>
<td>Zone of inhibition</td>
</tr>
</tbody>
</table>
CHAPTER 1

1 INTRODUCTION

1.1 Background

Plants contain useful chemical compounds which are of great medicinal importance (Das and Singh, 2012) and are sources of therapeutic agents (Cock, 2012). *Callistemon citrinus* is one of the plants with great medicinal and antimicrobial properties (Das and Singh, 2012). They contain secondary metabolites such as phenolic compounds, tannins, terpenoids, alkaloids and flavonoids. These phytochemicals can be extracted or isolated and used to develop antimicrobial agents (Cowan, 1999).

The genus *Callistemon* belongs to the family *Myrtaceae* and consists of 34 species and some of which have been introduced to other areas such as USA and Africa (Cock, 2012). The *Callistemon* flowers were used as a food source and the nectar was sucked out and used to make drinks (Cock, 2012). The leaves are used to cure infections such as the respiratory tract infections, this is due to the presence of terpenes in the leaves which are responsible for the efficacy in treatment of infections (Cock, 2012). Extracts of *Callistemon* species have been demonstrated to have antibacterial activities against a number of microorganisms (Cock, 2012).

*Callistemon citrinus* is commonly known as Red, Lemon and Crimson bottlebrush (Ahmed et al., 2015) and they is widely distributed in the wet tropics in Australia and tropical Asia (Ahmed et al., 2016). Its older name is *Callistemon lanceolatus*, *C. citrinus* has been reported to contain antimicrobial, herbicidal and relaxant properties (Ahmed et al., 2016), Ahmed et al., 2015). Various phytochemicals such as nitisinone, erythrodiol, betulinic acid, β-sitosterol-3-O-β-D-glucoside and taraxerol have been isolated from this plant (Ahmed
et al., 2016). *C. citrinus* was used as a medicine as it possesses anticough, antibronchitis and insecticidal effects (Netala et al., 2015). The oil of the plant have been used as an antibacterial and antifungal agent (Netala et al., 2015).

The leaves of *C. citrinus* have been investigated for their analgesic and antidiarrheal activities in mice (Ahmed et al., 2015). The methanol extract of the leaves was fed to the rats and analgesic and antidiarrheal activities were observed (Ahmed et al., 2015). The analgesic action was monitored by acetic acid-induced writhing method, mice model was prepared by injecting acetic acid to induce abdominal writhings (Ahmed et al., 2015). The methanol extract of the tested plant reduced the writhing as compared to the non-treated mice.

Antioxidant activity of the leaf extracts of the *Callistemon* species have been reported from previous investigations (Netala et al., 2015). The leaf extracts of *C. lanceolatus*, *C. comboynensis* and the methanolic extracts of the different parts of *C. citrinus* particularly the flowers, had active antioxidant properties (Netala et al., 2015). The aqueous leaf extracts of the plant have been reported to possess antifungal and antibacterial activity (Das and Singh, 2012).

1.2 Hard surface cleaning and disinfection

The hard surfaces become a reservoir for pathogens i.e. if they are contaminated and not thoroughly cleaned (Bouchra et al., 2017). In the hospital setup the external environment have been a source of health-care associated infections (HAIs) and the environment includes the air, food, water and surfaces (Bouchra et al., 2017). The contaminated surfaces lead to the transmission of nosocomial pathogens such as Methicillin resistant *Staphylococcus aureus*, Vancomycin resistant *Enterococcus, Pseudomonas, Acinetobacter* as well as some viruses (Bouchra et al., 2017).

A disinfectant is required to prevent the growth of microbes on materials and surfaces (Service, 2012). Proper disinfection procedures must be followed so as to minimize the risk
of infection in hospitals and other public areas. In hospital setups the environmental surfaces and surgical instruments are vehicles for infectious agents (Tibana et al., 2000).

1.3 Hospital acquired infections (HAIs) or nosocomial infections

HAIs have high social and economic impact (Lucia et al., 2010), they are the leading cause of death in patients as they cause emotional stress and reduces the quality of life (WHO, 2012). Many factors contribute to the infections such as prolonged stay of the patients in hospitals (WHO, 2012). The patient is exposed to a variety of microorganisms during their stay in the hospital (WHO, 2012). The factors which lead to the development of the nosocomial disease include the contact with the microbial agent. The microbial agent can either be bacteria, fungi or a virus (WHO, 2012). These are transmitted through various ways such as cross contamination with an infected patient or they can be acquired from other objects, surfaces and even staff members (WHO, 2012).

Most of these infections are caused by a number of organisms very common such as Staphylococcus aureus, coagulase-negative Staphylococci, Enterococci and Enterobacteriaceae (WHO, 2012, Kramer and Assadian, 2016). The patient’s immune status may also be a contributing factor to HAIs. The extremes of life such as infants and old people have decreased resistance to infection and those with chronic infection such as renal failure, diabetes mellitus and AIDS are also susceptible to opportunistic agents. Skin injuries and mucous membranes may also cause the patient to be susceptible to infection (WHO, 2012). Therapeutic procedures such as biopsies, catheterization, suction and surgical procedures also increase the risk of an infection (WHO, 2012). The environment can also be a contributing factor as well as the resistance if the microbes to the antimicrobial drugs (WHO, 2012). Nosocomial infections include urinary infections, nosocomial pneumonia, sinusitis, gastroenteritis and endometritis among others (WHO, 2012). Infection control
programs with the hospitals must be implemented to reduce the occurrence of nosocomial infections.

Disinfection of surfaces has been reported to reduce HAI’s (Rutala, 2013) as well as thorough cleaning of the patient environment (Kaur et al., 2018). Environmental surfaces play an important role in the transmission of pathogens such as Methicillin-resistant *Staphylococcus aureus*, *Acinetobacter* species, norovirus and rotavirus among others (Rutala, 2013). The selection of the disinfecting and cleaning method as well as materials for disinfecting are important (Kaur et al., 2018). The disinfectants reduce bacterial colony counts and the efficiency of the disinfectant is dependent on a number of factors (Kaur et al., 2018).

### 1.4 Disinfectants and microbial resistance

Different antimicrobial agents are used to kill or inhibit the growth of microbes and these include antibiotics and biocides (Health and Commission, 2009). Antibiotics are used to fight against infections, biocides examples include disinfectants, antiseptics and preservatives (Health and Commission, 2009). Antimicrobial agents are usually used in concentrations that are sufficient enough to kill or inhibit the growth of the microbes (Health and Commission, 2009, Fleming, 1945). The resistance mechanisms of microorganisms to biocides have also contributed to resistance to other antimicrobial agents (Health and Commission, 2009). If bacteria are able to survive and grow at concentrations sufficient to kill or inhibit the growth of the bacteria they are said to be resistant (Health and Commission, 2009). Bacterial resistance to biocides has been reported when the biocide was stored incorrectly or a low concentration was used. Biocides used in hospitals such as peracetic acid, glutaraldehyde were found to be ineffective due to the resistance developed by microbes which grow as biofilms attached to surfaces (Health and Commission, 2009).
There is a relationship between antibiotic resistance and resistance to a biocide. The bacteria is exposed to biocides and therefore the resistant strains only survive which increases their resistance to the antibiotics (Health and Commission, 2009).

1.5 *Callistemon citrinus* (older name - *Callistemon lanceolatus*)

*Callistemon citrinus* is a plant that belongs to the family *Myrtaceaee* and it is broadly utilized for forestry, ornamental agriculture, and essential oil recovery (Oyedeji *et al.*, 2009). Most of the *Callistemon* species are well known in traditional medicine and the essential oils have been used as anti-microbial and antifungal agents (Salem *et al.*, 2013). *C. citrinus* possesses numerous pharmacological and biological activities such as antibacterial and antifungal activity (Blesson *et al.*, 2014), antioxidant activity (Puranik, 2014) and larvicidal activity (Misvar and Aneesh, 2014, Hamed *et al.*, 2017).

The different parts of the plant have been used as a remedy for treatment of diarrhoea, dysentery and rheumatism (Cock, 2012). It is used as an anticough, antibronchitis herb and as an insecticide (Mohmoud *et al.*, 2012). Various phytochemicals have been isolated from *C. citrinus* and these include steroids, triterpenes, flavonoids, tannins and essential oils (Hamed *et al.*, 2017) (Krishna *et al.*, 2012). Flavonoids isolated from *C. citrinus* exhibit antioxidant, antiviral, antibacterial, anti-inflammatory and anticancer activity (Hamed *et al.*, 2017). They also inhibit lipid peroxidation and platelet accumulation (Hamed *et al.*, 2017).
1.5.1 Classification

Table 1.1: Classification of *C. citrinus*

<table>
<thead>
<tr>
<th>KINGDOM</th>
<th>PLANTAE</th>
</tr>
</thead>
<tbody>
<tr>
<td>DIVISION</td>
<td>Magnoliophyta</td>
</tr>
<tr>
<td>CLASS</td>
<td>Magnoliopsida</td>
</tr>
<tr>
<td>FAMILY</td>
<td>Myrtaceae</td>
</tr>
<tr>
<td>GENUS</td>
<td>Callistemon</td>
</tr>
<tr>
<td>SPECIES</td>
<td>Lanceolatus/ Citrinus</td>
</tr>
<tr>
<td>COMMON NAMES</td>
<td>Bottlebrush (English), isiDwadwa, inDalu, uBande</td>
</tr>
</tbody>
</table>

(Hamed et al., 2017, Ferreira, 1983)

1.5.2 *Callistemon citrinus* uses

*C. citrinus* flowers are used to make a tan dye which does not require a mordant (Baishya et al., 2012). The flowers have also been used as a food source as the nectar was sucked and used to make sweet drinks (Radulović et al., 2015). The leaves of the plant can also be used to make cinnamon dye. The natural dye obtained is non-toxic and reduces pollution and has less allergic effects (Singh and Srivastava, 2015), this makes *C. citrinus* an important plant in the textile industry as the dye is used to colour the fabrics.

