**CHAPTER ONE**

**INTRODUCTION**

Antibiotics to a large extent have helped protect people from infections and have become widely available; this unique class of drugs is losing its effectiveness from overuse. The more exposure bacteria have to an antibiotic, the more likely they will develop resistance to the antibiotic. Increasing resistance of microorganisms to antibiotics and other orthodox drugs has resulted in the search for more organic molecules from plant source with antimicrobial properties. Therefore African medicinal plants’ study and investigation is of great significance. Phytochemical composition of plants are abundant with tannins, saponins, alkaloids, flavonoids, cardiac glycosides and phytosterols (Nair *et al,* 2007), which serve as bioactive precursors have shown to exhibit antimicrobial characteristics. In ethnopharmacology research, the antimicrobial susceptibility test (AST) is used to determine the efficacy of potential antimicrobial species. AST methods are used to screen plant extracts for antimicrobial activity but are largely used to determine the usefulness of an antimicrobial in combating infections by determining its minimum inhibitory concentration. In clinical research in vitro susceptibility tests are particularly important if an organism is suspected to belong to a species that has shown resistance to frequently used antimicrobial agents of which the study organisms are part of them. (Kamba *et al.,* 2010). The array of secondary metabolites produced by plants is daunting, with wide ranging chemicals, physical and biological activities. These constitute a source of bioactive substances which are hypothetically the reason for the perceived recognition being antimicrobial. Antimicrobial extracts serve as plant defense mechanism against predation by microorganisms, insects and herbivores. Some such as terpenoids give plants their odors, others (quinones and tannins) are responsible for plant’s pigments (Cowan, 1999). Therefore, analogously, these metabolites and extracts are meant to be used initially to prevent microbial infestation of the plants (a defense mechanism) but these extracts are not discriminatory, that is to say that when prudently extracted, it would still inadvertently serve the same original purpose (inhibit microbial growth or rather prevent microbial survival). Therefore, for these extracts to be harnessed, the particular regions of the plant where the defenses are stationed or most concentrated should be recognized. These regions are characterized often by eccentric tastes (stems) or colorfulness (leaves).The inclining principle is that for the intricacies of plants to be sterile, they must have “something” that keeps it that way. That “something” should be extracted and tested against certain microorganisms to discover the antimicrobial potencies. Interestingly, African mango (*Irvingia gabonensis*) leaf and root extracts have documented inhibitory activity against several bacterial and fungi (Kubmarawa *et al.,* 2012). For instance, leaf extract of *lrvirgia gabonensis* used as a febrifuge. In Cameroon, preparation mainly from the bark are used to treat hernia and yellow fever and as an antidote for poisoning. Kernels of *Irviginia gabonensis* are used to treat diabetes. Preparations from the bark are rubbed on to the body to relieve pains and are applied to sores and wound and against toothache. They are also taken to treat diarrhea (Harris, 1996). Potential mechanism of action include membrane disruption by terpenoids and inactivation of microbial adhesion, enzymes and cell envelope transport proteins by ellagic acid-like compounds (Kuete *et al.,* 2017). *Dialium guineense* is reported to possess antimicrobial activities in the cure of diarrhea, palpitations as well as fever (Lamien *et al.,* 2010). Okwu and Ekeke (2003) reported in their studies that the plant contains saponins which are presumed to add to the cleaning effect of teeth and at the same time prevent caries and plaques on the teeth of the user.  *D. guineense* leaf and bark extracts have been reported by Orji *et al.,*(2012) to show antimicrobial properties against *Staphylococcus aureus* and *Klebsiella pneumoniae* at varying concentrations. The antibacterial activities of both the aqueous and ethanolic leaf and bark extracts of *D. guineense* were evaluated while the phytochemical analysis reveals the presence of flavonoids, alkaloids, tannin and saponin. Also, the methanolic crude leaf extract of *D. guineense* was found by Akinpelu (2011) to possess bioactivity against fourteen out of eighteen environmental strains of *Vibrio* species. Phytochemical analysis of the plant extract revealed some phenolic compounds. These phenolic compounds include phenolic acids, flavonoids, tannins, saponins and cardiac glycosides among others. Phenolic compounds from medicinal herbs and dietary plants play important roles in health in addition to enhancing antimicrobial activities in these plants*. D. guineense* stem is used as chewing stick (indigenous tooth brush) among the Nigerian populace.

Oral infections caused by microorganisms have led to increased risk of oral health problems such as Dental Caries (DC), periodontitis and Oral Candidiasis (OC). *Streptococcus mutans* and *Candida albicans* are the primary organisms responsible for DC and OC, respectively. It has been demonstrated that the initiation and progression of oral disease is primarily due to the increased proliferation of opportunistic microorganisms (Gazim *et al.,* 2008). A large variety of microorganisms are associated with oral disease, however, the increased proliferation of *S. mutans* , present in carious lesions, is the primary cause for the initiation and the progression of DC, and hence is of primary interest in the presented research (Gazim *et al.,* 2008; Mojab *et al.,*2008). The relationship between this causative organism and pathogenesis is not clear, although it has been suggested that *S. mutans* proliferation leads to acid production, promoting tooth decay and DC (Kuete *et al.,* 2017). OC, also termed thrush, is a yeast infection of the genus Candida, most often caused by *C. albicans.* The current methods for minimizing the incidence of DC focus on prevention techniques such as proper oral hygiene, drinking water fluoridation and application of dental sealants (Singleton, 1999). Unfortunately, as the statistics suggest, these prevention methods have shown only limited success. OC is typically controlled by anti-fungal drugs such as amphotericin B (Vogt, 2005). If not established as a biofilm, *C. albicans* is usually susceptible to most anti-fungals, but mechanical disruption of a biofilm prior to anti-fungal application may be required. A well-known problem is that long-term exposure to anti-fungal agents promotes acquired resistance (Bentley and Maganathan, 1982). The documented levels of resistance in oral*Candida* are indeed on the rise (Bentley and Maganathan, 1982). To address these shortcomings, an alternative therapy is necessary for controlling the incidence of DC and OC.

**1.2 STATEMENT OF PROBLEM**

* High resistance of microorganisms to antibiotics.
* High prevalence of *Candida albicans* and *Streptococcus mutans* with oral infection.
* Lack of adequate research of Nigerian medicinal plants in the treatment of oral infection.

**1.3 AIMS AND OBJECTIVES**

**AIM**

* The aim of this study is to determine the antimicrobial effect of two ethnobotanical Nigerian plants *Dialium guineense* (“Icheku”) and *Irvingia gabonensis* (“Ujiri”) against two microorganisms (*Streptococcus mutans* and *Candida albicans*) associated with oral infection.

