ANTIMICROBIAL ACTIVITY, PROXIMATE COMPOSITION AND PHYTONUTRIENT ANALYSIS OF ETHANOLIC EXTRACTS OF THE YOUNG AND MATURE LEAVES OF *Greenwayodendron suaveolens*

BY

FRANKLIN SOPULU EZE
LCS1505101

DEPARTMENT OF SCIENCE LABORATORY TECHNOLOGY

FACULTY OF LIFE SCIENCES

UNIVERSITY OF BENIN

BENIN CITY

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LCS1505101

A PROJECT WORK SUBMITTED TO THE DEPARTMENT OF SCIENCE LABORATORY TECHNOLOGY, FACULTY OF LIFE SCIENCES, UNIVERSITY OF BENIN, BENIN CITY IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE AWARD OF BACHELOR OF SCIECE DEGREE (BSc) IN SCIENCE LABORATORY TECHNOLOGY (MICROBIOLOGY OPTION).

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CERTIFICATION

This is to certify that this project work was carried out by **Franklin Sopulu EZE** with matriculation number **LSC1505101** of the Department of Science Laboratory Technology, Faculty of Life Sciences, University of Benin, Benin City.

____________________________ ________________________

Dr. E. O. Oshomoh Date
(Associate Professor)
(Project Supervisor)

___________________________ ________________________

Prof. A. B. O. Ogedegbe Date
(Project Coordinator)

____________________________ ________________________

Dr. E. O. Oshomoh Date
(Associate Professor)
(Head of Department)

DEDICATION

This project work is dedicated to God almighty.

ACKNOLEDGEMENT

It is my pleasure to acknowledge all persons who contributed to the success of this project work, especially my parents, Mr. I. E. Eze and Mrs. S. N. Eze, my siblings Chiamaka, Onyinye, Ejike and Mmesoma for their support. I cannot be grateful enough.

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ABSTRACT

The antimicrobial activity, proximate composition and phytonutrient contents of the ethanolic extracts of the young and mature leaves of *Greenwayodendron suaveolens* were analyzed using standard microbiological methods. The moisture contents of the young and mature leaves were 9.9% and 11.882% respectively. The ash contents of the young and mature leaves were 6.142% and 6.418% respectively. The crude fiber content of the young and mature leaves was 5.550% and 5.080% respectively. The crude fat content of the young and mature leaves was 14.750% and 18.870% respectively. The crude protein content of the young and mature leaves was 1.338% and 1.025% respectively. The carbohydrate content of the young and mature leaves was 72.220% and 68.607% respectively. The phytonutrient analysis carried out indicated that percentage alkaloid was 24.43 and 11.45 in the young and mature leaf extracts respectively. Phenolic contents were 64.00 µg/mL and 51.00 µg/mL in the young and mature leaf extracts respectively. Flavonoid content was 39.50 µg/mL and 13.75 µg/mL in the young and mature leaf extracts at respectively. Saponin content at 1,147 µg/mL and 1,205 µg/mL in the young and mature leaf extracts respectively. While tannin content was 65.00 mg/mL and 47.25 mg/mL in the young and mature leaf extracts at respectively. The ethanolic leaf extracts of *Greenwayodendron suaveolens* inhibited the growth of all the test organisms at the concentration of 25 mg/mL. However, the minimum inhibitory concentration (MIC) of the young and mature leaf extracts against *Bacillus subtilis* and *Trichoderma harzianum* was 6.25 mg/mL while the minimum inhibitory concentration (MIC) of the young leaf extract against *Acinobacter calcoaceticus* and *Candida albican* was also 6.25 mg/mL. The minimum inhibitory concentration (MIC) of the young and mature leaf extracts against *Aspergillus tamari* and *Aspergillus niger* was 12.5 mg/mL. The

antimicrobial results indicated that the ethanolic leaf extracts of *Greenwayodendron suaveolens* are active against bacteria and fungi.

CHAPTER ONE

INTRODUCTION

Traditional medical system is widely recognized in West Africa and some parts of Asia. During the last century, the practice of herbalism became widespread throughout the world. In spite of great advances observed in modern medicine, plants still make an important contribution to health care. This is due to the recognition of the value of traditional medical systems and the identification of medicinal plants from indigenous pharmacopoeias, which have significant healing power. Medicinal plants are distributed worldwide, but they are most abundant in tropical countries (Adebolu and Salau, 2005). Numerous past studies have underlined the pleotropic pharmacological activities of the crude extracts and isolated compounds from Annonaceae species. For centuries, Annonaceae species are employed in traditional medicines to cure various pathological conditions including snakebite, analgesic, astringent, diarrhea, dysentery, arthritis pain, rheumatism, neuralgia, and weight loss etc. (Attiq *et al.*, 2017). Plants synthesize chemicals as part of their defense against pathogens. Many such compounds occur in nature as antifeedant and antimicrobials, and are found to be effective against microbes (Gibbons, 2004; Tan and Vanitha, 2004). The biosphere is the richest source of bioactive phytochemicals and natural nutraceuticals. Enormous work done during the past fifty years has shown that these phytochemicals play an important role in the routine healthcare systems worldwide (Kochel *et al.,* 2016).

In Nigeria and most other West African Countries, a wide range of plant is used in nutrition and medicine because they constitute a wide variety of compounds which exhibit some medicinal and nutritive properties. Investigations have been made on the proximate, phytonutrient, vitamin and mineral content of most of these plants and they have been found rich in carbohydrates,

proteins, ash, crude fiber, crude fat, vitamins, minerals, alkaloids, phenolics, terpenoids and tannins (Akinmoladun *et al.,* 2007). The concentration of constituents of plant is actually determined by the nature and amount of nutrients available in the soil in which the plant are growing, thus fertilizer treatment affect the contents of the plants (Cristiana *et al.,* 2006).

Research carried out by Ojewuyi *et al.* (2014) revealed that young leaves of *Polyalthia longifolia* contained 9% protein, 4% ash, 0.21% lipid, 25% fiber, 8% moisture and 54% carbohydrate while the mature leaves contained 10% protein, 5% ash, 0.26% lipid, 19% fiber, 9% moisture and 57% carbohydrates. The quantitative phytonutrient analysis revealed that the young leaves contained 3.91 (ppm) tannins, 0.34 (ppm) phenols and 62% flavonoids with the mature samples showing a relative result of 3.69 (ppm) tannins, 0.33 (ppm) phenols and 63% flavonoids. The nutritional composition of medicinal plants is of immense importance. *Polyalthia longifolia* and all other medicinal plant species have their own nutrient composition besides having pharmacologically important phytonutrients (Ojewuyi *et al.*, 2014). Chemical compounds such as alkaloids, phenols and acetogenins have been identified and isolated from *Annona muricata*. Using *in vitro* studies, extracts and phytochemicals of *Annona muricata* have been characterized as an antimicrobial, anti-inflammatory, anti-protozoan, antioxidant, insecticide, larvicide and cytotoxic to tumor cells (Ana *et al.*, 2018). *Annona muricata* were shown to possess anxiolytic, anti-stress, anti-inflammatory, contraceptive, anti-tumoral, antiulceric, wound healing, hepato-protective, anti-icteric and hypoglycemic activities. In addition, clinical studies support the hypoglycemic activity of the ethanolic extracts of *Annona muricata* leaves.

1.1 Aim and Objectives

1.1.1 Aim

The aim of this research work is to analyze the antimicrobial activity, proximate composition and phytonutrient contents of the ethanolic extract of the young and mature leaves of *Greenwayodendron suaveolens*.

1.1.2 Objectives

The objectives of this study are:

i. To determine the antimicrobial sensitivity of some certain bacteria to the ethanolic extracts of young and mature leaves of *Greenwayodendron suaveolens.*
ii. To determine the proximate composition of the ethanolic extracts of young and mature leaves of *Greenwayodendron suaveolens.*
iii. To determine the phytonutrients of the ethanolic extracts of young and mature leaves of *Greenwayodendron suaveolens.*
iv. To compare the antimicrobial activity, proximate composition and phytonutrient contents of the mature leaves to the young leaves.

CHAPTER TWO

LITERATURE REVIEW

2.1 Ethnomedicinal Uses

Ajayi *et al.* (2013) reported that the leaf of *Greenwayodendron suaveolens* along with its root and bark is used in traditional medicine for the treatment of fever, rheumatic pains, oedema, swollen glands, headache, stomach ache, constipation, hernia, facilitation of child birth, fertility and as anthelmintic and aphrodisiac. In Nigeria the leaf has been recorded to be taken internally for menorrhagia. Leaf decoctions or macerations serve to treat hepatitis and pains, and are applied externally to treat rheumatism (Jiofack, 2011). Bark decoctions are taken to treat stomach-ache and other pains, gonorrhea and infertility, as diuretic, purgative and aphrodisiac, and to facilitate childbirth. Bark ash is rubbed into scarifications on the forehead to treat psychosis, and bark pulp is applied externally against rheumatism, headache, epilepsy and toothache. In Cameroon bark is applied to scarifications to treat malaria, and also in Gabon the bark is used for the treatment of malaria. Root decoctions are taken to treat liver complaints and headache, and root sap is administered as anthelmintic and aphrodisiac, and to treat oedema and swollen glands. *Polyalthia cerasoides* on the other hand, was used as medicinal plant in Thailand. Traditional medicinal uses of *Annona muricata* have been identified in tropical regions to treat diverse ailments such as fever, pain, respiratory and skin illness, internal and external parasites, bacterial infections, hypertension, inflammation, diabetes and cancer (Ana *et al.*, 2018).

