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KUMASI

BIOACTIVITY GUIDED INVESTIGATION OF THE STEM BARK AND LEAVES OF *VOACANGA AFRICANA* (APOCYNACEA)

BY

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DECLARATION

I wish to declare that the work done in this thesis is my work. I further wish to declare that to the best of my knowledge, this thesis does not contain any material that has been previously published by anyone except where due acknowledgement has been made in the text.

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ABSTRACT

Plants since time immemorial have been used for their beneficial effects such as antimicrobial, antioxidant and bacterial resistance modulation. This project presents work on *Voacanga africana* (Apocynaceae) a local Ghanaian plant, in which the leaves and stem barks were screened for antioxidant and resistant modulatory properties. The antioxidant assay was determined using DPPH scavenging, total phenolic count and total antioxidant capacity. The antimicrobial and bacterial resistance modulation activity was determined using the broth dilution and agar well diffusion methods using *S. aureus, E. coli, S. typhi, P. auregenosa, B. subtilis and Candida albicans*. Further work was performed by looking at the phytochemical constituents of the extracts and isolates.

The antioxidant activity as seen from the DPPH assay was concentration dependent with the methanolic extract of the stem bark showing more activity than the leaf, with Ic_{50} of 70 and 1605 respectively, whilst that of the standard (ascorbic acid) was 6.980. Antioxidant activity was also found to be very closely related to the amounts of phenolic compounds which were present in both extract from the correlation plots obtained after the total phenolic and antioxidant assays.

The leaf extract also showed no antimicrobial activity whilst the stem bark extract showed very significant activity against the chosen microorganisms, with MIC's of 4mg/ml against a majority of the microorganisms. Also in this work, isolates were chromatographically obtained from the methanolic extracts and bulked into six fractions labelled VAMA01, VAMA02, VAMA03, VAMA04, VAMA05 and

VAMA06. Amongst the isolates, VAMA01 and VAMA02 showed significant activity when combined with most used antibiotics including amoxicillin. Most significant result was when 3mg/ml of the extract VAMA01 was combined with 4mg/ml of amoxicillin against *E. coli* when the individual MIC's were 4mg/ml and 32mg/ml respectively. Also combining 3mg/ml of VAMA02 with 8mg/ml of amoxicillin was enough to inhibit the growth of *P. aeruginosa* where the initial MIC's were 8mg/ml and 64mg/ml respectively. Thus the extracts VAMA01 and VAMA02 were found to possess very significant resistance modulatory activity.

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GLOSSARY

MIC	Minimal Inhibitory Concentration
DMSO	Dimethyl Sulfoxide

DPPH	2, 2-Diphenyl-1-Picrylhydrazyl
Ic_{50}	Inhibitory concentration 50
SEM	Standard Error of Mean
MTT	3-(4, 5-dimethylthiazole-2-yl-2, 5-diphenyltetrazolium bromide

CHAPTER ONE

1 INTRODUCTION

1.1 MEDICINAL PLANTS: THE MODERN ALTERNATIVE

The use of plants dates back to the early days of man. Early records from the Bible show evidence of plants being used for both their nutritional and medicinal values; some chewed their leaves, others boiled their roots and some even swallowed the seeds whole (The Holy Bible King James Version Ezekiel chapter 47 verse 12). Some were successful while others were either dangerous or futile.

Records from ancient Egypt showed that fever and headaches were cured by chewing the bark of the Willow bark tree (*Salix safsaf*). (http://reshafim.org.il/ad/egypt/botany/willow.htm). Several centuries after, scientists discovered salicylic acid in the bark of the willow tree. (http://chestofbooks.com/health/materiamedica-drugs/Pharmacy-Pharmacology-And-Therapeutics/Salicinum-SalicinWillow.html) Salicylic acid is now used to prepare aspirin which serves the same purpose as Salicylic acid yet with fewer side effects. Quinine which is also an antimalarial drug: used in the management of Malarial symptoms is obtained from the Cinchona tree bark. Its use also dates back to the 1570's in Peru. The bark of the Cinchona tree was ground and sweetened with water to offset its bitter taste. The early Peruvians used this remedy to stop shivering caused by low temperatures (Flückiger & Hanbury, 1874).

Not all pre-historic plant uses have proved rational or successful, for instance medieval doctors believed rubbing an onion on the scalp could help cure baldness (http://www.plantsandus.org.uk/medicine.htm).

Silphium, which is a plant used mostly in pre-historic Egypt has lost its significance in the modern world due to the inability of recognizing the plant (Parejko, 2003). Hippocrates reported that it was used in the treatment of sore throat, fever, cough, aches, pains, indigestion, and many other illness (Aly, 2014) (http://data.perseus.org/citations/urn:cts:latinLit:phi0978.phi001.perseuseng1:22.49).

Most compounds derived from plants are still in use today. The foxglove plant (*Digitalis lanata*) is known to produce a poison, digitoxin. This compound has been used in the management of cardiac arrhythmias, and currently more drugs with relatively fewer side effects have been produced upon progressive studies of this compound. Digoxin a drug which was obtained upon purification of Digitalis, (Hollman, 1996) is now one of the most essential drugs on the World Health Organizations list of essential medicines (Behring). Another compound, well established in cardiology is atropine, which is obtained from *Atropa belladonna* and popularly referred to as the deadly nightshade plant (http://www.plantsandus.org.uk/medicine.htm). Atropine is used in the management of bradycardia,(Field et al., 2010) poisoning and to induce salivation during surgical procedures (Fry & Burr, 2011).

Plants do and will continue to contribute to medicine and are seen as the future lifeline. Prickly succulent plant (*Hoodia gordonii*) have been used to stave off hunger by Bushmen in the Kalahari. Although infamous in this present times, it may in the near future, become a suitable therapy in the control of weight gain.

Another compound which may in the future become useful in medicine is the daffodil bulbs. It could possibly prove the best cure for Alzheimer's disease (http://www.plantsandus.org.uk/medicine.htm).

Although beneficial, not all plants provide health benefits. Quite a large number of them have proven harmful. Plant effects include induction of immune response, damaging body organs or even causing deaths. Trees and grasses that produce pollen especially pose a great health hazard to most people who have compromised respiratory systems due to their ability to trigger allergic responses. Worst of them all is tobacco smoking, which is associated with millions of disabilities and in some situations death across the world. The World Health Organization reports that close to 6 million deaths that occur every year worldwide results from tobacco use, and with current statistics and estimations its use may result in over 8 million deaths per year by 2030 (Organization, 2011).

Going forward, Sofowora wrote "most of the existing texts on traditional medicine in Africa deal only with medicinal plants and their uses, ignoring chemical and pharmacological studies" (Sofowora, 1982). This alert has been rapidly addressed by the scientific community which has now engineered most of its efforts towards investigation into the chemical and pharmacological properties of most medicinal plants.

1.2 ROLE OF PLANTS IN MICROBIOLOGY

Use of medicinal plants have not been limited to cardiology and hunting but also in the fight against microbial infections and their resistance. Rather unfortunately the most pressing concern in health care is infections, and this problem is heightened

by the availability of microbes everywhere. Presence of microbes wouldn't have been an issue if they didn't take up residence in our bodies and most significantly cause harm to their host. Climate change has also played the major role in the emergence of new infections and this is worsened by the fact that most old plagues are still with us (Morse, 1995).

Auspiciously, a majority of plants including several herbs have exhibited antimicrobial properties. A vast number of these plants are dietary herbs and spices, like thyme, ginger, garlic, and cinnamon (White, 2016). For instance, Ginger (*Zingiber officinale*) has been backed scientifically as suitable for use in the management of nausea (Ernst & Pittler, 2000). Ginger is also believed to offer some benefits to those suffering from diarrheal infections. Strains of bacteria that are often implicated in intestinal infections have been proven to be annihilated by the alcoholic extract of the ginger root. Such bacteria strains include *Escherichia coli, Helicobacter pylori, Vibrio cholera, Salmonella typhimurium and Pseudomonas aeruginosa.* Microorganisms that affect the skin and other soft tissues such as *Staphylococcus aureus,* as well as those that affect the respiratory tract especially *Haemophilus influenzae, Streptococcus pneumoniae* and *Streptococcus pyrogenes* are also inactivated by the same alcoholic extract of ginger. The use of Ginger is not limited to microbial infections only, it possesses laudable antifungal activity most often against *Candida albicans* and as well as intestinal worms (White, 2016).

Garlic (*Allium sativum*), another culinary herb is known to possess activity against some disease causing bacteria which include Staphylococcus, and

Mycobacterium. It is also known to inactivate some microbial species associated with diarrhea most common of them being, *Bacillus subtilis, Salmonella, Klebsiella, Clostridium* and *Escherichia coli* (White, 2016).

Artemisinin, also known as ***Qing Hao Su,*** and its endoperoxides derivatives are currently the standard treatment for the treatment of Malaria caused by *Plasmodium falciparum* (Organization, 2006). Artemisinin is isolated from a Chinese plant *Artemisia annua*, sweet wormwood tree. The medicinal value of the plant has been known to the Chinese for over 2000 years until the fourth century when its use has become popular (Etymologia, 2014 Jul).

The popular fruit Mango (*Magnifera Indica*) with its pleasant taste has also proved to possess some health benefits which include anti-bacterial, anti-viral, astringent, anti-syphyllic, anti-parasitic, diaphoretic, vermifuge, hypotensive, antiinflammatory, cardiotonic, slightly laxative and diuretic properties. It is also known to be a good appetite stimulant (www.speedyremedies.com/mangobenefits.html). All parts of the plant are known to be very beneficial to health. (mdidea.com/products/herbextract/mangiferin/data03.html).

1.3 *VOACANGA AFRICANA* AS A MEDICINAL PLANT

Voacanga africana, of the family Apocynaceae is a West African plant mostly found in the rainforests. The plant is easily identified by its smallish and tropical nature and also by its white or yellow flowers. The leaves of the plant are characteristically evergreen hanging from a brownish stem whose height reaches 6

meters and spreads over 2 meters. *Voacanga africana* has an erect stem which shows some branching; the leaves which are oval in nature appear broad and reach a length of 30 cm. Several irregularly shaped brown seeds are found in its cluster shaped berries to resemble a human brain. *V. africana* grows well in rich, composted soils as well as partly shady areas. The plant is tender to frost and drought and propagated via cuttings and often times fresh seeds (entheology.com/plants/vocanga-vocanga/)

Iboga alkaloids are mostly found in the seeds and bark of the Voacanga tree. The plant is used in Ghana, and other West African countries for several purposes, ranging from poisons, aphrodisiacs, stimulants and psychedelic agents. Judging from its ability to affect the central nervous system its suitability for consumption is highly questionable, regardless of the form. Nonetheless *Voacanga africana* is classified in most literature as a stimulant, cognitive enhancer, energizer, and visionary plant whose effect may persist for several days (Erowid, 1985).

The use of the plant can be dated back to ancient Africa. Although its use have been centered around its ability to affect the central nervous system it has remained one of the important plants for African traditional healers. The milky latex obtained from the stem bark has been used as a wound applicant in most parts of Nigeria and Senegal, it has also found use as an adulterant for rubber. In Africa the people of Diola use *Voacanga africana* in the treatment of infectious diseases. (entheology.com/plants/vocanga-vocanga/) To relieve tiredness and shortness of breath the leaves are used to make Tea (decoction), which is known to serve as a strengthening potion. Painful menstruation and hernia are treated using Voacanga

extracts. In most African communities it is used to prevent premature childbirth and manage heart troubles. Its use is not only limited to Africa, in Europe, it is used as a precursor for vincamine synthesis due to its high tabersonine content. Vincamine produced from tabersonine is used in the management of neural deficiencies especially those that occur in the elderly (Voogelbreinder, 2009).

1.4 *VOACANGA AFRICANA* AS A TRADITIONAL PLANT

Voacanga africana along with other species of Voacanga has been a wellprotected secret amongst the magic healers in Africa. Very little is known with regard to the exact use of its various parts: leaves, barks and fruits included (including *V. africana*). Regardless, when it comes to rituals, it is of high significance as a plant. Its seeds are appreciated due to the psychedelic effects which produces visionary experiences obtained from them. These few traditional uses are what have been documented about this plant (Rätsch, 2005).

