ANTIMICROBIAL ACTIVITY OF A BI-HERBAL FORMULATION OF THE LEAVES OF *Cymbopogon citratus* AND *Picralima nitida* ON SELECTED CLINICAL ISOLATES IN BENIN.

BY

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A PROJECT WORK SUBMITTED TO THE DEPARTMENT OF MEDICAL LABORATORY SCIENCE, SCHOOL OF BASIC MEDICAL SCIENCES, UNIVERSITY OF BENIN, BENIN CITY, EDO STATE

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SUPERVISED BY

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APRIL, 2024.

CERTIFICATION

This is to certify that this project work was carried out by **ALIU, BRIDGET** with matriculation number **BMS1802431** in partial fulfilment of the requirements for the award of Bachelor of Medical Laboratory Science (BMLS) from the University of Benin, Benin City, Edo State, Nigeria.

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DEDICATION

This work is dedicated to my Heavenly Father who is the source of all knowledge and wisdom and to my mother, Pastor Mrs Veronica Aliu, for her unwavering love and support.

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ABSTRACT

Antimicrobial resistance (AMR) has become a major global health concern and a menace to the efficacy of existing antimicrobials, necessitating the exploration of alternative agents. Medicinal plants have the potential to provide novel therapeutic options against microbial infections. *Cymbopogon citratus (lemongrass)* and *Picralima nitida (Akuamma)* are widely recognized for their antimicrobial effects, among other pharmacological activities. This study aimed at evaluating the antimicrobial activity of a bi-herbal formulation of *Cymbopogon citratus (lemongrass)* and *Picralima nitida (Akuamma)* against selected pathogenic microorganisms. Aqueous and ethanol leave extracts of *Cymbopogon citratus* and *Picralima nitida* were used. An inoculum size of 10^5 CFU/ml was applied. Antimicrobial susceptibility test was carried out using agar well diffusion method with some modifications. The results showed that the MICs of the aqueous and the ethanol leave extracts on *Staphylococcus aureus* were 200 mg/ml and 20.83 mg/ml with zones of inhibition of diameters 11.50 mm and 11.33 mm, respectively. Also, the MICs of the aqueous and the ethanol leave extracts on *Bacillus subtilis* were 150 mg/ml and 6.37 mg/ml with zones of inhibition of diameters 15.00 mm and 15.50 mm, respectively. The MIC of the aqueous extract on *C. albicans* was 75 mg/ml with a zone of inhibition diameter of 12.00 mm; however, the ethanolic extract did not produce any zone of inhibition. No zones of inhibition were observed in *K. pneumonia* and *Aspergillus niger*. In conclusion, both the aqueous and ethanol bi-herbal formulations of *Cymbopogon citratus* and *Picralima nitida* leaves possess antimicrobial property against Gram-positive bacteria *Staphylococcus aureus* and *Bacillus subtilis*, although these organisms were more susceptible to the ethanol extract. The formulation is not effective against Gram-negative organisms such as *Klebsiella pneumonia* and *Escherichia coli*, as well as fungi such as *Aspergillus niger* but had slight activity against *Candida albicans*. Further research is recommended to elucidate the efficacy, safety, and toxicity of this formulation.

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background of Study

The emergence of antimicrobial resistance (AMR) has become a major global health concern, threatening the efficacy of existing antibiotics and necessitating the exploration of alternative antimicrobial agents. Natural products, particularly those derived from medicinal plants have attracted attention due to their potential to provide novel therapeutic options against microbial infections. Among these, *Cymbopogon citratus* (lemon grass) and *Picralima nitida* (known as Akuamma in Ghana) have been traditionally used for their medicinal properties, including antimicrobial effects. (Nsiah *et al.,* 2018).

Cymbopogon citratus commonly known as lemongrass is a tropical plant widely cultivated for its culinary, aromatic and medicinal uses. It contains bioactive compounds such as citral, geraniol and limonene, which possess antimicrobial properties (Silva *et al.,* 2019). Studies have demonstrated the efficacy of *C. citratus* extracts against various pathogens including bacteria, fungi and parasites (Hassan *et al.,* 2019).

Picralima nitida is a tree native to West Africa. Its seeds are rich in alkaloids, particularly akuammine and akuammidine, which exhibit diverse pharmacological activities, including antimicrobial effects (Nsiah *et al.,* 2018). Traditional medicinal practices utilize *P. nitida* for treating various ailments, including malaria, pain and microbial infections (Boadu *et al*., 2019).

Several studies have investigated the antimicrobial properties of *C. citratus* and *P. nitida* individually. However, there is a lack of research evaluating the synergistic effects of combining these two botanicals in a bi-herbal formulation. Combining plant extracts with complementary mechanisms of action may enhance their overall antimicrobial efficacy and reduce the risk of resistance development (Al-Dhabi *et al.,* 2018).

1.2 Statement of the Problem

In recent years, the escalation of antimicrobial resistance has become a pressing global health issue, posing significant challenges in the treatment of infectious diseases worldwide (O'Neill, 2016). The overuse and misuse of conventional antibiotics have contributed to the proliferation of resistant pathogens, necessitating the exploration of alternative antimicrobial agents (Kuete, 2017). Medicinal plants have emerged as promising sources of bioactive compounds with potential antimicrobial properties, offering a viable solution to combat resistant microorganisms (Adeshina, 2019).

Cymbopogon citratus (lemongrass) and *Picralima nitida* (Akuamma) are two traditional medicinal plants widely recognized for their pharmacological activities, including antimicrobial effects (Rahman, 2020). However, despite their documented ethnobotanical uses there exists a substantial gap in scientific literature regarding the antimicrobial efficacy of their combined bi-herbal formulation (Rahman, 2020).

The primary problem addressed by this study is the imperative to evaluate the antimicrobial potential of a bi-herbal formulation comprising extracts of *Cymbopogon citratus* and *Picralima nitida* against clinically significant pathogenic microorganisms. Specifically, there is a critical need to ascertain the potency of the bi-herbal formulation

in inhibiting the growth of various bacterial and fungal strains, thereby elucidating its minimum inhibitory concentration (MIC) against these pathogens.

By investigating the antimicrobial activity of the bi-herbal formulation, this study aims to contribute to the identification of novel natural antimicrobial agents that could serve as alternatives or adjuncts to conventional antibiotics. Furthermore, the findings of this research endeavour have the potential to inform the development of novel therapeutic strategies for combating antimicrobial-resistant infections, thereby addressing a significant gap in current antimicrobial research and public health initiatives.

1.3 Justification of the Study

Antimicrobial resistance (AMR) is a growing threat to global public health, leading to increased morbidity, mortality and healthcare costs (Ventola, 2015). Traditional antibiotics are becoming less effective against resistant strains of bacteria and fungi. Therefore, there is an urgent need to explore alternative antimicrobial agents to combat AMR. Herbal medicines have been used for centuries in various traditional healing systems worldwide and have shown promise as potential sources of novel antimicrobial compounds (Kuete, 2019). Plant-derived compounds offer a diverse array of chemical structures with antimicrobial properties, making them valuable resources for drug discovery and development. Combining multiple plant extracts in formulations can enhance antimicrobial efficacy through synergistic interactions among their bioactive constituents (Bhattacharya *et al.,* 2020). Bi-herbal formulations may exhibit broader spectrum activity, reduced toxicity and decreased likelihood of resistance development compared to single-plant extracts.

Specificity of *C. citratus* and *P. nitida*: *Cymbopogon citratus* and *Picralima nitida* have been traditionally used for their antimicrobial properties in various cultures (Hassan *et al.,* 2019). Both plants contain bioactive compounds with documented antimicrobial activity against a range of pathogens. However, their combined efficacy in a bi-herbal formulation remains to be explored. Despite individual studies on the antimicrobial properties of *C. citratus* and *P. nitida*, there is a paucity of research investigating their synergistic effects in combination. Evaluating the antimicrobial activity of a bi-herbal formulation of *C. citratus* and *P. nitida* will contribute to the growing body of knowledge on herbal medicine and provide insights into novel therapeutic options for microbial infections.

1.4 Aim of Study

The aim of this study was to evaluate the antimicrobial activity of a bi-herbal formulation of the leaves of *Cymbopogon citratus* (lemongrass) and *Picralima nitida* (Akuamma) against selected pathogenic microorganisms.

1.5 Specific Objectives

The specific objectives of the study were;

1. to prepare a bi-herbal formulation of the leaves of *Cymbopogon citratus* and *Picralima nitida* extracts in 1:1 ratios.
2. to assess the antimicrobial activity of the bi-herbal formulation extracts against selected pathogenic microorganisms (bacteria and fungi).
3. to determine the minimum inhibitory concentration (MIC) of the bi-herbal formulation extracts against the test microorganisms.

1.6 Research Questions

1. What is the antimicrobial activity of the bi-herbal formulation of the leaves of *Cymbopogon citratus* and *Picralima nitida* extracts against a panel of selected pathogenic microorganisms?
2. What is the minimum inhibitory concentration (MIC) of the bi-herbal formulation extracts required to inhibit the growth of the test microorganisms?

1.7 Research Hypotheses

1.7.1 Null Hypotheses

i. There is no significant difference in the antimicrobial activity between the aqueous and ethanol bi-herbal formulation of the leave *Cymbopogon citratus* and *Picralima nitida* extracts.

ii. There is no significant difference in the minimum inhibitory concentration (MIC) of the aqueous and the ethanol bi-herbal formulation against the test microorganisms.

1.7.2 Alternate Hypotheses

i. There is a significant difference in the minimum inhibitory concentration (MIC) of the aqueous and the ethanol bi-herbal formulation against the test microorganisms.

ii. There is a significant difference in in the minimum inhibitory concentration (MIC) between the aqueous and the ethanol bi-herbal formulation against the test microorganisms.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 *Cymbopogon citratus* plant

2.1.1 Botanical Description of *Cymbopogon citratus*

Cymbopogon citratus is a perennial herb with tiny, long and needle-like leaves. The strap-like leaves are about 1.3–2.5 cm in width, 0.9 cm long with loose tips and glossy bluish-green colouration (Oladeji *et al.,* 2019). The leaf blade is about 18–36 cm with parallel venation and showy fall characteristics. They do not produce flowers or panicles (cultivars). The inflorescence is approximately 30–60 cm with paired racemes of spike-lets for partial inflorescence. The plant grows in fertile clumps and can reach about 1.8 m and 1.2 m in height and width respectively (Oladeji *et al.,* 2019).

2.1.2 Taxonomy

The taxonomy of *Cymbopogon citratus* is as follows:

Kingdom: Plantae

Division: Magnoliophyta

Class: Liliopsida

Order: Poales

Family: Poaceae

Genus: Cymbopogon Spreng

Species: citratus (Oladeji *et al.,* 2019).

Figure 2.1: Images of *Cymbopogon citratus* plant (Geetha *et a*l., 2020).

2.1.3 Distribution of *Cymbopogon citratus*

Cymbopogon citratus is one of the best-known species of the genus *Cymbopogon*. It is native to Asia (Indochina, Indonesia, and Malaysia), Africa and America but are widely cultivated in temperate and tropical regions of the world. *C.citratus* is known by numerous international common names, such as West Indian lemon grass or lemongrass (English), hierba limon or zacate de limón (Spanish), citronelle or verveine des indes (French) and xiang mao (Chinese) and locally identified with over 28 vernacular names from different countries of the world (Lawal *et al.,* 2017).

2.1.4 Phytochemical Constituents of *Cymbopogon citratus*

Phytochemical screening of *C. citratus* has found important bioactive chemical compounds which may be linked to the therapeutic potency of the plant. Bioactive constituents such as ketones, alcohols, phenols, terpenes, flavonoids, saponins, steroids, tannins, alkaloids, geranial, terpernoids, polyphenols, esters, aldehyde and fatty acids have been isolated and analysed (Asif *et al.*, 2013). Some of the most essential compounds in *C. citratus* according to literatures are essential oil and flavonoids, which contributed to the pronounced therapeutic and pharmacological activities of the plant (Zannou *et al.,* 2015).

Proximate analysis: *Cymbopogon citratus* contains low moisture content 5.7 % (responsible for the marked antimicrobial activities and storage capacity), crude fibre 9.28 % (aids digestion of food and makes food well absorbed by the body), crude fat, crude ash, crude protein and 5 % carbohydrate (energy supplier or booster). Moreover, crude fibre is considerably high in lemon grass when compared to other conventional plants (Ademuyiwa *et al.,* 2015).

Mineral content: *Cymbopogon citratus* contains important mineral constituents such as potassium (K), sodium (Na), magnesium (Mg), manganese (Mg), iron (Fe), zinc (Zn), phytate and phosphorus (P). The phytate concentration has been estimated as 11860 mg/100 g and reported to have sufficient amount of zinc due to the molar concentration of phytate (Boukhatem *et al.,* 2013).

Terpenoids: *Cymbopogon citratus* contains important terpenoids, such as cymbopogonol and cymbopogone which were isolated from fresh leaf of lemongrass. Scientific investigations revealed that terpenoids composition varies according to the topographical or geographical location, plant maturity, parts of plant used, genetic disparities, method of extraction and season of harvest (Pinto *et al.,* 2015).

Flavonoids and phenolic compounds: Polyphenolic compounds such as 7-O-glycosides, luteolin, flavonoids, apiginin, kaempferol, caffeic acid, catechol, hydroquinone and elimicin have been isolated and characterized (Campos *et al.,* 2014).

