Title page

EFFECT OF *PARINARI CURATELLIFOLIA* PLANCH. EX BENTH. LEAF EXTRACT ON SOME CAUSATIVE AGENTS OF EPIGLOTTITIS

BY

EZE, HENRY TOCHUKWU
(M.TECH/BC/08/0356)

A THESIS SUBMITTED TO THE DEPARTMENT OF BIOCHEMISTRY, MODIBBO ADAMA UNIVERSITY OF TECHNOLOGY, YOLA IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE AWARD OF M.TECH (HONS) BIOCHEMISTRY. DEPARTMENT OF BIOCHEMISTRY SCHOOL OF PURE AND APPLIED SCIENCES.

NOVEMBER, 2012

DECLARATION

I hereby declare that this thesis was written by me and it is a record of my own research work. It has not been presented before in any previous application for higher degree. All references cited have been duly acknowledged.

Eze, Henry T.

DEDICATION

This thesis is dedicated to Almighty God for His grace and love upon my life throughout the period of my studies.

APPROVAL

This thesis entitled “Effect of *Parinari curatellifolia* Planch. ex Benth. Leaf Extract on Some Causative Agents of Epiglottitis” meets the regulations governing the award of Master of Technology of Modibbo Adama University of Technology, Yola and is approved for its contribution to knowledge and literary presentation.

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Dr. A.U., Wurochekke Date

(Supervisor)

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Internal Examiner Date

(Name and Signature)

---------- ----------

External examiner Date

(Name, address and Signature)

---------- ----------

Dr, Mrs. M.S., Nadro Date

(Head of Department)

---------- ----------

Prof. Remi O. Date

(Dean, Postgraduate School)

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TO GOD BE THE GLORY.

ABSTRACT

The medicinal effect of *Parinari curatellifolia* leaf extract on epiglottitis was investigated. Phytochemical screening of the extract revealed the presence of saponins, cardiac glycosides, alkaloids, flavonoids, steroids, and tannins. The antibacterial activities of the various solvent extract against some of the causative agents of epiglottitis showed that the acetic acid extract had the highest activity against the isolates used. The minimum inhibitory concentration (MIC) of the acetic acid extract was found to be 5mg/ml against *Pseudomonas sp* and *Streptococcus pyogenes*, and 50mg/ml against *Klebsiella sp* and *Staphylococcus aureus*. The activity of the acetic acid extract was compared to some standard antibiotics some of which are utilized in the treatment of epiglottitis. The result showed that there is no significant difference between Leofloxacin which gave the highest activity among the antibiotics used and the acetic acid extract ($p<0.05$). Four fractions were obtained on fractionation of the acetic acid extract using column chromatography (TiA, TiB, TiC, and TiD). With reference to susceptibility of the test strains, TiC recorded the highest inhibition zones against the test strains used. On TLC, the fraction gave three bands with the following Rf values 0.38, 0.36, 0.23. The extract showed activity against gram negative and gram positive organisms indicating a broad spectrum of activity. This research justified the use of this plant by traditional healers in the treatment and management of epiglottitis.

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CHAPTER ONE
INTRODUCTION

1.1 Introduction

Epiglottitis is the inflammation of the epiglottis-the flap tissue that sits at the base of the tongue, which keeps food away from going into the trachea (windpipe) so that one does not cough or choke after swallowing. Due to its place in the airway, swelling of the structure interferes with breathing and constitutes a medical emergency. Infections can cause the epiglottis to either obstruct or completely close off the windpipe making the condition life-threatening. The advent of Hemophilus influenza type b (Hib) vaccine has reduced the incidence of epiglottitis but it has not been eliminated, (Keyers, 1994; McEwan *et al.*, 2003).

Epiglottitis involves bacterial infection of the epiglottis, most often caused by *Haemophilus influenza* type b, although some cases are attributable to *Streptococcus pneumonia, Streptococcus agalactiae, Staphylococcus aureus, Streptococcus pyogenes, Pseudomonas spp, mycobacterium tuberculosis, Klebsiella spp,* Viruses, local trauma. *Streptococci* are becoming the major pathogen in acute epiglottitis now (Faden, 2006). Patients with epiglottitis may present with any of the following; sore throat, muffled voice, drooling, fever, anterior neck tenderness, cough, irritability, ear pain, cervical lymphadenopathy, odynophagia. The patient often appears acutely ill, anxious and has a very quiet shallow breathing with head held forward, insisting on sitting up in bed. The early symptoms are insidious but rapidly progressive, and swelling of the throat may lead to cyanosis and asphyxiation (Parson *et al*., 1996; Wong and Berkowitz, 2001). With more severe epiglottitis, dyspnoea, dysphagia, dysphonia, stridor (late finding indicates airway obstruction), respiratory distress may occur.

Intubation is required in over 30% of all cases of epiglottitis and prophylactic intubation may be carried out in patients with dyspnoea or stridor, as deterioration in symptoms may be rapid (usually if >50% airway destruction is present) (Hafidth *et al*., 2006). Treatment with antibiotic should be initiated without waiting for the swab/blood culture results. The chosen antibiotic should have a broad spectrum of action which covers both *Haemophilus influenza* type b and *Streptococcus spp*. Due to increasing resistance to ampicillin, third generation cephalosporin such as cefotaxime are now preferred as the first line agents. Corticosteroids are often given for their ant-inflammatory properties although there is yet little evidence that their uses influence the course of the disease (Wong and Berkowitz, 2001). Surgical tracheotomy may be required in patients with severe airway

obstruction in whom intubation has not been possible. Abscess formation is been increasingly seen as epiglottitis in adults increases (Berg *et al*., 2003). Drainage may be required in some patients.

Epiglottitis is life-threatening and usually occurs in both children and adults. The disease occurs at any time, there is no one season that it is more prevalent. Death may occur rapidly if the condition is not recognized and complete airway obstruction occurs (Berg *et al*., 1996). During treatment, the stimulating procedures of either orotracheal intubation or tracheotomy performed under the local anesthesia may precipitate sudden lost of the airway. General anesthesia performed with an inhalational induction can be complicated by a relatively prolonged excitation phase in adults. Bag and mask ventilation worsens or completely occludes the airway (Friedman *et al.*, 1998).

The use of herbal medicine by the traditional practioners for the treatment of diseases remains the main stay of health care system and is gaining increasing popularity especially among the rural populace in developing countries. Many of the herbal remedies used by herbal practioners are also employed therapeutically in orthodox medicine after the crude extract has been greatly improved upon. In recent times more research programs have been going on to assay and improve the medicinal principles found in plants for use in the development of new pharmacotherapeutic agents in the management and cure of diseases (Sofowora, 1993)

1.2 Justification of the Study

Parinari curatellifolia is a valuable and cherished medicinal plant in which different parts of the plant are widely used by the traditional herbalist in the treatment of diabetes and other disease conditions and has been evaluated for its anti-diabetic activities (Ogbonnia *et al*., 2009). The leaf of this plant as claimed by the traditional herbalist is utilized in the treatment of epiglottitis. However, there is no existing scientific evidence about the efficacy of the leaf of this plant. This work was therefore designed to investigate the pharmacological effects of the leaf extract of this plant with emphasis on its effect on the causative agents of epiglottitis.

1.3 Aims and Objectives

1. To determine the phytochemical components of the crude extract of the leaf of *Parinari curatellifolia.*
2. To determine the antimicrobial activity of the crude extract of the leaf of *Parinari curatellifolia* on some selected microorganisms.
3. To compare the efficacy of the crude extract of the leaf of *Parinari curatellifolia* with some selected antibiotics.
4. To partially fractionate the crude extract of the leaf of *Parinari curatellifolia* for possible identification of the active component responsible for the observed activity.

CHAPTER TWO
LITERATURE REVIEW

2.1 Medicinal Plants

Medicinal plants are plants with therapeutic activities. They remain the main source of active drugs from natural sources. Herbal or phytomedicine are medicine derived from plants. They remain the most common form of alternative medicine and are used by about 60% of the world population both in developing and developed countries where modern medicine are predominantly used (Rickert *et al*., 1999; Ogbonnia *et al*., 2008).

Phytomedicine is most often a polyherbal formulation prepared from many plant parts obtained from various plant species and families and may contain many bioactive constituents that could be difficult to characterize. The bioactive principles in most herbal preparation are not always known and there could be possibilities of interaction with each other in solution. The quality as well as the safety criteria for herbal drugs may be based therefore on a clear scientific definition of the raw materials used for such preparations. Also herbal medicine may have multiple physiological activities and could be used in the treatment of a variety of disease conditions (Pieme *et al*., 2006).

The bioactive constituents of plants are known as secondary metabolites and mankind have used many species for centuries to treat a variety of diseases (Cragg *et al*., 1999). Secondary metabolites are biosynthesized in plants for different purposes including growth regulation, inter and intra-specific interactions and defense against predators and infections. Many of these natural products have been shown to present interesting biological and pharmacological activities and are used as chemotherapeutic agents or serve as the starting point in the development of modern medicines (Verpoorte, 1998, 2000). Many plant secondary metabolites were found to interfere with different immune system functions, including the activation of cell mediated immunity (Wagner 1993).

Medicinal plants are of great importance to the health of individuals and communities. The medicinal value of these plants lies in some chemical substances (bioactive constituents) that produce a definite physiological action on the human body. The most important of these bioactive constituents of plants are alkaloids, tannins, flavonoids, and phenolic compounds (Hill, 1952). Many of these indigenous medicinal plants are used as spices and food. They are also sometimes added to foods meant for pregnant and nursing mothers for medicinal purpose (Okwu, 1999, 2001).

2.2 Bioactive Components of Medicinal Plants

2.2.1 Alkaloids

Alkaloids are a group of naturally occurring chemical compounds that contain mostly basic nitrogen atoms. This group also includes some related compounds with neutral and even weakly acidic properties (McNaught and Wilkinson, 1997; Manske, 1965) Also some synthetic compounds of similar structure are attributed to alkaloids (Robert, 1998). In addition to carbon, hydrogen and nitrogen, alkaloids may also contain oxygen, sulfur and more rarely other elements such as chlorine, bromine, and phosphorus.

Alkaloids are produced by a large variety of organisms, including bacteria, fungi, plants, and animals, and are part of the group of natural products (also called secondary metabolites). Many alkaloids can be purified from crude extracts by acid-base extraction. Many alkaloids are toxic to other organisms. They often have pharmacological effects and are used as medications, as recreational drugs, or in entheogenic rituals. Examples are the local anesthetic and stimulant cocaine; the psychedelic psilocin; the stimulant caffeine; nicotine; the analgesic morphine; the antibacterial berberine; the anticancer compound vincristine; the antihypertension agent reserpine; the cholinomimeric galatamine; the spasmolysis agent atropine; the vasodilator vincamine; the anti-arrhythmia compound quinidine; the anti-asthma therapeutic ephedrine; and the antimalarial drug quinine. Although alkaloids act on a diversity of metabolic systems in humans and other animals, they almost uniformly invoke a bitter taste (Rhoades, 1979).

The boundary between alkaloids and other nitrogen-containing natural compounds is not clear-cut. Compounds like amino acid peptides, proteins, nucleotides, nucleic acid, amines, and antibiotics are usually not called alkaloids (McNaught and Wilkinson, 1997). Natural compounds containing nitrogen in the exocyclic position (mescaline, serotonin, dopamine, etc.) are usually attributed to amines rather than alkaloids (Leland, 2006). Some authors, however, consider alkaloids a special case of amines (William, 1999; Raj, 2004; Aniszewski, 2007).

The name "alkaloids" (German: *Alkaloide*) was introduced in 1819 by the German chemist Carl F.W. Meissner, and is derived from late Latin root *Latin:* alkali (which, in turn, comes from the Arabic *al-qalwī* – "ashes of plants") and the suffix *Greek: -οειδής* – "like" (Andreas, 2009).

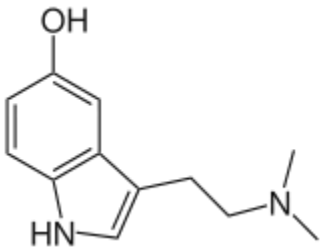
However, the term came into wide use only after the publication of a review article by O. Jacobsen in the chemical dictionary of Albert Ladenburg in the 1880s (Hesse, 2002).

There is no unique method of naming alkaloids (Hesse, 2002). Many individual names are formed by adding the suffix "ine" to the species or genus name. For example, atropine is isolated from the plant Atropa belladonna, strychnine is obtained from the seed of Strychnine tree (*Strychnos nux-vomica* L.). If several alkaloids are extracted from one plant then their names often contain suffixes "idine", "anine", "aline", "inine", etc. There are also at least 86 alkaloids containing the root "vin" (extracted from the Vinca plant). Alkaloid-containing plants have been used by humans since ancient times for therapeutic and recreational purposes. For example, medicinal plants have been known in the Mesopotamia at least around 2000 BC (Hesse, 2002).

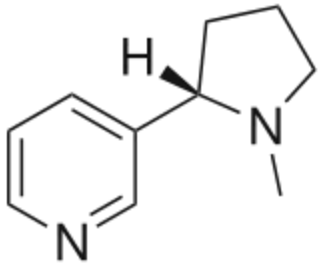
The *Odyssey* of Homer referred to a gift given to Helen by the Egyptian queen, a drug bringing oblivion. It is believed that the gift was an opium-containing drug. A Chinese book on houseplants written in 1st–3rd centuries BC mentioned a medical use of Ephedra and opium poppies. Also, coca leaves have been used by South American Indians since ancient times. Extracts from plants containing toxic alkaloids, such as aconitine and tubocurarine, were used since antiquity for poisoning arrows. Studies of alkaloids began in the 19th century. In 1804, the German chemist Friedrich Sertürner isolated from opium a "soporific principle" (Latin: *principium somniferum*), which he called "morphium" in honor of Morpheus, the Greek god of dreams; in German and some other Central-European languages, this is still the name of the drug. The term "morphine", used in English and French, was given by the French physicist Joseph Louis Gay-Lussac). A significant contribution to the chemistry of alkaloids in the early years of its development was made by the French researchers Pierre Joseph Pelletier and Joseph Bienaimé Caventou, who discovered quinine (1820) and strychnine (1818). Several other alkaloids were discovered around that time, including xanthine (1817), atropine (1819), caffeine (1820), coniine (1827), nicotine (1828), colchicine (1833), sparteine (1851), and cocaine (1860) (Hesse, 2002).

