PHYTOCHEMICAL AND ANTIBACTERIAL INVESTIGATIONS OF THE FRUITS OF *Nauclea latifolia* Smith (FAMILY: RUBIACEAE)

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By

LABAKE AJOKE FADIPE

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AHMADU BELLO UNIVERSITY, ZARIA
NIGERIA.

FEBRUARY 2016

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Ph.D/Pharm Sci/9006/2009-2010

A DISSERTATION SUBMITTED TO THE SCHOOL OF POSTGRADUATE STUDIES,

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DECLARATION

I declare that the work reported in this dissertationentitled **Phytochemical and antibacterial investigations of the fruits of *Nauclea latifolia* Smith (Rubiaceae)** has been carried out by me in the Department of Pharmaceutical and Medicinal Chemistry. The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this dissertation was previously presented for another degree or diploma at this or any other Institution.

Labake Ajoke FADIPE _____ _____

Name of Student Signature Date

CERTIFICATION

This dissertation entitled PHYTOCHEMICAL AND ANTIBACTERIAL INVESTIGATIONS OF THE FRUITS OF *Nauclea latifolia* Smith (RUBIACEAE) by Labake Ajoke FADIPE meets the regulations governing the award of the degree of Doctor of Philosophy in Pharmaceutical and Medicinal Chemistry of the Ahmadu Bello University, and is approved for its contribution to knowledge and literary presentation.

Prof. A. K. Haruna (Signature)_____

Chairman, Supervisory Committee Date

Prof. M. Ilyas (Signature)_____

Member, Supervisory Committee Date

Prof. U. U. Pateh (Signature)_____

Member, Supervisory Committee Date

Dr. A. M. Musa (Signature)_____

Head of Department Date_____

Prof. Kabir Bala (Signature)_____

Dean, School of Postgraduate Studies Date_____

DEDICATION

This piece of work is dedicated to my love, AYOMIDE JEMIMA. Thanks for making all the difference.

ACKNOWLEDGMENT

I wish to express my sincere and unreserved gratitude to my supervisors- Professor Haruna Kaita, Professor Mohammad Ilyas and Professor Usman Umar Pateh, for their encouragement, support and tireless optimism that kept me going in the course of this work. Thank you sirs for your faith in me! My deep appreciation and gratitude goes to all academic, technical and non-teaching staff of the department of Pharmaceutical Chemistry, you all motivated me and kept encouraging me even when I was almost giving up the programme. Thank you for believing in me.

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This manuscript would have been an impossible task without the platform provided by Prof. Ermias Dagne of the African Laboratory for Natural Products (ALNAP), Department of Chemistry, Addis Ababa University, Ethiopia. My tireless, Prof, I am most grateful. I also want to thank my fellow researchers in ALNAP: Hanna, Mesfin, Minbale, Yadessa, Haftu, Tsegun and Ta'ame. Thanks for always helping out. To my other Ethiopian friends: Mrs Dagne (Ariti herbal), Engr Adey and Aberash… You all made my stay worth the while.

To my dependable brothers, sisters and friends, Dr. Essang Uno, Pastor Abiodun Adeleke, Bro Olu Adedire, Iyabo Phillips, Joy Bakut, Deborah Godwin, Funke and Tayo Aborisade, Ayomide Idowu and Nike Odeyemi … I owe a load of gratitude.

Most of all, to the Author and the Finisher of my faith, I am most indebted! Your grace and mercy, has been and will always be my strength! Thank you JESUS.

ABSTRACT

Ripe and unripe fruits of *Nauclea latifolia* Smith (Family Rubiaceae) used traditionally in the treatment of dysentery, diarrhoea and other bacterial infections were investigated for their phytochemical content and antibacterial efficacy. Extraction, partitioning and further re-extraction of the air-dried ripe (r) and unripe (u) fruits gave rise to extracts (coded rP, uP, rM, uM), partitioned-soluble fractions (coded C, E, B, A) and soluble fractions (coded rD, uD, uC, rC, uE, rE, rA, uA, rR, uR). Phytochemical screening of extracts and fractions using standard methods revealed the presence of alkaloids, flavonoids, steroidal nucleus, saponins, coumarins and tannins. Purification of the ethyl acetate partitioned-soluble fraction of the methanol extract of the ripe fruits (E), its column fraction (E-2) and column sub-fraction (E-2f) led to the isolation of a benzaldehyde derivative, identified as 2- (2'-ethyl-3'-tertbutoxypropyl) benzaldehyde (coded E-2f1a). Similar purification of the acetone soluble-fraction of the ripe fruits (rA), its column fraction (rA-5) and sub-fraction (rA-5a) afforded a phthalate derivative, identified as di- (ethylhexyl) phthalate, (DEHP), coded rA-5a1. Chromatographic separations of the ethyl acetate-soluble fraction of the methanol extract of the unripe fruits (uE), its column fractions (uE-1 to uE-5) and column sub-fractions (uE-2 and uE-3) led to the isolation of (i) same phthalate as was isolated from the ripe fruits, also identified as DEHP, coded uE-2a1 (but of lesser quantity than in the ripe fruits), (ii) an unsaturated fatty acid ester derivative, identified as ethenyl pentadecanoate, coded uE-2a2, and (iii) a phytosterol, identified as β-sitosterol, coded uE-3a2a. Characterization of all compounds was based on the use of physical, colour reactions, spectroscopic parameters (IR, UV, ^{1}H-NMR,^{13}C-NMR, DEPT-135 and GC-MS) and literature search.

Antibacterial assay of the extracts (100 mg/ml) and soluble fractions (50 mg/ml) in comparison with chloramphenicol, erythromycin and tetracycline (1 mg/ml each) against two Gram-positive (*Bacillus subtilis* and *Staphylococcus aureus*) and four Gram-negative bacteria (*Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Salmonella typhi*) was carried out using the agar-well diffusion method. The diameter of zones of inhibition (active, if ≥ 14 mm), calculated percent activities, calculated bacterial susceptibility index, minimum inhibitory concentrations, and minimum bactericidal concentrations for the soluble fractions of the unripe fruits ranged from 12.3 – 21.2 mm, 50 - 100 %, 75 – 100 %, 6.25 – 25 mg/ml and 12.5 – 50 mg/ml respectively, while, it ranged from 7.33 – 21.2 mm, 0 – 83.3 %, 0 – 50 %, 6.25 – 25 mg/ml, 12.5 – 50 mg/ml; 6.33 – 17.6 mm, 0 – 66.7 %, 0 – 50 %, 12.5 – 50 mg/ml and 12.5 – 50 mg/ml for the soluble fractions and partitioned-soluble fractions of the ripe fruits respectively. The values for the crude extracts of both fruits also ranged from 6.67 - 15.3 mm, 0 - 50 %, 0 - 25 %, 12.5 - 25 mg/ml and 100 mg/ml. All values obtained in comparison with those of the standard whose values ranged from 9.60 – 26.2 mm, 83.3 – 100 %, 33.3 – 100 %, 0.20 – 0.50 mg/ml, 0.20 – 0.50 mg/ml respectively, indicates that the soluble fractions of the unripe fruits displayed higher activity than the soluble- and partitioned-soluble fractions of the ripe fruits and crude extracts of both ripe and unripe fruits. The diethyl ether- (uD) and ethyl acetate- (uE) soluble fractions of the unripe fruits displayed higher broad-spectrum activity better than that exhibited by some of the standard drugs against some of the test organisms.

The column fractions (rA-1 to rA-8; 6.21 – 19.0 mm) and column sub-fractions (rA-5a to rA-5c; 5.22 – 18.5 mm) of the acetone-soluble fraction of the ripe fruits (rA) at 20 mg/ml each, showed zones of inhibition (mm) that was higher than rA (7.0 - 10.1 mm)

at 50 mg/ml. Also, the column fractions (E-1 to E-6; 6.80 – 18.8 mm) and sub-fractions (E-2a to E-2h; 5.70 – 18.3 mm) of the ethyl acetate partitioned-soluble fraction of the ripe fruits (E) also at 20 mg/ml showed higher zones of inhibition (mm) than E (8.25 – 17.6 mm) at 50 mg/ml, while the column fractions (uE-1 to uE-5; 8.50 – 14.8 mm) and column sub-fractions (uE-2a; 6.6 -13.8 mm) of the ethyl acetate soluble fraction of the unripe fruits (uE), also at 20 mg/ml showed lower antibacterial activity than uE (17.3 – 21.5 mm) at 50 mg/ml. An activity that was of broad-spectrum when compared with that displayed by erythromycin (9.60 - 26.2 mm) at 1 mg/ml

DEHP (100 µg/ml) exhibited similar antibacterial activity against *B. subtilis* (17.0 mm) in comparison with erythromycin (15.1 mm) at 1 mg/ml, while it was less active against *S. aureus* (15.3 mm; erythromycin, 22.2 mm). β-sitosterol at 100 µg/ml was not too active (10.4 - 13.5 mm) against all tested strains (erythromycin, 9.60 - 26.2 mm) but was only moderately active against *E. coli* (15.1 mm; erythromycin, 22.6 mm).

The antibacterial studies of the ripe and unripe fruits of *N. latifolia* validate the ethnomedicinal uses of the fruits of the plant.

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Abbreviations and Symbols

^{o}C:	Degree Celsius
$CDCl_3$:	Deuteriated chloroform
n- Hex:	normal Hexane
PE:	Petroleum ether
Et_2O :	Diethyl ether
EtOAc:	Ethyl acetate
Me_2CO:	Acetone
MeOH:	Methanol
n- BuOH:	normal Butanol
EtOH:	Ethanol
UV:	Ultraviolet
^{1}H-NMR:	Proton Nuclear Magnetic Resonance
^{13}C-NMR:	Carbon-13 Nuclear Magnetic Resonance
DEPT-135:	Distortionless Enhancement Polarized Transfer at 135^{0}
GC-MS:	Gas chromatography coupled with mass spectrometry
s:	Singlet
d:	Doublet
t:	Triplet
q:	Quartet
p:	Pentet
m:	Multiplet
dd:	Doublet of doublets
δ:	Chemical shift
m/z:	Mass to charge ratio

mg: Milligram

µg: Microgram

ml: Millilitre

ppm: Parts per million

TLC: Thin layer chromatography

PTLC: Preparative thin layer chromatography

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List of Plate

Abbreviations and Symbols

°C: Degree Celsius

$CDCl_3$: Deuteriated chloroform

n- Hex: normal Hexane

PE: Petroleum ether

Et_2O : Diethyl ether

EtOAc: Ethyl acetate

Me_2CO: Acetone

MeOH: Methanol

n- BuOH: normal Butanol

EtOH: Ethanol

UV: Ultraviolet

^{1}H NMR: Proton Nuclear Magnetic Resonance

^{13}C NMR: Carbon-13 Nuclear Magnetic Resonance

DEPT-135: Distortionless Enhancement Polarized Transfer at 135^{0}

GC-MS: Gas chromatography coupled with mass spectrometry

s: Singlet

d: Doublet

t: Triplet

q: Quartet

p: Pentet

m: Multiplet

dd: Doublet of doublets

δ: Chemical shift

m/z: Mass to charge ratio

mg: Milligram

µg: Microgram

ml: Millilitre

ppm: Parts per million

TLC: Thin layer chromatography

PTLC: Preparative thin layer chromatography

CHAPTER ONE

1.0 INTRODUCTION

1.1 Traditional/Herbal Medicine

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The use of medicinal plants in most societies of the world is both old and prevalent. Over 60 % of the world's population rely wholly or partly on plants as their primary source of medication in the treatment of parasitic diseases, diarrhoea, fever, colds etc. (Sofowora, 1993a; Stary, 1998; Cordell, 2000) while over 80 % of people living in developing countries rely entirely on plant medicines as an important component of primary health care (Farnsworth *et al*., 1985). Nearly all cultures and civilizations from ancient times to the present day have used herbal medicines to cure infections (Erdemeier *et al.,* 1996; –Lino and Deogracious, 2006). Plants have been the basic source of sophisticated traditional medicine system for thousands of years and were instrumental to early pharmaceutical drug discovery and industry (Elujoba *et al*., 2005). Traditional medicine and herbal remedies (an integral of traditional medicine) are popular and gaining high patronage even in the western world. In countries with poor economy, where infectious diseases are very rife coupled with poverty, there is a strong resurgence in the use of herbal preparations to treat diseases (Harmmer-Beem *et al.,* 2006).

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The importance of traditional medicine as a source of primary health was first officially recognized by the World Health Organization (W.H.O) in the Primary Health Care Declaration of Alma Ata (1978), and has defined traditional medicine as the "sum total

of all the knowledge and practices whether explicable or not, used in diagnosis, prevention and elimination of physical, mental or social imbalance and relying exclusively on practical experience and observation handed down from generation to generation whether verbally or in writing" (WHO, 2000). The body described traditional healing system as one of the surest means to achieve total health care coverage of the world's population and encourages and support the inclusion of traditional/herbal remedies of proven safety and efficacy in the national health care programmes of countries of the world. Although relegated for a long time to a marginal place in the health sector of most countries, traditional systems of health care has undergone a major revival in the past 20 years (WHO, 2003).

Undoubtedly, medicinal plants are of great importance because they provide drugs that help widen the therapeutic arsenal (Jaramillo, 1989; Hamburger and Hostettmann, 1991; Cox and Balick, 1994; Lewis and Elvin-Lewis, 1995; Clark, 1996; Agosta, 1997; Cordell, 2000; Strohl, 2000). They equally help bridge the gap between the availability and the demand for modern medicines. There is a growing interest by consumers in the utilization of phytoceuticals in lieu of synthetic compounds due to their availability, effectiveness, reduced toxicity, minimal side effects in clinical experience, relatively low cost (Marin-Bettolo, 1980; Momin, 1987; Namiki, 1990; Valiathan, 1998; Boullata and Nace, 2000; Bandaranayake, 2006) and coupled with the fact that global diseases including cancer, malaria, tuberculosis and certain fungal, viral and bacterial infections are showing significant patterns of resistance to the conventional prescription drugs (Henry, 2000).

1.1.1 Growing demand of herbal/traditional medicine

Ethnomedicine has played an important role in Africa and western societies (Falodun *et al*., 2007). The use of herbal products, phytonutrients, supplements and nutraceuticals has increased greatly over the past decades with many people now resorting to these products for the treatment of various diseases and ailments in different national healthcare settings (WHO, 2004). The therapeutic use of herbs and phytomedicines become so popular, that by 1988, about 5.4 million prescription were written for a single phytomedicine, *Ginkgo biloba* extract; for the treatment of asthma, stress and cerebal insufficiency (Tyler, 1996). In Nigeria, especially at the primary healthcare level, traditional medicine is recognized as an intergral component of health care delivery system (FMOH, 2004). Majority of hypertensive patients receiving conventional treatment at a tertiary health facility in Lagos, Nigeria, also make use of traditional remedies (Amira and Okubadejo, 2007). It was reported that in under-developed countries like Nigeria, Ghana, Mali and Zambia, herbal remedy is the first line of treatment for children with high fever (WHO, 2002b). About 70 % of Ghananians (Robberts, 2001) and majority of the black population of South Africans (Lekotjolo, 2009) depend primarily on traditional medicine.

Over the past decade, over 40 % of healthcare delivered in China were of herbal remedies, while in Australia, Canada, U.S.A, Belgium France, UK, Chile and Colombia, it is estimated that 48 %, 70 %, 42 %, 38 %, 72 %, 40 %, 71 % and 40 % of the population of these countries respectively has used herbal remedies at least once (Foster *et al*., 2000; WHO, 2000; WHO, 2002a; Amzat and Abdullahi, 2008). The number of American adults who used alternative therapies increased from 60 million in 1990 to 83 million in 1997 (Blumenthal *et al.,* 1999) while, by 1998, the population increased by

70 % with three-fifths of consumers taking two or more products on daily basis (Eisenberg *et al.,* 1998).

The economic importance of traditional medicine in the global market has increased significantly (WHO, 2000; Cordell *et al.,* 2001; Ali, 2011). An average of 25 % of prescription drugs sold in the U.S.A during the period 1959-1980 contained active principles extracted from higher plants (Farnsworth *et al.*, 1985). Of the 520 drugs in various classes which were approved by the United States Food and Drugs Administration (USFDA) from 1983 to 1994, 30 were natural products and 173 were either semi- synthetic based on a natural product core, or modelled on a natural pharmacophore (Cragg *et al.*, 1997; Wachtel-Galor and Benzie, 2011). From the period, January-September, 1993, 57 % of the top 150 brand name products prescribed in the U.S.A, contained at least one major active compound now or once derived from plants (Grifo *et al.,* 1997).

It was reported that of the 20 best-selling non-protein drugs, 9 were natural products or products derived from them with combined annual sales of up to 16 million US dollars. There are well over 100 natural products derived pharmaceuticals used in medicine (Verpoorte, 1998; Harvey, 2000). In the U.S.A., there are well over 500 herbs marketed as bulk plants or portions of plants, or as teas powders, liquid, extracts, tablets and capsules (Boullata and Nace, 2000). De Smet (1999) reported that of the 25 drugs with the largest sales in Germany in 1996, 47.9 % of the total sales were for products with a natural origin. There are well over 700 different plant drugs sold singly and in combination in Germany. It was observed that the herb sector of the dietary supplements

market represents one of the biggest financial investment opportunities since the advent of the high-technology industry (Wachtel-Galor and Benzie, 2011).

The global use and booming sales of herbal remedies continue to grow even in developed countries because, among many reasons, they strongly believe it will promote healthy living and are often viewed as a balanced and moderate approach to healing (WHO, 2002a; Kong *et al*., 2003; Bandaranayake, 2006; Ekor, 2013).

1.1.2 Challenges and prospects of herbal/traditional medicine

Despite its existence and expansive use over many centuries, traditional medicine including herbal medicines, in most African countries has not been officially recognised and the regulation of herbal medicines has not been well established, although, in most African countries, more than 80 % of the population rely on traditional medicine for their primary health care needs (Rukangira, 2001a). Research into the chemistry and bioactive components of medicinal plants has not received due support, attention and public awareness so that traditional healing systems continue in their undeveloped form to provide majority of the population their basic health needs especially in areas where it is the only system available (Anokbonggo, 1992).

Challenges in the development as well as implementation of the regulation of herbal/traditional remedies in most countries, especially, Africa, has to do with regulatory status, assessment of safety and efficacy, quality control, safety monitoring/pharmacovigilance and adequate information about traditional and herbal remedies (WHO, 2005). Presently, there are few crude plant formulations that exist in some countries of the world. In such countries, such as African countries, there is the absence or weak regulation of herbal medicines, so that there is the need to monitor

safety (Rodrigues and Barnes, 2013), while in most of the developed countries, all crude plants extract formulations available are chemically standardized and follow norms recorded in national pharmacopoeias (Freiburghaus *et al.*, 1996). In the United States, for example, natural products are regulated under the Dietary Supplement Health Education Act (USFDA, 2012).

Quality assessment of herbal remedies has become a concern to consumers, regulatory authorities and healthcare professionals (Ekor, 2013). Assessment of safety and efficacy of herbal remedies will entail having to isolate each phytoconstituent(s) present in each medicinal plant that makes up the herbal medicine. This will be very cumbersome and may be practically impossible when an herbal product is a mixture of two or more herbs (WHO, 2005; Ekor, 2013). The quality of source of materials used in the production of these herbal remedies, which includes, plant selection, cultivation and environmental factors makes it difficult to perform quality controls on the raw and finished herbal mixtures (WHO, 2004; 2005). It is hoped that with improved quality, traditional medical system will be integrated into the mainstream of healthcare services, so as to improve accessibility to healthcare (Okigbo and Mmeka, 2006). This can be improved upon by taking inventory and documenting the various medicinal plants and herbs which are used to treat common diseases in each country; setting up a network of laboratories with adequate facilities for the assessment of the efficacy and toxicity of these medicinal plants; establishing dosage norms and; production of the most efficacious of herbal extracts, whether in the tablet, capsule, powder, syrup, liquid or other forms (Rukangira, 2001b).

It is of no doubt that the pharmaceutical potentials of African medicinal plants is immense and if well utilized can help constitute great economic and strategic value for the African continent. Although, these medicinal plants are in abundance, there is lack of proper scientific information on their chemical constituents, efficacies, toxicology and biological activities (Cordell, 1990a, 1990b, 1993, 1995a, b, 2000; Valiathan, 1998; Diallo *et al*., 2001). The continual search for, and the interest in natural plant products for use as medicines has therefore acted as the catalyst for exploring methodologies involved in obtaining the required plant materials and hence probing their constituents. It is also of necessity for researchers to investigate the claims of efficacy or potential therapeutic effects of the extracts of these plants so as to help contribute to the general health and well-being of mankind and equally help in the discovery of new biologically and industrially useful compounds which could be useful against diseases for which suitable cures are not yet available (Olaniyi and Ogunlana, 1989; Cox and Balick, 1994; Moran 1996; Shu, 1998; Cordell *et al*., 2001; Sanchez-Medina *et al*., 2001). Documentation of the medicinal plants of Africa is highly necessary and urgent as over exploitation resulting from increased demand, increased population, habitat destruction, excessive commercialization and indiscriminate harvesting of the plants has led to the loss of some valued species before adequate (or even any) biological/chemical evaluation could take place (Akerele *et al.*, 1991; Nkunya, 1996)

Natural products derived from plants, marine organisms and microorganisms exhibit interesting anti-microbial, anti-viral, anti-inflammatory, anti-infectives, anti-tumor and other biological potentials. These bioactivities make natural products an important source for the discovery of new pharmaceuticals that are highly effective with low toxicity. This search is driven by the development of resistance in infectious

microorganisms to existing drugs and by the menacing presence of naturally resistant organisms. The appearance of life threatening virus-related diseases, such as acquired immune deficiency syndrome (AIDS), ebola virus disease (EVD), severe acute respiratory syndrome (SARS), zika virus disease; the recurrent problems of diseases in persons with organ transplants and the tremendous increase in the incidence of fungal infections in world's population, all underscore our inadequacy to cope with these medical problems and therefore the need for discovery and development of new drugs to combat them (Montefiore *et al.*, 1989; Strobel *et al*., 2004).

More than 60 % of anti-tumor and anti-infective agents that are commercially available, or in the last stages of clinical trials, are of natural product origin (Wachtel-Galor, and Benzie, 2011). Some oustanding discoveries made in the field of medicinal plant research to mention but a few includes:

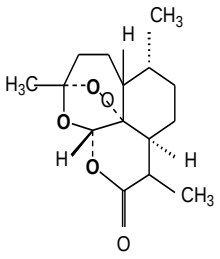
i) Artemisinine (Quinghaosu) (1), a novel active drug for the treatment of cerebral malaria has been derived from *Artemisia annua* (Klayman, 1985).

ii) The alkaloids, Vinblastine (II) and Vincristine (III) isolated from the Madagascar periwinkle *Catharanthus roseus* are now extensively used, most often in combination with other drugs in the treatment of different cancer disease types (Neuss and Neuss, 1990; Portier *et al.*, 1996).

iii) Prostratin (IV), a phorbol compound isolated from *Homalanthus nutans* used by healers for the treatment of yellow fever has been reported to possess potent anti-HIV activity, inhibiting cell killing caused by the virus (Gustafson *et al.*, 1992).

iv) An inactive hypoxoside isolated from *Hypoxis rooperi* was converted to a more lipophilic biologically active aglycone, rooperal (Theron *et al.*, 1994) which has proved useful in the treatment of lung cancer (Smit *et al.*, 1995).

v) Paclitaxel, commonly known as Taxol (V) is a novel drug isolated from *Taxus brevifolia* and was approved by US Food and Drug Administration in 1992 and 1994 respectively, as a treatment for advanced ovarian cancer and metastatic breast cancer (Wall and Wani, 1996).



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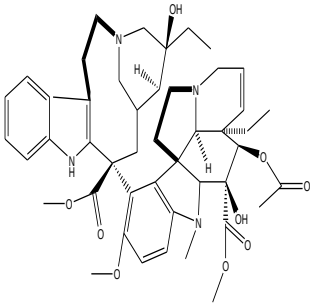

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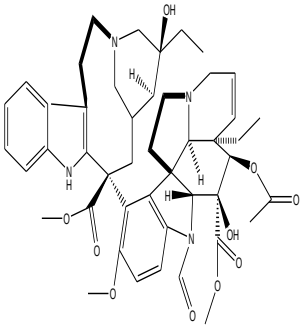

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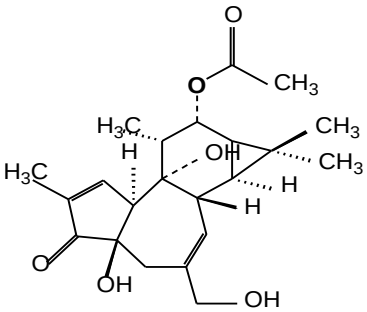

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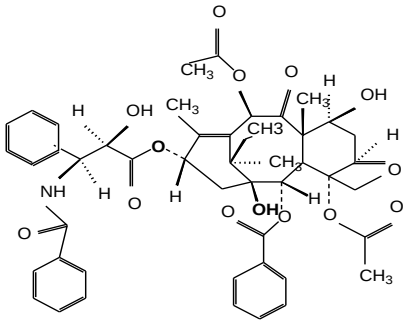

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1.2 Secondary Metabolites in Fruits of Plants

Plants synthesize a variety of metabolites/low molecular weight compounds that are involved in or produced during metabolism. They are majorly classified as primary or

secondary metabolites. Primary metabolites, which are involved in primary metabolic pathways, such as the Krebs cycle and glycolysis are compounds that are present in all plants and have essential roles associated with photosynthesis, respiration, reproduction survival, growth and development. They include, sugars, sterols, nucleic acids, enzymes, amino acids, proteins and fatty acids (Spiegel, 1998; Croteau *et al*., 2000, Agostini-Costa *et al*., 2012; Kabera *et al*., 2014). The secondary metabolites also referred to, as phytochemicals/natural products/plant constituents are structurally diverse and distributed among a limited number of species (especially higher plants) in the plant kingdom and are responsible for the medicinal properties of plants to which they belong (Kabera *et al*., 2014). Many of them are considered as end products of primary metabolism that are not involved in metabolic activity (Irchhaiya *et al*., 2015). Their function in plants, includes, protection of plants from herbivores and microbial infection, attractants for pollinators and seed dispersing animals, as allelopathic agents, UV protectants, signal molecules in formation of nitrogen-fixing root nodules in legumes and pigmentation. They are equally useful as dyes, fibres, glues, waxes, flavouring agents, dietary constituents, drugs and perfumes and are potential sources of new natural drugs, antibiotics, insectides and herbicides (Croteau *et al*., 2000; Dewick, 2002). These compounds, which are an extremely diverse group of natural products are not only synthesized by plants but by fungi, bacteria, algae and animals by specialized cells at particular developmental stages (Agostini-Costa *et al*., 2012). Many of them are known as bioactive substances and have been identified through studies of plants used in, for example, traditional medicine. Many of these bioactives are normally considered undesirable in human food due to their 'toxic' effects. However, a low daily intake of these 'toxins' (anti-nutritional factors) may be an important factor in the search for their beneficial effects on human health (Brandt, 2004).

Based on their biosynthetic pathways, phytochemicals can be divided into three major groups:

(i) Flavonoids and allied phenolic and polyphenolic compounds such as, various classes of flavonoids, tannins, condensed and hydrolysable tannins, lignins, lignans, phenolic acids, coumarins, and stilbenes.

(ii) Terpenoids, such as, monoterpenes, sesquiterpenes, diterpenes, triterpenes, made up of the sterols and saponins, and tetraterpenes, the iridoids, cardiac glycosides and carotenoids.

(iii) Nitrogen-containing alkaloids and sulphur-containing compounds such as, various classes of alkaloids, amines, cyanogenic glycosides, glucosinolates, alkamides and non-protein amino acids.

Other classes include, phenylpropanoids, polyacetylenes, fatty acids, waxes, anthraquinones and other polyketides, carbohydrates and organic acids (Mahmoud and Croteau, 2002; Agostini-Costa *et al*., 2012; Irchhaiya *et al*., 2015). These metabolites are accumulated by plant cells in lesser quantities, than primary metabolites, thus, making their extraction and purification not too easy a task (Irchhaiya *et al*., 2015). A wide range of separation techniques using various solvent systems and spray reagents coupled with spectroscopic approaches to structure elucidation can help separate and identify the various classes (Harbone, 1998; Croteau *et al*., 2000; Agostini-Costa *et al.*,

2012). The study of plants as natural products, is a continuous process, principally for the isolation, characterization, structural elucidation and biological or pharmacological testing of their secondary metabolites. These phytochemicals are usually stored in the storage organs of plants, such as roots, leaves, bark, seeds, skin of fruits (ripe and unripe), fruit rind, flowers, flowering shoots, aerial parts and other parts (Spiegel, 1998; Gordon and David, 2001; Crozier *et al*., 2007).

Fruits, which are one of the oldest forms of food known to man are widely consumed fresh and in commercial products, such as juices, jams and wines. They are made up of seeds, seed coats, pulp and peels, no wonder they are described as 'unusually effective generator of bioactive chemicals' (Levey *et al*., 2007) with their phytochemical composition varying with varieties, season and species. There has been a great demand for various fruit parts or whole fruit in the treatment of various illnesses (Unnisa *et al*., 2012). Production of secondary metabolites is said to be tightly controlled, especially during the ripening process (Lund and Bohlman, 2006). For example, concentration of emodin, an anthraquinone derivative is highest in unripe fruits (Tsahar *et al*., 2002) while, capsaicinoids, an amide derivative, is highest in ripe fruits (Estrada *et al*., 2000). The edible part of most fruits contain considerable amounts of saccharides, polyphenols, proteins, and some vital minerals (Levey *et al.*, 2007; Unnisa *et al*., 2012; Jelodarian *et al*., 2013). The presence of phytochemicals in ripe fruits help to prevent spoilage and act as signals in the form of colour, aroma and flavour for animals who feed on such fruits, thereby helping to disperse the seeds (Pichersky and Gang, 2000). However, unripe fruits does not elicit much of this character (Cipollini, 2000). Many fruits have a high level of tannin content, which is typically encountered in the outer cell layers. These tannins, which are extremely astrigent, render plant tissues inedible, but as the fruits mature and the seeds ripen, there is a decline in the level of tannin and astringency

(Crozier *et al*., 2007). The highest alkaloid concentrations are typically found in ripe fruits, but in some fruits, it is reported that the alkaloidal content changes with time (Koyama *et al*., 2003; Zheng *et al*., 2004).

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1.3 Bacterial Infections and Antibacterial Agents

1.3.1 Pathogenic bacteria

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Bacteria are microscopic, single-celled organisms with a rigid wall living in environments within us, such as, mouth, mucous membranes, blood stream, digestive, reproductive, urinary tracts, skin, and teeth and within our environments, soil, water, seawater, foods, animals and all over the earth surfaces and beyond. There are several thousands of kinds of bacteria; most of which are harmless, helping the body to digest food, provide essential nutrients and fight disease causing-microbes with only a few being able to cause disease in humans, called the pathogens/pathogenic bateria. They are most often associated with many illnesses and conditions. Their shape, cell wall composition and ability to grow with or without oxygen (aerobes/anaerobes) help in their identification. They range from the spiral, curved-shaped (*Campylobacter, Helicobacter* spps) to the rods, (*Bacillus, Escherichia, Salmonella, Pseudomonas, Shigella* spps) to the round cocci (*Staphylococci, Enterococci, Streptococci* spps) in shape (Timbury *et al*., 2002; Fischer *et al.*, 2007). These pathogens, because of difference in the composition of their cell walls, give different colours when Gram stained. The Gram-positive bacteria stain blue, while the Gram-negative stain pink. Both of them cause different infections and therefore, require different antibiotics in their treatments. *Bacillus subtilis, Clostridum botulinium, Enterococcus faecalis, Listeria*

monocytogenes, Streptococcus pneumoniae, Staphylococcus aureus, are examples of Gram-positive bacteria; while *Eschericia coli, Pseudomonas aeruginosa, Klebsiella pneumoniae, Bordetella pertussis, Brucella canis, Campylobacter jejuni, Helicobacter pylori, Neisseria gonorrhoea, Vibro cholera, Shigella sonnei* and *Salmonella typhi* are examples of Gram-negative bacteria. The Gram-positive bacteria are more susceptible to antimicrobial agents because they are composed of peptidoglycan cell wall (Scherrer and Gerhardt, 1971; Enwuru *et al*., 2008; Salman *et al.,* 2008), while, the Gram-negative bacteria possess an outer phospholipidic membrane that makes their cell wall imprenetrable to antimicrobial agents and lipophilic solutes (Nikaido and Vaara, 1985), therefore, they tend to have higher intrinsic resistance to most antimicrobial agents (Ndukwe *et al.,* 2005; Karou *et al.,* 2006).

1.3.2 Forms of bacterial infections

There are several forms of bacterial infections, to which dysentery and diarrhoea belongs. These include;

(i) Respiratory tract infections: both upper (UTI) and lower (LTI) tract infections, such as throat, middle ear infections, pneumonia caused by *Streptococci*, *Haemophilus* spps and *Staphyloccus aureus*. Also, tuberculosis, an infection caused by *Mycobacterium tuberculosis* (Dai *et al.,* 1998; Echave *et al*., 2003).

(ii) Gastrointestinal tract infections (GTI): dysentery is the one of the oldest known GTIs. It is an inflammatory disorder of the intestine, especially of the colon causing diarrhoea with blood. It can result from viral infections, bacterial infections or parasitic infestations. It is a leading cause of morbidity and mortality worldwide

(WHO/CDS/CSR/EDC/99.8, 1999; Marignani *et al*., 2004). Amoebic dysentery (intestinal amoebiasis) and bacillary dysentery (shigellosis) are the two major types of dysentery. Amoebic dysentery is caused by a protozoon- *Entamoeba histolytica*, while bacillary dysentery is caused by the *Shigella* spps. Other common bacterial pathogens of dysentery are *Campylobacter jejuni*, *Salmonella* spps (salmonellosis) and *Helicobacter pylori* (Gold and Eisenstein, 2000; Conte, 2002; Bultzler, 2004). *E. coli*, an organism, which is a normal inhabitant of the human and animal intestine, is also a common cause of diarrhoea (not all strains). Food intoxication sometimes caused by *S. aureus*also results to dysentery, but is usually short and self-limiting, making treatment sometimes unnecessary (Timbury *et al*., 2002).

