**PHYTOCHEMICAL ANALYSIS AND ANTIMICROBIAL ACTIVITY OF *Acalypha wilkesiana* EXTRACTAGAINST CLINICAL ISOLATES OF *Candida albicans***

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# APPROVAL PAGE

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# DEDICATION

To Cephas , whose existence however brief brought the greatest joy to my life.

To Israel, Abigail, Marvelous, Mildred, Anna, Scholastica, Kate and little Divine.

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**ABSTRACT**

The increased, sustained interest in the production of plant-based drugs for the treatment of many diseases has become a significant reason why people have become more interested in the use of traditional medicine for the treatment of mild and serious illness. Due to increase in the thrust for the production of plant-based antimicrobials, this study was performed on *Acalypha wilkesiana* against clinical isolates of *Candida albicans*. The plant extracts were prepared by using ethanol, methanol and hot water. The extracts were evapourated to dryness using a water bath set at 50oC. The plant extracts were tested for the presence of bioactive compounds such as tannins, saponins, glycosides, flavonoids, alkaloid, and steroids. The antimicrobial sensitivity assay of the extracts was studied by agar diffusion method against *Candida albicans.* The minimum inhibitory concentration was determined by broth dilution method and microtiter plate method.The results of this study showed the presence of the bioactive compounds tested for with the exception of alkaloid. It also showed that in the agar diffusion method the aqueous extracts did not display antimicrobial activity against the test organism, while the ethanol and methanol extracts were effective against the test organism, the methanolic extract demonstrated the highest activity against the test organisms with a mean zone of inhibition of 18mm. The study revealed that the plant contains active bioactive constituents which may hinder the growth of fungi causing skin irritation especially those in the genus of *Candida*.

**CHAPTER ONE**

**1.0 INTRODUCTION**

The use and history of herbs dates back to the time of early man, who had the crudest tools as his implements and used stones to start his fire. Herbs were used in a raw state and cooked forms to keep fit. Since that time, the use of herbs has been known and accepted by all nations and has been known also as the first art of treatment available to man (Kafaru, 1994). The search for natural products to cure diseases has received considerable attentions in which plants have been the most important source (Okwu, 2001). Herbal preparations form the basis for many therapeutic drugs and are the first line treatment for many of the world’s population, being readily available and relatively inexpensive (Olaniyi, 1998; Okpara *et al.,* 2007). Herbal medicinal products are assuming greater roles in the lives of the people across the world in the face of global upsurge of drug resistance, toxicity, adverse effects and increasing costs of synthetic products (Mbi, 1998). In Nigeria, several thousands of plant species have been claimed to possess medicinal properties and employed in the treatment of many ailments (Oludare, 2011). Many of these indigenous medicinal plants are used as spices and food plants and for medicinal purposes (Nwaogu, 20l7). Medicinal plants are believed to be an essential source of new chemical substances with potential therapeutic effects (Winston, 2008). Medicinal plants are defined as plants in which one or more of its organs contain substances that can be used for therapeutic purposes or which its precursors for the manufacturing of drugs are useful for disease therapy (Sofowora, l982; Balandrin, 1985). Since medicinal plants do not just nearly save people from the effect of the pathogenic organism but permit them to emerge unscathed, they deserve investigation. The local use of natural plants as primary health remedies as a result of their pharmacological properties is quite common in Asia, Latin America, and Africa (Bibitha, 2002). The importance of herbs in the management of human ailments cannot be over emphasized. It is clear that the plant kingdom harbours an inexhaustible source of active ingredients invaluable in the management of many intractable diseases. Furthermore, the active components of herbal remedies have the advantage of being combined with other substances that appear to be inactive. However, these complementary components give the plant as a whole a safety and efficiency much superior to that of its isolated and pure active components (Ahmad, 2001). An increasing reliance on the use of medicinal plants in the industrialized societies has been traced to the extraction and development of several drugs and chemotherapeutics from these plants as well as from traditionally used rural herbal remedies (UNESCO, 1998). Moreover, in these societies, herbal remedies have become more popular in the treatment of minor ailments; this is partly because of the increasing costs of personal health maintenance. Indeed, the market and public demand have been so great that there is a great risk that many medicinal plants today face either extinction or loss of genetic diversity (Idu, 2007). There is no plant that does not have medicinal value. The active components are normally extracted from all plant structures, but the concentrations of these components vary from structure to structure. However, parts known to contain the highest concentration of the bioactive components are preferred to therapeutic purposes and it can either be the leaves, stems, barks, roots, bulks, corms, rhizomes, woods, flowerers, fruits or the seeds (Kafaru, 1994). The bitter tastes, pungent and repulsive smell in some plants; have been found to have repressive ability over the metabolic activities of a wide range of microorganisms (Mitscher *et al.,* 1992). The genus *Acalypha* comprises of about 570 species (Riley, 1963). *Acalypha wilkesiana* belongs to Euphorbiaceae family. The plant is popularly used for the treatment of malaria, dermatological disorders, gastrointestinal disorders (Akinde and Odeyemi, 1987) and for its antimicrobial property (Adesina *et al.,* 1980; 2000, Kabir *et al.,* 2005, 0ladunmoye, 2006; Erute and Oyibo, 2008). It is widely used in southern Nigeria as a remedy for the treatment of skin infections in children (Alade and Irobi, 1992). *Candida albicans* belongs to the family *Saccharomycetaceae,* it is an opportunistic pathogenic and a common member of the human gut flora. It does not proliferate outside the human body. It is detected in the gastrointestinal tract and mouth in 40 – 60% of healthy adults. It is usually a commensal organism, but can become pathogenic in immunocompromised individuals under a variety of conditions. Children at their tender stage suffer a lot of skin irritation caused by fungi especially *Candida,* among these infections is “Nlacha” as proudly called by the Igbo speaking part of Nigeria. *Acalypha wilkesiana* have been used by people including educated and local women in treating this infection, this necessitated this research work to find out the active components of this plant that confers such therapeutic agent. Also, not many studies have been conducted on this plant to know its antimicrobial activity against *C. albicans.*

