**ANTIMICROBIAL EFFICACY OF GUAVA (*Pisdium guajava*) AND ORANGE (*Citrus* *sinensis*) STEMS EXTRACT AGAINST *Streptococcus mutans* and *Candida albicans***

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**APPROVAL PAGE**

This project has been presented to and approved by Godfrey Okoye University, Enugu in partial fulfilment of the requirement for the award of Bachelor of Science (B.Sc), and degree in Microbiology from the Department of Microbiology.

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**DEDICATION**

I dedicated this project work to God almighty and to my sponsor (daddy) Prof. Dr. Christian, N. Okeke and to my late Mum.

**ACKNOWLEDGMENTS**

I wish to express my gratitude to God Almighty for his indescribable love, presence, provision, mercies and grace upon my life. I will ever remain grateful and indebted to my loving and caring sponsor, father (daddy), prof. Dr. C. N. Okeke and his family for providing financial and emotional support which without this work would have not been completed. To my brothers and sisters Obinna Udeze, Ujunwa Udeze, Onyedika Udeze, Jude, Ebuka, Peter Udeze, and cousins for their love and support throughout my year in school. I also appreciate my late mum, Mrs. Anthonia Udeze, who was so kind to me and also supported me intense of prayers, unconditional love, and endless joy. May her soul rest in perfect peace Amen!!!.

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**ABSTRACT**

This research was conducted in Microbiology Laboratory of Godfrey Okoye University to determine the antimicrobial efficacy of aqueous, methanol, ethanol of *Pisdium guajava* and *Citrus x sinensis* on the pathogenic *Streptococcus mutans* and *Candida albicans*. The Dimethyl sulfoxide was used for dissolving the plant extracts. *Pisdium guajava* showed antimicrobial activity against *C. albicans* with the exception of methanol showing no zone of inhibition to any of the isolates. The aqueous and methanol plant extract of *Citrus x sinensis* showed antimicrobial activity against *S. mutans* and *C. albicans*. *Citrus x sinensis* showed highest inhibition with MIC concentration of 0.256g/ml, 0.064g/ml, 0.032g/ml showed slight growth while concentration 0.016g/ml and 0.008g/ml showed heavy growth, there were scanty growths in the MBC and MFC plates. Phytochemical screening, proximate, chromatographic analyses and the antimicrobial activities of tender stem of *Psidium guajava* and *Citrus x sinesis*, were carried out. The phytochemical screening revealed the presence of all metabolites and compounds tested for such as flavonoids, tannins, reducing sugar, terpenes, saponins, anthraquinones and alkaloids. The antimicrobial screening of methanol extract showed activity against the tested organisms. The antimicrobial screening of ethanol and aqueous also showed activity against the tested organisms. The result indicated that the plants had a potential antimicrobial activity and was concentration dependent. The chromatographic analysis of the extracts showed presence of variety of compounds. This therefore, supports the traditional medical use of *Psidium guajava* and *Citrus x sisnesis*