**OBJECTIVES**

* To determine the antimicrobial sensitivity of *Dialium guineense*(“Icheku”) and *Irvingia gabonensis* (“Ujiri”) against *Streptococcus mutans* and *Candida albicans* using agar well diffusion method.
* To determine the minimum inhibitory concentration and minimum bactericidal concentration of *Dialium guineense* (“Icheku”) and *Irvingia gabonensis* (“Ujiri”) against the test organisms
* To determine the phytochemical properties of *Dialium guineense* (“Icheku”) and *Irvingia gabonensis* (“Ujiri”)

**CHAPTER TWO**

**LITERATURE REVIEW**

**2.1 DESCRIPTIONAND ECOLOGY OF THE RESPECTIVE PLANTS**

*Dialium guineense* is a tree about 30 m high, with a densely leafy crown, but often shrubby. Bole without buttresses, bark smooth, grey; slash reddish, yielding a little red gum. Leaves sometimes finely hairy, with a common stalk 5-13 cm long, with an odd terminal leaflet and usually 2 pairs of opposite or alternate leaflets, the lower pair being somewhat smaller; leaflets mostly 3.5-10 x 2.5-5 cm, elliptic to broadly elliptic, sometimes slightly obovate; blunt at the apex or abruptly and shortly acuminate, symmetrical and rounded or slightly cuneate at the base; leathery, glabrous above and with the midrib slightly sunken, sometimes finely hairy beneath. Flowers usually whitish, in large terminal, or occasionally axillary, panicles up to 30 cm long; branches spreading out widely and more or less horizontally; the whole infloresence at first covered with very short, brownish hairs; individual flowers with short stout stalks, the buds about 2 mm long. Fruits usually abundant, more or less circular and flattened, but sometimes almost globose, up to 2.5 cm in diameter, densely velvety, black; each fruit with a stalk about 6 mm long with a little collar near the apex, with a brittle shell enclosing 1 seed (or exceptionally 2), embedded in a dry, brownish, sweetly acidic, edible pulp.

**2.1.1TAXONOMIC HIERARCHY OF *Dialum guineense***

Kingdom: Plantae

Phylum: Angiosperms

Class: Eudicots

Subclass: Rosids

Order: Fabales

Family: Fabaceae

Subfamily: Caesalpinioideae

Genus: Dialium

Species: *D. guineense*

In Nigeria, the tree flowers from September to October and fruits from October to January. In Ghana, in September to November the tree is covered with small white flowers in panicles; fruit ripens in March to May but may be earlier and may persist longer. Animals, which like to eat the pulp in which the seeds are embedded, help disperse the fruit. However, the fruit can also be transported by water since it floats; transport by sea currents may lead to long-distance dispersal.

*Irvingia gabonensis* grows straight, up to a height of 40 m (130 ft) and 1 m (3 ft 3 in) in diameter. (AFT database, 2006) It has buttresses to a height of 3 m (9.8 ft). The outer bark is smooth to scaly with grey to yellow-grey color. The crown is evergreen, spherical and dense. Leaves are elliptic, one margin is often a little rounder than the other, acuminate, dark green and glossy on the upside. Flowers are yellow to greenish-white in small panicles .(AFT database, 2006) The flowers are bisexual. The fruit is nearly spherical, green when ripe with a bright orange pulp. The stone is woody and contains one seed. Seedling germinates epigeally .(AFT database, 2006)

**2.1.2 TAXONOMIC HIERARCHY OF *Irvingia gabonensis***

Kingdom: Plantae

Clade : Angiosperms

Clade : Eudicots

Clade : Rosids

Order: Malpighiales

Family: Irvingiaceae

Genus: Irvingia

Species: *I. gabonensis*

*Irvingia gabonensis* is pollinated by Coleoptera, Diptera, Hymenoptera and Lepidoptera.(AFT database, 2006) It flowers from March to June and has two fruiting seasons: from April to July and from September to October. (National Academies Press, 2006) Seeds are dispersed by specialized vertebrates as elephants and gorillas. By reducing the number of those animals, the spread and regeneration of dika decreases and it becomes dependenton human planting. (Tchoundjeu and Atangana, 2007)

Humans eat the fruits fresh, leading to the misnomer, African mango. (AFT database, 2006) The fruits are processed into jelly, jam,juice and sometimes even wine. (Ecocrop, 1993) The pulp has also been used to prepare black dye for cloth coloration. The seed coat has to be cracked open to get to the endosperm. Seeds, also called dika nuts, are eaten raw or roasted. Mostly however they are pounded to butter- or achocolate-like block. (Tchoundjeu and Atangana, 2007) Seeds can be pressed to produce an edible oil (solid at ambient temperatures) or margarine used for cooking. The oil can also be processed further to soap or cosmetics. (Tchoundjeu and Atangana, 2007) The press cake can be used as cattle feed or as thickening agent for soup. Seeds can be groundor crushed and used as a thickening and flavoring agent insoups and stews.(AFT database, 2006) They can also be made into a cake called "dika bread" for preservation. (AFT database, 2006)

**2.2 *Streptococcus mutans and Candida albicans:* ROLE IN ORAL CARIES AND MECHANISM OF BIOFILM FORMATION.**

The human mouth with its diverse niches and ample supply of nutrients is undoubtedly conducive for the unrestricted formation of natural microbial biofilms. The oral microbial communities are some of the most complex microbial floras in the human body, consisting of more than 700 different bacterial species (Dewhirst *et al.*,2005). Occurrence of disease results from disturbance of the equilibrium of this complex ecosystem, where population shifts lead to overrepresentation of pathogenic species which contribute to the onset and progression of the most common oral diseases, caries and periodontal disease (Kuboniwa *et al*., 2012) Culture-independent molecular methods such as proteomics and 16S rRNA sequencing aiming to determine the bacterial diversity in the human oral cavity have demonstrated that in the supragingival plaque, *S. mutans* was the dominant species, with elevated levels of other streptococci including *S. sanguinis*, *S. mitis*, and *S. salivarius* in addition to lactobacilli and Veillonella. In contrast, the subgingival plaque was made up primarily of Gram-negative anaerobic bacteria such as *Fusobacterium nucleatum, Porphyromonas gingivalis*, and *Prevotella intermedia* which are known to be periodontal pathogens (Kuboniwa *et al*., 2012). The dental tissues—enamel, dentin, and cementum—constitute the oral solid surfaces coated by a pellicle to which the microbial cells attach. The primary colonizers and secondary organisms stick to each other on the surface of teeth and generate a matrix of exopolysaccharide within which cells grow, forming a community with a collective physiology (Kidd and Ferjerskov, 2004). The resulting biofilm formed, known as dental plaque, subjects the teeth and gingival tissues to high concentrations of microbial metabolites which result in dental disease (Jenkinson and Lamont, 2005; Kolenbrander *et al.,* 2002). The interactions between the various species in these mixed biofilms can be synergistic in that the presence of one microorganism generates a niche for other pathogenic microorganisms which can serve to facilitate the retention of organisms, an oral phenomenon known as coaggregation (Kuboniwa *et al*., 2012; Rickard *et al., 2003)*. The bacteria in the biofilm are always metabolically active which causes fluctuations in pH and loss of minerals from the tooth, ultimately resulting in dissolution of the dental hard tissues and formation of lesions known as dental caries (Kidd and Ferjerskov, 2004; Lemos *et al.,* 2013). Interestingly, metabolic communications among oral bacteria may occur where the excretion of a metabolite by one organism is used as a nutrient by other organisms and breakdown of a substrate by enzymatic activity of one organism creates available substrates for different organisms (Hojo *et al.,* 2009; Kleinberg, 1999).

