TITTLE

DEVELOPMENT AND COMPARATIVE STUDY OF PHYTOSOMES OF ETHANOL EXTRACTS OF *Bridelia ferruginea* AND *Bryophyllum pinnatum* LEAVES FOR ENHANCED ANTIMICROBIAL AND ULCER PROTECTIVE PROPERTIES

A THESIS SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR AWARD OF DEGREE OF DOCTOR OF PHILOSOPHY (Ph.D.) IN PHARMACOLOGICAL BIOCHEMISTRY

BY

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CERTIFICATION

Amadi, Emenike Benjamin, a postgraduate student with the registration number PG/Ph.D./15/78178 of the Department of Biochemistry has satisfactorily completed the requirements for the course work and research for the degree of Doctor of Philosophy in Pharmacological Biochemistry. The work embedded in this report is original and has not been submitted in part or in full for any other diploma or degree of this or any other University.

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DEDICATION

This work is dedicated to Almighty God.

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ABSTRACT

Herbal products have been found to show less activity in *in vivo* studies when compared to their activity in *in vitro* studies. Most of the phytochemicals in plants such as flavonoids, terpenoids and polyphenols are highly polar in nature and are unable to cross the highly lipid-rich biological membranes which lead to poor bioavailability. Phytosome is a natural product obtained by reaction of a stoichiometric amount of plant extract and phospholipids using a suitable solvent. *Bridelia ferruginea* (Euphorbiaceae) and *Bryophyllum pinnatum* Lam. (Crassulacea) are locally used in the treatment of various diseases. This study was based on the development of phytosomes from ethanol extracts of *Bridelia ferruginea* and *Bryophyllum pinnatum* leaves for enhanced antimicrobial and ulcer protective properties. The phytochemical screening of both plant extracts revealed the presence of tannins, carbohydrates, hydrogen cyanide, phenols, flavonoids, reducing sugar, alkaloids, steroids, terpenoid, saponin and glycosides. Higher concentration of phenols (1911.01 ± 2.308 mg/100g) was found in *Bridelia ferruginea* extract than that of (66.67 ± 12.73 mg/100g) found in *Bryophyllum pinnatum* extract. Both extracts (*B. pinnatum and B. ferruginea*) showed free radical-scavenging activity against 2, 2, diphenyl-1-picryl hydrazyl (DPPH), total antioxidant capacity (TAC) and ferric reducing antioxidant power (FRAP) in all tested concentrations (15.63 – 1000 µg/ml). *B. ferruginea* and *B. pinnatum* showed their highest ferric reducing antioxidant power of 174.29 ± 22.42 µMfe^{2+} /g and 672.46 ± 23.05 µMfe^{2+} /g respectively at the concentration of 1000 µg/ml. Scanning electron microscopy revealed that *Bridelia ferruginea* phytosome complexes have rougher surface morphology when compared to surface morphology of *Bryophyllum pinnatum* phytosome complexes at x1000 magnification. The Fourier Transform Infrared Spectroscopy spectrum peaks revealed interaction between individual extracts and phospholipid in the formulation of phytosomes. The presence of functional groups was detected which include alcohols (O-H) at 3324.8 cm^{-1} – 3358 cm^{-1}, aliphatic compound (C-H) at 2922.2 cm^{-1}, 2918.5 cm^{-1}, carbonyl compounds (aldehydes and ketones; C=O) at 1710.8 cm^{-1} - 1736.9 cm^{-1}. The entrapment efficiency showed that all the complexes have more than 90% entrapment. The highest entrapment of 97.42% was found in 1:5 phytosome complex of *Bryophyllum pinnatum* and the least entrapment of 94.14% was found in 1:5 phytosome complex of *Bridelia ferruginea. In vitro* drug release was higher in *Bridelia ferruginea* phytosome complexes than in the *Bryophyllum pinnatum* phytosome complexes within 3 h of investigation. It was observed that 1:1 *Bridelia ferruginea* phytosomes released 65.87% of its extract within 3 h of investigation while 1:1 *Bryophyllum pinnatum* phytosome released 13.47% within the same time interval. *Bryophyllum pinnatum* and *Bridelia ferruginea* showed no zone of inhibition with the selected bacterial isolates. The acute toxicity study on both extracts was greater than 5000 mg/kg. Both plants and their phytosome complexes exhibited ulcer protective property. The highest percentage protection was found among groups pretreated with 400 mg/kg of 1:3 phytosome complexes of *Bridelia ferruginea* which gave 78.14% mucosa protection and *Bryophyllum pinnatum* that produced 77.86% protection. There was a significant increase ($p < 0.05$) in pH, HDL, SOD, CAT and GPx among groups pretreated with extracts and phytosomes when compared to the pH, HDL, SOD. CAT and GPx activity of the untreated group (control). However, significant decrease ($p < 0.05$) in gastric volume, total acidity, urea, creatinine, ALT, AST, ALP and MDA was observed among the pretreated groups when compared to the ulcer untreated group (control). Histological examination of the stomach tissues of rats revealed various degrees of necrosis on the mucosa tips of indomethacin-induced ulcerated rats.

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ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
AP	Alternative pathway
AST	Aspartate aminotransferase
ATP	Adenosine Triphosphate
CAT	Catalase
COX	Cyclooxygenase
CFU	Colony forming unit
DPPH	2,2-diphenyl-1-picrylhydrazyl
FRAP	Ferric reducing antioxidant power
FTIR	Fourier transform infrared spectroscopy
GPX	Glutathione peroxidase
HDL	High density lipoprotein
LDL	Low density lipoprotein
MDA	Malondialdehyde
NSAIDs	Non-steroidal anti-inflammatory drugs
PC	Phosphatidylcholine
PG's	Prostaglandins
ROS	Reactive oxygen specie
RNS	Reactive nitrogen species
SEM	Scanning electron microscopy
SGF	Simulated gastric fluid
SIF	Simulated intestinal fluid
SOD	Superoxide dismutase
TAG	Triacylglycerol
TAC	Total antioxidant capacity
TCA	Trichloroacetic acid
TBA	Thiobarbituric acid

CHAPTER ONE

INTRODUCTION

Curative use of medicinal plants is referred to as herbal or traditional medicine. Traditional medicine has developed in various communities in Nigeria due to the health needs of the people. In Nigeria today, various forms of traditional medicine are practiced using mainly plants for treatment recipes. Medicinal plants constitute the major sources of the active drug in both traditional and modern medicine. These plants have been shown to have genuine utility and about 80% of the rural population depends on them for primary health care needs (Akinyemi, 2000). Medicinal plants have been used as sources of therapeutic solutions for the treatment of many diseases and people of all continents especially Africa has this old practice. Inspite of the great progress made in synthetic organic medicinal products of the twentieth century, more than 25% of prescribed drugs in developed countries are in one way or the other derived from plants (Newman *et al*., 2000). In developing countries, notably in West Africa, up to 80% of the population uses medicinal plants as remedies due to the unaffordable price of commercial drugs (Hostellmann and Marston, 2002). Traditional medicine might be considered as a solid amalgamation of dynamic medical know-how and ancestral experience. In Africa, millions of lives were saved by the use of herbal medicine. World Health Organisation (WHO) had described traditional medicine as one of the surest ways to achieve total health care coverage to secure world population. Many medicines have been derived from the knowledge of tropical medicinal herbs and more of these plant derrivatives will be discovered in the near future.

Ethno medicine plays a central role in the search for and development of new drugs (Heinrich, 2000). Many herbal remedies have been used traditionally for treatment of diseases in Nigeria. Different bioactive compounds from different sources have been isolated and characterized. Analysis of plants for new bioactive composition with the much needed therapeutic potential to fight against pathogens (for example, *Salmonella typhi, Klebsella pneumonia, Staphylococcus aureus,* etc.) is an important step in the development of new drugs. Peptic ulcer diseases comprise heterogeneous disorders, which manifest as a break in the lining of the gastrointestinal mucosa bathed by acid and pepsin. Several orthodox pharmaceutical drugs such as anticholinergic drugs, histamine H_2-receptor antagonists, antacids, and more recently, proton-pump inhibitors have been employed in the management of peptic ulcers, but they provoke many adverse effects. In recent years, there has been

growing interest in alternative therapies especially from plant sources due to their perceived lower side effects, ease of accessibility and affordability. Plants are the most attractive source of new drugs, and some have been shown to have promise for the treatment of gastro duodenal ulcer with little or no side effects (Alkofahi and Atta 1999).

Bridelia ferruginea (Family: Euphorbiaceae) is an indigenous medicinal plant in Nigeria. It is commonly found in savannah. The plant extracts have been used as purgative and vermifuge (Cimanga *et al*., 1999; Djoueche *et al*., 2011).

Bryophyllum pinnatum Lam. (Crassulacea) is a perennial herb that grows in many parts of Nigeria. This plant is well known as an agent for wound healing. It has been accepted as a herbal remedy in almost all parts of the world (Gupta *et al.,* 2010; Igwe and Akunyili 2005). *Bryophyllum pinnatum* is a crassulescent herb of about a metre in height, with smooth and fleshy leaves (Ojewole, 2002), distributed all over the world but grows primarily in the rain forest (Ursula *et al*., 2006). Ethno pharmacologically, it is used for the treatment of diarrhea and vomiting, earache, burns, abscesses, gastric ulcers, antibacterial and insect bites, (Ofokansi *et al*., 2005). The pharmacological activities of *Bryophyllum pinnatum* have been found to include antimicrobial (Akinpelu, 2000); wound healing (Nayak *et al.,* 2010); anti-inflammatory and antidiabetic (Ojewole, 2005) and antihypertensive (Ghasi1 *et al*., 2011; Ojewole, 2002). It is very unfortunate that the herbal drugs comprising major amount of active constituents with excellent bioactivity *in vitro* express less activity *in vivo* because of their poor lipid solubility and improper size of the molecules. This results in poor absorption and bioavailability of active constituents from herbal extract. A phytophospholipid complex vesicle (phytosome) is the promising emerging technique applied to pharmaceuticals for the enhancement of bioavailability of herbal extracts and phytoconstituents. Phytosomes are advanced forms of herbal formulations which contain the bioactive phytoconstituents of herbal extract and have good ability to transition from a hydrophilic into the lipophilic environment of the cell membrane, exhibit better pharmacokinetic and pharmacodynamics profile than conventional herbal extracts. They can be formulated in the form of various dosage forms like suspensions, capsules, creams, gels etc.

This study is therefore designed to enhance antibacterial and ulcer-protective properties of *Bridelia ferruginea* and *Bryophyllum pinnatum* ethanol leaf extracts by application of their various phytosomes in the treatment of bacterial infection and ulcer diseases.

1.1 *Bridelia ferruginea* Plant

Bridelia ferruginea belongs to the family Euphorbiaceae which is commonly found in Savannah regions (Ekanem *et al*., 2008). *Bridelia ferruginea* is a twisted shrub that reaches the size of a tree if it grows in a suitable condition.

The common names of *Bridelia ferruginea* in different Nigeria languages are Kizni (Hausa language), Iralodan (Yoruba language), and Ola (Igbo language) and were mostly found in Savannah regions. It is about 6-15 m high. The bark is dark grey, rough and often marked scaly (Rashid *et al*., 2000).

1.1.1 Binomial Nomenclature of *Bridelia ferruginea* Plant

Plant taxonomy

Kingdom	Plantae
Division	Angiospermae
Class	Archichlamydeae
Order	Geraniales
Family	Euphorbiaceae
Genus	Bridelia
Specie	ferruginea

1.1.2 Description, Ecology and Geographical Distribution of *Bridelia ferruginea* Plant

Bridelia species belong to the family of Euphorbiaceae and comprises approximately 60-70 species found in Asia, Africa and Australia (Rashid *et al*., 2000; Ngueyem *et al.*, 2009). It occurs commonly in the guinea savannah and coastal plains of Africa particularly Burkina Faso, Cote d'Ivoire, Ghana, Nigeria and Togo as well as Asia and Australia (Addae-Mensah, 1992).

Bridelia ferruginea is a shrub or straggly tree of 15 m high with crooked bole up to 1.80 m in girth, the commonest Bridelia species of the savanna woodland occurring from Guinea and Mali to Southern Nigeria, and throughout the wooded savanna regions of Africa. It is fire-

resistant. The leaves may be small to medium sized, simple, alternate, spiral or distichous, broadly elliptic and pubescent. They are also pinnately veined with entire margin and an acute apex (Ghana Herbal Pharmacopoeia, 1992). The wood is brown. It is said to be termite-proof, and is used for this reason in the soudano-guinean region to make grain warehouse. In Sierra Leone it is used primarily as structural timber. In the Central African Republic, it is recognized as good firewood.

Figure 1: *Bridelia ferruginea* plants (the leaves)
Source: www.tropical.theferns.info/plantimages

1.1.3 Nutritional Values of *Bridelia ferruginea* Plant

In West Africa, especially areas prone to drought, previous studies have demonstrated the importance of edible wild plants as food sources (Sena *et al*., 1998). Drought is associated with inadequate food intake and disease, where food scarcity and inadequate dietary intakes have led to increased incidence of malnutrition and famine (Franke and Chasin, 1980). Use of edible wild species in combination with domesticated foods has remained a hallmark of many African agro-societies. According to the research carried out by Adesina Adeolu Jonathan and Akomolafe Seun Funmilola (2014), *Bridelia ferruginea* plant (stem back) has been shown to contain crude protein, ash content, crude fat, Crude Fibre and Carbohydrate and minerals which are good for energy requirement. The anti-nutrients such as phytate, oxalates, tannins levels found in *Bridelia ferruginea* bark are low to significantly interfere with the nutrients utilization. The anti-nutrients present are below the established toxic level (Nkafamiya and Manji, 2006). This shows that *Bridelia ferruginea* plant can contribute

useful amount of nutrients to human diet. Interestingly, the anti-nutritional content of the sample is low, much lower than is obtainable in most Nigerian vegetables. This implies that, its overall nutritional value will not be affected.

1.1.4 Ethnomedical Uses / Applications

In traditional African medicine, *Bridelia ferruginea* is utilized in treating disease conditions such as arthritis, bruises, boils, dislocation, burns, fever, headaches, stiffness, rheumatic pains and oedema (Olumayokun *et al.,* 2012). Other uses include intestinal disorders, diabetes, thrush, epilepsy, infectious diseases including sexually transmitted diseases, skin diseases and eruption, skin cancers, roundworm (Cimanga *et al*., 2001). Other pharmacological applications include antibacterial activities against some micro-organisms known to cause enteric and secondary upper respiratory tract infections; anti-inflammatory activity (Olajide *et al*., 1999); antidiabetic (Taiwo *et al*., 2012) and its potential for water treatment has been reported by Kolawole and Olayemi (2003). Studies carried out by various workers have shown that this plant has several properties, which justify the plant's medicinal use.

1.2 *Bryophyllum pinnatum* Plant

Bryophyllum pinnatum (crassulacea) popularly known as "Resurrection plant" is a perennial herb used in folkloric medicine in tropical Africa. It is classified as weed and the plant grows well throughout the southern part of Nigeria (Gill, 1992). The plant is widely used in traditional medicine for the treatment of variety of ailments.

1.2.1 Binomial Nomenclature of *Bryophyllum pinnatum* Plant

Kingdom	Plantae
Phylum	Tracheophyta
Class	Magnoliopsida
Order	Saxifragales
Family	Crassulaceae
Genus	*Bryophyllum*
Species	*pinnatum*

1.2.2 Description, Ecology and Geographical Distribution of *Bryophyllum pinnatum* Plant

The plant, *Bryophyllum pinnatum* (Crassulaceae) is commonly known as air plant, love plant, miracle leaf, life plant, etc. This plant (*Bryophyllum pinnatum)* has been accepted as an herbal remedy in almost all parts of the world (Gupta *et al*., 2010). *Bryophyllum pinnatum* is a crassulescent herb of about 1 metre in height, with opposite, glabrous leaves (with 3–5 deeply crenulated, fleshy leaflets) (Ojewole, 2002). It is distributed worldwide but growing primarily in the rain forest. It grows widely and is used as folk medicine in tropical Africa, India, China, Australia and tropical America, Madagascar, Asia and Hawaii (Lans, 2006).

Figure 2. *Bryophyllum pinnatum* plant.
Source: www.homeremedies.com/ayurvedic-plant

1.2.3 Nutritional Values of *Bryophillum pinnatum* Plant

Some plants with good bioactive properties have useful minerals and food value for human and animal consumption. The result of proximate composition of *Bryophyllum pinnatum* leaves carried out by Nwali *et al*., (2014) revealed that *Bryophillum pinnatum* leaf contains ash, fat and oil, crude fibre and carbohydrate. The study also shows that carbohydrate value was the highest and ash had the least value. Also the presence of Ca, K, Mg, Na, Zn, Cd, Ni, Pb and Fe were detected.

1.2.4 Ethnomedical Uses of Bryophillum pinnatum Plant

The leaves and bark of *Bryophyllum pinnatum* are bitter tonic, astringent, analgesic and carminative, ethno pharmacologically used for the treatment of diarrhea and vomiting, earache, burns, abscesses, gastric ulcers, insect bites, and lithiasis (Ofokansi *et al*., 2005). The juice from fresh leaves is used to treat smallpox, otitis, cough, asthma, palpitatious,

headache, convulsion and general lack of strength. Leaves powder used as wound dressing and sold as 'Jakhmehayat'. In Southeastern Nigeria, the herb is used to facilitate the dropping of the placenta of newly born baby (Okwu, 2007). The antibacterial activity of the leaf juice of *B. pinnatum* was reported by Obaseiki-Ebor (1985). *Bryophyllum pinnatum* is also well-known as an agent of wound healing. Flavonoids, polyphenols, and triterpenoids have been identified from the leaves of *B. pinnatum* (Ojewole, 2005).

1.3 Plant Phytochemicals

Phytochemicals (from the Greek word phyto, meaning plant) are biologically active, naturally occurring chemical compounds found in plants, which provide health benefits for humans more than those attributed to macronutrients and micronutrients (Harsler and Blumberg, 1999). They protect plants from disease and damage and contribute to the plant's colour, aroma and flavor. In general, the plant chemicals that protect plant cells from environmental hazards such as pollution, stress, drought, UV exposure and pathogenic attack are called phytochemicals. Recently, it is clearly known that they have roles in the protection of human health, when their dietary intake is significant. Phytochemicals accumulate in different parts of the plants, such as in the roots, stems, leaves, flowers, fruits or seeds. These compounds are known as secondary plant metabolites and have biological properties such as antioxidant activity, antimicrobial effect, stimulation of the immune system, decrease of platelet aggregation, modulation of hormone metabolism and anticancer property. Researches have demonstrated that many phytochemicals can also protect human against diseases (Narasinga, 2003).

1.3.1 Classification of Plant Phytochemicals

In recent years' phytochemicals are classified as primary or secondary constituents, depending on their role in plant metabolism. Primary constituents include the common sugars, amino acids, proteins, purines and pyrimidines of nucleic acids, chlorophylls etc. Secondary constituents are the remaining plant chemicals such as alkaloids, terpenes, flavonoids, lignans, plant steroids, curcumines, saponins, phenolics, flavonoids and glycosides (Hahn, 1998).

Alkaloids: Alkaloids are natural product that contains heterocyclic nitrogen atoms, are basic in character. The name of alkaloids derives from the "alkaline" and it was used to describe any nitrogen-containing base. They were known as the vegetable alkalis or alkaloids and these alkaloids are used as the local anesthetic and stimulant as cocaine. Almost all the

alkaloids have a bitter taste. Alkaloids are so numerous and involve such a variety of molecular structure that their rational classification is difficult. Alkaloids are normally classified according to the heterocyclic ring system they possess, but some authors prefer a classification based on their biosynthetic origins from amino acids, e.g. phenylalanine, tyrosine or tryptophan (Jack, 2001).

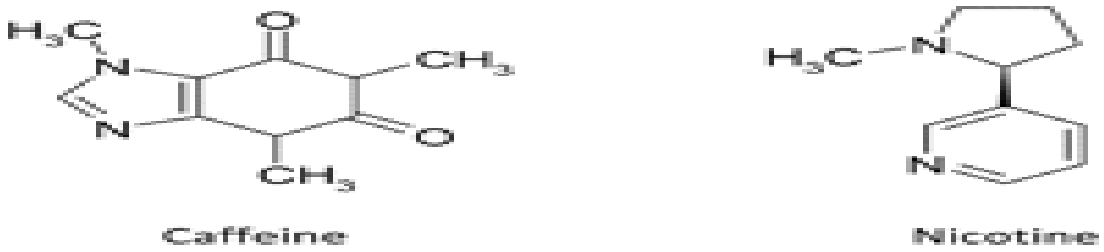


Fig. 3: Structures of Alkaloids
Source: (Zulak *et al*., 2006)

Alkaloids have many pharmacological activities including antihypertensive effects (many indole alkaloids), antiarrhythmic effect (quinidine, spareien), antimalarial activity (quinine), antibacterial, antifungal and anticancer actions (dimeric indoles, vincristine, vinblastine) (Wink *et al.,* 1998).

Terpenoids: The terpenoids are a class of natural products which have been derived from five-carbon isoprene units. Most of the terpenoids have multi cyclic structures that differ from one another by their functional groups and basic carbon skeletons. These types of natural lipids can be found in every class of living thing, and therefore considered as the largest group of natural products. Terpenes are widespread in nature, mainly in plants as constituents of essential oils. Their building block is the hydrocarbon isoprene, CH2=C(CH3)-CH=CH2. Terpenoids can have medicinal properties such as anticarcinogenic (e.g. perilla alcohol), antimalarial (e.g. artemisinin), anti-ulcer, antimicrobial or diuretic (e.g. glycyrrhizin) activity and the sesquiterpenoid antimalarial drug artimisinin and the diterpenoid anticancer drug taxol (Dudareva *et al*., 2004).

Phenolics: Phenolic phytochemicals are the largest category of phytochemicals and the most widely distributed in the plant kingdom. The three most important groups of dietary phenolics are flavonoids, phenolic acids, and polyphenols. Phenolic are hydroxyl group (-OH) containing class of chemical compounds where the (-OH) bonded directly to an aromatic hydrocarbon group. They are plant secondary metabolites, and they have an important role as defense compounds. Phenolics exhibit several properties beneficial to humans and its antioxidant properties are important in determining their role as protecting

agents against free radical-mediated disease processes. Flavonoids are the largest group of plant phenols and the most studied (Dai and Mumper, 2010).

Phenolic acid: The term "phenolic acids", in general, designates phenols that possess one carboxylic acid functional group. These compounds have been studied mainly for their properties against oxidative damage leading to various degenerative diseases, such as cardiovascular diseases, inflammation and cancer. Indeed, tumour cells, including leukaemia cells, typically have higher levels of reactive oxygen species (ROS) than normal cells so that they are particularly sensitive to oxidative stress (Mandal *et al.*, 2010). Varied biological activities of phenolic acids were reported. Increased bile secretion, reduction in blood cholesterol and lipid levels and antimicrobial activity against some strains of bacteria such as *staphylococcus aureus* are some of biological activities of phenolic acids. Phenolics acid possesses diverse biological activities, for instance, antiulcer, anti- inflammatory, antioxidant, cytotoxic and antitumor, antispasmodic, and antidepressant activities (Ghasemzadeh *et al*., 2010).

Flavonoids: Flavonoids are polyphenolic compounds that are ubiquitous in nature. The flavonoids appear to have played a major role in successful medical treatments of ancient times, and their use has persisted up to now.

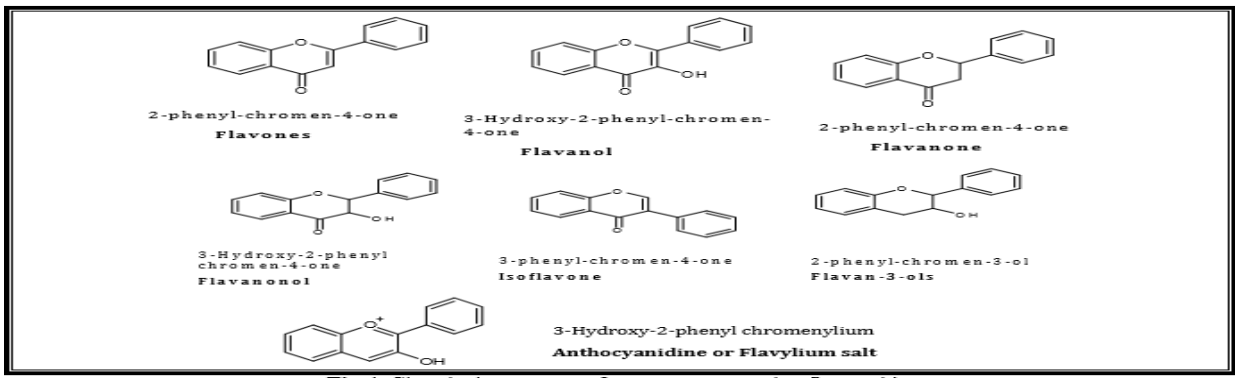


Fig. 4: Structures of flavonoids
Source: Pretorius, (2003).

Flavonoids have been reported to exert multiple biological property including antimicrobial, cytotoxicity, anti-inflammatory as well as antitumor activities but the best-described property of almost every group of flavonoids is their capacity to act as powerful antioxidants which can protect the human body from free radicals and reactive oxygen species. The capacity of flavonoids to act as antioxidants depends upon their molecular structure. The position of hydroxyl groups and other features in the chemical structure of flavonoids are important for

their antioxidant and free radical scavenging activities. Flavonoids have been stated to possess many useful properties, which include anti-inflammatory activity, enzyme inhibition, antimicrobial activity, oestrogenic activity, anti-allergic activity, antioxidant activity, vascular activity and cytotoxic antitumor activity (Tapas *et al.*, 2008).

Polyphenol

Polyphenols, secondary plant metabolite are the most abundant antioxidants in human diets. These compounds are designed with an aromatic ring carrying one or more hydroxyl moieties. Several classes can be considered according to the number of phenol rings and to the structural elements that bind these rings. Two main groups of polyphenols, termed flavonoids and non-flavonoids, have been traditionally adopted.

Tannins

From a chemical point of view, it is difficult to define tannins since the term encompasses some very diverse oligomers and polymers. It might be said that the tannins are a heterogeneous group of high molecular weight polyphenolic compounds with the capacity to form reversible and irreversible complexes with proteins (mainly), polysaccharides (cellulose, hemicellulose, pectin, etc.), alkaloids, nucleic acids and minerals, etc. (Schofield *et al*., 2001). In medicine, especially in Asian (Japanese and Chinese) natural healing, the tannin-containing plant extracts are used as astringents, against diarrhoea, as diuretics, against stomach and duodenal tumours and as anti-inflammatory, antiseptic, antioxidant and haemostatic pharmaceuticals (Dolara *et al*., 2005). Tannins have shown potential antiviral, antibacterial (Akiyama *et al*., 2001) and antiparasitic effects. It was also reported that certain tannins are able to inhibit HIV replication selectivity and is also used as diuretic.

Glycosides

Glycosides are colourless, crystalline carbon, hydrogen, and oxygen-containing (some contain nitrogen and sulfur) water-soluble phytoconstituents found in cell sap (Fig.5). Chemically, glycosides contain a carbohydrate (glucose) and a non-carbohydrate part (aglycone or genin) (Kar, 2007; Firn, 2010). Alcohol, glycerol or phenol represents aglycones. Glycosides are classified on the basis of type of sugar component, chemical nature of aglycone or pharmacological action. Drugs like ouabain and digoxin are examples of cardiac glycosides.

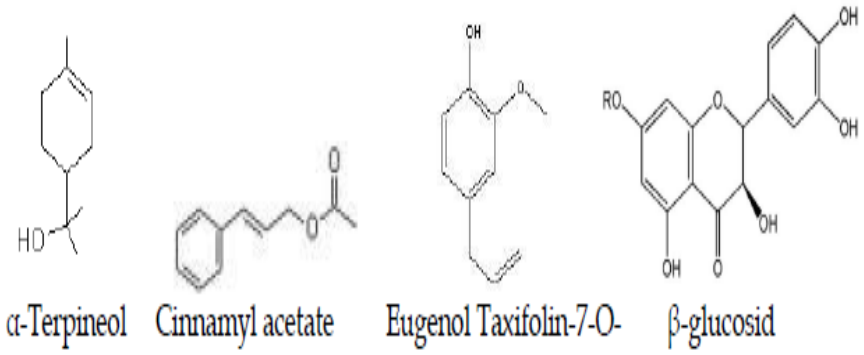


Fig: 5 Basic structures of pharmacologically important plant-derived glycosides.
Source: (Sarkar and Nahar, 2007).

Digoxin found in the foxglove plant is used clinically, while ouabain is used only experimentally due to its extremely high effectiveness. Cardiac glycosides are known to work by inhibiting the Na^+/K^+ pump. This causes an increase in the level of sodium ions in the myocytes, which then lead to a rise in the level of calcium ions. This inhibition increases the amount of calcium ions available for contraction of the heart muscle, which improves cardiac output and reduces distention of the heart; thus, they are used in the treatment of congestive heart failure.

1.4 Free Radicals

A free radical can be defined as any molecular species capable of independent existence that contains an unpaired electron in an atomic orbital. The presence of an unpaired electron results in certain common properties that are shared by most radicals. Many radicals are unstable and highly reactive. They can either donate an electron to or accept an electron from other molecules therefore free radicals behave as oxidants or reductants (Cheeseman and Slater, 1993). In recent years, there has been a great deal of attention toward the field of free radical chemistry. Free radical reactive oxygen species and reactive nitrogen species are generated by our body by various endogenous systems, exposure to different physiochemical conditions or pathological states. A balance between free radicals and antioxidants is necessary for proper physiological function. If free radicals overwhelm the body's ability to regulate them, a condition known as oxidative stress results. Free radicals thus adversely alter lipids, proteins, and DNA and trigger a number of human diseases. Hence application of external source of antioxidants can assist in coping with oxidative stress.

1.5 Plant Antioxidant

Use of plant extracts in traditional medications have been in the practice for long years and has exhibited a great potential in relieving the symptoms of many diseases (Maqsood *et al.*, 2010). Antioxidants and plants have a close relationship and many plants are used as antioxidants in decoctions made in medicines. Consumption of edible plant as greens and vegetables has been in the practice for long years as a source of vitamins, fibers, antioxidants, and other medicinal values. Natural antioxidants in the form of raw extracts, decoctions or isolated chemical constituents are reported to be very effective to prevent the destructive processes caused by oxidative stress (Zengin *et al.*, 2011). Therefore, many studies are performed in obtaining scientific information on the antioxidant properties of medicinal plants and a large number of medicinal plants have been investigated. The imbalance between antioxidants and free radicals in our body is responsible for most of our problems/diseases. Reactive oxygen species (ROS) are continuously being produced during cell metabolism and under normal condition; they are scavenged and converted to non-reactive species by different intracellular enzymatic and non-enzymatic antioxidant system (Shao *et al*., 2008). Antioxidants play an important role in neutralizing the effect of reactive oxygen species (ROS), reactive nitrogen species (RNS) and other oxidants that lead to numerous diseases and complications. The human body is equipped with its own inherited antioxidant defense system that involves many enzymes as superoxide dismutase, glutathione peroxidase, glutathione reductase, and catalases. These enzymes help to neutralize free radicals and reactive oxygen species and also to protect against free radical-induced cell damage. However, to accommodate excessive radical or reactive species produced due to many disease conditions it requires the use of antioxidants in the form of supplement. Many naturally occurring compounds such as phenolic compounds, flavonoids, tannins ascorbic acid etc. contribute to the antioxidant properties of herbal plants, vegetables, and fruits.

1.5.1 Sources of Antioxidants

Many antioxidant compounds, naturally occurring in plant sources have been identified as free radical or active oxygen scavengers. Based on the mechanism of action, antioxidants can be divided as either chain breaking antioxidants or preventive antioxidants. Different types of biological antioxidants exist, which include glutathione, vitamin C and E, cystine, and many others. Also, many plant-derived substances known as phytonutrients or phytochemicals possess antioxidant properties. Phenolic compounds such as flavonoids are such chemicals. These are found in several fruits, vegetables and green tea extracts.

1.5.2 Functions of Antioxidants

Antioxidants act as radical scavenger, hydrogen donor, electron donor, peroxide decomposer, singlet oxygen quencher, enzyme inhibitor, synergist, and metal-chelating agents. Both enzymatic and non-enzymatic antioxidants exist in the intracellular and extracellular environment to detoxify ROS (Frie *et al.*, 1988).

1.5.3 Mechanism of Antioxidants

Two principal mechanisms of action have been proposed for antioxidants. The first is a chain- breaking mechanism by which the primary antioxidant donates an electron to the free radical present in the systems. The second mechanism involves removal of ROS/reactive nitrogen species initiators (secondary antioxidants) by quenching chain-initiating catalyst. Antioxidants may exert /their effect on biological systems by different mechanisms including electron donation, metal ion chelation, co-antioxidants, or by gene expression regulation (Krinsky, 1992).

1.6 Phospholipids and Cell membrane

1.6.1 Phospholipids: Biological lipids are a chemically diverse group of compounds, the common and defining feature is their insolubility in water. Fats and oils are the principal stored forms of energy in many organisms. Phospholipids and sterols are major structural elements of biological membranes. The fats and oils used almost universally as stored forms of energy in living organisms are derivatives of fatty acids. Fatty acids are carboxylic acids with hydrocarbon chains ranging from 4 to 36 carbons long. Phospholipids are lipids that have glycerol as the backbone which contain a polar head group joined to the non-polar or hydrophobic moiety by a phosphodiester linkage.

1.6.2 Structure of Phospholipids (phosphatidylcholine)

A phospholipid is made up of two fatty acid tails and a phosphate group head. Fatty acids are long chains that are mostly made up of hydrogen and carbon, while phosphate groups consist of a phosphorus molecule with four oxygen molecules attached. These two components of the phospholipid are connected through a third molecule, glycerol. Phospholipids are able to form cell membranes because the phosphate group head is hydrophilic (water-loving) while the fatty acid tails are hydrophobic (water-hating). They automatically arrange themselves in a certain pattern in water because of these properties.

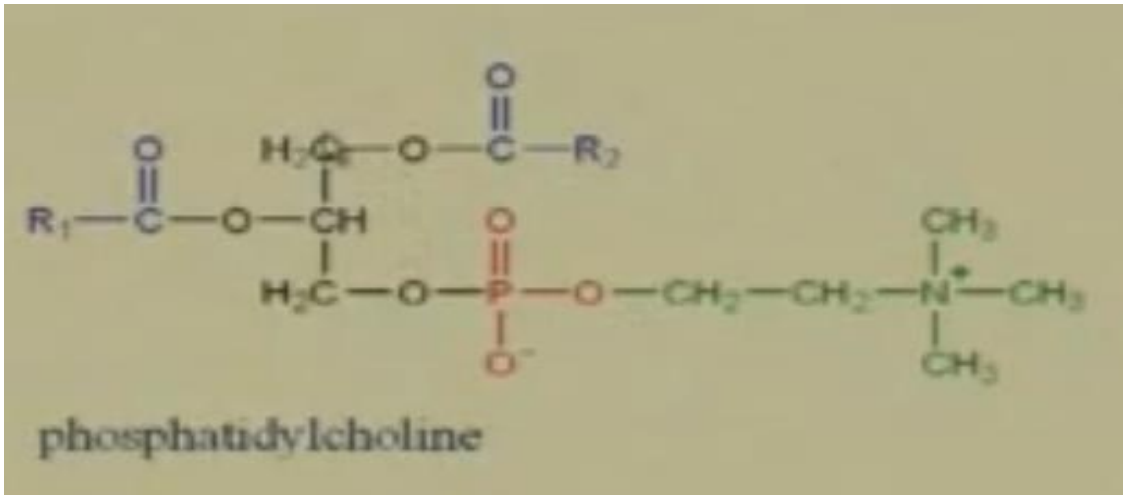


Figure 6 Structure of Phospholipids (phosphatidylcholine)

1.6.3 Functions of Phospholipids

Apart from being the major component of biological membrane, phospholipids can be used as a source of energy. Phospholipids can be broken down in the cell and used for energy. They can also be split into smaller molecules called chemokines, which regulate a variety of activities in the cell such as production of certain proteins and migration of cells to different areas of the body. Additionally, they are found in areas such as the lung and in joints, where they help lubricate cells. In pharmaceuticals, phospholipids are used as part of drug delivery systems, which are systems that help transport a drug throughout the body to the area that it is meant to affect. This is the basis of this study by utilization of phosphatidylcholine from soybean in formulation of phytosome which in turn promotes and improves the therapeutic effect of the herbal extract.

1.6.4 Cell Membrane

Lipids as a class of molecules display a wide diversity in both structure and biological function. A primary role of lipids in cellular function is in the formation of the permeability barrier of cells and subcellular organelles in the form of a lipid bilayer.

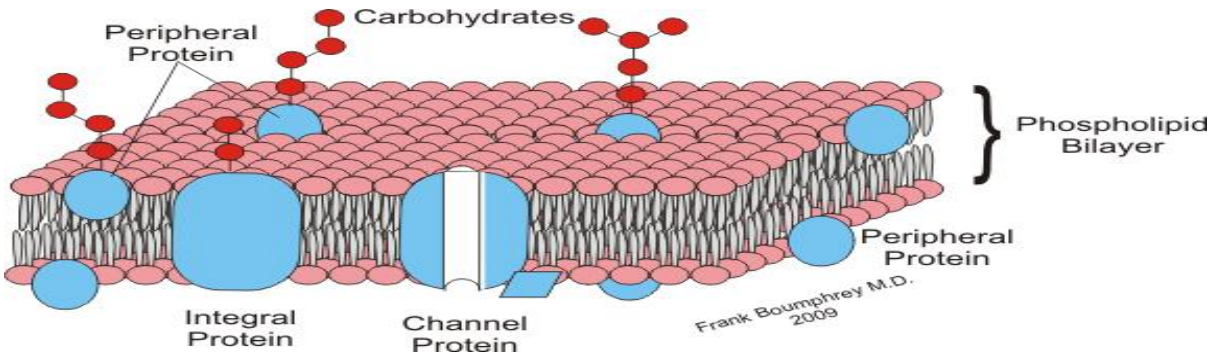


Fig.7: Structure of plasma membrane

Source: Singer and Nicolson (1972).