2.2 Nutraceuticals

The term 'nutraceutical' was coined from 'nutrition' and 'pharmaceutical' in 1989 by DeFelice and was originally defined as a food (or part of the food) that provides medical or health benefits, including the prevention and/or treatment of a disease (Kalra, 2003). A nutraceutical may be a naturally nutrient-rich food such as spirulina, garlic, soy or a specific component of a food like omega-3 oil from salmon. They are also known as medical foods, nutritional supplements and dietary supplements. It ranges from isolated nutrients, dietary supplements, genetically engineered foods, herbal products, and processed products such as cereals and soups. They have received considerable interest because of their presumed safety and potential nutritional and therapeutic effects (Garima and Manoj, 2016). Nutraceuticals have been recorded to have helped conditions such as cardiovascular diseases, diet related diseases, heart attack, lung cancer, diabetes, Alzheimer’s disease, Parkinson’s disease, osteoarthritis and adrenal dysfunction. Flavonoids which block the enzymes that produce estrogen reduces estrogen-induced cancers while alkaloids, glycosides, tannins, flavonoids, sterol fractions and other compounds have shown anti-inflammatory activities (Balch *et al.*, 2003; Garima and Manoj, 2016)

2.3 Antimicrobial Activity

Plants synthesize chemicals as part of their defense against pathogens. Many such compounds occur in nature as antifeedant and antimicrobials, and are found to be effective against microbes (Gibbons, 2004; Tan and Vanitha, 2004). The stem extract of *Polyathia longifolia* was found to inhibit the growth of gram positive and gram negative bacteria (Faizi et al. 2003) while its methanol extract of leaves and green berries were found to possess promising antibacterial

activity (Faizi et al. 2008). The seed extract of *Greenwayodendron suaveolens* has antimicrobial activities on *Staphylococcus aureus, Escherichia coli* and *Bacillus subtilis* (Idu *et al.*, 2017).

2.4 Phytonutrient Composition

Phytonutrients are biologically active, naturally occurring chemical compounds found in plants, which provide health benefits for humans as medicinal ingredients and nutrients They protect plants from disease and damage, and also contribute to the plant's color, aroma and flavor. In general, the plant chemicals that protect plants from environmental hazards such as pollution, stress, drought, UV exposure and pathogenic attack are called as phytochemicals. Annonaceae plant species are well known for their biological activity and produces many promising phytocomponents (Faizi *et al*. 2003; Lertyot *et al*., 2011; Patoomratna *et al*., 2000). Phytochemical analysis of Annonaceae family have reported the occurrence of alkaloids, flavonoids, triterpenes, diterpenes and diterpene flavone glycosides, sterols, lignans, and annonaceous acetogenin characteristically affiliated with Annonaceae species (Attiq *et al.,* 2017). Research carried out by Odaro and Sobotie (2019), showed that saponins, phenols and flavonoids are present in the aqueous leaf extract of *Greenwayodendron suaveolens*, but alkaloid was not detected. Idu *et al.* (2017) reported that the seed extract of *Greenwayodendron suaveolens* contains saponin, flavonoid, alkaloids, cardic glycosides and terpenoids while tannins, phlobatannins and steroids were absent. Phytochemical screening results of experiment conducted by Jothy *et al.* (2013) revealed the presence of alkaloids, triterpenoids, tannins, saponin, anthraquinones, and glycosides in the leaf extract of *Polyathia longifolia.* The crude extracts of the leaves of *Polyalthia longifolia* were subjected to phytochemical investigations by Uzama *et al.* (2011). The tests indicated the presence of alkaloids, tannins, saponins and

glycosides. The results for the quantitative phytochemicals analysis conducted by Ojewuyi (2014) revealed that the young leaves of *Polyalthia longifolia Sonn.* contained 3.91 (ppm) tannins, 0.34 (ppm) phenols and 62% flavonoids with the mature samples showing a relative result of 3.69 (ppm) tannins, 0.33 (ppm) phenols and 63% flavonoids.

2.5 *Greenwayodendron suaveolens*

Greenwayodendron suaveolens is member of the Annonaceae family and species of plant in the genus *Greenwayodendron*. It is a deciduous tree circumscribed to Tropical Africa and is mostly found in Southern Nigeria, Western Uganda, Northern Tanzania, Southern Democratic Republic of Congo and Cabinda in Angola (Odaro and Sobotie, 2019). It comprises two species, namely: *Greenwayodendron oliveri* (Engl) Verdc. and *Greenwayodendron suaveolens* (Engl. & Diels) Verdc. *Greenwayodendron* was formally classified under *Polyalthia* but was later separated from *Polyalthia*, which is a genus of about 120 species. Although wood anatomical characteristics are quite s *Greenwayodendron suaveolens Greenwayodendron suaveolens* imilar, molecular studies showed that *Greenwayodendron* is probably not closely related to *Polyalthia*, supporting the status as separate genus.

2.5.1 Morphology of *Greenwayodendron suaveolens*

The mature tree is usually medium sized to large sized- up to 35–45 meters tall (Idu *et al.,* 2017). Some of its Nigerian native names include: ewai (Edo), eleku (Isekiri), Osharo (Urhobo), Agudugbu (Yoruba). (Odaro and Sobotie, 2019). The leaves of *Greenwayodendron suaveolens* slightly vary in shape but usually have a narrow elliptic shape with an elongated leaf tip and are mostly glabrous. The young leaves have the color ranging from brown to greenish brown to light green as the leave gradually mature, while the old leaves have a dark green color. The midrib of

the leaves has a greenish yellow color which becomes more distinct as the leaves mature. The inner part of the bark of *Greenwayodendron suaveolens* is fibrous and has a resinous smell. It also has a yellow to orange or pale brown color which becomes brownish or blackish when exposed to the atmosphere (Odaro and Sobotie, 2019). The heartwood is yellow to brown when dry and usually not distinctly demarcated from the sapwood. The wood is lustrous and of medium-weight, with a density of 750–790 kg/m^3 at 12% moisture content. The wood is only moderately durable, being liable to termite, *Lyctus* and marine borer attacks and is easy to impregnate with preservatives (Dauby *et al.* 2010). The fruit has a very long fruiting season and is green when immature but turns very dark- almost black- when ripe and is widely consumed by many animals (Oseyemi *et al.* 2018).

Plate 1: *Greenwayodendron suaveolens* Tree

Photo Credit**:** Franklin

Plate 2: Young Leaves of *Greenwayodendron suaveolens*

Photo Credit**:** Franklin

Plate 3: Mature Leaves of *Greanwaydendron Suaveolen*
Photo Credit**:** Franklin

2.5.2 Taxonomy of *Greenwayodendron suaveolens*

Kingdom:	Plantae
Subkingdom:	Tracheobionta
Superdivision:	Spermatophyta
Division:	Magnoliophyta
Class:	Magnoliopsida
Subclass:	Rosidae
Order:	Magnoliales
Family:	Annonaceae
Genus:	*Greenwayodendron*
Species:	*Greenwayodendron suaveolens*

(Source: USDA)

CHAPTER THREE

MATERIALS AND METHOD

3.1 Sample Collection

The young and mature leaves of *Greenwayodendron suaveolens* were collected from the Department of Plant Biology and Biotechnology, Faculty of Life Sciences, University of Benin, Benin City. The young leaves were collected towards the tip of the branches while the mature leaves were collected towards the base of the branches. The sample was identified by Prof. E. O. Oshomoh of the Department of Science Laboratory Technology, Faculty of Life Sciences, University of Benin, Benin City.

3.2 Sample Preparation

The young and old leaves were weighed and air-dried separately in the Phytomedicine and Drug Discovery Laboratory in the Department of Science Laboratory Technology, Faculty of Life Sciences, University of Benin, Benin City. They was pulverized and weighed with a standard weighing balance. 1 kg each of the dried samples was soaked in 1.5 L of ethanol and was left to stand for four (4) days. They were then filtered and the filtrate were decocted and transferred into air-tight containers and stored in the refrigerator.

3.3 Antimicrobial Analysis

Antimicrobial analysis of the young and mature leaves of *Greenwayodendron suaveolens* was carried out on some selected bacteria and fungi in the Microbiology Laboratory, Department of Science Laboratory Technology, Faculty of Life Sciences, University of Benin, Benin City.

