ANTIBACTERIAL EFFECT OF FERMENTED EXTRACT OF *Citrullus colocynthis*, *Syzgium aromaticum* AND *Adansonia digitata* AGAINST *Neisseriae gonorrhoeae* and *Esherichia coli*

PROJECT REPORT

BY

AKINDUNTIRE, FUNMILAYO SARAH

(MCB/14/2316)

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THE DEPARTMENT OF MICROBIOLOGY,

FACULTY OF SCIENCE,

FEDERAL UNIVERSITY OYE-EKITI, EKITI STATE, NIGERIA.

SUPERVISOR

DR. MRS R.A. GABRIEL-AJOBIEWE

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CERTIFICATION

This is to certify that this project report was carried out by AKINDUNTIRE, FUNMILAYO SARAH with the Matric number; MCB/14/2316, of the department of microbiology, Faculty of science, Federal University Oye-Ekiti, Ekiti state, Nigeria under the supervision of Dr. Mrs. R.A. Gabriel-Ajobiewe.

Student — Date: 11/04/2019

Dr. Mrs. R.A. Gabriel-Ajobiewe
(SUPERVISOR) — Date: 11/04/2019

Prof. Bryan Ogeneh
(H.O.D) — Date: 11/4/19

DEDICATION

I dedicate this project to the Almighty God, for his favour and mercy since the beginning of this project and also to my parents Mr and Mrs Akinduntire for their support morally, spiritually and their financial support.

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ABSTRACT

The resistance of bacterial pathogenic strains to antibiotics is a major concern around the world and *Neisseriae gonorrhoeae* is not an exception to this. The use of ethanopharamaceutical product is being employed in Africa for the treatment of Sexually Transmitted disease (STD), of which gonorrhea infection is a major concern. The antibacterial effect of the major ethanopharmaceutical product in the southwestern Nigeria is being studied, *Citrullus colocynthis, Syzygium aromaticum* and *Adansonia digitata*. There were six treatments of the combinations plus the control with fresh palm wine and distilled water as fermenting solvent. The crude extracts were obtained by evaporating using rotary evaporator and reconstituted in DMSO. Different concentrations of the extracts (100, 200, 300, 400 and 500mg/ml) were used to check the sensitivity and susceptibility assay, using the agar well diffusion method against *Neisseriae gonorrhoeae* and *Esherichia coli* were carried out. The DMSO extracts of the combination fermented with palm wine (CPA) showed better activity against *Neisseriae gonorrhoeae* by inhibiting the growth by showing a zone of inhibition of 24.00mm at 500mg/ml, 20mm at 400mg/ml and 14mm at 300mg/ml and inactive against *Esherichia coli*. The Minimum Inhibitory Concentration (MIC) was analyzed using the checkerboard method, which was used to determine the synergy between the plant extracts. Different concentration of each extract was serially distributed along the ordinate, while the second extract was distributed along the abscissa on a microtitre plate. The resulting checkerboard contains each combination of two extract on each well of the microtitre plate which showed synergy, indifference and antagonism. The Fractional Inhibitory Concentration ($\sum$FIC) index of the combination of Water extract of CP-CPA is 0.33 which shows an additive effect against *N. gonorrhoeae*. The $\sum$FIC index of DMSO extract of CP-CPA is 1.00, which shows an additive effect on *N. gonorrhoeae*. The MBC of the extract, showed no bactericidal effect on both organisms. It is concluded that *Citrullus colocynthis* extracts showed good antibacterial effect, by inhibiting the growth of *Neisseriae gonorrhoeae* but not on *Esherichia coli.*

CHAPTER ONE

INTRODUCTION

1.1 Background of the study

Sexually transmitted diseases (STDs) are a group of infectious or communicable diseases in which sexual contact is the primary mode of transmission (Gilson *et al.*, 2001) and are among the major causes of illnesses in the world especially in the developing countries. Examples of common sexually transmitted diseases include: Gonorrhea, herpes, Chlamydia, trichomoniasis, Hepatitis B virus, HIV and syphilis (WHO, 2006). Gonorrhea is sexually transmitted caused by the bacterium *Neisseria gonorrhea*, which main symptom is the discharge from the vagina which is purulent and mildly odorous. Lagos was found to have the highest gonorrhea rate in the world. Gonorrhea has progressively developed resistance to the antibiotic drugs prescribed to treat it (Ogunbanjo, 1989).

A potent source of natural antimicrobial product are medicinal plants. Medicinal plants constitute the main source of new pharmaceuticals and healthcare products (Ivanova *et al.*, 2005). Plants has been used as source of remedies for treatment of infectious disease. The use of medicinal plants in the industrialized societies has been traced to the extraction and development of several drugs from these plants as well as from traditionally medicine. Due to the popular belief that green medicine is safe for the treatment of many diseases, herbal medicine has become popular. Also, because it is safe, little or no side effect and easily obtained. Indeed, the market and public demand has been so great that there is a great risk that many medicinal plants today, face either extinction or loss of genetic diversity (Misra, 2009). The use of medicinal plants all over the world predates the introduction of antibiotics and other modern drugs into Africa continent (Haslam *et al.*, 1989). Plants have been used in traditional medicine for many centuries as arbotifacients, contraceptives,

for menstrual regulation, fertility control, as well for the treatment of ailments of both microbial and non-microbial origins (Gill and Akinwunmi,1986). The Nigeria flora is rich in medicinal plants which are usually exploited by herbal doctors otherwise called ''native doctor''. The indigenous population in Southwest, Nigeria for example has developed a vast knowledge on the use of plants as traditional remedies (Ekundayo,1986). Some of the plants collections are used against a variety of diseases such as typhoid fever gastroenteritis, dysentery, malaria and others which are typical diseases of tropical countries (Sofowora,1993; Nick *et al.,* 1995).

Citrullus colocynthis L. belongs to the cucurbitaceae family, which usually consists of a large number of varieties that are generally known as melon. The fruit of *Citrullus colocynthis* is commonly called Colocynth/ Bitter Apple in English. It is a perennial plant and grows wild in sandy shore under xerophitic conditions. The young fruits are fleshy, mottled with dark green and usually turn yellow when ripe. It resembles a common watermelon vine, but bears, small, hard fruit with a bitter pulp. This is perennial herbs usually trailing. It is among the 300 species of melon found in trophical Africa and it is cultivated for its seeds, which are rich in oil (53%) and protein 28%. The seeds popularly known as 'egusi'. They are consumed in 'egusi soup', melon ball snacks and ogiri, 'a fermented condiment'. It has pharmaceutical, nutraceutical, ornamental and medicinal purposes, the latter derived primarily from the fruit pulp. This plant is a traditional medicine, and a well-known remedy for the treatment of diabetes, jaundice and asthma (Qureshi *et al.,* 2010). Recently, a number of studies have been conducted on the phytochemistry, toxicology and pharmacology (Salama, 2012; Ali *et al.,* 2013).

1.2 Statement of Problem

In 2017, the World Health Organization released its report on surveillance of antimicrobial resistance gonorrhea revealing that this is an increasing global threat and putting our capacity to treat common sexually transmitted infection at risk. This growing problem was characterized by the infectious diseases caused by multidrug-resistant Gram-negative bacteria that challenge the public health policies worldwide at the point of creating the WHO Global Gonococcal Surveillance Programme (WHO GASP), monitors trends in drug-resistant gonorrhea.

