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DEPARTMENT OF PHARMACEUTICAL CHEMISTRY

PHYTOCHEMICAL, ANTIMICROBIAL, AND ANTIOXIDANT CONSTITUENTS OF THE STEM BARKS OF *TRICHILIA MONADELPHA* (THRONN) J.J. DE WILDE AND *TRICHILIA TESSMANNII* (HARMS) BOTH OF THE FAMILY MELIACEAE

by

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DECLARATION

I wish to declare that this thesis is my own work. I further declare that to the best of my knowledge, this thesis does not contain any material previously published by any person or any material which has been accepted for the award of any degree of a university, except where due acknowledgement has been made in the text

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Dr. Isaac Ayensu ....

(Supervisor) Signature Date

Certified by:

Dr. Isaac Ayensu ....

(Head of department) Signature Date

DEDICATION

To my family

ABSTRACT

In this present work, the stem barks of two medicinal plants, *Trichilia tessmannii* (Harms) and *Trichilia monadelpha* (Thonn) JJ De Wilde both of the family Meliaceae were screened for their phytochemical constituents and evaluated for their antimicrobial and antioxidant activities with the sole objective of isolating the active principles present. The phytochemical investigation revealed the presence of plant secondary metabolites such as; Tannins, Reducing sugars, Anthraquinones, Alkaloids, Saponins glycosides, Cardiac glycosides, Terpinoids, Cumarins and Phytosterol.

The antimicrobial activity was determined by broth dilution method using standardized bacteria suspensions of *Staphylococcus aureus, Bacillus subtilis, Escherichia coli, Pseudomonas aeruginosa* and the fungus, *Candida albicans.*

Broad-spectrum antimicrobial activity was observed for all extracts except the petroleum ether extract of *T. tessmannii*. A minimum inhibitory concentration (MIC) of 0.625 mg/mL was determined as the highest activity for the ethyl acetate extract of *T. tessmannii* against *Pseudomonas aeruginosa* and *Candida albicans.* Lowest MIC of >10 mg/mL was however recorded by the petroleum ether extract of *T. tessmannii* against *Bacillus subtilis, Escherichia coli, and Pseudomonas aeruginosa*.

Antioxidant activity was determined by the use of DPPH free radical scavenging assay, Total phenol content assay and Total antioxidant capacity assay on the total crude methanol extract of the stem bark. The total crude methanol extract was found to possess DPPH free radical scavenging activity of EC_{50} values 0.0207 mg/mL and 0.0099 mg/mL for the extracts of *T. tessmannii* and *T. monadelpha* respectively and 0.004806 mg/mL for the ascorbic acid used as the reference drug. The values obtained from the estimation of the Total antioxidant capacity assay were 444.7 ± 28.58 mg/g of ascorbic acid and 324.40 ± 20.41mg/g of ascorbic acid for *T. tessmannii* and *T. monadelpha* respectively, and the Total phenol content values

for *T. tessmannii* and *T. monadelpha* were 970.2 ± 78.69 mg/g of tannic acid and 884.2 ± 61.00 mg/g of tannic acid respectively. These findings provide scientific justification for the use of the stem barks of *T. tessmannii* and *T. monadelpha*, in various traditional medicines, for the treatment and management of diseases associated with oxidative stress and infectious conditions. The chromatographic separation of extracts done so far was by open column silica gel chromatography using the gradient elution method. Chromatographic separation of the ethyl acetate extract of the stem back of T. *Monadelpha* (TME) has yielded an isolate 'A_1', which is yet to be further purified and characterized.

ACKNOWLEDGEMENT

"I will extol the Lord at all times; his praises will always be on my lips." Psalm 34:1

Thank you Lord for your abundant grace and mercy throughout the period of this research.

My sincere gratitude goes to Doctor Isaac Ayensu my supervisor, who kindly supervised this research. His ideas, advice and tireless encouragement have been most valuable. May you abound in the grace and blessings of God. I also appreciate greatly the support of all the lecturers of the Department of pharmaceutical chemistry and to Professor Abraham Yeboah Mensah and Professor Merlin Mensah of the Departments of Pharmacognosy.

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To my lovely parents, Mr. and Mrs. Quartey and my dear brothers Emmanuel Quartey, Duke Quartey and Jonathan Quartey, may God richly bless you for all your support and encouragement.

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CHAPTER ONE

INTRODUCTION

1.1 GENERAL INTRODUCTION

The use of plants as medicines predates written human history (Rios and Recio, 2005). Plants are great sources of medicines, especially in traditional medicine, which are useful in the treatment of various diseases. Many of the herbs and spices used by humans to season food have also yielded useful medicinal compounds (Lai and Roy, 2004). Traditional medicine has not only played a vital role in providing healing but has also contributed to the discovery of most pharmaceutically active substances (Ngo *et al.*, 2013) which have been used in the commercial production of drugs. Some of these pharmaceutically active single chemical entities include: aspirin, morphine, quinine, artemisinin, amodiaquine and many others (Iwu, 2014).

During the twentieth century, the interest of the pharmaceutical industry shifted from the use of these natural based products (for the discovery of drugs) to synthetic chemistry (combinatorial chemistry). This trend has dominated research and developments in the industry during the period. Thus, this has replaced natural extracts with synthetic molecules that have no connection to natural products. Though this has helped treat, prevent and saved many lives over the years, these synthetic drugs have serious side effects. Also the discovery of *de novo* entities are very low with combinatorial chemistry (Newman and Cragg, 2012). These setbacks of the combinatory chemistry trend have awakened interest in the use of natural products especially medicinal plants as source of pharmaceutical agents. It is believed that natural products provide greater structural diversity than standard combinatorial chemistry and so they offer major opportunities for finding novel low molecular weight lead structures that are active against a wide range of assay targets (Harvey, 2000).

However, as the environment changes and the world develops as well, areas of high biodiversity are losing species. The rate of plant species extinction is increasing rapidly, the multitude of potentially useful phytochemical structures which could be obtained and used directly as medicinal agents or used as templates for the synthesis of chemically active agents are at risk of being lost permanently (Cowan, 1999).

This project therefore seeks to scientifically investigate and isolate the antimicrobial and antioxidant agents present in two medicinal plants namely; *Trichilia monadelpha* (Thronn) J.J. de wilde and *Trichilia tessmannii* (Harms) both of the family Meliaceae.

1.1.1 Natural products as bioactive agents for drug discovery

Natural products have long been recognized as a major source of new and therapeutically effective medicines. Many successful drugs currently in use were originally synthesized to mimic the action of molecules found in them. They are the most consistently successful source of drug leads (Kingston, 2010). Natural product scientists claim that these products form the basis for many of the drugs currently in commercial use or in development (Cragg *et al.*, 1997).

Newman *et al.*, (2003) analyzed a number of drugs obtained from natural products which were present in the total drug launches from 1981 to 2002. From their research, they concluded that natural products still play an important role in sourcing for novel drug moieties, especially antihypertensive and anticancer agents.

Among the top 35 worldwide selling ethical drugs sales of 2000, 2001 and 2002, drugs obtained from natural products are well represented percentage-wise. Drugs derived from these products represented approximately 40 %, 24 % and 26 % in 2000, 2001 and 2002 respectively (Butler, 2008).

Between 2000 and 2003, about 15 drugs derived from natural products were launched. These included; new anti-malarial agent arteether, anti-fungal agent caspofungin (Cragg and

Newman, 2013), , anti- Alzheimer's drug and anti-bacterial drug (Graul, 2004). Most of these drugs or agents have progressed into phase III clinical trials and are hoped to be on the market soon for use (Butler, 2008).

Of the 20 best-selling non-protein drugs in 1999, nine were either derived from or developed as the result of leads generated by natural products. Examples include; simvastatin, lovastatin, enalapril, pravastatin, atorvastatin, augmentin, ciprofloxacin, clarithromycin and cyclosporin with combined annual sales of US$16 billion. Newer developments based on natural products include the antimalarial drug artemisinin and the anticancer agents taxol, docetaxel and camptothecin (Harvey and Waterman, 1998; Grabley and Thiericke, 1999). The use of natural products has been the single most successful strategy for the discovery of new medicines. In addition to this historical success in drug discovery, natural products are likely to continue to be sources for new commercially viable drug leads (Harvey, 2000).

1.1.2 Medicinal plants as sources of drugs

In plants, the chemical reactions that sustain life, is known as 'metabolism'. This process produces a number of different chemicals, which are called 'metabolites'. Plants have the ability to synthesize a wide variety of chemical compounds (primary and secondary metabolites) that are used to perform important biological functions, and to defend against attack from predators such as insects and other herbivorous mammals (Cowan, 1999).

These secondary metabolites are organic compounds that are not directly involved in the normal growth development or reproduction of an organism. Unlike primary metabolites, absence of secondary metabolites does not result in immediate death, but rather in long-term impairment of the organism's survivability (Fraenkel, 1959).

Most biologically active natural product compounds are secondary metabolites with complex structures. Humans use these secondary metabolites such as alkaloids, terpenoids, glycosides, natural phenols and saponins as medicines (Baliah and Astalakshmi, 2014).

It is estimated that about 60% of the world's population depend basically on plants for medication (Hafidh *et al.*, 2009). The World Health Organization (WHO) estimates that 80 percent of the population of some Asian and African countries presently use herbal medicine for some aspect of primary health care, e.g. new anti-malarial drugs were developed from the discovery and isolation of artemisinin from *Artemisia annua* L., a plant used in China for almost 2000 years (WHO, 2002)

Plants supply most of the active ingredients of traditional medicinal products, and plant extracts have long been used in screening programmes in pharmaceutical companies and university research institutes (Harvey, 2000). It might be thought that most of the plant kingdom has been thoroughly examined in the search for biologically active molecules. However, this is not the case. It is believed that only about 10% of the known 250,000 species of plants in the world have been tested for any active molecules and less than this have been studied extensively (Verpoorte, 1998). There are many habitats which are yet to be explored. Recent research shows that new species are being discovered from these habitats (Harvey, 2000). It is likely that these areas could yield useful drugs.

In spite of the increasing use of medicinal plants, their future, apparently, is being threatened by complacency concerning their conservation. Reserves of these medicinal plants in developing countries are diminishing and in danger of extinction (Hoareau and DaSilva, 1999). As more diseases are discovered, a continual need for new sources of drugs is crucial.

Plants are very likely to continue to be important in the search of these new agents.

1.1.3 Why plants are used as source of medicines

The role of plants in the development of medicine cannot be underestimated. Over the past centuries, numerous purified compounds have proven to be indispensible in the practice of modern medicine (Singh and Barrett, 2006). Examples of these agents include; cardiac glycoside (digitalis), opiate (codeine and morphine), cinchona alkaloids (quinine and

quinidine) and diterpenoid (taxol) which are all well-known agents used as drugs (Li and Vederas, 2009).

It has been reported that about 25 % of the drugs prescribed worldwide come from plants (Rates, 2001). Out of the over 252 drugs that the World Health Organisation (WHO) considers as basic and essential, 11 % are exclusively of plant origin and a significant number of many of the synthetic drugs are obtained from natural precursors (WHO, 1992). The WHO also states that about 80% of the people in developing countries rely on traditional medicine for their primary health.

There are several reasons why there has been great interest in drugs of plant origin. These include:

- It is believed that plants are readily accessible to folks for use (WHO, 2002).
- That conventional medicine can be ineffective and abusive or incorrect use of synthetic drugs results may be accompanied by severe side effects.
- A large percentage of the world's population does not have access to conventional pharmacological treatment (WHO, 2002).
- Therapies from these sources are considered to be affordable especially for the world's poorest patients. They are sometimes considered to be cheaper and sometimes not even paid for or maybe paid for in kind (WHO, 2002).
- Plants can be used as therapeutic agents in several ways. They can be used as tinctures, teas, extracts and other homemade remedies (Rates, 2001).
- The patronage and use of plants accords with most customs and cultural practices of non-urban/rural dwellers worldwide (WHO, 2002).

1.1.4 Organic chemistry in the development of drugs from natural sources

Natural products have inspired many developments in organic chemistry (Wilson and Danishefsky, 2006). There has been the advancement in synthetic methodologies and this has led to the development of analogues of original lead compound with improved pharmacological or pharmaceutical properties (Newman, 2008).With the application of various techniques to create analogues and derivatives of natural products, it has become possible to derive novel compounds that can be patented (Raskin *et al.*, 2002).

There has been new approaches to add value to natural products by the application of advances in fractionation techniques to isolate and purify these natural products (Wu *et al.*, 2008). Furthermore, improvements in analytical techniques in the screening of natural product mixtures has helped to determine their structures with a very short time (Singh and Barrett, 2006; Harvey, 2007).

There have been advances in NMR techniques; complex structures can now be elucidated with less than 1 mg of compound (Quinn *et al.*, 2008). Several alternative approaches are also being explored in efforts to increase the speed and efficiency with which natural products can be applied to drug discovery. Ethnobotany is now recognized as an effective way to discover future medicines.

1.1.4 Useful drugs from medicinal plants

The use of plants as medicines by humans predates written human history. In present history plants as medicines still play very crucial role in the treatment of various diseases (Singh and Barrett, 2006).