*C. citrinus* leaves can be used as a substitute for tea with a refreshing flavour (Palanikumar et al., 2017). *C. citrinus* is a very rich source of bioactive compounds and different polyphenols (phenolic acids, flavonol, glycosides, hydrolysable gallo- and ellagitannins and tannins) and terpenes have been isolated (Radulović et al., 2015). *C. citrinus* contain phytochemicals which possess special biological activities (Palanikumar et al., 2017). Phytochemicals present in the plant include carbohydrates, tannins, saponins, flavonoids, quinones,
terpenoids, phenols and steroids (Palanikumar et al., 2017). It has also been used an insecticide against mosquito vectors and an alternative to the available insecticides (Palanikumar et al., 2017). The methanol extract of *C. citrinus* have shown good larvicidal activity against *Culex quinquefasciatus* (K and Aneesh, 2003). Phytochemical investigations have resulted in the isolation of 1,8-cineole and α-terpineol as the major compounds specifically from the flowers and leaves of the plant (Ahmed et al., 2016). Nitisinone was isolated from *C. citrinus* and it exhibited herbicidal activity (Ahmed et al., 2016).

### 1.5.3 Pharmacological properties of *Callistemon citrinus*

*C. citrinus* have been used for ethno-medicinal purposes and used in the treatment of many diseases such as those with symptoms involving inflammation, gastrointestinal disorders, pain relief, bacterial, viral, fungal and parasitic infections (Radulović et al., 2015). The plants are a great source of chemical compounds which can be used for drug making (Das and Singh, 2012).

The phytochemicals present in *C. citrinus* have been reported to possess hepatoprotective activities (Jain et al., 2007). This was demonstrated in an experiment where the rats were fed with the methanolic extract of *C. lanceolatus* leaves resulting in reduced level of serum enzymes therefore showing protective action (Jain et al., 2007). The leaves also have wound healing activity (Palanikumar et al., 2017). The leaves of *C. citrinus* have been used to cure respiratory tract infections (Palanikumar et al., 2017). *C. citrinus* possesses anti-inflammatory activity and this was demonstrated by an in vitro experiment with Diclofenac sodium was used as the standard drug (Kabdal and Singh, 2016). Anti-inflammatory activity of *C. citrinus* extract (chloroform, ethanol and aqueous) was higher than that of Diclofenac there by proving that the extracts of the plant can be used to relieve inflammation (Kabdal and Singh, 2016).
1.6 Biocides

The European legislation has defined a biocide as a substance that is intended to destroy, deter, render harmless or have a controlling effect over any harmful organism either by chemical or biological means (Health Commission, 2009),(EU, 2012).

1.6.1 Classification of biocides

Biocides are classified into four main groups which include the disinfectants and general biocidal products such as those used in human hygiene, veterinary hygiene and food and feed area disinfectants as well as disinfectants for water. The second group are preservatives used during storage of products such as wood, film and fibrous or polymerised material such as leather, rubber and paper (EU, 2012). The third main group are used in pest control for example insecticides and repellents (EU, 2012). The last main group of biocides are antifouling products and products used for the disinfection and preservation of animal corpses (EU, 2012).

Biocides are used in the health care industry, they are used to decontaminate the skin of patients, instruments, surfaces that can harbour dirt and health professionals (Health and Commission, 2009). Surfaces such as floors, walls, tables and bedrails are disinfected using biocides as they contribute to the spread of infections such as MRSA and Clostridium difficile. Antimicrobial wipes are now commonly used in hospitals (Health and Commission, 2009).

Biocides are also added to cosmetics and personal care products such as toothpastes, laundry detergents and other general disinfectants (Health and Commission, 2009). These prevent the growth of microorganisms, cleaning products as well as laundry detergents contain preservatives (Health and Commission, 2009). Triclosan is the common biocide that is in
cosmetics, toothpastes and other products for dental hygiene and in deodorants (Health and Commission, 2009).

Biocides are used in the food industry to disinfect production plants, processing areas and containers (Health and Commission, 2009). This reduces microbial growth, drinking water is also disinfected so that it is safe to drink (Health and Commission, 2009).

1.6.1.1 Main group 1: Disinfectants

Product-type 1: Products in this group are used for human hygiene purposes. They can be applied on the skin or scalps (EU, 2012).

Product-type 2: These are disinfectants and algaecides which are not used to apply directly to humans and animals (EU, 2012). The products are mainly used for the disinfection of surfaces, equipment, materials and furniture. They are used in swimming pools, walls, floors, work areas, public and industrial areas (EU, 2012).

Product-type 3: These are disinfectants used for the purpose of veterinary hygiene and the products include disinfecting soaps and hygiene products with anti-microbial function (EU, 2012). They are used to clean animal houses to disinfect materials (EU, 2012).

Product-type 4: These are products used to disinfect food and feed area, to disinfect the equipment, utensils, surfaces and containers for humans and animals (EU, 2012).

Product-type 5: These are used to disinfect drinking water for both humans and animals (EU, 2012).

1.6.2 Modes of action of disinfectants

Disinfectants act in different ways such as inhibition of growth of the microorganisms i.e. bacteriostasis, fungistasis or lethal action i.e. bactericidal, fungicidal or virucidal (Maris, 1995). The disinfectants contain complex formulations comprising of solvents, chelating
agents, acidic and alkalinic agents and they vary in pH, hardness and salinity (Maris, 1995).
The action of the disinfectants is divided into the primary stage and the secondary stage (Maris, 1995). The primary stage is the mode of action and the secondary stage is the result of the action (Maris, 1995).

The disinfectant can act on the external membrane of the bacterial wall which consists of phospholipids and lipopolysaccharides (Maris, 1995). The membrane is also stabilized by magnesium and calcium cations. The molecules in the disinfectants disturb the membrane orientation. They can also act on the bacterial wall as it offers rigidity, Gram positive and negative bacteria have different cell walls therefore different responses to disinfectants (Maris, 1995). They also act on the cytoplasmic membrane and penetrate the membrane through passive diffusion and active transport (Maris, 1995). The structures on the bacteria can make the bacteria resistant to the disinfectant. Bacterial spores can make the bacteria resistant to disinfectants and only the highly oxidising products can destabilise the structure (Maris, 1995). The table below shows the action of the different disinfectants on the cells of microorganisms.

**Table 1.2: Disinfectants and cell structures they affect**

<table>
<thead>
<tr>
<th>Disinfectant</th>
<th>Cell structure targeted and effects</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Aldehydes</strong></td>
<td>Antibacterial, antifungal, antiviral and sporicidal activity</td>
</tr>
<tr>
<td></td>
<td>Inhibit the activity of enzyme and denature proteins</td>
</tr>
<tr>
<td><strong>Alcohols</strong></td>
<td>Antiseptic and antiviral. Denatures cell wall proteins</td>
</tr>
</tbody>
</table>
Chlorine and sodium hypochlorite  | Sporicidal and oxidizing agent. Reacts with the enzymes of the cells
---|---
Phenols  | Antiviral and antifungal activity. Denatures proteins and enzymes
Hydrogen peroxide  | Oxidizing agent. Reacts with proteins and DNA in the cell. High concentrations are effective against bacterial spores and virus
Benzalkonium Chloride  | Increases cell permeability and coagulation of cell contents
Chlorhexidine gluconate  | Antiseptic, coagulates cell content

(Rutala et al., 2008)

1.6.3  Factors affecting the effectiveness of a disinfectant

A number of factors determine the effectiveness of the disinfectant. The factors include the types of the contaminating microorganism (Dvorak, 2008). The microorganisms vary in their degree of susceptibility to the disinfectants (Dvorak, 2008). Gram-positive bacteria are more susceptible to chemical disinfectants than Gram-negative bacteria because of the single peptidoglycan layer found in Gram-positive bacteria. Mycobacteria or bacterial endospores are more resistant because of the thick, liquid rich cell envelope (Dvorak, 2008). The complex cell wall provides an effective barrier for the entry of the agents. The non-enveloped viruses (adenoviruses, picornaviruses, reoviruses, rotaviruses) are more resistant to disinfection than the enveloped viruses (coronaviruses, herpesviruses, orthomyxoviruses, paramyxoviruses, retroviruses) (Dvorak, 2008).
Other factors include the degree of contamination which also determines the quality of disinfectant required and the contact time (Dvorak, 2008). The amount of protein in the microorganism also affect the effectiveness of the disinfectant (Dvorak, 2008) as the proteins absorb and neutralize the chemical disinfectants. The nature of the chemical disinfectant also affects the effectiveness of the disinfectant. This is because the different chemicals in the disinfectants have different modes of action (Bruce, 2003).