1.5 JUSTIFICATION

A majority of the Voacanga genus have undergone scientific studies due to the presence of several alkaloids in most of their parts. Chemically *Voacanga africana* has proven to be a typical member of the genus possessing numerous alkaloids which if explored may present to be of economic relevance.

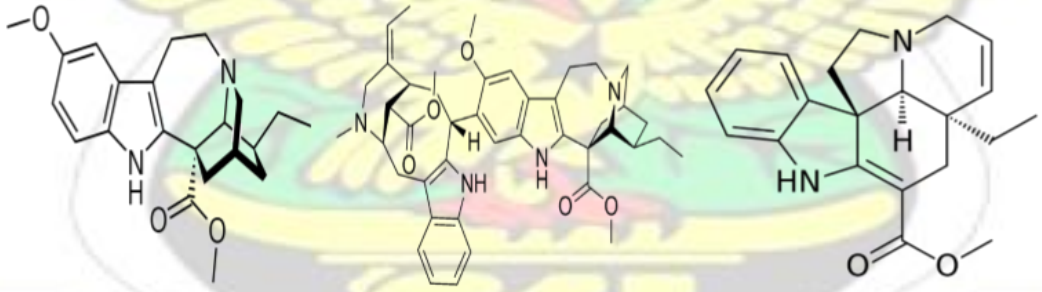
Voacanga africana has not been totally neglected by the scientific community; phytochemical studies performed on it indicated the presence of several alkaloids. The bark is known to contain latex, iboga and related alkaloids have been identified in the seeds and barks as well which rationalizes its use in most

Western African communities as a poison, stimulant, aphrodisiac as well as a psychedelic. A number of these constituents found in the Voacanga plant possess significant pharmacological activities and thus are used pharmaceutically (Hussain, Hussain, Al-Harrasi, & Green, 2012). Voacangine, an alkaloid is a typical example of such pharmaceutically important constituents. It is used in the semi synthesis of Ibogaine (a drug used in anti-addiction therapy) as a forerunner in the semi synthetic process. This is very necessary due to the insufficient amounts of natural Ibogaine which can be obtained via direct extraction from the plant. Thus if the plant is to be used for its anti-addictive effect then larger quantities must be consumed which would also increase the tendency for higher side effects. Voacangine and Ibogaine are not the only alkaloids present in the plant, other alkaloids present are coronaridine and voacamine and these may possess relatively weak anti-addictive tendencies.

Due to presence of several alkaloids that are closely related to iboga, Tabernanthe, and many other alkaloids that elicit psychoactive responses, it is of no doubt that *Voacanga africana* as a plant has become of major significance in this fast paced developing community where search for readily available solutions is the order of the day. Voacanga is generally known to increase stamina, endurance and aid magical and religious activities upon ingestion. It is vastly used as a hunting aid as well as a stimulant in Western African communities, where they ingest extracts of the bark. *Voacanga africana* is acclaimed to have a highly potent aphrodisiac activity. Other species of its genus are also known for their pharmacological activity; the barks of *Voacanga bracteata* from several reports is used as a substitute for marijuana in Gabon. [Puiseux et al. 1965 cited in (Rätsch, 2005) 588].

Amongst the several uses of Voacanga amongst the Diola people who are located in West Africa; is the use of Voacanga to treat infectious diseases. This small but significant fact forms the basis for this research which aims at substantiating this claim (drabeny.com/exports/vocanga.php).

Close to 10% indole alkaloids which are related to iboga, including voacangine, voacamine, tabersonine (designated as a, b, and c respectively in figure 1-1) and many other related compounds, can be found in the seeds of *Voacanga africana*. Although similar alkaloids can be found in the bark, they are only present at very low levels (usually below 2%) (White, 2016). When ingested such indole alkaloids can cause mild to strong persistent stimulation which last for several hours. Strong hallucinogenic effects have been associated with ingestion of higher doses of these indole alkaloids (White, 2016).



a. Voacangine b. Voacamine c. Tabersonine

Figure 1-1 most abundant alkaloids found in Voacanga africana.

Evolution suggests that continuous exposure of microorganisms to antimicrobials would cause the development of resistance strains in the near future. With chronic use of antibiotics, formerly susceptible strains will begin to develop resistance and

increase the burden on the scientific community. Resistance to a particular drug may develop instantly after a short period of use or may even take up to several years to develop. Thus it is not strange why some older antimicrobials are still in use as chemotherapeutic agents whilst others have been abandoned. Voacanga has been known to be able to manage some infections and although much is not known, this research aims at authenticating this claim.

1.6 AIMS AND OBJECTIVES

The aim of this project is to isolate biologically active constituents from the methanolic extracts of the leaves and bark of *Voacanga africana*, and to further identify them and possibly characterize those possessing significant biological activity.

The following objectives were set to help achieve the aim above

- To determine if *V. africana* has antioxidant properties and its degree of antioxidation compared using.
    - 2, 2-Diphenyl-1-Picrylhydrazyl method
    - Total phenolic test
    - Total antioxidant assay
- To determine if *V. africana* has antibiotic activity and its subsequent Minimal inhibitory concentration using;
    - Broth dilution method
- To determine if *V. africana* has resistant modulatory activity against selected strains of microbes using selected antibiotics and; ○ Broth dilution method

- To isolate and characterize the components of the methanolic extracts of the leaves and stem bark of *V. africana*.
    - Column chromatography to isolate components
    - Thin layer chromatography to identify difference
- To screen extract with activity for phytochemical constituents

CHAPTER TWO

2 LITERATURE REVIEW

2.1 THE FAMILY APOCYNACEA

Apocynaceae forms a family of shrubs, trees and in some cases herbs, they are characterized by a milky sap and are made up of 200 genera as well as about 5000 independent species. Their plants have simple leaves which are often whorled or decussate and opposite; shows no presence of stipules.

The flowers in this family, Apocynaceae are actinomorphic and bisexual or occasionally weak zygomorphics. They possess a synsepalous calyx which is usually lobed with a sympetalous corolla, distinct stamens, with numerous alternating corolla lobes, eventually adnating to the perigynous zone (corolla tube). They have introrse anthers which are very often adhered to the exterior of the stigma. Individual compound pistils of binary carpels (may be distinct at the superior level) or seldom partially inferior ovary, united by a singular style are found in the gynoecium. Each ovary when fully differentiated has several ovules located on marginal placentae; connate ovaries however have intruded parietal or axile placentation. (botany.hawaii.edu/faculty/carr/apocyn.htm)

At the base of the ovary is a nectary comprising of either an annular ring or 5 glands. Fruits of plants found in this family present as a follicle, capsule or berry, having flat and winged seed or sometimes seeds having a tuft oh hairs at an end. (botany.hawaii.edu/faculty/carr/apocyn.htm)

2.2 THE GENUS (VOACANGA)

In the Apocynaceae family there are about 200-400 genera which contains close to 5000 individual species located all over the world, and is most commonly found in tropics, southern Africa and subtropical South America. Plants of this genus have served several purposes, a majority of them are cultivated for ornamental purposes (including Asclepias, Hoya, Nerium, Plumeria, Stapelia), others for their phytoconstituents. Cardiac glycosides produced by members of this family have been used to control heart function in medicine and in hunting as arrow poison, and as a defensive mechanism for insects (Offinga, 2012)

Voacanga is a genus of plants in the Apocynaceae family found in Africa, Southeast Asia, New Guinea, and Australia (Cunningham, 1993). Striking similarities exist between the various species of the genus, characterized by white or yellow flowers having 5 united petals. Most plants in this genus are often times smaller in cultivation, possessing evergreen leaves and heights reaching up to 6 meters with a 2 metre spread which is very characteristic of the larger family to which they belong. Their stems are erect and branch to liberate broad and oval leaves up to 30 cm long.

Irregularly shaped brown seeds (which grow in a cluster that can shape out as a brain) are located in their berries and latex can be found in the bark of nearly all species of this genus (Schultes & Hofmann, 1992).

Plants in this genera include *Africana, Bracteata, Dregei, Grandiflora, Thoursii,* and *Foetida* amongst others.

2.3 MEDICINAL USES OF VOACANGA SPECIES

A majority of its documented use is observed in the African communities. In Senegal and Nigeria the practice of applying its milky latex to wounds is a very common amongst the local folks. Shortness of breath as well as fatigue have been relieved by drinking tea made from its leaves which is believed to be a strengthening portion (entheology.com/plants/vocanga-vocanga/). Voacanga species are also used by certain communities to treat painful menstruation and to prevent premature childbirth and hernias. In other parts of Africa it is used to manage heart troubles. In Europe it has gained much recognition due to a very high tabersonine content. Tabernosine serves as a precursor for the synthesis of vincamine, an alkaloid employed in the treatment of neural deficiencies found in elderly people (Voogelbreinder, 2009).

2.4 COMMON SPECIES OF THE VOACANGA GENUS

The Voacanga genus comprises of several independent species which contribute largely to the plant medicines. Several plants from these genus including the few discussed below have been exploited for their potential benefits.

2.4.1 Voacanga africana

Voacanga africana of the family Apocynaceae, is one of the common members of the Voacanga genus mostly found in Africa. *Voacanga africana* contains several alkaloids a number of which are very significant. One major alkaloid of *V. africana*, Voacangine is used in the semi synthesis of ibogaine as a precursor

(drabeny.com/exports/vocanga.php). Images of the *Voacanga africana* plant can be seen in figure 2-1, and its botanical classification is provided in table 2-1 below.

Figure 2-1 images of Voacanga africana

Table 2-1 Scientific classification of the plant Voacanga africana

RANK	**NAME**
Kingdom	Plantae
Unranked	Angiosperms
Unranked	Eudicots
Unranked	Asterids
Order	Gentianales
Family	Apocynaceae
Genus	Voacanga

Species	*V. africana*

2.4.2 Voacanga foetida

Voacanga foetida is another plant species from the Voacanga genus which unlike *Voacanga africana* is mostly found in Indonesia, Malaysia and Philippines. It grows to almost three times the height of *Voacanga africana*. Like all members of the Voacanga genus it is also rich with indole and ibogaine alkaloids and is mostly used for its psychedelic benefits.

Figure 2-2 Image of Voacanga foetida

2.4.3 Voacanga thouarsii

Voacanga thouarsii is characterized by fragrant flowers which exhibit pale green to cream or white corolla. Its dark green spotted fruits possess paired follicles. Vernacular names include "wild frangipani". *Voacanga thouarsii* best grows in forest and savanna areas. Its numerous local medicinal uses include treatment of wounds, sores, gonorrhea, eczema, heart problems, hypertension, rheumatism,

stomach-ache and snakebite (Abalaka, Fatihu, Ibrahim, & Ambali, 2014). *V. thouarsii* is found in Senegal, the Gambia, Guinea-Bissau, Guinea, and several other African countries The roots and bark contain the alkaloids voacamine, vobtusine and voacangine which are hypotensive, cardiotonic and sympatholytic (Burkill, 1985).

Figure 2-3 Images of Voacanga thouarsii

2.5 PHYTOCHEMICAL CONSTITUENTS OF VOACANGA SPECIES

Phytochemical screening of various species of the plant have been carried out by various independent researchers. The plant have been identified to contain several phytochemical constituents which include alkaloids, phenols, cardiac glycosides, saponins, tannins and some amount of starch. A majority of these constituents are produced due to the several biochemical and metabolic processes plants undergo in other to survive. Some of these phytochemical constituents are useful to the plant itself whilst others are not. Not to the plants alone can these phytochemical agents present useful. Man has benefited immensely from these metabolic products and by

products, from taking some as food to using some as aids and even to the extent of commercializing a majority of them.

2.5.1 Alkaloids

These are the most common amongst the various species of this genus; *V. africana, V. globosa,* (Vital & Rivera, 2011) and (Duru & Onyedineke, 2010). Several types of alkaloids exist amongst members of the genus including, indole, ibogaine and in some cases vinca. Fig 1-1 shows some alkaloids that can be obtained from Voacanga. Other alkaloids which are present in *V. africana* include tabernanthin and ibogamine.