(iii) Skin infections: which includes, cellulitis, boils and complications from burns. Common pathogens are *S. aureus, P. aeruginosa* and group A *Streptococci* (Wysocki, 2002; Baggett *et al.*, 2004).

(iv) Surgical wounds: common pathogens are *Staphyloccus* spps, *Enterococci* spps, *P. aeruginosa* and *E. coli* (Goldmann *et al.*, 1996).

1.3.3 Antibiotics and antibotic-resistance

Bacterial infections can be treated with antibiotics, whose main goal are to kill by invading the bacteria (bactericidal) or prevent the growth of the bacteria (bacteriostatic). These antibacterial agents act through several mechanisms, depending on the type of antibiotic. For example; vancomycin and penicillin, used in the treatment of infections caused by *Enterococcus* spp., *Staphylococcus* spp. and *Streptococcus* spp. act by inhibiting the bacterial cell walls, while drugs like erythromycin, tetracycline and chloramphenicol used in the treatment of infections caused by *Helicobacter* spp.,

Mycoplasma spp., *Neisseria* spp. and *Salmonella* spp. act by interrupting the synthesis of proteins. Others, like sulpha drugs, useful in the treatment of *Pseudomonas* spp. act by inhibiting bacterial metabolism, while, others interfere with DNA synthesis, for example, ciprofloxacin and rifampin useful in the treatment of infections caused by *Haemophilus* spp., *Mycobacterium* spp., *Escherichia* spp., *Salmonella* spp. and *Shigella* spp. (Conte, 2002).

Most often, bacteria develop resistance to conventional antibiotics, either by acquiring genes from other bacteria that have become resistant or by gene mutation, so that the antibiotics become ineffective against them, making the bacteria to survive. Such surviving-bacteria reproduce over and over again and become dominant. For example, after the development of the drug, penicillin in the 1940's for the treatment of infections, it was discovered that some strains of *Staphylococcus aureus* were penicillin-resistant. Chemists then altered the penicillin molecule, by making a different but similar drug to penicillin - 'methicillin'. Over the years, some strains of *Staphylococcus aureus* have equally become resistant to methicillin, referred to as methicillin-resistant *Staphylococcus aureus* (MRSA). The indiscriminate, inappropriate and prolonged use of antibiotics has contributed in no small measure to the resistance of these drugs (van der Waaij and Nord, 2000; Petrosillo *et al.*, 2002). Inappropriate sanitary conditions in the hospitals and around us also contribute to their resistance (Levin *et al.*, 2003).

1.3.4 Combating bacterial infections by the use of medicinal plants

Quite a number of bioactive components present in medicinal plants have been proved to possess ability to inhibit bacterial infection/antibacterial activity, either by being bacteriostatic or bactericidal. These constituents produced by plants possess medicinal properties (Cowan, 1999) and act as defence chemicals against predation by

microorganisms, insects and herbivores. They are known to play important roles in bioactivity of medicinal plants, producing definite physiological actions on human body, which implies that the medicinal values of medicinal plants lie in these phytochemical compounds (Akinpelu *et al*., 2008). Amongst the plant phytochemicals, tannins, phenolic compounds, saponins, alkaloids and flavonoids have been linked to or suggested to be involved with antimicrobial activity (Palombo, 2006). Traditionally, herbs that have tannins, because of their astringent nature are used for the treatment of intestinal disorders, such as, diarrhoea and dysentery (Kokate, 1988; Dharmananda, 2003), while, other phytoconstituents, such as alkaloids and flavonoids have also been reported to possess antidiarrhoeal activities (Mallikharjuna *et al*., 2007).

Natural products, such as bee propolis and honey have been used in the treatment of bacterial wounds infections, long before the advent of antibiotics (Miorin *et al.,* 2003) and they have displayed significant *in-vitro* activity against *H. pylori, M. tuberculosis, S. aureus* and *P. aeruginosa* (Boyanova *et al.,* 2003). *Hibiscus sabdariffa*, commonly called 'Zobo' reportedly contains a range of constituents that prevent *E. coli* from adhering to the walls of the urinary tract, especially in women. The constituents caused a reduction in UTIs (Allaert, 2009). The antibacterial activity of commonly used food spices, such as, thyme, ginger and garlic has been reported. Thyme, an essential herbal oil, inhibited the growth of many strains of *E. coli* (Marino *et al.,* 1999), while, ginger, made up of volatile oils displayed analgesic, antipyretic, antibacterial and gastrointestinal tract motility effects (Thongston *et al.,* 2004). Allicin, a bioactive constituent found in garlic is effective against antibiotic-resistant strains of *Staphylococcus* spps., *Streptoccus* spps. and *H. pylori* (Tsao *et al.,* 2003).

Some of these phytoconstituents in the form of nutritional and dietary supplements, known as 'phytonutrients' help to stimulate immune response and increase susceptibility to infection, such that, there are reduced infections, particularly in the elderly, malnourished and critically ill individuals (Chandra, 1999). These phytonutrients, such as, flavonoids and carotenoids, are commonly found in fruits and vegetables and act as immune boosters, antioxidants, modulation of detoxifying enzymes, modulation of cholesterol synthesis, reduction of blood pressure and antibacterial agents (Craig, 1999).

This shows that natural products, a never out-dated subject today and a very long time to come, thus, constitute a practical endless source of novel substances able to enrich therapeutics (Portier *et al.*, 1996).

1.3.5 Antibacterial activity of fruit extract of plants

The antibacterial activity of various fruit extracts against various organisms have been reported, with the Gram-positive strains, often beenmost susceptible (Iwu *et al*., 1999; Silvia *et al*., 2004; Parekh and Chanda, 2007; Chanda *et al*., 2010; Anshika and Neeraj, 2011; Unnisa *et al*., 2012; Manzoor *et al*., 2013; Umer *et al*., 2013). The unripe fruits extract in some cases exhibited better antibacterial activity than the ripe fruits (Tahera *et al*., 2014). It was suggested that the observedactivity was probably as a result of the high content of tannins and alkaloids which are known to be cytotoxic towards bacterial cells (Jones *et al*., 1994). Earlier antibacterial activity studies carried out by Emeruwa (1982) on the ripe and unripe fruits of *Carica papaya* separated into epicarp, endocarp and seeds revealed that all the extracts exhibited significant activity on both Gram-positive and Gram-negative strains.

1.4 Statement of Research Problem

Pathogenic bacteria are known to contribute to globally important diseases, such as pneumonia,typhoid fever,tuberculosis, urinary, gastrointestinal and respiratory tract infections (Timbury *et al*., 2002). Dysentery and diarrhoea is a type of gastrointestinal infection (gastroenteritis) that can be caused by a number of infectious agents ranging from bacteria and viruses to protozoa and parasitic worms and chemical irritations, but bacterial infections caused by *Shigella, Campylobacter, E. coli and Salmonella* species are by far the most common cause (WHO, 2014). It is a symptom marked by rapid and frequent passage of semisolid or liquid faecal material through the gastro intestinal tract. It has been recognized as one of the most important health problems in the developing countries (Syder and Merson, 1982). More than 5-8 million annual deaths have been recorded for infants and small children less than 5 years worldwide as a result of diarrhoea (Park, 2000), while, in adults, it could be self-limiting (Timbury *et al*., 2002).

Most of these infections can be treated by the use of various antibiotics, but unfortunately, the increasing prevalence of bacterial resistance to these antibiotics, and the appearance of multidrug resistant strains with reduced susceptibility to commonly used antibiotics have necessitated the urgency for the search for newer and alternative compounds (Davis, 1994; Service, 1995; Sieradski *et al*., 1999). Antimicrobials of plant origin have been known not to be associated with many side effects and have an enormous therapeutic potential to heal many infectious diseases (Iwu *et al.*, 1999).

Nauclea latifolia possesses fruits, which are ethnomedicinally used in the treatment of dysentery, diarrhoea and other conditions relating to bacterial infections (Abbiw, 1990; Iwu *et al.*, 1999). In Chinese Pharmacopeia, it is recorded that the skins of *N. latifolia* fruits are recommended for the treatment of diarrhoea, while, the unripe or half ripe fruits of the plant is prescribed by Hindu physicians for the treatment of diarrhoea and

dysentery because of their astringent, digestive and stomachic properties (Bakru, 1997). No detailed phytochemical investigations have been carried out on the fruits of the plant. This work was aimed at investigating the phytochemical constituents and evaluating the antibacterial potentials of the ripe and unripe fruits of *Nauclea latifolia*.

1.5 Justification

The fruits of *N. latifolia* have reportedly been used in folkloric medicine in the treatment of dysentery, diarrhoea and other bacterial infections. However, this claim based on literature review has not been investigated, neither, has there been reported isolation and characterization of constituents of the fruits, although quite alot of work has been carried out on other parts of the plant. This research work is therefore, geared towards investigating the ripe and unripe fruits of *Nauclea latifolia* for their anti-dysenteric, anti-diarrhoreal potentials and thereby validating the ethnomedicinal claim of the plant. Furthermore, attempt will be made at isolation and characterization of some of the constituents present in the fruits and investigating their antibacterial potentials.

At the end of this study, it is believed that the antibacterial investigations of the extracts, fractions and the isolated constituents from the ripe and unripe fruits of *N. latifolia* will contribute to the continual search for natural plant products for use as medicines. It will also help to justify which of the fruits (ripe or unripe) are better antibacterial agents.

1.6 Hypothesis of the Study

The ripe and unripe fruits of *N. latifolia* contain bioactive secondary metabolites that possess antibacterial potentials that can be isolated and characterized using standard procedures.

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1.7 Aim of the Study

This work aims at the phytochemical and *in-vitro* antibacterial investigations of the ripe and unripe fruits of *N. latifolia.*

1.8 Objectives of the Study

The main objectives of the study are:

(i) To extract the phytochemicals present in both the ripe and unripe fruits of *N. latifolia*

(ii) To partition the methanol extract and re-extract the partition insoluble residue of both fruits using various organic solvents

(iii) To phytochemically screen the crude extracts, partitioned-soluble fractions and soluble fractions of both fruits

(iv) To fractionate, separate and purify some of the partitioned-soluble fractions and soluble fractions of both fruits

(v) To isolate, purify and characterize some of the phytoconstituents present in both fruits

(vi) To investigate the antibacterial activity of the crude extracts, partitioned-soluble fractions and soluble fractions, column fractions, column sub-fractions and isolated compounds in comparison with standard antibiotics

(v) To validate the ethnomedicinal claim for the use of both fruits in the treatment of dysentery, diarrhoea and other bacterial-related conditions.

(vi) To justify which of the fruits exhibit better antibacterial potency.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 *Nauclea latifolia* Smith (Rubiaceae)

2.1.1 Plant taxonomy

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Family: Rubiaceae (sometimes called “Naucleaceae”)

Genera: *Nauclea*

Specie: *latifolia*

Synonyms: *Nauclea esculenta* (Afzel. ex. Sabine), *Sarcocephalus esculentus* (Afzel. ex. Sabine), *S. latifolius, S. sassandrae* (A. Chev.) and *S. russeggeri* (Kotschy ex. Schweinf.).

Common names: Pin-cushion tree, Guinea peach, Country figure and African peach.

Local names: Tafashiya/Tabashiya (Hausa); Ovoro-ilu/Ogwu-iba (Igbo) and Egbeshi/ Egbeyesi (Yoruba). (Gbile, 1984; Iwu, 1993; Mann *et al.*, 2003; Akpanabiatu *et al.*, 2005a; Igoli *et al.*, 2005; Madubunyi, 1995; Ogbonna *et al.*, 2008).

2.1.2 Plant morphology and geographical distribution

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Nauclea latifolia Smith is a straggling or scandent shrub/a small spreading tree of about 7 m high but could grow up to 35 m in closed forests. It has a short thick trunk. Bark is glabrous with a reddish slash and yellow wood. The leaves, 17 x 12 cm, are glabrous, opposite, rounded-ovate, sharply triangular acuminate at the apex, shortly cuneate to rounded at the base, glabrous, glossy green with tufts of hairs in the axis of the lateral nerves below. Midrib is hairy in young leaves, 10 × 25 × 1 - 15 cm with 6 - 8 pairs of pale uncurved lateral nerves. Petiole is 1 - 3 cm long with darker upper surface while the stipules are broad, ovate and persistent. The plant has sweet- scented white or white-yellow flower with thick spherical dense heads of 5 - 6 cm across with long projecting stamens forming the most conspicuous part of the inflorescence. The calyx lobes are triangular, pubescent with four oblong and overlapping petals (Iwu, 1993).

The fruit is an indehiscent syncarp with a characteristic pitted surface (5 - 6 cm in diameter). The fruits are red when ripe, resembling hard strawberry and yellow, when unripe. These fruits are usually fleshy, shallow-pitched, with numerous embedded seeds

surrounded by a pink edible, sweet- sour pulp. The seeds are usually small, ovoid, numerous and brownish with a pleasant taste but could be emetic if taken in excess (Iwu, 1993; Neuwinger, 1996; Iwu *et al*., 1999). The plant is native to Tropical Africa and Asia. It is generally a Savannah-woodland plant, commonly found along forest margins, often on shady moist places. The plant is well distributed in many parts of Nigeria, for example, Akwa-Ibom and Cross-Rivers (Akpanabiatu *et al.*, 2005b), Sokoto, Jos, Minna and Benin (Falodun *et al.*, 2007).

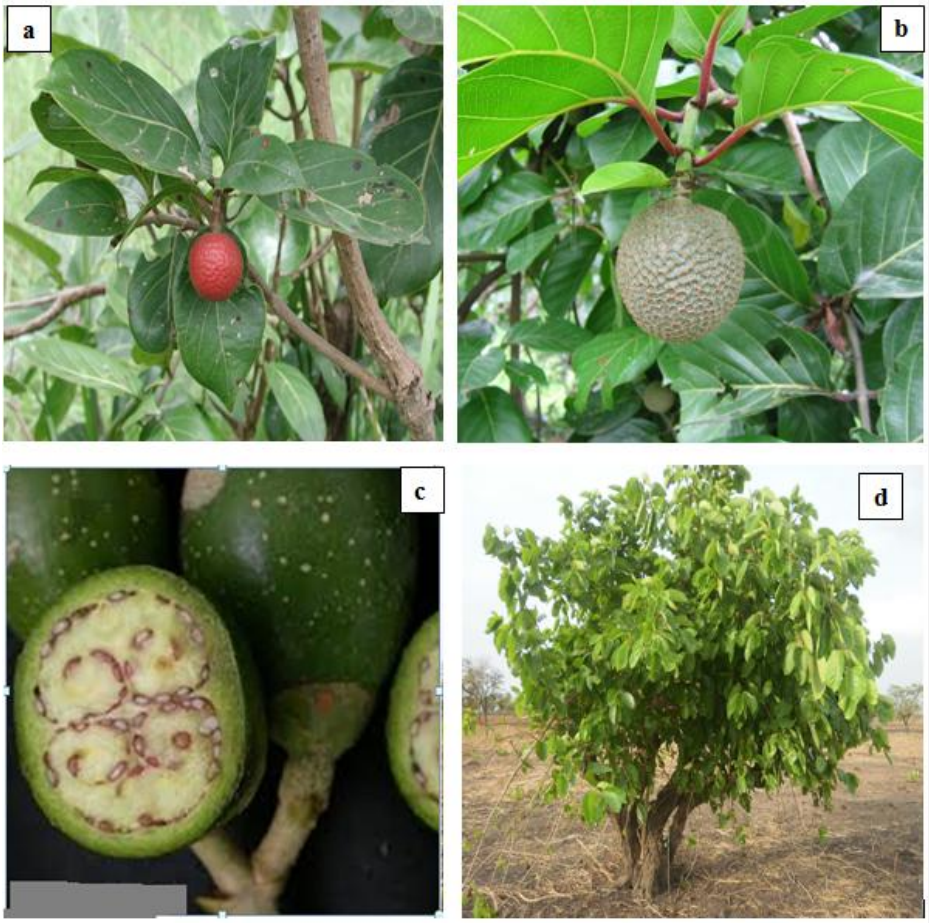

Plate I: (a) Ripe Fruit (b) Unripe Fruit (c) Seeds and Pulp of a Fruit (d) Whole Plant of *N. latifolia*

2.1.3 Medicinal and non-medicinal uses

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The leaf, stem bark, roots, and fruits of *N. latifolia* have been used traditionally in different localities for the treatment of different diseases. In Nigeria and some other parts of Africa, a decoction of the plant is used as a tonic and a remedy for fever and malaria. No wonder it is known as 'African cinchona' or 'African quinine' (Abbiw, 1990). Nigerian researchers have developed herbal cures in the form of a potent 'antimalarial' cocktail from plants such as *Morinda lucida* (local cinchona), *N. latifolia* leaves, *Cymbopogon citratus* (lemon grass), male *Carica papaya* leaves (pawpaw), *Moringa olifera, Magnifera indica* leaves and bark (mango), *Garcinia kola* (bitter cola) and *Psidium guajava* leaves (guava) in equal quantities. Also, the roots of *N. latifolia* soaked in corn water for 3 days has been claimed to clear the 'fever' in yellow fever (Muanya, 2009). Parts of the plant are commonly prescribed traditionally as a remedy for diabetes mellitus in Northern Nigeria (Gidado *et al.*, 2005). The plant is used in the treatment of sleeping sickness (Kerharo, 1974), hypertension (Akabue and Mittal, 1982), gastrointestinal tract disorders (Madubunyi, 1995) and stoppage of prolonged menstrual flow (Elujoba, 1995). The plant is used to treat bacterial infections, stomachache and diabetes mellitus. The root of the plant has been reported useful as an abortifacient and as a purgative (Vasileva, 1969). It is also used as a chewing stick and

in the treatment of toothaches, dental caries and septic mouth (Asubiojo *et al*., 1982; Iwu, 1993; Lamidi *et al.*, 1995). The root bark of the plant has been reported useful as an antimalarial (Iwu, 1993; Lamidi *et al.*, 1995; Benoit-vical *et al.*, 1998; Ogbonna *et al*., 2008). Roots of the plant are widely used in West Africa in the treatment of gastrointestinal troubles and for their antipyretic and antihelminthic properties (Duez *et al.*, 1994). In Ogun state, Nigeria, West Africa, the roots and bark of the plant is reportedly used in the treatment of cancer locally (Soladoye *et al.*, 2010).

The dried fruit is used traditionally in the treatment of dysentery, diarrhoea and piles (Abbiw, 1990; Iwu *et al.*, 1999). The charred roasted succulent ripe fruits are used to treat measles (Lawal *et al.*, 2010). The stem bark is useful as a hemostatic, analgesic, anthelminthic and diuretic. A lotion made from the stem bark is effective in the treatment of a complex skin disease resembling cutaneous leishmaniasis (Duke, 1983; Iwu *et al*., 1999). Herbalist in Southern Nigeria combines the leaves and roots for various medications (Udoh, 1998). The Igede people of Benue state of Nigeria use the macerated leaves or its decoction for the treatment of dysentery and measles while the stem bark is used against infertility (Adjanohoun *et al*., 1991; Igoli *et al*., 2002; Tor-Anyiin *et al*., 2003; Igoli *et al.*, 2005). It has been reported as the 6th most prescribed medicinal plant among the Igede people (Igoli *et al.*, 2005). In Benin-city, Nigeria, roots of *N. latifolia* are claimed by some traditional medicine practitioners to have antihypertensive effect (Nworgu *et al*., 2008).

Ghanaians reportedly use the shrub of *N. latifolia* by pounding the roots and adding lemon juice and palm wine. This concoction, or boiled leaves or sometimes the raw fruits of the plant is taken in the treatment of malaria (Asase *et al*., 2005). The roots and leaves are also used for stomach complaints and also for the treatment of sores (Abbiw,

1990). Decoction of the roots is used against sexual weakness (Addo-Fordjour *et al*., 2008). In Sudan, a root infusion is used for the treatment of gonorrhoea, fever and as a purgative (Abbiw, 1990). The roots are also used for the treatment of dysentery and as a tonic, whereas the bark is used for abdominal colic. The flowers and the bark of the plant are used in the relief of swollen knees. The plant is also used as an antipyretic and in the treatment of malarial fevers (El-Kamali, 2009). In Sierra Leone, the plant is used to manage veneral diseases and constipation (Abbiw, 1990) while in Southern Benin, the plant is reportedly used to alleviate malaria symptoms (Hermans *et al*., 2004). The bark of the plant is used in the treatment of diarrhoea while the root is used against sores, rheumatism, body pains, stomach pains, fever and sometimes, diabetes in the Gambia (Madge, 1998).

In Burkina-Faso, the plant is used for liver disorders, while in Kinshasa (Congo), the leaves of *N. latifolia* is traditionally used as an antidiarrhoeal (Tona *et al*., 1999). In some other parts of Cameroon, the plant is also used to treat fever, malaria, insomnia, anxiety and epilepsy (Bum *et al.*, 2009). Eating fruits of the plant is said to enhance indigestion, while the bark is used for threatened abortion (Jiofack *et al*., 2010). In some parts of Mali, the plantis used as an antimalarial (Traore-Keita *et al.* 2000a; Azas *et al.* 2001; Diallo *et al.*, 2004). Also, the decoction of the roots is used against abdominal pains and malaria (Maiga *et al.*, 2005). In Guinea, the stem bark steep is used in the treatment of infectious diseases, such as sexually transmitted diseases by oral route (Magassouba *et al*., 2007). In Togo, roots decoction in combination with *Peliocarpa mutica* is used to treat ascariasis by the oral route. Crushed roots in association with palm nuts are used to induce abortion (Adjanohoun *et al.*, 1986).

The plant is also regarded as a source of food in Northern Nigeria (Aiyela'agba *et al.*, 1996).

2.2 Phytochemistry of *N. latifolia*

2.2.1 Chemical constituents of various parts of the plant

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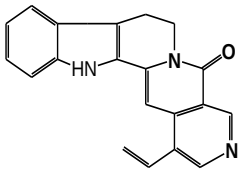
Work carried out on various parts of *Nauclea latifolia* revealed that the plant is very rich in alkaloids. Several indole-quinolizidine alkaloids and glycoalkaloids such as, angustine **(VI)**, angustoline **(VII)**, nauclefine **(VIII)** and naucletine **(IX)** have been isolated from the roots of the plant (Iwu, 1999). Atta-ur-Rahman (2003) also reported the presence of unusual trinitrogenated alkaloids and complex indole alkaloids including glucoindole alkaloids, which are restricted to a few iridoid-containing families such as the Rubiaceae. The strong presence of saponins in the plant has also been reported (Iwu *et al*., 1999). The presence of alkaloids (Hottellier *et al.*, 1979; Abreu and Pereira, 2001; Nkafamiya *et al*., 2006), -terpenes (Morah, 1995), -some organic compounds (Asubiojo *et al.*, 1982), tannins, oxalates and phytates (Nkafamiya *et al*., 2006; Omale and Haruna, 2011) in *N. latifolia* have been reported. Monoterpenes indole alkaloids called naucleamides have also been isolated from the plant (Shigemori *et al*., 2003; Kakuguchi *et al.*, 2009). Nauclefoline **(XI)**, a novel indole alkaloid was isolated from the roots of the plant. Other isolates from the roots, were triterpenic compounds such as rotundic acid **(X)**, α-L-rhamnoquinovic acid, 3-O-β-glucopyranosyl-β-sitosterol **(XII)**, squalene **(XIII)** and sitosterol-3-O-6'-stearoyl-β-D-glucopyranoside (Ngnokam *et al.*, 2003).

Phytochemical investigation of the root extract of the plant led to the isolation of new indole alkaloids, such as; 21-O-methylstrictosamide aglycone **(XIV)** and 21-O-ethylstrictosamide aglycone **(XV)** together with strictosamide **(XVI)**, angustine **(VI)**,

nauclefine **(VIII)**, angustidine **(XVII)**, angustoline **(VII)**, 19-O-ethylangustoline **(XVIII)**, naucleidinal **(XIX)**, 19-epi-naucleidinal, quinovic acid-3β-O-β-D-fucopyranoside, quinovic acid-3β-O-α-L-rhamnopyranoside, scopoletin **(XX)** and β-sitosterol **(XXI)** (Abreu and Pereira, 2001). Phytochemical screening of the petroleum and methanolic extracts of the leaves and stem bark of the plant revealed the presence of alkaloids, tannins, saponins and some sterols (Agyare, 2006), while the screening of the ethanolic extract of the roots revealed the presence of sugars, saponins and flavonoids. Alkaloids, tannins and cardiac glycosides were reportedly absent (Nworgu *et al*., 2008). Brown *et al* (1977) isolated strictosidine lactam from the heartwood of the plant. Isah *et al* (2014) characterized a mixture of two terpenoids, β-sitosterol **(XXI)** and β-sitostenone **(XXII)** from the methanolic extract of the stem bark of the plant, while a pentacyclic triterpenoid, betulinic acid **(XXIII)** has also been isolated from the methanol extract bark of the plant (Isah *et al.*, 2012).



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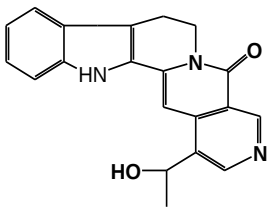

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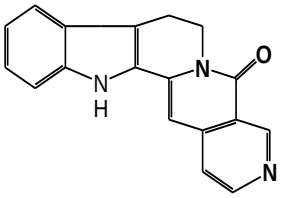

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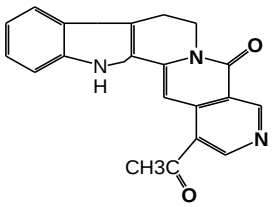
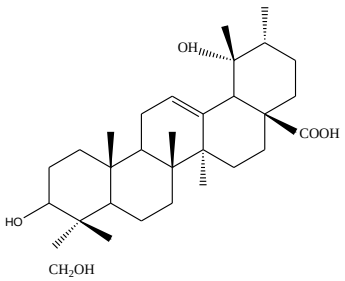
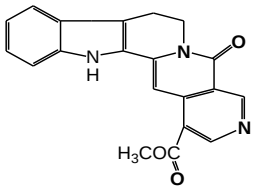
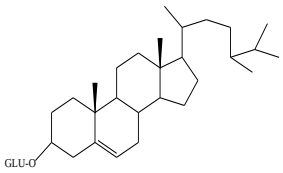
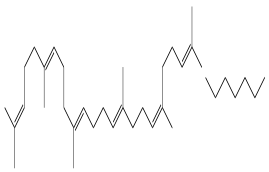




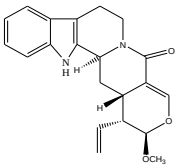
(IX) **(X)**

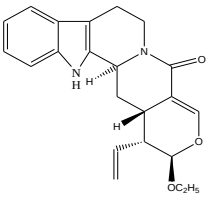


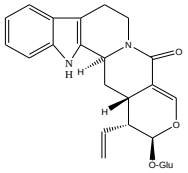




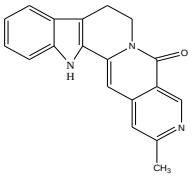
(XI) **(XII)** **(XIII)**

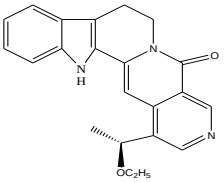


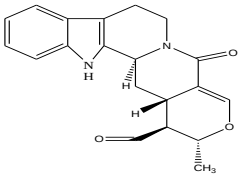




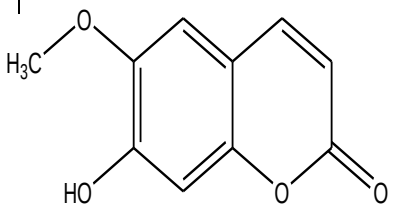
(XIV) **(XV)** **(XVI)**

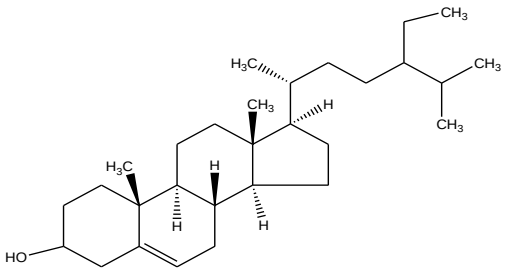






(XVII) **(XVIII)** **(XIX)**





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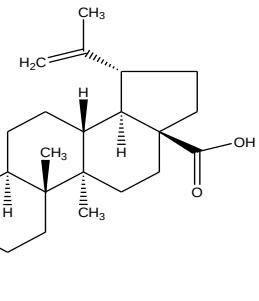

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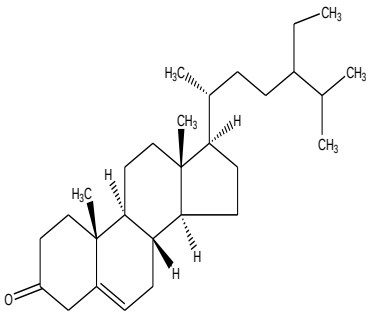

(XXII)

2.2.2 Chemical constituents of *N. latifolia* fruits

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Biochemical and nutritional evaluation of the fruits revealed that fruits of the plant are reportedly rich in vitamin C and this has made them a good source of fruit juice (Amoo and Lajide, 1999). Biochemical evaluation of the fruits of the plant revealed high values of calcium, phosphorous and magnesium, suggesting that they could be useful in the development of bones and teeth and could serve as an anti-clotting agent. The level of iron was reportedly high (1.8 - 6.7 mg/100 g), a range 2 - 5 times higher than that obtained in fruits like oranges and mangoes. The fruit is said to contain adequate

amounts of vitamins A, B_1, B_2, C and E and was therefore, suggested to be important in the prevention of night blindness (Nkafamiya *et al.*, 2006).

Literature review revealed no isolation and characterization of any phytochemical constituent(s) from the ripe or unripe fruits of the plant.

2.3 Biological and Pharmacological Activities of *Nauclea latifolia*

2.3.1 Activity of various parts of the plant

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(i) Antimicrobial Activity

The extracts of roots of *N. latifolia* have been reported to be most effective against *Corynebacterium diphtheriae, Streptobacillus* spp., *Streptococcus* spp., *Neisseria* spp., *Pseudomonas aeruginosa* and *Salmonella* spp. (Deeni and Hussaini, 1991) and *Klebsiella pnuemoniae* (Tona *et al*., 1999). The antibacterial activity of the roots of the plant against Gram-positive and Gram-negative bacteria and the usefulness of the plant as an antifungal agent has also been reported (Iwu, 1993). Okoli and Iroegbu (2004) revealed that the ethanol, cold and hot water extracts of the whole root were bacteriostatic to both Gram-positive and Gram-negative bacterial isolates from cases of non-gonococcal urethritis. The petroleum spirit, chloroform, methanol and aqueous extracts of the stem bark of the plant reportedly inhibited the growth of *Escherichia coli* (Omer *et al*., 1998; Abreu *et al*., 1999), with the petroleum spirit extract being the most effective followed by chloroform, methanol and water extracts being the least with almost the same potency (Umeh *et al.*, 2005). Chloroform and methanol extracts of the stem bark revealed significant antibacterial and antifungal effects against different organisms of clinical isolates- *Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumoniae, Bacillus subtilis, Streptococcus varidans, Staphylococcus aureus, Penicillum notatum, Candida albicans* and *Aspergillus niger*. The chloroform extract showed higher antimicrobial activity both in spectrum (broad) and potency against the test organisms, while the methanol extract was active only against *S. aureus* and *P. aeruginosa* (Falodun *et al*., 2007).

Antibacterial studies carried out on the ethanol and aqueous extracts of the leaves, bark and roots of the plant against some pathogenic bacteria such as *P. aeruginosa, K. pneumoniae, E. coli, S. aureus* and *Shigella dysenteriae* revealed that these extracts were quite active against these organisms with the ethanol extract being more potent than the aqueous extract of the plant, while the root extract showed more potency than the leaves and bark in terms of zones of inhibition. Isolates of *E. coli* and *P. aeruginosa* were reportedly not too susceptible to extracts of the plant (El-Mahmood *et al.*, 2008). Antimicrobial screening of the petroleum ether and methanol extracts of the leaves and stem bark of *N. latifolia* revealed that the methanol extract exhibited better antibacterial and antifungal activities against *E. coli, S. aureus, P. aeruginosa, B. subtilis* and *C. albicans* (Agyare *et al*., 2006). *In-vitro* studies of the leaves and root extracts of *N. latifolia* using agar diffusion method revealed that both the aqueous and alcoholic extracts of the plant showed appreciable inhibitory effect against *S. aureus* and *P. aeruginosa*, while *S. typhi* and *E. coli* were resistant to the extracts. The alcoholic extracts showed larger zones of inhibiton on the test organisms than the aqueous while the leaf extracts gave a higher percentage of inhibition (Okwori *et al.*, 2008).The antimicrobial potency of 50 % methanolic extract of the leaves of *N. latifolia* and its various soluble portions has also been reported (Okei *et al.*, 2011). The hexane, methanolic and aqueous extracts of the leaves, stem bark and roots of the plant grown in Hong, Adamawa state, Nigeria, exhibited moderate activity against both Gram-positive and Gram-negative bacteria (Maitera *et al*., 2011). The chloroform extract of the stem bark of the plant reportedly inhibited the growth of both Gram-positive and Gram-negative bacteria (Anowi *et al*., 2012).