3.3.1 Experimental Microorganisms

Staphylococcus aereus, Candida albicans , Mucor mucedo and *Penicilium chrysogenum* were obtained from the Pharmaceutical Microbiobiology Laboratory, Faculty of Pharmacy; *Bacillus subtilis*, *Pseudomonas aeruginosa*, *Micrococcus luteus*, *Enterococcus faecalis*, and *Klebsiella pneumoniae* were obtained from Microbiology Laboratory, Faculty of Life Sciences; *Providencia rettgeri*, *Arthrobacter globiformis*, *Acinetobacter calcoaceticus*, were obtained from Science Laboratory Technology Laboratory, Faculty of Life Sciences; *Aspergillus tamari*, *Aspergillus niger*, *Trichoderma harzianum* and *Escherichia coli* were collected from the Plant Biology and Biotechnology Laboratory, Faculty of Life Sciences, all in the University of Benin, Benin City. Each of these microorganisms was already identified by the various laboratory technologists/scientist before collection. The microorganisms were stored in the refrigerator.

3.3.2 Preparation of Extract Concentrations

0.5 g each of both young and mature leaf extracts was weighed into different sterile beakers containing 10 ml of ethanol which were taken as the stock concentrations. Further concentrations were made from the stock concentration by double serial dilutions; for each extract, 5 ml of the stock concentration was transferred into a test tube containing 5 ml of ethanol to get 25 mg/ml of the extracts. 5 ml was transferred from the 25 mg/ml concentrations into test tubes containing 5 ml of ethanol to obtain 12.5 mg/ml of the samples. 5 ml was transferred from the 12.5 mg/ml concentrations into test tubes containing 5 ml of ethanol to obtain 6.25 mg/ml of the samples. Finally, 5 ml was transferred from the 6.25 mg/ml concentrations into test tubes containing 5 ml of ethanol to obtain 3.125 mg/ml of the samples.

3.3.3 Preparation of Culture Media

The media used were Nutrient Agar for the bacteria, Potato Dextrose Agar for the fungi and Peptone Water. Both were prepared according to the manufacturer's instruction. The conical flasks containing the media solutions were properly labeled and corked with cotton wool wrapped with foil paper. The media was then sterilized using the autoclave at the temperature of 121 ^{o}C for 15 minutes.

3.3.4 Sterilization of Materials

The glass wares used for this study were thoroughly washed with mild detergent and rinsed with distilled water. Petri dishes, test tubes and pipettes were wrapped with aluminum foil and appropriately sterilized in the hot air oven at 160 ^{o}C for 1 hour. The culture media (Potato Dextrose Agar, Nutrient Agar and Peptone Water) were sterilized in an autoclave at 121 ^{o}C for 15 minutes. Inoculating wire loop and cork borer were sterilized by dipping in 70% ethanol and then flamed in Bunsen flame. Bench tops and other surfaces were sterilized by wiping with cotton wool dampened in 70% ethanol.

3.3.5 Preparation of Microbial Inoculum

Different universal bottles containing sterile peptone water were properly labeled according to the various microorganisms. The sterile Peptone water in different universal bottles was inoculated with the various appropriate test microorganisms. It was then incubated for 24 hours at 37 ^{o}C for the bacteria and 48 hours at 25 ^{o}C and the fungi.

3.3.6 Antimicrobial Plating

Antimicrobial plating was carried out as described by Elek and Hilson (1954). In an aseptic condition, the petri dishes were properly labeled according to the organisms, samples and the concentrations of the sample. The plates were all prepared in triplicates (R_1, R_2 and R_3). The sterilized media are allowed to cool down to the temperature of about 45 ^oC before pouring about 20 mL to each plate- Nutrient Agar for the bacteria and Potato Dextrose Agar for the fungi. It was allowed to totally solidify and then aliquots of the inoculums were dropped on the agar surfaces and spread evenly using sterile bent glass rod or hockey stick. Agar wells were made using a sterile 6 mm cork borer. With sterile Pasteur pipette a drop of molten agar were dropped in the wells to seal the bottom of the wells. The plates were allowed to stand for about 15 minutes before the wells were filled with the appropriate concentrations of the extracts according to the labeling. The plates for the control were prepared replacing the extracts with concentrations of standard antibiotics- ciprofloxacin for bacteria and metronidazole for fungi (positive control). The plates for blank were prepared for all the organisms also replacing the extracts with water (negative control). The Bacteria plates were incubated at 37 ^oC for 24 hours while the fungi plates were incubated at 25 ^oC for 48 hours.

The Inhibition Zone Diameter (IZD) was measured after incubation using a meter rule.

3.4 Proximate Analysis

Proximate analysis was also carried out on the young and mature leaves of *Greenwayodendron suaveolens* in the Organic Chemistry Laboratory, Department of Chemistry, Faculty of Physical Sciences, University of Benin, Benin City.

3.4.1 Determination of Moisture Content

The empty moisture can was weighed and recorded as W_0. About 2 g of the leaf sample was added to the moisture can and weighed. The weight was recorded as W_1. It was dried in the hot air oven at 105 °C for 24 hours and cooled in the desiccator. The can and the dried sample was weighed and recorded as W_2. The dried sample was returned to the oven and further dried for another 24 hours. This was repeated until W_2 was constant (AOAC., 1997).

The percentage moisture is calculated thus:

$$\text{Moisture \%} = \frac{W_1 - W_2}{W_1 - W_0} \times 100$$

3.4.2 Determination of Ash Content

The empty crucible was weighed and recorded as W_0. The sample was added to the crucible and weighed (W_1). It was the ashed at 500 °C for 3 hours and then cooled in the desiccator. The crucible is weighed along with the dry sample (W_2) (AOAC., 1997).

Calculation for percentage ash is done thus:

$$\text{\% Ash} = \frac{W_2 - W_0}{W_1 - W_0} \times 100$$

3.4.3 Determination of Crude Fiber

2 g of the leaf sample was weighed into the 100 mL conical flask (W_0). 200 mL of H_2SO_4 was Added and boiled for gently for 5 minutes using cooling fingers to maintain a constant volume. After 5 minutes, it was filtered through the poplin cloth stretched over 9 cm Buchner funnel, It was then rinsed with hot distilled water. The sample was scraped back into the flask with a spatula. 200 mL of NaOH was added and boiled gently for 30 minutes using cooling finger to

maintain a constant volume. It was then filtered through a poplin cloth. The residue was washed thoroughly with hot distilled water. It was then rinsed with 10% HCl followed by ethanol. It was finally rinsed with petroleum ether (BP 40 – 60 ^oC). It was allowed to drain and then the residue was scraped the crucible. The residue was dried overnight in the oven at 105 ^oC and was cooled in the desiccator. The sample was weighed (W_1) and then ashed in the muffle furnace at 550 ^oC for 90 minutes. It was finally cooled in the desiccator and weighed again (W_2) (Tel and Hagarty, 1984).

Calculation for percentage crude fiber is done thus:

$$\% \text{ Crude Fiber} = \frac{W_1 - W_2}{W_0} \times 100$$

3.4.4 Determination of Crude Fat

The 250 mL boiling flask was dried in the oven at 105 ^oC and allowed to cool in the desiccator before weighing (W_2). 2 g of the ground sample was weighed into a labeled porous thimble and was covered with clean cotton wool. 200 mL of petroleum ether was added was added to boiling flask. The covered porous thimble was placed into the condenser and then the apparatus was assembled. Extraction was done for about 5 – 6 hours and then the porous thimble was removed with care. The petroleum ether was collected in the top container (tube) for reuse. The boiling flask was removed from the water bath when it is almost free of petroleum ether. The boiling flask containing the oil is oven dried at 105 ^oC for 1 hour. It was then cooled in the desiccator and weighed (AOAC., 1997).

Calculation for percentage crude fat is done thus:

$$\% \text{ Fat} = \frac{W_3 - W_2}{W_1 - W_0} \times 100$$

Where W_0 = weight of empty porous thimble, W_1 = weight of thimble + ground sample, W_2 = weight of empty boiling flask and W_3 = weight of boiling flask + ether.

3.4.5 Determination of Crude Protein

The estimation of crude protein was done by determining the total nitrogen in the samples by Kjeldahl method. The amount of crude protein was obtained by multiplying the nitrogen content by 6.25. This is based on the assumption that all feed protein contains 16% nitrogen and that all the nitrogen in tissue is present as protein. Technical digester which is an alternative method of Kjeldahl digester was used.