2.5.2 Phenols

Most experiments performed on some species of the Voacanga genus have shown the presence of high antioxidant activity which is usually due to phenols which may be present in variant quantities amongst several independent species. Several kinds of phenols appear in plants including flavonoids (Barnes, 1999). Voacanga as a plant expresses phenols such as α-tocopherol.

2.5.3 Flavonoids

These metabolites form the most abundant naturally occurring phenols found in plants. Although about 2000 types exist a large majority of them exist as bound to other metabolites such as glycosides. Their forming elements include 3 acetate units and a phenylpropane unit. Their name originates from Latin text *flavus* which means yellow since they are yellow compounds whose color intensity increases with increasing pH, this forms the basis of their identification (Barnes,

1999).

2.5.4 Tannins

Tannin are large molecular weight plant metabolites that have the ability to combine with proteins of animal hides and prevent their putrefication and convert them to leather. Based on this fact the gold beaters test was developed as a simple test for tannins. In this test tannins are quantitatively determined by their adsorption on standard hide powder. Two major types of tannins are often identified in plants these are the hydrolysable tannins and the condensed tannins (Barnes, 1999).

2.5.5 Glycosides

Glycosides are naturally formed by the interaction of the nucleotide glycosidesfor example, uridine diphosphate glucose (UDPglucose) with the alcoholic or phenolic group of a second compound. Such glycosides are often referred to as Oglycosides and are the most numerous ones found in nature. Other glycosides do, however occur in which the linkage is through sulphur (S-glycosides), nitrogen (N-glycosides) or carbon (C-glycosides). Glycosides therefore consist of two factions- the sugar moiety (glycone) and the non-sugar moiety (aglycone). Several kinds of glycosides exist in nature from the cardiac glycosides to the cyanogenic glycosides. While glycosides do not themselves reduce Fehling's solution, the simple sugars they produce on hydrolysis will do so with precipitation of red cuprous oxide, this forms the basis for their identification(Barnes, 1999).

2.5.6 Saponins

Saponins are high molecular weight glycosides, which are highly polar in nature.

They often occur as complex mixtures which differ only with the nature of sugar present. They have haemolytic properties and thus can be highly toxic when injected directly into the blood stream. Despite their haemolytic tendencies when taken orally saponins are comparatively harmless. An example is Sarsarpiralla which is very rich in saponins yet used in the preparation of many alcoholic beverages. The name Saponins is due to their characteristic nature of producing a frothing aqueous solution upon agitation. This property is relied upon in their identification (Barnes, 1999).

2.5.7 Phytosterols

Phytosterols are constituents of plants that are related structurally to cholesterol, except that they are differentiated from cholesterol by the structure of their side chain (Cantrill & Kawamura, 2008). Two main classes exist: the sterols and stanols. They have asteroid backbone, a C-3 hydroxyl group on ring A, as well as an aliphatic side chain located at C- 17 atom on the rind D. Phytosterols possess a double bond, typically between C-5 and C-6 of the sterol moiety, whereas this bond is saturated in phytostanols (Cantrill & Kawamura, 2008).

2.5.8 Triterpenoids

These constituents have 30 carbons and are very abundant in nature. They may either occur as esters or even glycosides and can be presented as aliphatic, tetracyclic or pentacyclic. Their glycosides have been shown to have antileukemic, antiviral and anti-inflammatory activity (Barnes, 1999).

2.6 ANTIMICROBIAL RESISTANCE

The treatment of microbial infections have become increasingly demanding, and this comes as no surprise to the microbiological community which is fully aware of the rate at which antibiotics are been used and abused.

The search for antibiotics begun when microorganisms were first implicated in causing certain diseases and infections. This search has yielded much fruits and even led to the creation of more powerful antibiotics. The choice of antibiotic for the management of infections essentially depended on its in vitro activity, pharmacologic characteristics and clinical evaluation. Any antibiotic that is approved and accepted for use clinically, should have exhibited in-vitro activity by inhibiting or retarding the proliferation, of some target group of microorganisms. This activity should be achieved with certain proportions which can be ingested with acceptable toxic effects. The dose that was enough to do the killing or prevention of growth of microorganisms was termed the minimal inhibitory concentration (MIC). Those microorganisms that were unable to survive in the presence of these agents at their respective MIC's were termed, ***susceptible*** (sensitive) whilst those that still survived in the presence of similar doses of these agents were termed ***resistant***. Minimal inhibitory concentrations are often found lying between the susceptible and resistant concentrations. The term intermediate is used to refer to strains of microorganisms that appear on the borderlines of the MIC, depending on the convention or exact values of the reporting systems the terms **moderately sensitive** as well as **moderately resistant** may be used (Willey, 2008).

2.6.1 Origin, enhancement and spread of resistance

For some time antibiotics were seen as the giants in healthcare. They have been useful for the management of a number of infections and prevention of many as well. Nonetheless this hard won respect has eroded as time passed by with the appearance of new strains of microorganisms resistant to the actions of established antibiotics. This resistance may either be intrinsic (developed in a new strain) or acquired (developed in an already existing susceptible strain which has mutated or acquired new genes).

2.6.2 Intrinsic resistance

Some microorganisms express natural resistance to certain antimicrobials due to the mechanism of action of the antimicrobial. For example, some bacteria strains of streptococci, enterococci, and anaerobes are naturally resistant to aminoglycosides, and this is due to the lack of necessary oxidative pathways for transport of aminoglycosides.

2.6.3 Acquired resistance

With the constant use of antibiotics most bacteria have adapted to several ways of surviving them. Several mechanisms have been proposed of which enzymatic inactivation is one, most significantly is the production of *B*-lactamase, an enzyme capable of inactivating a *B*- lactam ring found in penicillins. Since antibiotics work via different mechanisms it is not surprising that every antimicrobial agent will have specific range of organisms which it can kill or control growth of, this makes some species naturally resistant.

The selective effect of antimicrobials used as chemotherapeutic agent cannot be overlooked as the chief cause of increasing resistance; this has been aided by extensive use of antimicrobials in the management of numerous infections. To complement matters of resistance is the increasing spread of infections and reinfections. To complement the situation microorganisms are now capable of crossing species and in some respects generic lines, this has vastly contributed to the development of resistance (Willey, 2008).

2.6.4 Resistance mechanisms

Resistance mechanisms have usually not been in the same manner. The main mechanisms of resistance have been

- accumulation barriers to an antimicrobial agent due to impermeability or active efflux;
- Enzymatic inactivation of the antimicrobial agent by microorganism.
- Target site alteration in the microorganism; rendering the microorganism insusceptible.
- Modification of the normal metabolic pathways in the microorganism leading to inefficacy of antimicrobial agent.

Antimicrobial resistance has led to many implications and complications in healthcare. These include the inability to effectively manage certain conditions, further compounded by the current need for polypharmacy in the management of simple conditions, a grave situation leading to patient non-compliant. The development of antimicrobial resistance has also led to ***superinfections,*** which are

a significant problem because of the existence of multiple drug-resistant bacteria that often produce drug-resistant respiratory and urinary tract infections.

2.6.5 The fight against antibiotic resistance

Despite attempts by stakeholders to control the development of resistance it has grown stronger as antimicrobial demands have increased. Several approaches have been considered in the fight against resistance. These approaches include; increasing antibiotic dose, practice of polypharmacy, and employing other chemotherapeutic agents as resistance modulators to enhance the activity of the antibiotic. These methods are briefly introduced in the following paragraphs.

In the past it seemed necessary to simply increase the dose of the antibiotics and although it helped in some respects it was a risky option because some antibiotics were already being used at their maximum doses permissible and thus doses could not be increased.

Further it became necessary to give multiple drugs acting via different mechanisms with the hope that this will help curb the problem of resistance, but rather it succeeded only in reducing patient compliance. Another approach was to use chemotherapeutic agents only when definitely necessary, that meant that before an antimicrobial agent can be used, the pathogen involved in the infection must have been identified and tested or known to be susceptible to that antimicrobial agent. Although this measures are still been employed it has become highly necessary to go back to the drawing board to synthesis new and more efficacious compounds that will replace these existing “worn-out” antibiotics.

Nonetheless a more current approach which has seen much acceptance by the scientific community is the use of ***resistance modulators***. Resistances modulators are chemical compounds which when used either alone or in combination with other drugs are able to decrease or eliminate resistance to treatment. Currently drug designs are channeling more efforts into obtaining resistance modulators, rather than synthesizing newer drugs.

2.7 IN VITRO ANTIMICROBIAL ASSAYS

Two main types of microbiological assays are often employed in the quest for better and safer antimicrobial agents as well as continuous monitoring of existing ones, these are;

- Agar Plate/disk diffusion assays,
- Broth and Agar dilutions

2.7.1 Agar Plate Diffusion Assays

In performing such test, a 'drug substance' or material whose activity is to be determined is slowly diffused in a microorganism seeded nutrient agar and incubated for a period of time with the appropriate conditions. The region on the nutrient agar plate surface where there is no growth is recorded as the "zone of growth inhibition". Different types of this assay exist and they are classified as one-dimensional, two-dimensional or three-dimensional (Kar, 2008).

The results of Agar diffusion are recorded as zones of inhibition. To achieve accuracy and utmost precision in recording the zones of inhibition a magnifying zone reader is used and the results calibrated most often by any one of the two

universally accepted methods; either a 2-By-2-Assay or a Standard Curve(Kar, 2008).

2.7.2 Broth and Agar dilutions

This assays are aimed at finding the minimal concentration of the assayed substance (usually an antimicrobial agent) enough to prevent the visible proliferation of a giving microorganism (used in the test). The results are documented as Minimal inhibitory concentrations (MIC) and is often expressed in either microgram per milliliter (μg/ml) or milligram per milliliter (mg/litre). Unfortunately this result is not always representative of the absolute value since the 'true' MIC is a point between the lowest test concentration that inhibits the growth of the bacterium and the next lower test concentration. Thus an inherent variation of one dilution could be factored when a series of dilutions are employed in MIC determinations (Silley, 2012).

Fortunately, these procedures are very useful due to their high reproducibility and ability to produce quantitative results compared to the agar disk diffusion assays. Yet, the fact that double dilutions are employed in testing can produce inaccurate MIC results. Thus, any analyst wishing to perform such determinations must use his own reagent-grade antimicrobials, reagents and antibiotic dilutions, which should be obtained, prepared and maintained in an appropriate stock solution sufficient for the entire determination. Working dilutions should also be generated on a regular basis, whilst organisms to be used are controlled to assure accuracy and standardization of the methods (Silley, 2012).

2.7.3 Broth dilution assay

This assays involves testing a suspension of bacterium (appropriate concentration) against the drug substance to be tested for prepared at different concentrations (oftentimes twofold serial dilutions) in a suitable solvent medium whose formulation has been predetermined and documented. Assays of this nature may either be performed in microtitration plates with smaller volumes (microdilution) or even test tubes containing volumes not less than 2ml. (macrodilution).

2.7.4 Agar dilution

In this procedure variant concentrations of the drug whose antimicrobial activity is to be tested for is incorporated into a nutrient agar medium, (concentrations are usually in twofold serial dilutions). This is immediately followed by the inoculation of a defined microorganism unto the nutrient agar surface. Results obtained from such assays are often upheld for their reliability in determination of

MIC's of a test antimicrobial/bacterium combination. Indicator solution are often employed to aid in achieving more accurate results during determination of assay results. One typical indicator used is 2-(4-iodophenyl)-3-(4-nitrophenyl)-5phenyltetrazolium chloride (INT) dissolved in a 70% ethanolic solution at a concentration of (2mg/mL) (Valgas, Souza, Smânia, & Smânia Jr, 2007).

Agar dilution has an advantage of being able to test for more than one bacteria strain (except swarming bacteria) using a single set of agar plate in a single determination. It also has the ability of improving the minimal inhibitory concentration endpoint identification as well as increasing extending the concentration of the antibiotics

used. Agar dilution is often recommended as a standardized Antibiotic Sensitivity Test method used for fastidious organisms

(CLSI, 2006c), examples of which will be helicobacter species and anaerobes (Silley, 2012).