**1.2 AIM AND OBJECTIVES**.

**Aim:** To determine the phytochemical and antimicrobial activity of methanolic, ethanolic and aqueous extracts of the of *Acalypha wilkesiana*

**Objectives:**

1. To determine the phytochemical components of *Acalypha wilkesiana*

2. To determine the susceptibility of common human pathogen of clinical origin to extracts of *Acalypha wilkesiana*

3. To determine the minimum inhibitory concentration and minimum fungicidal concentration of the plant extracts against the test organism.

**CHAPTER TWO**

**2.0 LITERATURE REVIEW.**

**2.1 HISTORIC USE OF PLANTS AS ANTIMICROBIALS**

Historically, plants have provided a source of inspiration for novel drug compounds, as plant derived medicines have made large contributions to human health and well-being. Their role is twofold in the development of new drugs: (1) they may become the base for the development of a medicine, a natural blueprint for the development of new drugs, or; (2) a phytomedicine to be used for the treatment of disease. There are numerous illustrations of plant derived drugs (Maurice and lwu, 1999). The isoquinoline alkaloid emetine obtained from the underground part of *Cephaelis ipecacuanha*, and related species, has been used for many years as an amoebicidal drug as well as for the treatment of abscesses due to the spread of *Echerichia coli* and *Entamoeba histolytica* infections. Another important drug of plant origin with a long history of use is quinine. This alkaloid occurs naturally in the bark of *Cinchona* tree. Apart from its continued usefulness in the treatment of malaria, it can be also used to relieve nocturnal leg cramps. Currently, the widely prescribed drugs are analogs of quinine such as chloroquine. Some strains of malaria parasite have become resistant to the quinines therefore antimalarial drugs with novel mode of action were required (Maurice and Iwu, 1999). Similarly, higher plants have made important contributions in the areas of anti-infectives, such as cancer therapies. Early examples include the antileukaemic alkaloids, vinblatine and vincristine, which were both obtained from the Madagascan periwinkle (*Catharanthus roseus* syn. *Vinca roseus*) (Nelson, 1982). Other cancer therapeutic agents include taxol, homoharringgtonine and several derivatives of camptothein like; a well-known benzylisoquinoline alkaliod, papaverine, has been shown to have a potent inhibitory effect on the replication of several viruses including cytomegalovirus, measles and HIV (Turano, 1989). Three new atropisomeric naphthylisoquinoline alkaloid dimers, michellamines A, B, and C were isolated from a newly described species *Ancistrocladus korupensis* fromthe rainforest of Cameroon. The three compounds showed potential anti HIV with michellamine B being the most potent and abundant member of the series. These compounds were capable of complete inhibition of the cytopathic effects of HIV-1 and HIV-2 on human lymphoblastoid target ban in vitro (Boyd, 1994). Due to the lack of adequate medical facilities in developing countries like Nigeria particularly, especially in rural areas, people make use of concoctions from medicinal plants in treatment of disease and aliments (UNESCO, 1998). The pharmacotherapeutic effect of medicinal plants is based on their phytochemical composition. Different plant parts of *Alcalypha wilkesiana* possess bioactive constituents; much research work has not been done to validate its antimicrobial active. *Acalypha wilkesiana* is frequently used as a traditional medicine or as a major constituent of many herbal preparations for the management or treatment of hypertension among its use in the treatment of skin infections.

**2.2 *Acalypha wilkesiana***

*Acalypha wilkesiana* is a member of the spurge family *Euphorbiaceae* belonging to the genus *Acalypha* and is commonly called copper leaf, Joseph’s coat and fire dragon (Makoshi *et al.,* 2016). *Acalypha wilkesiana* is a popular outdoor plant native to Fiji and nearby islands in the South Pacific, but has spread to most parts of the world, especially the tropics of Africa, America and Asia. Despite advancement in medical sciences, millions of people in various traditional systems still resort to the use of medicinal plants to treat their ailments. In southern Nigeria, expressed juice or boiled decoction of leaves of *A. wilkesiana* is used in traditional health care practice, for management of fungal skin infection, hypertension and diabetes and skin diseases, it is also used in the treatment of headache, swelling, cold (Akinyemi *et al.,* 2005). The plant is also popularly used for the treatment of malaria, dermatological and gastrointestinal disorders (Akinde and Odeyemi, 1987) and known for its antimicrobial property (Adesina *et al.,* 1980; 2000; Kabir *et al.,* 2005, Oladunmoye, 2006, Erute and Oyibo, 2008). It is also used as a remedy for the treatment of undefined skin infections in children (Alade and Irobi, 1992). Antimicrobial screening has been carried out on the leaves of *A. wilkesiana.* Adesina and coworkers, (2000) reported a seasonal variation in the distribution of the three natural antimicrobial phenols (geraniin, corilagin and gallic acid) in the *Acalypha*.