The first complete synthesis of an alkaloid was achieved in 1886 by the German chemist Albert Ladenburg. He produced coniine by reacting 2-methylpyridine with acetaldehyde and reducing the resulting 2-propenyl pyridine with sodium (Hesse, 2002). The development of the chemistry of alkaloids was accelerated by the emergence of spectroscopic and chromatographic

methods in the 20th century, so that by 2008 more than 12,000 alkaloids had been identified (Begley, 2009).



Bufotenin, an alkaloid from some toads, contains an indole core and is produced in living organisms from the amino acid tryptophan.



The nicotine molecule contains both pyridine (left) and pyrrolidine rings (right).

Classification of alkaloids

Compared with most other classes of natural compounds, alkaloids are characterized by a great structural diversity and there is no uniform classification (Hesse, 2002). First classification methods have historically combined alkaloids by the common natural source, e.g., a certain type of plants. This classification was justified by the lack of knowledge about the chemical structure of alkaloids and is now considered obsolete (Orekhov, 1955). More recent classifications are based on similarity of the carbon skeleton (e.g., indole-, isoquinoline-, and pyridine-like) or biogenetic precursor (ornithine, lysine, tyrosine, tryptophan, etc.). However, they require compromises in borderline cases; for example, nicotine contains a pyridine fragment from nicotinamide and pyrrolidine part from ornithine and therefore can be assigned to both classes (Hesse, 2002; Aniszewski, 2007; Dewick, 2002).

Alkaloids are often divided into the following major groups:

True alkaloids, which contain nitrogen in the heterocycle and originate from amino acids. Their characteristic examples are atropine, nicotine, and morphine. This group also includes some alkaloids that besides nitrogen heterocycle contain terpene (e.g., evonine) or peptide fragments (e.g. ergotamine. This group also includes piperidine alkaloids coniine and coniceine, although they do not originate from amino acids.

Protoalkaloids, which contain nitrogen and also originate from amino acids. Examples include mescaline, adrenaline and ephedrine.

Polyamine alkaloids – derivatives of putrescine, spermidine, and spermine.

Peptide and cyclopeptide alkaloids.

Pseudalkaloids – alkaloid-like compounds that do not originate from amino acids. This group includes, terpene-like and steroid-like alkaloids, as well as purine-like alkaloids such as caffeine, theobromine and theophylline. Some authors classify as pseudoalkaloids such compounds such as ephedrine and cathinone. Those originate from the amino acid phenylalanine, but acquire their nitrogen atom not from the amino acid but through transamination.

Some alkaloids do not have the carbon skeleton characteristic of their group. So, galantamine and homoaporphines do not contain isoquinoline fragment, but are, in general, attributed to **isoquinoline alkaloids**. (Aniszewski, 2007; Hesse, 2002; Dewick, 2002; Plemenkov, 2001)).

Properties of alkaloids

Most alkaloids contain oxygen in their molecular structure; those compounds are usually colorless crystals at ambient conditions. Oxygen-free alkaloids, such as nicotine or coniine are typically volatile, colorless, oily liquids. Some alkaloids are colored, like berberine (yellow) and sanguinarine (orange). Most alkaloids are weak bases, but some are amphoteric, for example theobromine and theophylline. Most alkaloids are poorly soluble in water but readily dissolve in organic solvents, such as diethyl ether, chloroform and 1,2-dichloroethane. However, caffeine dissolves well in boiling water. With acids, alkaloids form salts of various strengths. Those salts are

usually soluble in water and alcohol and poorly soluble in most organic solvents. Exceptions include scopolamine hydrobromide, which is soluble in organic solvents and water-soluble quinine sulfate. (Hesse, 2002; Grinkevich, 1983).

Most alkaloids have a bitter taste. It is believed that plants evolved the ability to produce these bitter substances, many of which are poisonous, in order to protect themselves from animals; however, animals in turn evolved the ability to detoxify alkaloids (Aniszewski, 2007). Some alkaloids can produce developmental defects in the offspring of animals that consume them but cannot detoxify them. A characteristic example is the alkaloid cyclopamine, which is present in the leaves of corn lily. During the 1950s, up to 25% lambs born by sheep that had grazed on corn lily suffered serious facial defects. Those defects ranged from deformed jaws to cyclopia. After decades of research, in 1980s, the substance that was responsible for the deformities was identified as the alkaloid 11-deoxyjervine, which was renamed cyclopamine (Thomas, 2004).

Distribution of alkaloids in nature

Alkaloids are generated by various living organisms, especially by higher plants – about 10 to 25% of these contain alkaloids (Aniszewski, 2007; Orekhov, 1955). Therefore, in the past the term "alkaloid" was associated with plants (Hesse, 2002). The alkaloids content in plants is usually within a few percent and is inhomogeneous over the plant tissues. Depending on the type of plants, the maximum concentration is observed in the leaves (black henbane), fruits or seeds (Strychnine tree), root (Rauwolfia serpentina) or bark (cinchona). Furthermore, different tissues of the same plants may contain different alkaloids (Grinkevich, 1983; Orekhov, 1955).

Beside plants, alkaloids are found in certain types of fungi, such as psilocybin in the fungus of the genus Psilocybe, and in animals, such as bufotenin in the skin of some toads (Hesse, 2002). Many marine organisms also contain alkaloids (Fattorusso, 2008). Some amines, such as adrenaline and serotonin, which play an important role in higher animals, are similar to alkaloids in their structure and biosynthesis and are sometimes called alkaloids (Aniszewski, 2007).

Extraction of Alkaloids

Because of the structural diversity of alkaloids, there is no single method of their extraction from natural raw materials. Most methods exploit the property of most alkaloids to be soluble in

organic solvents but not in water, and the opposite tendency of their salts. Most plants contain several alkaloids. Their mixture is extracted first and then individual alkaloids are separated. Plants are thoroughly ground before extraction. Most alkaloids are present in the raw plants in the form of salts of organic acids. The extracted alkaloids may remain salts or change into bases. Base extraction is achieved by processing the raw material with alkaline solutions and extracting the alkaloid bases with organic solvents, such as 1,2-dichloroethane, chloroform, diethyl ether or benzene. Then, the impurities are dissolved by weak acids; this converts alkaloid bases into salts that are washed away with water. If necessary, an aqueous solution of alkaloid salts is again made alkaline and treated with an organic solvent. The process is repeated until the desired purity is achieved (Grinkevich, 1983; Hesse, 2002)

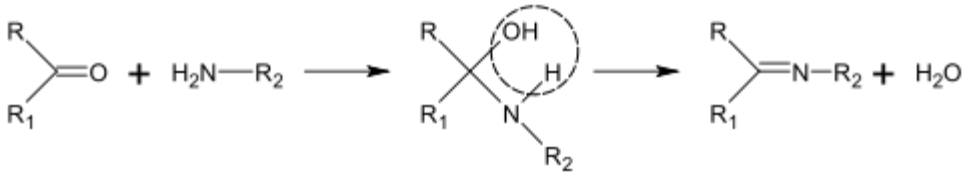
In the acidic extraction, the raw plant material is processed by a weak acidic solution (e.g., acetic acid in water, ethanol, or methanol). A base is then added to convert alkaloids to basic forms that are extracted with organic solvent (if the extraction was performed with alcohol, it is removed first, and the remainder is dissolved in water). The solution is purified as described above. Alkaloids are separated from their mixture using their different solubility in certain solvents and different reactivity with certain reagents or by distillation (Grinkevich, 1983; Hesse, 2002).

Biosynthesis of alkaloids

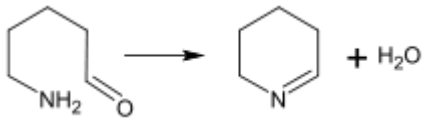
Biological precursors of most alkaloids are amino acids, such as ornithine, lysine, phenylalanine, tyrosine, tryptophan, histidine, aspartic acid, and anthranilic acid. Nicotinic acid can be synthesized from tryptophan or aspartic acid. Ways of alkaloid biosynthesis are too numerous and cannot be easily classified. However, there are a few typical reactions involved in the biosynthesis of various classes of alkaloids, including synthesis of Schiff bases and Mannich reaction (Plemenkov, 2001; Begley, 2009).

Synthesis of Schiff bases

Schiff bases can be obtained by reacting amines with ketones or aldehydes . These reactions are common method of producing C=N bonds.

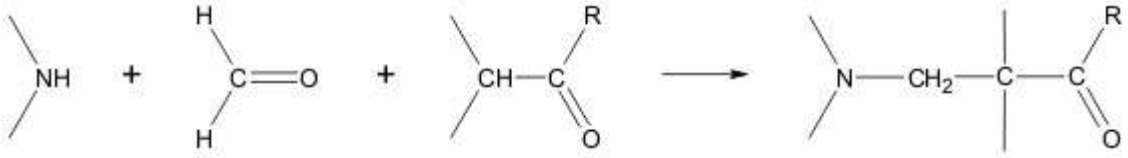


In the biosynthesis of alkaloids, such reactions may take place within a molecule, such as in the synthesis of piperidine (Plemenkov, 2001; Dewick, 2002)

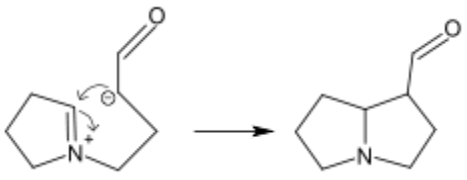


Mannich reaction

An integral component of the Mannich reaction, in addition to an amine and a carbonyl compound, is a carbanion, which plays the role of the nucleophile in the nucleophilic addition to the ion formed by the reaction of the amine and the carbonyl.



The Mannich reaction can proceed both intermolecularly and intramolecularly (Plemenkov, 2001; Dewick, 2002).

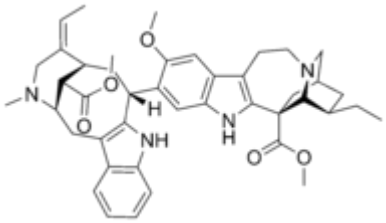


Dimer alkaloids

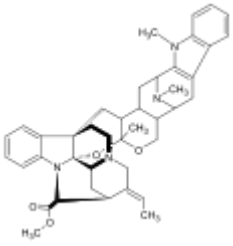
In addition to the described above monomeric alkaloids, there are also dimeric, and even trimeric and tetrameric alkaloids formed upon condensation of two, three, and four monomeric

alkaloids. Dimeric alkaloids are usually formed from monomers of the same type through the following mechanisms (Hesse, 2002).

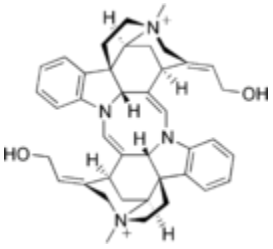
- Mannich reaction, resulting in, e.g., voacamine
- Michael reaction (villalstonine)
- Condensation of aldehydes with amines (toxiferine)
- Oxidative addition of phenols (dauricine, tubocurarine)
- Lactonization (carpaine).

- 

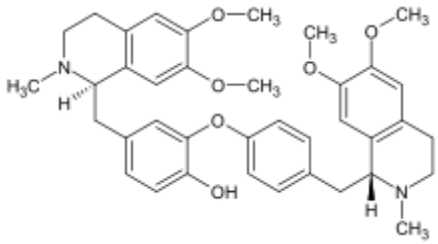
  Voacamine

- 

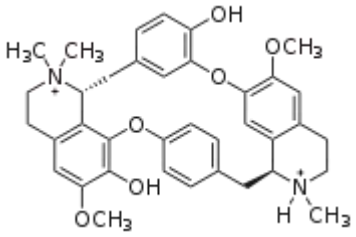
  Villalstonine

- 

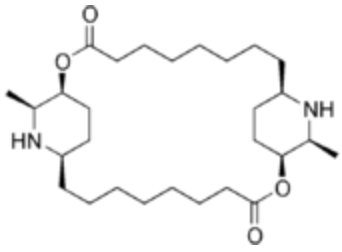
  Toxiferine

- 

  Dauricine

- 

  Tubocurarine

- 

  Carpaine

The biological role of alkaloids

The role of alkaloids for living organisms that produce them is still unclear. It was initially assumed that the alkaloids are the final products of nitrogen metabolism in plants, as urea in mammals. It was later shown that alkaloid concentrations vary over time, and this hypothesis was refuted. Most of the known functions of alkaloids are related to protection. For example, aporphine alkaloid liriodenine produced by the tulip tree protects it from parasitic mushrooms. In addition, presence of alkaloids in the plant prevents insects and chordate animals from eating it. However, some animals adapted to alkaloids and even use them in their own metabolism. Such alkaloid-related substances as serotonin, dopamine and histamine are important neurotransmitters in animals. Alkaloids are also known to regulate plant growth ((Aniszewski, 2007; Hesse, 2002).