The concentration of the disinfectant is important to come up with the best results (Dvorak, 2008). Some of the disinfectants are more potent at higher concentrations, over diluting the disinfectant will reduce its effectiveness (Dvorak, 2008). The application method also affects the end result of the process. Various methods are used for the application of the disinfectant such as wiping, brushing, spraying (Dvorak, 2008). The contact time of the disinfectant also determines its effectiveness for example 70% isopropyl alcohol destroys mycobacterium tuberculosis in 5 minutes and 3% phenol requires 2-3 hours to destroy the same microorganism (Dvorak, 2008). The storage of the disinfectant also affects its stability as some disinfectants lose stability more quickly than others (Dvorak, 2008). In order to maximize the shelf life of the products they must be stored in an appropriate place according to the manufacturer instructions (Dvorak, 2008).
1.7 Test microorganisms

1.7.1 *Staphylococcus aureus*

*Figure 5.1* Gram positive *S. aureus*. Source: www.infectiousdiseaseadvisor.com

*S. aureus* is a gram-positive spherical bacterium, it is non spore forming and subdivided into 32 strains (Loir, 2012). It is common in both community acquired and nosocomical infections. The optimum temperature for growth of *S. aureus* is 37 °C and optimum pH is between 6-7 and it can survive in food stored below -20 °C (Loir, 2012). It is a facultative anaerobe but the growth is much slower under anaerobic conditions (Loir, 2012). The genus *Staphylococcus* is traditionally divided in two groups based on the ability to produce coagulase which is an enzyme that causes blood clotting (Costa *et al.*, 2013). *S. aureus* colonizes the skin and mucous membranes of individuals (Costa *et al.*, 2013). It causes food poisoning when a person ingests food contaminated with the bacterium. The symptoms include nausea, vomiting and diarrhoea. In the severe cases headaches and muscle cramping
may occur (Loir, 2012). It can also cause other non-food related health issues such as mastitis, inflammations, wound sepsis and respiratory infections (Loir, 2012). There has been an increase in the prevalence of the antibiotic resistant strains such as methicillin resistant \textit{S. aureus} (Rao et al., 2015).

1.7.2 Virulence factors of \textit{S. aureus}

The virulence factors enable the bacteria to cause infections in humans. They include:

a) Cell surface factors

\textbf{Table 1.3 Cell surface factors of \textit{S. aureus}}

<table>
<thead>
<tr>
<th>Staphylococcal protein A</th>
<th>Binds to IgG and interferes with opsonisation and phagocytosis.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fibronectin-binding proteins</td>
<td>Attaches to fibronectin and plasma clot</td>
</tr>
<tr>
<td>Collagen binding protein</td>
<td>Adheres to collagenous tissues</td>
</tr>
<tr>
<td>Clumping factor protein</td>
<td>Clumping and adherence to fibrinogen</td>
</tr>
<tr>
<td>Capsular polysaccharides</td>
<td>Reduce phagocytosis and enhance bacterial colonization on mucosal surfaces</td>
</tr>
<tr>
<td>Staphyloxanthin</td>
<td>Enhances resistance to neutrophil reactive oxidant phagocytosis</td>
</tr>
</tbody>
</table>

(Costa \textit{et al.}, 2013)

b) Secreted factors

Secreted factors include super antigens such as staphylococcal enterotoxins B, C, D, E, G and Q. It activates the T cells and antibody presenting cells (Costa \textit{et al.}, 2013).

c) Cytolytic toxins
**Table 1.5: Cytolytic toxins**

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>α-hemolysin</td>
<td>Causes the lysis of a number of cells such as platelets and monocytes</td>
</tr>
<tr>
<td>β- hemolysin</td>
<td>Hydrolyses sphingomyelin and makes the cells susceptible to lytic agents</td>
</tr>
<tr>
<td>γ-hemolysin</td>
<td>Causes lysis of erythrocytes and leukocytes</td>
</tr>
<tr>
<td>Leukocidin E/ D</td>
<td>Induces the lysis of leukocytes</td>
</tr>
</tbody>
</table>

*(Costa et al., 2013)*

Various exoenzymes such as lipases, nucleases, proteases, hyaluronidases and staphylokinases are other virulence factors of the bacterium *(Costa et al., 2013)*.

### 1.8 *Escherichia coli* 0157

![Figure 1.2 E. coli bacterium. Source: www.bacteria.cz](image)
It is a bacterium that lives in the intestines of humans and animals (Neill, 2002), it is considered to be part of the normal flora (Padhye and Doyle, 2016). There are four main groups of \textit{E. coli} which are associated with diarrhoea and food borne illnesses. They include enteropathogenic \textit{E. coli} (EPEC), Enteroinvasive \textit{E. coli} (EIEC), enterotoxigenic \textit{E. coli} (ETEC) and enterohemorrhagic \textit{E. coli} (EHEC) (Padhye and Doyle, 2016). EPEC is associated with neonatal and infantile diarrhoea many adults are carriers of this organism but they do not show the symptoms of illness. They invade epithelial cells and produce verotoxins/ shiga toxins (Padhye and Doyle, 2016).

EIEC invade the colonic epithelial cells and causes the necrosis of the cells and resulting in bloody diarrhoea (Padhye and Doyle, 2016). ETEC adhere to small intestinal epithelial cells and produces enterotoxins which causes watery diarrhoea (Padhye and Doyle, 2016). The fourth group of EHEC consists of \textit{E. coli} O157: H7 and \textit{E. coli} 026: H11 (Padhye and Doyle, 2016). They cause bloody diarrhoea, unusual gastro enteritis, hemorrhagic colitis and hemolytic uremic syndrome (Padhye and Doyle, 2016).

It is an important enteropathogen which causes human infections resulting in watery diarrhoea and life threatening outbreaks of haemorrhagic colitis and haemolytic uraemic syndrome (Padhye and Doyle, 2016), (Law, 2000). Over 100 serotypes of \textit{E. coli} are capable of producing Shiga-toxins. It is more virulent than other \textit{E. coli} strains producing Shiga toxins (Law, 2000).

It produces toxins such as Shiga toxin which causes severe diarrhoea and kidney damage (Neill, 2002). \textit{E.coli} 0157 is more virulent than other Shiga toxin producing \textit{E. coli} (Costa et al., 2013). It produces Shiga Toxin 2 and an adhesin called intimin (Costa et al., 2013). STEC has been associated with outbreaks of diarrhoea worldwide and in Japan over 9 000 children have been affected (Law, 2000). The organism causes haemolytic uraemic
syndrome (HUS) and this condition complicates all the infections caused by *E. coli* 0157. There are some serotypes of *E.coli* 0157 which do not produce Shiga toxins and these have been isolated from cases of sporadic diarrhoea and HUS (Law, 2000). It has a low infectious dose which ranges from 20- 700 organisms and in some cases less than 100 organisms.

The serotype of *E. coli* 0157 which produces Shiga toxin more than the others is *E. coli* 0157: H7. Most of the isolates produce Shiga toxin 2 and those which produce Shiga toxin 1 only are rare. To identify the organism the plasmid in *E. coli* 0157 (pO157) is hybridized with a DNA probe called CVD419 (Levine *et al.* 1987). The pO157 plasmid have been sequenced and used to identify the virulence factors of *E.coli* (Law, 2000).

1.8.1  Epidemiology

*E. coli* O157 was reported to be a human pathogen in 1983 with increased incidence of infections caused by the organism (Tarr, 2016). Most of the infections are food borne, there were two major outbreaks in Utah and Minnesota in 1987 and 1988 respectively. Ground beef was the major vehicle for the transmission of the organism (Padhye and Doyle, 2016). Hamburger were the suspected vehicle for the organism and surveys were done on retail meats and the organism was isolated from about 2 % of the pork, beef and chicken samples (Padhye and Doyle, 2016). Raw milk was also a vehicle for the transmission of the organism and individuals developed hemorrhagic colitis and HUS after drinking the raw milk.
1.9 *Candida albicans*

*Figure 1.3 Candida albicans. Source: www.thecandidadiet.com*

*Candida* is normally a non-pathogenic fungi as it is part of the normal flora of the mucous membranes of upper respiratory tract, female genital tracts and also of gastrointestinal tract (Singh, Gurjeet, Raksha, 2013). It becomes a pathogenic yeast once it invades the mucous membrane and causes candidiasis in immunocompromised individuals (Singh et al, 2013). These infections increase with the use of broad-spectrum antibiotics, cytotoxic chemotherapies, transplantation and the use of intravenous catheters. It has contributed to nosocomial blood infections and causing a mortality rate of about 39% (Singh et al, 2013). The virulence factors of *Candida* assist in colonization, invasion, and pathogenesis of the fungi (Singh et al, 2013). The have the ability to evade host defences, adhere to cells forming biofilms and the production of hydrolytic enzymes such as haemolysin, phospholipases and proteases (Fusco-Almeida et al., 2012). The yeasts have developed resistance to the available
anti-fungal drugs and this is due to the ability of the candida species to form drug resistant biofilms (Fusco-Almeida et al., 2012).