Gonorrhea, one of the common sexually transmitted diseases (STDs), is caused by a bacterium called *Neisseria gonorrhoeae*. It is an important pathogenic bacterium which belongs to the genus Neisseria because of the quantity and types of invasive infections it produces, as well as the mortality associated. The number of gonorrhea cases is rising in worldwide, and an increasing proportion of cases are multi-drug resistant. The rate is higher among patients infected with resistant strains and received inappropriate treatment (WHO, 2007). Multidrug-resistant microorganism, is increasing very fast, among susceptible persons, due to the indiscriminate use of antimicrobials by people who practice self-medication. The emergence of antibiotic resistance and related toxicity issues limit the use of these drugs, and generate a rebirth in phytotherapy research. To address this challenge, there is growing interest in identifying and evaluating antimicrobial compounds in extracts of medicinal plant as a new source of drugs and alternative treatment approach (Alirol *et al.,* 2017).

1.3 Aim and Objective

Many studies have studied about the antibacterial effect of different extracts of *Citrullus colocynthis* against different organisms. Despite these researches, literature reveals that relatively little is known about the fermented extract of *C. colocynthis* on gonorrhea. Consequently, this research aims to study the fermented extract of *Citrullus colocynthis* on *Neisseriae gonorrheae* and other gram negative rods in order to validate scientifically the inhibitory activity attributed by their popular use as ethno medicine against gonorrhea and to propose new sources of antimicrobial agents.

The specific objectives of this research includes;

i. To determine the sensitivity of *Neisseria gonorrheae* and *E. coli* to the fermented extract of *Citrullus colocynthis.*

ii. To determine the Minimum Inhibition Concentration(MIC) of fermented *Citrullus colocynthis* on *Neisseria gonorrheae* and *E. coli*.

iii. To establish the Minimum Bactericidal Concentration (MBC) of fermented *Citrullus colocynthis* on *Neisseria gonorrheae* and *E. coli*

CHAPTER TWO

LITERATURE REVIEW

2.1 Fermentation

Fermentation is an ancient method of food preservation in the world, which bring about changes in the texture, aroma, taste, flavor in food products. It increases the shelf life of the food products and also increases the nutritional value of the food. Natural microorganisms are involved in the preservation of various kinds of food. The food source which can be that of plant or animal are under the action of microorganisms and their enzymes, which breaks large polymers into small monomers. This brings desirable change to the food and modify the food quality. The use of microorganisms for the fermentation has been an age long tradition. Fermented food products include saukeraut, Ogiri, Iru, Fish sauce, Prickles, Cheese, Youghurt, Wine, Kimchi, etc. Fermentation increases the palatability of the food, Vitamin and mineral level of the food (Campbell-platt, 2005).

2.2 Citrullus colocynthis

Citrullus colocynthis commonly known as the colocynth, bitter apple, bitter cucumber, desert gourd, egusi, or vine of Sodom belongs to the Cucurbitaceae family, a large plant family which consists of nearly 120 genera and 825 species (Milind and Kulwat, 2011). Egusi is among the 300 species of melon found in tropical Africa and it is cultivated for its seeds, which have been reported to be rich in oil and protein. It is a perennial herbaceous vine and it produces small flowers which are yellow and seen on the axils of the leaves. It is monecious, single and pedunculated. Each plant produces15–30 round fruits, about 7–10cm in diameter, green with undulate yellow stripes, becoming yellow all over when dry. The fruit is bitter and globular with smooth texture. It is hard and has a rind around it and contains 200–300seeds/gourd (Fig. 1).

Seeds are small (6mm in length), ovoid, compressed, smooth and brownish when ripe. Curcubits are known for their high protein and oil content with about 35% protein and 50% oil (Achu, 2005). Generally average mass of *Citrullus colocynthis* fruit is 506g and mass of pulp is almost 50% of the mass of fruit, while the seed contents is 71.8g (Aviara et al., 2007).

Several metabolites have been isolated from the fruits of *C. colocynthis* and it has been reported that these fruits have very interesting antibacterial and antifungal activities. As reported by Giwa *et al.*, 2010, in Nigeria only, ''egusi'' is cultivated over an area of 361,000 ha with a production figure of 347,000 tonnes (as seeds) in 2002. It is used both as condiment and thickener in Nigerian local soup, and the industrial scale production of the oil is yet to be utilized despite the huge potential. Various studies have reported predominantly high linoleic fatty acid content in egusi melon seed oils. Due to the unsaturated fatty acid composition of its oil, it has been reported to resemble that of safflower (Yaniv *et al.*, 1999), corn, cottonseed, sunflower, soybean and sesame oil (Oluba *et al.*, 2008).

2.3 Medicinal benefit of *Citrullus colocynthis*

The use of plants as source of remedies for the treatment of many diseases dated back to prehistory and people of all continents have this old tradition. The search for agents to cure infectious diseases began long before people were aware of the existence of microbes. It has been reported that *C. colocynthis* can be used as a natural insecticide and its biological activity has been tested on insect pest. The plant has various compounds which show insecticidal, antibacterial, larvicidal, deterrent, antifeedant, growth regulating and antifertility effects. The plant can be used as an insect repellant (Pravin *et al.*, 2013). This plant is used for the treatment of many diseases such as amenorrhea, rheumatism, hypertension, tumor and various contagious diseases, including dermatological problems and gynecological, urinary and pulmonary infections. The fruits are extensively used

medicinally, to a large extent for stomach pains due to the presence of glucosides such as colocynthin in the pulp, it is also an effective cathartic and laxative. Fruit juice with sugar is a house hold remedy in dropsy. It is more pronouncedly used in anti-cancerous drug. It is effective in leukemia and joint pains. It is used as antidiabetic, antihypersensitive, immunostimulant and antioxidant. Root extract of this plant can be used against jaundice and rheumatism. (Soam *et al.*, 2013).

2.3.1 Antimicrobial activity: Antimicrobial activity of aqueous and methanol extracts of *Citrullus colocynthis* was determined against different organisms like *E. coli, Staphylococcus aureus, Baclillus subtilis, Klebsiella pneumonia, Streptococcus pyogenes, Salmonella typhi* etc. It was revealed that the aqueous extract showed high antibacterial activity against *E. coli* and *S. aureus* and less effect against *B. subtilis* and *K. pneumonia*e and no antibacterial activity at all on the other organisms. On the other hand, methanol extract of the plant showed high antibacterial activity against *B. subtilis*, less activity on *S. faecalis* and no effect on the other organisms (Memon *et al.*, 2003). Also, the antifungal activity was determined against six fungi. The methanolic extract of the plant was reported to show high antifungal activity against *Aspergillus fumigatus, Ifueor sp.* and *Aspergillus flavus*. No antifungal activity against *Candida albicans, Pencillium sp.* and *Rhizopus sp* (Gurudeeban et al., 2010).

2.3.2 Antidiabetic activity: The insulin secretory effects of these different extracts of *C. colocynthis* seed components were evaluated in vitro in the isolated rat pancreas and isolated rat islets in the presence of 8.3 111M glucose. The investigation revealed that different *Citrullus colocynthis* seed extracts have an insulin tropic effect which could at least partially account for the antidiabetic activities of these fruits (Nmila *et al.*, 2000). It has been hypothesized that the

petroleum ether extract of *Citrullus colocynthis* fruits might be promising for the development of a standardized phytomedicine for the treatment of diabetes mellitus (Jayaraman *et al.*, 2009).

