Assessment of Phytochemical and Proximate Compositions of *Murraya koenigii (*curry leaf) and its Antibacterial Activities on *Salmonella* and *Shigella* species

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A THESIS WRITTEN IN THE DEPARTMENT OF MICROBIOLOGY AND SUBMITTED TO THE SCHOOL OF POSTGRADUATE STUDIES IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE AWARD OF THE DEGREE OF MASTER OF SCIENCE OF THE UNIVERSITY OF BENIN, BENIN CITY.

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CERTIFICATION

We certify that this work was carried out by Wilfred Omorodion ENOBAKHARE in the Department of Microbiology, University of Benin, Benin City.

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CERTIFICATION OF THESIS

We the under designated attest and declare that the thesis titled Antibacterial Activities of *Murraya koenigii (*curry leaf) against *Salmonella* and *Shigella* species 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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ABSTRACT

Spices are food supplements or food products commonly used as flavouring and colouring agents, preservatives and/or herbs in folk medicine. *Murraya koenigii* (Linn, Spreng), (Family-Rutaceae) is a type of spice commonly called Curry leaves found in tropical and sub-tropical region and cultivated in China, Australia and Nigeria. It known as *efirin oso* in *Yoruba* and *marugbo sanyan* in Hausa. The aim of this study was to access the antibacterial activities of *Murraya koenigii* against *Salmonella* and *Shigella* species.

Samples of commercial *Murraya koenigii* leaves were analysed and their phytochemical, phytocomponents and proximate components were assayed using standadrd methods. Also antibacterial activities of the olant extracts were investigated using well-in-agar diffusion methods. Data obtained for the different parameters were subjected to statistical analysis using the analysis of variance.

The results of the phytochemical analysis revealed the presence flavonoid (8.81 ± 0.09 mg/100g), tannins (20.28 ± 0.53 mg/100g) and phenolic (44.83 ± 1.18 mg/100g) in aqueous extracts and flavonoid (67.1 ± 0.49 mg/100g), tannins (55.5 ± 1.98 mg/100g) and phenolic (68.0 ± 1.40 mg/100g) in ethanolic extract. Gas Chromatography-Mass Spectrometry confirmed the presence of Dodocanoic acid (0.40%), Tridecanoic acid (0.69%), Decanoic acid (0.29%), Tetramethyl-2-hexadecon-1-01 (1.65%), Octadecanoic acid (0.45%), Hexadecanoic acid (1.04%), Phthalic acid (1.14%), n-Hexadecanoic acid (29.6%), Hexadecanol (3.35%), Vacconic acid (6.23%), Octacosane (2.78%), Squalene (2.52%), Tetratetracontane (3.18%) and Cholesterol (1.57%). Zone of inhibition of the aqueous leave extract of *Murraya koenigii* on *Salmonella* sp and *Shigella* sp ranged from 0.10 ± 0.00 - 1.37 ± 0.03mm while zone of ethanolic extract ranged from 0.10 ± 0.00 - 1.67 ± 0.03mm respectively. The Minimum Inhibitory Concentration ranged from 9.17 ± 2.20 - 45.0 ± 2.88 mg/ml for aqueous extract and 15.0 ± 7.64 - 90.0 ± 5.77 mg/ml for ethanolic extract. Minimum Bactericidal Concentration (MBC) were negative in both aqueous and ethanolic extracts. The proximate analysis revealed the presence of Moisture (8.69 ± 0.52 %), Protein (19.73 ± 0.30%), Ash (1.95 ± 0.00 %), Fibre (4.31 ± 0.29 %), Lipid (6.53 ± 0.50 %) and carbohydrate (43.48 ± 1.72%). *Shigella* sp and *Salmonella* sp were resistant to septrin, ciprofloxacin, amoxicillin and perfloxacin and susceptible to sparfloxacin, augmentin and gentamycin with *Salmonella* sp having the highest multiple antibiotic resistance index of 0.5. The isolates were found to harbor plasmids. Plasmid profile of the bacterial isolates after curing showed that *Shigella* sp was totally cured while presence of visible bands was observed for *Salmonella* sp. signifying inherent resistance to antibiotics. The antibacterial activities observed in *Murraya koenigii* leaves extract is due to the presence of phytochemicals. The use of *Murraya koenigii* in folk medicine is therefore recommended

CHAPTER ONE

INTRODUCTION

1.1 Background of Study

Spices are food supplements or food products, which have been used not only as flavoring and coloring agents, but also as food preservatives and herbs in folk medicines for thousands of years in Africa, Asia and other parts of the world (Srinivasan, 2005). They are consumed as whole spices or ground into powder and mixed with diets containing cereals, legumes, nuts, fruits, vegetables, milk and milk products. They are also used in soup preparation in various homes and serve as ingredients in the preparation of several traditional delicacies (Nelson *et al*., 2017). Spices are utilized as herbs, mainly in the form of isolates from their extracts. Spices are considered to be good contributors to the total nutrient intake of protein, carbohydrates, fats, vitamins and minerals, thereby enhancing the nutritional quality of diets (Pradeep *et al*., 1993). Apart from the nutrients supplied by spices, they possess many phytochemicals which are potential sources of natural antioxidant such as phenolic diterpenes, volatile oils, flavonoids, terpenoids, carotenoids, phytoestrogens, and phenolic acids (Cai *et al*., 2004; Suhaj, 2006; Kennedy *et al*., 2011).

Spice phytochemicals such as curcumin (turmeric), capsaicin (red chillies), eugenol (cloves), linalool (coriander), piperine (black pepper), zingerone (zinger) and cuminaldehyde (cumin) have been reported to inhibit lipid peroxidation (Shobana and Naidu, 2000; Oboh and Rocha, 2007). In recent times, spice antioxidants have raised considerable interest among food scientists, manufactures, and consumers because of their natural antioxidants (Lu *et al*., 2011). Consumers

are increasingly aware of the risk posed by synthetic antioxidants due to their high volatility and instability at elevated temperatures. Therefore, focus has been shifted to the use of natural antioxidants in food preservation (Odukoya *et al*., 2005; Oboh and Rocha, 2007; Adefegha and Oboh, 2011).

Food oxidation is considered a major cause of food deterioration and spoilage, causing rancidity in food (Sherwin, 1990). The resultant effect is noticed in the decreased nutritional quality, color, flavor, texture and safety of foods. Many spices have also been recognized to possess digestive stimulant action, carminative effect, antimicrobial activity, antioxidant capacity, anti-inflammatory property, antimutagenic ability and anticarcinogenic potential (Srinivasan, 2005). Spices contribute greatly to the daily antioxidant intake in most diets, especially in dietary cultures where spices are used as whole meal (Carlsen *et al*., 2010). Many spices have been shown to confer health benefits and have been proven to counteract oxidative stress *in vitro and in vivo* (Oboh *et al*., 2005, Oboh *et al*., 2010, Oboh *et al*., 2012; Shan *et al*., 2005; Wojdylo *et al*., 2007; Adefegha and Oboh, 2012). They are common sources of phenolic compounds which have been reported to show superior antioxidant capacity to fruits, cereals, and nuts (Pellegrini *et al*., 2006; Carlsen *et al*., 2010). The main active components in spices are phenolic acids, flavonoids and volatile or essential oils (Shan *et al*., 2005; Wojdyło *et al*., 2007; Viuda-Martos *et al*., 2011; Lu *et al*., 2011).

In Nigeria, over 100 indigenous spices are used as important components of the "African/Nigerian dishes", bringing original favors and desirable sensory properties to food. Essentially, "Banga soup" is famous for its sensory, aromatic, attractive, pungency and spicy flavor resulting from the use of the messocarp of banga seed extract, clove, alligator pepper,

curry leaf, ginger, black pepper, garlic, Ethiopian pepper, chili peppers and other spices (Oboh *et al*., 2008). Common spices, such as sweet basil, clove, black pepper, turmeric, chili pepper, and ginger are usually part of daily African household meals and also used as traditional African medicine. Numerous studies have reported that spices are important source of natural antioxidant, possessing digestive stimulant action, bioavailability enhancement nature, carminative attribute, antimicrobial activity, hypolipidemic property, antidiabetic influence, anti-inflammatory ability, anticarcinogenic potential and neuroprotective effect (Shan *et al*., 2005; Srinivasan, 2005; Adefegha and Oboh, 2011).

1.2 Aim and Objectives

The aim of the study was to assess the nutraceutical potentials of *Murraya koenigii (*curry leaf).

The objectives of this study were to;

1. evaluate phytochemical content of *Murraya koenigii* leaf extract
2. determine antibacterial activity of the aqueous and ethanolic extracts of *Murraya koenigii* leaves.
3. determine the spectrum profile of chemical composition in different crude extracts of *Murraya koenigii* leaves using gas chromatography-mass spectrometry (GC-MS).
4. Evaluate the proximate composition of aquoues and ethanol extracts of *Murraya koenigii* leaf
5. determine antibiotic susceptibility pattern on *Salmonella* and *Shigella* sp.
6. determine the plasmid profile of the bacterial isolates

CHAPTER TWO

LITERATURE REVIEW

2.1 Overview on Different Spices

There is no particular definition of spices, mostly because they are derived from different parts of the plants, such as cardamom from seed, bay leaf from leafs, clove from flower bud, pepper from fruit, cinnamon from bark or ginger from rhyzome. Furthermore, there is no a common method to classify spices. They can be classified by their flavor and colour, i.e., hot (pepper), pungent (garlic), aromatic (cinnamon, clove), colouring (turmeric) and herbaceous (rosemary, sage), or according to their taste, such as sweet, spicy, sour, bitter and astringent (Aiyelaagbe *et al*., 2006). Numerous studies have been published on the antimicrobial activities of plant extracts against different types of microbes, including foodborne pathogens (Beuchat, 2004; Nakatani, 2004).

Indigenous Nigerian spices consist of part of trees, shrubs and grasses which abound in the tropical rain forest and savannah grassland zone. These are readily available, inexpensive and form the major ingredients in soup preparation a meal item regularly consumed in Nigeria. In Nigeria, there are as many varieties of traditional soups as there are tribes. The difference between the taste and quality of one soup from another depends on the type of spices used in their preparation (Aiyelaagbe *et al*., 2006).

Plants, including herbs and spices, contain products of secondary metabolism such as phenolics, phenolic acids, quinones, flavonoids, tannins (Lai and Roy, 2004). Many of these

phytochemicals are rich sources of antioxidants and provide defense mechanisms to plants against predation by infectious organisms and insects (Deans and Ritchie, 2007). A number of studies have reported a high correlation between antimicrobial efficacy and the level of phenolic components present in certain herb and spice preparations. Indeed, compounds such as eugenol, carvacrol and carnosic acid present in clove, oregano and rosemary respectively, have been identified as being responsible for antimicrobial activity (Dorman and Deans, 2000; Moreno *et al*., 2006). In general, crude extracts of herbs and spices are mixtures of several or even dozens of phytochemicals, with the major bioactive compounds constituting up to 85%, while other components are found at trace levels (Burt, 2004; Lai and Roy, 2004). These bioactive compounds may involve multiple modes of antimicrobial action (Lambert *et al*., 2001) including degradation of the cell wall, disruption of the cytoplasmic membrane, leakage of cellular components, alteration of fatty acid and phospholipid constituents, changes in the synthesis of DNA and RNA and destruction of protein translocation (Shan *et al*., 2007). Hence it is possible that combining spice and herb extracts could lead to synergistic antimicrobial effects against both food spoilage and pathogenic microorganisms. Information on the occurrence of synergy in herb and spice extracts is slowly emerging, and some combinations of herbal extracts have been reported to display stronger inhibitory effects on food-borne bacteria than when used alone (Lambert *et al*., 2001). Recently, there has been considerable emphasis on studies involving essential oils of spices as a means of inhibiting the growth of microbes. However, in the manufacture of ready meals there is limited research on the inhibitory effect of different commercial spice extracts including oleoresins and aqueous resins of herbs and spices.