2.7.5 Resistance modulatory assays

Resistance modulatory assays are gradually becoming famous due to the continuous spread of resistance to antibacterial agents and the search for alternative pathways to curb this problem. Resistance modulatory assays often employs agar and broth dilution techniques with slight modifications. Serial concentrations of both the resistance modulator and the antibiotics below their respective MIC's are used against standard organisms. The methods employs either of the nutrient broth or agar dilution protocols and just incorporates the agent in variant concentrations usually below the minimal inhibitory

concentration.

2.8 ANTIOXIDATION

Oxygen, although highly required by the body can be very toxic. About 3% - 5% of the consumed oxygen ends up being converted to free radical Oxygen, alongside the free radical oxygen that is accidentally produced by the body during its numerous metabolic activities.

Excess free radical oxygen species lead to a high susceptibility to cellular injury, disease or worsening of diseases. Radical oxygen cause cellular dysfunction by extracting electrons from lipids, proteins, carbohydrates and DNA.

Consequently the human body has antioxidant defense mechanisms in place that help dissipate this seemingly damaging effect of oxygen radicals. Dietary and endogenous antioxidants (free radical scavengers) have served as the main source of defense. Although a majority of the work is done by these antioxidants, cellular compartmentalization, metal sequestration, and repair of damaged cellular components further helps to prevent oxidative damage.

Any substance which can delay or inhibit significantly, the oxidation of a substrate when present in low concentrations is referred to as an antioxidant (Halliwell & Gutteridge, 1990). To aid recognition, antioxidants have been divided into two traditional classes as the chain breaking/primary antioxidants and preventive/secondary antioxidants (Jadhav, Nimbalkar, & Kulkarni, 1996).

Chain-breaking antioxidants occur in nature, yet they can be synthesized when need be such as is the case of the gallates. Most often than not larger quantities of synthetic antioxidants are used in the food industry and do end up in the human diet (Arcella, Le Donne, Piccinelli, & Leclercq, 2004) .Although cost effective synthetic antioxidants are giving way to natural antioxidants (an example which is plant bio phenols) due to increasing concerns of safety(Iverson, 1995), (Williams, Iatropoulos, & Whysner, 1999) and the benefit of a relatively higher antioxidant activity (Beutner et al., 2001), (Velioglu, Mazza, Gao, & Oomah, 1998)

Preventative antioxidants are those compounds that generally slow down the rate at which oxidation occurs. This often occurs in a couple of ways which includes substrate removal or even by simple quenching (Sochor et al., 2010).

Plant extracts rich in phenolic compounds (phenolic extracts), and essential oils have long been shown to possess antimicrobial activity and were frequently studied and reviewed. They are especially interesting as they are generally recognized as safe and have a potential to be used as preservatives in food products (Kähkönen et al., 1999).

2.8.1 Antioxidant Assays

To measure antioxidant activity, a compounds ability to scavenge (in lipophilic and aqueous phases) free radicals is of prime focus. Thus, the potential to mop up specific radicals such as superoxide radical, nitric oxide radical or hydroxyl radical, may be targeted (Aruoma et al., 1997), (Chu, Chang, & Hsu, 2000). In one technique the free radical is generated and its inhibition due to the test antioxidant is measured (Cano, Hernandez-Ruiz, Garcia-Canovas, Acosta, & Arnao, 1998). Current trends involve accelerated oxidation and involves the manipulation of one or more variables in the test system by using "initiators" (Antolovich, Prenzler, Patsalides, McDonald, & Robards, 2002).

2.8.2 Determination of antioxidant activity DPPH•

This determination is based on the capability of a stable free radical; 2, 2diphenyl-1-picrylhydrazyl to react in the presence of species that donate hydrogen. An intense Ultraviolet-Visible absorption spectrum is displayed by the radical DPPH•. The test is aimed at decolorizing the radical after it has been reduced with an antioxidising agent, which is expressed with the reaction below:

$AH + DPPH^{\bullet} \rightarrow DPPH^{\bullet}\text{-}H + A^{\bullet}$, or $R^{\bullet} + DPPH^{\bullet} \rightarrow DPPH^{\bullet}\text{-}R$. (Parejo,

Codina, Petrakis, & Kefalas, 2000)

For performing analysis manually, this procedure is very quick and simple.

The prepared DPPH solution can be used for up to seven days if stored appropriately (4 °C and away from light). The absorbance of the sample after incubation is then recorded using any suitable means. It is always necessary to maintain a reference standard, one common example is Gallic acid.

2.8.3 The total phenolic test using Folin Ciocalteu Assay

This assay which is also referred to as the Gallic acid equivalent test is used for the determination of phenolic and polyphenolic antioxidants. The reagent used (folin ciocalteu) reacts with any reducing substance present in a substrate. Thus it is appropriate for the measurement of the total reducing ability of a substrate, and this includes nitrogen-containing compounds (Ikawa, Schaper, Dollard, & Sasner, 2003). Phenolic reactivity towards Folin Ciocalteu may be increased by copper complexation (Everette et al., 2010). The Reaction with all phenol containing compounds results in the formation of chromogens which can be spectrophotometrically detected.

2.8.4 The Total antioxidant assay

The chemical diversity of phenolic antioxidants makes it difficult to separate and quantify individual antioxidants (that is parent compounds, glycosides, and many isomers) from the vegetable matrix. Most antioxidant assays employ spectrophotometric electron transfer procedures. This assays measure the capacity of an antioxidant in the reduction of an oxidant, which changes colour when reduced.

The degree of colour change (either an increase or decrease of absorbance at a given wavelength) is correlated to the concentration of antioxidants in the sample.

CHAPTER THREE

3 MATERIALS AND METHOD

3.1 MATERIALS

3.1.1 Plant material

Leaves and stem bark of *Voacanga africana* was obtained in august 2015 from the Faculty of Pharmacy, KNUST campus and was identified at the Department of Pharmacognosy, Faculty of Pharmacy and Pharmaceutical sciences, KNUST, in Kumasi Ghana.

3.1.2 Chemicals

All solvents which were employed in extraction, dissolution, chromatographic (column and thin layer) analysis, microbial screening and phytochemical analysis, which including methanol, distilled water, petroleum ether, 2, 2-Diphenyl-1Picrylhydrazyl, Folin-Ciocalteu reagent and ethyl acetate, were all of analytical grade and were obtained from the department of Pharmaceutical Chemistry as well as the Department of Pharmacognosy and Microbiology all in the Faculty of Pharmacy, KNUST.

3.1.3 Chromatographic materials

For the column chromatography, the stationary phase material used was Silica gel 60 (70-230 mesh ASTM, Merck Germany). The thin Layer chromatography separation was achieved using an Aluminum pre-coated silica gel plates 60 F254 (0.25 mm thick). Silica gel powder (12-13% calcium sulphate as binder, Fisons) was used as a preparative agent in the preparative thin layer chromatography.

3.2 METHODS

3.2.1 Preparation and extraction of Plant material

The plant materials was dried in open air under sunlight for two weeks. With a commercial grinder, the dried stem bark and leaves were pulverised into fine powder and held in distilled methanol for 5 days at room temperature. The mixture was filtered, rotary evaporated at 50^{o}C degrees and air dried to obtain the pure extract.

3.2.2 Distillation of Methanol

Methanol obtained from the Department of Pharmaceutical chemistry was distilled by heating at 50^{o}C and collecting the distillate in a clean round bottom flask.

3.2.3 Chromatography

Silica gel (70-230 mesh ASTM) was packed gently to about half the length of a glass column (45cm ×2cm). Silica gel was added to 2.5 grams of the methanolic extract of the stem bark which had been dissolved in a small amount of methanol. The silica packed column was left standing so as to dry whilst stirring the mixture to obtain homogeneity. The sample mixed in silica was gently spread unto the packed column and cotton wool was placed on the surface with the aim of protecting the integrity of the surface during solvent delivery. To achieve optimum separation Gradient elution was carried employed. [Eluate: petroleum ether: ethyl acetate: methanol (100:0:0% - 0:0:100%)] and approximately 15 fractions of 30ml were collected. Fractions were bulked according to their TLC profiles to give six (6) bulked fractions.

3.2.4 Thin Layer chromatography Detection

Spot detection were made under ultraviolet light at 254nm as well as 365nm. Iodine as well as Anisaldehyde solution 0.5%w/v in ethanol concentrated Sulphuric acid and Methanol in the ratio (10:5:85%v/v/v) to spray the plates, and this was completed by warming for 5-10 minutes at 105 °C.

3.2.5 Antioxidant assays

Preparation of DPPH solution.

0.95mmol/L solution of radical DPPH• which is equivalent to 0.00374 grams dissolved in 100ml. 0.00374 grams of DPPH is first dissolved in 50ml of DMSO and mixed properly. The solution is made up to a volume of 100ml with distilled water.

Evaluation of antioxidant ability DPPH assay

The method used for this assay was the **2, 2-Diphenyl-1-Picrylhydrazyl (DPPH) radical** method written by Antalovich et al (2001) with slight modification. The methanolic extract of the leaves and stem bark of *Voacanga africana* were dissolved in distilled methanol to make an initial concentration of 1mg/ml from which the subsequent concentrations were prepared and used in the DPPH radical scavenging activity determination upon serial dilution. Ascorbic acid was used as a control in the analysis. The plant extracts were diluted in stepwise manner and 10 µL of the dilute solution was pipetted into a 96-well plate. 185 µL of DPPH solution prepared in a 50% ethanol was introduced into each well, and carefully shaken for 5 minutes at room temperature. The plates were incubated for 15 minutes after

which the absorption was measured at 517 nm using a Multilabel counter. Percentage inhibition was measured using the formula stated below:

Percentage Inhibition = (Aa-As/ Aa) ×100

Where Aa is absorbance of Ascorbic acid (control) and As is the absorbance of the sample

Total phenolic test: using Folin-Ciocalteu Assay.

The quantity of phenolics which were present in the extracts were determined using the Folin-Ciocalteu assay method. The extracts were prepared to the requisite concentrations using methanol for dilution. 2%w/v sodium carbonate solution was also prepared in water. 0.5ml of the individual extracts were added to 0.1ml of the Folin-Ciocalteu and incubated (by keeping in the dark) for 15 minutes. After incubation 2.5 ml of saturated sodium carbonate solution was added to each concentrate and further incubated for 30 minutes. 200 µL of each concentrate was transferred into a 96-well plate and the absorbance was measured at a wavelength of 760 nm using a Multilabel counter. The standard used was tannic acid which was prepared at a relatively lower concentration. Intensities of the color developed is shown as EGCG equivalents per gram of sample (µmol/g).

Total antioxidant assay.

The individual concentrations was prepared in methanol and the reagents to be used in the assay was also prepared in distilled water: 26mM disodium hydrogen phosphate, 4mM ammonium molybdate and 0.6m hydrogen phosphate. All reagents were mixed in a beaker and held in a volumetric flask. A volume of 3ml

of the reagent mix was taken and added to 1ml of the extract concentrations and incubated in an oven for 90 minutes. The resultant mix was decanted and its absorbance measured at 695nm. Ascorbic acid was used as standard.

3.2.6 Antibacterial screening

Media, antibiotics and organisms

Nutrient agar and broth used for the microbial assays were obtained from the Department of Microbiology in the Faculty of Pharmacy. Analytical grade [3-(4, 5-dimethylthiazole-2-yl-2, 5-diphenyltetrazolium bromide) (MTT)] was also used for the analysis and was also obtained from the department. *Staphylococcus aureus* (ATCC 25923), and *Bacillus subtilis* (NTCC 10073), *Pseudomonas aeruginosa* (ATCC 4853), *Escherica coli* (ATCC 25922), and *Candida albicans* (clinical strain) which were used for the microbial assays were also obtained from the Department of Microbiology. Five standard antibiotics: amoxicillin, ciprofloxacin, erythromycin, tetracycline and ketoconazole were used in the assay. Methanolic extracts of both the leaves and stem bark were tested independently against the organisms, and based on the results the

chromatographic isolates of the methanolic bark extracts were tested for on the organisms after which the modulatory assays were performed. All five strains of microorganisms were cultured overnight in nutrient broth at 37°C, prior to the determinations. Microorganisms used consisted of a fungus (*Candida albicans*), two gram positive and two gram negative; *Salmonella typhi, Pseudomonas aeruginosa, Escherichia coli*, and *Pseudomonas aeruginosa*.