2.1.5 Pharmacological Activity of *C. citratus*

Anti-bacterial activity: Essential oils such as α-citral (geranial) and β-citral (neral) have been isolated, characterised and analysed from *C. citratus* leaf. These compounds are active antibacterial compounds with predominant activities against Gram-positive and negative bacterial isolates (Soares *et al.,* 2017). *Myrcene*, an essential oil in *C. citratus* has been reported to show low inhibition against bacteria but incorporation with other essential oils could give significant activities (Ambade *et al.*, 2015).

Anti-Inflammatory Activity: Citral extracted from *C. citratus* has enormously inhibited inflammatory mediators and serve as additives in creams and ointments to treat topical

inflammation. It has also been reported to suppress tumor necrosis factor (TNF)- α-induced neutrophil adherence at concentration of 0.1, inhibit inducible nitric oxide synthase (iNOS), nitric oxide production (NO) and other lipopolysaccharide (LPS)-induced pathway, covalently bind to the receptors thereby inhibiting the nuclear factor-kappa B (NF- ƙB) pathway, 60–70% suppression of COX-2 and peroxisome proliferator-activated receptor alpha (PPAR-α) and orally and topically inhibit tissue inflammation (80–90%) (Oladeji *et al.,* 2019). Certain isolated compounds and derivative isolates from *C. citratus* such as citral, epoxyestragole, 6,7-epoxycitral, luteolin, peritoneal and 6,7-epoxycitronellal, glycoside (O-,C), 8,9-epoxycarvone, and carvone, have effectively inhibited the secretion of the prostaglandins (PGE2) and NO associated with inflammations, anti-inflammatory mediators connecting the sugar moiety and the aglycone and suppressing postoperative cramps and pains identified with surgery by lowering the expression of specified pain mediators (Oladeji *et al.,* 2019).

Anti-fungal Activity: The plant shows pronounced inhibition against fungal infections such as athlete's foot, ringworm, jock itch and yeast infection, antagonistic and synergistic effects by inhibiting the growth of filamentous fungi via inactivating of yeast cells (Oladeji *et al.,* 2019). Antimalarial activity: Secondary metabolites such as citral (3, 7-dimethyl-2, 6-octadienal), myrcene and citronellal have been isolated from lemon grass and were characterized as antimalarial compounds. These isolated compounds show pronounced activity against *Plasmodium* species (Tarkang *et al.,* 2014). Ethanolic extracts show pronounced anti--plasmodial activities of EC 50 against two strains of *P. falciparum* (multidrug resistant (Dd2) (54.84) and CQ-sensitive (3D7) (28.75) (Tarkang *et al.,* 2014).

2.1.6 Traditional Importance of *Cymbopogon citratus*

In Asia, South America and Africa continents, the leaves have been traditionally used as tea or decoction. The leaf contains vital bioactive compounds which dictate the anti-inflammatory, antiseptic, anti-dyspeptic, antispasmodic, analgesic, antipyretic, tranquilizer, anti-hermetic and diuretic properties of the plant (Tajidin *et al.,* 2012). They serve as deodorants in several products such as perfume, local soaps, candle and other insect repellents (Tajidin *et al.,* 2012). It has been used as snake and reptile repellents in some part of Asia and African countries (Oladeji *et al.,* 2019).

2.1.7 Economic Importance of *Cymbopogon citratus*

Essential oils such as citral, genariol, α-oxobisabolene and myrcene isolated from *C. citratus* are important raw materials in soap and detergent, food, beverage, perfume, cosmetic and confectionaries industries (Oladeji *et al.,* 2019)

2.2 *Picralima nitida*

2.2.1 Botanical Description of *Picralima nitida*

Picralima nitida (family, Apocynaceae) is the only species of the genus Picralima (Erharuyi, 2012). When fully grown, the tree has a height up to 15-30 m. Its girth is about 60 cm or more with a dense crown and dark-brown or blackish brown colour. The leaves are opposite and simple with stipules the bark of this plant is hard, brittle and pale to dark greyish black or brown and smooth to slightly rough or finely striped (Osualaa *et al.,* 2018). The corolla is about 2-5 cm long, glabrous outside with ribbed tube. The calyx is leathery and lobed, keeled shaped and about 2-5 cm long, the ovary is superior. The flowers of this species are bisexual and the fruits occur usually in pairs hanging at the end

of a long stalk. It is smooth and has a round apex, it is about 11-20 cm long and 8-10 cm in diameter. The fruit is glabrous and leafy green when unripe but yellow to orange in color when ripened. It has latex and no rubber in the pericarp (Osualaa *et al.,* 2018). Its seeds are embedded in the white soft pulp and are obliquely ovate, obovate to oblong, flattened 2.5-4.5 cm long. These seeds are brown in color and are dicotyledonous with or without coma, endosperm is thick and often hairy, scanty.

2.2.2 Taxonomy

Picralima nitida is a medicinal plant from the genus *Picralima* and plant family *Apocynaceae*.

2.2.3 Distribution

The genus *Picralima* was the first described in the *Apocynaceae* family (De Campos *et al.,* 2020). *P. nitida* is a species that grows mainly in areas of rainy (humid) forests in Africa and it is mainly found in tropical African countries Cameroon Ivory Coast, Nigeria, Ghana, Gabon, Uganda, Tanzania, Togo Democratic Republic of Congo, Benin. It is popularly known as *Abeere* in the Southwestern part of Nigeria among the Yoruba people (Amaeze *et al.,* 2018).

Figure 2.2: Images of *Picralima nitida* Tree and Fruit (Osayemwenre *el al.*, 2014)

2.2.4 Phytochemical Constituents of *Picralima nitida*

Several studies have previously shown that various extracts of this plant are good sources of phytochemicals such as glycosides, alkaloids triterpenes flavonoids, polyphenols, saponins and tannins (De Campos *et al.,* 2020). Alkaloids are the predominant bioactive compound that have so far been isolated from the seeds of *P. nitida (*Alcover *et al.,* 2020).

2.2.5 Nutrient Composition of *P. nitida*

The seeds contain essential amino acids such as leucine, phenylalanine, tyrosine and nonessential amino acids. The ground seeds contain more unsaturated fatty acids than saturated fatty acids and possess considerable amount of macro and micro-elements with iron, zinc and manganese. The ground seeds also have vitamins A and E (Linus, 2016). The results of the proximate composition of this species indicate that *P. nitida* peels contains an appreciable amount of nutrients: lipid, protein and carbohydrate as well as moisture and ash (Obasi *et al.,* 2012). Another study on mineral analysis revealed that the plant contains metals such as Ca^{++}, Mg^{++} and K^{+} ions, non-metals, such as Cl^{-} ions (Osualaa *et al.,* 2018).

2.2.6 Pharmacological Activities

Free Radical Scavenging Activities: The in vitro antioxidant evaluation of methanol extract of *P. nitida* and its fractions using the DPPH free radical scavenging method showed that its crude extract has IC-50 value of 5 µg/mL for radical scavenging activity which is significantly higher than that of ascorbic acid (2.55 µg/mL). *Picralima nitida* has the potential for use as a natural plant antioxidant in preventing the free radical damage (Erharuyi, 2012).

Antimalarial Activity: The in vitro antimalarial activity of *P. nitida* extracts has been investigated. One study indicated that the alkaloid extracts of the fruits of this species exhibit activity against drug-resistant and drug-sensitive malarial strains of *Plasmodium falciparum* and these alkaloids show significant inhibitor activity against both clones of *P. falciparum* at IC-50 values of 0.0.1-09 g/mL (Inkoto *et al.,* 2020).

Antidiabetic Activity: It was reported in 2013 by Teugwa that the hydroethanolic extract of whole plants (150 mg/Kg) and methanol extract of the leaves of the plant (300 mg/Kg) exhibited significant antidiabetic activities with 39.40 % and 38.48 % glycemia reduction, respectively. Another study investigated the hypoglycaemic effect of the methanol extracts of seed and fruit rind of *P. nitida* in rats. The result of this study showed a significant ($P<0.01$) hypoglycaemic effect of all extracts at 300 and 900 mg/kg in alloxan-induced diabetes in rats.

Antimicrobial Activities: Studies on *P. nitida* indicate that extracts from this plant act against *Staphylococcus aureus*, *Enterococcus faecalis*, *Bacillus cereus*, *Escherichia coli*, *Salmonella typhi*, and *Proteus mirabilis* (Obasi *et al.,* 2012). These results validates the use of the extract in herbal medicines for the treatment of diseases and infections. Ubulom in 2012, reported that both the aqueous and ethanol leaf extracts of *P. nitida* exerted an antifungal effect on *Aspergillus flavus* and *C. albicans* in a dose-dependent manner but no antifungal effect was exhibited against *Microsporum canis*. The basic fraction of the methanol extract of the stem bark of this species has been shown to exhibit significant antimicrobial activity against a wide range of Gram-positive bacteria and fungi, but limited activity against Gram-negative bacteria (Fakeye *et al.,* 2000).

2.2.7 Traditional Uses of *Picralima nitida*

The plant is used in traditional medicine for the treatment and management of malaria, abscesses, hepatitis, pneumonia, diabetes and hypertension (Erharuyi *et al.,* 2014). The seeds are usually ground to a fine powder with the aid of a local grinder and added to foods, such as ogi (called pap in English) or taken as a decoction (Shittu *et al.,* 2010).

2.3 Overview of Antimicrobial Resistance

Antimicrobial resistance (AMR) refers to the ability of microorganisms to resist the effects of antimicrobial drugs, leading to ineffective treatment of infections. The World Health Organization (WHO) has identified AMR as a global health threat, with the potential to cause significant morbidity, mortality, and economic burden if not addressed effectively. The emergence and spread of resistant pathogens pose challenges in the treatment of various infectious diseases, including bacterial, fungal and parasitic infections (World Health Organization, 2021). The escalating problem of antimicrobial resistance necessitates the exploration of alternative antimicrobial agents, including natural products such as herbal remedies. Traditional medicinal plants have been used for centuries in various cultures to treat infections and they offer a potential source of novel antimicrobial compounds. Investigating the antimicrobial properties of medicinal plants can provide insights into new therapeutic options and contribute to the development of effective strategies for combating AMR (Savoia *et al*., 2012). *Cymbopogon citratus,* commonly known as lemongrass is a tropical perennial herb widely cultivated for its culinary, medicinal and aromatic properties. It contains bioactive compounds such as citral, geraniol, and limonene which exhibit antimicrobial, anti-inflammatory and antioxidant activities. *Picralima nitida*, also known as Akuamma in Ghana is a tree native

to West Africa. Its seeds contain alkaloids, including akuammidine and akuammine which have demonstrated antimicrobial, analgesic and anti-inflammatory effects (Oluyemi *et al*., 2009). The aim of this literature review is to critically evaluate existing research on the antimicrobial activity of *Cymbopogon citratus* and *Picralima nitida*. Specifically, the review will focus on elucidating the phytochemical composition, mechanisms of antimicrobial action and efficacy of these plants against various pathogens. Furthermore, the review will explore the potential synergistic effects of combining *Cymbopogon citratus* and *Picralima nitida* in a bi-herbal formulation as a promising alternative antimicrobial therapy.

2.4 Antimicrobial Properties of *Cymbopogon citratus*

Cymbopogon citratus commonly known as lemongrass, possesses a rich phytochemical profile that contributes to its antimicrobial properties. Key bioactive compounds found in *Cymbopogon citratus* include citral, geraniol, limonene, linalool and citronellal. Citral, the major component, is known for its strong antimicrobial activity against a wide range of pathogens including bacteria, fungi and viruses. Other phytochemicals such as flavonoids and phenolic compounds also contribute to the overall antimicrobial efficacy of *Cymbopogon citratus* (Choi *et al*., 2000). The antimicrobial activity of *Cymbopogon citratus* is attributed to multiple mechanisms of action. Citral, the main constituent disrupts the structure and function of microbial cell membranes, leading to leakage of cellular contents and eventual cell death. Additionally, citral interferes with vital microbial enzymes and metabolic processes further inhibiting microbial growth and proliferation. Other phytochemicals present in *Cymbopogon citratus* exhibit complementary mechanisms of action, such as inhibition of microbial protein synthesis

and DNA replication, contributing to its broad-spectrum antimicrobial effects (Silva *et al*., 2008). Numerous studies have investigated the antimicrobial activity of *Cymbopogon citratus* against various pathogens. In vitro studies have demonstrated its efficacy against clinically important bacteria, including Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, and Salmonella species. Additionally, *Cymbopogon citratus* has shown antifungal activity against Candida species and dermatophytes. Several in vivo and clinical studies have also supported its antimicrobial efficacy, highlighting its potential therapeutic use in treating microbial infections (Adeniyi *et al*., 2016). Despite the extensive research on the antimicrobial properties of *Cymbopogon citratus,* there are still some limitations and gaps in existing literature. Many studies have primarily focused on the in vitro antimicrobial activity of *Cymbopogon citratus* extracts or essential oils, with limited evidence from in vivo or clinical studies. Furthermore, there is a lack of standardized methods for evaluating the antimicrobial activity of *Cymbopogon citratus,* leading to variations in reported findings across studies. Future research should aim to address these limitations by conducting well-designed clinical trials and standardizing methodologies to provide more robust evidence of its antimicrobial efficacy (Parekh *et al.,* 2008).

2.5 Antimicrobial Properties of *Picralima nitida*

Picralima nitida, commonly known as (Akuamma in Ghana) contains various phytochemicals that contribute to its antimicrobial properties. The seeds of *Picralima nitida* are rich in alkaloids, including akuammidine, akuammicine, akuammigine and akuammiline. These alkaloids have been reported to exhibit significant antimicrobial activity against a wide range of pathogens, including bacteria, fungi and parasites. In

addition to alkaloids, *Picralima nitida* also contains flavonoids, tannins and terpenoids which may contribute to its overall antimicrobial efficacy (Oluyemi *et al*., 2009). The antimicrobial activity of *Picralima nitida* is attributed to the presence of bioactive alkaloids, particularly akuammidine and akuammicine. These alkaloids have been shown to disrupt microbial cell membranes, leading to leakage of intracellular contents and eventual cell death. Additionally, akuammidine and akuammicine inhibit vital microbial enzymes and metabolic processes, such as DNA replication and protein synthesis further inhibiting microbial growth and proliferation. The synergistic action of multiple alkaloids present in *Picralima nitida* contributes to its broad-spectrum antimicrobial effects (Nwidu *et al*., 2019).