**2.2.1 PHARMACOTHERAPEUTIC USES OF *Acalypha wilkesiana***

The large armamentarium of diseases reportedly treated using *A. wilkesiana* has necessitated scientific inquiry into the biochemical basis of its therapeutic value. Gotep *et al.* (2010) carried out *in vitro* antimicrobial screening using ethanol extracts of *A. wilkesiana* and reported that the ethanol extract of the plant had varying antimicrobial activity against *Staphylococcus aureus*, *Yersinia enterocolitica*, *Escherichia coli*, *Salmonella typhi*, *Pseudomonas aeruginosa* and *Klebsiella aerogenes*. Since some of these organisms have been implicated in gastrointestinal diseases and skin diseases, their results provide insight into the acclaimed therapeutic effect of this plant on skin and gastrointestinal related diseases. The use of *A. wilkesiana* in the treatment of diabetes and cardiovascular related diseases, spurred investigation by Ikewuchi and Ikewuchi who examined the effect of the plant extract administration on blood sugar and cholesterol levels using rat model (Ikewuchi *et al.,* 2010). It was reported that the aqueous extract of *A. wilkesiana* had a lowering effect on blood cholesterol level as well as blood sugar, thereby explaining its use in the treatment of cardiovascular related diseases. Further studies on fractions of the plant extract reported its inhibitory effects on methicillin-resistant *Staphylococcus aureus* (Santiago *et al.,* 2015) as well as bactericidal activities (Din *et al.,* 2013) and antioxidant activities (Anokwuru *et al.,* 2015). Oxidative stress, a condition where generation of free radicals and reactive oxygen species overwhelms physiological antioxidant capacity, has been implicated in a number of diseases, including the aging process. Some plants like curcumin, tumeric among others are currently known as good antioxidant sources (Sharma *et al.,* 2001). Ogbuehi *et al.* (2014) investigated the protective effect of *A. wilkesiana* on biomarkers of oxidative stress in liver homogenates. 70% methanol was used for the extraction of *A. wilkesiana* leaves and the rats were intraperitoneally administered 50 mg/kg and 100 mg/kg of the extract for 14 days (Ogbuehi *et al.,* 2014). The results showed significant decreases in malondialdehyde levels in the liver. There was a significant increase in the liver activity of superoxide dismutase and catalase in both the 50 mg/kg and 100 mg/kg administered groups compared to control. There was an insignificant increase in glutathione peroxidase activity in the *A. wilkesiana* administered groups compared to control and an increase in glutathione levels in liver homogenates of *A. wilkesiana* administered groups compared to control. The results suggested that *A. wilkesiana* enhanced the antioxidant capacity of the animals and decreased reactive oxygen species mediated oxidation of lipids.

**2.2.2 TOXICITY STUDIES ON *Acalypha wilkesiana***

A plant with great therapeutic potential has no potential for use as a drug candidate if it has a high toxic effect on vital organs at the reported therapeutic dose. Many plants reportedly used in herbal medicine systems, have not been subjected to extensive toxicity studies (Makoshi *et al.,* 2016). Studies carried out by Makoshi *et al.* (2016) examined the toxic effect of *A. wilkesiana* at doses of 300, 600, 1200mg/kg using rats models. The results obtained showed a dose dependent increase in serum aspartate amino transferase (AST), alkaline phosphatase (ALP) and alanine amino transferase (ALT) levels and decrease in serum albumin level at 300, 600 and 1200 mg/kg compared to the control group administered distilled water, suggesting hepatocellular damage at the doses administered. Liver histology results of the same animals showed necrosis, hemorrhage centrilobular degeneration and sinusoidal dilatation at all doses of our study when compared to. The damage to liver cytoarchitecture observed is consistent with the increase in some serum markers of tissue damage (AST, ALT), and decreased albumin concentration, further control suggesting that the leaf decoction was hepatotoxic at all doses of the study. Sule *et al.* (2012) tested the effect of *A. wilkesiana* leaf inclusion on dietary performance and serum biochemical profiles in Albino rats. At 30% diet inclusion for 28 days, the results showed significant increases in serum AST, ALT, ALP and lactate dehydrogenase levels compared to the control, suggesting possible liver and extra hepatic damage at that level and duration of use. Ikewuchi *et al.* (2011) evaluated the effect of subcutaneous administration of aqueous extract of *A. wilkesiana* on hepatoprotection. The results showed that there was a decreased AST, ALT and ALP level in Albino rats administered 100mg/kg *A. wilkesiana* compared to control. However for the rats treated with 200 and 300mg/kg *A. wilkesiana*, there were elevated levels of ALT, AST and ALP compared to control. The results showed that *A. wilkesiana* provided protection against carbon tetrachloride induced hepatotoxicity, but only at 100 mg/kg. Ogbuehi *et al.* (2014) investigated the protective effect of A. wilkesiana on malaria infected rats. 70% methanol was used for the extraction of *A. wilkesiana* leaves and the rats were intraperitoneally administered 50 mg/kg and 100 mg/kg of the extract for 14 days. From their results, there was increase in AST and ALT levels, while the increase in ALP levels was significant in the *A. wilkesiana* administered group compared to control. Their histology results did not indicate liver damage as the histology results showed no infiltration by inflammatory cells or fatty degeneration. The normal physiological architectural integrity of the rats was maintained despite slight increases in AST, ALT and ALP, suggesting safety to the liver of the rats at doses of 50 and 100 mg/kg of the extract *A. wilkesiana* at doses of 300, 600 and 1200 mg/kg using rats as at doses of 50 and 100mg/kg of the extract.