0.1 g of the dried leaf sample was weighed into a micro kjeldahl flask. 2 mL of concentrated H_2SO_4 was added followed by 1 tablet of selenium catalyst. This was heated gently in a digester until froting ceased and then it was strongly heated until the solution was clear. It was then filtered into a 100 mL volumetric flask through a Whatman No. 42 filter paper and made up to mark. From the filtrate, 1 mL was taken into 100 mL volumetric flask. 1 mL of alkiali-phenate solution was added followed by 2 mL of sodium potassium tartrate ($KNaC_4H_4O_6 \cdot 4H_2O$) and 1.5 mL of sodium hypochloride (bleach) and was made up to 25 mL mark. The solution was allowed to stand for 10 minutes so the color develops and it was read in a spectrophometer at 630 nm. A blank was prepared and also subjected to the same processes as the sample. A series of Nitrogen standards of 2, 4, 6, 8 and 10 ppm was prepared and read at the same wavelength. A graph of OD against ppm was drawn and slope reciprocal (SR) was found (Tel and Hagarty, 1984).

Calculation for Nitrogen content was done thus:

$$\% \text{ Nitrogen} = \frac{\text{IR x SR x FV x CD x 100}}{\text{Wt. x Aliquot x 1,000,000}}$$

Where IR = OD or Instrumental reading, FV = Final volume, CD = Color development, Wt. = Weight of sample used, Aliquot = Volume of solvent used.

Then Crude Protein is calculated thus:

Crude Protein = % Nitrogen x 6.25

3.4.6 Determination of Soluble Carbohydrate

This was obtained as the difference between the sum of percentage of other contents of the leaf samples and 100%. It is then calculated thus:

Soluble Carbohydrate = 100 – (% fat + % ash + % crude fiber + % Crude protein)

(AOAC., 1997).

3.5 Phytochemical Analysis

Phytochemical analysis was carried out on the young and mature leaves of *Greenwayodendron suaveolens* in the Organic Chemistry Laboratory, Department of Chemistry, Faculty of Physical Sciences, University of Benin, Benin City.

3.5.1 Determination of Alkaloids Content

The total alkaloid content was measured using the method described by Harborne (1973). 5g of the extract was weighed into a 250 mL beaker and 100 mL of 20% acetic acid in ethanol was added and covered to stand for 2 hours. This was filtered and the extract was concentrated using a water bath to one quarter of the original volume. Concentrated ammonium hydroxide (NH_4OH) was added drop by drop to the extract until the precipitation was complete. The whole solution

was allowed to settle and the precipitate was collected by filtration, washed with 1% ammonia solution, dried and weighed. All samples were analyzed in triplicates.

$$\text{Alkaloid (\%)} = \frac{\text{Weight of Residue}}{\text{Weight of Sample}} \times 100$$

3.5.2 Estimation of Total Saponins Content

The saponin content of the samples was determined by double extraction gravimetric method described by Harborne (1973).

5 g of the powdered leaf sample was mixed with 50 mL of 20% aqueous ethanol solution in a flask. The mixture was heated with periodic agitation in water bath for 90 minutes at 55 ^{o}C. It was then filtered through Whatman filter paper (No. 42). The residue was extracted with 50 mL of 20% ethanol and both extract were poured together and the combined extract was reduced to about 40 mL at 90 ^{o}C and transferred to a separating funnel where 40 mL of diethyl ether was added and shaken vigorously. Re-extraction by partitioning was done repeatedly until the aqueous layer become clear in color. The saponins were extracted with 60 mL of normal butanol. The combined extracts were washed with 5% aqueous sodium chloride (NaCl) solution and evaporated to dryness in a pre-weighed evaporation dish. It was dried at 60 ^{o}C in the oven and reweighed after cooling in a desiccator. The process was repeated two more times to get an average. Saponin content was determined by difference and calculated as a percentage of the original sample thus:

$$\text{\% Saponin} = \frac{W_2 - W_1}{\text{Weight of Sample}} \times 100$$

3.5.3 Estimation of Tannins Content

Determination of tannin was done using the AOAC (1984) methods. 1 g of dry ground sample was weighed into a conical flask and 100 mL of distilled water was added. This was boiled gently for 1 hour and filtered through a No. 44 Whatman filter paper into a 100 mL volumetric flask. The paper was washed with distilled water and extract diluted to mark, then cooled. The sample was prepared just before shortly before color development. For color development, 50 mL of distilled water and 10 mL of the diluted extract was pipetted into a 100 mL conical flask followed by addition of 5.0 mL of Folin-Denis reagent and 10 mL of saturated Na_2CO_3 solution. The volume was made up to mark with distilled water. After thorough mixing, the solution was allowed to stand for 30 minutes in water bath at 75 ^{o}C. The optical density was measured at 700 nm using the spectrophotometer and optical density (absorbance) compared on a standard tannic acid curve.

The tannic acid standard curve was prepared by dissolving 1 g of tannic acid in distilled water and diluting to the 200 mL mark (1 mg/mL). Varying concentrations (0.1:1.0 mg/mL) of standard tannic acid solution were pipetted into 10 different 100 mL conical flask. 5 mL of Folin-Denis reagent and 100 mL of saturated Na_2CO_3 distilled water. The solution is left to stand for 30 minutes at 25 ^{o}C. Optical density was measured at 700 nm with the aid of a spectrophotometer. A plot of optical density (absorbance) against tannic acid concentration is made, with the line passing through the origin. This is the standard tannic acid curve.

The tannic content is calculated thus:

$$\text{Tannic acid (mg/100g)} = \frac{\text{C (mg) x Extract Volume}}{\text{Aliquot Volume (mL) x Wt. of Sample}} \text{ x } 100$$

Where C (mg) = Concentration of tannic acid read of the graph

3.5.4 Flavonoid Content Determination

The flavonoid content was determined on triplicate aliquots of the homogenous cabbage extract (1.5 g) (Ilahy *et al*., 2011). 30 µL aliquots of the ethanolic extract were used for flavonoid determination. Samples were diluted with 90 µL of ethanol, 6 µl of 10% Aluminum chloride (AlCl3). 6 µl of 1 mol Sodium acetate (CH_3CO_2Na) were added and finally 170 µL of methanol was added. The absorbance was read at 415 nm after 30 minutes. Quercetin was used as a standard for calculating the flavonoid content (µg Qe/g).

3.5.5 Determination of Total Phenolic Contents

The amount of total phenolics in the leaf extracts were determined with Folin Ciocalteu reagent according to the method of Singleton and Rossi (1965) with slight modification using tannic acid as a standard. Briefly, 1.0ml of extract solution (250 µg/ml) was added in a test tube. Then, 1.0 ml of Folin Ciocalteu reagent was added and the content of the flask was mixed thoroughly. After 5 minutes, 15.0 ml Na_2CO_3 (20 %) was added and 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 determined as µg of Tannic Acid Equivalent (TAE) using an equation obtained from the standard tannic acid calibration graph.