Media preparation

Four (4) grams of nutrient broth powder was weighed into a clean dry beaker with the aid of an analytical balance in other to prepare Double strength Nutrient Broth. 50mL distilled water was added to the weighed nutrient broth and dissolved by gradual stirring. More distilled water was added to the solution and made up to 100ml. this was transferred into a clean dry bottle and firmly plugged with cotton wool. The solution was sterilized in an autoclave for 15 minutes at a temperature of 121°C.

General screening of extracts: Agar diffusion assay

The agar disk diffusion method was used to initially screen the extracts for antimicrobial activity.

Microorganisms were prepared as described above and diluted in the sterilized nutrient agar, and incubating at 37 °C. sterilized nutrient agar which was in the molten state at 45 °C was introduced into a sterile petri dish and allowed to set (solidify), after which a ml each of the dilute cultures was inoculated unto the solidified nutrient agar: spreading them gently over the nutrient agar bed. Using methanol the extracts as well as standard antibiotic were reconstituted and soaked unto a sterile paper disc (diameter o.5mm) for close to 15 minutes. The disc was air dried under the screen after which they were placed on the solidified agar. The extract and drug was allowed to diffuse gently into the nutrient agar for an hour and incubated at 37°C for one day. The zone of inhibition which was recorded as the diameter around the disk where there was no growth, triplicate determinations were made and the mean zone of inhibition calculated.

Broth dilution assay

Isolates that showed activity in the agar diffusion method were subjected to minimum inhibitory assays employing the method proposed by Ellof (1998) with slight modifications. The extracts were screened at concentrations beginning at 1 mg/ml and doubling the strength in a milliliter of solution to prepare eight different concentrations. The assay was carried out in 96 micro titre wells and each concentration was determined in triplicates. Into each well was introduced 100 µL of double strength nutrient broth 10µl of organism, and a given volume of the extract that produced the desired concentration in 200µl of solution and made up to 200µL with sterile water. The 96 micro titre well plates were incubated at 37 °C for 24 hours. To help obtain accurate result 10µL of a 5 % solution of tetrazolium salt (MTT) was introduced into the wells and incubated for further 15 minutes. The presence of microorganisms were confirmed by the observation of a Dark/purple color in the wells. This result was due to dehydrogenase enzymes present in living bacteria reacting with the tetrazolium salt to produce a purple complex.

Resistance modulatory assays.

The method employed for this assay is similar to the method used in the minimum inhibitory concentration determinations with only a slight modification. In this method 100µL of double strength nutrient broth 10µl of organism, and a given volume of the extract as well as a given volume of the antibiotic that produced the desired concentrations in 200µl of solution was introduced into a 96 micro titre well plates and made up to 200µl with sterile water. Similar conditions were observed as was done in the minimum inhibitory concentration determinations.

3.2.7 Phytochemical screening

Phytochemical screening was performed on the bulked chromatographic isolates of the methanolic extract obtained from the *V. africana* bark. This was to help identify the phytochemicals responsible for the respective activities. Although these test were general test they helped narrow down in drawing possible constituents that possessed biological activity.

The phytochemical screening was carried out under the tests below:

Test for Saponnins

About 50mg of the various isolates were dissolved in water, contained in a test tube and shaken vigorously. The resultant was allowed to stand for 10 minutes and the observations were recorded. A saponin will produce a foamy lather which persisted for the entire duration of the test.

Test for glycosides

About 50mg of the isolate were taken into a conical flask, about 5ml of dilute Sulphuric acid was added as well as a similar volume of water was added and heated on a water bath for ten minutes, this was to hydrolyze the glycoside to release the sugar moiety. The solution was filtered of and 3ml quantities of Fehling's solutions 1 and 2 was added to the filtrate. Observation of reddish brown precipitates indicates the presence of reducing sugars, which resulted from hydrolysis.

Test for triterpenoids

About 50mg of the isolate was dissolved in chloroform and filtered off gently. 3ml of concentrated sulfuric acid was added to the filtrate which was held in a test tube

under a fume chamber. Formation of a reddish brown ring on the walls of the test tube indicated the presence of triterpenoids.

Test for flavonoids

About 50mg of the isolate was dissolved in a small volume of water and allowed to stand for 5 minutes. A clean and dry filter paper was inserted into the sample and allowed to air dry at room temperature. When dry the filter paper was brought near a strong ammonia solution. The appearance of a bright yellow color indicated the presence of flavonoids.

Test for phytosterols

About 50mg of the sample was dissolved in chloroform and filtered off. To the filtrate about 2 ml of acetic anhydride and also 2ml of concentrated sulfuric acid was added. (NB care should be taken during procedure to avoid explosion) a green ring formed between two layers of solvents indicates the presence of phytosterols.

Test for alkaloids

About 50mg of the sample was dissolved in dilute Sulphuric acid in a test tube. About 2ml of Dragendorff's reagent (solution of potassium bismuth iodide) was added to the solution. Alkaloids will precipitate to produce a reddish brown solution.

Test for tannins: the Goldbeaters test.

About 50mg of the sample to be tested was dissolved in a test tube with water. 1% ferric chloride solution was added to the mixture and allowed to stand. Formation of a dark green color denotes the presence of tanni

CHAPTER FOUR

4 RESULTS

The work involved determination of the antioxidant properties of methanolic extracts of the stem bark and leaves of *Voacanga africana*, using three basic tests.

The results for these assays are expressed in the following paragraphs.

4.1 ANTIOXIDANT ASSAY

4.1.1 DPPH scavenging activity

The antioxidant ability of the methanolic extracts obtained from both the leaves and the stem bark were determined using the DPPH scavenging activity test. The results showed a concentration dependent activity which is seen in the table and graphs that follow.

Table 4-1 Results for DPPH scavenging activity

Extract/drug	Ic_{50} (mg/ml)
Leaf extract	1605.00
Stem bark extract	70.00
Ascorbic acid(standard)	6.98

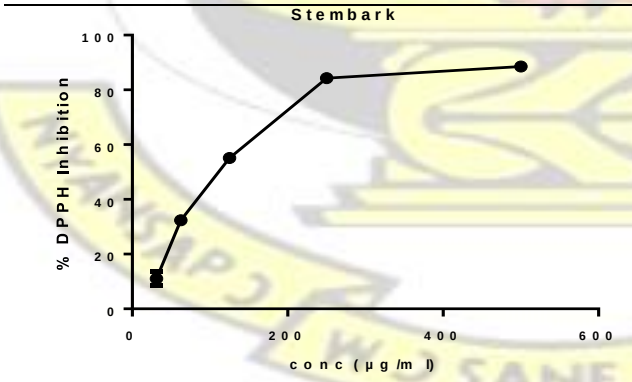


Figure 4-1 %DPPH inhibition versus concentration curves for stem bark

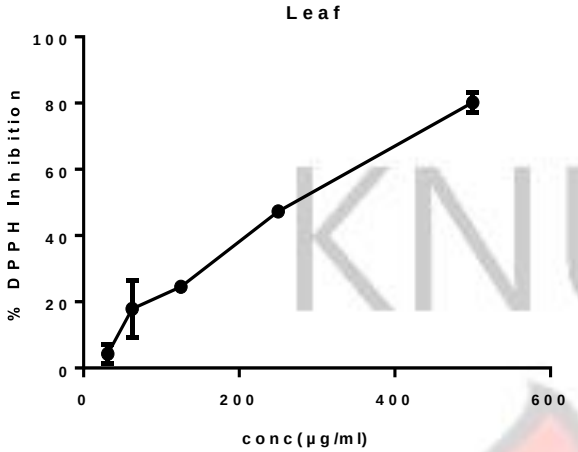


Figure 4-2 %DPPH inhibition versus concentration curves for leaf

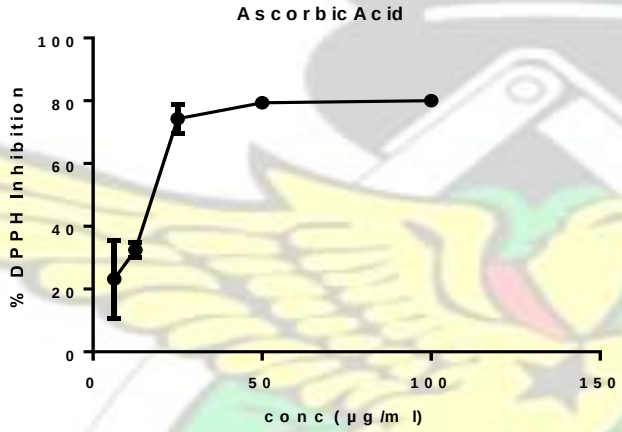


Figure 4-3 %DPPH inhibition versus concentration curves for standard

4.1.2 Total antioxidant capacity

The total antioxidant test involved comparing the degree of antioxidation obtained from the extracts against that of a standard antioxidant in this case Ascorbic acid. The results obtained are presented in the table 4-2 that follows.

Table 4-2 Results for total antioxidant capacity of V. africana

Extract	Mg/g equivalent of ascorbic acid

Stem bark	**23850.1**
Leaf	**24869.8**

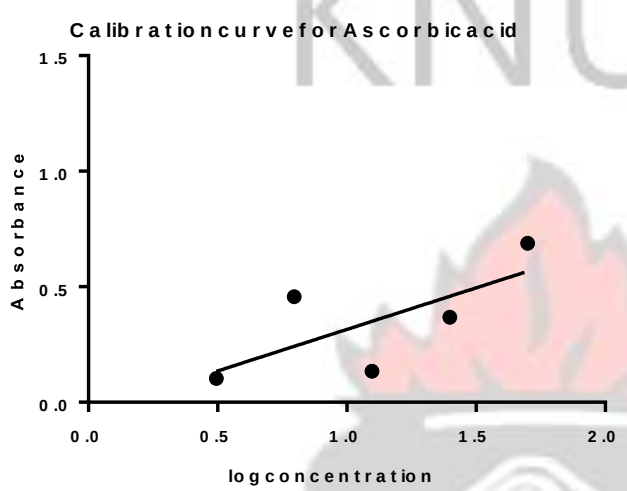


Figure 4-4 Calibration curve for ascorbic acid

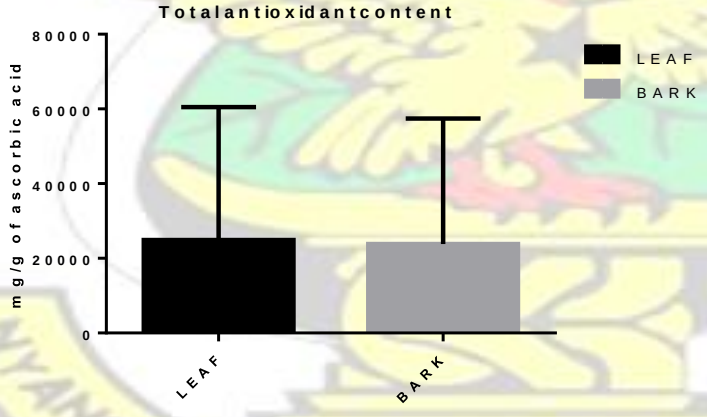


Figure 4-5 Total antioxidant content

4.1.3 Total phenolic content

Folin Ciocalteu was used to determine the total phenolic content of both extracts.

Table 4-3 Results for total phenolic content of V. africana

Extract	Mg/g equivalent of tannic acid
Stem bark	**11738.30**
Leaf	**13609.60**

When the total antioxidant capacity was plotted against the total phenolic content it was also observed that the antioxidation achieved was highly dependent on the activity of phenolic compounds present in the extract. This is observed in the figure below.