**2.3 *Candida albicans***

*Candida albicans* is opportunistic pathogenic yeast and also a normal flora of the human gut (Gow, 2017). It does not proliferate outside the human body (Odds, 1988). It is detected in the gastrointestinal tract and mouth in 40-60% of healthy adults (Kerawala *et al.,* 2010). It is usually a commensal organism, but can become pathogenic in immunocompromised individuals under a variety of conditions (Erdogan *et al.,* 2015). It is one of the few species of the genus *Candida* that causes the human infection candidiasis, which results from an overgrowth of the fungus (Martins *et al.,* 2014). Candidiasis for example is often observed in HIV-infected patients (Calderone *et al.,* 2012). *C. albicans* is the most common fungal species isolated from biofilms either formed on implanted medical devices or on human tissue (Kumamoto, 2002).

**2.3.1 ETYMOLOGY of *C. albicans***

*Candida albicans* can be seen as a tautology. *Candida* comes from the Latin word candidus, meaning white. Albicans itself is the present participle of the Latin word albicō, meaning becoming white. This leads to white becoming white, making it a tautology. It is often shortly referred to as thrush, candidiasis or candida. More than a hundred synonyms have been used to describe *C. albicans* (Simi, 1998). Over 200 species have been described within the *Candida* genus. The oldest reference to thrush most likely caused by *C. albicans*, dates back to 400 B.C. in Hippocrates' work of the Epidemics describing oral candidiasis (McCool, 1998).

**2.3.2 SCIENTIFIC CLASSIFICATION**

Kingdom Fungi

Division Ascomycota

Class Saccharomycetes

Order Saccharomycetales

Family Saccharomycetaceae

Genus *Candida*

Species *Candida albicans*

**2.3.3 MORPHOLOGY OF *C. albicans***

*C. albicans* exhibits a wide range of different morphological phenotypes due to phenotypic switching and bud to hypha transition. The yeast to hyphae transition (filamentation) is a rapid process and induced by environmental factors. Phenotypic switching is spontaneous, happens at lower rates and in certain strains up to seven different phenotypes are known. The best studied switching mechanism is the white to opaque switching (an epigenetic process). Other systems have been described as well. Two systems (the high frequency switching system and white to opaque switching) were discover by David R. Soll and colleagues (Slutsky *et al.,* 1985). Switching in *C. albicans* is often, but not always, influenced by environmental conditions such as the level of CO2, anaerobic conditions, medium used and temperature (Soll, 1992). In its yeast form *C. albicans* ranges from to 10-12 microns. Spores can form on the pseudohyphae called chlamydospores in order to survive when put in unfavourable conditions such as dry or hot seasons (Foss, 2013).

**2.3.4 GENOME OF *C. albicans***

The genome of *C. albicans* is almost 16Mb for the haploid size (28Mb for the diploid stage) and consists out of 8 sets of chromosome pairs called chr1A, chr2A, chr3A, chr4A, chr5A, chr6A, chr7A and chrRA. The second set (*C. albicans* is diploid) has similar names but with a B at the end. Chr1B, chr2B, and chrRB. The whole genome contains 6198 Open Reading Frames (ORFs). 70% of these ORFs have not yet been characterized. The whole genome has been sequenced making it one of the first fungi to be completely sequenced (next to *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*) (Calderone *et al.,* 2012). All open reading frames (ORFs) are also available in gateway adapted vectors. Next to this ORFrames; there is also the availability of a GRACE (gene replacement and conditional expression) library to study essential genes in the genome of *C. albicans* (Roemer *et al.,* 2003). The most commonly used strains to study *C. albicans* are the WO-1 and SC5314 strains. The WO-1 strain is known to switch between white-opaque forms with higher frequency while the SC5314 strain is the strain used for gene sequence reference. One of the most important features of the *C. albicans* genome is the high heterozygosity. At the base of this heterozygosity lies the occurrence of numeric and structural chromosomal rearrangements and changes as means of generating genetic diversity by chromosome length polymorphisms (contraction/expansion of repeats), reciprocal translocations, chromosome deletions, Nonsynonymous single-base polymorphisms and trisomy of individual chromosomes. These karyotypic alterations lead to changes in the phenotype, which is an adaptation strategy of this fungus. These mechanisms are further being explored with the availability of the complete analysis of the *C. albicans* genome (Jones *et al.,* 2004). An unusual feature of the genus *Candida* is that in many of its species (including *C. albicans* and *C. tropicalis*, but not, *C. glabrata*) the CUG codon, which normally specifies leucine, specifies serine in these species. This is an unusual example of a departure from the standard genetic code, and most such departures are in start codons or, for eukaryotes, mitochondrial genetic codes (Ohama *et al.,* 1993). This alteration may, in some environments, help these Candida species by inducing a permanent stress response, a more generalized form of the heat shock response (Santos *et al.,* 1999). However this different codon usage makes it more difficult to study *C. albicans* protein-protein interactions in the model organism *S. cerevisiae*. To overcome this problem a *C. albicans* specific two-hybrid system was developed (Stynen *et al.,* 2010). The genome of *C. albicans* is highly dynamic, contributed by the different CUG translation, and this variability has been used advantageously for molecular epidemiological studies and population studies in this species. The genome sequence has allowed for identifying the presence of a parasexual cycle (no detected meiotic division) in *C. albicans* (Butler *et al.,* 2009). This study of the evolution of sexual reproduction in six *Candida* species found recent losses in components of the major meiotic crossover-formation pathway, but retention of a minor pathway (Butler *et al.,* 2009). The authors suggested that if *Candida* species undergo meiosis with reduced machinery, or different machinery, and indicated that unrecognized meiotic cycles may exist in many species. In another evolutionary study, introduction of partial CUG identity redefinition (from *Candida* species) into *Saccharomyces cerevisiae* clones caused a stress response that negatively affects sexual reproduction. This CUG identity redefinition, occurring in ancestors of *Candida* species, was thought to lock these species into a diploid or polyploid state with possible blockage of sexual reproduction (Silva *et al.,* 2007).

