ISOLATION AND CHARACTERIZATION OF ACTIVE PRINCIPLE IN LEAVES OF *ABELMOSCHUS ESCULENTUS* (OKRA) AND *CARICA PAPAYA* (PAW-PAW)

By

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CERTIFICATION

This is to certify that this research work “Isolation and characterization of active principle in leaves of *Abelmoschus esculentus* (okra) and *Carica papaya* (paw-paw)” were carried out by Ukachukwu Veronica Ifeoma (20114775418) in partial fulfillment for the award of the degree of Master of Science (M.Sc) in Pharmaceutical Chemistry in the Department of Chemistry of the Federal University of Technology, Owerri.

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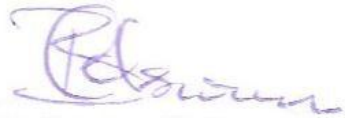

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DEDICATION

This work is dedicated to God Almighty for His mercies, kindness, provision, protection and direction to me throughout the course of this study and my stay in the University.

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ABSTRACT

This study was aimed at the isolation, purification and characterization of the bioactive compound present in the neutral metabolites of *Abelmoschus esculentus* (Okro) and *Carica papaya* (Paw paw) leaves. The study has adopted both chemical analysis and biological assay to achieve this objective. Samples of *Abelmoschus esculentus* and *Carica papaya* leaves were dried at room temperature, pulverized and separately extracted with 250ml of ethanol using the Soxhlet extractor. Each crude extract was analyzed for its photochemical composition and then fractionated into acidic, basic and neutral metabolites. Antimicrobial analysis carried out on the crude extract and various metabolites clearly indicated that both plants possessed pharmacological properties. The neutral metabolites of both plants were found to be the most active of all and were purified by column chromatography using silica gel. The different fractions obtained from *Abelmoschus esculentus* leaf extract were labeled FA1, FA2, FA3 and FA4 whereas the fractions from *Carica papaya* leaf were labeled FC1, FC2, FC3 and FC4.

A second antimicrobial evaluation was carried out on the purified fractions and the fractions which possessed the best antimicrobial potential (Fraction FA2 from neutral metabolite of Okro leaf and FC3 from neutral metabolite of paw-paw leaf) were selected for spectroscopic identification and structural elucidation using IR and GC/MS spectroscopic methods of analysis. Data analysis revealed that the bioactive compound in neutral metabolite of Okro leaf was Methyl oleate while that found in the neutral metabolite of pawpaw leaf was bis heptenoyl phythalate.

(Key word: Isolation, purification, extraction, bio-active compound, characterization, abelmoschus esculentus, carica papaya).

CHAPTER ONE

INTRODUCTION

1.1 Background of the study

Pharmaceutical Chemistry is “the chemistry of drugs” which utilizes the general laws of chemistry to study drugs. The word drug is derived from the French word “drogue” which means a dry herb and it is defined as any substance used for the purpose of diagnosis, prevention, relief or cure of a disease in man or animal. The action of a drug is not only determined by its chemical structure alone but also its physic-chemical properties. All living organisms are prey to infection. Humans, being no exception to the rule, are susceptible to disease caused by viruses, bacteria, protozoa, fungi and helminthes which are collectively referred to as pathogens.

However, various medicinal compounds have been extracted from plant resources. Many researchers have confirmed herb medicine very effective for the treatment of much illness. These plants often exhibit a wide range of biological and pharmacological activities, such as anti-fungi properties (Ajayi*et al.* 2011).

Medicinal plants such as *Abelmoschusesculentus*which is a botanical name of okra and *Carica papaya* which is also a botanical name of pawpaw are believed to be an important source of new classical substances with potential therapeutic benefits.

1.1.1 Ecology of the *Abelmoschusesculentus*plant

Abelmoschusesculentus is a binomial name of okra. The word okra is of Nigerian origin and is cognate with okwuru in the Igbo language spoken in Nigeria. Okra is known in many English speaking countries as ladies fingers. It is a flowering plant in the mallow family *malvaceae*. It is valued for its edible green seed pods. The leaves are 10-20cm long and broad palmately lobed with 5-7 lobes. The plant is cultivated in tropical, subtropical and warm temperature regions around the world for its fibrous fruits or pods containing round, white seeds. It is among the most heat and drought tolerant soils with heavy clay and intermittent moisture but frost can damage the pods. In cultivation, the seeds are soaked overnight prior to planting to a depth of 1-2cm.Germination occurs between six days (soaked seeds) and three weeks. Seedlings require ample water. The fruits are harvested when immature and eaten as a vegetable (Nilesh, 2012).

For classification and easy identification, plants are divided into different into different taxonomical groups: Kingdom - Phylum - Class - Order - Family – Genius - Species.

***Abelmoschusesculentus* belongs to;**

Kingdom	*Plantae*
Division	*Magnoliophyta*
Class	*Magnoliopsida*
(Unranked)	*Rosids*
Order	*Malvales*
Family	*Malvaceae*
Genius	*Abelmoschus*
Species	*A. esculentus*
Botanical Name	*Abelmoschusesculentus*

Synonyms *Hibiscus esculentus L.*

In *Abelmoschusesculentus*, the most common disease afflicting the okra plant is *verticilliumwilt*, which often causes a yellowing and wilting of the leaves. Other diseases include powdery mildew in dry tropical regions, leaf spots, and root-knot nematodes.

Fig. 1.1: *Abelmoschusesculentus* (okra) plant

1.1.2 Ecology of the *CaricaPapaya*Plant

Carica papaya is a binominal name of pawpaw which is the fruit of the plant family*caicaceae*. It is a large tree-like plant, with a single stem growing from 5-10m (16 to 30ft) tall. The leave is spirally arranged which confined to the top conspicuously scarred where leaves and fruit were borne. Usually for

such large plants, the trees are dioecious. The flowers appear on the axils of the leaves, maturing into large fruit. The *Carica papaya* plants and their fruits are known by different names around the English-speaking countries such as papaya or pawpaw. It is cultivated in most tropical countries. In cultivation, it grows rapidly, fruiting within three 2.3 years. It prefers sandy, well-drained soil as standing water will kill the plant within 24 hours (Mahmuda,2014).

***Carica papaya* belongs to;**

Kingdom	*Plantae*
(Unranked)	*Angiosperm*
(Unranked)	*Eudicots*
(Unranked)	*Rasids*
Order	*Brassicales*
Family	*Caricaceae*
Genius	*Carica*
Species	*C. papaya*
Botanical Name	*Carica papaya*

Carica papaya is susceptible to the papaya ring spot virus (PRV), which causes premature molting and malformation of the leaves. This virus threatened to wipe Hawaii's papaya industry completely in the 1990s. The papaya is also susceptible to fruit flies, which lay eggs in the fruit.

Fig. 1.2:*Carica papaya* (paw paw) plant

1.1.3 Importance of the *Abelmoschusesculentus* Plants

Okra is a popular health food due to its high fiber, vitamin C and folate content. Okra is also known for being high in antioxidants. Okra is also a good source of calcium and potassium. Greenish-yellow edible okra oil is pressed from okra seeds (Kochlar, 1986). It has a pleasant taste and odour and is high in unsaturated fats such as oleic acid and linoleic acid and suitable for use as a bio-fuel. In Nigeria, okra is a delicacy, especially deep fried in oil, after breading. Okra is widely used in a stew made with

vegetables and meat. The cooked leaves can also be used as a powerful soup thickener.

Abelmoschusesculentus is believed to provide a natural lining to the intestinal *muscosa*, prevent leakages from ails such as ulcerations, bacterial imbalances and general dyspepsia (Sathish*et al.* 2013). Thus it has excellent results in lowering inflammation, increasing active transportation of nutritional conversion (uptake) and reduces fluid retention. Unspecified parts of the plant were reported to possess diuretic properties which are referenced in numerous sources associated with herbal and traditional medicine. Also, some studies are being developed targeting okra extract as remedy to manage diabetes.

1.1.4 Importance of the *Carica Papaya*

Papaya can be used as a food, a coking aid and in traditional medicine. The stem and back may be used in rope production. Both the green papaya fruit and tree's latex are rich in papain, a protease use for tenderizing meat and other proteins (Mahmuda,2014). Papaya fruit is a source of nutrients such as vitamin A, vitamin C, carotenoids, foliate and dietary fiber. The ripe fruit of the papaya is usually eaten raw, without skin or seeds. The unripe green fruit can be eaten cooked, usually in curries, salads and stews. *Papayas* have a relatively high amount of pectin, which can be used to make jellies.

The black seeds of the *papaya* are edible and have a sharp, spicy taste. They are sometimes ground and used as a substitute for black pepper.

Carica papaya leaves are made into tea as a treatment for malaria (Aravind*et al*. 2013). Antimalaria and antiplasmodial activities have been noted in some preparations of the plant, but no treatment method based on these results has been scientifically proven. Papaya may be used as a medicine for dengue fever. It is also applied topically for the treatment of cuts, rashes, stings and burns (Aravind*et al.* 2013). Women in India, Bangladesh, Pakistan and other countries have long used green papaya as an herbal medicine for contraception and abortion. Preliminary research in animals has provided evidence for the potential contraception and abortion capabilities of *papaya*. Phytochemicals in papaya may suppress the effect on liver cancer cells, possibly due to lycopene or immune system stimulation. *Papaya* seeds might contain antibacterial properties against pathogens such as *Escherichia coli*, *Staphylococcus aureus* or *Salmonella typhi*.

1.1.5 Phytochemistry of the Plants

Phytochemicals are chemical compounds available in plants. Plants produce chemicals known as secondary metabolites that are not directly involved in the process of growth but acts as deterrents to insects and

microbial attack. Phytochemicals constitute one of the most numerous and widely distributed groups of substances in the plant kingdom. Alkaloids, tannins, terpenoids and phenolic compounds all fit in this category. Phytochemicals that possess many ecological and physiological roles are widely distributed as plant constituents.

Oxalic acid is a naturally occurring colourless organic acid found in many plants including okra. The toxicity of oxalic acid is due to kidney failure, which arises from precipitation of solid calcium oxalate, the main constituent of kidney stones. Oxalic acid can also cause joint pain due to the formation of similar precipitate in the joints.

Papaya skin, pulp and seeds also contain a variety of phytochemicals including lycopene and polyphenols. In preliminary research, danielone, a phytoalexin found in papaya fruit, showed antifungal activity against *Colletotrichumgloesporioides*, a pathogenic fungus of papaya (Mahmuda,2014).

1.2 **Statement of the problem**

It is true that there are a lot of antibodies in use today but these pathogens such as S*almonella thyphi, Coliform bacilli, Streptococcus spp, Staphylococcus aureus*and *Escherichiacoli* are adapting to the past and present drugs, developing into resistant species of themselves.

However, the problems of resistance are now acute and major worry. Presently, the emergence of new and previously eradicated disease leads to a pressing need for new and more potent antimicrobial compounds of natural origin to antimicrobial.

Therefore, the study will focus on the Isolation and characterization of active principle of *Abelmoschusesculentus* (okra) and *Carica papaya* (paw-paw).