Biological membranes are sheet-like structures composed mainly of lipids and proteins. All biological membranes have a similar general structure. Membrane lipids are organized in a bilayer (two sheets of lipids making up a single membrane) that is approximately 60 to 100 Å thick. The proteins, on the other hand, are scattered throughout the bilayer and perform most membrane functions. Membranes are two-dimensional fluids: both lipids and proteins are constantly in motion. All biological membranes are made of the same basic structure. They are composed of molecules called Phospholipids, which form a Phospholipid Bilayer. Phospholipids are fats. They are composed of two Fatty Acid 'tails' and a Phosphate 'head'. The Phosphate 'heads' are Hydrophilic whereas the Fatty Acid 'tails' are Hydrophobic, meaning that Phospholipids are amphipathic. A Phospholipid Bilayer consists of two layers of Phospholipids where the 'tails' point inwards and the 'heads' point outwards, towards water. One layer is like a mirror image of the other (fig. 7).

Lipid profile test is a kind of blood test often used to assess risk of heart disease. Abnormalities in lipid profile are common complications in ulcer disease. Such abnormality represents the risk factors for coronary heart diseases. Cholesterol, a fat-like substance can build up inside arteries when it is present in greater than normal amounts. High levels are a major risk factor for heart and blood vessel disease. Low-density lipoprotein (LDL) known as “bad” cholesterol contributes to artery-clogging plaque buildup. High-density lipoprotein (HDL) referred to as “good” cholesterol helps protect against heart disease. Triacylglycerol (TAG) is storage form of fat released in bloodstream. High levels are also a major risk factor for heart and blood vessel disease.

1.6.5 Components of Membrane

The basic components of biological membrane are lipids and proteins. Some membranes also contain carbohydrates. The composition of lipids, proteins and carbohydrates varies from one membrane to another. The major lipids in biological membrane are phospholipids, glycolipids and cholesterol.

1.7 Drug Delivery System

Drug delivery refers to approaches, formulations, technologies, and systems for transporting a pharmaceutical compound in the body as to safely deliver its contents to achieve the desired therapeutic effect. It may involve scientific site-targeting within the body, or it might involve facilitating systemic pharmacokinetics; therefore, it is typically concerned with both quantity and duration of drug presence. Drug delivery is often approached via a drug's chemical

formulation, but it may also involve medical devices or drug-device combination products. Drug delivery technologies modify drug release profile, absorption, distribution and elimination for the benefit of improving product efficacy and safety, as well as patient convenience and compliance (Wang and Von Recum, 2011).

1.7.1 Types of Novel Drug Delivery System

Bioavailability of plant drugs has remained an important issue to resolve since many years and much work has been done in this direction to make better use of plant drugs (Acharya *et al*., 2011). There are different approaches in which plant or herbal extracts could be delivered into the body to improve their efficacy. Such drug delivery systems are as follows:

Liposomes: Liposomes are artificial microscopic vesicles consisting of an aggregate of water-soluble substance (extract) and phospholipid, used to convey vaccines, drugs, enzymes or other substances to target cells or organs.

Nanoparticles: These are particles of less than 100 nm in diameter that exhibit new or enhanced size-dependent properties compared with larger particles of same material (Jain, 2005).

Microemulsion: Miroemulsion is a thermodynamically stable dispersion of two immiscible liquids, stabilized by surfactants. A microemulsion is an emulsion whose particles are less than 1 micron in size.

Transferosomes: A transferosome carrier is artificially designed to be like cell vesicle or a cell engaged in exocytosis and thus suitable for controlled and potentially targeted drug delivery.

Phytosome: Phytosome is a newly introduced patented technology developed to incorporate standardized plant extracts or water soluble phytoconstituents into phospholipids to produce lipid compatible molecular complexes. Phytosomes are also known as herbosome. The term “Phyto” means plant while “Some” means cell-like. Phytosome protects valuable components of herbal extract from destruction by digestive secretion and gut bacteria and thus they show better absorption which produces better bioavailability and improved pharmacological and pharmacokinetic parameters (absorption, distribution, metabolism and elimination) than conventional herbal extract (Pawar and Bhangale, 2015). Out of the several approaches for improving the bioavailability of plant drugs, complexation with naturally occurring phospholipid molecules has emerged as a successful novel carrier system for improving the bioavailability of poorly absorbed plant extracts. The major reasons behind this seem to be the exclusive structure of dietary phospholipids, which are similar to the lipid content of the mammalian cell membrane (Bernard and Lecithins, 2005). Phospholipids are amphipathic molecules having considerable solubility in aqueous and oily mediums. Phytosome is a patented process developed by Indena to

incorporate phospholipids into standardized extracts and so vastly improve their absorption and utilization.

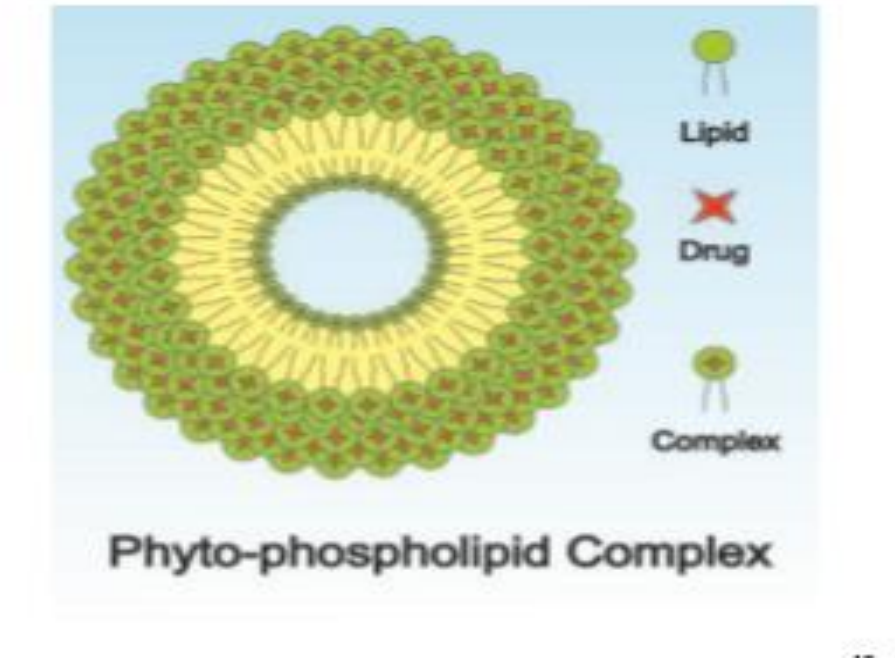


Fig. 8: Molecular organization of a Phytosome.
Source: Biju *et al*., 2006

1.7.2. Principle of Phytosome

Phosphatidylcholine (or phosphatidylserine) is a bi-functional compound. The phosphatidyl moiety is lipophilic and the choline (serine) moiety is hydrophilic in nature. This dual solubility of the phospholipid makes it an effective emulsifier. Thus, the choline head of the phosphatidylcholine molecule binds to the compound (e.g extract) while the lipid soluble phosphatidyl portion comprising the body and tail of the phospholipid then surrounds the choline bound material. Hence, the phytoconstituents produce a lipid-compatible molecular complex with phospholipids (Kumar *et al.,* 2010).

1.7.3. Physical, Chemical and Biological Properties of Phytosome

Physico-chemical Properties

1. Phytosome are prepared by reaction of stoichiometric amount of phospholipid with the standardized plant extracts as substrate. The spectroscopic data reveals that the phospholipid-substrate interaction is due to the formation of hydrogen bond between the polar head (i.e. Phosphate and ammonium group) and the polar functionalities of the substrate (Tripathy *et al*., 2013).

2. The size of phytosome varies from 50 nm to a few hundred µm (Patel *et al*., 2013).
3. Phytosome, when treated with water, assumes a micellar shape resembling liposome. Photon correlation spectroscopy (PCS) reveals this liposomal structures acquired by phytosome (Jain, 2005)
4. The H^{1}NMR and C^{13} NMR data deduced that the fatty chain gives unchanged signals both in free phospholipid and in the complex, which indicates that long aliphatic chains are wrapped around the active principle producing lipophilic envelope (Dayan and Touitou, 2000).
5. The complexes are often freely soluble in aprotic solvents, moderately soluble in fats, insoluble in water and relatively unstable in alcohol. But the phytosomes of certain lipophilic phytoconstituents like curcumin has shown increase in water solubility upon complexation with phospholipid (Maffei-Facino *et al*., 1994).

Biological Properties: Phytosome are novel complexes which are better absorbed and utilized; hence they produce more bioavailability and better result than the conventional herbal extract or non-complex extract, which has been demonstrated by pharmacokinetic studies or by pharmacodynamic tests in experimental animals and in human subjects (Maffei-Facino *et al*., 1994).

1.7.4. Advantages of Phytosome

Advantages of phytosome are:

1. Improved absorption in gastro intestinal tract (GIT).

2. Increased bioavailability which in turn gives improved therapeutic effect.

3. Less dose requirement due to high bioavailability.

4. Higher stability.

5. High lipophilicity causes high penetrability; hence phytosomes are used in cosmetics over liposomes

6. Greater clinical benefits.

7. Phosphatidylcholine acts as liver protective other than a carrier (Dhandapani *et al*., 2014).

1.7.5 Disadvantage of Phytosome: Phytoconstituent or the extract is rapidly eliminated from phytosome (Saha *et al*., 2013).

1.8 Pathogenicity and Bacterial Infection

This is the ability of a parasite to gain entry into host tissues and cause disease. Organisms that can cause disease are referred to as pathogens**.** Infections are caused by microorganisms such as bacteria, viruses, fungi and larger organisms like macro parasites such as ticks, nematodes etc. Hosts fight infections using their immune system. Mammalian host react to infections with an innate response, often involving inflammation, followed by adaptive response. Bacterial infection occurs when there is parasitic association between bacteria and the host organism. Bacteria in this kind of association are classified as pathogens. They cause diseases such as tuberculosis, typhoid fever, cholera, ulcer etc. Each species of pathogen has a characteristic spectrum of interaction with its human hosts. Some organisms, such as staphylococcus or streptococcus, can cause skin infection, pneumonia, meningitis and even overwhelming sepsis, a systemic inflammatory response producing shock, massive vasodilation and death (Fish, 2002). Bacteria constitute a large domain of prokaryotic microorganisms. They inhabit soil, water, hot springs, radioactive waste, and deep portions of earth's crust (Fredrickson *et al*., 2004). Bacteria also live in plants and animals. Bacteria display a wide diversity of shapes and sizes called morphology.

1.8.1 Bacterial Association with Human

It is not every interaction between bacteria and human being that results in harm or infection. Man could also benefit immensely by interaction with some bacteria depending on the position of such bacteria in the body.

Normal Flora

A diverse group of microbes (mostly bacteria) are adapted to live in the human body and on the skin surface. Many of the normal floras of the large intestine (*Escherichia coli* for example) are actually very beneficial to man and their relationship can be characterized as mutualistic symbiosis. However, *Staphylococcus aureus* (*S. aureus*) that typically colonize the skin can be dangerous if allowed to grow systemically.

Symbiosis

This is a relationship in which two organisms interact in close association usually with benefits to both species. Humans give *Escherichia coli* (*E coli*) a place to live and feed, while the *E. coli* in return gives protection against pathogens and a significant source of vitamin K.

Parasitism

This is the opposite of a mutualistic relationship. In this relationship, one organism benefits (parasite) and the other organism suffers (host). When *S. aureus* grows systemically in a human, the bacteria get food and a place to grow, while the human gets nothing in return. The worse in this relationship is that humans are being harmed by the exotoxins and exoenzymes these bacteria produce.

1.8.2 Routes of Entry of Pathogens

Human beings are infected by pathogens when these pathogenic microbes gain access to the body. There are different routes through which pathogens get to the body to cause harm. Portal of entry refers to the site at which the parasite enters the host. *Clostridium tetani* spores can cause tetanus when introduced into anaerobic tissue in a wound but will not cause an infection in the intestine if ingested. Certain parasites have multiple portals of entry (M. tuberculosis may enter the body by respiratory droplets, contaminated food and milk, and skin wounds.). The source of the infectious agent can be exogenous, from outside the host's normal flora, and/or endogenous, from the host's own normal flora.

(i) Infectious Agents that Enter the Skin: Infectious agents can enter the skin through cuts, nicks, wounds, and burns, such organisms include *Staphylococcus aureus*. Other infectious agents can create their own entry through the skin by the use of digestive enzymes. Some enter through bites of insects and other animals, or through needle puncture.

(ii) The Gastrointestinal Tract as Portal: Pathogens usually enter through ingestion of food and drinks. Enteric pathogens can survive digestive enzymes and pH changes. Bacteria such as Salmonella, Shigella, and Vibrio; Viruses; and enteric protozoans, such as Giardia, can be enteric pathogens.

(iii) The Respiratory Portal of Entry: The oral and nasal cavities are the most likely sites of entry for the respiratory system. These anatomical locations are lined by mucous membranes which can carry microbes deeper into the respiratory system. Infectious agents can infect the upper respiratory and lower respiratory tracts.

(iv) Urogenital Portals of Entry: This is a portal of entry for infectious agents that are transmitted by sexual contact. These diseases are called sexually transmitted infections (STI) or sexually transmitted diseases (STD).

1.9 Antibiotics

Antibiotics are a product produced by a microorganism or a similar substance produced wholly or partially by chemical synthesis, which in low concentrations, inhibit the growth of other organisms.

1.9.1 Classes of Antibiotics

Antibacterial drugs are commonly classified based on their mechanism of action, chemical structure, or spectrum of activity. Most antibiotics target bacterial functions or growth processes (Fig.8). Those that target the bacterial cell wall (e.g penicillins) or the cell membrane (polymyxins), or interfere with essential bacteria enzymes (sulfonamide) have bactericidal activities. Those that target protein synthesis (tetracyclines) are usually bacteriostatic, with the exception of bactericidal aminoglycosides. Further categorization is based on their target specificity. "Narrow spectrum" antibacterial antibiotics target specific type of bacteria, such as Gram positive or Gram negative bacteria whereas broad-spectrum antibiotics affect a wide range of bacteria (Srivastava *et al*., 2011)

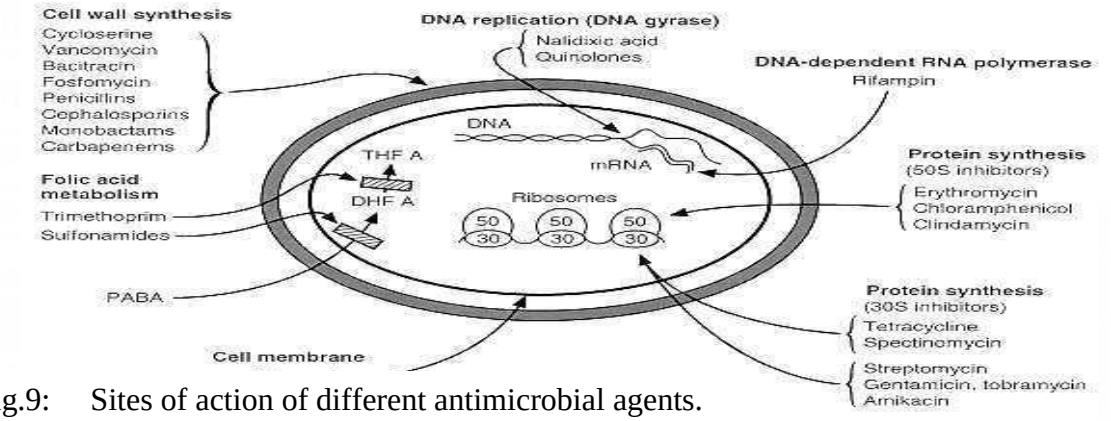


Fig.9: Sites of action of different antimicrobial agents.
Source: Gale *et al.*, (1981).

Antibacterial are screened for any negative effects on humans or other mammals before approval for clinical use, and are usually considered safe and most are well tolerated. However, some antibacterial have been associated with a range of adverse effects. Side effects range from mild to very serious complications depending on the antibiotics used, the microbial organism targeted, and the individual patient (Stams *et al.,* 2006).

1.9.2. Antimicrobial resistance

Infections have been the major problem throughout the history of human population. With the discovery of antibiotics, it was thought that health problems caused by infections would be a forgotten issue. However, due to abuse of these antibiotics, bacteria have been able to

evolve to become resistant to available drugs (Livermore, 2003). The increase in antibiotic resistance has also been attributed to a combination of microbial characteristics, abuse, social and technical changes that enhance the transmission of resistant organisms. The growing threat from resistant organisms calls for concerted efforts of researchers/ scientists to prevent the emergence of new resistant strains and the spread of existing ones (Okeke *et al.*, 2005).

1.9.3 Biochemistry of Antibiotic Resistance

Resistance of antibiotic differs in different species of microorganisms but the resistance is driven by few mechanisms as follows:

(i) Antibiotic inactivation (ii) Target modification (iii) Efflux pumps and outer membrane permeability changes. (iv) Target bypass.

Antibiotic Inactivation: The defence mechanisms within the category of antibiotic inactivation include the production of enzymes that degrade or modify the drug itself. Biochemical strategies include hydrolysis, group transfer, and redox mechanisms. In hydrolysis, antibiotics are made of susceptible hydrolysable bonds such as amides and esters. Bacteria create enzymes that hydrolyse the bonds of these antibiotics with amides and ester bonds therefore destroy the antibiotic before reaching its target site. β-lactamase from bacteria hydrolyses lactam ring in Penicillin (Poole, 2004). In group transfer mechanism, the most diverse family of resistant enzymes is the group of transferases. These enzymes inactivate antibiotics (aminoglycosides, chloramphenicol, streptogramin, macrolides or rifampicin) by chemical substitution (adenylyl, phosphoryl or acetyl groups are added to the periphery of the antibiotic molecule). By this action, the modified antibiotics are affected in their binding to a target. The Chemical strategies include O-acetylation (O-phosphorylation-nucleotidylation etc.), N-acetylation and thiol transfer. In redox process, bacteria have been exploiting this mechanism of oxidation and reduction of antibiotics to gain resistance over such drugs such as the oxidation of tetracycline antibiotics by the TetX enzyme.

Target Modification: This involves the modification of the antibiotic target sites and prevents the antibiotic from gaining proper binding with the target site. However, it is possible for mutational changes to occur in the target that reduce susceptibility to inhibition whilst retaining cellular function (Spratt, 1994). This mechanism includes peptidoglycan structure alteration, protein structure interference and DNA structure interference. In peptidoglycan structure alteration, the peptidoglycan component of the bacterial cell wall provides an excellent selective target for the antibiotics. It is essential for the growth and

survival of most bacteria. Consequently, enzymes involved in synthesis and assembly of the peptidoglycan component of the bacterial cell wall provide excellent targets for selective inhibition. The presence of mutations in the penicillin-binding domain of penicillin-binding proteins (PBPs) results in decreased affinity to β-lactam antibiotics. Alterations among PBPs result in ampicillin resistance among *Enterococcus faecium*, and penicillin resistance among *Streptococcus pneumoniae* (Kosowska *et al.,* 2004).

Efflux pump and outer membrane permeability: The efflux pumps are the membrane proteins that export antibiotics out of the cell and keep its intracellular concentrations at low levels. Efflux pumps affect all classes of antibiotics, especially the macrolides, tetracyclines, and fluoroquinolones because these antibiotics inhibit different aspects of protein and DNA biosynthesis and therefore must be intracellular to exert their effect. Gram-negative bacteria possess an outer membrane consisting of an inner layer containing phospholipids and an outer layer containing the lipid a moiety of lipopolysaccharides (LPS). This composition of the outer membrane (OM) slows down drug penetration, and transport across the outer membrane is achieved by porin proteins that form water-filled channels. Drug molecule can penetrate outer membrane by (i) diffusion through porin, (ii) through the bilayer and (iii) through self-promoted uptake. For example, hydrophilic compounds either enter the periplasm through porins (e.g. β-lactams). As a result of this, changes in porin copy number, size or selectivity will alter the rate of diffusion of these antibiotics.

1.9.4 Mechanism of Action of Antifungal Drugs and Resistance

Antifungal drugs are drugs that inhibit the growth or kill fungal organisms. They are drugs used for the treatment of fungal infections. The important factor in these drugs is the mechanism by which they attack the structure and cell function of fungal cells. There are four groups of antifungal drugs. They are as follows:

Polyenes: These are antifungals that work through disruption of fungal cell membrane. Example of such drug is Amphotericin-B and the resistance mechanism is by defective ergosterol biosynthesis, due to mutation in the ERG3 gene.

Azoles: These antifungal drugs act by inhibition of ergosterol synthesis. They interfere with the ergosterol biosynthesis by inhibiting the activity of 14 α-lanosterol demethylase which is involved in the conversion of lanosterol to ergosterol (Prasad, 2016). This inhibition results in the depletion of cellular membrane ergosterol components and therefore affects cell growth and cell division. Examples of these drugs are Fluconazole, Itraconazole, Voriconazole, Efinaconazole (approved in 2014), Terconazole, Posaconazole, Imidazole,

Isavuconazonium (a triazole-approved in 2015) (Ali *et al*., 2017). Resistance of this drug is by modification of the target enzyme due to point mutations in ERG11 and active efflux of the drug out of the cell through the activation of efflux transport protein.

Flucytosine: Inhibits DNA and protein synthesis. Fungi resist these drugs by the alterations in the target enzymes (Cytosine permease and Cytosine deaminase), or increased production of pyrimidines.

Echinocandins: e.g. Caspofungin. They work by inhibition of β-1, 3-D-glucan synthesis and fungi drug resistance mechanism is by generating insufficient amount of the target enzyme, β-1, 3-D-glucan synthase (Sanglard and Odds, 2002).

1.10 Physiology and Anatomy of Gastro Intestinal Tract (GIT)

The stomach plays a crucial role in the digestion of foods consumed. This organ can resist to a great extent the varieties of detrimental factors such as hydrochloric acid, alcohol, refluxed bile salts, and other irritating agents. Maintaining this high resistance to damage is possible because of the presence of a number of physiological defensive mechanisms as well as the ability of rapid repair of injured mucosa when such occurs. The stomach consists of three topographic and two functional (oxyntic and pyloric gland) areas. The three topographic areas include the fundus, corpus and antrum. The oxyntic gland area, the hallmark of which is the oxyntic or parietal cell, made up of 80% of the organ (fundus and corpus). The pyloric gland area, the hallmark of which is the gastrin or G cell, comprises 20% of the organ (antrum) (Schubert and Peura, 2008).

1.10.1 Gastric Secretions

Gastric acid is a fluid formed in the stomach, which plays an important role in digestion of proteins by activating digestive enzymes. The main constituent of gastric acid is hydrochloric acid, which is produced by parietal cells in the gastric gland in the stomach. The pH of gastric acid is 1.5–3.5 in the stomach lumen. Gastric acid is established as one of the major ulcerogenic factor for the induction of gastric ulcer disease. It has been reported that about 50% of gastric ulcer patients are pepsin and acid hyper secretors. But, on the other hand, gastric acid plays a good role in gastric defense. It is the first line of mucosal defense to prevent bacterial colonization and reduced bacteria ability to enter and gain access to the mucosal layer. The key substrate in the production of gastric acid is carbon iv oxide ($CO_{2)}$. The hydrogen ion concentration in parietal cell secretions is roughly 3 million fold higher than in blood, and chloride is secreted against both a concentration and electric gradient.

Thus, the ability of the parietal cell to secrete acid is dependent on active transport. The key player in acid secretion is H^+/K^+ ATPase or "proton pump" located in the cannalicular membrane. Hydrogen ions are generated within the parietal cell from dissociation of water. The hydroxyl ions formed in this process rapidly combine with carbon dioxide to form bicarbonate ion, a reaction catalyzed by carbonic anhydrase. The bicarbonate formed is transported out of the basolateral membrane in exchange for chloride. The outflow of bicarbonate into blood results in a slight elevation of blood pH known as the "alkaline tide". This process serves to maintain intracellular pH in the parietal cell. Chloride and potassium ions are transported into the lumen of the cannaliculus by conductance channels, and such is necessary for secretion of acid. Hydrogen ion is pumped out of the cell, into the lumen, in exchange for potassium through the action of the proton pump; potassium is thus effectively recycled. Accumulation of osmotically-active hydrogen ion in the cannaliculus generates an osmotic gradient across the membrane that results in outward diffusion of the gastric juice. The gastric juice is 155 mM Hydrochloric acid (HCl) and 15 mM Potassium Chloride (KCl) with a small amount of Sodium Chloride (NaCl) (Samuelson and Hinkle, 2003).

1.10.2 Regulation/ Control of Gastric Secretion

Gastric acid secretion is a complex, continuous process controlled by multiple central (neural) and peripheral (endocrine and paracrine) factors (Fig.10). Each factor attributes to a common final physiological event, the secretion of H^+ by parietal cells. The parietal cell is located in the body and fundus of the stomach. Neuronal (acetylcholine), paracrine (histamine), and endocrine (gastrin) factors play important roles in the regulation of acid secretion. Histamine type-2 receptors (H_2R) appear to represent the key receptor, as H_2R antagonists inhibit not only histamine-stimulated acid secretion, but also gastrin- and acetylcholine-stimulated acid secretion. Histamine released from enterochromaffin-like (ECL) cells is a critical regulator of acid production through the H_2 subtype of receptors. Histamine stimulates the parietal cell directly by binding to H_2-receptors coupled to activation of adenylate cyclase and generation of adenosine 3', 5'-cyclic monophosphate (cAMP). It is also important to note that the enterochromaffin-like cell (ECL) is the only source of gastric histamine in gastric acid secretion. Gastrin is produced from the D cell. Two major cholecystokinin (CCK) receptors have been characterized. They are CCK_1 receptor which recognizes only CCK and CCK_2 receptor which recognizes both CCK and gastrin with very high affinity. Gastrin has three major effects:

(i) Gastrin stimulates acid secretion directly from the parietal cell by activation of cholecystokinin receptor (CCK)

(ii) It also indirectly stimulates activation of the CCK2 receptor on the ECL cell with release of histamine (Schmitz et al., 2001).

(iii) Finally, gastrin stimulates the release of Somatostatin from gastric D cells.

Parietal cell secretion is achieved by activation of intracellular cAMP- and calcium-dependent signalling pathways that activate downstream protein kinases, ultimately leading to fusion and activation of H+/K+-ATPase, the proton pump. Somatostatin is the main inhibitor of acid secretion; it is localized in the antral D cells. In stomach, somatostatin cells are closely coupled to their target cells (e.g., parietal, ECL, and gastrin cells) either directly via cytoplasmic processes or indirectly via the local circulation. The release of Somatostatin is the key regulation of gastric acid production as it inhibits the release of histamine from the ECL cell, inhibits gastrin and the parietal cell. Figure10 below shows the sites of secretion and regulation points.

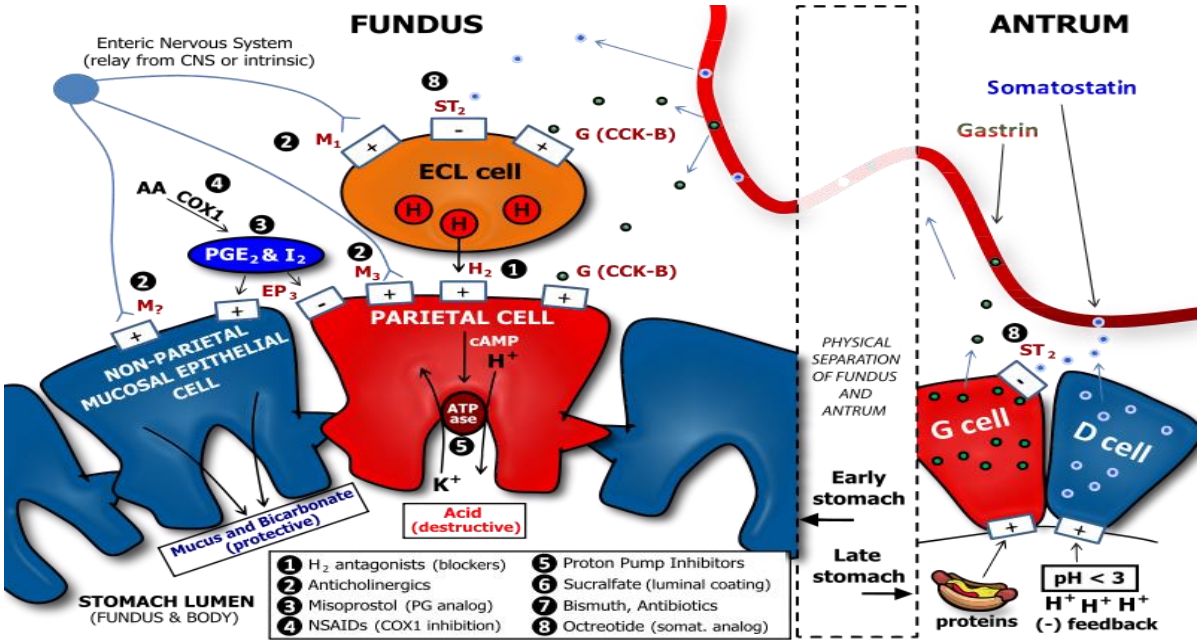


Fig.10: Physiological and pharmacological regulation of gastric secretions

Source: Schubert and Peura (2008).

1.11 Peptic Ulcer Disease (PUD)

Peptic ulcer is a sore or hole in the lining of the stomach or duodenum. It is a mucosal defect that its depth of wound reaches the muscularic mucosa and beyond. It is usually associated with severe complications including hemorrhage, perforations, gastrointestinal obstruction,

and malignancy. Different factors as the age, gender and geographical location may affect the incidence rates of peptic ulcer and its complications. Important aggressive factors include hydrochloric acid together with pepsin secreted by the gastric mucosa and also bile and pancreatic enzymes. Among aggressive factors of exogenous origin are cigarette smoking, NSAIDs and steroids (Abdel-Salam *et al.*, 2001). Also protective factors include mucus, prostaglandin, mucosal blood flow, nitric oxide and bicarbonate. In normal stomach, mucosa maintains its integrity because of the natural balance between these protective and aggressive factors affecting the stomach mucosa. The inhibition of gastric acid secretion is still a key therapeutic target for the ulcer diseases of any cause. Peptic ulcer may be gastric (if the ulceration is on the stomach) or duodenal (if the ulceration occurs in the duodenum or first part of the intestine.) depending on the site of injury.

1.11.1 Aetiology of Peptic Ulcer Disease

There are many factors that contribute to the development of peptic ulcer disease. These predisposing factors include uncontrolled gastric acid secretion, *Helicobacter pylori*, Non-steroidal anti-inflammatory drugs (NSAID), Alcohol and stress.

Mechanism of mucosal injury by Non-Steroidal Anti-Inflammatory Drugs (NSAID): NSAIDs are valuable therapeutics that works not only as anti-inflammatory, but also as analgesics and antipyretics. They are used in a wide variety of clinical conditions, including arthritis and other musculoskeletal disorders. The pathophysiology of gastric injury associated with NSAID administration depends partly on cyclooxygenase inhibition and partly on cyclooxygenase-independent mechanisms, which result mainly from local direct actions (Scarpignato and Hunt, 2010). NSAID induce injury by suppression of a number of gastric prostaglandin-mediated protective functions. For instance, prostaglandins reduce the activation of neutrophils and the local release of reactive oxygen species (ROS). NSAIDs inhibit cyclooxygenase (COX), which is the rate-limiting enzyme required for the conversion of arachidonic acid to prostaglandins. Two distinct isomers of COX have been identified and referred to as COX-1 and COX-2. The inducible COX-2 is an important regulator to generate prostaglandins that mediate inflammation and pain; it is expressed in high concentrations at sites of inflammation and carcinogenesis. The constitutive COX-1 is produced in virtually all body tissues, and it is responsible for maintenance of the integrity of gastric mucosa (by regulation of mucus and bicarbonate secretion, mucosal blood flow etc), platelet aggregation, sodium and water balance. The anti-inflammatory, antipyretic and analgesic properties of NSAIDs are mediated through inhibition of COX-2, whereas adverse effects, such as

gastroduodenal ulceration, occur as a result of suppression of COX-1 (Dickman and Ellershaw, 2004). Taking certain other medications along with NSAIDs, such as steroids, anticoagulants, low-dose aspirin, selective serotonin reuptake inhibitors (SSRIs), alendronate (Fosamax) and risedronate (Actonel), can also greatly increase the chances of developing ulcers.

Helicobacter pylori: It is a gram negative bacterium. All *Helicobacter spp*. cause some degree of persistent inflammation in the mammalian stomach. *H. pylori* is able to survive and multiply in gastric environment due to the numerous adaptations that permit its survival in the acidic medium of the stomach. H. *pylori* get its protection by production of urease which converts urea to ammonia and carbon dioxide and these products elevate the pH level and hence neutralize the acid in the stomach where it causes inflammation and triggers gastric ulcer disease. *H. pylori* infection stimulates over-expression of pro-inflammatory cytokines (*e.g*., interleukin (IL)-1β, IL-6, tumor necrosis factor (TNF)-α, and IL-8) in gastric epithelial cells, which act as neutrophil-activating chemokines and lead to leukocyte infiltration. Once the organism is established in the gut, pathogenic effects on the host may be produced by one or several means; examples are physical effects, elaboration of enzymes or toxins, and competition with the host for nutrients. *H. pylori* causes ulceration through the following ways:

Binding to mucus and epithelial cells: *H. pylori* have cell wall associated lectins which allow them to bind selectively to mucus and epithelial cells. Targets of *H. pylori* lectins exist in the gastric mucus as glycoproteins and glycolipids. *H. pylori* appears to bind to all of these, including sulfated (acid) mucins, L-fructose, D-galactose and sialic acids. *H. pylori* lectins also attaches to red blood cells of various animal species (Emody *et al*.,1988).

Tight attachment to cells: *H. pylori* attaches tightly to the epithelial cell, and a characteristic structure called an ‘attachment pedestal’ forms. This attachment causes localized cellular damage characterized by increment of microvilli and disruption of cytoskeletal elements of the cell (Goodwin *et al*., 1986).

Release of enzymes and toxins:

(A) **Urease:** Most common and virulence producing enzyme produced by *H. pylori* is urease. This enzyme is highly active between the pH of 5 and 8. It hydrolyses urea into ammonia. The generated ammonia act as a potent cellular toxin in 3 ways: Firstly, it combines with α-ketoglutarate to form glutamine, thus depleting Kreb’s cycle of an essential intermediate

substrate. Secondly, it interacts with hypochlorus acid to form mono-n-chloramine, which also acts as potent cellular toxin (Graham *et al*., 1992). Thirdly, its toxic effect may also be mediated by neutrophil-generated oxygen radicals because it is inhibited by anti-neutrophil serum. Thus, urease enzyme plays an important role in virulence of *H. pylori* and its colonization in gastric mucosa. In the course of *H. pylori* infection, accumulation of phagocytic cells in the gastric mucosa occurs through two distinct mechanisms: (i) neutrophil recruitment through Interleukin (IL) 8 production which is then released by the gastric epithelial cells, and (ii) release by the bacterium itself of substances with chemotactic activity able to attract phagocytes. These phagocytic cells ingest the microorganism and, as in the case of other pathogens, destroy it through oxygen-dependent and oxygen independent mechanisms. The release of free oxygen radicals by the neutrophils might play a role in the genesis of chronic inflammation and in the development of peptic ulcer (Salim, 1993).

(B) Phospholipases A_2 and C: The epithelial cell membrane consists of a phospholipid bilayer. Phospholipids are similar to triglycerides except that one of the terminal fatty acids is replaced by a phosphate group and hence the name -**phospholipids**. Phospholipase A_2 of *H. pylori* is an enzyme from *H. pylori* that removes a long-chain fatty acid group from the second carbon. It also attacks membrane phospholipids to liberate arachidonic acid which may then be converted to leukotriene, prostaglandin or thromboxane. These compounds are known to cause mucus release, chemotaxis of inflammatory cells and altered membrane permeability. Phospholipase C removes the phosphate group from the third carbon of the phospholipids. The resultant compounds, diacyl glyceride and particularly lysolecithin, are incapable of forming the normal phospholipid bilayers and may form micellar structures instead, potentially affecting the integrity of the epithelial cell membrane (Marshall, 1991).

(C)Production of Toxins: *H. pylori* also produces certain chemotoxins, known as vacuolating cytotoxin (VacA) which directly act on epithelial cell surface and damage the defence system.

Alcohols and stress

Alcohols and stress are also factors that contribute to the development of stomach ulcer. The epithelial lining of the gastric and intestinal mucosa is continually exposed to varied changes in chemical substances arising from intake of foods, drugs and drinks. As a result, tremendous disturbances that may culminate in pathological conditions such as ulcers, cancers could arise (Rang *et al*., 1995). Pathogenesis of ulcer has been attributed to effect of acid/ethanol (Goulart *et al*., 2005). Ethanol causes stomach ulceration by solubilization of the

stomach lining, hence making it easier for penetration of other chemicals and injurious agents. Ethanol damage to the gastrointestinal mucosa starts with micro-vascular injury, namely disruption of the vascular endothelium resulting in increased vascular permeability, oedema formation and epithelial lifting. These effects are secondary to ethanol induced slowing or cessation of gastric mucosal flow. Alcohol causes the stomach cells to over secrete both acid and histamine which make the stomach linings vulnerable to ulcer formation. Ethanol also reduces prostaglandin levels, increases the release of histamine and influx of calcium ions. Ethanol also produces a marked contraction of the circular muscles of fundic strip. Such a contraction can lead to mucosal compression at the site of the greatest mechanical stress, at the crests of mucosal folds leading to necrosis and ulceration. This reduces the secretion of bicarbonates and production of mucus and also leads to increased neutrophil infiltration into the gastric mucosa. Ethanol induces the damage of endothelia cell through the release of proteases, leukotriene and oxygen free radicals. The formed oxygen free radicals also cause increased lipid peroxidation which cause damage to the cell and cell membrane, thereby playing a major role in the pathogenesis of acute mucosal injury induced by ethanol (Shetty *et al*., 2000). Stress has also been found to be among the causes of stomach ulceration.