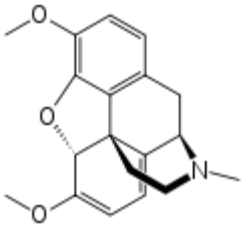
Applications of Alkaloids;

In medicine

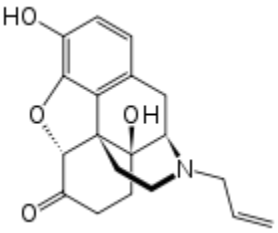
Medical use of alkaloid-containing plants has a long history, and, thus, when the first alkaloids were isolated in the 19th century, they immediately found application in clinical practice. Many alkaloids are still used in medicine, usually in the form of salts, including the following

Alkaloid	**Action**
Ajmaline	antiarrhythmic
Atropine, scopolamine, hyoscyamine	anticholinergic
Vinblastine, vincristine	antitumor
Vincamine	vasodilating, antihypertensive
Codeine	cough medicine
Cocaine	anesthetic
Colchicine	remedy for gout
Morphine	analgesic
Reserpine	antihypertensive
Tubocurarine	Muscle relaxant
Physostigmine	inhibitor of acetylcholinesterase
Quinidine	Antiarrhythmic
Quinine	antipyretics, antimalarial
Emetine	antiprotozoal agent
Ergot alkaloids	sympathomimetic, vasodilator, antihypertensive

Many synthetic and semisynthetic drugs are structural modifications of the alkaloids, which were designed to enhance or change the primary effect of the drug and reduce unwanted side-effects. For example, naloxone, an opioid receptor antagonist, is a derivative of thebaine that is present in opium (Dewick, 2002; Hesse, 2002).

- 

Thebaine

- 

Naloxone

In agriculture

Prior to the development of a wide range of relatively low-toxic synthetic pesticides, some alkaloids, such as salts of nicotine and anabasine, were used as insecticides. Their use was limited by their high toxicity to humans (György *et al*., 2002).

Use of alkaloids as psychoactive drugs

Preparations of plants containing alkaloids and their extracts, and later pure alkaloids, have long been used as psychoactive substances. Cocaine and cathinone are stimulants of the central nervous system (Veselovskaya,, 2000 ; Hesse, 2002). Mescaline and many of indole alkaloids (such as psilocybin, dimethyltryptamine and ibogaine) have hallucinogenic effect (Geoffrey, 2001). Morphine and codeine are strong narcotic pain killers.

There are alkaloids that do not have strong psychoactive effect themselves, but are precursors for semi-synthetic psychoactive drugs. For example, ephedrine and pseudoephedrine are used to produce methcathinone and methamphetamine (Veselovskaya, 2000).

The first medically useful example of an alkaloid was morphine, isolated in 1805 from the opium poppy *Papaver somniferum* (Fessenden and Fessenden, 1982). Codeine and heroin are both derivatives of morphine. Diterpenoid alkaloids, commonly isolated from the plants of the *Ranunculaceae*, or buttercup family are commonly found to have antimicrobial properties (Pantazis, 1996). Berberine is an important representative of the alkaloid group. Alkaloids are found to intercalate into cell wall and/or DNA and therefore disrupt the activities of microorganisms (Phillipson and Neill, 1987).

2.2.2 Tannin

Tannin is a general descriptive name for a group of polymeric phenolic substances capable of tanning leather or precipitating gelatin from solution, a property known as astringency. Their molecular weights range from 500 - 3,000 (Haslam, 1996), and they are found in almost every plant part: bark, wood, leaves, fruits, and roots (Scalbert, 1991). They are divided into two groups, hydrolysable and condensed tannins. Hydrolysable tannins are based on gallic acid, usually as multiple esters with D-glucose; while the more numerous condensed tannins (often called proanthocyanidins) are derived from flavonoid monomers. Tannins may be formed by condensations of flavan derivatives which have been transported to woody tissues of plants. Alternatively, tannins may be formed by polymerization of quinone units (Geissman, 1963). This group of compounds has received a great deal of attention in recent years, since it was suggested that the consumption of tannin-containing beverages, especially green teas and red wines, can cure or prevent a variety of ills (Serafini *et al*., 1994). Many human physiological activities, such as stimulation of phagocytic cells, host-mediated tumor activity, and a wide range of anti-infective actions, have been assigned to tannins (Haslam, 1996).

Tannins have traditionally been considered antinutritional but it is now known that their beneficial or antinutritional properties depend upon their chemical structure and dosage. The new technologies used to analyze molecular and chemical structures have shown that a division into condensed and hydrolysable tannins is far too simplistic (Muller-Harvey and McAllan, 1992). Recent studies have demonstrated that products containing chestnut tannins included at low dosages (0.15–0.2 %) in the diet can be beneficial (Schiavone *et al.*, 2008). Some studies suggest that chestnut tannins have been shown to have positive effects on silage quality in the round bale silages, in particular reducing NPNs (non protein nitrogen) in the lowest wilting level (Tabacco *et al*., 2006). Improved fermentability of soya meal nitrogen in the rumen has also been reported by F.

Mathieu and J.P. Jouany (1993). Studies by S. Gonzalez *et al.* (2002) on *in vitro* ammonia release and dry matter degradation of soybean meal comparing three different types of tannins (quebracho, acacia and chestnut) demonstrated that chestnut tannins are more efficient in protecting soybean meal from *in vitro* degradation by rumen bacteria.

Condensed tannins inhibit herbivore digestion by binding to consumed plant proteins and making them more difficult for animals to digest, and by interfering with protein absorption and digestive enzymes. Many tannin-consuming animals secrete a tannin-binding protein (mucin) in their saliva. Tannin-binding capacity of salivary mucin is directly related to its proline content. Advantages in using salivary proline-rich proteins (PRPs) to inactivate tannins are:

- PRPs inactivate tannins to a greater extent than do dietary proteins; this results in reduced fecal nitrogen losses
- PRPs contain non specific nitrogen and nonessential amino acids; this makes them more convenient for an animal to exploit rather than using up valuable dietary protein

Tannin is a component in a type of industrial particleboard adhesive developed jointly by the Tanzania Industrial Research and Development Organization and Forintek Labs Canada (Bisanda *et al.*, 2003). *Pinus radiata* tannins have been investigated for the production of wood adhesives (Li and Maplesden, 1998). Condensed tannins, i.e. quebracho tannin, and Hydrolysable tannins, i.e., chestnut tannin, appear to be able to substitute a high proportion of synthetic phenol in phenol-formaldehyde resins for wood particleboard. Tannins can be used for production of anti-corrosive primer, sold under brand name-Nox Primer for treatment of rusted steel surfaces prior to painting, rust converter to transform oxidized steel into a smooth sealed surface and rust inhibitor. The use of resins made of tannins has been investigated to remove mercury and methyl mercury from solution (Torres *et al*., 1999). Immobilized tannins have been tested to recover uranium from seawater (Takashi and Akira, 1987).

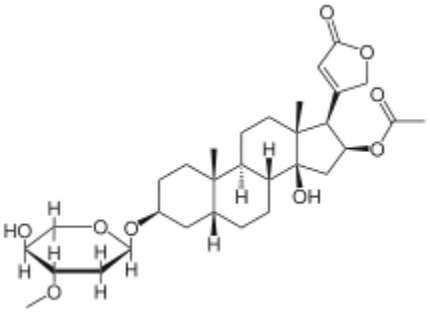
Tannins can also be effective in protecting the kidneys. When incubated with red grape juice and red wines with a high content of condensed tannins, the poliovirus, herpes simplex virus, and various enteric viruses are inactivated (Bajaj, 1988). Tannins have shown potential antiviral, antibacterial and antiparasitic effects (Lü *et al*., 2004; Akiyama *et al*., 2001; Kolodziej and Kiderlen, 2005).

It is believed that tannins isolated from the stem bark of *Myracrodruon urundeuva* may have neuroprotective functions capable of reversing 6-hydroxydopamine-induced toxicity. The plant has shown promise as a potential therapeutic agent, which may be beneficial in patients with neurological disease (Nobre-Junior, *et al.*, 2007). It was discovered that the tannins isolated from the stem bark also have anti-inflammatory and anti ulcer activity in rodents, showing a strong antioxidant property with possible therapeutic applications. Foods rich in tannins can be used in the treatment of hereditary hemochromatosis, a hereditary disease characterized by excessive absorption of dietary iron, resulting in a pathological increase in total body iron stores (Souza *et al.*, 2006).

Tannin has been reported to exhibit antiviral, antibacterial, and ant-tumor activities. It has also been reported that certain tannins are able to inhibit HIV replication and is also used as diuretic (Halsam, 1996). Plant tannins have been recognized for their pharmacologic properties and are known to make trees and shrubs a difficult meal for many caterpillars.

2.2.3 Cardiac Glycosides

Cardiac glycosides are drugs used in the treatment of congestive heart failure and cardiac arrhythmia. These glycosides are found as secondary metabolites in several plants, but also in some animals, such as the milkweed butterflies.



Chemical structure of oleandrin, one of the cardiac glycosides.

Therapeutic uses of cardiac glycosides primarily involve the treatment of cardiac failure. Their utility results from an increased cardiac output by increasing the force of contraction. By increasing intracellular calcium as described below, cardiac glycosides increase calcium-induced calcium release and thus contraction. Drugs such as ouabain and digoxin are cardiac glycosides. Digoxin from the foxglove plant is used clinically, whereas ouabain is used only experimentally due to its extremely high potency. Normally, sodium-potassium pumps in the membrane of cells (in this case, cardiac myocytes) pump potassium ions in and sodium ions out.

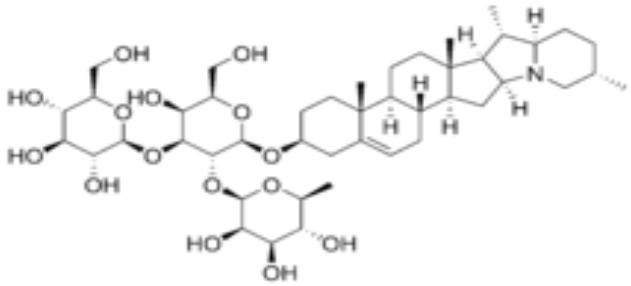
Cardiac glycosides inhibit this pump by stabilizing it so that sodium cannot be extruded: intracellular sodium concentration therefore increases. A second membrane ion exchanger known as Na^+/Ca^+ exchanger (NCX) is responsible for 'pumping' calcium ions out of the cell and sodium ions in (3Na/Ca); raised intracellular sodium levels inhibit this pump, so calcium ions are not extruded and will also begin to build up inside the cell. Increased cytoplasmic calcium concentrations cause increased calcium uptake into the sarcoplasmic reticulum via the Sarco- Endoplasmic Reticulum Ca^{2+}-ATPase (SERCA2) transporter. Raised calcium stores in the SR allow for greater calcium release on stimulation, so the myocyte can achieve faster and more powerful contraction by cross-bridge cycling. The refractory period of the atrioventricular (AV) node is increased, so cardiac glycosides also function to regulate heart rate. Binding of cardiac glycoside to Na-K ATPase is slow, and also, after binding, intracellular calcium increases gradually. Thus, the action of digitalis (even on IV injection) is delayed. Raised extracellular potassium decreases binding of cardiac glycoside to Na-K ATPase. Consequently, increased toxicity of these drugs is observed in the presence of Hypokalemia. This inhibition increases the amount of Ca^{2+} available for contraction of the heart muscles, which improves cardiac output and reduces distension of the heart. Thus, they may be used in the treatment of congestive heart failure and cardiac arrhythmia. They are also used to strengthen a weakened heart and allow it to function more efficiently, though the dosage must be carefully controlled since the therapeutic dose is close to the toxic dose (Dewick, 2002)

If sarcoplasmic reticulum (SR) calcium stores become too high, some ions are released spontaneously through SR ryanodine receptors. Then after-depolarization this effect leads initially to bigeminy: regular ectopic beats following each ventricular contraction. If higher glycoside doses are given, rhythm is lost and ventricular tachycardia ensues, followed by fibrillation. ("Digoxin oral", Retrieved 7 May 2012).

2.2.4 Saponins

Saponins are a class of chemical compounds, one of many secondary metabolites found in natural sources, with saponins found in particular abundance in various plant species. More specifically, they are amphipathic glycosides grouped, in terms of phenomenology, by the soap-like foaming they produce when shaken in aqueous solutions, and, in terms of structure, by their composition of one or more hydrophilic glycoside moieties combined with a lipophilic triterpene derivative (Hostettmann, 1995).

The aglycone (glycoside-free) portions of the saponins are termed sapogenins. The number of saccharide chains attached to the sapogenin/aglycone core can vary – giving rise to another dimension of nomenclature (monodesmosidic, bidesmosidic, etc. – as can the length of each chain. A somewhat dated compilation has the range of saccharide chain lengths being 1–11, with the numbers 2-5 being the most frequent, and with both linear and branched chain saccharides being represented. Dietary monosaccharides such as D-glucose and D-galactose are among the most common components of the attached chains (Hostettmann, 1995).



Chemical structure of the saponin, solanin

The lipophilic aglycone can be any one of a wide variety of polycyclic organic structures originating from the serial addition of 10-carbon (C10) terpene units to compose a C30 triterpene skeleton, often with subsequent alteration to produce a C27 steroidal skeleton. The subsets of saponins that are steroidal have been termed saraponins; Aglycone derivatives can also incorporate nitrogen, so some saponins also present chemical and pharmacologic characteristics of alkaloid natural products (Hostettmann, 1995; Foerster, 2006)

Saponins have historically been understood to be plant-derived, but they have also been isolated from marine organisms Saponins are indeed found in many plants, and derive their name from the soapwort plant (genus *Saponaria*, family Caryophyllaceae), the root of which was used historically as a soap. Saponins are also found in the botanical family Sapindaceae, with its defining genus *Sapindus* (soapberry or soapnut), and in the closely related families Aceraceae (maples) and Hippocastanaceae (horse chestnut). It is also found heavily in *Gynostemma pentaphyllum* (*Gynostemma*, Cucurbitaceae) in a form called gypenosides, and ginseng or red ginseng (*Panax*, Araliaceae) in a form called ginsenosides. Within these families, this class of chemical compounds is found in various parts of the plant: leaves, stems, roots, bulbs, blossom and fruit. Commercial

formulations of plant-derived saponins, e.g., from the soap bark (or soapbark) tree, *Quillaja saponaria*, and those from other sources are available via controlled manufacturing processes, which make them of use as chemical and biomedical reagents (Liener, 1980; Riguera, 1997; Hostettmann, 1995)

In plants, saponins may serve as anti-feedants, and to protect the plant against microbes and fungi. Some plant saponins (e.g. from oat and spinach) may enhance nutrient absorption and aid in animal digestion. However, saponins are often bitter to taste, and so can reduce plant palatability (e.g., in livestock feeds), or even imbue them with life-threatening animal toxicity. Data make clear that some saponins are toxic to cold-blooded organisms and insects at particular concentrations. There is a need for further research to define the roles of these natural products in their host organisms, which have been described as "poorly understood" to date (Foerster, 2006). Most saponins, which readily dissolve in water, are poisonous to fish (http://www.jstor.org/pss/4107559, Fish-poison plants). Therefore, in ethnobotany, they are primarily known for their use by indigenous people in obtaining aquatic food sources. Since prehistoric times, cultures throughout the world have used piscicidal plants, mostly those containing saponins, for fishing. Although prohibited by law, fish poison plants are still widely used by indigenous tribes in Guyana. On the Indian Subcontinent, the Gond tribes are known for their use of plant extracts in poison fishing. Many of California's Native American tribes traditionally used soap root, (genus *Chlorogalum*) and/or the root of various yucca species, which contain saponin, as a fish poison. They would pulverize the roots, mixing in water to create foam, and then add the suds to a stream. This would kill or incapacitate the fish, which could be gathered easily from the surface of the water. Among the tribes using this technique were the Lassik, the Luiseño, the Yuki, the Yokut, the Chilula, the Wailaki, the Miwok, the Kato, the Mattole, the Nomlaki and the Nishinam (Campbell, 1999).

