IN-VITRO ANTIOXIDANT AND ANTI-ARTHRITIC ACTIVITIES OF THE AQUEOUS AND ETHANOL LEAF AND ROOT-BARK EXTRACT OF *ALAFIA BARTERI* PLANT

BY

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A PROJECT SUBMITTED TO THE BIOLOGICAL SCIENCES DEPARTMENT, COLLEGE OF BASIC AND APPLIED SCIENCES, MOUNTAIN TOP UNIVERSITY, IBAFO, OGUN STATE
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CERTIFICATION

This is to certify that this project titled **IN-VITRO ANTIOXIDANT AND ANTI-ARTHRITIC ACTIVITIES OF THE AQUEOUS AND ETHANOL LEAF AND ROOT-BARK EXTRACT OF *ALAFIA BARTERI* PLANT** was compiled and written by AWOYERA AYOMIDE ESTHER with matriculation number of 16010102005 and submitted to the Department of Biological Sciences, College of Basic and Applied Sciences, Mountain Top University, Ogun State, under the supervision of Mrs. I.O Kolawole, having met the standard as required by the Institution and approved as to contents and style by:

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DEDICATION

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ABSTRACT

A medicinal plant is any plant that produces substances in one or more of its parts which may be used for therapeutic purposes or that are precursors to the synthesis of useful drugs.

Alafia barteri (Apocyanaceae) is a medicinal plant used for the treatment of fever, malaria and inflammation related disorders. The aim of this research was to investigate the in-vitro antioxidant and anti-arthritic activities of the aqueous and ethanol leaf and root bark extract of *Alafia barteri* plant. In-vitro antioxidant methods used were DPPH scavenging assay, reducing power activity and hydrogen peroxide scavenging assay while the anti-arthritic activity was evaluated using the inhibition of protein denaturation method. Results revealed that the aqueous and ethanol roots extracts had higher DPPH scavenging and reducing power activity while the ethanol leaf and root extracts had good anti-arthritic activities.

Key words: *Alafia barteri*, Medicinal plant, Anti-arthritic, Anti-oxidant

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CHAPTER ONE

1.0. INTRODUCTION

1.1. Medicinal plants and Arthritis

A medicinal plant is any plant which, in one or more of its components, contains substances that can be used for medical purposes or are precursors to the production of useful drugs. This description makes it possible to differentiate between therapeutic effects of medicinal plants and constituents have been scientifically identified and plants which have not been yet extensively scientifically studied but are considered medicinal.

In most developed countries, the use of herbal medicine and medicinal plants as a standard basis for the preservation of good health has been commonly observed. In addition, growing dependence in developed societies on the use of medicinal herbs has also been traced to the extraction and production of many drugs and chemotherapeutics from these plants, as well as from historically used rural herbal remedies. Herbal remedies have also become very common in the cure of different diseases in the modern world, thus raising their risk of extinction or loss of genetic diversity. (UNESCO, 1998)

In the developed world, infectious diseases are significant causes of mortality and morbidity and account for about 50 percent of all deaths. Today, the human population is affected by numerous diseases such as cardiovascular diseases, neurological diseases, metabolic diseases, etc. Among metabolic diseases, one of the oldest is arthritis, which mostly affects the joints.

Arthritis is a progressive inflammatory condition that affects the joints primarily. (Subramoniam *et al*, 2013).Inflammation is a process that helps protect the body's white blood cells and immune proteins from infection and foreign substances such as bacteria and viruses. However, in certain infections, where there are no foreign substances to tackle, the body's protective system (immune system) causes an inflammatory response, causing damage to its own tissues. These diseases are known as autoimmune diseases.

There are different forms of arthritis with different causes and approaches to therapy, but osteoarthritis and rheumatoid arthritis are the two most common types. Rheumatoid arthritis is the most common type of chronic inflammatory arthritis. The RA-associated inflammatory process expresses itself mainly in the synovial tissue and often in other parts of the body.

Rheumatoid arthritis is an autoimmune disease that causes inflammatory joint signs at certain stages of the body. Rheumatoid arthritis is a complex condition with a number of symptoms and complications for each patient. Rheumatoid arthritis symptoms are often confused with osteoarthritis symptoms. This misunderstanding typically exists in the first symptoms of arthritis. Despite the fact that all forms of arthritis cause joint discomfort, separate diagnoses are made between the two disorders.Interestingly, as all are chronic and non-curable, there are distinct disorders with different causes, signs, prognosis, and treatments. Symptoms of arthritis typically develop over time, but can also occur abruptly. Arthritis is most often seen in people over 65 years of age, but can also occur in teenagers, adolescents and younger adults.Arthritis is much more severe in women than in men and in those who are overweight. Osteoarthritis usually occurs later in life. (Samir Kumar 2018).

Biological molecules, such as reactive oxygen species (ROS) and reactive nitrogen species (RNS) are prone to oxidative damage by free radicals, which in turn contributes to different disease conditions, including arthritis.

In the management of RA, four main treatment interventions are recognized, including medication, physical activity, joint safety and improvements in lifestyle, and surgical intervention. Exercise therapy remains an essential part of care among all of these, (NiemanDC 2000).

It has been found that the common conventional modern drugs (NSAIDS) only minimize the symptoms, but with multiple side effects such as stomach irritation, kidney failure, liver disorders, ulcers, and haematological abnormalities.

Many studies have confirmed that one of the causes of rheumatoid arthritis is protein denaturation.In certain rheumatic diseases, the development of auto antigens could be attributable to in vivo denaturation of proteins whose denaturation process is likely to include electrostatic, hydrogen, hydrophobic and disulphide bonding alterations.

1.2. Aim of the study

The aim of this research is to investigate the antioxidant and anti-arthritic activity of the aqueous and ethanol leaf and root extract of *Alafia barteri* plant in-vitro.

1.3. Objectives of Study

- To evaluate the antioxidant activity of aqueous and ethanol leaf and root extract of *Alafia barteri*plant using 3 in-vitro models
- To evaluate the anti-arthritic activity of aqueous and ethanol leaf and root extract of *Alafia barteri*plant using the inhibition of protein denaturation method

CHAPTER TWO

2.1. Oxidative stress

Oxidative stress refers to the imbalance in the body between free radicals and the antioxidant enzymes in their stabilizing agents.Normal cellular metabolism can generate reactive oxygen species or free radicals and react with biomolecules such as proteins, lipids, and DNA to cause cellular damage and degenerative changes.In physiological regulation and cellular signalling pathways, free radicals play a critical role at low concentrations, while their presence in large amounts may cause harm to the cell.In contrast, antioxidants lower the level of oxidants by donating its own electron to stabilize free radical, thereby forming unreactive compounds so as to minimize the harmful effects generated by these radicals in the cell.

