DETERMINATION OF THE MINERAL ELEMENT, PROXIMATE CONTENT, PHYTOCHEMICAL COMPOSITION AND THE ANTIBACTERIAL PROPERTIES OF THE AQUEOUS AND ETHANOLIC EXTRACT OF *Citrus sinensis* ON SOME BACTERIAL ISOLATES

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BENIN CITY

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A THESIS WRITTEN IN THE DEPARTMENT OF SCIENCE LABORATORY, FACULTY OF LIFE SCIENCES AND SUBMITTED TO THE COLLEGE OF POSTGRADUATE STUDIES IN PARTIAL FULFILLMENT OF THE REQUIREMENT FOR THE AWARD OF POSTGRADUATE DIPLOMA (PGD) IN SCIENCE LABORATORY TECHNOLOGY, UNIVERSITY OF BENIN, BENIN CITY.

DECEMBER, 2025.

CERTIFICATION

We certify that this work was carried out by Martha Obhakhomen EKHEGBESELA (Mrs) in the Department of Science Laboratory Technology, Faculty of Life Sciences, University of Benin, Benin Cit.

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CERTIFICATION OF THESIS

We attest and declare that the thesis titled Determination of the Mineral Element, Proximate Content, Phytochemical Composition and the Antibacterial Properties of the Aqueous and Ethanolic Extract of *Citrus sinensis* on some Bacterial Isolates has successfully passed the anti-plagiarism test and does not violate any copy right regulation.

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DEDICATION

This project work is dedicated to God Almighty, who has made it possible for me to be alive till this day and has provided for me and sustained me.

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My heartfelt gratitude goes to God Almighty for His grace and blessings throughout my time at the University of Benin.

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I want to appreciate my lovely husband for his encouragement and financial support throughout this program**.** I also appreciate my elder brother and love ones for their assistance encouragement all through this program, and all my classmate that made it a success. God bless you all.

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ABSTRACT

Plants have long been used as medicine to cure a wide range of illnesses and humans have depended on nature to meet their basic needs. This study was aimed at determining the mineral content, proximate content, phytochemical composition and the antibacterial properties of the aqueous and ethanolic extract of *citrus sinensis* on some bacterial.

The aqueous and ethanolic extracts were evaluated for mineral elemental analysis, proximate content and phytochemical properties using the method of A.O.A.C. Antibacterial activity was evaluated using an agar well dilution techniques against five bacterial isolates using the Mueller Hinton Agar (MHA) method. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the extract were determined using the MIC and MBC.

The mineral element of *Citrus sinensis* of aqueous and ethanolic extract for Sodium (Na) was (250.333 ± 0.333 mg/kg) and (260.300 ± 0.3111 mg/kg), Potassium (K) (3415.667 ± 2.500 mg/kg) and (2215.060 ± 2.400) mg/kg, Calcium (Ca) (1.412.077± 1.400 mg/kg) and (1,521.047 ± 1.453 mg/kg), Phosphorus (P) (0.2100 ± 0.005 mg/kg) and (0.1900± 0.005 mg/kg), Iron (Fe) (1.1400 ± 0.004 mg/kg) and (1.2400 ± 0.009 mg/kg), Nitrogen (N) (0.1400 ± 0.001 mg/kg) and (1.2000 ± 0.009 mg/kg), Magnesium (Mg) (129.100± 0.700 mg/kg) and (131.000± 0.577 mg/kg). The result for proximate content of aqueous and ethanolic extract of *Citrus sinensis* showed varying composition of moisture content, ash content, crude fibre, crude fat, protein and carbohydrate. The qualitative phytochemical result revealed the presence of phenols, terpenoids, alkaloids, eugenols, flavonoids, tannins, and reducing sugars. The quantitative phytochemical result revealed the presence of alkaloid to be (5.4063 ± 0.2985) for aqueous and (6.3427±0.329) for ethanolic, total saponins, (5.9620 ± 3.6112) for aqueous and (7.0867±0.002) for ethanolic, total phenolic, (0.2077 ± 0.0012) for aqueous and (0.2140±0.001) for ethanolic, total tannins (10.1533 ± 0.0023) for aqueous and (12.7747±0.161) for ethanolic and for total flavonoids, (6.9993 ± 0.0127) for aqueous and (6.5827±0.031) for ethanolic. The antibacterial activity of the crude extract against selected bacterial isolates was also evaluated using the MHA method. It was demonstrated that at a concentration of 200 mg/kg of both the aqueous and ethanolic extract, the extract of *Citrus sinensis* expressed bactericidal potential against the bacterial isolates. The result from this research shows the potency of the extract of *Citrus sinensis* being able to the utilized for drug formations with antibacterial effects.

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background of the Study

Plants have long been used as medicine to cure a wide range of illnesses and humans have depended on nature to meet their basic needs (Cragg and Newman, 2013). The use of medicinal plants to treat illnesses has existed since the beginning of human history; that is, people have always looked to their surroundings for a means of healing from illnesses, using plants as their only option (Halberstein, 2005). Since ancient times, people have used nature to treat their own illnesses. Nowadays, the foundation of many early medications is the clinical, pharmaceutical and chemical research of these medications, which are mostly derived from plants such as Aspirin (from willow bark), Digoxin (from Foxglove), Morphine (from Opium poppy), Quinine (from Cinchoria skin) and Pilocarpine (from Maranham Jaborandi). Currently, it is estimated that over 50 % of the available drugs are somehow derived from medicinal drugs (Yarnell *et al*., 2002; Harvey, 2008). A wide range of plants with therapeutic qualities are referred to as medicinal plants. These plants have therapeutic qualities. The chemicals found in these plants are abundant and can be utilized to create new drugs (Rasool, 2012). Due to issues with accessibility, price and antimicrobial resistance to chemical medications, traditional medicines continue to be valued as significant and long-term therapy options (Patwardhan, 2005). Thus, researchers are compelled to investigate substances that may have antibacterial qualities and come from natural sources, particularly from traditionally used medicinal plants (Dhama *et al*., 2014; Dilbato *et al*., 2019). The unique characteristics of plants, including their wide range of chemical compounds (Gurib-Fakim, 2006), the proven therapeutic efficacy of their extracts in longstanding traditional medical practices around the world (Yang *et al*., 2014), their accessibility and the possibility of mutually beneficial effects between phytochemicals (Caesar and Cech, 2019), have made

them the preferred choice, particularly when it comes to addressing the problem of drug resistance. The emergence of chemistry and the isolation, purification and determination of plant chemicals coincided with the start of development of herbal medicines (Shakya *et al*., 2012). According to WHO, traditional medicines and primarily natural plant products provide the majority of developing country populations with primary healthcare. The medicinal power of ancient plant species is found in phytochemical components that have a specific pharmacological effect on the human body (Naseem *et al*., 2014). The primary phytochemical components found in medicinal plants are Tannins, alkaloids, saponins, cardiac glycoside, steroids, terpenoids, flavonoids, phylonatanins, anthraquinones and reducing sugars. Terpenoids has significant pharmacological properties such as antiviral, antibacterial, antimalarial, anti-inflammatory, cholesterol synthesis inhibition and anticancer properties (Boroushaki *et al*., 2016). The components of medicinal plants can be applied to many plant components, such as seeds, roots, fruit, skins, flowers, or even the entire plant. The majority of medicinal plants contain active chemicals that are employed as medicinal agents and have either direct or indirect therapeutic impact. The increasing prevalence of antibiotic resistance has become a significant challenge in the treatment of infectious diseases globally. As a result, the exploration of alternative sources of antimicrobial agents, particularly from plants, has garnered much attention. Plants are rich in bioactive compounds that have long been used in traditional medicine to treat various ailments. These natural substances, including alkaloids, flavonoids, saponins, and tannins, have demonstrated significant antimicrobial, anti-inflammatory, and antioxidant properties. *Citrus sinensis*, commonly known as the sweet orange, is one of the most widely cultivated fruit species worldwide. *Citrus sinensis* is mostly enjoyed for its sweet flavour and abundant vitamin C, yet the peel, leaves, and seeds of the plant have also been researched for their medicinal properties. Studies have indicated that the peel of plant contains multiple bioactive compounds, which contribute to its antimicrobial

properties, antioxidant effects, and potential health benefits (Aboaba and Efuwape, 2001; Alams *et al*., 2005). The antibacterial potential of plant extracts, including those of *Citrus sinensis*, has been documented in various studies. For instance, the methanolic extracts of citrus peels have exhibited antibacterial activity against a wide range of bacteria, including *Staphylococcus aureus* and *Escherichia coli* (Nair *et al*., 2005; Lawal *et al*., 2013). The potential of *Citrus sinensis* as a natural treatment for illnesses brought on by both Gram-positive and Gram-negative bacteria is highlighted by these findings.