1.9.1 Epidemiology

The *Candida* species colonize the skin and mucosal surfaces of humans. Immunocompromised individuals are more likely to develop both the superficial and life threatening infections (Fusco-Almeida et al., 2012). AIDS patients are more prone to the infections and they develop oropharyngeal candidiasis which eventually lead to malnutrition and interferes with the absorption of medication (Fusco-Almeida et al., 2012). The *albicans* species are causing public health challenges and are of economic importance due to high mortality rates caused by the yeast. There has been an increase in infections caused by the non albicans species such as *C. glabrata, C. parapsilosis, C. tropicalis* and *C. krusei* (Fusco-Almeida et al., 2012). In North America there has been an increase in non- albicans infections. In European countries more than half the cases of *candaemia* were caused by *C. albicans* (Fusco-Almeida et al., 2012). Some of the yeast are found in combination with others and these include *Candida dubliniensis* which is usually associated with *C. albicans*. *C. dubliniensis* has been isolated from oral cavities, vagina, urine, skin and the gastrointestinal tract of both the HIV negative and positive patients (Fusco-Almeida et al., 2012).

1.10 Plant extraction methods

Various methods are used to extract the bioactive compounds from the plants and they are categorized based on temperature and how they work (Mtewa et al., 2018).
1.10.1 Low or room temperature methods

In the cold extraction method is used for extraction and the dried parts of the plant are placed in various solvents with shaking. After the exposure of the sample to the solvent the samples are filtered using a Whatman filter paper (Mtewa et al., 2018). Maceration is a good example of the cold extraction method (Mtewa et al., 2018). Coarsely grounded powder of the plant parts are added to the solvent with regular shaking (Mtewa et al., 2018), this releases the soluble matter in the plant. This low or room temperature method is an easy, cheap and environmentally friendly method and it can be done anywhere.

Enzymes are also used in the extraction process (Mtetwa et al., 2018). This method reduces the use of solvents and it is non degrading to the compounds (Mtewa et al., 2018). Plant tissue homogenization is another low temperature method where the fresh, wet or dried plant parts are used and they are soaked in solvents (Mtewa et al., 2018). The extract filtrates are centrifuged and concentrated. The ionic liquid extraction method is used to obtain organic and inorganic ligands in high yields (Mtewa et al., 2018).

1.10.2 High temperature extraction methods

These are conducted on thermally stable compounds, these include decoction. Decoction is when the plant material is boiled and strained, this method yields many oil soluble compounds (Mtewa et al., 2018).

1.10.3 Optional temperature methods

The serial exhaustive extraction method is an example of the optional temperature methods, it extracts compounds from the least polar to the most polar solvents (Mtewa et al., 2018). Infusion and digestion is when the infusions are prepared freshly by maceration and digestion gentle heat is applied to the maceration process (Mtewa et al., 2018). This is done to enhance the release of the active compounds in the plant material (Banu and Catherine, 2015).
1.11 Antimicrobial susceptibility tests

1.11.1 Microbroth dilution

A technique done using microtiter plates filled with broth, it is used to test for antimicrobial activity of substances (Luc, 2015). The procedure involves preparing two-fold dilutions of antibiotics in a liquid growth medium dispensed in tube. The antibiotic-containing tubes are inoculated with a standardized bacterial suspension of $1–5 \times 10^5$ CFU/mL (Jorgensen and Ferraro, 2009).

Following overnight incubation at 37°C, the wells are examined for visible bacterial growth as evidenced by turbidity (Jorgensen and Ferraro, 2009). The lowest concentration of antibiotic that prevents the growth is the minimal inhibitory concentration (MIC).

The principal disadvantages of the macro dilution assay is the tedious, manual task of preparing the antibiotic solutions for each test, the possibility of errors in preparation of the antibiotic or antimicrobial solutions, and the relatively large number of reagents and space required for each test (Jorgensen and Ferraro, 2009).

1.11.2 MTT assay

The MTT assay is based on the conversion of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) to formazan crystal and this is due to the activity of the mitochondria (Meerloo and Cloos, 2011). It is a colorimetric assay, the yellow 3-(4,5-dimethylthiazol2-yl)-2,5-diphenyl tetrazolium bromide (MTT) is reduced by mitochondrial succinate dehydrogenase (Sigma-aldrich, 2013). The MTT enters cells into the mitochondria and it is reduced to an insoluble(dark purple) formazan (Sigma-aldrich, 2013).

It is used to measure viable cells and to determine cell cytotoxicity (Meerloo and Cloos, 2011). The activity of the mitochondria is determined by the conversion of the tetrazolium salt into formazan crystals (Meerloo and Cloos, 2011). The assay is carried out in 96 well
plates and the concentration of the formazan crystals is detected by measuring the formazan concentration using microplate reader at 540nm and 720 nm (Meerloo and Cloos, 2011).

The MTT assay can be applied to study cross resistance between related and unrelated drugs (Hubeek et al, 2006). It is also used to measure drug sensitivity to determine the clinical outcomes (Meerloo and Cloos, 2011). Sensitivity testing of new drugs can also be performed using this technique. Drug combination and screening can be done using the MTT assay (Klumper, 1995) (Meerloo and Cloos, 2011).

1.11.3 Agar disk diffusion method

It is one of the simplest and reliable method for susceptibility testing (Jorgensen and Ferraro, 2009). It is performed by applying a standard inoculum of 1-2 × 10^8 CFU/ml to an agar plate (Jorgensen and Ferraro, 2009). Filter paper disks are placed on the agar surface and the plates are incubated for 16-18 hours at 35 °C (Jorgensen and Ferraro, 2009). The zones of inhibition around the disks are measured to the nearest millimeter (Jorgensen and Ferraro, 2009).
2 CHAPTER TWO

2.1 Rationale of the project

The emergence of microbial resistance is of global concern as there is need of ways to minimize its occurrence (Aweke and Yeshanew, 2016). New, safe and effective antimicrobial agents with potent biological functions are needed. (Aweke and Yeshanew, 2016). Antibiotic resistance has been observed in S. aureus which have developed resistance to oxacillin, vancomycin, linezolid (Rutala, n.d.). Enterococcus have developed resistance to penicillin, aminoglycosides, vancomycin and linezolid (Rutala, n.d.). Candida spp have developed resistance to fluconazole (Rutala, n.d.). Studies have also shown that the use of disinfectants have also contributed to antibiotic resistance.

Callistemon citrinus is a rich source of bioactive compounds and many have been isolated (Radulović et al., 2015). These phytochemicals are responsible for the biological activities that are associated with C. citrinus which include wound healing, anti-inflammatory, antidiabetic, antioxidant, antithrombotic among others (Radulović et al., 2015).

2.2 Problem Statement

The synthetic chemical disinfectants have caused problems as they are toxic and not degradable. They contribute to environmental contamination whereas the herbal disinfectants are eco-friendly as they are biodegradable and most suitable for use in households. Some chemical disinfectants have also contributed to resistance in some microorganisms. If the disinfectant is used in lower concentration that is sufficient to kill or inhibit the growth of the microorganism, some of the organisms may be able to survive and grow at these concentrations. Therefore, there is need for new disinfectants to eliminate the microorganism that have developed resistance to the available chemical disinfectants.
2.3 Justification

*Callistemon citrinus* is a source of natural anti-microbial compounds which could be alternatively used in biocide manufacturing. *Callistemon citrinus* was reported to contain steroids, triterpenes, flavonoids (Jeong *et al*, 2009), tannins, phenolic compounds (El Dib and El Shenawy, 2008), (Gohar *et al*, 2013) and essential oils (Liu *et al*, 2010). Studies done have showed that the chloroform, ethanolic leaf extracts of *Callistemon citrinus* were exhibiting good antimicrobial activity against gram positive and Gram-negative microorganisms (Krishna *et al*, 2012). Bacteria most susceptible to the extracts of *C. citrinus* include *Bacillus subtilis*, *Bacillus pumilis*, *Escherichia coli*, *Staphylococcus aureus* and *Candida albicans*. Herbal disinfectants are eco-friendly and degradable and therefore they minimize environmental pollution. *Callistemon citrinus* is also a cheap source of antimicrobial compounds such as tormentic acid and it is a reliable source of the leaves as it is an evergreen tree or shrub (Waterman, 1983).

2.4 Hypothesis

*Callistemon citrinus* leaf extracts and compounds have biocidal activity on the microorganisms that reside on the floor surfaces such as *S. aureus*, *E. coli* and *C. albicans*.

2.5 Objectives of the study

2.5.1 Main objective

The main objective of the study is to determine if a biocide made from the leaf extracts and compounds from *C. citrinus* is effective against floor microbes.

2.5.2 Specific objectives

The specific objectives are:

1. To collect and prepare *C. citrinus* leaf extracts
2. To verify the antimicrobial activity of the extracts on *Staphylococcus aureus*, *Escherichia coli* and *Candida albicans*.

3. To determine the use-dilution concentration for hard surface disinfection for which the preparation is active and use it in biocide formulation

4. To perform *in-loco* or *in-situ* tests for the use of the biocide
CHAPTER THREE

3 MATERIALS AND METHODS

3.1 Plant sample, chemicals and other materials

The *Callistemon citrinus* leaves were collected from the University of Zimbabwe biochemistry car park (Geographic coordinates, Latitude: 17.7840° S, Longitude: 31.0530° E). Different solvents which include DMSO, hexane, dichloromethane, ethyl acetate, acetone, ethanol and methanol were purchased from Sigma Aldrich (Germany) and used in plant extraction.