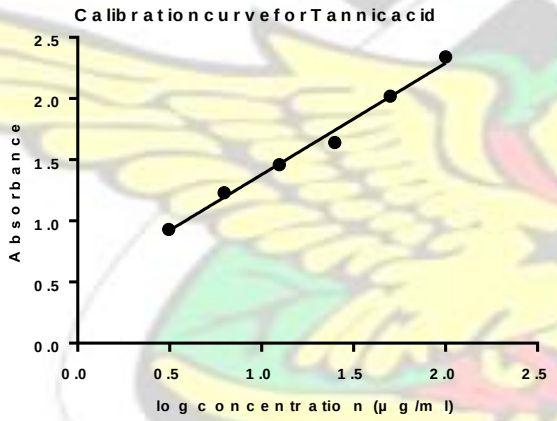


Figure 4-6 Calibration curve for tannic acid

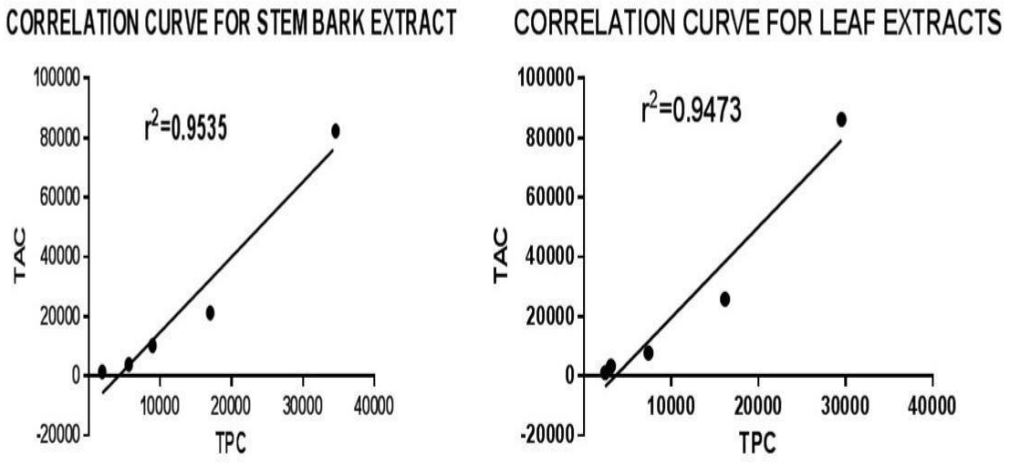


Figure 4-7 Correlation curves for Stem bark and leaf

4.2 CHROMATOGRAPHIC SEPARATION PRODUCTS

Column chromatography performed on the methanolic extract of the bark resulted in the realization of seventeen different eluents based on the solvent combinations. The elution was performed starting with a fully non polar solvents petroleum ether and a tapering down the polarity by preparing different concentrations of the solvent with polar solvents until the most polar condition was attained in using methanol. The collected eluents were combined into six different solutions with the aid of Thin Layer chromatography where samples that gave very close retardation factors were bulked. The bulked solvents were labelled VAMA**01** through to VAMA**06**. Phytochemical screening of the isolates are expressed in tables 4-4 and 4-5.

Table 4-4 Phytochemical screening of VAMA01 - VAMA03

	VAMA01	**VAMA02**	**VAMA03**
Tannins	+	-	-
Glycosides	-	-	PARTIAL

Alkaloids	+	+	-
Flavonoids	+	+	-
Triterpinoids	-	+	-
Phytosterols	-	+	-
Saponnins	+	+	-

Table 4-5 ***Phytochemical screening of VAMA04 – VAMA06***

	VAMA04	**VAMA05**	**VAMA06**
Tannins	-	-	+
Glycosides	-	-	+
Alkaloids	+	+	-
Flavonoids	-	-	-
Triterpinoids	-	-	-
Phytosterols	-	-	-
Saponnins	-	-	-

4.3 ANTIBACTERIAL ASSAYS

The various antimicrobial assays described in chapter 3 was used in the assay and the results obtained are described in the paragraphs that follow.

4.3.1 Antimicrobial assays of extracts from stem bark and leaves.

As stated earlier, the advantage agar disk diffusion assay presented was the major consideration for it to be the choice method in this assay. The extracts were prepared in methanol and sonicated for 20 minutes to ensure adequate dissolution was obtained to a concentration of 10mg/ml. Ciprofloxacin was used as positive control and sterile distilled water was used to dissolve the ciprofloxacin and made the extracts to volume. The assay results is found in table 4-6 below.

Table 4-6 Results of agar disk diffusion assay

	Mean diameter (zone) of inhibition (mm) ± SEM			
Organism	Stem bark extract	Leave extract	Ciprofloxacin	Ketoconazole
S. aureus	29.40 ± 0.43	1.45 ± 2.43	33.21 ± 1.23	2.54 ± 0.45
E. coli	33.67 ± 0.75	2.33 ± 3.12	29.45 ± 1.14	4.45 ± 3.96
B. subtilis	28.35 ± 4.21	1.32 ± 2.34	24.75 ± 1.32	3.06 ± 2.54
P. auregenosa	27.47 ± 3.24	0.90 ± 0.98	29.30 ± 2.19	0.98 ± 1.54
C. albicans	25.96 ± 3.23	1.45 ± 1.54	10.45 ± 3.21	30.79 ± 2.76

4.3.2 Antimicrobial screening of isolates obtained from the stem bark

The isolates were also screened for activity and the results are documented below.

Table 4-7 Results for agar disk diffusion assay of isolates

	Minimum inhibitory concentration (MIC)/mg/mL		
Organism	**VAMA01**	**VAMA02**	**VAMA03**
S. aureus	18.56±2.10	17.43±1.45	10.40±2.78
E. coli	24.32±1.98	22.32±2.33	4.50±1.45
B. subtilis	17.23±3.21	15.32±1.78	7.87±0.98
P. auregenosa	12.45±2.12	10.23±1.40	5.87±0.79
C. albicans	16.43±2.34	9.43±1.23	6.56±1.12

Table 4-8 Results for agar disk diffusion assay of isolates

	Minimum inhibitory concentration (MIC)/mg/mL		
Organism	**VAMA04**	**VAMA05**	**VAMA06**
S. aureus	7.32±3.34	2.33±1.10	2.33±0.90
E. coli	3.91±1.54	4.26±2.34	2.45±1.67
B. subtilis	1.44±0.99	3.34±1.23	2.54±1.22
P. auregenosa	7.43±1.45	3.78±0.22	1.56±0.98

C. albicans	2.34±0.98	2.13±0.98	3.45±0.89

4.3.3 Minimum inhibitory assays

The results obtained from the agar disk diffusion assay lead to a further determination of the minimal inhibitory concentrations of both the extracts and the isolates. The minimum inhibitory assay was aimed at identifying the lowest concentration of the extract/isolate/standard antibiotic that was enough to inhibit the growth of given volume of organism.

Table 4-9 MIC determination of standard antibiotics and major extracts of V. africana

	Minimum inhibitory concentration (MIC)/mg/mL				
Organisms	*S. aureus*	*B. subtilis*	*E. coli*	*P. auregenosa*	*C. albicans*
Drugs					
Amoxicillin	16	32	32	64	NA
Ciprofloxacin	4	2	2	4	NA
Erythromycin	8	8	4	8	NA
Tetracycline	16	16	8	8	32
Ketoconazole	NA	NA	NA	NA	32
Extracts					
Bark extract	4	4	4	8	8
Leave extract	NA	NA	NA	NA	NA

NA= not active

Table [1]-10 MIC determination of chromatographic extracts of V. africana

[1] .3.4 Resistance modulatory assays

ORGANISMS	Minimum inhibitory concentration (MIC)/mg/mL				
	S. aureus	*B. subtilis*	*E. Coli*	*P. auregenosa*	*C. albicans*
Stem Bark extracts					
VAMA01	4	4	4	8	8
VAMA02	4	4	8	8	NA
VAMA03	8	8	8	16	NA
VAMA04	16	16	8	32	NA
VAMA05	NA	NA	NA	NA	NA
VAMA06	NA	NA	NA	NA	NA

The results obtained from the minimal inhibitory assays are documented in the tables that follow

Table

4-11 Modulatory assay for VAMA01 with amoxicillin

	Minimum inhibitory concentration (MIC)/mg/mL			
ORGANISMS	*S. aureus*	*B. subtilis*	*E. Coli*	*P. auregenosa*
Extract(conc**)** + **Antibiotic (**conc**)** mg/ml	-- -	-- -	-- -	-- -
VAMA01(12**)** + **Amoxicillin(**16**)**	---	---	---	---
VAMA01 (6**)** + **Amoxicillin(**16**)**	---	---	---	NA
VAMA01(3**)** + **Amoxicillin(**16**)**	NA	NA	NA	NA
VAMA01(1.5**)** + **Amoxicillin(**16**)**	---	---	---	---
VAMA01(12**)** + **Amoxicillin(**8**)**	---	---	---	NA
VAMA01(6**)** + **Amoxicillin(**8**)**	---	---	NA	NA
VAMA01(3**)** + **Amoxicillin(**8**)**	NA	NA	NA	NA
VAMA01(1.5**)** + **Amoxicillin(**8**)**	---	---	---	---
VAMA01(12**)** + **Amoxicillin(**4**)**	---	---	---	NA
VAMA01(6**)** + **Amoxicillin(**4**)**	---	---	---	NA
VAMA01(3**)** + **Amoxicillin(**4**)**	NA	NA	NA	NA
VAMA01(1.5**)** + **Amoxicillin(**4**)**				

Table 4-12 Modulatory assay for VAMA01 with erythromycin

	Minimum inhibitory concentration (MIC)/mg/mL			
ORGANISMS	*S. aureus*	*B. subtilis*	*E. Coli*	*P. auregenosa*

Table Modulatory assay for VAMA

Extract(conc**) + Antibiotic (**conc**)** mg/ml	-- -	-- -	-- -	
VAMA01(12**) + Erythromycin(**8**)**				NA
VAMA01(6**) + Erythromycin(**8**)**	---	---	---	NA
VAMA01(3**) + Erythromycin(**8**)**	---	---	---	NA
VAMA01(1.5**) + Erythromycin(**8**)**	---	---	---	NA
VAMA01(12**) + Erythromycin(**4**)**	---	---	---	---
VAMA01(6**) + Erythromycin(**4**)**	---	NA	---	NA
VAMA01(3**) + Erythromycin(**4**)**	---	NA	---	NA
VAMA01(1.5**) + Erythromycin(**4**)**	NA	NA	---	NA
VAMA01(12**) + Erythromycin(**2**)**	---	---	---	---
VAMA01(6**) + Erythromycin(**2**)**	---	---	---	NA
VAMA01(3**) + Erythromycin(**2**)**	NA	NA	NA	NA
VAMA01(1.5**) + Erythromycin(**2**)**	NA	NA	NA	NA

4-13 01 with tetracycline

	Minimum inhibitory concentration (MIC)/mg/mL			
ORGANISMS	*S. aureus*	*B. subtilis*	*E. Coli*	*P. auregenosa*
Extract(conc**) + Antibiotic (**conc**)**		-- -	-- -	-- -
VAMA01(12**) + Tetracycline(**16**)**	---			
VAMA01(6**) + Tetracycline(**16**)**	---	---	---	---

VAMA01(3**) + Tetracycline(**16**)**	---	---	---	---
VAMA01(1.5**) + Tetracycline(**16**)**	---	---	---	NA
VAMA01(12**) + Tetracycline(**8**)**	---	---	---	---
VAMA01(6**) + Tetracycline(**8**)**	---	---	---	---
VAMA01(3**) + Tetracycline(**8**)**	---	---	---	---
VAMA01(1.5**) + Tetracycline(**8**)**	NA	NA	---	NA
VAMA01(12**) + Tetracycline(**4**)**	---	---	---	NA
VAMA01(6**) + Tetracycline(**4**)**	---	---	---	NA
VAMA01(3**) + Tetracycline(**4**)**	NA	NA	---	NA