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**CHAPTER ONE**

**1.0 INTRODUCTION**

Medicinal plants constitute an effective source of both traditional and modern medicine. These plants have been shown to have genuine utility and about 80% of the rural population depends on them as primary health care (Akinyemi, 2000). Plants have been used as sources of remedies for the treatment of many diseases since ancient times and people of all continents especially Africa have this old tradition. Infections caused by pathogenic bacteria and fungi remain an important public health concern particularly in developing countries because of factors such as: emergence of bacterial and fungal strains that are resistant to most useful antibiotics (Abad *et al*., 2007; WHO, 2007), HIV/AIDS pandemic (Wagate *et al*., 2008) and unavailability of vaccine (Assob *et al*., 2011). Conventional drugs are expensive and the western health facilities are also inaccessible to rural people (Matu and Staden, 2003; Wagete *et al*., 2008). Medicinal plants have been used since time immemorial to treat and prevent human ailments because they have components of therapeutic value (Hassan *et al*., 2006; Gulluce *et al*., 2006; Parekh and Chanda, 2007). Domesticated and non-domesticated animals in ordinary settings unconsciously treat themselves when sick by eating various parts of medicinal plants such as leaves, stems, barks and roots (Sindiga *et al*., 1995). They may also treat their skin conditions by briskly rubbing themselves against suitable plants with curative properties (Sindiga *et al*., 1995). WHO estimates that up to 80% of the world`s population relies on plants for their primary health care needs (Doughari, 2006; Turker and Usta, 2008; Verma *et al*., 2011). Such a large population depends on traditional medicine due to factors such as: Increase in resistance to the commonly used antibiotics, high cost and inaccessibility to antibiotics especially in rural areas. It is however noted that medicinal plants are readily available, they have little side effects and there is extensive local knowledge on herbal medicine amongst the communities (Rojas et al., 2006; Doughari *et al*., 2008). There are about 20,000 plant species used for medicinal purposes (Gulluce *et al*., 2006). From which at least 121 chemical substances are extracted (Olila *et al*., 2007). Some of the known good sources of pharmacologically active compounds are natural products from fungi and higher plants (Olila *et al*., 2001). Many of the effective drugs such as anti-malarial, anticancer, anti-diabetic and antibiotics such as atropine and ergometrine compounds have been purified from medicinal plants (Olila *et al*., 2001; Samie et al., 2005). Medicinal plants are also sources of many active ingredients in the pharmaceutical industries (Maundu and Tengnas, 2005). The popularity of plants medicine is increasing because of their biodegradability, least persistence and less toxic to non-target organisms, economic and easy availability. Guava plant (*Psidium guajava*) Linn.belonging to family *Myrtaceae,* a traditionally used plant because of its food and nutrition value. Guava is widely grown in tropical and many areas like India, Bangladesh, Florida, and West Indies. Different parts of the *Psidium guajava* are reported to be used in folk medicine. Various parts of the plant like root, bark, leaves and fruits are found to possess many pharmacological properties as it is used in the treatment of various disorders. Various evidences depict that the leaves and bark of *P. guajava* tree possess a long history of medicinal uses. The aqueous extract of guava leaves has been reported to be efficacious in the treatment of various types of gastrointestinal disturbances such as diarrhoea, inhibition of the peristaltic reflex and gastroenteritis. Moreover the whole plant is used as skin tonic and is employed in the treatment of female related disease like dysmenorrhoea, miscarriages, uterine bleeding and premature labour. Recent studies on the pharmacological properties of the bark, fruit and leaves depicts antibacterial, hypoglycaemic, anti-inflammatory, antipyretic, spasmolytic and central nervous system depressant activities. Bark tincture showed fungicidal activity at different concentrations but exhibit only fungistatic property in case of *Candida albicans*. Leaf extract of *psidium guajava* also reported for the anti-bacterial activity on *staphylococcus aureus* due to the protein degrading activity of the leaf extract. The aqueous extract was more potent in inhibiting the growth of *E.coli,* *staphylococcus aureus* and *Pseudomonas aeroginosa* than the organic extracts. The Gram negative bacteria were less susceptible to the effect of crude drugs. The genus *Citrus* belongs to the family of *Rutaceae* and is native tropical and subtropical areas in Southeast Asia. The citrus plants are grown worldwide and ranks top in production and trade among the fruit trees. *Citrus* fruits are richer sources of bioactive compounds having beneficial effect on human health such as vitamin C, carotenoids, flavonoids, limonoids, essential oils, acridone alkaloind, minerals and vitamin B complex. Majority of *citrus* fruits are eaten fresh such as sweet orange, mandarins, grapefruits etc (metallurgy 2011).

* 1. **AIM AND OBJECTIVES**

Aim

The aim of this study is to determine the antimicrobial efficacy of two medicinal plants (*Psidium guajava* and *Citrus x sinensis*) against two microorganisms (*Streptococcus mutans* and *Candida aldicans*).

OBJECTIVES

1. To determine the Minimum Inhibitory Concentration and Minimum Bactericidal Concentration of *Psidium guajava* and *Citrus x sinensis* against the test organisms
2. To determine the phytochemical properties of *Psidium guajava* and *Citrus x sinensis*.

**CHAPTER TWO**

**2.0 LITERATURE REVIEW**

**2.1 The history of medical plants**

Medicinal plant is defined as any plant with one or more of its organs containing substance that can be used for therapeutic purpose or which can be used as precursors for the synthesis of antimicrobial drugs (Bouayed *et al.,* 2007). Plants are presently the sources of medicines for many people of different age in many country of the world, where diseases are treated primarily with traditional medicines obtained from plants. Herbal medicine has been used in Africa for hundreds of years even before colonization. Currently it is still being used and about 80 % of the population relies on it for basic health care services (Okigbo and Mmeka, 2008). Even though colonialists discouraged the use of herbal medicine in Africa, it is still used (Orwa *et al.,* 2008). Localized treatment for different diseases have been used since time immemorial in rural Africa and herbal medicines possessing antimicrobial activity have significant advantages in the societies that greatly used them (Parker *et al.,* 2007).