In addition to its antibacterial properties, the *Citrus sinensis* plant is known for its rich content of bioactive compounds. Proximate analysis, which evaluates the nutritional content of plant materials, is essential for understanding the potential health benefits of *Citrus sinensis*. The nutritional components typically assessed in proximate analysis include moisture content, crude protein, fat, fiber, and ash content (Dubey *et al*., 2011). These components are important not only for understanding the nutritional value of the plant but also for determining its suitability for various therapeutic applications. The demand for alternative antibacterial agents has increased due to the growing worry over antibiotic resistance. Although synthetic antibiotics remain the mainstay in treating bacterial infections, their overuse has led to resistance, making infections harder to treat (Vahabi *et al*., 2011). The search for natural antimicrobial agents from plant sources offers a promising solution to this problem. *Citrus sinensis*, with its wide availability and reported medicinal properties, remains an understudied source of potential antimicrobial agents, especially when compared to other more commonly studied plants.

1.2 Aim of study:

This study was aimed to determine the mineral element, proximate content, phytochemical composition and the antibacterial properties of the aqueous and ethanolic extract of *citrus sinensis* on some bacterial

1.3 The specific objectives of this research were to:

- determine the mineral element of the aqueous and ethanolic extract of *Citrus sinensis* fruit.
- determine the proximate content of the aqueous and ethanolic extracts of *Citrus sinensis*
- analyze the qualitative and quantitative phytochemical properties of the aqueous pomace extract of *Citrus sinensis*
- determine the quantitative phytochemical properties of aqueous and ethanolic extracts of *Citrus sinensis*
- determine the zone of inhibition of the aqueous extract of *Citrus sinensis*
- determine the zone of inhibition of the ethanolic extract of *Citrus sinensis*
- determination of the minimum bactericidal concentration (MBC) of the aqueous extract of *Citrus sinensis* on bacterial isolates
- determination of the minimum bactericidal concentration (MBC) of the ethanolic extract of *Citrus sinensis* on bacterial isolates

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Medicinal plants

A wide range of plants with therapeutic qualities are referred to as medicinal plant. Medicinal plants are a valuable source of a wide variety of chemical molecules having different structures and functionalities that exhibit important biological activities and are linked to a multitude of beneficial properties such as antimicrobial, anticancer, antiviral, antioxidant and enzyme inhibitory, anti-aging, anti-inflammatory, antihypertensive, neuroprotective and anticoagulant effects (Ali *et al*., 2019; Lesellier *et al*., 2021). Medicinal plants are very important all around the world, both on their own and in combination with conventional medicine. According to Tlili and Sarikurkcu (2020), the use of natural goods is becoming more popular due to the impressive number of publications on the therapeutic qualities of medicinal plants and extensive expertise in folk medicine. Research into novel bioactive substances is even more important now because of the rise of germs that are resistant to antibiotics. Many sources claim that 25–50 % of presently manufactured pharmaceuticals used in healthcare are derived from medicinal plants (Mahmood *et al*., 2019; Sinan *et al*., 2020), and international efforts are underway to find novel bioactive chemicals from both familiar and exotic plants (Fettach *et al*., 2019). Research in this field is expected to continue for new medicines derived from natural products (Cádiz-Gurrea *et al*., 2021).

2.2 Importance of medicinal plants

Medicinal plants have been vital to human health for thousands of years, not only treating various ailments but also serving as the basis for many modern medications. Ancient civilizations, including the Egyptians and Native Americans, utilized plants like garlic and aloe vera for their antimicrobial and healing properties (Abalaka and Bello, 2006). Similarly, willow bark was used by the Greeks and Romans and led to the creation of aspirin (Vane, 2003).

Plants have medicinal qualities because of their complex chemical makeup, which contains substances like flavonoids and alkaloids that have beneficial effects on the body. In Traditional Chinese Medicine, for instance, ginseng has long been used to increase immunity and vigour (Kennedy *et al*., 2001), while Native Americans have traditionally used Echinacea to treat respiratory infections (Mills and Bone, 2005). Other plants with anti-inflammatory and antioxidant properties include ginger and turmeric (Chandran and Goel, 2012; Lumb *et al*., 2009). The fact that many contemporary medications, like quinine and morphine, are made from plant chemicals shows how important plants are still in pharmacology (Katzung *et al*., 2012). Recent research into natural remedies has revived interest in plants like St. John's Wort, which may help with depression, and moringa, praised for its nutritional value and anti-inflammatory properties (Linde *et al*., 2008). In summary, medicinal plants not only provide therapeutic benefits but also contribute to cultural heritage, sustainability, and biodiversity, emphasizing the deep connection between nature and human health. Citrus peels are abundant in nutrients and a variety of phytochemicals, according to their chemical makeup (Adewusi and Afolayan, 2010: Al-ani *et al*., 2010). Rarely found in other plants, flavanone and many polymethoxylated flavones are abundant in citrus peels (Anitha *et al*., 2016). The peel of citrus fruits yields an essential oil with significant global economic worth, yet they are primarily utilized as desserts. According to Bourgou *et al*. (2012), it is employed in the pharmaceutical industry as a flavouring ingredient to cover up bad tastes. They are used as

flavour in food industries (Caccioni *et al*., 1998). A wide number of plant extracts possess antimicrobial properties which are used as natural alternatives to treat several diseases. Scientific studies available on medicinal plants indicate that promising phytochemical can be developed for many health problems (Dhiman *et al*., 2012). There is a great demand of fruit juices in the treatment of various illnesses such as arthritis, heart diseases, muscle aches and drug addictions (Droby *et al*., 2008). Hamendra and Anand, 2007; Ehler, 2011) reported its use as antidiabetic and also as an antimicrobial (Espina *et al*., 2011). The medicinal potency of *C. sinensis* is due to its high content of Vitamin C which is believed to stimulate the production of white blood cells, primarily neutrophils which attack the foreign antigens such as bacteria and viruses. It does boost the immunity by production of antibodies and interferon, the proteins that helps protects against viral invaders and cancer cells (Forbes *et al*., 2007).

Citrus sinensis peel has many medicinal properties and is widely used against various ailments, such as colic, upset stomach, cancer, diuretic, cormunative, immuno – enhancing, stomachic, tonic to digestive system, immune system and skin. It is also used to treat and prevent vitamin deficiencies, colds, flu, and scurvy and helping to fight viral and bacterial infections. Antibacterial effects of orange peel have been demonstrated in the literature (Lawal *et al*., 2013). Dubey *et al*. showed potent antibacterial activity (against *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Staphylococcus epidermidis, Shigella flexineri, Bacillus subtilis and Escherichia coli*) of extract from fruit of Orange peels using disk diffusion method (Dubey *et al*., 2011). Chabuck *et al*. (2011) observed orange peel extract to be effective against *Klebsiella pneumonia*.

2.3 *Citrus sinensis*

The Rutaceae family includes the genus *Citrus sinensis*, which can be found worldwide in a variety of forms, including trees, shrubs and herbs. The citrus genus is one of the most significant commercial fruit crops grown on every continent in the globe and it is the most

widely grown and traded variety in garden plants. *Citrus sinensis* is cultivated for its edible and therapeutic fruits at a high cost. About 70 % of the annual production of Citrus species is produced by *Citrus sinensis*, one of the main citrus cultivar groupings cultivated worldwide (Flamini *et al*., 2003). Originally from Asia, *Citrus sinensis* is now found throughout the Pacific and warm regions of the world. *Citrus sinensis* is a blooming, evergreen tree. Orange trees often reach a height of 9 to 10meters, and their branches have prominent spines. The alternating leaves have narrowly winged petioles that are 3-5mm wide and 6.5-15cm long. The blades can be elliptical, rectangular, oval, and blunt-toothed and they emit a distinct citrus scent because of the presence of copious oil. The auxiliary flowers have five white petals and twenty to twenty-five yellow stamens and they can be displayed individually or in whorls of six (5cm wide). The fruit ripens to orange or yellow and can be globose to oval (6.5 to 9.5cm broad). The fruit's anatomy is divided into two parts: the endocarp, or pulp containing juice sac glands and the pericarp, often known as the peel, skin or rind (Orwa *et al*., 2009). The epidermis of the epicuticular layer, which is made up of many tiny, aromatic oil glands, is what gives the skin its distinct scent. The outer flavedo or epicarp, which is primarily composed of parenchymatous cells and cuticle, makes up the pericarp (Goudeau *et al*., 2008). The tissue max squeezed into the intercellular space is made up of tubular-like cells that make up the albedo or mesocarp that lies beneath the flavedo (Rao, 2011). The fruit is perennial, and it has adapted to a wide range of climates. Citrus fruits have long been prized for their healthful nutritional and antioxidant qualities and are a major source of vital phytochemical elements. *Citrus sinensis* has many health benefits for people. Their applications include the treatment of arteriosclerosis, cancer prevention, stomach ulcers, kidney stones, cholesterol reduction, immune system strengthening and high blood pressure. Vitamins, particularly vitamin C and phytochemical compounds such as synephrine, limonoids, hesperidin, flavonoids, polyphenols, pectin, etc., are responsible for these health

advantages (Etebu and Nwauzoma, 2012). *Citrus sinensis* is grown not only for its ripe, delicious fruit but also for its seeds, leaves, roots and blossoms, all of which have long been used to make a variety of medicinal concoctions.