Saponins complex with cholesterol to form pores in cell membrane bilayers. In addition, the amphipathic nature of saponins give them activity as surfactants that can be used to enhance penetration of macromolecules such as proteins through cell membranes. Saponins have also been used as adjutants in vaccines ("Saponin from quillaja bark". Sigma-Aldrich. Retrieved 23 February 2009).

Medical uses of saponins

Saponins are being promoted commercially as dietary supplements and nutriceuticals. There is evidence of the presence of saponins in traditional medicine preparations, where oral administrations might be expected to lead to hydrolysis of glycoside from terpenoid (and obviation of any toxicity associated with the intact molecule). But as is often the case with wide-ranging commercial therapeutic claims for natural products:

- the claims for organismal/human benefit are often based on very preliminary biochemical or cell biological studies; and
- mention is generally omitted of the possibilities of individual chemical sensitivity, or to the general toxicity of specific agents, and high toxicity of selected cases.

While such statements require constant review (and despite the myriad web claims to the contrary), it appears that there are very limited US, EU, etc. agency-approved roles for saponins in human therapy. In their use as adjuvants in the production of vaccines, toxicity associated with sterol complexation remains a major issue for attention. Therapeutic benefit is a result of careful administration of an appropriate dose. Very great care needs to be exercised in evaluating or acting on specific claims of therapeutic benefit from ingesting saponin-type and other natural products. (Asl and Hossein 2008; Xu *et al.*, 1996; Skene and Philip, 2006).

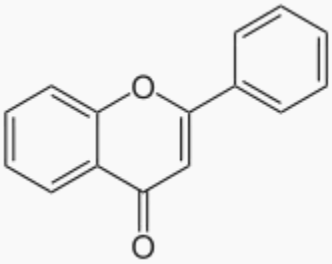
Saponins are group of two expectorant elements that induce hormonal activity. Saponins are similar to naturally occurring hormones found in the human body. Saponins have been reported to have an effect of cholesterol reduction (McDonald *et al*., 1995). Saponins have also been reported to have other effects such as, reduction of cancer, pathogen control and immunity booster (Cheeke, 1998).

2.2.5 Flavonoids

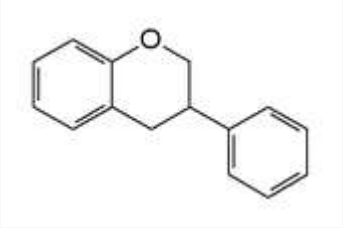
Flavonoids (or bioflavonoids) (from the Latin word *flavus* meaning yellow, their colour in nature) are a class of plant secondary metabolites. Flavonoids were referred to as Vitamin P (probably due to the effect they had on the permeability of vascular capillaries) from the mid-1930s to early 50s, but the term has since fallen out of use (Mobh, 1938).

According to the IUPAC nomenclature, flavonoids can be classified into:

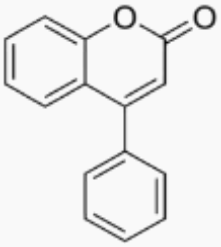
- *flavones*, derived from 2-phenylchromen-4-one (2-phenyl-1,4-benzopyrone) structure (examples:quercetin, rutin).
- *isoflavonoids*, derived from 3-phenylchromen-4-one (3-phenyl-1,4-benzopyrone) structure
- *neoflavonoids*, derived from 4-phenylcoumarine (4-phenyl-1,2-benzopyrone) structure.



Molecular structure of the flavone backbone (2-phenyl-1, 4-benzopyrone)



Isoflavan structure



Neoflavonoids structure

(McNaught and Wilkinson, 1997)

The three flavonoid classes above are all ketone-containing compounds, and as such, are flavonoids and flavonols. This class was the first to be termed "bioflavonoids." The terms flavonoid and bioflavonoid have also been more loosely used to describe non-ketone polyhydroxy polyphenol compounds which are more specifically termed flavanoids, flavan-3-ols (or catechins).

In plants, flavonoids are widely distributed in plants fulfilling many functions. Flavonoids are the most important plant pigments for flower coloration producing yellow or red/blue pigmentation in petals designed to attract pollinator animals. In higher plants, Flavonoids are involved in UV filtration, symbiotic nitrogen fixation and floral pigmentation. They may act as a chemical messenger or physiological regulator. They can also act as cell cycle inhibitors. Flavonoids secreted by the root of the host plant help *Rhizobia* in the infection stage of their symbiotic relationship with legumes like peas, beans, clover, and soy. Rhizobia living in soil are able to sense the flavonoids and this triggers the secretion of Nod factors, which in turn are recognized by the host plant and can lead to root hair deformation and several cellular responses such as ion fluxes and the formation of a root nodule. In addition, some flavonoids have inhibitory activity against organisms that cause plant disease e.g. *Fusarium oxysporum* (Galeotti *et al*., 2008)

Flavonoids (specifically flavanoids such as the catechins) are "the most common group of polyphenolic compounds in the human diet and are found ubiquitously in plants" (Spencer, 2008). Flavonols, the original bioflavonoids such as quercetin, are also found ubiquitously, but in lesser quantities. The widespread distribution of flavonoids, their variety and their relatively 2low toxicity compared to other active plant compounds (for instance alkaloids) mean that many animals, including humans, ingest significant quantities in their diet. Preliminary research indicates that flavonoids may modify allergens, viruses, and carcinogens, and so may be biological "response modifiers". *In vitro* studies show that flavonoids also have anti-allergic, anti-inflammatory, anti-microbial, anti-cancer, and anti-diarrheal activities (Cushnie and Lamb, 2005, 2011; de Sousa *et al*., 2007; Schuier *et al*., 2005).

Flavonoids (both flavonols and flavanols) are most commonly known for their antioxidant activity in vitro. At high experimental concentrations that would not exist in vivo, the antioxidant abilities of flavonoids in vitro may be stronger than those of vitamin C and E, depending on concentrations tested (Bagchi *et al*., 1999). Consumers and food manufacturers have become interested in flavonoids for their possible medicinal properties, especially their putative role in inhibiting cancer or cardiovascular disease. Although physiological evidence is not yet established, the beneficial effects of fruits, vegetables, tea, and red wine have sometimes been attributed to flavonoid compounds.

A research team at the Linus Pauling Institute and the European Food Safety Authority state that flavonoids, inside the human body, are of little or no direct antioxidant value (Lotito and Frei, 2006; Williams *et al*., 2004). Body conditions are unlike controlled test tube conditions, and the flavonoids are poorly absorbed (less than 5%), with most of what is absorbed being quickly metabolized and excreted. The increase in antioxidant capacity of blood seen after the consumption of flavonoid-rich foods may not be caused directly by the flavonoids themselves, but is probably due to increased production of uric acid resulting from excretion of flavonoids from the body.

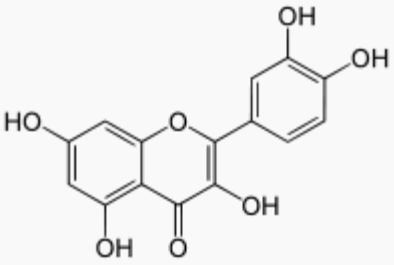
Flavonoids might induce mechanisms that affect cancer cells and inhibit tumor invasion. In preliminary studies, UCLA cancer researchers proposed that smokers who ate foods containing certain flavonoids, such as the flavan-3-ols (*catechins*) found in strawberries and green and black teas, kaempferol from brussel sprouts and apples, and quercetin from beans, onions and apples, may have reduced risk of developing lung cancer (UCLA news May 2008). Flavonoids were found to be

strong topoisomerase inhibitors and induce DNA mutations in the MLL gene, which are common findings in neonatal acute leukemia (Thirman *et al*., 1993; Strick *et al*., 2000). The DNA changes were increased by treatment with flavonoids in cultured blood stem cells (Barjesteh *et al*., 2007). In one study, a high flavonoid-content diet in mothers seemed to increase risk of MLL+ acute myeloid leukemia in neonates. This result was not statistically significant though, and when the data on all types of Natural phenols (flavonoids in one set of experiments and delphinidin in another) were found to be strong topoisomerase inhibitors, similar to some chemotherapeutic anticancer drugs including etoposide and doxorubicin (Esselen *et al*., 2009; Bandele *et al*., 2008). This property may be responsible for both an anticarcinogenic-proapoptotic effect and a carcinogenic, DNA damaging potential of the substances.

Examples of flavonoids are quercetin and epicatechin**.**

Quercetin

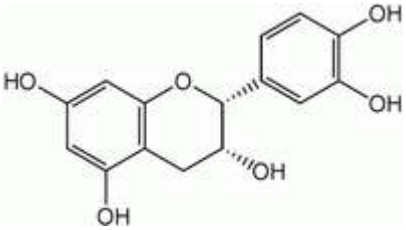
Quercetin, a flavonoid and more specifically a flavonol, is the aglycone form of other flavonoid glycosides, such as rutin and quercitrin, found in citrus fruit, buckwheat and onions. Quercetin forms the glycosides, quercitrin and rutin, together with rhamnose and rutinose, respectively. Although there is preliminary evidence that asthma, lung cancer and breast cancer are lower among people consuming higher dietary levels of quercetin, the U.S. Food and Drug Administration (FDA), EFSA and the American Cancer Society have concluded that no physiological role exists. The American Cancer Society states that dietary quercetin "is unlikely to cause any major problems or benefits (Paul *et al*., 2002).



Quercetin

Epicatechin

Epicatechin may improve blood flow and has potential for cardiac health. Cocoa, the major ingredient of dark chocolate, contains relatively high amounts of epicatechin and has been found to have nearly twice the antioxidant content of red wine and up to three times that of green tea in vitro (Lee, 2003). In the test outlined above, it appears the potential antioxidant effects in vivo are minimal as the antioxidants are rapidly excreted from the body.



Epicatechin (EC)

Good sources of flavonoids include all citrus fruits, berries, ginkgo biloba, onions (particularly redonion), parsley, pulses, tea (especially white and green tea), red wine, seabuckthorn, and dark chocolate (with a cocoa content of seventy percent or greater) (Tsushida and Suzuki,1996; Slimestad *et al.*, 2007; Marotti and Piccaglia, 2002; Justesen and Knuthsen 2001; Ewald *et al.*, 1999).

A variety of flavonoids are found in citrus fruits, including grapefruit. The citrus bioflavonoids include hesperidin (a glycoside of the flavanone hesperetin), quercitrin, rutin (two glycosides of the flavonol quercetin), and the flavone tangeritin. In addition to possessing *in vitro* antioxidant activity and an ability to increase intracellular levels of vitamin C, rutin and hesperidin may have beneficial effects on capillary permeability and blood flow. They also exhibit anti-allergy and anti-inflammatory benefits of quercetin from *in vitro* studies. Quercetin can also inhibit reverse transcriptase, part of the replication process of retroviruses (Spedding *et al.*, 1989). The therapeutic relevance of this inhibition has not been established.

Other dietary sources of flavonoids include tea, wine, dark chocolate. Flavonoids exist naturally in cacao, but because they can be bitter, they are often removed from chocolate, even dark chocolate (Lancet, 2007). Although flavonoids are present in milk chocolate, milk may interfere with their absorption (Serafini *et al*., 2003).

Flavonoids have been referred to as nature's modifier because of the strong experimental evidence of their inherent ability to modify the body's reaction to allergies, virus and carcinogens.

They show anti-allergic, anti-inflammatory, and anti-cancer activity. Flavonoid also binds to adhesins (Perrett *et al.,* 1995)

2.2.6 Anthraquinone

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

Industrially, 9,10-Anthraquinone is obtained by the oxidation of anthracene, a reaction that is localized at the central ring. It is also prepared by the Friedel-Crafts reaction of benzene and phthalic anhydride in presence of $AlCl_3$. The resulting O-benzoylbenzoic acid then undergoes cyclization, forming anthraquinone. This reaction is useful for producing substituted anthraquinones. The Diels-Alder reaction of naphthoquinone and butadiene followed by oxidative dehydrogenation will also produce 9,10-anthraquinone. Lastly, BASF has developed a process that proceeds via the acid-catalyzed dimerization of styrene to give a 1,3-diphenylbutene, which then can be transformed to the anthaquinone (Vogel, 2005). It also arises via the Rickert-Alder reaction, a retro-Diels-Alder reaction.