The *C. citrinus* leaves were oven dried at 60 °C for 72 hours. The dried leaves were ground to a powder and stored in an airtight container.

Other chemicals, media and drugs used in the study were ciprofloxacin, miconazole, nutrient broth, nutrient agar and 3- (4,5-dimethylthiazolyl)-2,5-diphenyltetrazolium bromide [MTT, thiazolyl blue] were also purchased from Sigma Aldrich (Germany).

3.2 Extraction of phytochemicals

The dried leaf powder was used for extraction. Total extraction and serial exhaustive extraction were performed. Two mixtures were made and for total extraction a mixture of 50 % dichloromethane and 50 % methanol and 50 % ethanol and 50 % water. The dried leaf sample was weighed i.e. 20g and mixed with 100 ml of the solvents. The mixture was left for 72 hours with shaking for at least three times per day before collecting the extract. After 72 hours filtration was done using Whatman filter paper number 1. The extracts were air dried and stored in a 50 ml Falcon tubes until they were required for use.

The serial exhaustive extraction was done as for the total extraction but the solvents were added sequentially. The first solvent was added and after the residue had dried the next
solvent was added up to the last solvent. After 24 hours the mixture was pre-filtered with
cotton wool followed by a Whatman No. 1 filter paper. The residue was allowed to dry
before it was mixed with another solvent. The extract obtained after each extraction process
was air dried and stored. The mass of each extract was weighed and the percentage yield was
expressed as below:

\[
\text{Extraction yield (\%) = } \frac{\text{Weight of dried plant extract (g)}}{\text{Weight of dried plant material (g)}} \times 100
\]

3.3 Microorganisms

Three different microorganisms were used one gram negative, one Gram positive bacteria
and one fungus. Laboratory strains of *Staphylococcus aureus* NCTC 6571, *Escherichia coli*
0157 and *Candida albicans* NCPF 3255 were purchased from the United Kingdom in Bristol.
The microorganisms were obtained as lenticule discs. The lenticule discs of the test
microorganisms were kept in the refrigerator at -20 °C. The lenticule discs were resuscitated
in NB for the bacterial strains and SDB for the fungi. Pure isolates were obtained by
streaking on agar plates and were also stored as 750 µl of NB overnight culture and 250 µl of
50% glycerol at -80 °C in micro tubes. Prior to each assay the microbial strains were
resuscitated in nutrient broth for bacterial strains and Sabouraud dextrose broth for fungi and
agar plate were made. A single colony from the NB and SDB agar plates was obtained and
inoculated in 20ml of NB or SDB depending on the nature of the test microorganism.

3.4 Evaluation of antibacterial and antifungal activity of *C. citrinus* leaf extracts

3.4.1 Cell cultures preparation

The glycerol stocks of the test microorganisms were used to prepare the cell cultures. From
the glycerol stocks 40 µl was inoculated into 20ml of NB and SDB for bacteria and fungi
respectively. The overnight culture was sub cultured on agar plates and incubated overnight
for 24 hours at 37 °C. The overnight agar plates are kept in the refrigerator and the colonies are used for the antibacterial and antifungal susceptibility tests. Single colonies were picked and inoculated into 20 ml of broth incubated for 24 hours at 37 °C in a Lab-Companion incubator with shaking. A cloudy mixture indicated the growth of the microorganism. The cells were diluted and adjusted to make a concentration of $2 \times 10^6$ CFU/ml.

3.4.2 Cell Standardization

The turbidity of the bacterial cell culture was compared to Mc Farlands standard solution. A 0.5 McFarland’s solution was made using 0.05 ml of 1% barium chloride and 9.95 % ml of 1% Sulphuric acid. The solution is equivalent to $1.5 \times 10^8$ CFU /ml, the absorbance’s were measured using a spectrophotometer at 600nm.

3.4.3 Antibacterial and Antifungal Susceptibility Tests (Screening of extract and determination of the MIC and MBC)

The *Callistemon citrinus* extracts were dissolved in DMSO and made up to a concentration of 200 µg/ml. Firstly, 0,004g of the extract was dissolved in 1ml of DMSO to make a concentration of 4 mg/ml. From this concentration 500 µl was added to 9 500 µl of media to make a concentration of 200 µg/ml. The cells were standardized using McFarland’s standard to give a concentration of $2 \times 10^6$ cfu/ml.

Positive controls containing ciprofloxacin and miconazole were made up to a concentration of 2 µg/ml and 100/ml respectively in 9, 8 ml of media. Serial dilutions were performed to obtain 10 concentrations of the antibiotic. Each of the well was made to contain a total of 200 µl. Negative controls containing media only and media and extract only were set up. The pre incubation measurements were obtained by reading the absorbance at 590nm using the microplate reader. The post incubation measurements were also determined after incubation at 37 °C for 24 hours using a microplate reader.
Figure 6.1 A well labelled microtiter plate for antimicrobial testing of the extracts

3.5 Preparation of disinfectant formulation

The Dichloromethane extract was used for the formulation of the disinfectant because it was the most potent extract on the tested microorganisms. Two formulations were made using the DCM extract and tested for antimicrobial activity.

Formulation 1

Fifty millilitres of 96% linear alkyl benzene sulphonic acid (LABSA) was mixed with distilled water until the required viscosity was obtained. Sodium hydroxide was added to the diluted sulphonic acid and they were both mixed by stirring continuously. The extract was added to make 1%, 2% and 4% weight of the total volume. Sodium chloride was added to thicken the mixture and pH was measured using a pH meter. It was stored in a cool place until it was required for use.
**Formulation 2**

Glycerine was used as the base for the disinfectant. The extract was dissolved in 1 ml of ethanol, to make 1% of the disinfectant 0.020 g of the extract was dissolved in 1 ml of ethanol and 1 ml of the base was added to make a final volume of 2 mls. The same was repeated to make 2 % and 4 % weight of the total volume of the disinfectant.

### 3.6 Biocide testing

The biocide formulated was tested using the agar disk diffusion method. Bacterial and fungal cultures were prepared by making an overnight culture using agar plates stored at 4 °C. Sterile filter paper disks were used, the filter paper disks were autoclaved together with Nutrient agar and Sabouraud Dextrose Agar. The sterile filter paper disks were prepared by adding 1 µl of the disinfectants to be tested and allowed to dry overnight. The overnight culture was standardised using McFarland’s standard to reach to concentration of \( 1 \times 10^6 \) CFU/ml. The agar was allowed to cool to reach a temperature warm enough to inoculate it with the organism to be tested. The inoculum was added to the agar and swirled to evenly distribute it and the agar was poured onto plates. The plates were allowed to solidify before the disks were added. The disks were then placed on agar using sterilized forceps and they were gently pressed on the agar to ensure that they were attached to the agar. The plates with the paper disks were placed in the refrigerator at 4 °C for 2 hours. The agar plates were incubated for 24 hours at 37 °C and observed for zone of inhibition the following day.

### 3.7 In - loco tests of the biocide

The effectiveness of the biocide against floor microbes was determined using the in-loco biocide tests where the biocide was applied on floor surfaces and swabs were collected and cultured before and after application of the biocide. The tests were carried out in the Biochemistry department where a portion of the floors was marked and a swab was taken
before and after the application of the disinfectant. The bacteria from the floor tiles was 
recovered from the test sample by adding sterile distilled water. The colony forming units 
were determined using the dilution plate count and the plates were observed to determine the 
bacterial and fungal growth before and after application of the disinfectant. This was 
repeated four times to get accurate results.
CHAPTER FOUR

4 RESULTS

4.1 Plant extract preparation

The *Callistemon citrinus* leaves were obtained and ground to fine powder using a motor and pestle. The leaf powder was green in colour with a fresh minty smell. A well labelled airtight container was used to store the leaf powder at room temperature.

![Figure 4.1: Dried Callistemon citrinus leaves which were used for the extraction process.](image)

4.2 Plant extraction

The cold extraction method was used to obtain the extracts with a wide range of compounds and in this study, maceration was used. The leaf powder was stored in contact with the solvent with regular shaking. Nine extracts were obtained from the two methods used i.e. total extraction and serial exhaustive extraction performed. For the total extraction two different solvent mixtures were used which were 50% methanol and DCM and 50% ethanol and water. The mass of the extracts obtained were expressed as percentage yields as show by the table below.
Table 4.1: The mass and percentage yield of *C. citrinus* extract using different solvents

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Mass of extract (g)</th>
<th>% yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>50% DCM and 50% methanol</td>
<td>2.417</td>
<td>4.02</td>
</tr>
<tr>
<td>50% ethanol and 50 % water</td>
<td>2.165</td>
<td>3.6</td>
</tr>
<tr>
<td>Hexane</td>
<td>1.267</td>
<td>2.11</td>
</tr>
<tr>
<td>DCM</td>
<td>1.355</td>
<td>2.3</td>
</tr>
<tr>
<td>Ethyl acetate</td>
<td>1.804</td>
<td>3.00</td>
</tr>
<tr>
<td>Acetone</td>
<td>0.825</td>
<td>4.1</td>
</tr>
<tr>
<td>Ethanol</td>
<td>0.334</td>
<td>1.67</td>
</tr>
<tr>
<td>Methanol</td>
<td>0.736</td>
<td>3.68</td>
</tr>
<tr>
<td>Water</td>
<td>2.679</td>
<td>13.4</td>
</tr>
</tbody>
</table>