2.3.3 Anticancer activity: It has been reported that *Citrullus colocynthis* might be a good source of natural antioxidant. Enzymatic antioxidant activity was evaluated in the stem and leaves of *C. colocynthis*. Enzymatic antioxidants such as catalase, super oxide dismutase, glutathaione reductase, glutathaione reductase etc. were found to be present. It was suggested that cucurbitacin glucosides exhibit pleiotropic effects on cells, causing both cell cycle arrest and apoptosis. The results suggest that cucurbitacin glucosides might have therapeutic value against breast cancer cells as treated cells showed rapid reduction in the level of the key protein complex necessary to the regulation of G2 exit and initiation of mitosis (Ramanathan *et al.*, 2010 (1b).

2.3.4 Local anesthetic activity: Local anesthetics are well-known group of pharmaceutical agents used to relieve pam m specific parts of the organism, inhibiting propagation of signals along the nerves. The petroleum ether extract of *Citrullus colocynthis* was evaluated for its local anesthetic activity in the animal frog. Local anesthetics are intended to relieve pain by depressing or blocking the sensory nerves reversibly. The study reported that the leaves are equally effective as that of the synthetic standard chug xyclocaine when placed in sciatic nerve (Ramanathan *et al.*, 2010a).

2.4 Phytochemical composition of *Citrullus colocynthis*

Phytochemical has been used to differentiate plant chemicals that do not meet the classical definition of 'essential nutrients.' in recent years. Phytochemicals are defined as bioactive non nutrient compounds in fruits, vegetables, grains, and other plant foods that have been linked to reductions in the risk of major non communicable chronic diseases. Phytochemicals that produce

activity in biological system including humans are called 'bioactive phytochemicals' which include phenolic compounds, terpenoid compounds, and alkaloids (Liu, 2013).

Phytochemical screening of this plant revealed the presence of numerous chemicals including alkaloids, tannins, flavonoids, steroids, glycosides etc. These phytochemicals serve as defense mechanisms against predation by many microorganisms, insects and herbivores.

2.5 Nutritional composition of *Citrullus colocynthis*

C. colocynthis is a common component of daily meals in West Africa. Its seed is edible but bitter, nutty-flavored, is eaten whole or used as an oilseed. Different composition of its oil has been reported, which may be due to the quality of stock planted, climatic conditions, processing techniques, nature of the farmland and analytical methods. These factors may act independently or not. Interestingly, one thing that has been reported in unison is the melon oil predominantly contains unsaturated fatty acid (Schafferman *et al.* 1998).

In the view of this report, *C. colocynthis* has been noted to have a gross chemical composition of the oil content of the seeds is, 67-73% linoleic acid, 10-16% oleic acid, 5-8% stearic acid, 9-12% palmitic acid, 8.2% carbohydrate, 2.7% fiber, 3.6% ash and 8.25% protein. The biological files its protein quality has been depicted as: "lower than soybean however similar to or higher than generally oil seeds". These are good sources of essential amino acids such as arginine, lysine, leucine, tryptophan and methionine and vitamins (B 1, B2, Niacin) and Minerals such as Calcium 569mg/100g, Potassium 465mg/100g, Sodium 11.9mg/100g, Iron 11.6/100g, Copper 5.1mg/100g and Zinc 1.1mg/100g.

Plate 1 showing fruit of *Citrullus colocynthis*

2.6 *Adansonia digitata*

Adansonia digitata (African baobab) is a striking tree which is known for its extraordinary shape, thickened trunk, enormous size and tolerance to harsh climatic conditions (high temperatures and low rainfall). A distinctive feature of this tree is the ability of the bark to recover after severe damage caused by elephants or bark harvesting by humans. It can be considered to be a "tree of life" because of the many uses to people as a source of food, medicine, moisture and protection. The bast fibers of *A. digitata* is widely used by people for making ropes, cordage, harness straps, strings for musical instruments, baskets, bags, nets, snares, fishing lines, mats and cloth (Cunningham *et al.* 2014). *Adansonia digitata* is naturally distributed throughout semi-arid sub-Saharan Africa (Wickens & Lowe 2008). A large tree can hold up to 4,500 liters of water.

2.6.1 Traditional uses of *Adansonia digitata*

The leaves, fruit, and bark of *Adansonia digitata* are used for food and medicinal purposes in southern Africa. It has astringent properties and has been used traditionally to alleviate colds, fevers, and influenza (the decoction is made from the fresh bark which is taken as a beverage for one week to treat the flu). The wood, bark, and seeds of *A. digitata* are also known to have anti-inflammatory properties. The leaves may also be used as an antiperspirant, and they also have been used to treat fever, kidney and bladder diseases, as well as asthma and diarrhea. In African traditional medicine, *A. digitata* fruit pulp is used to treat fever, diarrhea, dysentery, smallpox, measles, hemoptysis (the coughing up of blood), and as a painkiller. For the treatment of infant diarrhea, a mixture made from the floury pulp mixed with millet flour and water is given to the child until cured. Local medicinal uses for baobab are richly varied. The bark, along with dried leaves, is made into a preparation called lalo which is used to induce sweating and reduce fever. The bark contains a quantity of edible, insoluble, acidic, tragacanth-like gum, which can be used

to disinfect skin ulcers and wounds. Mucilages made from baobab phloem sap in the bark are used as a remedy for gastrointestinal inflammation. The bark also is popular as a cardiotonic; this traditional use has been confirmed experimentally by researchers who demonstrated the positive inotropic effect of an ethanolic bark extract on isolated atrial muscles of rats. In Sierra Leone specifically, the leaves and bark are used as a prophylactic against malaria. In the Congo, a bark decoction is used to bathe children with rickets, and in Tanzania, as a mouthwash to treat toothache. In Ghana, the bark is used as a substitute for quinine in cases of fever. In southern Zimbabwe, the leaf is eaten as a vegetable, while in central Africa it is used as a diaphoretic (perspirant) against fevers, and the seeds as a remedy for dysentery (Simon, 2015).

2.6.2 Biological properties of *Adansonia digitata*

The pulp of baobab fruit contains astringent compounds (e.g., tannins and cellulose), which exert an antidysenteric action due to an osmotic effect and an inhibitory interaction with acetylcholine, the neurotransmitter that is responsible for gut spasms. The fruit has anti-inflammatory, febrifuge, and analgesic properties due to the presence of saponins and sterols; experimental data have also shown the fruit to have hepatoprotective effects. The leaves have both antihypertensive and antihistaminic properties, and the leaf powder, due to its antihistaminic properties, has been suggested as an anti-asthmatic (Muller, 2014).

2.6.2.1 Anticancer Activity

Anticancer activity is totally unheard of in plants in the family Malvaceae, yet research suggests that *A. digitata* may have antitumor properties. In Senegal and Guinea, both a decoction and a

poultice made from baobab fruit extract were shown to have antitumor activities. The specific bioactive constituents responsible for these actions have not yet been identified (Wickens, 2008).

2.6.2.2 Antisickling Activity

Sickle-cell anemia is a problem that has affected Africans for centuries. One Nigerian remedy is derived from a concoction of an aqueous extract of the bark of *A. digitata*, which is used locally for its antisickling activity. However, after testing various concentrations on washed sickle-cell blood samples, researchers in Nigeria found that the results did not support the anecdotal reports (Burlando *et al.*, 2010).