2.4 Mineral Content of Medicinal Plants

Medicinal plants have been utilized for centuries to cure a variety of ailments because of their high concentration of bioactive chemicals and necessary minerals. These herbs, which are widely used in traditional medicine, are gaining scientific interest for their potential in healthcare and illness prevention. Aside from phytochemicals, medicinal plants include essential minerals that benefit human health by supporting metabolic functions, enzyme activity and immunological responses (Rafiq *et al.,* 2018) Minerals including calcium, potassium, magnesium and zinc are essential for maintaining physiological balance in the body. A lack of certain micronutrients can result in serious health diseases such as anaemia, osteoporosis and immune dysfunction. Numerous health advantages of other plants, such as orange and its numerous parts have also been found. Useful, that orange has a significant amount of minerals, which may be necessary for the body's processes, metabolism and poultry health. Citrus fruits are excellent source of phosphorus, which works with calcium to help build bones and teeth (Takeda *et al*., 2004). Zinc is a micronutrient that is equally significant because it boosts immune systems and shields the body from oxidative stress (Chasapis *et al*., 2012). According to a research study by Turner and Burri (2013), Citrus fruits are rich in these minerals. In addition to human nutrition, Citrus sinensis can also be considered for animal feed applications. However, its mineral content suggests that additional

supplementation with dicalcium phosphate or other mineral sources may be necessary for poultry and livestock diets to ensure optimal nutrient balance

2.5 Proximate Content of Medicinal Plants

Numerous minerals are present in medicinal plants, which adds to their high nutritional value. Plants have different mineral composition that are beneficial to human health when taken, such as nettle (Bhusal *et al*., 2022), dandelion greens (Olas, 2022), parsley (Farzaei *et al*., 2013), basil (Calderon-Bravo *et al*., 2021), and fibre (Anderson *et al*., 2009), are examples of these plants. There are numerous health advantages of other plants, such as the orange and its numerous parts, have also been found.

2.6 Phytochemical Properties of Medicinal Plants

Medicinal plants contain a variety of bioactive compounds, such as flavonoids, alkaloids, terpenoids, and phenolic acids, which give them therapeutic properties. Both *Citrus sinensis* (sweet orange) and *Citrus aurantium* (bitter orange) share similar phytochemicals, but with distinct differences. Bitter orange has higher levels of alkaloids like synephrine, which are linked to weight loss and appetite suppression, while sweet orange has more antioxidant-rich flavonoids like hesperidin. Both species contain antimicrobial terpenoids, but bitter orange has a different essential oil profile with compounds like linalool, contributing to its calming effects. Additionally, bitter orange has higher concentrations of flavonoids and tannins in the peel, which are used for digestive health, while sweet orange is more commonly used for immune support. Despite their differences, both types of orange offer health benefits, with sweet orange focusing more on overall antioxidant effects and bitter orange being more specialized for digestive and weight loss purposes (Moyo *et al.,*2023). Numerous substances have been identified by the thorough analysis of sweet orange peels' chemical makeup; these compounds, in particular secondary metabolites, are the subject of this article. These

substances include alkanes, terpenoids, steroids, flavonoids, ethyl esters, and essential oils. Because of their varied impacts on the human body, citrus phytochemicals, which are found in different regions of the fruit, are acknowledged as health boosters. These phytochemicals have antibacterial, antifungal, antiviral, anticarcinogenic, antithrombotic, and anti-inflammatory actions, among other positive qualities. Their potential health advantages are further enhanced by the fact that they participate in the reduction of cholesterol levels (Moyo *et al.,*2023).

Flavonoids are key secondary metabolites commonly found in sweet orange peels, playing a significant role in imparting the distinctive citrus aroma. Research indicates that sweet orange peels extracted using dimethyl sulfoxide (DMSO) and the juice squeezed from the peel contain a group of flavonoids, including Eriocitrin, Narirutin, Hesperidin, and Naringin (Manthey, 2004). Citrus fruits are renowned for their abundance of flavonoids, which possess various physiological properties, including antiviral and antimicrobial activities. Flavonoids are typically present in the form of glycosides or aglycones, especially in citrus juices, where they are found as flavonoid glycosides (FGs). These flavonoids have demonstrated potential health benefits for humans, exhibiting promising effects in controlling chronic diseases and combating infections. Specific flavonoids, like Hesperidin and quercetin, are implicated in controlling viruses such as herpes, parainfluenza, and polioviruses. Moreover, metabolites of Naringin serve as a rich source of natural antimicrobials, demonstrating activity against both Gram-positive and Gram-negative bacteria (Loizzo *et al*., 2012). Citrus species are renowned for being abundant sources of aromatic compounds, particularly in their fruit peel. Citrus fruits contain approximately 400 volatile compounds. For instance, orange peel is composed of around 1.5 % phytoconstituents in the form of essential oils. Since ancient times, essential oils extracted from citrus fruits have been utilized for medicinal and aromatic purposes. In contemporary times, these essential oils find applications across various industries, including

pharmacy, cosmetics, and related fields, owing to their notable antispasmodic and antimicrobial properties (Sikdar *et al*., 2016; Jung *et al*., 2001). Phytochemicals, also known as bioactive organic chemical compounds, found in medicinal plants, are known to have protective effects against major chronic diseases, including both infectious and host metabolic or genetically dysfunctional diseases (Khan *et al*., 2009; Nayak *et al*., 2015; Gbolade and Adeyemi, 2008; Esposito *et al*., 2016). According to phytochemical screening, the amounts of flavonoids, alkaloids, and saponins in citrus peel extracts obtained through various solvents—such as aqueous, methanol, ethyl acetate, chloroform, and n-hexane—vary depending on the extraction method used. These primary components of citrus peels have been linked to numerous therapeutic benefits. Citrus peel extracts are rich in various physicochemical components that contribute to their health benefits. The major components include flavonoids, essential oils, pectin, and ascorbic acid (vitamin C). Flavonoids possess antioxidant, anti-inflammatory, and anti-cancer properties. For instance, Hesperidin has been linked to improved cardiovascular health by enhancing blood circulation and reducing blood pressure (Kumar *et al*., 2016). Essential oils exhibit antimicrobial and anti-inflammatory effects, with the potential to reduce the risk of certain cancers and improve digestive health (Bakkali *et al*., 2008). Pectin, a soluble fiber found in citrus peels, aids in digestion, helps regulate blood sugar levels, and lowers cholesterol, which can contribute to heart health (Roberfroid, 2007). The high vitamin C content in citrus peels supports immune function and acts as a powerful antioxidant, protecting cells from oxidative stress (Carr and Maggini, 2017).

2.7 Antibacterial Properties of Medicinal plant

Citrus sinensis, commonly known as sweet orange, has demonstrated significant antimicrobial properties, particularly through its essential oils and phytochemical components. Studies have highlighted the effectiveness of sweet orange extracts against a variety of

microorganisms, reinforcing its value as a medicinal plant. One of the primary contributors to the antimicrobial activity of *Citrus sinensis* is its essential oil. (Baba *et al*., 2018) explored the antibacterial effects of the fruit on clinical bacterial species isolated from wounds and found that the orange exhibited potent antibacterial properties, particularly against wound pathogens. The main ingredient in the essential oil, limonene, has been demonstrated to have bactericidal properties by rupturing bacterial cell membranes, which is primarily responsible for this. Citrus peel essential oils also dramatically reduced microbial growth in bread, according to research by Rehman *et al*. (2007), highlighting *Citrus sinensis*'s broad-spectrum antibacterial properties. The fruit's peel, in particular, has been studied for its antimicrobial activity, the researcher provided insights into the antimicrobial properties of citrus fruit peels, revealing their potential in combating various bacteria and fungi (Kumar *et al*., 2016). This supports the utilization of orange peel waste in developing natural antimicrobial agents, thus adding value to the fruit byproducts. Flavonoids and other phytochemicals are essential to *Citrus sinensis*'s antibacterial activity. The fruit contains a lot of flavonoids, such as hesperidin, which help to stop bacteria from growing. According to studies, these substances show antibacterial activity against microorganisms that cause oral infections, such as *Streptococcus mutans* (Terao *et al*., 2002). This implies that *Citrus sinensis* may have uses in oral health, assisting in the reduction of dangerous oral germs. Additionally, *Citrus sinensis* has been shown to have strong inhibitory effects on human diseases that are resistant to several drugs in a larger study by Ahmed and Beg (2001) on the antibacterial properties of various medicinal plants. This supports the idea that *Citrus sinensis* is a natural antibacterial agent that can be utilized to treat infections that are hard to cure with traditional medicines. Overall, *Citrus sinensis* possesses notable antimicrobial properties, largely due to its essential oils and flavonoid content. These compounds contribute to its broad-spectrum antibacterial and antifungal effects, supporting its use as a natural remedy in traditional and modern medicine.