1.3 Objective of the Study

The objective of this study is to extract, isolate, purify and characterize the bioactive compounds of *Abelmoschusesculentus* (okra) and *Carica papaya* (pawpaw) found to be most effective as an antimicrobial agent. The structural elucidation of the bioactive compound of these medicinal plants will also be carried out. This will be achieved through the following steps;

- To extract the crude extracts from samples of plant materials (Okra and Paw-paw).
- To analyze the phytochemical composition of the crude extracts from plant materials.
- To separate the crude extracts into acidic, basic and neutral metabolites.
- To evaluate the antimicrobial potentials of the crude extracts, acidic, basic and neutral metabolites.
- To purify the isolated principal bioactive compound.

- ❖ To evaluate the antimicrobial potentials of the purified fractions.
- ❖ To characterize the purified fraction with the best inhibitory effects on human pathogens.
- ❖ To interpret the spectral and structural elucidation of the compound.

1.4 Justification of the Study

Since, this study will focus on the effects of the fractions from the plants extract with the best inhibitive effects. This study hopes that the findings will be useful as a guide in development of new drugs for resistant pathogens and other microbes.

1.5 Scope of the Study

In order to achieve these objectives, the study shall determine the bioactive principles present in Okro leaves and paw paw leaves found in Imo State, Nigeria.The scope of this study is limited to the neutral metabolites of*Abelmoschusesculentus* and *Carica papaya*.

CHAPTER TWO

LITERATURE REVIEW

2.1 Details of previous works

Baines and Brocklehurst (1982) reported the Isolation and characterization of four major cysteine-proteinase components from *Carica papaya* latex using high-quality spray-dried latex of the plant, which was fractionated with SP-Sephadex-C50. The four major cysteine-proteinase components, namely; papain, chymopapains A and B and *papaya* peptidase–A were isolated and characterized by protein chemical methods. The authors studied their thiol derivatives using2,2′-dipyridyl disulfide as a two-protonic-state reactivity probe and found that papain and papaya peptidase–A contain one thiol group per molecule, which in each case is part of the catalytic site, as evidenced by their high reactivity toward2,2′-dipyridyl disulfide in acidic media. Chymopapains A and B each contain two thiol groups per molecule, although only one of them is essential for catalytic activity.

Jussi-Pekka*et al*. (2000) studied the “Antimicrobial effects of Finish plants extracts containing flavonoids and other phenolic compounds” and tested the antimicrobial screening of thirteen phenolic substances and twenty nine extracts prepared from Finish plant materials against selective microbes. The tests were done using diffusion methods with four to nine microbial

species (*Asperigilusniger, Baccilussubtilis, Candida albicans, Escherichia coli, Pseudomonas aeruginosa, Sachariomycescerevisiae, Staphylococcus aureus*and*Naringerin*). The extracts were effective in inhibiting the growth of the microorganisms. The most active plant extracts were purple loosestrife against *Candida albicans*, meadow sweet and willow herbs, against bacteria and white birch, pine and potato against *Staphylococcus aureus*.

Cimanga*et al*. (2002) studied the correlation between chemical composition and antibacterial activity of essential oils of some aromatic medicinal plants growing in the Democratic Republic of Congo and identified more than fifteen compounds in amounts higher than 0.1% in the essential oils. They found out that 1,8-Ciniole, alpha and beta-pipene, P-cymene, myrcene, gamma-terpinene, and limonene were prevalent constituents in more than ten selected plant species. Results from anti-bacterial tests using the diffusion method indicated that the essential oils (5ul per disc) inhibited the growth of selected bacteria to different extents. The authors concluded that the most active essential oils were those of the leaves of *Eucalyptus camadulensis* and *Eucalyptus sterticornis* which showed a pronounced inhibition of growth of *Pseudomonas aeruginosa*. The essential oils from the leaves of *Eucalyptus alba, Eucalyptus citriodora, Eucalyptus deglupta, Eucalyptus globules, Eucalyptus Saligna, Eucalyptus robusta, Aframomumstipulatum, Cymbopogon citrates, Ocimumannericarum* and that of the

seeds of *Monodoramyntica* also showed good antibacterial activities except the oils from *Eucalyptus propingua, Eucalyptus nirophylla*and*Ocimumgratissimum* which were less active against the selected bacteria.

Okwu (2005) investigated the “phytochemicals, vitamins and mineral contents of two Nigerian medicinal plants” namely; *Garcinia kola (Heckel)*and*Aframomiummelegueta*. The result revealed the presence of bioactive constituents comprising; flavonoids, phenols, saponins, tannins, while the medicinal vitamins contained ascorbic acid, macin, riboflorin and thanin. The plants also contain minerals such as; Ca, P, K, Mg, Na, Fe, Zn, Mn, and Cu. From the experiment carried out, the concentration of saponins was higher in *G. kola* than in *Aframomiummelegueta*, although *Aframomiummelegueta* contained more flavonoids and tannins than *G. kola*. Quantitative estimates of other phytochemicals showed that *G. kola* contained more alkaloids than *A. melegueta*. More phenols were detected in G. kola than A. *melegueta*.

Edeoga*et al.* (2005) studied the phytochemical composition of C. *papaya* extract. Chemical tests were carried out qualitatively on the extract using standard procedures to identify the amino acids and phytochemical constituents as earlier described (Sofowara, 1993; Trease and Evans, 1996; Harborne, 1973). The results showed that the *C. papaya* leaf extract

contained alkaloids, flavonoids, glycosides, tannins, saponins and anthraquinones.

Okwu and Josiah (2006), studied on micronutrient determination of *C. papaya*. The test was carried out for the presence of minerals: copper, iron, magnesium, manganese and zinc were done following standard procedures via atomic absorption spectroscopy with little modification. Proximate analysis of the plants showed that all the macronutrients were present, with carbohydrate being the most abundant in *C. papaya.* Vitamins A, C and B12 as well as Folic acid were present. The authors concluded that Papaya leaf extract contained only magnesium, since the other metals tested were not detected.

Kotzekidou*et al*. (2008) carried out analyses on the “antimicrobial activity of some plant extracts and essential oils against food borne pathogens in vitro and on the fat of inoculated pathogens in chocolate”. The efficiency of commercially available plant extracts and essential oils used as flavour ingredients in confectionery products were used as antimicrobials in laboratory media against the following microorganism, *Escherichia 1057; H7, Salmonella enteritidis, Salmonella typhimirium, Staphylococcus aureus, listeria monocytogenes*and*Bacillis cereus* using the disc diffusion method. Inhibition zones diameter equal to or greater than 20mm was observed. Lemon flavour applied on *Escherichia coli*, lemon grass essences against *Staphylococcus aureus* and strawberry flavour against *Listeria*

monocytogenes strain. *Escherichia coli* strains were the most susceptible microorganisms inhibited by eighteen extracts followed by *Salmonella typhimurrium* and *S. aureus* which were inhibited by seventeen extracts. Lemon flavour, lemon grass essences, pineapple and strawberry flavours inhibited the food borne pathogens at the lowest concentration. Plant extracts and essential oils with potent antimicrobial activities were tested in chocolate held at different temperatures to determine their efficacy on the fate of the inoculated pathogens. The inhibitory action observed by lemon flavour applied on chocolate inoculated with *E. coli* cocktail culture after storage at 20^{o}C for nine days. Plant extracts tested on chocolate showed an enhanced inhibitory effect indicating that their applications provide protection in case of storage at the above temperature or even higher.

Indran*et al.*(2008) studied the effects of *C. papaya* leaf aqueous extract on blood oxidative stress level in rats using 2, 2-Diphenyl-1-Picryl-Hydrazyl (DPPH) assay and found that the extract showed strong in vitro antioxidant nature. Biochemical analysis indicated that the extract offered some protection with reduction in plasma lipid peroxidation level. The authors concluded that *C*. *papaya* leaf may potentially serve as a good therapeutic agent for protection against oxidative stress.

Torkpo*et al.*(2008) studied the total protein and seeds storage protein diversity in okra. Fibre obtained from the stem was used as a substitute for jute and for making paper and textiles. The authors observed that when

used for paper the leaves were removed and the stem was steamed until the fibres could be stripped off; thenthe fibreswere cooked for 2 hours with lye and put in a ball mill for 3 hours. The paper obtained was cream coloured.

Ejele (2010) studied the “effect of some plant extract on the microbial spoilage of *cajanuscajan*” and experimented the effect of ethanolic extracts of seven plant sources on the microbial spoilage of C.*cajan*. According to the author, the extracts obtained from *Aloe vera*, Bitter leaf, *Garcinia kola*,*Ocimumgratissimum*(scent leaf) and *zingiberofficialae* (ginger) were effective against the spoilage of *C. cajan* extract caused by *Asperigilisorizone* and *Saccharmyces* species (fungi) and inhibited the activities of the microorganisms at room temperature. However, the extract from *Gorgronemalatifolium* (utazi in igbo) was ineffective against the microorganisms at the same temperature.

Perez *et al.*(2011) studied the composition of chloroform extract of C. papaya seed using GC-MS analysis and identified nineteen compounds in the seed extract with oleic (45.97%), palmitic (24.1%) and stearic (8.52%) acids being the main components. The insecticidal and insectistatic activities of the extract showed that the seed extract was effective against several insects.

Adejuwon *et al.*(2011) studied the anti-fungal and anti-bacterial activities of aqueous and methanolic root extracts of *C*. *papaya* linn (*Caricaceae*).

Eleven microorganism species consisting of seven bacteria and four fungi were tested for their sensitivity to the herbal preparations using the Agar Diffusion method. Ampicillin and tetracycline were used as positive control drugs for investigating the bacterial species, while griseofulvin was selected for the fungi and zones of inhibition were measured to determine the microbicidal properties of the test agents. Another set of plates was cultured to estimate the effect of combination therapy using the herbal drug together in varied concentrations with the standard drugs. The results obtained showed that both extracts possessed good antimicrobial activity against four of the bacteria species and three fungi. However, the organic preparation produced better and more significant efficacy than the aqueous preparation. The combination mixtures revealed synergistic effect between *C. papaya* and ampicillin whereas antagonism was observed with tetracycline. A wide range of secondary metabolites were also identified in both extracts with the methanolic extract containing a higher amount.

Ejele (2011), studied the effect of secondary metabolites of *G. kola* on the microbial spoilage of C. *cajan* extract and the results showed that the fungi *Asperigilis orizone* and *Saccharmyces species* attacked the basic metabolites after only one week; the neutral metabolite possessed antimicrobial potential, inhibited microbial growth and showed rancidity probably because it lacked antioxidant properties. The acidic metabolite completely inhibited microbial attack on *C. cajan* extract and was not

attacked by the microorganisms throughout the study period of one year, suggesting that the acidic metabolite of *G. kola* possessed antimicrobial, antifungal and antioxidant properties. Data analysis of antimicrobial screening of the acidic metabolite on some selected human pathogens such as *E. coli*, *S. aureus* and *C. bacilli*, showed that this metabolite was effective against all the human pathogens tested.

Nurul (2012), reported a study on the antioxidant activity of *C. papay*and its phenolic content using different parts of the plant. The total phenolic content of the extracts was determined by Folin-Ciocalteu method and antioxidant activity was assayed using DPPH method. The total phenolic contents and antioxidant activity of the extracts,determined as Gallic acid equivalent, were found to be highest in fresh extract.