Plants; its constituents and other products obtained from them may be used in different ways as sources of therapeutic agents (Fabricant and Farnsworth, 2001). The various ways of

applying the plant materials as therapeutic agents may be accessed in four different ways. These include; (a) To use the whole or part of the plant as crude herbal preparation. Examples include; senna leaves, garlic, ginseng, ginkgo biloba, cranberry, St. Jonh's wort, feverfew, aloe vera and saw palmetto. (b) The active constituents of the plant may be isolated and the isolates used directly as drugs. Examples include; taxol [**7**], vinblastine, vincristine, morphine [**9**], digoxin, digitoxin, reserpine [**2**], quinine [**13**] and quinidine [**6**] (Tables 1.1 and 1.2). (c) The produced bioactive or isolated compounds may serve as templates for the synthesis of new drugs to produce patentable agents of higher potency with lower toxicity. Examples include; aspirin [**10**], amodiaquine [**12**], artemether [**14**], oxycodon, metformin [**16**] and taxotere based on salicin [**11**], quinine [**13**], artemisinin [**15**], morphine [**9**], galegine [**17**] and taxol [**7**] respectively (Tables 1.1 and 1.2). (d) By using the agents obtained from these plants as pharmacological tools or probes. Examples; mescaline [**1**]; used for its hallucinogenic properties and yohimbine [**3**]; used as a probe for α-2 adrenoceptors. Tables 1.1 and 1.2 below shows some useful drugs with their structures obtained from plants.

There is the need to search for more pharmacologically active agents from plants with the hope of obtaining entirely new drugs or lead compounds. The study of plants with ethnopharmacological uses has yielded several useful drugs and it is believed that the continuous study of them may yield even newer drugs (Harvey, 2000). It is also important that research in drug discovery should not be viewed as a means only to discover new compounds, but also be seen as a way to develop the most promising plant therapies into unmodified natural products (crude extracts), semi-refined products and other acceptable formula (Stark *et al.*, 2013).

Table 1.1: Drugs: structures, plant source and medicinal use(s)

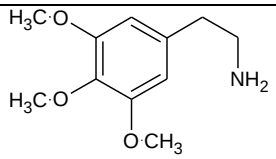
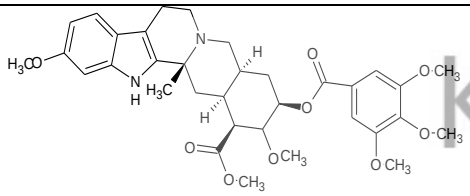
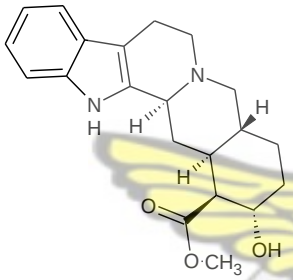
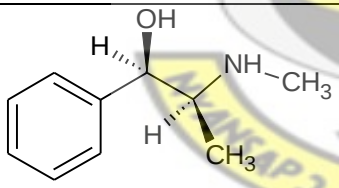
Drug name and structure	Plant source	Use(s)
 Mescaline [**1**]	*Lophophora williamsii* (Cactaceae)	Used for its hallucinogenic properties
 Reserpine [2]	*Rauwolfia vomitoria* (Apocynaceae)	Used in the management of essential hypertention and in certain Neuropsychiatric disorders.
 Yohimbine [**3**]	*Pausinystalia yohimbe* (Rubiaceae)	Used in the symptomatic treatment of erectile dysfunction. Used as a probe for α-2 adrenoceptors
 Ephedrine [**4**]	*Ephedra sinica* (Ephedraceae)	Sympathomimetic, decongestant, hypotension, appetite suppressant

Table 1.1 cont'd

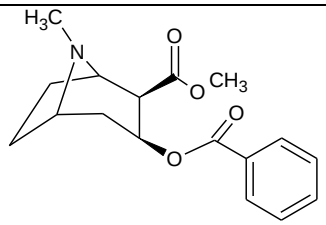
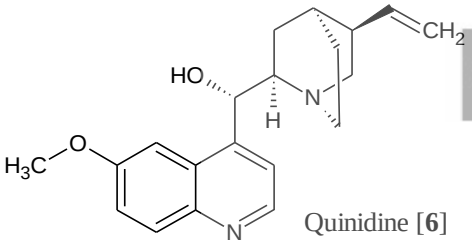
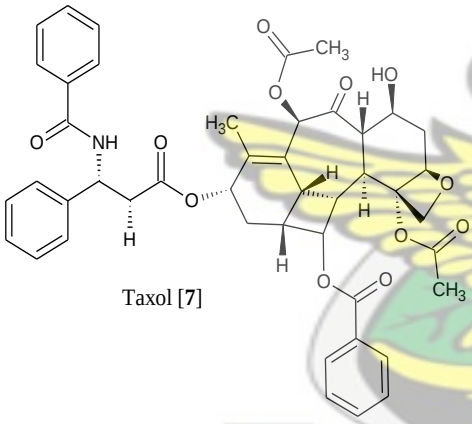
Drug name and structure	Plant source	Use(s)
 Cocaine [**5**]	*Erythroxylum coca* (Erythroxylaceae)	Stimulant, appetite suppressant and Local anaesthetic
 Quinidine [**6**]	Cinchona sucirhubra (Rubiaceae)	Used as an Antiarrhythmic agent.
 Taxol [**7**]	*Taxus brevifolia* (Taxaceae)	Used in the treatment of solid tumors such as ovarian cancers and breast cancers.

Table 1.2: Drugs: structures, lead compounds, plant source and medicinal use(s)

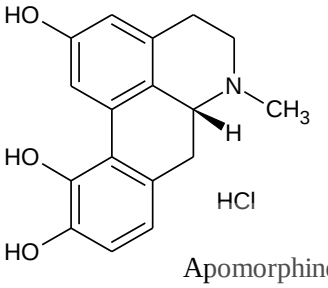
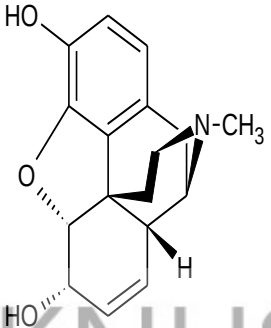
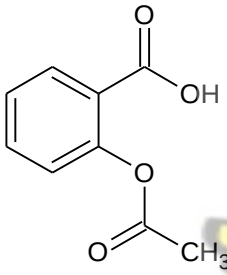
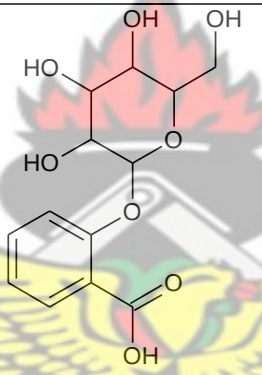
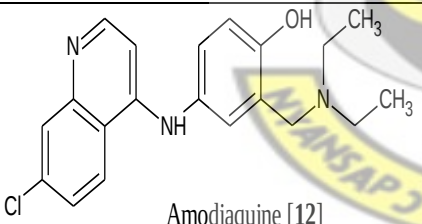
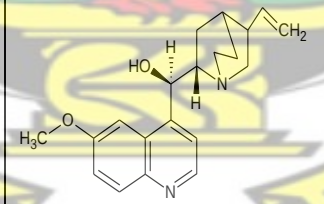
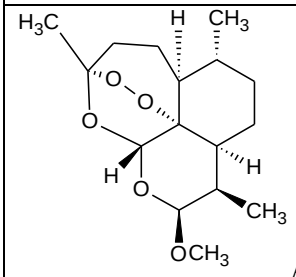
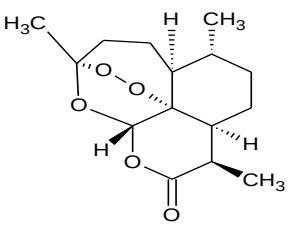
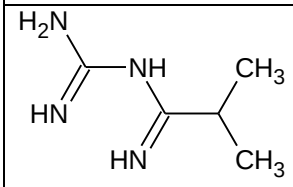
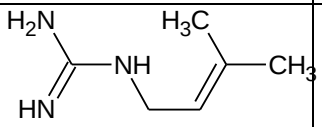
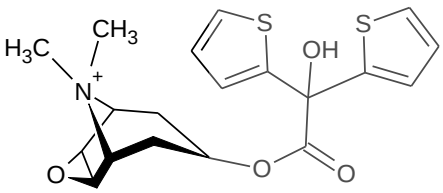
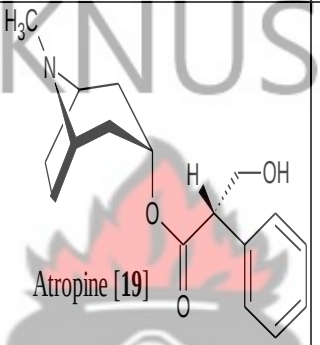
Drug name and structure	Lead compound	Plant source	Use(s)
 Apomorphine HCl [**8**]	 Morphine [**9**]	*Papaver somniferum* (Papaveraceae)	Used in the management of parkinson's disease.
 Aspirin [**10**]	 Salicin [**11**]	*Salix fragilis* and *Salix purpurea* (Salicaceae)	Analgesic, antipyretic and anti-inflammatory agent.
 Amodiaquine [**12**]	 Quinine [**13**]	*Cinchona succirubra* (Rubiaceae)	Used in the treatment of malaria.
 Artemether [**14**]	 Artemisinin [**15**]	*Artemisia annua* (Asteraceae)	Used in the treatment of malaria.

Table 1.2 cont'd

Drug name and structure	Lead compound	Plant source	Use(s)
 Metformin [**16**]	 Galegine [**17**]	*Galega officinalis* (Fabaceae)	Management of diabetes.
 Tiotropium [**18**]	 Atropine [**19**]	*Atropa belladonna* (Solanaceae)	Used in the treatment of bronchospasm associated with COPD.

1.2 JUSTIFICATION AND SIGNIFICANCES OF RESEARCH WORK

The emergence and the spread of multidrug-resistant pathogens are a major public health threat to the successful treatment of infectious diseases. As antibiotic resistance continues to grow and novel infections spread, the number of newly approved antibiotic agents is on the decline, as only six new antibiotics were approved since 2003 despite the medical need for them (Donadio *et al.*, 2010).

Since there is a perpetual need for antibiotics to treat infections, the discovery of new agents from underexplored natural products such as plants is worthwhile. The decline in the discovery of these antibiotics is as a result of low revenues obtained from the sale of these agents as compared to other drugs on the market. Secondly, this low revenue is due to the fact that antibiotic prescriptions last for only few weeks unlike that for chronic illnesses that lasts

for years (Fischbach and Walsh, 2009). Thirdly, as newly approved drugs can be prescribed without restrictions that of newly developed antibiotics are restricted. All these reasons assert to the decline in the development of new antibiotics despite the increase in resistance to old antibiotics (Nathan, 2003).

In view of this, there is therefore an urgent need for the discovery and development of new antimicrobial agents to solve the issue of multidrug resistance and the spread of novel and co-infections. The importance of natural products especially medicinal plants in the discovery of antimicrobial agents cannot be down played. Medicinal plants have a long history in traditional medicine and have served as promising reservoir for untapped molecules with diverse chemical structures for potentially new lead compounds for the development of antimicrobial agents.

In severe infections and chronic diseases there may be excess production of free radicals and so there is the need for antioxidants to mop up these free radicals to prevent excess damage to cells and tissues of the host organism.

Recently, the search for antioxidants that can counter these effects is on-going. Medicinal plants have taken a centre stage in this search since most dietary and other constituents of these plants such as, ascorbic acid (vit. c), tocopherol (vit. E), phenols, coumarins and flavonoids, have been found to have major antioxidant activity (Devasagayam *et al.*, 2004).

1.2.3 Aims

The aim of this research work is to scientifically investigate and compare the anti-infective and antioxidant potential of the stem bark of two medicinal plants namely *Trichilia monadelpha* (Thronn) J.J. de wilde and *Trichilia tessmannii* (Harms) both of the family Meliaceae with the sole objective of isolating the bioactive agents and characterizing them.

1.2.4 Specific objectives

In order to achieve this aim the following specific objectives were set;

- To perform phytochemical screening on the pulverized plant materials.
- To carry out crude extraction on the selected medicinal plants.
- To screen and compare both extracts obtained for anti-infective and antioxidant activities.
- To fractionate, isolate and elucidate the structures of the constituents of the active extracts.
- To test the isolated constituents for anti-infective and antioxidant activities.

CHAPTER TWO

LITERATURE REVIEW

2.1 INFECTIOUS DISEASES

2.1.1 The burden of infectious diseases

Infectious diseases are illnesses that are caused by pathogenic microorganisms, such as bacteria, viruses, protozoa, parasites or fungi or their toxins (Cohen and Williamson, 1991). The infection can be spread directly or indirectly, from one person to another. Zoonotic diseases are infectious diseases of animals that can cause disease when transmitted to humans. The root causes of these infections are attributed to poverty and its associated problems of unhygienic living conditions, malnutrition, illiteracy and poor access to clean water, toilet facilities and quality healthcare (Sinniah *et al.*, 2014) . According to the World Health Organisation (WHO), infectious diseases are the world's biggest killers, affecting both children and adults. Infectious diseases now account for one in ten deaths in the industrialized countries while in the developing countries, it accounts for six out of every ten deaths (WHO, 2004; Mathers *et al.*, 2008). It is believed that about 33% of all reported deaths are caused by infectious and parasitic disease in Ghana (Tabi *et al.*, 2006).

Against the constant background of established infections, epidemics of new and old infectious diseases periodically emerge, greatly magnifying the global burden of infectious diseases (Morens *et al.*, 2004). Due to this, there is an increase in the interest of global health issues and has led to increased levels of financial support, which combined with recent technological advances and research into finding new agents, are all geared towards curbing this public health issue.