(ii) Antimalarial Activity

Aqueous extracts of the stem and roots of *N. latifolia* when tested on *Plasmodium falciparum* FcB1- Colombia (chloroquine-resistant) and a Nigerian strain (chloroquine-sensitive) inhibited the FcB1 strain with the determined IC50 values being within the range already reported for other antimalarial plants such as *Azadirachta indica* A. Juss (Meliaceae) and *Artemisia annua* L. (Asteraceae). The antimalarial activity of the plant was associated with its cytotoxic activity (Benoit-Vical *et al*., 1998). The antimalarial potency of the plant against *Plasmodium falciparum* has also been reported (Traore-Keita *et al*., 1998). Further work carried out by Abreu and Pereira (2001) revealed that strictosamide **(XVI)**; an active constituent isolated from the roots of the plant displayed a moderate antiplasmodial activity against *P. falciparum.* Ethanol extracts of roots of *N. latifolia* was reported to have intrinsic antimalarial activity that was dose-dependent against chloroquine sensitive *Plasmodium berghel* in mice using the 4-day suppressive test procedure. It was observed that 500 mg/kg body weight of the extract produced 71.15 % suppression of the parasitaemia same as that produced by chloroquine. Ethanol extract (500 mg/kg) of combined roots of *N. latifolia,* roots of *Salacia nitida* and stem bark of *Enantia chlorantha* Oliv. (Three plants used traditionally in the treatment of malaria in the South-Eastern part of Nigeria) produced 77.46 % suppression of the parasitaemia, supporting the use of these plants in the treatment of malaria locally (Ogbonna *et al*., 2008). The use of the plant as an antimalarial has also been justified pharmacologically using rats and mice (Abbah *et al*., 2010). The toxicity and genotoxicity of the antimalarial alkaloid-rich extracts of *N. latifolia* has also been reported (Azas *et al*., 2001; Traore-Keita *et al*., 2000b; Ajaiyeoba *et al*., 2006). *N. latifolia* has been described as the most efficient antimalarial in the Rubiaceae family (Karou *et al*., 2011).

(iii) Hepatoprotective Activity

The polyphenolic- and not the saponin- or alkaloid-containing fractions of leaf extracts of the plant exhibited more than 70 % inhibition of acetylcholine and/ or KCl solution–induced contractions on isolated guinea pig ileum at a concentration of 80 μg/ml (Tona *et al*. 1999). Defatted ethanol extract of the root bark of *N. latifolia* reportedly had no effect on pentobarbitone-induced hypnosis in mice after i.p injection, while, oral administration of the extract (100 mg/kg) significantly reduced pentobarbitone–induced sleep in rats poisoned with CCl_4. Aqueous extract of the root bark showed a hepatoprotective activity against CCl_4-induced hepatopathy in NMR-1 mice. The ethanol extract also decreased the level of parasitaemia in mice infected with *Trypanosoma brucei brucei* (Madubunyi, 1995). Defatted methanol extract showed no significant effect on pentobarbital-induced sleep in rats after i.p injection. The extract significantly reduced pentobarbital-induced sleep time in paracetamol and CCl_4 intoxicated rats (Udem and Madubunyi, 2008).

(iv) Antidiabetic Activity

The ethanolic and aqueous extracts of the leaves of *N. latifolia* (200 mg/kg) significantly lowered glucose levels of alloxan-induced diabetics in rat by 45 % within 4 hours, but the extract showed no similar effect in normal glycaemic rats. The extracts also significantly lowered the fasting blood glucose levels of streptozotocin-diabetic rats in a dose-dependent manner with an activity comparable to that of glibernclamide at 1 mg/kg (Gidado *et al*., 2005; 2008), while butanolic extract of the stems and roots of the plant also decreased hyperglycemia in diabetic pregnant rats (Yessoufou *et al*., 2013). Crude ethanolic extract of *N. latifolia* reportedly reduced systolic, diastolic and mean arterial pressure in normotensive and in one-kidney one-clip hypertensive rats in a dose-dependent manner, while the chloroform fraction on column fractionation yielded

fractions which were active in lowering blood pressure of normotensive rabbits (Nworgu *et al*., 2009).

(v) Anti-inflammatory Activity

The aqueous and hydroalcoholic extracts of the aerial parts of *N. latifolia* were reported to produce analgesic effect when tested on acetic acid and hot plate-induced pains (Andissa-Okiemy *et al.,* 2004). The methanolic extract of the stem bark at low concentrations was found to have weak analgesic and anti-inflammatory effects (Otimenyin and Uguru, 2006). The plant reportedly displayed significant nociception and anti-inflammatory effects in rats and mice (Abbah *et al*., 2010).

(vi) Anticonvulsant Activity

Decoction of the rootbark of *N. latifolia* protected mice against maximal electroshock (MES), pentylenetetrazol (Sc-PTZ) and strychnine-induced seizures. The extract also strongly increased the total sleep time induced by diazepam (Bum *et al.*, 2009).

(vii) Other Activities

Treatment of sheep having natural acute/sub-acute parasitic gastro-enteritis with 400, 800 and 1600 mg/kg of crude aqueous extract of the stem bark of *N. latifolia* significantly reduced faecal egg counts in infected animals, an activity similar to that exhibited by albendazole at 5 mg/kg (Onyeyili *et al*., 2001). Extract of the plant reportedly produced *in-vitro* anthelminthic efficacy against gastrointestinal nematodes of sheep (Ademola *et al*., 2007). *In-vitro* and *in-vivo* estrogenic studies of the methanol extract of the plant confirmed that the plant has estrogenic activity (Onyeyili *et al*., 2001; Njamen *et al.*, 2008). The root extracts of *N. latifolia* reportedly has some anti-ulcer properties (Aguwa and Nwako, 1988).

Aqueous extract of the roots of the plant (50 - 200 mg/kg) significantly decreased the spontaneous motor activity (SMA) and exploratory behaviour in mice, while prolonged

pentobarbital sleeping time in rats was reduced (Amos *et al.,* 2005). Studies have shown that the plant contains heat-stable inhibitory activities against recombinant *Ascaris* and *Oncocerca* glutathione s-transferase *in-vitro* (Fakae *et al*., 2000).

2.3.2 Biological activity of *N. latifolia* fruits

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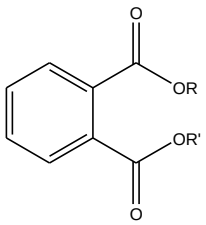
The fruit extract of the plant has been shown to be active against Human Immune Deficiency Virus (Hussein *et al.,* 1999). Controlled roasting of the ripe fruits of the plant in a peculiar way, gives a potent antiviral drug against measles (Morah, 1994). The fruit of *N. latifolia* is reported to possess hypocholesterolemic (cholesterol lowering effect) potential (Omale and Haruna, 2011).

Literature review has revealed no antibacterial investigations of either the ripe or unripe fruits of the plant.

2.4 Chemistry and Biological Activity of Phthalates

Phthalates or phthalic acid esters (PAEs) are diesters of phthalic anhydride or dialkyl or alkyl aryl esters of 1, 2-benzenedicarboxylic acid. They have the general chemical structure of a benzene dicarboxylic acid **(XXIV),** where R and R' could be the same or different, branched or unbra nched (alkyl, cycloalkyl, alkoxy, phenyl or benzyl). They are usually colourless/pale yellow, odourless/slight odour, low volatility, low melting

(-5.5 to -58^{o}C) and high boiling (230 - 486^{o}C) liquids at room temperature (Staples *et al*., 1997; Cousins *et al*., 2003). They, especially the dialkyl phthalates, are most often soluble in non-polar solvents (Woodward, 1988), but generally, their solubility decreases as length of the alkyl side chain increases (Stanley *et al.*, 2003). They, especially the dialkyl phthalates, are most often soluble in non-polar solvents, such as, petroleum ether, benzene, toluene, xylene, chloroform and diethyl ether (Woodward, 1988), but generally, their solubility decreases in these solvents as length of the alkyl side chain increases (Stanley *et al.*, 2003). They show high liphophilicity and low hydrophilicity, with their hydrophobicity increasing with increase in alkyl side chain (Chen *et al*., 2011).

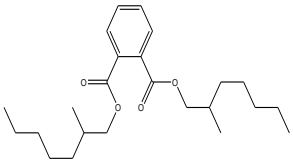


(XXIV)

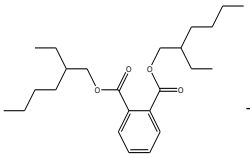
Studies have shown that phthalate esters, which are naturally produced extracellularly by micro organisms, such as bacteria, fungi and yeasts (Mahmoud *et al*., 2006) have been detected in bacteria, plants and the fatty acid fractions of certain species of marine macro-algae (Namikoshi *et al*., 2006). They are sometimes disregarded in considerations of risk (Rhind *et al*., 2005) because on the basis of chemical structure, it is considered that these compounds are readily degraded in the environment (Jianlong *et al*., 2004).

Phthalates have been isolated and characterized from several natural products. For example, from the leaves of *Pongamia pinnata*, bis-(2-methylheptyl) phthalate **(**BMHP,

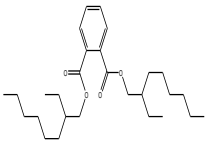
XXV) (Rameshthangam and Ramasamy, 2007); the methanolic extract of the variety of minor seeds of *Ricinus communis*, di-(2-ethylhexyl) phthalate (DEHP, **XXVI**) (Sani and Pateh, 2000) and from the flowers of *Calotropis gigantea* (Rowshanul *et al* ,. 2009) has all been isolated. Also, Gaikwad *et al* (2013) isolated DEHP from the leaves of *Cassia auriculata* and from the leaves of *Nauclea officinalis*, family Rubiaceae (Su *et al*., 2009). Compounds bis-(2-ethyloctyl phthalate (BEOP, **XXVII**) and bis-(2-ethylicosyl) phthalate (BEIP, **XXVIII**) have also been characterized from *Phyllanthus muellerianus* (Saleem *et al*., 2009). Efiom (2010) also isolated bis-(2-methoxyethyl) phthalate (BMEP, **XXIX)** from the root of *Cissampelos owariensis* (P. Beauv). Di-n-octyl phthalate (DNOP, **XXX**) and dibutyl phthalate (DBP, **XXXI**) have also been identified in the methanolic extract of the leaves of *Woodfordia fructicosa* Kuz (Grover and Patni, 2013), while, DNOP (**XXX**) and DBP, (**XXXI)** have also been isolated from the roots of *Leea indica* (Burm. F) Merr (Joshi *et al.*, 2013a). Joshi *et al* (2013b) also isolated DNOP (**XXX)** from the roots of *Ixora coccinea* Linn, family, Rubiaceae. Diethyl phthalate (DEP, **XXXII**) has been identified as the major compound in the young fruits of *Ficus palmata* (Alrumman *et al*., 2014). Phthalic acid, butyl octyl ester **(XXXIII)** has been identified in the chloroform extract of the leaves of *Acacia nilotica* L (Bai *et al*., 2014).



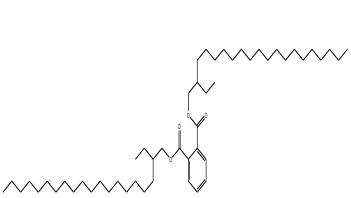
(**XXV**)



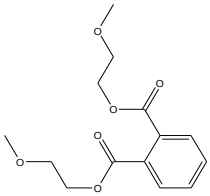
(**XXVI**)



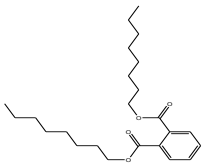
(**XXVII**)



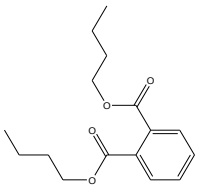
(**XXVIII**)



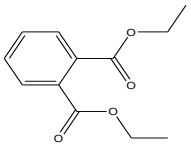
(**XXIX**)



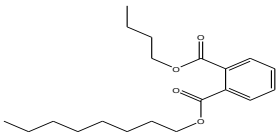
(**XXX**)



(**XXXI**)



(**XXXII**)



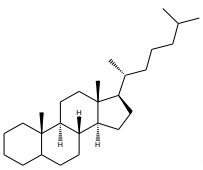
(**XXXIII**)

DEHP **(XXVI)** isolated from *Aloe vera,* reportedly exhibited anti-leukemic and anti-mutagenic effects (Lee *et al.*, 2000). The compound also exhibited significant inhibitory activity against some bacterial and fungal strains (Lyutskanova *et al.,* 2009). The compound also isolated from marine-derived fungus *Penicillium brevicompactum* was active against Gram-positive *B. subtilis* and Gram-negative *E. coli,* while, at 100 µg/ml it killed about 30 % of lung cancer cells (Atalla *et al*., 2011). DEHP from flowers of *Calotropis gigantea* was reported active against bacteria and fungi, while DEHP identified as the major compound in the young fruits of *Ficus palmata* exhibited moderate antimicrobial growth against some bacteria and fungi (Alrumman *et al*., 2014). Its toxicity against *Artemia salina* was also reported (Rowshanul *et al*., 2009). Bis-(2-ethyloctyl) phthalate (BEOP, **XXVII**) and bis-(2-ethylicosyl) phthalate (BEIP,

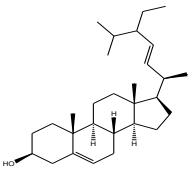
XXVIII) from *Phyllanthus muellerianus* also displayed significant antimicrobial potentials (Saleem *et al*., 2009).

2.5 Chemistry and Biological Activity of Steroidal Compounds

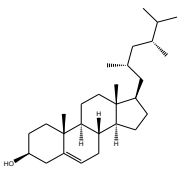
Sterols (a sub-group of steroids) are a group of steroidal alcohols/sugars that occur naturally in plants, animals and fungi. They are a type of lipid having a cholestane-derived framework **(XXXIV)** with a hydroxyl (aglycone) or glycosyl (glycoside) group at position 3. Commonly encountered plant sterols (phytosterols) include β-sitosterol (**XXI**), stigmasterol **(XXXV)** and campesterol **(XXXVI)** and all compounds that structurally resemble cholesterol (**XXXVII)**, a sterol obtained from animals (Kemal and Amar, 2006). They are present in various combinations in vegetables, legumes, nuts, wheat, seeds, fruits, medicinal plants and plant oils (Burg *et al*., 2013) as glycosidic compounds, fatty acid esters or in their free states (Ju *et al.*, 2004).



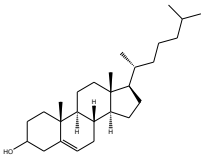
(XXXIV)



(XXXV)



(XXXVI)

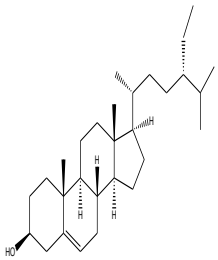


(XXXVII)

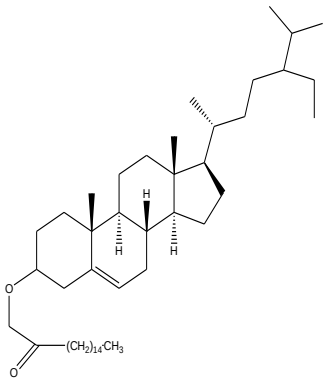
They are a group of unsaturated three fused cyclohexane rings with a hydrophenanthrene ring arrangement (**XXXIV)** but have different side chain configurations (Fernandes and Cabral, 2007). They are waxy, clear solids, melting points > 130^{o}C, soluble in most organic solvents but insoluble in water. They occur in all types of tissues, such as roots, stems, leaves, fruits, flowers (Yadav *et al*., 2014). Their isolation entails extraction using a~~n~~ non-~~a~~polar solvent, followed by

chromatographic separations/fractionation on silica gel column and monitoring the fractions on TLC by spraying with visualizing/chromogenic reagents, such as, Liebermann-Burchard, Salkowski, vanillin-sulphuric acid or anisaldehyde-sulphuric acid to confirm the presence of steroidal nucleus (Saeidnia *et al.*, 2014). Isolation of stigmasterol (**XXXV**) and β-sitosterol (**XXI**) has been reported by (Pateh *et al.*, 2008; Kamboj and Saluja, 2011; Chaturvedula and Prakash, 2012; Ahmed *et al*., 2013) from the rhizomes of *Stylochiton lancifolius*, petroleum ether extract of aerial parts of *Ageratum conyzoides*, dichloromethane extract of *Rubus suavissimus* and the hexane extract of the leaves of *Saurauia roxburghii* –respectively. Also, from the leaves of *Hygrophila spinosa* (Patra *et al.*, 2010) and *Momordica charantia* (Sen *et al*., 2012), β-sitosterol (**XXI**) was isolated. β-sitosterol and γ-sitosterol (**XXXVIII)** were among the phytoconstituents isolated from the roots of *Girardinia heterophylla* (Tripathi *et al*., 2013). Phytosterols have been identified from the 'Rubiaceae'family (Halilu *et al*, 2012; Vindhya *et al*., 2014), while, β-sitosterol (**XXI**) and β-sitosterol palmitate (**XXXIX**) were isolated from the leaves of *N. officinalis* (Su *et al*., 2009). From *Fadoga homblei* De Wild, β-sitosterol **(XXI)** and stgmasterol **(XXXV)** have also been isolated (Mohammed *et al*., 2013).

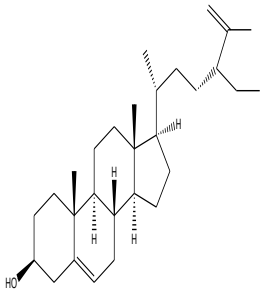
Phytosterols have also been identified and isolated from several fruits; Clerosterol **(XL**) was isolated from the hexane extract of the fruits of *Cassia fistula* (Sartorelli *et al*., 2007), while, from the fruits of *Pistacia atlantica*, campesterol (**XXXVI**), stigmasterol (**XXXV**), β-sitosterol (**XXI**) and Δ^5-avenasterol **(XLI**) have been isolated (Benhassaini *et al.*, 2007). A mixture of four sterols, including fucosterol **(XLII)**, racemosol, stigmasterol (**XXXV**) and stigmasta-7, 22-dien-3β, 4β-diol **(XLIII**) has also been isolated from the fruits of *Lagenaria siceraria* (Kalsait *et al*., 2011).



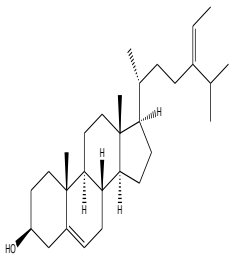
(XXXVIII)



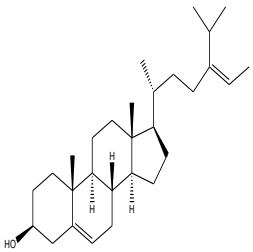
(XXXIX)



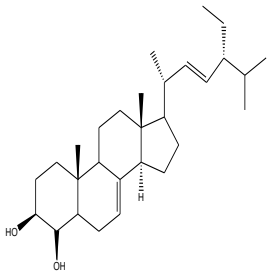
(XL)



(XLI)



(XLII)



(XLIII)

Phytosterols are widely applied in pharmaceutical, food and cosmetic industry due to their special biological-activity, their physical and chemical properties (Thomas *et al.*, 2002). They possess anti-cholesterolemic, antioxidant, anti-tumoral, hypoglycaemic, anti-inflammatory, anti-osteoarthritic, anti-trypanosomal and antibacterial properties (Hoet *et al*., 2007; Panda *et al*., 2009; Gabay *et al*., 2010; Tripathee *et al*., 2011; Sen *et al*., 2012; Isah *et al*., 2014). The antimicrobial properties of β-sitosterol from different

plants have recorded varying results (Saeidnia *et al*., 2014). For example, β-sitosterol (**XXI**) and β-sitosterol glucopyranoside (**XII**) isolated from the aerial parts of *Cissus sicyoides* exhibited significant antibacterial activity against *Bacillus subtilis* (Beltrame *et al*., 2002), while, β-sitosterol isolated from the methanol extract of *Senecio lyratus* (Asteraceae) exhibited moderate activity against Gram-positive *S. aureus* and Gram-negative *E. coli*, *K. pneumoniae* and *P. aeruginosa*, with an activity similar to that displayed by gentamicin (Sen *et al*., 2012). On the other hand, β-sitosterol (**XXI**) and stigmasterol (**XXXV**) both isolated from the roots of *Sida rhombifolia* (Malvaceae) exhibited low activity at 50 mg/ml against *S. aureus, E. coli, P. aeruginosa* and *S. typhimurium* in comparison with ciprofloxacin (Woldeyes *et al*., 2012).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Materials/Reagents/Equipments/Analytical Techniques

3.1.1 Chemicals, reagents, materials and instruments

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BDH chemicals, Ltd. Poole, England, Fischer chemicals, Fischer Scientific, UK and Riedel-de Haen, Sigma-Aldrich Laborchemikalien GmbH, supplied all organic solvents.

Silica gel for thin layer chromatography, Kieselgel 60 G (Merck, Darmstadt, Germany)

Silica gel 60 F_{254} Aluminium sheets (Merck, Darmstadt, Germany).

Silica gel for column chromatography, 60 - 120 mesh; 230 - 400 mesh (BDH Chemicals Ltd Poole, England).

Sephadex LH - 20 (Amersham Pharmacia Biotec AB, Sweden).

Rotary evaporator

Melting point apparatus (Gallenkamp)

Ultraviolet light (254 and 366 nm) (Model UVGL - 58, San Gabriel, U.S.A.)

GC - MS - QP 2010 plus Shimadzu, Japan.

Bruker ACQ 400 Avance spectrometer operating at 400 MHz (NMR)

Rudolph Autopol IV automatic polarimeter

T60 UV - Visible spectrophotometer

FTIR - Spectrolab MB3000

3.1.2 Analytical techniques

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(**I)** Chromatographic techniques

(i) Thin layer chromatography (TLC); Preparative thin layer chromatography (PTLC)

Technique: Ascending

Stationary phase: silica gel /pre coated plates

Thickness: 0.25 mm was used for TLC while 1.00 mm was used for PTLC.

Solvent systems: Details in text.

Spotting and development: Test compounds were spotted as spots manually on prepared (PTLC) and pre-coated TLC plates, using acetone-cleaned glass capillary tubes. Plates were developed in different solvent media (details in text) at room temperature using a glass tank.

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Visualization of spots: Dried developed plates (chromatograms) were visualized using daylight; UV - light (254 nm and 366 nm); iodine vapour in a closed iodine chamber and spray reagents/chromogenic reagents, such as;:

(a)

Vanillin - sulphuric acid: Vanillin (1 g) was dissolved in 95 ml MeOH (EtOH) and 5 ml of conc. H_2SO_4 acid (freshly prepared). Plates were heated at 120^oC until spots attained maximum colour intensity.

~~(a)~~ (b)

~~(b)~~ Anisaldehyde - sulphuric acid: 0.5 ml conc. H_2SO_4 + 9 ml EtOH + 0.5 ml anisaldehyde in 0.1 ml acetic acid (freshly prepared). Plates are heated at 100 - 105^oC until spots attained maximum colour intensity.

(ii) Column/ Flash chromatography

Technique: Gradient elution; Stationary phases: 60 - 120; 230 - 400 mesh silica gel (1 g of sample/20 g (30 g) of silica gel) and sephadex LH - 20.

Column 1 (for silica gel): Sintered glass column (30 x 3.5 mm; 60 x 7.5 mm; 150 x 18 mm; 280 x 35 mm) all packed by slurry method.

Column 2: 10 g of sephadex was soaked in 100 ml of methanol and stirred. Mixture was allowed to stand for 2 hrs before being packed by slurry method into a sintered glass column (30 x 3.5 mm).

Eluting solvents/mobile phases: Details in text.

Flow Rate: 1 drop/5 seconds.

(iii) Vacuum liquid chromatography (VLC)

Technique: Vacuum pressure, provided by a vacuum pump and controlled by a 3-way stop cork.

Stationary phase: Silica gel (60 - 120 mesh) was packed uniformly and compressed to a hard cake/layer under applied vacuum.

Apparatus: A sintered glass Buchner filter funnel (500 ml, 7184, porosity D) with fritted disk (ASTM 10 - 15 µ) and a t24/40 joint.

Eluting solvents/mobile phases: Details in text.

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(II) Spectroscopic techniques

(i) Gas chromatography-Mass spectrometry (GC - MS)

The identification of some of the compounds present in the ripe and unripe fruits of *N. latifolia* was based on direct comparison of the retention times and mass spectral data of standard compounds.

(ii) ^{1}H - NMR, ^{13}C - NMR and DEPT - 135

Each isolated compound was subjected to ^{1}H - NMR, ^{13}Carbon - NMR and DEPT - 135 spectrometry by dissolving samples in $CDCl_3$. TMS was used as an internal standard and chemical shifts (δ) recorded in parts per million (ppm).

(iii) Ultraviolet spectroscopy (UV)

Isolated compound (2 mg) was dissolved in 5 ml of $CHCl_3$ and the absorbance recorded.

(III) Physical parameters

(i) Optical rotation

Each isolated compound (5 mg) was dissolved in 10 ml of $CHCl_3$. The observed optical rotation and specific optical rotation at one or two different wavelengths was recorded.

(ii) Melting point

The melting point of each isolated compound was recorded in a glass capillary tube using the melting point apparatus. The melting points were compared with literature and were uncorrected.

(iii) Boiling point

The boiling points of the oily isolates were determined by the Siwoloboff's method (Furniss*et al*., 1989). Boiling points were uncorrected and compared with literature.

3.2 Extraction Procedures

3.2.1 Collection and drying of plant materials

Ripe and unripe fruits of *Nauclea latifolia* were collected from a farmland in Maikunkele, Bosso Local Government Area, Minna, Niger state, Nigeria in the month of October 2009 and January 2010 respectively and authenticated by Mallam Gallah of Herbarium section, Department of Biological Sciences, Ahmadu Bello University, Zaria, Kaduna state. A herbarium sample voucher (Number 40768) was deposited. Fruits were air-dried for several weeks and powdered.

3.2.2 Extraction of plant materials

Air-dried powdered ripe and unripe fruits of *N. latifolia* (2 kg each) were separately exhaustively extracted with methanol by continous extraction using a flask shaker. The resulting mixture was filtered and concentrated in vacuo using a rotary evaporator. The extract was further dried over a water bath and defatted with petroleum spirit (60 - 80^{o}C). The extracts were weighed and coded **rP** and **uP**, for the petroleum ether extracts of the ripe (r) and unripe (u) fruits respectively, while the residual portion was also concentrated in vacuo, dried, weighed and coded **rM** and **uM** for the methanol extracts of the ripe (r) and unripe (u) fruits respectively.

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3.2.3 Partitioning of crude methanol extract of ripe fruits (rM)

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Four hundred and fifty grams (450 g) of **rM** was suspended in 1 L of distilled water in a 2 L stoppered conical flask. The mixture was shaken vigorously for a few minutes and allowed to stand for 2 hrs. The mixture obtained was initially filtered using a glass wool and again filtered to give a clear filterate, which is the water-soluble (aqueous) portion of the crude methanol extract of ripe fruits of *N. latifolia*. The resulting filtrate was partitioned and extracted by adding 200 ml x 6 portions of chloroform, in a separatory funnel until a colourless organic phase was obtained. The resulting chloroform-soluble fraction was concentrated in vacuo, dried and coded **C**. The residual aqueous portion was again successively and exhaustively extracted with 200 ml x 7 portions of ethylacetate and 200 ml x 10 portions of n-butanol. The resulting organic portions were concentrated separately in vacuo and dried over a water bath to give the ethyl acetate-soluble fraction (coded **E**) and butanol-soluble fraction (coded **B**) respectively. The residual aqueous fraction was also concentrated under reduced pressure, dried and coded (**A)**. They were all stored in a dessicator until required.

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3.2.4 Further extraction of water-insoluble portions of both fruits

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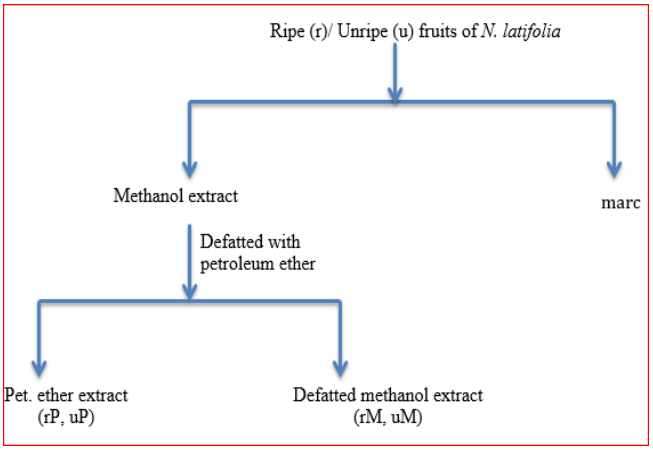
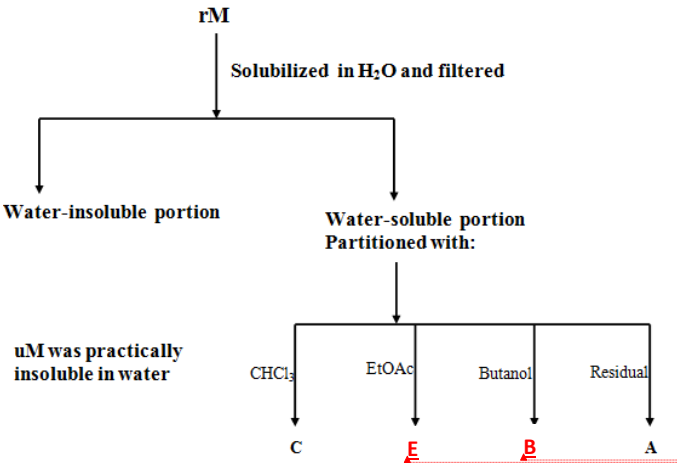
The water-insoluble portion (residue) of methanol extract of both ripe (**rM**) and unripe (**uM**) fruits of *N. latifolia* were both air-dried and separately re-extracted successively

and exhaustively by macerating it with 200 ml x 5 portions of diethyl ether in a stoppered extraction flask and kept in a refrigerator for 5 days. The resulting filtrate was concentrated, dried, weighed and labelled diethylether-soluble fraction, coded **rD** and **uD** for the ripe (r) and unripe (u) fruits respectively. The residual insoluble portion was again successively and exhaustively extracted with 200 ml x 7 portions each of chloroform, ethyl acetate and acetone respectively. The concentrated and dried fractions were labelled chloroform-soluble (coded **rC and uC**), ethylacetate-soluble (coded **rE and uE**) and acetone-soluble (coded **rA and uA**) fractions respectively. The residual water-insoluble portions for both ripe and unripe fruits were also concentrated, dried, weighed and coded **rR and uR** respectively, where **r** and **u** represents ripe and unripe fruits repectively. All were stored in a dessicator until required.

A schematic summary of the overall extraction procedures is presented in Scheme 3.1-3.3.



Scheme 3.1: Schematic representation of extracts obtained from the ripe (r) and unripe (u) fruits of *N. latifolia.*



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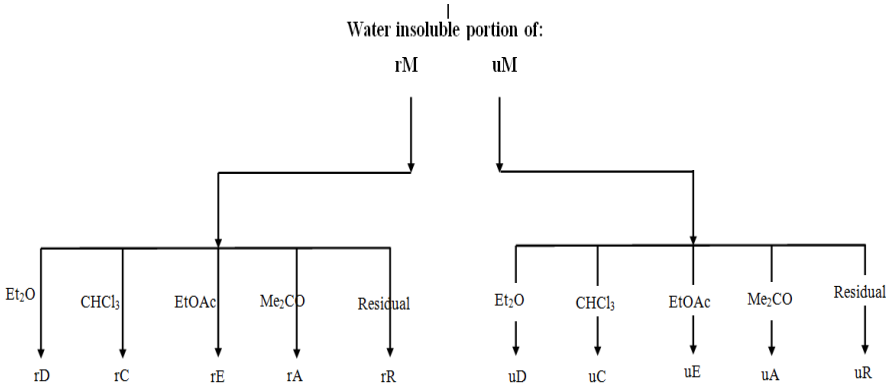
Scheme 3.2: Schematic representation of partitioned soluble-fractions obtained from the defatted methanol extract of ripe (r) fruits of *N. latifolia*



Scheme 3. 3: Schematic representation of fractions obtained from further extraction of water-insoluble residue of the methanol extract of the ripe (r) and unripe (u) fruits of *N. latifolia*

3.3 Preliminary Phytochemical Screening

The various extracts and fractions obtained from the ripe and unripe fruits of *N. latifolia* were screened for their various phytoconstituents using standard methods (Sofowora, 1993b; Evans, 1996; Harbone, 1998; Harbone 2001; Trease and Evans, 2002).

3. 4 Isolation of Compound E-2f1a from the Ethyl acetate Partitioned-soluble Fraction of the Ripe Fruits (E)

3. 4. 1 VLC of fraction E

Ethyl acetate -partitioned- soluble fraction of the methanolic extract of the ripe fruits of *N. latifolia*, **E** (ox-blood gummy mass, 11 g) was subjected to further purification by vacuum liquid chromatography (VLC). The column in the form of a sintered glass Buchner filter funnel was packed with 300 g of silica gel (mesh 60 - 120) and chloroform (300 ml) was poured onto the surface of the silica gel, and then pressure applied. Fraction **E** solubilised with few drops of chloroform was carefully introduced onto the surface of the packing and elution commenced with chloroform. Gradient elution continued with varying proportions of increasing polarity of $CHCl_3$: EtOAc~~chloroform-ethyl acetate~~. Eluates were collected in column fractions of 50 ml each and identical fractions pooled using TLC in various solvent systems to give six major column fractions (**E-1** to **E-6**).~~)~~

3.4.2 Further fractionation of column fraction E-2

Pooled column fraction **E-2** (deep brown gummy mass, 2500 mg) was further fractionated using column chromatography. Silica gel (90 g) was packed into a column

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with petroleum ether (100 %) by the slurry method. The dried **E-2** was homogeneously adsorbed on little silica gel mixed with petroleum ether and gently applied to the top of the prepared column. Elution commenced with petroleum ether and continued with increasing polarity of -PE ~~petroleum ether~~: $CHCl_3$ ~~chloroform~~ and~~;~~ $CHCl_3$ ~~chloroform~~: EtOAc~~ethyl acetate~~. The eluates were collected in fractions of 20 ml each and subjected to chromatographic identification using TLC and chromatograms were examined under sunlight, U.V light, iodine crystals and sprayed with vanillin - H_2SO_4. Identical fractions were combined and concentrated under vacuum to give eight major column sub-fractions (**E-2a** to **E-2h**).

3.4.3 Purification of column sub-fraction E-2f

Pooled sub-fraction **E-2f** (700 mg, golden brown flakes) was further purified by flash chromatography (30 g of silica gel, petroleum ether, slurry method). The dried **E-2f** was gently applied to the top of the prepared column and treated as in section 3.4.2 above using same eluting solvents to yield three major column sub-fractions (**E-2f1** to **E-2f3**). Further purification of sub-fraction **E-2f1** (yellowish-white flakes, 95mg) using flash chromatography yielded a major eluate collected from $CHCl_3$: EtOAc (19: 1). The eluate revealed a homogenous compound (based on TLC) that was concentrated in vacuo to yield colourless oil, coded compound **E-2f1a**.