**2.3.5 ROLE IN DISEASE**

*Candida* is found worldwide but most commonly compromises immunocompromised individuals diagnosed with serious diseases such as HIV and cancer. *Candida* is ranked as one of the most common groups of organisms that cause nosocomial infections especially among high risk individuals that have undergone surgery, a transplant or are in the Intensive Care Units (ICU) Brosnahan, (2013), *Candida albicans* infections is the top source of fungal infections in critically ill or otherwise immuncompromised patients (Syndor, 2011). These patients predominantly develop oropharyngeal or thrush candidiasis, which can lead to malnutrition and interfere with the absorption of medication (Sardi, 2016). *Candida* continues to be the fourth most commonly isolated organism in bloodstream infections (Vazquez, 2016). Healthy people usually do not suffer (severely) from superficial infections caused by a local alteration in cellular immunity as seen by asthma patients that use oral corticosteroids.

**2.3.5.1 Route of transmission:** this include mother to infant through childbirth, people-to-people; this most commonly occur in hospital settings where immunocompromised patients acquire the yeast from healthcare workers and has a 40% incident rate. Men can become infected after having sex with a woman that has an existing vaginal yeast infection (Brosnahan, 2013). Parts of the body that are commonly infected include the skin, genitals, throat, mouth, and blood (Tortora *et al.,* 2010)

**2.3.5.2 Pathogenesis**

The pathogenesis of *C. albicans* is mediated by certain virulence factors. Among these virulence factors, secreted aspartyl, proteases, adherence, pleomorphism are the most important feature. *C. albicans* infections of the skin and superficial mucosal sites are the results of interplay between the fungal virulence and the host defenses. *C. albicans* can express at least three adhesion molecules to colonize the host epithelial surfaces an aspartyl proteinase enzymes also facilitates its penetration of the keratinized cells. Deeper penetration of keratinized epithelia is assisted by the hypha formation, *C. albicans* hyphae may use contact sensing (thigomotropism) as a guiding mechanism. Pathogenesis requires differential expression of virulence factors at each new stage of the process; a propensity for rapid alteration of the expressed phenotype in *C. albicans* may therefore be a significant factor in establishing the comparatively high pathogenic potential of the organism.

**2.3.5.3 Prevention**

Candidiasis is mainly caused by overgrowth of the *Candida albicans*. Keeping a healthy lifestyle is one of the main keys in protecting an individual from being burdened by the microorganism. Good hygiene, proper nutrition, and careful antibiotic use prevent *C. albicans* from outcompeting other commensal microorganisms. Immunocompromised individuals such as HIV, cancer, ICU, surgical, and transplant patients can experience recurrent infections or candidemia, but anti-fungal drugs, such as clotrimazole (Lotrimin, Mycelex); can help in their situation (Romani, 2000).