Ejele*et al*. (2013) conducted a research on bioassay-guided isolation, purification and characterization of antimicrobial compound from acidic metabolite of *Piper umbellatum* seed extract and evaluated the antimicrobial potential of ethanol extract of the seeds and its acidic, basic and neutral metabolites using the disc diffusion method. The results showed that the acidic and neutral metabolites exhibited greater antibiotic activities against the tested microorganisms; C. *bacilli, S. typhi*and *S. aureus* and gave inhibition zone diameters greater than 28mm against both C. *bacilli* and S. *aureus*. These results were interpreted in terms of differences in phytochemical composition of these metabolites and indicated that an

effective anti-staphylococcal drug could be developed from *P. umbellatum* extract for chemotherapy of diseases caused by *S. aureus* which is known all over the world to develop resistance to most potent antibiotic drugs. Chromatographic purification of acidic metabolites gave five fractions whose antimicrobial potential against some pathogens were evaluated and compared with Amoxil, a positive standard antimicrobial drug. The results showed that all the chromatographic fractions possessed inhibitory activities against the tested microbes. Spectroscopic analysis using IR, ^{1}H and ^{13}C-NMR suggested the active principle in acidic metabolite of *P. umbellatum* could be Naringin, a naturally occurring polyphenol and antibiotic drug.

This study intends to extract, isolate, purify and characterize the bioactive compounds of *Abelmoschusesculentus* (okra) and *Carica papaya* (pawpaw) found to be most effective as an antimicrobial agent. Spectroscopic identification of the bioactive compound of these medicinal plants (Okra and Paw-paw) will also be carried out using IR, GC/MSwith a view to elucidate the structures of the compounds.

CHAPTER THREE

MATERIALS AND METHOD

3.1: Plant Material

Leaves of okraand pawpaw were collected from the farm in NtibunkaUmudaguMbieri in Mbaitolu Local Government Area of Imo State of Nigeria.

3.2: Reagents and Apparatus Used

The following solutes, solvents and apparatus are used for carrying out the experiments in the laboratory. They are 95% ethanol, dilute hydrochloric acid, chloroform, sodium hydroxide, 2,4-dinitraphonyl hydrazine, silica gel, Vaseline gel, distilled water, iron (III) chloride, concentrated tetraoxosulphate (IV) acid, Wagner's reagent, sodium trioxocarbonate (IV), soxhlet extractor, chromatographic column, burner, litmus paper, cotton wool, water bath, beaker, conical flask, measuring cylinder, stirrer, glass containers, spectrometers, test tubes etc.

3.3: Preparation of Crude Extract

Leaves of okra were collected, dried in a shade and pulverized. About 90g of the sample was extracted with 250ml of ethanol (EtOH) using the Soxhlet extractor. The extraction process lasted 12 hours. The ethanol extract was allowed to evaporate completely at room temperature to give a gel. Upon complete evaporation, the ethanol extract was de-waxed with a mixture of ethanol and water in the ratio of 4:1 and filtered. The filtrate was used for

further analysis. The same experiment was carried out on the leaves of pawpaw.

3.3.1: Extraction procedure:

Soxhlet reflux extractor was used for the extraction. The reagent used for the extraction was nascent ethanol. The pulverized material was packed with cotton wool and solvent heated. The ethanol vapour moved from the receiver into the condenser where it was condensed. The condensed solvent movedthrough the pulverized material to the receiver through the side arm.

3.4: Phytochemical Screening of Crude Extract

The phytochemical analyses of the crude extracts were carried out using A.O.A.C official methods of analysis (1990). The presence of tannins, alkaloids, flavonoids, saponins (steroids and terpenoids), phenols, carboxylic acid, aldehydes, ketones and reducing sugar were tested.

3.4.1:Test for Tannins

5ml of distilled water was added to 1ml of sample followed by addition of few drops of $FeCl_3$. Formation of bluish / greenish precipitate indicated presence of simple tannins.

3.4.2:Test for alkaloids

Wagner Reagent

2ml of Wagner's reagent was added to 1ml of sample and a dark-brown precipitate was formed, which indicated presence of alkaloids.

3.4.3: Test for flavonoids

1ml of sample was dissolved in 1ml of dilute NaOH and filtered. The filtrate was acidified by addition of drops of conc. HCl. Formation of precipitate indicated presence of flavonoid.

3.4.4: Test for Saponins

1ml of distilled water was added to 1ml of the extract and shaken vigorously (in the presence or absence of olive oil). Formation of persistent frothing foaming indicated presence of saponin.

3.4.4.1: Steroids

Salkowski test:

5ml of sample was shaken with equal volume of chloroform. The chloroform layer was removed into a clean test tube and 1ml of 5% H_2SO_4 was added cautiously down the side of the bent test tube. Formation of redish-brown ring indicated presence of steroid.

3.4.4.2:Terpenoids

Libermann's test:

0.5ml of sample was dissolved in 2ml of acetic acid and allowed to cool, followed by slow addition of 1ml of conc. H_2SO_4 along the line of the test tube which is bent at an angle. Two layers were formed and redish-brown ring at the interface indicated presence of terpenoid.

3.4.5:Test for Phenols

Ferric chloride test:

0.5ml of sample and 4ml of water weremixed and 3 drops of $FeCl_3$ solution were added to the mixture. Formation of dark-brown precipitate indicated the presence of phenols.

3.4.6:Test for Carboxylic Acid

a) Litmus test:

Blue litmus paper was dipped into the sample and examined for change in colour. Formation of faint red coloration indicated presence of acid.

b) Ester formation:

0.5ml of conc. H_2SO_4 was added to a warmed mixture of 1ml of sample and 2ml of 95% ethanol and cooled. 5ml of saturated Na_2CO_3 solution was added to the mixture and cautiously poured into evaporating dish. A sweet

smelling aroma indicated formation of esters due to presence of carboxylic acid or phenols.

3.4.7:Test for Aldehydes and Ketones

2ml of 95% ethanol was added to 2 drops of the sample followed by addition of 3ml of 2,4-dinitrophenyl hydrazine reagent and vigorous shaking of the mixture. A red precipitate indicated the presence of aldehydes, ketones and reducing sugar.

3.5 Separation of the Crude Extracts into Fractions

Each crude sample was separated into acidic, basic and neutral metabolites using the method described by Ejele&Alinnor(2010).

3.6: PREPARATION OF METABOLITES

3.6.1:Basic Metabolites

The metabolites were prepared as earlier stated (Ejele&Alinnor, 2010). The filtrate from the crude extracts were treated with dilute HCl and extracted with chloroform in a separating funnel. The lower chloroform layer was removed (and reserved for the preparation of other metabolites). The HCl layer was treated with dilute NaOH solution until the mixture became slightly basic. Then the mixture (with or without precipitate) was allowed to evaporate completely at room temperature to form a gel, which was

dissolved in ethanol and filtered. The filtrate was used without further purification.

3.6.2: Neutral Metabolites

The chloroform layer obtained above was placed in a separating funnel and treated with dilute NaOH solution. After equilibrating, the lower chloroform layer was removed and allowed to evaporate completely at room temperature to produce a gel.

3.6.3: Acidic Metabolites

The aqueous alkaline layer obtained above was treated with conc. H_2SO_4 until the solution became acidic. The mixture (with or without precipitate) was allowed to evaporate completely at room temperature to produce a gel, which was dissolved in ethanol and filtered. The filtrate was used without further purification.

3.7 Antimicrobial Evaluation of Extract, Acidic, Basic and Neutral Metabolites.

The crude extract, acidic, basic and neutral gel samples were each dissolved in ethanol and filtered. The filtrates obtained were used for antimicrobial experiments without further purification. The screening was carried out at the Department of Microbiology, Federal Medical Centre

(FMC), Owerri, Imo State, Nigeria, using the agar disk diffusion method as described by Garred&O'Graddy (1983). The test microbes used were *Staphylococcus aureus*, *Streptococcus spp*, *Salmonella typhi*, *Coliform bacilli* and *Escherichia coli*.

An inoculating loop was touched to five isolated colonies of the pathogen on an agar plate. The loop was used to inoculate a tube of culture broth, which was incubated at 35-37^{o}c until it became slightly turbid. This was diluted to match the turbidity standard. A sterile cotton swab was dipped into the standardized bacterial test suspension and was used to evenly inoculate the entire surface of the agar plate. After the agar surface has been dried for about 5 minutes, the appropriate antibiotic test disks were placed in a multiple applicator device. The agar plate was incubated at 35^{o}c to 37^{o}c for 16-18 hours. Later, the inhibition zone diameters (i.e. areas showing little or no microbial growth) were measured to the nearest millimeter (mm).

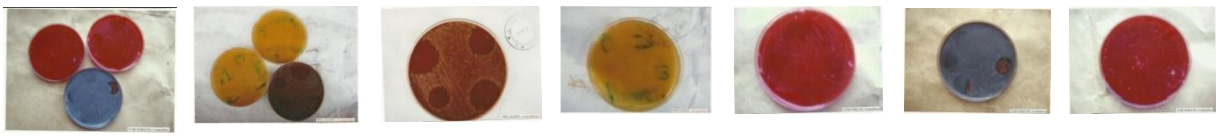


Fig. 3.1: Antimicrobial Evaluation of Extractsof various Metabolites.

Figure 3.1 showed the Agar plate and inhibition zone diameters (IZD) of various metabolites of *Abelmuschusesculentus*and *Carica papaya*. Thecoloured areas represented areas with little or no microbial growth, from which the IZD was determined

3.7.1:Determination of Minimum Inhibitory Concentration (MIC)

The determination of MIC was carried out to obtain the lowest concentration of extract and metabolites which would give the least visible growth of the micro-organism. The agar disc diffusion assay (a quantitative method based on the method of European Society of Clinical Microbiology and Infectious Diseases) was used for the evaluation of antimicrobial potential. Standard solutions of the samples were prepared in agar nutrient; 100mg/ml, 50mg/ml, 25mg/ml, 12.5mg/ml and 6.25mg/ml and poured into sterilized Petri-dishes and allowed to solidify.

The test microorganisms were spotted onto the surface of the solidified extract-agar mixture. The plates were inoculated, starting from the highest concentration to the lowest concentration and were allowed to dry for 30 minutes. They were incubated at 37^{o}C for 18 hours, after which the sample were examined for microbial growth. The lowest concentration of the extract which showed little or no visible growth of the micro-organism was taken as the MIC(Garred& O-Graddy,1983).

3.8 Chromatographic Purification of Bioactive Compound

The bioactive compound was isolated from the neutral metabolite as described by Ejele *et al*. (2012). The neutral metabolite was dissolved in

chloroform/ethanol mixture (ratio 1:1) and filtered. The filtrate was purified using silica gel packed in a chromatographic column. Slurry of finely powdered silica gel dispersed in chloroform were packed in glass column to a height of about 12m and loaded with 10ml of the isolate dissolved in ethanol. The purification was done by gradient elution with chloroform-ethanol mixture in a ratio of 1:2 and finally eluted with 95% ethanol. Different fractions were collected at intervals of one hour, although differently coloured compounds were collected in different conical flask. The solvent in each flask was allowed to evaporate at room temperature. The different fractions obtained were labeled F1A, F2A, F3A, F4A, etc for *Abelmuschus esculentus* and F1C, F2C, F3C, F4C, etc for *Carica papaya*. Thin layer chromatographic (TLC) analysis of the fractions showed only one spot suggesting that each fraction could be considered as a pure compound. The fractions werethen screened for antimicrobial activity and the fraction with best antimicrobial potential was selected for spectroscopic characterization and structural elucidation.