2.8 Orange Plant (*Citrus sinensis*)

The Rutaceae family includes the genus *Citrus sinensis*, which can be found worldwide in a variety of forms, including trees, shrubs and herbs. The citrus genus is one of the most significant commercial fruit crops grown on every continent in the globe and it is the most widely grown and traded variety in garden plants. *Citrus sinensis* is cultivated for its edible and therapeutic fruits at a high cost. About 70 % of the annual production of Citrus species is produced by *Citrus sinensis*, one of the main citrus cultivar groupings cultivated worldwide (Flamini *et al*., 2003). Originally from Asia, *Citrus sinensis* is now found throughout the Pacific and warm regions of the world. *Citrus sinensis* is a blooming, evergreen tree. Orange trees often reach a height of 9 to 10meters, and their branches have prominent spines. The alternating leaves have narrowly winged petioles that are 3-5mm wide and 6.5-15 cm long. The blades can be elliptical, rectangular, oval, and blunt-toothed and they emit a distinct citrus scent because of the presence of copious oil. The auxiliary flowers have five white petals and twenty to twenty-five yellow stamens and they can be displayed individually or in whorls of six (5cm wide). The fruit ripens to orange or yellow and can be globose to oval (6.5 to 9.5cm broad). The fruit's anatomy is divided into two parts: the endocarp, or pulp containing juice sac glands and the pericarp, often known as the peel, skin or rind (Orwa *et al*., 2009). The epidermis of the epicuticular layer, which is made up of many tiny, aromatic oil glands, is what gives the skin its distinct scent. The outer flavedo or epicarp, which is primarily composed of parenchymatous cells and cuticle, makes up the pericarp (Goudeau *et al*., 2008). The tissue max squeezed into the intercellular space is made up of tubular-like cells that make up the albedo or mesocarp that lies beneath the flavedo (Rao, 2011). The fruit is perennial, and it has adapted to a wide range of climates. *Citrus sinensis* is the world's most commonly cultivated fruit tree. It is commonly known as sweet orange which belongs to the *Rutaceae* family which comprises mandarins, limes, lemons, grapefruits, sour and sweet

oranges (Karoui and Marzouk, 2013). The sweet orange tree is a blooming, evergreen that typically reaches a height of 9 to 10 meters. Its fruit eliminates weariness and has laxative, anthelmintic, strengthening, and cardiotonic properties (Kirtikar and Basu 1984). According to Ramachandran *et al*. (2002), it has antibacterial, anti-inflammatory, and antioxidant qualities. Its leaves are shiny and leathery, arranged alternatively. Oranges are said to lower cholesterol and aid in the digestion of fatty foods (Cesar *et al*., 2010). Citrus fruits are of immense economic value, occupying the top position in fruit production. Orange trees are widely cultivated in tropical and subtropical climates for the sweet fruit, which is peeled or cut (to avoid the bitter rind) and eaten whole or processed to extract orange juice (Pandharipande and Makode, 2009; Kamar *et al*., 2011). Citrus fruits are known to contain substantial quantities of vitamin C, a potent water-soluble vitamin essential for healthy living. They are also known to contain other bioactive components such as carotenoids and a wide array of phenolic compounds. The consumption of citrus fruits is also believed to confer some protection against diseases such as cardiovascular disease and cancer (Guimaraes *et al*., 2010; Atolani *et al*., 2012). The orange fruit is composed of an external layer (peel) formed by flavedo (epicarp or exocarp) and albedo (mesocarp), and an inner material called endocarp that contains vesicles with juice (Liu *et al*., 2007). The seeds are usually embedded at the centre of the fruit, in direct contact with the juice sacs. The peels obtained from citrus fruits constitute between 50 and 65 % of the total weight of the fruits. When not processed further, this by-product becomes a very worrisome waste capable of causing serious environmental pollution (Mandalari *et al*., 2006; Hegazy and Ibrahim, 2012). In Nigeria, citrus fruit wastes are discarded carelessly in the environment. This leads to the release of odours, serving as fertile ground for insect proliferation and generally, the production of an unsightly environment with reduced aesthetic outlook.

Table 2.1: Taxonomical classification of *Citrus sinensis*

Rank	Scientific Name and Common Name
Kingdom	Plantae - Plants
Subkingdom	Tracheobionta -Vascular plants
Superdivision	Spermatophyta – Seed plants
Division	Magnoliophyta – Flowering plant
Class	Magnoliopsida - Dicotyledons
Subclass	Rosidae
Order	Sapindales
Family	Rutaceae
Genus	Citrus
Species	*Citrus sinensis*

Barett and Rhodes, (2008).

CHAPTER THREE

3.0 MATERIALS AND METHOD

3.1 Collection and Identification of Plant Materials

The sweet orange fruits were purchased from Ekiosa market in Benin city, Edo state, Nigeria. The fruits were identified by a taxonomist Prof. H.A. Akinnibosun in the Department of Plant Biology and Biotechnology, Life Sciences, University of Benin, Nigeria as Linnaeus Osbeck variety of *Citrus sinensis*.

3.1.1 Preparation of *Citrus sinensis* fruits

The *Citrus sinensis* fruits were thoroughly rinsed to get rid of impurities. The fruit was cut open and the juice removed. The plant was cut into small pieces and spread on a flat tray to air dry in the laboratory. It was also taken into the oven to dry at a temperature of 40 ^{o}C for six hours and the brought out to air dry in the laboratory for 3 weeks until it was brittle The sample was ground into a fine powder using an 8000 w high-power grinder. The grounded powder was soaked in aqueous and ethanolic solution separately for 72 hours and then filtered with a Whatman filter paper (No. 1).

3.1.2 Extraction of *Citrus sinensis*

800 g of the grounded *Citrus sinensis* fruit was added into 2 L aqueous and ethanolic solvent separately, they were allowed to soak for 72 hours. After 72 hours, each extract was sieved using the double sieve method. The filtrate was evaporated at 40 °C on a water bath, resulting in a concentrated extract with a sweet fragrance and a brown hue. The solid concentrate obtained from the filtrate was stored in a tightly sealed glass container that was kept in a refrigerator.

3.2: Mineral Analysis of *Citrus sinensis* Extract

3.2.1 Nitrogen Digestion

A modified method of micro-Kjeldahl was used for nitrogen digestion. Three grams (3 g) each of the defatted samples (aqueous and ethanolic extract) were separately weighed into micro-Kjeldahl digestion flasks together with few anti bumping granules. Two grams (2g) of catalyst mixture ($CuSO_4$: Na_2SO_4: SeO_2, 5:1:02 w/w) was added to each flask and then 10 mL nitrogen free concentrated H_2SO_4 was also added to each flask. The flasks were placed in inclined position on a heating mantle in a fume cupboard. The digestion began at temperature of 30 °C until frothing ceased and then heating was increased to 50 °C for another 30 min and finally at full heating (100 °C) until a clear solution was obtained. The flask and its content were left to simmer below boiling point for another 30 min to ensure complete digestion and conversion of nitrogen to ammonium sulphate. After digestion was completed, the samples were allowed to cool and then transferred quantitatively to 100 mL volumetric flasks with washing and cooling to room temperature. Volumes were made up to mark with distilled water (AOAC, 1990).

3.2.2 Nitrogen Determination

5 ml of the filtrate from the digest was transferred with the aid of a 10 ml pipette into a 25 ml standard flask. 2.5 ml of the alkaline phenate was added and the solution shaken to mix properly. Then 1 ml of Sodium Potassium Tartarate was added, shaken properly followed by the addition of 2.5 ml of sodium hypochlorite. Then, after the solution was made up to the 25 ml mark with distilled water and the absorbance of the resultant solution measured with the aid of UV/visible spectrophotometer, at 630 nm. The Nitrogen standards were treated the same way with the sample (AOAC, 1990).

Calculation

$$\text{Nitrogen (mg/kg)} = \frac{\text{Instrument. Reading. } \mathbf{X} \text{ Slope Reciprocal(mg/L) } \mathbf{X} \text{ Color Vol.(L) } \mathbf{X} \text{ Digest Vol.(L)}}{\text{Weight of Sample (kg) } \mathbf{X} \text{ Aliquot Taken (L)}}$$

3.2.3 Digestion of Other Elements

Three grams of the ethanolic sample was placed in a Kjeldahl flask. 10 ml of mixed acid (Nitric acid and perchloric acid mixture, ratio 3 to 1) was added to each flask. The flask and its content were mildly heated for about 20 minutes at a temperature of 40^{o}C and then increased to about 100^{o}C for another 40 minutes. The sample was allowed to cool, about 20 ml distilled water added and filtered into a standard flask. It was the made up to the 100 ml mark with distilled water (AOAC, 1990).