1.11.2 Symptoms and Diagnosis of Peptic Ulcer Disease

Symptoms: signs and symptoms of peptic ulcer include the following**:**

1. Abdominal Pain, classically epigastric strongly correlated to mealtimes. In case of duodenal ulcers, the pain appears about three hours after taking a meal;
2. Bloating and abdominal fullness (i.e. abdominal swelling)
3. Nausea and copious vomiting;
4. Loss of appetite and weight loss;
5. Haematemesis (vomiting of blood); this can occur due to bleeding directly from a gastric ulcer, or from damage to the esophagus from severe/continuing vomiting.
6. Melena (tarry, foul-smelling feces due to presence of oxidized iron from haemoglobin)
7. Rarely, an ulcer can lead to a gastric or duodenal perforation, which leads to acute peritonitis (inflammation of the abdominal lining), extreme stabbing pain and requiring immediate surgery.

Diagnosis of peptic ulcer disease: During diagnosis, peptic ulcer disease is typically suspected due to the presenting symptoms with confirmation by either esophagogastroduodenoscopy (EGD) or barium swallow. An esophagogastroduodenoscopy (EGD), a form of endoscopy, also known as a gastroscopy, is carried out on people suspected to have peptic ulcer. It is also the gold standard of diagnosis for peptic ulcer disease. By direct visual identification, the location and severity of an ulcer can be described (Lanas and Chan, 2017). *H. pylori* can be diagnosed by testing the blood for antibodies, a urea breath test, testing the stool for signs of the bacteria. Blood tests are not reliable for accurate peptic ulcer diagnosis on their own because of their inability to differentiate between past exposure to the bacteria and current infection. Additionally, a false negative result is possible with a blood test if the patient has recently been taking certain drugs, such as antibiotics or proton-pump inhibitors.

1.11.3 Management of Peptic Ulcer Disease

Gastric and duodenal ulcers occur because of an imbalance between aggressive factors (gastric acid and pepsin) and mechanisms that maintain mucosal integrity (mucosal defense and repair). There are many classes of drugs that are used in the management of peptic ulcer disease. These classes include H_2-receptor antagonists, proton pump inhibitors, mucosal protective agents and antibiotics (for treatment of *H. pylori*).

Proton pump inhibitors (PPIs): The gastric proton pump, H^+ / K^+ ATPase present in cytoplasmic membranes constitutes p2 type phosphoenzyme concentrated in resting parietal cells from where it secretes H^+ into the lumen of the gastric glands in electroneutral exchange for extracellular K^+. Gastric proton pump inhibitors (PPIs) are important as well as valuable pharmaceuticals in the treatment of peptic ulcer disease. PPIs act by inhibiting the action of H^+K^+-ATPase, an enzyme that occurs almost exclusively in the gastric parietal cell. The enzyme catalyzes the exchange of protons (H^+) for potassium ions at the cell membrane, i.e. the final step in the acid secretory process, sometimes called the proton pump. Therefore, they antagonize all stimulants of gastric secretion (Laurence and Bennett, 1992). PPIs bind to cysteine 813, resulting in covalent inhibition of the enzyme via formation of disulphide that stabilizes the enzyme in the E2 conformation (Sachs *et al*., 2006). Several salient features required for the activity are:

(a) The weak basicity of the compounds (pka » 4), allowing them to accumulate in the acid space adjacent to their site of action.

(b) The sulphoxides undergoes Smile's rearrangement to form spiro intermediate.

(c) The active thiophillic group binds to thiol group of enzymes and generates disulphide bridges to cause enzyme inactivation.

The protonation in the pyridine nitrogen initiates the formation of spiro intermediate and then sulfonamide. This sulfonamide binds covalently to sulfohydryl groups of cysteine of proton pump (Hardman *et al.*, 2001). Examples of proton pump inhibitors are omeprazole, lansoprazole, rabeprazole, pantaprazole etc.

H_2 receptor antagonists: These drugs reduce gastric acid secretion, and are used to treat peptic ulcer disease and gastric acid hypersecretion. Histamine receptor antagonists exhibit competitive inhibition at the parietal cell H_2 receptor. The volume of gastric secretion and concentration of pepsin are also reduced. Four H_2 receptor antagonists have been used worldwide - cimetidine, ranitidine, famotidine and nizatidine. Also roxatidine has been marketed in a number of regions. These agents are specific antagonists that inhibit acid secretion by competitively and reversibly blocking the H_2-receptors on the basolateral membrane of the parietal cell. H_2 antihistamines reduce acid secretion stimulated by histamine as well as by gastrin and cholinomimetic agents through two mechanisms. The first mechanism is that histamine release from enterochromafin-like cell (ECL cells) by gastrin or vagal stimulation is blocked by binding to the parietal cell H_2 receptor. Secondly, direct stimulation of the parietal cells by gastrin or acetylcholine results in diminished acid secretion in the presence of H_2 receptor blockade. It appears that reduced parietal cell cyclic adenosine monophosphate (cAMP) levels reduce or lessen the intracellular activation of protein kinases by gastrin and acetylcholine (Katzung, 2004).

Cytoprotectants/ ulcer protectives: These are chemical compounds used to provide protection to gastric cells against harmful agents. They combat ulcers not by reducing gastric acid but by increasing mucosal protection by binding to tissue proteins. Examples of gastric cytoprotective drugs include sucralfate (a complex sucrose salt in which hydroxyl group is substituted by aluminum hydroxide and sulphate), misoprostol and zinc L-carnosine. Sucralfate may act by two mechanisms:

In the gastric environment, aluminum hydroxide dissociates, leaving the polar sulphate anion, which can bind to positively charged tissue proteins found within the ulcer bed, and providing a physicochemical barrier impeding further tissue injury by acid and pepsin. Also,

it may induce a trophic effect by binding growth factors such as EGF, enhance prostaglandin synthesis, stimulate mucous and bicarbonate secretion, and mucosal defence and repair.

Antibiotics: Antimicrobial agents that are clinically effective against *H. pylori* are: amoxicillin, clarithromycin, tetracycline and metronidazole. However, any single drug is relatively ineffective because resistance develops rapidly, especially to metronidazole. Earlier, bismuth was used in the combination regimens for eradication of *H. pylori* but due to poor patient acceptability is infrequently used now. The use of antibacterial agents is also helpful in the eradication of some enteric bacteria. Combination of antibacterial and antisecretory agents is now being used as combination therapy for treatment of gastric ulcer.

1.12 Rationale of the study

Herbal products have been found to express less *in vivo* activity when compared to the *in vitro* activity. As a result of this, large and frequent dosing are often being used to achieve a therapeutic effect. This high and frequent dosing affects the acceptability of these herbal products by the public. Therefore, enclosing the plant products in a phospholipid via phytosome formulation will not only improve the absorption and bioavailability of the drug but also achieve high efficacy with a smaller dose.

1.13 Aim and Objectives of the Study

1.13.1 Aim of the Study

The aim of the study was to produce and compare the phytosomes of ethanol extracts of *Bridelia ferruginea* and *Bryophyllum pinnatum* leaves for enhanced antimicrobial and ulcer-protective properties.

1.13.2 Specific Objectives of the Study

Objectives of the study included:

1. Qualitative and quantitative determination of phytochemicals present in the ethanol extracts of *Bridelia ferruginea* and *Bryophyllum pinnatum* leaves.
2. Formulation and characterisation of phytosome complexes of ethanol extracts of *Bridelia ferruginea* and *Bryophyllum pinnatum*.
3. Assay for *in vitro* antioxidant activities (DPPH, TAC and FRAP) and *in vivo* (SOD, CAT, GPx and MDA) oxidative status of ethanol extracts of *Bridelia ferruginea* and *Bryophyllum pinnatum* leaves and their phytosomes.
4. Antimicrobial sensitivity tests using ethanol extracts of *Bridelia ferruginea, Bryophyllum pinnatum* leaves and their respective phytosome complexes.

5. Determination of the median lethal dose (LD_{50}) of ethanol extracts *of Bridelia ferruginea* and *Bryophyllum pinnatum* leaves
6. Determination of the ulcer-protective properties of ethanol extracts of *Bridelia ferruginea*, *Bryophyllum pinnatum* leaves and their phytosome complexes in indomethacin-induced ulcer model.
7. Determination of the effects of ethanol extracts of *Bridelia ferruginea*, *Bryophyllum pinnatum* and their phytosomes on the activities of liver enzymes (alanine aminotransferase, aspartate aminotransferase and alkaline phosphatase) and kidney markers (creatinine and urea concentrations) in the ulcerated rats.
8. Determination of the effects of ethanol extracts of *Bridelia ferruginea, Bryophyllum pinnatum* and their phytosome complexes on the lipid profile of the ulcerated rats.
9. The histological examination of the stomach tissues of the ulcerated rats.

CHAPTER TWO

MATERIALS AND METHODS

2.1 Materials

2.1.1 Plant Material

Plant collection and identification: The *Bridelia ferruginea and Bryophyllum pinnatum* plants were collected from Opi in Nsukka and Aku in Igbo-etiti Local Government Areas respectively, in Enugu State, Nigeria. Both plants were identified by Mr. Alfred Ozioko of Bioresources Development and Conservation Programme (BDCP) Nsukka Enugu State, Nigeria.

Phospholipid

Phospholipid (Phospholipon®90H) used, was obtained from Germany through Dr. P.O Nnamani; Department of Pharmaceutics, University of Nigeria Nsukka.

2.1.2 Microorganisms

Microorganisms used were clinical isolates obtained from Bishop Shanahan Hospital, Nsukka and Department of Microbiology, University of Nigeria Nsukka. Both Gram positive and Gram negative bacteria were used in this study. Test organisms used included *Escherichia coli, Klebsiella pneumonia, Staphylococcus aureus, Salmonella wangiadata, Enterobacter aerogenes, Liesteria ivanovi, Pseudomonas aeruginosa, Aspergillus fumigatus* and *Candida albicans.*

2.1.3 Experimental Animals

A total of one hundred and twenty (120) adult Wistar albino rats were used for the ulcer protective studies. Seventy-two (72) mice were used for the acute toxicity study (LD_{50}). All animals were obtained from the animal house of Department of Zoology and Environmental Biology, University of Nigeria, Nsukka.

2.1.4 Chemicals and Reagents

The chemicals and reagents used for this research work were of analytical grade. They included acetone (Sigma, London), aluminum chloride (BDH, England), chloroform (Sigma, London), cimetidine (Greenlife pharmaceuticals Nigeria), distilled water (Department of Industrial Chemistry, UNN), dichloromethane (Lobal Chemie Laboratory Reagents, India), ethanol (BDH, England), Fehling's solutions A and B (BDH, England), ferric chloride (Merck, Germany), glacial acetic acid (Sigma, London), hydrochloric acid (BDH, England),

iodine crystals (Merck, Germany), indomethacin (20 mg) (Greenlife Pharmaceuticals, Nigeria), lead acetate (BDH, England), mercuric chloride (Sigma London), million's reagent (BDH, England), monobasic potassium phosphate, nutrient agar (Life Save Biotech, USA), olive oil (Goya, Nigeria), peptone water (Life Save Biotech, USA), picric acid (Lab Tech Chemicals, London), potassium iodide (East Angelis, United Kingdom), Tween 80 (STC, UNN), sodium chloride (BDH, England), sulphuric acid (BDH, England).

2.1.5 Instruments and Equipment

The equipment's used were obtained from the Department of Biochemistry, University of Nigeria Nsukka and other scientific laboratories in Nsukka. They include beaker (Pyrex), centrifuge (PAC, Pacific), colorimeter (Techmel and Techmel), conical flask (pyrex), filter paper (Whatman), FT-IR spectrometer (Schimadzu 4300, Japan), incubator (Gallenkamp, England), Megapixel microscope camera, oven (Gallenkamp, England), refrigerator (Haier Thermocool), scanning electron microscope (Phenom ProX by Phenom World Eindhoven Netherlands), spectrophotometer (E312 model) (Jenway, England), thermometer (Zeal), water bath (Gallenkamp, England). Weighing balance (Metler HAS).

2.2 Methods

2.2.1 Preparation of Reagents

Ferric chloride solution (5% (w/v))

A quantity, 5.0 g ferric chloride, was dissolved in 100 ml of distilled water.

Ammonium solution

Stock concentrated ammonium solution (187.5 ml) was diluted in 31.25 ml of distilled water and then made up to 500 ml with distilled water.

Ethanol (45% (v/v))

A quantity, 45 ml of absolute ethanol, was mixed with 55 ml of distilled water.

Aluminium chloride solution

Aluminium chloride (0.5 g) was dissolved in 100 ml of distilled water.

Dilute sulphuric acid

A 50 ml aliquot of concentrated sulphuric acid was mixed with 5 ml of distilled water and made up to 100 ml.

Lead sub acetate solution

A quantity, 45 ml of 15% lead acetate (i.e. 15.0 g of lead acetate in 100 ml of distilled water) was dissolved in 20 ml of absolute ethanol and made up to 100 ml with distilled water.

Wagner's reagent

A known quantity, 2.0 g, iodine crystals and 3.0 g potassium iodide were dissolved in minimum amount of water and then made up to 100 ml with distilled water.

Mayer's reagent

A known weight, 13.5 g, of mercuric chloride was dissolved in 50ml of distilled water. Also, 5.0 g of potassium iodide was dissolved in 20 ml of distilled water. The two solutions were mixed and the volume was made up to 100 ml with distilled water.

Dragendorff's reagent

Bismuth carbonate (0.85 g) was dissolved in 100 ml of glacial acetic acid and 40 ml of distilled water to give solution A. Another solution designated solution B was prepared by dissolving 8.0 g of potassium iodide in 20 ml of distilled water. Both solutions were mixed to give a stock solution.

Hydrochloric acid (2% (v/v))

Measured volume, 2.0 ml concentrated hydrochloric acid was diluted with some distilled water and made up to 100 ml.

Picric acid (1% (w/v))

Picric acid (1.0 g) was dissolved in 100 ml of distilled water.

Normal saline

Normal saline was prepared by dissolving 0.9 g sodium chloride in distilled water and made up to 100 ml.

Trichloroacetic acid (TCA) (25%)

A known weight, 25 g trichloroacetic acid was dissolved in distilled water and made up to the 100 ml mark with distilled water in a measuring cylinder.

Thiobarbituric acid (TBA) (1%)

One (1 g) thiobarbituric acid was dissolved in distilled water and made up to the 100 ml mark with distilled water in a measuring cylinder.

Sodium hydroxide (NaOH) (0.3%)

Sodium hydroxide (0.3 g) was dissolved in a small quantity of water and made up to 100 ml mark with distilled water in a measuring cylinder.

Sodium hydroxide (0.1M NaOH)

Sodium hydroxide (4 g) was dissolved in small quantity of distilled water and then made up to one litre. Also 0.01N NaOH was prepared by dissolving 0.4 g of NaOH in little quantity of distilled water and then made up to one litre.

Sodium dodecyl sulphate (SDS) (20%)

A quantity, (20 g) sodium dodecyl sulphate was dissolved in some quantity of distilled water and made up to the 100 ml mark with distilled water in a measuring cylinder.

Glacial acetic acid (10%)

A known quantity, 10 ml of glacial acetic acid, was made up to 100 ml with distilled water in a measuring cylinder.

Phosphate buffer (pH 7.4).

Phosphate buffer was prepared by dissolving (5.67 g) anhydrous monopotassium phosphate in 1 litre of distilled water and the pH adjusted.

Thymol blue indicator

This was prepared by dissolving 0.1 g of thymol blue in 2.15 ml of 0.1 M NaOH solution. A quantity of 20 ml of 95% ethanol was added to it and finally, made up to 100 ml mark with distilled water.

Simulated gastric fluid (pH 1.2)

A given quantity of 1 g of sodium chloride (NaCl) was dissolved in 7 ml of concentrated hydrochloric acid and the volume of the whole content was made up to 1000 ml mark with distilled water. The pH was then adjusted to 1.2.

Simulated intestinal fluid (pH 6.8)

A given quantity of 2 g of monobasic potassium phosphate was dissolved in 190 ml of 0.2 N sodium hydroxide solution, the pH was adjusted with pH meter and the volume of the content was made up to 1000 ml with distilled water.

Alcoholic buffer (pH 1.5)

Alcoholic buffer was prepared by mixing of simulated gastric buffer and ethanol together in the ratio of ethanol: simulated gastric buffer of 2:1.

0.5 MC Farland Standard

MC Farland turbidity standards were prepared by mixing various volumes of 1% barium chloride and 1% sulphuric acid to obtain a solution with specific optical densities. The 0.5 MC Farland turbidity standard provides optical density comparable to the density of a bacterial suspension 1.5 x 10^8 cfu/ml. This bacterial population turbidity standard was prepared by mixing 0.05 ml of 1% barium chloride with 9.95 ml of 1% sulphuric acid. The resulting turbid suspension of barium sulphate was tightly caped and stored at room temperature.

2.2.2 Preparation of Plant Materials: The leaves of *Bridelia ferruginea* and *Bryophyllum pinnatum* were air-dried and pulverized to fine powder.

2.2.3 Extraction of Plant Materials

A quantity, 1150 g of the pulverized *Bridelia ferruginea* leaves was macerated in 5 litres of absolute ethanol. The mixture was left for 48 h. Also 1000 g of *Bryophyllum pinnatum* dry leaves (powder) was soaked with 2.5 litres of absolute ethanol. The mixture was also left for 48 h. Each of the solutions was filtered using Whatman $N_0$1 filter paper. The filtrates were collected and concentrated with rotary evaporator at 60^0C.

2.2.4 Preparation of test Organisms

Inoculum size of 10^8 colony forming unit per milliliter (cfu/ml) of each of the isolates was prepared according to the method described by Irobi *et al. (*1994). This was effected by suspending loopful of inoculums from stock into test tubes containing 5ml of nutrient broth and incubated at 37ºC for 24 h. The resultant culture was diluted with fresh nutrient broth in order to achieve optical density corresponding to 10^8 cfu/ml. This was repeated for all the bacteria used for this study before use.

2.2.5 Qualitative Phytochemical Screening of *Bridelia ferruginea* and *Bryophyllum pinnatum* Leaves

Qualitative analysis was carried out according to the methods of Sofowora (1993), Trease and Evans (2002) and Harbone (1998).

Test for Carbohydrates

This was determined according to the method of Sofowora (1993). Few drops of Molisch's reagent were added to each of the portions of extract dissolved in water. This was followed by addition of 1ml of concentrated sulfuric acid (H_2SO_4) by the side of test tube. The mixtures were allowed to stand for two minutes and then diluted with 5ml of distilled water. Formation of red or dull violet colour at the interphase of the two layers indicated a positive test.

Test for Reducing Sugars

Extract (0.5g) was dissolved in distilled water and filtered; the filtrate was heated with 5ml of equal volume of Fehling's solutions A and B, formation of red precipitate of cuprous oxide was an indication of the presence of free reducing sugar (Sofowora, 1993).

Test for Tannins

A quantity of extract (0.5g) was boiled in 10ml of distilled water in a test tube and then filtered with Whatman No 1 filter paper. Then, 0.1% ferric chloride ($FeCl_3$) solution was added to 2ml of the filtrate. Occurrence of blue-green precipitate indicated the presence of tannins (Trease and Evans, 2002).

Test for Steroids

Ethanol (9ml) was added to 1g of the sample refluxed for a few minutes and filtered. The filtrate was concentrated to 2.5ml on boiling water bath, and 5ml of hot water was added. The mixture was allowed to stand for 1 hour, and the waxy matter filtered off. The filtrate was extracted with 2.5ml of chloroform using a separating funnel. To 0.5ml of the chloroform extract in a test tube was carefully added 1ml of concentrated sulphuric acid to form a lower layer. A reddish-brown interface showed the presence of steroids.

Test for Saponins

A quantity of the sample (1g) was boiled with 5ml of distilled water and filtered. To the filtrate, 3ml of distilled water was further added and shaken vigorously for 5 minutes. Frothing which persisted on warming was taken as evidence for the presence of saponins.

Test for Flavonoids

A quantity, 0.5g of sample, was boiled with distilled water and then filtered. To 2ml of the filtrate, few drops of 10% ferric chloride solution were added. A green-blue or violet colouration indicated the presence of phenolic hydroxyl group (Trease and Evans, 2002).

Test for Alkaloids

Plant sample (0.2g of extract) was boiled with 5ml of 2% hydrochloric acid (HCl) on a water bath. The mixture was filtered and 1ml portion of the filtrate was treated with 2 drops of of the following reagents:

(I) Dragendorff's reagent: An orange precipitate indicated the presence of alkaloids.
(II) Mayer's reagent: A creamy-white precipitate (ppt) indicated the presence of alkaloids.
(III) Wagner's reagents: A reddish–brown precipitate indicated the presence of alkaloids. (Sofowora, 1993).

Test for Glycosides

A quantity of the sample (2.0g) was mixed with 30ml of distilled water and heated on a water bath for 5 minutes and filtered. The filtrate was used for the following test. A quantity, 0.3ml

of Fehling's solutions A and B was added to 5ml of the filtrate until it turned alkaline (tested with litmus paper) and heated on a water bath for 2 minutes. A brick-red precipitate indicated the presence of glycosides (Harborne, 1998).

Test for Terpenoids

About 0.5 ml aliquot of the extract was evaporated to dryness on a water bath and heated with 3 ml of concentrated sulphuric acid for 10 minutes. A grey colour indicated the presence of terpenoids.

Test for Phenols

Distilled water (2 ml) was added to 0.1g of methanol extract, followed by addition of sodium carbonate (0.5 ml) and Folin Ciocalteau's reagent (0.5ml). Formation of green colour indicated the presence of phenols.

Hydrogen Cyanide (HCN)

The sample (0.5 g) was dissolved in 2 ml of glacial acetic acid, containing 1 drop of 1 % ferric chloride. Few drops of concentrated H_2SO_4 were added. Formation of a brown ring at the interface indicated the presence of hydrogen cyanide (Trease and Evans, 2002).

2.2.6 Quantitative phytochemical analyses of *Bridelia ferruginea* and *Bryophyllum pinnatum* Leaves

Hydrogen Cyanide Concentration

This was determined according to the method of El-Olemyl *et al.* (1994). A quantity (1 g) of the extract was macerated with 50 ml of distilled water and filtered. To (1 ml) filtrate, 4 ml of alkaline picrate solution was added, boiled for 5 min and cooled at room temperature. Measurement of the absorbance of the solution in the tube was at 490 nm.

$$\text{Hydrogen Cyanide conc.} = \frac{\text{abs} - \text{blank}}{\text{Slope}} \times \frac{\text{volume of solvent}}{\text{wt of sample}}$$

Alkaloids Concentration

The determination of alkaloid was as described by Harborne (1998). The sample (5.0g) was weighed into a 250ml beaker and 200ml of 10% acetic acid in ethanol was added, covered and allowed to stand for 2 hours. This was filtered and the extract was concentrated on a water bath to one-quarter of the original volume. Concentrated ammonium hydroxide was added drop-wise to the extract to obtain the precipitate which was collected and washed with

dilute ammonium hydroxide and then filtered. The residue was the alkaloid, which was dried and weighed.

Flavonoids Concentration

The sample (1.0 g) was macerated with 20 ml of ethylacetate for 5 minutes. Dilute ammonia (0.5 ml) was added to 5ml of the filtrate and shaken for 5 minutes. The upper layer was collected. The absorbance was measured at 490 nm.

Steroids Concentration

This was determined by the method described by Okeke and Elekwa (2003). A known weight of the sample was dispersed in 100ml of distilled water and homogenized in a laboratory blender. The homogenate was filtered and the filtrate was eluted with normal ammonium hydroxide solution (pH 7.4). About 2ml of the resultant solution was put in a test tube and mixed with 2ml of chloroform. About 3ml of ice-cold acetic anhydride was added to the mixture in the flask and 2 drops of concentrated tetraoxosulphate VI acid (H_2SO_4) was cautiously added. Standard sterol solution was prepared and treated as described above. The absorbances of standard and prepared sample were measured with a spectrophotometer at 420 nm.

Tannins Concentration

The method of Swain (1979) was used for the determination of tannin content. A quantity of sample (0.2 g) was measured into a 50 ml beaker. About 20 ml of 50% methanol was added and covered with paraffin and placed in a water bath at 77-80^{o}C for 1 hour and stirred with a glass rod to prevent bumping. The extract was filtered using a double layer of Whatman No 1 filter paper into a 50 ml volumetric flask. Then, 20 ml distilled water, 2.5 ml Folin-Denis reagent and 10 ml of 17% of sodium trioxocarbonate IV (Na_2CO_3) were added and allowed to stand for 20 minutes until a bluish-green colouration developed. Standard tannic acid solutions of range 0-10 ppm were treated similarly as 1 ml of sample above. The absorbances of the tannic acid standard solutions as well as samples were read after colour development at 760 nm. The tannin content was calculated using the formular:

$$\text{Tannin (mg/100 g)} = \frac{\text{Absorbance of sample x Average gradient x Dilution factor}}{\text{Weight of sample x 1000}}$$

Concentration of Reducing Sugars

Reducing sugar content of plant extract was determined according to the method of Harborne (1973). About 1 g of the sample was macerated with 20 ml of distilled water and 1ml of

alkaline copper reagent was added to the filtrate and boiled for 5 minutes. This was allowed to cool and 1 ml of phosphomolybdic acid reagent was added to the filtrate followed by 7 ml of distilled water and the absorbance of the solution was read at 420 nm.

Concentration of Glycosides

Glycoside concentration was determined according to the method of Harborne (1973). About 1 g of the sample was macerated with 20 ml of distilled water and 2.5 ml of 15% lead acetate was added to the filtrate. This was shaken vigorously with 2.5 ml of chloroform. Lower layer was collected and evaporated to dryness. Residue was dissolved with 3 ml of glacial acetic acid. About 0.1 ml of 5% ferric chloride and 0.25 ml of concentrated sulphuric (H_2SO_4) were added. The tube was kept in dark for 2 hours. The absorbance of the resulting mixture was read spectrophotometrically at 530nm.

Concentration of Saponins

Saponin content was determined by the method of Harborne (1973). This was done by macerating 5 g of the sample with 10 ml of petroleum ether in a beaker. The filtrates were combined and evaporated to dryness. The residue was dissolved with 6 ml of methanol. Then, 2 ml of the dissolved residue was taken in a test tube and 2 ml of chromate solution added to it. It was allowed to stand for 30 minutes and the absorbance of the solution was read at 550nm against an ethanol blank.

2.2.7 Assay for Free Radical-Scavenging Activity of Ethanol Extracts of *Bridelia ferruginea* and *Bryophyllum pinnatum* Leaves using 1,1-diphenyl-2-picryl hydrazyl (DPPH).

A stable radical DPPH was used to measure the free radical scavenging activity by the method of Blois (1958). Samples at various concentrations (15.63, 31.25, 62.50, 125, 250, 500 and 1000 µg/ml of extracts with respective organic solvents) were taken and the volume was adjusted to 100 µg/ml with methanol. About 5 ml of a 0.1 mM methanol solution of DPPH was added to the aliquots of different extracts of plant sample and standards. About 100 µl of methanol in 5 ml of DPPH solution was used as negative control. All the reaction mixtures were incubated for 20 min at 27^0C. Inhibition of DPPH radical by the plant extract was measured at 517 nm against the blank (methanol). All tests were performed in triplicate. Results were expressed as percentage of blank (100 %).

$$\% \text{ inhibition} = \frac{A_C - A_T}{A_C} \times 100$$

Where: A_C = Absorbance of the control

A_T = Absorbance of the test drug/ extract

2.2.8 Determination of Ferric Antioxidant Reducing Power (FRAP) of Ethanol Extracts of *Bridelia ferruginea* and *Bryophyllum pinnatum* Leaves.

Ferric reducing power capacity of the extract was determined by the method of Oyaizu (1986). Different concentrations (15.63, 31.25, 62.50, 125, 250, 500 and 1000 µg/ml) was mixed with 0.5 ml phosphate buffer (pH 6.6) and 0.5 ml 0.1 % potassium hexacyanoferrate, followed by incubation at 50 ^{0}C in water bath for 20 minutes. After incubation, 0.5 ml of 10 % TCA was added to terminate the reaction. The upper portion of the solution (1 ml) was mixed with 1 ml of distilled water and 0.1 ml 0.01 % $FeCl_3$ solution. The reaction mixture was left for 10 minutes at room temperature and the absorbance of the solution was read at 700 nm against blank solution. All tests were performed in triplicate. A higher absorbance of the reaction mixture indicated greater reducing power. Ascorbic acid was used as a positive control.

2.2.9 Determination of Total Antioxidant Capacity (TAC) of the Ethanol Extracts of *Bridelia ferruginea* and *Bryophyllum pinnatum* Leaves.

Total antioxidant capacity of the extract was determined using the phosphomolybdate method as reported by Saeed *et al*. (2012) using ascorbic acid as standard drug. An aliquot of 0.1 ml of the extract and ascorbic acid at varying concentrations (15.63, 31.25, 62.50, 125, 250, 500 and 1000 µg/ml) were mixed with 1 ml of reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The tubes were capped with aluminium foil and incubated in a water bath at 95 ^{0}C for 90 minutes. After the samples had cooled at room temperature, the absorbance of each mixture was read at a wavelength of 765 nm against a blank. A typical blank contained 1 ml of reagent solution and incubated under the same conditions. All tests were performed in triplicate. The IC_{50} of the extracts were determined. The antioxidant capacity was estimated using the following formula:

$$\% \text{ inhibition} = \frac{A_C - A_T}{A_C} \times 100$$

Where: A_C = Absorbance of the control

A_T = Absorbance of the test drug/ extract

2.2.10 Formulation of Phytosomes

Development of phytosome was achieved by solvent evaporation method as described by Maiti *et al*. (2007). *Bridelia ferruginea and Bryophyllum pinnatum* phytosome complexes were prepared in different ratios of extract: phosphatidylcholine (1:1, 1:3 and 1:5) by using

dichloromethane as a reaction medium. A specific quantity of phosphatidylcholine was dissolved in dichloromethane and added drop by drop to the standardized dichloromethane solution of plant extract with continuous stirring / refluxing at temperature (not above 40^{o}C) for 2 - 3 h. The resultant mixture was evaporated under vacuum at 40^{o}C. Then the residue was properly stored in air-tight container till use.

2.2.10.1 Scanning Electron Microscopy (SEM) of Phytosome Complexes Formed from Ethanol Extracts of *Bridelia ferruginea* and *Bryophyllum pinnatum* Leaves.

Phytosome complex was first placed on a sample stub, with a double adhesive attached. It was coated with 5 nm of gold using a sputter coater by quorum, afterwards was transferred to SEM machine, viewed via a NavCam after some adjustment of focusing and contrast was transferred to SEM mode where different magnification was stored in an USB stick. This produced the surface morphology of the formed phytosome complexes at different magnifications.

2.2.10.2 Fourier Infrared Spectroscopy of *Bridelia ferruginea* and *Bryophyllum pinnatum* Phytosome Complexes

FT-IR spectra were obtained by transmittance method using an FT-IR spectrometer (Shimadzu 4300, Japan). Ethanol leaf extracts of plant samples (*Bridelia ferruginea* and *Bryophyllum pinnatum*), phosphatidylcholine and various stoichiometric extract: phosphatidylcholine ratio (1:1, 1:3 and 1:5) phytosome complexes of *Bryophyllum pinnatum* and *Bridelia ferruginea* were mixed with potassium bromide, separately. The potassium bromide discs were prepared by compressing the powders at pressure of 15 tons for 10 min in hydraulic press. Scans / spectrum were obtained at a resolution of 8 cm-^{1}, from 4000 to 650 cm-^{1}.

2.2.10.3 Percentage Entrapment Efficiency of Phytosome

This was determined according to the method of Khalid and Khalid (2016). A total of 30 mg of each formulation was dissolved in freshly prepared phosphate buffer (50 mL/pH 6.8) and maintained on a mechanical shaker for 24 hours. The content was filtered with a filter paper and the concentration of the extract in the filtrate was determined using spectrophotometer at wavelengths of 340 nm for *Bridelia ferruginea* and at 420 nm for *Bryophyllum pinnatum* phytosome complexes respectively (n=3). The encapsulation efficiency is expressed in percentage and calculated according to the equation below:

% EE = $\frac{DT-DS}{DT}$ X 100 (Shahira *et al*.,2018).

Where DT= theoretical amount of extract used in formulation of the phytosome complex.

DS = the experimental detected amount of extract in the filtrate (n=3).

2.2.10.4 Determination of *in vitro* Drug Release Profile

The dialysis method was adopted in this study. The dissolution medium consisted of 200 ml of freshly prepared Simulated intestinal fluid (SIF p^H 6.8) maintained at 37°C (acceptor phase). The dialysis membrane was pre-treated by soaking in the dissolution medium 24 h before the experiment. A 300 mg of 1:1 phytosomes, 300 mg of 1:3 phytosomes, 300 mg of 1:5 phytosomes, and 300 mg of Cimetidine-loaded P90H, 150 mg of ethanol extracts and 150 mg of pure Cimetidine were analysed separately by placing each of them in a dialysis membrane and dissolution achieved with 5 ml of SIF (donor compartment). The dialysis membrane was securely tied with a thermo-resistant thread attached to a clamp in such a way as to suspend the dialysis membrane and its content in the recipient compartment. The stirrer was set at 50 rpm and a 5 ml aliquot was removed and replaced with an equal volume of fresh dissolution medium at different sampling time intervals (30, 60, 90,120, 150 and 180 min). The samples were then analysed for drug content spectrophotometrically (Spectrophotometer (E312 model) Jenway, England) at a wavelength of 340 nm for Cimetidine and *Bridelia ferruginea* and at 420 nm for *Bryophyllum pinnatum* complexes respectively. The above procedure was repeated three times.

2.2.11 Preparation of Stock 200 mg/ml from Extract

Stock of extracts or phytosomes of concentration of 200 mg/ml was prepared by dissolving 1.0 g of extract or phytosome in 5 ml of 2% dimethyl sulfoxide (DMSO). Other concentrations used in antimicrobial studies were obtained from serial dilution from this stock solution.

2.2.11.1 Preparation of Nutrient Agar

Nutrient agar preparation depends on the direction given by the manufacturer. For life save biotech, USA, 2.8 g of the nutrient agar was mixed with 100 ml of distilled water, swirled and allowed to soak for ten minutes. It was autoclaved at 121ºC for 15 minutes, cooled and mixed well before pouring into plate behind a bursen burner to avoid bubbles and contamination.

2.2.11.2 Tests for Identification of *Klebsiella pneumonia, Escherichia coli* and *Staphylococus aureus.*

Indole Test: Covac's reagent was added to a broth culture of suspected microorganism. A positive result is indicated by a pink/red layer forming on top of the broth. But the absence of red colour confirms that the organism is *Klebsiella,* instead of *Escherichia coli.*

Catalase test: Identification of *Staphylococcus aureus*

A smear of test organism was mixed with hydrogen peroxide on a glass slide. The presence of bubbles on mixing was observed, *staphylococcus aureus* was suspected.

Coagulation test: Agglutination observed on mixing the test bacteria, normal saline, and serum on glass tile confirmed the presence of *staphylococcus aureus.*

2.2.11.3 Antibacterial Activity Test (Sensitivity)

Agar well diffusion method was used. The plates were seeded with 24- hour old cultures of the isolates. Wells of 7mm in diameter were created in nutrient agar plate. In each well, 150 µl of the dissolved extracts or phytosome of various concentrations (200 mg/ml, 100 mg/ml, 50 mg/ml, 25 mg/ml, 12.5 mg/ml and 6.25 mg/ml) each was placed to fill each of the holes. A positive control or standard drug gentamicin (40 mg/ml) was also used, while dimethyl sulphoxide and distilled water were used as the negative controls. The plates were incubated at 37^{o}C for 24 h. Antibacterial activity was determined by measuring the diameter of the inhibition zone formed around the well by use of meter rule or Vernier caliper. The experiment was repeated three times and the average values were calculated for antibacterial activity.

2.2.12 Determination of Median Lethal Dose (LD_{50})

The acute toxicity test of ethanol extract of *Bridelia ferruginea and Bryophyllum pinnatum* leaves was conducted in accordance with Lorke's method (1983). The study was conducted in two phases using a total of eighteen mice. In the first phase, nine mice were divided into 3 groups of 3 mice each. Groups 1, 2 and 3 animals were given 10, 100 and 1000mg/kg body weight (b.w) of the extract respectively, to possibly establish the range of doses producing toxic effect. In addition, a fourth group of three mice was set up as control group and animals in this group were given 5ml/kg body weight of normal saline. In the second phase, further specific doses (1600, 2900 and 5000mg/kg b.w) of the extract were administered to the mice (three mice per group) to further determine the correct LD_{50} value. The extract was dissolved in normal saline and the route of administration was oral. After the administration, animals

were observed for signs of toxicity. None of the animals showed any sign of toxicity or death at the end of 24 h of administration of extract / phytosome complex. LD_{50} was calculated as follows:

$$LD_{50} = \sqrt{(D_0 \times D_{100})}$$

Where

D_0 = highest dose that gave no mortality

D_{100} = lowest dose that produced mortality

2.2.13 Ulcer Induction

Experimental animals were pre-treated with extracts and the formulated phytosome complexes for three (3) weeks before ulcer induction. Ulceration was induced according to the method of Urushidani *et al.* (1979) with slight modifications. Rats were fasted for 24 h having access to water *ad libitum*. Water was removed 1 h before treatment. All drugs were administered by oral route. Thirty minutes after treatment, animals were induced by oral administration of 50 mg/kg b.w of indomethacin. Then after 8 h, animals were sacrificed. The stomachs were removed, opened along the greater curvature and rinsed under a stream of water. Erosions formed on the glandular portion of the stomach were observed and each graded on a (0-3) scale based on the severity of the ulcer; 0 = normal; 1 = < 1 mm; 2 = 1- 2 mm; 3 = > 2 mm (Main and Whittle, 1975). Mean ulcer score for each group was calculated and expressed as the Ulcer Index (UI). Ulcer protection (%) was calculated using the relation:

% ulcer protection = $\frac{UC-Ut}{UC}$ x 100

Where Uc= ulcer index of control group, Ut = ulcer index of the test group.