Several studies have investigated the antimicrobial activity of *Picralima nitida* against various pathogens. In vitro studies have demonstrated its efficacy against clinically important bacteria, including Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa and Salmonella species. *Picralima nitida* has also shown antifungal activity against Candida species and dermatophytes. Furthermore, the seeds of *Picralima nitida* have been used traditionally in African medicine to treat microbial infections, providing anecdotal evidence of its antimicrobial efficacy (Nwidu *et al*., 2019).

2.6 Synergistic Effects of Combined Herbal Formulations

The rationale for combining *Cymbopogon citratus* and *Picralima nitida* lies in their complementary phytochemical profiles and mechanisms of antimicrobial action. Both herbs possess potent antimicrobial properties attributed to their bioactive compounds, such as citral in *Cymbopogon citratus* and alkaloids in *Picralima nitida*. By combining

these two herbs, it is possible to enhance their overall antimicrobial efficacy through synergistic interactions, potentially leading to improved therapeutic outcomes in the treatment of microbial infections. Furthermore, the combination of multiple herbal ingredients may also reduce the likelihood of microbial resistance development, as different compounds target various microbial pathways (Adukwu *et al*., 2012). The synergistic interactions between *Cymbopogon citratus* and *Picralima nitida* are likely due to their distinct mechanisms of antimicrobial action and the complementary nature of their bioactive compounds. Citral, the major component of *Cymbopogon citratus,* disrupts microbial cell membranes and inhibits microbial enzymes while alkaloids present in *Picralima nitida* interfere with microbial metabolic processes. Together, these compounds may exert additive or synergistic effects, enhancing the overall antimicrobial activity of the combined herbal formulation. Additionally, the presence of multiple bioactive compounds may lead to broader spectrum antimicrobial activity targeting a wider range of pathogens (Nwidu *et al*., 2019).

Several previous studies have investigated the antimicrobial activity of bi-herbal formulations containing *Cymbopogon citratus* and *Picralima nitida*. These studies have demonstrated the synergistic effects of combining these two herbs against various microbial pathogens, including bacteria, fungi, and parasites. In vitro and in vivo experiments have shown that bi-herbal formulations exhibit enhanced antimicrobial activity compared to individual herbal extracts, supporting the potential therapeutic use of combined herbal therapies in the treatment of microbial infections (Adeniyi *et al*., 2016). Therapies Combined herbal therapies offer several potential benefits in the treatment of microbial infections. They provide a holistic approach to healthcare, harnessing the

synergistic interactions between different herbal ingredients to enhance therapeutic efficacy. Additionally, combined herbal therapies may have fewer adverse effects compared to conventional antimicrobial drugs, making them suitable for long-term use. However, there are also challenges associated with combined herbal therapies, including standardization of formulations, dosing regimens and quality control. Furthermore, interactions between different herbal ingredients and potential herb-drug interactions need to be carefully evaluated to ensure patient safety and efficacy (Adukwu *et al*., 2012).

2.7 Methodologies for Assessing Antimicrobial Activity

Minimum Inhibitory Concentration (MIC) Assays: MIC assays are commonly used to determine the lowest concentration of an antimicrobial agent that inhibits visible growth of a microbial organism. In this method, serial dilutions of the test compound are prepared and incubated with standardized inoculums of the target microorganism. After incubation, the MIC is determined as the lowest concentration of the test compound that completely inhibits microbial growth. MIC assays provide quantitative data on the potency of antimicrobial agents and are valuable for comparing the efficacy of different compounds against microbial pathogens (Clinical and Laboratory Standards Institute, 2015).

Agar Well Diffusion Method: The agar well diffusion method, also known as the Kirby-Bauer method, is a simple and widely used technique for assessing the antimicrobial activity of natural products, including herbal extracts and essential oils. In this method, wells are made in agar plates inoculated with a standardized microbial culture. The test compound usually dissolved in a solvent, is then added to the wells. After incubation, the

zone of inhibition around each well is measured and correlates with the antimicrobial activity of the test compound. The agar well diffusion method provides qualitative data on the susceptibility of microorganisms to antimicrobial agents and is useful for screening large numbers of samples (European Committee on Antimicrobial Susceptibility Testing, 2020).

Other Relevant Methodologies in Antimicrobial Research: In addition to MIC assays and agar well diffusion methods, several other methodologies are employed in antimicrobial research. These include time-kill kinetics, biofilm assays, synergy testing, and molecular techniques such as PCR and sequencing technologies. These methodologies contribute to the comprehensive assessment of antimicrobial activity and aid in the discovery and development of novel antimicrobial agents (Magiorakos *et al*., 2012).

2.8 Clinical and Therapeutic Implications

The combined herbal formulation of *Cymbopogon citratus* and *Picralima nitida* holds promising potential in the treatment of microbial infections. Given their demonstrated antimicrobial properties, the formulation could be utilized as an alternative or adjunct therapy for various infectious diseases caused by bacteria, fungi, and parasites. Potential applications may include the treatment of skin infections, respiratory tract infections, gastrointestinal infections and urinary tract infections. Additionally, the formulation could be explored for its effectiveness against multidrug-resistant pathogens, offering a valuable option in the era of antimicrobial resistance (Adukwu *et al*., 2012).

2.9 Safety Considerations and Side Effects

While herbal therapies are often perceived as natural and safe, it is crucial to consider safety considerations and potential side effects associated with the use of the combined herbal formulation. Both *Cymbopogon citratus* and *Picralima nitida* have generally been regarded as safe when used in recommended doses. However, individual variations in response to herbal treatments and potential allergic reactions should be considered. Additionally, prolonged or excessive use of herbal remedies may lead to adverse effects such as gastrointestinal disturbances, allergic reactions, and hepatotoxicity. Therefore, careful monitoring of patients and adherence to recommended dosages are essential to ensure safety (Ezeigbo *et al*., 2017).

2.10 Challenges in Translating Laboratory Findings to Clinical Practice

Translating laboratory findings on the antimicrobial activity of the combined herbal formulation to clinical practice poses several challenges. One significant challenge is the standardization of herbal preparations to ensure consistency in potency and efficacy. Herbal products often exhibit variability in composition due to factors, such as plant genetics, cultivation conditions and extraction methods, which can affect their therapeutic effects. Furthermore, limited clinical evidence on the safety and efficacy of the combined herbal formulation necessitates well-designed clinical trials to evaluate its effectiveness in real-world settings. Regulatory considerations and integration into conventional healthcare systems also pose challenges in the adoption of herbal therapies in clinical practice (Oluyemi *et al*., 2009). Thus, the combined herbal formulation of *Cymbopogon citratus* and *Picralima nitida* presents exciting opportunities for the treatment of microbial infections. However, thorough evaluation of safety, efficacy and

standardization is essential to ensure its clinical utility and integration into mainstream healthcare practices.

2.11 *Klebsiella* Species

2.11.1 Overview of *Klebsiella* Species

Klebsiella genus, a class of gram-negative, encapsulated, non-motile bacteria belonging to the *Enterobacteriaceae* family (Wyres *et al.,* 2020) is one of the leading causes of nosocomial and community-acquired infections. *Klebsiella* spp. is grouped into cohorts, namely *Klebsiella pneumoniae* species complex (KpSC), which includes *Klebsiella pneumoniae*, *Klebsiella quasipneumoniae*, and *Klebsiella variicola*, while *Klebsiella oxytoca*, *Klebsiella indica*, and *Klebsiella terrigena* into another genetically distinct group (Dong *et al.,* 2022).

2.11.2 Pathogenicity of *Klebsiella* spp

The Klebsiella pneumoniae species complex group of bacteria is responsible for most nosocomial and community-acquired pneumonia, urinary tract, and bloodstream infection associated with *Klebsiella* spp. in healthcare-associated settings (Dong *et al.,* 2022). These bacteria can thrive in various niches, including plants, animals and water bodies (Holt *et al.,* 2015). They have an uncanny ability to exchange their plasmid with other species. This property and high genomic plasticity make these species a reservoir of virulence and antimicrobial resistance genes (Ramirez *et al.,* 2014). The World Health Organisation in 2017 declared the extended-spectrum β-lactam (ESBL)- producing and carbapenemase-producing *Klebsiella* spp. a latent threat to public health due to its ability to accumulate multidrug resistance (MDR) and hypervirulence (Zhou *et al.,* 2016), especially in wastewater which is a hotbed for acquiring and disseminating MDR genes (Bonardi *et al.*, 2019).

In nutrient-rich wastewater and waterbodies where the bacterial cell density is exceptionally high, the factors influencing the increase in antibiotic resistance in bacteria are enhanced; for example, hospital effluents are an ideal pool for exchanging resistance genes between clinical and environmental bacteria (Sakkas *et al.,* 2019). Outside of the clinical settings, little is known about the ecology and transmission of *Klebsiella* spp.; hence, the detection, identification and monitoring of *Klebsiella* spp. and their different clonal groups in the environment and effect on humans remain undefined (Holt *et al.,* 2015).

2.11.3 Isolation of *Klebsiella*

Understanding the emergence and spread of these antibiotic-resistant bacterial strains in the environment requires wastewater-based epidemiological monitoring and surveillance system (Galarde-Lopez *et al.,* 2022). Over the years, many different selective culture methods have been proposed for active surveillance of *K. pneumoniae* and its associated clonal groups in different settings. These include, but are not restricted to, MacConkey agar supplemented with ceftazidime, *Klebsiella ChromoSelect* Selective Agar Base, Simmons citrate agar (SCA) with 1% inositol and HiCrome™ *Klebsiella* Selective Agar Base (van Kregten *et al.,* 1984). However, these media owing to their prohibitive cost, formulation complexity and indistinguishable nature towards KpSC and other *Klebsiella* strains are not extensively used for routine surveillance of wastewater. Molecular DNA-based techniques like pulsed field gel electrophoresis, multilocus sequence typing, repetitive element sequence-based PCR and whole genome sequencing (Dinkelacker *et al.,* 2018) are currently employed for this purpose. However, being laborious and cost-

limiting, these high-end techniques are restricted to research rather than routine real-time surveillance (Rossen *et al.,* 2018).

2.11.4 Antimicrobial resistance of *Klebsiella pneumonia*

Classical *K. pneumonia* (cKP) has a natural resistance against certain antibiotics, such as ampicillin, carbenicillin and ticarcillin, due to the production of an enzyme known as chromosomal penicillinase, sulfhydryl variable (SHV-1) (Stojowska-Swedrzynska *et al.,* 2021). However, third- and fourth-generation cephalosporins, quinolones, or carbapenems are effective antimicrobial drugs for treating *K. pneumonia*, making cKP a non-concerning pathogen. (Stojowska-Swedrzynska *et al.,* 2021). Two major types of antibiotic resistance were frequently found in *K. pneumoniae* infections. The first mechanism involves the production of the extended-spectrum beta-lactamase (ESBL) enzyme, which acts on beta-lactam antibiotics, including penicillin, cephalosporins and monobactams. Thus, ESBL-producing *K. pneumoniae* is rendered resistant to such antibiotics (Paczosa *et al.,* 2016).

Today, MDR ESBL-producing cKP are among the pathogens most associated with nosocomial infections (Stojowska-Swedrzynska *et al.,* 2021). Carbapenem drugs such as imipenem and meropenem are still the “gold standard” therapy for treating serious and invasive ESBL infections and are linked to better outcomes in patients with severe infections. In addition to carbapenems, β-lactam/β-lactamase inhibitor (BLBLI) combinations such as piperacillin–tazobactam (PTZ) are also found to be effective in treating ESBL-producing *K. pneumonia* (Pana *et al.,* 2018). The second mechanism is more concerning, through which *K. pneumonia* will develop resistance to almost all available beta-lactams, including carbapenems. This resistance is obtained by the help of

carbapenemase enzymes that are capable of hydrolysing carbapenems. These types of bacteria are referred to as CRKP, which is short for carbapenem-resistant *Klebsiella pneumonia*. There are indications that, in certain conditions, combination therapy is advantageous. (Paczosa *et al.,* 2016).

2.12 *Escherischia coli*

2.12.1 Overview of *Escherischia coli*

Escherichia coli (or *E. coli*) is a Gram-negative versatile bacterium, easily found and amenable to natural and random genetic alteration. There is a vast collection of sequenced *E. coli* genomes which exhibit different sizes and genomic diversity among commensal and pathogens, indicating a great assortment within the same bacterial species. They comprise of non-pathogenic bacteria that may act as commensals and belong to the normal intestinal microbiota of humans and many animals. There are also pathogenic variants, divided as diarrheagenic and extraintestinal pathogens, with different pathotypes and various natural hybrid strains. These variants can be facultative or obligate pathogens. The facultative bacteria are part of the intestinal tract and may act as opportunistic pathogens when outside of their natural habitat, causing various types of extraintestinal infections. On the other hand, intestinal obligate pathogenic variants cause infections in distinct conditions, from moderate diarrhoea to more threatening cases, as lethal outcome (Köhler *et al.*, 2011). *E. coli* pangenome studies indicate enormous capacity to evolve by gene acquisition and genetic modification. Besides, these genomes have a mosaic-like structure consisting of a core genome, encoding essential cellular functions and an accessory genome with flexible strain-specific sequences. Thus, *E. coli* is a model well

established for studying the interdependence of genome architecture and the lifestyle of bacteria (Dobrindt *et al.,* 2010).