In a classic organic reaction called the Bally-Scholl synthesis, named after Oscar Bally and Roland Scholl (1905) , anthraquinone condenses with glycerol forming benzanthrone (Macleod and Allen, 1934). In this reaction, the quinone is first reduced with copper metal in sulfuric acid (converting one ketone group into a methylene group) after which the glycerol is added. Synthetic dyes are often derived from 9,10-anthraquinone, such as alizarin (Bien *et al.*, 2005). Important derivatives are 1-nitroanthraquinone, anthraquinone-1-sulfonic acid, and the dinitroanthraquinone (Vogel, 2005). Natural pigments that are derivatives of anthraquinone are found, inter alia, in aloe latex, senna, rhubarb, and Cascara buckthorn),fungi, lichens, and some insects.

9,10-Anthraquinone is used as a digester additive in production of paper pulp by alkaline processes, like the Kraft, the alkaline sulfite or the Soda-AQ processes. The anthraquinone is a redox catalyst. The reaction mechanism may involve single electron transfer (SET) (Samp, 2008). The anthraquinone is oxidizing cellulose and thereby protecting it from alkaline degradation (peeling). The anthraquinone is reduced to 9,10-dihydroxyanthracene which then can react with lignin. The lignin is degraded and becomes more water soluble and thereby more easy to wash away from the pulp, while the anthraquinone is regenerated. This process gives an increase in yield of pulp, typically 1-3% and a reduction in kappa number (Sturgeoff and Pitl, 1997). Sodium 2-anthraquinonesulfonate (AMS) is a water soluble anthraquinone derivative that was the first anthraquinone derivative discovered to have a catalytic effect in the alkaline pulping processes (http://smartech.gatech.edu/bitstream/1853/673/1/3370_001_071978.pdf.)

A large industrial application of anthraquinones is for the production of hydrogen peroxide. 2-Ethyl-9,10-anthraquinone or a related alkyl derivatives is used, rather than anthraquinone itself (Goor *et al.*, 2007). Derivatives of 9,10-anthraquinone include many important drugs (collectively called **anthracenediones**). They include

- Laxatives such as dantron, emodin, and aloe emodin, and some of the senna glycosides
- Antimalarials such as rufigallol
- Anti-neoplastics used in the treatment of cancer, such as mitoxantrone pixantrone, and the anthracyclines.

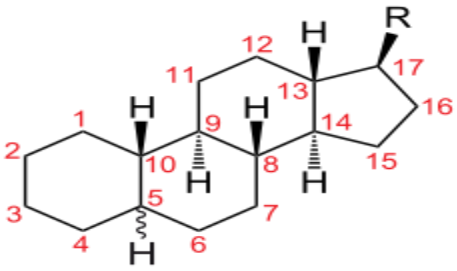
Anthraquinone is used as a bird repellant on seeds and as a gas generator in satellite balloons. Natural anthraquinone derivatives tend to have laxative effects. Prolonged use and abuse leads to melanosis coli (Müller-Lissner, 1993; Moriarty and Silk, 1988). Five anthraquinones have been shown to inhibit the formation of Tau aggregates and dissolve paired helical filaments thought to be critical to Alzheimer's disease progression in both mouse models and in vitro testing but have not been investigated as a therapeutic agent (Pickhardt *et al.*, 2005).

Several other isomers of anthraquinone are possible, including the 1,2-, 1,4-, and 2,6-anthraquinones. They are of comparatively minor importance. The term is also used in the more general sense of any compound that can be viewed as an anthraquinone with some hydrogen atoms replaced by other atoms or functional groups. These derivatives include substances that are

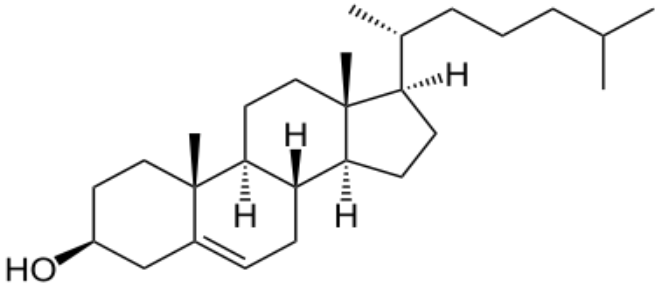
technically useful or play important roles in living beings. The enzyme encoded by the gene UGT1A8 has glucuronidase activity with many substrates including anthraquinones (Ritter *et al*., 1992).

2.2.7 Steroids

Steroids are a class of organic compounds with a chemical structure that contains the core of gonane or a skeleton derived therefrom. Usually, methyl groups are present at the carbons C-10 and C-13 – an alkyl side-chain at carbon C-17 may also be present.

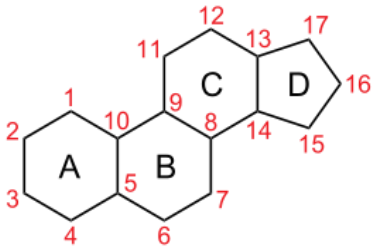


The basic skeleton of a steroid, with standard stereo orientation. R is a side-chain at C-17.

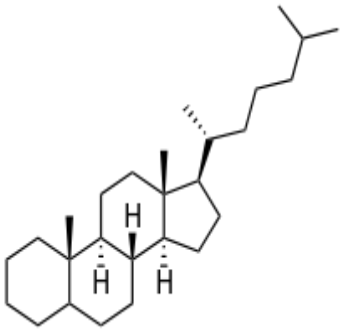


Cholesterol. This steroid is the precursor to other steroids in the steroidogenesis.

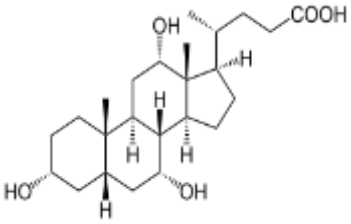
Gonane is the simplest possible steroid and is composed of seventeen carbon atoms, bonded together to form four fused rings. The three cyclohexane rings (designated as rings A, B, and C in the figure below) form the skeleton of phenanthrene; ring D has a cyclopentane structure. Hence, together they are called cyclopentaphenanthrene.



Numbering of rings and of carbon atoms in gonane, the simplest possible steroid.



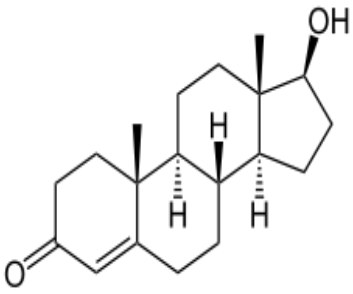
The structure of cholestane, one of the comparatively simpler steroids.



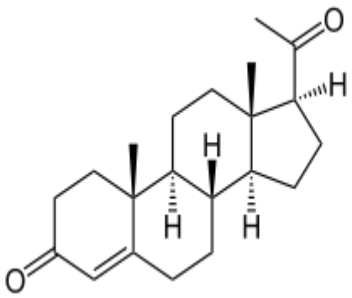
The complexer structure of cholic acid, a bile acid.

Commonly, steroids have a methyl group at the carbons C-10 and C-13 and an alkyl side chain at carbon C-17. Further, they vary by the configuration of the side chain, the number of additional methyl groups, and the functional groups attached to the rings. For example, sterols have a hydroxyl group attached at position C-3.

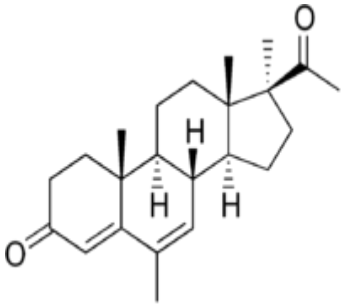
Some exemplary steroids with their structures:



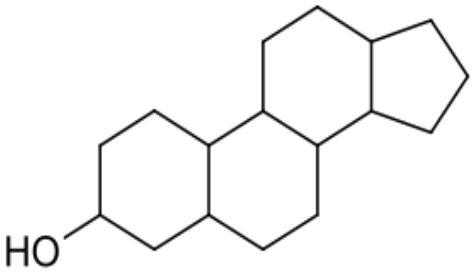
The anabolic steroid testosterone, the principal male sex hormone.



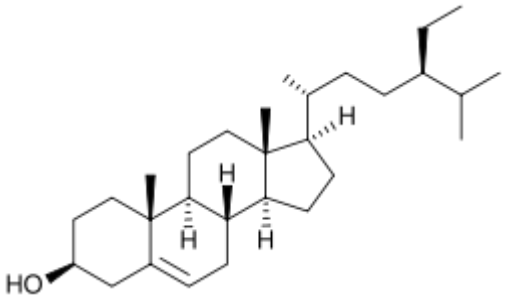
Progesterone, a steroid hormone involved in the female menstrual cycle, pregnancy and embryogenesis.



Medrogestone, a synthetic drug with similar effects as Progesterone.



An example of functional groups is the hydroxyl group at C-3 common to sterols.



β-Sitosterol, a phytosterol showing the hydroxyl group at C-3.

2.3 Description of *Parinari curatellifolia*

Parinari curatellifolia Planch. ex Benth. is a valuable and cherished medicinal plant in Yoruba and Igbo land (Southern part of Nigeria). The different parts of the plant are widely used by the traditional herbalist in the treatment of various diseases. In Igbo land especially in Eha-Amufu of Enugu State, *Parinari curatellifolia* as claimed by the traditional herbalist is used in the treatment of epiglottitis. It may be used alone or in combination with other herbs locally in the treatment of diabetes and other diseases and has been evaluated for its anti-diabetic activities (Ogbonnia *et al*., 2009).

Parinari curatellifolia is a spreading tree that is striking amongst surrounding vegetation because of its semi-circular, almost mushroom-shaped canopy depicting hues of blue-green and grey (figure 1b). It is an evergreen, medium to large tree, 10-13m high, although heights of 23-26m have been recorded in certain regions. The bark is rough and corky with yellow woolly hairs occasionally present in younger twigs and branches. Silica crystals in the wood are a common occurrence. The leaves are distinctly bicoloured, having a white-silver undersurface and a dark green-grey upper surface. These simple and alternate leaves are inwardly folded with a base and tip fairly rounded and with a herringbone venation pattern visible on both surfaces. Leaf shape is somewhat oblong having a square base with much tapering at the apex. Petioles are short with an entire leaf margin and velvety hairs covering the surfaces of younger leaves. Mature leaves appear to be darker and more rigid. Glands are usually present at the stalk of the leaf or the leaf base.

Fig 1a *Parinari curatellifolia* tree

Fig 1b *Parinari curatellifolia* stem

The sweetly scented inflorescences appear in shades of white, yellow or pink, and are covered in hairs. These small bell-shaped flowers are usually visible from July to November. The occurrence of terminal panicles is common in the axils, primarily in the smaller leaves. The fruit is a drupe, and is yellow-orange with grey speckles when ripe. The oval or rounded fruit which has a scaly texture may take up to a year to ripen and is found at twig ends. These plum-like fruits are about 50 mm long with a yellow edible flesh. The fruits taste pleasant when completely ripe and tend to ripen towards the end of the year (Lely, 1989). Within the fruit is the seed which is encapsulated by means of a lid or operculum. As the operculum ages, it allows moisture to enter, hence prompting seed germination. This process may last up to 2 years. It has been recorded that the mobola-plum tree occasionally produces a rather disagreeable smell, the reason for this is uncertain.

2.4 Scientific Classification of *Parinari curatellifolia*

Kingdom: Plantae

Division: Magnoliophyta

Class: Magnoliopsida

Order: Malpighiales

Family: Chrysobalanaceae

Genus: *Parinari*

Specie: *curatellifolia* Planch. ex Benth.

2.5 Distribution of *Parinari curatellifolia*

Parinari curatellifolia is an evergreen tropical tree of Africa, found in various kinds of deciduous wood land most frequently in poorly drained areas and inland at moderate altitudes. The tasty nature of the fruit causes it to be spared when woodland is cleared for cultivation (Coates, 1997).

It grows in the Guinea Savanna region of West Africa from Senegal across to Chad and then in seasonal woodland across the Equator through Kenya and Eastern side of the Continent in deciduous Miombo woodland inland to Zambia and Zimbabwe. Its Southernmost is just the tropics in the South Africa Lowveld, about 25^{o}S. In Nigeria, the plant can be found in the Eastern and Western part of the country. It is also found in some parts of Northern Nigeria.

2.6 Uses of *Parinari curatellifolia*

Parinari curatellifolia is a traditional food plant in Africa, the fruit of this plant has potential to improve nutrition, boost food security, and foster rural development and support sustainable land care (National Research Council, 2008). The wood is very hard and makes a good charcoal. The fruit appears early in the dry season and can be harvested over 3 or more months. It is used as snack and has very high oil content. The crush pulp of the fruit is an ingredient in drinks and since it ferments well, is often used to make alcoholic drinks as well (Storrs, 1979).

CHAPTER THREE
MATERIALS AND METHODS

3.1 Equipments

The following equipment were used;

Electronic Incubator – IT 9052 (Techmel and Techmel), Refrigerator – HR-135A (Haier Thermocool), Water Bath – TT42D (Techmel and Techmel), Microscope – CX21 (Olympus) Electronic Oven, Autocalve, Bunsen Burner, Inoculating Loop, Disposable Petri-dishes, Electronic Weighing Balance, Beakers, Conical Flasks, Anaerobic Jar, Glass Rod, Cork Borer, Filter Paper, Spatula, Soxhlet Extractor, Glass Chromatographic Tank, Spotters made from Microcapillary Tubes, Milling Maching, Biju Bottles, Test Tubes.

3.2 Reagents

The reagents used were of analytical grade.

3.3 Identification of the Plant Material

The plant was collected from Isu village in Eha-Amufu of Enugu State, Nigeria. It was identified and authenticated by a staff of Botany Department, University of Nigeria Nsukka. The voucher specimen was deposited at the biological science department of Modibbo Adama University of Technology, Yola.

3.4 Source of the Test Organism

The clinical isolates of *Streptococcus sp, Staphylococcus aureus, Pseudomonas sp, and Klebsiella sp* were obtained from Federal Medical Centre Yola, Nigeria. Each test bacterial strain was re-identified using standard bacteriological and biochemical methods (Cheesbrough, 1984). Stock cultures were maintained in nutrient agar slants at 4°C.