**2.3.5.4 Clinical manifestations**

There are 3 major types of infections caused by *Candida albicans*: oropharyngeal candidiasis, vulvovaginal (genital) candidiasis, and invasive candidiasis (candidemia). Oropharyngeal candidiasis is an infection in the mouth and throat area. Usually, it is characterized by the formation of white patches on top of the tongue and throughout the mouth, which is also known as “thrush”. Thrush can be removed with a blade or a cotton-tipped swab, but the underlying tissue will be irritable and show a distinct redness. This infected area will cause soreness and difficultly during eating (Romani, 2000). Vulvovaginal candidiasis is the infection of the genital region, typically the vaginal walls, in women. The vaginal yeast infection causes itchiness and a burning-sensation in the vagina and surrounding tissues. Also, a white discharge – described with an appearance similar to white cottage cheese – is typically present. Genital candidiasis is much more prevalent in women, but men can also contract it. Although it is not considered an STD, men are usually infected after sex with a woman having a vaginal yeast infection. Symptoms involved rash, irritation on the head and surrounding skin of the penis (Romani, 2000). Invasive candidiasis (or candidemia) is the infection of *C. albicans* into the bloodstream. This leads to its invasion of organs throughout the body, such as the kidney, liver, brain, and many more. Patients began to suffer from fevers, chills, fatigue, muscles aches, and abdominal pains. Typically, patients with compromised immune systems are only at risk, while healthy people are susceptible to oral/genital candidiasis. Compromised immune systems can be caused by chemotherapy, transplantation, broad-spectrum antibiotics, and much more (Romani, 2000).

**2.3.5.5** **Diagnosis**

The diagnosis is done either by microscopic examination or culturing. For microscopic examination, it is done by the use of light microscope. A scraping or swab of the affected area is placed on a slide. A drop of 10% potassium hydroxide (KOH) solution is then added to the specimen. The KOH dissolves the skin cells, but leaves the *Candida* cells intact, permitting the visualization of pseudohyphae and budding yeast cells which is a typical feature of *C. albicans*. For culturing method, a sterile swab is rubbed on the infected skin surface. The swab is then streaked on a culture medium for four to five days, to allow the development of yeast colonies. The characteristics such as formation of pseudohyphae and cream colour allows the diagnosis of C. *albicans*

**CHAPTER** **THREE**

**3.1 MATERIALS AND METHODS**

**3.1.1 Collection and maintenance of test microorganism**

The test organism used in this study which is *Candida albicans* was obtained from Solo Reference Laboratories, Agbani, Enugu. The isolate was collected in sterile agar plate and subcultured into sterile agar slant and broth, were incubated at 37oC for 48hrs, preserved as stock culture in the refrigerator set at 4oC.

**3.1.2 Collection of plant material**

Fresh, pesticide free leaves of *Acalypha wilkesiana* were obtained from Trans Ekulu, Enugu State in the month of April, 2018.

**3.1.3 Preparation of plant material**

The plant leaves of *Acalypha wilkesiana* were washed with distilled water and dried at room temperature. The dried leaves were pulverized using a clean big miller. The powder was stored in an air tight container. The grinded powder was extracted separately with ethanol, methanol and hot water. These were prepared using the method described by Oyagade *et al.* (1999).

**3.1.4 Hot water extraction of *A. wilkesiana***

One hundred grams of the finely ground leaves was weighed with a weighing balance and suspended in 500milliliter of boiled water, the extraction was done for 72hours. The extract was decanted and filtered using Whatman No. 1 filter paper. The filtrate was evaporated to dryness with the aid of a water bath at 60oC

**3.1.5 Ethanol extraction of *A. wilkesiana***

One hundred grams of the finely ground leaves were weighed with a weighing balance and suspended in 500milliliter of ethanol the extraction was done for 72hours. The extract was then decanted and filtered using Whatman No. 1 filter paper. The filtrate was evaporated to dryness with the aid of a water bath at 60oC.

**3.1.6 Methanol extraction of *A. wilkesiana***

One hundred grams of the finely ground leaves were weighed with a weighing balance and suspended in 500milliliter of methanol the extraction was done for 72hours. The extract was then decanted and filtered using Whatman No. 1 filter paper. The filtrate was evaporated to dryness with the aid of a water bath at 60oC.

**3.2 Phytochemical analysis (qualitative analysis)**

Chemical test for the screening and identification of bioactive chemical constituents in the leaves of the plant were carried out using the methods of Trease and Evans, (1989).

**3.2.1 Test for alkaloids**

Zero point five grams of the extract was stirred with 3ml of 1% aqueous hydrochloric acid on a steamed bath and filtered. 1ml of the filtrate was treated with few drops of the following reagents:

1. Mayer’s reagent

2. Picric acid solution

3. Dragendroff’s reagent

Precipitation with either of these reagents was taken as preliminary evidence for the presence of alkaloid.

**3.2.2 Test for flavonoids**

Two grams of powdered extract was detanned with acetone. The sample was placed on a hot water bath for all traces of acetone to evaporate. A colour change of the extract is taken as evidence for a positive result.

**3.2.3 Test for tannin and phenolic compounds:**

Zero point five grams of the extract was stirred with 1ml of distilled water and filtered. Ferric chloride solution was added to the filtrate. A blue-black, green or blue-green precipitate was taken as evidence for the presence of tannins.

**3.2.4 Test for saponins**

Zero point five grams of the extract was shaken with water in a test tube. Frothing which persists on warming was taken as evidence for the presence of saponins.

**3.2.5 Test for steroids**

Zero point five grams of extract was dissolved in 2ml of chloroform, sulphuric acid was carefully added to form a lower layer. A reddish brown colour at the interphase is indicative of the presence of steroidal ring.

**3.2.6 Test for glycoside**

Zero point five grams of extract was dissolved in 1ml of glacial acetic acid containing one drop of ferric chloride solution. One ml of concentrated sulphuric acid was added gently by the side of the test tube. A brown ring at the interphase was indicative of the presence of glycoside.