Many studies have concentrated exclusively on antimicrobial effects of one or few herb or spice extracts only. While these data are useful, this information is difficult to compare directly due to

a number of factors, such as variability in composition or content of active agents between plants due to origin from various geographical regions, harvesting seasons, growth and drying conditions, or using plant material of different maturity (McGimpsey *et al.,* 2004; Cosentino *et al*., 2009).

It has been reported that spices owe their antimicrobial properties mostly to the presence of alkaloids, phenols, glycosides, steroids, essential oils, coumarins and tannins (Shan *et al*., 2019). As reviewed by López-Malo *et al*. (2006), some of antimicrobial components that have been identified in spices and herbs are: eugenol from cloves, thymol from thyme and oregano, carvacrol from oregano, vanillin from vanilla, allicin from garlic, cinnamic aldehyde from cinnamon, allyl isothiocyanate from mustard, etc. Among these products, particular interest has been focused on essential oils (EOs) and their components (Elgayyar *et al*., 2001), because they are known to be active against a wide variety of microorganisms, including food-borne pathogens and spoilage bacteria (Hammer *et al*., 2009). EOs are aromatic and volatile oily liquids of an aromatic plant's secondary metabolism. They are normally formed in special cells or groups of cells, found in leaves and stems, and commonly concentrated in one particular region such as leaves, bark or fruit (Oussalah, 2006). EOs have long served as flavouring agents in food and beverages, but they are much more important because of their antimicrobial activity, which is assigned to a number of small molecules of terpenoids and phenolic compounds (thymol, carvacrol, eugenol) (Conner, 2003).

2.2 Some Commonly Used Spices in Nigeria

2.2.1 *Murraya koenigii* (curry leaf)

The scientific name of the curry plant is *Murraya koenigii* Spreng and it belongs to the Rutaceae family. The plant is native to India and is usually found in tropical and subtropical regions. It is cultivated in various other countries such as China, Australia, Nigeria and Ceylon. The most useful parts of this plant are the leaves. *Murraya koenigii* L. (curry leaves) It is reported to have antioxidant, antidiabetic, anticarcinogenic, antidysenteric, stimulant, hypoglycaemic and antimicrobial activities (Khanum *et al*., 2000; Yadav *et al*., 2002; Ningappa *et al.,* 2010).

Medicinally, these leaves found use to treat diarrhea, dysentery and to prevent vomiting. The Leaves and fruits are also a source of an essential oil which finds use as a fixative for heavy type of soap perfume. Leaves, root and bark are tonic, stomachic and carminative. Juice of roots provides relief from renal pain (Henry, 2015). Previous phytochemical investigations on this plant revealed the presence of carbazole alkaloids and coumarins (Anwer *et al*., 2013).

Murraya koenigii have lots of bioactive substances that mnakes it medicinally important plant but it has not got a commensurate attention by scientist. *Murraya koenigii* is used in a variety of forms such as extract, essential oil, or directly used due to the presence of following active constituent bismahanine, murrayanine, murrayafoline-A, bi-koeniquinone-A, bismurrayaquinone, mukoenine-A, mukoenine-B, mukoenine-C, murrastifoline, Murrayazolinol, murrayacine, murrayazolidine, murrayazoline, mahanimbine, girinimbine, koenioline, xynthyletin, koenigine-Quinone A and koenigine-Quinone B for therapeutic purpose by folk people (Tajkarimi *et al*., 2010). Many medicines such as digitalis, vinblastine, aspirin and quinine has plant as a source of origin for example foxglove (*Digitalis purpurea*), willow bark (Salix spp.), quinine bark (*Cinchona officinalis*). Medicinal plants are used either for therapeutic or prophylactic purposes medicinal plant are used. For the therapeutic properties of medicinal plants presence of

secondary metabolites plays a very important role such as alkaloids, flavonoids, terpinoids, vitamins, tannins etc., these all are the secondary metabolites of the plant as active constituent. These all secondary metabolites of plant physiologically affect the body at different stages of body development and make the body disease free. The plant *Murraya koenigii* belonging to the family Rutaceae that grows throughout the spring, summer and in rain fall season in every part of the tropical region up to the height of 1500 to 1655m from sea level (Sofia *et al*., 2007).

2.2.2 *Justicia flava*

Justicia flava (Forrsk, Vahl) ("ighereje" among Itsekiris and Urhobos), is a common hedge plant whose seeds are used for preparing pepper soup and banga soup. In Burkina Faso, the pulp of the leaves is rubbed on babies for treatment of convulsion, feverish aches and pains; in Ghana it is used for treating fever, yaws and diarrhea (Burkhill, 2005). Among the Urhobos and Itsekiris of Delta State, the leaf infusion is used for bathing new born babies to treat infantile jaundice.

A study carried out by Masete (2021) showed that the methanolic leaf extract reduced the mean arterial pressure significantly.

2.2.3 Nutmeg

African nutmeg (*Monodora myristica*), which belong to the Ananacea family is a berry with many seeds that grows well in the evergreen forests of West Africa (Burubai *et al*., 2007) and is very prevalent in the Southern part of Nigeria where it is variously known as Iwor amongst the Itsekiris; Ikposa (Bini); Ehiri (Ibo); and Ariwo (Yoruba). It is observed that almost every part of the tree has both economic and medicinal importance. However; the most economically

important parts are the seeds which are embedded in the white sweet-smelling pulp of the fruit. When grounded to powder, the seed is used as spice to prepare varieties of soup such as banga, pepper, Egusi, Ogbonor, black soups respectively and also used as stimulant to relieve constipation and to control passive uterine hemorrhage in women immediately after child birth (Udeala, 2001).

Monodora myristica is used widely in Nigeria for culinary purposes, and sometimes traditionally for its medicinal properties; not much is, however, documented of its antimicrobial and nutritional properties. Most documented reports on the seed are mostly on the antioxidant properties of essential oils (Owotokomo and Ekundayo, 2012).

2.2.4 *Parkia biglobosa*

Parkia biglobosa (Jacq) Benth and *Parkia bicolor* A. Chev belong to the plant family Mimosaceae of the order Leguminisae. In Yoruba, *P. bicolor* is referred to as Igba Odo; Dorowa, in Hausa, and in lbo as Origili Okpi. *P. biglobosa* popularly known as the African locust bean tree is known in Yoruba as Igba, or Irugba, in Hausa as Dorowa and in lbo as Origili. The fermented seeds of *P. biglobosa* are used in all parts of Nigeria and indeed the West Coast of Africa for seasoning traditional soups.

Similarly, both trees form a crown so are often grown as shade trees (Daziel, 2007). However, there are some distinctive characteristic differences. *P. bicolor* usually grows by the river bank and can grow up to about 100m high. On the other hand *P. biglobosa* is found commonly everywhere in the Savannah and it grows up to about 20m high. The pinnae of the former is

about 10 - 26 pairs while that of the latter is about 6 - 11 pairs. The leaflets of *P. bicolor* occur in 20 – 55 pairs while those of *P. biglobosa* in 14 – 30 pairs (Andrew, 2006).

Parkia species have found use traditionally as foods, medicinal agents and are of high commercial value. The pulverized bark of *P. bicolor* is employed in wound healing. *P. biglobosa* is known to provide an ingredient that is used in treating leprosy, and for treating hypertension. In Gambia, the leaves and roots are used in preparing a lotion for sore eyes. A decoction of the bark of *P. biglobosa* is also used as a bath for fever, as a hot mouthwash to steam and relieve toothache. The pulped bark is used along with lemon for wound and ulcers (Irvine, 2004). Parkia plants have been identified as source of tannins, saponins, gums, fuel and wood Seeds of various species of *Parkia* have also been investigated for their protein and amino acid contents (Fetuga *et al*., 2004).

2.2.5 *Piper guineense*

This is an African spice plant and is commonly called Ashanti pepper. The seeds, leaves and sometimes the stems are used in preparing soup. It imparts “heat” and a spicy pungent aroma to food. The medicinal properties of *Piper guineense* exert bacteriostatic and bacteriocidal effects on some bacteria. The leaves are considered aperitive, carminative and eupeptic (Arora and Kaur, 2009).

They are also used for the treatment of cough, bronchitis, intestinal disease and rheumatism (Burkhill, 2005). The leaves are also used to treat female infertility while the fruits are used as an aphrodisiac. It is a climbing plant that can grow up to 20m in length. The seeds are smooth and are prolate-elliptically shaped (Conner, 2003).

2.2.6 Ginger

Ginger, a common substance found increasingly in the diets of the global population, has known antimicrobial effects and is commonly used together in teas (Sebiomo *et al.,* 2011). Ginger has been used in centuries to fight infection. Its components are active against diarrhea which is the leading cause of infant death in developing countries (Sebiomo *et al.,* 2011).

Ginger is the rhizome of *Zingiber officinale* and is the part of the plant meant for consumption. Its characteristic odour and flavour is caused by a mixture of zingerone, shogaol and gingerols, volatile oils that compose 1 to 3 percent of the weight of fresh ginger (Jolad *et al*., 2005).

Fresh ginger contains 80.9% moisture, 2.3% protein, 0.9% fat, 1.2% minerals, 2.4% fibre and 12.3% carbohydrates. The minerals present in ginger are iron, calcium, and phosphorus. It also contains vitamins such as thiamine, riboflavin, niacin and vitamin C (McGee, 2004).

2.2.7 Turmeric

Turmeric is used mostly in savory dishes, but is also used in some sweet dishes, such as the cake. In India, turmeric plant leaf is used to prepare special sweet dishes, patoleo, by layering rice flour and coconut-jaggery mixture on the leaf, then closing and steaming it in a special copper steamer (Priyadarsini, 2014).

In recipes outside South Asia, turmeric sometimes is used as an agent to impart a golden yellow color. It is used in canned beverages, baked products, dairy products, ice cream, yogurt, yellow cakes, orange juice, biscuits, popcorn color, cereals, sauces, gelatins, etc. It is a significant ingredient in most commercial curry powders (Nelson *et al*., 2017).

Most turmeric is used in the form of rhizome powder. In some regions (especially in Maharashtra, Goa, Konkan, and Kanara), turmeric leaves are used to wrap and cook food. Turmeric leaves are mainly used in this way in areas where turmeric is grown locally, since the leaves used are freshly picked. Turmeric leaves impart a distinctive flavor (Chattopadhyay *et al*., 2004).

Although typically used in its dried, powdered form, turmeric is also used fresh, like ginger. It has numerous uses in East Asian recipes, such as pickle that contains large chunks of soft turmeric, made from fresh turmeric (Siewek, 2013; Tayyem *et al*., 2006).

Turmeric is used widely as a spice in South Asian and Middle Eastern cooking. Many Persian dishes use turmeric as a starter ingredient. Various Iranian *khoresh* dishes are started using onions caramelized in oil and turmeric, followed by other ingredients. The Moroccan spice mix ras el hanout typically includes turmeric (Vaughn *et al*., 2016; Daily *et al*., 2016).

In India and Nepal, turmeric is widely grown and extensively used in many vegetable and meat dishes for its color. It also is used in Nepal for its supposed value in traditional medicine. In South Africa, turmeric is used to give boiled white rice a golden colour. In Vietnamese cuisine, turmeric powder is used to color and enhance the flavors of certain dishes. The powder is used in many other Vietnamese stir-fried and soup dishes (Nagpal and Sood, 2013).

The staple Cambodian curry paste *kroeung*, used in many dishes including *Amok*, typically contains fresh turmeric. In Indonesia, turmeric leaves are used for Minang or Padang curry base of Sumatra, such as *rendang*, *sate padang*, and many other varieties. In Thailand, fresh turmeric

rhizomes are used widely in many dishes, in particular in the southern Thai cuisine, such as the yellow curry and turmeric soup.

In medieval Europe, turmeric became known as Indian saffron because it was used widely as an alternative to the far more expensive saffron spice (Singh and Bhanu, 2004).