2.12.2 Pathogenicity of *Escherischia coli*

Based on virulence factors in *E. coli* genomes and phenotypic traits, the human pathotypes of diarrheagenic *E. coli* (DEC) are differentiated from non-pathogenic (commensal) *E. coli* and extraintestinal pathogenic *E. coli* (ExPEC). The ExPEC are classified as uropathogenic *E. coli* (UPEC), sepsis-causing *E. coli* (SEPEC) and neonatal meningitis-associated *E. coli* (NMEC) (Kaper *et al.,* 2004). *Escherichia coli* is the leading cause of UTIs. Although *E. coli* is most closely linked to UTI, it can infect any extraintestinal site causing meningitis, skin structure infections, myositis, osteomyelitis and epididymo-orchitis. Severe *E. coli* infections, which often involve bloodstream infection (of which *E. coli* is a leading cause), frequently result in the systemic inflammatory response syndrome (SIRS) (Russo *et al.*, 2003).

2.12.3 Isolation of *Escherischia coli*

E. coli cells may grow on a solid or in a liquid growth medium under laboratory conditions. It may be grown in a basic minimum of media, which includes glucose as a carbon and energy source, ammonium salts as a nitrogen source, other salts and trace elements (Elbing *et al.*, 2019). As *E. coli* have simple nutritional requirements it can be easily cultured on a common medium, such as Nutrient agar, MacConkey agar, and Eosin Methylene Blue agar (EMB agar) (Bonnet *et al.,* 2019). *E. coli* can grow at temperatures ranging from 10°C to 40°C, although the optimum temperature for most strains is 37 °C however, some laboratory strains can proliferate at temperatures as high as 49°C (Fotadar

et al., 2005). *E. coli* can survive at 4.5–9.5 pH but the maximum growth is observed at 7.0 (neutral pH). Also, the pH requirements vary with the strains of *E. coli*.

2.12.4 Antimicrobial Resistance of *E. coli*

Many gram-negative bacteria have developed antibiotic resistant genes and *E. coli* is not an exception. These bacteria evolved different mechanisms that confer resistance to antibiotics. *E. coli* can produce extended-spectrum beta-lactamase (ESBL) that makes the bacteria resistant to beta lactams (e.g. cephalosporins, monobactams, etc.). Carbapenemase-producing *E. coli* strains, on the other hand have genes that confer carbapenem resistance (e.g., imipenem, ertapenem, and meropenem) failure (Gandra *et al*., 2019). ESBL producing *E. coli* are a rapidly evolving group of β-lactamases, produced by certain types of bacteria where *E. coli* are the major ones. These enzymes can break down the active ingredients by cleaving the beta-lactam ring of penicillin and cephalosporin antibiotics, resulting in the inactivation of these drugs. There are at least 200 different types of ESBL enzymes increasingly isolated as causes of complicated UTIs and remain an important cause of failure of therapy with cephalosporins and have serious infection control consequences. ESBL producing *Enterobacteriaceae* have been responsible for numerous outbreaks of infection throughout the globe and pose challenging infection control issues. These organisms are associated with multidrug resistance causing a high rate of mortality and treatment failure (Gandra *et al*., 2019).

2.13 Staphylococcus aureus

2.13.1 Overview of Staphylococcus aureus

Staphylococcus aureus, also known as "golden staph", is a gram-positive coccus belonging to the class *Bacilli*, order *Bacillales*, family *Staphylococcaceae* and genus *Staphylococcus* (Masalha *et al.,* 2001). It is a facultative anaerobe often positive for catalase and nitrate reduction and is coagulase variable, i.e., it may be coagulase positive or negative (Matthews *et al.,* 1997). The bacterium is non-motile, non-spore forming and appearing as bunch of grapes, microscopically. On blood agar it shows large, round, golden-yellow colonies often with haemolysis.

2.13.2 Pathogenicity of *Staphylococcus aureus*

Due to its ability to colonize a wide range of species (all mammals including rodents and lagomorphs), *Staphylococcus aureus* (*S. aureus*) can readily be transmitted from one species to another; from humans to animals and reverse. Staphylococcal infections are zoonotic in nature. *S. aureus* exhibits wide range of diseases (Raygada *et al.*, 2009). It causes diseases ranging from minor skin infections to life threatening conditions such as bacteraemia, endocarditis, necrotizing pneumonia, toxic shock syndrome, necrotizing fasciitis, necrotizing pneumonia, bone and joint infections accompanied by septic thromboembolic disease, purpura fulminans with or without Waterhouse-Friderichsen syndrome, orbital cellulitis and endophthalmitis, infections of the central nervous system owing to various virulent factors (Moran *et al.,* 2006). *Staphylococcus aureus* causes severe animal diseases; such as suppurative disease, mastitis, arthritis and urinary tract infections owing to the virulence factors, such as the production of extracellular toxins and enzymes (Waldvogel *et al.,* 1990).

2.13.3 Isolation of *Staphylococcus aureus*

Nearly all isolates of *S. aureus* produce coagulase enzyme, a virulence factor that also helps in identification of the organism (Brown *et al.,* 2005). The organism is salt tolerant, which is able to grow in mannitol-salt agar medium containing 7.5% sodium chloride. The organism is catalase positive and oxidase negative (Brown *et al.,* 2005).

2.13.4 Antimicrobial Resistance of *Staphylococcus aureus*

Staphylococcus aureus is resistant to almost all antibiotic drugs that are used so far (Gulzar *et al.,* 2018). The resistance is chromosome or plasmid mediated and is attributed

to transduction, transformation and conjugation. *blaZ* gene in the organism mediates the resistance to β-lactam antibiotics *i.e.,* penicillin and its derivatives (Oslen *et al.,* 2006). *mecA* gene which encodes the Phosphate binding protein which may have an altered binding capacity or new PBP (PBP2') may be responsible for resistance to methicillin and other β-lactam antibiotics. There are some strains which are named as Boderline Oxacillin resistant *S. aureus* (BORSA). These strains are β-lactamase hyperproducers and show resistance to Oxacillin in absence of *mecA* or *mecC*. Aminoglycosidal resistance may arise because of the mutations in the genes regulating the ribosome leading to the structural changes in the ribosomal proteins which may hinder the binding capacity of the antibiotic and further its action. Diminished uptake of the antibiotic or modification of antibiotic due to the cellular enzymes such as aminoglycoside acetyltransferases (AAC) or aminoglycoside phosphotransferases (APH) catalyzed by a bifunctional protein encoded by *aacA-aphD* gene may also be responsible for the aminoglycoside resistance. Tetracycline resistance is due to *tetK* and *tetL genes* that are located on the plasmid (Gulzar *et al.,* 2018). These genes control the active efflux. The ribosomal protection is mediated by *tetO* or *tetM* genes present on transposon or chromosom. Vancomycin intermediate resistance is due to *vraSR* operon and *graS* gene (Qureshi *et al.,* 2014). Acquisition of *Enterococci vanA* gene may lead to development of VRSA (Vancomycin resistant *S. aureus*). Altered peptidoglycan synthesis on exposure to Vancomycin may lead to an increase in the thickness of cell wall thus impairing the diffusion of drug into the bacterial cell. Inducible resistance to macrolide antibiotic is commonly due to *ermC* gene present on the plasmid (Gulzar *et al.,* 2018).

2.14 Bacillus spp.

2.14.1 Overview of Bacillus spp.

The *Bacillus* and related genera are some of the most prevalent bacteria in our surroundings and interact with humans via different means, such as soil, air, and plants and even reside in the human gut. Members of *Bacillus* species are Gram-positive, spore-forming, and facultative aerobes. Interestingly, the *Bacillus* genus contains both pathogens and non-pathogens and has a complex taxonomic relationship. *B. anthracis, B. cereus, and B. thuringiensis* are the most studied pathogenic bacteria of the genus *Bacillus* that are known to cause food poisoning, localized infections such as ocular and ear canal infections and systemic infections such as meningitis and bacteraemia.

2.14.2 Pathogenicity of *Bacillus spp.*

The pathogenesis of the *Bacillus* species depends upon the survival of spores in the non-host environment, as dormant spores are the main infectious form of the bacterium. Sporulation of *B. anthracis* occurs only in the non-host environment when vegetative cells are exposed to the air and the reasons behind this are not well understood. Furthermore, the environment poses a major challenge for host-to-non-host transition via temperature, moisture and nutrient availability (Baindara *et al.,* 2023). Interestingly, in the form of metabolically inactive spores, pathogenic bacteria can endure extreme challenges, including pressure, pH, and ultraviolet rays (Baindara *et al.,* 2023).

Human anthrax epidemiology involves livestock, wildlife, soil, and water. In the soil, B. anthracis spores can remain viable for decades and avail a potential source of transmission, causing infection to herbivores while grazing via inhalation or ingestion (Jiranantasak *et al.,* 2022). On the other hand, *B. cereus* mostly exists in soil and water;

however, it has been isolated from various mammals, birds, reptiles and invertebrates, including the guts of poultry, turtles and the udders of cattle. *B. cereus* strains have been associated with various food and dairy products, including the staple food rice. Notably, spores are resistant to pasteurization and heat treatment (Baindara *et al.,* 2023). Furthermore, spore hydrophobicity plays a critical role in surface adherence and later results in recurrent problems in *B. cereus* biofilms in milk tanks (Ehling-Schulz *et al.,* 2019). *B. anthracis* is the causative agent of anthrax, an acute infection in humans, economically important livestock and wild animals. Anthrax is lethal in humans and may cause severe gastrointestinal and pulmonary infections. *B. cereus* is known as one of the food poisoning agents and has been reported to cause localized wound and eye infections in humans. Some *B. thuringiensis* strains are entomopathogens that have been developed as biopesticides and can possibly be transmitted to humans, causing infections in immunocompromised patients (Baindara *et al.,* 2023).

2.14.3 Isolation of *Bacillus spp.*

Determination of morphological, physiological and biochemical traits is a long and often unreliable process. The most accurate method for examining the diversity of *Bacillus* spp. is their identification and characterization at the molecular level. In addition to standard molecular methods such as 16S rRNA analysis, RFLP (Restriction Fragment Length Polymorphism), RAPD (Random Amplified Polymorphic DNA), rep-PCR (Repetitive element sequence-based Polymerase Chain Reaction), MLSA (Multilocus Sequence Analysis), etc., different PCR methods with species-specific primers are increasingly used for reliable differentiation of Bacillus spp. (Sicuia *et al.,* 2015).

2.14.4 Antimicrobial Resistance of *Bacillus*

It has been reported that *Bacillus* from pasteurized milk showed resistance to some antibiotics including ampicillin, lincomycin, erythromycin and tetracycline (Liu *et al.,* 2020). For example, *Bacillus* strains were isolated from pasteurized milk samples collected from dairies in France and showed resistance to penicillin or erythromycin (Perrin-Guyomard *et al.,* 2005). Seventy *Bacillus cereus* strains were isolated form 258 pasteurized milk samples collected from 32 cities in China and most of the isolates were resistant to ampicillin (99 %), penicillin (99 %), and cefoxitin (95 %) (Gao *et al.,* 2018).

In *Bacillus*, the resistance to antibiotics could be either intrinsic or acquired. Generally, genes encoding acquired antibiotic resistance are located on mobile genetic elements such as plasmids or transposons, which could lead to the spread of antibiotic resistance among *Bacillus* and other clinical pathogens via horizontal gene transfer (Navaneethan *et al.*, 2021). The erythromycin resistance gene on plasmid pHT73 can be transferred from *Bacillus thuringiensis* ssp. *Kurstaki* KT0 to *B. cereus* and *Bacillus mycoides* by conjugation (Hu *et al.,* 2004). The tetracycline resistance gene *tet(M)* located on a Tn916-like transposon can be transferred from *B. cereus* R89 to *Enterococcus faecalis* JH2-2 and *Staphylococcus aureus* 8794RF by filter mating (Agerso *et al.,* 2002). Therefore, *Bacillus* strains in pasteurized milk could act as donors or reservoirs for antibiotic resistance genes (ARG), which can be transferred to pathogenic bacteria (von Wintersdorff *et al.,* 2016).

2.15 Aspergillus niger

2.15.1 Overview of Aspergillus niger

Aspergillus niger is a cosmopolitan representative of microscopic filamentous fungi. Although the main source of this strain is soil, it frequently occurs in various other sources, such as historical and archaeological objects (Aldosari *et al.,* 2019) or indoor environments (Omar *et al.,* 2018). The ability of *Aspergillus niger* to produce substances of various types, such as low molecular weight organic acids (e.g., gluconic, citric, itaconic, oxalic, malic, acetic, lactic, and others), enzymes (e.g. amylase, aryl-phosphatase, b-glucosidase, cellulase, lipase, and others) as well as other products of metabolism, has great use not only in the food, medicine, pharmaceutical and chemical industries but also in mineral biotechnology (Adeleke *et al.,* 2017).

2.15.2 Pathogenicity of *Aspergillus niger*

A. niger is generally regarded as a non-pathogenic fungus widely distributed in nature. Humans are exposed to its spores every day without disease becoming apparent. Only in few cases has *A. niger* been able to colonise the human body as an opportunistic invader and in almost all these cases the patients have a history of severe illness or immunosuppressive treatment (Poulsen *et al.,* 2021).