4.3 Screening for activity of the crude extracts

A two-fold micro broth dilution method was used to determine the activity of the crude extracts on the growth of bacterial and fungi strains used for the study. The MTT assay was used to determine the cell viability of the microorganisms. The mitochondrial activity of the cells is determined by this assay and therefore the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) is converted by the viable (living) cells to formazan crystals (Meerloo and Cloos, 2011). It is used to test for the cytotoxic effects of drugs and plant extracts at different concentrations on the cells (Meerloo and Cloos, 2011). The assays were done in 96 well micro titre plates, in this study it was used to determine the activity of the plant extracts on the test microorganisms.
The day before the assay the test microorganisms were resuscitated from agar plates and cultured in broth overnight. 15 ml falcon tubes are thoroughly cleaned and autoclaved together with the broth to be used in the assay. All the equipment to be used are autoclaved to minimize contamination. The extract was dissolved in a solvent and added to media to make a final concentration of 200 µg/ml. The dilution is performed and the turbidity of the bacterial and fungal solution is adjusted using the McFarland’s solution before they are placed on the microtiter plate. On the plate two columns are used as the controls i.e. positive and negative controls. One column contained media only which is the negative control as there is no bacteria or fungi. This shows the sterility of the media and ensures that only one microorganism is tested. The positive control is the column containing cells only and this helps us to know if the microorganism is growing properly.

4.3.1 Bioactivity of crude extracts against *S. aureus* NCTC 6571

In vitro bioactivity of all the extracts was evaluated using microbroth-dilution method to determine the MIC of each extract against *S. aureus*. Absorbance corresponding to different cell densities of *S. aureus* after incubation with different concentration of different extracts at 37 °C overnight are as shown in Figure 4.2.
Hexane extract

DCM extract

Ethylacetate extract

Acetone extract

Ethanol extract

Methanol extract
The microbroth dilution assay was used to determine the Minimum Inhibitory Concentration (MIC) of the extracts against the test bacteria. For ciprofloxacin the positive control the MIC was 0.50 µg/ml. The cells only were the positive control and the media only was the negative control. *S. aureus* was susceptible to the crude extracts shown by the inhibition of growth in the hexane, DCM, ethyl acetate and acetone extracts. The MIC was determined for the extracts and percentage inhibitions were calculated.
Figure 4.3 Activity of the different extracts used to screen *S. aureus*.

A total of 9 extracts were used in the microbroth dilution assay and no significant inhibition was observed in the ethanol, methanol and water extracts. From the results *S. aureus* was susceptible to the crude extracts and this was seen by the inhibition of growth in the hexane, DCM, ethyl acetate and acetone extracts as shown in Fig 4.3.
4.3.2 Bioactivity of the crude extracts against *E. coli*

In vitro bioactivity of all the extracts was evaluated using micro-dilution method to determine the MIC of each extract against *E. coli*. Absorbance corresponding to different cell densities of *E. coli* after incubation with different concentration of different extracts at 37 °C overnight are as shown in Figure 4.4.
Figure 4.4 The activity of the crude extracts against *E. coli* 0157 measured using the microbroth dilution assay
The microbroth dilution method was used to screen the extracts and determine the activity of the extracts against the *E. coli* 0157. Ciprofloxacin was used as the standard drug and the MIC was 0.032 µg/ml. The highest concentration of ciprofloxacin used was 1 µg/ml. The cells only column was used as the positive control showing the growth of the bacterium. The media only was the negative control as there was no bacteria added. Four different concentration of the extracts were used with the highest being 100 µg/ml and the lowest being 12.5 µg/ml. *E. coli* 0157 was not susceptible to the extracts and the percentage inhibitions were calculated as shown in Fig 4.5.

![Bioactivity of the crude extracts](image)

**Figure 4.5** The percentage inhibitions of the extracts used to screen *E. coli* 0157.

There was no significant inhibition of the growth of *E. coli* 0157 in all the extracts. There was no inhibition of growth in the methanol extract and the DCM extract had the highest inhibition of 32%.
4.3.3 Bioactivity of the crude extracts against *Candida albicans*

*In vitro* bioactivity of all the extracts was evaluated using microbroth-dilution method to determine the MIC of each extract against *C. albicans*. Absorbance corresponding to different cell densities of *C. albicans* after incubation with different concentration of different extracts at 37 °C overnight are as shown in Fig 4.6.  

![Graphs showing bioactivity of different extracts against Candida albicans](image)
Figure 4.6: The bioactivity of the 9 extracts determined by the microbroth dilution method with miconazole as the standard drug against *C. albicans*.

The microbroth dilution method was used to determine the activity of the extracts on *Candida albicans*. *C. albicans* and the extracts were incubated overnight and absorbances were measured using a microtiter plate reader and values were generated. The MIC for miconazole was 50 µg/ml as shown by the graph above. *Candida albicans* was less susceptible to the extracts as shown in Fig 4.6 except for the ethanol extracts which inhibited the growth of the fungi.
Complete inhibition of *C. albicans* cells was observed in the ethanol extract, the percentage inhibition was calculated and presented as shown in **figure 4.10**

**Figure 4.7**: Activity of the crude extracts at 100 µg/ml.

The ethanol extract inhibited the growth of *C. albicans* as shown in the graph. No significant inhibition was observed in the hexane, DCM, ethyl acetate and acetone extracts.

**4.3.4 Determination of Minimum Inhibitory Concentration and Minimum Bactericidal Concentration.**

The MIC was determined using the microbroth dilution method and MTT assay, ten different concentrations of the extract were used. Inoculums were taken from the 25, 50 and 100 µg/ml and cultured on agar plates so as to determine the MBC. The agar plates were incubated at 37°C overnight and observed the following day.
Figure 4.8: MIC for the ethyl acetate extract measured using the microbroth dilution assay against *S. aureus*. Inoculums were taken from the 25, 50 and 100µg/ml wells and cultured on agar plates as shown in Fig 4.9 below
Figure 4.9 Cultured agar plate from the microtiter plate used for MIC determination

The cultured agar plate from the microtiter plates of the wells with the concentration 25, 50 and 100 µg/ml. This was done to determine the Minimum Inhibitory Concentration as for the MBC it was more than 100 µg/ml. There was a reduction in the number of colonies of *S. aureus* with an increase in the concentration of the extract. Few colonies were observed in the concentration of 100 µg/ml as compared to the concentration of 25 µg/ml and 50 µg/ml.
Figure 4.10 MIC for the DCM extract against *S. aureus* determined by using microbroth dilution method.

The MIC for the DCM extracts against *S. aureus* was determined by the microbroth dilution method and the MTT assay. The extract was exposed to the bacteria using ten different concentrations as shown above. The cells were the positive control and the media was the negative control. There was inhibition of the growth of the bacteria and this was shown by the decrease in the absorbances. The MIC was 25 µg/ml, to determine the MBC the contents of the 12.5, 25, 50 and 100 µg/ml were cultured on agar palates and incubated overnight and the plates were observed.
Figure 4.11 The agar plates used to determine the MBC of the DCM extract against *S. aureus*

The agar plates were incubated for 24 hours and observed for growth of the bacteria. There was a reduction in the number of colonies with an increase in the concentration of the extract. Inoculums were taken from the 12.5 to 100 µg/ml and cultured on the plate. The 100 µg/ml had few colonies followed by the 50, then 25 and lastly 12.5 µg/ml.
4.4 Biocide formulation

The two formulations were prepared as shown by Fig 4.12 and Fig 4.13 below

**Figure 4.12 Formulation 1:** Disinfectant base containing sulphonic acid and sodium hydroxide

The formulation was a clear light brown mixture with semi-thick consistency. Sodium hydroxide was added to neutralise the sulphonic acid. Sodium chloride was added as a thickener. The mixture was stored in a cool place until it was required for use. Percentage weight / volume of 1, 2 and 4% of the extract was formulated using this base.

**Formulation 2**
Figure 4.13 Formulation 2: Glycerin based disinfectant

The disinfectant was formulated to achieve an overall percentage weight/volume of 1, 2 and 4% weight of the extract. The 1% had a lighter green colour and the colour intensity increased as the percentage weight increased. The stability of the disinfectant decreased as the % weight of the extract increased. There was no precipitation in the 1% as compared to the 2% and 4% as shown in Fig 4.13.

4.5 Biocide tests

The agar disk diffusion method was used to test the effectiveness of the disinfectant against *S. aureus*, *E. coli* and *C. albicans*. Sterile filter paper disks were used and 20 µl of the 1, 2 and 4% disinfectant were added to the disks. Disks containing the base only were also prepared and used as the control to test for the antimicrobial activity of the base on its own.
4.5.1 Formulation 1

Figure 4.14 Antimicrobial activity of Formulation 1 (Sulphonic acid base) against a - S. aureus, b- E. coli and C. albicans.