2.3 Phytochemical Constituents of Spices

The chemicals which are referred to as active principles or phytochemical substance include terpenes, flavonoid, bioflavonoid, benzophenones, xanthenes, as well as some metabolites such as tannins, saponins, cyanates, oxalates and anthraxquinones (Asaolu, 2003). Phytochemical screening on *Murraya koenigii* soup spice resulted in the isolation of several alkaloids and steroidal compounds with the potential to induce quinine reductase and to inhibit 7, 12 dimethylbenz(9)anthracene induced prenoplastic lesion in mouse mammary organ (Dinan *et al*., 2001; Jang *et al*., 2003).

Phytochemicals derived from plant source can act as larvicide, insect growth regulators, repellants and ovipositor attractant and have different activities (Jebasan, 2001). Polyphenol extract of *Murraya koenigii* has a weak antioxidant activity as observed in *in vitro* free radicals scavenging assays, but, the extract was very active on pathogenic bacteria and this activity may be influenced by the polymerization size of the phenolic compound (Jang *et al.*, 2003). Among the compounds isolated from *Murraya koenigii,* its alkaloids belonging to the family of indolquinolines appeared to be of great interest in pharmacological studies. Many investigations have been done on this family of compounds and the result showed that they are new leads in the

establishment of drugs against much disease. For example, cryptolepine 5-methylindo the main alkaloid of *Murraya koenigii* has been isolated (Gunatilaka *et al*., 2000; Yang *et al*., 2009). Cryptolepine itself is found to produce many pharmacological effect such as antimicrobial (Cimanga *et al*., 2008), antiprotozoa (Arzel *et al*., 2001), antihyperglycemic (Bierer *et al*., 2008) and cytotoxic effect through GC-rich DNA sequence intercalation that provides basis for design of new anticancer drug (Lisgarten *et al.*, 2002).

The presence of tannins in African nutmeg (*Monodora myristica*) show that the plant can be used as purgative (Gills, 2002). The presence of terpenoid revealed that *Monodora myristica* can act mainly as anti-feedant and growth disrupter and possesses considerable toxicity towards insect. Terpenoid also play an important role in wound and scar healing (Hayashi *et al*., 2003). It should be noted that steroidal compounds are of importance and interest due to their relationship with certain compounds such as sex hormones. The presence of steroidal compounds in *Monodora myristica* is an indication that the plants can be used by expectant or breastfeeding mothers to ensure their hormonal balance, since steroidal structures could serve aspotent starting materials in the synthesis of these hormone (Okwu, 2001). Phytochemicals exert their activity through different mechanisms.

2.3.1 Tannins

Tannin is an astringent, bitter plant polyphenolic compound that binds to and precipitates protein and various other organic compounds including amino acids and alkaloids. Tanin compound play major role in protection, and in plant growth regulation (Katie *et al.*, 2006). The astringency of tannin is what causes the dry and puckery in the mouth following the consumption of unripened fruits or red wine (McGee and Harold., 2004). Tannins have molecclar weights ranging from 500

to over 3,000 *Murraya koenigii* esters and up to 20,000 proanthocyanidins (Bate-smith and Swain, 2002). Tannins are mainly physically located in the vacuoles or surface wax of plants. Tannins are classified as ergastic substance i.e. non-protoplasm materials found in cells (Kadam *et al*., 1990). Tanins regulate the growth of tissues. Tanins can be effective in protecting the kidneys (Bajaj, 2008). When incubated with red grape juice and red wines with a high content of condensed tannins, the polio virus, herpes simplex virus and various enteric viruses are inactivated (Bajad, 2008).

Tannins extracted from *Murraya koenigii* (soup spice) have shown potential antiviral (Lu *et al*., 2004), antibacterial (Akiyama *et al*., 2001) and anti-parasitic effects (Kolodziej and Kiderlen, 2005).

2.3.2 Alkaloids

Alkaloids are a group of naturally occurring chemical compounds that contain mostly basic nitrogen atoms. This group also includes some related compounds with neutral (McNaught and Wilkinson, 2007) and even weakly acidic properties. Also some synthetic compounds of similar structure are attributed to alkaloids. In addition to carbon, hydrogen and nitrogen, alkaloids may also contain oxygen, sulfur and more rarely other elements such as chlorine, bromine, and phosphorus.

Alkaloids are produced by a large variety of organisms, including bacteria, fungi, plants, and animals, and are part of the group of natural products (also called secondary metabolites). Many alkaloids can be purified from crude extracts by acid-base extraction. Many alkaloids are toxic to other organisms. They often have pharmacological effects and are used as medications, as

recreational drugs, or in entheogenic rituals. Examples are the local anesthetic and stimulant cocaine; the psychedelic psilocin; the stimulant caffeine; nicotine; the analgesic morphine; the antibacterial berberine; the anticancer compound vincristine; the antihypertension agent reserpine; the cholinomimeric galatamine; the spasmolysis agent atropine; the vasodilator vincamine; the anti-arhythmia compound quinidine; the anti-asthma therapeutic ephedrine; and the antimalarial drug quinine. Although alkaloids act on a diversity of metabolic systems in humans and other animals, they almost uniformly invoke a bitter taste (Rhoades, 2009).

2.3.3 Saponins

Saponins are a class of chemical compounds, one of many secondary metabolites found in natural sources. They are amphipathic glycosides grouped in terms of phenomenology, by soap-like foaming they produce when shaken in aqueous solutions and in terms of structure, by their composition of one or more hydrophilic glycoside moieties combined with a lipophilic triterpene derivatives (Hostettmann and Marston, 2005).

In *Piper guineense* (soup spice) plant, saponins may serve as anti-feedant, and to protect the plant against microbes and fungi. Some plant saponins (e.g from oat and spinach) may enhance nutrient absorption and aid animal digestion (Foerster and Hartmut, 2006).

Saponins are being promoted commercially as dietary supplements (Marjan and Hossein, 2008) and nutriceuticals (Francis, 2002). Saponins have many health benefits like cholesterol reduction, reduce cancer risk, immunity booster, reduce bone loss and antioxidant activity (Skene *et al.*, 2006). Saponins have also been used as adjuvants in vaccines.

2.3.4 Anthraquine

Anthraquinone, also called anthracenedione or dioxoanthracene, is an aromatic organic compound with formula $C_{14}H_8O_2$ (Romanotto *et al*., 2017). Several isomers are possible, each of which can be viewed as a quinone derivative. The term anthraquinone, however, almost invariably refers to one specific isomer, 9,10-anthraquinone (IUPAC: 9,10-dioxoanthracene) wherein the keto groups are located on the central ring. It is a building block of many dyes and is used in bleaching pulp for papermaking. It is a yellow highly crystalline solid, poorly soluble in water but soluble in hot organic solvents (Bien *et al*., 2005). Anthraquinone is almost completely insoluble in ethanol near room temperature but 2.25 g will dissolve in 100g of boiling ethanol.

2.3.5 Cyanogenic Glycosides

Cyanogenesis is the ability of some plants to synthesize cyanogenic glycosides, which when enzymically hydrolyzed, release cyanohydric acid (HCN), known as prussic acid (Harborne, 2002).

Cyanogenic glycosides account for approximately 90% of the wider group of plant toxin known as cyanogens(Atkinson, 2006). In this case, the glycone contains a cyanide group. All of these plants have these glycosides stored in the vacuole, but, if the plant is attacked, they are released and become activated by enzymes in the cytoplasm (Lindhorst, 2007). These remove the sugar part of the molecule and release toxic hydrogen cyanide. Storing them in inactive forms in the cytoplasm prevents them from damaging the plant under normal conditions.

An example of these is amygdalin from almonds. They can also be found in the fruits (and wilting leaves) of the rose family (including cherries, apples, plums, almonds, peaches, apricots, raspberries, and crabapples). *Murraya koenigii*, an important spice in Africa and South America,

contains cyanogenic glycosides and, therefore, has to been used in many dishes. *Murraya koenigii* expresses cyanogenic glycosides in its seed and, thus, is resistant to pests such as rootworms. It was once thought that cyanogenic glycosides might have anti-cancer properties, but this idea was disproven (Sun *et al*., 2018).

2.3.6 Terpenes

Terpenes are a large and diverse class of organic compounds, produced by a variety of plants, particularly conifers though also by some insects such as termites (Nutting *et al*., 2011) or swallowtail butterflies, which emit terpenes from their osmeteria. They are often strong smelling and thus may have had a protective function.

They are the major components of resin, and of turpentine produced from resin. The name "terpene" is derived from the word "turpentine" (Zelena *et al*., 2009). In addition to their roles as end-products in many organisms, terpenes are major biosynthetic building blocks within nearly every living creature. Steroids, for example, are derivatives of the triterpene squalene.

When terpenes are modified chemically, such as by oxidation or rearrangement of the carbon skeleton, the resulting compounds are generally referred to as terpenoids. Some authors will use the term terpene to include all terpenoids. Terpenoids are also known as isoprenoids.

Terpenes and terpenoids are the primary constituents of the essential oils of many types of plants and flowers. Essential oils are used widely as natural flavor additives for food, as fragrances in perfumery, and in traditional and alternative medicines such as aromatherapy. Synthetic variations and derivatives of natural terpenes and terpenoids also greatly expand the variety of aromas used in perfumery and flavors used in food additives. Vitamin A is an example of a

terpene. Terpenes are released by trees more actively in warmer weather, acting as a natural form of cloud seeding. The clouds reflect sunlight, allowing the forest to regulate its temperature. The aroma and flavor of hops, highly desirable in some beers, comes from terpenes. Of the terpenes in hops myrcene, b-pinene, b-caryophyllene, and a-humulene are found in the largest quantities (Glenn, 2010).

In addition to their roles as end-product in many organisms, terpenes are major biosynthetic building blocks within nearly every living creatures (Juliana, 2009). Many terpenes are hydrocarbons, but oxygen-containing compounds such as alcohols, aldehydes or ketones are also found.

2.3.7 Reducing sugar

A reducing sugar is any sugar that either has an aldehyde group or is capable of forming one in solution through isomerism. The cyclic hemiacetal forms of aldoses can open to reveal an aldehyde and certain ketoses can undergo tautomerization to become aldoses. However, acetals, including those found polysaccharide linkages, cannot easily become a free aldehyde. The aldehyde functional group allows the sugar to act as a reducing agent (Campbell and Farrell, 2012).

2.3.8 Flavonoid compounds

Flavonoid are also hydroxylated phenolic substances but occur as a C6-C3 unit linked to an aromatic ring. Since flavonoids are known to be synthesized by plants in response to microbial infection, it should not be surprising that they have been found *in vitro* to be effective antimicrobial substances against a wide array of microorganisms (Dixon and Pasinetti, 2010).

Their activity is probably due to their ability to complex with extracellular and soluble proteins and to complex with bacterial cell walls. More lipophilic flavonoids may also disrupt microbial membranes (Tsuchiya *et al*., 1996).

2.4 Medicinal uses of Some Spices

Murraya koenigii herb plant is traditionally used in the treatment of malaria, diarrhea and many other disease Nalcouma and Ouedraogo, (Rana and Yamini, 2022). Research focused on malaria led to the identification of alkaloids, principally cryptolepine the major alkaloid of plants as its antimalaria agent (Rajendran *et al.*, 2014).

In Central America, *Murraya koenigii* is used to treat asthma, renal inflammation, colds, fever, headache, ulcers and worms (Vats and Singh, 2011). In Colombia, *Murraya koenigii* is known to treat snake bites (Tamokou *et al*., 2013) and also demonstrated that the ethanolic extract of the plant had an effective moderate activity against venom of Bothrax anthrax. *Murraya koenigii* has been reported to contain aphrodisiac, analgestic and anti-inflammatory properties (Das *et al*., 2011). Its extract is reported to be a tonic in nature. It affects central nervous system and provides relief from anxiety. It is also used to reduce the body weight (Patil *et al*., 2019). The intake of the extract of *Murraya koenigii* leaves is reported to regulate blood pressure and to improve the cardiac irregularity. It is useful in healing wounds and the extract of the whole plant is beneficial in spermatorrhoea. The bark of the stem is effective in sciatica and facial paralysis

and the consumption of the juice of sida is reported to improve sexual strength (Dhamane *et al*., 2019).