Recently, there has been the connection of certain diseases that were not thought to be associated with microbial infections to be now associated with them directly or indirectly such that some cancers are now being attributed to some viruses Coronary heart diseases have

also have been linked to some chronic infections with microorganisms (Vuorela *et al.*, 2004). Peptic ulcer and gastric carcinoma have been associated with helicobacter pylori (Chang *et al.*, 2005). Other chronic diseases such as hepatocellular carcinoma, diabetes and multiple sclerosis have also been associated with some microbial infections (Karin *et al.*, 2006) .

The spread of multidrug-resistant pathogen is also a major setback to the treatment of infectious diseases all over the world. Resistance to antibiotics is an inevitable challenge. There is therefore the need to develop new antibiotics and also manage the use of these new antibiotics to prevent resistance (Singh and Barrett, 2006). In these past years, the spectrum and frequency of antimicrobial-resistant infections have increased and the antibiotics in use are gradually being rendered useless (Iwu *et al.*, 1999). The Centre for Disease Control (CDC) based on these negative health trends of infectious diseases have renewed the strategies on the treatment and prevention of infectious diseases and have proposed solutions which include; prevention (such as vaccination); improved monitoring, and the development of new antimicrobials (Fauci, 2001).

There is therefore the need to search for new lead and novel compounds to solve this public health issue. Plants have provided a source of inspiration for novel lead compounds and drugs and these have contributed to human health and well-being and as such considering them for development of new antimicrobial may be worthwhile (Iwu *et al.*, 1999).

2.1.2 Antimicrobial agents from natural sources

Right from the "golden era" of antibiotics discovery such as the penicillins, cephalosporins, aminoglycosides, tetracyclines, erythromycin and related macrolides, vancomycin etc, natural products have played a major role in this dispensation (Walsh, 2003). Antibiotics from natural products tend to possess very complex architectural scaffolds and have substituents that are likely to interact with biological targets in the pathogenic bacteria. These

scaffolds have been excellent platforms for remodelling and re-engineering by medicinal chemists to create subsequent generation of semi-synthetic variants of natural products antibiotic in use today (Walsh, 2003). It is believed that much more of these may be obtained if natural products are continually tested or screened. Natural products have been the major source of chemical diversity for starting materials for driving pharmaceutical discovery over the past years (Harvey, 2007). Natural product research has enormous yet unexploited potentials.

From combination of research efforts in academic and industrial laboratories, significant numbers of novel structures of biologically active natural products have been discovered from various natural sources especially plants. Recently several hundreds of plants worldwide used in treatment of infectious diseases have been screened for antimicrobial activities which have been published by different authors (Martin and Ernst, 2003). It is believed that these antimicrobial agents from these plants are synthesized by the plants *de novo* following microbial attack to protect themselves from any other pathogenic microbes in the environment. These agents are believed to have entirely different mechanisms of action from the antibiotics in present use (Eloff, 1998).

2.1.3 Plants as source of anti-microbial agents

Many antimicrobial agents have been derived from microbial sources (example; the discovery of penicillin which later led to the discoveries of other antibiotics). Some soil microorganisms have also produced very useful antibiotics (example; streptomycin and aureomycin) (Iwu *et al.*, 1999).

On the other hand, most plants have been known to have healing potentials. These plants contain useful constituents that have been believed to possess antimicrobial principles long before mankind discovered the existence of microbes (Rios and Recio, 2005). With this

knowledge at hand, plant based antimicrobial agents still represent a major untapped sources of medicines (Iwu, 2014).

The search for newer antibiotics from other sources apart from microorganisms has become very necessary due to the following;

- The emergence of multiple drug resistances to human pathogenic organisms and
- The associated challenges from antibiotics derived from products of microorganisms and their synthesized derivatives.

At present, there is no single plant derived antimicrobial entity on the market (Gibbons, 2004). Plants have therefore been focused on to obtain new antimicrobial agents. The reason for this interest is as follows;

- Plants are believed to be effective in the treatment of some infectious diseases while simultaneously mitigating many of the side effects that are often associated with synthetic antimicrobial (Iwu *et al.*, 1999).
- The antibacterial constituents present in plants, used by humans for the cure of infections are synthesized *de novo* following microbial attack. These synthesized products are used by these plants to protect themselves from invasive and pathogenic microbes in the environment (Gibbons, 2004).
- It is believed that the antimicrobial activities of some of these plants may act by mechanisms that are totally different from the currently used antibiotics. This may be of clinical value in the treatment of resistant microbial strains (Eloff, 1998).

Currently there are several researches into this field, and authors have published a number of works that have demonstrated the antimicrobial activities of several plants. Some of these include; (a) flavonoid glycosides from *Margaritaria discoidea* which have been found to

have broad spectrum antibacterial activity (Ekuadzi *et al.*, 2013), (b) an isolated indolizinium alkaloid from *Aniba panurensis* has been found to possess potent antimicrobial activity (Klausmeyer *et al.*, 2004), (c) a diterpenes from *Eupatorium glutinosum* have also been found to possess antimicrobial activity (El-Seedi *et al.*, 2002), (d) the essential oils of the fruit extract of *Xylopia aethiopica* have also shown to possess broad spectrum antimicrobial activity and antifungal activity against *Candida albicans* (Fleischer *et al.*, 2008), (e) allinine in *Allium sativum* (garlic) has been found to have some antimicrobial activity (Iwu *et al.*, 1999), (f) the methanol and petroleum ether extracts of the leaves and stem bark of *Nauclea latifolia, Bridelia atroviridis,* and *Zanthoxylum gilletii* have exhibited antimicrobial activities (Agyare *et al.*, 2006) and (g) the stem bark extracts of *Harungana madagascariensis* have shown to possess some antimicrobial activity (Iwalewa *et al.*, 2009).

2.2 OVERVIEW OF IN VITRO ANTIBACTERIAL ASSAYS

Antimicrobial activity can be established by observing the growth response of various microorganisms to test samples (e.g. natural extract). The method for detecting this activity has been classified into two namely; Dilution and Diffusion methods.

The dilution method includes agar and broth dilution methods while the diffusion method consist of agar disc diffusion, agar well diffusion and bio-autography. These methods have been further described below;

2.2.1 Agar-diffusion method

Diffusion of the active sample (extract) from a reservoir into pre-inoculated solid agar medium is the basic principle underlining this method. The diameter of the clear zone around the reservoir is measured at the end of the incubation period.

The diffusion methods are usually qualitative and suitable in preliminary antimicrobial screening of compounds and medicinal plant extracts with potential antimicrobial activity.

This method gives no quantification of bioactivity (Langfield *et al.*, 2004); (Hammer *et al.*, 1999).

In this method, different types of reservoirs can be used. These include; filter paper disc, stainless steel cylinders placed on the surface and hole punched in the medium (Cos *et al.*, 2006). The diffusion method is not appropriate for testing non-polar samples and those that do not easily diffuse into agar (Fleischer *et al.*, 2013).

In the agar well diffusion, wells are made in a pre-inoculated agar with a sterile cork borer aseptically and these wells are filled with different concentrations of the test sample. The inoculated system is kept at a temperature of 25°c for several hours before incubation to allow the extract or test sample to diffuse into the agar to make contact with the seeded microorganisms. The diameter of the clear zone around the well is then measured after incubation (Norrell and Messley, 1997).

In the agar disc diffusion assay, sterile filter paper discs are impregnated with the extract or test sample to serve as the reservoir. The impregnated paper discs are placed on top of the solidified pre-inoculated agar. This is then incubated and the clear zones of inhibition around the reservoir are measured after the incubation period (Salie *et al.*, 1996). This method do not allow the determination of MICs (Parekh and Chanda, 2006).

2.2.2 Dilution method

Dilution methods of antimicrobial assay are an important tool for assessing the activity of potential antimicrobial agents. This method can employ both solid and liquid media and growth of the microorganisms can be measured in different ways.

In broth (liquid) dilution method, the turbidity and redox-indicators are used to measure the extent of growth by the microorganism. The turbidity can be estimated or measured visually

or by measuring the optical density of the resultant solution at 405 nm (Cos *et al.*, 2006). The redox indicators frequently used are; 3-(4,5-dimethylthiazole-2,-yl)-2,5-diphenyltetrazolium bromide (MTT) and resazurin (Gabrielson *et al.*, 2002); (Eloff, 1998; Umeh *et al.*, 2005).

One setback with the use of this method is the sedimentation of the broth and the extract in cases where the extract is not fully soluble. This causes interference in the turbidity readings. This method is appropriate for the assaying of the antimicrobial activity of both polar and non-polar extracts and compounds.

The use of micro-plate reader can make assay easy and reproducible. In both agar (solid) and broth (liquid) dilution, the MIC which is the lowest concentration able to inhibit any visible microbial growth can be measured after incubation (Cos *et al.*, 2006).

2.2.3 Bio-autographic method

This method is an easy way to detect the particular constituent present in an extract or test sample that possess antimicrobial activity. The method employs three different methods which include;

- Direct contact bio-autography, where the microorganisms grow directly on the developed thin layer chromatographic (TLC) plate (Hostettmann *et al.*, 2001).
- Contact bio-autography, where the antimicrobial compounds are transferred from the TLC plate onto an inoculated agar plate, so that the active constituent would have direct contact with the organisms (Cos *et al.*, 2006) and
- Agar overlay bio-autography, where a seeded agar medium is applied directly onto the TLC plate (Rahalison *et al.*, 1991).

The growth inhibition on the TLC plate is detected by spraying the surface with a microbial indicator such as MTT, a tetrazolium salt. Antimicrobial activity of the extract is detected as clear (white) zones on the TLC plate.

Some setbacks for this method include the fact that it is limited to microorganisms that can easily grow on TLC plates. Another setback is the residue of low volatile solvents such as ammonia and trifluoroacetic acid that need to be removed completely before the transfer of the active compounds from the TLC plate onto the agar surface.

However, this method supports a quick search for new antimicrobial agents through bioassay guided isolation. This is because the technique allows localized antimicrobial activity of the test sample on a chromatogram (Cos *et al*., 2006).

2.3 OXIDANTS AND ANTIOXIDANTS

2.3.1 Origins of the free radical theory

The pioneer of free radical research, Denham Harman made the first connection between free radical chemistry and aging in the early 1950's. His first study which showed that 2-MEA (2-mercaptoethylamine) a radiation protection compound synthesised by the Atomic Energy Commission could extend life span by decreasing the level of free radical reactions was presented as an abstract in 1956 at the American Federation of Clinical Research. However, little support was generated from his theories at that time (Harman, 1993).

A single common process was proposed by Harman, that the production of free radicals was responsible for aging and death of all living things. This theory was based on the chemical nature of free radicals and their ubiquitous prominent presence in living systems (Harman, 1992).

Oxygen-derived free radicals were cited in the free radical theory of aging as being responsible for age-associated impairment at cellular and tissue levels. Implying that cellular aging is associated with oxidative stress (Sies, 2000). This theory gained credibility with the discovery by McCord and Fridovich (1969) of superoxides dismutase (SOD), a natural enzyme that destroys superoxide free radicals in the body (McCord, 2000); (McCord and Fridovich, 1969).

2.3.2 Free radicals and their generation

Free radicals are atoms or molecules which have unpaired electrons and are capable of independent existence. They are unstable because the unpaired electron creates highly reactive molecule which stabilises itself by taking an electron away from a stable molecule. The previously stable molecule, on loss of an electron, becomes 'damaged' resulting in another free radical. This then starts up a destructive chain reaction. These free radicals are collectively known as Reactive Oxygen Species (ROS) or Oxygen Derived Species and may include both oxygen radicals and certain non-radicals that are oxidising agents, or are easily converted into radicals (Halliwell, 2006).

There is permanent damage caused even if the free radical regains its electron from a stable molecule. Thus the original form and function is lost permanently (Halliwell, 2006). Biological membranes are among the most common target for auto-oxidative and oxidative tissue injury and disease (Pendry *et al.*, 2005). Here a membrane lipid is attacked by a free radical by removing a hydrogen atom from a polyunsaturated fatty acid (PUFA) side chain, creating a carbon centred radical (CH-). An unpaired electron is left on the carbon since hydrogen atom has only one electron. The carbon then combines with molecular oxygen and yields a highly reactive peroxyl radical ($COO^{\cdot}$). This peroxyl then abstract hydrogen from adjacent fatty acids and generates new carbon centre, resulting in a chain reaction known as lipid peroxidation (Santanam *et al.*, 1998).

The accumulation of lipid hydroperoxides (-COOH) causes destabilization of the cell membrane leading to interrelated derangement of cell metabolism including DNA strand breakage, damage to membrane ion transporters, gene expression alteration and some other protein damage. The resulting damage may be the production of mutagenic products, which burden the immune system leading to diseases (Halliwell, 2006).

There is therefore a growing evidence that excessive production of free radicals can cause or exacerbate many human diseases (Halliwell, 2012).

2.3.3 Sources of free radicals

Free radicals may arise from various sources. They may arise from the environment which include; tobacco smoke, ozone derived from air pollution, automobile exhaust emissions, pesticides and excessive radiation. It may also arise from normal *in vivo* biological processes which involve chain reactions. Deep fried foods, hydrogenated oils and toxic metals when indigested may serve as sources of free radical (Diplock, 1997).

Nitrogen dioxide (NO_2) a destructive free radical for example, results from the reaction between nitric oxide ($NO^{\cdot}$) and oxygen (O_2) formed in cigarette smoke and vehicle exhaust and has been implicated in respiratory illnesses and irreversible lung damage (van der Vliet and Cross, 2000).

2.3.4 The role of oxidative stress in infection

Majority of complex life on earth require oxygen for existence. Oxygen is a highly reactive molecule that can damage living cells by producing reactive oxygen species (Kulkarni *et al.*, 2007). These reactive oxygen species do have useful cellular functions such as redox signalling but may be dangerous if not kept under control (Rhee, 2006). A fine biological balance must exist between the normal physiological formation of ROS/ RNS and their removal (Bergendi *et al.*, 1999).