3.4.4 Characterization of compound E-2f1a

Physical parameters, colour reactions and spectral analysis (proton NMR, carbon - 13 NMR, DEPT - 135 and GC - MS) were carried out to elucidate structure of compound

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E-2f1a.

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3.4.4.1 Colour reactions of compound E-2f1a

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(i) 2, 4-Dinitrophenylhydrazine test

To a few drops of compound **E-2f1a**, a solution of 2, 4-dinitrophenylhydrazine (3 ml) was added and shaken. Formation of an orange-red precipitate of phenylhydrazone indicates the presence of a carbonyl group (Furniss *et al*., 1989).

(ii) Ammoniacal silver nitrate test (Tollen's solution)

To 3ml of Tollen's solution in a test tube, a few drops of compound **E-2f1a** was added and warmed over a water bath. Formation of a silver mirror on the inside of the test tube is indicative of an aldehyde (Furniss *et al*., 1989).

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(iii) Fehling's test

To 2 drops of compound **E-2f1a,** 2mls of a mixture of Fehling's A and B solution was added and the mixture heated on a water bath for 3 minutes. Formation of a red precipitate of copper (1) oxide is taken as positive for aldehydes (Furniss *et al*., 1989)

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3.5 ——Isolation of Compound rA-5a1 from the Acetone-soluble Fraction of the Ripe Fruits (rA)

3.5.1 —Chromatographic separation of fraction rA

Acetone-soluble fraction of ripe fruits, rA (reddish-brown gummy mass, 40 g) was subjected to VLC separation as in section 3.4 to yield eight major column fractions coded, -rA-1 to rA-8. Further column chromatographic separation of column fraction rA-5 (deep brown gummy mass, 8 g) using silica gel (mesh 230 - 400, 160 g) and varying proportions of PE~~petroleum ether~~: $CHCl_3$, $CHCl_3$: EtOAc as the mobile phase gave three major sub-fractions (rA-5a to rA-5c). Flash chromatographic purification of column sub-fraction rA-5a (golden-yellow oily mass, 140 mg) yielded a single spot on TLC. Compound was coded rA-5a1.

3.5.2 Characterization of compound rA-5a1

Physical parameters and spectral analysis was carried out to elucidate structure of compound rA-5a1.

3.6 ————Isolation of Compounds uE-2a1 and uE-2a2 from the Ethyl acetate-soluble Fraction of the unripe fruits (uE)

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3.6.1 Separation of fraction uE

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Ethyl acetate-soluble fraction of methanol extract of the unripe fruits of *N. latifolia*, **uE** (greenish brown gummy mass, 30 g) was subjected to VLC. Five major column fractions were collected (**uE-1** to **uE-5**) and column fraction **uE−2** (6 g, dark green gummy mass) was further separated by flash column chromatography using silica gel (mesh 230 - 400, 120 g) and increasing polarity of PE~~petroleum ether~~: $CHCl_3$, $CHCl_3$: EtOAc and EtOAc (100 %). This yielded nine major column sub-fractions, coded **uE-2a** to **uE-2i**.

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3.6.2 Preparative TLC of sub-fraction uE-2a

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A solution of **uE-2a** in chloroform was applied in form of a transverse band across the chromatographic plate, air-dried and developed in hexane: $CHCl_3$ (6:4). Individual longitudinal bands were identified using UV lamp. The two major bands were scrapped individually and the resulting silica gel mixture triturated with acetone, filtered and concentrated in vacuo. This gave rise to two individual spots on TLC, coded compounds **uE-2a1** and **uE-2a2**.

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3.6.3 Characterization of compound uE-2a1

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-Structure of compound **uE-2a1** was elucidated using physical parameters and spectral analysis.

3.6.4 Characterization of compound uE-2a2

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Structure of compound **uE-2a2** was elucidated using physical parameters, colour reactions and spectral analysis

3.6.4.1 Colour reactions of compound uE-2a2

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(i) 2, 4-Dinitrophenylhydrazine test (section 3.4.4.1)

(ii) Hydrolysis of esters

To a little quantity of compound **uE-2a2**, 2 ml of water to which has been added 1 drop of 1 ml NaOH and a trace of phenolphthalein indicator was added. The solution was gently warmed on a water bath with further dropwise addition of NaOH until the pink colour was discharged. A very slow discharge of the pink colour indicates that an ester is been hydrolysed (Furniss *et al*., 1989).

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3.7 Isolation of Compound uE-3a2a from the Ethyl acetate-soluble Fraction of the Unripe Fruits (uE)

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3.7.1 Purification of column fraction uE-3

Column fraction **uE-3** (a golden brown mass, 9 g) obtained from **(**section 3.6.1) was further fractionated using flash column chromatography (silica gel mesh 60 - 120, 200 g) and increasing polarity of petroleum ether: $CHCl_3$ and $CHCl_3$: EtOAc. Solvent mixture $CHCl_3$: EtOAc (9:1) yielded a column sub-fraction, coded **uE-3a** (yellowish-white mass, 300 mg), 5 spots on TLC with $CHCl_3$: EtOAc (4:1) which on further flash chromatographic separation yielded a major promising sub-fraction from solvent system

$CHCl_3$: EtOAc (8:2) coded **uE-3a2** (whitish-yellow flakes, 71 mg). Its TLC revealed 2 major spots with PE: $CHCl_3$ (4:1) + 3 drops of EtOAc which on further purification using sephadex LH - 20 yielded a major isolate with slight impurities. The isolate was washed severally with methanol to yield a pure compound, coded **uE-3a2a.**

3.7.2 Characterization of compound uE-3a2a

The isolated compound was characterized based on physical, colour reactions and spectral parameters.

3.7.2.1 Colour reactions of compound uE-3a2a

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(i) Test for alcohol

Cerric ammonium nitrate (4 g) was dissolved on mild heating in 10 ml of 2N HNO_3. A few crystals of compound **uE-3a2a** were dissolved in 0.5 ml of dioxane and the solution added to 0.5 ml of cerric ammonium nitrate and diluted to 1 ml with dioxane solution and thoroughly shaken. Emergence of a yellow color that gradually changed to red indicates the presence of an alcoholic hydroxyl group (Harbone, 2001~~2~~).

(ii) Liebermann-Burchard's test

A few crystals of compound **uE-3a2a** were dissolved in chloroform and a few drops of concentrated sulphuric acid added followed by addition of 3 drops of acetic anhydride. A change in colour from violet-blue to green was taken as positive for the presence of a steroidal nucleus (Harbone, 1998, 2001~~2~~).

(iii) Salkowski's test

A few crystals of compound **uE-3a2a** were dissolved in chloroform and a few drops of conc sulphuric acid added gently down the side. Appearance of a reddish-brown color in the chloroform layer was taken as an indication of the presence of a steroidal compound (Harbone, 1998, 20012).

3.8 Antibacterial Studies

3.8.1 Standard drugs/test organisms

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(i) Reference/standard drugs

Chloramphenicol (Ningbo Shuangwei Pharmaceutical Ltd., Zhejiang, China)

Erythromycin (Falma Laboratories, India)

Tetracycline (La Tetra-250, Me-Cure Industries, Ltd, Lagos, Nigeria)

(ii) Test organisms

Gram-positive strains: *Bacillus subtilis and Staphylococcus aureus*

Gram-negative strains*: Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa and Salmonella typhi.*

All organisms were obtained from the Department of Microbiology, Federal University of Technology, Minna, Niger state, Nigeria.

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3.8.2 Preparation of materials and test organisms

(i) Nutient broth: Nutrient broth powder (3.25 g) was dissolved in 50 ml of distilled water by boiling. 5 mls and 20 mls of the prepared broth were dispensed into sterile universal bottles and autoclaved at 121^{o}C for 20 mins. Cooled nutrient broth was used as media for standardization of test organisms.

(ii) Standardization of test organisms: Cooled nutrient broth (5 ml) was inoculated with a loopful of organism and incubated for 24 hrs. 0.2 ml of the overnight culture of the organism was dispensed into 20 ml of sterile nutrient broth and incubated for 18 hrs to standardize the culture to 10^6 cfu/ml. This was done separately for all the test organisms used. A loopful of these standard cultures was used for the antibacterial assay (Olukoya *et al*., 1986; Babayi *et al*., 2004).

(iii) Concentration of test compounds: Petroleum ether-based test compounds were dissolved in petroleum ether (~~10, 50,~~ 100 mg/ml), while methanolic-based test compounds were dissolved in methanol (~~10,~~ 50 mg/ml). Petroleum ether and methanol were set up as negative controls. Reference drugs were dissolved in sterile water (1 mg/ml) and set up as positive controls.

3.8.3 Data analysis

The diameter of the zone of inhibition around each hole was measured and recorded at the end of incubation period. The average of duplicate independent readings for each test organism was recorded. Data were represented as mean ± standard error of the mean (SEM). Comparisons between groups were performed using analysis of variance (ANOVA) coupled with Duncan multiple range test where significance were observed. Analysiswas considered significant at $P < 0.05$ levels of significance. All the analyses

were performed using Microsoft Excel 2007 and Statistical Packages for Social Sciences, 20th version.

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3.8.4 Antibacterial assays

3.8.4.1 Determination of antibacterial activity of test compounds

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The agar-well diffusion method was employed (Dall' Agnol *et al.*, 2003) to screen the ~~various~~ extracts (100 mg/ml), partitioned-soluble fractions (50 mg/ml) and soluble fractions of the ripe (50 mg/ml) and unripe fruits (50 mg/ml) of *N. latifolia* (test compounds) for their antibacterial activity in comparison with some standard antibiotics (1 mg/ml)~~drugs~~.

Molten and cooled agar (20 ml) was poured into sterile Petri dishes asceptically. On solidification, plates were separately inoculated with a loopful of the standardized overnight cultures of the test organisms by the streaking method. Four holes (8 mm in diameter) were asceptically bored into the solidified agar using a sterile cork borer at equidistance. Each test compound ~~(10 mg/ml), standard drugs (1 mg/ml)~~ and solvent blanks (petroleum ether and methanol) were fed into each hole with the aid of a micropipette, ensuring that no spillage occurred. Plates were allowed to stand for an hour at room temperature for adequate diffusion of the test compounds into the agar. Plates were incubated at 37^{o}C for 24 hrs. Zones of inhibition around the holes were measured to the nearest millimetre using a metre rule. Procedure was repeated for extracts (50 and 100 mg/ml) and fractions (50 mg/ml) each. A test compound is considered 'active', when it has an inhibition zone of ≥ 14 mm (Mothana and Linderquist, 2005).

3.8.4.2 Determination of antibacterial percent activity (A %) and bacterial susceptible index (BSI)

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These parameters were determined for the various test compounds/test organisms using the equations below **(**Eloff, 2004; Mahlke *et al*., 2009)

$$A\% = \frac{100 \times \text{susceptibile bacterial strains to a specific extracts/fractions}}{\text{Total number of bacterial strains tested}}$$

$$BSI = \frac{100 \times \text{number of extracts/fractions effective against each bacterial strains}}{\text{Total number of extracts/fractions}}$$

3.8.4.3 Determination of minimum inhibitory concentrations (MIC)

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Test compounds that showed significant antibacterial activities (≥ 14 mm) from above were selected for the determination of their MICs using the microdilution/serial tube dilution technique (Reiner, 1982; Collins *et al.*, 1995; Andrews, 2001). This was determined by examining the test bacterial strain's ability to grow in broth cultures containing different concentrations of test compounds. A stock solution of each active extract (100 mg/ml), partitioned-fractions (50 mg/ml), soluble-fractions (50 mg/ml) was prepared using petroleum ether or methanol as diluant. Six tubes containing 1 ml of sterile nutrient broth and marked 1 - 6 were cotton plugged and sterilized in an autoclave for 30 mins. Tubes were allowed to cool and 1ml of the test compound was added to the 1st tube and mixed thoroughly. 1 ml of this content was transferred to the 2nd tube, then content of the 2nd tube was again thoroughly mixed and 1 ml of the mixture was transferred to the 3rd tube. This process of serial dilution was continued up

to the 6th tube. 10 µl containing 10^6 cells/ml of the inoculum was added to each of the six tubes and mixed thoroughly. All tubes were incubated at 37°C for 24 hrs and examined for visible growth or turbidity. The minimum inhibitory concentration (MIC) is a quantitative test used to determine the lowest concentration of the test compounds that inhibited the macroscopic growth of the organisms used in the study *in-vitro*. All determinations were carried out in duplicates and the mean recorded.

3.8.4.4 Determination of minimum bactericidal concentrations (MBC)

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This was determined by assaying for live organisms in those tubes from the MIC determination~~tests~~ of test compounds that showed no growth after 24 hrs of incubation. A loopful of broth was collected from each of those tubes that showed no turbidity, transferred and sub cultured into freshly prepared sterilized nutrient agar. Inoculated plates were incubated at 37°C for 24 hrs and examined for visible growth or turbidity. The highest dilution or lowest concentration that yielded no single bacterial colony on the solid medium after 24 hrs was taken as MBC (Nester *et al*., 2007).

3.8.4.5 Determination of observed antibacterial effects of test compounds

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The MBC/MIC ratio of the test compounds was calculated to determine whether the observed antibacterial effect(s) of the test compounds was bacteriostatic or bactericidal by adopting the method of Agnese *et al* (2001).

3.8.4.6 Determination of antibacterial activities of column fractions, column sub-fractions and some isolated compounds

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Column fractions **E-1** to **E-6**, **rA-1** to **rA-8** and **uE-1** to **uE-5** and column sub-fractions **E-2a** to **E-2h, rA-5a** to **rA-5c** and **uE-2a** to **uE-2i** obtained from the fractionation of the

ethyl acetate -partitioned--soluble fraction of the ripe fruits (**E**), acetone-soluble fraction of the ripe fruits (**rA**) and ethyl acetate-soluble fraction (**uE**) of the unripe fruits of *N. latifolia* respectively were screened for their antibacterial activity at 20 mg/ml each. Also, antibacterial activity of some of the isolated compounds; **rA-5a1, uE-2a1** and **uE-3a2a** at 100 μg/ml each were determined in comparison with that of erythromycin (1 ~~at 1~~ mg/ml).~~ml.~~

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CHAPTER FOUR

4. 0 RESULTS

4.1 ——Description of Extracts and Fractions of Ripe and Unripe Fruits

Extraction, partitioning and further extraction of the ripe (r) and unripe (u) fruits of *N. latifolia* yielded the following extracts, partitioned-soluble fractions and re-extracted soluble fractions as shown in Table 4.1

Table 4.1: Description and yields of crude extracts and fractions

*Extract / Fraction	Code	Colour and appearance	Weight (g)	% Yield
Petroleum ether extract	**rP**	Greenish-brown oily mass	12.37	0.62
Methanol extract	**rM**	Reddish-brown gummy mass	458.20	22.90
Petroleum ether	**uP**	Dark green oily mass	10.22	0.51
Methanol extract	**uM**	Brownish-orange jelly-like mass	421.20	21.10
Chloroform-soluble fraction	**C**	Deep-brown gummy mass	5.80	1.27
Ethyl acetate-soluble fraction	**E**	Ox-blood gummy mass	11.88	2.59
Butanol-soluble fraction	**B**	Deep-red gummy mass	63.30	13.80
Residual aqueous fraction	**A**	Reddish-brown gummy mass	162.84	35.50
Chloroform-soluble fraction	**rC**	Yellowish-red solid mass	5.20	1.23
Chloroform-soluble fraction	**uC**	Greenish-brown mass	26.81	6.37
Diethyl ether-soluble fraction	**rD**	Reddish-brown gummy mass	8.70	2.07
Diethyl ether-soluble fraction	**uD**	Dark brown gummy mass	18.44	4.38
Ethyl acetate-solublefraction	**rE**	Reddish-brown oily mass	8.59	2.04
Ethyl acetate-solublefraction	**uE**	Greenish-brown gummy mass	35.50	8.43
Acetone-soluble fraction	**rA**	Reddish-brown gummy mass	49.20	10.70
Acetone-soluble fraction	**uA**	Golden-brown gummy mass	57.70	13.70
Residual fraction	**rR**	Reddish-brown solid mass	140.90	30.80
Residual fraction	**uR**	Brownish-orange solid mass	280.89	66.70

*Extracts of ripe and unripe fruits (Petroleum ether, **rP** and **uP;** Methanol, **rM** and **uM**); partitioned-soluble fractions of ripe fruits (chloroform **C,** ethyl acetate **E,** butanol **B** and residual aqueous **A**); re-extracted soluble fractions of ripe (r) fruits (chloroform **rC,** diethyl ether **rD,** ethyl acetate **rE,** acetone **rA** and residual **rR**) and re-extracted soluble fractions of unripe (u) fruits (chloroform **uC,** di ethyl ether **uD,** ethyl acetate **uE,** acetone **uA** and residual **uR**) of *N. latifolia.*

4.2 ——Preliminary Phytochemical Screening

Result of preliminary phytochemical screening of extracts, partitioned-soluble fractions and re-extracted soluble fractions of both ripe and unripe fruits using standard methods is presented in Table 4.2

Table 4.2: Phytoconstituents present in extracts, partitioned-soluble -fractions and re-extracted soluble -fractions of ripe (r) and unripe (u) fruits of *N. latifolia*

*Extract/ Fraction	Carbohydrates	Tannins	Phlobatannins	Saponins	Flavonoids	Steroidal sapogenins	Anthraquinones	Coumarins	Tetraterpenoids	Alka
rP	–	–	–	–	–	+	–	+	–	–
uP	–	–	–	–	–	+	–	+	–	–
rM	+	+	–	+	+	+	+	+	–	+
uM	+	+	–	+	+	+	–	+	–	+
C	+	+	–	+	+	+	+	+	–	+
E	+	+	–	+	+	+	+	+	–	+
B	+	+	–	+	+	+	+	+	–	+
A	+	–	–	+	+	–	–	–	–	+
rC	–	–	–	–	+	+	–	+	–	–
uC	–	+	–	–	+	+	–	+	–	+
rD	–	–	–	–	+	+	–	+	–	+
uD	–	+	–	–	+	+	–	+	–	+
rE	+	+	–	+	+	+	–	+	–	+
uE	+	+	–	+	+	+	–	+	–	+
rA	+	+	–	+	+	+	–	+	–	+
uA	+	+	–	+	+	+	–	+	–	+
rR	+	+	–	+	–	–	–	+	–	–
uR	+	–	–	–	–	–	–	+	–	–

+ = Present; –= Absent

*Extracts of ripe and unripe fruits (Petroleum ether, **rP** and **uP;** Methanol, **rM** and **uM**); partitioned-soluble fractions of ripe fruits (chloroform **C,** ethyl acetate **E,** butanol **B** and residual aqueous **A**); re-extracted soluble fractions of ripe (r) fruits (chloroform **rC,** diethyl ether **rD,** ethyl acetate **rE,** acetone **rA** and residual **rR**) and re-extracted soluble fractions of unripe (u) fruits (diethyl ether **uD,** chloroform **uC,** ethyl acetate **uE,** acetone **uA** and residual **uR**) of *N. latifolia.*

4.3 Isolation of Compound E-2f1a

4.3.1 VLC separation of ethyl acetate partitioned-soluble fraction of methanol extract of ripe fruits (E)

Purification of E by VLC gave rise to 6 major fractions that were pooled based on similar TLC profiles as shown in Table 4.3

Table 4. 3: Description of major column fractions from **E**

Column fractions	Solvent system	Code	Physical appearance	Weight (g)
2-18	$CHCl_3$ (100%)	**E-1**	Brown mass	1.16
19-26	$CHCl_3$: EtOAc (9: 1)	**E-2**	Deep-brown mass	3.26
27-42	$CHCl_3$: EtOAc (8: 2)	**E-3**	Yellowish-brown mass	1.93
43-63	$CHCl_3$:EtOAc (5: 5)	**E-4**	Deep brown mass	0.72
64-69	$CHCl_3$: EtOAc (2: 8)	**E-5**	Deep brown mass	1.32
70-112	EtOAc (100%)	**E-6**	Reddish brown mass	2.10

4.3.2 Column chromatography of column fraction E-2

Column chromatographic separation of column fraction **E-2** yielded sub-fractions as shown in Table 4. 4

Table 4.4: Description of major column sub-fractions collected from separation of column fraction **E−2**

Column sub-fractions	Solvent system	Code	Colour and appearance	Weight (g)
5-13	PEet ether: $CHCl_3$ (9:1)	**E-2a**	Reddish-brown mass	0.08
-14- 21	PE:$CHCl_3$ (8:2)	**E-2b**	Deep-brown mass	0.11
22- 30	PE:$CHCl_3$ (7:3)	**E-2c**	Golden- brown mass	0.14
31- 49	PE:$CHCl_3$ (5:5)	**E-2d**	Deep- brown mass	0.17
50-64	PE:$CHCl_3$ (2: 8)	**E-2e**	Golden-brown mass	0.30
65-86	$CHCl_3$ (100%)	**E-2f**	Golden-brown flakes	0.85
87-93	$CHCl_3$: EtOAc (9:1)	**E-2g**	Brown flakes	0.52

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94-106	$CHCl_3$: EtOAc ,, (8: 2)	**E-2h**	Deep brown mass	0.24

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4.3.3 Chromatographic separation of sub-fraction E-2f

Flash chromatographic separation of sub-fraction **E-2f** yielded 3 promising column sub-fractions as shown in Table 4. 5

Table 4.5: Sub-fractions collected from separation of column sub-fraction **E-2f**

Column sub-fraction	Solvent system	Code	Number of spots observed on TLC
1	PE (100%)		No spot observed
2	PE: $CHCl_3$ (9:1)		,,
3	PE: $CHCl_3$ (8:2)		,,
4	PE: $CHCl_3$ (7:3)		,,
5	PE: $CHCl_3$ (6:4)		,,
6	PE: $CHCl_3$ (5:5)		,,
7	PE: $CHCl_3$ (3:7)		,,
8	PE: $CHCl_3$ (2:8)		Several spots (PE: EtOAc, 4:1)
9	$CHCl_3$ (100%)	**E-2f1**	1 major with 2 minor spots ($CHCl_3$: EtOAc, 4:1)
10	$CHCl_3$ (100%)	,,	,,
11	,,	,,	,,
12	,,	,,	,,
13	$CHCl_3$: EtOAc (9:1)	**E-2f2**	1 major spot (negligible quantity, $CHCl_3$: EtOAc, 4:1; R_f 0.52)
14	$CHCl_3$: EtOAc (9:1)	,,	,,

15	$CHCl_3$: EtOAc (8:2)	**E-2f3**	2 spots ($CHCl_3$: EtOAc, 4:1 + 3 drops of MeOH; R_f 0.62 and 0.64)
16	$CHCl_3$: EtOAc (7:3)	,,	,,

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4.3.4 Further fractionation of sub-fraction E-2f1

Further purification of column sub-fraction **E-2f1** using flash chromatography gave rise to a major column sub-fraction as shown in Table 4.6

Table 4.6: Sub-fractions from separation of sub-fraction **E-2f1**

Sub-fraction	Solvent system	Code	Number of spots observed on TLC
1	Pet ether (100%)		No spot observed
2	PE: $CHCl_3$ (8:2)		,,
3	PE: $CHCl_3$ (5:5)		,,
4	PE: $CHCl_3$ (2:8)		,,
5	PE: $CHCl_3$ (1:9)		2 spots ($CHCl_3$, 100%)
6	$CHCl_3$ (100%)		3 spots (PE: EtOAc 19:1)
7	$CHCl_3$ (100%)		,,
8	$CHCl_3$: EtOAc (19:1)	**E-2f1a**	1 spot (PE: EtOAc 9:1)
9	$CHCl_3$: EtOAc (19:1)	**,,**	,,
10	$CHCl_3$: EtOAc (19:1)	**,,**	,,
11	$CHCl_3$: EtOAc (19:1)	**,,**	,,
12	$CHCl_3$: EtOAc (9:1)		2 spots ($CHCl_3$: EtOAc 4:1)
13	$CHCl_3$: EtOAc (8:2)		No spot observed
14	$CHCl_3$: EtOAc (7:3)		,,

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4.3.5 Characterization of compound E-2f1a

4.3.5.1 Physical characterization

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Concentration of **E-2f1a** in vacuo gave rise to colourless oil (14.2 mg). The compound gave a single spot in petroleum ether: EtOAc (9:1) and petroleum ether: EtOAc (4:1) with R_f values of 0.42 and 0.68 respectively. The spot was colourless (sunlight), golden brown (I_2 crystals) and reddish-brown when sprayed with vanillin - H_2SO_4.

The compound was soluble in $CHCl_3$, EtOAc and Me_2CO slightly soluble in petroleum ether and insoluble in MeOH and H_2O.

The compound was found to have a melting and boiling points of 44 - 46.6^oC and 204 - 204.9^oC respectively. Optical rotation of the compound was calculated to be $[\alpha]^{20}_{D\,(589nm)}$+10.0 (c = 0.0005 g/ml of $CHCl_3$; 1dm^3; α = 0.005)

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4.3.5.2 Colour reactions

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Compound **E-21fa** gave an orange-red precipitate with 2, 4-dinitrophenylhydrazine (hydrazone formation), while, it gave a deposit of Ag^+ ions with the Tollen's reagent.

The compound did not form a red precipitate of copper (1) oxide with a solution of Fehling's A and B on warming (Furniss *et al*., 1989).

4.3.5.3 Spectral characterization

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The proton NMR, carbon - 13 NMR, Dept - 135 and GC - MS spectra of compound **E-2f1a** are presented in Figures 4.1 - 4.4, while a summary of the various peaks obtained in each spectra are presented in Tables 4.7 - 4.10 respectively.

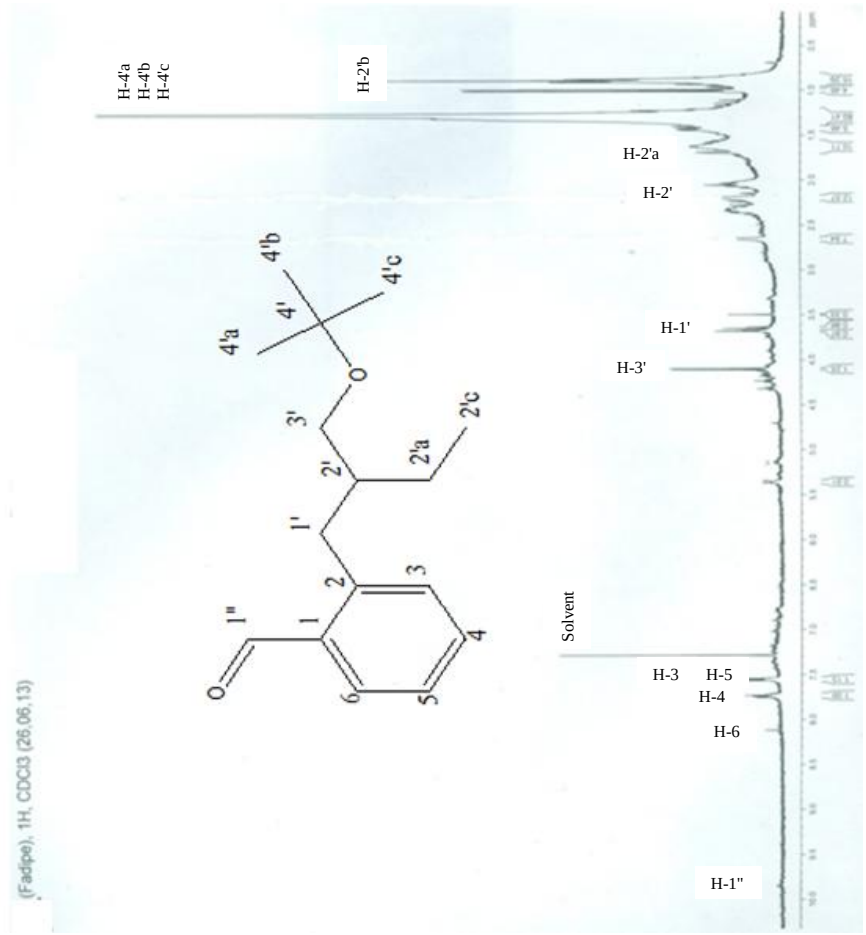

Figure 4. 1: ^{1}H - NMR spectrum of compound **E-2f1a**

Table 4.7: Summary of ^{1}H - NMR spectral data of compound **E-2f1a**

Position	^{1}H (ppm)	Assignment	Literature values (ppm)*
H-1"	9.85 (weak singlet)	-CHO	9.90
H-6	8.13 (weak singlet)	Ar-H	8.09
H-4	7.75 (weak triplet)	Ar-H	7.77
H-3 and H-5	7.54 (weak doublet of doublet)	Ar-H	7.48 - 7.60
	7.3 (singlet)	Solvent singlet of $CDCl_3$	
H-1'	3.66 (weak multiplet)	Ar-CH-	3.73, 3.66
H-2'	2.10 (multiplet)	$-CH-CH_2CH_3$	2.09
H- 3'	4.15 (multiplet)	-CH-O-	4.16, 4.35
H-4'a, b, c	1.36 (sharp singlet)	$-(CH_3)_3$	1.35
H-2'a	1.55 (mutiplet)	$-CH_2-CH-$	1.54
H-2'b	0.93 (triplet)	$-CH_2-CH_3$	0.94

*ACD/ChemDraw (Product Version 15); Abraham *et al*., 2002; Field *et al*., 2005; Abraham and Mobli, 2008; Tanc *et al*., 2014; Hussein, 2015

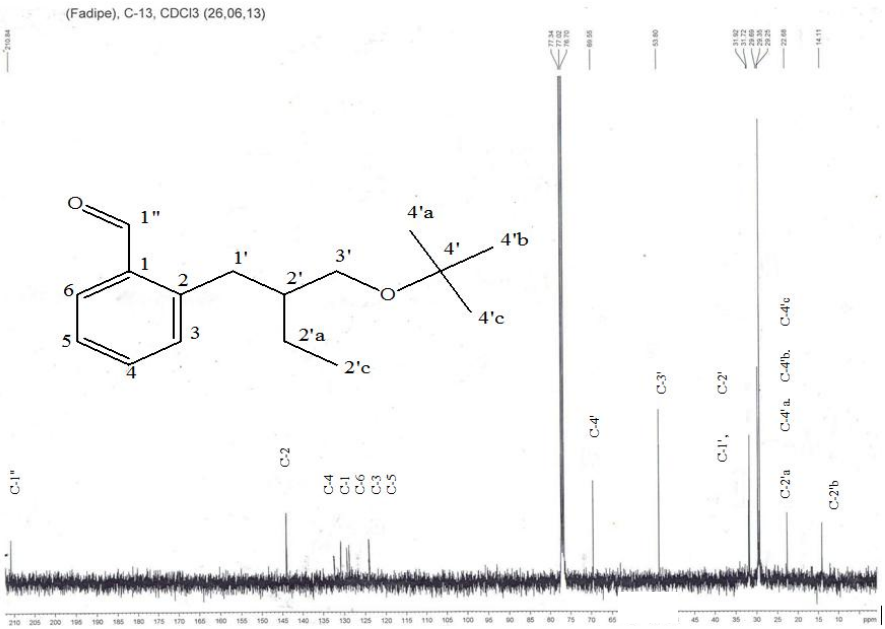

Figure 4. 2: ^{13}C - NMR spectrum of compound **E-2f1a**

Table 4.8: Summary of ^{13}C - NMR spectral data of compound **E-2f1a**

Position	^{13}C(ppm)	Assignment	Literature values (ppm)*
C-1"	210.8	-CHO	210.9
C-1	132.4	-Ar-C	132.6
C-2	144.1	,, (ortho-substituted)	144.1
C-3	127.3	,, (meta-position)	127.7
C-4	133.2	,, (para-position)	133.4
C-5	124.7	,,	124.5
C-6	129.5	,,	129.7
C-1'	31.9	Ar-CH_2 (ortho-position)	31.6
C-2'	31.7	-CH-CH_2 (CH_3)	31.4
C-3'	53.8	-CH_2-O-	53.8
C-4'	69.6	O-CR_3	70.4
C-4'a	29.7	-C (CH_3) $_3$	29.9
C-4'b	29.4	,,	29.8
C-4'c	29.3	,,	29.5
C-2'a	22.7	-CH (CH_2)-	22.5
C-2'b	14.1	-CH (CH_2CH_3)	13.9

*ACD/ChemDraw (Product Version 15), Abraham *et al*., 2002; Field *et al.*, 2005; Abraham and Mobli, 2008; Nummert *et al*., 2009; Tanc *et al*., 2014; Hussein, 2015.