Oxidative stress plays an important part in the progression of age-related diseases including arthritis, diabetes, dementia, cancer, atherosclerosis, vascular diseases, obesity, osteoporosis, and metabolic syndromes (Tan *et al*., 2015a; Liu *et al*., 2017). The production and elevation of reactive oxygen species has been associated with the worsening of these diseases via oxidative damage. (Dias *et al.*, 2013).Inside the biological system, their generation leads to the modulation of cellular activities such as inflammation.High ROS concentrations can cause oxidative stress by disrupting the balance of antioxidant and pro-oxidant levels due to reactivity. (Zuo*et al*, 2015).Emerging research evidence has shown that natural compounds can decrease oxidative stress and increase immune function.Indeed, oxidation damage is strongly dependent on genetic or acquired mutations in the enzymes involved in redox-mediated signalling pathways.The function of antioxidant-active medicinal plants to counteract oxidative stress is therefore worthy of study.

2.2. Medicinal plants as antioxidants

Antioxidants derived from plants are natural products with radical scavenging capacity or reducing properties which attract lots of attention due to their powerful preventive and therapeutic properties. Plants, especially dietary fruits and vegetables, are a rich source of antioxidants and combating oxidative stress occurs via several different mechanisms in the body.

The donation of protons to these plant-based antioxidants has been documented, thus reducing reactive oxygen species and preventing oxidative stress in human health. Previous studies have shown that in the general pathophysiology of diseases such as Alzheimer's

disease and Parkinson's disease and even arthritis, oxidative stress plays central role.These natural antioxidants prevent oxidation of proteins, lipid peroxidation and prevent generation of ROS, thus blocking oxidative stress. Antioxidants derived from plants are broadly distributed in foods and more specifically, medicinal plants with mainly vegetables, herbs, spices and mushrooms as major sources.

Flavonoids and provitamins such as vitamin A, exert various biological effects on the biological system. These biological effects are anti-inflammatory, anti-aging, anti-atherosclerosis, anticancer and anti-arthritic. Effective extraction and eventual bioactive isolation, along with proper evaluation of food antioxidants and medicinal plants, are critical for exploring the capacity of sources of antioxidants and for advancing their usage in functional foods, pharmaceuticals, and food additives.

Exogenous antioxidants have the potential to prevent the damage induced through oxidative stress by preventing the activation of oxidative reactions, acting as scavengers, quenchers of singlet oxygen and reducing agents (Baiano and Del, 2016). Antioxidants essentially slow down the oxidation of biomolecules even at a small concentration. The major sources of these antioxidants from plant and foods are mainly vegetables, herbs, spices and mushrooms (Fu *et al*, 2011). In addition, the industries processing agricultural by-products are equally good sources of natural antioxidants (Deng, 2012). Polyphenols (phenolic acids, flavonoids, anthocyanins, lignans and stilbenes), carotenoids (xanthophylls and carotenes) and vitamins (vitamins E and C) are primarily natural antioxidants extracted from plant materials. (Baiano, 2016)

Alam *et al* (2013) stated that There are about 19 in vitro and 10 in vivo approaches used to test the antioxidant function of plant samples.In many in vitro assays, plant extracts have demonstrated potent antioxidant activity. The innate capacity of the plant to synthesize non-enzymatic antioxidants such as ascorbic acid and glutathione, as well as secondary metabolites such as phenolic compounds, can be due to this.

Free radicals are essential mediators which trigger or sustain inflammatory processes and may also neutralize antioxidants and radical scavengers to attenuate inflammation.

Research has shown that antioxidants work by directly reducing ROS levels, which can induce biomolecule degradation if unaltered, leading in turn to cell oxidative damage and overexpression of oncogenes, development of mutagens, activation of atherogenic behavior,

or inflammation (Szymanska*et al*, 2016). Diseases such as cancer, arthritis, diabetes, disorders in the immune system are majorly initiated by several conditions such as oxidative stress.

2.3. Arthritis

Arthritis is a general term, derived from the Greek words **arthro-,** meaning "joint," and **-itis**, meaning "inflammation."

Arthritis is an inflammatory condition that affects one or more of the body's joints with a number of causative factors, including trauma, inflammation, autoimmune disorders, idiopathic causes, and aging. Depending on the source, the underlying pathophysiology includes the breakdown of cartilage, which protects the end surfaces of the bones in the joints, contributing to a loss of smooth joint glide during movement. This frictional rubbing results in joint discomfort, swelling and stiffness, and subsequent muscle strain due to difficulty moving the joint. (Arend WP 1997).

More than 100 common illnesses are arthritis and other rheumatic diseases affecting the joints and surrounding connective tissues (muscles, tendons and ligaments).These conditions are generally characterized by pain, aching, and stiffness in and around a joint. Inflammatory or systemic types of arthritis and other rheumatic conditions (e.g., rheumatoid arthritis, systemic lupus erythematosus) can affect multiple organ systems (e.g., cardiovascular, renal, respiratory) and often have an autoimmune component. Osteoarthritis and rheumatoid arthritis are the two predominant forms of arthritis. (Nevitt MC. 2001).

2.4. Rheumatoid arthritis (RA)

Rheumatoid arthritis (RA) is a chronic progressive inflammatory rheumatic condition that affects articular and extra-articular structures, resulting in pain, impairment and mortality. (Birch et al 2010). It is one of the most prevalent inflammatory disorders regulated by immunity in the society which is characterised by a series of pathological processes like severe inflammation of joints and progressive destruction of cartilage and sub-chondral bone (Yeom*et al* 2006).

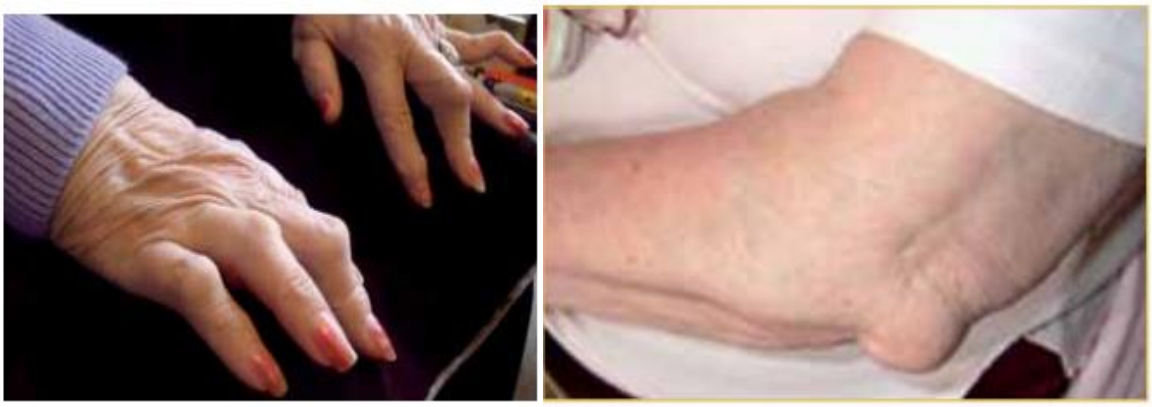

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Figure 1: **Rheumatoid nodules on the hands** Figure 2: **Rheumatoid nodule on the elbow**

Rheumatoid arthritis appears to symmetricallyaffect the hips, knees, elbows, ankles, spine, hands, and feet. Recovery cycles, accompanied by incremental exacerbations, in which individual joints become warm, swollen, and painful, define the progression of the disease.A signature characteristic of rheumatoid arthritis is morning stiffness, usually lasting about two hours. After extended periods of inactivity, patients with rheumatoid arthritis appear to complain about joint pain.Around 1% of the people has RA in the UK, making it more widespread than type 1 diabetes.It is possibly the most widespread autoimmune disease (Moots,2004). RA accounted for 12.3 percent of a total of 1,623 patients who reported rheumatologic symptoms to the clinic during the study time, according to Adelowo et al, 2010.Females, with an average age of 46.9 years, were mainly affected.