3.9 Spectroscopic Analysis of Best Bioactive Principle

The GC/MS and IR (infra-red) characterization of the purified fraction with the best antimicrobial activity were performed at National Institution of Science and Technology Research, Zaria, Nigeria.

CHAPTER FOUR
RESULTS AND DISCUSSION

4.1 Phytochemical Screening of the Crude Extracts

Table 4.1: Results of Phytochemical analysis of crude extracts

Phytochemicals tested	CEA	CEC
Alkaloids	Positive	Positive
Tannins	Positive	Positive
Flavonoids	Positive	Negative
Saponins	Positive	Positive
Carboxylic acids	Positive	Positive
Phenols	Positive	Positive
Steroids	Positive	Positive
Aldehydes/ketones	Negative	Positive
Terpenoids	Negative	Positive

CEA – Crude extract of *Abelmoschusesculentus* leaves. CEC – Crude extract of *Carica papaya* leaves.

The results of phytochemical screening of crude extracts of *Abelmoschusesculentus*and*Carica papaya* were presented in Table 4.1 from which it could be seen that the crude extract of *Abelmoschusesculentus* leaves contained alkaloids, tannins, flavonoids, saponins, carboxylic acid, phenols and steroids. Similarly, the crude extract of *Carica papaya* leaves contained alkaloids, tannins, saponins, carboxylic acid, tannins, phenols, steroids, terpenoids, aldehydes or ketones.The results also showedthat aldehydes/ketones and terpenoids were not found in *Abelmoschusesculentus* leaf while the *Carica papaya* leaf extract did not

contain flavonoids. Thus, a wide range of phytochemicals were identified in both extracts.

4.2 Results of Antimicrobial Evaluation of Okra leaf

Table 4.2.: Results of Antimicrobial Evaluation of Acidic Metabolites

Microorganism	100mg/ml	50mg/ml	25mg/ml	12.5mg/ml	6.25mg/ml
Staphylococcus aureus	0mm	0mm	0mm	0mm	0mm
Streptococcus Spp.	10mm	0mm	0mm	0mm	0mm
Salmonella typhi	05mm	0mm	0mm	0mm	0mm
Coliform bacilli	12mm	1mm	0mm	0mm	0mm
Escherichia coli	10mm	0mm	0mm	0mm	0mm

The results of antimicrobial screening of acidic metabolites of *Abelmoschusesculentus* leaf, presented in Table 4.2, showed that this metabolite was ineffective against the test human pathogens, with inhibition zone diameters (IZD) ranging between 5 – 12mm. This metabolite gave IZD of 0mm against *Staphylococcus aureus*, 5mm against*Salmonella typhi*,

10mm against *Streptococcus Spp* and*Escherichia coli*and 12mm against *Coliform bacilli* at 100mg/ml concentration.

Table 4.3: Results of Antimicrobial Evaluation of Basic Metabolites

Microorganism	100mg/ml	50mg/ml	25mg/ml	12.5mg/ml	6.25mg/ml
Staphylococcus aureus	22mm	10mm	0mm	0mm	0mm
Streptococcus Spp.	20mm	8mm	0mm	0mm	0mm
Salmonella typhi	18mm	7mm	0mm	0mm	0mm
Coliform bacilli	20mm	7mm	0mm	0mm	0mm
Escherichia coli	22mm	9mm	0mm	0mm	0mm

Table 4.3 showed results of antimicrobial screening of basic metabolites of *Abelmoschusesculentus* leaf, from which it could be seen that the basic metabolite was active against the test human pathogens, with inhibition zone diameters (IZD) ranging between 18 – 22mm. The metabolite was bacteriostatic against *Staphylococcus aureus* and *Salmonella typhi*with IZD of 22 and 18mm respectively, but bactericidal against *Streptococcus*

Spp.and *Coliform bacilli* with IZD of 20mm and *Escherichia coli* with IZD of 22mm at 100mg/ml concentration.

Table 4.4: Results of Antimicrobial Evaluation of Neutral Metabolites

Microorganism	100mg/ml	50mg/ml	25mg/ml	12.5mg/ml	6.25mg/ml
Staphylococcus aureus	20mm	8mm	0mm	0mm	0mm
StreptococcusSpp.	20mm	7mm	0mm	0mm	0mm
Salmonella typhi	19mm	5mm	0mm	0mm	0mm
Coliform bacilli	18mm	6mm	0mm	0mm	0mm
Escherichia coli	20mm	7mm	0mm	0mm	0mm

Results of antimicrobial screening of neutral metabolites of *Abelmoschusesculentus* leaf were presented in Table 4.4, from which we see that the neutral metabolite was also active against the test human pathogens, with inhibition zone diameters (IZD) ranging between 18 – 20mm. The metabolite was bacteriostatic against *Staphylococcus aureus*,*Salmonella typhi*and *Coliform bacilli*with IZD of 20, 19 and 18mm respectively, but bactericidal against *Streptococcus spp.* and *Escherichia coli* with IZD of 20mm at 100mg/ml concentration.

It wasobserved that the basic and neutral metabolites possessedbetter antimicrobial potential and ability to inhibit growth of test microorganisms than the acidic metabolite.

The crude extract showed zero activity against the test microorganisms at all concentrations.

4.3 Results of Antimicrobial Evaluation of Paw Pawleaf

Table 4.5: Results of Antimicrobial Evaluation of Acidic Metabolites

Microorganism	100mg/ml	50mg/ml	25mg/ml	12.5mg/ml	6.25mg/ml
Staphylococcus aureus	8mm	0mm	0mm	0mm	0mm
Streptococcus Spp.	10mm	0mm	0mm	0mm	0mm
Salmonella typhi	12mm	0mm	0mm	0mm	0mm
Coliform bacilli	05mm	0mm	0mm	0mm	0mm
Escherichia coli	0mm	0mm	0mm	0mm	0mm

Results of antimicrobial screening of acidic metabolites of *Carica papaya* leaf were presented in Table 4.5, from which we see that the acidic

metabolite was weak against*Staphylococcus aureus*and *Coliform bacilli* with IZD of 8 and 5mm, bacteriostatic against *Salmonella typhi* and *Streptococcus spp.* with IZD of 12and 10mmand 0mmagainst *Escherichia coli* at 100mg/ml concentration.

Table 4.6: Results of Antimicrobial Evaluation of Basic Metabolites

Microorganism	100mg/ml	50mg/ml	25mg/ml	12.5mg/ml	6.25mg/ml
Staphylococcus aureus	20mm	8mm	0mm	0mm	0mm
Streptococcus Spp.	20mm	7mm	0mm	0mm	0mm
Salmonella typhi	22mm	9mm	0mm	0mm	0mm
Coliform bacilli	20mm	7mm	0mm	0mm	0mm
Escherichia coli	18mm	8mm	0mm	0mm	0mm

Results of antimicrobial screening of basic metabolites of *Carica papaya* leaf were presented in Table 4.6, from which we see that the basic metabolite was active against the test human pathogens, with inhibition zone diameters (IZD) ranging between 18–22mm. The metabolite was

bacteriostatic against *Staphylococcus aureus* and*Escherichia coli* with IZD of 20 and 18mm respectively and bactericidal against *Streptococcus spp.,Salmonella typhi* and *Coliform bacilli* with IZD of 20mmat 100mg/ml concentration in each case.

Table 4.7: Results of Antimicrobial Evaluation of Neutral Metabolites

Microorganism	100mg/ml	50mg/ml	25mg/ml	12.5mg/ml	6.25mg/ml
Staphylococcus aureus	25mm	13mm	2mm	0mm	0mm
Streptococcus Spp.	22mm	9mm	0mm	0mm	0mm
Salmonella typhi	20mm	8mm	0mm	0mm	0mm
Coliform bacilli	22mm	8mm	0mm	0mm	0mm
Escherichia coli	18mm	7mm	0mm	0mm	0mm

Results of antimicrobial screening of neutral metabolites of *Carica papaya* leaf were presented in Table 4.7, from which we see that the neutral metabolite was also active against the test human pathogens, with inhibition

zone diameters (IZD) ranging between 18–25mm. The metabolite was bactericidal against *Staphylococcus aureus*, *Streptococcus spp., Salmonella typhi* and *Coliform bacilli* with IZD of 25,22, 20 and 22 respectively but bacteriostatic *Escherichia coli* with IZD of 18mm at 100mg/ml concentration.

These results showed that the basic and neutral metabolites of *Carica papaya* leaf have the greater potential to inhibit growth of microorganisms.

The crude extract showed zero activity against the test microorganisms at all concentrations.

4.4 Results of Antimicrobial Evaluation of Control Drugs

Table 4.8: Results of Antimicrobial Evaluation of Control Drugs

Microorganisms	Ciprofloxacin	Verofloxacin	Amoxil	Augumentin	Gentamycin
	100mg/ml	200mg/ml	200mg/ml	300mg/ml	100mg/ml
Staphylococcus aureus	20mm	23mm	18mm	____	12mm
Streptococcus spp.	22mm	24mm	21mm	____	16mm
Salmonella spp.	20mm	21mm	____	19mm	13mm
Coliform bacilli	17mm	20mm	____	21mm	20mm
Escherichia coli	22mm	16mm	____	20mm	18mm

Five standard drugs sourced from Federal Medical Centre, Owerri were used as to investigate the bacterial species, namely: Ciprofloxacin, Verofloxacin, Amoxil, Augumenti and Gentamycin. The results presented in Table 4.8, showed that the positive control drugs (at different concentrations) were active against the test human pathogens to different extents, with inhibition zone diameters (IZD) ranging between 13–24mm.