2.2.13.1 Experimental Design

Experimental animals were randomly divided into eighteen groups of four rats each. The animals were grouped as shown below: Group1- 6 serve as control groups for each of the plant. This was repeated for each of the plant and its phytosome complexes.

Group 1: Normal rats + 5 ml/kg of normal saline

Group 2: Ulcer + 5 ml/kg normal saline (control).

Group 3: Ulcer + 1% Tween 80 (vehicle control)

Group 4: Ulcer + 100 mg/kg of Phospholipid (P90H)

Group 5: Ulcer + 100 mg/kg of Cimetidine (Standard)

Group 6: Ulcer + 100 mg/kg Cimetidine enclosed in Phospholipid (P90H)

Group 7: Ulcer+ 100 mg/kg b.w of extract

Group 8: Ulcer+ 200 mg/kg b.w of extract

Group 9: Ulcer+ 400 mg/kg b.w of extract

Group 10: Ulcer+ 100 mg/kg b.w of extract: phospholipid (1:1) phytosome complex.

Group 11: Ulcer + 200 mg/kg b.w of extract: phospholipid (1:1) phytosome complex.

Group 12: Ulcer + 400 mg/kg b.w of extract: phospholipid (1:1) phytosome complex.

Group 13: Ulcer + 100 mg/kg b.w of extract: phospholipid (1:3) phytosome complex.

Group 14: Ulcer + 200 mg/kg b.w of extract: phospholipid (1:3) phytosome complex.

Group 15: Ulcer + 400 mg/kg b.w of extract: phospholipid (1:3) phytosome complex.

Group 16: Ulcer + 100 mg/kg b.w of extract: phospholipid (1:5) phytosome complex.

Group 17: Ulcer + 200 mg/kg b.w of extract: phospholipid (1:5) phytosome complex.

Group 18: Ulcer + 400 mg/kg b.w of extract: phospholipid (1:5) phytosome complex.

2.2.13.2 Tests for Antiulcer Mechanisms

Gastric Volume, pH and Total Acidity

Gastric volume: The stomach was opened along the greater curvature and the content was drained into a calibrated centrifuge tube. The gastric contents were centrifuged at 1000 rpm for 10 min. The supernatant was measured and the volume was recorded.

Gastric pH: then gastric content was measured using a pH meter. The pH reading for each gastric content was also recorded.

Total acidity: Total acidity was determined by the method of Kulkarni (1999). The gastric contents were centrifuged at 1000 rpm for 10 min. One ml of the supernatant liquid was pipetted out and diluted to 10 ml with distilled water. The solution was titrated against 0.01N NaOH using thymol blue as indicator until reddish orange colour changed to blue colour and volume of NaOH used was noted and was taken as corresponding to the total acidity. The level of acidity was measured by the amount of base (sodium hydroxide) used to neutralize 1 ml of the gastric content during the titration. Acidity was expressed as:

$$\text{Acidity (mmol/L)} = \frac{\text{Volume of NaOH used x Normality of NaOH (0.01) x 1000}}{\text{Volume of gastric content used}}$$

2.2.14 Kidney Function Test

Test for Creatinine Concentration

Principle: Creatinine reacts with picrate in alkaline solution to form a red-orange compound (Jaffe reaction). The intensity of the colour which is proportional to the concentration of the creatinine is measured using the end point. The alkaline picrate method was used (Fabing and Ertingshausen, 1971). Reagents were pipetted into three (3) test tubes labelled as reagent blank, standard and test sample respectively. About 1.5 ml of reagent 1 (picrate solution) was added in each of the three test tubes. This was followed by adding 0.2 ml of distilled water to the reagent blank tube, 0.2 ml of sample (serum) to the test sample tube and 0.2 ml of solution2 (Creatinine standard) to the standard tube. The tubes were centrifuged at 3,000 rpm for 10 minutes. Quantity of 1 ml of the supernatant was collected from each of the test tubes. This was followed by addition of 0.5 ml of solution 3 (sodium hydroxide 2N) to each of the tubes (reagent blank, standard and test sample) accordingly. The absorbance of the solution in the test tubes was read at 540 nm against the reagent blank and concentration of creatinine was calculated as shown below:

Creatinine conc.(mg/dl) = $\frac{Abs\ of\ Test}{Abs\ of\ Standard}$ x conc. of standard (2.0 mg/dl)

Test for Urea

Principle: This test was based on Urease-Berthelot method. Urea in serum is hydrolyzed to ammonia in the presence of urease. The ammonia formed was then measured by Berthelot's reaction.

Urea + H_2O ----→$2NH_3$ + CO_2----→ NH_3 + Hypochlorite + phenol ---→Indophenol (blue) This assay was carried out according to the method of Chaney and Marbach (1962).

Protocol:

Thre (3) test tubes were labeled as blank, standard and sample. About 10 µl of sample (serum) was added to the sample test tube, 10 µl of reagent (standard) was added to the satandard test tube, and 10 µl of distilled water was added to blank test tube accordingly. The contents of each tube were incubated at 37^0C in water bath for 10 minutes. About 2.5ml of reagent 2 (phenol) and distilled water (25 ml) were respectively added to each tube and the contents shaken followed by 2.5ml of reagent 3 (sodium hypochlorite + NaOH). Each tube was mixed and incubated for 15 minutes at 37^0C. The absorbance of the solution in each tube was read against the blank at 546 nm.

Calculation: The urea concentration in the serum was calculated as shown below:

Urea concentration (mg/dl) = $\frac{Absorbance\ of\ sample}{Absorbance\ of\ standard} \times 80$ (mg/dl)

2.2.15 Liver Function Tests

Liver Function Test (ALT, AST and ALP) was determined using the method of Reitman and Frankel (1957) and Klein *et al*. (1960).

Assay of Alanine Aminotransferase (ALT) Activity

Principle: ALT is measured by monitoring the concentration of pyruvate hydrazone formed with 2, 4-dinitrophenylhydrazine. The colour intensity was measured against the blank at 540nm. The whole blood sample collected from the animal was centrifuged at 4000 revolutions per minute for 5 minutes to get the serum.

Method: Two test tubes were labeled as blank and sample respectively. Quantity of 0.5 ml of solution R1 (buffer solution) was added to each of the test tubes. Then, 0.1 ml of sample (serum) was added to the sample test tube and mixed properly. The tubes were incubated for 30 minutes at 37^{o}C and pH of 7.4. About 0.5 ml of solution R2 (2, 4-dinitrophenlhydrazine) was added to each of the test tubes (i.e blank and sample) respectively. This was followed by addition of 0.1 ml of standard reagent into the blank test tube and mixed. The test tubes were allowed to stand for 20 minutes at the temperature of 25^{o}C. This was followed by addition of 5.0 ml of sodium hydroxide solution to each of the test btubes. The contents of each tube were mixed and the absorbance of the sample was read against blank at 540 nm after 5 minutes.

Assay of Aspartate Aminotransferase (AST) Activity

Principle: AST is measured by monitoring the concentration of Oxaloacetate hydrazone formed with 2, 4-dinitrophenylhydrazine. The colour intensity is measured against the blank at 540nm.

Procedure: The reagent blank and sample test tubes were set up. The sample, 0.1 ml of serum was pipetted into the sample test tube. Reagent1 (buffer) (0.5 ml) was pipetted into each of the test tubes (both sample and blank). The mixture was thoroughly mixed and incubated for 30 minutes at 37^{o}C and at pH of 7.4. Reagent 2, (2, 4-dinitrophenylhydrazine) (0.5 ml) was pipetted into each of the test tubes followed by 0.1 ml of standard into the blank tube. The tubes were mixed thoroughly and incubated for exactly 20 minutes at 25^{o}C. Sodium

hydroxide solution (5 ml) was added to each tube and mixed. The absorbance of the solution was read against the blank after 5 minutes at 540 nm.

Assay of Alkaline Phosphatase (ALP) Activity

Principle: The principle was based on the reaction involving serum alkaline phosphatase which hydrolyzes a colourless substrate of Phenolphthalein monophosphate giving rise to phosphoric acid and phenolphthalein which at alkaline pH values, turns into a pink colour that can be photometrically determined. This was determined using the method of Klein *et al.* (1960).

Method: The standard and sample test tubes were set up. Distilled water (1ml) was pipetted into each of the tubes. Standard solution (0.1ml) was added to the test tube for standard. The sample (0.1 ml) was added into the sample test tubes. The contents of each of the test tubes were mixed thoroughly and incubated at 37^0C for 20 minutes. Colour developer (5ml) was added to each of the test tubes. The absorbance of the solution was read at 540 nm.

Calculation: Alkaline phosphatase was calculated as follows:

$$\text{U/L of ALP} = \frac{\text{absoance of sample} \times}{\text{absorbance of standard}}\ 30\text{u/L}\,.$$

2.2.16 Determination of Oxidative Status

The stomach tissue of rats was homogenized in ice cold 0.1M phosphate saline buffer (1:4 w/v, pH7.4) and the homogenate was centrifuged at 2500 rpm for 10 min. The resulting supernatant was thereafter used for the assessment of oxidative status.

Determination of Malondialdehyde (MDA) Concentration

MDA concentration was measured spectrophotometrically as described by Wallin *et al.* (1993).

Principle: The principle for the estimation is based on the fact that thiobarbituric acid-reactive substance (TBARS) reacts with malondialdehyde (MDA) to give a red or pink colour, which absorbs maximally at 532 nm.

Method: About 0.1 ml of the stomach tissue homogenate was mixed with 0.9 ml of distilled water in a test tube. Then 0.5 ml of 25% TCA (trichloroacetic acid) and 0.5 ml of 1 ml TBA (thiobarbituric acid) in 0.3% NaOH were also added to the mixture. The mixture was boiled for 40 minutes in water bath and cooled in cold water. Then, 0.1 ml of 20% sodium dodecyl

sulphate (SDS) was added to the cooled solution and mixed properly. The absorbance of the solution was read at wavelengths 532 nm and 600 nm against a blank.

$$\text{MDA (mg/dl)} = \frac{A532-A600}{0.5271} \; x \; \frac{100}{1}$$

Assay of Catalase Activity: This was carried out according to the method of Aebi (1983). Principle: The ultraviolet absorption of hydrogen peroxide can be easily measured at 240nm. On the decomposition of hydrogen peroxide with catalase, the absorption decreases with time and from this decrease, catalase activity can be measured. Reagents: Phosphate buffer (pH 7.0), 0.2M H_2O_2, 5% Potassium dichromate and Glacial acetic acid.

Procedure: About 2.5 ml of phosphate buffer + 2 ml H_2O_2 + 0.5 ml of homogenate sample were pipetted into a test tube. To 1 ml portion of the reaction, 2 ml of Potassium dichromate and acetic acid were added. The absorbance of the solution was determined at 240 nm into 4 places at a minute interval. Catalase concentration was then calculated using the equation,

$$\text{Catalase concentration (Unit/L)} = \frac{0.23 \times (\log Abs1/\log Abs2)}{0.00693}$$

Assay of Glutathione Peroxidase Activity

This was carried out according to the method of Paglia and Valentine (1967).

Principle of test: The Cayman chemical glutathione peroxidase assay kit measures glutathione reductase activity indirectly by a coupled reaction with GPx. Oxidized glutathione GSSG, produced upon reaction with an organic hydroperoxide by GPx is recycled to its reduced state by glutathione reductase and NADPH. The oxidation of NADPH to $NADP^+$ is accompanied by a decrease in absorbance at 340 nm. The rate or decrease in A_{340} is directly proportional to the GPx activity in the sample.

$$2GSH + ROOH \xrightarrow{GPx} GSSG + ROH + H20$$

$$GSSG + NADPH + H+ \xrightarrow{GR} NADP+ + 2GSH$$

Test Procedure: A known volume, 0.1 ml of homogenate sample was pipette into a test tube; 1 ml of diluted R1 and 1 ml of R2 solutions were added to the test tube and mixed. The initial absorbance of the solution mixture was read after 30 seconds. The final absorbance of the solution mixture was read after 1 minute at 340 nm. Glutathione Peroxidase activity was calculated from the formula below.

U/L of haemolysate = 8412 x A 340 nm/minute.

Assay of Superoxide Dismutase (SOD) Activity

Superoxide dismutase activity was assayed according to the method of Arthur and Boyne (1985). Principle of test: The method employed xanthine and xanthine oxidase to generate superoxide radicals which reacted with 2(-4- iodophenyl)-3(- 4 – nitrophenol), – 5 – phenyltetrazolium chloride (INT) to form a red formazan dye. The superoxide dismutase activity was measured by the degree of inhibition of this reaction. One unit of superoxide dismutase activity is that which causes a 50% inhibition of the rate of reduction of 2(-4-iodophenyl)-3(- 4 – nitrophenol), – 5 – phenyltetrazolium chloride (INT) under the conditions of the assay.

Test procedure: Phosphate buffer (1 ml) was taken inside in all the test tube; 100 µl of stomach homogenate samples were added into test tubes. Then, 75 µl of Xanthine oxidase was added and mixed and the absorbance of the solution was taken after 30 seconds (initial absorbance). The final absorbance was taken after 3 minutes at 480 nm. The rate of change in absorbances of the solution was used to determined superoxide dismutase activity.

2.2.17 Lipid Profile Estimations

Determination of Total Cholesterol Concentration

The method of Abell *et al.* (1952) was followed. Principle**:** Cholesterol concentration was determined after enzymatic hydrolysis and oxidation. The indicator quinoneimine was formed from hydrogen peroxidase and 4-aminoantipyrine in the presence of phenol and peroxidase.

Test procedure: Three (3) test tubes were set up in a test tube rack and labelled blank, standard and sample respectively. To the blank, was added (10 µl) distilled H_2O, 10 ul standard specimen to the standard test tube and 10 ul sample (serum) to the sample test tube. To each of these test tubes was added 1000 µl of the cholesterol reagent. It was thoroughly mixed and incubated for 10minutes at room temperature (20-25^oC). The absorbance of the sample (A_{sample}) against the blank was read within 60 minutes, at 500nm.

Determination of Low Density Lipoprotein (LDL) Concentration

Principle: LDL-C can be determined as the difference between total cholesterol and the cholesterol content of the supernatant after precipitation of the LDL fraction by polyvinyl sulphate (PVS) in the presence of polyethylene glycol monomethyl ether. **Procedure:** The serum samples were kept at 2-8^0C. The precipitant solution (0.1ml) was added to 0.2ml of the

serum sample, mixed thoroughly and allowed to stand for 15 min. This was centrifuged at 2,000 x G for 15 min. The cholesterol concentration in the supernatant was determined. The estimation of the serum total cholesterol as described by Kameswara *et al.* (1999) was used.

Calculation:

LDL-C (mmol/L) = Total Cholesterol (mmol/L) – 1.5 x Supernatant Cholesterol (mmol/L).

Determination of High Density Lipoprotein (HDL) Concentration

The high density lipoprotein (HDL) was determined according to the method of Kameswara *et al.* (1999).

Principle: LDL and VLDL (low and very low density lipoproteins) were precipitated from serum by the action of a polysaccharide in the presence of divalent cations. Then, high density lipoproteins (HDL) cholesterol present in the supernatant was determined.
Procedure: The precipitant solution 0.1ml was added to 0.3ml of the serum sample and mixed thoroughly and allowed to stand for 15 min. This was centrifuged at 2,000 x G for 15 min. The cholesterol concentration in the supernatant was determined.

Determination of Triacylglycerol Concentration

Principle: The triacylglycerol was determined after enzymatic hydrolysis with lipases. The indicator was a quinoneimine formed from hydrogen peroxide, 4-aminophenazone and 4-chlorophenol under the catalytic influence of peroxidase.

$$\text{Triacylglycerol} + H_2O \xrightarrow{\text{lipases}} \text{Glycerol} + \text{fatty acids}$$

$$\text{Glycerol} + \text{ATP} \xrightarrow{\text{GK}} \text{Glycerol-3-phosphate} + \text{ADP}$$

$$\text{Glycerol-3-phosphate} + O_2 \xrightarrow{\text{GPO}} \text{Dihydroxyacetone phosphate} + H_2O_2$$

$$2H_2O_2 + \text{4-aminophenazone} + \text{4 chlorophenol} \xrightarrow{\text{POD}} \text{Quinoneimine} + HCl + 4H_2O$$

Procedure: A quantity, 0.1 ml of the sample, was pipetted into a clean labeled tube and 1.0 ml of trichloroacetic acid (TCA) was added to it, mixed and then centrifuged at 250 rpm for 10 minutes. The supernatant was decanted and reserved for use. The assay procedure was carried out by labeling 3 test tubes as blank, standard and sample and set them in test tube rack. To the blank, was added 0.5 mlof distilled water, 0.5 ml of TCA. To the standard test tube, 0.5 ml of standard solution and 0.5 ml of TCA were added. To the sample tube, 10 ml of the supernatant of the sample was added. To each of these tubes was added 1.0 ml of

reagent mixture. Each tube was mixed and allowed to stand for 20 minutes at 25 ^{0}C and the absorbance of the sample and standard were read against the blank at 540 nm.

The concentration of triacylglycerol in serum was calculated as follows:

$$\frac{\text{Absorbance of sample}}{Absorbance\ of\ standard}\ x\ Concentration\ of\ st\text{andard (mmol/l)} = \text{mmol/l}$$

2.2.18 Histological Examination of the Stomach Tissue

Tissue Preparation

At the end of the study period, sections of the stomach from all the experimental animals were collected for histopathological examination. The tissue samples were fixed in 10% phosphate buffered formalin for a minimum of 48 hours before commencement of tissue processing. The tissues were subsequently trimmed, dehydrated in 4 grades of alcohol (70%, 80%, 90% and absolute alcohol), cleared in 3 grades of xylene and embedded in molten wax. After solidifying, the tissue blocks were sectioned, 5µm thick with a rotary microtome, floated in water bathe and incubated at 60˚C for 30 minutes. The 5µm thick sectioned tissues were subsequently cleared in 3 grades of xylene and rehydrated in 3 grades of alcohol (90%, 80% and 70%). The sections were then stained with Hematoxylin for 15 minutes. Blueing was done with ammonium chloride, differentiation was done with 1% acid alcohol before counterstaining with Eosin. Permanent mounts were made on degreased glass slides using cover slips and a mountant, DPX.

Slide Examination and Photomicrography

The tissue sections on glass slides were examined and scored using a Motic™ compound light microscope with x4, x10 x16 and x40 objective lenses. The photomicrographs were captured using a Motic™ 9.0 megapixels microscope camera at x100 and x160 magnifications.

2.2.19 Statistical Analysis

Data were analysed using statistical product for service solution (SPSS) version 22. Results were expressed as mean ± SD and test of statistical significance was carried out using one-way analysis of variance (ANOVA), $p < 0.05$ was considered significant.

CHAPTER THREE

RESULTS

3.1 Qualitative and Quantitative Phytochemical Composition of Ethanol Extract of *Bridelia ferruginea* Leaves

Table 1 shows the presence of alkaloids, tannins, reducing sugars, carbohydrates, hydrogen cyanide, steroids, terpenoids, saponins, glycosides, flavonoids and phenols in ethanol extract of *Bridelia ferruginea* leaves. Alkaloids, tannins and carbohydrates were in low concentration, hydrogen cyanide, steroids, terpenoids, saponins, glycosides and reducing sugars were moderately present while phenols and flavonoids were present in high concentrations of 1911.01 mg/100g and 1891.95 mg/100g respectively. Higher concentrations of phenols, flavonoids, reducing sugars, steroids and tannins were found in *Bridelia ferruginea* leaves than the *Bryophyllum pinnatum* leaves.

3.2 Qualitative and Quantitative Phytochemical Composition of Ethanol Extract of *Bryophyllum pinnatum* Leaves

Table 2 shows the presence of reducing sugars, tannins, alkaloids, flavonoids, steroids, phenols, carbohydrates and terpenoids in ethanol extract of *Bryophyllum pinnatum* leaves at varying concentrations in which tannins has the least concentration of 2.36 mg/100g and terpenoids has the highest concentration of 476.89 mg/100g. *Bryophyllum pinnatum* leaves showed higher concentrations of alkaloids, carbohydrate and terpenoid than *Bridelia ferruginea* leaves.

Table 1: Qualitative and Quantitative Phytochemical Composition of Ethanol Extract of *Bridelia ferruginea* Leaves

PHYTOCHEMICAL	RELATIVE ABUNDANCE	CONC.(mg/100g)
Alkaloids	+	71.080 ± 2.517
Tannins	+	36.905 ± 0.003
Carbohydrates	+	7.214 ± 0.005
Hydrogen Cyanide	++	106.500 ± 0.004
Steroids	++	259.300 ± 0.003
Terpenoids	++	315. 656 ± 0.002
Saponins	++	124.600 ± 0.002
Glycosides	++	116.375 ± 0.004
Reducing Sugar	++	847.868 ± 0.002
Phenols	+++	1911.010 ± 2.308
Flavonoids	+++	1891.945 ± 0.015

Key: + = present in low conc.; + + = Present in moderate amount; +++= Present in high conc.

Results are expressed as mean ± SD (n=3)

Table 2: Qualitative and Quantitative Phytochemical Screening of Ethanol Extract of *Bryophyllum pinnatum* Leaves

PHYTOCHEMICALS	RELATIVE ABUNDANCE	CONC.(mg/100g)
Reducing sugars	+	7.82 ± 0.58
Tannins	+	2.36 ± 0.37
Alkaloids	++	171.58 ± 6.21
Flavonoids	++	269.64 ± 50.38
Steroids	++	11.31 ± 1.41
Phenols	++	66.67 ± 12.73
Carbohydrates	++	30.87 ± 2.56
Terpenoids	+++	476.89 ± 76.59

Key: + = present in low conc.; + + = Present in moderate amount; +++= Present in high conc.

Results are expressed as mean ± SD (n=3)

3.3 Effect of Ethanol Extract of *Bridelia ferruginea and Bryophyllum pinnatum* Leaves on 1,1-diphenyl-2- picryhydrazine (DPPH)

Bridelia ferruginea and *Bryophyllum pinnatum* extracts exhibited antioxidant property in reduction of 1,1 diphenyl-2-picrylhydrazine(DPPH) solution. The reduction power observed in *Bridelia ferruginea* leaves showed IC_{50} of 1.00, *Bryophyllum pinnatum* leaves with IC_{50} of 1.28 while the ascorbic acid standard showed IC_{50} of 1.37. Both plants showed higher antioxidant property than the standard ascorbic acid at all concentrations (Table 3). However, *Bridelia ferruginea* leaf extract showed higher reduction power than that of *Bryophyllum pinnatum*.

3.4 Ferric Reducing Antioxidant Power (FRAP) of Ethanol Extracts of *Bridelia ferruginea* and *Bryophyllum pinnatum* Leaves

The ferric reducing antioxidant power (FRAP) of ethanol extracts of *Bridelia ferruginea* and *Bryophyllum pinnatum* leaves increased with increase in concentration of the extracts. Table 4 showed the ferric reducing antioxidant power of the plant samples from concentration range of 15.63 µg/ml to 1000 µg/ml. *Bryophyllum pinnatum* leaf extract showed the highest ferric reducing power of 672.46 ± 23.05 $\mu MFe^{2+}/g$ at concentration of 1000 µg/ml, *Bridelia ferruginea* leaf extract showed 174.29 ± 22.42 $\mu MFe^{2+}/g$ and gallic acid standard showed 187.32 $\mu MFe^{2+}/g$ at the same concentration. *Bryophyllum pinnatum* leaf extract showed better ferric reducing antioxidant power when compared to *Bridelia ferruginea* leaf extract. In both plant extracts, reducing power increased with increase in the concentrations.

3.5 Total Antioxidant Capacity (TAC) of Ethanol Extracts of *Bridelia ferruginea* and *Bryophyllum pinnatum* Leaves

Table 5 shows the total antioxidant capacity (TAC) of ethanol extracts of *Bridelia ferruginea* and *Bryophyllum pinnatum* leaves. *Bridelia ferruginea, Bryophyllum pinnatum* and gallic acid standard showed highest percentage inhibition of 94.11 ± 0.85%, 89.01± 1.35% and 87.89% respectively at the concentration of 31.25 µg/ml. The percentage inhibition decreases with increase in the concentration of extracts in both *Bridelia ferruginea* and *Bryophyllum pinnatum*.

Table 3: Effect of Ethanol Extracts of *Bridelia ferruginea and Bryophyllum pinnatum* Leaves on 1,1-diphenyl-2- picryhydrazine (DPPH)

Conc (µg/ml)	% Inhibition *B. ferruginea*	% Inhibition *B. pinnatum*	% Inhibition Ascorbic acid
15.63	71.50 ± 1.30	62.5 ± 1.34	60.00 ± 1.97
31.25	73.95 ± 1.79	64.84 ± 11.65	62.30 ± 1.34
62.50	83.95 ± 1.35	68.10 ± 5.64	63.63 ± 0.78
125.00	94.42 ± 2.84	73.79 ± 4.93	68.42 ± 0.68
250.00	97.81 ± 0.00	77.24 ± 3.05	72.84 ± 0.77
500.00	94.10 ± 3.60	82.66 ± 0.42	80.14 ± 0.96
IC_{50}	1.00	1.28	1.37

Results are expressed as mean ± SD (n=3)

Table 4: Ferric reducing Antioxidant Power (FRAP) of Ethanol Extracts of *Bridelia ferruginea* and *Bryophyllum pinnatum* Leaves

Conc (µg/ml)	*B. ferruginea* (μMFe^{2+}/g)	*B. pinnatum* (μMFe^{2+}/g)	Std (Gallic acid) (μMFe^{2+}/g)
15.63	0.51 ± 0.31	1.69 ± 0.50	0.84 ± 0.07
31.25	1.12 ± 0.76	3.68 ± 0.65	3.01 ± 0.88
62.50	2.76 ± 0.99	6.14 ± 0.19	4.62 ± 1.17
125.00	7.76 ± 0.53	16.85 ± 0.67	10.46 ± 2.24
250.00	19.26 ± 1.30	71.10 ± 25.75	35.25 ± 10.39
500.00	55.83 ± 1.94	206.58 ± 36.06	60.98 ± 7.29
1000.00	174.29 ± 22.42	672.46 ± 23.05	187.32 ± 25.82

Results are expressed as mean ± SD (n=3)

Table 5: Total antioxidant Capacity (TAC) of Ethanol Extracts of *Bridelia ferruginea* and *Bryophyllum pinnatum* leaves

Conc (µg/ml)	% Inhibition *B.ferruginea*	% Inhibition *B.pinnatum*	% Inhibition Gallic acid
15.63	93.85 ± 2.21	87.92 ± 2.91	86.15
31.25	94.11 ± 0.85	89.01 ± 1.35	87.89
62.50	93.48 ± 0.90	86.29 ± 1.21	84.98
125.00	88.87 ± 2.19	84.42 ± 0.60	84.81
250.00	85.56 ± 4.00	71.21 ± 3.02	82.99
500.00	82.74 ± 0.77	73.05 ± 10.31	88.10
1000	76.85 ± 1.93	61.74 ± 3.77	81.75
IC_{50}	0.50	0.69	0.80

Results are expressed as mean ± SD (n=3)

3.6 Morphology of the Formed Phytosomes of *Bridelia ferruginea and Bryophillum pinnatum* Leaves (× 1000)

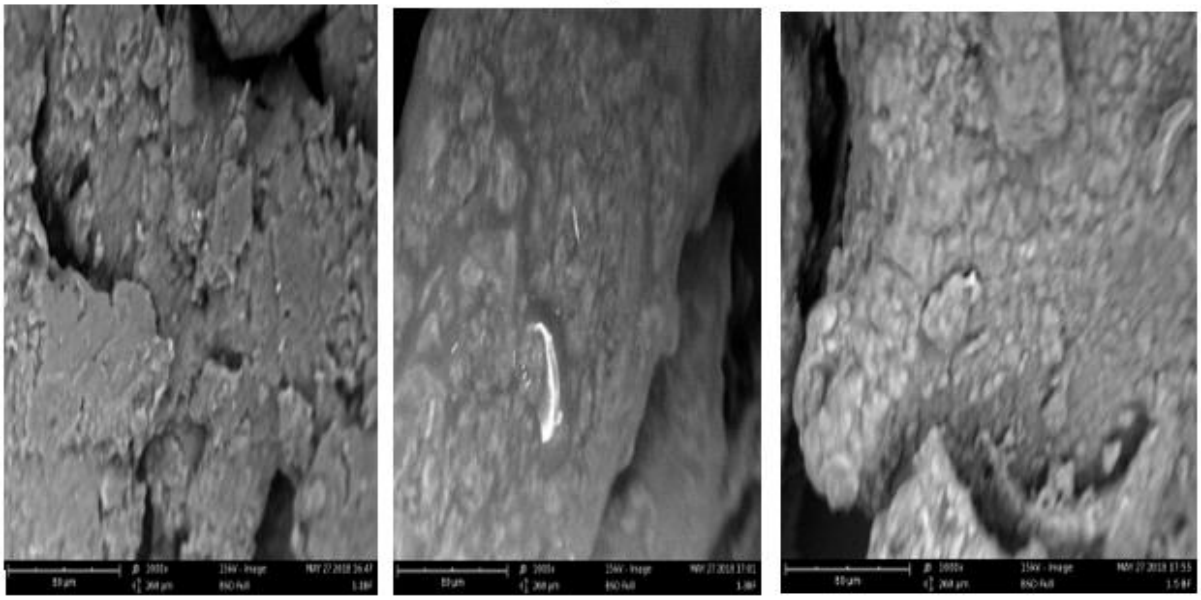
Scanning electron microscopy revealed the surface morphology of *Bridelia ferruginea and Bryophillum pinnatum* phytosome complexes. Phytosome complexes from *Bridelia ferruginea* showed rougher surface morphology while phytosome complexes from *Bryophillum pinnatum* revealed a smoother surface at magnification of 1000 **(**Figure 11).

3.7 Fourier Transform Infrared Spectroscopy of Ethanol Extracts of *Bryophillum pinnatum and Bridelia ferruginea* Leaves and their Phytosome Complexes.

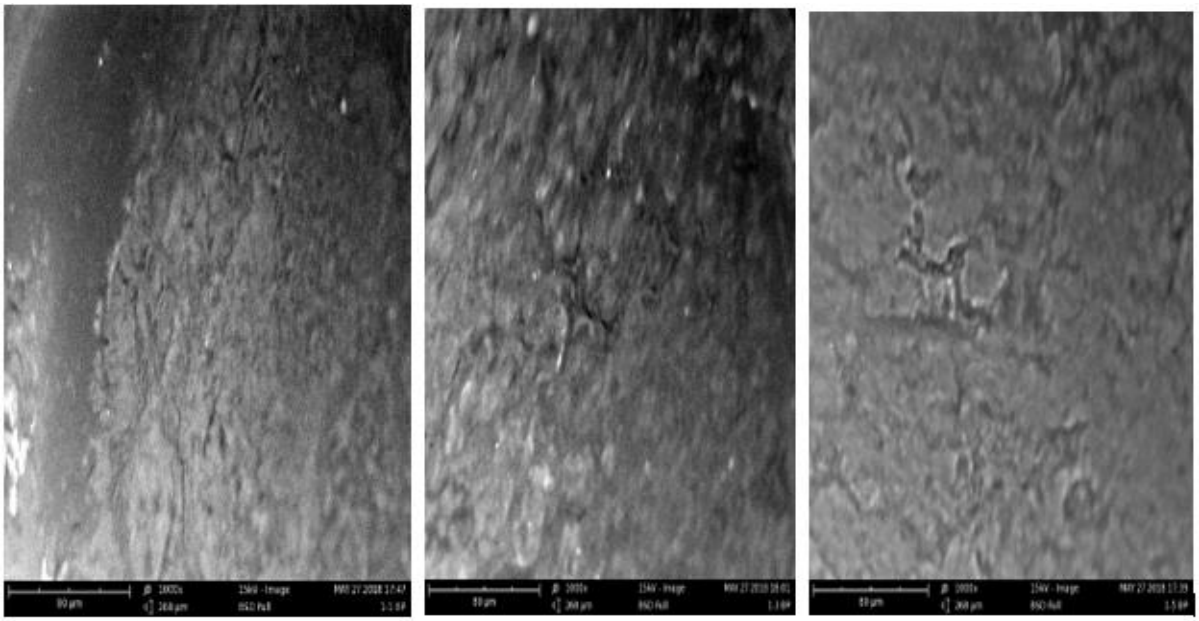
The spectrum peaks revealed various chemical bonds existing in both extracts and their various phytosome complexes. They revealed downfield shifts in the OH bonds of extract and the different phytosome complexes (Table 6). These field shifts in the infrared spectrum revealed interaction between phosphatidylcholine and plant extract in the formation of phytosome complex. Also Table 7 shows the various functional groups identified from functional group regions in the infrared spectrum of the extracts and phytosome complexes in both plants. Both *Bridelia ferruginea* and *Bryophillum pinnatum* leaf extracts showed the presence of alcohol/phenols, aliphatic compound (carboxylic acid), aldehydes and ketones.

3.8 Percentage Entrapment Efficiency of Phytosome Complexes.

Figure 12 shows the percentage entrapment efficiency of the formed phytosome complexes of extract: phospholipid ratios of (1:1, 1:3 and 1:5). This revealed that all the formed complexes had their entrapment efficiency more than 90%. The least entrapment efficiency of 94.14% was found in 1:5 *Bridelia ferruginea* phytosome complex while the greatest entrapment of 99.71% was observed in cimetidine encapsulated in phospholipid. The entrapment efficiency of *Bryophillum pinnatum* was greater than that of *Bridelia ferruginea* in all the formulated phtosome complexes except in extract: phospholipid ratio of 1:3 where *Bryophillum pinnatum* was entrapped up to 94.93% while *Bridelia ferruginea* was 97.33%.



1: 1 *B.ferruginea* phytosome 1: 3 *B.ferruginea* phytosome 1: 5 *B.ferruginea* phytosome



1:1 *B. pinnatum* phytosome 1:3 *B. pinnatum* phytosome 1:5 *B. pinnatum* phytosome

Figure 11: Surface Morphology of Phytosome Complexes formed from *Bryophillum pinnatum and Bridelia ferruginea* Leaves (×1000).

Table 6 Functional Groups and Field Shifts Showing Interactions between Phospholipid and individual Extracts.

Sample	OH	C-H	Si-H	C=C=C	C=C=N	C=O	C=C	C=CH	S-H	C=N	C=C=O
BF Extract	3350.5	2922.2				1710.8	1654.9				
BP Extract	3336	2922.2				1710.8	1654.9			2132.0	
Phospholipid	3358	2914.8	2098.5			1729.5					
BF 1:1	3328.5	2918.5				1736.9			2523.4	2176.8	2094.8
BF 1:3	3634.2	2918.5	2169.3	1938.2		1736.9			2523.4	2169.3	
BF 1:5	3337.0	2918.5				1729.5				2184.2	
BP 1:1	3324.8	2918.5		1927.0		1736.9	1654.9				
BP 1:3		2918.5		1927.0		1736.9		2124.6			
BP 1:5	3634.2	2918.5	2154.4		2016.5	1736.9			2542.0		

Table 7 Functional groups detected in *B. ferruginea* and *B. pinnatum* leaf extracts.