2.5. Other types of arthritis

- Gout: a type of arthritis caused by the accumulation within the joints of urate crystals. High levels of uric acid in the blood raise the chance of gout.

- Infectious arthritis: It is an inflammation that is caused in one of the joints by bacteria, viruses, parasites, or fungi which may lead to discomfort or swelling.Fever and chills sometimes follow this type of arthritis.

- Systemic lupus erythematosus (SLE): Another inflammatory disease that may affect the body's joints and other connective tissues.

- Juvenile arthritis (JA): Juvenile idiopathic arthritis (JIA), formerly referred to as juvenile rheumatoid arthritis, is the most common form. This is a category of autoimmune disorders that can affect children's joints.

2.6. Causes of Rheumatoid arthritis (RA)

RA is one of several chronic progressive diseases. It affects women three times more than men, (Silman et al, 2010). Despite much research, the trigger(s) that initiate the destructive process is still unclear, however, genetic predisposition and lifestyle have been considered to be underlying factors that can increase its risk. However, there is evidence of RA in Pima Indian (Native American) skeletons dating back to 4500 BC (Moots, 2004). However, it is possible that RA develops due to an interaction of factors, which are outlined below.

- **Immune response:** Rheumatoid Arthritis may be caused by an immune system dysfunction, but the reason behind this is unknown. In patients with RA, two elements of the immune system (the 'T' cells and the 'B' cells) become over-active for reasons yet to be understood. These immune cells play a part in RA-related inflammation and are in a family of immune cells called lymphocytes.(American Accreditation HealthCare Commission, 2010).

- **Genetic predisposition:** Over two thirds of RA sufferers have the gene HLA-DR4 indicating a potential genetic component to the disease. Thus, the absence of this gene implies an inability to be affected with the disease**.**

- **Hormones:** The occurrences of Rheumatoid Arthritis in women especially have been due to the decrease in activity of the hormones: oestrogen and progesterone. Women with RA often experience significant symptom improvements during pregnancy but these flare up again after childbirth. Also, low level of the cortisol hormone poses a risk of RA due to its anti-inflammatory property.

- **Age:** Rheumatoid arthritis can occur at any age, but for people between 30 and 50 years of age, it is more common. There is also childhood RA and it is called juvenile arthritis.

- **Viruses:** Bacterial or viral infections have been found to be a trigger for this disease. An example of the bacteria which may be involved is *Proteus mirabilis.* All these

contribution to the malfunctioning of the immune system, thereby posing a risk of arthritis

- **Smoking:** Long-term smokers appear to be at risk of developing a more severe form of RA with lumps of chronic inflammatory cells and fibrotic tissue that form under the skin (rheumatoid nodules) and a higher disability score. Healthy people who smoke have a higher presence of rheumatoid factor (Moots, 2004), which are antibodies produced during an inflammatory process.

- **Patient environments:** The incidence of certain autoimmune disorder, such as RA, increases in certain countries, indicating that lifestyle or diet may trigger RA. For example, in Scandinavian countries, the incidence of diabetes and arthritis is higher, perhaps due to insufficient vitamin D.

2.7. Treatment of Rheumatoid Arthritis

Without proper care, joints will continue to break down and become damaged, eventually leading to further loss of joint movement and disability Presently, no permanent cure for rheumatoid arthritis have been found, however, management options include the use of analgesics majorly to reduce pain and inflammation, although with the existence of side effects. Aspiration of fluid and injection of hydrocortisone (steroids) into a joint may also help but sepsis may occur as a risk. Treatment is multifaceted, and includes regular exercise, physical therapy, and dietary changes.

Patients should be trained by physiotherapists and occupational therapists to strengthen their joints while retaining muscle bulk. Splints may be provided to maintain functional positions and assist with tasks. Complementary therapy such as aromatherapy, hydrotherapy and acupuncture may be beneficial. In addition, surgery may be considered if joints are painful, damaged or deformed. Arthroscopy (examination and treatment of the interior of a joint) may help to diagnose RA, and remove bone and cartilage fragments that cause pain and inflammation. An osteotomy (removal of bone may be needed if joints are damaged and deformed. When conservative measures fail, a synovectomy removes a diseased joint lining, and joint replacement may be undertaken if RA destroys normal functioning. (Julie swann 2013)

2.8. General drugs used for arthritis

2.8.1. Paracetamol (acetaminophen): Paracetamol is active in many arthritic disorders and in all age groups. As the oral analgesic of choice, it has been recommended for mild to severe pain in OA and is typically well tolerated for periods of up to 12 months in osteoarthritic patients. Paracetamol is usually well absorbed and considered healthy, although the level of use has been independently reported to be associated with a mild increase in the risk of hypertension. (Forman JP *et al* 2007).Its mechanism of action remains unclear, but different claims have been made concerning inhibition of the activity of central cyclooxygenase, inhibition of the activity of the N-methyl-D-aspartate receptor, and stimulation of descending inhibitory pathways. (Libert F *et al* 2004).

2.8.2. Tramadol: Tramadol is a central-acting oral analgesic that has a unique dual mechanism of action involving a weak μ-agonist action as well as inhibition of the reuptake of noradrenaline (norepinephrine) and serotonin. It has received universal acceptance for use in both moderate and severe pain and is being used as an adjunctive remedy for arthritic pain. Tramadol is paired with paracetamol in a beneficial manner and decreases the use of NSAIDs without sacrificing analgesia.Side effects such as dizziness, nausea and constipation have been associated with the use of the drug. (Ripple MG, *et al*, 2000).

2.8.3. Non-steroidal anti-inflammatory drugs

The primary anti-inflammatory and anti-nociceptive effects of NSAIDs have been linked to an inhibitory effect on cyclo-oxygenase enzymes and a subsequent decrease in inflammatory prostaglandins such as $PGE_2$and prostacyclin. (Burian M, *et al* 2005).

It has been shown that NSAIDs are highly effective in treating acute pain and remain one of the key pharmacological agents for arthritic pain management. The published guidelines and expert opinion are split on the relative functions of NSAIDs versus paracetamol as a first-line analgesic medication for arthritic conditions.NSAIDs are also commonly used for symptomatic treatment for RA, but accompanied with few side effects (Towheed TE *et al* 2006).

2.8.4.Methotrexate: Due to its protection and usefulness in the treatment of rheumatoid arthritis and some other rheumatic diseases (such as some types of vasculitis, or inflammation of the

blood vessels), methotrexate is the chemotherapeutic drug most commonly used among rheumatologists. Its typical side-effects are relatively easy to control, treat and prevent.