Infection is the invasion of pathogenic microorganisms where they attack, multiply and induce a local or generalised reaction (Ochsendorf, 1999). During this process, a lot of free radicals are produced. Oxidative stress plays a dual role in the process of infections; free radicals may protect the living organism against invading microorganisms and they can also cause tissue damage during the resulting inflammation process that follows infection (Fleischer *et al.*, 2013).

In the process of infection, inflammation is initiated, this generates reactive species by myeloperoxidase, NADPH oxidase and nitric oxide synthase. The function of inflammation during the incident of infection is to eliminate the pathogenic effect and remove damaged tissue, to help restore tissue (Bonizzi *et al.*, 2000). Though inflammation is a protective mechanism, it is implicated in most diseases including rheumatoid arthritis, asthma, colitis, hepatitis and some cancers and may even cause perpetual tissue injury to healthy cells (Halliwell, 2006; Halliwell, 2012).

In a healthy body, the generation of pro-oxidants in the form of ROS is kept in check by the various levels of antioxidant defence. Oxidative stress is when there is imbalance, that is, when the ROS are overly produced that antioxidants come in handy to help prevent excessive damage to the body tissues. Antioxidants are molecules that inhibit the oxidation of other molecules. Antioxidants terminate the chain reactions caused by free radical and other oxidation reaction by being oxidized themselves. They are often referred to as reducing agents (Sies, 2000).

Medicinal plants have been found to possess potential source of natural antioxidant agents and so have found use in averting and reducing the damage caused by free radicals. Examples of these include; ascorbic acid, thiols and polyphenols (Sies, 2000).

Currently, medicinal plants are perceived as potential sources of new antioxidant agents due to the presence of constituents such as flavonoids, coumarins, phenols and other vitamins (vit. C and vit. E).

2.4 OVERVIEW OF IN VITRO ANTIOXIDANT ASSAYS

Different reactive oxygen species are produced as normal physiological activities take place in living cells. These reactive oxygen species have different reaction mechanisms and so attempting to use a single method to assay or evaluate the antioxidant activity of test sample would be inappropriate.

There are several in vitro methods developed to assess the antioxidant activity of compounds, extracts and nutritional supplements. These methods include; lipid peroxidation assay method, total antioxidant capacity assay method, total phenol content assay method, DPPH radical scavenging assay method, TLC autography technique method and ferric reducing power assay method, just to mention but a few. Some of the widely used methods are as described below;

2.4.1 Rapid antioxidant screening using DPPH (TLC autography technique)

This provides the easiest, effective and rapid way to study plant extract profile for antioxidant activity. It involves, developing TLC plate in an appropriate solvent system and spraying the plate with 2,2-diphenyl-1picrylhydrazyl (DPPH) reagent. The antioxidant activity is assessed by a light yellow to white bands against a purple background seen on the TLC plate after spraying with DPPH solution reagent (Cuendet *et al.*, 1997).

DPPH is a free radical stable at room temperature and produces a violet colour when dissolved in methanol. This solution changes to light yellow when it comes into contact with antioxidants. This is due to the fact that it accepts an electron or hydrogen in the presence of

antioxidants (reducing agents). The reducing agent causes the DPPH to loss its free radical property and the solution changes to yellow (Blois, 1958; Aliyu *et al.*, 2010).

This method of assay is more qualitative but may also be considered a semi quantitative method by measuring the diameter of the light yellow to white bands on the TLC plate and the intensity of the light yellow to white bands to give an idea of the concentration of antioxidants present (Balandrin *et al.*, 1993).

2.4.2 DPPH radical scavenging assay

This method is one of the most widely used assays for screening antioxidant activity of medicinal plants. This method involves the reduction of a purple methanolic DPPH solution to a yellow colour. DPPH is a stable free radical with a characteristic purple colour in methanol and maximum absorption at 517nm. This purple colour changes to light yellow when it comes into contact with a reducing agent by accepting an electron or hydrogen from the reducing (antioxidant) agent, thus forming 1,1 diphenyl-2-picrylhydrazine which is the yellow colour observed (Blois, 1958; Aliyu *et al.*, 2010).

The antioxidant activity is assayed by measuring the remaining DPPH at a maximum absorption of 517 nm with a spectrophotometer. A decrease in the absorbance of DPPH at its absorption maxima is proportional to the concentration of the free radical scavenger (antioxidant) added to the DPPH reagent solution (Aliyu *et al.*, 2010). This implies that a decrease in absorbance shows an increase in free radical scavenging activity of the extract.

The DPPH scavenging activity is expressed as effective concentration EC_{50} which denotes the concentration of sample required or needed to scavenge 50% of DPPH free radicals.

2.4.3 Total antioxidant capacity

This is a developed method for the quantitative determination of antioxidant capacity using spectrophotometry described by (Prieto *et al.*, 1999). This assay method involves the reduction of Mo (vi) to Mo (v) by the test sample (extract) and the subsequent formation of a green phosphomolybdenum (v) (phosphate/Mo (v)) complex at acidic PH.

The amount of complex formed is measured at absorbance of 695 nm. Solutions of a standard antioxidant such as ascorbic acid are used to obtain a standard curve. Values for total antioxidant capacity are expressed in terms of ascorbic acid equivalents (mg/g of the extracted compound) (Prieto *et al.*, 1999).

2.4.4 Total phenol

Researchers have shown that, polyphenolics which include; flavonols, anthocyanins and phenolic comounds are potent antioxidants and are abundant in fruits, vegetables and medicinal plants (Nabavia *et al.*, 2009).

The folin-ciocalteu method is used to estimate the total phenol compound content in medicinal plants as described by Singleton *et al*, (1999). This method depends on the reduction of folin-ciocalteu reagent by phenols in the test sample to blue oxides which have a maximum absorbance at 760 nm.

Phenolic compounds undergo a complex redox reaction with phosphotungstic and phosphomolybdic acids which are present in folin-ciocalteu reagent. The reaction is as follows;

$$\text{Folin : } Mo^{+6}\text{ (yellow) + e (from antioxidant)} \longrightarrow Mo^{+5}\text{ (blue).....Equation 2.1}$$

Where the oxidizing agent is a molybdophosphotungstic heteropolyacid comprising of $3H_2O.P_2O_5.13WO_3.5MoO_3.10H_2O$, which the hypothesized active centre is Mo^{+6}.

2.5 SELECTION OF MEDICINAL PLANTS

2.5.1 Trichilia monadelpha (Thonn.)JJ de Wilde

Figure 2.1: *Trichilia monadelpha* plant showing the stem and leaves

2.5.1.1 Description

Trichilia comprises about 90 species, most of them in tropical America. In continental Africa, 18 species occur, while 6 are found in Madagascar. *Trichilia* as a genus is made up of hard woody plants yielding pit-props and those useful for building boats and canoes (Irvine, 1961). *Trichilia monadelpha* (Thonn) JJ De Wilde is of the family meliaceae (local 'twi' name; Otanduro; meaning the hatred medicine). The Meliaceae family has shown to be of much interest among phytochemists because either it contains plants which produce very complex chemical structures, or because of their biological activity, mainly against insects.

Trichilia monadelpha (Thonn) JJ De Wilde is an evergreen, small to medium-sized tree up to12- 20 m tall with smooth, pale grey to greenish brown or dark brown bark surface and a spreading crown. The leaves are compound with 3–6 pairs of leaflets with stipules absent and petiole of about 4–13 cm long. leaflets opposite, ovate to obovate, 4–26 cm × 1.5–9 cm,

cuneate to obtuse at base, acuminate at apex, hairy below when young but glabrescent, pinnately veined. Flowers are unisexual, male and female flowers are very similar in appearance, may be greenish yellow or greenish white in colour and usually scented. The fruits are often found in clusters, with obovoid to globose capsule of about 1.5–2.5 cm in diameter, (2–4)-lobed, with up to 6-seeds (Irvine, 1961).

2.5.1.2 Ecology and geographical distribution

Trichilia monadelpha (local 'twi' name; Otanduro) is a tropical forest plant found in the lowland high forest up to 650 m altitude and evergreen semi-deciduous secondary jungles, and appears often near river banks (Burkhill, 2000), often found in wet places in Ivory Coast, Sierra Leone, Nigeria, Benin, Congo and Ghana (Irvine, 1961). In Ghana, the plant is found in the secondary rain forest in Western, Eastern, Ashanti and Brong Ahafo regions. The plant flourishes best on fertile and well drained soils where the annual temperature is between 17-28 °C with moderately high humidity (Burkhill, 2000).

2.5.1.3 Traditional medicinal uses

Trichilia monadelpha is an important medicinal plant, and particularly its bark is commonly used in traditional medicine. The various parts (leaves, stem bark and root bark) of *Trichilia monadelpha* have very useful medicinal uses. These parts have been used by the local people to treat different kinds of diseases and to improve on their health. These parts have different modes of preparations to meet their intended use. The preparations may be in the form of decoctions, infusions, tinctures etc.

The stem bark of the plant has been used in Ghanaian traditional medicine to treat pain, psychoses, epilepsy, and inflammation for many years and their efficacies are widely acclaimed in different communities in Ghana (Dokosi, 1998) and (Abbiw, 1990).

A bark decoction or the pulped bark is considered to have antiseptic and curative properties and so applied externally to wounds, cuts sores and skin affections including yaws (Abbiw, 1990).

Its analgesic property is applied in the treatment of arthritis, lumbago and oedema by massaging topically with the bark-pulp in Ghana (Abbiw, 1990) and also for the treatment for fever pains and intercostals pain in Ivory Coast .

A bark decoction is drunk to sooth cough making use of its sedative property; a bark decoction also has anthelmintic property (Abbiw, 1990).

A bark decoction is used to treat gonorrhoea by mouth or by baths and taken for syphilis and wash for chancroid and syphilitic sores, whereas small amounts of pulped bark are eaten or applied as an enema to treat gastro-intestinal complaints in Ghana (Abbiw, 1990); (Dalziel, 1937) .

Bark decoctions serve as an aphrodisiac, ecbolic and abortifacient (Irvine, 1961).

Trichilia bark extract has been reported to have therapeutic effect against microbes, dysentery, dyspepsia, sores/ulcers (Busia, 2007).

In Ivory Coast, the bark is given to women who are sterile and also to those who are suffering from amenorrhoea to help relieve stomach pain (Bouquet and Debray, 1974)

The bark powder is applied to promote bone strength in children with rickets (Abbiw, 1990).

A leaf decoction is taken to treat heart complaints (de Wilde) and pounded leaves to treat gonorrhoea and lumbago.

The roots are an ingredient in preparations to treat dysentery, and are considered aphrodisiac.

2.5.1.4 Non-traditional medicinal uses

In Sierra Leone and Ghana, the bark is used as reddish-brown dye for clothes and hides (Irvine, 1961).

It is used to make canoes and other wood carvings e.g. Masks because it is relatively light and easy to cut with simple tools and does not crack. The wood obtained from *Trichilia monadelpha*, is conceded to be quite hard and fairly durable (Irvine, 1961) and sometimes used for house-building, flooring, interior trim and in ship building. It is also used in the making of furniture, plywood, cabinet works and etc. It is burnt in firewood and also converted into charcoal in Ghana (Abbiw, 1990). *Trichilia monadelpha* is useful for soil protection and soil improvement.

2.5.1.5 Reported biological activity and bioactive agents/compounds from the plant

Although *Trichilia monadelpha* has many traditional medicinal uses especially its bark which is a common ingredient of traditional medicinal preparations, little research has been done on its phytochemistry and pharmacological activity. A few of the work done on the plant include the following;

- The ethanolic stem bark extract of *T. monadelpha* has been found to have antitrypanosomal activity as well as antiplasmodial activity against chloroquine and pyrimethamine-resistant *Plasmodium falciparum* strains (Kamanzi Atindehou *et al.*, 2004).
- Oral administration of the aqueous stem bark extract (400 mg/kg/day for 4 weeks) significantly reduced serum testosterone levels and increased both sperm motility and viability with no significant difference in the sperm counts in adult male albino rats (Oyelowo *et al.*, 2011).
- Ainooson *et al.*, (2012) have recently shown that the stem bark extracts of the plant inhibit carrageenan-induced foot-oedema in the 7-day-old chick and the oedema associated with adjuvant-induced arthritis in rats.

- Woode *et al*., (2012) have also shown that the stem bark extracts of the plant caused dose-related antinociception (analgesic properties) in chemical, thermal, and mechanical models of pain in animals.
- Ayandi *et al.*, (2012) have demonstrated that *Trichilia* bark extracted with ethanol as an organic solvent, had a relatively higher activity on excision wounds
- Ben *et al*. (2013), published work on Preliminary Phytochemical Screening and In vitro Antioxidant Properties of *Trichilia monadelpha* (Thonn.) J. J. de Wilde (Meliaceae).

2.5.2 Trichilia tessmannii (Harms)

Figure 2.2: *Trichilia tessmannii* plant showing the stem and leaves

2.5.2.1 Description

The distribution of *Trichilia tessmannii* (Harms) (local 'twi' name; Otanduro-nini; meaning the 'male' hatred medicine) is nearly the same as that of *Trichilia monadelpha* (Thonn) JJ

De Wilde (Lemmens, 2008). *Trichilia tessmannii* (Harms) is a medium-sized forest tree with a height of about 30 m. It has a straight cylindrical base of about 70 cm in diameter bearing a dense crown. The leaves are pari-pinnate with opposite leaflets with soft orange hairs beneath. The fruit is a three (3) chamber, sub-globbose, stalked capsule of a size of about 3 cm across. The fruits are pinkish to purple or purplish red in colour. There are two (2) seeds in each chamber of the fruit. This tree is easily recognized by the greyish and slightly scaly back which peels off in plates. The older tees slowly exude a little sweet-scented creamy or yellowish latex (Savill and Fox, 1967; Sofowora *et al.*, 2013).