2.6.2.3 Hepatoprotective Influence

In vitro studies in Saudi Arabia have shown that aqueous extracts of *A. digitata* pulp demonstrate hepatoprotective activity against carbon tetrachloride administered in rats. Consumption of certain Adansonia fruits may play an important role in human resistance to liver damage. The mechanism of action for liver protection is unknown, but it may be due to the triterpenoids or other phytochemical in the fruit (Simon, 2015).

2.6.2.4 Antiviral and antibiotic Effects

Researchers in Togo, western Africa, and Canada studied 19 medicinal plants of Togo and analyzed them for antiviral and antibiotic activity. Of the 19 species studied, 10 demonstrated significant antiviral activity, and all but two showed antibiotic activity. *A. digitata* was the most potent, exhibiting activity against each of the three tested viruses (herpes simplex, Sindbis, and polio).

2.6.2.5 Antioxidant Capacity

Epidemiological evidence has linked intake of vitamin C and other antioxidant micronutrients to health benefits, by virtue of their capacity to trap reactive oxygen species (ROS) that are associated with degenerative diseases and damage to biological systems. Current scientific evidence has helped boost consumer interest in supplementing the diet with antioxidants, especially those derived from natural sources. Baobab fruit pulp is a valuable source of vitamin C, while *A. digitata* leaves contain provitamin A. However, the exact antioxidant composition in baobab has not yet been determined (Elsayed, 2001).

2.7 Syzygium aromaticum (Clove)

It is a precious and valuable spice of the world. It belongs to the family Myrtaceae which is the same as that of guavas. *Syzygium aromaticum* are aromatic dried flower buds They are commonly used in pickles, salads etc. They have deep brown colour, Powerful fragrant odour warm, pungent, strong sweet and slightly astringent. It is used as spice in almost all Indian dishes. Half the world population of cloves is used to male kretek cigarettes in Indonesia, in which one part of clove is used to mix two parts of tobacco. They are harvested when 1.5-2 cm long.

2.7.1 Nutrient content of *Syzygium aromaticum*

The composition of clove varies according to the agro climatic conditions under which it is grown, processed and stored. Dried *Syzygium aromaticum* bud contains Carbohydrate, fixed oil, tannins, proteins, cellulose, pentosans, mineral elements, Steam volatile oils.

2.8 Role of medicinal plants in prevention of diseases

Medicinal plants have been used in healthcare since time immemorial. Plants are inevitable part of human existence since prehistoric times because of their diverse uses in daily livelihood and their immense capability of curing human diseases. Studies has been carried out to verify their efficacy and some of the findings have led to the production of plant-based medicines The emphasis on the use of medicinal plants had hitherto been placed on the treatment rather than prevention of diseases.

The development of new drugs relying purely on modern technology appears to be reaching something of a limit. In developing new drugs, the pharmaceutical industry has tended to adopt high-throughput synthesis and combinatorial chemistry-based drug development since the 1980s; however, the considerable efforts made in this direction has not resulted in the expected drug productivity. Challenges were faced in some large pharmaceutical companies in the development of new products. Increasing attention is being paid to natural products in the search for novel drugs in combination with new technology. Considering their incomparable chemical diversity and novel mechanisms of action, natural products has played a pivotal role in many drug development and research programs (Pravin *et al.,* 2013).

Natural products have undergone interesting and meaningful developments in their ability to interact with numerous, varied biological targets, and some have become the most important drugs in health care system. Among 69 small-molecule new drugs approved from 2005 to 2007 worldwide, 13 were natural products or originated from natural products, which underlines the importance of such products in drug research and development.

CHAPTER THREE

MATERIALS AND METHODS

3.1. Materials used:

Incubator, autoclave, petri dish, Bunsen burner, microtitre plates, inoculating loop, test tubes, micropipette, micropipette tips, microscope, weighing balance, distilled water, di-methyl sulfoxide, bijou bottles, syringe, test tube racks, nutrient agar, Muller-Hinton agar, Muller-Hinton broth, *Citrullus colocynthis* fruits, Clove, Bark of baobab tree, freshly tapped palm wine, distilled water, 0.8% saline, Ceftriaxone

3.2. Collection of plant materials:

Fresh fruits of *Citrullus colocynthis,* Clove and Bark client were collected from Oja bisi market, Ado, Ekiti, Ekiti state. Freshly tapped palm wine was collected from a palm wine tapper in a tightly capped keg on the day of sample preparation at Oye- Ekiti, Ekiti state.

3.3. Preparation of extracts:

The fruits, *Syzygium aromaticum* and *Adansonia digitata* were washed and rinsed with distilled water. After which they were chopped into tiny pieces to increase the surface area. The buckets for fermenting were also washed with distilled water and disinfected with 70% ethanol. A closed fermentation was carried out in a plastic bucket containing water, palm wine which was used as the fermenting solvent. The ratio of the solvent to the fruit in the bucket is 1:2.5, and that of fruit, clove and bark of baobab tree was 1:10:50. The first bucket contained the combination *C. colocynthis*, *Syzygium aromaticum* and *Adansonia digitata* with palm wine used as a fermenting solvent. The second bucket also contained the combination with water as a fermenting solvent labelled CWA. Each assay of *Citrullus colocynthis* fermentation was carried out using 480.0g of the fruit, 6.99g of *S. aromaticum* and 34.95g of *A. digitata*. 1500ml of the solvent was used. Three

controls were used, one contained the fruit and water, labelled CW, the second contained fruit and palm wine, also labelled CP and the third contained *Citrullus colocynthis*, clove and bark of baobab tree without any solvent, labelled (C). The fermentation process lasted five days. Fermented solvent from each buckets were evaporated using the rotary evaporator. Dried extracts were obtained and stored at 4^0C in an air tight container. The stock concentration of the extracts was prepared by using the formula C1V1=C2V2. Various concentrations of 100, 200, 300, 400 and 500mg/ml were prepared from the stock.

3.4 Bacterial strains and culture conditions:

Bacterial strains were obtained from standard laboratory. The antibacterial activity of the extracts was investigated using the strain of bacteria *Esherichia coli* and *Neisseria gonorrhea.* The typed cultures of bacteria were sub-cultured on Nutrient agar and stored at 4^OC until required for study.

3.5 Agar well diffusion assay for extracts:

The susceptibility of the reference organisms to the extract was carried out using the agar well diffusion method on Muller-Hinton agar as recommended by CLSI. *Citrullus colocynthis* fruits extracts were reconstituted in 0.5% dimethylsulfoxide (DMSO) and distilled water were diluted to the different concentrations of the extracts (100, 200, 300, 400 and 500) mg/ml. *Esherichia coli* and *Neisseria gonorrhea* plates were incubated on Nutrient agar at 36±0.1^{O}C. Standardization of the test organisms (*Esherichia coli* and *Neisseria gonorrhea*) was carried out to McFarland standard. A set Muller-Hinton agar, a hole of 6mm was bored on the agar and seeded aseptically with the reference strain and allowed to absorb at 37^0C for 3h. The wells were filled with the extracts, careful not to allow overflow of the suspension onto the surface of the Muller-Hinton agar plates. The plates were incubated at 35^OC for 24h in an anaerobic jar for *N. gonorrhea,* while

plates seeded with *E. coli* were incubated at 37^{O}C for 24h. Standard antibiotic ceftriaxone was used to determine the effect of the extracts on the bacterial isolates, which served as a control. The plates were placed in an incubator at 36±0.1^{O}C for 18-24 hours. Zone of inhibition were observed after.