4.5 Comparing IZD of Crude Extract and Various Metabolites against *Streptococcus spp* (Concentration = 100mg/ml).

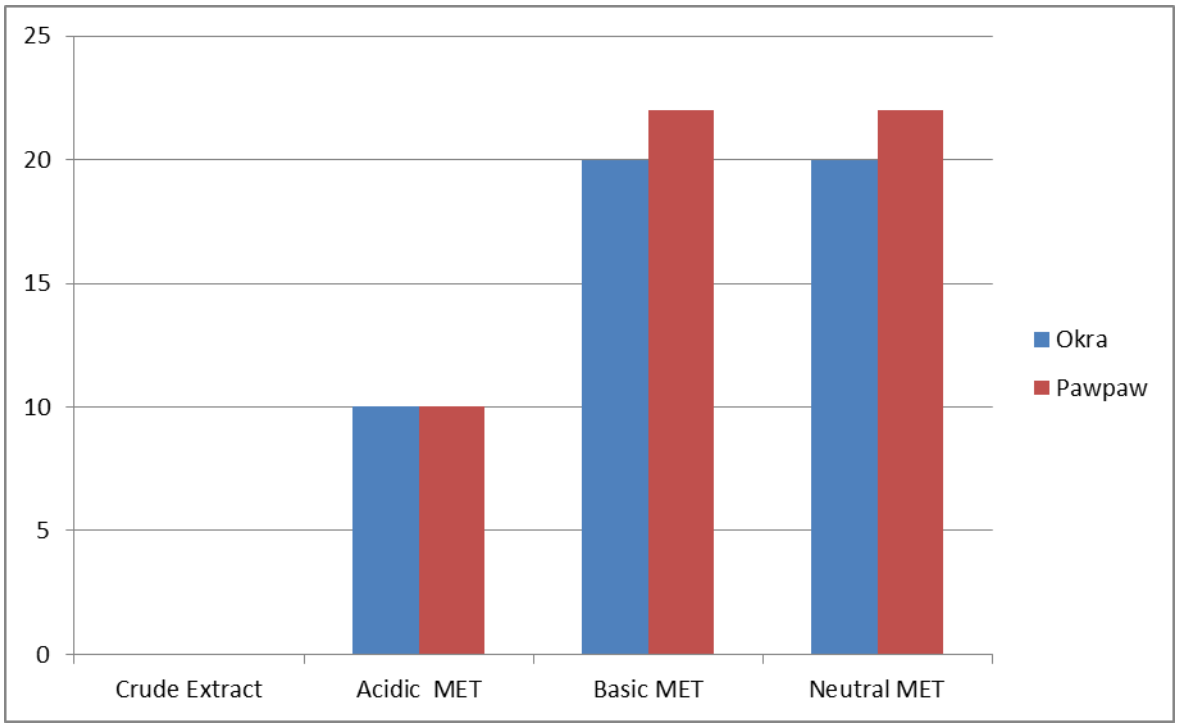


Fig. 4.1: IZD of Extract of Various Metabolites against *Staphylococcus aureus*

Figure 4.1 showedthe Histogram comparingthe IZD of various metabolites of *Abelmoschus esculentus* and *Carica papaya* leaves extract against *Streptococcus spp* at 100mg/ml concentration. It could be seen from the Histogram that the neutral metabolite of *Carica papaya* leaf extract gave the best antimicrobial activity against *Streptococcus spp* with IZD of 25mm. The minimum inhibitory concentration was 50mg/ml, since the IZD was between 9mm and 7mm. The neutral metabolite of both okra and pawpaw were compared with control drugs such as Ciprofloxacin and Gentamycin in 100mg/ml.

Definitions

i) **Ineffective Drugs**

A drug is considered to be ineffective when its IZD is between 0 and 10mm. Ineffective drugs usually do not have an effect on the growth and multiplication of the microorganism.

ii) **Bacteriostatic Drugs**

When the IZD is between 11 and 19mm, the drug is said to be bacteriostatic.This group of drugs can stop the growth and reproduction of microorganisms but cannot kill them.

iii) **Bactericidal Drugs**

When the IZD of a drug is 20mm and above, it is said to be bactericidal. This means the drug will not only stop the growth and multiplication of the microorganisms, but willcompletely kill and wipe them out.

However, for ***Staphylococcus aureus***, adrug is considered **Ineffective** when the IZD is between 0 and 15mm; **Bacteriostatic**, when IZD is between 16 and 25mm and**Bactericidal**, when the IZD is above 25mm.

4.6 Antimicrobial Evaluation of Purified Fractions.

Results of antimicrobial evaluation of purified fractions of neutral metabolites of leaves of *Abelmoschusesculentus* (FA1, FA2, FA3, FA4, etc) and *Carica papaya* (FC1, FC2, FC3, FC4, etc) was shown in Tables 4.6 below.

Table 4.9: Results of Antimicrobial Evaluation of Fraction FA1

Microorganism	100mg/ml	50mg/ml	25mg/ml	12.5mg/ml	6.25mg/ml
Staphylococcus aureus	0mm	0mm	0mm	0mm	0mm
Streptococcus Spp.	15mm	2mm	0mm	0mm	0mm
Salmonella typhi	22mm	8mm	0mm	0mm	0mm
Coliform bacilli	18mm	6mm	0mm	0mm	0mm
Escherichia coli	16mm	4mm	0mm	0mm	0mm

Results of antimicrobial screening of Fraction FA1 of *Abelmoschus esculentus* leaf were presented in Table 4.9. The Tableshowed that Fraction FA1 was active against the test human pathogens to various extents (except *Staphylococcus aureus*) with IZD ranging between 15–22mm. Fraction FA1 was bacteriostatic against *Streptococcus spp.,Escherichia coli*and *Coliform bacilli* with IZD of 15, 16 and 18mmrespectively but bactericidal against *Salmonella typhi* with IZD of 22mm at 100mg/ml concentration. This Fraction showed zero activity against *Staphylococcus aureus* at all concentrations.

Table 4.10: Results of Antimicrobial Evaluation of Fraction FA2

Microorganism	100mg/ml	50mg/ml	25mg/ml	12.5mg/ml	6.25mg/ml
Staphylococcus aureus	0mm	0mm	0mm	0mm	0mm
Streptococcus Spp.	30mm	14mm	3mm	0mm	0mm
Salmonella typhi	20mm	5mm	0mm	0mm	0mm
Coliform bacilli	15mm	2mm	0mm	0mm	0mm
Escherichia coli	25mm	10mm	0mm	0mm	0mm

Results of antimicrobial screening of Fraction FA2 of *Abelmoschus esculentus* leaf were presented in Table 4.10. This Table showed that Fraction FA2 was active against the test human pathogens to various extents (except *Staphylococcus aureus*) with IZD ranging between 15–30mm. Fraction FA2 was bacteriostatic against *Coliform bacilli* with IZD of 15mm but bactericidal against *Salmonella typhi, Escherichia coli* and *Streptococcus spp.* with IZD of 20, 25 and 30mm respectively at 100mg/ml

concentration. Fraction FA2 showed zero activity against *Staphylococcus aureus* at all concentrations.

Table 4.11: Results of Antimicrobial Evaluation of Fractions FA3 & FA4

Microorganism	100mg/ml	50mg/ml	25mg/ml	12.5mg/ml	6.25mg/ml
Staphylococcus aureus	0mm	0mm	0mm	0mm	0mm
Streptococcus Spp.	15mm	4mm	3mm	0mm	0mm
Salmonella typhi	25mm	10mm	0mm	0mm	0mm
Coliform bacilli	20mm	4mm	0mm	0mm	0mm
Escherichia coli	23mm	8mm	0mm	0mm	0mm

Results of antimicrobial screening of Fractions FA3&4 of *Abelmoschus esculentus* leaf were presented in Table 4.11. This Table showed that both Fractions were active against the test human pathogens to various extents (except *Staphylococcus aureus*) with IZD ranging between 15–25mm. Both Fractions were bacteriostatic against *Streptococcus spp.* with IZD of 15mm

but bactericidal against *Coliform bacilli*, *Escherichia coli* and *Salmonella typhi* with IZD of 20, 23 and 25mm respectively at 100mg/ml concentration. Fractions FA3&4 showed zero activity against *Staphylococcus aureus* at all concentrations.

Table 4.12: Antimicrobial Results of Fraction FC1& FC2

Microorganism	100mg/ml	50mg/ml	25mg/ml	12.5mg/ml	6.25mg/ml
Staphylococcus aureus	0mm	0mm	0mm	0mm	0mm
Streptococcus Spp.	12mm	0mm	3mm	0mm	0mm
Salmonella typhi	12mm	0mm	0mm	0mm	0mm
Coliform bacilli	10mm	0mm	0mm	0mm	0mm
Escherichia coli	12mm	0mm	0mm	0mm	0mm

Results of antimicrobial screening of Fractions FC1&FC2of *Carica papaya* leaf was presented in Table 4.12,which showed that both Fractions were active against the test human pathogens to various extents (except *Staphylococcus aureus*) with IZD ranging between 10–12mm at 100mg/ml

concentration. Both Fractions were weak and ineffective against *Coliform bacilli* with IZD of 10mm, *Streptococcus spp., Salmonella typhi* and *Escherichia coli* with IZD of 12mmin each case. Fractions FC1&2showed zero activity against *Staphylococcus aureus* at all concentrations.

Table 4.13: Antimicrobial Results of Fraction FC3

Microorganism	100mg/ml	50mg/ml	25mg/ml	12.5mg/ml	6.25mg/ml
Staphylococcus aureus	18mm	6mm	0mm	0mm	0mm
Streptococcus Spp.	20mm	6mm	3mm	0mm	0mm
Salmonella typhi	30mm	14mm	0mm	0mm	0mm
Coliform bacilli	23mm	8mm	0mm	0mm	0mm
Escherichia coli	23mm	8mm	0mm	0mm	0mm

Results of antimicrobial screening of Fraction FC3 of *Carica papaya* leaf were presented in Table 4.13, which showed that Fraction FC3 was active against the test human pathogens to various extents with IZD ranging

between 18–30mm at 100mg/ml concentration. Fraction FC3 was bacteriostatic against *Staphylococcus aureus* with IZD of 18mmbut bactericidal against and *Streptococcus spp., Coliform bacilli*, *Escherichia coli* and *Salmonella typhi* with IZD of 20, 23, 23 and 30mm respectively.

Table 4.14: Antimicrobial Results of Fraction FC4

Microorganism	100mg/ml	50mg/ml	25mg/ml	12.5mg/ml	6.25mg/ml
Staphylococcus aureus	0mm	0mm	0mm	0mm	0mm
Streptococcus Spp.	20mm	8mm	0mm	0mm	0mm
Salmonella typhi	18mm	6mm	0mm	0mm	0mm
Coliform bacilli	24mm	10mm	0mm	0mm	0mm
Escherichia coli	20mm	8mm	0mm	0mm	0mm

Results of antimicrobial screening of Fraction FC4 of *Carica papaya* leaf were presented in Table 4.14, which showed that Fraction FC4 was active against the test human pathogens to various extents (except *Staphylococcus aureus*) with IZD ranging between 18–24mm at 100mg/ml concentration. Fraction FC4 was bacteriostatic against *Salmonella typhi*, with IZD of 18mmbut bactericidal against *Streptococcus spp., Escherichia coli* and *Coliform bacilli* with IZD of 20, 20and 24mm respectively. Fraction

FC4 showed zero activity against *Staphylococcus aureus* at all concentrations.

Results of antimicrobial screening of chromatographic fractions of *Abelmoschus esculentus* and *Carica papaya* leaves extracts have shown that Fraction FA2 of *Abelmoschus esculentus* leaf extract exhibited the greatest potential to inhibit the growth of *Streptococcus spp.* with IZD of 30mm while Fraction FC3 of *Carica papaya* leaf extract exhibited the greatest potential to inhibit the growth of *Salmonella typhi*with IZD of 30mm. Therefore both Fractions FA2 and FC3 were selected for spectroscopic identification and structural elucidation.

The inhibition of growth and activity of microorganisms were the major reasons for use of chemical preservatives because they are able to inhibit microbial growth by interfering with cell membranes, enzymes activity or genetic mechanism of the microorganisms. Chemical preservatives may also be used as coating to keep out microorganisms, prevent loss of water and hinder undesirable microbial, enzymatic and chemical reactions (Fulton, 1981). On the other hand, plants have an almost limitless ability to synthesize aromatic substances most of which are phenols or their oxygen-substituted derivatives. These are secondary metabolites of which at least twelve thousand have been isolated, a number estimated to be less than 10% of the estimated total plant derived compounds (Hasham, 1996).