2.9. Medicinal Plants with anti-arthritic activity

Medicinal plants can be described as plants that are widely used to treat and prevent specific diseases and illnesses and are generally considered to be hazardous to humans. (Schulz et al 2001). These plants are either "wild plant species" that grow spontaneously in self-maintaining populations in natural or semi-natural conditions and may occur independently of direct human behaviour or the opposing "domesticated plant species" that have developed through human activities such as selection or breeding and rely on their survival management. (Calixto 2000)

Some plants have been known to have important antibacterial, antifungal, anti-cancer, antidiuretic, anti-inflammatory and anti-diabetic properties. (Timothy 2012). Venom neutralization with lupeol acetate derived from the root extract of *Hemidesmus indicus*, prevention of hypertension and lowering of blood sugar by serpentine removed from the root of *Rauwolfia serpentine* are other applications of herbal medicines.In modern medicine, the introduction of plant-derived medicines has been compared to the use of plant-derived materials as an indigenous cure in the traditional medicine system. (Igoli*et al* 2003).

- Examples of Medicinal Plants with Anti-arthritic Activity include:

2.9.1. *Alstoniascholaris* Linn (Apocynaceae): AS is frequently referred to as the tree of saptaparni or demon. Traditionally, the bark is used to treat rheumatism, malaria, pruritis,abdominal diseases, leprosy, asthma, bronchitis, and chronic ulcers.[Kalaria et al 2012].

2.9.2. *Boerhaaviadiffusa* Linn. (Nyctagineae): BD is found particularly during the rainy season throughout India.The root is a medically essential part of inflammatory disorder therapy such as arthritis as a powder in drachm or decoction or infusion doses.Also, for the

treatment of chronic alcoholism and various other diseases, including phthisis, insomnia and rheumatism, BD is used [Nadkarni et al 2009].

2.9.3. *Rutagraveolens* (Rutaceae): Rue is a perennial herbaceous plant originally cultivated in the Mediterranean region. It is traditionally used as an antiseptic, anthelmintic, antispasmodic, stimulant, expectorant, anti-rheumatic and abortifacient. [Nadkarni et al 2009].

2.9.4.*Phyllanthus amarus*(Euphorbiaceae): PA is a 10-60 cm tall herb growing in tropical and subtropical sandy regions.It is traditionally used for jaundice, diarrhoea, dysentery, urinary-genital disease, scabies, ulcers, and wounds. It is being used as an astringent, stomach, diuretic, antiseptic, bitter, and febrifuge.In the Hand Book of African Medicinal Plants it is reported that PA was traditionally use for its anti-inflammatory activity. Moreover, in Amazonia and Brazil, the whole plant was used for the treatment of different inflammatory disorders like arthritis. [Nadkarni et al 2009].

2.9.5. *Glycyrrhiza glabra(*Fabaceae):GY is a 2 m high herb/shrub occurring predominantly in subtropical or temperate areas, generally referred to as mulethi. The plant has been reported to be used historically in anaemia, gout, asthma, epilepsy, fever, cough, skin disease, rheumatism, paralysis, and haemorrhagic diseases.Under inflammatory conditions, as demulcent, roots are useful in the form of infusion, decoction, extract or lozenge.[Nadkarni et al 2009].

2.9.6. *Lantana camara (*Verbinaceae*): LC is native to India and is around 1-3 m high. LC is traditionally used to treat herpes, chicken pox, measles, fever, colds, rheumatism, asthma, ulcers, and high blood pressure. [Saxena 2012]

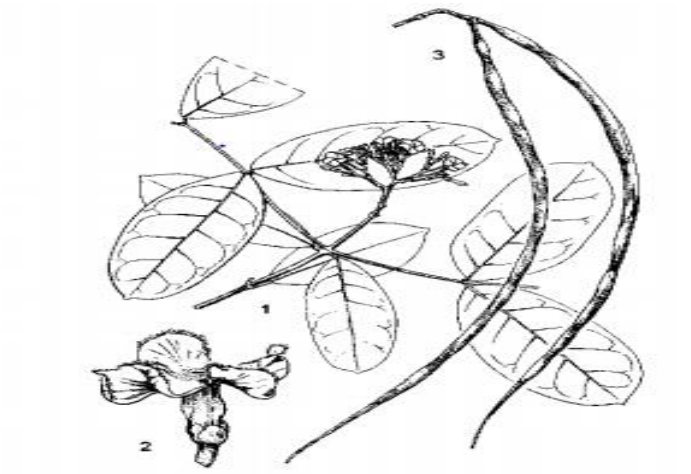
2.9.7.*Caesalpiniasappan* (Leguminosae): CP tree spreads to a height of 10 m and is cultivated with yellow flowers for its large, ornamental pencils. When they are interlaced, a very strong barrier is created by the branches. For the prevention of ulcers, leprosy,

rheumatism, skin disorder, diarrhoea, dysentery, epilepsy, seizures, diabetes, odontopathy, stomatopathy, and leucorrhoea, CP heartwood is typically used.

2.9.8. *Alafia barteri*

Alafia barteri is a vigorous, climbing shrub that produces stems that scramble over the ground or ascend into trees in the forest up to 35 meters high.In diameter, the stems may be 3cm. (Irvine, 1961).*Alafia barteri* occurs in the forests of West and Central Africa, from Guinea Bissau east to Cameroon and south to Congo.

Alafia barteri (Apocynaceae) is a high-climbing, scandent shrub with small, pure white or pink flowers. Its local names are agbarietu (Yoruba), loko or mende(Sierra leone),anyi (ivory coast), akan-asante or fante(ghana),obompa,otanza(igbo).



Alafia barteri — 1, flowering twig; 2, flower; 3, fruit.
Source: Flore analytique du Bénin

Taxonomy

Division: Angiosperm

Class: Eudicots

Subclass: Asterids

Order: Gentianales

Family: *Apocynaceae*

Subfamily: *Apocynoideae*

Tribe: *Malouetieae*

Genus: Alafia

Species: *A. barteri*

For local use as a binding material and medicine, the plant is harvested from the wild.For the prevention of sickle cell anaemia, rheumatism, eye infections, febrifuges, as chew-sticks and toothache, it is used in ethno-medicine. (*Leeuwenberg,et al* 1997). The twining stem of *A. barteriBarteri*is used as a binding material for roofs for the treatment of fever and inflammation. In Cote d'Ivoire, its leaf infusion is used to treat malaria. Also a decoction is usually taken to treat rheumatic pains in Nigeria.

Antifungal activity against *Aspergillus flavus, Aspergillus fumigatus, Aspergillus niger, Candida albicans, Microsporumaudoninii, Trichoderma viride* and *Trichophyton mentagrophytes* has been found in the ethanol and water extracts of the leaves of *Alafia barteri*. Extracts of ethanol were more effective than extracts of water. (Adekunle and Okoli, 2002).

CHAPTER THREE

3.1 MATERIALS AND METHODS

3.1.1. Materials / apparatus used: Beakers, Pipettes, Test tubes and test-tube rack, Burettes Centrifuge, Spectrophotometer, Water bath, Spatula.