**2.2 Guava plant *(Psidium guajava)***

Guavas are plants in the myrtle family *(Myrtacease).* Common names are guava (English), gioba (Hausa), goifa (Yoruba), gova (Igbo), guayaba (Spanish), goyave (French), goeajaaba (Dutch), (Burkhill 1997). A native of tropical America, it is now planted as a fruit tree in West Africa. The seeds are distributed by man, and animals, (mainly monkey and birds) and are cultivated throughout the tropics. A small tree of about 6- 8m high, bark is greyish brown, hard or very rough and resistant to termites. The fruits are up to 4 inches long, fleshy, globosely, ovoid or pear-shaped; generally yellowish or white when ripe. They contain a mass of small seeds embedded in the endocarp, though some are seedless or nearly so. It is said to be higher in vitamin C than citrus, it contains 80mg of vitamin C in 100g of fruit and also contains an appreciable amount of vitamin A (Burkhill, 1997). The seeds are numerous, flattened and kidney-shaped. The plant is angiospermic and dicotyledonous. Leaves are up to 4.5 inches long, opposite, elliptic/ellipsoidal and the veins are prominent on the lower surface. The tender leaves are a lighter shade of green than the matured leaves and are curly and unfurling. The leaves of *Psidium guajava* are aromatic when crushed.

**Scientific classification**

* Kingdom: Plantae
* Clade: Angiosperms
* Clade: Eudicots
* Clade: Rosids
* Order: Myrtales
* Family: Myrtaceae
* Genus: Psidium
* Species: *P. guajava*
* Binomial name: *Psidium guajava*

**Phytochemical constituents**

Guava is a rich source of dietary fibres, vitamin A, C, folic acid and various dietary minerals like potassium, copper, and manganese. Reports indicates that a single guava *(Psidium* *guajava)* fruit contains about four times the amount of vitamin C as an orange (Hassimotto, 2005). Further, guava also contain both carotenoids and polyphenols – which are reported as major classes of antioxidant pigments, thus guava provide relatively high potential antioxidant value among plant foods *(*Rincon *et al.,*2001*)*. The pulp and peel of the guava are a remarkable source of anti-oxidants and anti- oxidant dietary fibre (Linda *et al.,* 2004).

Guava is rich in tannins, phenols, triterpenes, flavonoids, essential oils, saponins, carotenoids, lectins, vitamins, fibre and fatty acids. Guava fruits are also a good source of pectin - a dietary fibre. However lot of pharmacological activity is attributed due to the presence of flavonoids, leutin, zeaxanthine and lycopene (Hobert *et al.,* 1998). The flavonoids have demonstrated to possess antibacterial activity. The active flavonoid compound quercetine-3-O-alpha-l-arabinopyranoside has been reported for the anti-plague activity (Limsong *et* *al.,* 2004). Further, it has been showed that the Guava's main plant contain various chemicals like alanine, alphalinolenic acid, ascorbic acid, Asiatic acid, aspartic acid, benzaldehyde, carotenoids, catechol-tannins, Dgalactose, D-galacturonic acid, ellagic acid, essential oils, flavonoids, gallic acid, glutamic acid, guaijavarin, guajiverine, guajivolic acid, histidine, hyperin, isoquercetin, lectins, limonene, linoleic acid, linolenic acid, lysine, myricetin, oxalic acid, pectin, polyphenols, quercetin, serine, tannins, terpenes (Joseph boby *et al.,* 2011). Leaves of the guava tree are a rich source of flavonoids, especially quercetin, which is mainly responsible for the antibacterial activity (Joseph boby *et al.,* 2011).

**Pharmacological Activity**

Studies indicates that a number of pharmacological active components are present in the *Psidium guajava* which are responsible for the various biological activities like anti-diabetic, anti-diarrhoeal, antimicrobial, anti-oxidant, cardio active, hepatoprotective, antipyretic, spasmolytic, immunomodulatory, and contractile effect.