The metabolic products of *Aspergillus niger* are mainly used in the biodegradation processes of environmental pollutants, such as crude oil and its products found in industrial water and wastewater (Gulzar *et al.,* 2017). The biosorption and bioleaching of heavy metals and toxic elements from solutions and soils are key processes in bioremediation (Xinhui *et al.,* 2018). This means the treatment of contaminated water,

soil and subsurface material by altering environmental conditions to stimulate the growth of microorganisms can degrade the target pollutants.

2.15.3 Antimicrobial resistance of *Aspergillus niger*

Infections due to *Aspergillus* species constitute an important challenge for human health. Invasive aspergillosis represents a life-threatening disease, mostly in patients with immune defects. Drugs used for fungal infections comprise amphotericin B, triazoles, and echinocandins. However, in the last decade, an increased emergence of azole-resistant *Aspergillus* strains has been reported, particularly belonging to *Aspergillus fumigatus* species (Shishodia *et al.,* 2019). While, *A. niger* is generally regarded as a non-pathogenic fungus widely distributed in nature. Humans are exposed to its spores every day without disease becoming apparent. Only in few cases has *A. niger* been able to colonise the human body as an opportunistic invader and in almost all these cases the patients have a history of severe illness or immunosuppressive treatment (Poulsen *et al.,* 2021).

2.16 Candida spp.

2.16.1 Overview of Candida spp.

Candida species are opportunistic pathogens that are a major cause of morbidity and mortality worldwide and, thus, represents a serious threat to public health (Pappas *et al.,* 2016). *Candida albicans* is the pathogenic species most frequently isolated. However, other species such as *C. glabrata*, *C. tropicalis*, *C. parapsilosis*, *C. krusei*, *C. famata*, *C. guilliermondii* and *C. lusitaniae* have been increasingly isolated, mainly in human immunodeficiency virus (HIV)-infected individuals (Barchiesi *et al.,* 2016).

2.16.2 Pathogenicity of Candida sp.

The pathogenicity of *Candida* species is poorly understood, and the rate of infections is increasing rapidly. The most frequent fungal disease affecting populations in the world is candidiasis (Vázquez-González *et al.,* 2013). There are several types of candidiasis as mucosal candidiasis, cutaneous candidiasis, onychomycosis and systemic candidiasis (Wächtler *et al.,* 2012). An important fact is that candidiasis is an infection that can affect both immunocompromised and healthy people (Raman *et al.,* 2013). Candidemia is another infection due *Candida* spp. and is the most relevant and prevalent nosocomial fungal infection associated with a high mortality rate (up to 49%) in patients with a compromised immune system (Sardi *et al.,* 2013). The association of *Candida* with bloodstream infections depends on patient's condition, age and geographic region. Candidemia is such an important infection that in 10–40 % of cases it is associated with sepsis or septic shock while *Candida* species as main agent of sepsis or septic shock are responsible for no more than 5 % of the total number of cases (Guery *et al.,* 2009).

2.16.3 Isolation of *Candida spp*.

Candida albicans is a species that presents high degree of flexibility, being able to grow in extremely different environment regarding to the availability of nutrients, temperature variation, pH, osmolarity and amount of available oxygen (Paramythiotou *et al.,* 2014). There are newer molecular techniques available for rapid yeast detection, such as fluorescence in situ hybridization (PNA-FISH). However, commercially available equipment for analysis does not differentiate between *C. albicans* and *C. parapsilosis*, *C. glabrata*, or *C. krusei*. These facts are relevant because increased mortality rate is associated with delays in initiating adequate antifungal therapy (Bassetti *et al.,* 2014).

2.16.4 Antimicrobial Resistance of *Candida albicans*

There are several classes of compounds that comprise the arsenal used to treat *Candida* infections. The polyenes, azoles, echinocandins, nucleoside analogues, and allylamines are used with varying efficacy depending on the type and site of infection and the sensitivity of the *Candida* species (Pappas *et al.,* 2016). The most commonly prescribed antifungal used for most *C. albicans* infections is fluconazole, a member of the azole class of antifungals (Pfaller *et al.,* 2010). Azoles inhibit 14- a-sterol demethylase, encoded by the *ERG11* gene, which is an enzyme involved in the biosynthesis of the fungal-specific membrane sterol ergosterol. As some NAC species exhibit intrinsic resistance to azoles, their use is likely a contributing factor to the more frequent incidence of infections caused by these NAC species (Lortholary *et al*., 2011). Moreover, many studies have documented the ability of *Candida* to develop high-level resistance to azole antifungals (*Lortholary et al.*, 2011). Infections caused by *C. albicans* are associated with varying levels of fluconazole resistance depending on the type of infection. *C. albicans* isolates from candidaemic patients have the lowest incidence of azole resistance (0–5 %) (Pfaller *et al.,* 2015). The incidence of fluconazole resistance in *C. albicans* isolates from oropharyngeal candidiasis (OPC) is higher and depends upon previous fluconazole treatment and prior OPC infections (Berberi *et al.,* 2015).

One mechanism of resistance identified in this species is the presence of point mutations in *ERG11*. Previous studies have identified amino acid substitutions that result in decreased fluconazole susceptibility and have noted that several of these critical allelic variations cluster in three “hot spot” regions within Erg11p (Marichal *et al.,* 1999). Another mechanism of fluconazole resistance in *C. albicans* is the increased expression

of *ERG11* due to activating mutations in the gene encoding the zinc-cluster transcriptional regulator Upc2p. Two other mechanisms of fluconazole resistance in *C. albicans* involve the over expression of drug efflux pumps Mdr1p and Cdr1p/Cdr2p. A less common mechanism of azole resistance in *C. albicans* is inactivation of the *ERG3* gene, which encodes the ergosterol biosynthesis enzyme sterol 15,6 desaturase. Erg3p catalyses one of the final steps in the pathway and also converts nontoxic 14α-methylated sterol intermediates, that accumulate during azole treatment, into the toxic sterol 14α-methyl ergosta-8,24(28)-dien-3β,6 α -diol. Inactivation or deletion of the *ERG3* gene, therefore, prevents such toxic sterols from being synthesized (de Oliveira Santos *et al.,* 2018).

CHAPTER THREE

METHODOLOGY

3.1 Study Area

This study was carried out to determine the antimicrobial activity of the leaves of *Cymbopogon citratus* and *Picralima nitida* when combined together in an herbal formulation.

3.2 Materials

3.2.1 Microbiological Media

Sabouraud dextrose agar, Nutrient agar, Nutrient broth and Miller Hinton agar all from Titan Biotec

3.2.2 Equipment

Portable autoclave (Model YX-2803, Lincoln Mark Medical, England), weighing balance, hot air oven (Gallenkamp, England), mortar, pestle, mechanical grinding machine, micropipette (P-1000) and incubator.

3.2.3 Glassware

Conical flask, bottles (MacCartney, universal and Bijoux) as well as test tubes, pipettes, glass stirrers, porcelain dish, pestle, maceration jars, glass funnels, beakers, measuring cylinders, and Petri dishes. All glassware were products of Pyrex, England.

3.2.4 Chemicals and Reagents

Tween 80, ethylacetate, distilled water, glycerol. Disinfectant: Dettol®, soap, detergent. Other apparatus includes Whatman filter paper, cotton wool, pipette tip, a corkborer (10mm in diameter), a transparent millimetre rule, grease pencil, sterile swab sticks, tripod stand, Bunsen burner, foil paper, air dried leaves.

3.2.5 Clinical Isolates and Resources

Clinical isolates include *Aspergillus niger, Candida albicans, Klebsiella pneumoniae, Bacillus subtilis, Escherichia coli, Staphylococcus aureus.*

3.3 Methods

3.3.1 Collection and Identification

The leaves of *Cymbopogon citratus* and *Picrilima nitida* were collected from Idunmwina before Oluku in Ovia North East, Edo State in the month of January. The leaves were authenticated by Professor Akinnibosun H. Adewale of the Department of Plant Biology and Biotechnology, University of Benin, Benin City and were allotted Voucher numbers; *Cymbopogon citratus* UBH-C451 and *Picrilima nitida* UBH-P424.

3.3.2 Preparation of Crude Extract

The leaves of *Cymbopogon citratus* and *Picralima nitida* plants were harvested, washed and air dried under shade for two weeks and were later dried properly in the laboratory oven at 50°C for 24 hours. The dried leaves were then pulverized using a commercial grinding machine. 100 g each of *Cymbopogon citratus* and *Picralima nitida* powder was macerated together in 2.5L of ethanol solvent and 100 g each of *Cymbopogon citratus*

and *Picralima nitida* powder was maceration together for 72hours. They were stirred at intervals to allow for proper permeation of extraction solvent. A double filtration using Whatman filter paper was carried out on the resultant solution so as to ensure that no leaf particles or residue was in the filtrate, after which the solvent was evaporated completely at 50 ºC in hot air oven. The extracts were obtained weighed and kept at 4 ºC in an airtight container until required for use.

3.3.3 Calculation of Percent Yield of Crude Extract

% Yield = [(weight of dried extract) / (weight of dried plant sample)] × 100 %

3.4 Antimicrobial Assay

3.4.1 Specimen Collection

Microorganisms used in this study were clinical isolates obtained from stock cultures from Pharmaceutical Microbiology Laboratory in the University of Benin and the University of Benin Teaching Hospital, Benin City, Edo state, Nigeria.

Gram positive cocci used; *Staphylococcus aureus*.

Gram positive bacilli used; *Bacillus subtilis*.

Gram negative bacilli used; *Klebsiella. Pneumoniae, Escherichia coli*

Fungi species used; *Candida albicans, Aspergillus niger.*

3.4.2 Preparation of Test Organisms

All test microorganisms were maintained in 20 % glycerol broth and frozen. Prior to use, test microorganisms were sub-cultured from stock into sterile nutrient agar plates for

bacteria and Sabouraud dextrose agar for fungi and were incubated overnight at 37°C for bacteria and 48 hours at room temperature for fungi. After incubation colonies from the overnight plates were suspended in sterile broth for 12 hours and adjusted to 0.5 McFarland standard to give an inoculum size of approximately 10^7 CFU/ml. The adjusted inoculum was diluted 1:100 to give inoculum size of approximately 10^5 CFU/ml.

3.4.3 Preparation of McFarland Standard

A 0.5 McFarland standard solution is prepared by adding 0.5ml of 1.175 % (weight/volume). Barium Chloride dihydrate salt ($BaCl_2.2H_2O$) to 9.95 ml of 1 % Sulfuric acid (H_2SO_4). The two solutions are mixed completely to form a turbid suspension in a test tube which is then placed in a test tube rack and kept at room temperature before use.

3.5 Antimicrobial Susceptibility Tests and Minimum Inhibitory Concentration (MIC)

Antimicrobial susceptibility test was carried out using agar well diffusion method (Murray *et al.,* 2009) with some modifications. Sterile nutrient agar was prepared and poured into different Petri dishes aseptically each containing 30ml and allowed to solidify. Excess moisture was removed from the prepared agar surface by drying in a hot air oven for 10min at 40°C. The agar plates were inoculated with the test organism using a sterile swab stick aseptically. A sterile cork borer (10mm) was used to bore 4 wells in each agar plate. The agar wells were sealed with two drops of molten agar. Four of the wells were filled with 50mg/200µL, 100mg/200µL, 200mg/200µL and 400mg/ 200uL of the extract respectively. The plates were incubated at 37°C for 24 hr. The procedure was repeated using Sabouraud agar with 400mg/200µL, 200mg/200µL and 100mg/200µL and

50mg/200uL of the extract in the 4 wells respectively. The plates were incubated at room temperature for 48hours. The zones of inhibition diameter were measured as an index of the killing or inhibitory action of the test agent against a given organism.

3.6 Ethical Approval

Ethical approval for the study was obtained from the school of basic medical sciences.

3.7 Statistical Analysis

Data was analysed using GraphPad Prism® version 9.5.1. Student *t* test or one-way analysis of variance (ANOVA), where appropriate, was conducted on the data. Tukey post hoc (multiple comparison) analysis was conducted on significantly different means. Results were presented as mean ± SEM (standard error of the mean) in tables and bar graphs. Microbial isolates that showed no zones of inhibition following treatment with the mixed extract were excluded from the analysis. These are shown as NZI (no zone of inhibition) on the tables.

CHAPTER FOUR

RESULTS

RESULTS

Table 4.1 shows the percent yield of crude extract, physical properties and yield of bi-herbal formulation of *Cymbopogon citratus* and *Picralima nitida*

The aqueous formulation of *Cymbopogon citratus* and *Picralima nitida* constituted in distilled water yielded 34.58% of extract, was dark brown in colour, had an aromatic odour, thick consistency, and a smooth texture. While the ethanol formulation of *Cymbopogon citratus* and *Picralima nitida* constituted in ethanol yielded 13.78% of extract, was dark green in colour, had an aromatic odour, thick consistency, and a smooth texture.