Zone of inhibition were observed around the disks with the formulation at 1, 2 and 4 % extract. The formulation inhibited the growth of S. aureus and C. albicans. E. coli was
resistant to the formulation, no ZOI was observed. The base only was used as the negative control but in this formulation, it had a ZOI.

\[ S. \text{ aureus} \]

![Graph showing Zone of Inhibition against S. aureus](image)

**Figure 4.15** The Zone of inhibition of formulation 1 against *S. aureus*

The ZOI were measured using a ruler and used to draw the graph. The negative control i.e. the base only had an average inhibition almost similar to that of the extract and even more. The ZOI was decreasing as the concentration was increasing as shown by the ZOI for the 4 % formulation.
Figure 4.16 Average Zone of inhibition of Formulation 1 against *C. albicans*

ZOI was observed for formulation 1 against *C. albicans*, the average ZOI was decreasing with an increase in the percentage of the extract in the formulation as shown by the graph. The base only which was the negative control had a slightly higher average ZOI.

4.5.2 Formulation 2

![Image a](image1.png)  ![Image b](image2.png)
The Glycerine based formulation inhibited the growth of *S. aureus* and *C. albicans*. The negative control i.e. the disks with the base only had no ZOI on all the microorganisms. *E. coli* was not susceptible to the formulation and no ZOI was observed.
Figure 7.18 Average Zone of inhibition of Formulation 2 (Glycerine based disinfectant) against *S. aureus*

The average ZOI was determined for the activity of the formulation against *S. aureus*. The highest inhibition was observed to be in the formulation containing 4% of the extract. The negative control had no activity against the Gram-positive bacterium. The activity of the formulation increased with an increase in the extract percentage.

Figure 4.19 Average Zone of inhibition of Glycerine based disinfectant against *C. albicans*
The formulation was able to inhibit the growth of *C. albicans* and this was explained by the ZOI observed around the disks containing the formulation. The negative control i.e. the base only had no ZOI.

### 4.5.3 In-loci biocide tests

![Image of petri dishes before and after treatment](image-url)
Figure 4.20 Agar plates cultured before and after application of the disinfectant based on the Glycerine based formulation.

The disinfectant was applied on the floor tiles in the BIA laboratory. Swabs were taken before the application of the disinfectants and added to autoclaved distilled water in a 10ml falcon tube. Serial dilutions were performed to count the colony forming units. The disinfectant was used to clean the floor tiles and a swab was taken after a contact time of 1 hour and cultured as above. The agar plates were incubated overnight and observed for growth of the floor microbes. The positive control was the application of the base and growth of the floor microbes was recorded even after the application of the base only.
CHAPTER FIVE

5 Discussion

The world is now focusing on herbal products as these are economically friendly and contain less chemicals (Pandya et al., 2017). Plants produce a number of secondary metabolites such as tannins, terpenoids, alkaloids, phenolic compounds and flavonoids (Khanam and Afsar, 2013). New antimicrobial agents are being made from the phytochemicals present in the plants as there is need, especially in the hospitals so as to treat new and reemerging infectious diseases (Sukanya et al., 2009).

The study focused on the development of a biocide using the phytochemicals present in the *Callistemon citrinus* leaf. Previous studies have shown that the plant has antimicrobial properties (Das and Singh, 2012).

5.1 Plant extraction

Plant extraction is an important step which must be done before biocide production in order to obtain the phytochemicals from the plant (Mtewa et al., 2018). The different solvents used for extraction extract different phytochemical groups from the plant.

The leaves were dried in the oven and not air dried as this preserves the plant and reduces the chances of contamination from the external environment. The oven drying method also helps to minimizes the effects of the environment, during air drying the plant material will be exposed to various contaminants and exposure to moisture due to changes in temperature (Azwanida, 2015). Weather changes can result in rainfall and the plant samples will be affected as the moisture will cause the plant material be affected with molds or even rot (Azwanida, 2015).
The air drying method requires a longer time to completely dry the plant material as compared to the time required for oven drying (Azwanida, 2015). The plant sample was oven dried at 60 °C as oven drying preserves phytochemicals if the temperature does not exceed 60 °C as high temperatures can affect the heat labile compounds. However the drying process can affect enzymatic processes and this lead to changes in the composition of the bioactive compounds such as phenolic compounds, ascorbic acid and pigments responsible for color such as carotenoids (Kamel et al., 2013).

After oven drying the plant samples were ground to a fine powder using mortar and pestle, this was done so as to increase the surface area between the plant samples and the extraction solvent so as to ensure efficient extraction (Azwanida, 2015). The extraction method used in the study was maceration, the plant samples were soaked in the extraction solvents at room temperature with regular shaking (Azwanida, 2015). This softens and breaks the plant cell walls and release soluble phytochemicals.

This is an easy and simple method for extraction however proper management of the waste is needed (Azwanida, 2015).

Nine extracts were obtained from *Callistemon citrinus* leaves and the different solvents used for the extraction process were a mixture of methanol and DCM as well as ethanol and water mixture for total extraction. For the serial exhaustive extraction, the solvents were added in the order of their polarity. The different solvents extract different phytochemicals from the plant material (Houghton and Raman, 1998). With the order of polarity, the low polar solvents such as hexane extract chemical classes which include waxes, fats and volatile oils (Houghton and Raman, 1998). Chloroform is another low polar solvent which extract alkaloids, aglycones and volatile oils (Houghton and Raman, 1998). Flavonoids are also extracted using this solvent. Dichloromethane extracts terpenoids. The methanol extracts
anthocyanins, terpenoids, saponins, tannins (Sukanya et al., 2009). The methanol extracts contain alkaloids, coumarins and tannins. Coumarins and tannins often have antibacterial and antihelminthic properties (Sukanya et al., 2009). The ethanol extract contains compounds such as tannins, polyphenols, polyacetylenes, flavonols, terpenoids, sterols, alkaloids and propolis.

*Callistemon citrinus* contains a wide range of compounds such as steroids, terpenoids, flavonoids, tannins and phenolic compounds (Hamed et al., 2017). Flavonoids have multiple biological activities such as antimicrobial, antioxidant and anti-inflammatory properties (Hamed et al., 2017).

According to Khanh et al., 2016 eight phenolic compounds have been isolated from the stems and leaves of *C. citrinus*. Two flavonoids have been isolated namely eucalyptine and 8- demethyleucalyptine. 8- demethyleucalyptine was isolated from the DCM fraction and eucalyptine was isolated from the hexane fraction of the methanolic extract (Khanh et al., 2016). Blumenol A an alcohol has been isolated from the ethyl acetate fraction of the methanol extract (Khanh et al., 2016).

### 5.2 Bioactivity of the extracts

The activity of the plant extracts was determined using selected microorganisms i.e. Gram positive bacteria *S. aureus*, Gram negative bacteria *E. coli* and a fungus *C. albicans*. The maximum concentration of the extract used was 100 µg/ml and the microbroth dilution method was used to determine the activity of the extracts. According to Eloff and McGaw (2014) 100 µg/ml is the highest concentration used for a compound or solution to be considered to have antimicrobial properties.

The media was autoclaved together with the other materials to be used. This was done to minimize contamination and be able to assess only one microorganism. During all the
practical’s the aseptic techniques were taken into consideration, the Bunsen burner was used during culturing to prepare overnight cultures as well as during the assay. According to Bykowski and Stevenson (2008) the use of the Bunsen burner is the easiest way to create a sterile environment. The inoculating loop was sterilized using the burner. The assays were done in the laminar flow unit which is another way to reduce contamination as it keeps the room air from entering the working area (Bykowski and Stevenson, 2008).

The methanol solvent extracts alkaloids, coumarins and tannins. Coumarins and tannins are known to have antibacterial properties (Sukanya et al., 2009). Eloff 1998 and Cowan 1999 have reported that the methanol extracts are more efficient than the acetone extracts but in this study the acetone extract exhibited total inhibition as compared to the methanol extract (Sukanya et al., 2009). This could be due to the solubility of the active compound in the *C. citrinus* leaf and the serial exhaustive extraction was done in which the solvents were added in order of increasing the polarity. The active antimicrobial compound in the leaf was extracted by acetone.

Total inhibition was observed in the DCM, ethyl acetate, acetone and hexane extract. This shows that the active compounds in the leaves extracted with these solvents have antimicrobial properties. According to Cowan (1999) the DCM solvent extracts terpenoids, the acetone solvents extract flavonols. Many compounds have been isolated from *C. citrinus* and among them is tormentic acid which is a triterpenoid and have exhibited antimicrobial activities.

Many compounds isolated from *C. citrinus* include essential oils, flavonoids, alcohols, benzoic acid derivatives, terpenoids and sterols. Essential oils isolated from this plant include 1,8-cineole, α-pinene, β-pinene, myrcene, limonene, linalool and methyl acetate (Misra et al., 2011)
*Staphylococcus aureus* is a Gram-positive bacterium and this makes it more susceptible to the extracts of *C. citrinus* extracts. This is due to the cell wall structure differences in both the Gram-positive and Gram-negative bacteria (Cock, 2008). MIC was determined for the DCM, Ethyl acetate and acetone extract against *S. aureus*. The DCM extract had an MIC of 25 µg/ml and dichloromethane is a solvent known to extract terpenoids (Sukanya et al., 2009).

*C. citrinus* contains terpenoids such as tormentic acid, which is a triterpenoid.