2.5 Antimicrobial Activity of Spices

A study investigated the antimicrobial activity of alkaloids from *Murraya koenigii* against Gram positive and Gram negative bacteria. The antibacterial assays were performed by the agar-well diffusion and the broth microdillution for the evaluation of inhibition zone, diameters, MIC and MBC values. The highest inhibition zone diameters were recorded with gram positive bacteria. The microdillution assay gave the range of MIC value of 16-400mg/ml and 80-400mg/ml in the case of MBC, for different strains. Hence, the two major alkaloids in the extract identified as cryptolepine and quindoline exhibited good antimicrobial activity against several test microorganism (Karou *et al*., 2006).

It was also noted that the ethanolic extract of *Murraya koenigii* had significant activity on *Streptococcus faecalis*, contrary to Anani *et al*. (2000), Rajacuruna *et al*. (2002), Saganuwan and Glumbe (2006) who reported that the ethanolic extract of *Murraya koenigii* had no inhibitory effect against *Streptococcus faecalis*.

2.6 Analgestic Activity

The analgestic activity of *Murraya koenigii* was evaluated by hot plate and tail immersion methods at three(3) different dose levels of 100, 300 and 500mg/ml in mice. However, *Murraya koenigii* extract only showed significant protection of 55% at the highest dose of 500mg/ml (Prakash *et al.*, 2006).

2.7 Antibacterial Activity

The antimicrobial screening of *Murraya koenigii* revealed that many compounds might be responsible for the activity of the plant. The first antimicrobial screening of the plant was conducted by Tamokou *et al*. (2013) using the disk diffusion assay. The authors found that the methanolic extract of the plant had significant activity on *Staphylococcus aureus, Escherichia coli, Bacillus subtilis* and *Mycobacterium phlei,* however the extract was not active on *Streptococcus faecalis*, *Klebsiella pneumoniae, Salmonella typhimurium, Pseudomonas aureginosa* and *Candida albicans.* Polyphenols and alkaloids of the plant were tested separately on several pathogenic bacteria including clinical strains and reference strains of *Enterobacteriaceae* and *Staphylococcaceae* families. The tests were performed on agar well diffusion (Perez *et al*., 1990). The results revealed that the phenolic compound had a good *in vitro* antimicrobial activity and this activity was much influenced by the storage of the extract probably because of the phenolic compound oxidation. The inhibition zones diameters varied from 11-25mm for 250µg/ml polyphnols and MBC values ranged from 20-2000µg/ml (Karou *et al*., 2006).

Alkaloids of *Murraya koenigii* also displayed a good antibacterial activity. The recorded inhibition zone diameters varied from 16-38mm for 100µg alkaloid and the MBC values from 80-400µg/ml (Karou *et al*., 2006).

Murraya koenigii contains phenolic compounds that are responsible for the activity of the plant. The current problem is the fact that they are vulnerable to polymerization in air through oxidation reactions. This oxidization may first affect the extractability of the phenolic compounds that is crucial in drug preparation (Das *et al.*, 2011). Secondly, an important factor

governing the activity of phenolic compounds is their polymerization size. Oxidized condensation of phenols may result in the toxification of microorganisms, while the adverse effects can be observed in some cases (Das *et al.*, 2011).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Sample Collection

Samples of the curry (*Murraya koenigii*) leaves were purchased from Uselu market in Benin City, Edo State. The samples were collected aseptically in sterile polyethylene bags and were transferred immediately to the laboratory for phytochemical constituents, antibacterial activities and proximate analysis.

3.2 Sample Preparations/Extraction

The leaves were washed, dried at 40 ^{o}C for 24 hrs and ground to powder in a milling machine (Kenstar model, India).

3.2.1 Extraction with Ethanol

Five hundred grams (500 g) of the curry powder was suspended in about 900ml of ethanol (100%) in a chromatographic tank for 24 hrs. the suspension was thereafter filtered through a filter paper (0.22µm Millipore filter). The filtrate was concentrated in an evaporating disc. The ethanol curry extract was thereafter preserved in the refrigerator at 4^{o} C until used (Handa *et al.,* 2008).

3.2.2 Extraction with Aqueous

Same process as applicable to ethanol was followed except the extraction process water. 1.2kg of the curry powder was suspended in about 1.7ml of distilled water in a chromatographic tank for 24 hrs. the suspension was thereafter filtered through a filter paper (0.22µm Millipore filter). The filtrate was concentrated in an evaporating disc. The ethanol curry extract was thereafter preserved in the refrigerator at 4^{o} C until used (Handa *et al.,* 2008).

3.3 Preparation of Different Concentrations

The method of Ekwenye and Elegalam (2005) was used. Concentration of 100mg/ml of the extract was prepared by dissolving 0.1g of the extract in 1ml of sterile water and ethanol respectively. Then concentrations of 50mg/ml, 25mg/ml 12.5mg/ml and 6.25mg/ml were prepared from the stock concentration (100mg/ml) by double dilution procedure.

3.5 Collection of Test Organisms

Bacterial isolates used for the analyses were collected from Medical Microbiology Laboratory, University of Benin Teaching Hospital (UBTH), Benin City. The isolates were thereafter subcultured for further analysis (Cheesbrough, 2006).

3.5.1 Nutrient Agar

Nutrient agar was prepared from the already sold dehydrated powder of nutrient agar. The preparation followed the dissolving of 28 g of the nutrient agar powder in 1000 ml of distilled water inside a conical flask sealed with cotton wool and aluminum foil paper. The mixture was shaken in other to attain an almost homogeneous solution. Sterilization was then carried out on the resulting mixture using the autoclave at 121^{o}C for 15 minutes. The medium was cooled to 45-50^{0}C which was then dispensed aseptically into sterile Petri dishes.

3.5.2 Mueller-Hinton agar

The medium was prepared from already sold dehydrated powder of Mueller-Hinton agar. The preparation followed the dissolving of 38 g of Mueller-Hinton agar in 1000 ml of distilled water inside a conical flask sealed with cotton wool and aluminum foil paper. The mixture was shaken in other to attain an almost heterogeneous solution. Sterilization was subsequently carried out using the autoclave at 121^{o}C for 15 min. The medium was cooled to 45^{0}C and then dispensed aseptically into sterile Petri dishes.

3.6 Determination of Antibacterial Properties of *Murraya koenigii* leave extracts

The antibacterial effects of *Murraya koenigii* extracts were investigated using the Agar well diffusion using Mueller Hinton agar. The surface of the plates containing agar was inoculated using standardized test bacterial inoculums (*Salmonella* sp and *Shigella* sp) obtained by comparing with 0.5 MCFARLAD turbidity level. A six millimiter (6mm) sterilized cork borer

was used to bore holes on the agar plates. Equal amount of the aqueous and ethanol extracts were dispensed into the holes and incubated at 37^0C for 24 hours. After incubation, the diameter for the various inhibition zones were determined using a transparent meter rule calibrated in millimeter (Hufford *et al*., 2003).

3.7 Determination of Minimum Inhibitory Concentration (MIC)

This was done by transferring 1 ml of the aqueous and ethanol extracts of all four concentrations respectively into separate test tubes. Subsequently, 1 ml of nutrient broth was also added followed by the addition of a loopful of the test bacterial already standardized. This was done for all concentrations (100mg.ml, 50mg/ml, 25mg/ml, 12.5mg/ml and 6.25mg/ml) for both ethanol and aqueous curry extracts. The bacteria was then incubated and then observed for turbidity (Ettebong and Nwafor, 2009; Ajaiyeoba *et al.,* 2003).

3.8 Determination of Minimum Bactericidal Concentration (MBC)

This was determined from the results of the MIC. Test tubes that showed no turbidity was first taken as the Minimum Inhibitory Concentration (MIC). Those tubes with no observable turbidity were then re-inoculated into sterile nutrient agar plates and incubated at 37°C for 24 hours. The plates that showed no growth were considered the minimum bactericidal concentration (Ajaiyeoba *et al.,* 2003).

3.9 Phytochemical Screening

The phytochemical screening of the aqueous and ethanol extracts were carried out as described in the method of Hufford *et al*. (2003). This was done using basic chemical tests to determine the

presence of plant metabolites such as phenols, tannins, flavonoids, alkaloids, saponins, steroids and glycoside, in the sample.

3.9.1 Qualitative Phytochemical Screening

Test for Saponins: Five (5.0) ml aliquot of the aqueous and ethanol extracts (100mg/ml) separately were mixed with 20 ml deionized water. The mixture was shaken vigorously and observed. Persistent foaming inferred that Saponin is present.

Test for Alkaloids: An aliquot of 0.1 ml of the stock extract were separately added to 6 ml of dilute hydrochloric acid which was then boiled, cooled and filtered. The filtrate was divided into three portions and subjected to the following tests. To the first portion, 2 drops of Dragendorff's reagent were introduced. The presence of a precipitate with a characteristic red colour was indicative of alkaloids been present. To the second portion, 2 drops of Meyer's reagent was added. The formation of a creamy white precipitate inferred that alkaloid was present. To the third portion, 2 drops of Wagner's reagent was introduced. The formation of reddish-brown precipitate inferred that alkaloid was present (Hufford *et al*., 2003)..

Test for Tannins: It was done by the addition of 1 ml of the extract to 10ml deionised water and subsequently treated with three drops of ferric chloride. The formation of a greenish-brown precipitate inferred that tannins were present (Hufford *et al*., 2003).

Test for Flavonoides: three (3) ml of 1% Aluminium chloride solution was mixed with 5 ml extract of the plant. The presence of a yellow colour indicated that flavonoid was present. Subsequently upon the addition of 5 ml dilute ammonia solution and concentrated H_2SO_4. Initial

yellow colouration disappears on standing which further confirmed the presence of flavonoids (Hufford *et al*., 2003).

Test for Steroids: Two (2) ml acetic anhydride was mixed with 2 ml extract of the sample upon which 2 ml H_2SO_4 was added carefully. The presence of steroids was confirmed in a colour change from violet to blue or green (Hufford *et al*., 2003).

Test for Phenols: One gram (1 g) of curry extract was mixed with 10 ml distilled water. It was subsequently boiled in water bath for 3 minutes and then filtered. From the filtrate, 2 ml aliquot was put in 3 test tubes each. One of the filtrate was diluted with distilled water in the ratio 1:4. The appearance of a blue or greenish colour indicated that phenol was present.

Test for Glycosides: Five (5) ml of dilute sulphuric acid was mixed with 0.1 g plant extract in test tube which was boiled for 15 minutes using water bath. The mixture was cooled and neutralized with 20% potassium hydroxide solution. 10 ml of a mixture of equal parts of Fehling's solution A and B was mixed with the mixture and boiled for 5 min. The formation of a dense brick red precipitate indicated that glycoside was present (Hufford *et al*., 2003).

3.9.2 Quantitative Phytochemical Analysis

Test for Saponins: One (1) gram of extract was soaked with 10 ml petroleum ether and decanted inside a beaker. A 10 ml of another petroleum ether was poured in the beaker followed by evaporation of the filtrate to dryness. 6 ml of ethanol was used to dissolve the residual substance. 2 ml of the solution was dispensed into a test tube were 2 ml of chromagen solution was added. The absorbance was read at 550 nm after it was left to stay for 30 minutes.

Test for Alkaloids: One (1) gram of the extract was soaked in 20 ml ethanol and 20% H_2SO_4 (1:1 v/v). One (1) ml of the filtrate was added to 5 ml of 60% H_2SO_4. After 5 min, 5 ml of 0.5% formaldehyde in 60% H_2SO_4 was mixed with the mixture and allowed to stay for 3 hours. 565 nm was read as the absorbance.

Test for Tannins: One (1) g of the extract was dispensed in the 50 ml of methanol and filtered. To the filtrate (5 ml), 0.3 ml of 0.1N ferric chloride in 0.1N HCl and 0.3 ml of 0.0008 M of potassium ferri cyanide were added were 720 nm of absorbance was read.