2.5.2.2 Ecology and geographical distribution

Trichilia tessmannii (Harms) (local 'twi' name; Otanduro-nini) is a forest tree found in the rain forests in lowlands and submontane altitudes, near river banks; from Guinea to south Nigeria and into Cameroon, Gabon, Zaire and Cabinda (Aubréville, 1959).

2.5.2.3 Traditional medicinal uses

Only a few medicinal uses have been reported. A decoction of the bark is used to treat stomach-aches and as a purgative (Lemmens, 2008).

2.5.2.4 Non-traditional medicinal uses

The wood from this tree is said to be termite proof, hence its' usage in house construction (Dalziel, 1937).

The cooked fruits are eaten in Zaire (de Wilde).

The seeds are used by the women in Nigeria for rattles and tambourines (Kennedy, 1936).

2.5.2.5 Reported biological activity and bioactive agents/compounds from the plant

There has been no reported scientific biological activities and isolation of any compound from this tree.

CHAPTER THREE

EXPERIMENTAL

3.0 MATERIALS AND METHODS

3.1 MATERIALS

3.1.1. Media and chemicals used

Nutrient broth and Sabouraud broth were obtained from Oxoid Ltd, Basingstoke UK. (3-(4,5-dimethylthiazole-2yl-2,5-diphenyltetrazolium bromide) (MTT), 2,2-diphenyl-1-picryl-hydrazyl (DPPH) (Sigma Aldrich USA), Tannic acid (Fluka, U.K), Disodium phosphate, Ammonium molybdate, Folin-ciocalteu reagent and Ascorbic acid all of analytical grade were obtained from BDH, Chemical Laboratory, (England, U.K). Chromatographic separation were achieved with analytical grade petroleum ether, ethyl acetate and methanol from BDH laboratory supplies (England). The detecting reagents anisaldehyde reagent, dragendorff's spray reagent and iodine vapour were used without further modifications.

The microorganisms used included; *Escherichia coli* (NCTC-9002), *Pseudomonas aeruginosa* (ATCC 27853)), *Staphylococcus aureus* (ATCC 25923, and *Bacillus subtilis* (NCTC 10073)) were obtained from the Department of Microbiology, Faculty of Pharmacy and Pharmaceutical Sciences, KNUST. The fungus; *Candida albicans* (ATCC 10231)) was a clinical strain obtained from Komfo Anokye Teaching Hospital (KATH), Kumasi Ghana.

3.2 METHODS

3.2.1 PLANT COLLECTION AND AUTHENTICATION

The stem barks of the two plants were collected with the help of a local herbalist. The collections were done in two different locations in Ghana at different periods.

Table 3.1: list and sources of plant materials used

Name of plant	Location of collection	Period of collection	Voucher number
Trichilia tessmannii	Asakraka in Kwahu (Eastern Region)	January, 2013	KNUST/TT/2013/S003
Trichilia monadelpha	Bomaa in Sunyani (Brong Ahafo Region)	February, 2013	KNUST/TM/2013/S006

The plant materials were authenticated at the Department of Herbal Medicine, Faculty of Pharmacy and Pharmaceutical sciences, Kwame Nkrumah University of Science and Technology (KNUST). Voucher numbers were given to the specimen and they were deposited in the department's herbarium.

3.2.2 Processing of Plant Material

The dead cells on the bark were scrapped off and the remaining portions of the stem barks washed with water to get rid of all unwanted materials. They were chopped into smaller pieces and air-dried at room temperature for a week. The dried material was coarsely powdered using a mechanical grinder.

3.2.3 Extraction of Plant Material

3.2.3.1 Extraction of plant material for preliminary antioxidant screening

200g of pulverized plant material of each plant was extracted by soxhlet extraction using 500 mL methanol for 48 hrs, filtered through No.1 Whattman's filter paper and evaporated to dryness using a rotary evaporator (R-114, Buchi, Switzerland) at reduced temperature and pressure. The concentrates were further dried to obtain the dried total crude methanol extracts which were used in the preliminary antioxidant screening.

3.2.3.2 Preparation of polar, semi-polar and non-polar fractions of plant materials

200g of pulverized plant material of each plant was extracted by successive extraction using the soxhlet apparatus. 500 mL of petroleum ether, ethyl acetate and methanol were used successively for 48 hrs. The extracts obtained were filtered through No.1 Whattman's filter paper and evaporated to dryness using a rotary evaporator (R-114, Buchi, Switzerland) at a temperature of 50°c and a rotational speed of 5 revolutions per minute (rpm). The concentrates were further dried to obtain the petroleum ether, ethyl acetate and methanol extracts which were used in the preliminary antimicrobial screening.

3.2.3.3 Bulk extraction of plant materials for chromatographic separation and isolation

About three kilograms (3 kg) of the pulverized plant material of each plant was packed into a cellulose thimble in a soxhlet apparatus. 5 L of three solvents, (petroleum ether, ethyl acetate and methanol) were used to serially extract the plant material. The three obtained extracts were then concentrated using a rotary evaporator (R-114, Buchi, Switzerland) at a temperature of 50 °c and a rotational speed of 5 rpm to obtain syrupy masses. The syrupy masses were further dried and stored in a desiccator until required for further analysis.

The percentage yields for all extracts were calculated as the ratio of the weight of dried extract to the weight of crude powdered drug used (% w/w).

3.2.4 Phytochemical Investigation

The pulverised plant materials were subjected to preliminary phytochemical screening for the identification of secondary plant metabolites using standard procedures as described by (Evans, 2009; Sofowora, 2010) and Harborne, (1998). Below are brief descriptions of the methods used for the detection of the various secondary plant metabolites.

Tannins

About 0.5 g of pulverized plant material was extracted by boiling with 20 mL of water for 5 minutes, cooled and filtered.1mL of the cooled filtrate was diluted to 10 mL with distilled water and 5 drops of 1% lead acetate solution were added.

Reducing sugars

About 0.2 g of plant material was extracted by warming with 5mL of dilute HCl on a water bath for 2 minutes. The mixture was filtrated and the filtrate was made distinctly alkaline with several drops of 20 % NaOH. 1mL of each of Fehlings solution A and B was added to the alkalined filtrate and heated on a water bath for 2 minutes.

Saponins

An amount of 0.2g of pulverised plant was shaken vigorously with about 10 mL of water in a stoppered test tube and observed for the presence of a persistent froth.

Anthraquinones/ anthracene glycosides

About 0.2 g of the pulverized plant material was boiled with 2 mL of dilute H_2SO_4 and 5 % aqueous $FeCl_3$ for 5 minutes. The mixture was filtered hot, cooled and shaken with an equal volume of chloroform. The chloroformic layer was separated and shaken with half its volume of dilute ammonia solution and observed for the formation of a rose-pink colouration.

Cyanogenetic Glycosides

0.2 g of the pulverized plant material was placed in a conical flask and moistened with a few drops of water. A strip of sodium picrate paper was suspended by means of a cork in the neck of the flask and warmed gently on a water bath. The change in colour of the test paper was observed to indicate the presence of cyanogenetic glycosides.

Cardiac glycosides

About 0.5 g of the pulverized plant material was extracted with 70 % alcohol and the mixture filtered. 5 mL of the alcoholic filtrate was mixed with 1 mL of glacial acetic acid with traces

of ferric chloride. Concentrated Sulphuric acid was carefully poured down the sides of the tube. A reddish brown ring is formed at the interface due to the presence of aglycone (steroidial) that indicates the presence of a cardiac glycoside.

Alkaloids

0.2g of pulverized plant material was extracted with ammoniacal alcohol, filtered and evaporated to dryness. The dry residue was extracted 1 % sulphuric acid and filtered. The filtrate as rendered distinctly alkaline with dilute NH_3 solution and then shaken with chloroform. The chloroformic layer was separated, evaporated to dryness and dissolved in 1 % sulphuric acid. To 1 mL of 1% H_2SO_4 extract formed, 5 drops of dragendorff's reagent was added. The presence of the characteristic orange brown precipitate indicates the presence of alkaloids. This is confirmed by the absence of a buff white precipitated upon addition of Mayer's reagent to 1 mL of the acidic extract.

Flavonoids

About 0.2 g of pulverized plant material was boiled with 5 mL of water for 2 minutes and filtered. To 1 mL of the filtrate, 20 % NaOH was added. There was a formation of an intense yellow colouration which was further exposed to fumes of concentrated HCl. The yellow colouration disappears or turns colourless, indicating the presence of flavonoids.

Lead acetate test: A few drops of lead acetate solution were added to 1mL of the filtrate. Formation of a yellow colour precipitate indicates the presence of flavonoids.

Coumarins

A small amount of the pulverized plant material was extracted with chloroform and filtered to obtain a chloroformic extract. 5 mL of the chloroformic extract was evaporated to dryness and the residue dissolved in hot distilled water and cooled. About 0.5 mL of 10% ammonia solution was added to the extract and observed under UV light (long wave length; 366nm).

The occurrence of an intense bluish green fluorescence under UV light indicates the presence of coumarins and their derivatives.

Steroids

Powdered plant material (0.2g) was shaken with chloroform and filtered. A few drops of acetic anhydride were added to1 mL of the filtrate after which concentrated sulphuric acid was carefully poured down the side of tube. The formation of a bluish- green colouration at the interface indicates the presence of a steroidal ring.

Terpenoids

A chloroformic extract was obtained by shaking 0.2 g of the pulverized plant material with chloroform and filtered. To 1 mL of the filtrate, a few drops of concentrated sulphuric acid was added, shaken and allowed to stand. Effervescence followed by the appearance of a clear reddish brown colour at the interface indicates the presence of terpenoids.

3.2.5 Antimicrobial Assay

3.2.5.1 Preparation of standard suspension of microorganisms

The five (5) microorganisms were selected and used in this research based on their implication in most infections.

The bacteria strains were cultured overnight at 37^{o}c in nutrient broth and the fungus was cultured overnight at 25^{o}c in sabourand dextrose agar.

Standardized suspensions of microorganisms were prepared from overnight broth cultures. Standardization was done by serial dilutions of cultures in sterile normal saline to achieve a suspension of equal turbidity with 0.5 Mc. Farland standards by visual comparison to achieve a dilution that contains approximately 10^5 CFU/mL (Govinden-Soulange *et al.*, 2014).

3.2.5.2 Preparation of media

Nutrient broth

25 g of nutrient broth powder was weighed into a beaker containing 500 mLs of distilled water and stirred to dissolve. Enough freshly prepared distilled water was added to produce 1000 mL. 10 mL quantities were poured into test tubes and plugged firmly with cotton wool. They were then sterilized by heating in an autoclave at 121°C for 15 minutes.

Saborand dextrose broth

65 g of the powder was weighed and dissolved in one litre of freshly prepared distilled water. The mixture was heated whiles stirring until it dissolved completely. Aliquot of 20 mL of the mixture was poured into test tubes, plugged with cotton wool and sterilised in an autoclave at 121°C for 15 minutes.

3.2.5.3 Micro-dilution assay (Broth dilution)

In the determination of the minimum inhibitory concentration (MIC), the method used was micro-well dilution as described by (Selim *et al.*, 2014) and (Oyono *et al.*, 2014).

In this method, the inocula of microorganisms were prepared from a 12 –hour broth cultures and serial dilutions were made to achieve a suspension of 10^5 CFU/mL.

The various plant extracts were solubilised with 2 %v/v dimethyl sulfoxide (DMSO) to obtain concentrations of between 0.312 mg/mL to 10 mg/mL. The 96-well sterile plates were prepared by dispensing into each well 100 µl of double strength nutrient broth, 100 µl of the test samples and 20 µl of the inoculums of standardized suspensions of the various cultures of test organisms.

The micro-plates were then incubated at 37°C (for bacteria) and 25°C (for fungus) for 24 hours. Growth of the microorganisms was determined by adding 20µl of a 0.125%w/v

solution of tetrazolium salt (3-(4,5-dimethylthiazole-2yl-2,5-diphenyltetrazolium bromide) (MTT) and incubating for further 30 minutes. Dark wells indicate the presence of microorganisms as the dehydrogenase enzymes in the live bacteria react to form a dark complex with the tetrazolium salt.

Ciprofloxacin and ketoconazole were used as positive control and DMSO was used as a negative control. All the experiments were triplicated.

3.2.6 Antioxidant Assay

3.2.6.1 Rapid antioxidant screening using DPPH.

A small amount of the extract was dissolved in an appropriate solvent. With the aid of a capillary tube, the dissolved extract was spotted on a TLC plate and allowed to dry. The TLC plate with the spot was then developed in an appropriate solvent system (Table 3.2). After the development of the TLC plate, it was removed from the solvent system and allowed to dry. The dried plate was then sprayed with DPPH of concentration, 20 mg/L in methanol. The Rf values of the spots were then determined.