Table 4-14 Modulatory assay for VAMA 02 with amoxicillin

	Minimum inhibitory concentration (MIC)/mg/mL			
ORGANISMS	*S. aureus*	*B. subtilis*	*E. Coli*	*P. auregenosa*
Extract(conc**) +**	--	--	--	--
Antibiotic (conc**)** mg/ml	-	-	-	-
VAMA02(12**) + amoxicillin(**16**)**	---	---	NA	NA
VAMA02 (6**) + amoxicillin(**16**)**	---	NA	NA	NA
VAMA02(3**) + amoxicillin(**16**)**	---	NA	NA	NA
VAMA02(1.5**) + amoxicillin(**16**)**	---	---	---	---
VAMA02(12**) + amoxicillin(**8**)**	---	---	NA	NA
VAMA02(6**) + amoxicillin(**8**)**	---	NA	NA	NA
VAMA02(3**) + amoxicillin(**8**)**	---	---	---	---
VAMA02(12**) + amoxicillin(**4**)**	---	---	NA	NA
VAMA02(6**) + amoxicillin(**4**)**	---	NA	NA	NA
VAMA02(3**) + amoxicillin(**4**)**	---	---	---	---
VAMA02(12**) + amoxicillin(**2**)**	---	---	NA	NA
VAMA02(6**) + amoxicillin(**2**)**				

Table 4-15 Modulatory assay for VAMA 02 with erythromycin

	Minimum inhibitory concentration (MIC)/mg/mL			
ORGANISMS	*S. aureus*	*B. subtilis*	*E. Coli*	*P. auregenosa*
Extract(conc**) + Antibiotic (**conc**)** mg/ml	-- -	-- -	-- -	
VAMA02(12**) + erythromycin(**8**)**				NA
VAMA02(6**) + erythromycin(**8**)**	---	---	---	NA
VAMA02(3**) + erythromycin(**8**)**	---	---	---	NA
VAMA02(1.5**) + erythromycin(**8**)**	---	---	---	NA
VAMA02(12**) + erythromycin(**4**)**	---	---	---	NA
VAMA02(6**) + erythromycin(**4**)**	---	NA	---	NA
VAMA02(3**) + erythromycin(**4**)**	---	NA	---	NA
VAMA02(1.5**) + erythromycin(**4**)**	NA	NA	---	NA
VAMA02(12**) + erythromycin(**2**)**	---	---	---	NA
VAMA02(6**) + erythromycin(**2**)**	---	---	NA	NA
VAMA02(3**) + erythromycin(**2**)**	NA	NA	NA	NA
VAMA02(1.5**) + erythromycin(**2**)**	NA	NA	NA	NA

Table 4-16 Modulatory assay for VAMA 02 with tetracycline

	Minimum inhibitory concentration (MIC)/mg/mL			
ORGANISMS	*S. aureus*	*B. subtilis*	*E. Coli*	*P. auregenosa*
Extract(conc**) + Antibiotic (**conc**)** mg/ml				
VAMA02(12**) + Tetracycline(**16**)**	---	---	---	---
VAMA02(6**) + Tetracycline(**16**)**	---	---	---	---
VAMA02(3**) + Tetracycline(**16**)**	---	---	---	---
VAMA02(1.5**) + Tetracycline(**16**)**	---	---	---	NA
VAMA02(12**) + Tetracycline(**8**)**	---	---	---	---
VAMA02(6**) + Tetracycline(**8**)**	---	---	---	---
VAMA02(3**) + Tetracycline(**8**)**	---	---	---	---
VAMA02(1.5**) + Tetracycline(**8**)**	NA	NA	---	NA
VAMA02(12**) + Tetracycline(**4**)**	---	---	---	NA
VAMA02(6**) + Tetracycline(**4**)**	---	---	---	NA
VAMA02(3**) + Tetracycline(**4**)**	NA	NA	---	NA
VAMA02(1.5**) + Tetracycline(**4**)**	NA	NA	NA	NA

Table Modulatory assay for VAMA

CHAPTER FIVE

5 DISCUSSION, CONCLUSION AND RECOMMENDATIONS 5.1 DISCUSSION

This project was aimed at determining the antioxidising potential of methanolic extracts of *Voacanga africana* leaves and barks, and further identify, isolate and characterize resistant modulatory compounds present in the extracts. As stated earlier, *Voacanga africana* plants possess a wide range of alkaloids along with other phytochemical constituents, as a result of the plants' metabolic activities. A larger majority of these extracts possess inherent biological activity.

5.1.1 Antioxidant assays

DPPH scavenging activity

DPPH scavenging activity measures the ability of an agent (extract) to scavenge free radicals. The results were documented as "Ic_{50}" which represents the concentration at which half the maximum inhibition was obtained. The results obtained from this assay is summarized in the table 4-1(in the previous chapter).

The activity of the extracts compared to the standard (ascorbic Acid) is a relatively good one bearing in mind that the ascorbic acid is a pure compound and all of its measured mass should contribute to its antioxidant potential. The stem bark extract exhibits better antioxidant abilities (with an Ic_{50} of 70) compared to the leaf extract (with an Ic_{50} of 1605).

Again it can be seen that the antioxidant ability was concentration dependent following the sequential rise in percentage DPPH inhibition for every increase in

concentration. This can be seen from figures 4-1 to 4-3 (in chapter 4) which shows the percentage DPPH inhibition against the concentration of the extract/ standard.

Total antioxidant activity

The total antioxidant activity of the extracts was compared against tannic acid, and is provided in table 4-2. Graphs provided in figure 4-4 and 4-5 express the results. And the results show that the antioxidant potential is dependent on the concentration of the extract. Thus, for every increase in concentration there was a consequential rise in degree of antioxidation.

Total phenolic assay

The extracts were chromatographically separated into bulk fractions and these fractions were determined for their resistant modulatory activity using standard assay procedures. The samples that produced prominent activity were then screened to aid in the identification of the possible phytochemical constituents and identification.

The total phenolic content was calculated in milligram of tannic acid equivalent per gram of the extract. The total phenolic content was identified to be expressed as tannic acid equivalent for the methanolic leaf extract and stem bark extract and was found to be 11738.3 and 13609.6 respectively. This further confirms that the stem bark is relatively more potent than the leaf extract.

5.1.2 Chromatographic products

The methanolic extracts obtained from the stem bark was chromatographically separated and bulked into six fractions. The isolates were bulked based on their thin

layer chromatography profiles chiefly their retardation factors. Six isolates were obtained and labelled VAMA and a suffix number. The solvents used (petroleum ether, ethyl acetate and methanol) were combined in various proportions starting from a single solvent and ending with a single solvent.

VAMA01

Deep brown solution that looks molten on drying. Very soluble in methanol and remains dissolved upon dilution. On heating in water it remains partially dissolved and settles immediately on cooling. Phytochemical screening revealed the presence of alkaloids possibly in large quantities due to the intensity of the pigmentation produced. Glycosides were also identified to be present in the isolate. Preliminary antimicrobial assays showed activity against a large spectrum of organisms including *E. coli and staphylococci.* Minimal inhibitory assay proved that even at relatively lower concentrations than standard antibiotics it had the ability to inhibit the growth of the organisms used in the assay: *E. coli, staphylococci, B. subtilis* and *Pseudomonas.*

VAMA02

This isolate presented as a clear brown solution in methanol and looked powdery on drying. The isolate was rapidly soluble in methanol but only partially soluble in the presence of water. On heating in water, most of it remained undissolved.

Phytochemical screening revealed the presence of Saponnins, triterpenoids, phytosterols and glycosides.

VAMA03

VAMA03 was a blackish solution which looks dark brown when dried with a smooth appearance. Was very soluble in methanol but rapidly forms colloids in the presence of water yet dissolved immediately when the temperature was raised. Phytochemical screening revealed the presence of glycosides.

VAMA04

The isolate labelled VAMA04 was a very dark solution which had patchy violet colors, with a mushy appearance. The isolate was slightly soluble in water but readily soluble in methanol. It was identified to contain alkaloids.

VAMA05

VAMA05 was a dark green solution which looks bright green upon drying. Was slightly soluble in methanol and very insoluble in water even at elevated temperatures. Phytochemical screening showed it had none of the major phytochemicals tested for which included Saponnins, tannins, triterpenoids, phytosterols, glycosides, flavonoids, and alkaloids.

VAMA06

VAMA06 presented as a light green solution which looks bright green upon drying, with a smooth appearance. The isolate was sparingly soluble in methanol but dissolved in large quantities, and remained undissolved in water.
Phytochemical screening revealed the presence of triterpenoids as well as phytosterols at large concentrations judging from the intensity of the bluish to violet ring that appeared.

5.1.3 Antimicrobial assay

Microbial resistance has become a major subject for global health concerns, with a majority of hospitalizations being as a result of infections. Due to this, it comes as no surprise that the scientific community is working relentlessly to find potent drug agents against this everyday havoc. The quest has led to the development of several newer antibiotics and although the list of antimicrobials keep increasing the situation keeps worsening due to the emergence of more resistant microbial strains. The onus now lies on the researchers to develop agents with the ability to overcome both the susceptible and resistant and further remain significant over time. Plants have proven to be a good source of anti-infective agents and thus this work seeks to complement existing findings on plant use by exploring the untapped abilities of the *Voacanga africana* plant.

Antimicrobial assays of extracts from stem bark and leaves

Preliminary antimicrobial assays performed involved agar disk diffusion assays which was used to test the antimicrobial abilities of the extracts as well as the isolates. Agar disk diffusion was chosen in order to eliminate the possibility of inadequate diffusion which occurs in agar well diffusion protocols. Agents that showed significant activity were further subjected to broth dilution assays to help identify their minimum inhibitory concentrations. Organisms used in this work included *Staphylococcus aureus, Escherica coli, Pseudomonas aeruginosa, Bacillus subtilis* and *Candida albicans.* The choice was biased towards those organisms which were well known to exhibit some degree of resistance to existing antimicrobial agents and thus the possibility of achieving a significant result

suggests the suitability of the extract, or isolate as being a good candidate for managing infections caused by such organisms in the future.

From the table 4-6 provided in chapter 4, it is evident that whilst the bark extract possessed very significant activity against the chosen organisms the leaf extract seemingly did not have any significant activity.

Compared to ciprofloxacin which is a standard antibiotic belonging to the Fluoroquinolone drug class and a very potent antibiotic, the bark extract showed very close mean diameter of inhibitions against almost all the strains of bacteria that were used in the assay. Because a fungus was used in the assay it was necessary that a standard antifungal agent be employed as well. The result obtained showed that the fungus (*Candida albicans*) was very susceptible to the standard antifungal agent (ketoconazole).

Antimicrobial screening of the isolates

Column chromatography and intermittent thin layer chromatography resulted in the production of six different isolates which exhibited different retention times on thin layer chromatographic plates and presented with different solubility characteristics. Each of this isolate was screened for antimicrobial properties and the results encouraged the further determination of minimum inhibitory assays and resistance modulatory assays. The results obtained for preliminary screening of the individual isolates are tabulated in the table 4-6.

As stated earlier the agar disk diffusion was used to determine if the substance used (isolate) will exhibit a significant antimicrobial activity. From the results obtained

the extracts VAMA01 and VAMA02 possessed high and very significant antimicrobial activity which made them suitable candidates for further determinations. Compared to the other isolates, VAMA01 and VAMA02 showed a relatively higher activity which suggested that their components were the likely constituents responsible for the general effect observed in the methanolic extract. For this reason the isolates VAMA01 and VAMA02 were the only isolates used in the antimicrobial resistance modulatory assays.

The results also shows a broad spectrum of antibacterial activity due to the ability of the isolates to inhibit both gram negative and gram positive bacteria as well as a very meaningful antifungal potential. *Escherica coli* was the most susceptible organism against the most active isolates (VAMA01 and VAMA02) whereas the less potent isolates exhibited little to possibly no activity.

Minimal inhibitory assays

Eight different concentrations of the extract, isolates and standard antibiotics were prepared and used for these determinations. Because of the plants inherent poisonous nature low concentrations were used in the assay. The concentrations of the isolates chosen started from as low as 0.5mg/ml increasing serially to 64mg/ml where each concentration was half of the succeeding concentration. The standard drugs were also prepared into eight different concentrations. For the standard antimicrobial agents the starting concentrations were antibiotic specific none the less the concentrations was increased exponentially by a value of two times the preceding. The results are summarized in tables 4-9 and 4-10.