3.5 Re-identification of the Clinical Isolates

The clinical isolates were re-identified using the following methods as described by Aneja, 2007; nature of growth in agar medium, gram staining, catalase test, oxidase test, Taxos A (bacitracin sensitivity testing).

Gram Staining

Thin smears of about 18 hours old cultures of the clinical isolates were made on clean glass slides and allowed to air dry. The smears were heat fixed using Bunsen burner and placed on the slide tray. Each smear was covered with crystal violet for 30 seconds and washed with distilled water for few seconds using wash bottle.

Each washed smear was covered with Gram's iodine solution for 60 seconds and washed off with 95 percent ethyl alcohol. The slides were then washed with distilled water and drained. Safranin (counter stain) was applied to each of the smears for 30 seconds. They were washed with distilled water and blot dried with absorbent paper.

Finally, the stained slides were viewed microscopically using oil-immersion objective.

Catalase Test

A colony of each isolate was touched with a capillary tube dipped into 3%H_2O_2. The tube was observed for bubbles indicating a positive reaction. *Staphylococcus spp* are catalase positive while *Streptococcus spp* are catalase negative.

Oxidase Test

Streaks of the bacteria isolates were made on Whatman filter paper. Few drops of oxidate reagent were added to the streaked bacteria isolates. Positive result turned the bacteria purple-violet with 1 to 30 seconds.

Coagulase Test – slide test

This test was used to distinguish *Staphylococcus aureus* (coagulase positive) from other *Staphylococcus sp* (coagulase negative). The slide test was preformed by preparing a suspension of bacterial cells mixed into a drop of plasma on a microscope slide. Positive result caused the plasma to clump.

Taxos A (bacitracin sensitivity testing)

This is a differential test used to distinguish between the β-hemolytic streptococci: *Streptococcus pyogenes* (bacitracin sensitive) and other *Streptococcus sp* (bacitracin resistant). A sample of the isolate; *Streptococcus pyogenes* was streaked on a chocolate agar plate and the antibiotic disk, bacitracin was aseptically placed on it using a flamed needle. It was incubated at 37^{o}C for 24 hours. Large zone of inhibition surrounding the disk indicated a positive result.

3.6 Brief description of the isolates used

Staphylococcus aureus is a gram positve coccus. It shows abundant, opaque, golden growth in agar slant. It is catalase positive and oxidase negative. *Klebsiella sp* is gram negative rod. It shows slimy, white, somewhat translucent, raised growth on slant agar. It is catalase positive and oxidase negative. *Pseudomonas sp* is gram negative rod. It shows abundant, thin, white medium turns green. It is catalase positive and oxidase positive. *Streptococcus pyogenes* is gram negative coccus occurring in chains. It is catalase negative and bacitracin sensitive.

3.7 Preparation of the plant extract

The acetic acid, ethyl acetate and methanolic extract of the leaf of the plant were prepared. The plant sample collected was air dried and ground using a milling machine. The powdered material was transferred into a Soxhlet apparatus and extracted in the Soxhlet extractor using hexane, ethyl acetate methanol, acetic acid, and water separately for 24hrs each (Harborne, 1973; Sofowora, 1982). The extracts were concentrated to dryness and the residues obtained. The residues were transferred into pre-weighed sample containers, and stored at 4^{o}C until when required for use.

3.8 Phytochemical Screening

The leaf extract of *P. curatellifolia* was analyzed for the presence of alkaloid, saponin, anthraquinone, steroids, tannin, flavonoid, reducing sugars and cardiac glycosides (Odebiyi and Sofowora, 1978; Sofowora, 1982; Harborne, 1973; Onwukeame *et al*., 2007).

3.8.1 Screening for alkaloids

Three grams of the leaf extract was stirred with ethanol containing 3% tartaric acid. The filtrate was shared into 3 beakers and tested for alkaloids as follows: into the first beaker, Hagar's reagent (appendix III) was added, into the second beaker, Mayer's reagent (appendix III) was added and into the last beaker, Marquin's reagent (appendix III) was added. Precipitation in any of the 3 test indicated the presence of alkaloids.

3.8.2 Screening for saponin

About 0.5 g of the plant extract was shaken with water in a test tube. Frothing, which persist on warming was taking as a preliminary evidence for the presence of saponin. Few drops of olive oil was added to 0.5 g of the extract and vigorously shaken. Formation of soluble emulsion in the extract indicated the presence of Saponin (Odebiyi and Sofowora, 1978; Ngbede *et al*., 2008).

3.8.3 Screening for tannin

About 0.5g of the extract was added to 10ml of freshly prepared potassium hydroxide (KOH) in a beaker and shaken to dissolve. A dirty precipitate was observed indicating the presence of tannin.

3.8.4 Screening for steroids (Salkowski's test)

About 100 mg of *P. curatellifolia* leaf extract was dissolved in 2ml of chloroform. Sulphuric acid was carefully added to form a lower layer. A reddish brown color at the interface was an indicative of the presence of steroidal ring (Sofowora, 1982).

3.8.5 Screening for flavonoid

About 2g of the powdered leaf was dissolved in acetone. The residue was extracted in warm water after evaporating the acetone in a water bath. The mixture was filtered while, still hot. The filtrate was cooled and used. About 5ml of 20% NaOH was added to equal volume of the detained water extract. A yellow solution indicated the presence of flavonoid.

3.8.6 Screening for anthraquinone (Borntrager's test)

About 0.5 g of the extract was taken into a dry test tube and 5ml of chloroform added and shaken for 5 min. The extract was filtered and the filtrate was shaken with equal volume of 10% ammonia solution. A pink violet or red color in the ammonical layer (lower layer) indicated the presence of anthraquinone.

3.8.7 Screening for cardiac glycosides (Keller Killiani's test)

About 100mg of extract was dissolved in 1ml of glacial acetic acid containing one drop of ferric chloride solution. This was under layered with 1ml of concentrated Sulphuric acid. A brown ring obtained at the interface indicated the presence of a de-oxy sugar characteristic of cardenolides.

3.9 Determination of Viable Cell

The viable cells were determined as described by Kanika, 2009. Serial dilutions of 24hour broth cultures were prepared. Dilution blanks in test tubes were labeled as follows; 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}. One millilitre of the sample was added into a 9ml dilution blank labeled 10^{-1} thus diluting the original sample 10 times. The contents were mixed by rolling the tube back and forth between the hands to obtain a uniform distribution of the organism (cells). From the first dilution, 1ml of the suspension was transferred while in motion to the dilution blank 10^{-2} with a sterile and fresh pipette diluting the original specimen/suspension 100 times. The procedure was repeated until the original sample was diluted 10,000,000 times. One millilitre of the suspension was inoculated into nutrient agar plates labeled according to the dilutions used. They were incubated in an inverted position at 37ºC for 24hrs. After the incubation, plates with number of colonies ranging from 30-300 were counted. Plates with spreaders were discarded.

Numbers of viable cells were calculated as follows;

$$\text{No of cells/ml} = {}^{\text{No of colonies}}/_{\text{Volume of sample}} \text{ x dilution factor}$$

3.10 Determination of Antimicrobial Activity of the Extract

The test for sensitivity of each organism was done by the disc diffusion technique (Cheesebrough, 2000). A number of sterile paper discs (diameter 5mm) were mixed completely with a reconstituted extract in a sterile 10ml glass beaker. The discs were allowed to remain in contact with the extract for at least an hour to enable them absorbs the extracts which became embedded in the discs. The discs were brought out and allowed to air dry. As control, a number of sterile paper discs (diameter 5mm) were also mixed in the solvents without the extract. Each organism was cultured by the spread plate technique (Pelczars and Chan, 1977). The test organisms were adjusted to 11 x 10^7cells/ml. They were spread evenly on the surface of the agar medium with a sterile glass hockey. Then using a flame needle, the prepared sensitivity test discs were carefully picked and placed on top of the inoculated plate at some distance from one another. The plates were allowed to stand for about 5 minutes and incubated at 37^{o}C in an electronic incubator. They were observed for 24hours for growth and possible clear zone around the disc as a mark of sensitivity to the test extract. The antimicrobial activity of the partially fractionated extract was also determined using disc diffusion method.

3.11 Determination of the Minimum Inhibitory Concentration (MIC) of the Extract

The minimum inhibitory concentration was determined as the least concentration of the extract which inhibits each test organism. Five hundred milligrams of the extract was separately reconstituted in sterile distilled water and diluted to concentrations of 50, 5, 0.5, 0.05, 0.005 mg/ml. The serially diluted extracts were used for the sensitivity test. After incubation, the plates were observed for inhibition zones. The list concentration which caused inhibition was taken to be the minimum inhibition concentration.

The efficacy of the plant extract was compared to that of cephalosporin (cefixime), leofloxacin, augumentine, oflaxicin, nitrofurantoin, cefuroxime, gentamicin, cefazidime. Some of these antibiotics are utilized in the management and treatment of the disease in the hospital.

3.12 Fractionation of the Extract of the Leaf of *Parinari curatellifolia*

The extract of the leaf of *Parinari curatellifolia* was fractionated using the Column and the Thin-Layer Chromatographic Techniques for possible identification of the active component responsible for the observed activity. For column chromatography, a piece of cotton wool was inserted into a column of length 50cm and diameter 1cm. The column was about two-third filled with Silica gel (grit size 200–300 mesh). The packed column was clamped onto a retort stand. About 30ml of solvent (ethyl acetate) was added into the column and gently tapped with a cork ring to ensure proper packing of the column. Dry sample of the extract was slowly poured on top of the column and continuously eluted with 40ml each of different solvents starting from the least polar to the most polar, in the following order; ethyl acetate, methanol, acetic acid, and water. The fractions obtained (TiA, TiB, TiC, TiD) were evaporated using a rotary vapor system. The residue obtained was investigated for its antimicrobial activity.

Analytic TLC was done with silica gel glass plates (5 × 15cm) as stationary phase and a mixture of acetic acid, ethyl acetate, and hexane (5:3:2) as solvents for the mobile phase. The glass plates were coated with the silica gel and allowed to air dry. The coated glass plates were activated by heating in an oven at 110^{o}C for 30 minutes. A thin pencil line mark was made on the plate at about 1cm from one end of the plate known as point of application. A small amount of the extract dissolved in the extracting solvent was spotted on the line using a spotter made from micro-capillary tube. The spot was allowed to air dry. The plate was put into a thin-layer chromatographic tank saturated with the solvent vapor. The plates were allowed to remain in the tank until the solvent is few centimeters away from the top of the plates. The distance moved by the samples and the solvents were noted. Pigments visible at daylight were documented by photographing.

3.13 Statistical Analysis

The results were subjected to a statistical software SPSS (version 15.0) for analysis. Results were expressed as Mean ± SEM. Significant differences were determined using the student's t-test. Differences were considered significant if $p<0.05$.

3.14 Ethical Consent

All experiments were examined and approved by the appropriate ethics committee and were performed in accordance with the ethical standards laid down in the 1964 declaration of Helsinki.

CHAPTER FOUR

RESULTS

Physical examination of the various leaf extracts of *Parinari curatellifolia* showed that the aqueous extract is a brownish-solid; methanol and ethyl acetate extract are greenish-black solid, while the acetic acid extract is a brownish-black solid. Out of 50g of the powdered leaf of *Parinari curatellifolia*, the percentage extracts recovered were as follows; water extract 5.6%, methanol extract 12.14%, ethyl acetate 18.62%, and acetic acid 21.12% (table 1)

The Phytochemical screening of the plant revealed the presence of saponin, alkaloids, flavonoids, steroids, tannins, cardiac glycosides (Table 2).

The antimicrobial activity of the various extracts revealed that there are zones of inhibition on the bacteria culture media (appendix 1), thus signifying the presence of antimicrobial activity of the extract against the microbial isolate used. The acetic acid extract gave inhibition zones ranging from 20.0-28.7mm that of ethyl acetate gave inhibition zones ranging from 14.7-15.3mm. The methanol extract gave an inhibition zones ranging from 0.9-13.0mm, while that of water ranges from 11.7-13.0mm (Table 3).

The Minimum Inhibitory Concentration(MIC) of the acetic acid extract which gave the highest zones of inhibition against the microbial isolates used revealed that the MIC for *Streptococcus pyogenes* and *Pseudomonas sp* is 5mg/ml while that for *Klebsiella sp* and *Staphylococcus aureus* is 50mg/ml (Table 4).

The efficacy of this plant extract was compared to some standard antibiotics. Among the antibiotics used, leofloxacin gave the highest inhibition zone of 29.3mm against *Pseudomonas spp,* while the acetic acid extract (500mg/ml) gave 28.7mm against the same organism. There is no significant difference between the two inhibition zones ($P<0.05$). However, other antibiotics as well as other solvent extracts gave some inhibition zones. Cefazidime gave the least inhibition zone of 10.3mm against *Pseudomonas sp* but showed no inhibition zone on the other isolates used. (Table 5 and 3)

Acetic acid extract which gave the highest zone of inhibition was fractionated using column and thin layer chromatography. Four fractions were obtained from the column (TiA, TiB, TiC, and TiD). With reference to susceptibility of the test strains, TiC recorded the highest activity against *Klebsiella spp* showing an inhibition zone of 17.0mm. However, the zone of inhibition of this

fraction was less than that observed with the crude extract (Table 6). On TLC, the fraction gave three bands with the following Rf values 0.38, 0.36, 0.23 (plate 1).

Table 1: Percentage recovery of extracts from 50g of *Parinari curatellifolia* leaf.

Extracting solvent	Amount of extract (g)	Percentage recovery (%)
Water	2.80	5.60
Methanol	6.07	12.14
Ethyl acetate	9.31	18.62
Acetic acid	10.56	21.12

Table 2. Results of the Phytochemical Screening of Different Leaf Extracts of *P.curatellifolia*

Extracting solvent	SAP	CG	ALK	FL	ST	TA	AQ
Water	+	+	+	+	+	+	-
Methanol	+	+	+	+	+	+	-
Ethyl acetate	+	+	+	+	+	+	-
Acetic acid	+	+	+	+	+	+	-

Note: + = present - = absent, SAP = Saponin, CG = Cardiac glycosides, ALK = Alkaloids, FL = Flavonoids, ST = Steroids, TA = Tannin, AQ = Anthraquinone.