3.6 Determination of Minimal Inhibitory Concentration (MIC)

Microdilution method was used to determine the MIC of the extracts. Standardization of the inoculum size at 10^5CFU/mL of each test strain was obtained using McFarland standard .50µl of sterilized Muller-Hinton broth were added to the wells of the microtitre plate. 50µl aliquot of different concentrations (100,200,300,400,500) mg/ml of the extracts were added to the 50µl of Muller-Hinton broth. 10µl of the bacterial suspensions (*N. gonorrhea, E. coli*) was added to the microtitre well. The microtitre plate was covered with the lid and incubated at 37^{O}C for 24 hours. Microbial growth observed as turbidity in tubes was looked out for and lowest concentration that showed no growth was considered the MIC.

3.7 Checkerboard assay

The assay was carried out as previously described by (Gani *et al.*, 2005). A total of 50µl of Mueller-Hinton broth was distributed into each well of the microtitre plates. The first extract (CPA) of the combination was serially diluted along the ordinate, while the second extract (CP) was diluted along the abscissa. Standardization of the test organisms (*Escherichia coli* and *Neisseria gonorrhea*) was carried out to McFarland standard. 50µl aliquot of different concentrations (0.1,0.2,0.3,0.4,0.5) µg/ml of the extracts were added to the 50µl of Muller-Hinton broth in the well. Each microtiter well was inoculated with 50µl of a bacterial inoculum, and the plates were incubated at 36±0.1°C for 48h. The resulting checkerboard contains each combination of two extract (CPA and CP, CWA and CW). According to the NCCLS guidelines for broth

microdilution, the MIC was defined as the lowest concentration of antibiotic that completely inhibited the growth of the organism as detected with the naked eye. The fractional inhibitory concentration (FIC) index ∑FICs were calculated as follows:

∑FIC= FIC A+ FIC B

Where,

$$\text{FIC A} = \frac{\text{MIC A\&B}}{\text{MIC A}}$$ (MIC A&B is the MIC of CPA in the combination of CP/ MIC of CPA alone)

$$\text{FIC B} = \frac{\text{MICA\&B}}{\text{MIC B}}$$ (MIC A&B is the MIC of CP in the combination of CPA/MIC of CP alone)

The combination is considered synergistic when the ∑FIC is ≤0.5, indifferent when the ∑FIC is >0.5 to ≤4, and antagonistic when the ∑FIC is >4.

3.8 Determination of Minimum Bactericidal Concentration (MBC)

The MBC of the plant extracts was derived by sub culturing 10µl from each culture that showed no growth after 24h of incubation and inoculated on fresh Muller-Hinton agar plates. These plates were incubated at 35-37°C for 48 hours and were observed daily for growth. The absence of growth at the end of incubation period signifies total cell death. The minimum concentration of the plant extracts that produces total cell death is taken as the MBC.

CHAPTER FOUR

RESULT

4.1. Susceptibility Test (Agar well diffusion method)

The ability of the plant extract to inhibit the growth of *Neisseriae gonorrheae* and *Esherichia coli* was determined in this study. The results revealed that 2 extracts exert antibacterial activity on *N. gonorrheae* and not on *E. coli*. The DMSO extracts of the combination fermented with palm wine (CPA) showed better activity against *Neisseriae gonorrhoeae* by inhibiting the growth by showing a zone of inhibition of 24.00mm at 500mg/ml, 20mm at 400mg/ml and 14mm at 300mg/ml and inactive against *Esherichia coli*. The water extract of the fruit only fermented with palm wine (CP) also showed antibacterial activity against *N. gonorrhoeae* by showing a zone of inhibition of 17mm at 300mg/ml, 20mm at 400mg/ml and 22mm at 500mg/ml and inactive against *E. coli*. The DMSO extract of CP also showed antibacterial activity against *N. gonorrhoeae* by showing zone of inhibition of 9mm at 200mg/ml, 11mm at 300mg/ml, 17mm at 400mg/ml and 20mm at 500mg/ml.

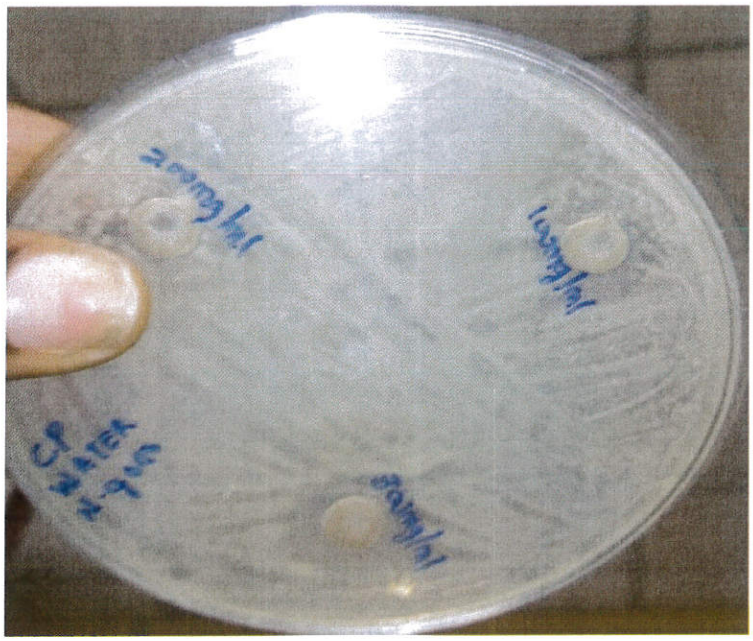

Plate 2: Water extract of CP against showing a zone of inhibition of 17mm at 300mg/ml against *N. gonorrhoeae*.

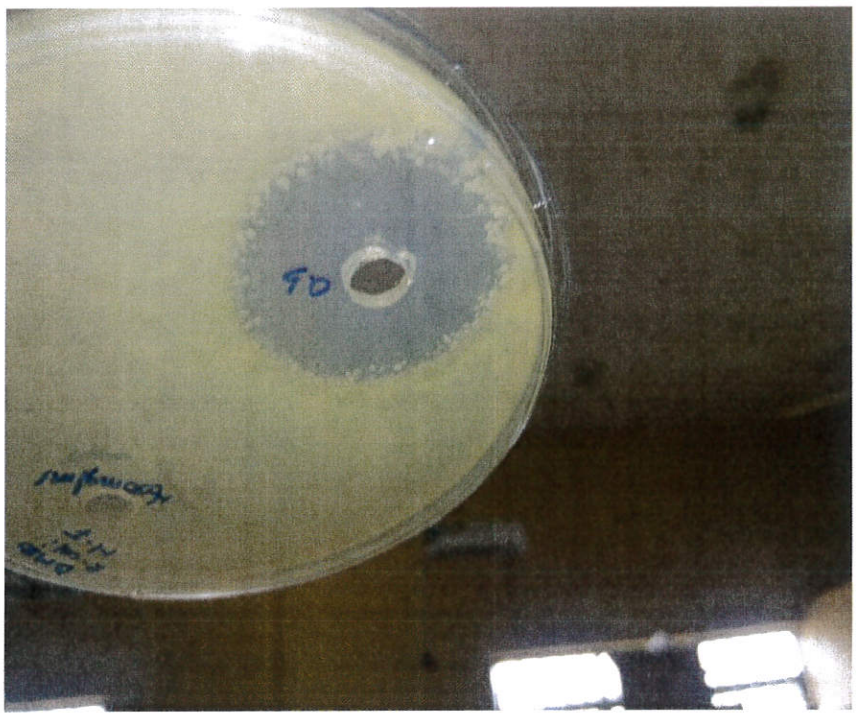

Plate 3 showing zone of inhibition of *N. gonorrhoeae* against ceftriaxone.