* **Anti-Diabetic Activity:** *Psidium guajava* has been reported to lower the blood glucose level. Guava fruit extract has been shown to significantly restore the loss of body weight and reduces the blood glucose level in the diabetic condition. In induced diabetic’s guava fruit extract, when administered at a dose of 125 and 250mg/kg. Fruit extract of guava protects the pancreatic tissues, including islet beta cells, against lipid oxidation and thus reduces the loss of insulin-positive beta cells and insulin secretion (Huang chin-shiu *et al.,* 2011). The ethanolic stem bark extract exhibited significant hypoglycaemic activity in alloxan-induced hyperglycaemic rats at an oral dose of 250mg/kg (Mukhtar *et al.,* 2011).
* **Anti-diarrhoeal Activity:** Diarrhoea is a major problem in the world. The ripe fruit of guava has been reported as laxative which is used to treat constipation. Studies indicate that guava fruit is an effective antidiarrhoeal when it is used with the peel but if taken unripe fruit in large quantity can cause indigestion, vomiting (Conway *et al.,* 2002). The leaf decoction of guava has been reported for the gastroenteritis and chronic diarrhoea, while the young leaves and shoots has been reported for dysentery and diarrhoea. Quercetine, the major component of the guava leaf extract is responsible for the inhibition of the intestinal movement and reduce capillary permeability in the abdominal cavity and inhibition of increased watery secretion that occur in the acute diarrhoeal disease (Tona *et al.,* 1999). Fresh leaf extract of the plant when administered at a dose of 0.2 ml/kg of morphine sulphate showed inhibition of propulsion. Flower buds and leaf extraction of the *Psidium guajava* consist of Quercetin and quercetin-3-arabinoside which are used in the treatment of diarrhoea in the Costa Rica. This extract at concentrations of 1.6ug/ml showed a morphine-like inhibition of acetylcholine release in the coaxially stimulated ileum, as well as an initial increase in muscular tone, followed by a gradual decrease (Ismail *et al.,* 1999).
* **Anti-microbial Activity:** Four antibacterial flavonoids (morin-3-O-lyxoside, morin-3-O-arabinoside, quercetin, and quercetin-3-Oarabinoside) of the leaf extract of *Psidium* *guajava* are found to be effective against the pathogenic bacteria including *Bacillus* *stearothermophilus, Brochothrix thermosphacta,* *Escherichia coli, Listeria monocytogenes, Pseudomonas fluorescens, Salmonella enterica, Staphylococcus aureus,* and *Vibrio cholera* (Pongsak *et al.,*2010). Studies showed that *P. guajava* leaf extract has trypanocidal properties which could be attributed in parts to the broad antimicrobial and iron chelating activity of f1avonoids and tannins respectively. Iron chelation has been suggested by several reports as an effective way of killing trypanosomes. The methanolic root extract of *Psidium guajava* has been found to possess fungicidal effect because of the quercetin which is present in the root extract. Bark tincture showed fungicidal activity at different concentrations but exhibit only fungistatic property in case of *Candida albicans.* Leaf extract of *psidium guajava* also reported for the anti-bacterial activity on *staphylococcus aureus* due to the protein degrading activity of the leaf extract. The aqueous extract was more potent in inhibiting the growth of *E.coli, staphylococcus* *aureus* and *Pseudomonas aeroginosa* than the organic extracts. The Gram negative bacteria were less susceptible to the effect of crude drugs (Abubakar *et al.,* 2009). Due to the presence of tannins the leaf extract of guava has been reported for antimicrobial activity against Gram-positive and Gram-negative organisms (*Sarcina lutea* and *Staphylococcus aureus*) and *Mycobacterium phlei*. Studies indicates that leaf extract of *psidium guajava* has potent anti-microbial activity against Propioni bacterium acnes and beneficial for the treatment of acne. The leaf extract of guava is effective against the agents which cause the infection in the human intestine like *Streptococcus mutatis, Pseudomonas* *aeurginosa, Salmonella enteritidis, Bacillus cereus, proteus* spp*. Shigella* spp. The aqueous and methanolic extract is effective against the spore formation and production of *Clostridium prefringens* type A. Further, four antibacterial compound has been reported from the methanolic root extract which was further separated by column Chromatograph. Three antibacterial substances have been detected in the leaves which are derivatives of quercetin.
* **Anti-Inflammatory Activity:** A decoction of *Psidium guajava* leaves is used for the treatment of various inflammatory ailments including rheumatism. Antiinflammatory and analgesic effects of the leaf extracts of *Psidium guajava* is due to the presence of polyphenolics compound and triterpenoids. Aqueous extract of *Psidium guajava* at a dose of 50800mg/kg, produce dose-dependent and significant inhibition of fresh egg albumin-induced acute inflammation (oedema) in rats.
* **Miscellaneous Activity:** Leaf extract of *Psidium guajava* is reported in the *Acne* *vulgaris*, a chronic inflammatory disease involving colonization of *Propionibacterium* *acnes*, plus activation of neutrophils and lymphocytes. *Psidium guajava* leaf extracts have potent antimicrobial activities against *Propionibacterium acnes* and may be beneficial in treating acne especially when they are known to have anti-inflammatory activities. Further, the aqueous leaf extract of *Psidium guajava* has been reported to be effective against dental caries and helpful in reducing dental plaque caused by *Staphylococcus sanguinis, Staphylococcus mitis* and *Actinomyces* sp. at a dose of 1mg/ml. Moreover Guava stem bark and leaf stem extract has been found to posssess antigiardiasic activity and inhibit growth of *Entamoeba histolytica* (Ojewole *et al.,* 2008).