Table 3. Results of Antibacterial Activity of Different Leaf Extracts of *P. curatellifolia.*

Microorganisms	Extracting solvent and inhibition zones (mm)			
	Water (mm)	Methanol (mm)	Ethyl acetate (mm)	Acetic acid (mm)
Streptococcus pyogenes	11.7 ± 1.2	09.0 ± 0.6	14.7 ± 0.9	20.0 ± 0.6
Staphylococcus aureus	13.7 ± 1.3	10.0 ± 0.6	16.3 ± 0.9	28.3 ± 0.3
Klebsiella sp	12.3 ± 1.5	12.0 ± 0.6	14.7 ± 2.3	22.3 ± 0.3
Pseudomonas sp	13.0 ± 1.2	13.0 ± 0.6	15.3 ± 1.8	28.3 ± 0.3

Results are express as mean ± SEM

Table 4: Minimum inhibitory concentration of the acetic acid extract

Microorganism	Extract concentration (mg/ml)				
	50mg/ml	5mg/ml	0.5mg/ml	0.05mg/ml	0.005mg/ml
Streptococcus pyogenes	-	-	+	+	+
Staphylococcus aureus	-	+	+	+	+
Klebsiella sp	-	+	+	+	+
Pseudomonas sp	-	-	+	+	+

Note: – indicates no growth, + indicates growth

Table 5. Results of antibacterial activities of some standard antibiotics used in treatment and management of epiglottitis.

Microorganisms	Standard Antibiotics and Inhibition Zones (mm)							
	AUG	GEN	LEO	CXM	NIT	OFL	CRX	CAZ
Streptococcus pyogenes	6.0±0.6	13.6±1.6	29.0±0.3	11.3±0.9	8.0±0.6	14.0±0.6	-	-
Staphylococcus aureus	-	16.3±0.9	25.0±0.6	-	22.3±0.9	-	-	-
Pseudomonas sp	-	14.7±0.3	29.3±0.9	24.0±0.6	15.7±1.2	24.7±0.9	-	10.3±0.9
Klebsiella sp.	-	13.7±0.9	29.3±0.3	-	22.3±0.9	-	-	-

Results are express as mean ± SEM

AUG=Augumentin, GEN=Gentamicin, LEO=Leofloxacin, CXM=Cefixime, NIT=Nitrofurantoin, CRX=Cefuroxime, CAZ=Cefazidime, OFL=Oflaxicin

- Indicates no inhibition zone

Table 6. Results of Antibacterial Activities of the Different Fractions of Acetic Acid Extract of the leaf of *P.curatellifolia*.

Microorganisms	Different Fractions and inhibition Zones (mm)			
	TiA (mm)	TiB (mm)	TiC (mm)	TiD (mm)
Streptococcus pyogenes	06 ± 0.6	0	10 ± 0.6	10 ± 0.6
Staphylococcus aureus	06 ± 0.6	0	15 ± 0.6	15 ± 0.5
Pseudomonas sp	08 ± 0.6	0	10.7 ± 0.9	0
Klebsiella sp.	07 ± 0.6	0	17.3 ± 0.9	10.7±0.3

Results are express as mean ± SEM

TiA = first fraction (ethyl acetate fraction), TiB = second fraction (methanol fraction), TiC = third fraction (acetic acid fraction), TiD = fourth fraction (water fraction)

Plate I: TLC of the Fraction (TiC) of the Leaf Extract of *Parinari curatellifolia*

CHAPTER FIVE

DISCUSSION

Since ancient times, herbs were the basis for nearly all medicinal therapies until synthetic drugs were developed. In recent times, herbal medicines have received greater attention leading to subsequent increase in their demand (Sushruta *et al*., 2006). *Parinari curatellifolia* is a valuable and cherished medicinal plant in which different parts of the plant are widely utilized in the treatment of many diseased conditions (Ogbonnia *et al*., 2009).

In the present study, the phytochemical constituents as well as the antimicrobial activity of the leaf extract of *Parinari curatellifolia* were evaluated. The efficacy of the crude extract was compared to some standard antibiotics, some of which are been utilized in the treatment and management of epiglottitis. The crude extract was also partially fractionated in an attempt to identify the active component responsible for its activity. The Phytochemical screening of the plant revealed the presence of saponin, alkaloids, flavonoids, steroids, tannins, and cardiac glycosides.

Tannin has received a great deal of attention in recent years, since it was suggested that the consumption of tannin-containing beverages, especially green teas and red wines, can cure or prevent a variety of ills (Serafini *et al*., 1994). Many human physiological activities, such as stimulation of phagocytic cells, host-mediated tumor activity, and a wide range of anti-infective actions, have been assigned to tannins (Haslam, 1996). The observed activity of the leaf extract of *Parinari curatellifolia* could be attributed partly to the presence of tannin in the leaf. Tannin is found to complex with proteins through so-called nonspecific forces such as hydrogen bonding and hydrophobic effects, as well as by covalent bond formation (Haslam, 1996; Stern *et al*., 1996). Thus, the mode of antimicrobial action of tannins may be related to their ability to inactivate microbial adhesins, enzymes, cell envelope transport proteins, and metal ion complexation etc. They also complex with polysaccharide (Ya *et al*., 1988). There is also evidence that tannins directly inactivate microorganisms (Brownlee *et al*., 1990).

The antimicrobial activity observed in this plant leaf extract may also be partly due to the presence of alkaloids in the leaf. Alkaloids are heterocyclic nitrogen containing compounds. Alkaloids are found to intercalate into cell wall and/or DNA and therefore disrupt the activities of microorganisms (Phillipson and Neill, 1987).

The antimicrobial activity observed in this plant could also be partly attributed to the presence of saponins in the leaf. Saponins are a class of chemical compounds, one of many

secondary metabolites found in natural sources, with saponins found in particular abundance in various plant species. More specifically, they are amphipathic glycosides grouped, in terms of phenomenology, by the soap-like foaming they produce when shaken in aqueous solutions, and, in terms of structure, by their composition of one or more hydrophilic glycoside moieties combined with a lipophilic triterpene derivative (Hostettmann and Marston,1995).

Saponins are found to complex with cholesterol to form pores in cell membrane bilayers. This complexation leads to cell lysis (Francis *et al*., 2002). In addition, the amphipathic nature of saponins make them act as surfactants that can be used to enhance penetration of macromolecules such as proteins through cell membranes (Zablotowicz *et al*., 1996).

Flavonoids are also hydroxylated phenolic substances but occur as a C_6-C_3 unit linked to an aromatic ring. Since they are known to be synthesized by plants in response to microbial infection (Dixon, 1983), it should not be surprising that the activity observed in this plant may be partly due to its presence in the leaf of this plant. Flavonoids have been found *in vitro* to be effective antimicrobial substances against a wide array of microorganisms. Their activity is probably due to their ability to complex with extracellular and soluble proteins and to complex with bacterial cell walls. More lipophilic flavonoids may also disrupt microbial membranes (Tsuchiya *et al*., 1996).

Steroids are a type of organic compound that contains a characteristic arrangement of four cycoalkane rings that are joined to each other. Steroids vary by the functional groups attached to the four ring core and by the oxidation state of the rings. Sterols are special forms of steroids with hydroxyl group at position 3 and a skeleton derived from cholestane (Moss, 1989). The activity of this plant extract may be as a result of the presence of steroids. Steroids have been reported to have antibacterial properties, the correlation between membrane lipids sensitivity for steroidal compound indicates the mechanism in which steroids specifically associate with membrane lipids and exerts its action causing leakages from liposomes (Raquel, 2007). The presence of all these secondary metabolites with different mechanism of antimicrobial activities makes this plant a potential source of antibiotics.

The difference in the zones of inhibition (Table 2) is an indication of the differences in the solubility of the active components of the leaf of this plant in different solvents. The result implies that the active components are more soluble in acetic acid compared to ethyl acetate, methanol, and water, thus making acetic acid to extract more of the active components compared to the other solvents used in this research. The antimicrobial activities observed in the leaf of this plant may be attributed to the presence of some secondary metabolites in the leaf extract.

The low Minimum Inhibitory Concentration (MIC) of the acetic acid extract observed against the isolates used in this research is an indication of the efficacy of the plant leaf extract against these bacteria.

The efficacy of this plant extract (500mg) was compared to that of some standard antibiotics; gentamicin (10µg), leofloxacin (5µg), nitrofurantoin (300µig), cefixime (5µg), ofloxacin (5µg), augumentine (30µg), cefuroxime (30µg), cefazidime. Some of these antibiotics are used in the treatment and management of epiglottitis. The leofloxacin gave the highest zone of inhibition of 29.3mm while the acetic acid extract gave 28.7mm against the targeted organisms (Table 3). There is no significant difference between the inhibition zones of the extract and leofloxacin ($p<0.05$). This shows that the leaf extract of this plant could be utilized in the treatment of epiglottitis, thus revealing why the traditional healers use this plant leaf to treat epiglottitis.

Fractionation of plant extract may result in improved activity or loss of activity (Nwodo *et al.*, 2010). Four fractions obtained on fractionation of the acetic acid extract using column chromatography (TiA, TiB, TiC, and TiD), were subjected to antimicrobial screening. Comparing the different fractions of the acetic acid extract and the crude acetic acid extract, the crude extract showed a higher activity against the tested strains. This is an indication that fractionation of this extract resulted in reduced activity. It is possible that the acetic acid extract of this plant contains some components which are soluble in other solvents used in the fractionation. This may have resulted in the separation of the active components. Therefore the various components of the crude acetic acid extract of the leaf of this plant (*Parinari curatellifolia*) might have acted synergistically to produce the observed effect of the crude extract. This could be attributable to the fact that combination of secondary metabolites enhances the activity of the combined agents and susceptible organisms (Marjorie, 1999). The efficacy of this extract against both gram positive and gram negative organism is an indication of broad spectrum activity of this extract. On TLC, the extracts gave three bands each with the following RF values 0.38, 0.36, 0.23 (appendix 3).

This plant studied may be seen as a potential source of useful drugs. This research, however, justified the use of this plant by the traditional healers in the treatment and management of epiglottitis. Further studies are going on to, investigate the toxicity of the acetic acid extract of this plant on rats, and to isolate, identify, characterize and elucidate the structure of the bioactive compounds that may be responsible for the observed activity.

CHAPTER SIX

SUMMARY, CONCLUSION AND RECOMMENDATION

6.1 Summary/Conclusion

It is obvious that the *in vitro* antimicrobial activity observed in this study may be attributed to any of the secondary metabolites found present in this plant. It is also apparent that the active component responsible for the activity is highly soluble in acetic acid than any other solvent. The actual component responsible for the observed activity, whether single or compound remains to be elucidated. The analytical TLC carried out with the acetic acid extract of the plant in this research is suggestive that there may be three main components in the leaf extract of this plant. However, the research justified the use of the leaf of *Parinari curatelliolia* by the traditional healers in the management and treatment of epiglottits.

6.2 Recommendations

The following recommendations are made;

1. Preparative TLC should be done and the various bands scrapped and tested for their antimicrobial activity
2. Further studies should be carried out to identify and elucidate the structure of the bioactive compounds that may be responsible for the observed activity.
3. The toxicity of the acetic acid extract of the leaf of the plant should be investigated.

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APPENDIX I

Plate II: Antibacterial Activity of Leaf Extracts of *P. curatellifolia* against *Staphylococcus aureus*

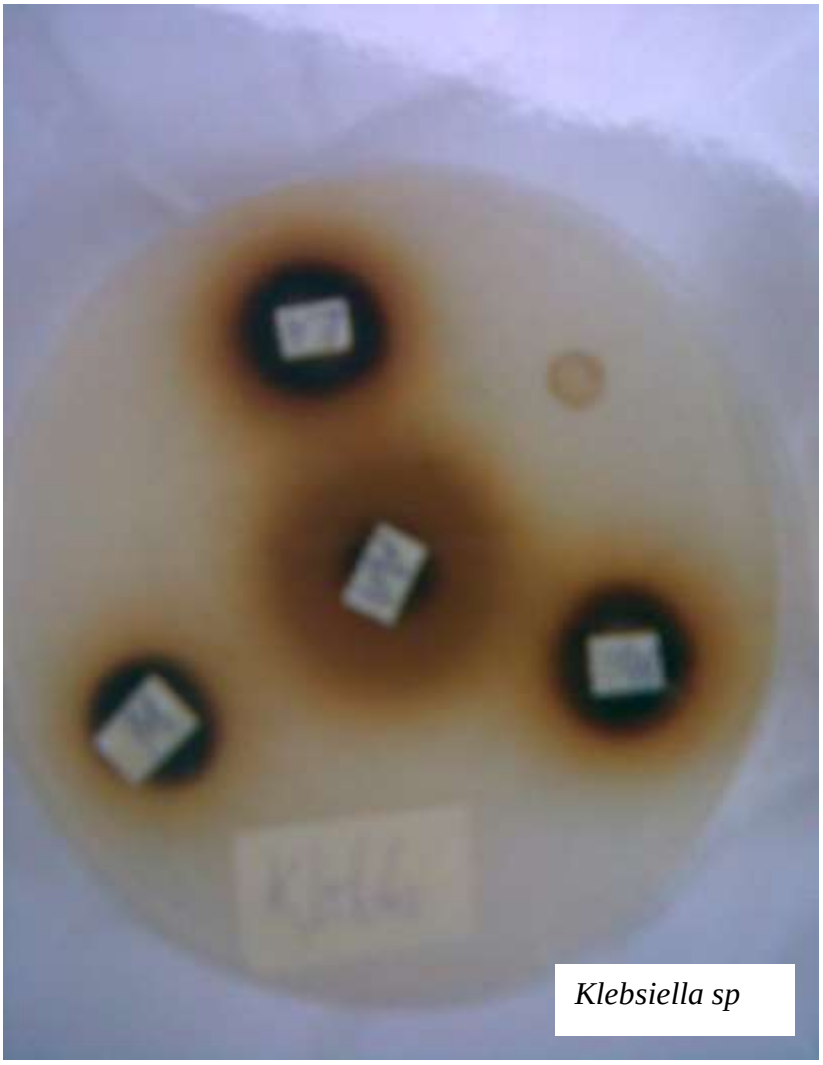

Plate III: Antibacterial Activity of Leaf Extracts of *P. curatellifolia* against *Klebsiella sp*