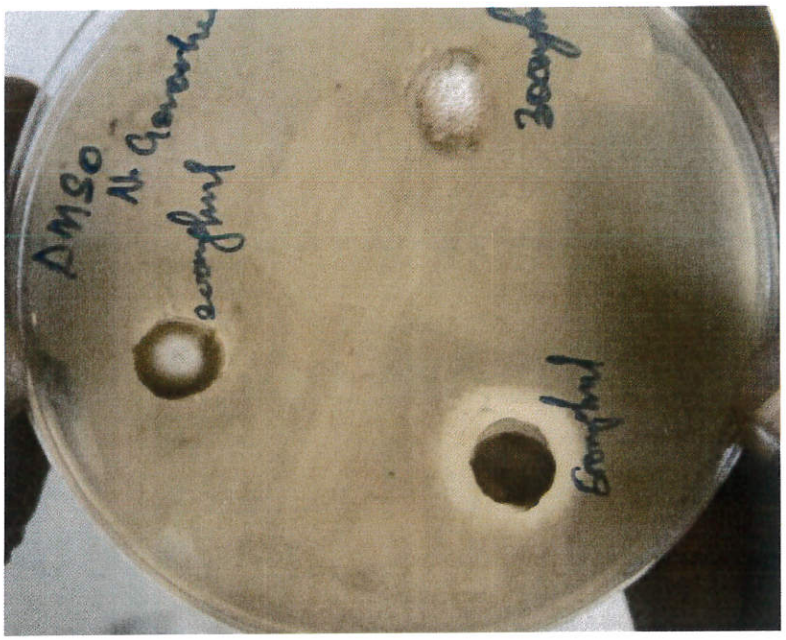

Plate 4: DMSO extract of CPA showing a zone of inhibition of 24mm at 500mg/ml against *N. gonorrhoeae*.

Plate 5 showing zone of inhibition of DMSO extract of CPA against *N. gonorrhoeae* at 400mg/ml and 500mg/ml.

Plate 6 showing zone of inhibition of DMSO extract of CP against *N. gonorrhoeae* at 400mg/ml and 500mg/ml

4.2 Minimum Inhibitory Concentration

The results of the Minimum Inhibition Concentration of the DMSO and water extracts against *Neisseriae gonorrheae* and *Esherichia coli* are shown in table 4.1 and 4.2

Table 4.1 showing MIC (mg/ml) values of *Citrullus colocynthis* extract (DMSO)against *Neisseria gonorrhea* and *Esherichia coli*

Extracts	*Neisseria gonorrhea*					*Esherichia coli*				
	(100	200	300	400	500) mg/ml	(100	200	300	400	500)mg/ml
CPA	+	+	+	-	-	+	+	+	-	-
CP	+	+	-	-	-	+	+	+	-	-
CWA	+	-	-	-	-	+	+	-	-	-
CW	+	+	-	-	-	+	+	+	+	-

KEY: + = Turbid (Growth), - Not Turbid (No Growth)

CPA- All ingredients and palmwine

CP- Fruits and palmwine only

CWA- All ingredients and distilled water

CW- Fruits and distilled water

Table 4.2 showing MIC (mg/ml) values of *Citrullus colocynthis* extract (Water) against *Neisseria gonorrheae* and *Esherichia coli.*

Extracts	*Neisseriae gonorrhea*					*Esherichia coli*				
	(100	200	300	400	500) mg/ml	(100	200	300	400	500) mg/ml
CPA	+	-	-	-	-	+	-	-	-	-
CP	+	+	-	-	-	+	+	-	-	-
CWA	+	+	-	-	-	+	+	-	-	-
CW	+	-	-	-	-	+	+	-	-	-

KEY: + = Turbid (Growth), = Not Turbid (No Growth)

CPA- All ingredients and palmwine

CP- Fruits and palmwine only

CWA- All ingredients and distilled water

CW- Fruits and distilled water

4.3 CHECKERBOARD METHOD

The results obtained with extract combinations by checkerboard method are shown in table 4.

By the checkerboard method, the ∑FIC results for the combination CPA-CP showed an additive effect against *N. gonorrheae*, also the water extract combination showed synergy against *N. gonorrheae*. The ∑FIC results for the other extract combinations CWA-CW by the checkerboard method and comparison of the results for synergy, indifference, and antagonism against *N. gonorrheae* and *E. coli* are presented in Tables 4.3a and b