**2.2.2 Orange plant *(Citrus X sinensis)***

*C. sinensis* is an orange, its shape is round and its tree has a length of 9-10 m. Leaves of these trees are in oval shape their barks appear to be green or brown in colour which is quite smooth. Leaves have a size of 4-10 cm if its length is taken in to account. The leaves of this tree are green and flower of this tree consists of mainly five petals which smell same as saccharine (Webber *et al*. 1903). *C. sinensis* fruit has seeds in between the parts where juices are present. The seeds are green or cream in colour. The fruit’s flesh is mostly made of the orange sweet juicy part (Valiant *et al.,* 2004).

The family of this fruit is Rutaceae which is found in tropical and subtropical areas in Southeast Asia. It originated from South East Asia but it is found worldwide. It is a great source of vitamin C. *C. sinensis* contains various bioactive compounds like acridone alkaloids, flavonoids, vitamin C, carotinoids, limonoids, essential oils, minerals and vitamin B complex. Sweet orange contains phytochemical nutrients which are important to our health such as flavanones, polyphenols, anthocyanins and hydroxycinnamic acids which are used mainly in pathological conditions like inflammation, high cholesterol related diabetes and cancer etc. (Milind *et al.* 2012).

**Scientific classification**

* Domain: Eukarya
* Kingdom: Plantae
* Subkingdom: Tracheobionta
* Division: ​Magnoliophyta
* Class: ​Magnoliopsida
* Subclass: ​Rosidae
* Order: ​Sapindales
* Valentina Perea: ​*Citrus Sinensis*
* Family: ​Rutaceae
* Genus: ​Citrus
* Species: ​*sinensis*

**Medicinal uses of *Citrus sinensis***

The *Citrus sinensis* fruit is low in calories, contains no saturated fats or cholesterol, but rich in dietary fiber, pectin. Pectin helps to protect the mucosa of the colon by its virtue as a bulk laxative. Oranges, especially the juice is rich in Vitamin C which helps in the antioxidant protection and immune Support and helps the body develop resistance against infectious agents that come from the blood. Also, compounds in orange peel can lower the Cholesterol and act as a cleaner of the interior part of the human body. Orange fruit also contains some minerals as potassium and calcium, potassium is an important component of cell and body fluids that helps control heart rate and blood pressure through countering pressing effects of sodium.

**Anti-cardiovascular diseases:** Orange natural product contains vitamin C, carotenoids and flavonoids, which are cardio defensive. As per WHO's late report, citrus natural products offer assurance against cardiovascular illnesses by diminishing levels of homocysteine (Guarnieri *et al.* 2007).

**Anti-cancer:** *C. sinensis* contains limonene which is known to diminish the tumor at the colon, lung, mouth, skin and bosom. The other constituent of sweet orange is hesperidin which showed anti-cancer property in many studies. Most part relies on cell reinforcement properties of the atoms causing anti-cancer activity, and also their capacity to regulate the action of detoxifying hepatic chemicals. The polymethoxylated flavones have demonstrated solid hostile to proliferative activity against malignancy cells and antigen initiated T-lymphocytes. β-cryptoxanthin (an orange-red carotenoid) is available in most astounding sums in oranges. It might essentially bring down one's danger of creating lung malignancy (Kurowska *et al.,* 2004).