4.6.1 Comparison of IZD of Various Fractions against *Streptococcus spp.*(Concentration = 100mg/ml)

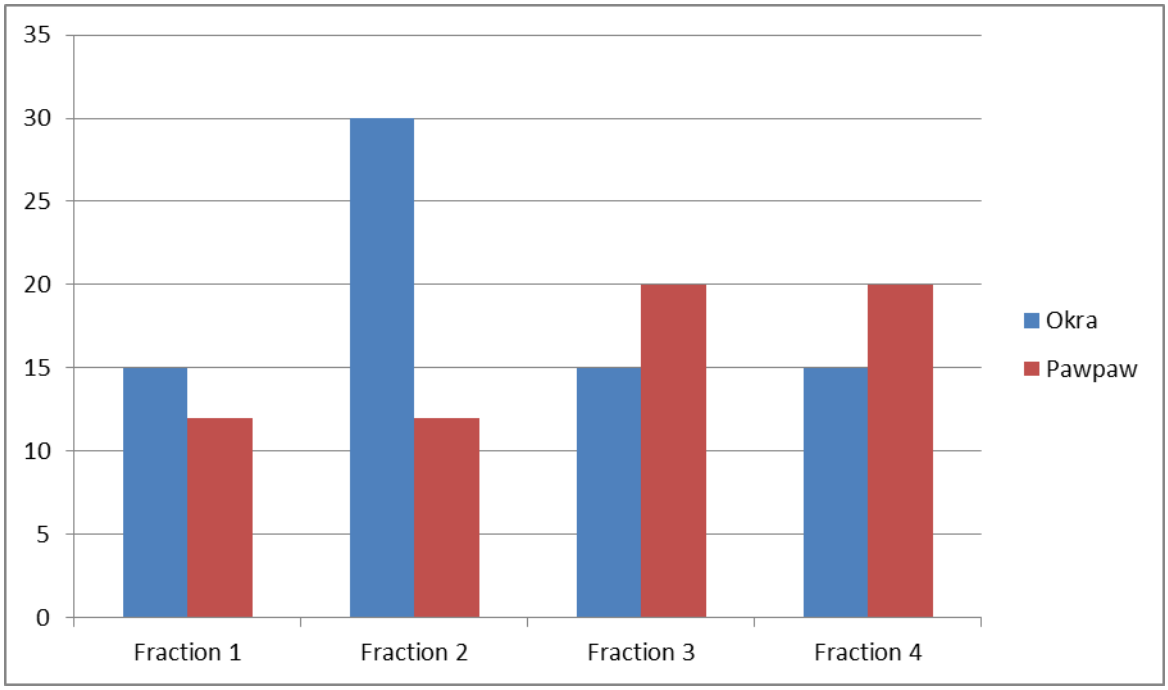


Fig.4.2: **IZD of Various Fractions against *Streptococcus spp.***

Figure 5 showed a comparison of IZD of various fractions of *Abelmoschus esculentus* and *Carica papaya* leaves extracts against *Streptococcus spp*.at 100mg/ml concentration. It could be seen that Fraction FA2 from neutral metabolite of *Abelmoschus esculentus* leaf extract gave the best antimicrobial activity against *Streptococcus spp*. with IZD of 30mm. The minimum inhibitory concentration was 50mg/ml, since the IZD was between 9mm and 7mm. The neutral metabolite of fraction 2(FA2) of okra and fraction 3 (FC3) of pawpaw were compared with control drugs such as Ciprofloxacin and Gentamycin in 100mg/ml.

4.7: Characterization and Structural Elucidation of Fraction FA2

4.7.1:Infra Red (IR)Spectrum of Fraction FA2

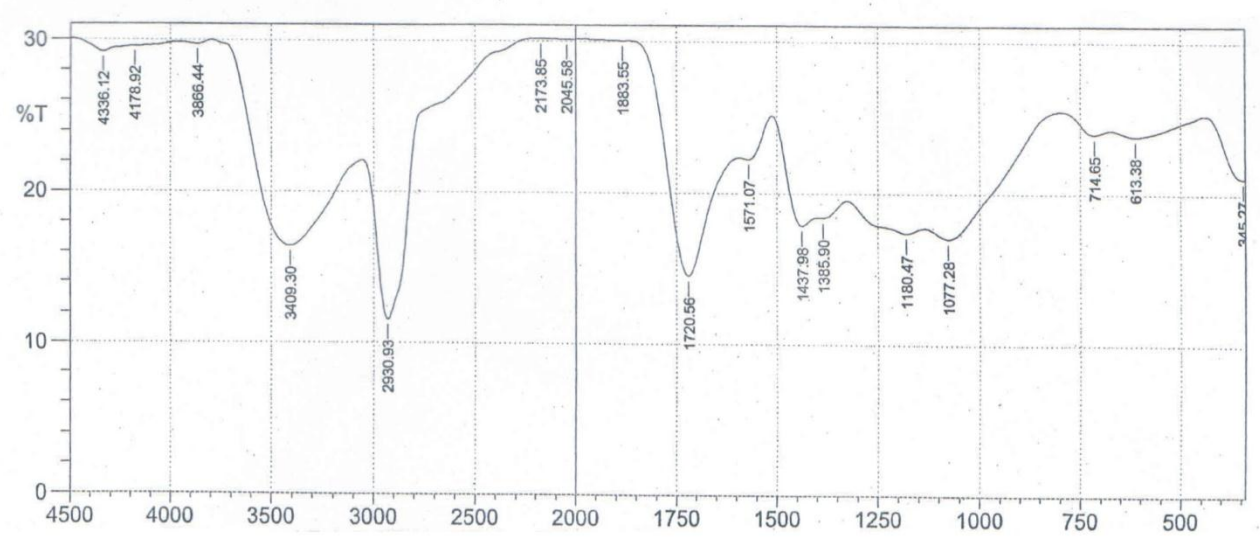


Fig.4.3: **Infra Red (IR) Spectrum of FA2**

The Infra Red (IR) spectrum of FA2 obtained from neutral metabolite of *Abelmoschus esculentus* (okra) leaf showed a broad peak at 3409cm^{-l}, which suggested the presence of hydrogen bonded –OH group of alcohol or phenol andasharp peak at 2930cm^{-l}, which suggested the presence of –CH group of aliphatic hydrocarbon. The strong sharp peak at 1720cm^{-1} suggested the presence of –C=O group of ketone or H-bonded –C=O of ester or lactone. The weak peak at 1571cm^{-1}could be due to the presence of –C=C–double bond while the twin peaks at 1437cm^{-1} and 1385cm^{-1} confirmed the presence of –CH_3 and –CH_2- groups of aliphatic hydrocarbon.

The appearance of the weak peak at 1180 cm^{-1} showed the presence of C–O–C group of ether or ester while the peak at 1077cm^{-1} confirmed presence of –OH of alcohol (probably used as solvent).Since no peaks appeared in the region of 2850–2750cm^{-1} or 1680–1700cm^{-1}, the –CHO group of aldehyde was excluded.

4.7.2 GC/MS Result of Fraction FA2 of Neutral Metabolite of Okra leaf

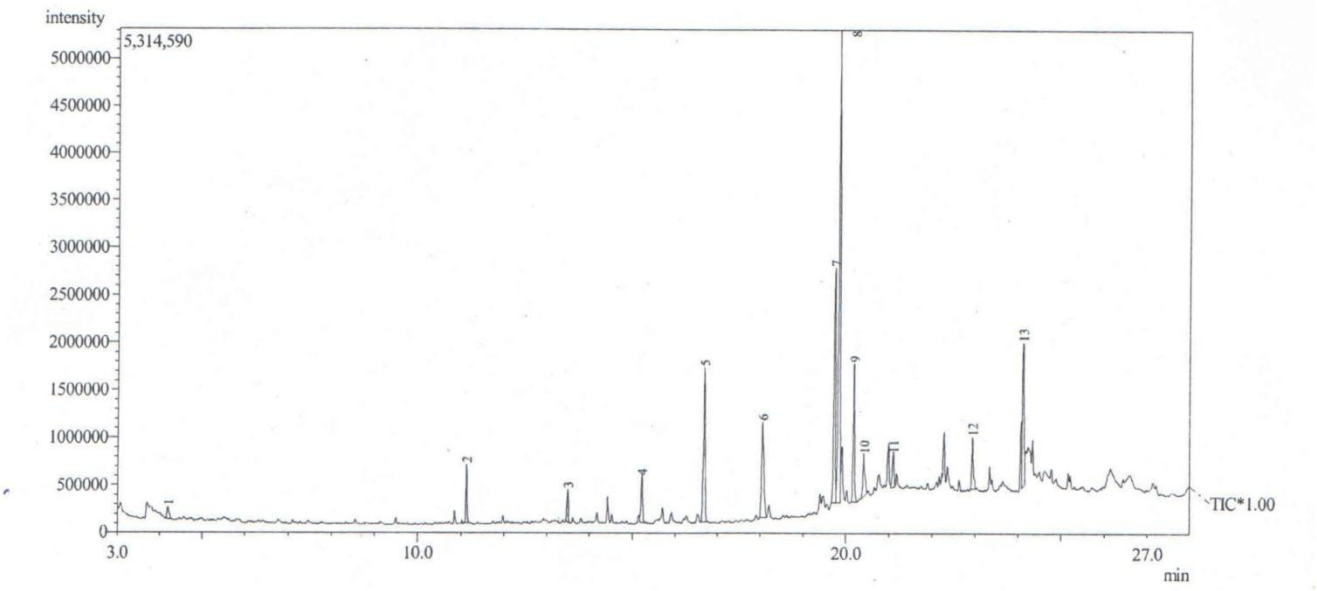


Fig.4.4: Gas Chromatogram (GC)Spectrum of Fraction FA2

The GC spectrums of Fraction FA2 from neutral metabolite of *Abelmoschus esculentus* (okra) leaf were shown in Figure 4.4. The spectrum showed one major peak at Line #8 with intensity of 5,314,590 and elution time of 20minand other minor peaks which suggested the presence of impurities.

This major peakat Line 8 was considered to be characteristic of a particular compound andthe Mass Spectrum (MS) of the compound were shown in Figure 4.5.