Test for Flavonoids: One (1) g of the extract was dispensed in 20 ml of ethylacetate for 5 minutes and filtered. 5 ml of the filtrate was added to dilute ammonia and was shaken for 5 min. The absorbance was read at 490 nm from the upper layer collected in the mixture.

Test for Steroids: One (1) g of the extract was dispensed in 20 ml of ethanol and filtered. 2 ml of chromagen solution was added to 2 ml of the filtrate while the absorbance was read at 550 nm after the mixture was left to stay for 30 minutes.

Test for Glycosides: One (1) g of the extract was dispensed in 50 ml of distilled water and filtered. 4 ml of alkaline pirate solution was added to 1 ml of the filtrate. It was then boiled for 5 minutes and allowed to cool. The absorbance was read at 490 nm.

Test for Phenols: One (1) g of the extract was soaked in 20 ml of 80% ethanol and then filtered. The filterate (5 ml) was added to 0.5 ml of folinciocalteus reagent and allowed to stay for 30 min. Subsequently, 2 ml of 20% sodium carbonate was added and absorbance measured at 650 nm.

3.10 Gas Chromatography-Mass Spectrometry (GC-MS) Analysis

The gaschromatography-mass spectrometry assay of *Murraya koenigi* leaves extracts was carried out with a 5.0. Ultraversion of Thermo GC-Trace and gas chromatograph interfaced with a mass spectrometer instrument. The oven temperature was maintained at 220ºC with a rate of 6ºC/min; the carrier gas having a flow rate of 1 ml per min. The split sampling technique was used to add the sample into a ratio of 1:10. Retention indices (RI) of the compounds were obtained by comparing the retention times of a series and identification of each compound was confirmed by the comparison of its retention index with data in the literature. The Mass-Spectrum was interpreted by using the data base of National institute Standard and Technology (NIST). The spectrum of the unknown compound was checked and analyse against with the spectrum of known compounds in the NIST library. The molecular weight, name, chemical structure and molecular formula of the compounds from the aqueous and ethanol plant extracts were ascertained.

3.11 Proximate Analysis

The moisture, ash, crude fats, proteins and carbohydrates content of the powdered sample was carried out using standard AOAC method (1990).

The moisture and ash were assayed by the difference of weight technique. Crude fat was extracted with a Soxhlet apparatus containing petroleum ether at a temperature of 40-60°C for 8 hours. Crude fibers were obtained by consecutive digestion of the defatted samples with 1.25% H_2SO_4 and 1.25% NaOH solutions. The amount of nitrogen, which is the precursor for the determination of protein in a substance, were assayed using micro Kjeldahl technique as described by Pearson (1976), which involved digestions, distillation and then titration of the sample. The amount of nitrogen was expressed in terms of protein content by the multiplication

of a factor of 6.25. Carbohydrate was assayed using the technique of difference. The carbohydrate was calculated using the technique of difference calculated as 100 - % (p + f + x + y + m) where p = protein, f = fat, x = fibre, y = ash, m = moisture (Pearson, 1976). All the values of the proximate analyses were interpreted as percentage (AOAC, 1990). The proximate analyses were done in triplicates.

3.11.1 Determination of moisture content:

Two (2) grams of the fresh sample of the plant material was put in a crucible at a temperature of 105˚ C until a stable weight was achieved. The value of the moisture was derived by the loss in mass of the original sample which was expressed in percentage (FAO, 2002).

3.11.2 Determination of crude protein:

The crude protein was assayed by the technique of Kjeldahl with a little changes (AOAC, 1990). The assay of crude protein had three steps which were; digestion, distillation and titration.

Digestion: One (1) g of the grounded sample was measured inside a digestion flask. Reagent blank and high purity lysine HCl was added to check for correctness of the digestion parameters. 15 g potassium sulfate, 0.04 g anhydrous copper sulfate, 0.5 to 1.0 g aluminum granules, 16.7 g K_2SO_4, 0.01 g anhydrous copper sulfate, 0.6 g TiO_s and 0.3 g pumice were added. Which was followed by the addition of 20 mL sulfuric acid, The flask was left on preheated burner (adjusted to bring 250 mL water at 25^{o}C to rolling boil in 5 minutes) and the mixture was heated till a

white fume clear bulb of flask was observed, it was swirled gently, while the heating process was on for 90 min with copper used as a catalyst. 250 mL of distilled water was carefully added to the mixture after cooling to room temperature.

Distillation: A mixture of 15 mL HCl and 70 mL H_2O was constituted to form a standard acid solution which was subsequently poured into the titration flask. To the reagent blank, 1 mL of acid and approximately 85 mL H_2O were added followed by three to four drops addition of methyl red indicator. Also, two to three drops of tributyl citrate, an antifoam agent was added to digestion flask to lower foaming. This was followed by the addition of another 0.5 to 1.0 g alundum granule. Slowly down side of flask, sufficient 45% NaOH solution (approximately 80 mL) was added to make mixture strongly alkali (pH 8.2). The flask was joined to distillation apparatus and was distilled in 150 mL distillate collected inside the titrating flask.

Titration: Sufficient acid was titrated with 0.1M NaOH standard solution to orange end point and the reading was taken to the nearest 0.01 mL (V NaOH). The reagent blank (B) was titrated in a similar way. Calculations were done in the following ways:

%N (DM basis) = [(V HCl x N HCl) - (V BK x N NaOH) - (V NaOH x N NaOH)]/1.4007 x W x

Lab DM/100

Where DM – dry matter; V NaOH = mL standard Sodium hydroxide needed to titrate sample; V HCl = mL

Standard Hydrochloric acid pipetted into titrating flask for sample; N Sodium hydroxide = Normality of Sodium hydroxide; N Hydrochloric acid = Normality of Hydrochloric acid; V BK = mL standard Sodium hydroxide needed to titrate 1 mL standard Hydrochloric acid minus B; B

= mL standard Sodium hydroxide needed to titrate reagent blank carried through method and distilled into 1 mL standard Hydrochloric acid; 1.4007 = milli equivalent weight of nitrogen x 100; W = sample weight in grams.

Calculation of percentage crude protein (CP):

Crude Protein (Dry Matter (DM) basis) = % N (DM basis) X F; where F = 6.25 (AOAC, 1990).

3.11.3 Determination of crude lipid:

This process was carried out with the Soxhlet extraction technique. 10 g of the powdered plant sample was measured and wrapped with a filter paper and put inside a thimble. The thimble was sealed with a cotton wool and put in the extraction column that was joined to the Condenser. 200 ml of n – hexane was used to extract the lipid (AOAC, 1990).

3.11.4 Determination of crude fibre:

Five (5) grammes of the powdered plant material and 200 ml of 1.25 % $H_2SO_4$for 30 min was heated and filtered with a Buchner funnel. The residual part was washed using distilled water till it became acid free. Two hundred millitre (200 ml) of 1.25% Sodium hydroxide was used to boil the residue for 30 minutes; it was then filtered and washed several times with distilled water till it became alkaline free. This was then rinsed with 10% Hydrochloric acid once and then twice using ethanol. Lastly it was rinsed three times with petroleum ether. The residual part was

poured in a crucible and dried at 105ºC in an oven over the night. A desiccator was used to cool and it was ignited in a muffle furnace at 550^{O}C for 90 minutes to attain the weight of the ash (AOAC, 1990).

3.11.5 Determination of ash content:

The whole ash content of the sample is the percentage of inorganic residue left after the organic matter has been burnt. 2 g of the powdered plant sample was put inside a crucible and was ignited in a muffle furnace at 550ºC for 6 hours. A desiccator was used to cool to room temperature were the weight of the ash was subsequently read (AOAC, 1990).

3.11.6 Determination of carbohydrate:

The carbohydrate content was determined by the subtraction from the total percentage compositions of moisture, protein, lipid, fibre, and ash contents (AOAC, 1990).

3.12 Determination of Susceptibility to Commercial Antibiotics

Susceptibility test was performed according to recommendation of Clinical and Laboratory Standards Institute (CLSI, 2006) by the agar diffusion technique on Mueller-hint on agar (Kirby-Bauer NCCLS modified disc diffusion technique) using 10 antibiotic disc corresponding to the drugs most commonly used in the treatment of human and animal infections caused by bacteria; The following commercially prepared antibiotic discs was used for susceptibility testing. Septrin (SXT) 30µg, amoxicillin (AM) 30µg, chloramphenicol (CH) 30µg, gentamicin (CN) 10µg, ciprofloxacin (CPX), pefloxacin (PEF) 30µg, Augmentin (AU) 30µg, Tarivid (OFX),

Streptomycin (S) 30µg and Sparfloxacin (SP) 10µg (Cheesbrough, 2006). Pure colony of the pathogenic bacteria isolate growing on a selective agar plate were picked to make a suspension in 1ml sterile normal saline. This was adjusted to an equivalence of a 0.5 McFarland standard. Sterile Mueller-Hinton agar plates was inoculated by spreading 0.1ml of the bacteria inoculum suspension on the entire surface of the plate. The plates was allowed to air-dry and within 15 minutes antibiotic sensitivity discs containing the above mentioned antibiotics were applied to the surface of the agar at 15mm equidistance to one another. Incubation was done at 37^{o}C for 24 hours. Thereafter, zones of growth inhibition was measured and interpreted according to recommended standard (CLSI, 2015). Obtained data was used to construct the phenotypic pattern of drug resistance for isolated bacteria. Results were interpreted as susceptible (S), intermediate (I) or resistant (R) according to standardized CLSI chart (Lauderdale, 2003).

3.13 Plasmid Profile of the Bacterial Isolates

3.13.1 Plasmid Isolation

A colony of test organisms, cultured on fresh agar plates was picked with the aid of a sterile wire loop and inoculated into sterile test tubes containing 8 ml of fresh nutrient broth and then incubated at 37^{o} C for 72hrs the antibiotic resistance plasmids in the cultures was isolated using the alkaline lyses protocol (Gyllensten, 1989). The broth culture was centrifuged in a microfuge at 6000 rpm for 10 minutes to pellet the cells, and the supernatant gently decanted leaving 50-

100µL together with cell pellet. The cells was completely pellet by at high speed. Tris EDTA/SDS (300µL TENS buffer) was added and vortexed for 2-5 seconds to respond the pellets. Thereafter, 150µl 3M sodium acetate, pH 5.2 was added and vortexes 2-5 to mi completely. The mixture was spun again at 6000 rpm for 10 min to pellet cell debris and chromosomal DNA. The supernatant was transferred into a fresh micro-test tube and mixed with 0.9ml of 95% ethanol which has been pre-cooled to 20^{o}C and further spun for 2 minutes to pellet plasmid DNA and RNA. The supernatant was also discarded, and the pellet rinsed twice with 1 ml of 70% EtOH and dried in vacuum. For the subsequent steps., the isolated plasmid DNA was re-suspended in 200µL of TE buffer, pH 8 and 200ng/µl RNAse was also added to aid dissolution of the pellet and the digestion of RNA.

3.13.2 Plasmid Separation and Profiling

Agarose gel. (0.8%) was prepared by weighing out 0.8 g of agarose and dissolving in 100ml of 1x /TBE (Tris-Boric /Acid-EDTA buffer) with the aid of heart. The hot gel solution was cooled until the glass conical flask could be touched without discomfort and then ethidium bromide (5 µl of 10 mg/ml stock) was added. The gel solution (100 ml) was poured into electrophoresis tray to which a comb was fixed to create holes on the solidified agarose gel. The combs was removed after gelling and, using the micropipette, the plasmid preparations (to which bromophenol blue/glycerol loading dye, 3µl was added) was loaded into the holes. A standard DNA ladder was also loaded in a hole between the sample plasmid preparations. The tray with the gel was buffered with TBA buffer and the plasmid preparations for the different bacterial isolates, added to the different holes, was set up to migrate towards the positive charged electrode. The gel was allowed to run for 3 h at 63 v. the gel containing the separated plasmids was removed and

visualized under UV light with the aid of UV goggle. A picture of the DNA was taken showing size and mobility on the agarose gel. The mobility (mm) and the size was determined relative to the standard DNA ladder loaded in between the sample plasmid preparation)

3.13.3 Plasmid Curing

The resistant isolates were cured with acridine orange to ascertain the involvement of plasmids in antibiotics resistance determination. A small inoculum of 100 to 300 cfu/ml was added to acridine orange nutrient broth (pH 7.6) in varying concentration of acridine orange up to 0.25 mg/ml and incubated at 37 °C for 24 hours. Cultures containing the highest concentration of acridine orange in which growth was clearly visible were diluted and spread on nutrient agar plates with appropriate antibiotics for susceptibility testing.