3.2.4 Determination of Phosphorus

A phosphorous standard stock solution 100 ppm was prepared by dissolving 0.44 g of potassium dihydrogen phosphate (K) in 250 ml of distilled water in a volumetric flask. Afterward, 2 ppm,4 ppm,6 ppm,8 ppm and 10 ppm are prepared by pipetting 2 ml, 4 ml, 6 ml,8 ml and 10 ml of the 100 ppm standard into five (5) respective 100 ml standard flasks already labelled and made up to mark with distilled water. From each standard solution, 1 ml was pipetted out and placed in the respectively labelled test tubes. 1 ml from the digested samples was also placed in an appropriate test tube and to each of the test tubes 8 ml of distilled water was added. This was followed by the addition of 1 ml phosphorous colouring reagent (which was prepared using 100 ml of 5N Sulfuric acid, 30 ml of ammonium molybdate solution and 10 ml of antimony potassium tartrate solution). The content of the flask was swirled gently to ensure proper mixing. Then 0.5 ml of the ascorbic acid solution (0.44 g/25 ml) was then added to the flasks. The absorbance of the mixtures read at 880 nm using a UV/VIS spectrometer (AOAC, 1990)..

$$\text{Phosphorous (mg/kg)} = \frac{\text{Instrument reading} \times \text{Slope} \times \text{Reciprocal (mg/L)} \times \text{Colour Vol. (L)} \times \text{Digest Vol. (L)}}{\text{Aliquot taken (L)} \times \text{weight (kg)}}$$

3.2.5 Analysis of Other Elements

The elements Sodium (Na) and Potassium (K) were assayed using Flame Photometer while Calcium (Ca), Magnesium (Mg), Iron (Fe), Copper (Cu), Lead (Pb), Cadmium (Cd), Nickel (Ni), Manganese (Mn) and Zinc (Zn) were assayed using Atomic Absorption Spectrophotometer (AOAC, 1990).

3.3 Proximate Determinations

3.3.1 Moisture content

2g of the sample was weighed and dried in an oven continuously. The dried sample was constantly re-weighed at 10 minutes intervals until a constant weight was obtained. The ratio of the change in weight to the original weight expressed in percentage gives the moisture content given by ---------- $\frac{W0-Wdry\ (\%)}{W0}$ (AOAC, 1990)..

3.3.2 Fat content

The Pearson (1973) method was used, which operates on the principle that non-polar components in samples can be efficiently extracted into organic solvents. In the procedure, 3 grams of each sample (moisture-free) were placed into fat-free thimbles, which were then weighed and sealed with glass wool. These thimbles were placed into Soxhlet extractors, each containing 160 mL of petroleum ether (b.p. 60 - 80 °C). Clean, dry receiver flasks were also weighed and attached to the extractors. After assembling the system, the water bath was heated to 60°C and cold water was cycled. It took eight hours to extract. The thimble containing the sample was then taken out and dried for three hours at 70 °C in an oven until its weight remained consistent. Finally, the weight of the thimble and its contents was measured using an analytical balance (AOAC, 1990).

Calculation: The crude fat was obtained as the difference in weight before and after the exhaustive extraction.

Hence the percentage fat was therefore calculated as:

% Fat = $\frac{X-Y}{Z}$

where,

x = Weight of sample and thimble and oil

Y = Weight of empty thimble

Z = Weight of sample

3.3.3 Ash content

2 grams of the dried sample were placed in a porcelain crucible, which was first weighed. The crucible was then placed into a preheated muffle furnace set to a temperature of 900 °C. The furnace was left running for one hour, after which the crucible and its contents were transferred to a desiccator to cool. Once cooled, the crucible and its contents were re-weighed, and the weight was recorded (AOAC, 1990). The percentage of ash content was then determined using the appropriate calculation.

$$\text{Ash} = \frac{100\ W_{ash}\ (\%)}{W_o}$$

W_{ash} = content weight after final drying.

W_o = the dried weight of the sample

3.3.4 Protein content

The crude protein content was determined using a modified micro-Kjeldahl method, as described by the AOAC (1990). For digestion, three grams of defatted samples were weighed into pre-weighed micro-Kjeldahl digestion flasks along with a few anti-bumping granules. Each flask received 2 grams of a catalyst mixture ($CuSO_4$: Na_2SO_4: SeO_2, 5.1:0.2 w/w), followed by 10 mL of nitrogen-free concentrated H_2SO_4. The flasks were placed in an inclined position on a heating mantle inside a fume hood. The digestion started at 30°C, continuing until frothing stopped, then the temperature was raised to 50°C for an additional 30 minutes. The temperature was finally increased to 100°C, and the heating continued until a clear solution formed. Digestion was carried on below boiling for another 30 minutes to ensure complete breakdown and conversion of nitrogen to ammonium sulfate. After digestion,

the samples were allowed to cool, then transferred quantitatively to 100 mL volumetric flasks, washed, and cooled to room temperature. The volumes were then adjusted to the mark with distilled water.

Next, 5 mL of the filtrate was transferred into a 25 mL standard flask using a 10 mL pipette. To this, 2.5 mL of Alkaline Phenate was added, and the solution was mixed thoroughly. Then, 1 mL of Sodium Potassium Tartrate was added, followed by shaking, and then 2.5 mL of sodium hypochlorite was added. The solution was made up to the 25 mL mark with distilled water, and the absorbance was measured at 630 nm using a UV/visible spectrophotometer. Nitrogen standards were processed in the same way as the sample.

Calculation

$$\%N = \frac{\text{Instrument. Reading. } \mathbf{X} \text{ Slope Reciprocal } \mathbf{X} \text{ Color Vol. } \mathbf{X} \text{ Digest Vol.}}{\text{Weight of Sample } \mathbf{X} \text{ Aliquot Taken } \mathbf{X} \text{ 10000}}$$

% Crude Protein = %Nitrogen×6.25

3.3.5 Crude Fiber

The procedure followed was based on (AOAC, 1990) method. In brief, 4 grams of each moisture-free sample were weighed into a 250 mL beaker, and 50 mL of 4% H_2SO_4 was added, followed by distilled water to reach a total volume of 200 mL. The mixture was then heated to boiling and kept boiling for exactly 30 minutes on a Bunsen flame, with constant stirring using a rubber-tipped glass rod to ensure all particles were removed from the sides of the beaker. The volume was maintained by adding hot distilled water. After 30 minutes of boiling, the contents were poured into a Buchner funnel fitted with ashless Whatman No. 40 filter paper and connected to a vacuum pump. The beaker was rinsed several times with hot distilled water, and the contents were transferred quantitatively using a jet of hot water. Washing continued on the funnel until the filtrate was acid-free, as indicated by litmus paper.

The acid-free residue was then quantitatively transferred from the filter paper into the same beaker, with any remaining traces removed using 5 % NaOH solution and hot water to a volume of 200 mL. The mixture was boiled for another 30 minutes with constant stirring, as previously described, and the volume was kept constant with hot water. The mixture was then filtered and washed as before until it was alkaline-free. Finally, the residue was washed twice with 2 mL of 95% alcoholThe filter paper remains were moved to a porcelain crucible that had been previously weighed. After being dried to a constant weight in an oven set at 110 °C, the contents of the crucible were let to cool in a desiccator. The crucible and its contents were chilled, then lit for eight hours at 550°C in a muffle furnace, cooled, and weighed. A triplicate analysis was performed for each sample. The percentage of crude fiber was then calculated.

Calculated as:

$$\% \text{ Crude Fibre } = \frac{100\ (y - a)}{X}$$

x = Weight of sample (g)

y = Weight of insoluble matter (g)

a = Weight of Ash (g)

3.3.6 Carbohydrate content

Estimation of total carbohydrate: The total carbohydrate content of the diet samples was determined by subtracting the combined percentages of crude protein, crude fat, moisture, fiber, and ash from 100.

3.4. Qualitative Phytochemical Screening

3.4.1. Detection of Alkaloids Content

2.0 ml of the plant extract was first evaporated to dryness. Then the resultant residues were dissolved in 5 ml of HCl (2 mol/dm^3) and filtered. Two test tubes were filled with the filtrate. A few drops of Mayer's reagent were added to the first test tube; the presence of alkaloids is indicated by the production of a yellow precipitate. The second test tube was treated with few drops of Wagner's reagent, and the brownish-red precipitate formation indicates alkaloids (AOAC, 1990).

3.4.2. Detection of Glycoside

This was carried out by dissolving 0.5 mg of the extract in approximately 1 mL of water, followed by the addition of an aqueous NaOH solution. The appearance of a yellow colour indicates the presence of glycosides (AOAC, 1990).

3.4.3. Detection of Tannins

1.0 ml of 1 % gelatine solution containing Sodium Chloride was added to 1.0 ml of the extract. The formation of a white precipitate indicates the presence of tannins.

3.4.4. Identifications of Phenolic Compounds

This was done by treating 1.0 ml of the plant extract with 4 drops of ferric chloride solution. The formation of a bluish-black colour indicates the presence of Phenols.

3.4.5. Detection of Saponins

The presence of saponins was detected using both the foam test and froth test methods. In the foam test, 0.5 g of the plant extract was shaken with 2.0 mL of distilled water. The formation of foam that lasts for 10 minutes indicates the presence of saponins. In the froth test, 5.0 mL of the extract was diluted with water to a total volume of 20.0 mL and shaken in a 50 mL graduated cylinder for 15 minutes. The formation of a 1 cm layer of foam indicates the presence of saponins (AOAC, 1990).

3.4.6. Screening for Flavonoids

Flavonoids were detected using the alkaline reagent test and the lead acetate test. In the alkaline reagent test, a few drops of a 2 mol/dm³ sodium hydroxide solution were added to the extract. The development of an intense yellow colour, which turns colourless upon the addition of dilute hydrochloric acid (2 mol/dm³), indicates the presence of flavonoids. In the lead acetate test, the plant extract was treated with a few drops of lead acetate solution. The appearance of a yellow precipitate indicates the presence of flavonoid (AOAC, 1990).