4.7.3:Mass Spectrum of Line #8 Component of FA2 of *Abelmoschusesculentus*

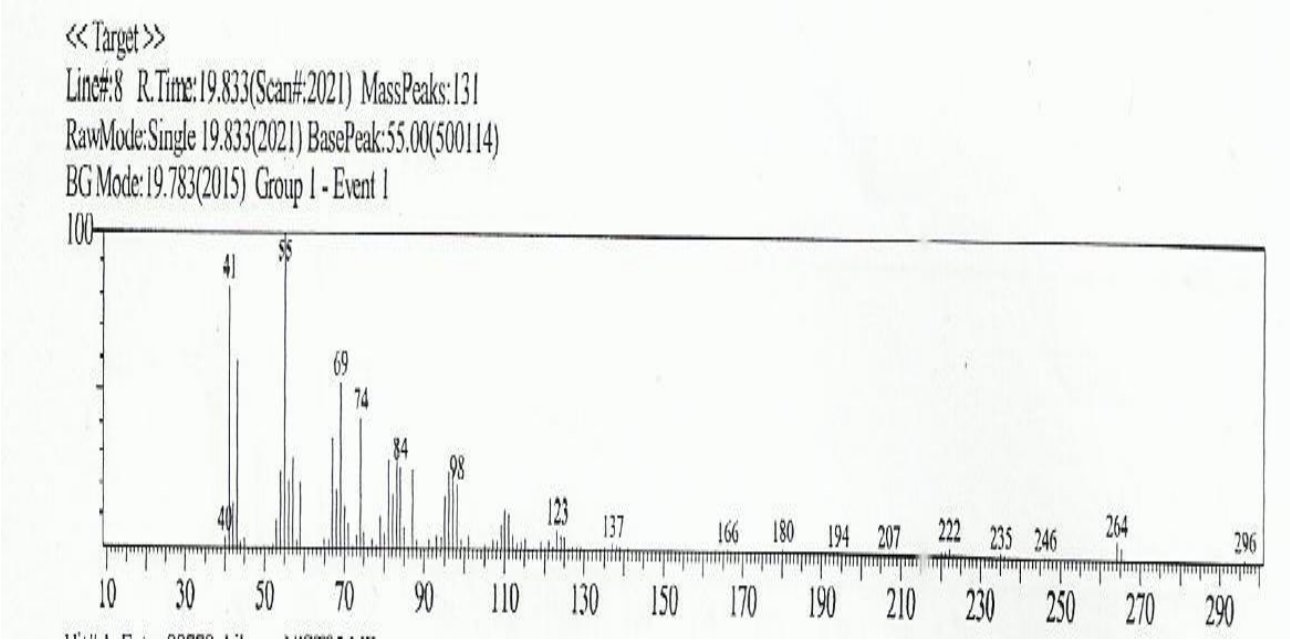


Fig. 4.5: **Mass Spectrum of Line #8 Component of FA2**

The mass spectrum of line #8 component of purified Fraction FA2 obtained from neutral metabolite of *Abelmoschusesculentus* (Okra)leaf showed the Molecular ion peak at m/e = 296, which is the peak of highest mass number corresponding to the molecular weight of the compound. In additionthe MS also showed theBase peak of m/e = 55, which is the peak assigned to the

ion of most abundance = 100%. This base peak at m/e = 55 is characteristic of ketones or esters.

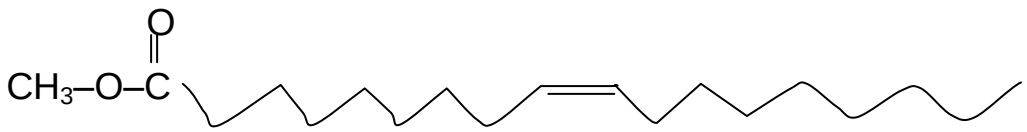
Since mass spectrum was a presentation of the masses of positively charged fragments (including the molecular ion) versus their relative abundance (concentrations), it could be seen that fragment ions occurred at m/e =41, m/e = 55 and m/e = 69 corresponding to the formula C_nH_{2n-1}; if n=3, formula is $C_3H_5^+$, n = 4,formulais $C_4H_7^+$ and n = 5,formula is $C_5H_9^+$ respectively. The formation of these ions showed loss of 14 mass units corresponding to loss of $–CH_2–$ units,which suggested that major component (Line #8) of the purified Fraction FA2 could be a linear aliphatic compound.

Although the molecular ions (m/e =296) was not clearly seen (probably due to reasons of stability), ions representing loss of the elements of methanol CH_3OH^+(m/e =264, which is $[M – 32]^+$), loss of McLafferty ion (m/e = 222) and the MCLafferty ion (m/e = 74) are obvious. The characteristic ion at m/e=180, which is $[M – 116]^+$, together with ions due to the homologous series(m/e = 166, 137 etc) were also diagnostic.

The first of these ions was formed by loss of a fragment containing the carboxyl group by cleavage between carbon 5 and 6 with addition of a rearranged hydrogen atom.

This compound could most likely be methyl oleate (cis-9-octadecenoate), whose chemical formula is $C_{19}H_{36}O_2$ (Hallgren *et al.* 1959).

The structure of the compound could be:



Methyoleate (cis-9-Octadecenoate)

4.8: Characterization and Structural Elucidation of Fraction FC3

4.8.1:Infra Red (IR) Spectrum of Fraction FC3

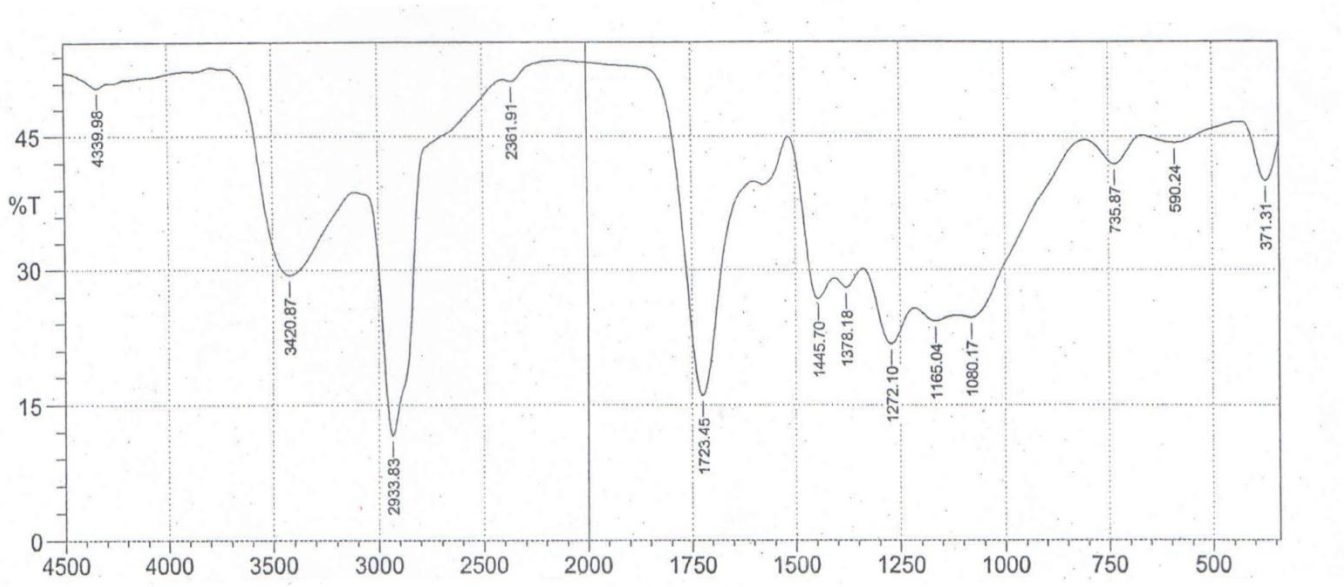


Fig.4.6: **Infra Red (IR) Spectrum of Fraction FC3**

The infrared (IR) spectrum of FC3 from neutral metabolite of *Carica papaya* (paw-paw) leaf extract showed a broad peak at 3420cm^{-1}which suggested presence of hydrogen bonded –OH group of alcohol or phenol. Thestrong sharp peak at 2933cm^{-l}suggested presence of –CH group of aliphatic

hydrocarbon. The strong peak at 1723cm^{-1} suggested the presence –C=O group of ketone or H-bonded –C=O of ester or lactone. The unlabeled peak at 1600cm^{-1} suggested the presence of –C=C–double bond. The twin peaks at 1445 and 1378cm^{-1} confirmed the presence of $–CH_3$ and $–CH_2$- groups of aliphatic hydrocarbon. The peak at 1272cm^{-1} confirmed presence of –C–Ogroup of ester. The peak at1165cm^{-1} showed the presence of C–O–C group of ether or ester. The peak at 1077cm^{-1} confirmed presence of –C-O- of alcohol (probably used as solvent).

4.8.2: Gas Chromatographic Spectrum of Fraction FC3

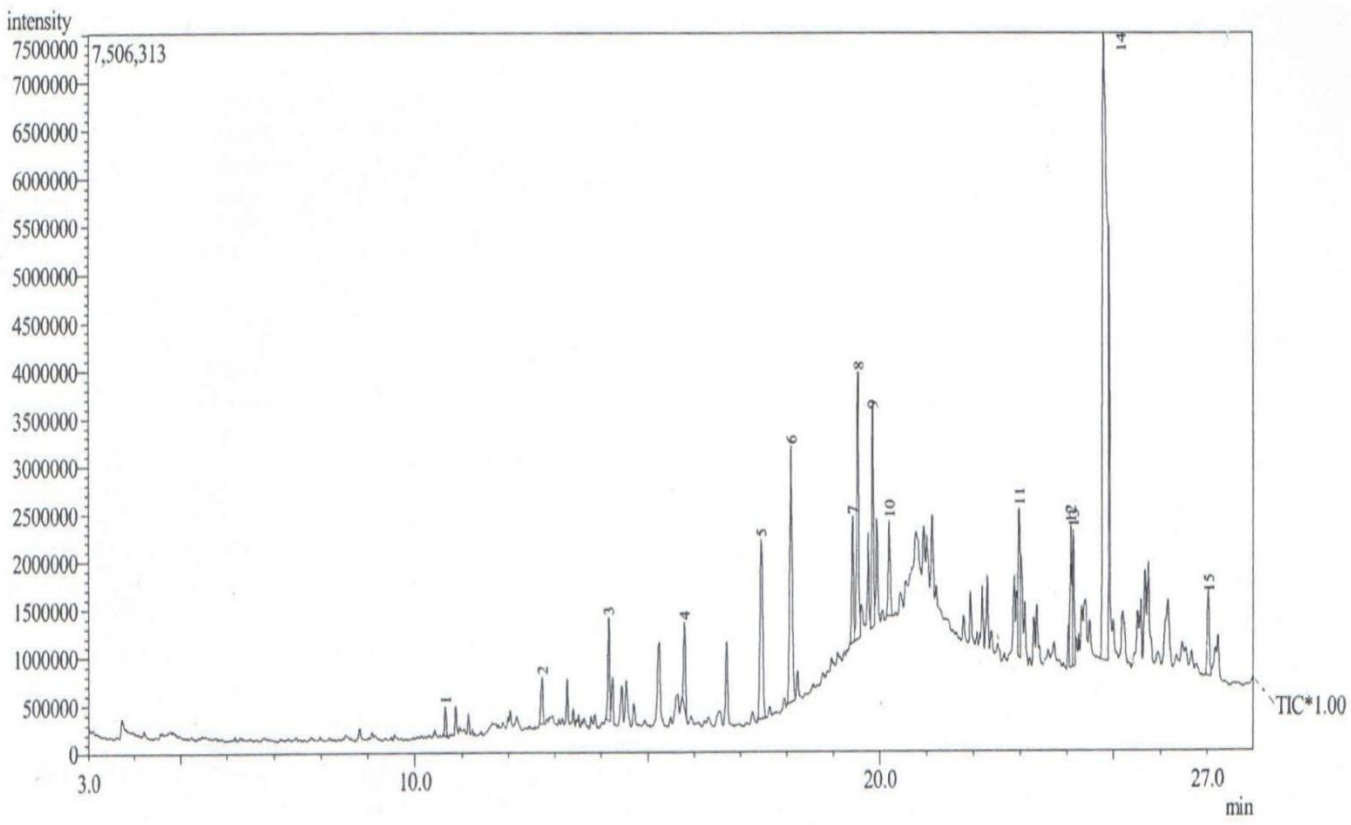


Fig. 4.7: Gas Chromatogram (GC) of Fraction FC3

The GCspectrum of Fraction FC3was presented in Figure 10 and showed one major peak at Line #14 with intensity of 7,506,313 and retention time of 25min plus other minor peaks (probably impurities). The major peak at Line #14 was considered to be characteristic of a particular compound whose Mass Spectrum was shown in Figure 4.8.