Table 4.1: Physical properties and yield of bi-herbal formulation of *Cymbopogon citratus* and *Picralima nitida*

Property	Aqueous extract	Ethanolic extract
Odour	Aromatic	Aromatic
Solvent	Distilled water	Ethanol
Texture	Smooth	Smooth
Colour of crude extract	Dark brown	Dark green
Consistency	Thick	Thick
Percentage yield	34.58 %	13.78 %

Table 4.2 shows the minimum inhibitory concentration of extract on the microbial isolates**.**

The aqueous formulation produced a minimum inhibitory concentration of 200 ± 1.00 mg/mL on *Staphylococcus aureus* while the ethanol formulation produced a minimum inhibitory concentration of 20.8 ± 4.17 mg/mL on the same. Whereas the aqueous formulation produced a minimum inhibitory concentration of 150.0 ± 50.00 mg/mL on *Bacillus subtilis*, the ethanol formulation produced a minimum inhibitory concentration of 6.4 ± 0.13 mg/mL on the same. Also, the aqueous formulation produced a minimum inhibitory concentration of 75.0 ± 25.00 mg/mL on *Candida albicans* while the ethanol formulation produced no zone of inhibition and, thus, no minimum inhibitory concentration *Candida albicans*. Both the aqueous and ethanol formulations of the herbal formulation produced no zones of inhibition on *Klebsiella pneumonia, Escherichia coli,* and *Aspergillus niger.*

Table 4.2: Minimum inhibitory concentration (mg/ml) of extract on the microbial isolates

Type of extract	*Staph. aureus*	*Bacillus Subtilis*	*Candida albicans*	*Klebsiella*	*E. coli*	*Aspergillus Niger*
Aqueous	200.00 ± 1.00	150.0 ± 50.00	75.0 ± 25.00	NZI	NZI	NZI
Ethanolic	20.83 ± 4.167	6.37 ± 0.125	NZI	NZI	NZI	NZI

- NZI = No zone of inhibition

Table 4.3 show zones of inhibition of microbial isolates following extract treatment (aqueous ethanolic**)**

The zones of inhibition produced by the minimum inhibitory concentrations of the herbal formulation on *Staphylococcus aureus* were 11.5 ± 0.50 mm and 11.3 ± 0.33, respectively. While, the zones of inhibition produced by the minimum inhibitory concentrations of the herbal formulation on *Bacillus subtilis* were 15.0± 0.10 mm and 15.5 ± 0.50, respectively. Also, the zones of inhibition produced by the minimum inhibitory concentrations of the herbal formulation on *Candida albicans* was 12.0 ± 0.10 mm; the ethanol formulation produced no zone of inhibition. Both the aqueous and ethanol formulations of the herbal formulation produced no zones of inhibition on *Klebsiella pneumonia, Escherichia coli,* and *Aspergillus niger.*

Table 4.3: Zones of inhibition of microbial isolates following extract treatment

Type of extract	3.125 mg/ml	6.25 mg/ml	12.50 mg/ml	25.00 mg/ml	50.00 mg/ml	100.00 mg/ml	200.00 mg/ml	400.00 mg/ml
Mg	***Staphylococcus aureus***							
Aqueous	NZI	NZI	NZI	NZI	NZI	NZI	11.50 ± 0.50	14.00 ± 1.00
Ethanolic	NZI	NZI	11.33 ± 0.33	12.00 ± 1.00	16.00 ± 1.73	18.00 ± 1.73	19.67 ± 1.453	21.67 ± 1.33
	Bacillus subtilis							
Aqueous	NZI	NZI	NZI	NZI	NZI	15.00 ± 1.00	14.5 ± 2.500	18.00 ± 3.00
Ethanolic	NZI	15.50 ± 0.50	23.00 ± 5.00	25.00 ± 5.00	24.00 ± 1.00	26.50 ± 0.50	27.50 ± 0.50	30.50 ± 0.50
	Candida albicans							
Aqueous	NZI	NZI	NZI	NZI	12.00 ± 1.00	12.50 ± 0.50	15.50 ± 0.50	20.00 ± 1.00
Ethanolic	NZI	NZI	NZI	NZI	NZI	NZI	NZI	NZI
	Klebsiella							
Aqueous	NZI	NZI	NZI	NZI	NZI	NZI	NZI	NZI
Ethanolic	NZI	NZI	NZI	NZI	NZI	NZI	NZI	NZI
	Escherichia coli							
Aqueous	NZI	NZI	NZI	NZI	NZI	NZI	NZI	NZI
Ethanolic	NZI	NZI	NZI	NZI	NZI	NZI	NZI	NZI
	Aspergillus niger							
Aqueous	NZI	NZI	NZI	NZI	NZI	NZI	NZI	NZI
Ethanolic	NZI	NZI	NZI	NZI	NZI	NZI	NZI	NZI

- NZI = No zone of inhibition

Figure 4.1 shows the minimum inhibitory concentration (MIC) of the aqueous and the ethanolic bi-herbal leaves extracts on *Staphylococcus aureus*

The ethanol formulation exhibited significantly smaller minimum inhibitory concentration on *Staphylococcus aureus* compared to the aqueous formulation.

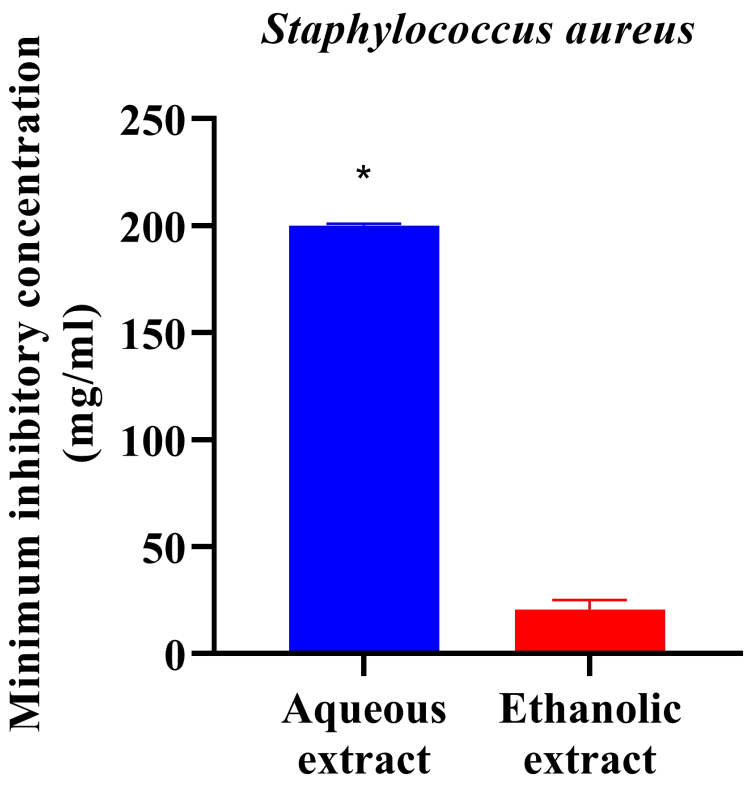

Figure 4.1: The minimum inhibitory concentration (MIC) of the aqueous and the ethanolic bi-herbal leaves extracts on *Staphylococcus aureus*, t (3) = 32.99, $p < 0.0001$.

Figure 4.2 shown the minimum inhibitory concentration (MIC) of the aqueous and the ethanolic bi-herbal leaves extracts on *Bacillus subtilis*

The ethanol formulation exhibited smaller minimum inhibitory concentration on *Bacillus subtilis* compared to the aqueous formulation, however, this was not statistically significant.

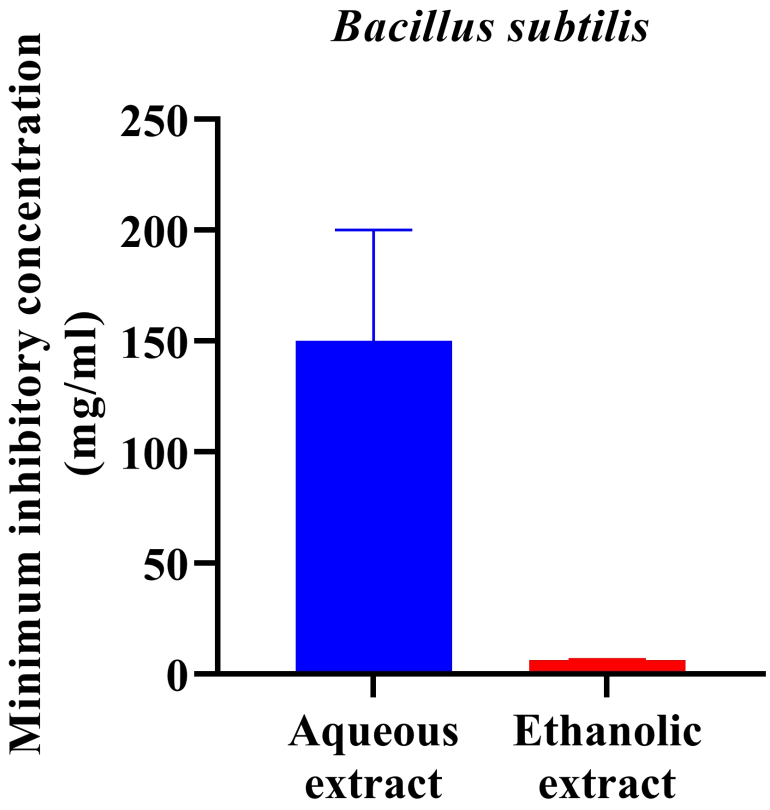

Figure 4.2: The minimum inhibitory concentration (MIC) of the aqueous and the ethanolic bi-herbal leaves extracts on *Bacillus subtilis*, t (2) = 2.872, p = 0.1028.

Figure 4.3 shows the minimum inhibitory concentration (MIC) of the aqueous and the ethanolic bi-herbal leaves extracts on *Candida albicans*.

The aqueous formulation exhibited a minimum inhibitory concentration of 75.0 ± 25.00 mg/mL on *Candida albicans*, whereas the ethanol formulation produced no zone of inhibition on *Candida albicans*.

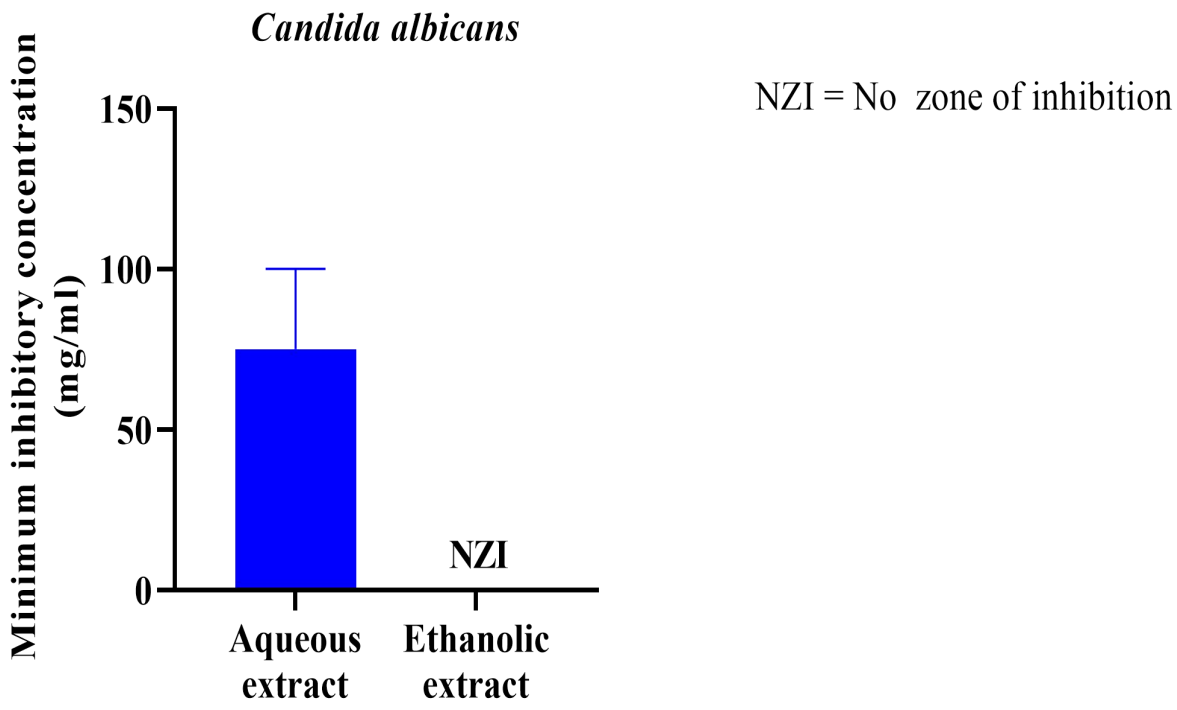

Figure 4.3: The minimum inhibitory concentration (MIC) of the aqueous and the ethanolic bi-herbal leaves extracts on *Candida albicans*.

Figure 4.4 shows the zones of inhibition of *Staphylococcus aureus* at different concentrations of the aqueous bi-herbal leaves extract

The 400 mg/ml of the aqueous formulation produced greater diameter of zone of inhibition on *Staphylococcus aureus* compared to the 200 mg/ml concentration of the same formulation, however, this difference was not statistically significant.