CHAPTER FOUR

RESULTS

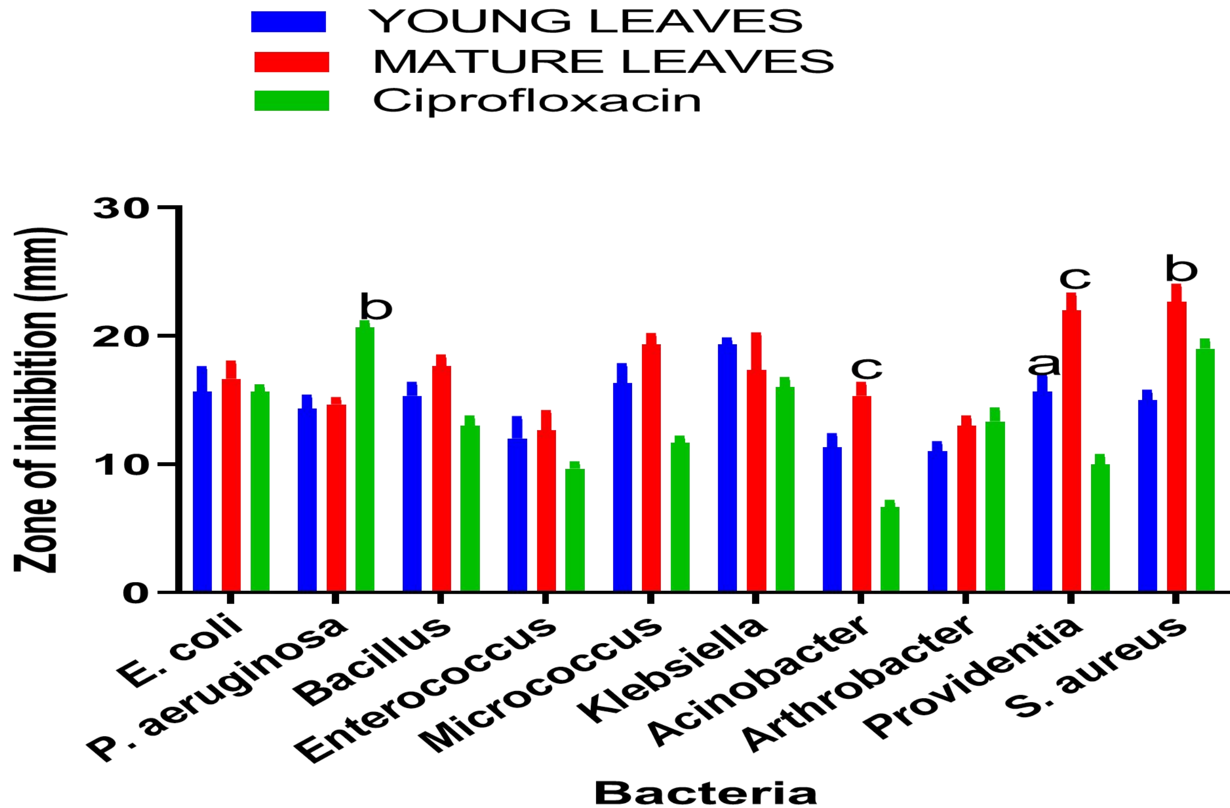

Figure 1: The antimicrobial activity of the ethanolic extracts of young and mature leaves of *Greenwayodendron suaveolens* at the concentration of 25 mg/mL against bacteria isolates in comparison with ciprofloxacin. Ciprofloxacin was more active against *Pseudomonas aeruginosa* compared to the young and mature leaf extracts of *Greenwayodendron suaveolens* ($^{b}p<0.0001$). The mature leaf extract of *Greenwayodendron suaveolens* was significantly more active against *Acinetobacter calcoaceticus, Providencia rettgeri* and *Staphylococcus aereus* compared to ciprofloxacin and the young leaf extract ($^{c}p<0.001$; $^{b}p<0.0001$). Values are represented as mean ± S.E.M. where n = 3.

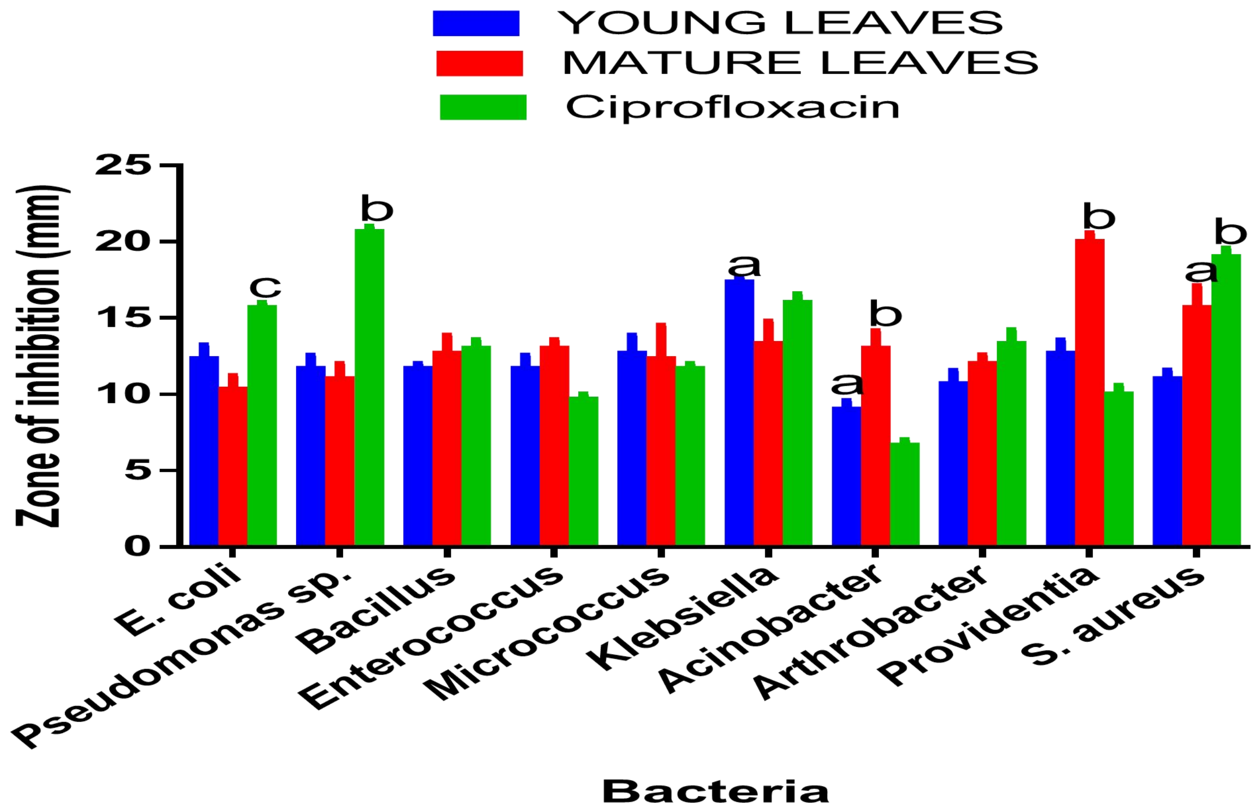

Figure 2: The antimicrobial activity of the ethanolic extracts of young and mature leaves of *Greenwayodendron suaveolens* at the concentration of 12.5 mg/mL against bacteria isolates in comparison with ciprofloxacin. Ciprofloxacin was significantly more active against *Escherichia coli, Pseudomonas aeruginosa, and Staphylococcus aureus* compared to the both leaf extracts ($^{c}p<0.001$; $^{b}p<0.0001$). The young leave extracts of *Greenwayodendron suaveolens* was slightly more active against *Klebsiella pneumonia* compared to the mature leaf extract and ciprofloxacin ($^{a}p<0.01$), while the mature leaf extract was significantly more active against *Providencia rettgeri* and *Acinetobacter calcoaceticus* ($^{b}p<0.0001$). Values are represented as mean ± S.E.M. where n = 3.

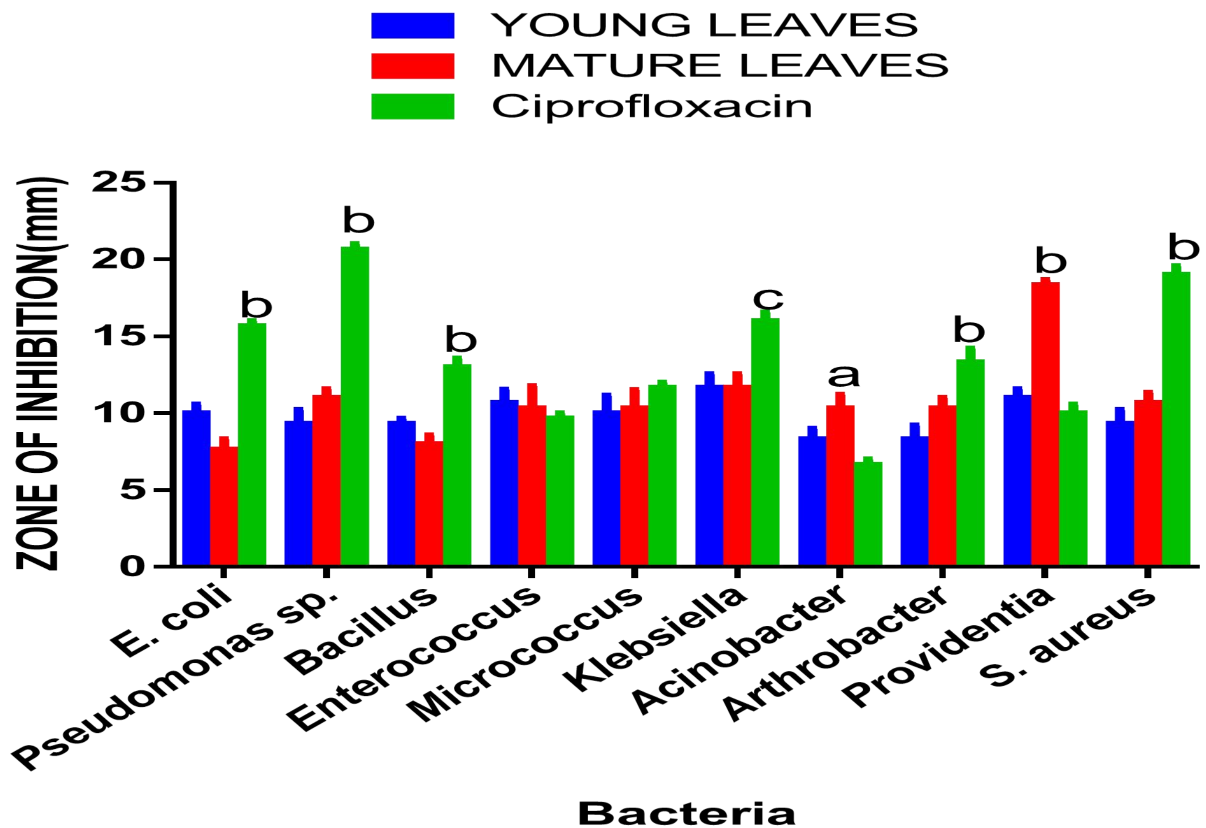

Figure 3: The antimicrobial activity of the ethanolic extracts of young and mature leaves of *Greenwayodendron suaveolens* at the concentration of 6.25 mg/mL against bacteria isolates in comparison with ciprofloxacin. Ciprofloxacin showed the greatest zone of inhibition against *Escherichia coli*, *Pseudomonas aeruginosa*, *Bacillus subtilis*, *Klebsiella pneumoniae* and *Staphylococcus aureus* compared to the both leaf extracts of *Greenwayodendron suaveolens* ($^{b}p<0.0001$; $^{c}p<0.001$). Mature leaf extracts of *Greenwayodendron suaveolens* was more active against *Providencia rettgeri*, and *Acinetobacter calcoaceticus* ($^{a}p<0.01$; $^{b}p<0.0001$). Values are represented as mean ± S.E.M. where n = 3.