3.4.7. Detection of Eugenols

5 mL of a 5% KOH solution was mixed with around 2 mL of the extract. Next, the aqueous layer was filtered and separated. A light yellow precipitate formed when a few drops of HCl were added to the filter, indicating a positive result.

3.4.8. Detection of Steroids

To 0.5 g of the extract, 2 mL of acetic anhydride and 2 mL of H_2SO_4 were added. A colour change from violet to blue or green in some samples indicated the presence of steroids.

3.4.9. Detection of Terpenoid

A 0.2 g portion of the plant extract was combined with 2 mL of chloroform ($CHCl_3$), and 3 mL of concentrated H_2SO_4 was carefully added to create a separate layer. The appearance of a reddish-brown colour at the interface indicates a positive result for the presence of terpenoids.

3.4.10. Detection of Reducing Sugars (Carbohydrates)

To ascertain this parameter, Fehling's test was employed. Fehling's solutions A and B were heated in equal volumes for one minute each, followed by the addition of a volume of the plant extract and another boiling for five minutes. A bricked- red precipitate is required (AOAC, 1990).

3.5 Quantitative Phytochemical Analysis

3.5.1 Determination of Total Phenolic Contents

The total phenolic content in the extract was determined using Folin-Ciocalteu reagent, following the method of Singleton and Rossi (1965) with slight modifications, using tannic acid as the standard. Briefly, 1.0 mL of the extract solution (250 µg/mL) was placed in a test tube. Then, 1.0 mL of Folin-Ciocalteu reagent was added, and the mixture was stirred thoroughly. After 5 minutes, 15.0 mL of 20% Na_2CO_3 was added, and the mixture was allowed to stand for 2 hours. The absorbance was measured at 760 nm using a UV-Vis spectrophotometer (Jenway 6100, Dunmow, Essex, U.K.). The total phenolic content was expressed in µg of tannic acid equivalents (TAE), based on a calibration curve prepared with standard tannic acid (AOAC, 1990).

3.5.2 Determination of Total Alkaloids Content

The total alkaloid content was determined using the method outlined by Harborne (1973). 100 mL of 20% acetic acid in ethanol was added to five grams of the extract in a 250 mL beaker, which was then covered and allowed to stand for two hours. Following the filtration process, the extract was concentrated to a quarter of its initial volume in a water bath. Prior to precipitation, concentrated ammonium hydroxide was progressively added. The mixture was allowed to settle, and the precipitate was collected by filtration, washed with 1% ammonia solution, dried, and weighed. All samples were analysed in triplicate (AOAC, 1990).

Alkaloid (%) = Weight of residues x 100 weight of sample

3.5.3. Flavonoid Content Determination

The flavonoid content was measured using triplicate samples of the homogenized cabbage extract (1.5 g) as described by Ilahy *et al*. (2011). Thirty-microliter aliquots of the methanolic extract were used for flavonoid determination. The samples were diluted with 90 µL of methanol, followed by the addition of 6 µL of 10 % Aluminum Chloride ($AlCl_3$), 6 µL of 1 mol/L Sodium acetate (CH_3CO_2Na), and 170 µL of methanol. After 30 minutes, the absorbance was measured at 415 nm. Quercetin was used as the standard to calculate the flavonoid content (µg Qe/g). For the lead test, the plant extract was treated with a few drops of lead acetate solution, and the formation of a yellow precipitate indicated the presence of flavonoids.

3.5.4. Estimation of Total Saponins Content

Estimation of total saponins content was determined by the method described based on vanillin-sulphuric acid colorimetric reaction with some modifications. About 50 µL of plant extract was added with 250 µL of distilled water. To this, about 250 µL of vanillin reagent (800 mg of vanillin in 10 ml of 99.5 % ethanol) was added. Then 2.5 ml of 72 % sulphuric acid was added and it was mixed well. This solution was kept in a water bath at 60 °C for 10 min. After 10 min, it was cooled in ice cold water and the absorbance was read at 570 nm. 0-25 ppm standard saponin solutions were prepared from saponin stock solution. The standard solutions were treated similarly as test samples (AOAC, 1990). The values were expressed as PPM.

3.5.5. Estimation of Tannins Content

Exactly 0.20 mL of sample was added to 20 mL of 50 % methanol and placed in a water bath at 77 ˚C – 80 ˚C for 1 hr and shaken. The extract was quantitatively filtered using a double layered Whatman No.1 filter paper and 20 mL of distilled water, 2.5 mL Folin Denis reagent

and 10 ml 17 % Na_2CO_3 were added and mixed. The mixture was allowed to stand for 20 min. A series of standard tannic acids solutions were prepared in methanol and their absorbance as well as samples was read after colour development on a UV/ Visible spectrophotometer at a wavelength of 760 nm. Total tannin content was calculated from calibration curve (AOAC, 1990).

3.6 Antibacterial Activity Assay

The antibacterial properties of *Citrus sinensis* were evaluated using the agar diffusion method in Mueller Hinton Agar (MHA), with microbial isolates obtained from the Department of Pharmaceutical Microbiology, Faculty of Pharmacy, University of Benin, Edo State, Nigeria. The microbial isolates used in the study included *Pseudomonas aeruginosa*, *Klebsiella*, *Bacillus subtilis*, *Escherichia coli* and *Staphylococcus aureus*.

3.6.1 Standardization of Test Organisms

The MacFarland 0.5 turbidity standard was prepared by mixing 0.5 mL of a 1% w/v solution of Barium Chloride dihydrate ($BaCl_2.2H_2O$) with 99.5 mL of 1% sulfuric acid (H_2SO_4). The mixture was thoroughly mixed and then divided into several test tubes. A sterile wire loop was used to pick a loopful of inoculum from a pure culture of the test organism, which was transferred into sterile normal saline in separate test tubes and standardized. The turbidity of the solution was then compared with the MacFarland turbidity standard, and adjustments were made by adding either more organism or sterile saline to match the standard (Zapata and Ramirez-Arcos, 2015).

3.6.2. Agar Well Diffusion Method

This method was carried out on Muller Hinton agar for selected bacterial and fungal organisms. After sterilizing the agar, it was allowed to cool, and then 30 mL of the agar was poured into sterile Petri dishes and left to solidify. After solidifying, the agar plates were

placed in an oven for ten minutes at 50 °C to eliminate any remaining moisture from the surface. A standardized inoculum suspension was then evenly swabbed onto the agar plates, including both bacterial and fungal inocula on the Muller Hinton agar. An 8-10 mm sterile cork borer was used to create wells in the agar, and the base of the Muller Hinton agar plate was sealed. Using a calibrated micropipette with a rubber teat, 0.2 mL of the plant extract was added to the well. The plates were allowed to stand for 30 minutes to allow for proper diffusion, then incubated at 37 °C for 24 hours. The control for bacterial organisms was Ciprofloxacin (0.5 µg/mL). The inhibition zone was measured using a meter rule after a 24-hour period.

3.6.3 Determination of Minimum Inhibitory Concentration

The agar dilution method (Afolayan and Meyer, 1997) was used for the determination of minimum inhibitory concentration (MIC) of the extracts and Ciprofloxacin as the standard antibacterial drug. The MIC was regarded as the lowest concentrations of extracts of ciprofloxacin that inhibited the growth of the test bacterial.

3.6.4 Determination of Minimum Bactericidal Concentration

The plates showing no visible growth after determining the MIC were swabbed and streaked onto fresh Muller Hinton agar plates containing the same predetermined concentrations of extracts. All the plates were then incubated at 37°C for 18-24 hours. The MBC was identified as the lowest concentration of extracts and ciprofloxacin that inhibited the growth of the test organisms.