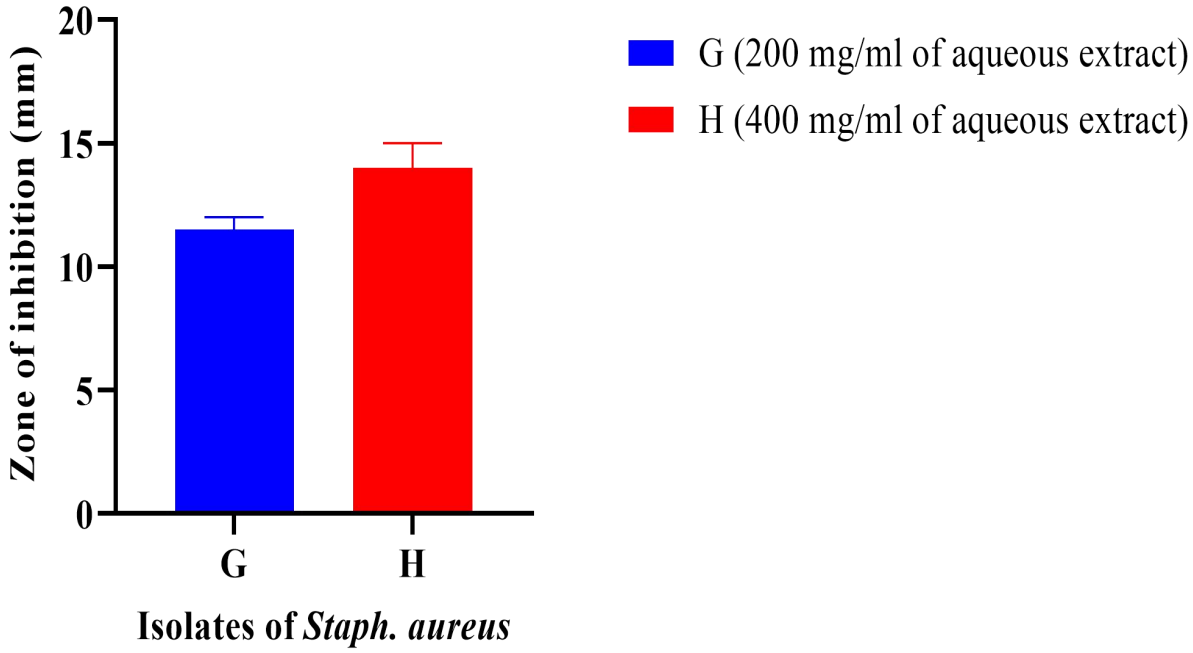

Figure 4.4: The zones of inhibition of *Staphylococcus aureus* at different concentrations of the aqueous bi-herbal leaves extract, t (2) = 2.236, p = 0.1548.

Figure 4.5 shows the Zones of inhibition of *Bacillus subtilis* by the different concentrations of the aqueous bi-herbal leaves extract**.**

Diameter of zone of inhibition produced by the 400 mg/ml concentration of the aqueous formulation on *Bacillus subtilis* was statistically not significantly higher than the 200 mg/ml and 100 mg.ml concentrations on *Bacillus subtilis*. Similarly, diameter of zone of inhibition produced by the 200 mg/ml concentration of the aqueous formulation on *Bacillus subtilis* was statistically not significantly higher than the 100 mg.ml concentration on *Bacillus subtilis*

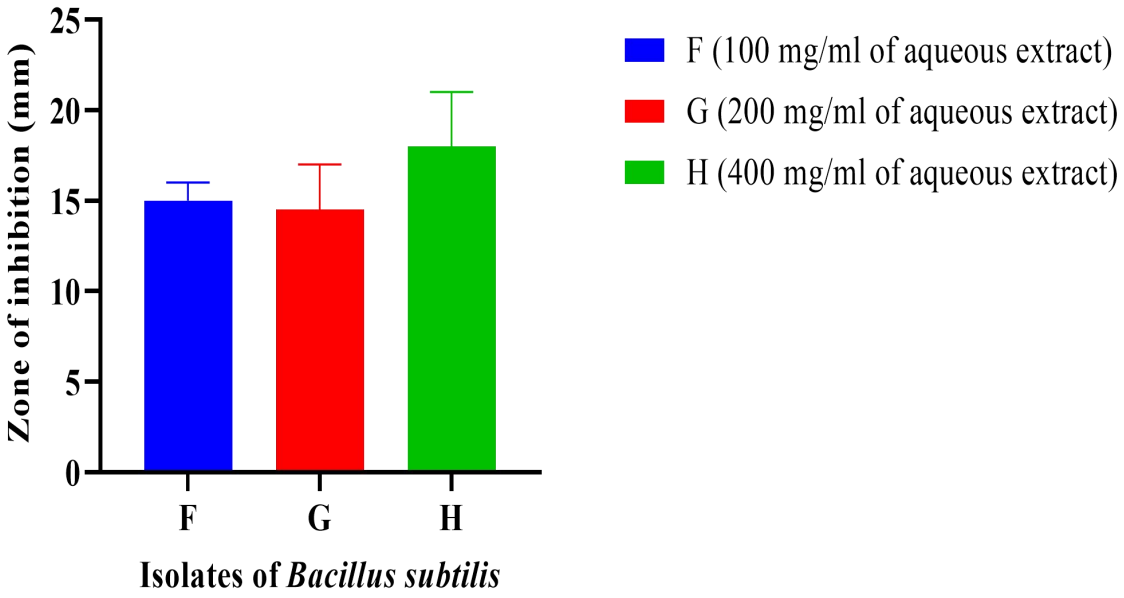

Figure 4.5: Zones of inhibition of *Bacillus subtilis* by the different concentrations of the aqueous bi-herbal leaves extract, F (2, 3) = 0.6615, p = 0.5781.

Figure 4.6 shows zones of inhibition of *Candida albicans* by different concentrations of the aqueous bi-herbal leaves extracts extract.

Diameter of zone of inhibition produced by the 400 mg/ml concentration of the aqueous formulation on *Candida albicans* was statistically significantly higher than the 200 mg/ml, 100 mg.ml, and 50 mg/ml concentrations on *Candida albicans*. The diameter of zone of inhibition produced by the 200 mg/ml, 100 mg.ml, and 50 mg/ml concentrations of the aqueous formulation on *Candida albicans* was statistically not significant when compared to one another.

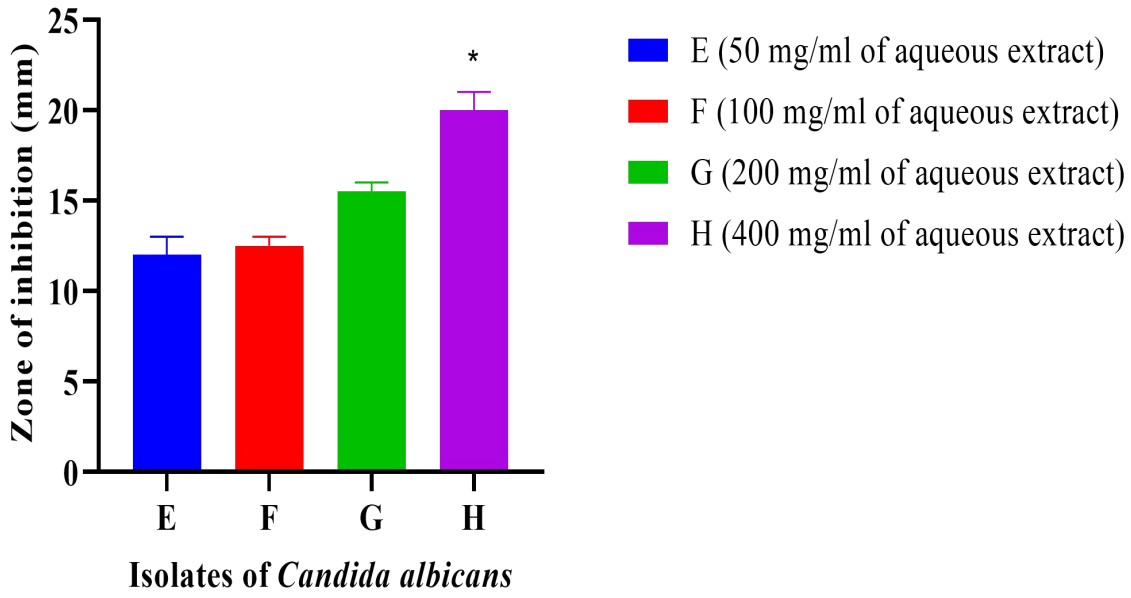

Figure 4.6: The zones of inhibition of *Candida albicans* by different concentrations of the aqueous bi-herbal leaves extracts extract, F (3, 4) = 0.0062, p = 0.9419.

Figure 4.7 shows the Zones of inhibition of *Staphylococcus aureus* by different concentrations of the ethanolic bi-herbal leaves extracts extract.

Diameter of zone of inhibition produced by the 400 mg/ml, 200 mg/ml, and 100 mg/ml concentrations of the ethanol formulation on *Staphylococcus aureus* was statistically significantly higher than that produced by the 12.5 mg/ml. Similarly, 400 mg/ml and 200 mg.ml concentrations on *Staphylococcus aureus* was statistically significantly higher than that produced by the 25 mg/ml. While the diameter of zone of inhibition produced by the 12.5 mg/ml, 25 mg/ml, and 50 mg/ml concentrations of the ethanol formulation on *Staphylococcus aureus* was not statistically different.

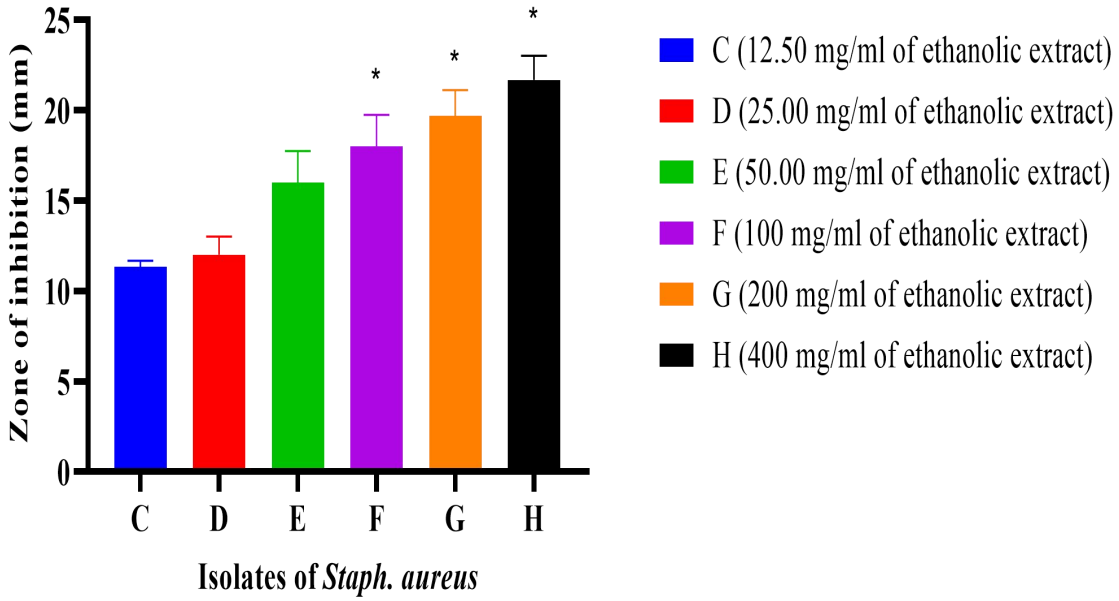

Figure 4.7: Zones of inhibition of *Staphylococcus aureus* by different concentrations of the ethanolic bi-herbal leaves extracts extract, F (5, 12) = 9.398, p = 0.0008.

Figure 4.8 shows the Zones of inhibition of *Bacillus subtilis* by different concentrations of the ethanolic bi-herbal leaves extracts extract.

The 400 mg/ml, 200 mg/ml, and 100 mg/ml, 50 mg/ml, 12.5 mg/ml, and 6.25 mg/ml concentrations of the ethanol formulation on *Bacillus subtilis* was statistically not significantly different when compared among one another.

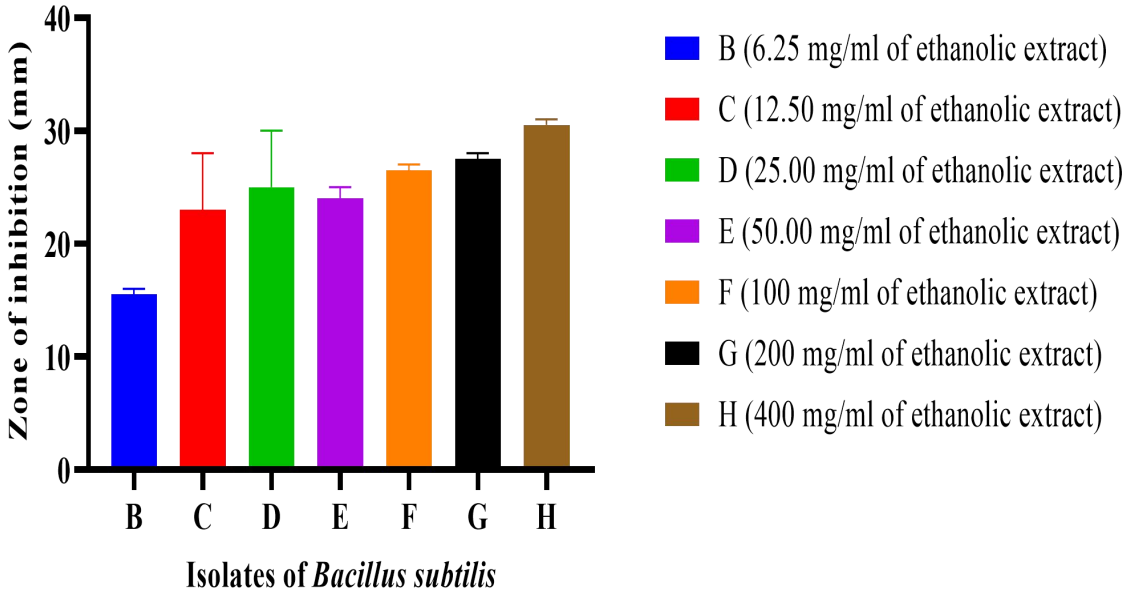

Figure 4.8: Zones of inhibition of *Bacillus subtilis* by different concentrations of the ethanolic bi-herbal leaves extracts extract, F (6, 7) = 2.978, p = 0.089