3.14 Data Analysis

The data generated were analyzed by one –way ANOVA (analysis of variance) using Genstat 12th edition analytical package as well as Non-Parametric T. test. Differences in mean were compared by Duncan's multiple range tests (Ogbeibu, 2015).

CHAPTER FOUR

4.0 RESULTS

Table 4.1 shows the qualitative phytochemical screening of the aqueous and ethanol extracts of *Murraya koenigii* leaves. The phytochemical screening showed that the aqueous extract of *Murraya koenigii* leaves contained saponins, coumarin, phenolics, flavonoids and terpenoids while the ethanol extract showed the presence of tannin, coumarin, phenolics, flavonoids and

terpenoids. Ethanol extract showed a wider range of phytochemicals than the aqueous extract. Quantitative phytochemicals identified as part of aqueous extracts were flavonoid (8.81 ± 0.09 mg/100g), tannins (20.28 ± 0.53 mg/100g) and phenolic (44.83 ± 1.18 mg/100g) while the ethanolic extract contained flavonoid (67.1 ± 0.49 mg/100g), tannins (55.5 ± 1.98 mg/100g) and phenolic (68.0 ± 1.40 mg/100g). Ethanol is a better extraction solvent for extracting phytochemicals from ground curry leaves than aqueous as shown in Table 4.2. this positive was observed for flavonoids, tannins and phenolic compounds in particular.

Table 4.3 shows the zone of inhibition of aqueous ethanol *Murraya koenigii* leaf extracts. The sensitivity of *Salmonella* spp to the aqueous extract ranged from 0.10 ± 0.00 - 1.37 ± 0.03 mm while ethanolic extract ranged 0.10 ± 0.00 - 1.67 ± 0.03 mm. The sensitivity of *Shigella* spp to the aqueous extract ranged from 0.21 ± 0.09 - 0.37 ± 0.03 mm while ethanolic extract ranged 0.10 ± 0.00 - 0.63 ± 0.03 mm. *Salmonella* spp and *Shigella* spp showed the highest susceptibility (1.67 ± 0.03 mm; 0.63 ± 0.03 mm) in the ethanolic extract at 100 mg/ml concentration compared to aqueous extract were highest susceptibility of 1.37 ± 0.03 mm and 0.37 ± 0.03 was observed at 100 mg/ml concentration respectively. The minimum inhibitory concentration of the aqueous extract ranged from 25 – 50 mg/ml while ethanolic extract ranged from 12.5 - 50mg/g. Minimum bactericidal concentration for aqueous and ethanol extracts were 100 mg/ml respectively. The result clearly indicates that the ethanol extract was more efficient in inhibiting and causing bacterial death at a lower concentration compared to the aqueous extract.

Figure 4.1 shows the GC/MS Chromatogram of *Murraya koenigi* ethanol leaf extracts, indicating the various compounds identified with their various peak area and retention time. Table 4.4 shows the compounds identified in ethanol extracts of *Murraya koenigi* leaves were Dodecanoic

acid (0.40%), Tridecanoic acid (0.69%), Decanoic acid (0.29%), Tetramethyl-2-hexadecon-1-01 (1.65%), Octadecanoic acid (0.45%), Hexadecanoic acid (1.04%), Phthalic acid (1.14%), n-Hexadecanoic acid (29.6%), Hexadecanol (3.35%), Vacconic acid (6.23%), Octacosane (2.78%), Squalene (2.52%), Tetratetracontane (3.18%) and Cholesterol (1.57%)

Table 4.5 shows the proximate composition of *Murraya koenigi* leaves. The results of the proximate analysis revealed the presence of Moisture (19.73 ± 0.30 %), Protein (8.69 ± 0.52 %), Ash (1.95 ± 0.00 %), Fibre (4.31 ± 0.29 %), Lipid (6.53 ± 0.50 %) and carbohydrate (43.48 ± 1.72%). Carbohydrate (43.48 ± 1.72 %) was found to be present in the largest amount while ash (1.95 ± 0.00 %) was present in the least amount.

Table 4.6 shows the antibiogram profile of bacterial isolates before curing. Bacterial isolates such as *Shigella* spp and *Salmonella* spp were resistant to septrin, ciprofloxacin, amoxicillin and perfloxacin and susceptible to sparfloxacin, augmentin and gentamycin with *Salmonella* spp having the highest multiple antibiotic resistance index of 0.5.

Table 4.1: Qualitative phytochemical screening of *Murraya koenigii* leaf Extract

Parameters	**Aqueous**	**Ethanol**
Saponin	+	-
Tannin	-	+
Alkaloids	-	-
Coumarin	+	+

Phenolics	+ +	+
Flavonoids	+	+ +
Terpenoids	+ +	+

Key:

- = not detected

+ = low

+ + = moderate

Table 4.2: Quantitative phytochemical screening of *Murraya koenigii* leaf extracts

Constituent	**Aqueous**		**Ethanol**		**P value**
	Absorbance	**Concentration (mg/g)**	**Absorbance**	**Concentration (mg/g)**	
Flavonoid	0.02±0.00	8.81±0.09[a]	0.96±0.01	67.1±0.49[b]	0.00
Tannins	0.32±0.01	20.28±0.53[a]	0.67±0.03	55.5±1.98[b]	0.03
Phenolic	0.62±0.02	44.83±1.18[a]	0.96±0.03	68.0±1.40[b]	0.00

Same letter in each row signify non significant difference (P>0.05) while different letter in each row signify significant difference (P<0.05)

Table 4.3: Zone of inhibition of aqueous ethanol *Murraya koenigii* leaf extracts

Extract concentration (mg/ml)	Solvent	*Salmonella* spp (mm)	*Shigella* spp (mm)
100	Aqueous	1.37±0.03	0.37±0.03
	Ethanol	1.67±0.03	0.63±0.03
50	Aqueous	0.42±0.03	0.21±0.09
	Ethanol	0.57±0.03	0.23±0.03
25	Aqueous	0.37±0.03	0.00±0.00
	Ethanol	0.40±0.00	0.10±0.00

12.5	Aqueous	0.10±0.00	0.00±0.00
	Ethanol	1.17±0.03	0.00±0.00
6.25	Aqueous	0.00±0.00	0.00±0.00
	Ethanol	0.10±0.00	0.00±0.00
Aqueous extract (mg/ml)	MIC	25 mg/ml	50 mg/ml
	MBC	100 mg/ml	100 mg/ml
Ethanol extract (mg/ml)	MIC	12.5 mg/ml	50 mg/ml
	MBC	100 mg/ml	100 mg/ml

Key: MIC= Minimum Inhibitory Concentration. MBC= Minimum Bactericidal Concentration. Inhibition zone diameters are expressed as Mean ± Standard Error of triplicate experiments.

Table 4.4: Phytocomponents identified in the different extracts of *Murraya koenigi* leaves by GC-MS Analysis

Peak	Name of the Compound	RT	Peak Area (%)
1	Dodecanoic acid (lauric acid)	11.4	0.40
2	Tridecanoic acid (tridecyl acid)	12.8	0.69
3	Decanoic acid (capric acid)	13.1	0.29

4	Tetramethyl-2-hexadecon-1-01	13.5	1.65
5	Octadecanoic acid (stearic acid)	13.6	0.45
6	Hexadecanoic acid (palmitic acid	13.6	1.04
7	Phthalic acid (benzene-1,2-dicarboxylic acid)	13.9	1.14
8	n-Hexadecanoic acid	14.1	29.6
9	Hexadecanol (cetyl alcohol or palmityl alcohol)	14.5	3.35
10	Vacconic acid (methylene glutaric acid)	15.5	6.23
11	Octacosane (n-octacosane)	19.5	2.78
12	Squalene (precursor acid)	21.2	2.52
13	Tetracontane (hydrocarbon)	21.9	3.18
14	Cholesterol (sterol)	23.8	1.57

KEY:

RT: Retention Time

Table 4.5: Proximate composition of *Murraya koenigi* leaves.

Parameter	**Concentration (%)**
Moisture	19.73±0.30
Protein	8.69±0.52

Ash	1.95±0.00
Fibre	4.31±0.29
Lipid	6.53±0.50
Carbohydrate	43.48±1.72

Values are expressed as Mean ± Standard Error of triplicate experiments.

Table 4.6: Antibiogram profile of *Salmonella* and *Shigella* species isolates before and after curing

	***Salmonella* spp**		***Shigella* spp**	
Antibiotic	**Before curing**	**After curing**	**Before curing**	**After curing**

S	19(S)	20(S)	11(I)	18(S)
SXT	10(R)	10(R)	9(R)	20(S)
CH	12 (I)	19(S)	18(S)	20(S)
SP	19(S)	19(S)	21(S)	19(S)s
CPX	09(R)	18(S)	10(R)	17(S)
AM	10(R)	20(S)	10(R)	18(S)
AU	18(S)	20(S)	19(S)	20(S)
CN	20(S)	20(S)	18(S)	19(S)
PEF	10(R)	19(S)	10(R)	15(I)
OFX	09(R)	19(S)	20(S)	20(S)
MARI	0.5	0.1	0.4	0.0

Key:

PEF: Pefloxacin (30ug), CN: Gentamycin(10ug), CPX: Ciprofloxacin (10ug), S: Streptomycin (30ug), SXT: Septrin (30ug), AM: Amoxicillin (30ug), AU: Augmentin (30ug), T: Tarivid (10ug), CH: Chloramphenicol (30ug), SP: Sparfloxacin (30ug)

R = Resistance, I = Intermediate, S = Susceptible.

Sensitivity standard for disc: 0-10mm resistant, 11-16mm intermediate, 17mm and above Susceptible.

MAR I: Multiple Antibiotic Resistant Index

Plasmid analyses before curing revealed that there were detectable plasmids in the bacterial isolates. *Shigella* sp. possessed double-sized plasmids while *Salmonella* sp had triple-sized plasmids (Plate 4.1). Plasmid analysis after curing revealed that *Salmonella* sp possessed single-sized plasmids (Plate 4.2). High antibiotic resistance was detected in isolates with high molecular weight plasmids.

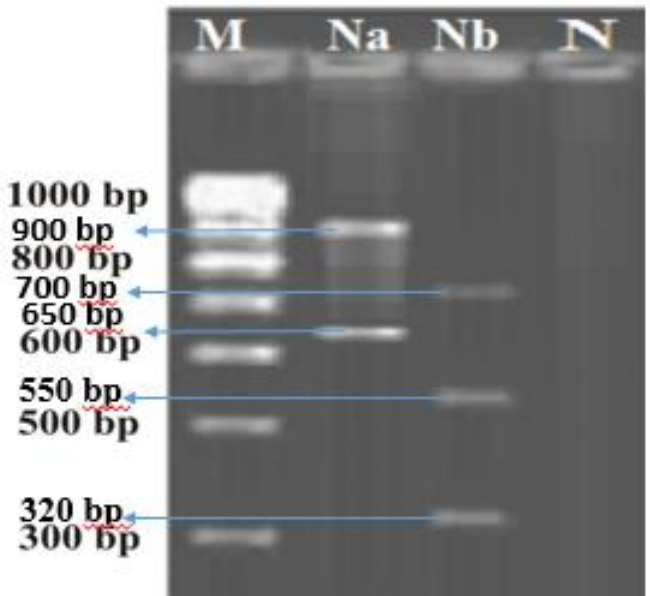
M
Na
Nb
N
1000 bp
900 bp
800 bp
700 bp
650 bp
600 bp
550 bp
500 bp
320 bp
300 bp

Plate 4.1: Gel electrophoresis of amplified plasmid gene before curing. Lane M-Molecular weight marker (1000 bp); Lane Na- *Shigella* sp; lane Nb- *Salmonella* sp; Lane N- negative control (Distilled sterilized water).