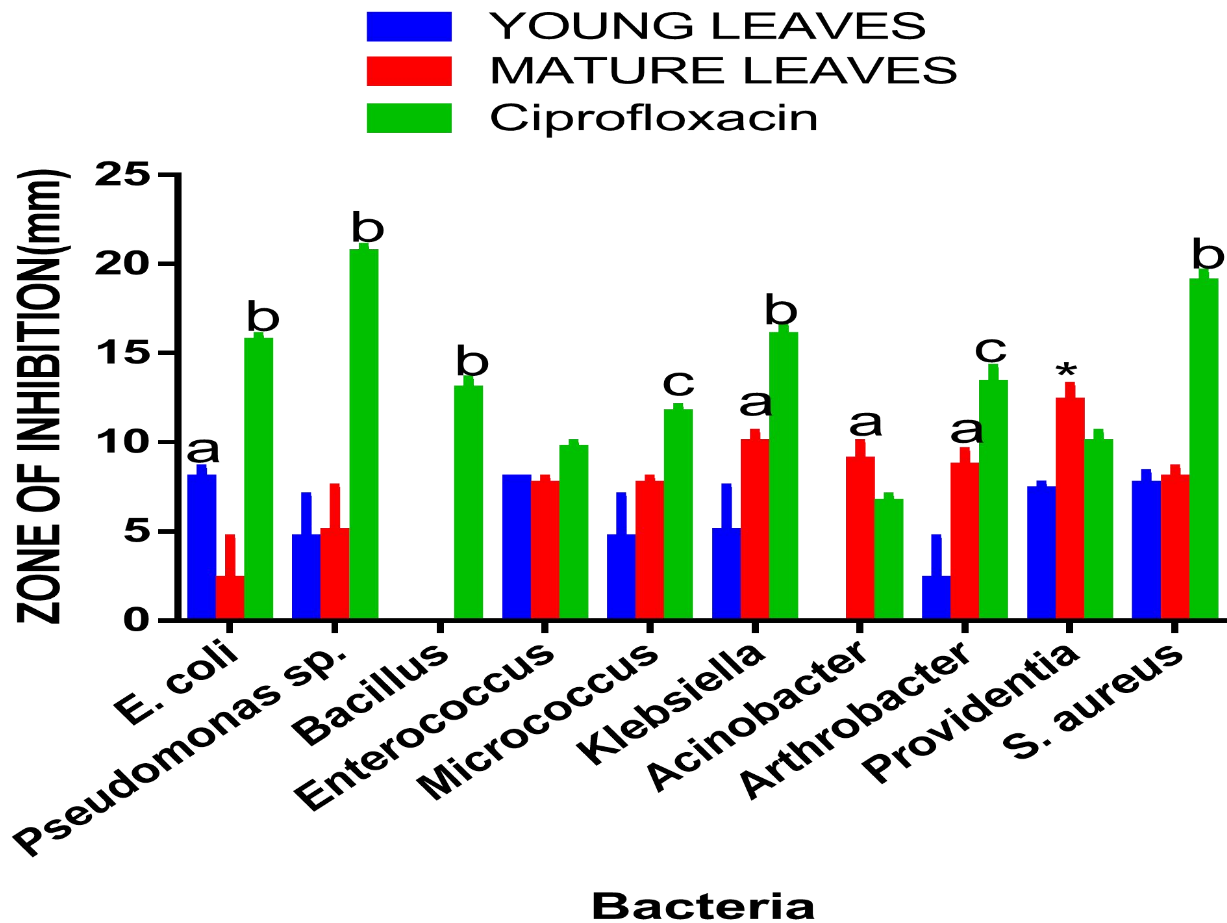

Figure 4: The antimicrobial activity of the ethanolic extracts of young and mature leaves of *Greenwayodendron suaveolens* at the concentration of 3.125 mg/mL against bacteria isolates in comparison with ciprofloxacin. Ciprofloxacin showed greater zones of inhibition than the both leaf extracts of *Greenwayodendron suaveolens* ($^{b}p<0.0001$; $^{c}p<0.001$). However, mature leaf extract was more active against *Providentia rettgeri* than ciprofloxacin and young leaf extract (*$p<0.05$). Young leaf extract was more active against E. coli than the mature leaf extract ($^{a}p<0.01$). Mature leaf extract was more active than the young leaf extract against *Klebsiella pneumoniae*, *Acinobacter calcoaceticus* and *Arthrobacter globiformis* ($^{a}p<0.01$). Values are represented as mean ± S.E.M. where n = 3.

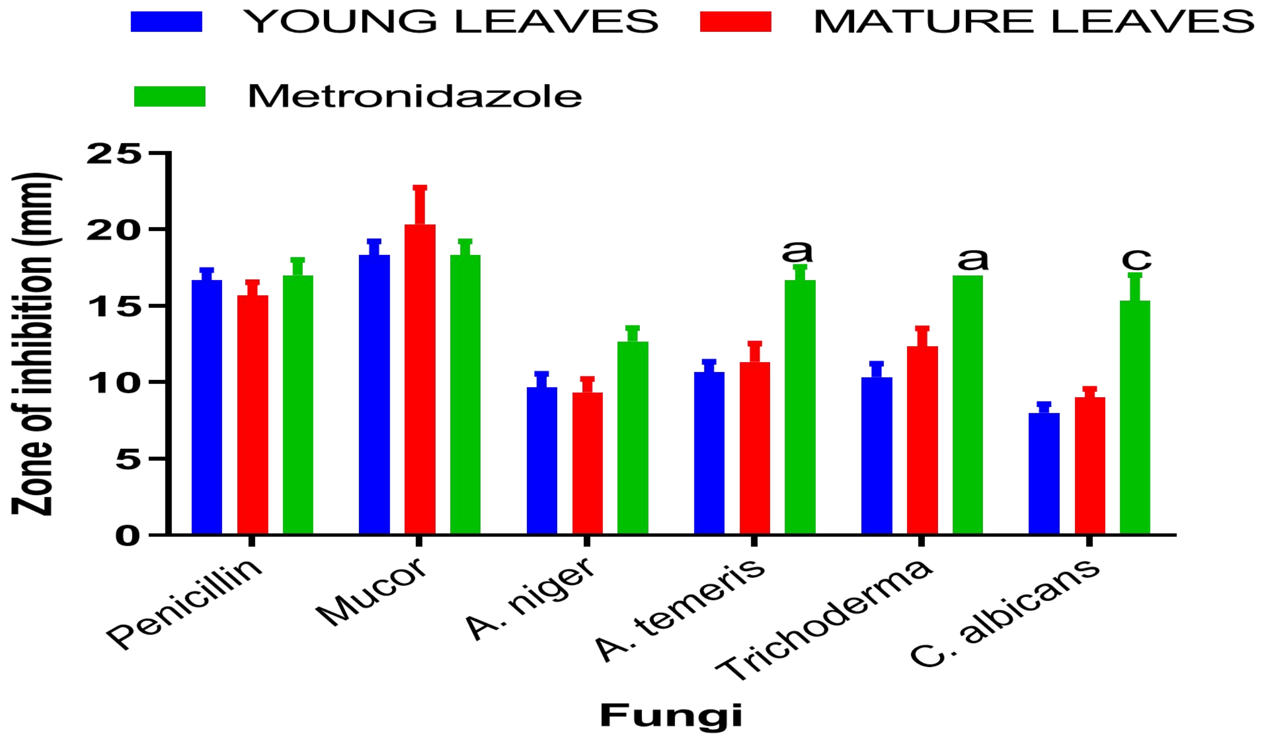

Figure 5: The antimicrobial activity of the ethanolic extracts of young and mature leaves of *Greenwayodendron suaveolens* at the concentration of 25 mg/mL against fungi isolates in comparison with metronidazole. Metronidazole was more active against *Aspergillus tamari, Trichoderma harzianum, Candida albicans and Aspergillus niger* compared to both young and mature leaf extracts of *Greenwayodendron suaveolens* ([a]$p<0.01$; [c]$p<0.001$). Values are represented as mean ± S.E.M. where n = 3.