4.8.3:Mass Spectrum of Line #14 Component of FC3

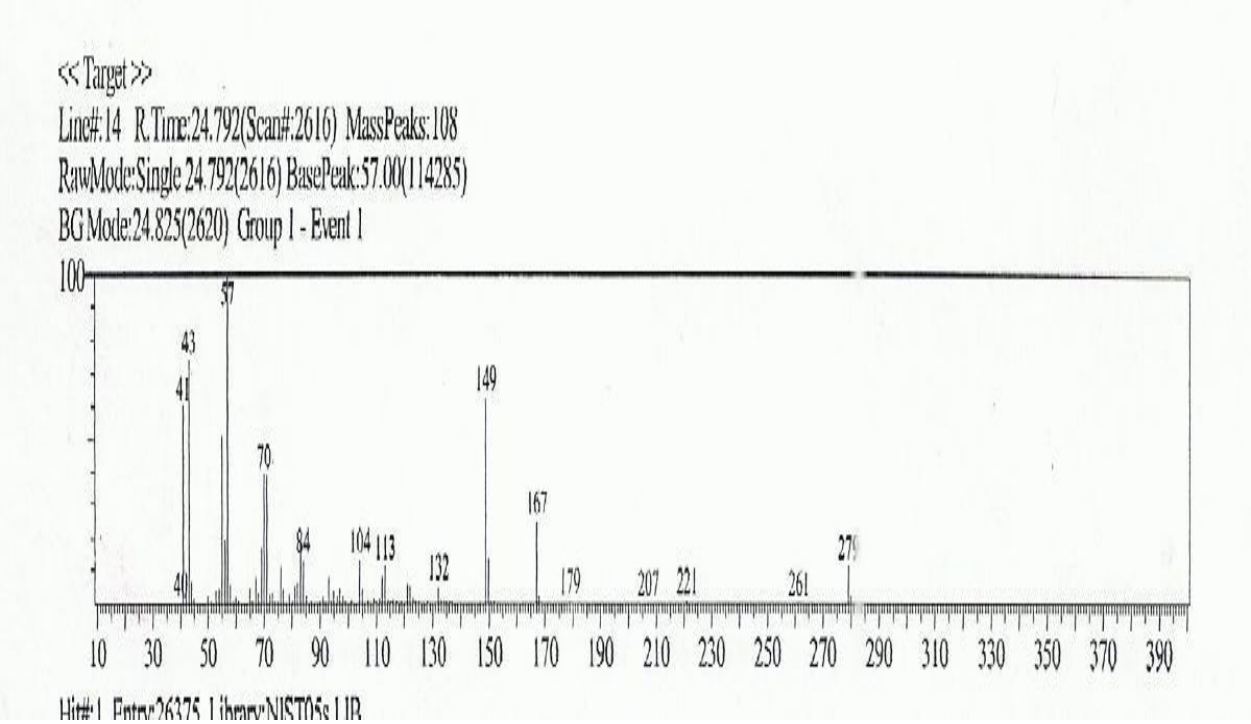


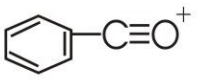
Fig. 4.8: Mass Spectrum of Line #14 Component of FC3

The mass spectrum (MS) of the major peak in fraction FC3 of neutral metabolite of *Carica papaya* leaf extract showed Molecular ion peak at m/e = 390 which represents molecular weight of the compound. In addition, the MS showed Base peak at m/e = 57 (which is peak of the most abundant ion assigned value of 100%). The Base peak could be due to fragment ion $CH_3CH_2C{\equiv}O^+$ (m/e = 57), which is characteristic of aliphatic ester or cyclicsix-member alcohol (cyclohexanol). The MS also showed a peak

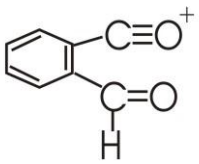
atm/e = 41 due to fragment ionCH_2=$CHCH_2^+$, which is characteristic of olefins. Similarly the peakat m/e = 43,probably due to fragment ion $CH_3C{\equiv}O^+$ (acetoxyl group) is characteristic of esteror ketone and while the peak at m/e = 70 suggested loss offragment ion $C_5H_{10}^+$.

Thus, we see the presence of an aliphatic chain containing at least six carbon atoms in a straight chain:CH_3CH=$CHCH_2CH_2C$=O.

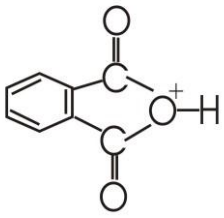
The peak atm/e = 104could be due to the fragmention $C_6H_5CO^+$



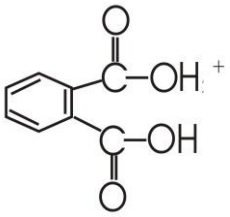
while that at m/e = 132 could be due to the fragment ion $C_8H_4O_2^+$



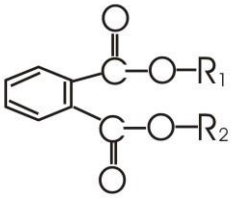
The peak at m/e = 149suggested the fragment ion $C_8H_5O_3^+$:



The peak at m/e = 167 suggested the presence of fragment $C_8H_6O_4^+$

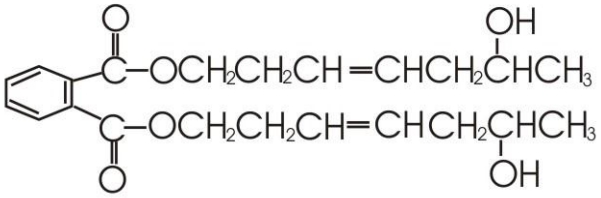


In the high mass region the peak at m/e = 279 represented loss of an alkyl group, which could be represented as$[M - 111]^+$. Thus the molecular weight of the compound could be represented as:



where R1 = R2 = $CH_2CH_2CH{=}CHCH_2CH(OH)CH_3$

These discussions suggested a compound of chemical formula of $C_{22}H_{30}O_6$ with a structure as shown below:



Bis (hept-4-en-2-oyl) phythalate

Sathish et al. (2013) in their study on Abelmoschusesculentus (okra) concluded that the fiber possessed excellent quantity of cellulose. Hence it could be used as cellulosic raw materials in cellulose based industries. It

also contains low percentage of lignin, which was responsible for its yellowing and photochemical degradation and exhibited some properties like colour fastness, tensile strength, anti-oxidant and memory enhancement activities. Thus, the extract can be used as a good medicine for alzhemeirs disease and also as chemical preservative.

The inhibition of growth and activity of microorganisms is one of the major purposes for the use of chemical preservatives in the food industry because these are able to inhibit microbial growth by interfering with cell membranes, enzyme activity or genetic mechanisms of microorganisms. Chemical preservatives may also be used as coating to keep out microorganisms, prevent loss of water and hinder undesirable microbial, enzymatic and chemical reactions (Fulton, (1981); Branen&Dandson,(1985)). Plants have an almost limitless ability to synthesize aromatic substances most of which are phenols or their oxygen-substituted derivatives (Hasham, 1996). Most are secondary metabolites of which at least twelve thousands have been isolated, a number estimated to be less than 10% of their total evaluation of new plant derived compounds.

The bactericidal effects of plant extracts and metabolites have been reported and several attempts made to destroy bacteria and their spores by the application of these substances (Jussi-Pekka *et al*. (2000); Okwu,

(2005); Kotzekidou *et al.* (2008); Ejele, (2010); Ugbogu, *et al.*(2010); Ejele & Akujobi, (2011); Ejele & Ogukwe, (2013).

CHAPTER FIVE

CONCLUSION AND RECOMMENDATIONS

5.1: CONCLUSION

This study was aimed at the isolation,purificationand characterization of the bioactive compound present in the neutral metabolites of *Abelmoschusesculentus* (Okro) and *Carica papaya* (Paw paw) leaves.

The study has adopted both chemical analysis and biological assay to achieve this objective. Samples of *Abelmoschusesculentus* and *Carica papaya* leaves were dried at room temperature,pulverized and separately extracted with 250ml of ethanol using the Soxhlet extractor. Each crude extract was analyzed for its photochemical composition and the results showed that both extractscontained alkaloids, tannins, flavonoids, saponins, carboxlic acids, phenols and steroids, although the paw-paw leaf extract lacked flavonoids.

The crude extracts were thenfractionated into acidic, basic and neutral metabolites using a modification of the method earlier described by Ejele&Alinnor,(2010).

Antimicrobial analysis carried out on crude extract, acidic, basic and neutral metabolites clearly indicated that both plants possessed pharmacological properties which varied with nature of the secondary metabolites. The neutral metabolite was found to be the most active of all and was purified by

column chromatography using silica gel packed in a chromatographic column. The different fractions obtained from *Abelmoschusesculentus* (Okro) leaf extract were labeled FA1, FA2, FA3 and FA4 whereas the fractions from *Carica papaya* (Paw-paw) leaf were labeled FC1, FC2, FC3 and FC4.

A second antimicrobial evaluation was carried out on the purified fractions and the fractionswhich possessed the best antimicrobial potential (Fraction FA2 from neutral metabolite of Okro leaf and FC3 from neutral metabolite of paw-paw leaf) were selected for spectroscopic identification and structural elucidation using IR and GC/MS spectroscopic methods of analysis.Data analysis showed that the bioactive compound found in neutral metabolite of Okro leaf was Methyl oleate (cis-9-octadecenonte) while that found in neutral metabolite of pawpaw leaf was Heptenoyl phythalate

5.2: RECOMMENDATIONS

The column chromatographic method of separation used in this study was slow and sluggish, hence it is recommended that the High Performance Liquid Chromatography (HPLC) should in future be used for the separation since it saves time and yields better and more sensitive and selective results. Efforts should be made to synthesis the compounds isolated from okra and pawpaw. Furthermore, studies should be carried out on other metabolites such as acidic metabolite and basic metabolite.

5.3: CONTRIBUTION TO KNOWLEDGE

a) This study represents the first attempt made to isolate and characterize the bioactive compounds responsible for antimicrobial potential of okro and paw-paw leaves.

b) Bis (heptenoyl phthalate)has not been isolated from paw-paw leaf before now, although its presence in the plant had earlier been reported by Pflegere*t al.* (1985).

c) Similarly, Methyl oleate (cis-9-octadecenonte) has not been isolated from Okra leaf before now.

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