Table 3.2: Solvent systems for TLC development

Extract	Solvent system
Petroleum ether	Petroleum ether: Ethyl acetate (8:2)
Ethyl acetate	Petroleum ether: Ethyl acetate (7:3)
Methanol	Ethyl acetate: Methanol (8:2)

3.2.6.2 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay

Various concentrations of the extract (total crude methanol) ranging between 0.00098 mg/mL to 0.125 mg/g were prepared and used in the experiment. The concentration of the DPPH solution used was 20 mg/L in methanol. In preparing the reaction mixture, 3.0 mL of DPPH

in methanol and 1.0 mL of the extract was used for each of the prepared concentration of the extract. The mixtures were then incubated for 30 minutes in the dark. After the incubation period, the absorbance for each concentration was measured at 517 nm using a UV-spectrophotometer (Cecil CE 7200 spectrophotometer, Cecil instrument limited, Milton Technical Centre, England). The positive control used was ascorbic acid with concentration ranging from 0.00098 mg/mL to 0.0625 mg/mL and methanol (vehicle) was used as the negative control. These were all taken through the same procedures and conditions as the extract. All the tests were carried out in triplicates. A graph of concentration against % DPPH scavenging is plotted to estimate the EC_{50}.

The percentage DPPH scavenging ability was calculated according to the equation blow;

% DPPH radical scavenging activity = $[(A_O - A_1)/ A_O]$ * 100.....Equation 3.1

Where; A_O is the absorbance of the negative control and

A_1 is the absorbance of extract or positive control.

3.2.6.3 Total antioxidant capacity

In this method, various concentrations of the extract were prepared between 0.00098 mg/mL to 0.125 mg/mL and taken through the experiment. A mixture of the standard reagent is prepared by adding 0.6 M sulphuric acid, 28 mM disodium phosphate and 4mM ammonium molybdate. 3 mL of this reagent is added to 1mL each of the various concentrations of the extracts and incubated at 95^0C for 24 hrs. After incubation, the mixtures were cooled at room temperature and centrifuged for 10mins. The absorbance of the supernatant liquid of the various concentrations was measured at 695 nm.

The reference standard used was ascorbic acid. Various concentrations of the standard drug were prepared in the range of 0.00098 mg/mL to 0.03125 mg/mL and taken through the same

procedure and conditions as the extract. A calibration curve was plotted with the values obtained from the measurement of the absorbance of the ascorbic acid. The total antioxidant capacity was expressed in terms of ascorbic acid equivalent (mg/g of the extracted compound). All tests were done in triplicates.

3.2.6.4 Total phenol

Extract concentrations ranging from 0.00098 mg/mL to 0.125mg/mL were prepared and taken through the experiment. For the reaction mixture, 0.5 mL of the extract was added to 0.1 mL of folin-ciocalteu reagent (with a concentration 0.5N). The mixture was incubated at room temperature for 15 minutes. 2.5 mL of saturated sodium carbonate was added to the mixture after the first incubation and then incubated for a second time for a further 30 minutes at room temperature. The absorbances for the various concentration of the extract with the reagent were taken at 760 nm using a UV-spectrophotometer.

The standard drug used was tannic acid. Various concentrations of 0.00098 mg/g to 0.125 mg/g of this drug (the positive control) were prepared and taken through the same conditions as the extract. The absorbances obtained were used in plotting a calibration curve. The total phenol content was expressed in terms of tannic acid equivalent (mg/g of the extracted compound) (McDonald *et al.*, 2001; Boudries *et al.*, 2014). All tests were done in triplicates.

3.2.7 Chromatography

Results from both antioxidant and antimicrobial assays showed various positive activities of the stem bark extracts of the two plants. As no reports of the chemical constituents of the stem bark of these plants are known, the various extracts were taken through fractionation and isolation to identify the compounds responsible for the observed activities.

3.2.7.1 Chromatographic techniques employed

Open column chromatography

The open column chromatography on silica method was employed for the fractionation of the extracts. The size of silica gel used was 70-230 mesh ASTM (Merck, Germany). The dry method of packing was used. A column with diameter 5 cm and a height of 50 cm was chosen and mounted vertically on a retort stand with the help of a clamp. The column was packed with the dried silica to about two-thirds full. 20 g of the extract was dissolved in the appropriate solvent (for instance if the extract is an ethyl acetate extract then ethyl acetate solvent is used as solvent) and triturated with equal amount of silica to obtain a uniform mixture. The mixture was allowed to dry to obtain a free flowing powder and then poured onto the top of the packed column. Gradient elution method was used. Eluents (mixtures of petroleum ether, ethyl acetate and methanol) of increasing polarities were added in fixed volumes (see Section 3.2.7.2, page 45). Fractions eluted from the column under gravity were collected in glass specimen bottle.

Development of analytical thin layer chromatography (TLC)

Pre-coated aluminium-backed silica gel plates F_{254} of 0.25 mm thickness (Merck, Germany) were used. The one way ascending technique was employed in running all the thin layer chromatography. In this method, samples were dissolved in suitable solvents (i.e. solvents that dissolves the particular extract.). The dissolved samples were spotted on the TLC plates with the aid of capillary tubes at one end of the plate about 2 cm above the edge and 1.5 cm away from the margins and developed in appropriate solvent systems (See Table 3.2) in a chromatank. The plates were dried and first observed under UV light at 254 nm (short wavelength) and then at 366 nm (long wavelength) for fluorescence.

The plates were then sprayed with the appropriate detecting reagents such as anisaldehyde, dragendorff's spray reagent and iodine vapour. The sprayed TLC plates were then heated at 105 ^{0}c for about 10 minutes.

3.2.7.2 Isolation of compounds from the stem bark extracts

Chromatographic separation of the ethyl acetate fraction of *T. monadelpha* (TME)

A glass column with diameter 5 cm and a height of 50 cm was packed with silica gel of mesh size 70-230 to about two-thirds of its height. 40 g of the ethyl acetate extract of stem bark of *T. monadelpha* was dissolved in a minimum amount of ethyl acetate solvent. An amount of 40 g of silica gel was added to the dissolved extract and triturated until a dry, flowing powder was obtained.

The dried flowing power obtained was poured on top of the packed column. A wad of cotton wool was placed on top of the packed column to prevent disturbing the surface of the packing as the various solvent systems are poured onto the packed column.

The column was eluted initially with 100 % petroleum ether. The polarity of the solute was gradually increased by 10 %, 20 %, 30 %, 50 %, 60 %, 70 %, 80 % and 90 % ethyl acetate in petroleum ether. This was then followed by 100 % ethyl acetate then 10 % and 20 % methanol in ethyl acetate.

About 140 aliquots of 100 mL each were collected in glass specimen bottles. With the aid of TLC analysis, these aliquots were bulked into about six (6) major fractions. Four of the bulked fractions were selected to be worked on based on their quantities and profile on the TLC plates. These four fractions were labelled as TME_1, TME_2, TME_3 and TME_4.

Fractionation of ethyl acetate sub-fraction of *T. monadelpha* TME_3

A glass column with diameter 5 cm and a height of 50 cm was packed with silica gel of mesh size 70-230 to about 2/3's of its height. The sub-fraction TME_3 of weight 5.46 g, obtained from the fractionation of the ethyl acetate extract of stem bark of *T. monadelpha* was dissolved in a minimum amount of ethyl acetate solvent. An amount of 10 g of silica gel was added to the dissolved extract and triturated until a dry, flowing powder was obtained.

The dried flowing power obtained was poured on top of the packed column. A wad of cotton wool was placed on top of the packed column to prevent disturbing the surface of the packing as the various solvent systems are poured onto the packed column.

The column was eluted initially with 100 % petroleum ether. The polarity of the solute was gradually increased by 10 %, 20 %, 30 %, 50 %, 60 %, 70 %, 80 % and 90 % ethyl acetate in petroleum ether.

70 aliquots of 50 mL each were collected in glass specimen bottles. With the aid of TLC analysis, these aliquots were bulked into about four (4) major sub-fractions. One of the bulked fractions labelled 'A' was selected to be worked on based on its quantity and its profile on the TLC plates. Using preparative TLC plates for further purification an unknown compound A_1 was obtained.

Chromatographic separation of the petroleum ether fraction of the stem bark of *T. monadelpha*

A glass column with diameter 5 cm and a height of 50 cm was packed with silica gel of mesh size 70-230 to about 2/3's of its height. 30 g of the petroleum ether extract of stem bark of *T. monadelpha* was dissolved in a minimum amount of petroleum ether solvent. An amount of 30 g of silica gel was added to the dissolved extract and triturated until a dry, flowing powder was obtained which was poured on top of the packed column. A wad of cotton wool was

placed on top of the packed column to prevent disturbing the surface of the packing as the various solvent systems are poured onto the packed column.

The column was eluted initially with 100 % petroleum ether. The polarity of the solute was gradually increased by 10 %, 20 %, 30 %, 50 %, 60 %, 70 %, 80 % and 90 % ethyl acetate in petroleum ether. This was then followed by 100 % ethyl acetate. About 120 aliquots of 100 mL each were collected in glass specimen bottles. With the aid of TLC analysis, these aliquots were bulked into about five (5) major fractions. Three (3) of the bulked fractions were selected to be worked on based on their quantities and their profile on the TLC plates.

CHAPTER FOUR

RESULTS

In this work, the phytochemicals present in the pulverised plant powders and of the two plants were investigated. The anti-microbial activity of these three extracts was also investigated. The anti-oxidant activity was assessed on the crude methanol extracts of the two plants. The ethyl acetate and petroleum ether extracts of the two plants were fractionated by the use of open column chromatography.

Below is the summary of the results of the percentage yield obtained after the extraction process, preliminary phytochemical screenings, anti-microbial and anti-oxidant activities and the chromatographic procedures.

4.1 PERCENTAGE YIELD

The results of the calculated percentage yields of the three extracts of the two plants are showed in Table 4.1 below.

Table 4.1: Percentage yields of different solvent extracts of the plant materials

Plant material	**Percentage yield (%w/w)**		
	Methanol Extract	**Ethyl acetate Extract**	**Petroleum ether Extract**
T. tessmannii	10.53	1.10	0.72
T. monadelpha	11.43	2.19	3.00

4.2 PHYTOCHEMICAL SCREENING

Results for the preliminary phytochemical screening performed on the dried powdered stem bark of each plant material are summarized in Table 4.2 below.

Table 4.2: Results of the phytochemical screening of dried pulverised stem barks of selected plants

Test	Results	
	T. tessmannii	*T. monadelpha*
Tannins	+	+
Reducing sugar	+	+
Saponin glycosides	+	+
Anthracene glycosides	-	+
Anthraquinones	+	-
Cyanogenetic glycosides	-	-
Cardiac glyciosides	+	+
General Alkaloids	+	+
Flavanoids	-	-
Terpenoids	+	+
Coumarins	+	+
Streroids	+	+

(+) indicates presence and (-) indicates absence of phytochemical

4.3 ANTIMICROBIAL ASSAY

The antimicrobial activity of the stem barks of the two selected plants were tested against five microorganisms of which, two (2) were Gram positive, two (2) Gram negative and One (1) fungus, by the use of broth dilution method. The MIC was taken as the least concentration that inhibited any visible growth of organisms after a 24 hour incubation period (Selim *et al.*, 2014).

(a) *T. tessmannii*

The antimicrobial activity was assessed on three different extracts of *T. tessmannii*. These extracts were; methanol, ethyl acetate and petroleum ether extracts. These extracts were tested against the five (5) selected microorganisms (two Gram positive, two Gram negative and one fungus). The results for the antimicrobial assays are summarized in Table 4.3 below.

Table 4.3: Minimum inhibitory concentrations (MIC) for the three extracts of *T. tessmannii*.

TEST ORGANISMS	**MIC (mg/mL)**		
	Methanol Extract	Ethyl acetate Extract	Petroleum ether Extract
Staph. Aures	2.50	1.25	10.00
B. subtilis	1.25	1.25	>10.00
E. coli	2.50	1.25	>10.00
P. aeruginosa	1.25	0.625	>10.00
C. albicans	1.25	0.625	10.00

(b) *T. monadelpha*

The antimicrobial activity was assessed on three different extracts of *T. monadelpha*. These extracts were; methanol, ethyl acetate and petroleum ether extracts. These extracts were tested against the five (5) selected microorganisms (two Gram positive, two Gram negative and one fungus). The results for the antimicrobial assays are summarized in Table 4.4 below.

Table 4.4: Minimum inhibitory concentrations (MIC) for the three extracts of *T. monadelpha.*

TEST ORGANISMS	**MIC (mg/mL)**		
	Methanol Extract	Ethyl acetate Extract	Petroleum ether Extract
Staph. Aures	2.50	10.00	10.00
B. subtilis	2.50	5.00	2.50
E. coli	1.25	5.00	2.50
P. aeruginosa	2.50	5.00	2.50
C. albicans	2.50	5.00	10.00

Table 4.5: MIC for reference drugs

TEST ORGANISMS	**MIC (µg/mL)**	
	Ciprofloxacin	Ketoconazole
Staph. Aures	0.25	NT
B. subtilis	0.10	NT
E. coli	0.10	NT
P. aeruginosa	0.10	NT
C. albicans	NT	5.00

4.4 DATA ANALYSIS

The concentrations responsible for 50 % of the maximum effect (EC_{50}) for the extract were determined using interactive computer least squares method, with the following non-linear regression (three-parameter logistic) equation.

$$Y = \frac{a+(b-a)}{1+10^{(\text{Log } EC_{50}-x)}} \quad \text{.....Equation 4.1}$$

Where x is the logarithm of concentration and Y is the response. Y starts at 'a' (the bottom) and goes to 'b' (the top) with a sigmoid shape. Graph pad prism windows version 6 (Graph pad software, San Diego, CA, USA) was used to estimate the EC_{50} of both the extract and the standard drug. With the aid of the Graph pad prism, the mg/g equivalent of the extract in terms of ascorbic acid and tannic acid were also calculated.