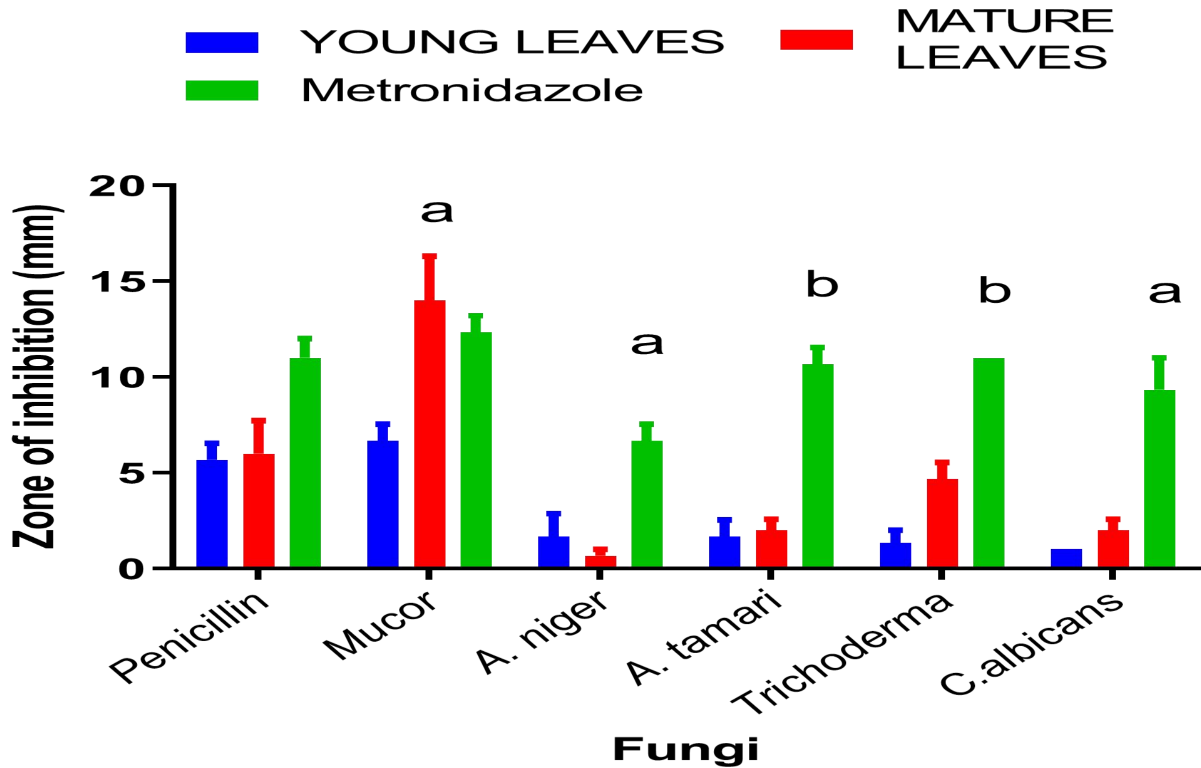

Figure 6: The antimicrobial activity of the ethanolic extracts of young and mature leaves of *Greenwayodendron suaveolens* at the concentration of 12.5 mg/mL against fungi isolates in comparison with metronidazole. Metronidazole was significantly more active against *Penicilium chrysogenum, Aspergillus niger, Aspergillus tamari, Trichoderma harzianum* and *Candida albicans* compared to the both leaf extracts of *Greenwayodendron suaveolens* ($^{a}p<0.01$; $^{b}p<0.0001$). *Mucor mucedo* was more susceptible to mature leaf extracts of *Greenwayodendron suaveolens* compared to metronidazole and young leaf extract of *Greenwayodendron suaveolens* ($^{a}p<0.01$). Values are represented as mean ± S.E.M. where n = 3.

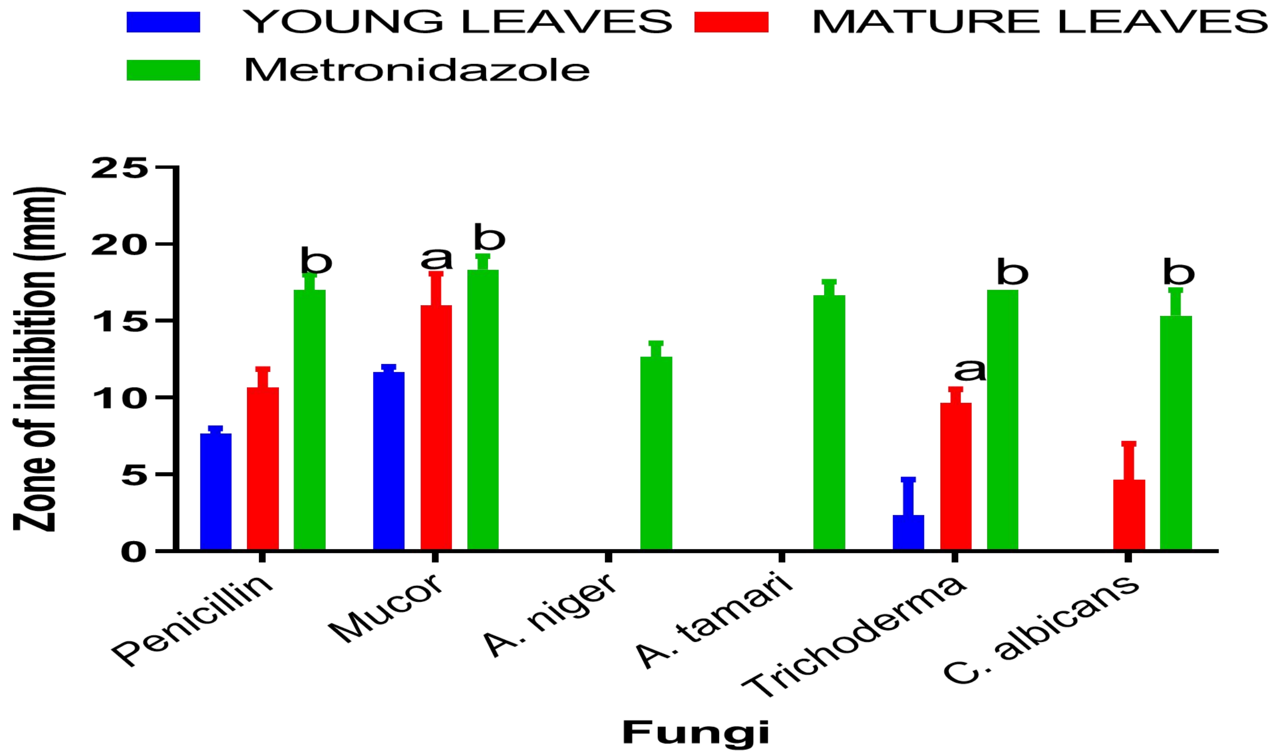

Figure 7: The antimicrobial activity of the ethanolic extracts of young and mature leaves of *Greenwayodendron suaveolens* at the concentration of 6.25 mg/mL against fungi isolates in comparison with metronidazole. Metronidazole generally showed higher activity against the fungi compared to the both leaf extracts of *Greenwayodendron suaveolens* ($^{b}p<0.0001$). The mature leaf extract was more active against *Penicilium chrysogenum, Mucor mucedo*, *Trichoderma harzianum* and *Candida albicans* ($^{a}p<0.01$). At the concentration of 6.25 mg/mL the both leaf extracts were not able to inhibit the growth of *Aspergillus niger* and *Aspergillus tamari.* Values are represented as mean ± S.E.M. where n = 3.