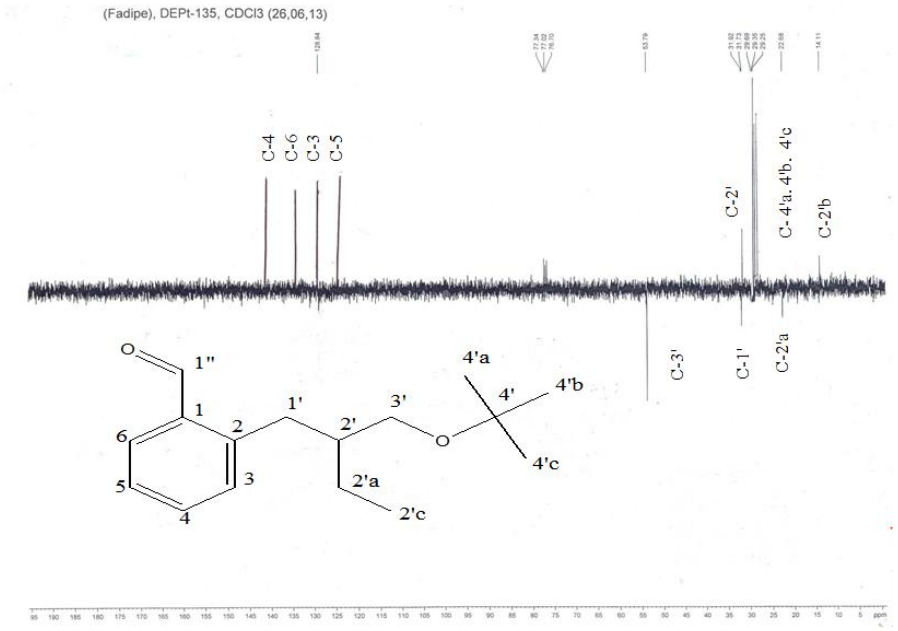

Figure 4. 3: DEPT - 135 spectrum of compound **E-2f1a**

Table 4.9: Summary of DEPT - 135 spectral data of compound **E-2f1a**

Position	DEPT (ppm)	Assignment	*Literature values
C-1"	210.8	Quartenary C, disappeared in the spectrum (-CHO)	210.9
C-1	132.4	,, (Ar-C)	132.6
C-2	144.1	,,	144.1
C-3	127.3	-CH- (above in the spectrum)	127.7
C-4	133.2	,,	133.4
C-5	124.7	,,	124.5
C-6	129.5	,,	129.7
C-1'	31.9	$-CH_2-$ (below in the spectrum)	31.6
C-2'	31.7	-CH- (above)	31.4
C-3'	53.8	$-CH_2-$ (below)	53.8
C-4'	69.6	Quartenary C, disappeared in the spectrum ($-CR_3$)	70.4
C-4'a	29.7	$-CH_3$ (above)	29.9
C-4'b	29.4	,,	29.8
C-4'c	29.3	,,	29.5
C-2'a	22.7	$-CH_2-$ (below)	22.5
C-2'b	14.1	$-CH_3$ (above)	13.9

*Tanc *et al.*, 2014; Hussein, 2015

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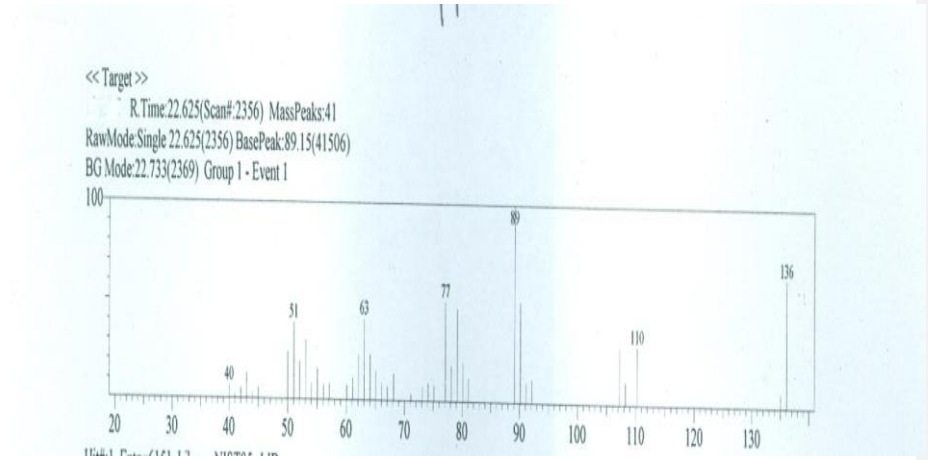

Figure 4.4: GC - MS spectrum of compound **E-2f1a**

Table 4.10: Summary of GC - MS spectral data of compound **E-2f1a**

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Molecular formular	m/z of fragment ion	Assignment
CHO^+	29	Generation of an aldehydic cation
$[C_3H_3]^+$	39	,, of a propyl cation from phenyl ion
$[C_4H_3]^+$	51	,, of a butyl cation from phenyl ion
$[C_4H_5]^+$	53	,,
$[C_5H_3]^+$	63	,, of a cyclopentadienyl cation
$[C_6H_5]^+$	77	,, of a phenyl (aryl) ion
$[C_7H_5]^+$	89 (base peak)	,, of a benzyl cation
$[C_7H_6]^+$	90	Formation of a tropylium ion

4.4 Isolation of Compound rA-5a1

4.4.1 VLC separation of acetone-soluble fraction of methanol extract of ripe fruits (rA)

VLC separation of **rA** using various solvents yielded 8 major column fractions as shown in Table 4.11

Table 4.11: Result of vacuum liquid chromatography of **rA**

Column fractions	Solvent system	Code	Physical appearance	Weight (g)	% Yield
1-14	Pet. ether (100%)	**rA-1**	Yellow oil	2.88	7.20
15-21	PE:$CHCl_3$ (80:20)	**rA-2**	Golden brown mass	0.92	2.30
22-34	$CHCl_3$:MeOH (70:30)	**rA-3**	Brown gummy mass	4.01	10.0
35- 52	$CHCl_3$:MeOH (50:50)	**rA-4**	Deep brown gummy mass	6.13	15.3
53- 85	EtOAc (100%)	**rA-5**	,,	10.09	25.2
86- 106	EtOAc:MeOH (90:10)	**rA-6**	,,	7.15	17.9
107- 121	EtOAc:MeOH (80:20)	**rA-7**	,,	3.67	9.18
122- 143	EtOAc:MeOH (50:50)	**rA-8**	Reddish-brown	4.28	10.7

			gummy mass

4.4.2 Fractionation of column fraction rA-5

Column chromatographic separation of pooled fractions 53 - 85 (**rA-5)** yielded 3 major column sub-fractions as shown in Table 4.12

Table 4.12: Description of sub-fractions from column fraction **rA-5**

Sub-fraction	Solvent system	Code	Number of spots observed in TLC
1	Pet ether (100%)		No spot observed
2	PE: $CHCl_3$ (8:2)		,,
3	PE: $CHCl_3$ (6:4)	**rA-5a**	2 spots (PE: $CHCl_3$, 1: 1)
4	PE: $CHCl_3$ (5:5)	,,	,,
5	PE: $CHCl_3$ (3:7)	**,,**	,,
6	PE: $CHCl_3$ (2:8)	**,,**	,,
7	$CHCl_3$ (100%)	**rA-5b**	4 spots ($CHCl_3$: EtOAc, 4:1)
8	$CHCl_3$: EtOAc (9:1)	**,,**	,,
9	$CHCl_3$: EtOAc (8:2)	**,,**	,,
10	$CHCl_3$: EtOAc (5:5)	**rA-5c**	2 spots ($CHCl_3$: EtOAc, 1:1)
11	$CHCl_3$: EtOAc (7:3)	**,,**	,,
12	$CHCl_3$: EtOAc (6:4)	**,,**	,,

13	$CHCl_3$: EtOAc (5:5)	„	„

4.4.3 Fractionation of sub-fraction rA-5a

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Pooled column sub-fractions rA-5a (golden-yellow oily mass, 140 mg) was further purified using flash chromatography. Collected sub-fractions are as shown in Table 4.13

Table 4.13: Column sub-fractions from separation of column sub-fraction **rA-5a**

Sub-fraction	Solvent system	Code	Number of spots observed in TLC
1	Hexane (100%)		No spot observed
2	Hex: $CHCl_3$ (19:1)		„
3	Hex: $CHCl_3$ (9:1)		„
4	Hex: $CHCl_3$ (8:2)		1 major spot (negligible quantity)
5	Hex: $CHCl_3$ (7:3)		2 spots (PE: $CHCl_3$, 1:1)
6	Hex: $CHCl_3$ (6:4)		„
7	Hex: $CHCl_3$ (5:5)		„
8	Hex: $CHCl_3$ (4:6)		1 major spot (negligible quantity)
9	Hex: $CHCl_3$ (3:7)		„
10	Hex: $CHCl_3$ (2:8)	**rA-5a1**	1 spot (PE: EtOAc, 9:1)
11	Hex: $CHCl_3$ (1:9)	„	„
12	$CHCl_3$ (100%)		2 spots (PE: EtOAc, 4:1)

4.4.4 Characterization of compound rA-5a1

4.4.4.1 Physical characterization

Concentration of column sub-fraction **rA-5a1** in vacuo gave rise to bright yellow oil (32.6 mg) with a slight odour. TLC profile of the compound revealed a single spot in Petroleum ether: $CHCl_3$ (4:1); R_f 0.45, Petroleum ether: EtOAc (9:1); R_f 0.52 and Petroleum ether: $CHCl_3$ (1:1); R_f 0.63. The spot was colourless (sunlight), UV (red), golden brown (I_2 crystals) and gave a red colouration on spraying with anisaldehyde-sulphuric acid.

The compound was soluble in petroleum ether, $CHCl_3$, Me_2CO and CH_3OH, while it was insoluble in water.

GC - MS revealed the molecular formula and molecular weight of the compound to be $C_{24}H_{38}O_4$ and 391 $gmol^{-1}$ respectively.

The compound was found to have a melting pointof -56 to -54.4^oC and boiling point of 382 – 383.6^oC. The optical rotations of the compound at 2 different wavelengths was calculated as $[\alpha]^{22}_{D\,(589nm)}$ 0.588 (c = 0.017 g/ml of $CHCl_3$; 1dm^3; α = 0.001) and $[\alpha]^{22}_{D\,(633nm)}$**:** 1.176 (c = 0.017 g/ml of $CHCl_3$; 1dm^3; α = 0.002).

4.4.4.2 Spectral characterization

The proton NMR, carbon - 13 NMR, DEPT - 135 and GC - MS spectra of compound

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rA-5a1 are presented in Figures 4.5 - 4.9, while a summary of the various peaks obtained in each spectra are presented in Tables 4.14 - 4.17 respectively.

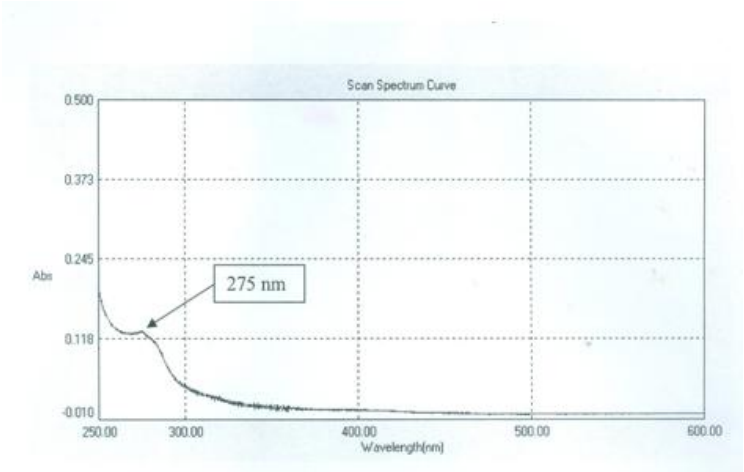

Figure 4.5: UV spectrum of compound **rA-5a1**

UV ($CHCl_3$) spectrum of compound **rA-5a1** indicated a characteristic peak at λ max 275 nm

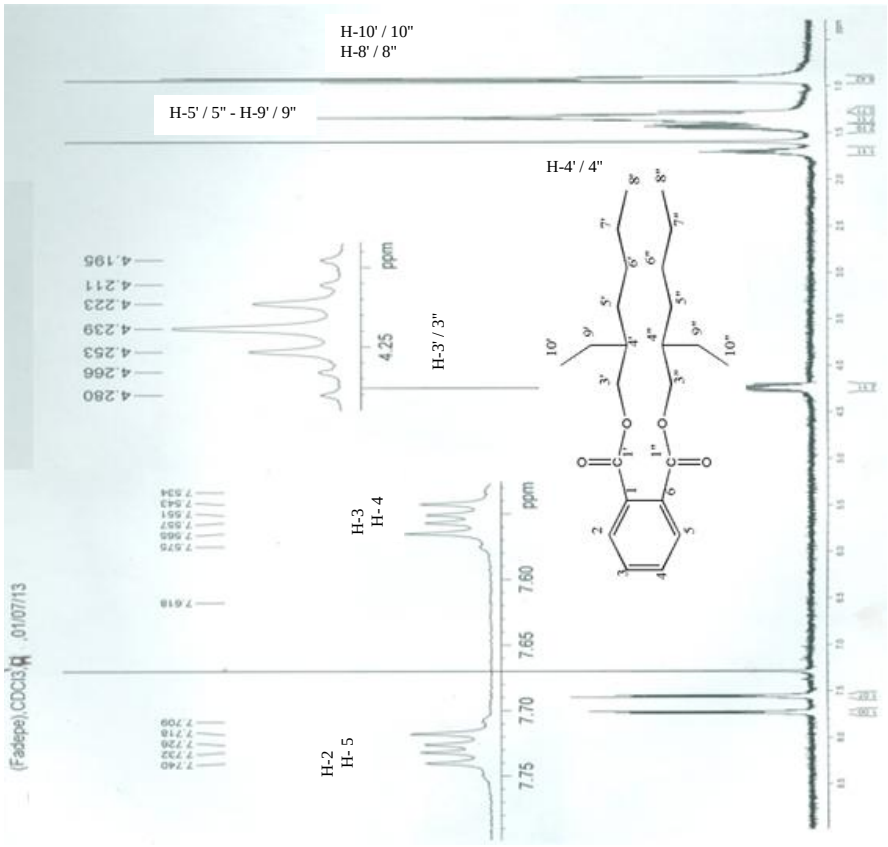

Figure 4.6: ^{1}H - NMR spectrum of compound **rA-5a1**

Table 4.14: Summary of ^{1}H - NMR spectral data of compound **rA-5a1**

Position	^{1}H (ppm)	Assignment	Literature Values (ppm)*
H-2 and H-5	7.726 (dd)	Ar–H	7.78
H-3 and H-4	7.557 (dd)	,,	7.58
	7.256 (singlet)	Solvent singlet of $CDCl_3$	
H-3'/H-3"	4.239 (septet)	-O-CH_2–	4.09 - 4.34
H-4'/H-4"	1.652 (pentet)	-C-CH_2-	1.67
H-5'/H-5"	1.355	Overlapping signals of several methylene protons in almost same enviroment	1.25 - 1.46
H-6'/H-6"	(Complex multiplet)		
H-7'/H-7"		(–CH_2–CH_2-) $_n$	
H-9'/H-9"			

H-8'/H-8"	0.905 (multiplet)	Overlapping signals of	0.96
H-10'/H-10"		$-(C-C-)_nC-CH_3$	

*ACD/ChemDraw (Product Version 15); Amade *et al*., 1994; Rao *et al*., 2000; Alim Al-Bari *et al*., 2006; Habib and Karim, 2009; Lyutskanova *et al*., 2009; El-Sayed, 2012)

The peak area integration ratio = 1.00: 1.07: 2.11: 1.11: 7.11: 0.71: 6.42

~2H: 2H: 4H: 2H: 14H: 1H: 13H =~38 protons

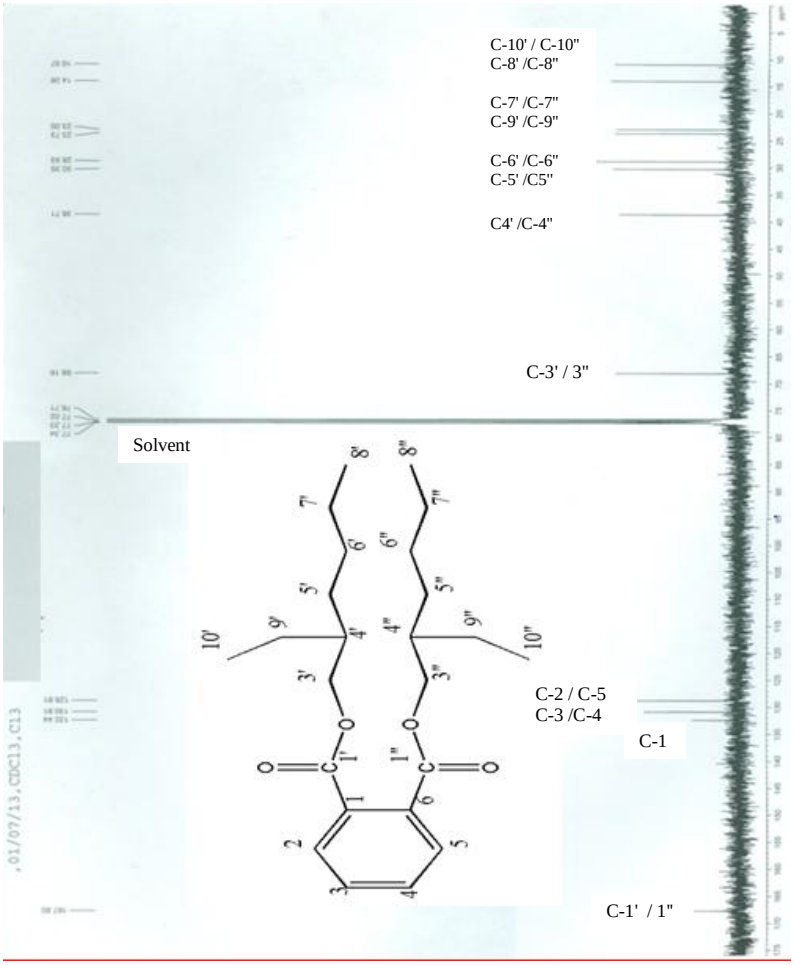

Figure 4.7: ^{13}C - NMR of compound **rA-5a1**

Table 4.15: Summary of ^{13}C - NMR spectral data of compound **rA-5a1**

Position	^{13}C (ppm)	Assignment	Literature values (ppm)*
C-1'/C-1"	167.75	-CHO	167.2
C-1/C-6	132.47	Ar-C	132.4
C-3/C-4	130.87	,,	130.3
C-2/C-5	128.80	,,	129.1
C-3'/C-3"	68.16	-CO-CH_2-	68.4
C-4'/C-4"	38.74	-C-CH (CH_2)-	39.0
C-5'/C-5"	30.37	-C-CH_2-	30.5
C-6'/C-6"	28.93	,,	29.1
C-9'/C-9"	23.75	,,	24.0
C-7'/C-7"	22.98	,,	23.1
C-8'/C-8"	14.04	-C-CH_3	14.1
C-10'/C-10"	10.95	,,	11.0

*ACD/ChemDraw (Product Version 15); Amade *et al*., 1994; Rao *et al*., 2000; Alim Al-Bari *et al*., 2006; Habib and Karim, 2009; Lyutskanova *et al*., 2009; El-Sayed, 2012

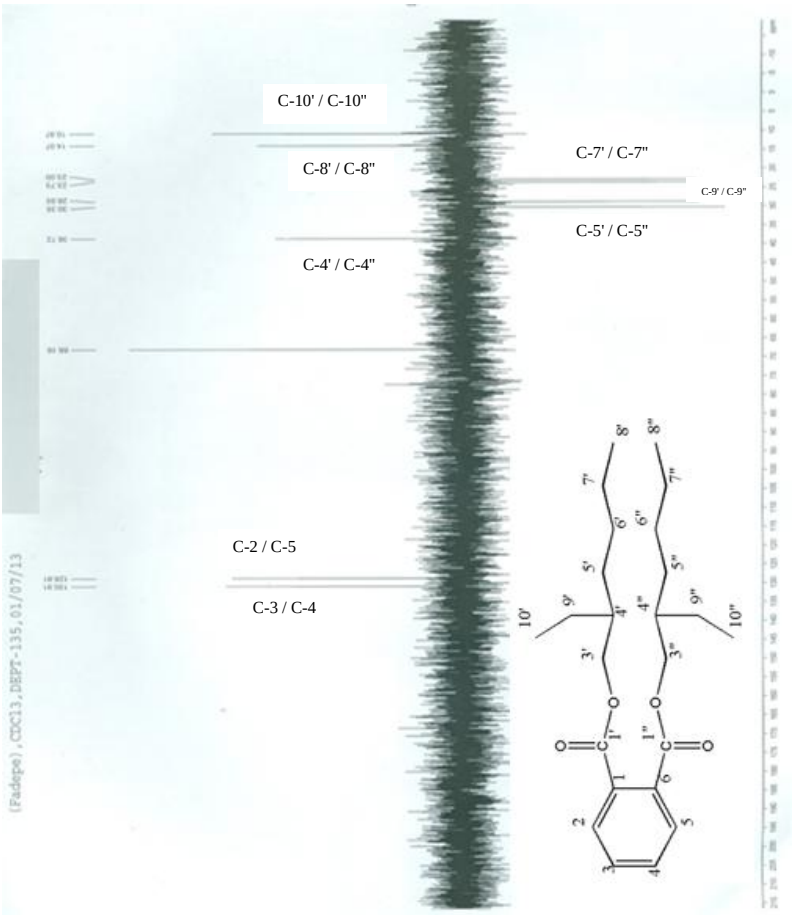

Figure 4.8: DEPT - 135 spectrum of compound **rA-5a1**

Table 4.16: Summary of DEPT - 135 spectral data of compound **rA-5a1**

Position	DEPT (ppm)	Assignment	*Literature values (ppm)
C-1'/C-1"	167.75	Quaternary carbon, disappeared in the spectrum (-CO)	167.2
C-1/C-6	132.47	Quaternary carbon, disappeared in the spectrum (Ar-C)	132.4
C-3/C-4	130.87	Ar-H (above in the spectrum)	130.3
C-2/C-5	128.80	,,	129.1
C-3'/C-3"	68.16	O-CH_2- (below)	68.4
C-4'/C-4"	38.74	-CH- (above)	39.0
C-5'/C-5"	30.37	-CH_2- (below)	30.5
C-6'/C-6"	28.93	,,	29.1
C-9'/C-9"	23.75	,,	24.0
C-7'/C-7"	22.98	,,	23.1
C-8'/C-8"	14.04	-CH_3 (above)	14.1
C-10'/C-10"	10.95	,,	11.0

*ACD/ChemDraw (Product Version 15); Amade *et al*., 1994; Rao *et al*., 2000; Alim Al-Bari *et al*., 2006; Habib and Karim, 2009; Lyutskanova *et al*., 2009; El-Sayed, 2012

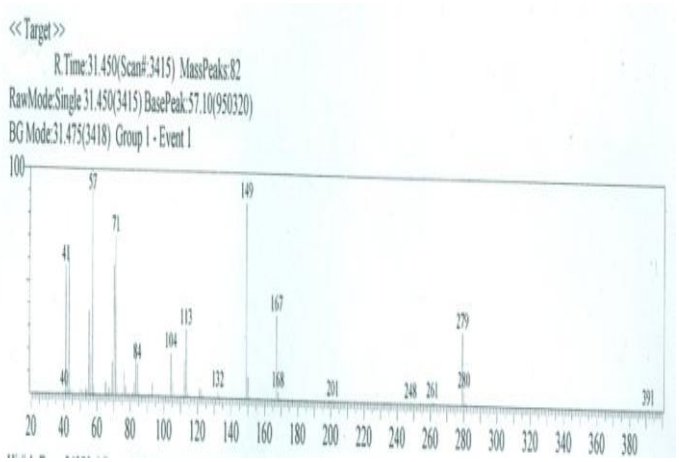

Figure 4.9: GC - MS spectrum of compound **rA-5a1**

Table 4.17: Summary of GC-MS spectral data of compound **rA-5a1**

Molecular formular	m/z of fragment ion*	Assignment
$[C_{24}H_{38}O_4] + 1$	391	Molecular ion peak +1 $[M + H]^+$
$[C_{24}H_{38}O_4]$	390	Molecular ion peak [M]
$M- [C_8H_{17}O]$	261	Elimination of ^-OR
$[C_8H_8O_4]^+$	168	Rearrangement of two H atoms with elimination of an allylic radical
$[C_8H_7O_4]^+$	167	McLafferty rearrangement
$[C_8H_5O_3]^+$	149	Cleavage of two esters with shift of two H atoms, followed by elimination of H_2O
$[C_8H_{17}]^+$	113	Retention of the positive charge by the alkyl group (R^+)
$[C_7H_4O]^+$	104	Elimination of $Ar–CO^+$
$[C_6H_{12}]^+$	84	,,–Elimination of a hexyl cation
$[C_5H_{11}]^+$	71	Elimination,, -of a pentyl cation
$[C_4H_9]^+$	57	Elimination,,– of a butyl cation (Base peak ion)
$[C_3H_5]^+$	41	Elimination,,– of a propyl cation
$[C_2H_3]^+$	27	Elimination,,– of an ethyl cation

*Amade *et al*., 1994; Rao *et al*., 2000; Alim Al-Bari *et al*., 2006; Habib and Karim, 2009; Lyutskanova *et al*., 2009; El-Sayed, 2012

4.5 Isolation of Compounds uE-2a1 and uE-2a2

4.5.1 VLC separation of fraction uE

Vacuum liquid chromatography of uE using varying ratios of different solvents, revealed the presence of several fractions that were pooled based on their TLC similarities in different solvent media as shown in Table 4.18

Table 4.18: Vacuum liquid chromatographic separation of fraction **uE**

Column fractions	Solvent System	Code	Physical appearance	Weight (g)	% Yield
1-28	$CHCl_3$ (100%)	**uE-1**	Dark Brown mass	4.35	14.5
29-55	$CHCl_3$:MeOH (90:10)	**uE-2**	Dark Green mass	7.04	23.5
56-82	$CHCl_3$:MeOH (80:20)	**uE-3**	Golden-brown mass	9.65	32.2
83- 97	$CHCl_3$:MeOH (50:50)	**uE-4**	Deep-Brown crystals	3.22	10.7
98- 125	MeOH (100%)	**uE-5**	Deep-brown mass	4.97	16.6

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4.5.2 Fractionation of column fraction uE-2

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Column Chromatographic separation of column fraction **uE-2** yielded column sub-fractions that were bulked based on TLC similarities as shown in Table 4.19

Table 4.19: Major column sub-fractions from purification of column fraction **uE-2**

Solvent system	Code	Weight (mg)	Number of spots observed on TLC
PE~~et ether~~:$CHCl_3$ (9:1)	**uE-2a**	850	2 distinct spots (PE: $CHCl_3$, 4:1)
PE:$CHCl_3$ (7:3)	**uE-2b**	320	3 spots (PE: $CHCl_3$, 1:1)
PE:$CHCl_3$ (5:5)	**uE-2c**	580	4 spots (PE: $CHCl_3$, 1:1)
$CHCl_3$ (100%)	**uE-2d**	700	4 spots ($CHCl_3$: EtOAc, 4:1)
$CHCl_3$:EtOAc (9:1)	**uE-2e**	550	,,
$CHCl_3$:EtOAc (8:2)	**uE-2f**	1100	6 spots ($CHCl_3$: EtOAc, 4:1)
$CHCl_3$:EtOAc (7:3)	**uE-2g**	360	4 spots ($CHCl_3$: EtOAc, 1:1)

$CHCl_3$:EtOAc (5:5)	**uE-2h**	300	Several spots ($CHCl_3$: EtOAc 4:1+ 3 drops of MeOH)
EtOAc (100%)	**uE-2i**	250	Several spots (EtOAc: Me_2CO 4:1)

4.5.3 PTLC of column sub-fraction uE-2a

Preparative TLC of sub-fraction **uE-2a** (300 mg, golden yellow oil, 2 major spots on TLC) gave rise to a major and minor band. PTLC purification of these bands yielded compounds **uE-2a1 (**major) and **uE-2a2** (minor).

4.5.4 Characterization of compound uE-2a1

4.5.4.1 Physical characterization

Concentration of the major band gave rise to oil (golden yellow, 27.4 mg) with slight odour. Compound yielded a single spot on TLC using petroleum ether: $CHCl_3$ (1: 1) and hexane: $CHCl_3$ (9:1 + 3 drops of EtOAc) with R_f values 0.45 and 0.59 respectively. The spot was colourless under sunlight, pink at 254 nm and blue at 366 nm, while it was golden brown on exposure to crystals of iodine and deep blue when sprayed with vanillin - sulphuric acid.

Compound was soluble in $CHCl_3$, Me_2CO, MeOH and insoluble in H_2O.

Its molecular formula and weight were revealed by GC - MS to be $C_{24}H_{38}O_4$ and

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391 gmol^{-1} respectively. Melting point and boiling point of the compound was found to be -55 to -52.6^{o}C and 382 - 383.8^{o}C respectively. Optical rotation of the compound at 2 different wavelengths was calculated to be $[\alpha]^{22}_{D\ (589nm)}$ 0.585 (c = 0.017g/ml of $CHCl_3$1dm^3; α = 0.001) and $[\alpha]^{22}_{D\ (633nm)}$ 1.178 (c = 0.017 g/ml of $CHCl_3$; 1dm^3; α = 0.002)

.

4.5.4.2 Spectral characterization

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^{1}H - NMR,^{13}C - NMR and DEPT - 135 spectra of compound **uE-2a1** (Figures 4.10 – 4.13) are similar to those obtained for compound **rA-5a1** (Figures 4.5 – 4.9)

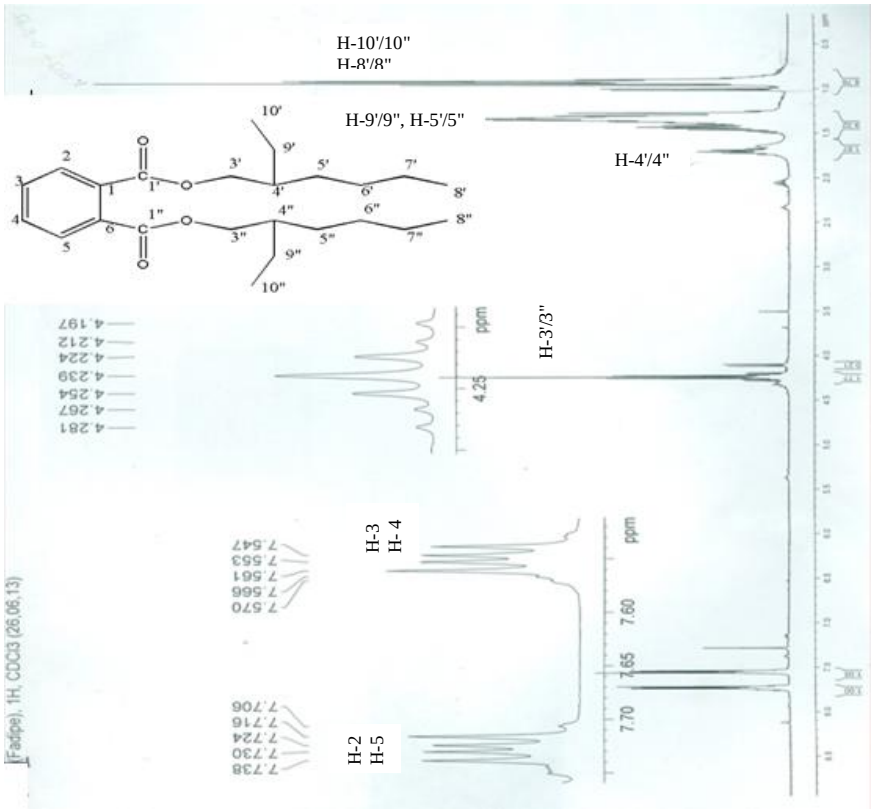

Figure 4.10: ^{1}H - NMR spectrum of compound **uE-2a1**

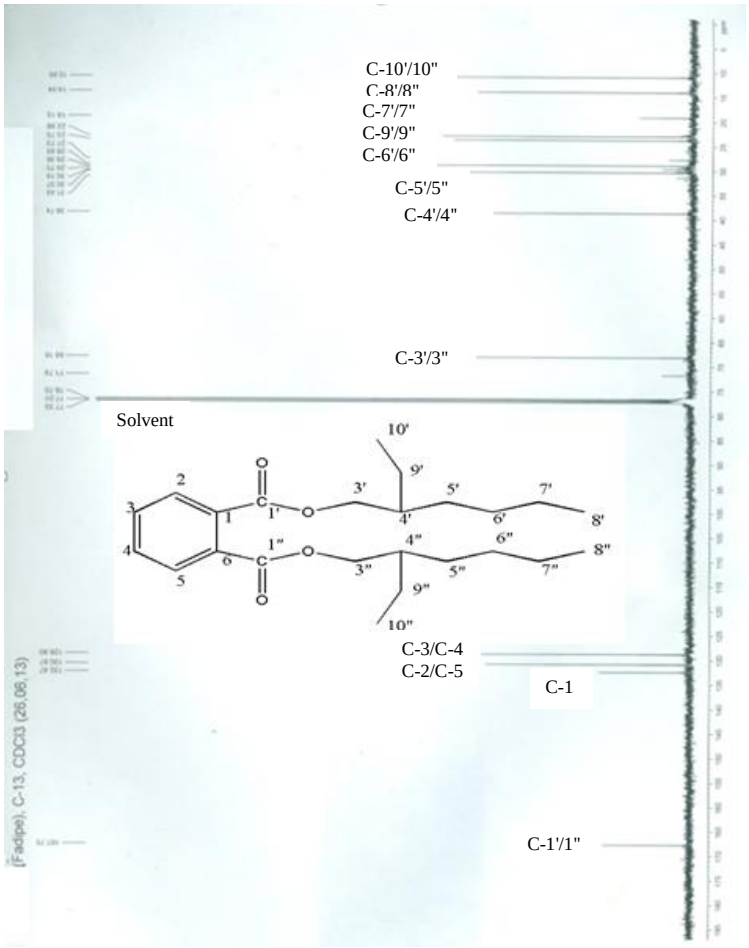

Figure 4.11: —^{13}C - NMR spectrum of compound **uE-2a1**

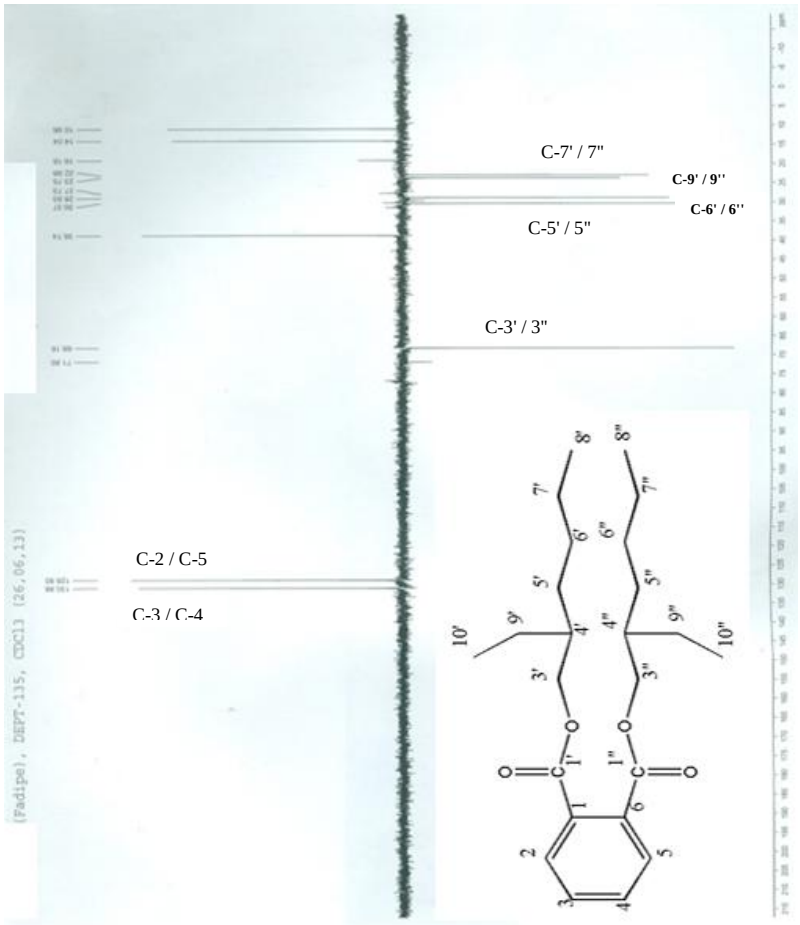

Figure 4.12: DEPT - 135 spectrum of compound **uE-2a1**

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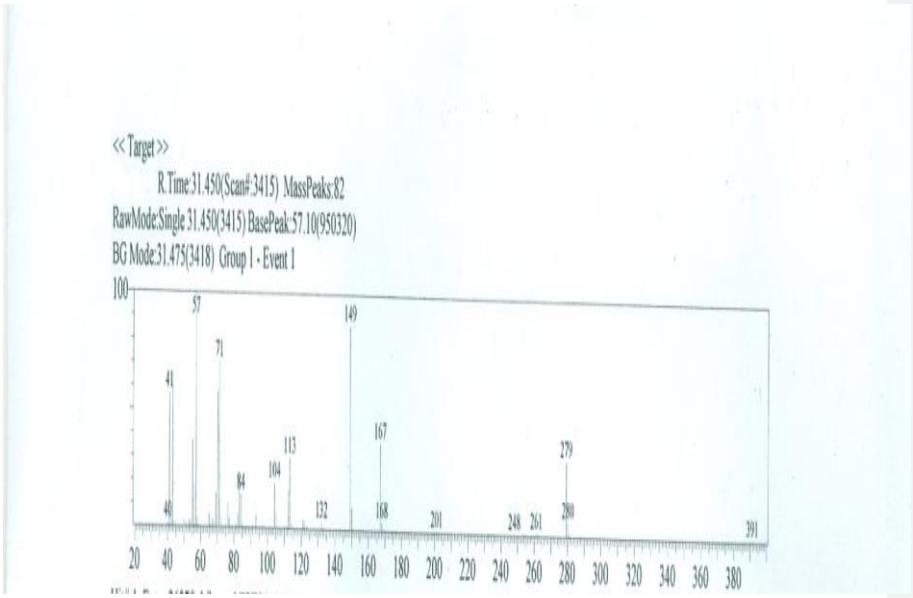

Figure 4.13: GC - MS spectrum of compound **uE-2a1**

4.5.5 Characterization of compound uE-2a2

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4.5.5.1 Physical characterization

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PTLC of band 2 afforded a white amorphous substance (12.8 mg), which on TLC using hexane: $CHCl_3$ (4: 1) and hexane: EtOAc (9: 1) revealed a homogeneous spot, R_f 0.46 and R_f 0.66 respectively. Spot was colourless (sunlight), UV (pink, 254 nm and blue, 366 nm) and golden brown (I_2 crystals).