**Micro-organisms:** Oranges are eaten to cure fever. The cooked squash is prepared as a poultice for skin ailments. The fresh peel is rubbed on skin break out. A decoction of the dried leaves and blooms is taken in Italy and France as an antispasmodic, cardio-protective and antagonistic to emetic ailments in China. Orange peel oil creates lethal effect on bugs, fire ants, and houseflies as a result of its 90% limonene. Orange peel is used as herbal drugs against living beings (Honow *et al.,* 2003).

**Anti-inflammation:** Physical injuries which tend to open skin or ruptures tissues of the skin due to any physical injuries are called wounds. The healing property of orange depends on upon wide blend of phytonutrients, for instance, citrus flavones (hesperidin and naringenin), anthocyanins, hydroxycinnamic acids, and a blended pack of polyphenols. There are reports on the biological activity of *C. sinensis* as antioxidants (Tripoli *et al.,* 2007).

**Anti-ulcers:** There are studies which have shown the effect of *C. sinensis* peel on the ulcers. The ulcer diminishes due to decreased occurrence of Helicobacter pylori (H. pylori) (Sharma *et al.,* 2008).

**Anti-diabetes:** Against diabetic kineticism of orange is a result of bioflavonoids, for instance, hesperidin and naringin present in citrus natural item peels. These peels play against diabetic role in mice by betokens of regulation of glucose managerial impetuses. They lessen the activity of glucose-6-phosphatase and phosphoenol pyruvate. The anti-diabetic capacity of orange peel and juice have one of the reserves of being intervened by betokens of against peroxidation, check of α-amylase impetus kineticism that is responsible for the change of involute starches to glucose, extended hepatic glycogen content, actuation of insulin release, and restoration of secretory disfigurements of pancreatic β-cell (Faturi *et al.,* 2010).

**2.3 Test Organisms**

**2.3.1 *Candida albicans***

*Candida albicans* is a commensal fungal found on oral cavity, gastrointestinal tract, and genitourinary tract. However, it is able to cause severe and recurrent mucosal infections such as oral and vaginal condidosis as well as invasive and fatal infections in both immunocompromised and immunocompetent individuals (Mitchell *et al.,* 2011).

*C. albicans* is the most pathogenic and prevalent specie of the *Candida* genus, followed by *Candida tropicalis, Candida krusi* (Back-Brito *et al.,* 2012). *Candida albicans* is the third organism responsible for nosocomial bloodstream infection with morality rates over 40% (Pelroth *et al.,* 2007). The pathogenic condition is recognized by the host cells when the fungal burden increases and yeast turn into hyphal forms activating immune response of the first line of defence, epithelial cells.

**Scientific classification**

* Kingdom : Fungi
* Phylum : Ascomycota
* Class : Saccharomycetes
* Order : *Saccharomycetales*
* Fasmily : *Saccharomycetaceae*
* Genus : *Candida*

**Polymorphism**

*Candida albicans* is a polymorphism fungus that can grow either as ovoid-shaped budding yeast, as elongated ellipsoid cells with constraints at the septa or as parallel- walled true hyphae (Berman, 2002). Further morphologies include white and opaque cells formed during switching, and chlamydospores which are thick-walled spore-like structures (Sudbery *et al.,* 2004). A range of environment cues affect *C. albican*s cells predominantly grow in yeast form, while at high pH, hyphal growth in induced. A number of conditions including starvation and physiological temperature promote the formation of hyphae (Sudbery, 2011).

**Methods of isolation**

Techniques available for the isolation of *Candida albican* include the use of a smear, swab (Martin *et al.,* 2009), an imprint culture collection of whole saliva the concentrated oral rinse and mucosal biopsy. Quantitative estimation of fungal load can be done using imprint, concentrated oral rinse, and culturing of oral rinse as a means of differentiating between commensal carriage and pathogenic (Marsh *et al.,* 2009).

**Medium for isolation**

The most frequently used primary isolation medium for *Candida* is sabouraud dextrose agar (SDA) which permits growth of *Candida* suppresses the growth of many species of bacteria due to its low pH. *Candida* grows on SDA as cream, smooth, pasty convex colonies.