**3.3 Preparation of media used**

**Sabouraud dextrose agar:** 2.8g of SDA was dissolved in 60ml of water and autoclaved at 121oC for 15minutes for sterilization.

**Mueller hinton agar:** 5.32g of MHA was dissolved in 80ml of water and autoclaved at 121oC for 15minutes for sterilization.

**Sabouraud dextrose agar slant:** 0.93g of SDA was dissolved in 20ml of water and pour in a sterile bijou bottle and autoclaved at 121oC for 15minutes for sterilization.

**3.4 Antimicrobial sensitivity assay**

**3.4.1 Agar well diffusion method**

Zero point five grams of the plant extract was dissolved in 2ml of Dimethyl sulfoxide (DMSO). Zero point one ml of the broth organism was inoculated into plates of SDA using spread method. A ditch was aseptically dung on the agar plate using a sterile 6mm cork borer. The ditch was filled with 0.2ml of the homogenous mixture of the plant extract dissolved in DMSO. The Petri dishes were allowed to set for 30 minutes. The plates were incubated at 37oC for 24 hours.

**3.5 Determination of Minimum Inhibitory Concentration (MIC)**

Two different methods were used to determine the MIC.

First method was by **broth dilution method**: 0.512g of plant extract was weighed into a test tube containing 1ml DMSO and allowed to dissolve. One ml of DMSO was measured into seven different test tubes, serial dilution of the concentration was done by taking 1ml from 0.512g test into the first of the seven test tubes and further. The microbial standard was prepared to match McFarland (0.5%) solution. This was prepared by adding 0.05ml of 1%BaCl2 and 0.95ml of 1%H2SO4. 1ml was taken from the microbial solution into each of the six test tubes containing the plant extract and was incubated for 18 hours at 37oC.

Second method was by **the use of 96-well microtiter plate**: 10% (v/v) DMSO was prepared by measuring 10ml of DMSO in 90ml of water. 2000µg of plant extract was dissolved in 1ml of the 10%DMSO, a twofold dilution of the extract to six places in a 96 well plate. 20µl of overnight broth suspension of the organisms was added to 180µl of the plant extract dilution in the well plate and incubate for 18 hours at 37oC.

**3.6 Determination of Minimum Fungicidal Concentration (MFC)**

The minimum fungicidal concentration of the tested organism was determined by sub-culturing the test dilution without growth on a fresh solid medium of SDA and MHA and incubated further for 18-24 hours. The lowest dilutions that yielded no fungal growths on solid medium were taken as MFC.

**CHAPTER FOUR**

**4.0 RESULT**

The phytochemical analysis of the ethanolic, methanolic and aqueous extracts of *Acalypha wilkesiana* shows that the extract contains high level of Tannins, Flavonoids and Steriod. The result shows that Glycosides and Saponins were present at lower levels and also shows the absence of Alkaloid as shown in table 1 below. The antimicrobial activity and potency of the extracts were determined by the presence or absence of zones of inhibition. The extract inhibited the growth of the test organism in varying degrees indicated by the zone of inhibition with exception of aqueous extract showing no zones of inhibition. The methanol extract showed the highest activity against the test organism. The ethanol extract had a varying range of inhibition on the test organism. Fig. 1 and 2 shows the antimicrobial activities of ethanol and methanol extracts, while fig. 3 illustrates the mean zones of inhibition of the ethanol, methanol and hot water extracts on Sabouraud dextrose agar (SDA) after incubation for 24 hours at 37oC. The minimum inhibitory concentration and Minimum fungicidal concentration of the extracts were determined. In table 2 and 3, 0.256g/ml and 0.128g/ml had no growth which was indicated by the absence of turbidity, concentrations 0.016g/ml and 0.008g/ml showed heavy growth. There was no growth in the MFC for the methanolic and scanty growth was seen with ethanolic extract which are shown in table 6 and 7 respectively.

**Table 1:** Qualitative phytochemical screening of the aqueous, ethanol, and methanol extracts of *Acalypha wilkesiana*

|  |  |  |  |
| --- | --- | --- | --- |
| Phytochecmicals | Aqueous | Ethanol | Methanol |
| Tannins | + | ++ | +++ |
| Flavonoids  Steroids | +  + | ++  ++ | +++  ++ |
| Saponins | + | + | + |
| Glycosides | + | + | + |
| Alkaloids | - | - | - |

(+) = minimum amount (++) = maximum amount (-) = not present

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**Figure 1:** Antimicrobial activity of ethanol extract

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**Figure 2:** Antimicrobial activity of methanol extract

**Zones of inhibition (mm)**

Plant against microbial isolate

**Figure 3:** Mean zones of inhibition (mm) of three replicates of crude extract of *A. wilkesiana*.

**Table 2:** Minimum inhibitory concentration (MIC) of ethanol extracts by broth dilution method

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| Test organism | 0.256g/ml | 0.128g/ml | 0.064g/ml | 0.032g/ml | 0.016g/ml | 0.008g/ml |
| *C.* *albicans* | - | - | + | + | ++ | ++ |