Wave number	Functional group	Assignment
33000 – 3500	Hydroxyl group (O-H)	Alcohols and Phenols
2922.2	Carbonyl carbon of carboxylic acid (C-H)	Aliphatic groups
1938.2	Carbon-carbon bond (C=C=C)	Alkene
2016.5	(C=C=N)	Ketenimines
1710.8	(C=O)	Aldehyde and ketones
1654.9	C=C	Vinyl esters
2124.6	C=CH	Monosubstituted Alkyne

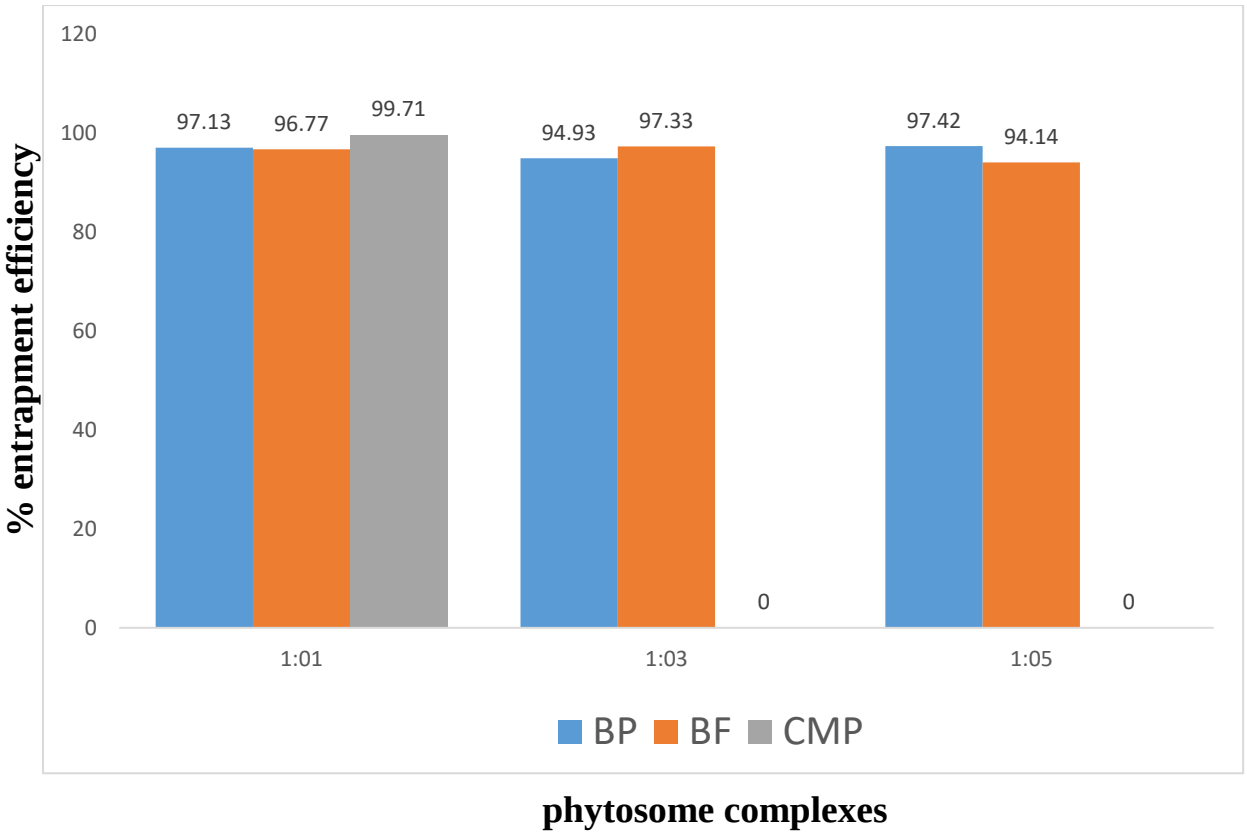


Figure 12: Percentage Entrapment Efficiency of Phytosome Complexes

Key: BP= *Bryophyllum pinnatum*, BF = *Bridelia ferruginea*, CMP = Cimetidine enclosed in phospholipid.

3.9 *In vitro* Drug Release in Simulated Intestinal Fluid (pH 6.8)

Figure 13 shows the *in vitro* release profile of conventional extracts and the formulated phytosome complexes in simulated intestinal fluid (SIF) at temperature of 37^0C and pH 6.8 for 3 h. *Bridelia ferruginea* extract and its phytosome complexes exhibited greater release than *Bryophyllum pinnatum* extract and its phytosome complexes. Figure 13 shows that *Bridelia ferruginea* extract and its phytosome complexes reached their peak activity faster than *Bryophyllum pinnatum* extract and its phytosome complexes that showed slow and sustained release profile. *Bryophyllum pinnatum* phytosome complexes were released higher than the ordinary conventional extract unlike the *Bridelia ferruginea* extract that showed higher release than its phytosome complexes except 1:1 *B. ferruginea* phytosome complex. Moreover, cimetidine enclosed in phospholipid also revealed a significant and higher release of 26.13% of its contents than the marketed cimetidine that released 12.93%.

3.10 Antibacterial Activities of Ethanol Extract of *Bryophyllum pinnatum* Leaves

Table 8 shows the antibacterial activity of ethanol extract of *Bryophyllum pinnatum* leaves on some selected bacteria and fungi, using 40 mg/ml of gentamicin and 200 mg/ml of ketoconazole as standard drugs for bacteria and fungi respectively. Table 8 showed that ethanol extracts of *Bryophyllum pinnatum* leaf had no antibacterial property on the tested strain of bacteria and fungi.

3.11 Antibacterial Activities of Ethanol Extract of *Bridelia ferruginea* Leaves

Table 9 shows the antibacterial activity of ethanol extract of *Bridelia ferruginea* leaves on some selected bacteria and fungi, using 40 mg/ml of gentamicin and 200 mg/ml of ketoconazole as standard drugs for bacteria and fungi respectively. Zone of inhibition was not observed at the end of 24 h antibacterial activity study of the extract on the selected bacteria and fungi.

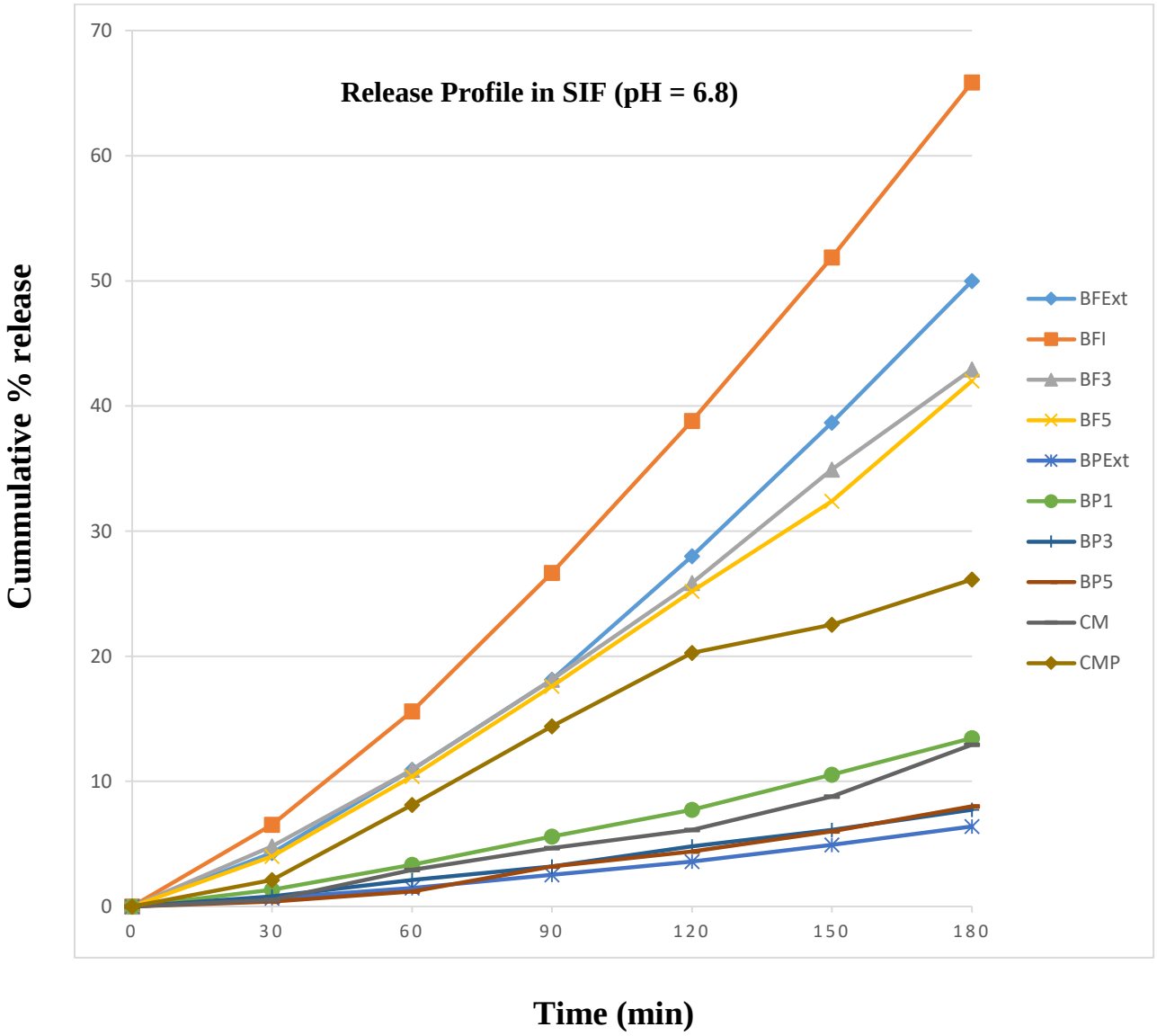


Figure 13: Cummulative Drug Release Profile of 300 mg of Phytosome Complexes in SIF

Key: BF1=1:1 phytosome complex of *Bridelia ferruginea*, BF3 = 1:3 phytosome complex of *Bridelia ferruginea*, BF5 = 1:5 phytosome complex of *Bridelia ferruginea*, BP1= 1:1 phytosome complex of *Bryophyllum pinnatum*, BP3= 1:3 phytosome complex of *Bryophyllum pinnatum*, BP5 = 1:5 phytosome complex of *Bryophyllum pinnatum*, CM = Cimetidine

Table 8 Antibacterial Activities of Ethanol Extract of *Bryophyllum pinnatum* Leaf

	Zone of inhibition (mm) at various concentrations (mg/ml)							
Organisms	**200**	**100**	**50**	**25**	**12.5**	**6.25**	**D.H_2O**	**Gent. (40mg/ml)**
Bacteria								
K.pneumonia	—	—	—	—	—	—	—	29.00 ± 1.00
E- coli	—	—	—	—	—	—	—	30.67 ± 2.08
L. ivanovi	—	—	—	—	—	—	—	31.33 ±1.15
Staph. aureus	—	—	—	—	—	—	—	31.67 ±2.08
E. aerogenes	—	—	—	—	—	—	—	29.00 ±1.67
P. aeruginosa	—	—	—	—	—	—	—	33.33 ±2.89
S.wangiadata	—	—	—	—	—	—	—	29.67 ±1.53
Fungi	**Zone of inhibition (mm) at various concentrations (mg/ml)**							**Ket. (200mg/ml)**
A. fumigatus	—	—	—	—	—	—	—	—
C.albican	—	—	—	—	—	—	—	21.67 ± 7.64

Experiment was performed in triplicate and values were expressed as mean ± SD (n=3).

Table 9 Antibacterial Activities of Ethanol Extracts of *Bridelia ferruginea* Leaf

	Zone of inhibition (mm) at various concentrations (mg/ml)							
Organisms	**200**	**100**	**50**	**25**	**12.5**	**6.25**	**D.H_2O**	**Gentamicin**
Bacteria								
K.pneumonia	—	—	—	—	—	—	—	27.00 ± 2.65
E- coli	—	—	—	—	—	—	—	29.00 ± 1.00
L. ivanovi	—	—	—	—	—	—	—	30.00 ±1.00
Staph. aureus	—	—	—	—	—	—	—	30.33 ±4.04
Enterobacter	—	—	—	—	—	—	—	28.67 ±2.08
P. aeruginosa	—	—	—	—	—	—	—	26.67 ±4.16
S.wangiadata	—	—	—	—	—	—	—	34.67 ±0.58
Fungi	**Zone of inhibition (mm) at various concentrations (mg/ml)**							**Ketoconazole**
A. fumigatus	—	—	—	—	—	—	—	—
C.albican	—	—	—	—	—	—	—	24.33±8.14

Experiment was performed in triplicate and values were expressed as mean ± SD (n=3).

3.12 The Median Lethal Dose of Ethanol Extracts of *Bryophyllum pinnatum and Bridelia ferruginea* Leaves

Table 10 shows the median lethal dose of ethanol extracts of *Bryophyllum pinnatum and Bridelia ferruginea* leaves. This indicated that ethanol extracts of *Bryophyllum pinnatum and Bridelia ferruginea* leaves were not toxic to the experimental animals up to 5000 mg/kg body weight.

3.13 Ulcer Protective Effects of *Bridelia ferruginea, Bryophyllum pinnatum* Extracts and their Phytosome Complexes on Indomethacin-induced Ulcer in Rats

Table 11 shows the ulcer protective properties of ethanol extracts of *Bridelia ferruginea***,** *Bryophyllum pinnatum* and their phytosome complexes on indomethacin-induced ulcer in rats. There were significant decreases ($p < 0.05$) in the ulcer index of groups pretreated with extracts and phytosome when compared to the ulcer untreated groups. Both plant extracts exhibited ulcer protection property. The extract and phytosome complexes from each of the plants also showed dose-dependent effect in the protection of stomach linning. The 1:1 phytosome complex of *Bridelia ferruginea* showed its maximum ulcer protective property of 70.29% at the dose of 200 mg/kg body weight. Ulcer protective property was greater in *Bridelia ferruginea* conventional extract dosage forms when compared to the *Bryophyllum pinnatum* extract. However, both *Bridelia ferruginea* and *Bryophyllum pinnatum* showed comparable ulcer protective effect in their phytosome dosage forms. Highest ulcer protection of 78.14% and 77.86% was found in group preatreated with 400 mg/kg of 1:3 *Bridelia ferruginea* and *Bryophyllum pinnatum* phytosomes respectively. The groups pretreated with phytosome complexes in both plants showed higher percentage ulcer protection when compared to the groups pretreated with ordinary extracts.

Table 10 The Median Lethal Dose of Ethanol Extracts of *Bryophyllum pinnatum and Bridelia ferruginea* Leaves

Phase I	**Dosages (mg/kg body weight)**	**Mortality (*Bridelia ferruginea)***	**Mortality (*Bryophyllum pinnatum*)**
Group 1	10	0/3	0/3
Group 2	100	0/3	0/3
Group 3	1000	0/3	0/3
Phase II			
Group 1	1600	0/3	0/3
Group 2	2900	0/3	0/3
Group 3	5000	0/3	0/3

Table 11 Ulcer Protective Effects of *Bridelia ferruginea, Bryophyllum pinnatum* Extracts and Their Phytosome Complexes on Indomethacin-induced Ulcer in Rats

Treatment groups	Dose (mg/kg)	Ulcer index		Percentage ulcer protection	
		B. ferruginea	*B. pinnatum*	*B. ferruginea*	*B. pinnatum*
Normal	5 ml	0.00^{a}	0.00^{a}	-	-
Ulcer control	5 ml	7.00 ± 3.69^{c}	7.00 ± 3.69^{d}	0.00	0.00
1% tween 80	5 ml	6.98 ± 1.11^{c}	6.98 ± 1.11^{d}	0.29	0.29
Phospholipids	100	6.95 ± 2.09^{c}	6.95 ± 2.09^{d}	0.71	0.71
Cimetidine	100	1.75 ± 1.30^{ab}	1.75 ± 1.30^{ab}	75.00	75.00
Cimetidine in lipids	100	1.63 ± 1.08^{ab}	1.63 ± 1.08^{ab}	76.71	76.71
Extract	100	3.53 ± 0.73^{b}	4.70 ± 0.78^{c}	49.57	32.86
Extract	200	2.93 ± 1.20^{b}	4.43 ± 1.55^{c}	58.14	36.71
Extract	400	2.60 ± 0.47^{b}	2.85 ± 1.03^{bc}	62.86	59.29
1:1 Complex	100	2.23 ± 0.85^{ab}	3.60 ± 1.22^{bc}	68.14	48.57
1:1 Complex	200	2.08 ± 0.61^{ab}	1.88 ± 0.74^{ab}	70.29	73.14
1:1 Complex	400	2.50 ± 1.13^{b}	1.85 ± 0.85^{ab}	64.29	73.57
1:3 Complex	100	2.15 ± 0.47^{ab}	2.72 ± 0.66^{bc}	69.29	61.14
1:3 Complex	200	1.83 ± 0.76^{ab}	1.58 ± 0.49^{ab}	73.86	77.43
1:3 Complex	400	1.53 ± 1.04^{ab}	1.55 ± 0.44^{ab}	78.14	77.86
1:5 Complex	100	2.73 ± 1.66^{b}	2.75 ± 1.56^{bc}	61.00	60.71
1:5 Complex	200	2.30 ± 1.12^{b}	2.65 ± 1.23^{bc}	67.14	62.14
1:5 Complex	400	1.78 ± 0.13^{ab}	1.98 ± 1.51^{ab}	74.58	71.71

Values down the column with the same superscript are not statistically different. ($p > 0.05$). Results are expressed as mean ± SD (n= 4).

3.14 Effects of Extracts of *Bridelia ferruginea, Bryophyllum pinnatum* and their Phytosome Complexes on Gastric pH, Volume and Total acidity in Gastric Ulcerated Rats.

Table 12 shows the effects of *Bridelia ferruginea and Bryophyllum pinnatum* extracts and their phytosome complexes on the gastric volume, pH, and total acidity on indomethacin-induced ulcer in rats. A significant increase ($p < 0.05$) in pH was observed among the groups treated with extract and phytosomes when compared to the pH of untreated group. There was a significant decrease ($p < 0.05$) in the gastric volume and total acidity among the treated groups when compared to the gastric volume and total acidity of the disease control group. The pH of groups treated with 1:3 and 1:5 phytosomes complexes of both plants at 200 and 400 mg/kg respectively were comparable to the pH of groups treated with the standard drug, Cimetidine. A marked reduction of total acidity was found in the groups treated with *Bridelia ferruginea* extract when compared to groups treated with *Bryophyllum pinnatum* extract. *Bridelia ferruginea* phytosmes are highly effective in the reduction of total acidity and are equipotent when compared to the total acidity of those treated with *Bryophyllum pinnatum* phytosomes. However, there was a significant decrease ($p < 0.05$) in total acidity in groups treated with phytosomes of *Bryophyllum pinnatum* when compared to the groups treated with the ordinary extract. But groups treated with phytosomes complexes of *Bridelia ferruginea* showed a non- significant decrease ($p > 0.05$) in total acidity when compared to their extract dosage forms.

3.15 Effects of *B. ferruginea* and *B. pinnatum* Extracts and their Phytosome Complexes on Kidney Markers.

Table 13 shows a significant ($p < 0.05$) decreases in urea and creatinine concentrations among the groups treated with extracts and phytosomes when compared to the urea and creatinine concentrations of the ulcer untreated group. Groups treated with various doses of extracts and phytosomes showed non-significant ($p > 0.05$) decrease in creatinine level when compared to the groups treated with cimetidine (standard drug) in both plants. *Bridelia ferruginea* extract showed a better reduction of urea concentration when compared to the urea concentrations of groups treated with *Bryophyllum pinnatum* extract dosage forms. However, groups treated with phytosomes showed more and efficient reduction in urea and creatinine concentrations when compared to the urea and creatinine concentrations of group treated with conventional extract forms in both plants. The 400 mg/kg b.w of 1:1 phytosome of *Bridelia ferruginea* showed the least urea concentration (41.97 mg/dl) while the 200 mg/kg b.w of 1:3 phytosome of *Bryophyllum pinnatum* showed least urea concentration (42.10 mg/dl).

Table 12 Effects of extracts of *Bridelia ferruginea*, *Bryophyllum pinnatum* and Their Phytosome Complexes on Gastric pH, Volume and Total acidity in Ulcerated rats

Treatment	Dose (mg/kg)	pH		VOLUME (ml)		TOTAL ACIDITY (mmol/l)	
		B. ferruginea	*B. pinnatum*	*B. ferruginea*	*B. pinnatum*	*B. ferruginea*	*B. Pinnatum*
Normal	5 ml	5.50 ± 0.58^{b}	5.50 ± 0.58^{ab}	2.03 ± 0.56^{a}	2.03 ± 0.56^{a}	6.50 ± 2.74^{a}	6.50 ± 2.74^{a}
Ulcer control	5 ml	4.01 ± 0.82^{a}	4.01 ± 0.82^{a}	4.03 ± 0.60^{c}	4.03 ± 0.60^{e}	18.25 ±1.71^{c}	18.25 ±1.71^{e}
1%tween 80	5 ml	4.20 ± 0.98^{ab}	4.20 ± 0.98^{ab}	3.98 ± 0.46^{c}	3.98 ± 0.46^{de}	18.07 ± 5.83^{c}	18.07 ± 5.83^{de}
Phospholipids	100	4.35 ± 1.58^{ab}	4.35 ± 1.58^{ab}	3.75 ± 0.83^{c}	3.75 ± 0.83^{de}	17.34 ± 5.67^{c}	17.34 ± 5.67^{de}
Cimetidine	100	5.45 ± 0.64^{b}	5.45 ± 0.64^{ab}	2.37 ±1.08^{ab}	2.37 ±1.08^{abc}	7.50 ± 3.11^{ab}	7.50 ± 3.11^{a}
Cimetidine in lipids	100	5.52 ± 0.96^{b}	5.52 ± 0.96^{ab}	2.25 ± 0.06^{ab}	2.25 ± 0.06^{ab}	7.52 ± 3.82^{ab}	7.52 ± 3.82^{a}
Extract	100	5.00 ± 0.00^{ab}	5.00 ± 0.00^{ab}	3.78 ± 0.68^{c}	3.60 ± 0.49^{de}	11.50 ± 2.89^{b}	14.00 ± 3.74^{cde}
Extract	200	5.25 ± 0.50^{b}	5.25 ± 1.26^{ab}	3.40 ± 0.16^{c}	3.55 ± 0.53^{de}	10.25 ± 1.26^{ab}	13.25 ± 1.50^{bcd}
Extract	400	5.31 ± 0.86^{b}	5.38 ± 1.10^{ab}	3.40 ± 0.16^{c}	3.43 ± 0.42^{cde}	8.00 ± 1.63^{ab}	10.25 ± 0.5^{abc}
1:1 Complex	100	5.25 ± 0.50^{b}	5.00 ± 00^{ab}	3.80 ± 0.24^{c}	3.10 ± 0.78^{abcde}	9.50 ± 3.87^{ab}	9.00 ± 2.16^{ab}
1:1 Complex	200	5.50 ± 0.58^{b}	5.20 ± 1.60^{ab}	3.23 ± 1.03^{bc}	3.05 ± 0.77^{abcde}	8.26 ± 1.49^{ab}	8.50 ± 3.00^{bcd}
1:1 Complex	400	5.37 ± 1.25^{b}	5.40 ± 1.45^{ab}	3.10 ± 0.38^{bc}	2.85 ± 0.72^{abcd}	8.01 ± 0.82^{ab}	8.00 ± 0.82^{a}
1:3 complex	100	5.25 ± 0.50^{b}	5.41 ± 0.70^{ab}	3.63 ± 0.96^{c}	3.30 ± 0.26^{bcde}	9.50 ± 1.00^{ab}	8.13 ± 3.06^{a}
1:3 complex	200	5.25 ± 0.50^{b}	5.52 ± 0.96^{ab}	3.10 ± 0.38^{bc}	3.18 ± 0.77^{bcde}	7.50 ± 1.91^{ab}	7.00 ± 1.63^{a}
1:3 complex	400	5.52 ± 1.69^{b}	5.54 ± 0.92^{b}	2.98±0.54^{abc}	3.03 ± 0.54^{abcde}	6.78 ± 2.71^{a}	6.74 ± 2.88^{a}
1:5 Complex	100	5.25 ± 0.50^{ab}	5.25 ± 0.50 ^{ab}	3.60 ± 0.49^{c}	3.50 ± 1.23^{cde}	10.25 ± 0.50^{ab}	10.00 ± 2.45^{abc}
1:5 Complex	200	5.43 ± 0.67^{b}	5.25 ± 0.96^{ab}	3.48 ± 0.73^{c}	3.35 ± 0.41^{bcde}	9.50 ± 3.41^{ab}	8.50 ± 1.73^{a}
1:5 Complex	400	5.37 ± 1.25^{b}	5.41 ± 1.18^{ab}	3.20 ± 0.52^{bc}	3.00 ± 0.71^{abcde}	7.48 ± 2.64^{ab}	7.72 ± 1.31^{a}

Table 13 Effects of *B. ferruginea*, *B. pinnatum* Extracts and Their Phytosome Complexes on the Kidney Markers

Treatment	Dose (mg/kg)	UREA(mg/dl)		CREATININE (mg/dl)	
		B.ferruginea	***B. pinnatum***	***B. ferruginea***	***B. pinnatum***
Normal	5 ml	40.42± 7.28^{a}	40.42 ± 7.28^{a}	0.69 ± 0.31^{a}	0.69 ± 0.31^{a}
Ulcer control	5 ml	65.02± 5.87^{c}	65.02± 5.87^{c}	1.82 ± 0.90^{b}	1.82 ± 0.90^{b}
1%tween 80	5 ml	63.41±35.71^{bc}	63.41±35.71^{c}	1.84 ± 0.12^{b}	1.84 ± 0.12^{b}
Phospholipid	100	58.36±25.69^{abc}	58.36±25.69^{abc}	1.20 ± 0.08^{ab}	1.20 ± 0.08^{ab}
Cimetidine	100	46.49±0.82^{abc}	46.49±0.82^{abc}	0.90 ± 0.22^{a}	0.90 ± 0.22^{a}
Cimetidine in lipids	100	46.20±8.59^{abc}	46.20±8.59^{abc}	0.84 ± 0.27^{a}	0.84 ± 0.27^{a}
Extract	100	63.05 ± 4.47^{bc}	62.41±11.65^{bc}	0.69 ± 0.20^{a}	0.64± 0.09^{a}
Extract	200	56.24±1.07^{abc}	60.01±5.60^{abc}	0.65 ± 0.15^{a}	0.83 ± 0.04^{a}
Extract	400	43.63 ± 2.84^{ab}	47.27±7.20^{abc}	0.63 ± 0.12^{a}	0.62 ± 0.09^{a}
1:1 Complex	100	60.14± 0.66^{abc}	53.06±0.73^{abc}	0.59 ± 0.06^{a}	0.66± 0.08^{a}
1:1 Complex	200	50.86±13.06^{abc}	50.12±2.86^{abc}	0.61 ± 0.09^{a}	0.66 ± 0.10^{a}
1:1 Complex	400	41.97 ± 2.49^{a}	43.27±4.06^{ab}	0.61 ± 0.13^{a}	0.61 ± 0.11^{a}
1:3 complex	100	50.42±9.51^{abc}	50.48 ± 8.48^{abc}	0.59 ± 0.06^{a}	0.56 ± 0.05^{a}
1:3 complex	200	42.54 ± 2.52^{a}	42.10 ± 2.67^{a}	0.67 ± 0.08^{a}	0.63 ± 0.08^{a}
1:3 complex	400	42.53 ± 4.64^{a}	40.65 ± 3.19^{a}	0.65 ± 0.09^{a}	0.72 ± 0.04^{a}
1:5 Complex	100	53.02±11.93^{abc}	58.71 ± 3.43^{abc}	0.69 ± 0.09^{a}	0.65 ± 0.10^{a}
1:5 Complex	200	42.07 ± 1.58^{a}	43.59 ±3.74^{ab}	0.67 ± 0.09^{a}	0.68 ± 0.07^{a}
1:5 Complex	400	43.52 ± 2.64^{ab}	43.05 ± 3.76^{ab}	0.59 ± 0.08^{a}	0.61 ± 0.05^{a}

3.16 Effects of *B. ferruginea* and *B pinnatum* Extracts and their Phytosome Complexes on Liver Enzymes of Indomethacin-induced Ulcer in Rats

Table 14 shows significant decreases ($p < 0.05$) in AST, ALT and ALP activities in groups treated with various doses of extracts and phytosome complexes when compared to the AST, ALT and ALP activities of the ulcer untreated group (control). Groups treated with various doses of extracts of both plants showed a comparable effect on the activities of liver enzyme with groups treated with 100 and 200 mg/kg b. w of 1:1 phytosome complexes in both *Bridelia ferruginea* and *Bryophyllum pinnatum*. Also 400 mg/kg b.w of phytosome complexes (1:1, 1:3 and 1:5) of *Bridelia ferruginea* and *Bryophyllum pinnatum* produced best improved activities of ALT and ALP when compared to the ALT and ALP activities of groups treated with other doses of phytosome complexes.

3.17 Effects of *B. ferruginea, B. pinnatum* Extracts and their Phytosome Complexes on the Antioxidant Status.

Table 15 shows significant increases ($p < 0.05$) in the activities of antioxidant enzymes (SOD, CAT, and GPx) in groups treated with extract and their phytosome complexes when compared with the activities of antioxidant enzymes (SOD, CAT and GPx) of the ulcer untreated group (control). A significant decrease ($p > 0.05$) in the malondialdehyde (MDA) concentration was observed in the groups treated with the extracts and the phytosome complexes when compared with the malondialdehyde (MDA) concentration in the ulcer untreated group (control). Groups treated with phytosomes of *Bridelia ferruginea* showed a significant increase ($p < 0.05$) in the activities of SOD, CAT and GPx when compared to the activities of SOD, CAT and GPx in the groups treated with extract. However, the phytosome complexes of *Bryophyllum pinnatum* showed significant increase in SOD and CAT activities when compared to the SOD and CAT activities of group treated with the conventional extract. An exception to this is groups treated with varying doses of 1:1 *Bryophyllum pinnatum* phytosome complex.

Table 14 Effects of *B. ferruginea*, *B pinnatum* and Their Phytosome Complexes on Liver Enzyme Activities in Indomethacin-induced Ulcer Rats

Treatment	Dose (mg/kg)	AST(IU/L)		ALT(IU/L)		ALP (IU/L)	
		B. ferruginea	*B. pinnatum*	*B.ferruginea*	*B. pinnatum*	*B. ferruginea*	*B. pinnatum*
Normal	5 ml	9.33 ± 1.73^{a}	9.33 ± 1.73^{a}	9.75 ± 0.44^{a}	9.75 ± 0.44^{a}	80.11 ± 4.71^{a}	80.11 ± 4.71^{a}
Ulcer control	5 ml	14.12 ± 2.77^{c}	14.12 ± 2.77^{c}	12.50 ± 2.60^{b}	12.50 ± 2.60^{b}	95.44 ± 3.00^{b}	95.44 ± 3.00^{b}
1%tween 80	5 ml	14.04 ± 6.59^{c}	14.04 ± 6.59^{c}	11.86 ± 3.70^{ab}	11.86 ± 3.70^{ab}	84.89 ± 6.79^{ab}	84.89 ± 6.79^{ab}
Phospholipid	100	13.68 ± 4.57^{bc}	13.68 ± 4.57^{bc}	10.74 ± 1.50^{ab}	10.74 ± 1.50^{ab}	80.74 ± 4.88^{a}	80.74 ± 4.88^{a}
Cimetidine	100	10.65 ± 0.73^{abc}	10.65 ± 0.73^{abc}	11.82 ± 2.77^{ab}	11.82 ± 2.77^{ab}	88.16 ± 4.20^{ab}	88.16 ± 4.20^{ab}
Cimetidine in lipids	100	9.52 ± 2.02^{a}	9.52 ± 2.02^{ab}	11.72 ± 2.33^{ab}	11.72 ± 2.33^{ab}	86.42 ± 8.53^{ab}	86.42 ± 8.53^{ab}
Extract	100	10.78 ± 1.55^{abc}	10.87 ± 0.72^{abc}	10.41 ± 1.82^{ab}	10.28 ± 1.46^{ab}	83.03 ± 3.99^{a}	84.01 ± 3.47^{ab}
Extract	200	10.70 ± 1.07^{abc}	10.70 ± 1.75^{abc}	9.79 ± 0.87^{ab}	9.99 ± 0.81^{ab}	81.26 ± 5.54^{a}	83.25 ± 3.86^{ab}
Extract	400	9.94 ± 1.39^{ab}	10.50 ± 1.04^{abc}	9.67 ± 0.47^{a}	9.95 ± 0.78^{ab}	80.67 ± 5.40^{a}	81.26 ± 12.13^{a}
1:1 Complex	100	10.61± 0.71^{abc}	10.50 ± 2.33^{abc}	10.13 ± 1.02^{ab}	10.00 ± 0.57^{ab}	83.13 ± 7.81^{a}	83.25 ± 3.86^{ab}
1:1 Complex	200	10.34 ± 1.81^{abc}	10.30 ± 2.00^{abc}	9.89 ± 1.12^{ab}	9.95 ± 1.20^{ab}	82.18 ± 5.42^{a}	82.86 ± 4.65^{a}
1:1 Complex	400	9.89 ± 1.60^{ab}	10.29 ± 2.38^{abc}	9.44 ± 0.86^{a}	9.68 ± 0.62^{a}	81.16 ± 8.94^{a}	80.67 ± 8.89^{a}
1:3 complex	100	10.79 ± 1.16^{abc}	10.73 ± 0.93^{abc}	9.92 ± 0.67^{ab}	10.04 ± 0.52^{ab}	81.26 ± 5.65^{a}	82.45 ± 4.33^{a}
1:3 complex	200	9.73 ± 0.65^{ab}	10.62 ± 1.64^{abc}	9.79 ± 0.25^{ab}	9.80 ± 0.80^{a}	81.12 ± 8.47^{a}	81.88 ± 7.59^{a}
1:3 complex	400	9.08 ± 2.72^{a}	10.50 ± 1.78^{abc}	9.52 ± 1.07^{a}	9.28 ± 0.56^{a}	80.51 ± 5.81^{a}	80.25 ± 7.50^{a}
1:5 Complex	100	10.50 ± 1.72^{abc}	10.43 ± 1.27^{abc}	9.96 ± 0.59^{ab}	9.85 ± 0.40^{ab}	83.38 ± 8.12^{a}	80.38 ± 8.01^{a}
1:5 Complex	200	9.77 ± 1.43^{ab}	10.43 ± 1.31^{abc}	9.78 ± 0.85^{ab}	9.83 ± 0.45^{a}	81.13 ± 7.40^{a}	80.50 ± 10.51^{a}
1:5 Complex	400	9.46 ± 1.38^{a}	9.74 ± 0.68^{ab}	9.40 ± 0.41^{a}	9.22 ± 0.64^{a}	80.38 ± 5.34^{a}	80.50 ± 6.09^{a}

Table 15 Effects of *B. ferruginea, B. pinnatum* Extracts and Their Phytosome Complexes on the Antioxidant Status of indomethacin-induced Rats.

GROUPS	SOD (IU/L)		CAT(IU/L)		GPX (IU/L)		MDA (mg/dl)	
	B. ferruginea	*B. pinnatum*	*B. ferruginea*	*B. pinnatum*	*B. ferruginea*	*B. pinnatum*	*B. ferruginea*	*B. pinnatum*
5 ml Normal	12.01 ± 0.41^{c}	12.01 ± 0.41^{e}	1.17 ± 0.22^{f}	1.17 ± 0.22^{b}	11.00 ± 1.90^{b}	11.00 ± 1.90^{c}	1.19 ± 0.23^{a}	1.19 ± 0.23^{a}
5 ml ulcer control	8.17 ± 1.64^{a}	8.17 ± 1.64^{a}	0.86 ± 0.15^{abc}	0.86 ± 0.15^{a}	8.03 ± 1.77^{a}	8.03 ± 1.77^{a}	3.45 ± 1.10^{f}	3.45 ± 1.10^{f}
5 ml 1% tween 80	8.20 ± 2.76^{a}	8.20 ± 2.76^{a}	0.84 ± 0.26^{ab}	0.84 ± 0.26^{a}	8.07 ± 1.60^{a}	8.07 ± 1.60^{a}	2.44 ± 0.31^{de}	2.44 ± 0.31^{bcde}
100 mg/kg phospholipid	8.43 ± 1.56^{a}	8.43 ± 1.56^{ab}	0.90 ± 0.29^{abcde}	0.90 ± 0.29^{ab}	8.10 ± 0.72^{a}	8.10 ± 0.72^{ab}	2.33 ± 0.57^{cde}	2.33 ± 0.57^{abcd}
100 mg cimetidine	11.85 ± 0.64^{c}	11.85 ± 0.64^{e}	1.07 ± 0.05^{abcde}	1.07 ± 0.05^{ab}	10.94 ± 1.93^{b}	10.94 ± 1.93^{bc}	1.26 ± 0.20^{ab}	1.26 ± 0.20^{a}
100 mg/kg cimetidine (lipids)	11.87 ± 0.65^{c}	11.87 ± 0.65^{e}	1.11 ± 0.02^{cdef}	1.11 ± 0.02^{ab}	11.00 ± 1.90^{b}	11.00 ± 1.90^{c}	1.20 ± 0.25^{a}	1.20 ± 0.25^{ab}
100 mg/kg Extract	8.72 ± 2.75^{ab}	8.26 ± 2.91^{a}	0.84 ± 0.21^{ab}	0.87 ± 0.29^{a}	8.40 ± 1.78^{ab}	8.50 ± 1.67^{abc}	3.04 ± 0.46^{ef}	3.83 ± 0.94^{f}
200 mg/kg Extract	9.04 ± 2.03^{ab}	8.73 ± 0.49^{abc}	0.92 ± 0.07^{abcdef}	0.97 ± 0.20^{ab}	9.09 ± 2.60^{ab}	8.79 ± 3.24^{abc}	3.00 ± 1.01^{ef}	3.02 ± 1.63^{cdef}
400 mg/kg Extract	10.00 ± 1.33^{abc}	9.02 ± 2.56^{abcd}	1.04 ± 0.20^{abcdef}	1.14 ± 0.04^{ab}	9.87 ± 0.54^{ab}	9.22 ± 2.09^{abc}	2.41 ± 0.13^{de}	2.31 ± 1.24^{abcd}
100 mg/kg 1:1 complex	9.51 ± 1.58^{abc}	8.40 ± 0.57^{ab}	0.95 ± 0.24^{abcdef}	0.92 ± 0.15^{ab}	8.94 ± 2.26^{ab}	8.90 ± 3.18^{abc}	3.01 ± 0.21^{ef}	3.24 ± 0.34^{abc}
200 mg/kg 1:1 complex	9.94 ± 1.67^{abc}	9.10 ± 1.51^{abcd}	0.81 ± 0.05^{a}	0.94 ± 0.21^{ab}	10.34 ± 1.41^{ab}	9.34 ± 0.71^{abc}	2.10 ± 0.98^{bcd}	2.07 ± 0.33^{abc}
400 mg/kg 1:1 complex	10.28 ± 1.28^{abc}	9.48 ± 2.19^{abcde}	1.13 ± 0.19^{def}	1.13 ± 0.06^{ab}	10.69 ± 1.17^{ab}	10.84 ± 1.19^{abc}	1.37 ± 0.23^{ab}	1.25 ± 0.06^{a}
100 mg/kg 1:3 complex	10.75 ± 0.72^{abc}	10.84 ± 0.48^{bcde}	0.92 ± 0.04^{abcdef}	0.97 ± 0.11^{ab}	9.47 ± 1.24^{ab}	9.62 ± 0.73^{abc}	2.01 ± 0.73^{abcd}	1.23 ± 0.11^{a}
200 mg/kg 1:3 complex	11.29 ± 0.13^{bc}	11.29 ± 0.17^{e}	1.08 ± 0.11^{bcdef}	1.17 ± 0.25^{b}	10.35 ± 0.71^{ab}	10.29 ± 0.62^{abc}	1.51 ± 0.33^{abc}	1.25 ± 0.06^{a}
400 mg/kg 1:3 complex	12.05 ± 1.42^{c}	11.87 ± 0.91^{e}	1.18 ± 0.05^{f}	1.17 ± 0.19^{b}	11.02 ± 1.10^{ab}	10.57 ± 0.84^{abc}	1.20 ± 0.04^{a}	1.21 ± 0.07^{a}
100 mg/kg 1:5 complex	10.21 ± 1.42^{abc}	9.74 ± 2.03^{abcde}	0.88 ± 0.08^{abcd}	1.03 ± 0.12^{ab}	9.62 ± 0.71^{ab}	9.43 ± 0.64^{abc}	2.36 ± 0.25^{de}	2.11 ± 0.73^{abc}
200 mg/kg 1:5 complex	11.38 ± 0.23^{bc}	10.17 ± 1.06^{abcde}	1.11 ± 0.03^{bcdef}	1.09 ± 0.05^{ab}	10.34 ± 0.71^{ab}	10.31 ± 0.96^{abc}	1.19 ± 0.02^{a}	1.26 ± 0.08^{a}
400 mg/kg 1:5 complex	12.00 ± 0.84^{c}	11.25 ± 0.14^{cde}	1.15 ± 0.09^{ef}	1.10 ± 0.06^{ab}	11.08 ± 1.91^{ab}	10.37 ± 0.71^{abc}	1.25 ± 0.08^{ab}	1.25 ± 0.08^{a}

3.18 Effects of *B. ferruginea*, *B. pinnatum* Extracts and Their Phytosome Complexes on Lipid Profile of Indomethacin-induced Rats

The groups treated with varying doses 1:3 and 1:5 *Bridelia ferruginea* and *Bryophyllum pinnatum* phytosomes showed significant decreases ($p < 0.05$) in cholesterol concentrations when compared to the cholesterol concentrations of groups treated with extracts. All groups treated with 1:1 phytosome complexes of both plants showed non-significant ($p > 0.05$) decrease in in cholesterol concentration when compared with the cholesterol concentrations of groups treated with extract doses.