**2.3 ORAL CARIES**

Oral caries or tooth decay is among the most prevalent human diseases, second only to the common cold (Isalm *et al*., 2007). Caries is a chronic disease that progresses slowly and is characterized by localized and irreversible destruction of the tooth (Rouabhia and Chmielewski, 2012; Zero *et al.,* 2009). Despite scientific advancements in cariology in the past 150 years, dental caries remains a serious issue worldwide, particularly in children where it is the primary source of tooth loss. In the United States, 42% of children of ages between 2 to 11 have had dental caries in their primary teeth, and in the adult population, dental caries and periodontal diseases affect 60–90% of individuals worldwide (Rouabhia and Chmielewski, 2012). People with disabilities and lower socioeconomic status suffer from the highest prevalence and pathogenicity of dental caries. Caries results from the complex interactions among the microbial species adhering to the tooth surface, with dietary, salivary, and genetic influences. The metabolic microbial interactions that take place in the dental biofilm result in acid production and extracellular glucan formation which promote microbial attachment to teeth (Kidd and Ferjerskov; 2004; Isalm *et al*., 2007; Rouabhia and Chmielewski, 2012). Ninety percent of carious lesions occur in the pits and fissures of permanent posterior teeth and molar teeth as the biofilm tends to stagnate and mature in these areas which are relatively protected from mechanical wear by the tongue, cheeks, and tooth brushing (Kidd and Ferjerskov; 2004). The recognition of acid as the central etiological agent in dental caries initiated a search for the causative microorganisms in the oral microbiota, and in the early 1960s, the bacterial species *Streptococcus mutans* became the main focus of caries research, assumed to be the specific cariogen (Klinke *et al.,* 2009).*Streptococcus mutans* are Gram-positive bacteria that reside in the human mouth and, more specifically, in the multispecies biofilms on the surfaces of teeth (Zero *et al.,* 2009). *Streptococcus mutans* are major cariogenic organisms—the result of their ability to produce large quantities of glucans as well as acid, exceeding the salivary buffering capacities, which gives the bacteria an advantage to outcompete noncariogenic commensal species at low pH environments (Lemos *et al.,* 2013; Falsetta *et al.,* 2012). This ability to survive in an acid environment by modulating sugar metabolic pathways coupled with irreversible binding to teeth is a key component to *S. mutans* pathogenesis.

Bacteria have evolved numerous defenses against antimicrobial agents, and drug resistant pathogens are on the rise. This resistance is conferred by multidrug resistance pumps (MDRs), membrane translocases that extrude structurally unrelated toxins from the cell. These protect microbial cells from both synthetic and natural antimicrobials (Stermitz *et al.,* 2000).The use of plant extracts and phytochemicals can be of great significance in therapeutic treatments and could help curb the problem of these multi-drug resistant organisms. In a study done with *Pseudomonas aeruginosa*, which is resistant to different antibiotics, its growth was inhibited by extracts from clove, jambolan, pomegranate and thyme (Nascimento *et al.,* 2000).

**2.4EXPERIMENTAL APPROACHES**

There are multiple factors that may affect the outcome of susceptibility tests and standardized methods are more likely to be reproducible than unstandardized methods. Standardization is required for intra- and interlaboratory reproducibility as results may be significantly influenced by the method used (EUCAST, 2003). Standard criteria for evaluation of plant antimicrobial activity are lacking and results greatly differ between authors. Sometimes it is difficult to compare results obtained, when dealing with plant extracts, with published results in the literature because several variables influence the results, such as the environmental and climatic conditions under which the plant grew, choice of plant extracts, choice of extraction method, antimicrobial test method and test microorganisms (Nostro *et al.,* 2000; Hammer *et al.,*1999). The beneficial medicinal effects of plant materials typically result from the secondary products present in the plant although it is usually not attributed to a single compound but a combination of the metabolites. The medicinal actions of plants are unique to a particular plant species or group, consistent with the concept that the combination of secondary products in a particular plant is taxonomically distinct (Parekh *et al.,* 2005). They also vary between tissues (higher concentrations occur in bark, heartwood, roots, branch bases and wound tissues), among species from tree to tree and from season to season (Gottlieb, 1990). In their work, Mitscher *et al.*(1972), found that extracts are generally richest in antibacterial agents after the flowering (sexual) stage of their growth is complete, and that plants taken from stressful environments were particularly active.

**2.4.1Plant extract preparation**

Extraction methods, used pharmaceutically, involve separation of medicinally active portions of plant tissues from the inactive/inert components by using selective solvents with appropriate extraction technology. During extraction, solvents diffuse into the solid plant material and solubilise compounds with similar polarity (Green, 2004). The basic parameters influencing the quality of an extract are:

1. The plant part used as starting material,
2. The solvent used for extraction and
3. The extraction technology.

Effect of plant material depends on the nature of the plant material; its origin; degree of processing; moisture content and particle size, while variations in extraction method include type of extraction; time of extraction and temperature. The nature of solvent as well as solvent concentration and polarity will also affect quantity and secondary metabolite composition of an extract (SEA, 2006).

* + 1. **Plant material**

Fresh or dried plant material can be used as a source for secondary plant components. However, most scientists working on the chemistry of secondary plant components have tended to use dried plant material for several reasons. Differences in water content may affect solubility of subsequent separation by liquid-liquid extraction and the secondary metabolic plant components should be relatively stable, especially if it is to be used as an antimicrobial agent. Furthermore many plants are used in the dry form (or as an aqueous extract) by traditional healers. Plants are usually air dried (Dilika *et al.,* 1996; Baris *et al.,* 2006) to a constant weight but other researchers dry the plants in the oven at about 40oC for 72hours (Salie *et al.,* 1996). Also, plants will have different constituents depending on the climatic conditions in which it is growing. The choice of plant material used in the extract preparation is usually guided by the traditional use of the plant and the ease of handling of the different plant parts like the leaves, stems etc.