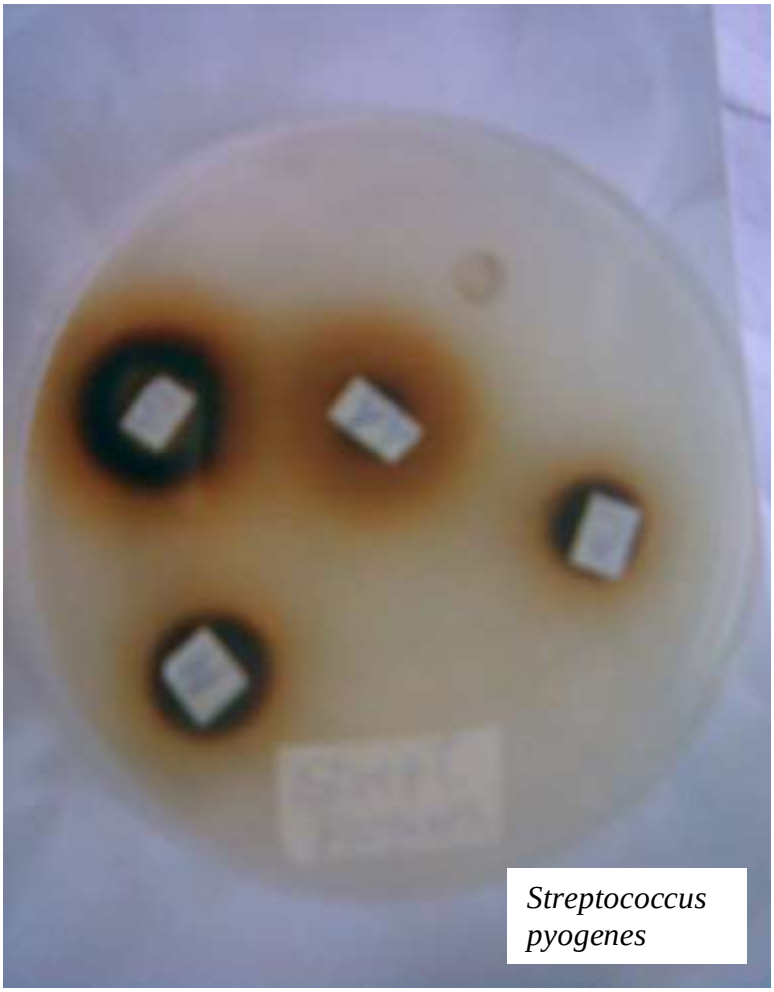

Plate IV: Antibacterial Activity of Leaf Extracts of *P. curatellifolia* against *Streptococcus pyogenes*

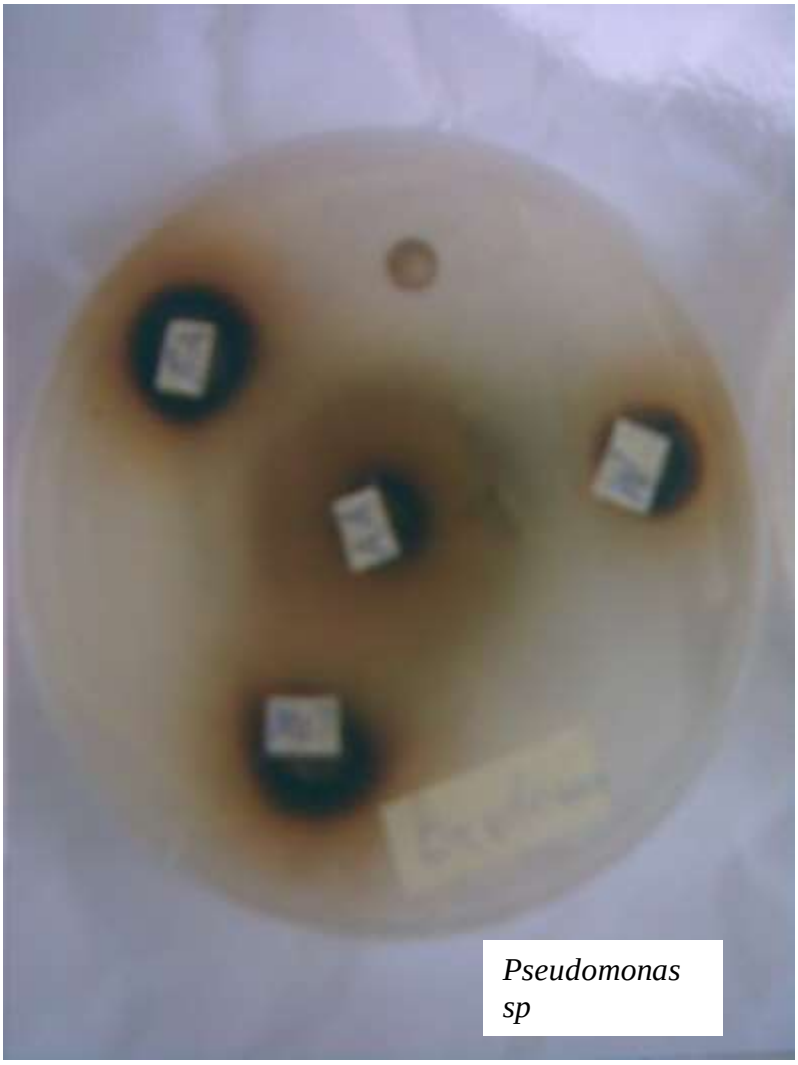

Plate V: Antibacterial Activity of Leaf Extracts of *P. curatellifolia* against *Pseudomonas sp*

APPENDIX II

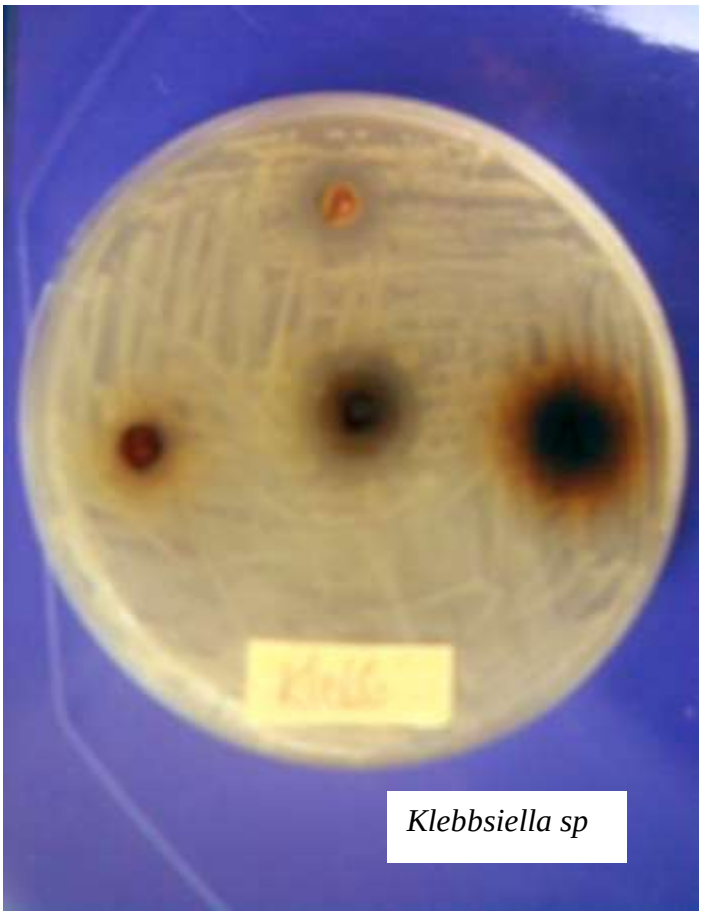

Plate VI: Antibacterial Activities of the Different Fractions of Acetic Acid Extracts of the Leaf of *P.curatellifolia* against *Klebbsiella sp*

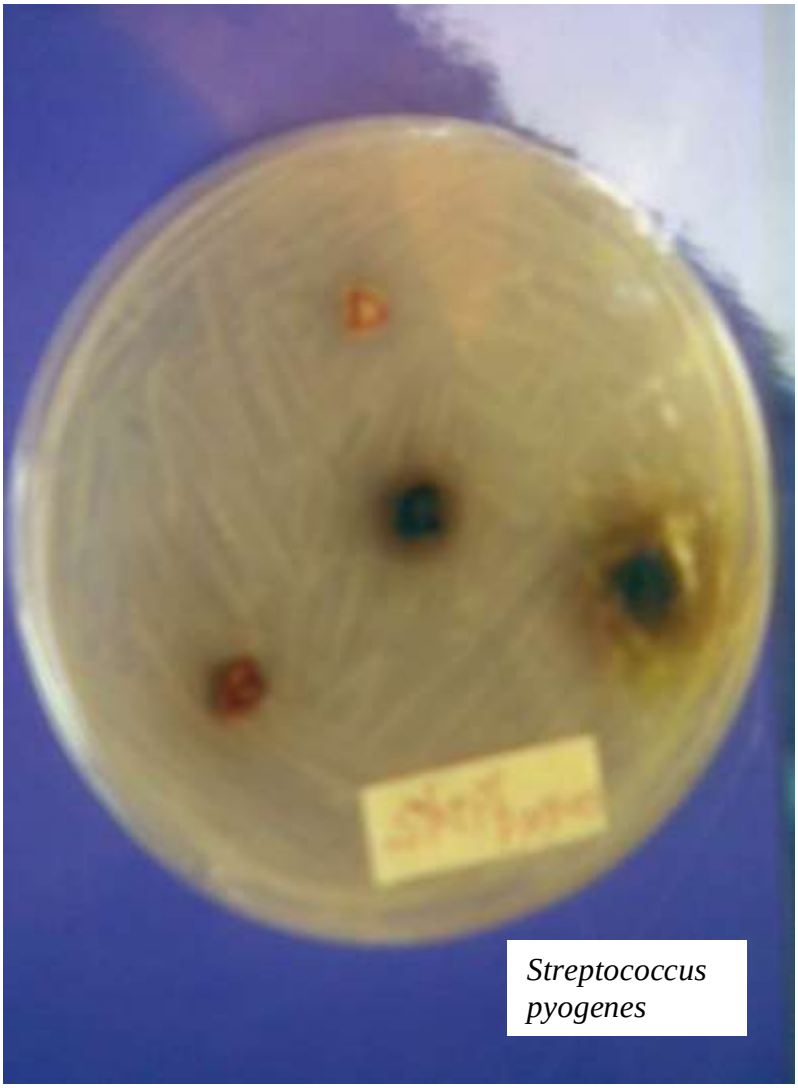

Plate VII: Antibacterial Activities of the Different Fractions of Acetic Acid Extracts of the Leaf of *P.curatellifolia* against *Streptococcus pyogenes*

Plate VIII: Antibacterial Activities of the Different Fractions of Acetic Acid Extracts of the Leaf of *P.curatellifolia* against *Pseudomonas sp*

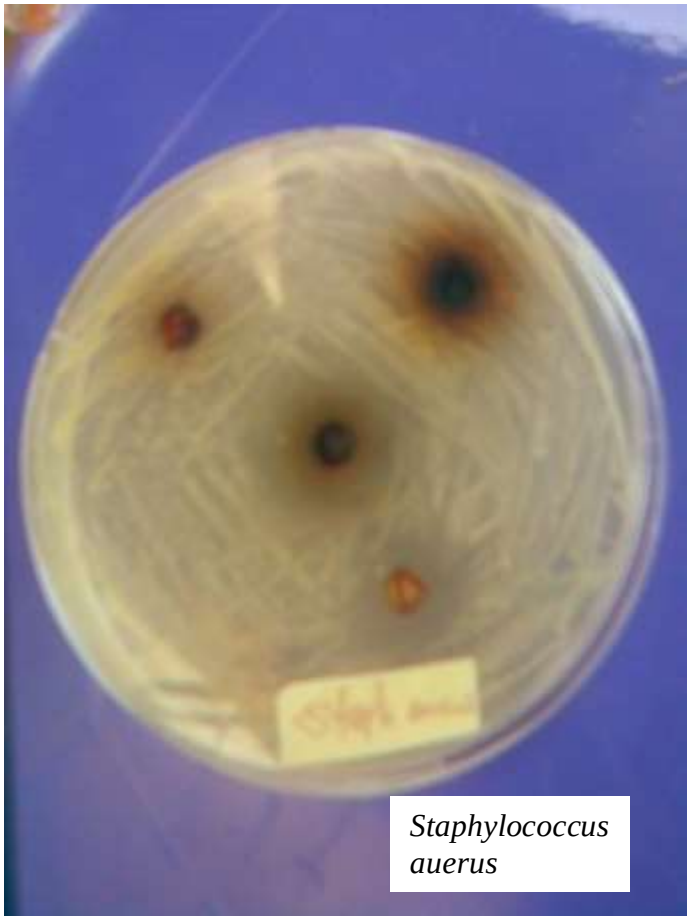

Plate IX: Antibacterial Activities of the Different Fractions of Acetic Acid Extracts of the leaf of *P.curatellifolia* against *Staphylococcus aureus*

APPPENDIX III

Reagent Preparations

Hager's reagent: Dissolve 1g of picric acid in 100ml of H_2O

Mayer's reagent: Dissolve 1.358g of $HgCl_2$ in 60ml of water and pour into a solution of 5g of KI in 10ml of H_2O. Add sufficient water to make 100 ml.

Marquis' reagent: Add 10ml of formaldehyde solution to 50ml of sulfuric acid.