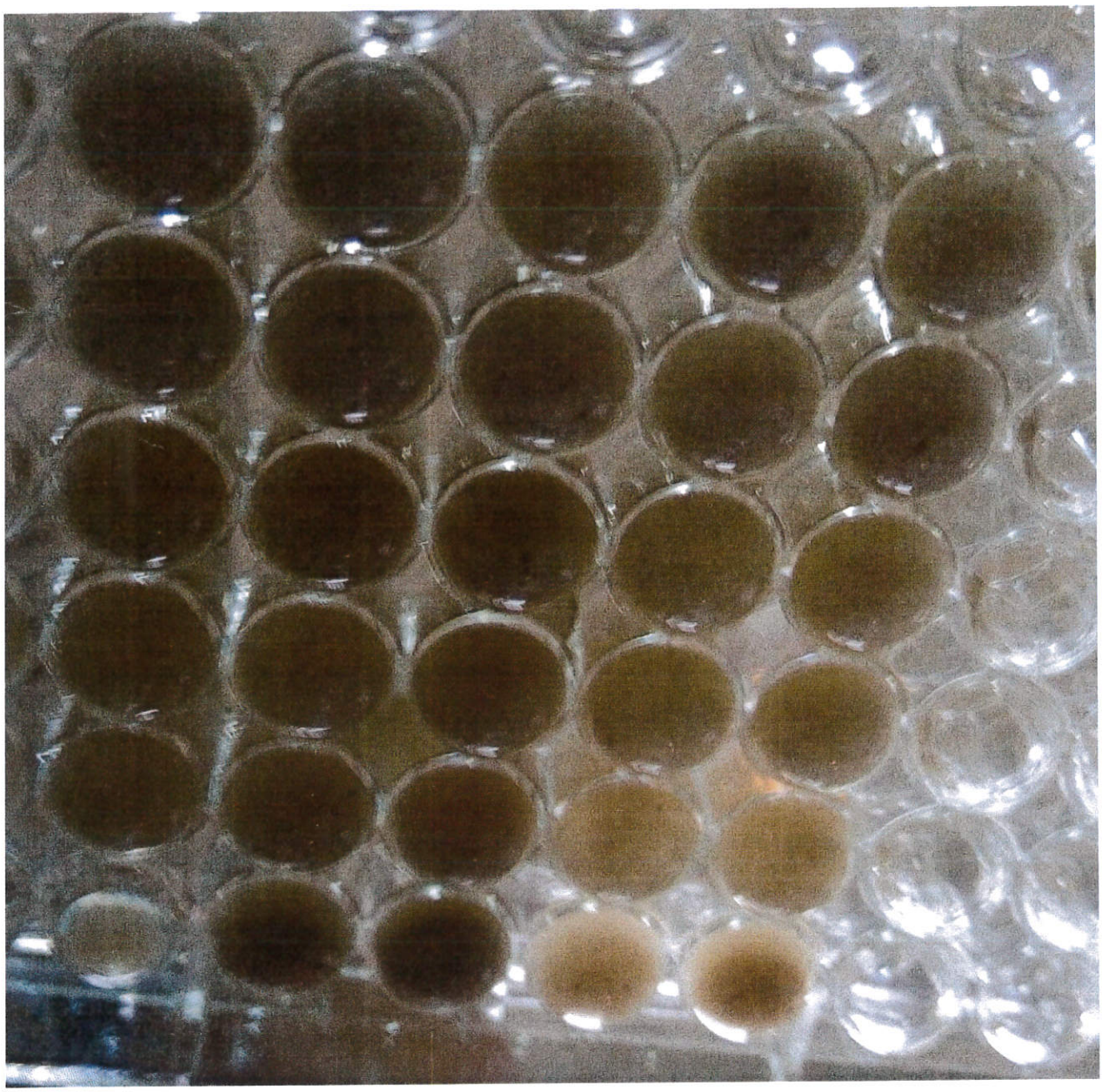

Plate 7: Checkerboard method showing the synergy of two extract combination CPA-CP

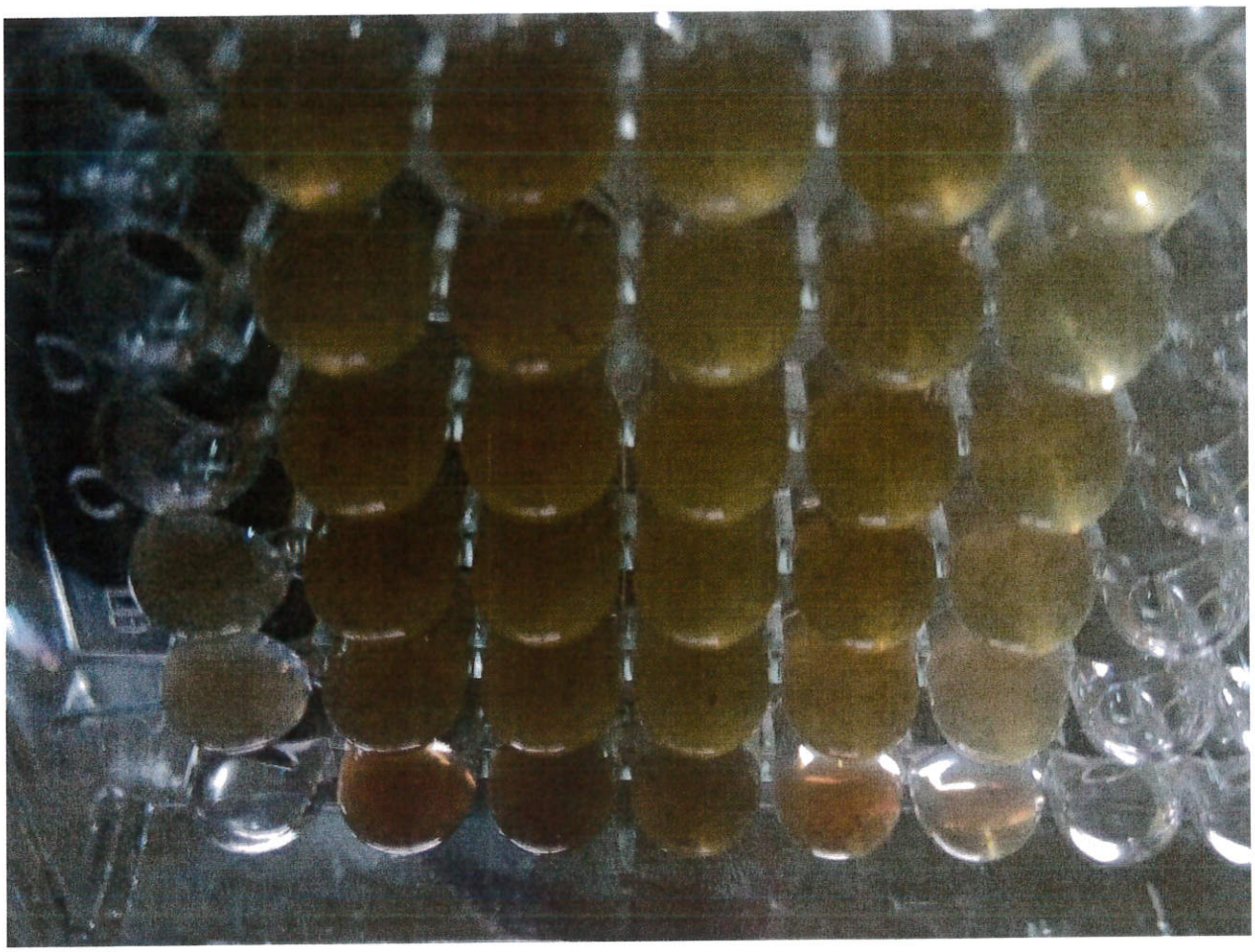

Plate 8: Checkerboard method showing the synergy of two extract combination CWA-CW

Table 4.3: showing results obtained by extract (DMSO) combination by checkerboard method

Organism	CPA+CP		CWA+CW	
	∑FIC	Activity	∑FIC	Activity
N. gonorrhea	1.00	A	3	I
E. coli	1.5	I	5.5	A

KEY: S=Synergy; A=antagonism, I=indifferent

CPA- All ingredients and palmwine

CP- Fruits and palmwine only

CWA- All ingredients and distilled water

CW- Fruits and distilled water

∑FIC- Fractional Inhibitory Concentration

Table 4.4: showing results obtained by extract (Water) combination by checkerboard method

Organisms	CPA-CP		CWA-CW	
	∑FIC	Activity	∑FIC	Activity
N. gonorrhea	0.3	S	1.33	I
E. coli	2	I	6.33	A

KEY: S=synergy, A=antagonism, I=indifferent

CPA- All ingredients and palmwine

CP- Fruits and palmwine only

CWA- All ingredients and distilled water

CW- Fruits and distilled water

∑FIC- Fractional Inhibitory Concentration

CHAPTER 5

DICUSSION

Traditionally, *Citrullus colocynthis* has been utilized as a plant of medicinal significance at various nativities in different parts of the world. It can be prepared as a decoction or infusion in the treatment of various diseases like gonorrhea, ulcer etc. As far as the pharmacological studies is concerned, the following activities have been evaluated and they include antioxidant activity, anti-hyperlipidemic effect, anti-fertility effects, anti-ulcer activity, anticonvulsant activity, antimicrobial effect, antifungal activity, insecticidal activity, anticandidal activity, antidiabetic activity and analgesic effect. Research on the antibacterial activity practiced by traditional people using the fermented fruit extract of *Citrullus colocynthis, Syzygium aromaticum* and *Adansonia digitata* tree to treat gonorrhea has been elucidated.