**2.4.3 Choice of solvent**

Successful determination of biologically active compounds from plant material is largely dependent on the type of solvent used in the extraction procedure. Properties of a good solvent in plant extractions include low toxicity, ease of evaporation at low heat, promotion of rapid physiologic absorption of the extract, preservative action and inability to cause the extract to complex or dissociate (Hughes, 2002). The choice of solvent is influenced by what is intended with the extract. Since the end product will contain traces of residual solvent, the solvent should be non-toxic and should not interfere with the bioassay. The choice will also depend on targeted compounds. In a study where the optimal conditions for extraction of tannins and other phenolics, aqueous acetone was better at extracting total phenolics than aqueous methanol (Cork and Krochenberger, 1992). In another study where twenty different solvents were valuated, chloroform was found to be the best solvent for the extraction of non-polar, biologically active compounds from the roots of *Angelica archangelica* (Harmala *et al.,* 1992). If the extraction is for general phytochemical analysis or screening then the larger the variety of compounds the extractant will extract the better, because there is a better chance that biologically active compounds will be present (Eloff, 1998). Traditional healers use primarily water but plant extracts from organic solvents have been found to give more consistent antimicrobial activity compared to water extracts (Parekh *et al.,* 2005). Polyphenolic compounds such as flavonols and most other reported bioactive compounds are generally soluble in polar solvents such as methanol (Houghton *et al.,* 1998). Most antimicrobial active components that have been identified are not water soluble and thus organics solvent extracts have been found to be more potent (Parekh *et al.,*2006). Water soluble flavonoids (mostly anthocyanins) have no antimicrobial significance and water soluble phenolics are only important as antioxidant compounds (Yamaji *et al.,* 2005; Nang *et al.,*2007).The most commonly used solvents for investigations ofantimicrobial activity in plants are methanol, ethanol, andwater (Parekh *et al.,*2005; Bisignino *et al.,* 1999; Lourens*et al.,* 2004; Salie *et al.,* 1996; Rojas*et al.,* 2006).Dichloromethane has also been used by a number ofresearchers (Dilika and Meyer, 1996; Freixa *et al.,* 1996).Some authors use a combination of these solvents to obtain the best solvent systems for extraction (Nostro *et al.,* 2000). Acetone, although not a very commonly used solvent, has been used by a number of authors (Basriand Fan, 2005; Dilika *et al.,* 1996; Lourens *et al.,* 2004; Mathkega *et al.,* 2006). In a study by Masoko and Eloff (2006) where they investigated the antifungal activity of Combretum species, from the extractants used, which included hexane, dichloromethane, acetone and methanol, they discovered that acetone and methanol extracted more chemical compounds from the leaves than the other solvents. Both acetone and methanol were found to extract saponins which have antimicrobial activity. Eloff (1998) examined a variety of extractants for their ability to solubilise antimicrobials from plants, rate of extraction, ease of removal, toxicity in bioassay, among other things, and acetone received the highest overall rating. It gave the lowest minimum inhibitory concentration for gram positive organisms tested and the largest number of different components and inhibitors from two plants tested, but Eloff does note that different results may be obtained with other plants and generalization cannot be made on the usefulness of acetone as an extractant.

**2.4.4 The extraction methods**

Variations in extraction methods are usually found in the length of the extraction period, solvent used, pH, temperature, particle size and the solvent-to-sample ratio. The longer the contact between solvent and material the more is extracted until all possible materials have been extracted. The extraction period can be shortened by grinding the plant material finer as this will increase the surface area for extraction thereby increasing the rate of extraction. Shaking the plant material-solvent mixture will also increase the rate of extraction. In the study by Eloff (1998), 5 min extractions of very fine particles of diameter 10 µm gave higher quantities than values obtained after 24 h in a shaking machine with less finely ground material. In one study, sequential extraction with various solvents at room temperature was compared with extraction in a water bath at 37°C for 30 min with distilled water adjusted to pH 2.0 with HCI and then neutralized with NaOH before extraction with diethyl ether. The authors concluded that the latter method had higher activity which was ascribed to the acidified aqueous environment which promoted easy extraction (Nostro *et al.,* 2000). The solvent-to-sample ratio affects the quantity and quality of constituents obtained. In a study to identify the optimal conditions for extracting sugars from nondefatted soybean a solvent ratio of 5:1 at 25◦C or 50◦C

for 15 min was found to give the best yield of sugar (Giannoccano *et al.,* 2006). In some studies solvent to sample ratios of 10 ml : 1 g solvent to dry weight ratio has been used and reported as ideal (Green, 2004). The method that has widely been used by researchers investigating antimicrobial activity is homogenization in solvent (Meyer and Dilika, 1996; Basri and Fan, 2005; Parekh *et al.,* 2005). Dried plant material is ground in a blender, put in solvent and shaken vigorously for 5 min or left for 24 hours after which the extract is filtered and fresh solvent added to the residue for another 24 hours. Some authors report shaking unhomogenized dry leaves in solvent for about 5 min, followed by filtering and concentrating under reduced pressure to obtain an epicuticular extract (Mathekga, 2001). This actually gave a higher yield and bioactivity than using the same method but with homogenized (macerated) extract (Dilika *et al.,*1996; Mathekga, 2001). Of interest are the results obtained by Meyer and Dilika (1996) using these different methods on the same plant. They found that the homogenized dichloromethane extract generally had higher activity than the shaken extract of the same solvent. The trend was the same for the aqueous extract for the same microorganisms tested. One other common method is serial exhaustive extraction which involves successive extraction with solvents of increasing polarity from a non-polar (hexane) to a more polar solvent (methanol) to ensure that a wide polarity range of compounds could be extracted (Green, 2004). This is ideal when the aim is to screen the plant for a variety of compounds. Some methods are employed when a particular class of compounds is targeted. For example when one is interested in essential oils, then the method of choice would be steam distillation, volatile solvent extraction or supercritical fluid extraction (Lemberkovics *et al.,* 2002). Maceration, maceration with sonication, Soxhlet extraction and SFE with hexane or CO2 was compared for the extraction of low-polarity compounds from Mikania glomerata and SFE-hexane proved to be the most effective. These newer methods, which also include microwave assisted methods, are proving to be more efficient than the conventional methods (Vilegas *et al.,* 2002). Other researchers employ soxhlet extraction of dried plant material using organic solvents (Kianbakht and Jahaniani, 2003). In soxhlet extraction, the sample is continually exposed to fresh solvent, which improves the efficiency of the method. The method works well for compounds that can withstand the temperature of the boiling solvent, but cannot be used for thermolabile compounds as prolonged heating may lead to degradation of compounds (de Paira *et al.,* 2004). Other common extraction methods include maceration (for fluid extract) where whole or coarsely powdered plant-drug is kept in contact with the solvent in a stoppered container for a defined period with frequent agitation until soluble matter is dissolved; percolation; and infusion which is prepared by immersing the plant material for some time in cold or hot water (Handa, 2006).