CHAPTER FOUR

RESULTS

The mineral content of *Citrus sinensis* extracts in Table 4.1 shows that aqueous and ethanolic extract showed for Sodium (Na) (250.333 ± 0.333 mg/kg) and (260.300 ± 0.3111 mg/kg), Potassium (K) (3415.667 ± 2.500 mg/kg) and (2215.060 ± 2.400) mg/kg, Calcium (Ca)

(1.412.077± 1.400 mg/kg) and (1,521.047 ± 1.453 mg/kg), Phosphorus (P) (0.2100 ± 0.005 mg/kg) and (0.1900± 0.005 mg/kg), Iron (Fe) (1.1400 ± 0.004 mg/kg) and (1.2400 ± 0.009 mg/kg), Nitrogen (N) (0.1400 ± 0.001 mg/kg) and (1.2000 ± 0.009 mg/kg), Magnesium (Mg) (129.100± 0.700 mg/kg) and (131.000± 0.577 mg/kg). Table 4.2 revealed the results of the proximate content of aqueous and ethanolic extract of *Citrus sinensis*. Table 4.3 revealed that all the phytochemicals tested showed present for both aqueous and ethanolic extracts. Table 4.4 revealed the varying composition of alkaloid to be (5.4063 ± 0.2985) for aqueous and (6.3427±0.329) for ethanolic, total saponins, (5.9620 ± 3.6112) for aqueous and (7.0867±0.002) for ethanolic, total phenolic, (0.2077 ± 0.0012) for aqueous and (0.2140±0.001) for ethanolic, total tannins (10.1533 ± 0.0023) for aqueous and (12.7747±0.161) for ethanolic and for total flavonoids, (6.9993 ± 0.0127) for aqueous and (6.5827±0.031) for ethanolic. Table 4.5 to 4.8 showed the different response of the bacteria to the aqueous and ethanolic extract of *Citrus sinensis*

Table 4.1: Result for mineral concentration of the aqueous and ethanolic extracts of *Citrus sinensis*

Parameters (mg/kg)	Aqueous	Ethanolic
Sodium (Na)	250.333± 0.333	260.300± 0.3111
Potassium (K)	3415.667 ± 2.500	2215.060 ± 2.400

Calcium (Ca)	1.412.077± 1.400	1,521.047± 1.453
Phosphorus (P)	0.2100± 0.005	0.1900± 0.005
Iron (Fe)	1.1400± 0.004	1.2400± 0.009
Nitrogen (N)	0.1400± 0.001	1.2000± 0.009
Magnesium (Mg)	129.100± 0.700	131.000± 0.577

Table 4.2: Result for proximate content of aqueous and ethanolic extract of *Citrus sinensis*

Parameters (%)	Aqueous	Ethanolic
Moisture Content	51.0837 ± 0.1310	50.0337 ± 0.1220

Ash Content	4.8953 ± 0.0707	4.2953 ± 0.0232
Crude Fat	8.1967 ± 0.1073	7.9967 ± 0.1230
Crude Fibre	2.1111± 0.000	2.0121±0.1001
Protein	7.1567 ± 0.1027	7.2117 ± 0.1221
Carbohydrate	34.7532 ± 0.0669	28.4509 ± 0.9110

Table 4.3: Result for qualitative phytochemical screening for aqueous and ethanolic extract of *Citrus sinensis*

S/N	Parameters	Test	Aqueous	Ethanolic
1	Glycosides	General Test	+	+

2	Saponins	Frothing Test	+	+
3	Phenols	Ethanol/Ferric Chloride	+	+
4	Eugenols	Ethanol/Ferric Chloride	+	+
5	Terpenoids	Salkowski Test	+	+
6	Steroids	KOH Test	+	+
7	Alkaloids	Pieric Test	+	+
8	Flavonoids	Lead Acetate	+	+
9	Tannins	Ferric Chloride	+	+
10	Reducing Sugars	Fehlings A&B	+	+

Key: = - Absent, = + Present

Table 4.4: Result for quantitative phytochemical analysis of aqueous and ethanolic extract of *Citrus sinensis*

Parameters	Unit	Aqueous	Ethanolic
Alkaloid	%	5.4063 ± 0.2985	6.3427±0.329
Total saponins	Mg/kg	5.9620 ± 3.6112	7.0867±0.002

Total phenolic	TAE mg/kg	0.2077 ± 0.0012	0.2140±0.001
Total tannins	TAE mg/kg	10.1533 ± 0.0023	12.7747±0.161
Total flavonoids	QE mg/kg	6.9993 ± 0.0127	6.5827±0.031

Key: Tannic Acid Equivalent (TAE)

Table 4.5: Result for Zone of Inhibition concentration (mm) of aqueous extract of *Citrus sinensis*

Organism	CIP (Control)	200 (mg/kg)	100 (mg/kg)	50 (mg/kg)	25 (mg/kg)
Escherichia coli	NG	NG	NG	14.2±0.20	11.4±0.22

Pseudomonas aeruginosa	NG	NG	NG	14.3±0.13	10.5±0.12
Bacillus subtilis	NG	NG	NG	14.4±0.12	10.4±0.10
Staphylococcus aureus	NG	NG	NG	15.2±0.04	11.2±0.11
Klebsiella spp.	NG	NG	NG	12.2±0.22	12.4±0.11

Key: CIP = Ciprofloxacin, NG = No Growth, G = Growth

Table 4.6: Result for Zone of Inhibition concentration of ethanolic extract of *Citrus sinensis*

Organisms	CIP	200 (mg/kg)	100 (mg/kg)	50 (mg/kg)	25 (mg/kg)

	(Control)				
Escherichia coli	NG	NG	NG	18.5±0.10	12.5±0.12
Pseudomonas aeruginosa	NG	NG	NG	15.5±0.13	11.5±0.02
Bacillus subtilis	NG	NG	NG	13.5±0.22	11.5±0.10
Staphylococcus aureus	NG	NG	NG	16.1±0.02	12.4±0.13
Klebsiella spp.	NG	NG	NG	14.4±0.12	11.5±0.11

Key: CIP = Ciprofloxacin, NG = No Growth, G = Growth

Table 4.7: Result for minimum bactericidal concentration for aqueous extract of *Citrus sinensis*

Organisms	CIP (Control)	200 (mg/kg)	100 (mg/kg)
Escherichia coli	NG	NG	NG
Pseudomonas aeruginosa	NG	NG	G
Bacillus subtilis	NG	NG	G
Staphylococcus aureus	NG	NG	G
Klebsiella spp.	NG	NG	G

Key: CIP = Ciprofloxacin, NG = No Growth, G = Growth

Table 4.8: Result for minimum bactericidal concentration (mm) for ethanolic extract of *Citrus sinensis*

Organisms (mg/kg)	CIP (Control)	200 mg/kg	100 mg/kg
Escherichia coli	NG	NG	NG
Pseudomonas aeruginosa	NG	NG	NG
Bacillus subtilis	NG	NG	NG
Staphylococcus aureus	NG	NG	G
Klebsiella spp.	NG	NG	NG

Key: CIP = Ciprofloxacin, NG = No Growth, G = Growth

CHAPTER FIVE

DISCUSSION

The use of medicinal plants have provided quite a number of natural remedies to treat and manage diseases in both human and livestock (Rios and Receo, 2005). Through studies, the potency of these plants have been established and have been identified to contain certain bioactive compounds which are responsible for their therapeutic effects on the human body.

The findings in Table 4.1 show that there were minor variances in the mineral composition from the aqueous and ethanolic extract of *Cirtus sinensis* in this study. The minerals analysed were potassium (K), sodium (Na), Calcium (Ca), Phosphorus (P), Iron (Fe), Nitrogen (N) and Magnesium (Mg). Potassium is essential for maintaining electrolyte balance and supporting muscle and nerve function. Iron is crucial for the formation of haemoglobin and prevention of anaemia. The research of (Weaver, 2013 and Waldvogel-Avramowski *et al*., 2014) confirm the presence of minerals in plants as reported in this study. The result in Table 2 revealed the proximate result of the aqueous and ethanolic extract of *Cirtus sinensis* in this study. The result showed the close composition of proximate from both extracts. The high moisture content in the aqueous extract (51.08 %) and ethanolic extract (50.03 %) is consistent with common plant-based extracts, where moisture aids in enhancing nutrient solubility and bioavailability. The carbohydrate content for aqueous is (34.75 %) and ethanolic extract is (28.45 %), positioning the extract as an efficient energy source. Although the protein content is moderate as aqueous is (7.15 %) and ethanolic (7.21 %), suggesting that the pomace may provide supplementary protein but is unlikely to be a primary protein source compared to other more protein-dense plant extracts. The proximate result in this study aligns with previous plants results from the research of (Osagie, 1998; Murray *et al*., 2002; Uraku, 2015; Oikeh *et al*. (2013). These reports revealed that citrus pomace contains significant macronutrients essential for human health. Uraku (2015) found that orange peel has a high carbohydrate content (61.07 %), supporting the findings of this study that citrus pomace can serve as an energy source. Additionally, Oikeh *et al*. (2013) demonstrated that citrus seeds

have notable protein content (6.13 %), which is comparable to the range of (7.1 - 7.21) % found in this study. The results in Table 4.3 and 4.3 revealed the qualitative and quantitative phytochemical constituents in both extracts. The phytochemical quantification reveals the presence of various bioactive compounds in the aqueous and ethanolic extracts of *Citrus sinensis*. The presence of the various bioactive compounds in the extracts include alkaloids, saponins, phenolics, tannins and flavonoids, suggesting that these substances may have a role in the therapeutic benefits of *Citrus sinensis* (Cha *et al*., 2001). The extracts tested positive for alkaloids, saponins, phenolics, tannins, flavonoids. This suggests the presence of compounds with various pharmacological activities such as synephrine, liminoids, hesperidin, flavonoids and polyphenols, pectin (Etebu and Nwauzoma 2012). Alkaloids are known for their pharmacological activities, including antimalarial, antibacterial and analgestic effect (Aye *et al*., 2019). Tannins are known for their antimicrobial and antioxidant properties (Sung *et al*., 2012). Flavonoids have been extensively studied for their antioxidant and antimicrobial effects (Pandey and Rizvi, 2009). The detection of phenols and terpenoids further supports the findings of Rao *et al*. (2013), who reported that these compounds contribute to anti-inflammatory and antimicrobial properties. The results in this study are consistent with studies of (Dubey *et al*. 2011; Moyo *et al.,* 2023), which highlighted that *Citrus sinensis* contains flavonoids, alkaloids, essential oils, and phenolic acids known for their medicinal benefits.