In this study, plate 4 and 5 shows the susceptibility test of the DMSO extract of the combination (*C. colocynthis*, *Syzygium aromaticum* and *Adansonia digitata*) extract fermented with palm wine (CPA) showed antibacterial effect by showing a zone of inhibition of 24.00mm at 500mg/ml, 20mm at 400mg/ml and 14mm at 300mg/ml inhibit the growth of *Neisseriae gonorrhoeae*. This same extract showed no zone of inhibition on *Esherichia coli*. Water extract of CPA showed no antibacterial effect on both test organisms. Also, the susceptibility test of the DMSO and Water extract of the combination fermented with water (CWA) showed no antibacterial effect at all on *Neisseriae gonorrhoeae* and *Esherichia coli*. Plate 2, which is the water extract of fruit fermented with palm wine (CP) showed moderate antibacterial effect by showing a zone of inhibition of 17mm at 300mg/ml, 20mm at 400mg/ml and 22mm at 500mg/ml. DMSO extract of CP also showed antibacterial activity against *N. gonorrhoeae* by showing zone of inhibition of 9mm at 200mg/ml, 11mm at 300mg/ml, 17mm at 400mg/ml and 20mm at 500mg/ml. DMSO and Water extract of fruit fermented in water (CW) showed no antibacterial activity on *N. gonorrhoeae* and

E. coli. Also, the DMSO and Water extract of the combination fermented in water only (CWA) showed no antibacterial effect on the test organisms.

The use of combinations of extracts in the treatment of infections is a common trend lately due to the frequency of resistance among microorganisms. Extract combinations are used so that the synergistic and additive potentials of each of the combinants can be exploited. They are also used to improve efficacy and further retard the development of resistance to the combinants by microorganisms. The use of these combinations however, can only be justified if they produce a synergistic or at least an additive effect against the organisms. The theoretical value of synergy of an FIC index is ≤ 0.5, Antagonism is defined as an FIC index of >4.0, while an FIC index of >0.5 to $\leq$ is additive and >1 to 4.0 is indifferent (Hsieh *et al.,* 1993). The goal of synergy testing is to assess the in vitro interaction of extract combinations to determine whether the effect of the two extracts is greater than the sum of their individual activity. The theoretical definition of additive or indifferent is in which neither help nor hinder one another's activity. The checkerboard microtiter plate assay is used to test the activities of the extracts in combination against *Neisseriae gonorrhoeae* and *Esherichia coli* strains by determining the Fractional Inhibitory Concentration ($\sum$FICs) of all combinations tested. The FIC index of the combination of the DMSO extract of CP-CPA on N. gonorrhoeae is 1.00 which means it shows an additive effect against the organism. The FIC index of the combination of the Water extract of CP-CPA on *N. gonorrhoeae* is 0.33, which shows a synergistic effect against the organism. Also, the FIC index of the combination of DMSO extract of CW-CWA on *N. gonorrhoeae* is 3 which shows indifferent effect against the organism. The FIC index of the combination of the Water extract of CW-CWA on *N. gonorrhoeae* is 1.33 which shows an indifferent effect against the organism. The FIC index The FIC index of the combination of the Water extract of CP-CPA on *E. coli* is 1.5, which shows an indifferent effect

against the organism. Also, the FIC index of the combination of DMSO extract of CW-CWA on *E. coli* is 5.5 which shows antagonistic effect against the organism. The FIC index of the combination of the Water extract of CW-CWA on *E. coli* is 6.33 which shows an antagonistic effect against the organism. The FIC index of the combination of the Water extract of CP-CPA on *E. coli* is 2 which shows an indifferent effect against the organism

CONCLUSION AND RECOMMENDATION

5.2 CONCLUSION

The antibacterial screening of fermented extracts of the fruit of *C. colocynthis* revealed it exhibit potential antibacterial effect on *Neisseriae gonorrhoeae* and no antibacterial effect on *Esherichia coli*. The palm wine extract of the fermented extract showed antibacterial activity by inhibiting the growth of *N. gonorrhoeae* against that of water extract. Furthermore, the results obtained through the checkerboard method in study shows synergy, antagonism, additivity and indifference of the extracts combined together.

5.3 RECOMMENDATION

Plant is an important source of medicine and plays a key role in world health. Medicinal plants have been known to be an important potential source of therapeutics or curative aid. China for example is able to provide adequate healthcare for its urban and rural population due to the process of uniting medicinal plant and synthetic drug. Some approaches are needed for the development of medicinal plant. There should be more contribution made by traditional medicine towards the modern system of medicine. The research in herbal medicine in Nigeria needs to be improved. Government should provide funding and patency for the research. There should be more awareness on the importance of herbal medicine

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APPENDIX 1

PREPARATION AND COMPOSITION OF MEDIA USED FOR MICROBIAL ISOLATION AND IDENTIFICATION

Nutrient agar (NA)

Composition g/L	
Peptone	5.00
HM peptone B #	1.50
Yeast extract	1.50
Sodium chloride	5.00
Agar	15.00
Final pH (at 25^{O} C)	7.4±0.2

14 grams of powdered NA was dissolved in 500ml of distilled water and homogenized in the conical flask which was corked with cotton wool and sterilized in an autoclave at 121^{O}C for 15 minutes.

Muller-Hinton Agar (MHA)

Composition g/L	
Casein hydrolysate	17.5
Starch	1.5
Agar	17.0

Beef dehydrated infusion from	300.0
pH	7.3 ±0.1

26.6g of powdered Muller-Hinton agar was dissolved in 700ml of distilled water and brought to dissolve gently to dissolve the agar and thereafter, dissolved in McCartney bottles for sterilization in the autoclave at 121^{O}C for 15 minutes.

Thayer-Martin Medium (TMM)

Composition g/l

Beef dehydrated infusion from	300.0
Starch	1.5
Sodium Chloride	5.0
Casein hydrolysate	17.5
Agar	17.0
pH	7.3±0.1

18g of MTM agar base was added to 235ml of distilled water and brought to heat dissolve gently to dissolve the agar. It was sterilized by autoclaving at 121^{O}C for 15 minutes.

Thayer-Martin Medium with GC supplement

Composition g/l

Special peptone	15.0
Sodium chloride	5.0

Di-potassium hydrogen phosphate	4.0
Potassium dihydrogen phosphate	1.0
Agar	10.0
pH	7.2±0.2

2% solution of soluble hemoglobin was prepared by adding 250ml of warm water distilled water To 5g of hemoglobin powder. The solution was stirred continuously during the addition of water. It was sterilized in the autoclave at 121^{O}C for 15 minutes. After sterilization, 250ml of the sterile hemoglobin solution was added to 50^{O}C cooled Thayer medium aseptically and mixed gently to avoid trapping air bottles in the agar.

Muller-Hinton broth (MHB)

Composition g/l	
Acid casein peptone (H)	17.50
Beef infusion	2.00
Corn starch	1.50
pH	7.4± 0.2

10.5g was dissolved in 500ml of distilled water. It was mixed well and dissolved by heating with frequent agitation and thereafter boiled for one minute for complete dissolution. It was dispensed into McCartney bottles and sterilized in an autoclave at 121^{O}C for 15 minutes

Composition g/L

Peptone	5.00
HM peptone B #	1.50
Yeast extract	1.50
Sodium chloride	5.00
Agar	15.00
Final pH (at 25^{O} C)	7.4±0.2

14 grams of powdered NA was dissolved in 500ml of distilled water and homogenized in the conical flask which was corked with cotton wool and sterilized in an autoclave at 121^{O}C for 15 minutes.