Compound was completely soluble in $CHCl_3$, slightly soluble in petroleum ether and Me_2CO and insoluble in MeOH and H_2O

The uncorrected melting point of the compound was 11-13.5^oC

4. 5. 5. 2 Colour reactions

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Compound **uE-2a2** gave a negative test with 2, 4-dinitrophenylhydrazine (no hydrazone formed), while alkaline hydrolysis of the compound was very slow (Furniss *et al*., 1989).

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4.5.5.3 Spectral characterization

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The proton NMR, carbon - 13 NMR, DEPT - 135 and GC-MS spectra of compound **uE-2a2** are presented in Figures 4.14- 4.17, while a summary of the various peaks obtained in each spectra are presented in Tables 4.20 - 4.23 respectively.

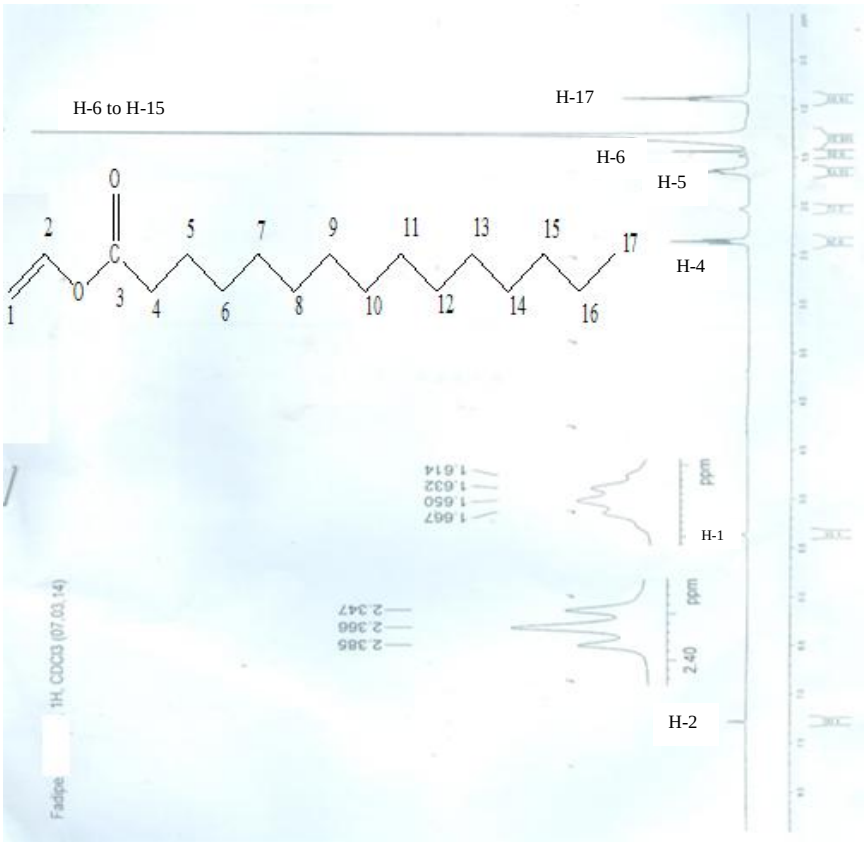

Figure 4.14: ^{1}H - NMR spectrum of compound **uE-2a2**

Table 4.20: Summary of ^{1}H - NMR spectral data of compound **uE-2a2**

Position	^{1}H (ppm)	Assignment	Literature values (ppm)*
H-2	7.28 (weak singlet)	C=CH-O	7.28
H-1	5.35 (weak singlet)	CH_2=C	5.38, 5.56
H-4	2.365 (triplet)	-CO-CH_2-	2.33
H-5	1.627 (quintet)	-CO-C-CH_2-	1.60
H-16	1.375 (triplet)	-CH_2-C	1.35
H-6 to H-15	1.224 (strong peak, singlet)	(-CH_2-CH_2-) $_n$	1.21
H-17	0.956 (triplet)	-C-CH_3	0.96

*ACD/ChemDraw (Product Version 15); Chang *et al*., 2008; Kim and Chung, 2009; Abozid and Ahmed, 2013; Khan *et al*., 2013; Su *et al*., 2013

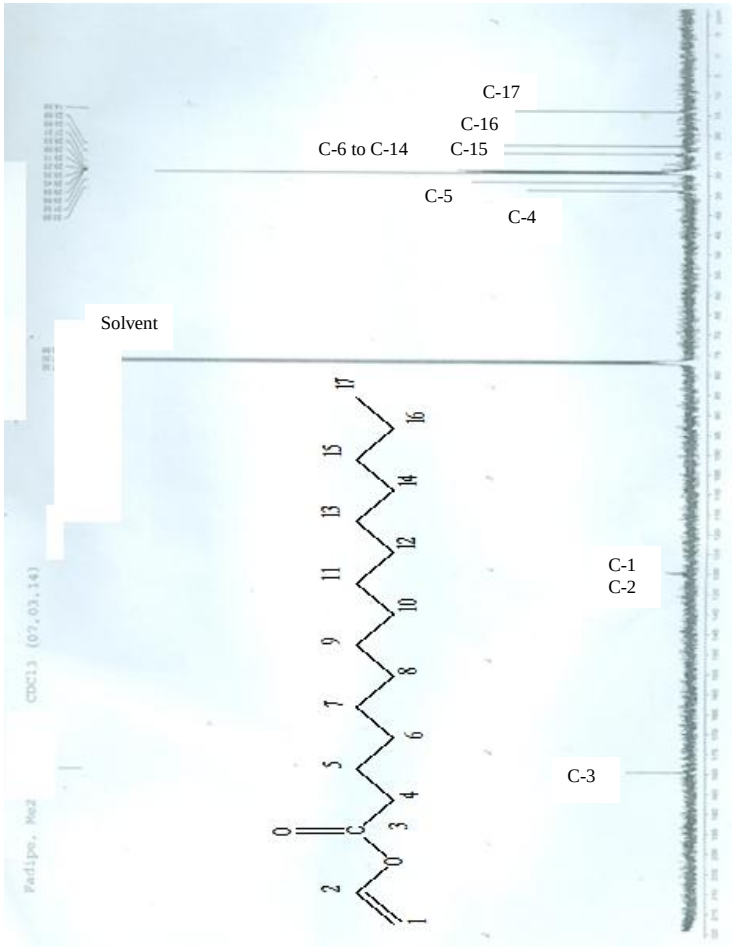

Figure 4.15: ^{13}C - NMR spectrum of compound **uE-2a2**

Table 4.21: Summary of ^{13}C - NMR spectral data of compound **uE-2a2**

Position	^{13}C (ppm)	Assignment	Literature values (ppm)*
C-3	179.61	-OC=O	179.2
C-2	130.5	-C=C	131.4
C-1	129.72	-C=C-O	129.4
C-4	33.98	-CO-CH_2-	34.0
C-5	31.92	-CO-C-CH_2-	30.2
C-6 to C-14	29.3	(-CH_2-CH_2-) $_n$	29.1
C-15	27.21	(-C-C-) $_n$-CH_2-	27.4
C-16	22.68	(-C-C-) $_n$-C-CH_2-	22.8
C-17	14.09	(-C-C-) $_n$-C-C-CH_3	14.1

*ACD/ChemDraw (Product Version 15); Chang *et al*., 2008; Kim and Chung, 2009; Abozid and Ahmed, 2013; Khan *et al*., 2013; Su *et al*., 2013.

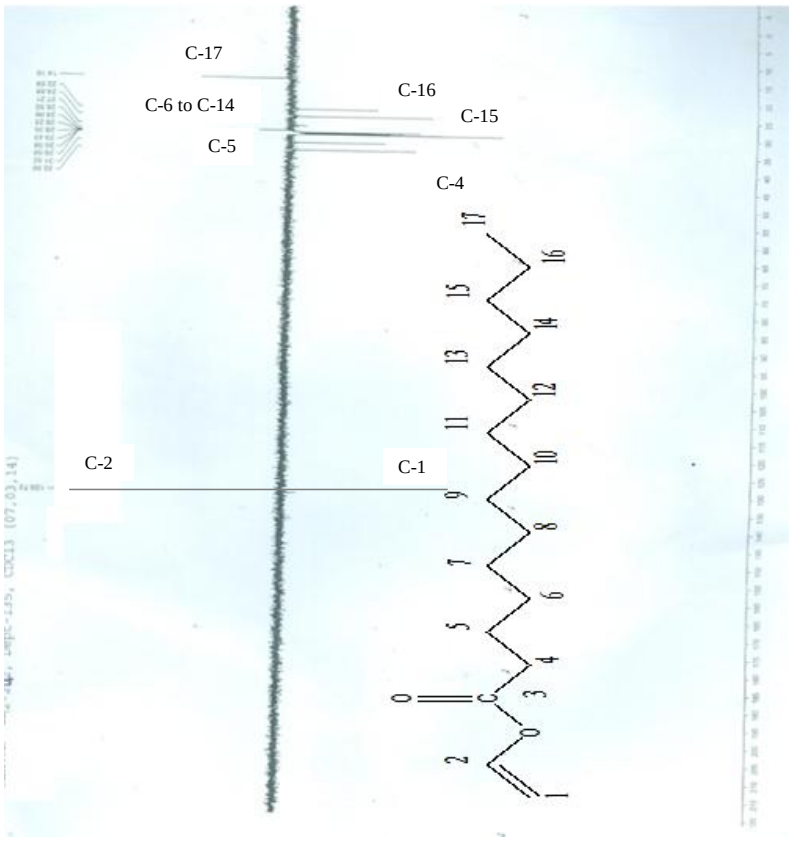

Figure 4.16: DEPT - 135 spectrum of compound **uE-2a2**

Table 4.22: Summary of DEPT-135 spectral data of compound **uE-2a2**

Position	DEPT (ppm)	Assignment	Literature values (ppm)*
C-3	179.61	Quartenary C, disappeared in the spectrum	179.2
C-2	130.5	-CH- (above in the spectrum)	131.4
C-1	129.72	$-CH_2-$ (below in the spectrum)	129.4
C-4	33.98	,,	34.0
C-5	31.92	,,	30.2
C-6 to C-14	29.3	,,	29.1
C-15	27.21	,,	27.4
C-16	22.68	,,	22.8
C-17	14.09	$-CH_3$ (above in the spectrum)	14.1

*Chang *et al*., 2008; Kim and Chung, 2009; Abozid and Ahmed, 2013; Khan *et al*., 2013; Su *et al*., 2013

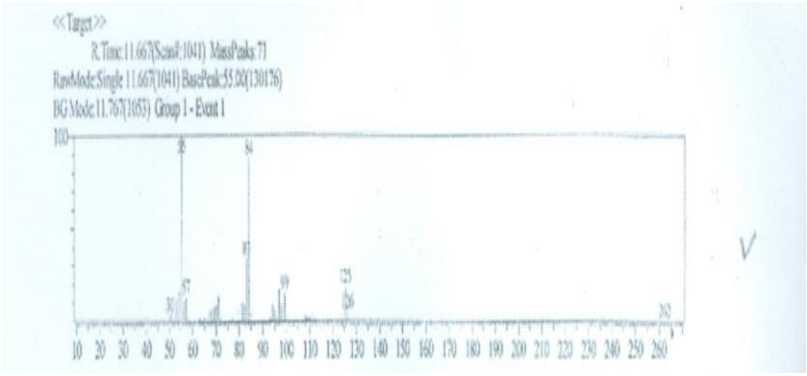

Figure 4.17: GC - MS spectrum of compound **uE-2a2**

Table 4.23: Summary of GC - MS spectral data of compound **uE-2a2**

Molecular formular	m/z of fragment ion*	Assignment
$[C_2H_3O]^+$	126	Generation of an alkoxyl cation
$[C_2H_4O]^+$	125	Loss of a proton from the alkoxyl cation
$[C_5H_7O_2]^+$	99	Generation of ethenylethylester group
$[C_4H_4O_2]^+$	84	Mc-Lafferty re-arrangement
$[C_3H_3O_2]^+$	71	Generation of ester cation, ($COOR^+$)
$[C_4H_8]^+$	56	Generation of a butyl cation
$[C_4H_7]^+$	55 (base peak)	Generation of a butenyl cation
$[C_2H_3O]^+$	43	Elimination of RCO cation
$[C_3H_7]^+$	43	Generation ofpropyl cation
$[C_3H_5]^+$	41	Generation of a propenyl cation
$[C_2H_5]^+$	29	Generation of an ethyl cation
$[C_2H_3]^+$	27	Generation of an ethenyl cation

*Li *et al*., 2011; Csoka *et al*., 2013

4.6 Isolation of Compound uE-3a2a

4.6.1 Fractionation of column fraction uE-3

Further purification of column fraction **uE-3** by the use of a series of columns and flash chromatographic separation gave rise to a mixture that was purified over sephadex LH-20 which on washing severally with methanol afforded a major compound, ~~coded~~ **uE-3a2a.**

4.6.2 Characterization of compound uE-3a2a

4.6.2.1 Physical characterization

This was obtained as white crystals (34 mg) re-crystallized from methanol. TLC of the compound in solvent systems- petroleum ether: EtOAc (9:1) petroleum ether: EtOAc (4:1) and $CHCl_3$: EtOAc (4:1) gave R_f values 0.33, 0.54 and 0.69 respectively. The spot was colourless (sunlight), UV inactive (254 and 366 nm), purple and reddish-brown when sprayed with anisaldehyde - H_2SO_4 and vanillin - H_2SO_4 respectively.

Compound was soluble in $CHCl_3$, EtOAc and Me_2CO, slightly soluble in petroleum ether and insoluble in MeOH and H_2O. GC-MS revealed its molecular formula to be $C_{29}H_{50}O$ and molecular weight as 414 $gmol^{-1}$. Its melting point was shown to be 136-138^oC, while its optical rotation at 20^oC was $[\alpha]^{20}_{D\ (589nm)}$**:** -100 (c = $5x10^{-5}$ g/ml of $CHCl_3$; 1dm^3; α = -0.005).

4.6.2.2 Colour reactions

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Compound **uE-3a2a** gave a positive Liebermann-Burchard's test for steroidal nucleus (green colouration), Salkowski's test for sterols (reddish colouration in the upper chloroform layer) and cerric ammonium test for alcohols (yellow colouration that gradually changed to red)

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4.6.2.3 Spectral characterization

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^{1}H - NMR, ^{13}C - NMR and DEPT-135 spectra of compound **uE-3a2a~~2~~** are presented in Figures 4.18 – 4.21, while a summary of the various peaks obtained in each spectra are presented in Tables 4.24 - 4.27 respectively.

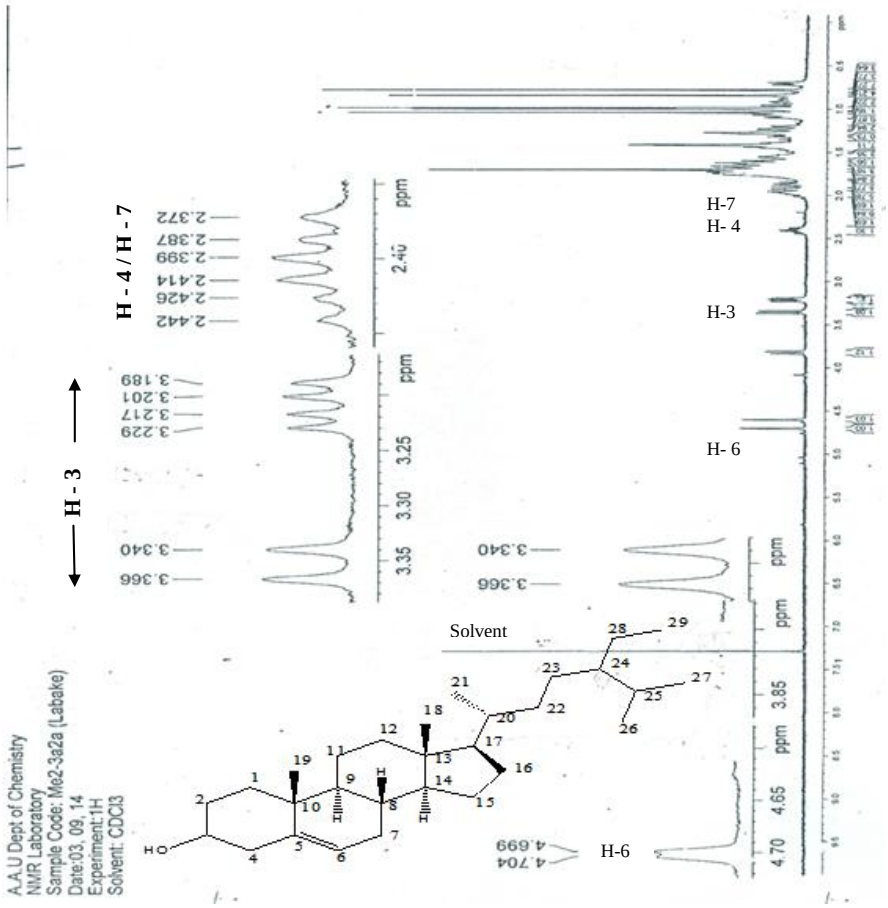

Figure 4.18: ^{1}H - NMR spectrum of compound **uE-3a2a**

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Table 4.24: Summary of ^{1}H - NMR spectral data of compound **uE-3a2a**

Position	^{1}H (ppm)	Assignment	Literature values (ppm)*
H-1	1.453 (multiplet)	$-CH_2-$	1.43, 1.38 (m)
H-2	1.552 (multiplet)	$-CH_2-$	1.57, 1.32 (t)
H-3	3.340 (dd) 3.217 (dd) 3.189 (dd)	-CH- (H-3α) i.e	(Tripledoublet of doublets, tdd)
H-3 (OH)	2.038 (multiplet)	HO- at C-3	2.00 (m)
H-4	2.414(triplet)	$-CH_2-$	2.43, 1.98 (t)
H-5	-	$-CR_3$	-
H-6	4.699 (doublet)	Olefinic H	4.71 (t)
H-7	2.372 (triplet)	$-CH_2-$	2.34, 1.79
H-8	1.675 (triplet)	-CH-	1.65 (m)
H-9	1.492 (triplet)	-CH-	1.47 (m)
H-10	-	$-CR_3-$	-
H-11	1.534 (triplet)	$-CH_2-$	1.54, 1.27 (m)
H-12	1.487 (triplet)	$-CH_2-$	1.49, 1.24 (m)
H-13	-	$-CR_3-$	-
H-14	1.384 (multiplet)	-CH-	1.40 (t)
H-15	1.623 (doublet)	$-CH_2-$	1.61, 1.35 (d)
H-16	1.796 (doublet)	$-CH_2-$	1.78, 1.35 (d)
H-17	1.514 (doublet)	-CH-	1.49 (d)
H-18	0.723 (singlet)	Angular $-CH_3$ protons on C-13	0.71 (s)
H-19	0.991 (doublet)	Angular $-CH_3$protons on C-10	1.06 (s)
H-20	1.627 (multiplet)	-CH-	1.64 (t)
H-21	0.954 (doublet)	$-CH_3$	1.02 (d)
H-22	0.916 (multiplet)	$-CH_2-$	1.01 (m)
H-23	1.084 (multiplet)	$-CH_2-$	1.05 (m)
H-24	1.602 (triplet)	-CH-	1.61 (m)
H-25	1.435 (doublet)	-CH-	1.42 (d)
H-26	1.121 (doublet)	$-CH_3$	1.11 (d)
H-27	1.082 (doublet)	$-CH_3$	1.01 (d)
H-28	1.304 (multiplet)	$-CH_2-$	1.29 (m)

H-29	1.005 (singlet)	$-CH_3$	0.96 (s)

*ACD/ChemDraw (Product Version 15)

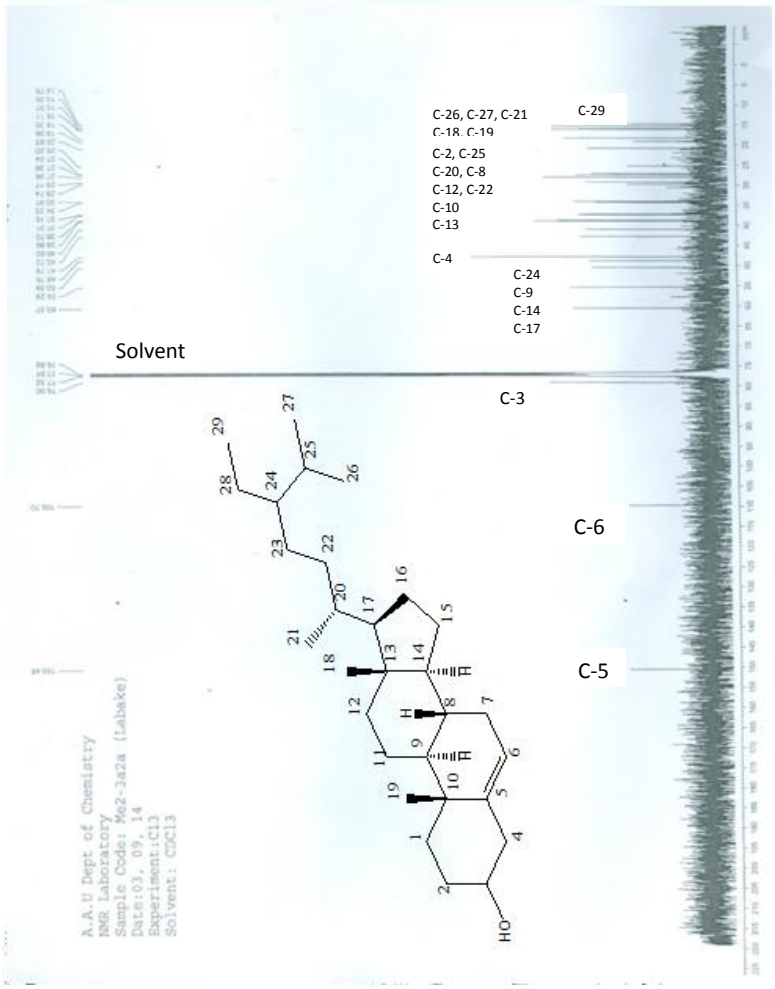

Figure 4.19: ^{13}C - NMR spectrum of compound **uE-3a2a**

Table 4.25: Summary of ^{13}C - NMR spectral data of compound **uE-3a2a**

Position	^{13}C (ppm)	Assignment	Literature values (ppm)*
C-1	29.74	-C- of cyclohexane (Ring A)	30.1
C-2	34.23	,,	33.8
C-3	79.0	-C-bearing OH (Ring A)	78.7
C-4	47.79	-C- of cyclohexane (Ring A)	46.9
C-5	150.48	3^oCarbon of olefinic group in cyclohexene (Ring B)	148.9
C-6	109.69	-C- of ethylene of cyclohexene	110.9
C-7	29.17	-C- of cyclohexene (Ring B)	30.0
C-8	37.16	-C-of cyclohexane (Ring C)	37.9
C-9	50.39	,,	50.8
C-10	40.92	-CR_3 of cyclohexene (Ring B)	39.8
C-11	20.82	-C- of cyclohexane (Ring C)	20.7
C-12	38.86	,,	38.2
C-13	42.72	-CR_3 of cyclopentane (Ring D)	42.0
C-14	55.29	-C- of cyclopentane (Ring D)	55.5
C-15	27.38	-C-of cyclopentane (Ring D)	27.7
C-16	27.04	-C-of cyclopentane (Ring D)	27.3
C-17	60.57	-C-of cyclopentane (Ring D)	59.3
C-18	18.30	-C- attached to C-13	18.7
C-19	19.08	-CR_3 attached to C-10	19.0
C-20	37.31	-C- at C-20	37.1
C-21	16.11	-C- at C-21	16.4
C-22	38.70	-C-at C-22	38.9
C-23	27.98	-C- atC-23	27.9
C-24	48.76	-C- at C-24	48.1
C-25	33.97	-C- at C-25	33.7
C-26	15.97	-C-at C-26	15.6
C-27	15.36	-C- at C-27	15.1
C-28	25.20	-C- at C-28	25.6
C-29	14.76	-C- C-29	12.2

*ACD/ChemDraw (Product Version 15)

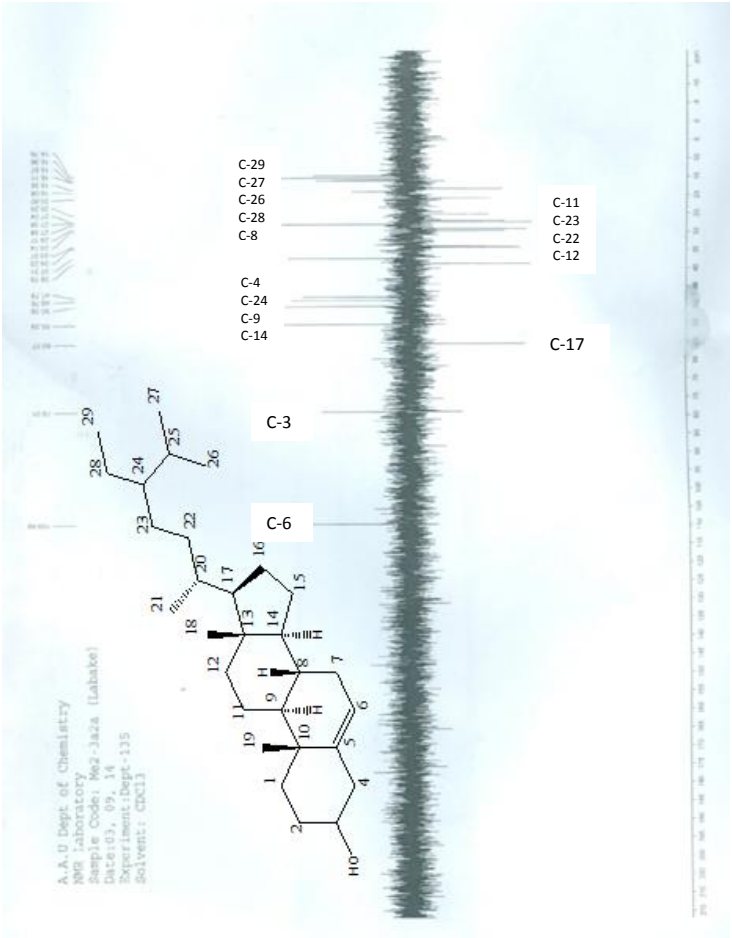

Figure 4.20: DEPT 135 spectrum of compound **uE-3a2a**

Table4.26: Summary of DEPT - 135 spectral data of compound **uE-3a2a**

Position	DEPT (ppm)	Assignment	Literature values (ppm)*
C-1	29.7	$-CH_2-$ (Ring A)	30.1
C-2	34.2	,,	33.8
C-3	79.0	-CH- (Ring A)	78.7
C-4	47.8	$-CH_2-$ (Ring A)	46.9
C-5	150.9	Quartenary C (Ring B)	148.9
C-6	109.7	-CH- (Ring B)	110.9
C-7	29.2	$-CH_2-$ (Ring B)	30.0
C-8	37.2	-CH- (Ring C)	37.9
C-9	50.4	,,	50.8
C-10	40.9	Quartenary C (Ring B)	39.8
C-11	20.8	$-CH_2-$ (Ring C)	20.7
C-12	33.9	$-CH_2-$ (Ring C)	38.2
C-13	42.7	Quartenary C (Ring D)	42.0
C-14	55.3	$-CH_2-$ (Ring D)	55.5
C-15	27.4	$-CH_2-$ (Ring D)	27.7
C-16	27.0	,,	27.3
C-17	60.6	-CH-(Ring D)	59.3
C-18	18.3	$-CH_3$	18.7
C-19	19.1	$-CH_3$	19.0
C-20	37.3	-CH- (Ring D)	37.1
C-21	16.1	$-CH_3$(Ring D)	16.4
C-22	38.7	$-CH_2-$ (Ring D)	38.9
C-23	27.9	$-CH_2-$ (Ring D)	27.9
C-24	48.8	-CH- (Ring D)	48.1
C-25	33.9	-CH- (Ring D)	33.7
C-26	15.9	$-CH_3$ (Ring D)	15.6
C-27	15.4	,,	15.1
C-28	25.2	$-CH_2-$ (Ring B)	25.6
C-29	14.8	$-CH_3$ (Ring D)	12.2

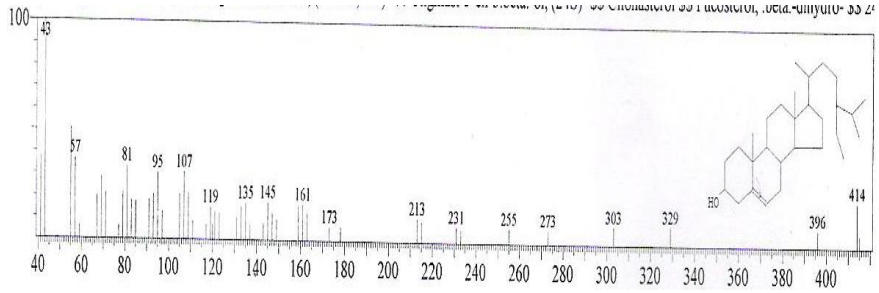

Figure 4.21: GC - MS spectrum of compound **uE-3a2a**

Table 4.27: Summary of GC-MS spectral data of compound **uE-3a2a**

Molecular formular	m/z of fragment ion	Assignment
$[C_{29}H_{50}O]$	414	Molecular ion peak, $[M]^+$
$[C_{29}H_{48}]^+$	396	Loss of a water molecule from the molecular ion
$M-[C_5H_9O]^+$	329	Formation of a resonance-stabilized 3^oC^+ group (ring A)
$M-[C_7H_{11}O]^+$	303	Formation of a 1^oC^+ (rings A and B)
$M-[C_9H_{15}O]^+$	275	Opening of ring B
$M-[C_{10}H_{21}]^+$	273	Loss of alkyl side chain at C-17by β bond cleavage to give a positively charged ring fragment
$M-[C_{13}H_{27}]^+$	231	Loss of alkyl side chain + opening of ring D
$[C_{19}H_{27}]^+$	255	Dehydration of residual ring fragment
$[C_{17}H_{27}]^+$	231	Loss of ethenyl group from dehydrated molecule
$[C_{16}H_{21}]^+$	213	Loss of propenyl group from dehydrated molecule
$[C_{13}H_{17}]^+$	173	Retention of positive charge by R groups (R^+)
$[C_{12}H_{17}]^+$	161	,,
$[C_{11}H_{13}]^+$	145	,,
$[C_{10}H_{15}]^+$	135	,,
$[C_9H_{11}]^+$	119	,,
$[C_8H_{13}]^+$	109	,,
$[C_8H_{11}]^+$	107	,,
$[C_7H_{11}]^+$	95	,,
$[C_6H_9]^+$	81	,,
$[C_4H_9]^+$	57	Elimination of butyl cation
$[C_3H_7]^+$	43 (base peak)	Generation of resonance-stabilized allylic C^+ in ring B; Typical of cycloalkenes
$[C_3H_5]^+$	41	Elimination of propyl cation

4.7 Antibacterial Assays

4.7.1 Antibacterial assay of extracts, fractions and standards (test compounds)

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In-vitro antibacterial assessment of the extracts (100 mg/ml each), partitioned-soluble ~~and~~ fractions (50 mg/ml each) and re-extracted soluble fractions (50 mg/ml each) of both the ripe and unripe fruits of *N. latifolia* in comparison with standard ~~some~~ antibiotics (1 mg/ml each) is as shown in Table 4.28

Table 4.28: Antibacterial activity of test extracts and fractions against test organisms

*Test Compound	Diameter of zones of inhibition against test organisms (mm)**					
	B. subtilis	*S. aureus*	*E. coli*	*K. pneumoniae*	*P. aeruginosa*	*S. typhi*
rP	–	–	–	–	3.11±1.15^{q2}	6.67±0.88^{r1}
uP	12.1±0.58^{k4}	–	15.0±1.00^{f2}	14.6±0.95^{g3}	–	15.3±0.36^{i1}
rM	7.05±0.67^{n4}	11.0±0.00^{j3}	–	12.3±2.52^{j1}	5.33±0.58^{o5}	12.2±2.00^{l2}
uM	–	–	12.3±1.18^{i2}	12.0±0.00^{k4}	13.3±2.06^{i1}	12.1±0.82^{m3}
C	–	–	6.33±1.53^{m4}	12.7±3.06^{i1}	7.67^{m3}±2.52	11.5±1.00^{n2}
E	8.25±0.58^{m5}	–	11.3±1.53^{k4}	17.6±0.83^{b1}	12.2±1.00^{k3}	17.3±0.58^{e2}
B	12.2±0.38^{j5}	14.7±2.08^{i4}	–	15.3±0.58^{d3}	16.4±1.00^{e2}	17.3±2.33^{e1}
A	–	–	–	7.33±1.15^{q2}	–	9.22±0.82^{o1}
rD	17.5±0.36^{b2}	21.2±1.00^{c1}	11.7±0.95^{j6}	16.1±1.18^{c5}	16.7±1.53^{d3}	16.3±1.53^{g4}
uD	17.3±0.46^{c4}	17.5±0.92^{d3}	18.4±1.18^{d1}	15.3±1.62^{d3}	18.3±0.94^{a2}	16.1^{h5}±0.74
rC	14.2±0.38^{h4}	17.1±0.16^{e1}	–	9.14±1.15^{o5}	12.3±2.08^{j3}	12.7^{k2}±1.15
uC	14.8±0.83^{g4}	16.0±0.00^{h2}	13.1±1.30^{h6}	15.0±0.00^{e3}	14.2±0.63^{h5}	17.2±0.46^{f1}
rE	10.7±0.25^{l3}	7.33±1.15^{k6}	7.36±1.53^{l5}	11.7±1.53^{l2}	15.3±1.53^{f1}	7.67±1.15^{p4}
uE	20.4±0.82^{a2}	21.5±0.92^{b1}	18.3±1.24^{e3}	17.7±0.42^{a4}	17.3±0.76^{b6}	17.6±0.64^{d5}
rA	–	–	–	10.1±1.00^{m1}	7.00±2.00^{n3}	7.33±0.58^{q2}
uA	14.1±0.15^{i3}	16.5±0.68^{g1}	14.2±0.46^{g2}	12.8±0.95^{h5}	12.3±0.98^{j6}	13.1±0.42^{j4}
rR	–	–	–	–	3.33±0.58^{p2}	7.33±1.53^{q1}
uR	4.20±0.45^{o2}	3.82±1.18^{l3}	–	2.48±0.64^{r4}	–	4.33±0.58^{s1}
Chloramphenicol	15.5±1.05^{e4}	16.6±0.32^{f3}	20.2±2.00^{c1}	14.8±2.00^{f5}	9.60±0.45^{l6}	19.3±0.00^{c2}
Erythromycin	15.1±2.05^{f5}	22.2±0.24^{a3}	22.6±0.56^{a2}	9.60±1.06^{n6}	17.1±0.62^{c4}	26.2±1.41^{a1}
Tetracycline	17.2±1.00^{d3}	16.6±0.20^{f4}	22.0±0.00^{b1}	8.50±0.83^{p6}	14.3±0.70^{g5}	19.8±0.52^{b2}

Keys: − = No measurable zone of inhibition, **= mean values of two replicates with standard error shown as ±.