All the groups treated with phytosome complexes (1:1, 1:3, and 1:5) of *Bridelia ferruginea* showed significant decreases ($p < 0.05$) in TAG concentrations when compared to the TAG concentrations of groups treated with its extract. However, the groups treated with 100 mg/kg of all the phytosome complexes (1:1, 1:3, and 1:5) of *Bridelia ferruginea* showed non-significant decrease ($p > 0.05$) in TAG concentration when compared to the TAG concentration of groups treated with extract.

Groups treated with 1:3 and 1:5 phytosome complexes of *Bridelia ferruginea* and *Bryophyllum pinnatum* showed significant increases ($p < 0.05$) in HDL concentrations when compared to the HDL concentrations of groups treated with extracts. Groups treated with 1:1 phytosome complexe of both plants showed non-significant ($p > 0.05$) increase in HDL concentration when compared to the HDL concentration of groups treated with ordinary extract dosage form.

All groups treated with 1:3 and 1:5 phytosome complexes of *Bridelia ferruginea* and *Bryophyllum pinnatum* showed significant decreases ($p < 0.05$) in LDL concentrations when compared to the LDL concentrations of groups treated with extracts. However, groups treated with 100 mg/kg of 1:1 *Bridelia ferruginea* and *Bryophyllum pinnatum* phytosome complexes showed non-significant decrease ($p > 0.05$) in LDL concentration when compared to the LDL concentration of groups treated with conventional plant extracts.

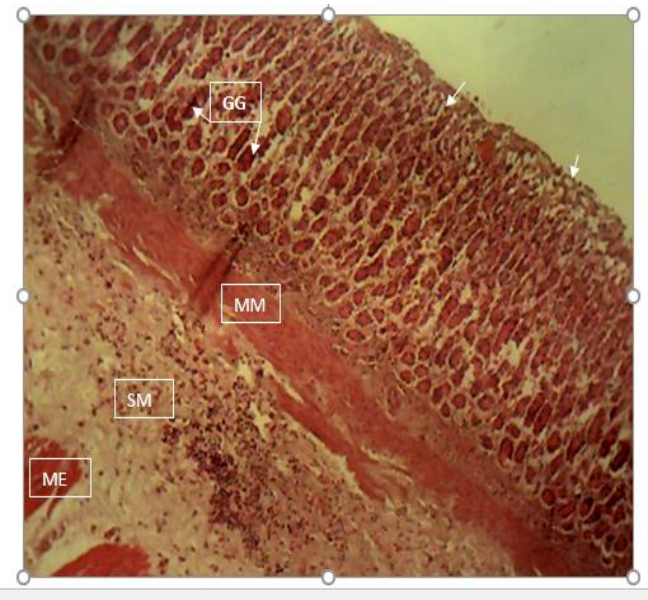
Generally, 1:3 and 1:5 phytosme complexes of *Bridelia ferruginea* and *Bryophyllum pinnatum* improved lipid profile of indomethacin-induced ulcer in rats when compared to the lipid profile of groups treated with ordinary plant extracts. The phytosomes of *Bridelia ferruginea* performed better than *Bryophyllum pinnatum* phytosomes in the improvement of the TAG concentration of ulcerated rats.

Table 16: Effects of *B. ferruginea*, *B. pinnatum* Extracts and Their Phytosome Complexes on Lipid Profile of Indomethacin-Induced Ulcerated Rats

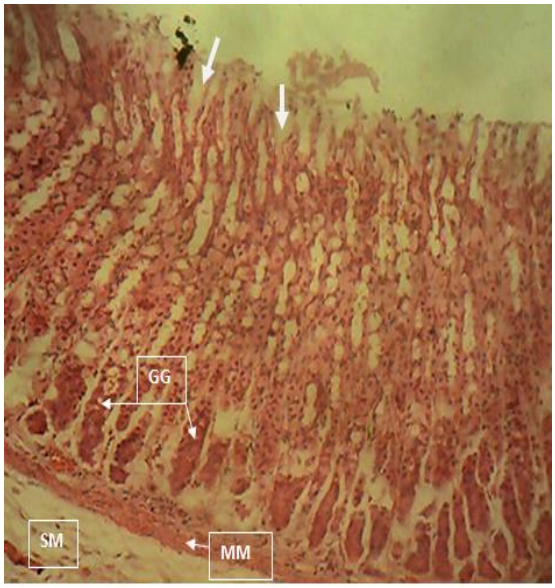
GROUPS	CHOL(mmol/l)		TAG (mmol/l)		HDL (mmol/l)		LDL (mmol/l)	
	B. ferruginea	***B. pinnatum***	***B. feruginea***	***B. pinnatum***	***B. ferruginea***	***B. pinnatum***	***B. ferruginea***	***B. pinnatum***
5 ml (N/s)Normal	2.49 ± 1.47^{a}	2.49 ± 1.47^{a}	1.07 ± 0.27^{a}	1.07 ± 0.27^{a}	2.05 ± 0.56^{d}	2.05 ± 0.56^{b}	1.46 ± 0.75^{ab}	1.46 ± 0.75^{abc}
5 ml(N/s) ulcer control	4.76 ± 1.58^{c}	4.76 ± 1.58^{c}	3.24 ± 0.81^{b}	3.24 ± 0.81^{b}	0.63 ± 0.29^{a}	0.63 ± 0.29^{a}	3.15 ± 1.39^{de}	3.15 ± 1.39^{d}
5 ml 1% tween 80	3.64 ± 0.49^{abc}	3.64 ± 0.49^{abc}	3.24 ± 1.32^{b}	3.24 ± 1.32^{b}	0.87 ± 0.37^{ab}	0.87 ± 0.37^{a}	2.54 ± 0.21^{bcde}	2.54 ± 0.21^{bcd}
100mg/kg phospholipid	3.00 ± 0.83^{ab}	3.00 ± 0.83^{ab}	1.50 ± 0.68^{a}	1.50 ± 0.68^{a}	1.00 ± 0.23^{abcd}	1.00 ± 0.23^{a}	1.94 ± 1.16^{abc}	1.94 ± 1.16^{abcd}
100mg/kg cimetidine	2.53 ± 1.04^{a}	2.53 ± 1.04^{a}	1.18 ± 0.03^{a}	1.18 ± 0.03^{a}	1.30 ± 0.51^{abcd}	1.30 ± 0.51^{ab}	1.52 ± 0.56^{ab}	1.52 ± 0.56^{abc}
100mg/kg cimetidine (lipid)	2.58 ± 0.79^{a}	2.58 ± 0.79^{a}	1.29 ± 0.17^{a}	1.29 ± 0.17^{a}	1.34 ± 0.46^{abcd}	1.34 ± 0.46^{ab}	1.48 ± 0.44^{ab}	1.48 ± 0.44^{abc}
100 mg/kg Extract	4.44 ± 1.73^{bc}	4.37 ± 1.33^{bc}	3.05 ± 0.28^{b}	2.10 ± 0.34^{a}	0.64 ± 0.24^{a}	0.73 ± 0.41^{a}	3.24 ± 0.17^{e}	3.07 ± 0.60^{d}
200 mg/kg Extract	4.28 ± 0.45^{bc}	4.01 ± 0.91^{abc}	3.12 ± 0.82^{b}	2.04 ± 0.71^{a}	0.84 ± 0.31^{ab}	0.90 ± 0.34^{a}	3.25 ± 0.92^{e}	3.00 ± 1.53^{d}
400 mg/kg Extract	3.07 ± 0.23^{ab}	3.03 ± 0.51^{ab}	2.24 ± 0.20^{ab}	1.94 ± 0.50^{a}	1.52 ± 1.15^{abcd}	1.12 ± 0.64^{a}	2.75 ± 1.14^{cde}	1.92 ± 0.66^{abcd}
100 mg/kg 1:1 complex	4.31 ± 1.00^{bc}	4.02 ± 1.49^{abc}	3.01 ± 1.89^{b}	2.11 ± 0.79^{a}	0.76 ± 0.41^{a}	0.94 ± 0.14^{a}	2.86 ± 0.42^{cde}	3.04 ± 0.45^{d}
200 mg/kg 1:1 complex	3.04 ± 0.85^{ab}	3.38 ± 0.76^{abc}	1.32 ± 0.31^{a}	1.41 ± 0.63^{a}	1.27 ± 0.51^{abcd}	1.06 ± 0.14^{a}	2.04 ± 0.91^{abcd}	2.29 ± 0.78^{d}
400 mg/kg 1:1 c0mplex	3.17 ± 0.80^{ab}	2.82 ± 0.45^{a}	1.47 ± 0.28^{a}	1.38 ± 0.40^{a}	2.00 ± 0.66^{cd}	1.32 ± 0.62^{ab}	1.49 ± 0.65^{ab}	1.52 ± 0.39^{abc}
100 mg/kg 1:3 complex	2.66 ± 0.25^{a}	3.41 ± 1.06^{abc}	2.24 ± 0.97^{ab}	1.78 ± 0.67^{a}	0.97 ± 0.52^{abc}	1.34 ± 0.59^{ab}	2.06 ± 0.57^{abcde}	2.08 ± 1.22^{abcd}
200 mg/kg 1:3 complex	2.60 ± 0.46^{a}	2.65 ± 0.31^{a}	1.19 ± 0.23^{a}	1.22 ± 0.08^{a}	1.86 ± 1.09^{bcd}	2.01 ± 0.39^{b}	1.37 ± 0.04^{ab}	1.21 ± 0.20^{ab}
400 mg/kg 1:3 complex	2.47 ± 1.07^{a}	2.52 ± 0.45^{a}	1.10 ± 0.30^{a}	1.20 ± 0.40^{a}	2.04 ± 1.18^{d}	2.05 ± 0.80^{b}	1.44 ± 0.42^{ab}	1.05 ± 0.10^{a}
100 mg/kg 1:5 complex	3.20 ± 0.53^{ab}	3.09 ± 0.77^{ab}	2.17 ± 0.72^{ab}	2.00 ± 1.14^{a}	0.85 ± 0.22^{ab}	1.45 ± 0.62^{ab}	2.49 ± 0.75^{bcde}	2.75 ± 1.31^{cd}
200 mg/kg 1:5 complex	2.57 ± 0.52^{a}	2.54 ± 0.94^{a}	2.04 ± 0.18^{ab}	1.31 ± 0.17^{a}	1.29 ± 0.53^{abcd}	2.02 ± 0.32^{b}	2.00 ± 0.47^{abcd}	1.56 ± 0.57^{abc}
400 mg/kg 1:5 complex	2.49 ± 0.44^{a}	2.62 ± 0.66^{a}	1.20 ± 0.66^{a}	1.08 ± 0.77^{a}	2.02 ± 0.77^{cd}	2.00 ± 0.71^{b}	1.26 ± 0.42^{a}	1.28 ± 0.83^{ab}

3.19 Photomicograph of the Stomach Tissues of Indomethacin-induced ulcerated Rats

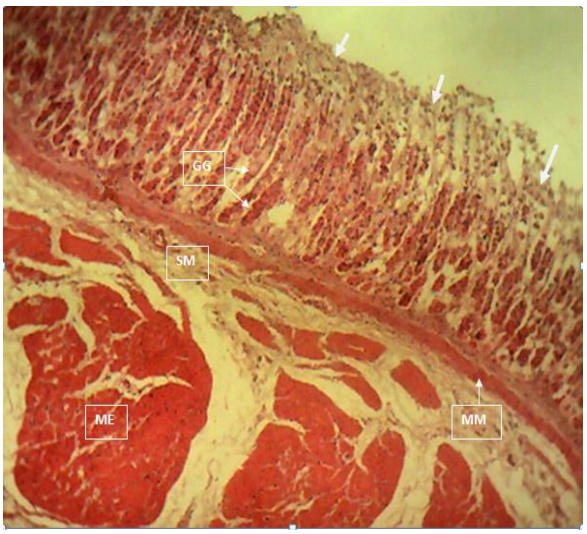
The histopathology photomicrograph revealed normal gastric pit in the normal rats and also showed some degrees of necrotic attacks on the tips of the mucosa on the stomach tissues of ulcerated rats. Reduced level of necrosis was observed among the groups treated with phytosome complexes (Figure 14)



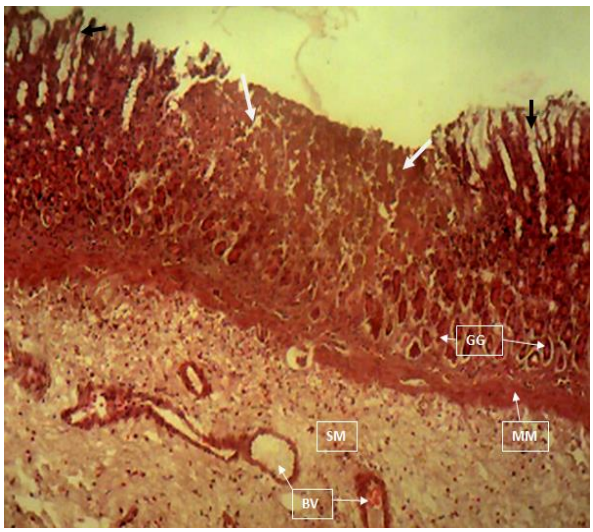
Normal group: shows normal gastric pits (white arrows). The gastric pits are continuos to the gastric gland (GG). Separating the mucosa (M) from underlying submucosa (SM) is the muscularic mucosa (MM). (X 100)



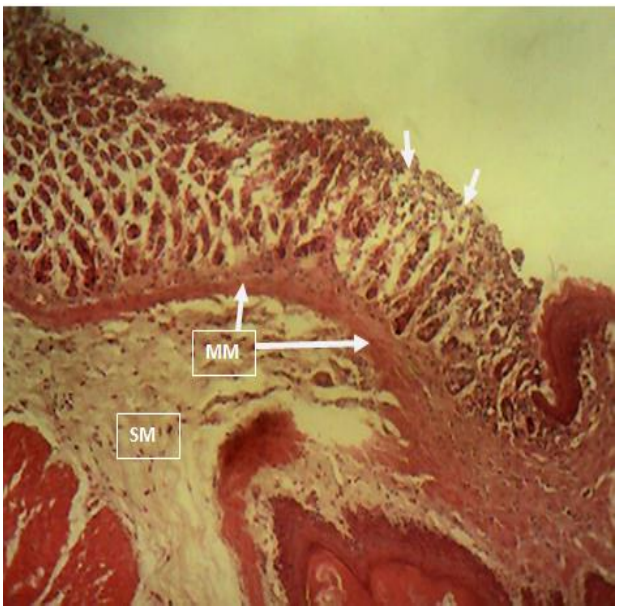
Group treated with Cimetidine: shows relatively normal histomorphology(arrows). The gastric pits are continuos to the gastric gland (GG). Separating the mucosa (M) from underlying submucosa (SM) is the muscularic mucosa (MM). (X 100)



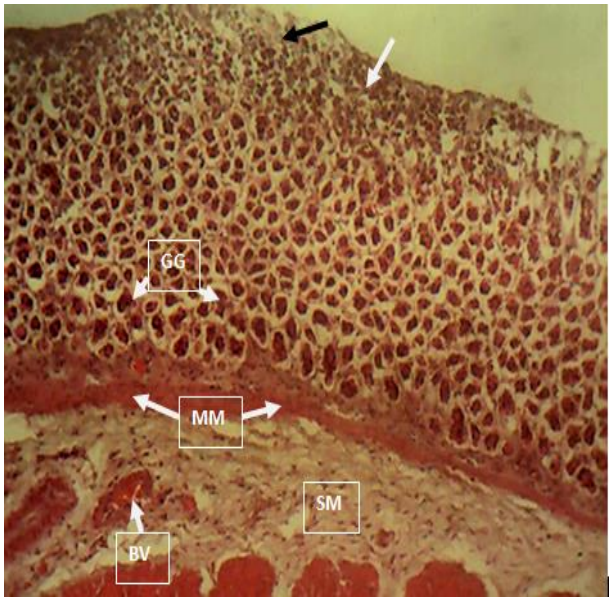
Ulcer untreated: showed a multifocally widespread necrosis of the mucosal tips, at the luminal surfaces as well as necrosis of the epithelia of the gastric pits (white arrow. (X 100)



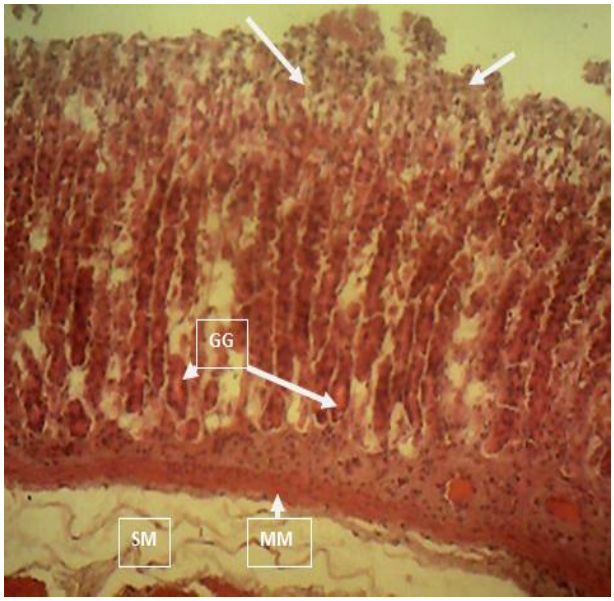
Groups treated with 100 mg/kg phospholipid: Also showed Multifocally widespread necrosis of the mucosal tips. (arrows). However, the deeper areas of the mucosa and the gastric glands (GG) do appear relatively normal. (X 100).



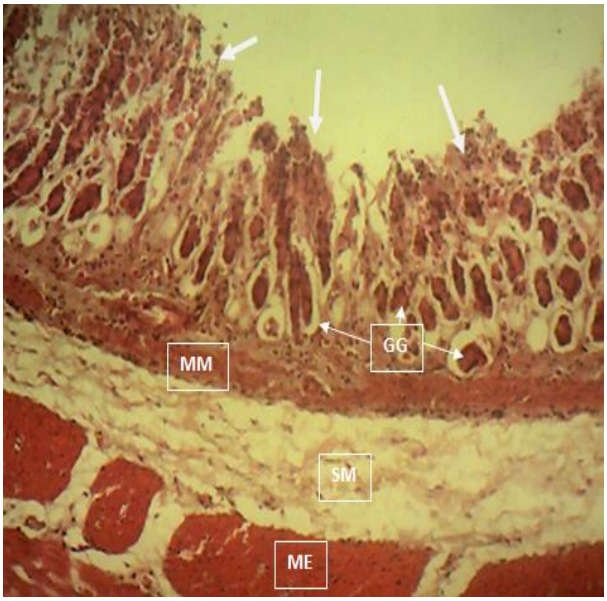
100 mg/kg *B.ferruginea* extract: showed multifocal areas of gastric mucosal necrosis (arrow). Note; Mucosal Necrotic foci are precursors to gastric ulcers. (x100)



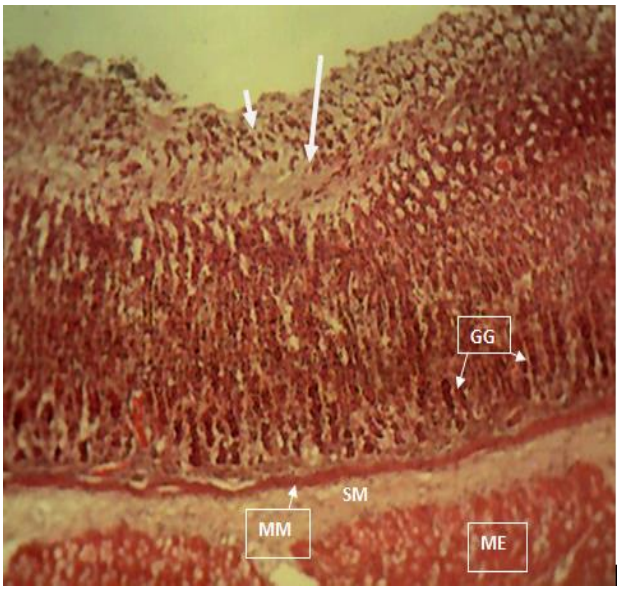
100 mg/kg *B.pinnatum* extract: showed multifocal areas of gastric mucosal necrosis (arrow). Gastric glands (GG); Muscularis mucosa (MM); Sub mucosa (SM); Blood vessel (BV). (x100)



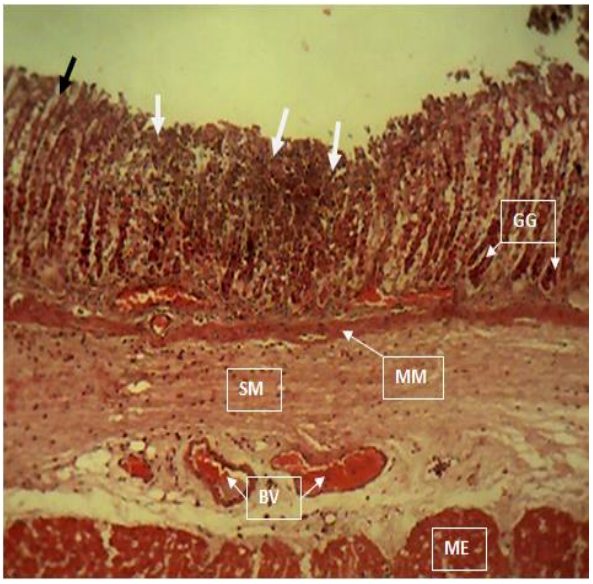
100 mg/kg 1:1 *B.ferruginea* phytosome: showed marked necrosis of the mucosal tips (arrows) (x100)



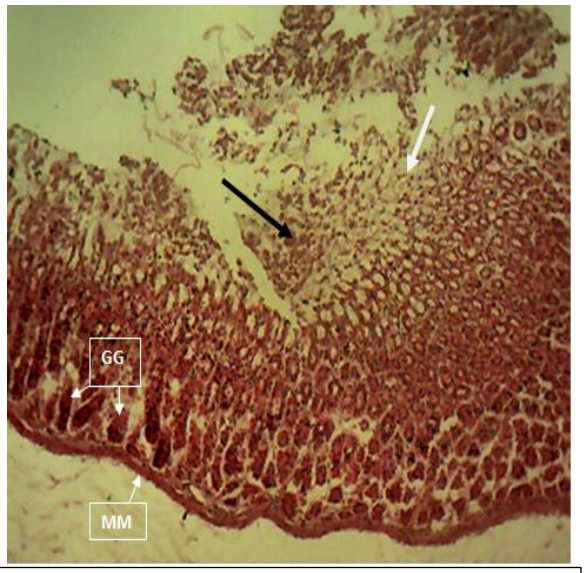
100 mg/kg 1:1 *B.pinnatum* phytosome: showed marked necrosis of the mucosal tips (arrows) and multifocal erosions and ulcerations of the gastric mucosal (arrows) (x100)



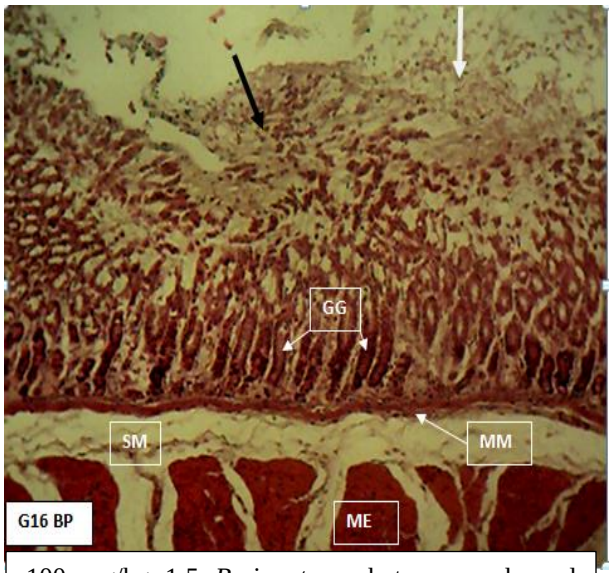
100 mg/kg 1:3 *B.ferruginea* phytosome: showed multifocal necrosis of the mucosal tips (arrows)(x100)



100 mg/kg 1:3 *B.pinnatum* phytosome: showed multifocal necrosis. Areas of necrosis (white arrows); Normal areas showing gastric pits (black arrow) (x100).



100 mg/kg 1:5 *B.ferruginea* phytosome: Also showed multifocal necrosis of the mucosal tips (white arrows). Normal gastric pit are represented with black arrow (x100)



100 mg/kg 1:5 *B.pinnatum* phytosome: showed multifocal necrosis. Areas of necrosis (white arrows); Normal areas showing gastric pits (black arrow) (x100).

Figure 14: Photomicographs of Stomach Tissues of Indomethacin-induced ulcerated Rats

CHAPTER FOUR

DISCUSSION

Human beings, over the years, have been faced with various acute and chronic diseases which have been cured using plant products. Plants grow naturally in the environment while others are used as foods. According to an estimate of the World Health Organization, about 80% of the world population still uses plant-based drugs for their primary health care needs. Traditional medicine is more prevalent in some countries than others. These traditional medicines have gained immense popularity and tolerance of patients not only in developing nations, but the developed western countries like US and UK have also become potential buyers of such medicines. This popularity of phytomedicine is due to its affordability, accessibility among the rural settings. The major drawback of traditional medicine has been found to be low bioavailability and poor absorption across the lipid bilayer due to their hydrophilic nature. There is need for the development of new approaches to maximize the potentials of these plants for treatments of diseases in which one of such approaches includes development of phytosome.

The phytochemical screening of the ethanol extracts of *Bryophyllum pinnatum* and *Bridelia ferruginea* leaves revealed the presence of tannins, carbohydrates, hydrogen cyanide, phenols, flavonoids, reducing sugars, alkaloids, steroids, terpenoids, saponins and glycosides. Large amounts of phenols, flavonoids, and terpenoids were detected in both plant extracts. Higher concentrations of tannins, steroids, reducing sugars, phenols and flavonoids were found in *Bridelia ferruginea* leaves than *Bryophyllum pinnatum*. However, higher concentrations of alkaloids, carbohydrate and terpenoids were found in *Bryophyllum pinnatum* than the amount found in *Bridelia ferruginea* leaves. These phytochemicals found in these leaves have been found to be effective in the treatment of various diseases which include bacterial infections and ulcer diseases (Ofokansi *et al*., 2005, Dudareva *et al.,* 2004).

The phyto-constituents like flavonoids, tannins, terpenoids, and saponin have been reported in several anti-ulcer literature as possible gastroprotective agents. Flavonoids, tannins and terpenoids are among the cytoprotective active materials for which anti ulcerogenic efficacy have been extensively confirmed.

Tannins may prevent ulcer development due to their protein-precipitating ability and vasoconstriction effects (Pandian *et al.,* 2002). But saponins and glycosides were not detected in *Bryophyllum pinnatum* extract which may be due to the plant part used, age or geographical location of the plant. The phytochemical compositions observed in *Bridelia ferruginea* extract were in line with the findings of Akinsetel *et al*. (2017) and that of *Bryophyllum pinnatum* was also consistent with the findings of Kanika (2011); Okwu and Josiah (2006) who had reported the presence of these phytochemicals in their study. Phytochemicals accumulate in different parts of plants, such as in the roots, stems, leaves, flowers, fruits or seeds (Costa *et al*., 1999). The absence of saponins and glycosides observed in *Bryophyllum pinnatum* leaves could be as a result of the part of plant used for study. Many phytochemicals, particularly the pigment molecules are often concentrated in the outer layers of the various plant tissues. Concentrations of phytochemicals vary from plant to plant depending upon the variety, processing, cooking and growing conditions of plants (King and Young, 1999).

Reactive oxygen or nitrogen species are detrimental to biological molecules in the cells, hence destroying cell membranes, nucleic acids and proteins which in turn lead to ageing and other diseases. Vegetables and fruits are good sources of free radical scavengers or antioxidants. Antioxidants have been found to be useful in the protection of the human body against free radical-induced damage. Phytochemicals in plants carry out this function by acting as oxygen scavengers (Buyukokuroglu *et al*., 2001). Most of the antioxidants are part of our diet including the polyphenolic variety such as flavonoids (Hanninen *et al*., 2000). There was dose dependent increase in DPPH and FRAP scavenging activity in *Bridelia ferruginea* and *Bryophyllum pinnatum* leaf extracts. The plant extracts exhibited strong antioxidant activities. The DPPH radical scavenging assay enabled the measurement of the electron-donating ability of the extracts and the scavenging of radicals was indicated by the extent of the decolourisation of the DPPH solution (Krishnaiah *et al*., 2011). The IC_{50} of the *Bridelia ferruginea* extract was 1.00 and that of *Bryophyllum pinnatum* was 1.28 as against that of the standard ascorbic acid that was 1.37. This indicated that both plants have strong antioxidant capacity with that of *Bridelia ferruginea* extract being stronger than that of *Bryophyllum pinnatum* extract. Scavenging activity of *Bridelia ferruginea* in DPPH assay at concentration of 500 µg/ml was reduced. Since the DPPH assay is based on spectrometry, this reduced scavenging activity could be as a result of the turbidity of the extract which may be darker than the DPPH solution on increasing concentration.

Phenolic compounds are known as powerful chain breaking antioxidants. Phenols are very important plant constituents, because of their scavenging ability due to the presence of hydroxyl groups they may contribute directly as antioxidative agents. It is suggested that polyphenolic compounds have inhibitory effects on mutagenesis and carcinogenesis in humans. These *in vitro* assays indicated that the ethanol extracts of *Bridelia ferruginea* and *Bryophyllum pinnatum* leaves are good sources of natural antioxidant compound which are helpful in preventing oxidative stress by donating electrons to unstable radicals. The reducing activities of the plant extracts are comparable to that of gallic acid. The *Bryophyllum pinnatum* extract exhibited a better reducing activity than *Bridelia ferruginea* activity. These results might imply that the ethanol extract of both *Bryophyllum pinnatum* and *Bridelia ferruginea* leaves carry chemical constituents with the ability of donating hydrogen to a free radical and thereby preventing the potential damage of biological molecules caused by unstble radicals. These results were in line with the findings of Orisakeye *et al*, (2015) and Olaide *et al*. (2014) that earlier reported the antioxidant activities in roots and stem barks of *Bryophyllum pinnatum* and *Bridelia ferruginea* respectively.

From the result, it was observed that phytosome from *Bryophyllum pinnatum* has a finer surface than phytosomes from *Bridelia ferruginea*. This helps to reveal the surface structure of the phytosomes. The finer surface morphology observed in *Bryophyllum pinnatum* phytosome complexes may be an indication of greater surface area to volume ratio for greater interaction of the extract and the phosphatidylcholine in the phytosome. This indicates that there was higher interaction between the *Bryophyllum pinnatum* extract and the phosphatidylcholine than the extract-lipid interaction obtained for the *Bridelia ferruginea* phytosome complex. This shows that more of the *Bryophyllum pinnatum* extracts was entrapped by the phospholipid than that of the *Bridelia ferruginea* extract for greater enhancement of the efficacy. This SEM result is in agreement with the results obtained from the entrapment efficiency in which all the phytosome complexes from *Bryophyllum pinnatum* extract showed higher percentage entrapment efficiency than the entrapment efficiency of *Bridelia ferruginea* phytosome complexes. The scanning electron microscopy (SEM) morphology in this work is in agreement with the findings of Rudra and Ramakant (2015) which revealed that drug particles associated with the phospholipid form complexes with irregular particle shape and crystalline structures. Also, the findings of Temitayo *et al*. (2017) using silver nanoparticles synthesized with *Bridelia feruginea* bark revealed a

pentagonal shape similar to the surface morphology of *Bridelia ferruginea* phyosome complexes obtained in this study.

Fourier transform infrared spectroscopy was carried out in this work for confirmation of Phytosome formation. Infrared (IR) spectroscopy is a technique used to identify the structure of chemicals based on the interaction of atoms with infrared radiation. Molecular vibration and rotation can be excited by the absorption of radiation in an infrared region. Such molecular vibrations and rotations can directly be measured as absorbance or transmittance in the infrared spectrum. When infrared radiation interacts with an organic compound, certain frequencies of energy are absorbed while others are transmitted or reflected. Since the finger print region characterizes the property of the whole compound involved, the interpretation of this result was limited to the peaks found at the functional group regions in the various spectra. The frequencies absorbed or transmitted are determined by the functional groups present in the substance. The molecular vibrations are localized within the functional groups and do not extend over the rest of the molecules. Such functional groups can be identified by their absorption bands (Manfred *et al.,* 1997). Hence, IR can be used as a means for identifying the chemical composition of a material.

Formation of Phytosome complexes of *Bryophyllum pinnatum* and *Bridelia ferruginea* were confirmed by comparing the peaks in the phytosome complexes with that of the original individual components (extracts of *Bryophyllum pinnatum, Bridelia ferruginea* and the Phosphatidylcholine). There was a shift in the absorption spectra of O-H and C-H groups in the individual components when compared to the various spectra in *Bryophyllum pinnatum* and *Bridelia ferruginea* complexes (Table 6). This downward shifts observed in the complexes is an indication of bond formation. The O-H or N-H absorption between 3200 and 3600 cm^{-1}, indicates an alcohol, N-H containing amine or amide, or carboxylic acid. O-H stretch from phenols and alcohols occur at the peak 3300 – 3500 cm^{-1} (hydrogen bonded-broad) and at 3600-3650 cm^{-1} (when they are free). The O-H stretching vibration from carboxylic acids occurs at 2500 – 3300 cm^{-1}. Therefore, the O-H stretching vibrations (3350.5, 3336, 3358, 3328.5, 3634.2, 3337.0, 3324.8 and 333.2 cm^{-1}) observed in the peaks of extracts, phosphatidylcholine and various phytosome complexes are as a result of O-H stretch from alcohols, phenols since the spectra were broad unlike N-H stretch. The absorption bands of the N-H stretching may

sometimes be confused with those of hydrogen bonded O-H. The N-H absorption is usually sharper because of a much weaker tendency of the N-H group to form hydrogen bonds (Manfred *et al*., 1997).

Therefore, O-H bond exists in the extracts of *Bryophyllum pinnatum, Bridelia ferruginea*, Phosphatidylcholine and the various Phytosome complexes. The peaks at 2851.4cm^{-1}, 2922.2 cm-^{1} and 2914.8 cm-^{1} in all (*B. ferruginea* extract, *B. pinnatum extract*, phosphatidylcholine and various complexes at 2918.5 cm-^{1}) indicate asymmetric (2922.2 cm-^{1}) and symmetric (2851.4cm^{-1}) stretching vibrations of C-H alkane group which are seen as shoulders in the FTIR spectrum in all the samples. Also all the samples showed peaks at 1736.9 -1710.8 cm^{-1} denoted to the carbonyl stretching of carboxylic acid. Both *B. ferruginea* and *B. pinnatum* extracts showed peaks at 1654.9 cm^{-1} attributed to the aromatic skeleton of C=C bond vibration. This result shows that both *B. ferruginea* extract and *B. pinnatum* extracts have almost common functional groups in them above finger print region except that *B. pinnatum* exhibited wavenumber at 2132.0 cm-^{1} attributed to C=N bond (cyanoguanidine).