3.1.2. Reagents Used: Ethanol, Phosphate buffer, Potassium ferricyanide, Trichloro acetic acid, Ferric chloride, Ascorbic acid, Hydrogen peroxide, Methanol, 2, 2-diphenyl-1-picrylhydrazyl (DPPH), Hydrochloric acid, Bovine serum albumin, Aspirin .

3.2 Methods

3.2.1. Collection of plants

Alafia barteri leavesand root barks were collected fromIkire in Osun State. The plant materials were identified in the department of Botany University of Lagos where it was given the voucher number LUH8657.

3.2.2. Aqueous extract preparation

Leaves after air drying for two weeks were ground to powder using the blender while the root bark were chopped into small pieces.
100g of each ground and chopped part (that is, leaf and stem bark) was mixed with 500mls of distilled water and was allowed to stand at room temperature with occasional stirring for 72 hours. The mixtures were filtered and the filtrate was collected separately in a clean beaker. The extracts were evaporated, using laboratory oven to dryness.

3.2.3. Ethanolextract preparation

100g of each ground part (that is, leaf and stem bark) was mixed with 500mls of ethanol and was allow standing at room temperature with occasional stirring for 72 hours. The mixtures were filtered and the filtrate was collected separately in a clean beaker. The extracts were evaporated, using laboratory oven to dryness.

3.3. In-vitro antioxidant assays

3.3.1 Reducing power Assay (Jayanthi*et al,* 2011)

50-300ug/ml of the plant extracts was prepared respectively. To each of these extracts, 2.5mls of phosphate buffer and 2.5mls of potassium ferricyanide was added. The mixtures were kept at 50ºC in water bath for 20 minutes. 2.5 ml of 10 % trichloro acetic acid was added to each sample after cooling, and centrifuged for 10 min at 3000 rpm.With distilled water (2.5 ml) and a freshly prepared ferric chloride solution (0.5 ml), the upper layer of each solution (2.5 ml) was mixed.At 700 nm, the absorbance was measured.Excluding samples, controls were prepared in a similar manner.The standard used was ascorbic acid (AA) at different concentrations.

$$\%\text{increase in reducing power} = \frac{A\ test - A\ blank}{A\ blank} \times 100$$

3.3.2. Hydrogen peroxide scavenging activity (Ruch et al 1989)-

The extract's scavenging behavior against hydrogen peroxide radicals was calculated by themodifiedmethod of Dehpour. In phosphate buffer pH7.4, a solution of hydrogen peroxide (40Mm) was prepared. 1 ml of hydrogen peroxide was added to 0.1 mg/ml of each extract using a UV spectrophotometer against a blank solution containing phosphate buffer without hydrogen peroxide and absorbance was measured.at 560nm.

The hydrogen peroxide percentage scavenged by the extract and the standard compound was determined using the given formula:

$$\text{Percentage scavenged } [H_2O_2] = 1 - \frac{Abs\ (standard)}{Abs\ (control)} \times 100$$

Where, Abs control is the absorbance of the control (without extract) at560nm;

Abs sample is absorbance in the presence of the extract at 560nm.

The experiment was repeated in triplicates.

3.3.3 DPPH Free Radical Scavenging Activity (W. Brand et al 1995)

The ability to donate hydrogen atoms from the various plant extracts was determined by the decolorization of2, 2-diphenyl-1-picrylhydrazylhydrazyl methanol solution.

0.2 ml of each extract was added to 3 mls of 0.1 mM DPPH solution, and absorbance was read at 517 nm. The reduction in absorption was associated with the percent inhibition of samples. The percentage of inhibition was calculated by the following:

% antioxidant capacity= [Ac – As /AC] x100

Where: Ac = absorbance of control and

As = absorbance of sample.

3.4. In-vitro anti-arthritic activity

3.4.1. Inhibition of protein denaturation method (Kar, 2012)

In this experiment, four sets of solutions were prepared:

- Test solution: 0.45 ml of BSA (bovine serum albumin) (5% w/v) (aqueous solution) and 0.05 ml plant extract (50-300ug/ml) respectively
- Test control solution: 0.45 ml of BSA (5% w/v) (aqueous solution) and 0.05 ml distilled water
- Product control solution: 0.45 ml distilled water and 0.05 ml of plant extract (500 µg/mL)
- standard solution consist of 0.45 ml 0f BSA (5% w/v) (aqueous solution) and 0.05 ml diclofenac sodium (500 µg/mL)

All the above solutions were adjusted using 1N hydrochloric acid to pH 6.3. Samples were incubated at 37 °C for 20 min and the temperature was raised to hold the samples at 57 °C for 3 min. After cooling, 2.5 ml of phosphate buffer saline was applied and the absorbance was measured at 416 nm.The percentage of inhibition of protein denaturation was determined using the following formula:

$$\% \text{ inhibition} = 1 - \frac{\text{O.D of test solution} - \text{O.D of product control}}{\text{O.D of test control}} 100$$

The control represents 100% protein denaturation. The results were compared with diclofenac sodium (500µg/mL). Each experiment was done in triplicates and the average was taken.

CHAPTER FOUR

RESULTS

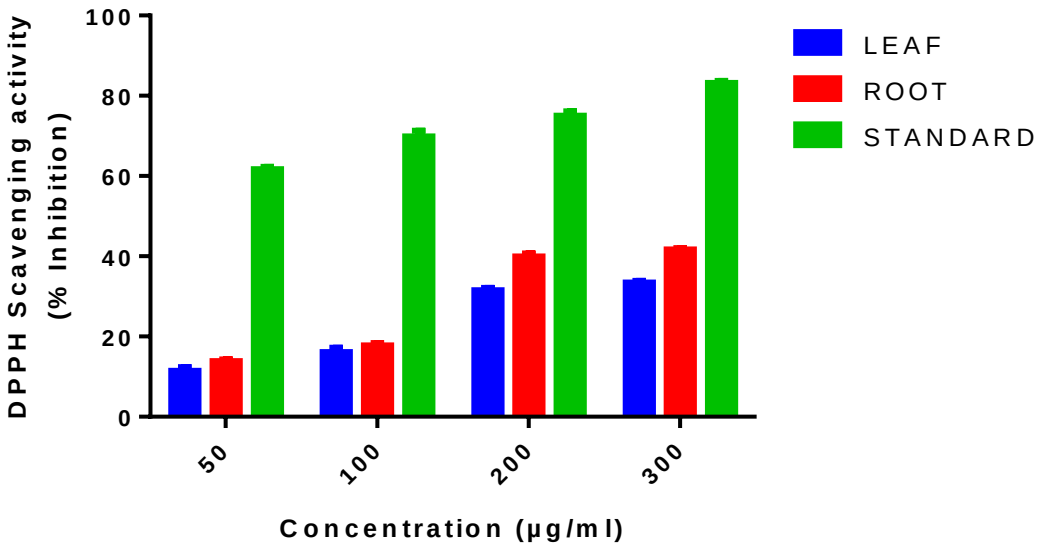


Figure 1: DPPH Scavenging activity of the aqueous leaf and root extracts of *Alafia barteri*