4.5 ANTIOXIDANT ASSAY

DPPH scavenging activity

The DPPH scavenging activity was carried out on the total crude methanol extracts of both plants. The results of the DPPH scavenging activity is as shown in Table 4.5 below. The DPPH scavenging assay determines the ability of the agent (extract) to scavenge free radicals. From the results obtained it may be observed that the DPPH free radical scavenging activity was concentration dependent. The EC_{50} values recorded were 0.0207 mg/mL and 0.009854 mg/mL for the extracts of *T. tessmannii* and *T. monadelpha* respectively. A value of 0.004806 mg/mL was obtained for ascorbic acid used as the reference drug.

Table 4.6: EC_{50} values of the stem bark total crude methanol extracts of *Trichilia tessmannii*, *Trichilia Monadelpha* and Ascorbic acid.

Extract / drug	**EC_{50} (mg/mL)**
T. tessmannii	0.02078
T. monadelpha	0.009854
Ascorbic acid	0.004806

Total Antioxidant capacity (TAC)

In the total antioxidant capacity assay, ascorbic acid was used as reference drug. The antioxidant activity was expressed as mg of ascorbic acid equivalent per g of the extract. The results for the Total antioxidant capacity showed a concentration dependant increase. There was an increase in the Total antioxidant capacity as the concentration of the extract increased. The total antioxidant capacities of the extracts were estimated to be 444.7 ± 28.58 mg/g for *T. tessmannii* and 324.4mg/g for *T. monadelpha*, both expressed as ascorbic acid equivalent. The standard curve plotted had the following; ($y = 5.744 * x + 0.1079$, $r^2 = 0.9930$).

Table 4.7: Total antioxidant capacity of the stem bark total crude methanol extracts of *T. tessmannii* and *T. monadelpha*, expressed as milligram ascorbic acid equivalent per gram of extract.

Extract / drug	**mg /g equivalent of ascorbic acid**
T. tessmannii	444.7 ±28.58
T. monadelpha	324.4 ±20.41

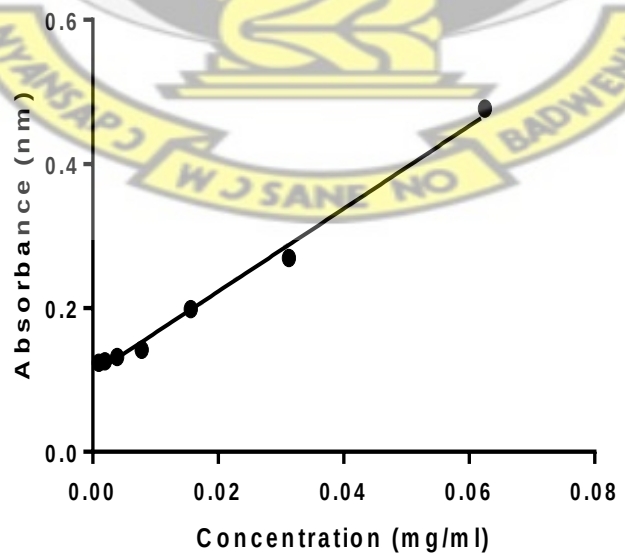


Figure 4.1 Calibration curve for ascorbic acid showing absorbance versus concentration

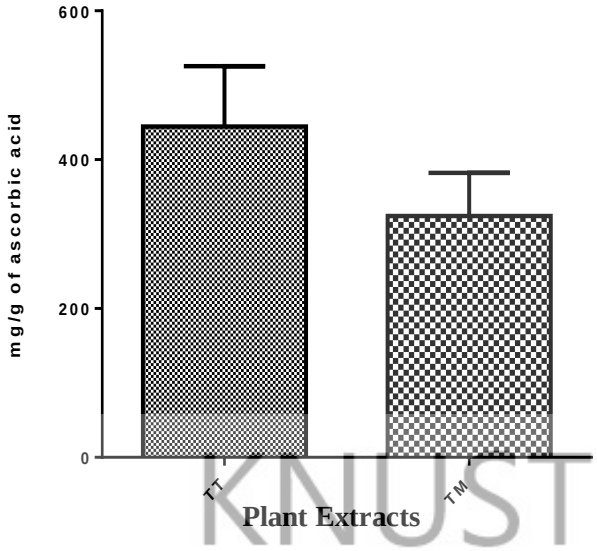


Figure 4.2: Total antioxidant capacity of *T. tessmannii* (TT) and T. *Monadelpha* (TM)

Total Phenol content (TPC)

The total phenol content of the extract was determined by using the folin-ciocalteu reagent with tannic acid used as the reference drug. The Total phenol content was expressed as mg of tannic acid equivalent per g of the extract (McDonald *et al*., 2001). The total phenol content of the extract was estimated to be 970.2 ± 78.69 mg/g expressed as tannic acid equivalent. The standard curve plotted had the following; ($y = 7.314 * x + 0.07054$, $r^2 = 0.9873$).

Table 4.8: Total phenol content of the stem bark total crude methanol extracts of *T. tessmannii* and *T. monadelpha*, expressed as mg tannic acid equivalent per gram of extract.

Extract / drug	**mg /g equivalent of tannic acid**
T. tessmannii	970.2±78.69
T. monadelpha	884.2±61.00

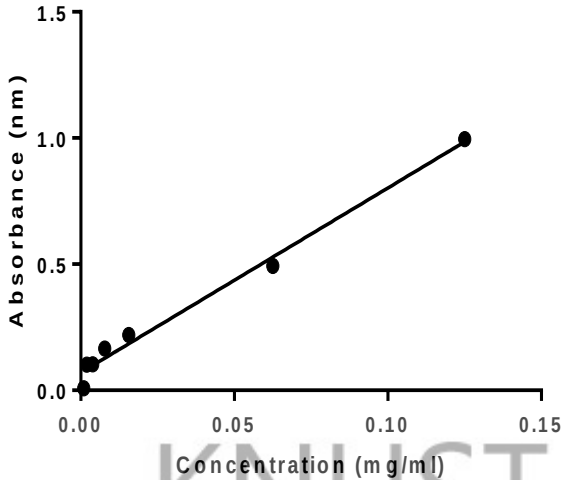


Figure 4.3: Calibration curve for tannic acid for Total Phenol content.

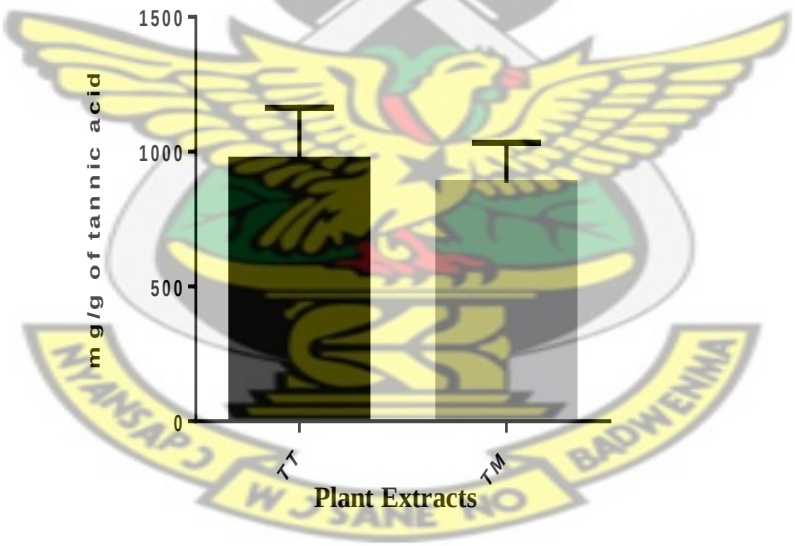


Figure 4.4 Total phenol content of *T. tessmannii* (TT) and T. *Monadelpha* (TM)

4.6 Chromatographic separation

Chromatographic separation of the ethyl acetate extract of the stem back of T. *Monadelpha* (TME) produced four main bulked fractions of interest. Below is a Schematic representation of fractionation of *T. monadelpha* stem bark (ethyl acetate extract) showing the various

fractions, sub-fractions and the isolated compound (unknown) with their corresponding weights.

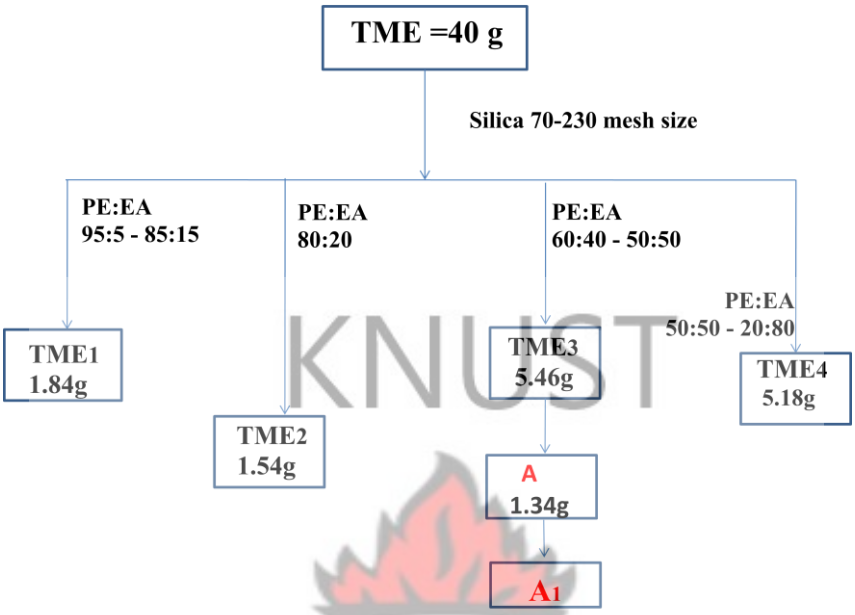
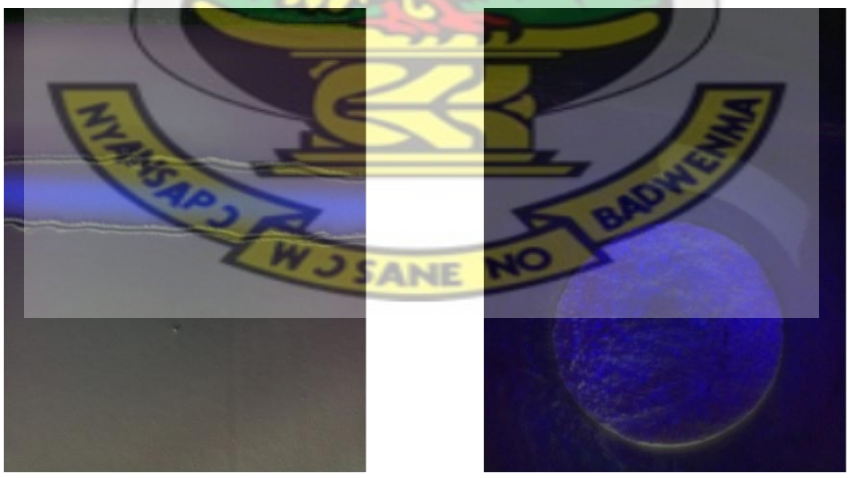


Figure 4.5: Schematic representation of fractionations of *T. monadelpha* stem bark (ethyl acetate extract)

4.6.1 Isolation of 'A_1'



Preparative TLC plate viewed under UV (366 nm)

After scraping off marked portion on the prep. TLC plate

Figure 4.5: shows the isolation process of 'A_1' using preparative TLC plates viewed under UV and the results after scraping off the marked portion on the plate.

CHAPTER 5

DISCUSSION AND CONCLUSION

5.1 DISCUSSION

This study for the first time, reports on the phytochemical constituents, the antimicrobial and antioxidant activities of the stem bark of *T. tessmannii*. It also reports for the first time, the antimicrobial activity of the stem bark *T. monadelpha*. The discussion looks at the preliminary phytochemical screening, the percentage yield obtained after extraction, the antimicrobial assay, the antioxidant assay and the chromatographic separations.

5.1.1 Phytochemical screening

The pulverized plant materials of the stem bark of both *T. tessmannii* (local 'twi' name: Otanduro-nini (meaning the 'male' Hatred medicine)) and *T. monadelpha* (local 'twi' name: Otanduro (meaning the Hatred medicine)) were taken through various tests prescribed by Sofowora (Sofowora, 2010) to investigate the phytochemicals present in them. The phytochemical screening revealed the presence of alkaloids, tannins, saponins, coumarins, steroids, cardiac glycosides, reducing sugars and terpenoids in the pulverizsed plant materials of both plants. There was the absence of cyanogenetic glycosides and flavonoids in both plants (Table 4.2). However, previous work done on *T. monadelpha* revealed the presence of flavonoids (Woode *et al.*, 2012; Ben *et al.*, 2013) but this study shows otherwise. This may be due to the differences in the time and place of collection. Leterme *et al*. (2006) reported that the variability in the mineral composition depends on the cultivation conditions, such as soil fertility and pH, water supply, climate and seasonal variations.

The only difference between the phytochemicals of the two plants was the presence of anthraquinone in *T. tessmannii* and its absence in *T. monadelpha* and the presence of anthracene glycosides in *T. monadelpha* and its absence in *T. tessmannii* (Table 4.2).

Many thousands of secondary plant metabolites have been isolated and many of them have powerful physiological effects in humans and are used as medicines (Newman, 2008; Iwu, 2014).