The implication of this result is that it has revealed that *B. ferruginea* extract, *B. pinnatum extract*, phosphatidylcholine and the various formulated phytosome complexes have functional groups of alcohol, aldehyde, ketones, alkyne, alkene, thiols, amines and esters. These functional groups are present in the active compounds (extracts) which accounts for good pharmacological activities found in the extracts. It has also confirmed the presence of the phytochemicals as revealed in the qualitative and quantitative phytochemical screening carried out on the extracts. Moreover, the absorptions at 3358 cm-^{1} in phospholipid and 2922.2 cm-^{1} in individual extracts shifted to the lower field in the spectra of complexes, indicating the formation of hydrogen bond and existence of electrostatic interactions between extracts and phospholipid (phosphatidylcholine) (Singh, *et al.,* 2014). The C-H, O-H and C=C bonds found in the *Bridelia ferruginea* extract and its complexes in this work were in line with the findings of Temitayo *et al*. (2017) who reported the presence of these bonds in silver nanoparticle made from ethanol extract of *Bridelia ferruginea* bark. FTIR results in this work on *B. pinnatum* extract also agreed with the findings of Esther *et al.* (2016) who analyzed aqueous, methanol, and dichloromethane leaf extracts of *Bryophyllum pinnatum* using Fourier Transform Infrared Spectroscopy and also

reported the presence of O-H stretching bond and carboxylic acid stretching bond (C-H) at 2922 cm^{-1}.

Perfect loading of extracts on the phytosomes and minimal loss of it throughout the preparation steps were reflected in the entrapment efficiency. The entrapment efficiency shows the percentage amount of the extract that actually binds with the phospholipid to form a phytosome. The entrapment efficiency result in *Bryophyllum pinnatum* complexes of extract: lipid ratio of (1:1, 1:3, and 1:5) are 97.13 ± 1.4%, 94.93 ± 2.6 % and 97.42 ± 0.7 %, respectively. This showed good encapsulation efficiency in all the formulations. 1:5 phytosome complex of *Bryophyllum pinnatum* had the highest encapsulation efficiency when compared to the encapsulation efficiency of other phytosome complex formulations. This indicates that the higher increase in phospholipid also increases encapsulation except the decrease observed in 1:3 complex which may be due to losses of the extract during formulation process. The more phospholipid added in the formulation, the more *Bryophyllum pinnatum* extract entrapped by the lipid. This result is in agreement with the findings of Anwar and Farhana (2018); Zahra *et al* .(2015) and Sarita *et al*. (2019) who worked on the Preparation and evaluation of Phytosomes containing ethanolic extract of leaves of *Bombax ceiba* for hepatoprotective activity and observed their optimum encapsulation at extract: lipid ratio of 1:1.5. The entrapment efficiency of the *B. ferruginea* complexes of extract: lipid ratio of (1:1, 1:3, and 1:5) are as follows: 96.77 ± 1.15%, 97.33 ± 1.74% and 94.14 ± 2.30 % respectively.

The lowest entrapment was found in 1:5 phytosome complex which was expected to have the highest entrapment. Entrapment efficiency in *Bridelia ferruginea* complexes followed a pattern in which the increase in phospholipid (phosphatidylcholine) did not have a corresponding increase in the encapsulation efficiency of the Phytosome. This implies that further increase in phospholipid did not guarantee an increase in encapsulation. It also indicates that extract: lipid ratio of 1:3 with the highest encapsulation efficiency is the optimum for formulation of *Bridelia ferruginea* complexes. With further increase in the lipid concentration in 1:5 phytosome complex, the entrapment efficiency decreased to 94.14% and this indicated that increased amount of lipid did not help in entrapping more extracts. By comparing the entrapment efficiency in the two plants, the entrapment efficiency in 1:1 and 1:5 phytosome complexes of *B. pinnatum* had more encapsulation of the extract when compared to the 1:1and 1:5 phytosome

complexes of *Bridelia ferruginea.* Therefore, *Bryophyllum pinnatum* can be said to have better encapsulation efficiency than *Bridelia ferruginea* complexes.

In vitro dissolution was carried out in this study to predict the potential *in vivo* performance of phytosome complexes of *Bridelia ferruginea* and *Bryophyllum pinnatum* oral dosage forms and as quality control test, it demonstrates the dissolution pattern of these drug products. Results and interpretation of dissolution data can be achieved only when the biopharmaceutical and physical properties of the drug products are well understood. Release of *Bridelia ferruginea /Bryophyllum pinnatum* extracts from phytosome complexes was studied using the dialysis method. The drug release at different time intervals was measured using a visible spectrophotometer. The drug release in the simulated intestinal fluid (pH 6.8) showed that none of the phytosome or the plant extract released up to 10% of their active content within the first 30 min of the study. At the end of the 3 h study, *Bridelia ferruginea* phytosome complexes of extract: phospholipid ratio (1:1, 1:3 and 1:5) has cummulative release of 65.87%, 42.93% and 42% respectively while *Bryophyllum pinnatum* phytosome complexes of extract: phospholipid ratio (1:1, 1:3 and 1:5) released only 13.47%, 7.73% and 8% respectively. *Bryophyllum pinnatum* phytosomes were released higher than its ordinary extract unlike the *Bridelia ferruginea* extract that showed higher release than its phytosomes complexes except 1:1 *B. ferruginea* phytosome.

The less release observed among the *Bryophyllum pinnatum* phytosome complexes when compared to the *Bridelia ferruginea* counterparts could be due to stickiness of *Bryophyllum pinnatum* which affects the release and permeability of the active component of the phytosome complex through the dialysis bag. Although, this delayed release observed in the *Bryophyllum pinnatum* phytosome complexes did not affect the *in vivo* performance of the drug. According to Chandrasekan (2011), *in vitro* drug release may be non-discriminate or over-discriminate when compared to the *in vivo* performance. In comparison of this *in vitro* study with the *in vivo*, *Bryophyllum pinnatum* phytosomes expressed an over-discriminate effect. *Bryophyllum pinnatum* phytosomes showed improved release at the end of 3 h when compared to their conventional extract forms that had released 6.4% in SIF (pH 6.8). Unlike the *Bryophyllum pinnatum, Bridelia ferruginea* phytosome complexes showed a different release pattern when compared to *Bryophyllum pinnatum*.

All the phytosome complexes showed a delayed and sustained release than the ordinary herbal extract that was free and uncomplexed in lipid. This might imply that the bioavailability of the ordinary extract may reach its peak within a short time and the effect will not be sustained for a longer period. The delayed effects of the complexed phytosomes could be due to the fact that phyto-phospholipid complex powders have not got good technological properties because of their limited flowability, potential stickiness and low apparent density as reported by Aysegul and Fatih (2015).

Plant-derived natural products are the source of most active components of medications, which play a significant role in the treatment or prevention of different diseases that affect human which include bacterial infections. This will help in the development of new, sustainable, natural and affordable drugs. Antimicrobial study was carried out to investigate the ability of ethanol leave extracts of *Bryophyllum pinnatum, Bridelia ferruginea* and phytosome complexes from these plants in inhibition of bacterial growth especially commonly encountered pathogens and enteric bacteria. No zone of inhibition was found in ethanol extracts of *Bryophyllum pinnatum, Bridelia ferruginea* leaves and the formulated phytosomes complexes from these plants which implies that they have no antibacterial activity with the selected strains of bacteria and fungi. This could also be affected by the processing, geographical location of plant, age of plant and part of the plant used for this study. Zones of inhibition were observed with 40 mg/ml of gentamicin (standard drug) in the tested strains of bacteria. It is no surprise that the phytosome complexes showed no zone of inhibition since the active components (Bryophyllum *pinnatum* and *Bridelia ferruginea)* of the phytosomes have not shown any activity against test bacteria. This result is in line with the findings of Abhishek *et al.* (2013) who investigated antibacterial potential of aqueous and methanol extracts of *Bryophyllum pinnatum* leaves and observed no zone of inhibition of the extracts with the tested strains of both Gram positive and Gram negative bacteria which include *Alcaligenes faecalis, Bordetella bronchiseptica, Serratia marcescens Bacillus subtilis, Corynebacterium diphtheriae and Micrococcus luteus*. It was only *Alcaligenes faecalis* and *Bacillus subtilis* that showed zones of inhibition with the aqueous extract.

Also the result of Azuonwu *et al.* (2017) on aqueous extract of *Bryophyllum pinnatum* leaf indicated no zone of inhibition with *Escherichia coli, Staphylococcus aureus and pseudomonas aeruginosa* but showed only 1 mm zone of inhibition in ethanol extract. This result is also in line

with the findings of Adebayo and Ishola (2009) who reported no zones of inhibition with methanol extracts of leave and stem bark of *Bridelia ferruginea* with *Escherichia coli, Klebsiella pneumoniae, Staphylococcus aureus, B. anthracis, Salmonella typhi, Pseudomonas aeruginosa, Proteus mirabilis and Candida albicans* at concentrations ranging from 10 mg/ml - 250 mg/ml of the extracts. Only *Candida albican* and *Staphylococcus aureus* were sensitive to the stem bark. Owoseni *et al*. (2010) reported no zone of inhibition in the water and ethanol leave extracts of *Bridelia ferruginea* on the tested bacteria and fungi except *Citrobacter, B. subtilis* and *Staphylococcus aureus* that showed sensitivity in ethanol leaf extract.

The resistance by *Aspergillus fumigatus* to ketoconazole in both *Bryophyllum pinnatum* and *Bridelia ferruginea* plants could be as a result of resistance of *Aspergillus fumigatus* to azoles drugs observed in different regions (e.g.UK, China, Canada and USA) in the recent years. This resistance to ketoconazole as observed in this study might be due to mutation in the cyp51A gene, leading to alterations in the target protein (Ahmed *et al*., 2010).

The experimental animals showed no sign of toxicity or death after treatment of animals with different phytosome complexes from both ethanol extracts of *Bridelia ferruginea* and *Bryophyllum pinnatum* leave. The lethal dose was found to be above 5000 mg/kg body weight.

Graded doses of different phytosome complexes from both plants (1:1, 1:3 and 1:5), ethanol extracts of *Bridelia ferruginea* and *Bryophyllum pinnatum* leaves exerted anti-ulcer effect on the indomethacin-induced ulcer model, with maximum gastric protection found among groups treated with graded doses of 1:3 phytosome complexes of *Bridelia ferruginea* and *Bryophyllum pinnatum* respectively. This antiulcer activity could be due to the presence of active phytochemical constituents as revealed in Tables 1 and 2. Phenols, flavonoids and terpenoids which are known to be rich antioxidant play important role in antiulcer activity were in high concentrations in both plants. Also tannins present in both plants (*Bridelia ferruginea* and *Bryophyllum pinnatum*) are known to “tar” the outermost layer of the gastric mucosa rendering it less permeable and more resistant to chemical and mechanical injury (Asuzu and Onu, 1990).

The highest percentage ulcer protection of 78.14% and 77.86% were also observed among the groups treated with 1:3 phytosome complexes of *Bridelia ferruginea* and *Bryophyllum pinnatum* respectively. This shows that ethanol extracts of *Bridelia ferruginea* and *Bryophyllum pinnatum* leave and their various phytosome complexes were effective in decreasing haemorrhagic

lessions. It was also observed that ulcer index decreased among the groups treated with phytosome complexes from each of the plant than the conventional extract forms. This implies that the various phytosome complexes enhanced gastric mucosa protection of the extract forms. This increased activity could be due to increased absorption and bioavailability of the active components (extract) in the phytosomes which was achieved by incorporation of amphiphilic (lipophilic and hydrophilic) property on the hydrophilic extracts in the formulation of phytosomes. The result of this research indicates that ethanol extracts of *Bridelia ferruginea* and *Bryophyllum pinnatum* leaves and their various phytosomes probably might have antagonized the aggressive factors like pepsin and acid secretions which are vital in the pathogenesis of gastric ulcer. Since the leaf ethanol extracts of *Bridelia ferruginea* and *Bryophyllum pinnatum* have no antibacterial activity (Azuonwu *et al.,* 2017; Adebayo and Ishola, 2009), this implies that the ulcer protective property of these plants and their phytosome complexes could not be by inhibition of bacterial growth such as *Helicobacter pylori*. This result corroborates the findings of Akuodor *et al. (*2012) who reported ulcer protective effect of aqueous stem bark of *Bridelia ferruginea.*

Peptic ulcer occurs due to overproduction of gastric acid (or) decrease in gastric mucosal protection. Indomethacin (NSAID) induce ulcer by nonselective inhibition of cyclooxygenase (COX1 and COX2 isoforms) which are responsible for mucosal blood flow, mucus and bicarbonate production, epithelial cell renewal and neutrophil activation. This will lead to generation of reactive oxygen species and increased aggressive factors (such as pepsin and acid secretions) that result to the imbalance between aggressive and defensive factors and cause ulceration. Indomethacin-induced ulceration by inhibition of cyclooxygenase also prevents prostaglandin biosynthesis and this in turn inhibits the release of mucus, which is a defensive factor against gastrointestinal damage (Dengiz and Gursan, 2005). Inhibition of prostaglandin synthesis and free radical formation are the biochemical bases in the pathogenesis of gastric ulceration (Inas *et al*., 2001). Gastric analysis of biochemical indices (gastric volume, pH and total acidity) of stomach is often used to ascertain the stomach's integrity following administration or exposure to pharmacological agents (Biplab *et al*., 2011). The pH gives an idea of the level of gastric acidity. Low pH value is a manifestation of increased hydrogen ion concentration in gastric juice. This has been linked to ulcer genesis and gastric damage in experimental animals (Lüllmann *et al.,* 2000).

The significant increase in ulcer index, gastric juice volume and gastric total acidity following oral administration of indomethacin in the ulcerated control rats might be attributed to either free radical formation or inhibition of prostaglandin synthesis. Decreased prostaglandin concentration has been linked to impaired gastroprotection and also implicated in increased gastric acid secretion which is an important event in the pathogenesis of mucosal ulceration. This study agrees with the reports of Bech *et al.* (2000), Biplab *et al*. (2011) and Muhammed *et al*. (2012) where indomethacin was reported to have caused alterations in gastric secretions of rats. A significant decrease ($p < 0.05$) in total acidity and gastric volume was observed in all the treated groups in both *Bryophyllum pinnatum* and *Bridelia ferruginea* when compared to the untreated ulcer control group. The least acidity was detected among the groups treated with 400 mg/kg body weight of 1:3 phytosome complexes of both plants with *B. ferruginea* acidity at 6.78 ± 2.71 mmol/l and *B. pinnatum* at 6.74 ± 2.88 mmol/l.

Among the pretreated animals, highest pH value, least volume and acidity was detected among groups pretreated with various doses (100 mg/kg, 200 mg/kg and 400 mg/kg) of 1:3 phytosome complexes. This implies that 1:3 phytosome complexes exerted the most ulcer protective activity among the whole phytosome formulations from both plant extracts. The decrease in gastric ulcer index, volume and acidity observed among the pretreated groups (extract, 1:1, 1:3 and 1:5 phytosome complexes) were comparable and equipotent as that of the standard drug (Cimetidine) especially among the groups treated with phytosomes. This could indicate that *Bryophyllum pinnatum* and *Bridelia ferruginea* ethanol leaf extracts and their various phytosome complexes have inhibitory effect on gastric acid secretion and their inhibitory action might mimic/ imitate cimetidine effect on gastric acid secretion. The extracts and their phytosomes might have acted by blocking H_2 receptor and prevented histamine from binding causing decrease in gastric acid secretion. It is established that inhibition of histamine release as a result of blockage of H_2 receptors, inhibit intracellular adenylate cyclase, Na^+-K^+ ATPase and proton pump of gastric parietal cells, reduce the gastric acid secretion (Sasaki *et al*.,2000; Ayada *et al*., 2003).

However, *Bridelia ferruginea* and *Bryophyllum pinnatum* ethanol leave extracts and their various phytosome complexes may exert their ulcer protective activity by increasing the formation of endogenous prostaglandin synthesis by trophic effect in which they binds to the epithelial growth factor (EGF). This in turns promote regulation of gastric secretion and enhance the mucosal

barrier against the damaging effect of indomethacin on the gastric mucosa. The result of this study is in agreement with the findings of Akuodor *et al.* (2012) who reported gastroprotective effect with aqeous stem bark extract of *Bridelia ferruginea* plant. The findings of Syed and Syed (2016) who reported decreased volume and total acidity on treatment of ulceration with herbal extract which also agrees with this result. The comparable percentage ulcer protection found among the groups pretreated with phytosomes of *Bryophyllum pinnatum* to the groups pretreated with phytosomes of *Bridelia ferruginea* (Table 11) is in agreement with the findings of Chandrasekaran (2011) who investigated the *in vitro* and *in vivo* performance of slow, medium and fast dissolution phytosomes. He demonstrated the bioequivalence of fast, medium and slow dissolution drugs. He found that all the drugs (fast, medium and slow dissolution) produced the same potency *in vivo* with their corresponding formulations which he reported that *in vitro* drug release may over- discriminate drug performance. This effect can be noted by comparing Table 11 and Figure 13.

Consumption of herbal products or traditional medicines involves some challenges and drawbacks, including effects, which put some doubts on the safety of herbal remedies (Elvin-Lewis, 2001). Nephrotoxicity may be assessed using the values of urea and creatinine concentrations in an organism. Creatinine and urea concentrations increase in biological system in response to muscle and tissue degradation. Creatinine and urea concentrations are used as markers for assessment of kidney function. A significant decrease ($p < 0.05$) in urea and creatinine concentration was observed in all the treated groups in both *Bridelia ferruginea* and *Bryophyllum pinnatum* extracts and their various complexes (1:1, 1:3, 1:5) when compared to the urea and creatinine concentration in the ulcer untreated group (control). This increase in the urea and creatinine concentrations in the ulcer-induced rats may be attributed to the nonselective inhibition of cyclooxygenase by indomethacin which in turn leads to decreased blood flow, epithelial cell renewal and decreased prostaglandin concentration that favoured the aggressive factors resulting in gastric ulceration.

The highest creatinine and urea values in the ulcer treated groups were found among the groups pretreated with plant extracts of *Bridelia ferruginea* and *Bryophyllum pinnatum*. Groups pretreated with 200 mg/kg and 100 mg/kg of 1:1 and 1:3 phytosomes in both plants showed a non-significant ($p < 0.05$) increase in urea concentration when compared to the urea

concentration in group pretreated with cimetidine (standard drug). Urea, the end product of protein breakdown, and its concentration are influenced by the rate of excretion, while creatinine is the waste product of muscle breakdown. Renal diseases that diminish the glomerular filtration rate lead to urea and creatinine retention in the serum.

The significant reduction ($p < 0.05$) in the serum concentrations of urea and the creatinine in the extract and phytosome-treated rats is an indication that the kidney was able to clear waste products from the system efficiently; this is also indicative that the phytosome complexes and ethanol extracts of *Bridelia ferruginea* and *Bryophyllum pinnatum* had no deleterious effect on the kidney. This result is in line with the findings of Kolawole and Sunmonu (2010); Olufunsho *et al*. (2015) who reported safety of methanolic and aqeous bark extract of *Bridelia ferruginea* on the kidney respectively. The result of this study is also in line with findings of Kanika, (2011) and Nwali *et al*. (2014), who reported some phytochemical constituents of *Bryophyllum pinnatum* to include; vitamin E, selenium, vitamin C, taurine and the carotenoids (beta-carotene, lutein and lycopene) which have the potentials to improve the glomerular filtration rate and the severity of the tubular damage (Ekor *et al*., 2006). Sule and Arhoghro (2016) had also reported protective potentials of ethanol extract of *Bryophyllum pinnatum* leaf against gentamicin-induced hepatic and nephrotic damage in Wistar albino rats. Moreover, (Harlaka and Patil, 2007; Majaz *et al*., 2011) had reported that aqueous extract of *Kalanchoe pinnata* leaves, a plant in the same family to *Bryophyllum pinnatum* possess potent nephroprotective activity in gentamicin induced nephrotoxicity in rats.

In this study, ulceration induced a significant increase in serum activities of aspartate aminotransferase, alanine aminotransferase and alkaline phosphatase (AST, ALT and ALP) which are sensitive biomarkers of liver injury and hepatic necrosis. ALT activities are relatively low in other tissues, very specific marker of hepatocellular injury, produced in hepatocytes and hence more specific than AST activity in diagnosis of liver toxicity. Rise in ALT activity may occur with the use of certain drugs or during periods of strenuous exercise. AST, a cytosolic isoenzyme is present in skeletal muscle, heart muscle and kidney tissue. Caution must be exercised in its use to evaluate hepatocellular damage. AST activity usually rises in conjunction with ALT activity to indicate hepatocellular injury. ALP, a group of isoenzymes that act to dephosphorylate a variety of molecules throughout the body is produced in the membranes of

cells lining bile ducts and canaliculi and released in response to the accumulation of bile salts or cholestasis. Non-hepatic production occurs in the kidney, intestine, leukocytes, placenta and bone. Physiological rise is experienced in pregnancy or in growing children. Pathological rise may occur in Paget's and renal diseases (Philip and Johnny, 2012). During hepatocellular damage, these enzymes are released into the blood flow from the cytoplasm after the rupture of plasma membrane of the hepatic cells. These elevations in the activities of liver enzymes observed in the ulcerated rats were due to intake of indomethacin above therapeutic level. Liver is prone to injury in the body because of its central role in the metabolism and detoxification of all toxins that enter the body. On administration of extract / phytosome complexes, there was a significant decrease ($p < 0.05$) in the activities of liver enzymes (AST, ALT, and ALP) in all the groups pretreated with ethanol extracts of *Bridelia ferruginea and Bryophyllum pinnatum* leaves and their various phytosome complexes when compared with the untreated ulcer group (control). Ethanol extracts of *Bridelia ferruginea and Bryophyllum pinnatum* leaves showed a reduction in the activities of liver enzymes more than the groups treated with cimetidine. Also, much appreciable decreases in the activities of AST, ALT and ALP were observed among the groups treated with phytosomes in both plants. This implies that treatment of indomethacin-induced ulcer with ethanol extrtacts and phytosome complexes of *Bridelia ferruginea and Bryophyllum pinnatum* leaves at doses of 100 mg/kg, 200 mg/kg and 400 mg/kg does not portend danger to the activities of liver enzymes. This study corroborates the hepatoprotective activity of *Bryophyllum pinnatum* leaf against gentamicin and carbon tetrachloride -induced hepatic and nephrotic damage in Wistar albino rats as reported by Sule and Arhoghro (2016); Devbhuti *et al*. (2008) respectively. This result is also in agreement with the findings of Momoh *et al*. (2012) and Olufunsho *et al.* (2015) who evaluated anti-venom activity of ethanol leave extract and the toxicological effect of aqueous stem bark extract of *Bridelia ferruginea* (Euphorbiaceae) in rodents respectively. The non-significant increase in the activities of AST, ALT and ALP in groups pretreated with phytosome complexes when compared with the AST, ALT and ALP activities in the normal group is an indication that phosphatidylcholine used in preparation of phytosomes, besides acting as a carrier also acted as a hepatoprotective agent. This is in line with the reports of Kareparamban *et al*. (2012); Mascarella (1993) who treated hepatitis patients with silybin phytosome at a dose of 120 mg/kg twice daily for up to 120 days and liver function returned to normal.

In order to explore the effects of antioxidant defences on the ulceration process in all gastric tissues, the activities of antioxidant enzymes were evaluated. (Table15). Long term use of NSAIDs such as indomethacin leads to a decrease in the prostaglandin (PG) level by inhibition of cyclooxygenase (COX) enzyme which leads to damage in gastric mucosa by production of free radicals (Baigent *et al*., 2009). Recent studies have shown that the inhibition of the PG secretion is not the only significant pathogenic factor for the mucosal damage. Oxidative stress induces generation of reactive oxygen species (ROS) and it's over production is one of the prime etiologic factors that cause gastric ulcer. As a result of overproduction of ROS, an imbalance of antioxidant system occurs and some relevant biochemical marker condition gets affected as peroxidation of lipids, proteins and nucleic acids, which may lead to cellular damage and cell death (Bhattacharyya *et al.,* 2014). MDA, a product of polyunsaturated fatty acids peroxidation and related esters, is a suitable index of oxidative tissue damage (Blandizzi *et al*., 2005). The increase in malondialdehyde (MDA) concentration in ulcerated rats was caused by administration of indomethacin and generated free radicals caused lipid peroxidation which led to membrane fluidity and increased in influx of Ca^{2+} ions.

As a result, membrane integrity was reduced of surface epithelial cells and led to gastric ulcer. Superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) are some of the antioxidant enzymes that contribute to the enzymatic defence mechanisms. SOD plays important role in the protection of gastric mucosa by partially preventing oxidative damage. It destroys the highly reactive radical superoxide (O^{2-}) by converting it into the less reactive hydrogen peroxide (H_2O_2), which can be destroyed by a CAT or GPX reaction. CAT is a highly reactive enzyme that reacts with H_2O_2 to form water and molecular oxygen. It can also form methanol, ethanol, formic acid, or phenols by donating hydrogen. The GPx is an enzyme which acts as catalyst, converting H_2O_2 to water and oxygen with oxidation of reduced glutathione (GSH) to oxidized glutathione (GSSG) helping in the other pathway of antioxidant mechanism (Ferreira and Matsubara, 1997). These are the various mechanisms these enzymes offered gastric mucosal protection to oxidative damage.

A significant increase ($p < 0.05$) was observed in the activities of antioxidant enzymes (SOD, CAT, and GPX) in groups pretreated with various doses of *Bridelia ferruginea* and *Bryophyllum pinnatum* leave extracts and their phytosome complexes when compared with the ulcer untreated

group (control). However, significant decrease ($p < 0.05$) in the MDA concentration was observed in the groups treated with various doses (100, 200 and 400 mg/kg) of extract and their phytosome complexes when compared with the ulcer untreated group (control). This implies that the pretreated animals exhibited body defence against oxidative stress damage caused by indomethacin and this also supports the earlier result on the *in vitro* antioxidant test carried out on the *Bridelia ferruginea* and *Bryophyllum pinnatum* leave extracts (Tables 3, 4 and 5). The activities of antioxidant enzymes among the groups pretreated with ordinary *Bridelia ferruginea* and *Bryophyllum pinnatum* leave extracts have a comparable effect to each other. The highest antioxidant enzyme activities were found among the groups pretreated with various doses (100, 200 and 400 mg/kg) of different phytosomes, which implies that phytosome improved the efficacy of the ordinary plant extracts by increased absorption and bioavailability. The decreased activities of SOD, CAT and GPX and increased concentration of MDA found among the untreated animals was an indication of oxidative stress among the untreated ulcer control groups which led to tissue damage. The results of this study supported previous findings that reported decreased SOD, CAT and GPX activities in rat stomach tissues by NSAIDs (Halici *et al*., 2005; Basiviredy *et al*., 2003 and Bilal *et al*., 2017).

Dyslipidemia is seen in many diseases (Heidarian and RafieianKopaei, 2013). In this study, 50 mg/kg body weight of indomethacin administration on the starved experimental animals caused an increase in serum concentrations of CHOL, TAG, and LDL and a decrease in serum concentration of HDL in all the ulcerated groups. A significant decrease ($p < 0.05$) in CHOL, TAG and LDL concentrations were observed in groups pretreated with various doses (100 mg/kg, 200 mg/kg and 400 mg/kg) of *Bridelia ferruginea* and *Bryophyllum pinnatum* extracts and their phytosome Complexes when compared with CHOL, TAG and LDL concentrations in the ulcer untreated group (control). However, a significant ($p < 0.05$) increase in HDL concentration was observed among the pretreated groups when compared to the HDL concentration in the ulcer control group. This indicates that gastric mucosal damage could significantly influence lipid metabolism *in vivo*. A non-significant decrease ($p > 0.05$) in TAG concentration was observed among groups pretreated with 100 mg/kg and 200 mg/kg of *Bridelia ferruginea* extract when compared to the TAG concentration in the untreated control rats. In the same vein, a non-significant increase ($p > 0.05$) in HDL concentration was observed among groups pretreated with 100 mg/kg, 200 mg/kg and 400 mg/kg of *Bryophyllum pinnatum* leaf

extract when compared to the HDL concentration in the untreated group (control). This implies that it was not all the doses of ordinary extracts were able to significantly return the altered lipid concentration to normal within three weeks of pretreatment. However, phytosome complexes (of extract to lipid: ratio of 1:1, 1:3 and 1:5) of *Bryophyllum pinnatum* and *Bridelia ferruginea* improved the lipid profile after three weeks of pretreatment. Indomethacin induce ulceration through overproduction of ROS which lead to oxidative stress that changes the normal lipid profile as dyslipidemia was observed among the ulcerated animals. This result is in line with the findings of Ansari *et al.* (2010) who reported that mean concentrations of cholesterol, triglyceride, LDL with gastritis due to *H. pylori* infection were significantly increased compared to controls, while HDL concentration was decreased significantly. Kim *et al.* (2011); Fatima *et al*. (2016) had also reported that cholesterol and LDL concentrations were significantly increased and HDL concentration was significantly lower in *H. pylori* infected male as compared to male controls.

Histological examination of the stomach tissues of rats revealed various degrees of necrosis on the stomachs of indomethacin-induced ulcerated rats. The untreated control group showed severe erosion of the surface epithelium more than the pretreated groups. Groups pretreated with either extract or phytosome showed an ulcer protective property when compared to the lessions in the ulcer control that did not receive any pretreatment. However, less necrotic attack on the mucosa tissues was observed among the groups pretreated with phytosome complexes especially 1.3 phytosome complexes of *Bridelia ferruginea and Bryophyllum pinnatum*.

4.2 Conclusion

Ethanol extracts of *Bryophyllum pinnatum* and *Bridelia ferruginea* leaves posses good antioxidant and ulcer protective properties but none of them showed antibacterial property on the tested strains of bacteria isolates. Different stoichiometric formulated phytosomes (1:1, 1:3 and 1:5) from both plants improved ulcer-protective property of their individual extracts. However, *Bridelia ferruginea* extract showed a better therapeutic efficacy than the *Bryophyllum pinnatum* extract. But enhanced activity was greater in the *Bryophyllum pinnatum* phytosomes when compared to *Bridelia ferruginea* phytosome complexes. Enhanced ulcer protective property in phyosomes of both plants might be due to protection of the extract and amphiphilic nature of the

phosphatidyl choline which increased absorption and bioavailability of the hydrophilic plant extracts.

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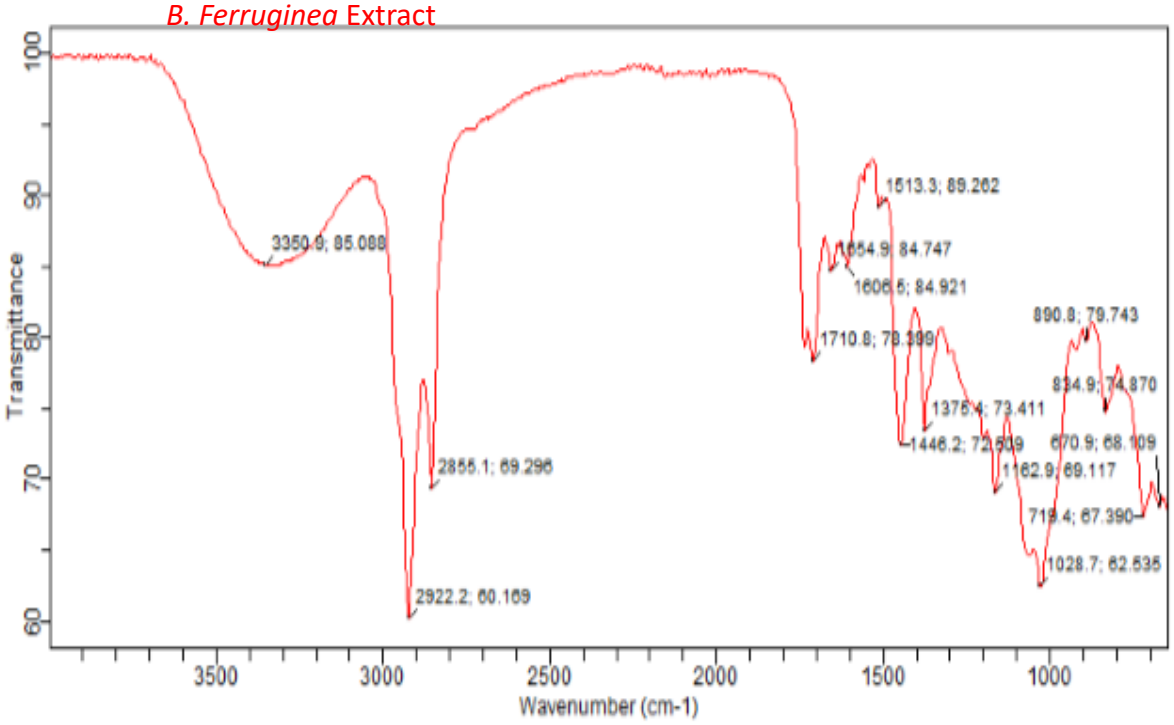
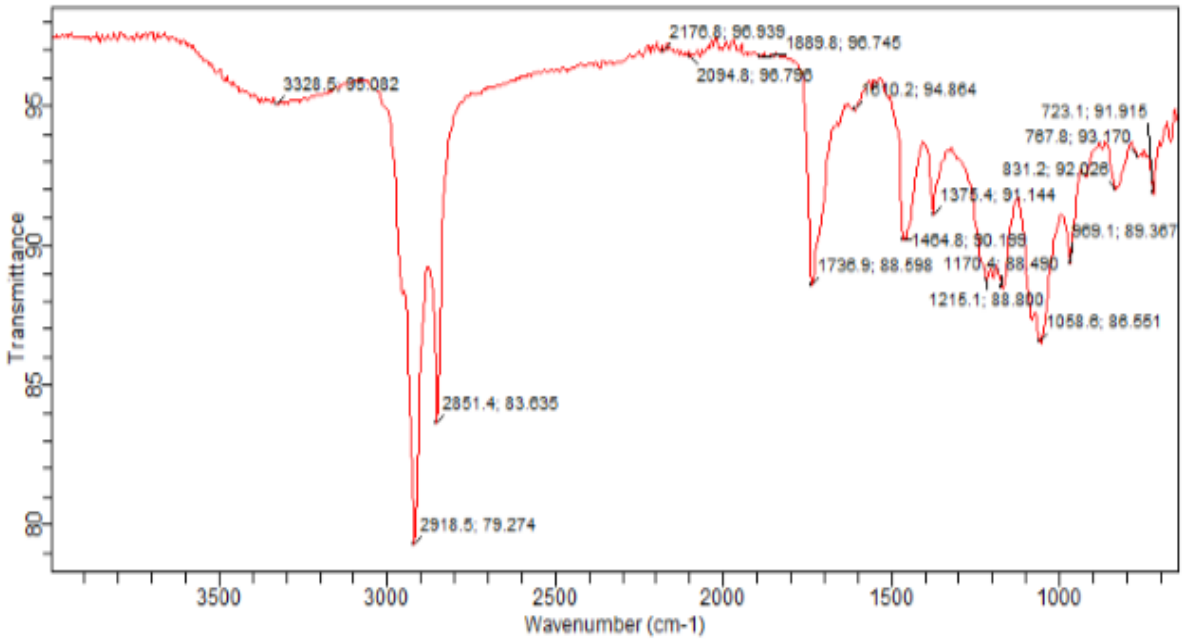
APPENDICES

Appendix I: Fourier Transform Infrared Spectroscopy of *Bridelia ferruginea* Leaves and All its Phytosomes Complexes