The antibacterial assay of the aqueous and ethanolic extract evaluated through the agar well diffusion method at varying concentrations (Table 4.5-4.6), showed promising results against various bacterial strains. The Minimum Inhibitory Concentration (MIC) of an antibiotic agent is the lowest concentration of an antibacterial agent that stops observable bacteria growth. At 200 mg/ml, 100 mg/ml, 50 mg/ml and 25 mg/ml, the ethanolic extract of *Citrus sinensis* was evaluated against *Escherichia coli*, *Pseudomonas aeruginosa*, *Bacillus subtilis*,

Staphylococcus aureus and *Klebsiella spp*. Effective inhibition was demonstrated by the varying concentrations, which showed no microbial growth at 200 mg/ml and 100mg/ml. Nevertheless, all studied species showed bacterial growth at 50 mg/ml and 25mg/ml, indicating that these dosages were not enough to totally stop microbial activity. The extracts demonstrated inhibition effect against all bacterial strains tested, with the inhibition better as the concentration of the extract increased. This suggest the extract ability to be dose dependent. These results align with previous research with Ahmed and Beg (2001), who found that citrus extracts required moderate concentrations to exhibit bactericidal effects. The concentration-dependent nature of the antibacterial effect further supports past research on the necessity of higher extract concentrations for stronger antibacterial action.

The result in Table 7 and 8 revealed the ability of the extracts to cause death at varying concentrations. The results showed that the ethanolic extracts was a little more bactericidal than the aqueous extract, this could be its ability to be a better extracting solvent. It appeared that the ethanolic extract of *Citrus sinensis* has strong antibacterial qualities at higher concentration because growth was absent at 200 mg/ml and 100 mg/ml. The inclusion of bioactive substances with well-documented antibacterial properties, including flavonoids, tannins, alkaloids and essential oils may be the cause of this. *Citrus sinensis* extracts have been found to include substance that can break down bacterial cell membranes, interfere with enzyme activity and prevent the creation of proteins. These compounds may account for the bacterial action seen at greater doses. Oikeh *et al*. (2020) evaluated in his report the antibacterial properties of fresh and dried ethanol extracts of *Citrus sinensis* peels and performed a quantitative phytochemical study. According to the study, the presence of phenolic content in the peels gave the extracts their remarkable antibacterial qualities against a variety of bacterial strains. Another recent study by Iwuji *et al*. (2022) evaluated the antibacterial activities of sweet orange (*Citrus sinensis*) juice against isolates of *Klebsiella*

species, Pseudomonas species, *Staphylococcus aureus and Escherichia coli*., with the zone of inhibition that was seen at increasing concentrations, it was obvious the juice had inhibitory effects on these bacteria. These investigations support the results of this present study that has showed that *Citrus sinensis* extract has antibacterial properties. It appears that *Citrus sinensis* include bioactive chemicals with broad-spectrum antibacterial action based on the reported inhibitory effects across different bacterial species. This is consistent with the findings of the current investigation which showed that at doses of 100 and 200 mg/ml, the extracts of *Citrus sinensis* significantly inhibited *Escherichia coli, Pseudomonas aeruginosa, Bacillus subtilis, Staphylococcus aureus* and *Klebsiella* spp.

The result in Table 4.7 and 4.8 of the minimum bacterial concentration (MBC) of the extract of *Citrus sinensis* showed that the extracts were active against *Bacillus subtilis, Klebsiella species, Escherichia coli, Pseudomonas aeruginosa* and *Staphylococcus aureus*. The findings imply that the extracts of *Citrus sinensis* has potent antibacterial action against *Escherichia coli* because the bacterium did not develop at 200 mg/ml for both extracts. This aligns with earlier research by Oikeh *et al*., (2020), that found that orange extracts significantly inhibited *Escherichia coli*, Due to their ability to damage bacterial membrane and interfere with cellular activities, flavonoids, phenolic compounds and essential oils may be responsible for the significant inhibition (Kumar and Bhardivaj, 2012). Conversely, *Pseudomonas aeruginosa, Bacillus subtilis, Staphylococcus aureus* and *Klebsiella* spp. showed growth at both 100 mg/ml for both extracts Similar findings were reported by Kumar and Bhadivaj, (2012) where ethanolic extracts of *Citrus sinensis* demonstrated considerable suppression of *Staphylococcus aureus*, with efficacy improving at higher concentrations. Previous studies support the selective antibacterial activity observed in this research.

5.1 CONCLUSION

The aqueous and ethanolic extract of *Citrus sinensis* demonstrated superior phytochemical properties making it a more potent candidate for medicinal and antibacterial applications. The aqueous and ethanolic extract of *Citrus sinensis* pomace appears to be a promising source of bioactive compounds, including flavonoids, alkaloids, and phenolics, which contribute to its potential therapeutic properties. Its nutritional composition, particularly its high carbohydrate content, indicates that it could serve as an energy source. The extract's antibacterial activity, particularly against *Staphylococcus aureus*, suggests that it may have applications in combating bacterial infections. However, further research is needed to optimize its antimicrobial efficacy and to explore its potential against fungal pathogens.

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APPENDIX

Plate 1: Picture of an orange fruit and the leaves

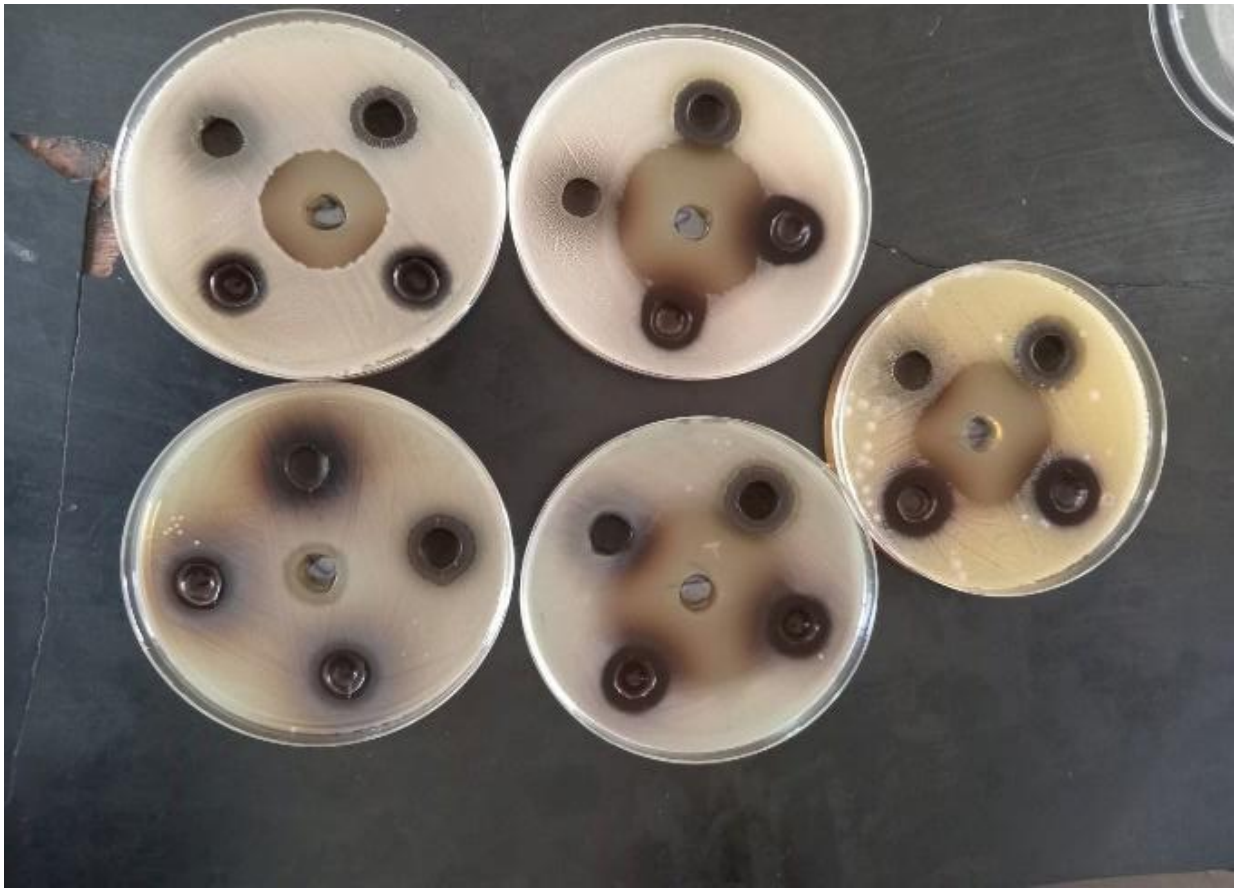

Plate 2: Petri dish with well bored holes for the *Citrus sinensis* pomace extract after which bacterial strain was seeded in it