Alkaloids, a group of compounds containing nitrogen have been proven to have useful medicinal properties that improve the health of humans. Some of these useful isolated compounds include morphine and cocaine which act on the nervous system (Charbogne *et al.*, 2014); vincristine and vinblastine used in the treatment of some cancers (Sun *et al.*, 2014)and quinine which is used in the treatment of malaria (Kessler *et al.*, 2014).Some terpenoids (diterpense; taxol) are also used in the treatment of some solid tumors.

Saponins and some alkaloids have been known to possess selective antibacterial effects and have proven useful in the treatment of many diseases (Osbourn, 2003; Wallace, 2004). Some terpenoids (diterpense; taxol) are also used in the treatment of some solid tumors.

Also some phenolic compounds such as salicylic acid have been found to have anti-fungal properties. Aspirin, a derivative of salicylic acid have been use to reduce inflammation, pain and fever (Corey *et al.*, 2012).

Some flovonoids have also been proven to have important anti-inflammatory, anti-allergic anti-cancer activities (Abdel-Hadi, 2014) and have been shown to have potent antioxidant properties too (Lotito *et al.*, 2011).

The difference observed and the amount of each phytochemical present in each plant may have accounted for the difference observed in the antimicrobial and antioxidant activities of

the plants. In addition, the presence of these phytochemicals may be responsible for the acclaimed traditional medicinal uses of the plants under investigation.

5.1.2 Percentage yield

The pulverized plant material from both plants were separately serially extracted by the soxhlet apparatus using three solvents; petroleum ether, ethyl acetate and methanol. After the extraction, three extracts were obtained each for *T. tessmannii* and *T. monadelpha*. The percentage yields for the extracts were calculated as the ratio of the weight of crude dried extract to the weight of crude powdered drug used. The results from the calculation showed that *T. monadelpha;* (methanol extract = 11.43 %w/w, ethyl acetate = 2.19 %w/w and petroleum ether = 3.00 %w/w) had better yields in all the three extracts obtained as compared to those of *T. tessmannii* (methanol extract = 10.53 %w/w, ethyl acetate = 1.10 %w/w and petroleum ether = 0.72 %w/w).

It may also be seen from the above that with extracts of *T. monadelpha*, the methanol extract had a highest yield followed by the petroleum ether extract and lastly by the ethyl acetate extract.

The methanol extract *T. Tessmannii* had the highest yield followed by the ethyl acetate extract and finally by the petroleum ether extract. The petroleum ether, ethyl acetate and methanol extracts are known to contain non-polar, semi-polar and polar constituents respectively. In both plants the methanol extracts were the highest in yield meaning there are much more polar compounds present in these plants.

5.1.3 Antimicrobial assay

The antimicrobial activity was assessed on the three extracts obtained from serial extraction each of the stem bark of the two selected plants; *T. tessmannii* and *T. monadelpha*. The broth

dilution method was used and the Minimum Inhibitory Concentrations (MICs) were determined (Selim *et al.*, 2014).

The results from the antimicrobial assay performed showed that the three extracts of *T. tessmannii* (Table 4.3) exhibited varying inhibitory effects against the five selected microorganisms (two Gram positive, two Gram negative and one fungus). The best results were observed with the use of the ethyl acetate extract (this contains the semi-polar constituents of the plant) against all the selected microorganisms. The minimum inhibitory concentrations (MICs) were between the ranges of 0.625 mg/mL to 1.25 mg/mL (Table 4.3). The highest activity observed with the use of ethyl acetate extract was against *P. aeruginosa* and *C. albicans* with MIC of 0.625 mg/mL. The next best extract was the methanol extract (this contains the polar constituents of the plant) which also showed varied inhibitory effect against all the selected microorganisms. The MICs obtained by this extract was between the ranges of 1.25 mg/mL to 2.5 mg/mL (Table 4.3). This extract showed higher activity against *B. subtilis*, *P. aeruginosa* and *C. albicans* at MIC of 1.25 mg/mL.

The petroleum ether extract (this contains the non-polar constituents of the plant) showed the lowest activity against all the selected microorganisms (Table 4.3). There was no inhibition of the extract against *B. subtilis*, *P. aeruginosa* and *E. coli* even at a concentration of 10 mg/mL. There was however inhibition of *Staph. aures* and *C. albicans* at MIC as high as 10 mg/mL. The petroleum ether extract had the poorest activity among all the three extracts used. It may therefore be said that the antimicrobial activity of *T. tessmannii* resides more in the ethyl acetate extract (the semi-polar portion) since it gave the most potent activity and had the lowest MICs among all the three extracts.

The results of the antimicrobial activities from the three extracts of *T. monadelpha* (Table 4.4) also exhibited varying inhibitory effects against the five selected microorganisms. The

best results were observed with the use of the methanol extract (this contains the polar constituents of the plant) against all the selected microorganisms. The minimum inhibitory concentrations (MICs) were between the ranges of 1.25 mg/mL to 2.5 mg/mL. The highest activity observed with the use of methanol extract was against *E. coli* with MIC of 1.25 mg/mL.

The next best extract was the petroleum ether extract (this contains the non-polar constituents of the plant). This also showed varied inhibitory effect against all the selected microorganisms. The MICs obtained by this extract was between the ranges of 2.5 mg/mL to 10 mg/mL. This extract showed higher activity against *B. subtilis*, *P. aeruginosa* and *E. coli* at MIC of 2.5 mg/mL.

The ethyl acetate extract (this contains the semi-polar constituents of the plant) showed the lowest activity against all the selected microorganisms. There was inhibition of B. subtilis, P. *aeruginosa*, *E. coli* and *C. albicans* at MIC of 5 mg/mL and *Staph. aures* at MIC of 10 mg/mL. The ethyl acetate extract had the poorest activity among all the three extracts used. It may therefore be said that the antimicrobial activity of *T. monadelpha* is highest in the methanol extract (the polar portion) since it gave the most potent activity and had the lowest MICs among all the three extracts used.

Comparing the antimicrobial activities of the various extracts for both plants; the methanol extract for *T. tessmannii* (Table 4.3) gave better MICs as compared to *T. monadelpha* (Table 4.4) with both extracts showing broad spectrum antimicrobial activity. The ethyl acetate extract for *T. tessmannii* also showed a better activity against all the microorganisms compared to *T. monadelpha* with both extracts showing broad spectrum antimicrobial activity. Furthermore, comparing the activity of the petroleum ether extract of *T. tessmannii*

to *T. monadelpha*, a better result was observed for *T. monadelpha* which showed a broad antimicrobial activity than *T. tessmannii* with a narrow antimicrobial activity.

Comparing the activity of the extracts of both plants to the reference drugs, it may be observed that all the extracts with the exception of the petroleum ether extract of *T. tessmannii* showed both broad spectrum antibacterial and antifungal activity while ciprofloxacin (reference drug) only showed antibacterial activity and ketoconazole also showed only antifungal activity. In terms of the antimicrobial spectrum, the extracts from both plants may be said to have better antimicrobial spectrum than each of the reference drugs since the extracts showed both antibacterial and antifungal properties.

In relation to the MICs, those of reference drugs were better than the extracts. However the reference drugs are single compounds while the extracts are crude; thus the extracts are a mixture of compounds. Therefore the effects observed by these extracts may be as a result of one or more compounds. The other compounds may be masking or antagonising the effect produced by these extracts. Again, the higher MICs of the extracts as compared to the lower MICs for the reference drugs could be as a result of the responsible compound(s) in the extracts being in lower concentration. In other to obtain the full effect of the extracts, isolation and purification of the active compound(s) responsible for the observed activity may be necessary.

A number of researchers have shown that some phytochemicals such as terpenoids (El-Seedi *et al.*, 2002), coumarins (Ojala *et al.*, 2000), saponins (Osbourn, 2003; Wallace, 2004), tannins and alkaloids (Klausmeyer *et al.*, 2004) identified in this work possess certain antimicrobial properties. The presence of these phytochemicals in these plants may be responsible for the observed antimicrobial activities.

5.1.4 Antioxidant assay

Antioxidant is an agent that neutralizes or terminates the chain reaction initiated by free radicals either by providing extra electrons needed to make the pair or by breaking down the free radical molecule to make it harmless (Barazesh, 2008). Antioxidants stop the chain reaction of free radical formation and improve our health by boosting our immune system (Sandeep Prabhu *et al.*, 2004).

The antioxidant properties were assessed on the total crude methanol extract of each of the two selected plants; *T. tessmannii* and *T. monadelpha* using assays such as the DPPH scavenging assay, Total antioxidant assay and Total phenol content assay.

From the results of the DPPH scavenging assay, it was observed that there was an increase in the percentage DPPH scavenging activity with increase in the concentration of the extracts of both *T. tessmannii* and *T. monadelpha* and the standard drug (ascorbic acid). There was a decrease in DPPH absorbance as the concentration of the extracts increased. The EC_{50} values recorded were 0.0207 mg/mL and 0.0099 mg/mL for the extracts of *T. tessmannii* and *T. monadelpha* respectively and 0.004806 mg/mL for the ascorbic acid used as the standard drug. The extracts may be said to contain some constituents that may have the ability to quench free radicals by donating hydrogen atoms or electrons to make the DPPH radical stable; thus reducing it to form DPPH.H which is a yellow coloured product (Blois, 1958; Aliyu *et al.*, 2010). The donation of the electrons to the free radical helps in the termination of the chain reaction that the free radicals initiate.

Among the two plants, *T. monadelpha* may be said to have a better DPPH scavenging activity than *T. tessmannii* due to the low EC_{50} value obtained.

Total antioxidant capacity assay involves the reduction of Mo(vi) to Mo(v) by the test sample

(extract) and the formation of a green phosphomolybdenum(v) complex at acidic pH (Prieto *et al.*, 1999; Das *et al.*, 2014). The absorbance of the complex formed is measured at 695 nm using a spectrophotometer. The values obtained from the estimation of the Total antioxidant capacity assay were 444.7 ± 28.58 mg/g of ascorbic acid and 324.40 ± 20.41mg/g of ascorbic acid for *T. tessmannii* and *T. monadelpha* respectively. The above results imply that for

every 1 g of *T. tessmannii* and *T. monadelpha* extract taken; only 44.70% and 32.40% respectively behave like ascorbic acid, a standard antioxidant drug. These values may also imply that, these two plants possess some antioxidant activity and can may be used in disease states that involve oxidative stress.

Total phenol content assay depends on the reduction of folin-ciocalteu reagent (made up of phosphotungstic and phosphomolybdic acids) by phenols present in the extract to blue oxides which have a maximum absorption at 760nm (Kamboj *et al.*, 2014). The Total phenol content values for *T. tessmannii* and *T. monadelpha* were 970.2 ± 78.69 mg/g of tannic acid and 884.2 ± 61.00 mg/g of tannic acid respectively. Research have shown that polyphenolic and phenol compounds are potent antioxidant and these are abundant in medicinal plants and so the need to estimate their content (Ozgová *et al.*, 2003).

Current researches have proven that certain secondary metabolites of medicinal plants such as polyphenols, coumarins, terpenoids possess potent antioxidant properties and these may be used in the management of diseases that are linked with oxidative stress (Sies, 2000).

Also some polyphenols, and vitamins such as E and C have been proven to help in the delay of aging by neutralizing the actions of free radicals (Bolann and Ulvik, 1997). This may suggest that the antioxidant potentials of this plant may be as a result of the presence of certain phytochemicals such as coumarins, terpenoids polyphenol and other phenolic

compounds. Further studies would therefore be required to fractionate, isolate and characterize the active principles responsible for the antioxidant activity observed.

5.1.5 Chromatographic separation

Results from the chromatographic separation reviewed the isolation of an unknown compound from a sub-fraction TME_3 from the ethyl acetate extract of *T. monadelpha*. The isolated compound now awaits further purification and characterization.

5.2 CONCLUSIONS AND RECOMMENDATIONS

5.2.1 Conclusions

The present study was set out to investigate the phytochemical constituents, antimicrobial and anti-oxidant constituents of two Ghanaian medicinal plants namely *Trichilia tessmannii* and *Trichilia monadelpha*. The first part of the study was to screen the plants for phytochemical constituents. The phytochemical screening revealed the presence of major secondary metabolites such as alkaloids, tannins, saponins, coumarins, sterols, cardiac glycosides, reducing sugars and terpenoids in the pulverizsed plant materials of both plants.

The results of this study has also shown that the stem bark extracts of *Trichilia tessmannii* and *Trichilia monadelpha* have some antimicrobial activity against selected Gram positive, Gram negative bacterial as well as fungus *C. albicans*. The best antimicrobial results of *Trichilia tessmannii* were observed with the use of the ethyl acetate extract against *P. aeruginosa* and *C. albicans* with MIC of 0.625 mg/mL. The best results for *Trichilia monadelpha* were observed with the use of the methanol extract against *E. coli* with MIC of 1.25 mg/mL.

The total crude methanol extract of the plant materials also possessed potent antioxidant activity after being screened for free radical scavenging activity, total antioxidant content and

total phenol content. The best free radical scavenging activity was exhibited by *Trichilia monadelpha* with EC_{50} 0.0099 mg/mL, that of total antioxidant content was by *T. tessmannii* with 444.7 ± 28.58 mg/g of ascorbic acid and that of total phenol content was by *T. tessmannii* with 970.2 ± 78.69 mg/g of tannic acid. These biological effects observed may be possibly due to the presence of secondary metabolites in the plants.

This study has shown that the ethno-botanical use of the selected medicinal plants for the treatment of various disease conditions is thus justified to some extent by the results of this study. Finally, an unknown compound 'A_1' has been isolated.

5.2.2 Future Laboratory Work

Work to be done include;

- To continue isolation of the rest of the extracts
- To perform a bioassay on the isolated compounds and
- To characterise these compounds.

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