From table 4-9 which documents the minimal inhibitory concentration results obtained when the various antibiotics and extracts were determined against the cultured organisms. It was evident that the methanolic leave extract showed no activity against all the microorganisms used in the assay at all the respective concentrations after the incubation period. This could be possibly due to the lack of any metabolites which could possess any significant antimicrobial activity. Contrary, the methanolic bark extract showed very significant antimicrobial activity with relatively very low minimal inhibitory concentrations which represents an ability to inhibit growth at even small concentrations. When used against all five organisms a minimal inhibitory concentration of >4mg/ml was observed against *S. aureus, E. coli and B. subtilis.* Against *P. aeruginosa* and *Candida albicans* the minimal inhibitory concentrations recorded was >8mg/ml which is relatively significant compared to the MIC's recorded for the standard antibiotics which were used in the assay.

Amoxicillin possessed some degree of activity against the used microbes at various concentrations of which *P. aeruginosa* exhibited the highest MIC which was >64mg/ml. When used for Candida albicans it showed no activity and a MIC of >16mg/ml was recorded when it was used against *S. aureus* as well as >32mg/ml when it was used against *E. coli* and *B. subtilis*. The broad spectrum activity of amoxicillin makes it very suitable for clinical use.

Ciprofloxacin with as low as >2mg/ml showed the lowest minimal inhibitory concentrations amongst all the antibiotics used in the assay against two of the test organisms thus *B. subtilis* and *E. coli.* The other strains of bacteria used in the assay

were inhibited at a MIC of >4mg/ml except for *Candida* which was a fungus and could not be inhibited by the ciprofloxacin in the assay. Ciprofloxacin like amoxicillin is a broad spectrum antibiotic and thus its ability to inhibit all strains of bacteria at such low concentrations justifies its everyday use as a first choice antibiotic for resistant infections and general infections in patients who are penicillin sensitive.

Erythromycin, a macrolide antibiotic was also used in the assay and it also showed some level of activity against all the strains of bacteria that was used but not against the fungi (*Candida albicans*). Tetracycline exhibited some antibacterial activity and surprisingly against *Candida albicans* but at a relatively higher concentration. This degree of activity is of interest because it was the same minimal inhibitory concentration observed when the standard antifungal agent (ketoconazole) was used. Ketoconazole, the only antifungal agent employed in the assay showed no activity against the bacteria used in the assay but an activity against the fungi (*C. albicans*) was observed.

The isolates obtained from the chromatographic separation of the methanolic stem bark extract also showed some degree of antibacterial activity,(this can be seen from table 4-9) with the most polar ones having a minimal inhibitory concentration as low as >4mg/ml. From table 4-9 it can be observed that as the isolates were becoming non polar their activity against the organisms diminished gradually, the last observation of activity was made for isolate VAMA04 which required a relatively higher concentration to elicit an effect against the same strains of bacteria

used in the assay. Isolates VAMA05 and VAMA06 showed no activity at all neither against the bacteria nor the fungi employed in the assay.

Resistant modulatory assays

Microorganisms chosen for this assay were based on current resistant patterns documented in recent findings. Of major interest was *Staphylococcus aureus* which has been implicated in Multidrug Resistance and more commonly referred to as a "superbug". *Staphylococcus aureus* is known to be able to pump out antibiotics through the efflux pump mechanisms. *Pseudomonas aeruginosa* is also another micro bacteria which has exhibited strong resistance to existing antibiotics. It is known to form "*biofilms*" which serves as a protective coating to its cell structure and thus preventing the antibacterial agent from getting to it cell surface.

E. coli and *B. subtilis* are also known to show some resistance to commonly used antibiotics and this is why they are also employed in the assay.

The resistance modulatory assay aimed at identifying possible activity would when both the standard antibiotic and the isolate were combined at concentrations below their Minimal inhibitory concentrations. To achieve this it was ensured that the final concentrations in the well were just a step above the MIC and at the MIC and then sequentially reduced to six further half dilutions below the MIC making a solution of eight different concentrations of the standard antibiotic whilst the chromatographic isolates were maintained at only four concentrations also a step above the MIC and three below. Based on the results obtained from the agar disk diffusion assays as well as the broth diffusion assay two of the chromatographic

isolates were further subjected to resistance modulatory assays; VAMA01 and VAMA02. The chromatographic isolates were tested for synergistic activity with only three of the used antibiotics and these were amoxicillin, erythromycin and tetracycline. The results obtained from the assay were recorded in table 4-11, 412, 4-13, 4-14, 4-15 and 4-16.

Resistance modulatory assay results for chromatographic isolate VAMA01

From table 4-11, very significant result was observed when the isolate was combined with amoxicillin at various concentrations. When amoxicillin at amoxicillin (MIC: >16mg/ml) was combined with isolate VAMA01 (MIC: >4mg/ml) at concentrations of 8mg/ml and 3mg/ml there was inhibition of Staphylococcal growth. The exact mechanism by which this occurs is unknown but a possible synergistic effect is observed when both agents are used in combination.

Against *Bacillus subtilis* a combination of 3mg/ml of the chromatographic isolate VAMA01 (MIC: >4mg/ml) and 8mg/ml of the amoxicillin (MIC: >32mg/ml) was enough to inhibit the growth of the organism. A similar combination was also sufficient to inhibit the growth of *Escherica coli* with a similar MIC's. A relatively higher concentration was required of both the extract and the isolate, yet there was an inhibition of microbial growth below the initial MIC.s observed when the agents were used alone in the nutrient broth dilution assay. *Candida albicans* was not inhibited below the MIC obtained for the individual agents. Thus the isolate does not exhibit any synergistic effects when giving with amoxicillin on *Candida albicans*, these results are showed in the table 4-11.

Combined with Erythromycin (table 4-12) there was no significant activity below the minimal inhibitory concentrations when tested against four of the organisms; *B. subtilis, E. coli, P. aeruginosa* and *Candida albicans*. The only activity observed was against *Staphylococcus aureus* which is known to be among the superbug family. This outcome could possibly be due to synergistic effect produced upon combination of the two agents.

The extract was combined with tetracycline at concentrations below the minimal inhibitory concentration. Although it showed no activity against *Pseudomonas aeruginosa* and *Candida albicans* it produced some level of inhibition when used against *Staphylococcus aureus, E. coli* and *B. subtilis*. For *Staphylococcus aureus,* it required 3mg/ml of the chromatographic isolate (MIC >4mg/ml) and 8 mg/ml of Tetracycline (MIC > 16mg/ml) the same was observed for B. subtilis at the same concentrations and with the MIC's being the same. Similar explanations can be given as above for the results that are observed in table 4-13

Resistance modulatory assay results for chromatographic isolate VAMA02

Chromatographic isolate VAMA02, obtained from the methanolic extract of the stem bark was also tested for the ability to modulate resistance in the same manner as the isolate VAMA01.

When combined with amoxicillin it was not capable of inhibiting the growth of *Escherica coli, Bacillus subtilis* and *Candida albicans* at any of the concentrations below the minimal inhibitory concentrations. *Staphylococcus aureus* was well inhibited by the combination at concentrations of 3mg/ml and 4mg/ml of the

chromatographic isolate (MIC: > 4mg/ml) and amoxicillin (MIC: > 16mg/ml) respectively. This activity means that the combination is capable of overcoming the active efflux pumps of the *Staphylococcus aureus* although the mechanism by which this occurs is not covered in this study. *Pseudomonas aeruginosa* a biofilm forming organism was also inhibited by the combination of the chromatographic isolate VAMA02 (MIC: > 8mg/ml) and amoxicillin (MIC : >64mg/ml) at lower concentrations below the individual MIC's thus 3mg/ml for the former and 8mg/ml for the latter. The results for this assay are expressed in table 4-14.

The chromatographic isolate was also combined with Erythromycin at various concentrations below the individual MIC's. Against *Bacillus subtilis, Pseudomonas aeruginosa* and *Candida albicans* there was no inhibition of growth when the two agents were combined. Significant inhibition was achieved when tested against *Staphylococcus aureus* at concentrations of 3mg/ml and 4mg/ml of the chromatographic isolate (MIC: >4mg/ml) and erythromycin (MIC: >8mg/ml).

Also against *Escherica coli* there was inhibition of growth at concentrations below the MIC's when 6mg/ml of the chromatographic isolate (MIC: > 8mg/ml) was combined with 2mg/ml of Erythromycin (MIC: > 8mg/ml). These observations can be seen in table 4-15. Thus the extract can be combined with Erythromycin to significantly inhibit the growth of *S. aureus* and *E. coli*.

When the chromatographic isolate was also combined with tetracycline there was significant inhibition of the growth of *Staphylococcus aureus*. This was achieved using a 3mg/ml of the chromatographic isolate (MIC: >4mg/ml) combined with 8mg/ml of tetracycline (MIC: >16mg/ml). None of the combinations below the

MIC's was enough to inhibit the growth of *Bacillus subtilis*, *Pseudomonas aeruginosa* and *Candida albicans*. Yet a 3mg/ml and 4mg/ml combination of the chromatographic isolate VAMA02 (MIC: >8mg/ml) and Tetracycline (MIC: >8mg/ml) respectively. The results for this assay can be observed in the table 4-16.

5.2 CONCLUSION

Tenacious efforts are been made every day to combat the seeming threat that microbial infections and their resistance pose to the health of mankind. This work seeks to supplement the enormous work done so far. It was aimed at identifying and isolating any biologically active makers which are present in either the leaf or stem bark of the methanolic extract of *Voacanga africana*.

To achieve this the antioxidant potential of both extracts were compared and their antimicrobial activity determined, as a progress step isolates of the extracts were obtained and determined for their resistant modulatory activity and their phytochemical constituents determined.

The antioxidant assay revealed a concentration dependent antioxidant activity where the activity obtained from the methanolic extract of the stem bark (Ic_{50} = 70) was very significant compared to that obtained from the methanolic leaf extract (Ic_{50} = 1605), with ascorbic acid as a standard (Ic_{50} = 6.980). Antioxidant activity was also found to be very closely related to the amounts of phenolic compounds which were present in both extract.

In vitro antimicrobial action revealed that methanolic extracts of the stem bark possessed very significant antimicrobial activity at relatively lower concentrations whereas the methanolic leaf extracts showed no activity at similar concentrations. This led to the chromatographic separation of the methanolic stem bark extract; producing six bulk solutions based on their thin layer chromatography profiles.

The extracts labelled with a prefix of VAMA and a suffix of 01 to 06 were screened for phytochemicals. The screening revealed the presence of various metabolites in the individual extracts including alkaloids, glycosides, tannins, Saponnins, phytosterols, flavonoids and triterpinoids.

Amongst the isolates VAMA01 and VAMA02 were found to possess good antimicrobial activity and resistant modulatory activity which is summarized in the table 5-1 (provided in the appendix).

5.3 RECOMMENDATIONS

Following the work done so far, the under listed recommendations are made for further work

In vivo assays be carried out on the isolates that exhibited significant resistance modulatory activity. Identification of the compounds responsible for the activities Characterization of the chemical compounds present in the isolate.

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APPENDICES

Summary of resistance modulatory results for VAMA01 and VAMA02

	Minimum inhibitory concentration (MIC)/mg/mL			
ORGANISMS	S. aureus	B. subtilis	E. Coli	P. auregenosa
VAMA01				
Amoxicillin	(4) **3 : 8** (16)	(4) **3 : 4** (32)	(4) **3 : 4** (32)	(8) **6 : 16** (64)
Erythromycin	(4) **3 : 4** (8)	TG	TG	TG
Tetracycline	(4) **3 : 8** (16)	(4) **3 : 8** (16)	(4) **3 : 4** (8)	TG
VAMA02				
Amoxicillin	(4) **3 : 4** (16)	TG	TG	(8) **3 : 8** (64)

Erythromycin	(4) **3 : 4** (8)	TG	(8) **6 : 2** (8)	TG
Tetracycline	(4) **3 : 8** (16)	TG	(8) **3 : 4** (8)	TG

KEY;(MIC of isolate) concentration of extract : concentration of antibiotic (MIC of antibiotic)