CHAPTER FIVE

DISCUSSION

The emergence of antimicrobial resistance (AMR) has become a major global health concern, threatening the efficacy of existing antibiotics and necessitating the exploration of alternative antimicrobial agents. *Cymbopogon citratus* and *Picralima nitida* are two traditional medicinal plants widely recognized for their pharmacological activities, including antimicrobial effects (Rahman, 2020). Both plants contain bioactive compounds with documented antimicrobial activity against a range of pathogens. However, their combined efficacy in a bi-herbal formulation remains to be explored. To the best knowledge of the author, the present study is the first research investigating the synergistic effects of a combination *C. citratus* and *P. nitida* on common Gram-positive, and Gram-negative bacteria as well as some fungi. This study investigated the antimicrobial effects of the bi-herbal formulation of *C. citratus* and *P. nitida* on *Staphylococcus aureus, Bacillus subtilis, Escherichia coli, Klebsiella pneumoniae, Candida albicans,* and *Asperiluus niger.*

In this study, of the ethanolic formulation of *C. citratus* and *P. nitida* was more potent in inhibiting *Staphylococcus aureus* than the aqueous formulation. This greater potency is evidenced by the minimum inhibitory concentration (MIC) which was many times lower than that of the aqueous formulation. However, *Staphylococcus aureus* was more susceptible to the ethanolic formulation as the zones of inhibition were greater compared to that produced by aqueous formulation. Thus, the ethanol formulation was more effective than aqueous formulation against *Staphylococcus aureus.* Increasing doses of the aqueous formulation did not produce better inhibition; whereas, increasing doses of

the ethanol formulation produced better inhibition, evidenced by larger diameter of zones of inhibition, may be achieved with concentrations of the ethanol extracts much higher than the MIC. A recent study observed that *Staphylococcus aureus* was susceptible to both the aqueous and the ethanol extract of lemongrass (*Cymbopogon citratus*) with an MIC of on *Staphylococcus aureus* to both be 4 mg/ml, whereas the essential oil from *C. citratus* showed a MIC of 0.5 mg/dl (Kabotso *et al.,* 2022). Although there appears to be a paucity of information on the antibacterial effect of the leaf extract of *Picralima nitida,* Fakeye *et al.* (2000) had observed a MIC of 1.25 µg/ml of the stem bark of *P. nitida* on *Staphylococcus aureus*. Thus, our study is consistent with previous finding. In the present study, *Staphylococcus aureus* was more susceptible to the ethanolic extract of the bi-herbal formulation of *Cymbopogon citratus* and *Picralima nitida.*

The ethanolic formulation of *C. citratus* and *P. nitida* was more potent in inhibiting *Bacillus subtilis* than the aqueous formulation, even though this difference did not reach statistical significance. This is evidenced by the minimum inhibitory concentration (MIC) which was lower than that of the aqueous formulation. However, *Bacillus subtilis* seems to be susceptible to the ethanolic formulation as the zone of inhibition at any given dose was greater for the ethanol extract compared to the aqueous formulation. Thus, the ethanol formulation was more effective than aqueous formulation against *Bacillus subtilis.* Increasing the doses of the aqueous formulation did not produce better inhibition; similarly, increasing doses of the ethanol formulation did not produce better inhibition on *Bacillus subtilis*. Thus, the ethanol formulation appears to be more effective than the aqueous formulation, however, increasing the doses of the extract seems to not confer greater effectiveness on the extract than that achieved at the minimum inhibitory

concentration (MIC). Earlier, Fakeye *et al.* (2000) had observed a MIC of 40 µg/ml of the stem bark of *P. nitida* on *Staphylococcus aureus*. Shendurse *et al.* (2021) observed that the essential oil extract from leaf of *Cymbopogon citratus* had antimicrobial activity on *B. subtilis.* Therefore, the antimicrobial activity of each plant is intact in the combined formulation. However, proper evaluation of the activity in each is difficult due to non-uniformity in the studies.

Application of the aqueous and ethanolic extract mixture of *Cymbopogon citratus* and *Picralima nitida* at the concentrations used in this study did not produce any zones of inhibition on *Klebsiella pneumonia*. This finding contrasted with the finding of Kolawole *et al.* (2023), who found that *K. pneumonia* was susceptible to the essential oil of *Cymbopogon citratus* among other organisms. There is a paucity of data on the antimicrobial effect of the leaf extract of *P. nitida* on *K. pneumonia.* Nonetheless, a previous study found that *P. nitida* fruit extract possessed antimicrobial efficacy against *K. pneumonia* (Shaik *et al.,* 2014). Thus, the combination of *Cymbopogon citratus* and *Picralima nitida* may undermine their antibacterial effect on *K. pneumonia.* Otherwise, it is possible that the *K. pneumonia* strains used in this study are different and/or resistant to the antibacterial effect of the bi-herbal formulation of *Cymbopogon citratus* and *Picralima nitida.* Further research is required to establish this hypothesis.

Similarly, the application of the aqueous and ethanolic extract mixture of *Cymbopogon citratus* and *Picralima nitida* at the concentrations used in this study did not produce any zones of inhibition on *Escherichia coli.* Thus, *Escherichia coli* exhibited resistance and lack of susceptibility to the antimicrobial activity of the aqueous and the ethanol bi-herbal formulation of *Cymbopogon citratus* and *Picralima nitida.* Recently, Shendurse *et al.*

(2021) observed a similar finding that the essential oil extract from leaf of *Cymbopogon citratus* did not produce any zone of inhibition on *E. coli.* Much earlier, Obasi *et al.* (2012) had observed that the aqueous extract of *P. nitida* peel had antimicrobial activities against *E. coli.* Thus, *E. coli* may exhibit resistance to the antimicrobial action of *P. nitida* or the combination of *P. nitida* with *C. citratus* may have obliterated this effect. Further research is required to prove or refuse this hypothesis.

The aqueous extract of *Cymbopogon citratus* and *Picralima nitida* was effective against *Candida albicans*; however, the ethanolic extract did not produce any zone of inhibition at the various concentrations used up to 400 mg/ml. The zones of inhibition produced by greater concentration of the aqueous extract was significantly higher than the MIC. Thus, better efficacy of treatment, evidenced by larger diameter zones of inhibition, may be achieved with concentrations of the ethanol extracts much higher than the MIC. This finding contrasts with previous findings with the single herbal extracts. Abe *et al.* (2003) had observed that lemongrass essential oil and citral (its components) in the range of 25 µg/ml and 200 µg/ml inhibited the mycelial growth but allowed yeast-like growth. Cordoba *et al.* (2019) found that the essential oil of *Cymbopogon citratus* showed antifungal activity on *Candida* species. Ubulom *et al.* (2011) observed that *P. nitida* aqueous and ethanol extract showed inhibitory activities on *C. albicans* with the zone of inhibition generally increasing with increase in concentration. In that study 25 mg/ml, 50 mg/ml, 100 mg/ml, and 200 mg/ml were used and only the least concentration of the aqueous extract did not show a zone of inhibition. However, in the present study, *Candida albicans* exhibited resistance and lack of susceptibility to the antifungal activity

of the aqueous and the ethanol bi-herbal formulation of *Cymbopogon citratus* and *Picralima nitida.*

Application of the aqueous and ethanolic extract mixture of *Cymbopogon citratus* and *Picralima nitida* did not produce any zones of inhibition on *Aspergillus niger.* Helal *et al.* found that *Cymbopogon citratus* essential oil applied by fumigation or contavt method at 1.5 – 2.0 µL/ml completely inhibited the mycelial growth of *Aspergillus niger* (2000) Fakeye *et al.* (2000) had observed that stem bark extract of *P. nitida* had a lower MIC for *A. niger* than that of tiaconazole. Thus, *Aspergillus niger* exhibited resistance and lack of susceptibility to the antimicrobial activity of the aqueous and the ethanol bi-herbal formulation of *Cymbopogon citratus* and *Picralima nitida.* The combination of *P. nitida* with *C. citratus* may have obliterated this effect. Further research is required to prove or refuse this hypothesis.

CONCLUSION

In summary, both the aqueous and ethanol bi-herbal formulations of *Cymbopogon citratus* and *Picralima nitida* possess antimicrobial property against Gram-positive bacteria *Staphylococcus aureus* and *Bacillus subtilis*, although the organisms were more susceptible to the ethanol extract. The formulation is not effective against Gram-negative organisms such as *Klebsiella pneumonia* and *Escherichia coli,* which are resistant to the antibacterial activity. The formulation had no antifungal action against *Aspergillus niger* but had slight activity against *Candida albicans.*

RECOMMENDATIONS

Therefore, this formulation should be considered for further research and application in the treatment of infections caused by Gram-positive organisms. Further research using the crude extract formulation and not just on the extract of the essential constituents is needed to better understand the differences that the synergistic effects of *Cymbopogon citratus* and *Picralima nitida* may make in the treatment of drug-resistant infections. There is also the need to proffer a standard approach to the investigation of the antimicrobial activity of herbal agents on pathogenic organisms. For example, a study using single extracts of *Cymbopogon citratus* and *Picralima nitida* as well as the combined extract of both plants will reveal the advantages, or otherwise, of one treatment over the other. This will allow for easy comparison of research data and extrapolation of research findings. Also, further research is recommended to elucidate the efficacy, safety, and toxicity of this formulation.

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APPENDIX

1. Gram reaction

It was carried out to differentiate organisms by the Gram stain

Method:

A Clean slide was made grease free by passing through a gentle flame of Bunsen burner. A smear of the organism was made using normal saline and allowed to air dry. The smear was best fixed by passing through a gentle flame of Bunsen burner. It was stained with crystal violet for 1 minute, washed with water, flooded with Lugol iodine for I minute, washed with water, decolourised with acetone immediately, washed immediately with water, counter stained with neutral red for 1 minute, washed with water, allowed to air dry then examined microscopically using 100 objectives

2. Motility

It was carried out to examine the growth for motile bacteria.

Method (hanging drop technique):

A glean slide was made grease free hy passing through flames of Bunsen burner. A ring of plasticine was made at the centre of the slide. To a clean cover slip, a loopful of the broth culture of grown organism was placed. The ring of plasticine was gently pressed unto the cover slip. With a quick movement, the slide was inverted so that the cover slip will be uppermost. The drop was then examined microscopically using 10 objective lens to focus and 40 objective lens to view

3. Catalase test

It was carried out to differentiate bacterial that produce catalase from non-catalase producing bacteria.

Method (tube method):

2-3ml of 3% hydrogen peroxide was poured into a clean test tube. Using a sterile stick, a growth of the test organism was immersed into hydrogen peroxide. The tube was observed for bubble.

4. Coagulase test

It was carried to differentiate bacteria that produce coagulase from those that do not produce coagulase

Method:

On a clean microscope slide, a thick suspension of the organism in a drop of normal saline was made. One loopful of undiluted pooled human plasma was added. It was then observed for visible clot formation.

5. Oxidase test

It was carried out to determine the oxidase reaction of organisms in culture growth. It would help in the identification of Pseudomonas aeruginosa.

Method:

To an overnight peptone water culture, 0.5ml of Kovac's reagent was added, it was shaken and examined red coloring on the surface layer

PREPARATION OF REAGENTS AND CULTURE MEDIA

Nutrient Agar

To 1 litre of sterile distilled water, 39 grams of Mueller Hinton powder was weighted and added aseptically. This was allowed to dissolve for 10 minutes and sterilized by autoclaving a121°efor15minutes. The Agar was cooled to 50°e, mixed and poured aseptically into Petri dish and allowed to set

Mac Conkey Agar

This medium is best prepared from ready to use dehydrated powder available from most suppliers of culture media. The medium is usually used at a concentration of 32.2g in

every 1000ml distilled water. It was prepared according to manufacturer's instructions. It was sterilized by autoclaving at 121°C for 15mins and allowed to cool at 55°C and then dispensed aseptically in sterile petri dish and store at 2-8°C

Normal saline

8.5g of the sodium chloride was weighed and transferred to a leak proof bottle pre-marked to hold 1 litre. Distilled water was added to the 1 litre mark. The mixture was mixed until the salt was fully dissolved. The bottle was labelled and stored at room temperature.

Method:

To an overnight peptone water culture, 0.5ml of Kovac's reagent was added, it was shaken and examined for red coloring on the surface layer

PREPARATION OF REAGENTS AND CULTURE MEDIA

Nutrient Agar

To 1 litre of sterile distilled water, 39grams of Mueller Hinton powder was weighted and added aseptically. This was allowed to dissolve for 10 minutes and sterilized by autoclaving at121"efor15minutes, The Agar was cooled to 50°, mixed and poured aseptically into Petri dish and allowed to set.

Mac Conkey Agar

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Normal saline

8.5g of the sodium chloride was weighed and transferred to a leak proof bottle pre-marked to hold 1 litre, Distilled water was added to the 1 litre mark. The mixture was

mixed until the salt was fully dissolved. The bottle was labelled and stored at room temperature.

Peptone water

1.5 g Oxoid peptone was dissolved in 100 ml of distilled water. This was mixed and distributed into bijou with screw caps and sterilised by autoclaving at 121 ° C for 15 minutes.

APPENDIX II

Standard biochemical test of the bacterial isolates

Biochemical tests (Validation tests)	*Staphylococcus Aureus*	*Escherichia coli*	*Klebsiella pneumoniae*	*Bacillus Subtilis*
Gram	+	-	-	+
Motility	-	+	-	+
Catalase	+	-	+	+
Coagulase	+	-	-	-
Oxidase	-	-	-	-
Indole	-	+	-	-
Urease	-	-	+	-
Citrate utilisation	-	-	+	+
Germ tube test	-	-	-	-

KEYWORD

+ = Positive

- = Negative

Standard biochemical test of the fungal isolates

Characteristic	*Candida albicans*	*Aspergillus niger*
Morphology	Oval to elongated budding yeast cells	Septate hyphae with conidiophores
Colony appearance	Creamy-white smooth colonies on agar	Dark green to black colonies
Germ tube formation	Positive	Negative