**2.3.2 *Streptococcus mutans***

*S. mutans* gives its name to group of seven species: *S. criceti, S. ratti, S. mutans, S. sobrinus, S. macacae, S. downei,* and *S. orisratti* closely related and collectively referred to as the *mutans streptococci*. The primary habitats for *S. mutans* are mouth, pharynx, and intestine (Loesche *et al.,* 1986). Several factors, such as adherence to enamel surface, production of acidic metabolites, the capacity to build up glycogen reserves and the ability to synthesize extracellular polysaccharide are present in dental caries (Trahan *et al.,* 1995). *S. mutans* and *streptococcus sobrinus* have a central role in the etiological dentals because these can adhere to the enamel salivary pellicle and to other plaque bacteria (Lamont *et al.,* 2004).

**Scientific classification**

* Domain : Bacteria
* Phylum : Firmicutes
* Class : Bacilli
* Order : *Lactobacillales*
* Family : *Streptococcaceae*
* Genus : *Streptococcus*
* Species : *S. mutans*
* Binomial : *Streptococcus mutans*

**Media for streptococcus mutans**

Clinical studies that quantitatively relate *S.mutans* to the total number of recoverable bacteria must use a nonselective medium example; blood agar for enumeration of the total flora, the agar has been used to assess the *S. mutans* population within the sample. This is not necessary when sucrose agar medium is used. These brain heart infusion medium are nonselective, but *S. mutans* can be identified on the basis of its colonial morphology. The purpose of this study was to compare the growth ability of *S. mutans*. Since different serotypes of *S. mutans* do not have identical biochemical characteristics, data are also presented regarding the ability of these media to support growth of strains of various serotypes.

**CHAPTER THREE**

**3.0 MATERIALS AND METHODS**

**3.1 Collections of Plant Materials and Processing**

The stems of plants *Pisdium guajava* (Guava) and *Citrus x sinensis* (Orange) were collected from Obinofia Ndiuno, Ezeagu Local Government Enugu. The plant materials were examined and authenticated by a taxonomist from University of Nigeria Nsukka. The plants were size reduced with sterilized knife in order to facilitate drying and milling. The plant parts were dried at room temperature in order to prevent loss of active constituents which may be thermo-labile. Both stems were separately grounded using sterile mortar and pestle and then milled to fine particles using a big miller, after which they were stored in air tight containers separately and properly labeled.

**3.2 Qualitative phytochemical screening**

Phytochemical examinations of the extracts were carried out for alkaloids, saponins, flavonoids, and tannins using the standard methods as described by Akinpelu *et al.* (2011); Prashant e*t al.* (2011); Essiett *et al.* (2011).

**Test for alkaloids**Zero point five gram of the plant extracts were 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"**

Zero point five grams of extracts were shaken with 2 ml of water. If foam produced persists for ten minutes it indicates the presence of saponins.

**Test for flavonoids**

Zero point two grams of the extracts were 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 coloration was indicative of the flavonoids.

**Test for tannins**

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

**3.3 Collection of Microbial Isolates**

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

**3.4 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.

**3.5 Antimicrobial sensitivity assay**

**Agar well diffusion method:** Muller Hinton agar was poured aseptically into petri dishes and was allowed to gel. The surface of the plates was then streaked with standardized inoculum 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 100µl of 0.5g of the plants extracts dissolved in 1ml dimethyl sulfoxide (DMSO). The plates were allowed to stand for about 30 minutes for pre-diffusion of the plants extracts, and the plates were incubated at 37ºC for about 24 hours. The inhibition zone diameters were then measured after incubation.

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

**96-well Microtitre plate**

This was carried out using the method described by Rosas-Pinon *et al.* (2012). Two-fold serial dilution was performed in a 96-well microtitre plate starting with 2000µg of the plant extracts dissolved in 1ml of 10% DMSO. 180µl of the extracted was placed into plate which was seeded with 20µl of overnight microbial suspension. This was incubated at 37oC for 18h. The MIC was determined as the lowest concentration of test agent which was able to inhibit the growth of the test organisms.

**Broth dilution method**

This test was carried using two-fold serial dilution. Briefly, 0.512g of each of the plant extracts was dissolved in 1ml of DMSO, one milliliter was dispensed into six test tubes, two-fold serial dilution was performed up to the sixth test tube starting with 0.512g/ml. One milliliter of microbial broth prepared using McFarland standard was added each to the tests. They were incubated at 37oC for 18h.

**3.7 Determination of Minimum Bacteriocidal Concentration (MBC) and Minimum Fungicidal Concentration (MFC)**

The MBC and MFC were determined by selecting tubes that showed no growth during MIC determination; a wire loop full of broth from each tube was sub-cultured onto extract-free Mueller-Hinton agar plates, incubated for another 24 h at 37°C. The plate with no growth was taken to be the MBC or MFC as the case may be.