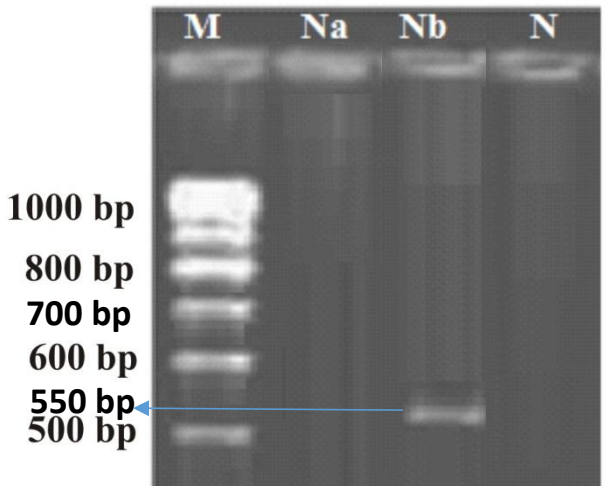

Plate 4.2: Gel electrophoresis of amplified plasmid gene after curing. Lane M-Molecular weight marker (1000 bp); Lane Na- *Shigella* sp; lane Nb- *Salmonella* sp; Lane N- negative control (Distilled sterilized water).

CHAPTER FIVE

DISCUSSION

Phytochemical analysis of *Murraya koenigii* aqueous and ethanol extracts revealed the presence of saponins, Tannin, Alkaloids, coumarin, phenolics, flavonoids and terpenoids (Table 4.1).

Usman *et al.* (2013) had earlier reported the presence of tannins, alkaloids, saponins, terpenes and flavonoids in *Murraya koenigii* leave methanolic and aqueous extracts.

The presence of tannins in the leaves of *Murraya koenigii* implies the extract can be pharmacologically useful as astringents. Singhal *et al*. (2010) reported that the astringent activity of tannins is by precipitating proteins, thereby protecting the underlying tissue leading to improvement of wound healing. Tannins inhibit microbial proliferation by denaturation of enzymes involved in microbial metabolism (Ncube *et al*., 2008). Tannins also have shown potential antiviral (Barrett, 2004), antibacterial (Akiyama *et al.,* 2001), antiparasitic and anticancer effects (Fumal *et al*., 2004; Talukdar *et al*., 2010).

Flavonoids present in *Murraya koenigii* leaf extract have been referred to as nature's biological response modifiers because of its efficiency in the modification of the body's response to allergies, viruses, and carcinogens. They display antiallergic, anti-inflammatory microbial and anti-cancer activity (Yamato and Ganor, 2002). The presence of flavonoids in the leaf of *Murraya koenigii* may also account for its usage as an antinflammatory agent (Ekwueme *et al.*, 2011). It also implies that the plant could be useful in preventing damages caused by free radicals in the body (Dweck and Mitchell, 2002) and also for the treatment of diarrhoea (Schuier *et al*., 2005).

Saponins have antibacterial activities (Birk and Petri, 2000) and have been applied in treating microbial infections. Alkaloids have a broad range of pharmacological properties which include antimalarial, antiasthma, anticancer properties as reported by Kittakoop, *et al* (2014). Glycosides have been useful in the treating of congestive heart failure, constipation, edema and microbial infections (Franstisk, 2001).

Phenols have also been reported to posses antibacterial properties (Adamu *et al*., 2017). Findings from this work showed that the quality and quantity of phytochemicals present in *Murraya koenigii* leaves differ for both extracts (Table 4.1 and 4.2). This may be due to the type and nature of extraction solvent used as suggested by Dai and Mumper (2010). The result as shown in table 4.1 and 4.2 therefore suggest that ethanol can extract a wider range and quantity of phytochemicals from *Murraya koenigii* leaves than water.

This study showed that *Murraya koenigii* extracts are effective inhibitors of microbial growth. The extracts showed varying levels of activity against the Gram negative bacteria (*Salmonella* spp and *Shigella* spp). Ethanolic extracts of *Murraya koenigii* showed strong antibacterial activity against the bacterial isolates at 100mg/ml and moderate activity against 25mg/ml (Table 4.3). It was observed that, increased concentration of the extracts, led to increased antibacterial activities. This is in agreement with the work of Abiy (2016) who reported that ethanolic and aqueous extracts of *Murraya koenigii* had antibacterial activity against *Staphylococcus aureus*, *Salmonella* spp., *Escherichia coli* and *Shigella* spp. Another observation showed that the ethanolic extract of *Murraya koenigii* had the broadest spectrum of activity on the test organisms compared to the aqueous leaf extract. This may be due to the ability of ethanol to extract a wider range of antibacterial properties than the aqueous solvent. The presence of ethanol in addition to achieving better extraction may also enhance the efficacy of the active ingredients (Ali *et al*., 2018).

The bacterial isolates were resistant to the lowest concentration of the ethanol and aqueous extracts (Table 4.3). This is in accordance with the results from previous studies by Packia *et al*. (2015) who stated that low concentrations of plant extracts lead to low antibacterial potency. The zones of inhibition were greater in the plates inoculated with *Salmonella* spp than *Shigella* spp

indicating that *Salmonella* spp is more sensitive to the extracts than *Shigella* spp. This difference in sensitivity may also be attributed to the fact that Gram negative organisms possess an outer membrane in their cell it, this outer membrane may be responsible for the difference in the degree of sensitivity of these organisms to the ethanolic and aqueous extracts of *Murraya koenigii*. The outer membrane prevents a substantial amount of the extract having contact with the cell wall. Also, the activity of the extracts against *Salmonella* spp justifies the use of *Murraya koenigii* in the treatment of typhoid and is in agreement with the work of Shobana *et al*. (2009) who stated that *Salmonella* spp were susceptible to *Murraya koenigii* ethanolic and aqueous leave extracts. The results of the overall sensitivity patterns show that there is a mild difference in the sensitivity pattern of this study and earlier reports (Benkeblia *et al*., 2004; Karuppiah and Rajaram, 2012) who stated that bacterial were susceptible to plant extracts due to the presence of phytochemicals such as tannins, flavoinoids, alkaloids, steroids etc.

The broad spectrum of activity of the extracts on the bacterial isolates coupled with its low minimum inhibitory concentration (MIC) on the bacteria tested makes it a good source as antibacterial agents. Minimum Bactericidal Concentration (MBC) values obtained for the extracts against the clinical isolates were higher than MIC, indicating that the extracts are bacteriostatic at lower concentrations and bactericidal at higher concentrations (Table 4.3). This suggests that the plant extracts, when used traditionally as antibacterial agent, inhibit bacteria growth without necessarily killing the bacteria and since most of the traditional preparations lack specific concentrations, this may thus account for the use of large quantity of the extracts by traditional medical practitioners for the treatment of their patients (Onyeagba *et al*., 2004).

The antibacterial potential of *Murraya koenigii* leaves was tested against some clinical isolates. The tested bacteria isolates were much more susceptible to conventional antibiotics than

Murraya koenigii extracts. This may be due to the fact that the active ingredients in commercial antibiotics are in refined and purified forms, whereas the active ingredient in the plant extract is in a crude, impure, unrefined form and in a mixture with other unknown compounds (Altuner *et al*., 2012). The growth observed in the minimum bactericidal test is an indication that the active ingredient is bacteriostatic rather than being bactericidal. This is also attributable to the impure unrefined state of the active ingredient in the plant extract or that the active ingredient in the extract is not quantified and concentrated to the level that will make it bactericidal. It is therefore my suggestion that further studies on extracts of *Murraya koenigii* should involve refining or purifying and concentrating the active ingredient to determine its real effect on pathogenic microorganisms. The results of this study are in conformity with the results obtained by earlier researchers (Shruti *et al.,* 2018; Muhammad and Idris, 2019) who reported bioactivities of *Murraya koenigii* extracts against microbial isolates due to the presence of biochemical compounds such as alkaloids, cynogenic glycoside, flavonoids etc.

Of the compounds detected by GC- MS in the medicinal *Murraya koenigi* leaves extracts, several compounds have been reported to have medicinally important bioactivities and also used for the production of various bioactive products.

Dodecanoic acid (lauric acid) which was obtained from the ethanolic extract (Table 4.4) increases total serum cholesterol more than many other fatty acids, but mostly high-density lipoprotein known as the good blood cholesterol (Mensink *et al*., 2003). In general, a lower total/HDL serum cholesterol ratio has been shown to correlate with a decrease in atherosclerotic risk (Thijssen and Mensink. 2005).

Hexadecanoic acid was reported to have antioxidant, antiallergic (Santos *et al.*, 2013) antinociceptive and anti-inflammatory activities (Ryu *et al.,* 2011). Recent studies have shown

that hexadecanoic acid is an excellent immunostimulant. It is superior to a good amount of commercial adjuvants in terms of long-term memory induction and activation of both innate and acquired immunity (Lim *et al.,* 2006) Hexadecanoic acid has also been found to have antimicrobial activity against *Mycobacterium tuberculosis* and *Staphylococcus aureus* (Saikia *et al.,* 2010). Similarly Mary (2011) revealed the presence of hexadecanoic acid in the leaves of *Lantana camara* and Hexadecanoic acid was observed to have antibacterial activities against *Staphylococcous aureus* by causing damage to cell membranes as a result there is a leakage of potassium ions from bacterial cells.

The Hexadecanol have antibacterial and antifungal properties and inhibits melanin production.

Other compounds such as 5-nonanol a fatty alcohol is used as Pheromones in the form of 4-Methyl-5-nonanol (Golemanov *et al.*, 2006).

Decanoic acid a fatty acid is used as Food additive (Usually flavouring agent) while

Octacosane has been reported by Carla *et al*. (2015) to exhibit mosquitocidal and antimicrobial activity against *Proteus mirabilis* and *Bacillus subtilis*

Squalene has been reported to inhibit the growth of fungal such as *Aspergillus niger* and *Pennicilium notatum* 2-octenoic acid a fatty acid is used as Surfactant and Emulsifier in the industry and also as flavouring agent.

Cholesterol is present food and drugs. Tetratetracontane is also antifungal against dermatophytes; anti-tumor, analgesic, antibacterial, anti-inflammatory; anticoagulant properties; reduces liver damage; effective in killing cancer cells and treating rheumatoid arthritis ( ).

The presence of these compounds may have contributed to the bioactivities of *Murraya koenigi* leaves.

Proximate analysis of food is the detection of various food components which include moisture, protein, fat, ash, crude fibre and total carbohydrate (Alfred and Patrick, 2005). Proximate analysis is a technique of assaying also know as "conventional analysis" that is used to detect the nutritional quality of a substance which is the gross components (protein, fat, carbohydrate, ash etc.) of the food substance rather than individual nutrients (amino acid, fatty acids, monosaccharides, mineral, etc.) are detected (Prohp *et al*., 2006).

Nutrients are needed for living and to maintain the body's health; these nutrients can be obtained from different food types. Basically, nutritional functions include fuel (energy) source for various biological activities and functions, building materials for body structures and regulation and control of body processes. The proximate analysis showed that *Murraya koenigi* contain Moisture 8.69±0.52 %, Protein 19.73±0.30%, Ash 1.95±0.00 %, Fibre 4.31±0.29 %, Lipid 6.53±0.50 % and carbohydrate 43.48±1.72% (Table 4.5). The presence of nutrients such as these could serve as source of energy and nutrients for the body metabolic activities in addition to the medicinal potentials of *Murraya koenigi* leaves.

The carbohydrates and proteins in the plant may be a conglomerate of biologically active sugars, glycoproteins or proteins that provides the plant its medicinal properties against certain diseases. Some plants have been found to posses certain sugars which are bioactive against certain diseases (Sanni, 2007).