*E. coli 0157* was less susceptible to all the extracts of *C. citrinus*, with the highest inhibition having 32 % inhibition of the DCM extract. In a study done before *E. coli* strain was resistant to the methanolic and ethanolic extracts of *C. citrinus* (Seyyed et al., 2010). The microorganism was resistant to all the extracts as no significant inhibition was observed. This is due to the cell membrane permeability of Gram negative bacteria and other genetic factors (Seyyed et al., 2010).

*Candida albicans* was less susceptible to the extracts *C. citrinus*. The ethanol extract had significant inhibition as total inhibition was observed. This is because *C. albicans* produces hydrolytic enzymes such as haemolysin, phospholipases and proteases which increases its virulence (Fusco-Almeida et al., 2012). The fungus is also capable of forming biofilms and this may have increased it resistance to the extracts (Fusco-Almeida et al., 2012).

MIC determination was done for the DCM, Ethyl acetate and acetone extract against *S. aureus* The DCM extract had an MIC of 25 µg/ml. Dichloromethane is a solvent known to extract terpenoids (*C. citrinus* contains terpenoids such as tormentic acid, which is a triterpenoid.

The MBC was further determined by plating the inoculums from the wells of 25, 50 and 100 µg/ml. The *S aureus* was only inhibited and no MBC was observed as the number of colonies from the plate increased with the decrease in concentration of the extract. The MBC
may be higher than 100 µg/ml and if higher concentrations are used the extracts may have bactericidal effects.

The Ethyl acetate extract had an MIC of 50 µg/ml and no MBC was recorded against \textit{S. aureus} at 100 µg/ml. The acetone extract had an MIC of 100 µg/ml showing that the highest concentration of the extract was the one which inhibited the growth of \textit{S. aureus}.

5.3 **Biocide Formulations**

5.3.1 **Formulation 1**

Linear alkyl benzene sulphonylic acid was the surfactant used in disinfectant formulation 1. It is used as a major ingredient in making household detergents (Ivanković and Hrenović, 2010). It is an anionic surfactant which has good performance. It is biodegradable and therefore environmentally friendly. It is used as a foaming agent and as an emulsifier (Ivanković and Hrenović, 2010).

It has been incorporated in formulations and used for hard surface cleaning. It is a surface cleaning active ingredient and that made it suitable for use in biocide formulation (Nahler, 2009). The surfactant used in this formulation was acidic and therefore it was neutralized by sodium hydroxide. Sodium hydroxide was added so that the pH was adjusted to 7 to make sure that the active compound was not exposed to acidic or alkaline conditions.

5.3.2 **Formulation 2**

Glycerin was used in formulation 2 because of its versatile and unique chemical and physical properties. It is nontoxic to the environment and to humans therefore can be used anywhere. Glycerin is water soluble, colorless, viscous and hygroscopic liquid. It is abundant in nature in the form of triglycerides. It is used in formulations as it is highly stable and miscible with various substances. It also has high solvent power and solubility which makes it suitable for
use in formulations. It has high viscosity and therefore used as a thickening or bodying agent in liquid preparations.

5.4 Biocide tests: Agar disk diffusion method

5.4.1 Formulation 1

The formulations had antimicrobial properties as a zone of inhibition was observed around the disks containing the base only i.e. sulphonic and sodium hydroxide. The mixture is suitable for use as a surface cleaner but in this case, it had antimicrobial properties and therefore made it difficult to assess the activity of the extract included in the formulation.

The linear alkyl benzene sulphonate have been reported to be effective against suspensions of *E. coli, Staphylococcus aureus* and *Streptococcus agalactiae* after a contact time of 2 minutes (Nahler, 2009). This explains the ZOI around the disks containing the base only i.e. sulphonic acid and sodium hydroxide.

96 % sulphonic acid was used and it was very strong as compared by the 1.94% used by Nahler (2009). Sodium hydroxide is an alkali used for cleaning in food and dairy industries. It also have antimicrobial activities and this explains the ZOI observed around the disk containing the base only (Jeffrey, 2016).

Therefore, the combined antimicrobial effects of sulphonic acid and sodium hydroxide were responsible for the antimicrobial properties of the base. This made the research difficult as the effect of the extract in the formulation was not clear.

*S. aureus* was more susceptible to formulation 1 as compared to the Gram-negative bacterium *E. coli*. ZOI were observed in plates containing the bacterium this is due to the cell wall differences between the Gram-positive and Gram-negative bacteria (Cock, 2008)
Sulphonic acid is an anionic surfactant and according to Moore (1997), the site of damage of the anionic surfactants is the cytoplasmic membrane. The permeability of the Gram-positive bacterium (S. aureus) caused the bacterium to be susceptible to the formulation.

This is due to the differences in the cell membrane structure and composition of the Gram negative and positive bacteria (Wu et al., 2016). S. aureus is a Gram-positive bacterium and therefore has a single peptidoglycan layer.

According to Wu et al. (2016) Gram negative bacteria E. coli was resistant to the formulation as it has a thick lipopolysaccharide layer which reduces the permeability of the membrane. The anionic surfactant used targeted the cytoplasmic membrane and with the reduced permeability of the membrane the formulation could not pass to the cytoplasmic membrane.

This shows that the bacterium is easy to control using the formulation as compared to Gram negative bacterium E. coli which was less susceptible to the formulation. Therefore the S. aureus is easy to control as compared to E. coli and the diseases caused by the resistant bacteria will continue to be on rise.

5.4.2 Formulation 2

The formulation was able to inhibit the growth of S. aureus and C. albicans as shown by the ZOI on the agar plates with the microbes. E. coli was resistant to the formulation and no ZOI was observed. The disks containing the base only which is glycerin had no zone of inhibition thereby explaining the fact that the base had no antimicrobial properties.

This shows that it was a good base and therefore is suitable for use as a base for disinfectant formulations. The use of the glycerine base made it possible and easy to evaluate the activity of the extract in the formulation.
S. aureus was susceptible to the formulation at 1, 2 and 4 % weight of the extract. The extract was able to inhibit the growth of the bacterium this is due to the presence of phytochemicals in the extract i.e. the DCM extract used in the formulation. The extract has antimicrobial activities as reported by Hamed et al. (2017).

The phytochemicals in the extract disrupted the cell walls (Simoes et al., 2009). Hydrophobicity is the common characteristic of the plant extract and it causes the membrane to be permeable increasing the susceptibility of the test organism to the extract, causing leakage of ions and cell death (Lalitha and Jayanthi, 2012).

The Gram-negative bacteria was less susceptible to the formulation as no ZOI was observed. The cell wall of Gram negative bacteria acts as a barrier for many substances and this increase their resistance to disinfectants (Cock, 2012).

5.4.3 In loco biocide tests for Formulation 2

The floor microbes were susceptible to the disinfectant formulated and this was explained by the reduction in bacterial and fungal colonies before and after the application of the disinfectant. Glycerine has no antimicrobial properties and this explains the result obtained after its application on the floor. It was not able to inhibit the growth of the floor microbes. Microbes such as E. coli, S. aureus, and P. aeruginosa reside on hard surfaces as well as fungal spores in the presence of moisture. Cleaning and disinfectant application reduces the chances of contamination from the microbes from the floor surfaces (Gupta, 2017).
6 CHAPTER SIX

6.1 Conclusion

The extracts of *Callistemon citrinus* leaves have antimicrobial properties against *S. aureus* and *Candida albicans*. *S. aureus* was more susceptible to the extracts whereas the Gram-negative bacteria was resistant to the extracts. The Dichloromethane extract can be used in disinfectant formulation using bases such as glycerine which does not alter the active compound in the extract. The biocide produced was active against *S. aureus* and *C. albicans* whereas *E. coli* was resistant to the disinfectant.

6.2 Recommendations

The research must be expanded and come up with new formulations for use to disinfect various surfaces. Other formulations for basic human hygiene can also be made to manufacture sanitizers and herbal soaps. The study can also be improved by making use of microbes which reside on floor surfaces, starting by isolating them and using them to carry out the disinfectant tests. The antimicrobial tests done on the extracts can also be improved by using automated antimicrobial testing machines to improve accuracy.
References


https://doi.org/10.9734/ejmp/2016/27987


https://doi.org/10.4172/2167-0412.1000196


https://doi.org/10.1002/9780471729259.mca04ds11

Cock, I., 2008. Antibacterial Activity of Selected Australian Native Plant Extracts Link to published version Antibacterial Activity of Selected Australian Native Plant Extracts. J.

https://doi.org/10.5530/pc.2012.3.11


https://doi.org/10.1099/jmm.0.045054-0

Gupta, M., 2017. No Title.


Health, D.G., Commission, E., 2009. Effects of Biocides on antibiotic resistance Level 2 -
Details on Effects of Biocides 1–25.


Kramer, A., Assadian, O., 2016. Survival of Microorganisms on Inanimate Surfaces. https://doi.org/10.1007/978-3-319-08057-4


Sigma-aldrich, 2013. MTT assay 実験レシピ 7335.


comparative 193–199.


Appendices

Appendix 1: Effect of the standard drug on *E. coli* 0157

![Effect of ciprofloxacin on E. coli](image)

Appendix 2: Effect of standard drug on *S. aureus*

![Effect of ciprofloxacin on S. aureus](image)
Appendix 3: Effect of the standard drug on *C. albicans*

![Graph showing effect of miconazole on C. albicans](image)

Appendix 4: Microtitre plates showing bioactivity of the extracts

*S. aureus*