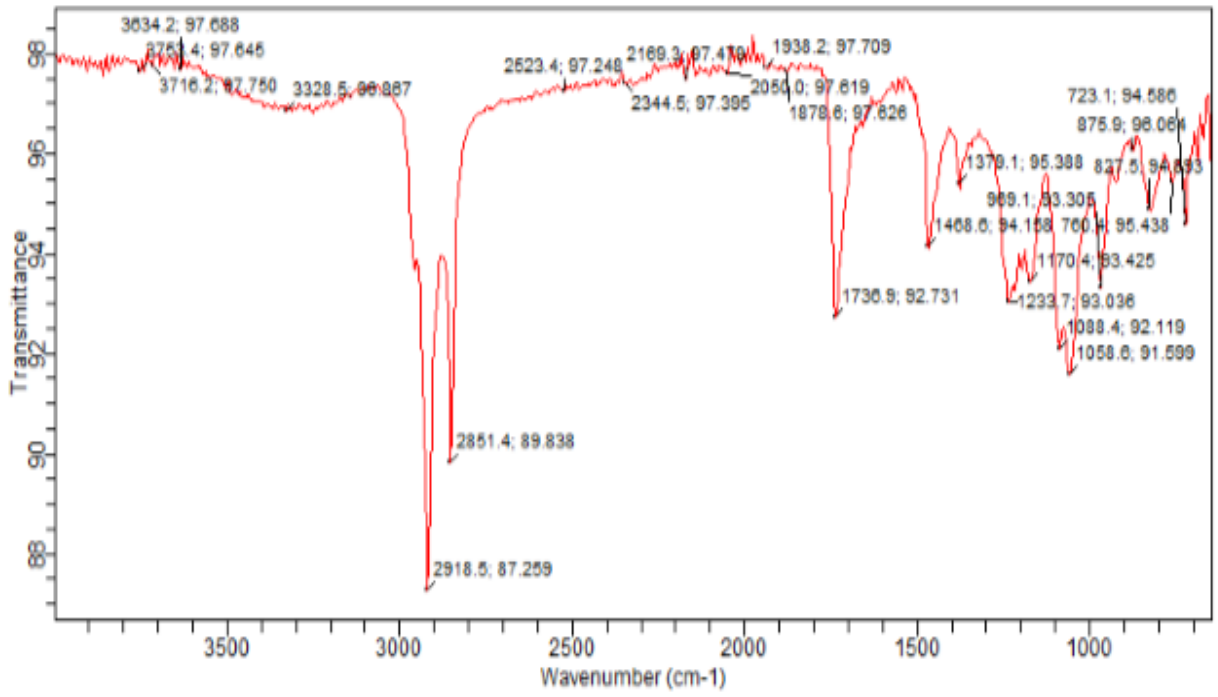
B. Ferruginea Extract



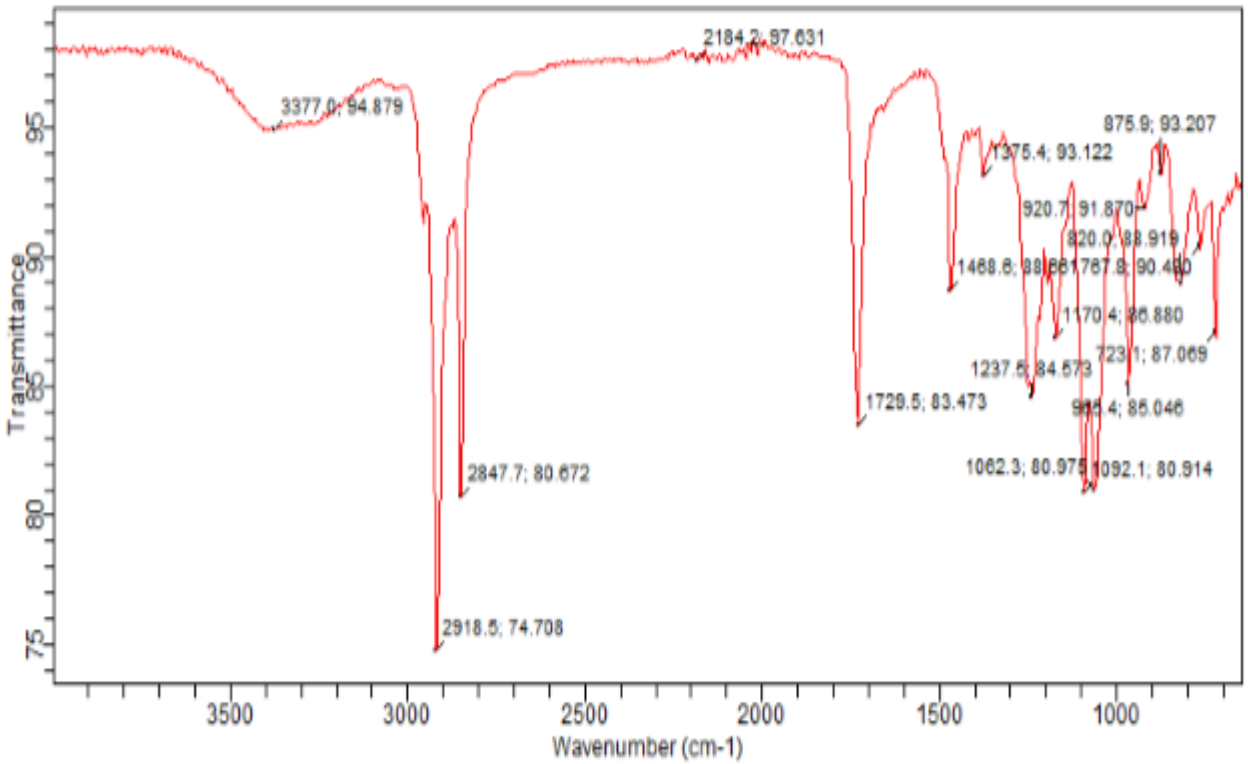
1:1 ferruginea phytosome



1:3 ferruginea phytosome

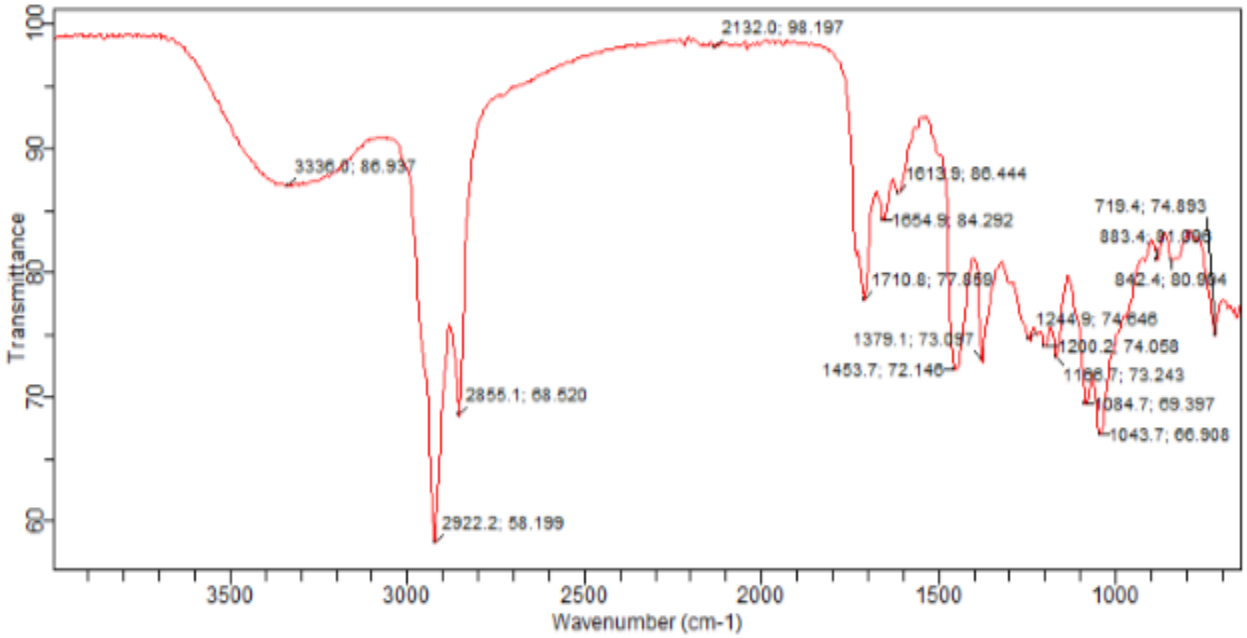


1:5 ferruginea phytosome

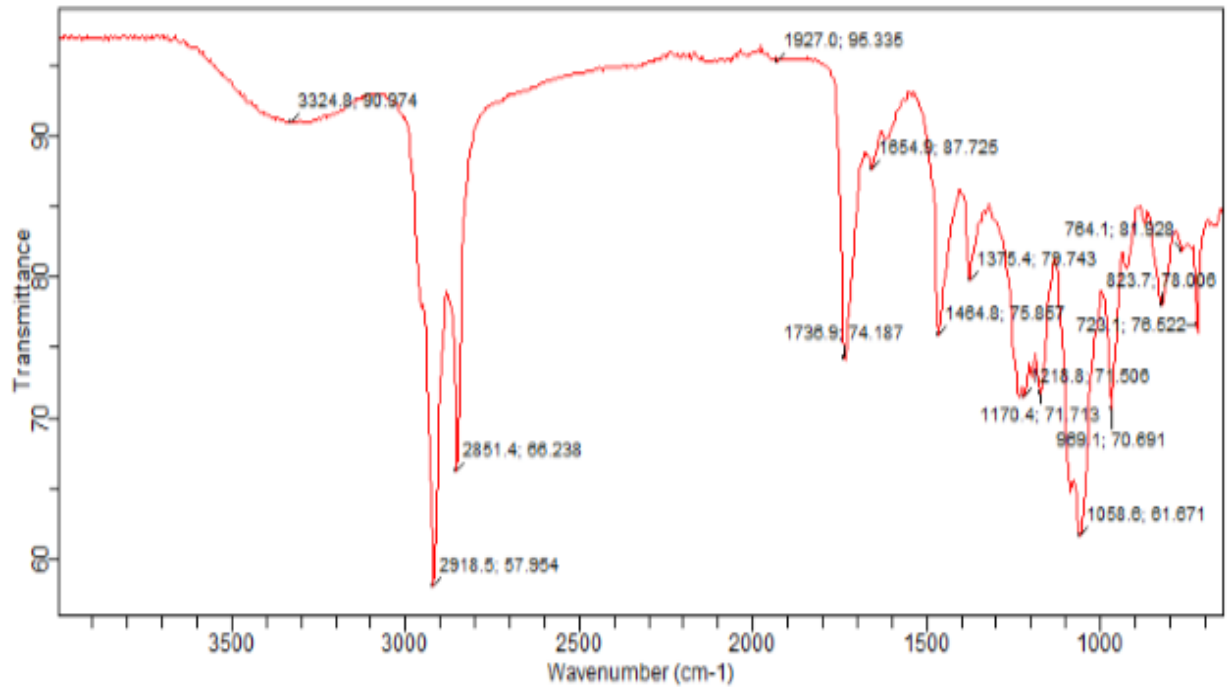


Appendix II: Fourier Transform Infrared Spectroscopy of *Bryophyllum pinnatum* Leaves and All its Phytosomes Complexes

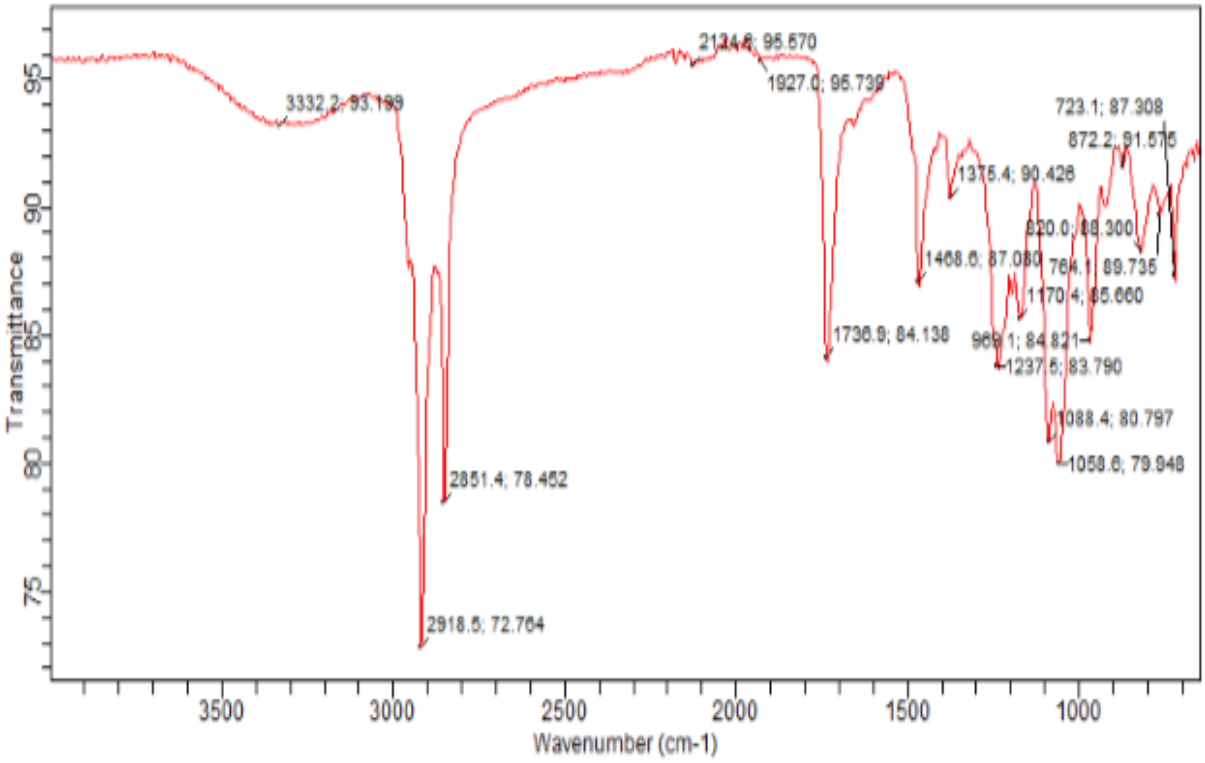
B. pinnatum Extract



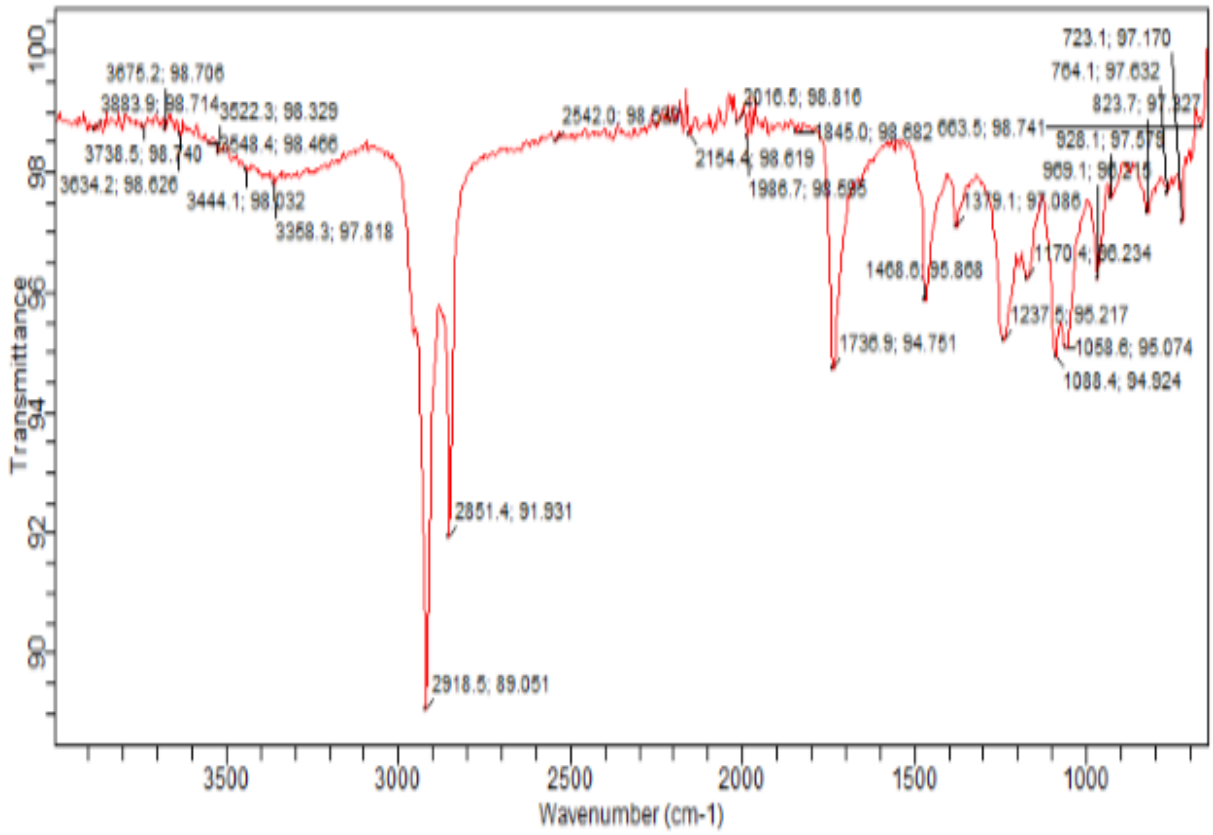
1:1 *B. pinnatum* phytosome



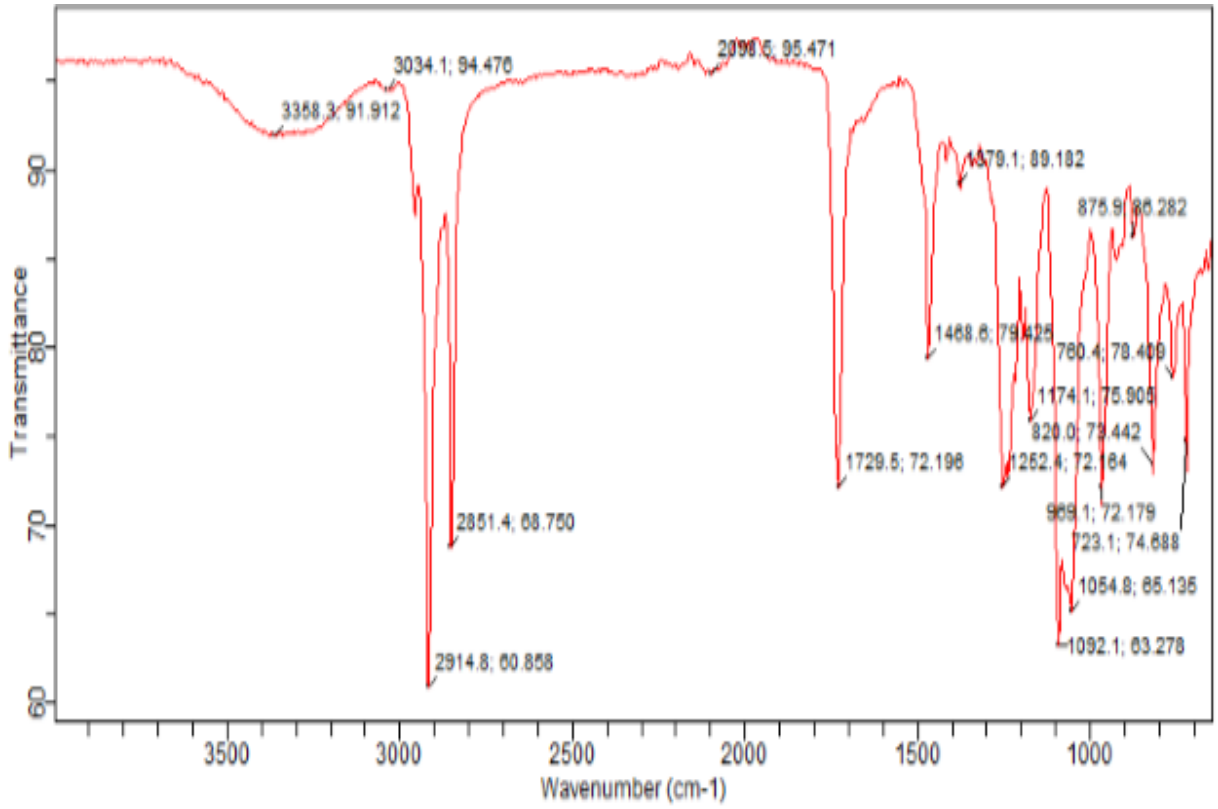
1:3 *B. pinnatum* phytosome



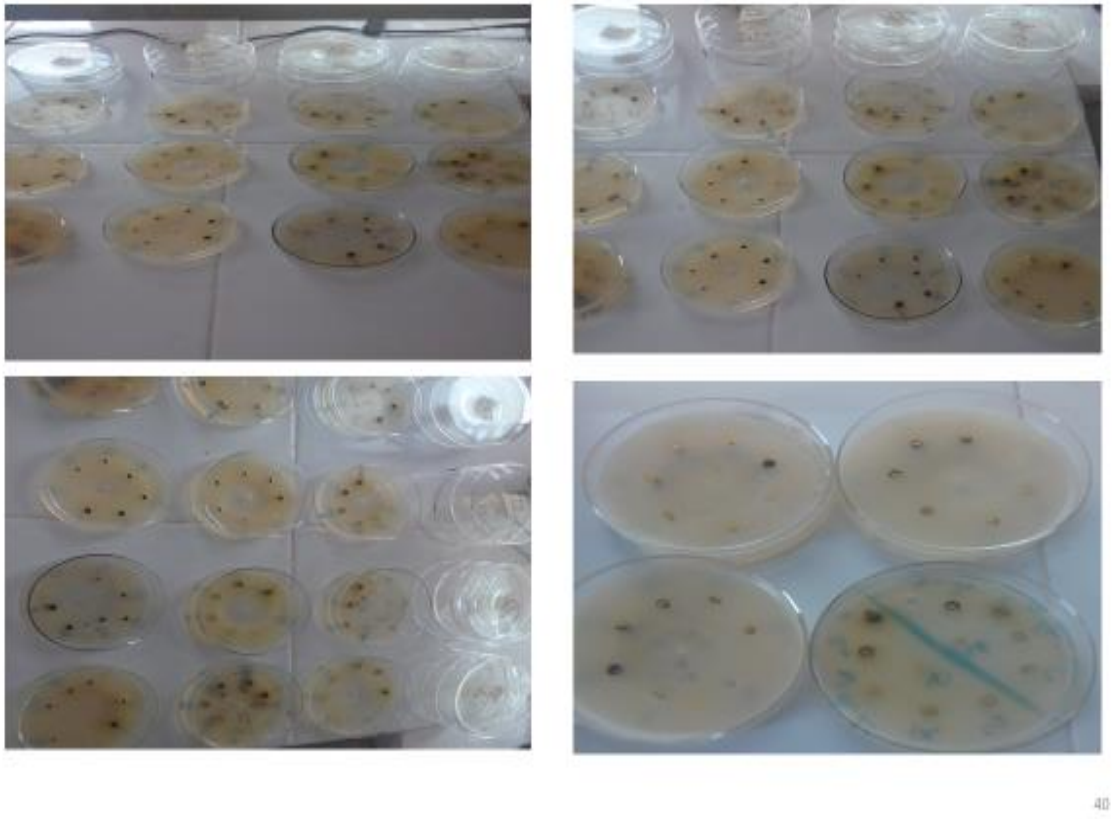
1:5 *B. pinnatum* phytosome

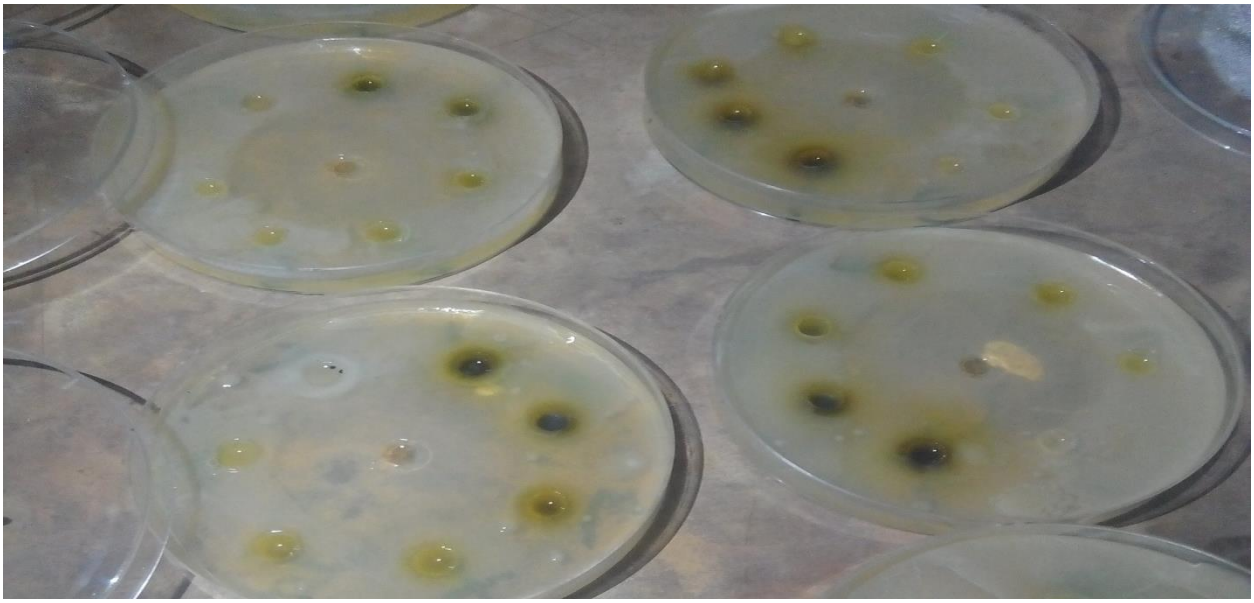


Phospholipid (phosphatidylcholine)

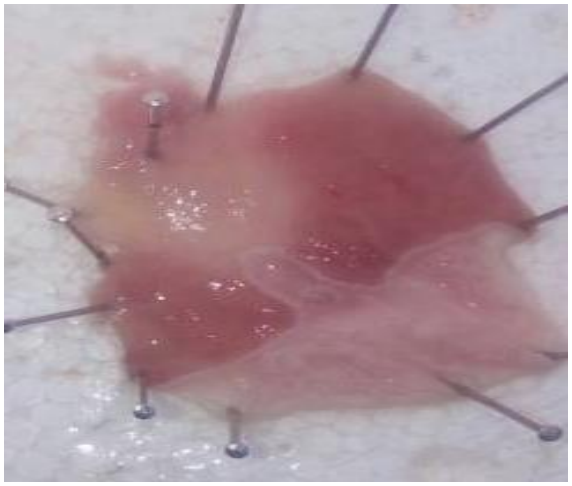


Appendix IV: Antimicrobial Sensitivity Plates of *Bridelia ferruginea* and *Bryophyllum pinnatum* Ethanol Leave Extracts.

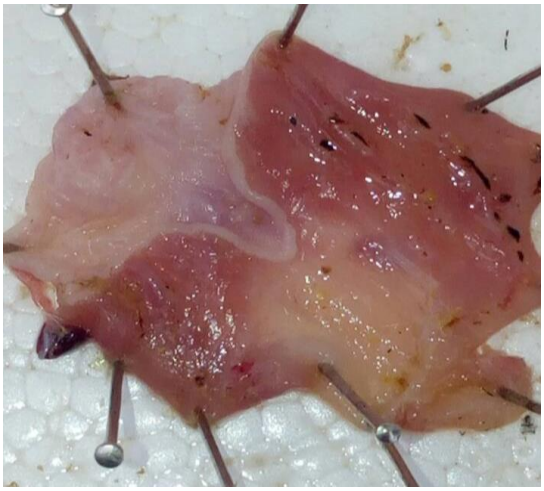




Appendix V: Ulcer Protective Studies on *B. ferruginea, B. pinnatum* and Their Phytosome Complexes.

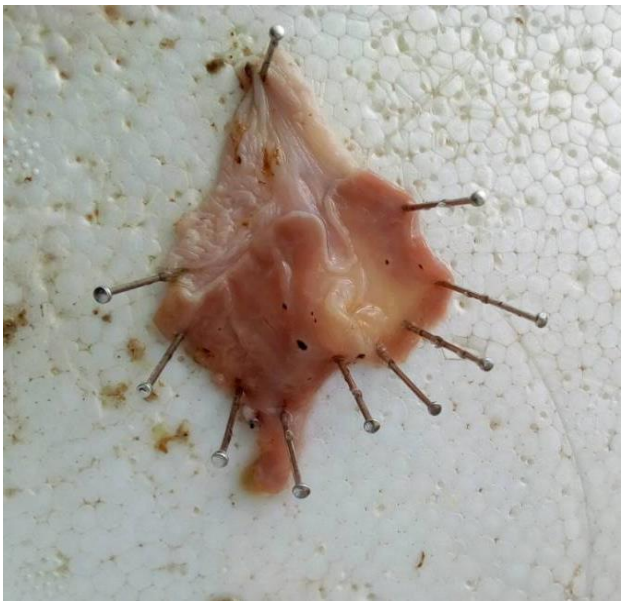


A= Normal rat
B=Ulcer untreated (control)

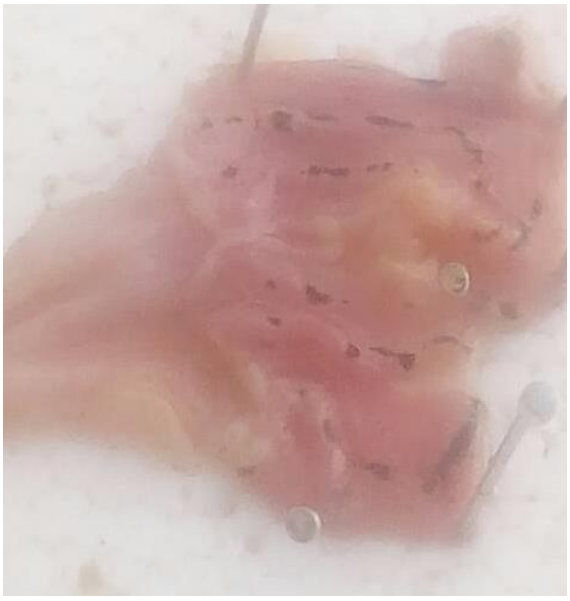


C= 100 mg/kg Cimetidine

D=400 mg/kg of *B. ferruginea* extract

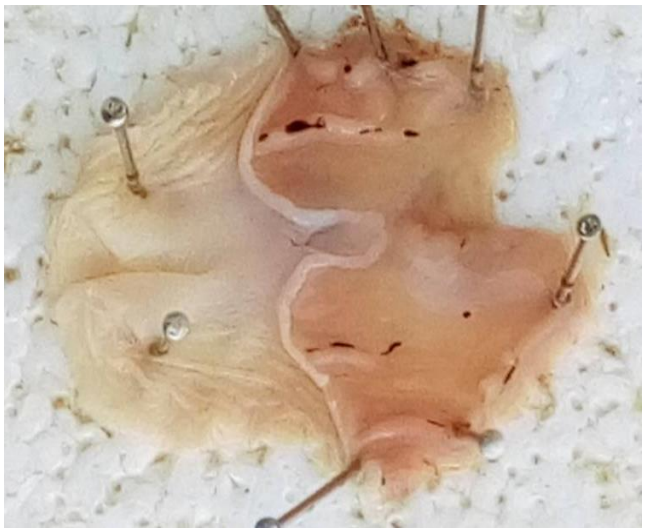


E= 400 mg/kg 1:3 B. ferruginea phytosome

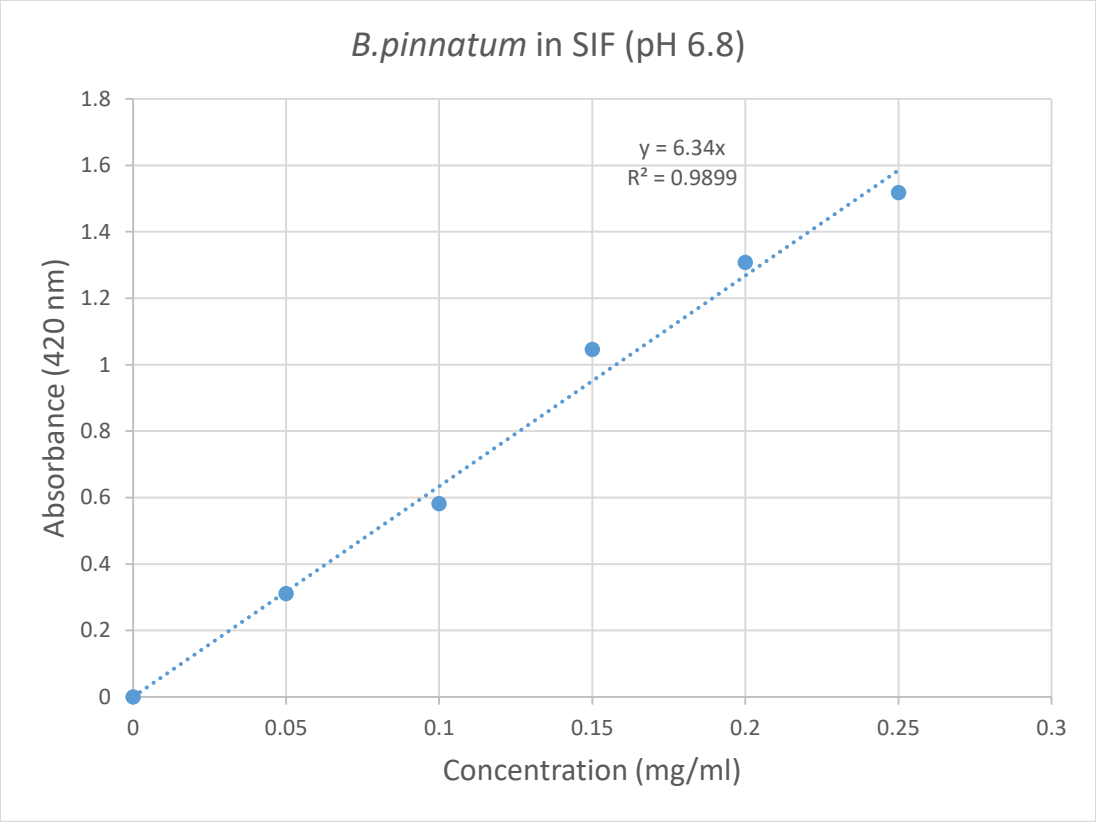


F= 400 mg/kg 1:1 B. ferruginea phytosome

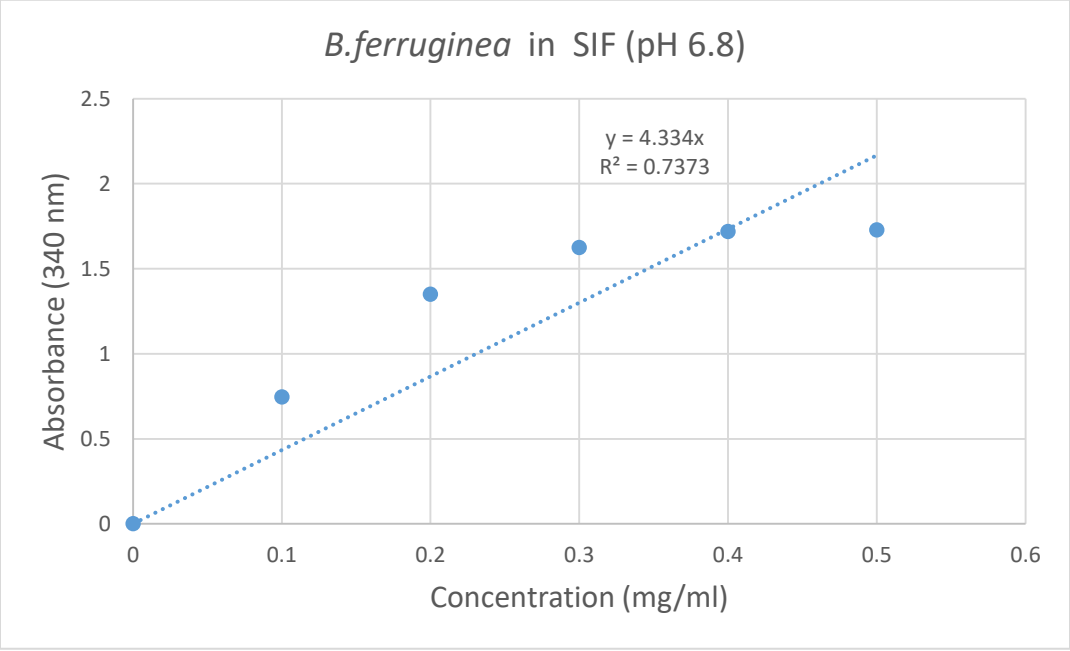
400 mg/kg 1:1 *B. pinnatum* phytosome



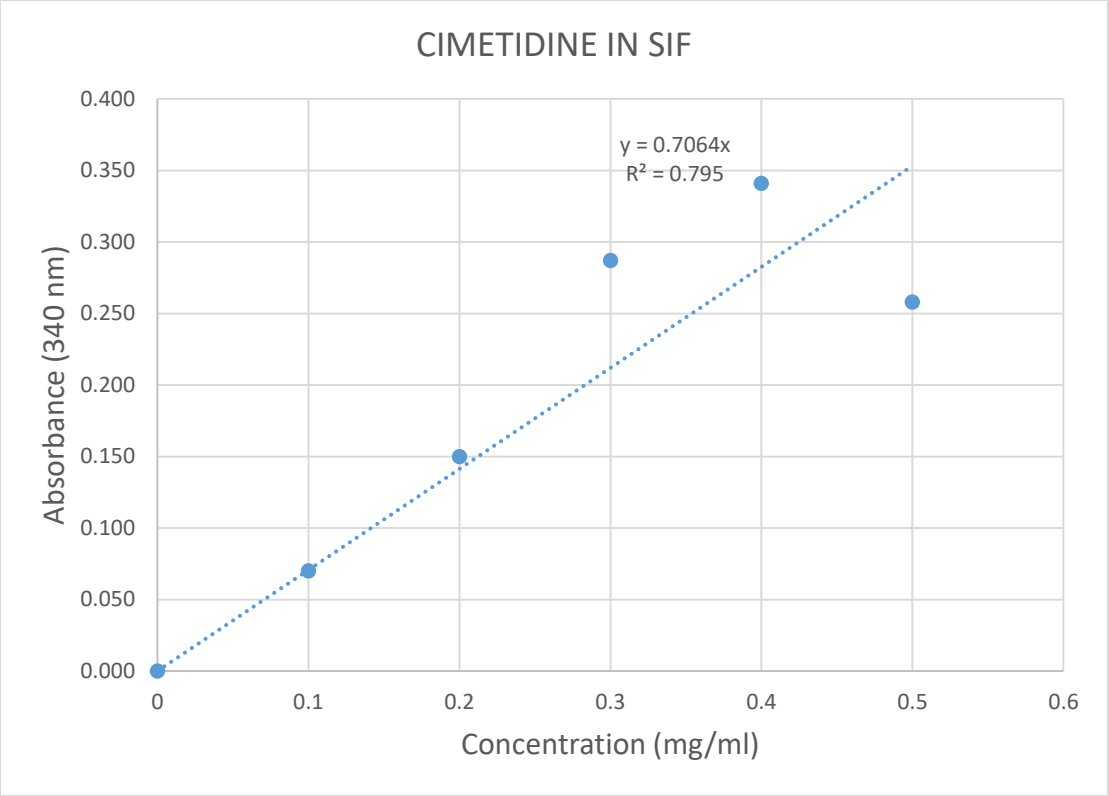
400 mg/kg 1:5 *B. pinnatum* phytosome



Standard curve of *Bryophyllum pinnatum* in Simulated interstitial fluid



Standard curve of *B. ferruginea* in Simulated interstitial fluid



Standard curve of Cimetidine in Simulated interstitial fluid