**2.5 STUDIES IN ANTIMICROBIAL SUSCEPTIBILITY TESTING**

In ethnopharmacology research the antimicrobial susceptibility test (AST) is used to determine the efficacy of potential antimicrobials from biological extracts against a number of different microbial species. AST methods are used to screen plant extracts for antimicrobial activity but are largely used to determine the usefulness of an antimicrobial in combating infections by determining its minimum inhibitory concentration (MIC). In clinical research in vitro susceptibility tests are particularly important if an organism is suspected to belong to a species that has shown resistance to frequently used antimicrobial agents. They are also important in epidemiological studies of susceptibility and in comparisons of new and existing microbial agents (EUCAST, 2003). Successful discovery of novel natural antimicrobials has necessitated the development of new bioassay techniques which are sensitive enough to detect small amounts of biologically active chemicals (Lampinen, 2005). Standardized in vitro tests are essential for screening plant extracts or compounds and more studies should be conducted for MIC determination of natural products in order to get results that are comparable tothose of currently used antibiotics (Devienne and Raddi,2002). Evaluation of the performance of a susceptibility test should include criteria such as ease of use, reproducibility, i.e. the ability to yield the same result on repeat testing, test sensitivity and specificity (Struelens *et al.,* 1995). Although current standard methods, approved by various bodies like the National Committee for Clinical Laboratory Science (NCCLS) [now known as Institute of Clinical Laboratory Standards (ICLS)], British Society for Antimicrobial Chemotherapy (BSAC) and the European Committee for Antimicrobial susceptibility testing (EUCAST), exist for guidelines of antimicrobial susceptibility testing of conventional drugs, these might not be exactly applicable to plant extracts and modifications have to be made (Hammer *et al.,* 1999). AST standard tests can be conveniently divided into diffusion and dilution methods. Common diffusion tests include agar well diffusion, agar disk diffusion and bioautography, while dilution methods include agar dilution and broth micro/macrodilution. The broth and agar based methods are the conventional reference methods for AST (Tenover *et al.,* 1995). There are other commercial custom-prepared methods like the agar screen plate, Epsilometer test and the Vitek system which could be used in place of the standard reference methods but these are not common in routine AST (Joyce *et al.,* 1992) and are not common for testing activity of plant extracts. The major problem in the diffusion and dilution based AST is one of availability of the active principles which is a function of the solubility of the test compound.

**2.5.1 Agar disk diffusion assay**

Agar diffusion techniques have been widely used to assay plant extracts for antimicrobial activity (Freixa *et al.,* 1996; Salie *et al.,* 1996), although there are limitations with the technique. Disk diffusion is suitable for identification of leads but not effective for quantification of bioactivity (Hammer *et al.,* 1999; Nostro *et al.,* 2000;Langfield *et al.,* 2004). These diffusion techniques generally do not distinguish bactericidal and bacteriostatic effects. The MIC cannot be determined and these areusually used for preliminary screening (Parekh *et al.,*2006; Tepe *et al.,* 2004) that is, as qualitative tests, since the amount of extract that adheres to the disk is not quantitatively determined. Some researchers however have reported MIC values obtained by the agar diffusion method (Dilika *et al.,* 2000; Leite *et al.,* 2006) although high activity in the disk diffusion assay does notnecessarily correlate to low MIC values in the microtitre plate method (Lourens *et al.,*2004). The agar disk diffusion technique can only be used for AST of pure substances because when it is applied to mixtures containing constituents, which exhibit different diffusion rates, results may be unreliable (Silva *et al.,* 2005).In the method, 6 mm paper disks, saturated with filter sterilized (Salie *et al.,*1996) plant extract at the desired concentration, are placed onto the surface of a suitablesolid agar medium. Muller Hinton is usually the medium of choice although Tryptone soy agar (Lourens *et al.,*2004) or Nutrient agar (Doughari, 2006) have sometimes been used by other researchers. The media is preinoculated with the test organism and authors have reported inoculum sizesof 1 x 108cfu/ml of bacteria for inoculating diffusion plates (Baris *et al.,* 2006). There have been some variations noted on whether the disks are impregnated with antimicrobial substances after or before placing on the inoculated plate. Some impregnate before placing on the agar (Lourens *et al.,*2004; Salie *et al.,*1996) while others place the disk on the plate first before impregnating (Nostro*et al.,*2000; Baris *et al.,*2006). In a report by Mbata *et al* (2006), the paper disks were soaked in the leaf extract for about 2 hours while Basri and Fan (2005) left the disks to dry under a laminar flow cabinet overnight. Other authors refrigerate the plates for an hour or two at 4oC to allow pre-diffusion of the extracts from the disk into the seeded agar layer before incubation (Lourens *et al.,* 2004; Tepe *et al.,* 2004; Schmourlo *et al.,*2004). The plates are then incubated at 37oC for 24 hours for bacteria and 48 hours for fungi (Salie *et al.,* 1996; Baris *et al.,*2006). Some incubate for 18 h at 37oC for the same bacteria (Nostro *et al.,* 2000; Lourens *et al.,* 2004). Zones of inhibition are then measured from the circumference of the disks to the circumference of the inhibition zone or recorded as the difference in diameter between the disks and the growth free zones around the disks (Salie *et al.,*1996).

**2.5.2 Agar well diffusion**

The principle of the agar well diffusion is the same as that of the agar disk diffusion method. A standardized inoculum culture is spread evenly on the surface of gelled agar plates. Wells of between 6 and 8 mm are aseptically punched on the agar using a sterile cork borer allowing atleast 30 mm between adjacent wells and the Petri dish.Fixed volumes of the plant extract are then introducedinto the wells. The plates are then incubated at 37oC for24 h for bacteria (Mbata *et al.,* 2006).

**2.6.3 Bioautography**

This is a variation of the agar diffusion method where the analyte is adsorbed onto a Thin Layer Chromatography (TLC) plate. Bioautography is also employed as a preliminary phytochemical screening technique, by bioassay guided fractionation, to detect active components (Nostro *et al.,* 2000; Schmourlo *et al.,* 2004). Bioautography overcomes the challenge of isolating antimicrobial compounds from crude extracts with complex chemical components by simplifying the process of their isolation and identification. It relatively uses very little amount of sample which is ideal for plant extracts andalso allows the determination of the polarity of the active compounds (Runyoro *et al.,* 2006). In their study, Silva *et al.* (2005) compared different methods of AST, and they concluded that bioautography is a practical, reproducible test which is easy to perform. In the bioautography agar overlay procedure, a determined amount of the extract is applied to silica 60gel plates and developed with an appropriate solvent system. A suspension of the test bacteria is sprayed onto the TLC plate. Some authors reported using an inoculums of 0.84 absorbance at 560 nm (Meyer and Dilika, 1996),while others report using a suspension of 106 CFU/ml (Schmourlo *et al.,* 2004). The bioautograms are then incubated at 25oC for 48 hours in humid conditions. Microbial indicators (usually tetrazolium salts) are used as a growth detector (Silva *et al.,* 2005). These are sprayed onto the plates after which the plates are reincubated at 25oC for24 hours (Dilika and Meyer, 1996) or at 37oC for 3 – 4 hours (Dilika *et al.,* 1996; Runyaro *et al.,* 2006). Clear (white)zones on the TLC plate indicate antimicrobial activity of the extracts. Some authors’ state that direct bioassay on TLC plates is not an ideal method for the quantification of bioactivity of plant extracts. They suggest that TLC causes disruption of synergism between active constituents in an extract thereby reducing its activity (Schmourlo *et al.,* 2004).