**CHAPTER FOUR**

**RESULTS**

Phytochemical screening shows that some of the natural products tested for were present in the plant material. Tannins and saponins were present in all solvent extract of both *Pisdium guajava* and *Citrus x sinensis*, glycosides were absent in all solvents of *Citrus x sinensis* extract. This shows the generality of the components in medicinal plants. Biological actions are primarily due to these components in a very complicated concert of synergistic or antagonistic activities. Mixtures of such chemicals show a broad spectrum of biological effects and pharmacological properties. To a large extent, the phonological age of the plant, percentage humidity of the harvested material, place and time of harvest, and the method of extraction are possible sources of variation for the chemical composition, toxicity and bioactivity of the extracts (Felix, 1982)

**Table1.** Qualitative phytochemical screening of the aqueous, ethanol and methanol extracts of *Pisdium guajava* plant.

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

(+) = minimum amount

(++) = maximum amount.

**Table2.** Qualitative phytochemical screening of the aqueous, ethanol and methanol extracts of *Citrus x sinensis* plant.

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

(+) = minimum amount

(++) =maximum amount

(-) = absent.

**Antimicrobial Activity of the Extracts**

The stem extracts of *P. guajava* 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 *C. sinensis* 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 *C. sinensis* with 22 mm zone of inhibition. While *S. mutans* showed zone of inhibition to *P. guajava.*

**Figure1.** Avrage zones of inhibition (mm) of the crude extracts of *P. guajava* and *C. sinensis* on microbial isolates

**Table3;** Minimum inhibitory Concentration (MIC) broth dilution method; *P. guajava* and *C.* *sinensis* aqueous extract.

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| 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\*\** | + | + | + | + | + | Nil |

\*Indicates against *P. guajava* extract

\*\*Indicates against *C. sisnensis*

+Indicates growth

-Indicates no growth

**Table4;** Minimum inhibitory Concentration (MIC) broth dilution method; *P. guajava* and *C.* *sinensis* ethanol extract.

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| 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\*\** | + | - | - | + | + | + |

\*Indicates *P. guajava* extract

\*\*Indicates *C. sisnensis* extract

+Indicates growth

-Indicates no growth

**Table5;** Minimum inhibitory Concentration (MIC) broth dilution method; *P. guajava* and *C.* *sinensis* methanol extract.

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| 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\*\** | + | + | + | - | + | Nil |

\*Indicates *P. guajava* extract

\*\*Indicates *C. sisnensis* extract

+Indicates growth

-Indicates no growth

**Chapter five**

**Discussion**

Phytochemical screening (Table 1 and 2) revealed the presence of flavonoid, tannins, saponins, steroid and alkaloid. The chemical constituents present in the extracts have some therapeutic values. Tannins are plant metabolites well known for their antimicrobial properties, flavonoids have both antifungal and antibacterial activities, and they possess anti-inflamatory activity (Ogundaini *el at.,* 2005). Flavonoid, and alkaloid are known to have antimicrobial and bactericidal properties against several infections. In the antimicrobial studies, the majority of the organisms were more sensitive to methanolic extract (Trease el at., 1987), the anti-bacterial activity and inhibitory effect of plant extracts may be due to the presence of secondary metabolites. The methanolic extract of tender leaves of *P. guajava* was active against the entire microorganisms. The 0.259g/ml shows growth of Candida albicans in MIC but the presence of growth in the MBC and MFC shows that the ethanol of orange inhibit the growth of *Candida albicans,* therefore, the orange plant extract is bacteriostatic and fungistatic respectively. Ceftriaxone had effect on *S. aureus* while fluconazole had no effect on *C. albicans* which could be a result of acquired resistance against the antibiotics. The different in the antimicrobial effect of the different plant extract could be the content level of their difference active components. The reason for the difference in the antimicrobial effect of the plant extracts on the test organisms compared to the work of Adegboye *et al.,* 2008. Biswas *et, al.,* (2002), and Rao *et al.,* (2002) cloud be different in the concentration of extract used for the antimicrobial tests.

**Conclusion**

This study contributes to the existing literature and the scientific justification of the medicinal potential of this plant. The findings in this research validate its use among the low income people such as farmers, villagers and native communities. It is also likely to establish a scientific bases and proof for its potential use in folk medicine for the treatment of infectious diseases produced by common pathogenic microorganisms.