Plant proteins are sources of food nutrient most importantly for the under privileged population in developing nations such as Nigeria. Proteins are one of the macromolecule and it is a reserved source of energy when other energy sources are not readily available ( ). They are building block components and useful in making important hormones, important brain chemicals, antibodies, digestive enzymes, and important elements for the production of DNA. Some proteins are

implicated in structural support; others are concerned with bodily movement, or in defense against germs (Bailey, 2008).

Fibre clears the digestive tract by clearing potential carcinogens from the body and the prevention of absorpting excess cholesterol. Fibre also contributes bulk in diet and prevents the intake of too much starchy food (Mensah *et al*., 2008) and thus guard against metabolic situations which include hypercholesterolemia and diabetes mellitus (Henry, 2004). Dietary fiber has a useful effect in the control of diabetes by controlling post-prandial hyperglycemia. It slows down gastric emptying or adds to the viscosity of gastro-intestinal tract content thereby retarding the digestion of carbohydrate and delays its absorption. The presence of fibre in *Murraya koenigii* leaves indicates they are useful in maintaining the function and health of the digestive system. Fibre helps and increases the excretion of waste and toxins from the body, therefore not allowing them to accumulate in the intestine or bowel for a long time, which can result in the build-up and leading to certain diseases (Hunt *et al.*, 1980). Sufficient intake of dietary fibre can bring down the serum cholesterol level, risk of coronary heart disease, hypertension, constipation, diabetes, colon and breast cancer (Rao and Newmark, 1998; Ishida *et al*., 2000).

Ash content is an indication of the mineral composition present in the leaves of plants. The ash content of *Murraya koenigii* leaves was lower compared to previously studied plant leaves such as *Vernonia colorate* (15.86%) and *Moringa oleifera* (15.09%) (Lockeett *et al*., 2000; Antia *et al*., 2006). This result therefore implies that this nutrient can be supplemented for by other sources.

Murraya koenigii had carbohydrate value of 43.48±1.72%. Thus the carbohydrate content which is the highest proportion of nutrient present in *Murraya koenigii* leaves increases the energy level

of *Murraya koenigii*. Carbohydrates are important in maintaining life of both plants and animals and also provide raw materials for different industrial processes (Ebun-Oluwa and Alade, 2007). Carbohydrates produced by plants form one of the three main energy sources in food, along with protein and fat. When animals consume plants, energy stored as carbohydrates is released by the process of respiration; the glucose produced which is the end product of carbohydrate can also be used by animal cells in the manufacturing of several substances necessary for growth (Westman, 2002).

Crude lipid present in *Murraya koenigii* leaves; also add to the energy level of *Murraya koenigii* (Table 4.5). Dietary fat increases the palatability of food by absorbing and sustaining flavours (Antia *et al*., 2006). The adequate amount of fat implies that *Murraya koenigii* leaves is not a source of lipid accumulation which can cause arteriosclerosis, aging (Antia *et al*., 2006). It is low compared to previously studied plants such as *Talinum triangulare* (5.90%), *Baseila alba* (8.71%) and *Acalypha racemosa* (6.30%) (Ifon and Bassir, 1980; Akindahunsi and Salawu, 2005).

The moisture content (5.73 ± 0.52%) of *Murraya koenigii* leaves was lower than the moisture content (11.01 ± 0.82%) of *Murraya koenigii* leaves conducted in earlier studies which was reported to be low moisture content, considered to, hinder the growth of spoilage microorganisms and improve shelf life (Abere *et al*., 2007).

The antibiotic sensitivity test in this study revealed that *Shigella* spp and *Salmonella* spp were susceptible to sparfloxacin, augmentin and gentamycin (Table 4.6). This is similar to the studies of Uwimbabazi *et al*. (2015) who reported that *Pseudomonas aureginosa, Shigella* spp *Staphylococcus aureus, Escherichia coli* and *Salmonella* spp were susceptible to septrin, sparfloxacin, augmentin amoxicillin and gentamycin. The resistance of *Shigella* spp and

Salmonella spp to septrin and ciprofloxacin in this work however is in agreement with the study of Liu *et al*. (2017).

The resistance of the bacterial isolates to few antibiotics could probably be due to chromosomally or plasmid mediated resistant genes in the bacteria genetic make-up. This could be associated with indiscriminate usage of antibiotics. This result is in agreement with the work of Raja *et al*. (2018); Hala *et al*. (2015) who reported that *Escherichia coli, Shigella* spp., *Enterococcus faecalis* and *Salmonella* spp were resistant to streptomycin, septrin, ciprofloxacin gentamycin and ceftazidime.

In this study, both *Shigella* spp and *Salmonella* spp showed multiple antibiotic resistance to the tested antibiotics, especially *Salmonella* spp which had the highest multiple antibiotic resistance index (0.5) (Table 4.6). This agrees with the studies of Hafsat *et al*. (2015) who reported *Salmonella* spp and *Staphylococcus aureus* to be multidrug resistant to sparfloxacin, pefloxacin, augmetin, gentamycin, and zinnacef. In addition, these results provide evidence that there is an increased emergence of antibiotic resistance, a finding which is in agreement with the reports of Ndip *et al*. (2015) who found increasing emergence of antibiotic resistance in bacterial isolates wound infected patients.

The plasmid profiles of the tested clinical bacterial isolates is presented in plate 1. Plasmid profiles have been reported to be useful in tracing the epidemiology of antibiotic resistance (Opera and Ojo, 2013). In this study, plasmid profiles were detected which indicates that plasmid profiling can also be used as an epidemiological tool for typing *Salmonella* sp. and *Shigella* spp. as described by Opera and Ojo (2013). Samie *et al*. (2011) stated that generally epidemiologically unrelated isolates contain different plasmid profiles whereas related isolates could also display variation in plasmid profiles. The more plasmids exist in an organism, the

more specific is the plasmid profile as a marker for a single isolate. Bacterial antibiotics resistance patterns are sometimes associated with the presence of large plasmids and ability of plasmids for conjugation process (Onuoha, 2018). However, for other isolates that had no plasmid, they also showed the multiple antibiotics resistance patterns with high number of antibiotics which indicates that resistance to most of these antibiotics is of chromosomal origin or on mobile genetic elements that may help in the dissemination of the resistant genes to other bacteria of human clinical significance (Mbim *et al*., 2016). According to Adetunji *et al.* (2014) and Ishola *et al.* (2016), the antibiotic resistance in *Salmonella* spp that seem not to possess plasmids maybe associated with chromosome and/or transposons instead of being plasmid-mediated.

5.1 Contribution to Knowledge

This work has further shown that *Murraya koenigii* leaves possesses phytochemicals and other numerous biochemical compounds that have antibacterial properties, and would potentially add to healthy living of those that consume it.

5.2 Conclusion and Recommendation

The results of this study showed that *Murraya koenigii* leaf extracts has antimicrobial effects. The presence of bioactive chemicals in the extracts may have been responsible for this antimicrobial effects and the ethnopharmacological usage in traditional medicine. This study also provides data on the solvent concentration and indicates that ethanolic extract of *Murraya koenigi* leaves can offer significant potential for the development of antibacterial therapies as compared to aqueous extract. The antibacterial properties of *Murraya koenigii* leaves can therefore be enhanced when further purified.

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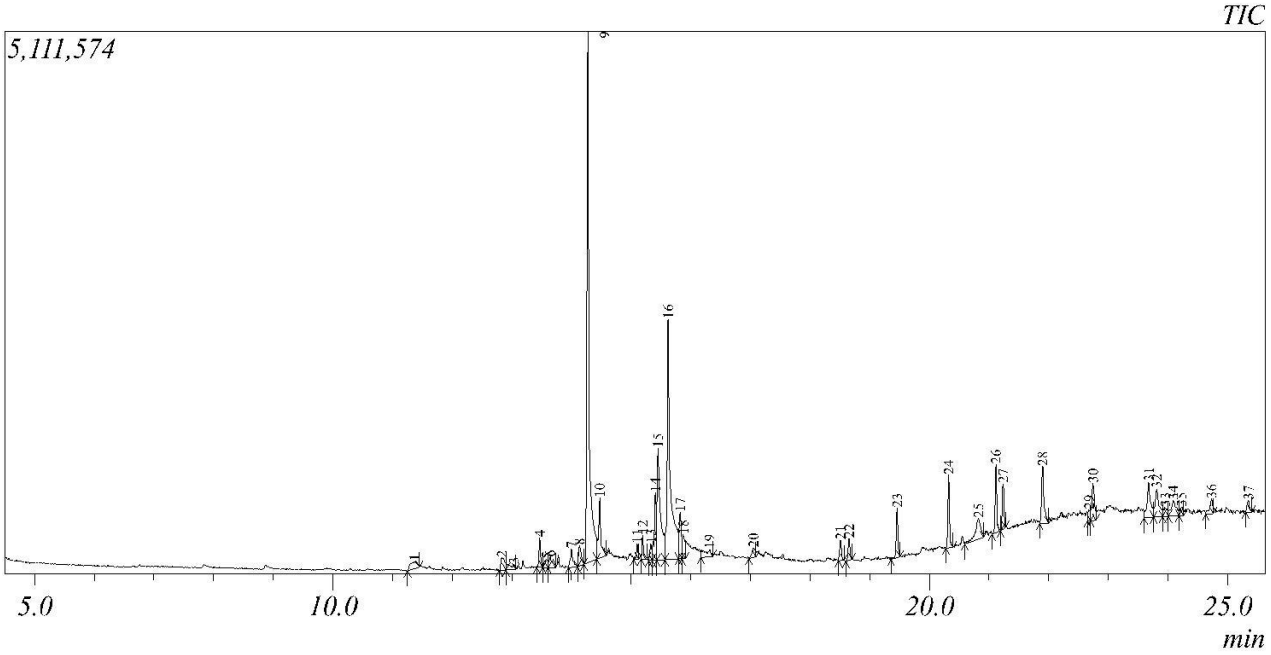

APPENDIX I

Figure 1: Gas Chromatography-Mass Spetrometry Chromatogram of *Murraya koenigi* ethanolic Leaf Extract

APPENDIX II

CULTURE MEDIA

Nutrient agar

Beef extract	3.0g
Agar No.2	12.0g
Peptone	5.0g
Sodium chloride	8.0g
Distilled water	1000ml

Mueller Hinton Agar

Beef Extract ..... 2 g

Acid Hydrolysate of Casein ..... 17.5 g

Starch ..... 1.5 g

Agar ..... 17 g

Final pH 7.3 ± 0.1 at 25°C

APPENDIX III

ANOVA TABLES

Zone of inhibition of aqueous and ethanolic leaf extract of *Murraya koenigii*

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean	
						Lower Bound	Upper Bound
Ethanolic	1.00	3	.6333	.05774	.03333	.4899	.7768
	2.00	3	.2067	.16166	.09333	-.1949	.6082
	3.00	3	.0000	.00000	.00000	.0000	.0000
	4.00	3	.0000	.00000	.00000	.0000	.0000
	5.00	3	.0000	.00000	.00000	.0000	.0000
	Total	15	.1680	.26282	.06786	.0225	.3135
Aqueous	1.00	3	.3667	.05774	.03333	.2232	.5101
	2.00	3	.2333	.05774	.03333	.0899	.3768

3.00	3	.1000	.00000	.00000	.1000	.1000
4.00	3	.0000	.00000	.00000	.0000	.0000
5.00	3	.0000	.00000	.00000	.0000	.0000
Total	15	.1400	.15024	.03879	.0568	.2232

MIC and MBC of aqueous and ethanolic leaf extract of *Murraya koenigii*

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean	
						Lower Bound	Upper Bound
Salmonella	Aqueous	3	9.167	3.8188	2.2048	-.320	18.653
	Ethanol	3	15.000	13.2288	7.6376	-17.862	47.862
	Total	6	12.083	9.2759	3.7869	2.349	21.818
Shigella	Aqueous	3	45.000	5.0000	2.8868	32.579	57.421
	Ethanol	3	90.000	10.0000	5.7735	65.159	114.841
	Total	6	67.500	25.6418	10.4682	40.591	94.409