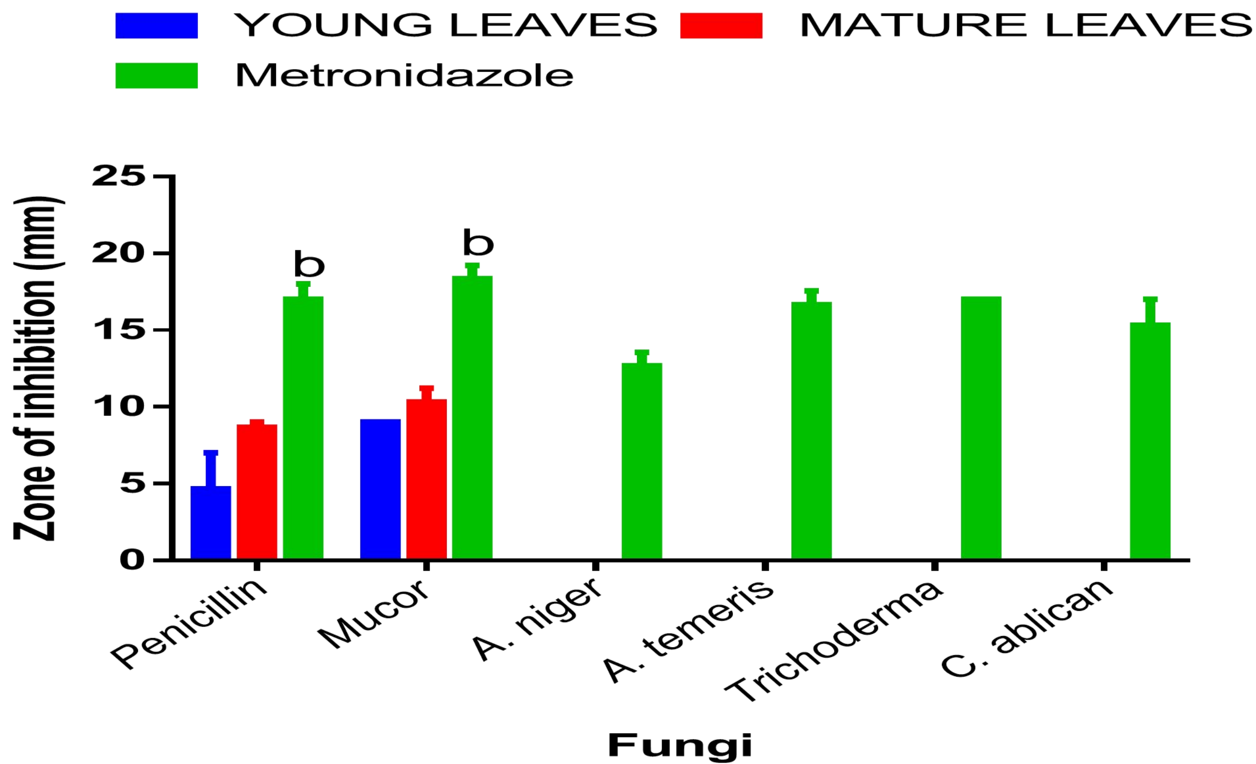

Figure 8: The antimicrobial activity of the ethanolic extracts of young and mature leaves of *Greenwayodendron suaveolens* at the concentration of 3.125 mg/mL against fungi isolates in comparison with metronidazole. At the concentration of 3.125 mg/mL metronidazole was active against all the fungi ($^{b}p<0.0001$), while the both leaf extracts were only active against *Penicilium chrysogenum* and *Mucor mucedo.* Values are represented as mean ± S.E.M. where n = 3.

Table 1: Proximate composition of the young and mature leaves of *Greenwayodendron suaveolens.*

Parameters	Young Leaves	Mature Leaves
Moisture Content (%)	9.98±0.0.29	11.88±0.003
Ash Content (%)	6.14±0.001	6.42±0.002
Crude Fiber (%)	5.55±0.012	5.08±00.012
Crude Fat (%)	14.75±0.012	18.87±0.012
Crude Protein (%)	1.34±0.001	1.023±0.001
Carbohydrate (%)	72.27±0.024	68.61±0.06

Values are represented as mean ± S.E.M. where n = 3.

Table 2: Phytonutrient analysis of the young and mature leaves of *Greenwayodendron suaveolens*.

Parameter	Young Leaves	Mature leave
Total Alkaloids (%)	24.41±0.34	11.66±0.14
Total Phenolics (μg/mL)	63.97±0.49	51.57±0.35
Total Flavonoid (μg/mL)	38.50±0.51	14.15±0.33
Total Saponin (μg/mL)	1150.67±4.70	1221.00±9.24
Total Tannin (μg/mL)	65.77±0.41	47.58±0.22

Values are represented as mean ± S.E.M. where n = 3.

CHAPTER FIVE

DISCUSSION

Following the ethanolic extraction of the young and mature leaves of *Greenwayodendron suaveolens*, the antimicrobial activity was determined. The results of antimicrobial analysis show that the bacteria were all susceptible to the ethanolic extracts of *Greenwayodendron suaveolens* at the concentration of 25 mg/mL (Figures 1). Idu *et al.* (2017) also reported that the aqueous and chloroform seed extract of *Greenwayodendron suaveolens* were remarkably active against *Staphylococcus aureus*, *Bacillus subtilis*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Aspergillus niger* and *Candida albicans*. At the concentration of 3.125 mg/mL, the extracts were not able to inhibit the growth of most of the bacteria (Figure 4). At the concentration of 6.25 mg/mL and below, the extracts were not able to inhibit the growth of most of the fungi (Figures 7; 8). This trend could be attributed to the presence of phytochemicals such as alkaloids, saponins, tannin, flavonoids and phenolics (Idu *et al.*, 2017). Aruoma (2003) reported that mixtures of such chemicals have been known to exhibit a broad spectrum of biological effects and pharmacological properties. The bacteria are more susceptible to the mature leaf extract than the young leaf extract. The young and mature leaves of *Greenwayodendron suaveolens* were active against *Escherichia coli* and are therefore conceivable that these extracts can be used in medicine to treat cases of diarrhea caused by *Escherichia coli* (Ellis *et al.*, 2020). *Klebsiella pneumoniae* showed one of the highest susceptibility to these extracts. This can indicate the possibility of treating infections such as pneumonia, blood stream infections, wounds and surgical site infection which are caused by *Klebsiella pneumonia* with extracts of young and mature leaves of *Greenwayodendron suaveolens*. Idu *et al.* (2017) revealed that the seed extracts of *Greenwayodendron suaveolens* were active against *Staphylococcus aureus*, *Escherichia coli,*

Bacillus subtilis and *Pseudomonas aeruginosa.* However, *Aspergillus niger* and *Candida albicans* were not susceptible to the aqueous extract of *Greenwayodendron suaveolens* seeds. This shows some conformation with the relatively low susceptibility of these two organisms to the leaf extracts of *Greenwayodendron suaveolens* (Figure 6; 7).

The results of phytonutrient analysis of the young and mature leaves of *Greenwayodendron suaveolens* show that they contain alkaloid (24.43±0.02% and 11.45±0.04% respectively); phenolics (64.00±0.03 µg/mL and 51.00±0.33 µg/mL respectively); flavonoid (39.50±0.07 µg/mL and 13.75±0.23 µg/mL respectively); saponins (1,147±4.67 µg/mL and 1,205±3.33 µg/mL respectively); tannin (65.00±0.22 µg/mL and 47.25±0.13 µg/mL respectively). However, phytonutrient analysis carried out by Odaro and Sobotie (2019) reported that saponins, phenols and flavonoids are present in the aqueous leaf extract of *Greenwayodendron suaveolens*, but alkaloid was not detected contrary to the result of phytonutrient analysis in Table 2. This could owe to the fact that the content and level of phytochemicals and macronutrients in plants can be affected by the various factors as like varieties, climatic conditions, cultural practices, and level of maturity at harvest and storage conditions (Jannatul *et al.*, 2020). Odaro and Sobotie (2019) made use of mature leaves and this can be related to the much lower alkaloid content (11.45±0.04%) as shown in Table 2. The young leaf extract of *Greenwayodendron suaveolens* contains all the phytonutrients in higher proportion than the mature leaf extract except saponin which is higher in mature leaf extracts (Table 2). This result is supported by the result of analysis conducted on the different developmental stages of *Clausena lansium* leaf by Chang *et al.* (2018). The total and free phenolic content of leaf buds, young leaves, mature leaves and old leaves of *Clausena lansium* were analyzed and it showed a significant decreased as the leaves got older.

The mature leaves have higher moisture content (11.882±0.003%) than the young leaf leaves (9.980±0.001%). Since high moisture content is favorable for microbial attack and is also an index of spoilage, the mature leaves tend to spoil faster than the young leaves. The ash content of the young leaves was slightly lower which implies that minerals establish as the leaf mature and so the mature leaves will provide more minerals for animals when used as fodder (Bamishaiye, 2011). The carbohydrate content is very high in both young (72.27±0.024) and mature (68.61±0.06) leaves though there is a slight decrease in the mature leaves. This can be related to the findings of Bamishaiye *et al.* (2011) who analyzed the proximate composition of the leaves of *Moringa oleifera* and reported that the carbohydrate content showed a slight decrease as the leaves mature. There was no significant difference in the crude fiber content of the young (5.55±0.012) and mature (5.08±00.012) leaves of *Greenwayodendron suaveolens* though the young leaves have a slightly higher fiber content. *Greenwayodendron suaveolens* has higher crude fiber content than *Cnidoscolus chayamansa* (0.92%), *Solanium nodiflorum* (0.78%), *Senecio biafrae* (0.92%) and lower crude fiber content compare with *Acalypha hispada* (10.25%,), *Acalypha racemosa* (7.20%) and *Acalypha marginata* (11.50%) as reported by Bamishaiye *et al.* (2011). The young leaves are higher in crude protein while the mature leaves are higher in crude fat.

CONCLUSION

The results of this work indicate that the young and mature leaf extracts of *Greenwayodendron suaveolens* have active ingredients which are effective against the test organisms used. This shows that these active ingredients can be utilized in treating infections caused by these test organisms. The young leaves of *Greenwayodendron suaveolens* are relatively richer in nutrients while the mature leaves prove to have better antimicrobial properties than the young leaves.

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