Mean values on the same column with same superscript alphabets are not significantly different (p>0.05), while those with different superscript numbers on the same row are significantly different (p<0.05).

*Extracts of ripe and unripe fruits (Petroleum ether, **rP** and **uP;** Methanol, **rM** and **uM**); partitioned-soluble fractions of ripe fruits (chloroform **C,** ethyl acetate **E,** butanol **B** and residual aqueous **A**); re-extracted soluble fractions of ripe (r) fruits (chloroform **rC,** di ethyl ether **rD,** ethyl acetate **rE,** acetone **rA and** residual **rR**) and re-extracted soluble fractions of unripe (u) fruits (chloroform **uC,** diethyl ether **uD,** ethyl acetate **uE,** acetone **uA** and residual **uR**) of *N. latifolia*.

4.7.1.1 Percent antibacterial activity of test compounds (A %)

Calculated antibacterial percent activities of extracts/fractions/standard (A %)* against test organisms are expressed in Figure 4.22

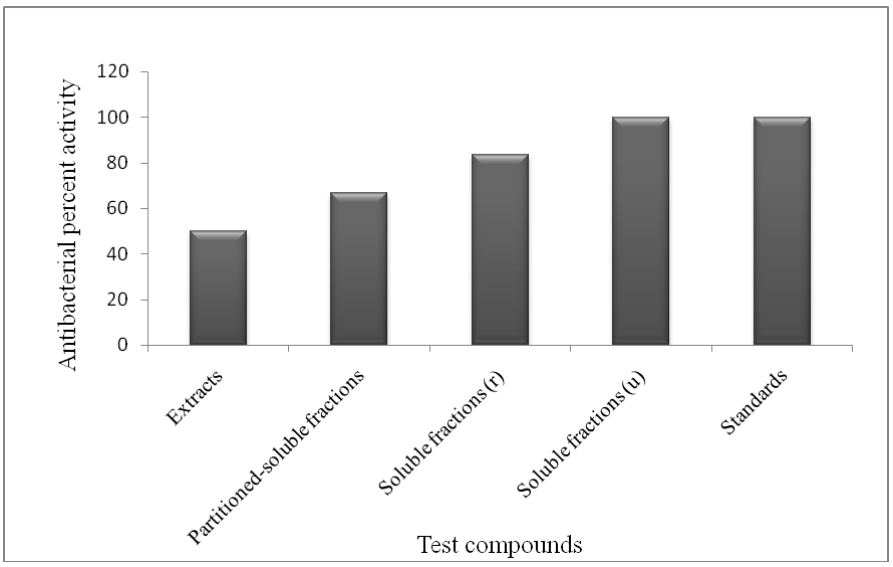

Figure 4.22: Maximum ——A % of each test compound~~s in comparison with standard drugs~~ against test bacterial strains.

*A % of extracts of both fruits ranged from 0 - 50 %

A % of partitioned-soluble fractions of ripe fruits ranged from 0 - 66.7 %

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A % of re-extracted soluble fractions of unripe fruits ranged from 50 - 100 %

A % of re-extracted soluble fractions of ripe fruits ranged from 0 - 83.3 %

A % of standard antibiotics - 100 %

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4.7.1.2 Bacterial susceptible index of test organisms (BSI)

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Calculated BSI of the test compounds showing the degree of susceptibility of test organisms to extracts/fractions/ standards are expressed in Figure 4.23

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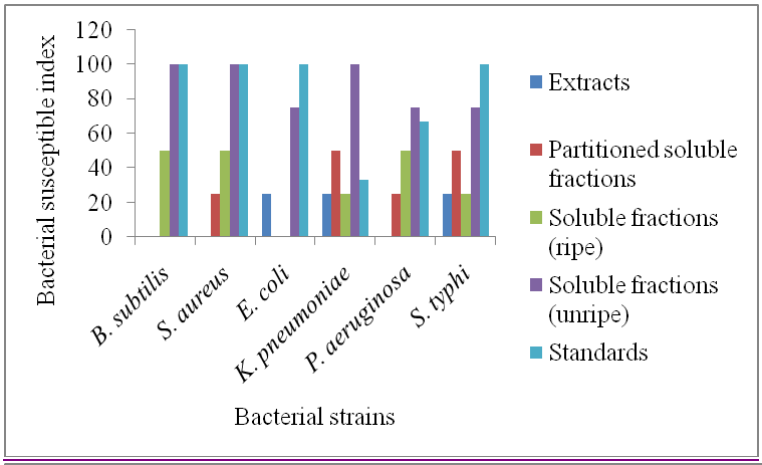

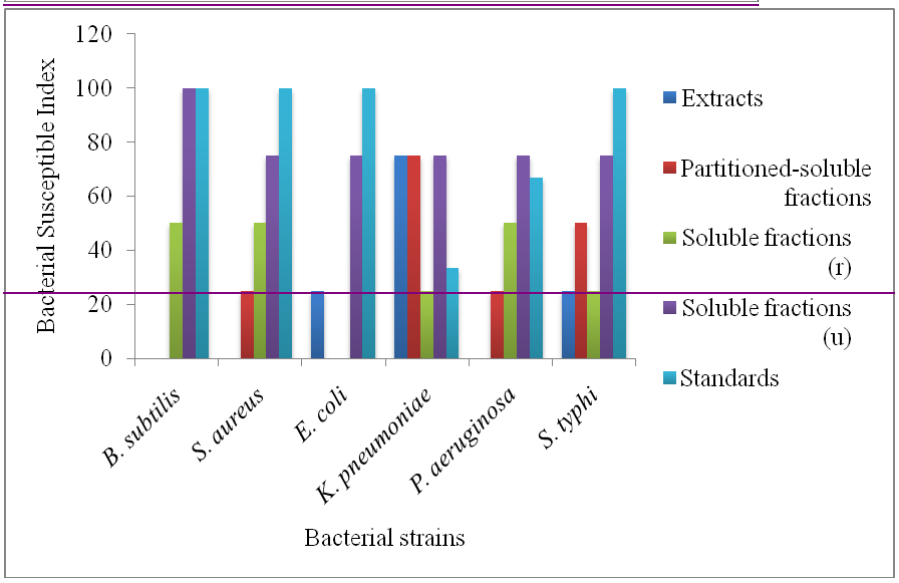

Figure 4.23: –Degree of susceptibility of test bacterial strains to the test compounds in comparison with standard drugs

4.7.1.3 Minimum inhibitory concentrations of test compounds (MIC)

The minimum inhibitory concentrations for active extract/fractions/standards are presented in Table 4.29

Table 4.29: MICs of active test compounds against test organisms

	MICs (mg/ml) of test compounds against test organisms					
Test Compound*	*B. subtilis*	*S. aureus*	*E. coli*	*K. pneumoniae*	*P. aeruginosa*	*S. ty*
uP	ND	ND	12.5	25	ND	25
E	ND	ND	ND	12.5	ND	12.5
B	ND	25	ND	12.5	12.5	12.5
rD	25	6.25	ND	25	12.5	12.5
rC	12.5	12.5	ND	ND	ND	ND
rE	ND	ND	ND	ND	25	ND
uD	12.5	12.5	12.5	25	12.5	25
uC	12.5	12.5	ND	25	25	25
uE	6.25	6.25	12.5	12.5	12.5	12.5
uA	50	12.5	12.5	ND	ND	ND

Chloramphenicol	0.40	0.30	0.30	0.40	ND	0.30
Erythromycin	0.40	0.20	0.20	ND	0.30	0.20
Tetracycline	0.530	0.30	0.30	ND	0.50	0.20

ND = Not Determined; *Extract of unripe fruits (Petroleum ether, **uP**); partitioned-soluble fractions of ripe fruits (ethyl acetate **E,** butanol **B**); re-extracted soluble fractions of ripe (r) fruits (diethyl ether **rD,**chloroform **rC,** and ethyl acetate **rE**) and re-extracted soluble fractions of unripe (u) fruits (chloroform **uC,** di ethyl ether **uD,** ethyl acetate **uE** and acetone **uA**) of *N. latifolia*.

4.7.1.4 Minimum bactericidal concentrations of test compounds (MBC)

The minimum bactericidal concentrations for active extract/fractions/standards are presented in Table 4.30

Table 4.30: MBCs of active test compounds against test organisms

	MBCs (mg/ml) of test compounds against test organisms					
Test Compound*	*B. subtilis*	*S. aureus*	*E. coli*	*K. pneumoniae*	*P. aeruginosa*	*S. typhi*
uP	ND	ND	100	100	ND	100
E	ND	ND	ND	25	ND	12.5
B	ND	50	ND	25	25	12.5
rD	25	12.5	ND	50	50	50
rC	12.5	12.5	ND	ND	ND	ND
rE	ND	ND	ND	ND	50	ND
uD	12.5	12.5	12.5	50	12.5	50
uC	25	25	ND	50	50	25
uE	6.25	6.25	12.5	12.5	12.5	12.5

uA	50	25	50	ND	ND	ND
Chloramphenicol	0.30	0.20	0.20	0.20	ND	0.20
Erythromycin	0.30	0.20	0.20	ND	0.30	0.20
Tetracycline	0.520	0.20	0.30	ND	0.30	0.20

ND = Not Determined; *Extract of unripe fruits (Petroleum ether, **uP**); partitioned-soluble fractions of ripe fruits (ethyl acetate **E,** butanol **B**); re-extracted soluble fractions of ripe (r) fruits (diethyl ether **rD,**chloroform **rC,** and ethyl acetate **rE**) and re-extracted soluble fractions of unripe (u) fruits (chloroform **uC,** diethyl ether **uD,** ethyl acetate **uE** and acetone **uA**) of *N. latifolia*.

4.7.1.5 Antibacterial effect of test compounds (MBC/MIC)

The bacteriostatic or bactericidal effect of the active extract/fractions/standards against the tested organisms are presented in Table 4.31

Table 4.31: MBC/MIC of active test compounds against test organisms

	**MBC/MIC ratios of test compounds against test organisms					
Test Compound*	*B. subtilis*	*S. aureus*	*E. coli*	*K. pneumoniae*	*P. aeruginosa*	*S. typhi*
uP	ND	ND	8.00	4.00	ND	4.00
E	ND	ND	ND	2.00	ND	1.00
B	ND	2.00	ND	2.00	2.00	1.00
rD	1.00	2.00	ND	2.00	4.00	4.00
rC	1.00	1.00	ND	ND	ND	ND
rE	ND	ND	ND	ND	2.00	ND
uD	1.00	1.00	1.00	2.00	1.00	2.00
uC	2.00	2.00	ND	2.00	2.00	1.00
uE	1.00	1.00	1.00	1.00	1.00	1.00
uA	1.00	2.00	4.00	ND	ND	ND

Chloramphenicol	0.75	0.66	0.66	0.50	ND	0.66
Erythromycin	0.75	1.00	1.00	ND	1.00	1.00
Tetracycline	1.00~~0.66~~	0.66	1.00	ND	0.60	1.00

ND = Not Determined; *Extract of unripe fruits (Petroleum ether, **uP**); partitioned-soluble fractions of ripe fruits (ethyl acetate **E,** butanol **B**); re-extracted soluble fractions of ripe (r) fruits (chloroform **rC,** di ethyl ether **rD** and ethyl acetate **rE**) and re-extracted soluble fractions of unripe (u) fruits (diethyl ether **uD**, chloroform **uC,** ethyl acetate **uE** and acetone **uA**) of *N. latifolia.*

**MBC/MIC ratios >1= Bacteriostatic effect; < 1= Bacteriocidal effect

4.7.2 Antibacterial investigation of ethyl acetate partitioned-soluble fraction of ripe fruits (E); its column fractions and column sub-fractions

4.7.2.1 Antibacterial activity of E

Antibacterial activity of ethyl acetate partitioned-soluble fraction of methanol extract of ripe fruits (**E**) in comparison with a standard drug is presented in Figure 4.24

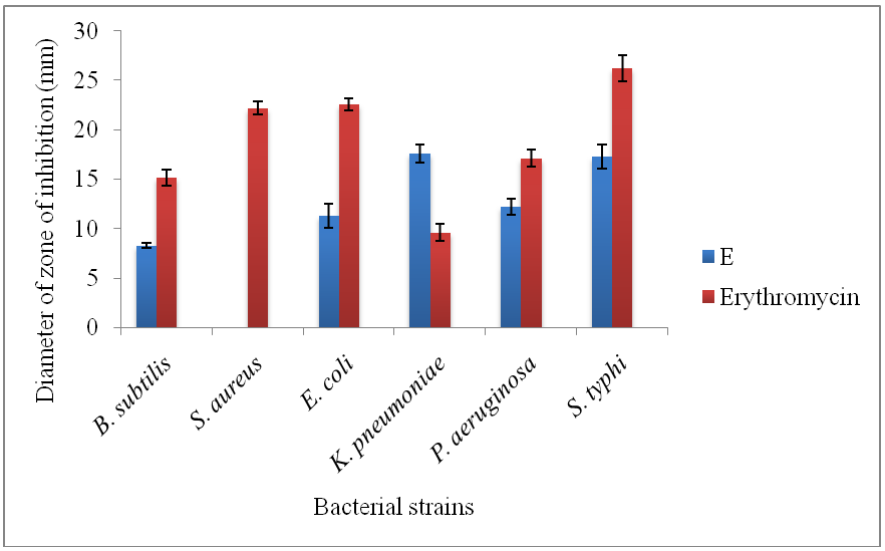

Figure 4.24: Antibacterial activity of ethyl acetate partitioned-soluble fraction of ripe fruits of *N. latifolia*, **E** (50 mg/ml) in comparison with erythromycin (1 mg/ml)

The error bars represent the standard error of the mean of duplicate readings.

4.7.2.2 Antibacterial activity of column fractions from partitioned-soluble fraction E

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Antibacterial activity of fractions (**E-1** to **E-6**) obtained from column fractionation of ethyl acetate partitioned-soluble fraction of methanol extract of ripe fruits (**E**) is presented in Figure 4.25

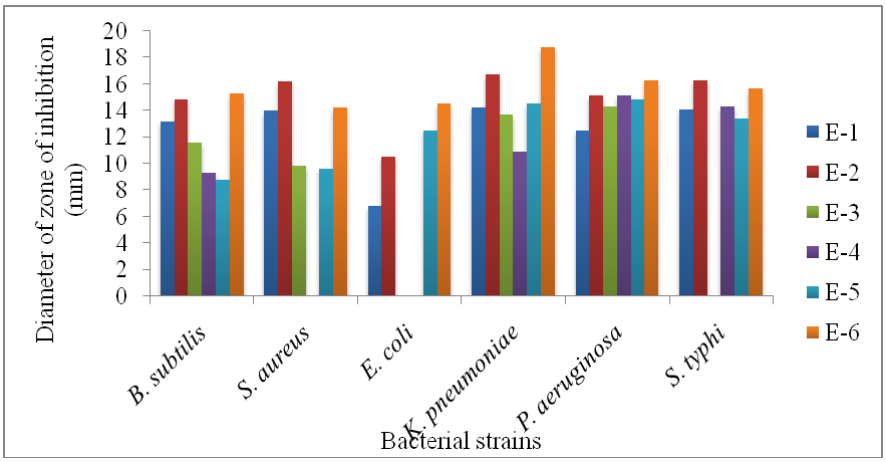

Figure 4.25: Antibacterial activity of the 6 major column fractions from fraction **E** (20 mg/ml) against test bacterial strains.

4.7.2.3 Antibacterial activity of sub-fractions from column fraction E-2

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Antibacterial activity of column sub-fractions (**E-2a** to **E-2h**) obtained from further fractionation of column fraction (**E-2**) obtained from fractionation of ethyl acetate partitioned-soluble fraction of methanol extract of ripe fruits (**E**) is presented in Figure 4.267

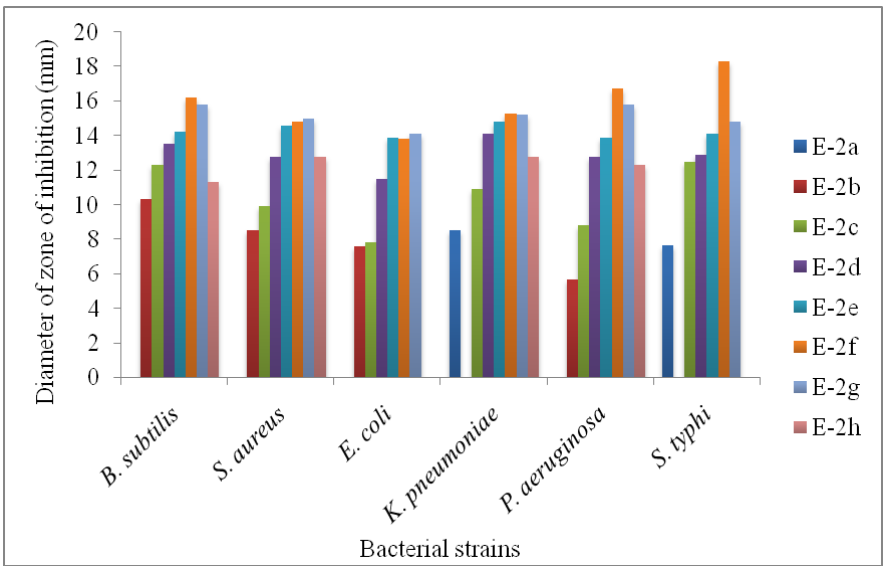

Figure 4.26: Antibacterial activity of the 8 major column sub-fractions (20 mg/ml) from fraction **E-2**

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4.7.3 Antibacterial investigations of acetone-soluble fraction of ripe fruits (rA); its column fractions, sub-fractions and isolated compound

4.7.3.1 Antibacterial activity of rA

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Antibacterial activity of acetone soluble fraction of methanol extract of ripe fruits (**rA**) in comparison with a standard drug is presented in Figure 4.27

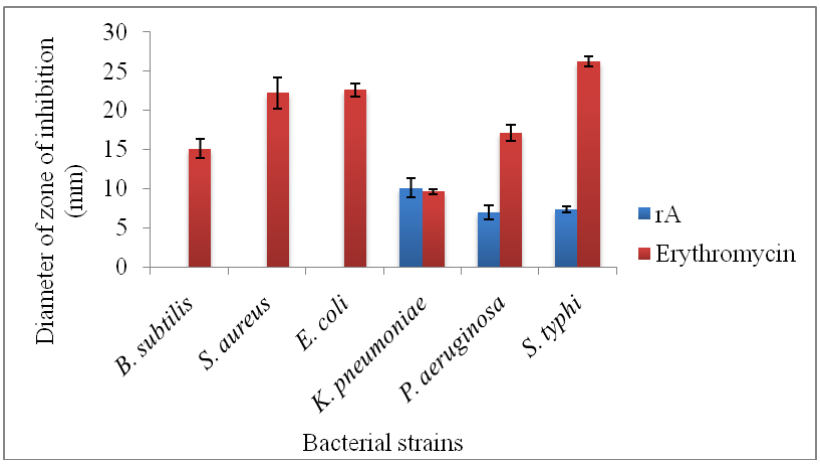

Figure 4.27: Antibacterial activity of acetone-soluble fraction of ripe fruits of *N. latifolia*, **rA** (50 mg/ml) in comparison with erythromycin (1 mg/ml)

The error bars represent the standard error of the mean of duplicate readings.

4.7.3.2 Antibacterial activity of column fractions from rA

Antibacterial activity ~~of column~~of column fractions (**rA-1** to **rA-8**) obtained from fractionation

of acetone soluble fraction of methanol extract of ripe fruits (**rA**) is presented in Figure 4.28

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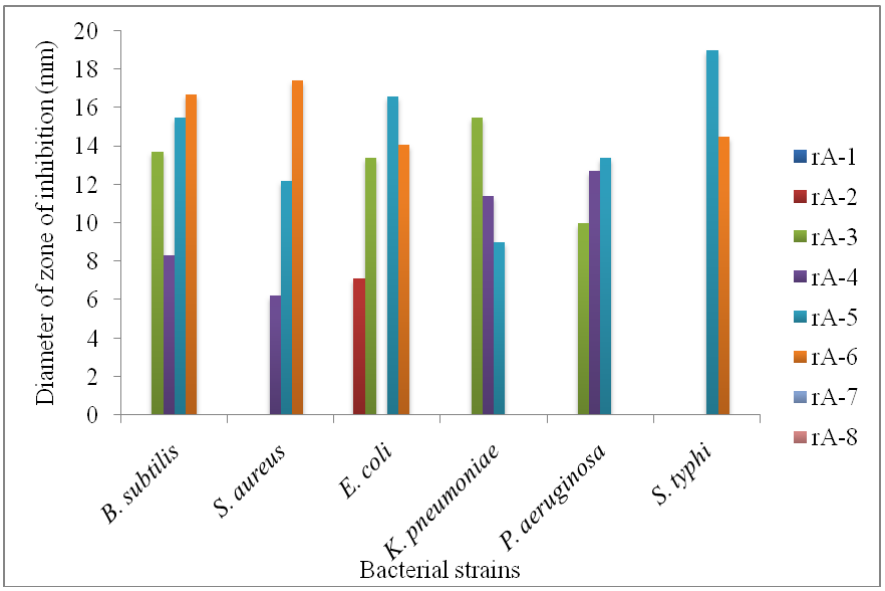

Figure 4.28: Antibacterial activity of the 8 column fractions (20 mg/ml each) against test bacterial strains

4.7.3.3 Antibacterial activity of column sub-fractions from rA-5

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Antibacterial activity of the 3 column sub-fractions (**rA-5a** to **rA-5c**) obtained from fractionation of column fraction **rA-5** of acetone soluble fraction of methanol extract of ripe fruits (**rA**) is presented in Figure 4.29

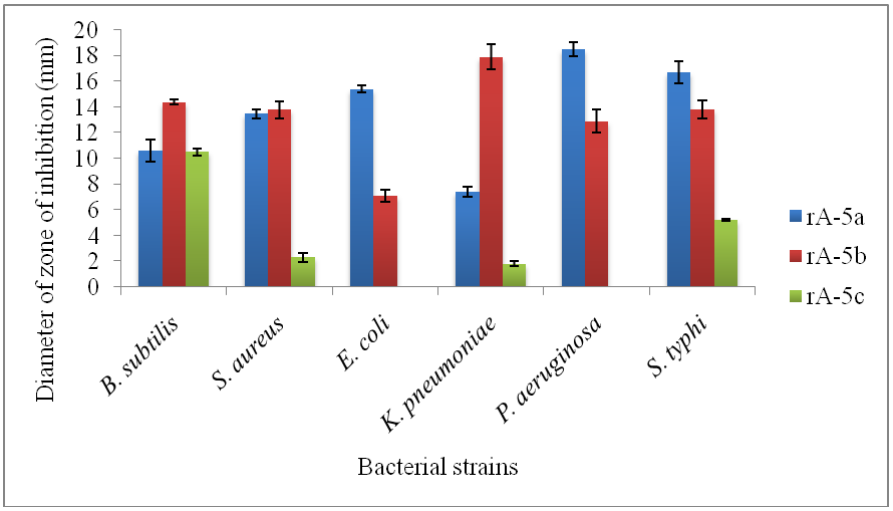

Figure 4.29: Antibacterial activity of the 3 major column sub-fractions (20 mg/ml each) against test bacterial strains

The error bars represent the standard error of the mean of duplicate readings.

4.7.3.4 Antibacterial activity of compound rA-5a1

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Antibacterial activity of DEHP (compound **rA-5a1**) obtained from purification of column sub-fraction **rA-5a** of acetone soluble fraction of methanol extract of ripe fruits

(**rA**) in comparison with a standard drug is presented in Figure 4.30

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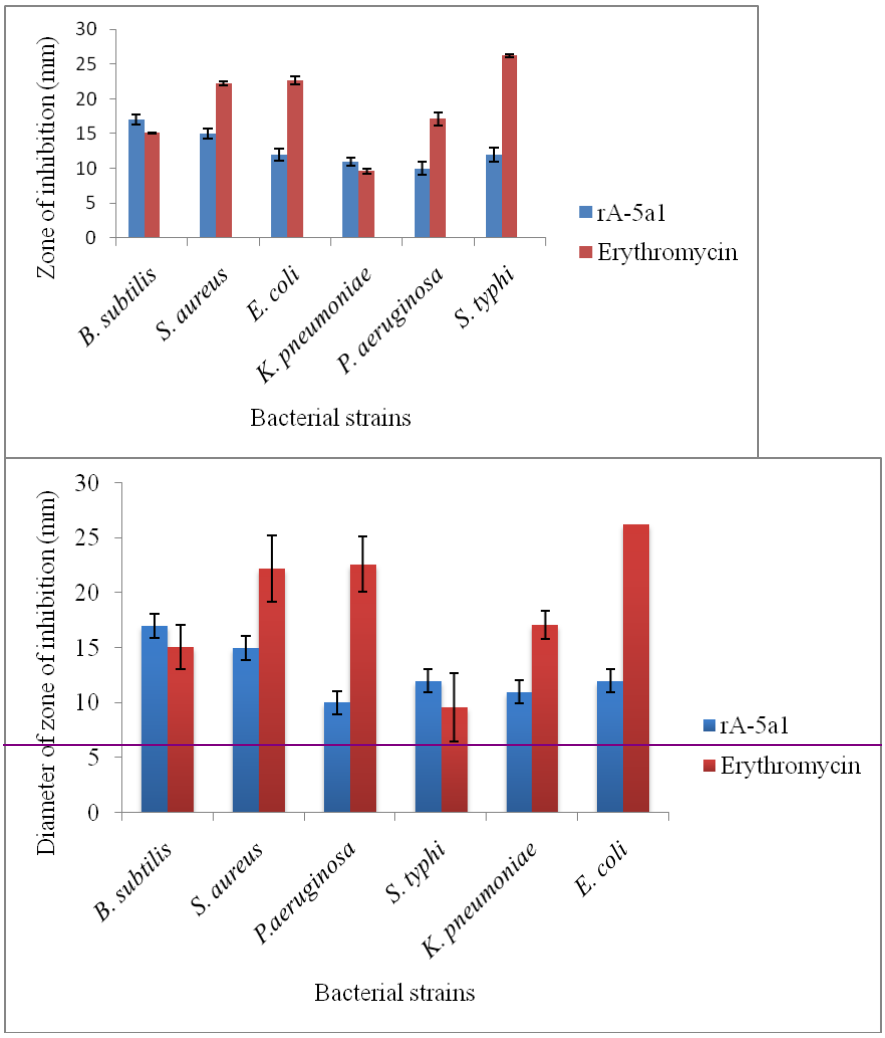

Figure 4.30: Antibacterial activity of compound **rA-5a1** (100 µg/ml) in comparison with erythromycin (1 mg/ml) against test bacterial strains

The error bars represent the standard error of the mean of duplicate readings.

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4.7.4 Antibacterial investigation of ethyl acetate-soluble fraction of unripe fruits (uE); its column fractions, sub-fractions and isolated compounds

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4.7.4.1 Antibacterial activity of uE

The antibacterial activity of ethyl acetate-soluble fraction of methanol extract of unripe fruits, **uE** in comparison with a standard is shown in Figure 4.31

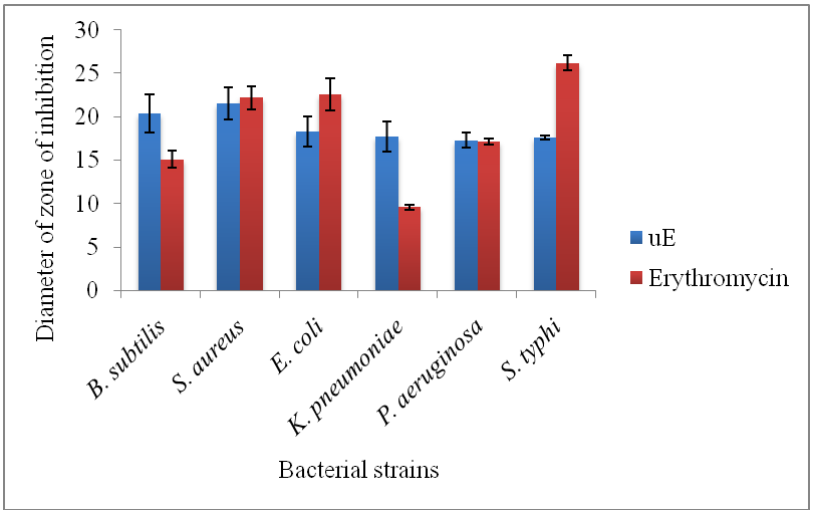

Figure 4.31: Antibacterial activity of ethyl acetate-soluble fraction of unripe fruits of *N. latifolia,* **uE** (50 mg/ml) in comparison with erythromycin (1 mg/ml)

The error bars represent the standard error of the mean of duplicate readings.