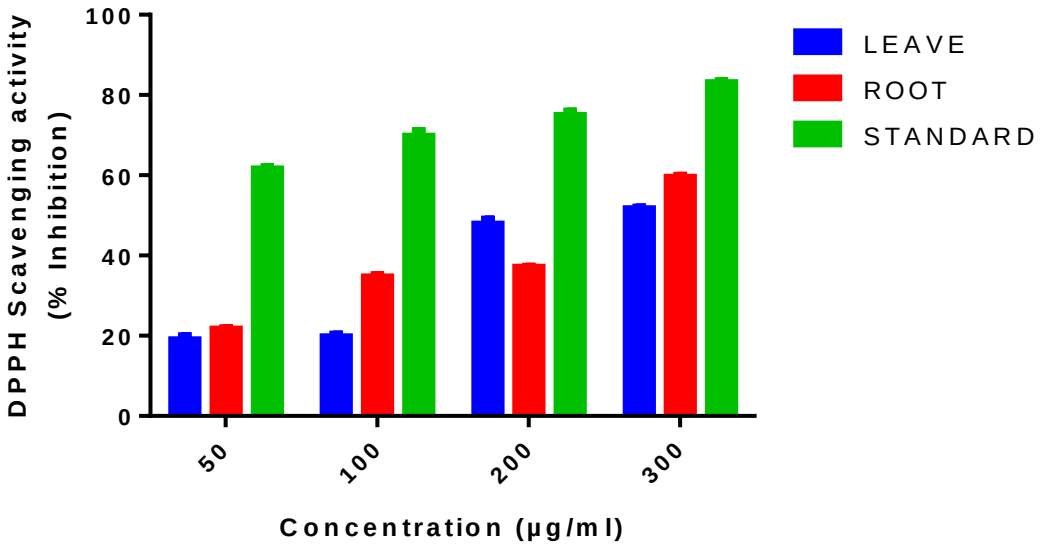


Figure 2: DPPH Scavenging activity of the ethanol leaf and root extracts of *Alafia barteri*

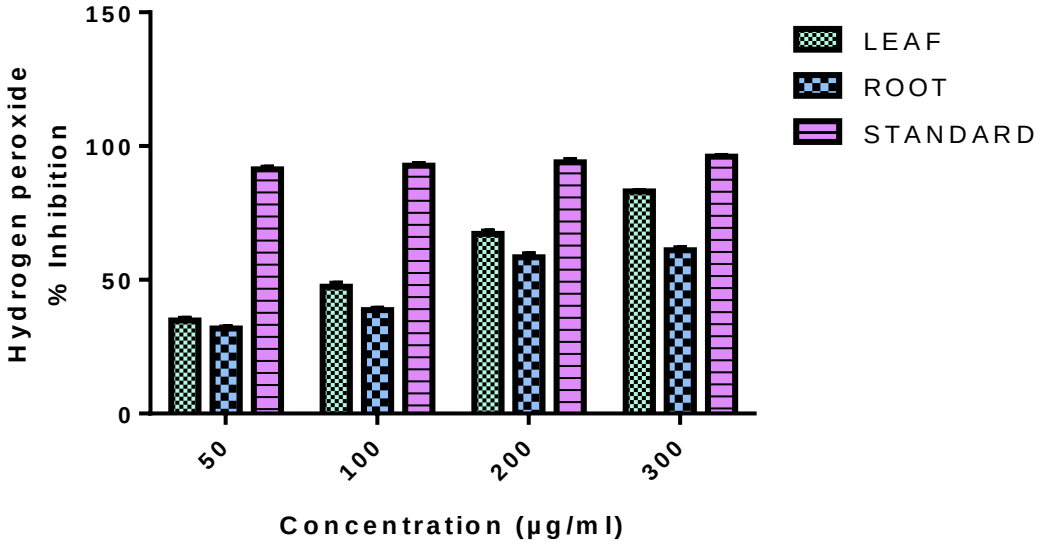


Figure 3: Hydrogen Peroxide scavenging activity of aqueous leaf and root extracts of*A.barteri*

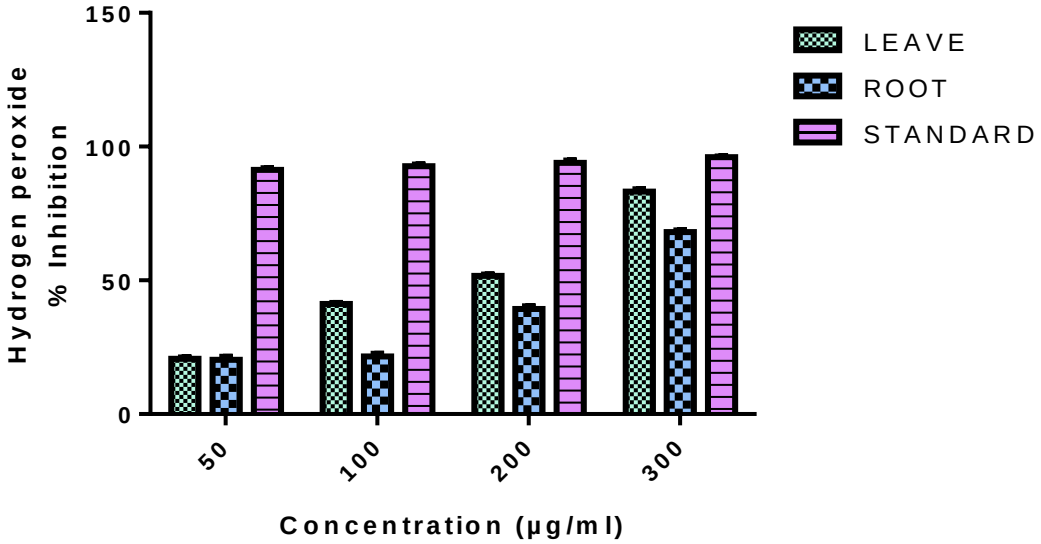


Figure 4: Hydrogen Peroxide scavenging activity of ethanol leaf and root extracts of *A. barteri*

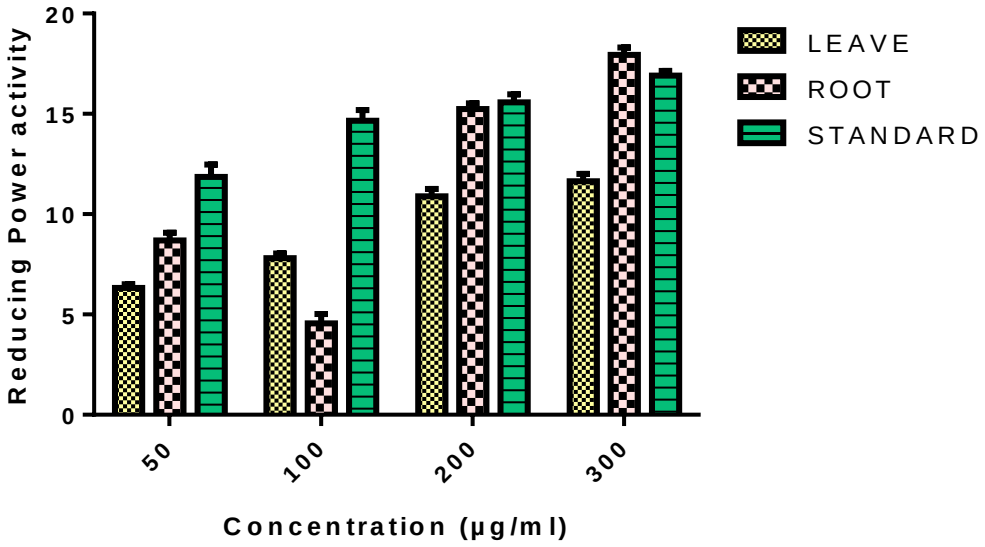


Figure 5: Reducing power activity of the aqueous leaf and root extracts of *A. barteri*