**2.6.4 Broth microdilution**

The micro-titre plate or broth microdilution method has provided a potentially useful technique for determining MICs of large numbers of test samples. Its advantages over diffusion techniques include increased sensitivity for small quantities of extract which is important if the antimicrobial is scarce as is the case for many natural products; ability to distinguish between bacteriostatic and bactericidal effects; and quantitative determination of the MIC (Langfield *et al.,* 2004). This method can also be used for a wide variety of microorganisms, it is not expensive and it presents reproducible results. In the micro-titre plate method, a stock solution of the extract is first obtained in solvent, usually the solvent used forextraction (Grierson and Afolayan, 1999) or in DMSO (Salie *et al.,* 1996; Nostro *et al.,* 2000; Baris *et al.,* 2006). Methanol and acetone are sometimes chosen as solvents because, in addition to dissolving the extracts completely they show no inhibition of the microorganisms even at 2%final concentration (Meyer and Afolayan, 1995; Afolayan and Meyer, 1997; Mathekga *et al.,* 2000). Most authors report on filter sterilizing with a 0.22 or 0.45 µm membrane filter before the procedure (Meyer and Afolayan,1995; Kianbakht and Jahaniani, 2003). The EUCAST (2003) document states that when membrane filtration is used the samples, before and after sterilization, should be compared by assay to ensure that adsorption has not occurred. Mueller Hinton Broth or water are often used as diluents in the wells of the microtitre plate before transferring an equal volume of stock solution to the plate. EUCAST (2003) recommends cation-supplemented Muller-Hinton broth for non-fastidious microorganisms. Kianbakht and Jahaniani (2003) discovered that the MIC values for *Tribulus terrestris* L. did not depend on the type of media used when comparing the performance of Brain Heart Infusion Broth and Muller-Hinton Broth. Two fold serial dilutions are then made from the first well to obtain a concentration range. For full range MIC 5- 8 concentrations representing achievable concentrations for the antimicrobial are usually tested (Mendoza,1998), although some authors have reported from even 3concentrations. An equal volume of a fixed bacterial culture is added to the wells and incubated at 37oC for 24 hours (Lourens *et al.,* 2004); the EUCAST recommended temperature is 35oC–37oC in air for 16–20 hours for non-fastidious organisms (EUCAST, 2003). The inoculum size for the microtitre plate procedure is usually 1×106cfu/ml(Lourens *et al.,* 2004; Basri and Fan, 2005). Others have used a microbial culture with an optical density of 0.4(log-phase) at 620 nm or a 12 h broth culture adjusted toa 0.5 McFarland turbidity standard (Baris *et al.,* 2006).

EUCAST (2003) recommends that plates be inoculated within 30 min of standardization of inoculum, to avoid changes in inoculum density. Plates are then examined for changes in turbidity as an indicator of growth. The first well that appears clear is taken to be the MIC of the extract. Some researchers use indicators (Umeh *et al.,* 2005) or spectrophotometry to determine presence of growth in microtitre plates (Devienne and Raddi, 2002, Matsumoto *et al.,* 2001).Indicators (usually tetrazolium salts or resazurin dye) are added after the incubation period and left for about 6 hand changes in color or absence of color, depending on the indicator, is used to detect the MIC breakpoint. The use of calorimetric indicators eliminates the need for a spectrophotometric plate reader and avoids the ambiguity associated with visual comparison or measurement of growth inhibition rings on agar plates. When the spectrophotometric method is used the absorbance, usually at 620 nm with the negative control as a blank, is used to detect the breakpoint (Salie *et al.,* 1996). The concentration at which there is a sharp decline in the absorbance value (Devienne and Raddi, 2002), or the lowest concentration which gives a zero absorbance reading (Salie *et al.,* 1996) is deemed to be the MIC. The minimum bactericidal concentration (MBC) is determined by subculturing the preparations that would have shown no evidence of growth in the MIC determination assay. These subcultures are made either in broth or in agar plates. In broth, the MBC is regarded as the lowest concentration of extract which does not produce an absorbance reading at 620 nm relative to the negative control (Salie *et al.,* 1996). On agar the lowest concentration showing lack of growth represents the MBC.

**2.6.5 Agar dilution assay**

The agar dilution test is more versatile than the broth dilution assay and does not present problems encountered with the latter that is, sample solution, contamination and determination of MIC breakpoints (Silva *et al.,* 2005).In this method a stock solution of the extract is prepared in its extracting solvent, filter-sterilized (0.22 µm) and then incorporated in molten agar, cooled to 50oC in a water bath, to obtain different concentrations of the extract in the agar. Usually Muller-Hinton (EUCAST,2003) is used although some authors have used nutrient agar (Grierson and Afolayan, 1999; Meyer and Afolayan,1995). Inoculum preparation also differs between authorsand others have used overnight culture dilutions of 1:100(Meyer and Afolayan, 1995) or 1:10 (Meyer and Dilika,1996) in broth. EUCAST (2003) recommends an inoculum density of about 107cfu/ml and using replicator pins, micropipette or standard loop to transfer about 1 µl (104CFU/ml) of the inoculum. Some publications have reported leaving the plates overnight, before streaking, to allow the solvent to evaporate (Grierson and Afolayan, 1999). The organisms are streaked in radial patterns on the agar plates and incubated at 37oC for 24 to 48 h. The MIC is defined as the lowest concentration of the extract inhibiting the visible growth of each microorganism on the agar plate (Nostro *et al.,* 2000; Hammer *et al.,* 1999).

**2.7 BIOLOGICAL ACTIVITIES OF PHYTOCHEMICALS**

The most commonly encountered secondary metabolites of plants (phytochemicals) are saponins, tannins, flavonoids, alkaloids, anthraquinones, cardiac glycosides, cyanogenic glycosides, phlobatannins, resins, balsam and volatile oils (Soetan *et al.,* 2008; Olutayo *et al.,* 2013). The presence of these secondary metabolites in plants probably explains thevarious uses of plants for traditional medicine, because most of them play important role in the defence against free radicals.

Tannins are complex phenolic polymers which can bind to proteins and carbohydrates resulting in reduction in digestibility of these macromolecules and thus inhibition of microbial growth (Nwogu *et al.,* 2008). Tannins from the bark, roots and other parts of many plants especially Euphorbiaceae are used to treat cells that have gone neoplastic. Tannins are also reported to have astringent properties on mucous membranes (Egunyomi *et al.,* 2009).Alkaloids are basic natural products occurring primarily in many plants. They are generally found in the form of salts with organic acids and they are haemolytically active and are also toxic to microorganisms. Alkaloids, comprising a large group of nitrogenous compounds are widely used as therapeutic agents in the management of cancer. Alkaloids also interfere with cell division. An alkaloid isolated from *Hibiscus sabdariffa* demonstrated its ability to prevent mutagenesis.