(-) = no growth, (+) = scanty growth, (++) = medium growth

**Table 3:** Minimum inhibitory concentration (MIC) of methanol extracts by broth dilution method

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| Test organism | 0.256g/ml | 0.128g/ml | 0.064g/ml | 0.032g/ml | 0.016g/ml | 0.008g/ml |
| *C*. *albicans* | - | - | + | + | ++ | ++ |

(-) = no growth, (+) = scanty growth, (++) = medium growth

**Table 4:** Minimum inhibitory concentration (MIC) of ethanol extracts by microtiter plate method

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| Test organism | 0.256g/ml | 0.128g/ml | 0.064g/ml | 0.032g/ml | 0.016g/ml | 0.008g/ml |
| *C.* *albicans* | + | ++ | ++ | ++ | +++ | +++ |

(-) = no growth, (+) = scanty growth, (++) = medium growth, (+++) = heavy growth

**Table 5:** Minimum inhibitory concerntration (MIC) of methanol extracts by microtiter plate method

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| Test organism | 0.256g/ml | 0.128g/ml | 0.064g/ml | 0.032g/ml | 0.016g/ml | 0.008g/ml |
| *C.* *albicans* | + | + | + | + | ++ | ++ |

(-) = no growth, (+) = scanty growth, (++) = medium growth, (+++) = heavy growth

**Table 6:** Minimum fungicidal concentration (MFC) of ethanol extract

|  |  |  |
| --- | --- | --- |
| Test organism | Concentration | Growth |
| *C.* *albicans* | 0.128g/ml | + |

(+) = scanty growth

**Table 7:** Minimum fungicidal concentration (MFC) of methanol extract

|  |  |  |
| --- | --- | --- |
| Test organism | Concentration | Growth |
| C. *albicans* | 0.128g/ml | - |

(-) = no growth

**CHAPTER FIVE**

**5.1 DISCUSSION**

The antimicrobial properties of plants have been investigated by a number of studies worldwide and many of them have been used as therapeutic alternatives because of their antimicrobial properties (Adriana, 2007). Plants are the cheaper and safer alternative sources of antimicrobial (Doughari, 2007). Phytochemical research based on ethnopharmacological information is generally considered an effective approach to the discovery of antinfective agents in higher plant (Kloucek *et al.,* 2005). Srinivasan, (2001) stated that the presence of bioactive substances confer resistance to plants against bacteria, fungi and pests and that is in accordance with this particular study of which the plant used in this study was able to resist the growth of *C. albicans* probably due to presence of tannins, steroids, saponins, flavonoid and glycoside. The results of the phytochemical analysis of this study showed that the ethanolic, methanolic and aqueous extracts had high levels of Tannins, Flavonoids and Steroids, while Glycosides, Saponins were present in lower levels with the absence of Alkaloid as stated in table 1, this differs slightly from Gotep *et al.* (2009) who reported the absence of Saponins in the extract, this may be due to the different locations where the plant was collected. There is a relationship between the chemical composition of plant and geographical location. The presence of zones of inhibition on the seeded agar plates as shown in figure 1 showed that the ethanolic and methanolic plant extract had antimicrobial activity against the test organism while the aqueous extract showed no zones of inhibition; this may be due to the better solubility of the active components in organic solvents (de Boer, 2005). Although the zones of inhibition are lower than when compared to zones of inhibition by standard drugs as reported by (Gotep *et al.,*2009), this could also be due to the fact that the plant extract is crude and contains other components that do not possess antifungal properties, also the ability of the extract to diffuse into the gel, may be hindered by the presence of large molecules (stearic hindrance), at higher concentration of the extract the zones of inhibition will be comparable with standard drugs. Figure 1 also shows that the methanolic plant extract has higher antimicrobial activity than the ethanolic extract. In table 2, 0.128g/ml of the ethanolic extract showed no growth of the test organism in the MIC, but the presence of growth in the MFC is an indication that the ethanolic *A. wilkesiana* extract inhibited the growth of the test organism; therefore the ethanolic extract is fungistatic but not fungicidal. In table 3, the 0.128g/ml of the methanolic extract showed no growth of the test organism in the MIC and the absence of growth in the MFC is an indication that the methanolic extract of the plant had a cidal effect on *Candida albicans*: therefore the methanolic extract is fungicidal. Table 4 and 5 showed growth of the test organism in both the ethanolic and methanolic at all concentration of the extract in contrast with the results of the broth dilution method in table 2 and 3, this could be due to the differences in concentration of the plant extracts, indicating that the plant extracts are more effective at higher concentration. The difference in the antimicrobial activities of the ethanolic and methanolic extracts of the plant could also be due to the content level of their bioactive compound, which could be as a result of the better solubility of the plant extract in a particular organic solvent.

**CONCLUSION**

This study has provided information that the leaves of *Acalypha wilkesiana* contain many bioactive compounds such as Tannins, Saponins, Flavonoids, Glycosides, and Steroids. The ethanolic and methanolic extracts of the plants had antimicrobial properties against the test organism (*C. albicans*) at varying degrees. It could therefore be concluded that the demonstration of antimicrobial activity against the test organism is an indication that the plant is a potential source for the production of drugs. The results of the study also supports the traditional application of the plant and suggests that the plant extracts possess compounds with antifungal properties that can be used as antifungal agents in novel drugs production for the treatment of ailments associated with the fungi especially *Candida*.

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