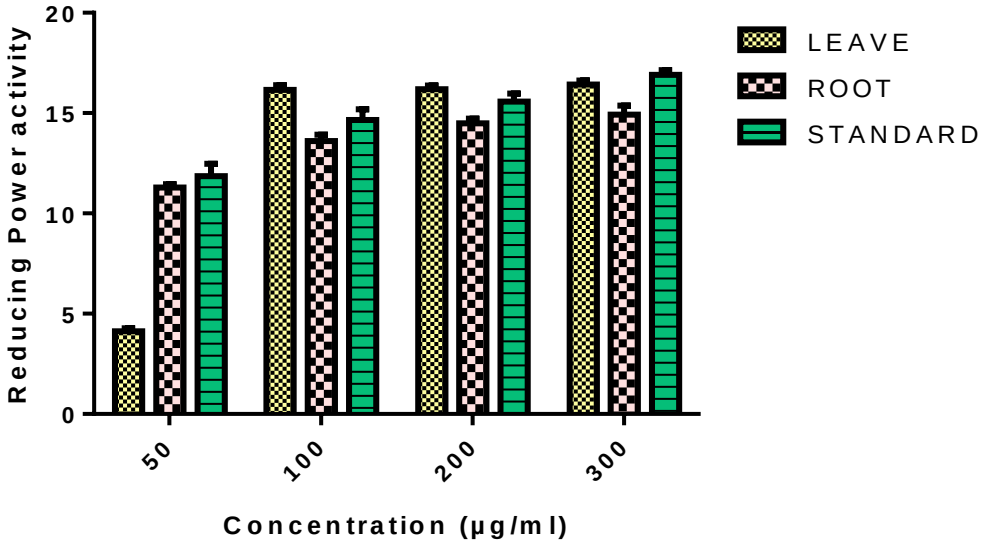


Figure 6: Reducing power activity of the ethanol leaf and root extracts of *A. barteri*

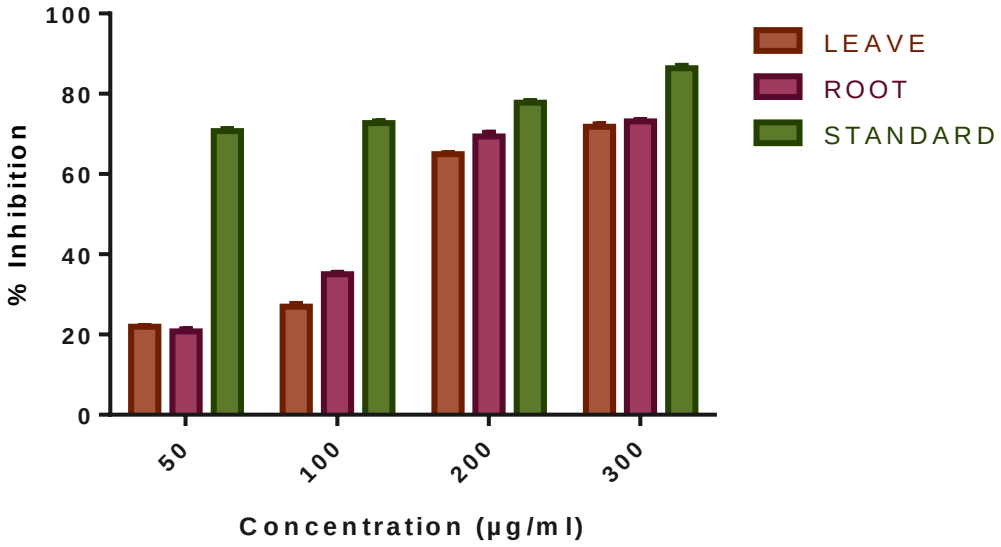


Figure 7: Effect of aqueous leaf and root extract of *A.barteri* protein denaturation using egg albumin

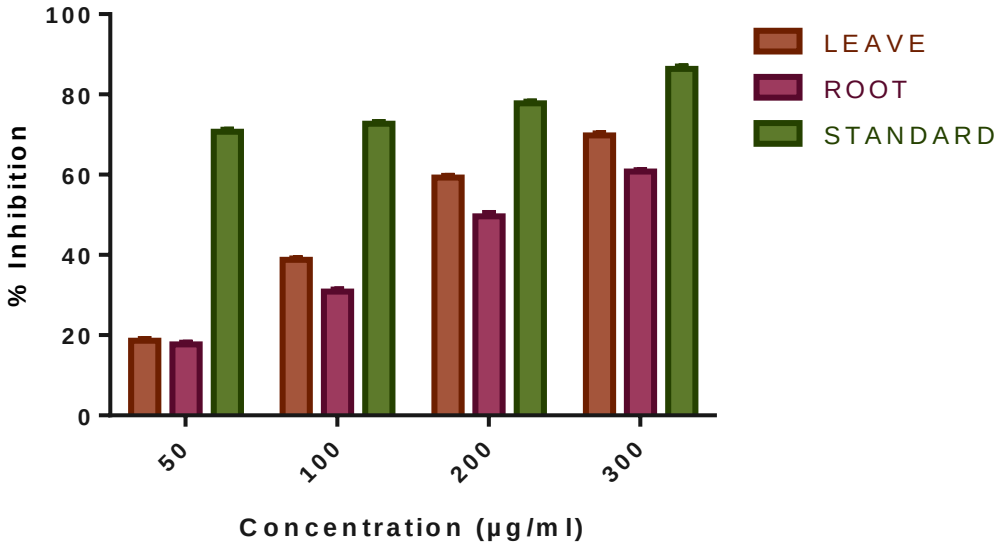


Figure 8: Effect of ethanol leaf and root extract of *A.barteri* protein denaturation using egg albumin

Figure 1 and 2 shows the DPPH scavenging activity of aqueous and ethanol leaf and root extract compared to the standard, ascorbic acid. Aqueous and ethanol root extracts of *Alafia barteri* showed higher activity than the leaf extracts in comparison with ascorbic acid which had the highest activity.

Hydrogen peroxide scavenging activity as seen in figures 3 and 4 were expressed as percentage inhibition. The aqueous and ethanol leaf extracts had higher percentage inhibition compared to other extracts though the standard still had the highest. Figure 5 and 6 reveals that the reducing power activity of the extracts increased with increase in concentration,however, the ethanol leaf and root extracts had higher activity.

No significant difference was observed between the percentage inhibition of protein denaturation by the aqueous leaf and root extract, however the ethanol leaf extract had higher percentage than the root extract.(figure 7 and 8).