**CHAPTER THREE**

**MATERIALS AND METHODS**

**3.1 Collections of Plant Materials and Processing**

The stem of plants*Irvingiagabonensis* (Bush mango tree) and *Dialium guineense* were collectedfrom Akwuke, Awkunanaw, Enugu. The plant materials were examined and authenticated by Mrs. Onyinye of Microbiology Department, Godfrey Okoye University, Enugu and also were size reduced with sterile knife in order to facilitate drying. The plant parts were dried at room temperature in order to prevent loss of active constituents which may be thermo-labile and drying was continued until constant weights obtained. Both stems were separately grounded using sterile mortar and pestle and then electric grinder was used to further grind it into fine powder. It was then stored for further use.

**3.2 Collection of Microbial Isolates**

Standard type cultures of *Candida albicans* (ATCC MYA-2676) and clinical isolates of *Streptococcus mutans* were obtained from Associate Prof. Mrudula Patel of University of the Witwaterand, Johannesburg, south Africa.

**3.3 Qualitative phytochemical screening**

Phytochemical examinations of the extract were carried out for alkaloids, saponins, flavonoids and tannins and oxalates using the standard methods as described by Akinpelu *et al.,* 2011; Prashant *et al.,* 2011; Essiett, Edet and Bala, 2011).

**Test for alkaloids**

About 0.5 g of the plant extract was dissolved in 5 ml of 1% HCl on steam bath. A millilitre of the filtrate was treated with drops of Dragendorff’s reagent. Turbidity or precipitation was taken as indicative of the presence of alkaloids.

**Test for saponins using "foam test"**

About 0.5 g of extract was shaken with 2 ml of water. If foam produced persists for ten minutes it indicates the presence of saponins.

**Test for flavonoids**

About 0.2 g of the extract was dissolved in 2 ml of methanol and heated. A chip of magnesium metal was added to the mixture followed by the addition of a few drops of concentrated HCl. The occurrence of a red or orange colouration was indicative of the flavonoids.

**Test for tannins**

About 1 g of the extract was dissolved in 20 ml of distilled water and filtered. 2 to 3 dropsof 10% of FeCl3 was added to 2 ml of the filtrate. The production of a blackish-blue or blackish-green colouration was indicative of tannins.

**Test for oxalates**

To 0.5 g of the extract was added two to three drops of 80% H2SO4 was added. The formation of Bright crystal disappeared on the addition of reagents confirms the presence of oxalate in calcium oxalate form.

**3.4 ANTIMICROBIAL SENSITIVITY ASSAY**

Muller-Hinton agar was poured aseptically into petri dishes and was allowed to gel. The surface of the plates was then streaked with standardized inoculums of the test organism (0.1ml). thereafter, a sterilized 6mm cork borer was used to create holes on the agar plates and the holes were filled with 100ul of 0.5g of the plant extracts dissolved in 1ml of dimethyl sukfoxide (DMSO). The petri dishes were allowed to sit for 30 minutes. For pre-diffusion of the plant extracts, and the plates were incubated at 37oC for about 24 hours. The inhibition zone diameters were then measured after incubation.

**3.5 MINIMUM INHIBITORY CONCENTRATION**

Two different methods will be used for the MIC.

First method is by broth dilution:0.512g of plant extract was weighed out into a test tube containing 1ml DMSO and allowed to dissolve. 1ml of DMSO was measured into seven different test tubes, and the concentration was serially diluted by taking 1mlfrom 0.512g/ml test tube into the first seven test tubes. The microbial standard was prepared to match McFarland (0.5%) solution. 1ml was taken from the solution into each of the test tubes above and incubated for 18hrs at 37oC.

McFarland (0.5%) solution was prepared by adding 0.05mlof1%BaCl2 and 0.95mlof 1%H2SO4.

Second method: 10%(v/v) DMSO was prepared by measuring 10mlofDMSO in 100ml of water. 2000µg of plant extract was dissolved in 1ml of the10%DMSO, twofold dilution of this extract were made to eight places in a 96 well plate. 20µl of microbialsuspension was added into the microtitre plate followed by 180µl of the antibacterial dilution and incubated.

**3.6 DETERMINATION OF MINIMUM BIOCIDAL CONCENTRATION (MBC)**

The MBCs were determined by first selecting tubes that showed no growth during MIC determination; they were inoculated into Muller-Hinton agar plates by spread method and incubated at 37oC for 24 hours.

**CHAPTER FOUR**

**RESULTS**

The phytochemical screening showed that some of the natural products tested for were present in the plant material. Tannins and saponins was present in all solvent extract of both *Dialium guineense* and *Irginvia gabonensis*. Alkaloids were only absent in aqueous extract of*Dialium guineense* and methanol extract of *Irginvia gabonensis*..

**Table 1.** Qualitative phytochemical screening of the aqueous, ethanol and methanol extracts of *Dialium guineense* stem.

|  |  |  |  |
| --- | --- | --- | --- |
| Phytochemicals | Aqueous | Ethanol | Methanol |
| Tannins | +++ | ++ | ++ |
| Saponins | + | + | + |
| Alkaloids | + | + | + |
| Flavanoids | ++ | - | ++ |
| Oxalates | + | ++ | ++ |

**Table 2.** Qualitative phytochemical screening of the aqueous, ethanol and methanol extracts of *Irvingia gabonensis* fruit pulp.

|  |  |  |  |
| --- | --- | --- | --- |
| Phytochemicals | Aqueous | Ethanol | Methanol |
| Tannins | + | ++ | ++ |
| Saponins | + | + | + |
| Alkaloids | + | + | - |
| Flavanoids | + | + | + |
| Oxalates | - | - | - |

**ANTIMICROBIAL ACTIVITY OF THE EXTRACTS**

The stem extracts of *D*. *guineense* showed antimicrobial activity against *C. albicans* with the exception of methanol extract showing no zone of inhibition to any of the isolates. The Aqueous and methanol stem extracts of *I.gabonensis* showed antimicrobial activity against *C. albicans* while the ethanol and methanol stem extracts showed antimicrobial activity against *S. mutans. C*.*albicans* showed the highest inhibition to the crude ethanol extract of *I. irvingia* with 22 mm zone of inhibition. While *S. mutans* showed no inhibition to *D. guineense.*

**Figure 1:** Average zones of inhibition (mm) of the crude extracts of *D. guineense* and *I. gabonensis* on microbial isolates

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **Concentration** | **256mg/ml** | **128mg/ml** | **64mg/ml** | **32mg/ml** | **16mg/ml** | **MBC mg/ml** |
| ***C. albicans\**** | - | + | + | + | + | 256 |
| ***S. mutans\**** | + | + | + | + | + | nil |
| ***S. mutans\*\**** | + | + | + | + | + | nil |
| ***C. albicans\*\**** | - | - | + | + | + | 128 |