CHAPTER FIVE

5.1. Discussion

Due to their possible use in the treatment of various chronic and infectious diseases, medicinal plants having antioxidant and anti-arthritic properties have been on the rise.Medicinal plants can give an alternative source of agents with significant anti-arthritic and antioxidant activity due to the risk of adverse effects experienced when using synthetic agents.

The outcome of the antioxidant analysis showed that the extract could be an effective DPPH antioxidant, models of scavenging activities for reducing power and hydrogen peroxide.The radical scavenging activity of1, 1-diphenyl-2-picrylhydrazyl (DPPH) is a simple and commonly used method to test natural compounds or plant extracts for in-vitro antioxidantactivity. DPPH, purple in colour, is a stable free radical at room temperature. Its potential to minimize the absorption of an electron or a hydrogen radical from antioxidants is determined by the measurement of a decrease in its absorption at 517 nm.

In this analysis, DPPH radical scavenging activity of the aqueous and ethanol leaf and root extract of *Alafia barteri* was compared with standard ascorbic acid. While the standard antioxidant had higher scavenging activity than the extracts at all tested concentrations, the extract still showed good free radical scavenging activity because the inhibition percentage also increased as the concentration increased. The aqueous root extract of the plant had higher activity compared to the aqueous leaf extract while in ethanol extract the leaves have the highest percentage inhibition after the standard (Figure 1).

As a traditional medicine, the free radical scavenging property of *Alafia barteri* may be one of the mechanisms by which this plant is effective. The use of *Alafia barteri* plant may be beneficial for the prevention of degenerative diseases associated with oxidative stress.

H_2O_2 is extremely essential since it is capable of penetrating biological membranes. At first, H_2O_2 is not very reactive, but often it can be toxic to cells because in the cell it can give rise to hydroxyl radicals (OH-) [Gülçin et al 2010]. Phenolic that can donate electrons to H_2O_2 can be attributed to H_2O_2 scavenging by the extract, thereby neutralizing Hydrogen to water (H_2O). The results show that *Alafia barteri's* aqueous and ethanol leaf and root extract had a strong H_2O_2 scavenging activity that could be attributed to the presence of antioxidant compounds. Since good electron donors are the antioxidant components present in the extracts, they can speed up the conversion of H_2O_2 to H_2O. The results indicate a good inhibition percentage that increases as the extract concentration increased, with a high scavenging activity of the standard drug. The standard for each extract have the highest percentage inhibition followed by the aqueous leaf and also ethanolic leaf.

The presence of antioxidants in the aqueous and ethanol leaf and root-bark extract of *Alafia barteri* in the reducing power assay can lead to a reduction in the form of the Fe3+/ferricyanide complex.The reducing power of compound can serve as an important indicator of its potential antioxidant activity. (Meir *et al.* 1995). The reducing power of the aqueous and ethanol leaf and root extract of *Alafia barteri* was determinedby comparing with that of ascorbic acid. It wasfound that the reducing powers activity increased with the increase in theirconcentrations.At 300 µg/ml, the aqueous extract of the root has the highest percentage inhibition than that of the standard while in ethanol extract the leaves have the highest percentage inhibition. This implies that these extracts are ableto react significantly to

free radicals in order to turn them into more stable non-reactive species and to end radical chain reactions.

In in-vitro anti-arthritic activity by inhibition of protein denaturation, the protein denaturation assay was selected for in vitro assessment of anti-arthritic properties of aqueous and ethanol leaf and root-bark extract of *Alafia barteri* with a wide range of dose concentrations. The present findings showed a concentration dependent inhibition of protein (bovine serum albumin) denaturation by the extract throughout the concentration range of 50,100,200 and 300µg/ml. Aspirin (at the concentration range of 50,100,200 and 300 µg/ml) was used as the standard drug, which also showed concentration dependent inhibition of protein denaturation. The aqueous leaf and root extracts almost inhibited protein denaturation equally with no significant difference.

It has been stated that protein denaturation plays a role in the progression of rheumatoid arthritis. The development of auto-antigens may be due to in vivo denaturation of proteins in some rheumatic diseases. (Arya D 2013). Denaturation mechanisms are likely to involve changes in electrostatic, hydrogen, hydrophobic and disulphide bonding. It can be stated from the results of this study that aqueous and ethanol leaves and root parts of *Alafia barteri* are capable of regulating auto antigen production and inhibiting protein denaturation in rheumatic diseases (Arya D, et al 2014)

5.2. CONCLUSION

This study reveals that the aqueous and ethanol leaf and root extract of *Alafia barteri* possesses radical scavenging and anti-arthritic activities as determined by the inhibition of protein denaturation assay, DPPH scavenging, reducing power and hydrogen peroxide scavenging assay.

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APPENDIX

Percentage Inhibition Values for DPPH Scavenging Activity of Aqueous Leaf and Root Extracts of *A. barteri.*

Concentration	Leaf	Root	Standard
50	11.42	13.89	61.70
100	16.11	17.75	69.92
200	31.56	39.95	75.03
300	33.43	41.67	83.22

Percentage Inhibition Values for DPPH Scavenging Activity of Ethanol Leaf and Root Extract of *A. barteri.*

Concentration	Leaf	Root	Standard
50	19.09	21.79	61.70
100	19.89	34.78	69.92
200	48.00	37.20	75.03
300	51.77	59.59	83.22

Percentage Inhibition Values for Hydrogen Peroxide Scavenging Activity of Aqueous Leaf and Root Extracts of *A. barteri.*

	Leaf	Root	Standard
50	34.79	31.86	91.30
100	47.48	38.68	92.75
200	67.21	58.38	93.88
300	83.05	61.05	96.04

Percentage Inhibition Values for Hydrogen Peroxide Scavenging Activity of Ethanol Leaf and Root Extract of *A. barteri.*

	Leaf	Root	Standard
50	20.63	20.37	91.30
100	41.22	21.56	92.75
200	51.73	39.37	93.88
300	83.17	68.11	96.04

Percentage Inhibition Values for Reducing Power Activity of Aqueous Leaf and Root Extracts of *A. barteri.*

	Leaf	Root	Standard
50	6.33	8.69	11.85
100	7.81	4.56	14.66
200	10.89	15.24	15.58
300	11.65	17.94	16.91

Percentage Inhibition Values for Reducing Power Activity of Ethanol Leaf and Root Extracts of *A. barteri.*

	Leaf	Root	Standard
50	4.12	11.30	11.85
100	16.15	13.62	14.66
200	16.20	14.49	15.58
300	16.43	14.92	16.91

Percentage Inhibition Values for Inhibition of protein denaturationby the aqueous leaf and root extracts of *A. barteri.*

	Leaf	Root	Standard
50	21.97	20.75	70.70
100	26.98	35.08	72.70
200	64.93	69.41	77.79
300	71.81	73.13	86.39

Percentage Inhibition Values for Inhibition of Protein Denaturationby the ethanol Leaf And Root Extracts of *A. barteri.*

	LEAF	ROOT	STANDARD
50	18.66	17.74	70.70
100	38.83	30.90	72.70
200	59.28	49.65	77.79
300	69.79	60.75	86.39