**Table 3:**Minimum inhibitory Concentration (MIC) broth dilution method; aqueous extract.

\* Indicates “against Icheku extract”

\*\* Indicates “against Ujiri extract”

+ indicates growth

- Indicates no growth

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **Concentration** | **256mg/ml** | **128mg/ml** | **64mg/ml** | **32mg/ml** | **16mg/ml** | **MBC mg/ml** |
| ***C. albicans\**** | + | + | + | + | + | nil |
| ***S. mutans\**** | + | + | + | + | + | nil |
| ***S. mutans\*\**** | - | - | + | + | + | nil |
| ***C. albicans\*\**** | - | - | + | + | + | 128 |

**Table 4:** Minimum inhibitory Concentration (MIC) broth dilution method; ethanol extract.

\* Indicates “against Icheku extract”

\*\* Indicates “against Ujiri extract”

+ indicates growth

- Indicates no growth

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **Concentration** | **256mg/ml** | **128mg/ml** | **64mg/ml** | **32mg/ml** | **16mg/ml** | **MBC mg/ml** |
| ***C. albicans\**** | + | + | + | + | + | nil |
| ***S. mutans\**** | + | + | + | + | + | nil |
| ***S. mutans\*\**** | + | + | + | + | + | nil |
| ***C. albicans\*\**** | - | + | + | + | + | 256 |

**Table 5:**Minimum inhibitory Concentration (MIC) broth dilution method; methanol extract.

\* Indicates “against Icheku extract”

\*\* Indicates “against Ujiri extract”

+ indicates growth

- Indicates no growth

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **Concentration** | **1000ug/ml** | **500ug/ml** | **125ug/ml** | **62.5ug/ml** | **31.25ug/ml** | **MBC mg/ml** |
| ***C. albicans\**** | + | + | + | + | + | nil |
| ***S. mutans\**** | + | + | + | + | + | nil |
| ***S. mutans\*\**** | + | + | + | + | + | nil |
| ***C. albicans\*\**** | - | + | + | + | + | nil |

**Table 6:** Minimum inhibitory Concentration (MIC) broth microdilution method; aqueous extract

\* Indicates “against Icheku extract”

\*\* Indicates “against Ujiri extract”

+ indicates growth

- Indicates no growth

**Table 7:** Minimum inhibitory Concentration (MIC) broth microdilution method; ethanol extract.

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **Isolate** | **1000ug/ml** | **500g/ml** | **125ug/ml** | **62.5ug/ml** | **31.25ug/ml** | **MBC mg/ml** |
| ***C. albicans\**** | + | + | + | + | + | nil |
| ***S. mutans\**** | + | + | + | + | + | nil |
| ***S. mutans\*\**** | - | + | + | + | + | nil |
| ***C. albicans\*\**** | + | + | + | + | + | nil |

\* Indicates “against Icheku extract”

\*\* Indicates “against Ujiri extract”

+ indicates growth

- Indicates no growth

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **Concentration** | **1000ug/ml** | **500ug/ml** | **125ug/ml** | **62.5ug/ml** | **31.25ug/ml** | **MBC mg/ml** |
| ***C. albicans\**** | + | + | + | + | + | nil |
| ***S. mutans\**** | + | + | + | + | + | nil |
| ***S. mutans\*\**** | + | + | + | + | + | nil |
| ***C. albicans\*\**** | + | + | + | + | + | nil |

**Table 8:**Minimum inhibitory Concentration (MIC) broth microdilution method; methanol extract

\* Indicates “against Icheku extract”

\*\* Indicates “against Ujiri extract”

+ indicates growth

- Indicates no growth

**CHAPTER FIVE**

**DISCUSSION**

The multidrug resistance of microorganisms is a major medical concern; screening of natural products in a search for new antimicrobial agents that would be active against these microorganisms is the need of the hour (Prany and Rishabh, 2011). The results obtained from this study revealed that the stem of *D. guineense* and *I. gabonensis* contain bioactive agents that exhibit antimicrobial properties against both organisms associated with oral caries (*Candida albicans*  and *Streptococcus mutans*). The aqueous and ethanol crude extracts of the plants relatively showed greater range of inhibition to the microbial isolates with *C. albicans* showing the highest sensitivity and *Streptococcus mutans* unsensitive to *D. guineense* extract. Orji et al., (2012) reported positive antimicrobial properties of the crude aqueous and ethanol leaf and bark extracts of *D. guineense* against *Staphyloccus aureus* and *Klebsiella pneumonia*. Akinpelu et al., (2011) also reported the bioactivity of the methanolic crude leaf extract of *D. guineense* on fourteen environmental strains of Vibrio species. The ethanolic extract of the leaves of three wild strains plant species of *D. guineense* has been reported by Osaugwu and Eme, (2012). Nevertheless, Orji et al., (2012) reported MIC values for the crude leaf and stem back aqueous and ethanol extract of D. *guineense* to *S. aureus* and *K. pneumoniae* also at high concentration of 200mg/ml. This variation could results from the variety of stains of microbial isolates used, extraction methods as well as varying phytochemical components of plant parts. Although, medicinal plants are natural products of nature, as a result, they have little or no side effects when consumed for therapeutic purposes. Also, they are efficacious in combating many pathogens exhibiting multi-drug resistant traits to synthetic drugs. However, consuming medicinal plants could also results to adverse medical conditions among perpetual users, therefore, cautions and international standards should be employed while using them. The adverse conditions could arise via consuming herbs with toxic ingredients, unintentional substitution of herbs with toxic species, intentional addition of drugs, environmental contamination of toxic substances during preparation, and combination of herbs with synthetic drugs (Abas, 2001). For instance, Bernard and Clovis (2014), reported that red spinach (also known as Chinese spinach, *Amaranthus dubius*) which is used in treating high blood pressure, kidney infections and obesity could induce acute kidney injury and pulmonary injury and other complications. Also, South African geranium (*Pelargonium sidoides*) commonly used for the treatment of respiratory tracts infections and irritation and, gastrointestinal disorders could also cause gastrointestinal disorders, skin rashes, and allergic reactions. Nonetheless, this study has been able to strengthen the traditional uses of the fruit pulp of *D. guineense* and suggest that it contains bioactive that could be used as both antibacterial and antifungal agents.

**CONCLUSION**

This study has revealed that the aqueous, ethanol and methanol stem extract of *D. guineense* and *I. gabonensis* have antimicrobial properties against *C. albicans* and *S. mutans* while *D. gabonensis* showed no inhibtion against *S. mutans.* More so, the stem extracts were shown to possess significant amount of phytochemicals such as saponins, tannins, flavonoids, oxalate and alkaloids. These findings are interesting essentially at the present time where the problems of emerging and re-emerging resistant strains of microorganism are becoming the order of the day.

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