4.7.4.2 Antibacterial activity of fractions from uE

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The antibacterial activity of column fractions (**uE-1** to **uE-5**) collected from fractionation of ethyl acetate-soluble fraction of methanol extract of unripe fruits, **uE** is shown in Figure 4.32

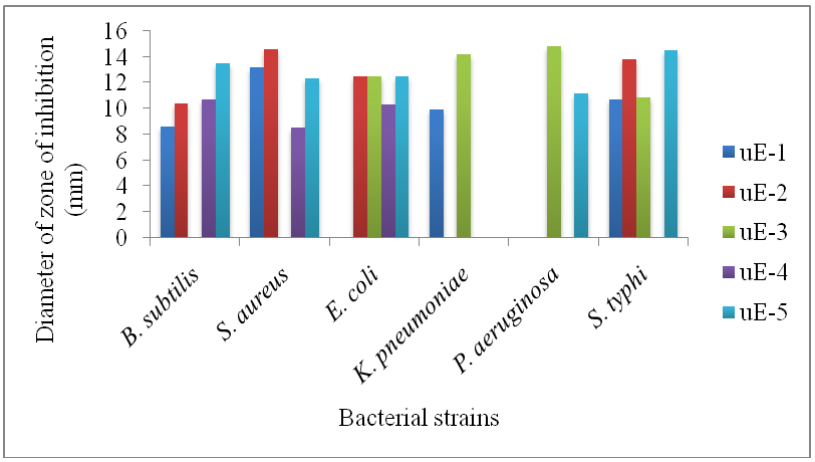

Figure 4.32: Antibacterial activity of the 5 column major fractions (20 mg/ml each) against test bacterial strains

4.7.4.3 Antibacterial activity of column sub-fraction uE-2a

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The antibacterial activity of a column sub-fraction (**uE-2a**) collected from fractionation of column fraction **uE-2** of ethyl acetate-soluble fraction methanol extract of unripe fruits, **uE** is shown in Figure 4.33

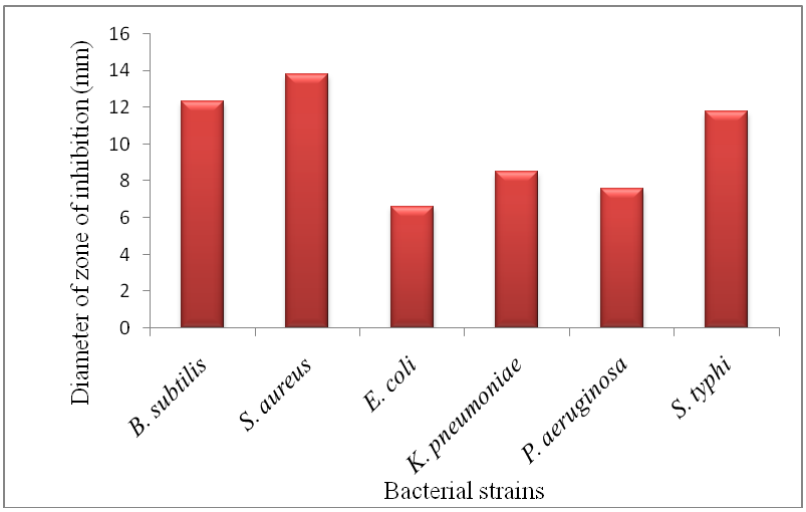

Figure 4.33: Antibacterial activity of column sub-fraction **uE-2a** (20 mg/ml) against test bacterial strains

4.7.4.4 Antibacterial activity of compound uE-2a1

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The antibacterial activity of DEHP (compound **uE-2a1**) isolated from purification of column sub-fraction **uE-2a** of ethyl acetate-soluble fraction of methanol extract of unripe fruits, **uE** in comparison with the standard is shown in Figure 4.34

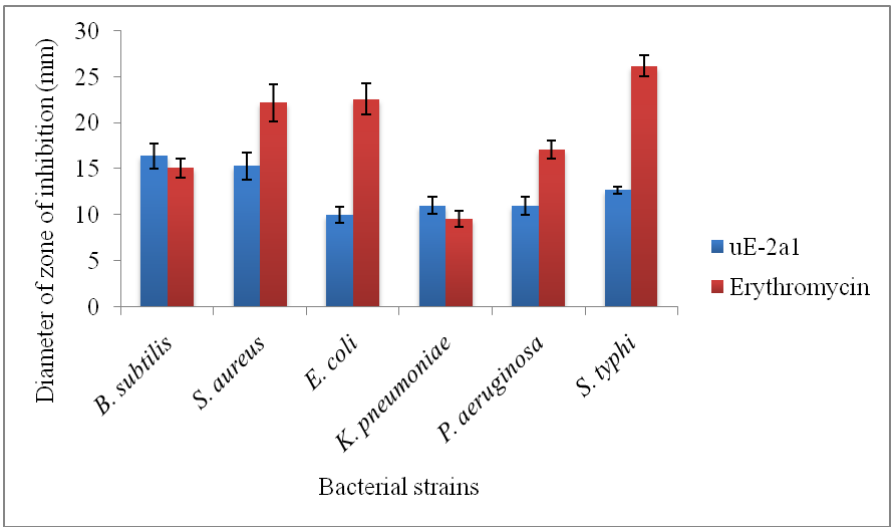

Figure 4.34: Antibacterial activity of compound **uE-2a1** (100 µg/ml) in comparison with erythromycin (1 mg/ml) against test bacterial strains

The error bars represent the standard error of the mean of duplicate readings.

4.7.4.5 Antibacterial activity of compound uE-3a2a

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The antibacterial activity of β-sitosterol (compound **uE-3a2a**) isolated from purification of column sub-fraction **uE-3a2** of ethyl acetate-soluble fraction of methanol extract of unripe fruits **uE** in comparison with the standard is shown in Figure 4.35

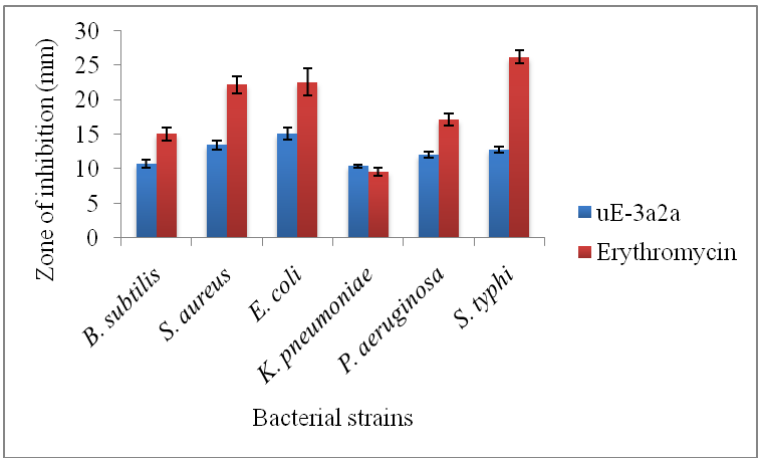

Figure 4.35: Antibacterial activity of compound **uE-3a2a** (100 µg/ml) in comparison with erythromycin (1 mg/ml) against test bacterial strains

The error bars represent the standard error of the mean of duplicate readings.

CHAPTER FIVE

5.0 DISCUSSION

5.1 Extraction and Yield of Phytoconstituents

Different solvents have been demonstrated to have the ability to extract different phytoconstituents, which depends on their polarity and solubility in the solvents (Marjorie, 1999). Often, low polarity solvents yield more of liphophilic compounds, whereas, alcohol extracts yield both non-~~a~~polar and mid-polar components, while water extracts yield only polar components (Yrjonen, 2004). The methanol extracts of both ripe and unripe contained more of the extractives than the petroleum ether extracts of both fruits as shown in Table 4.1. Generally, methanol has been reported to be a better solvent for more consistent extraction of plant phytoconstituents from medicinal plants compared with other solvents such as petroleum ether, chloroform, ethanol and water (Ahmad *et al*., 1998; Eloff, 1998; Lin *et al*., 1999; Gulluce *et al*., 2004; Masoko *et al.,* 2008; Koday *et al*., 2010).

Successive partitioning of the crude methanol extract of the ripe fruits using non-~~a~~polar and mid-polar solvents showed that more of the phytoconstituents were extracted into the polar solvents (n-butanol and water) with the residual water fraction (**A**) possessing the highest quantity of the phytoconstituents. This implies that the phytoconstituents of the methanolic extract of the ripe fruits are more of polar components than non-polar, since `like dissolves like'. Attempt to partition the methanol extract of the unripe fruits using same solvents as above, yielded no significant quantities, implying that the phytoconstituents of the unripe fruits may likely not be hydrophilic in nature. Partitioning between solvents is a method of preliminary separation of complex plant matrices (Mahlke *et al*., 2009).

Exhaustive successive further extraction of the water-insoluble portions (partitioned in-soluble) of both ripe and unripe methanol extracts using polar and non-polar solvents, again showed that the bioactives were extracted more into the polar solvents. This still confirms that both the ripe and unripe fruit extracts contain more of polar than apolar phytoconstituents. Generally, polarity of solvents affect the quantity and types of bio-molecules eluted from extracts, more polar solvents most often elute more active molecules (Eloff, 1998).

5.2 Preliminary Phytochemical Screening

Phytochemical screening of the petroleum ether extracts of both the ripe (**rP**) and unripe (**uP**) fruits of *N. latifolia* using standard methods as shown in Table 4.2 revealed only a strong presence of steroidal/triterpenoidal aglycones (sapogenins), all other group of constituents were practically absent or just fairly present. The methanolic extracts of the ripe (**rM)** and unripe (**uM)** fruits, their partitioned-soluble fractions and re-extracted soluble fractions all revealed the presence of almost the same class of phytoconstituents. The presence of carbohydrates, tannins (higher in unripe fruits), saponins and its aglycones, flavonoids, alkaloids and coumarins was observed. Phlobatannins, combined anthraquinones and tetraterpenoids were absent in all the extracts. All observed phytoconstituents serve as lead compounds in drug discovery and design (Chakravarthy and Gode, 1985; Ebi and Ofoefule, 2000).

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5.3 Compound E-2f1a

This compound was obtained from the ethyl acetate partitioned-soluble fraction of the defatted methanol extract of the ripe fruits of *N. latifolia* as colourless oil. TLC of the oil in two different solvent systems revealed that it as a single spot, fluorescencing

compound under UV light and on spraying with vanillin - H_2SO_4. Its solubility in mid-polar organic solvents, indicates that it is a mid-polar compound, while its optical rotation indicates that it is a dextrorotatory enantiomeric compound, with stereocentres likely at C-1, C-2, C-3, C-4, C-5, C-6 and C-2'. Its melting and boiling points is within the range of those obtained for substituted aromatic aldehyde derivatives, melting point> 45^{o}C and boiling point > 199^{o}C (Criddle and Ellis, 1976; Furniss *et al.*, 1989; Jones, 1997).

The formation of a phenylhydrazone on addition of 2, 4-dinitrophenylhydrazine to compound **E-2f1a,** suggests that the compound could be an aldehyde or a ketone, while, its formation of a silver mirror with Tollen's reagent confirmed that it is an aldehyde and not a ketone. Generally, only aldehydes reduce tollen's reagent. Also, its inability to give a red precipitate with Fehling's A and B shows that it is not an aliphatic aldehyde, but rather an aromatic. Most often, aliphatic aldehydes give positive test with Fehling's (Furniss *et al.*, 1989).

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The proton NMR of **E-2f1a** (Figure 4.1) revealed the presence of a weak deshielded singlet at δ9.85, indicating the proton of an aldehyde. Usually, the protons attached to a carbonyl usually resonate at lowest field position (Williams and Fleming, 1987; Abraham *et al.*, 2002; Field *et al.*, 2005) and the aldehydic proton produces a weak signal due to lack of neighbouring proton, while protons ortho- (position 2 and 6) to the aldehyde are more deshielded than protons in the meta- position (3 and 5) giving rise to a doublet of doublets, while that in the para- position is splitted by two adjacent protons

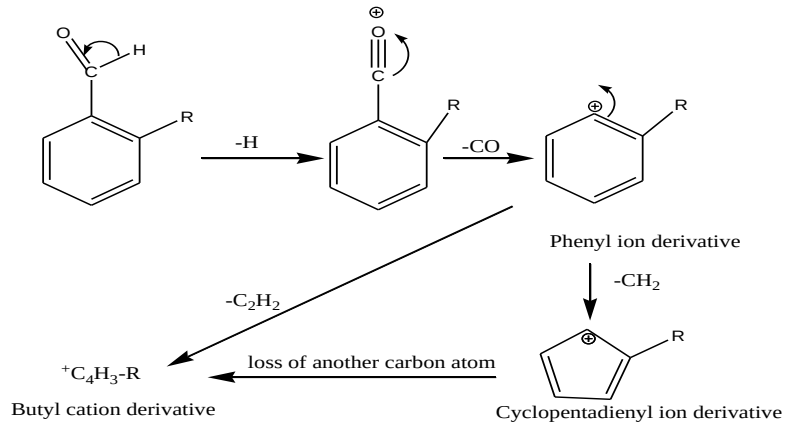
into a triplet signal (Abraham and Mobli, 2008). Lack of signals for C-1 and C-2 (ortho-position) is an indication that they lack protons on them: 3^{o}carbon atoms bearing no H atom. The presence of an electronegative oxygen atom in the moleculecaused the methylene protons at C-3' to resonate as multiplets at lowfield (δ4.10 -4.35) because of anisotropic effect. Another anisotropic effect was observed in the methylene protons (C-1') attached to the phenyl group (δ3.60 -3.75). The less shielded intense singlet at δ1.3 indicates three overlapping methyl groups in similar environment (Abraham *et al*., 2002; Field *et al*., 2005).

The proton de-coupled ^{13}C-NMR spectrum of **E-2f1a** (Figure 4.2) revealed fourteen peaks of which few are of lowfield (deshielded) while most are of highfield (shielded). The weak peak at δ210.8 is attributable to an aldehydecarbon (C-1"), a value slightly higher than that of an isolated aldehyde (Carey, 2003). This indicates the presence of an ortho substituent (alkyl) in an aromatic aldehyde. Usually, the steric effects of ortho- and alkyl substituents cause the deshielding of the carbonyl carbon. The electron-donating substituents increase the electron density at the carbonyl carbon (Nummert *et al*., 2009; Bruice, 2011). The weak peaks at δ144.1 to δ124.7 are attributed to the presence of aromatic carbon atoms in which C-1 and C-2 are tertiary C atoms, while C-3 and C-5 (methine carbons) are almost in the same environment. A weak peak at δ 69.55 is as a result of increased downfield effect of a 3^{o} carbon attached to an electronegative oxygen atom at C-4' (Silverstein *et al.*, 1991).

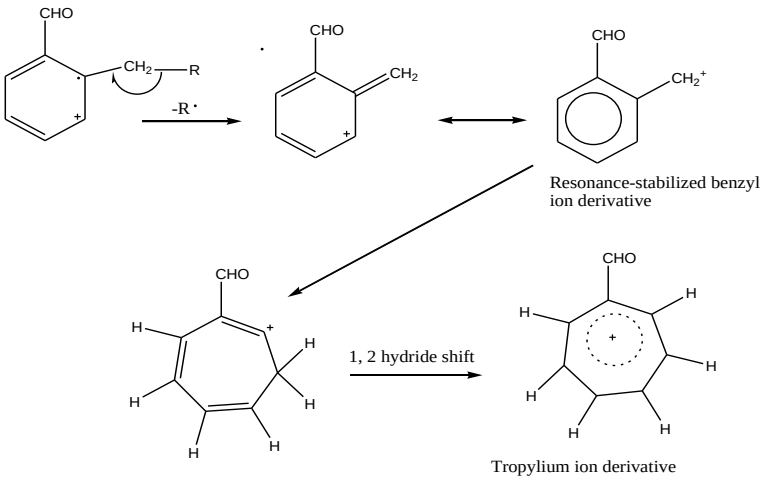
Among the 14-carbon singlet resonances (Figure 4.3) obtained from distortionless enhancement of polarization transfer at pulse 135^{0} (DEPT - 135), it was revealed that 4 peaks were nulled, 3 appeared inverted, while the remaining peaks retained their normal configuration, out of which 5 are methines and 4 are methyls. Generally, DEPT helps to

distinguish the various types of carbon atoms in a given molecule according to the number of hydrogens attached to each carbon (Carey, 2003).

Ionization of compound **E-2f1a** (Figure 4.4) by electron impact and dissociation into various smaller fragments as shown in its GC-MS fragmentation patterns revealed the compound to be a substituted aromatic aldehyde (di-substituted benzene), with characteristic positive ion peaks as mass-to-charge ratios, m/z at 29, 51, 63, 77, 89 and 110. Aromatic aldehydes generally loose the aldehyde proton with further elimimation of CO to give the phenyl ion at m/z 77, which in turn eliminates a methylene group to give the cyclopentadienyl cation derivative at m/z 63 (Scheme 5.1). In aromatic compounds possessing alkyl substituents, cleavage is favoured at the substituted carbon atom, to generate stable carbocations. These cleavages are most probable at the bond β to the ring, giving rise to a resonance-stabilized benzyl ion derivative or the tropylium ion (Scheme 5.2).

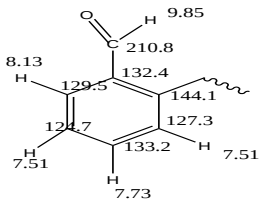


Scheme 5.1: —Fragmentation patterns typical of an aromatic aldehyde (Silverstein *et al*., 1991; Mohan, 2010).

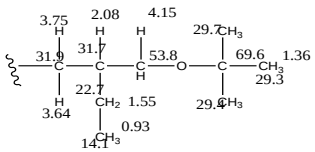


Scheme 5.2: ~~—~~Generation of a resonance-stabilized benzyl ion by cleavage of bond β to the ring (Silverstein *et al*., 1991)

From the spectral~~obtained~~ data, the structure of compound **E-2f1a** was proposed~~proposed~~ as Figure 5.1



(i)



(ii)

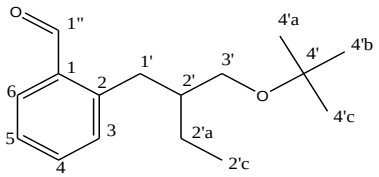


Figure 5.1: 2- (2'-Ethyl-3'-tertbutoxypropyl) benzaldehyde

Based on correlation of physical, chemical and spectral parameters of compound **E-2f1a** with literature; the structure of compound **E-2f1a** was concluded~~deduced~~ as 2- (2'-Ethyl-3'-tertbutoxypropyl) benzaldehyde (Figure 5.1). The compound and its derivatives have been synthesized and characterized from different sources (Abraham *et al*., 2002; Field *et al*., 2005; Abraham and Mobli, 2008; Nummert *et al*., 2009; Tanc *et al*., 2014; Hussein, 2015).

5.4 Compound rA-5a1

This compound was obtained as bright yellow-coloured oil, with a slight odour from solvent system hexane: chloroform (2:8) on purification of the acetone-soluble fraction of the methanol extract of the ripe fruits of *N. latifolia* (**rA**). Solublity of the oil in mid-polar organic solvents indicates that it is a mid-polar organic compound. GC - MS revealed it to be a high molecular weight compound with very low melting point and a high boiling point, indicating a low volatility (Staples *et al*., 1997; Cousins *et al*., 2003). Thin layer chromatography in different solvent systems revealed a single spotcompound, which fluorescened at 245 nm and 366 nm, an indication that it is contains an aromatic ring (Yrjonen, 2004). Its positive response to anisaldehyde-H_2SO_4 and I_2 vapour indicates that it is not a saturated alkane (Kovar and Morlock, 1996). Its observed small optical rotation at two different wavelengths, indicates that the molecule is only slightly chiral/almost achiral/a meso compound (Jones, 1997), with carbon 4'/4" being the only chiral centre.

Spectral characterization of compound **rA-5a1** showed the compound to be an aromatic carbonyl compound. Its UV spectrum (Figure 4.5) showed absorption at 275 nm, typical

of an n-π* transition of a carbonyl compound (Mohan, 2010). Proton NMR (Figure 4.6) revealed the presence of aromatic protons (two doublets of a doublet) at δ 7.74-7.72 and δ 7.58 - 7.54, indicating 4 protons in which a pair (H's at positions 2 and 5) and (H's at postions 3 and 4) are in the same chemical environment (di-substituted benzene). The septet at ~δ4.28- 4.19 is most likely the result of a CH_2 group (position 3'/3") germinal to an electronegative oxygen and the carbon atom bearing CH_2 and CH_3 protons, while, a pentet at ~δ1.63 is indicative of a CH (position 4'/4") bonded to two methylenes (n + 1 rule). A complex multiplet at ~δ1.85 to ~δ1.48 indicates several CH_2 groups (positions 5'/5", 6'/6", 7'/7" and 9'/9") resonating in the same chemical environment, hence the overlapping signals. Intense peaks at ~δ0.89 to 1.00 indicate a terminal CH_3 attached to a CH_2 (positions 8'/8" and 10'/10"). The peak integration ratio showed that the compound is made up of 38 protons. The obtained peaks for the unknown compound are typical of those of a dialkyl phthalate (Lide and Milne, 1994).

Carbon - 13 NMR of the compound (Figure 4.7) revealed a weak signal in a characteristic downfield region at ~δ167 indicating a carbonyl (C-1'/C-1") that is not aldehydic, since no signal was observed in the ^1H - NMR. The weak and medium peaks at ~δ132 to ~δ128 show the presence of aromatic carbon atoms in which C-1 and C-6, C-3 and C-4 and C-2 and C-5 resonated in the same chemical environment respectively. A medium peak at δ 68.1 is as a result of increased downfield effect as a result of a CH_2 group being attached to an electronegative oxygen atom (C-3'/C-3"), while a medium peak occurring upfield of 68.1 ppm ie at ~δ38.7 indicates the occurrence of a CH groupas a neighbour to a CH_2 benefitting from the effect of anisotropy (C-4'/C-4"). The obtained peaks for the unknown compound are typical of those of a dialkyl phthalate (Lide and Milne, 1994).

Although the ^{13}C spectra revealed the presence of only 12 carbons atoms, its GC - MS spectra revealed 24 carbon atoms (molecular formular $C_{24}H_{38}O_4$), an indication that the compound consisted of two identical parts, which are mirror images of each other (Alim Al-Bari *et al.,* 2006), that is, there is the presence of a plane of symmetry in the molecule, confirming that it is a meso compound.

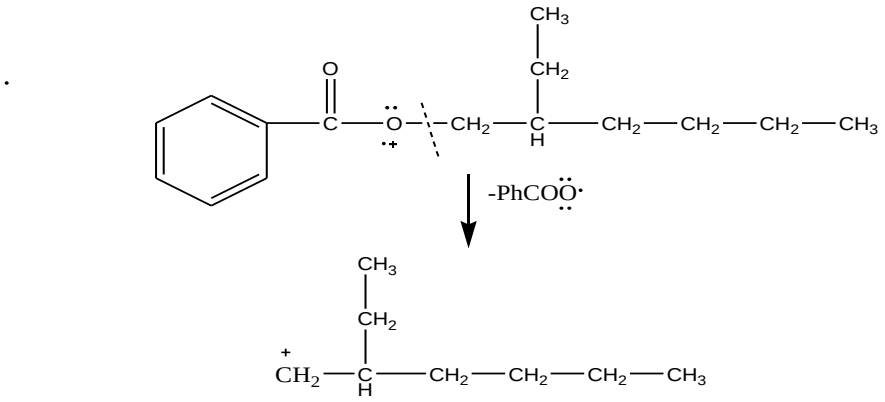
Among the 12 carbon resonances obtained, DEPT - 135 (Figure 4.8) revealed that 2 were quaternary (disappeared in the spectrum), 3 were methine (normal peaks), 5 were methylenes (negative peaks) and two were methyl (above in the spectrum). Thisconfirms the nature/multiplicity of carbon atoms in compound **rA-5a1**.

The GC - MS fragmentation patterns(Figure 4.9) revealed the compound to be a phthalate, with characteristic peaks at m/z 149, 167 and 168 (Hites, 1985). Generally, phthalates undergo four common modes of cleavage, which account for some of their major fragmentions (Silverstein *et al.,* 1991) as shown in Schemes 5.3 - 5.6. Rearrangement ions such as that formed by McLafferty rearrangement are as a result of intramolecular atomic rearrangement involving migration of hydrogen atoms in certain molecules during fragmentation to generate prominent characteristic peaks as fragment ions. Usually, only molecules that posses:

(i) An appropriately located heteroatom (e.g O, N, S)

(ii) A π electron system (sp^2 or sp hybridized) and;

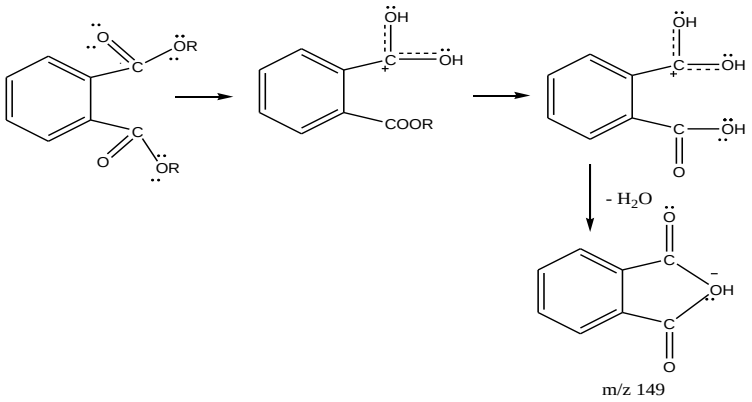
(iii) An abstractable H atom γ to the C bearing the heteroatom undergoes this rearrangement (Silverstein *et al*., 1991).



m/z 113

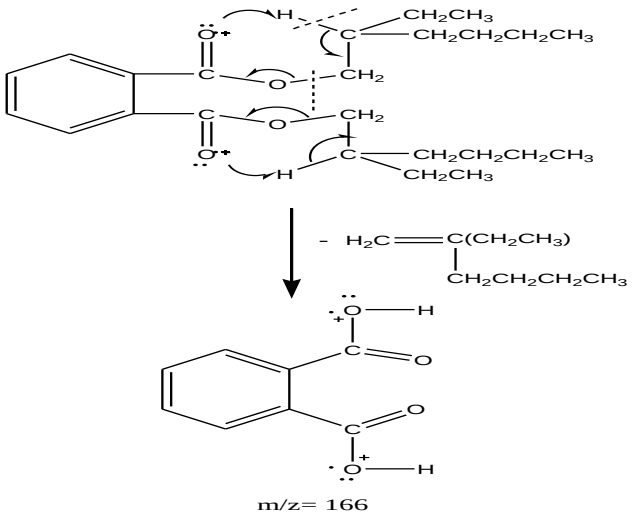
Formation of a 1^{o} carbocation

Scheme 5.3: Retention of positive charge by the alkyl group of the phthalate (William and Fleming, 1987; Silverstein *et al*, 1991; Mohan 2010)

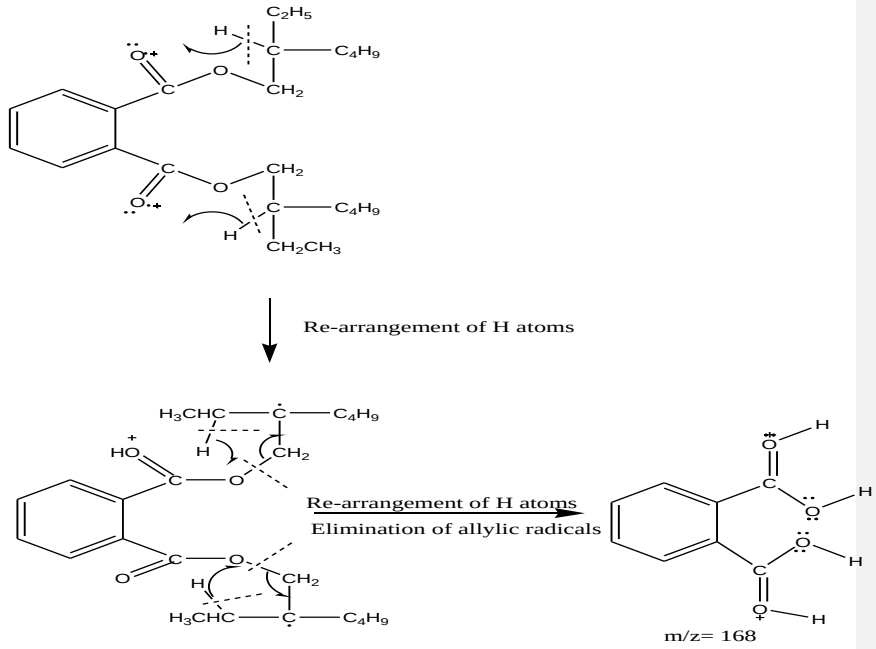


m/z 149

Scheme 5.4: Ester cleavage involving two hydrogen atoms and then another hydrogen atom, followed by dehydration (William and Fleming, 1987; Silverstein *et al*, 1991; Mohan 2010)



Scheme 5. 5: McLafferty rearrangement (William and Fleming, 1987; Silverstein *et al*, 1991; Mohan 2010)



Scheme 5.6: Rearrangement of two hydrogen atoms with elimination of an allylic radical (William and Fleming, 1987; Silverstein *et al*, 1991; Mohan ——2010)

From the obtained data, it can be concluded~~deduced~~ that the structure of compound **rA-5a1** is as shown in Figure 5.2

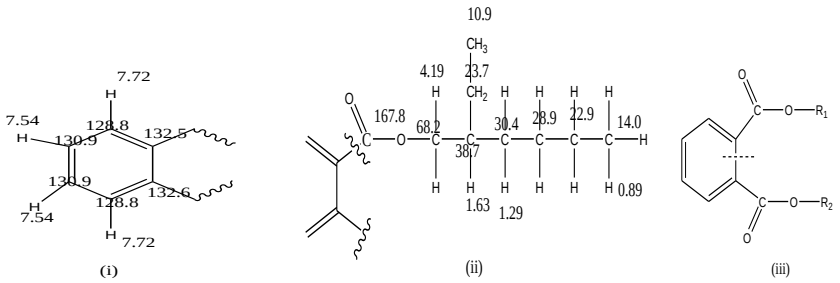


Figure 5.2: Di- (ethylhexyl) phthalate (DEHP) /Bis- (2-ethylhexyl) phthalate (BEHP)/1, 2-Benzenedicarboxylic acid, dioctyl ester.

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By comparison of the obtained physical and spectral data with those published in literature (Amade *et al.*, 1994; Rao *et al.*, 2000; Alim Al-Bari *et al.*, 2006; Habib and Karim, 2009; Lyutskanova *et al*., 2009; El-Sayed, 2012), the compound was characterized and identified as Di- (ethylhexyl) phthalate (DEHP), Figure 5.2. This is the first report of the isolation and characterization of DEHP from the ripe fruits of *N. latifolia*, although DEHP~~such a phthalate~~ and other phthalates have been isolated and characterized from quite a lot of medicinal plants as reviewed earlier (Section 2.4).

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5.5 Compound uE-2a1

The compound was obtained from the ethyl acetate-soluble fraction of the methanol extract of the unripe fruits, **uE** as golden yellow oil with a slight odour. Like compound **rA-5a1** above, it was shown to be a mid-polar compound, with very similar optical rotations, melting and boiling points. Its positive response to vanillin-sulphuric acid indicates that it possesses an aromatic ring (Yrjonen, 2004). Spectral characterization of the oil (Figures 4.10 - 4.13) revealed that the compound gave almost same signals with

compound **rA-5a1.** Therefore, it was concluded that compound **uE-2a1** is same as compound **rA-5a1.**

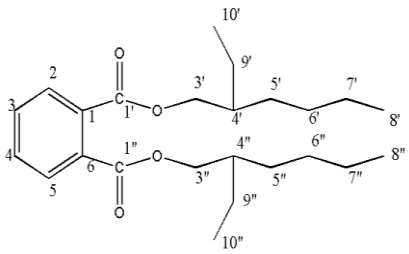

Figure 5.3: Di-(ethylhexyl) phthalate (DEHP) /Bis- (2-ethylhexyl) phthalate (BEHP)/ 1, 2-Benzenedicarboxylic acid, dioctyl ester.

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These findings revealed that DEHP (Figures 5.2 and 5.3) is present in both the ripe and unripe fruits of *N. latifolia*, with the former possessing greater quantity (32.6 mg) than the latter (27.4 mg).

5.6 Compound uE-2a2

Scrapping, filtration and concentration of the minor band obtained from PTLC of column fraction **uE-2a** of soluble-fraction **uE** gave rise to a white amorphous compound coded (**uE-2a2**). Its physical characterization shows that it is a single spotted, low melting, non-polar, organic compound. Functional group identification of the compound revealed its negative response to 2, 4-dinitrophenylhydrazine test for aldehydes and ketones, though its spectral data (Figures 4.14 - 4.17) showed that it possesses a carbonyl group, an indication that the compound might not be an aldehyde or ketone, but an ester or acid anhydride. Slow approach of compound **uE-2a2** to alkali hydrolysis confirms that it is an ester and not an anhydride. Since anhydrides discharge the colour of phenolphthalein 'in situ' (Furniss *et al*., 1989).

Spectral characterization of the compound revealed that it is an alkene containing a carbonyl group in the form of an ester with several methylene groups joined together. Proton NMR of the compound (Figure 4.14) suggests the presence of deshielded protons downfield δ5.35 and 7.28, indicating a vinylic group at C-1 and C-2 (β- and α-positions respectively). Generally, π bonds are effective in influencing the chemical shift of nearby atoms, so that vinylic H's are shifted downfield/higher δ values (Williams and Fleming, 1987; Abraham and Mobli, 2008). Usually, the β proton (C-1) of a vinyl group attached to an oxygen atom is shifted upfield than the α proton (C-2) because of a higher electron density around the β proton as shown in Figure 5.4 (Silverstein *et al*., 1991; Mohan, 2010).

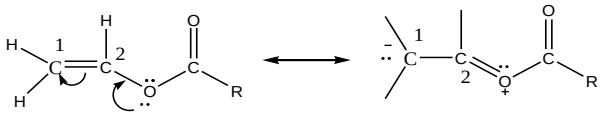


Figure 5.4: Delocalization of electrons in a α, β-unsaturated ester

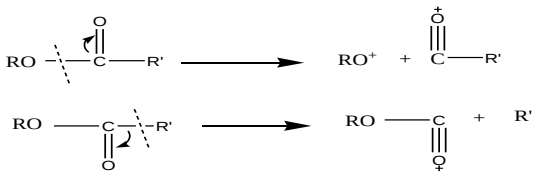
The triplet signal that appeared at δ2.34 - 2.35 is most likely due to methylene protons at position 4 attached to an electron-withdrawing carbonyl group at position 3. The quintet at C-5 is likely as a result of neighbouring group effect from 2H (C-3) and 2H (C-6) atoms, the n+ 1 effect, while other protons appearing at upfield/ lower δ values are likely due to other protons on methylene and methyl groups, since they are not attached to any electronegative specie. A sharp methylene singlet signal at about 1.45 ppm is an indication that there are several methylene protons resonating at the same frequency as a result of being in the same environment (positions C-6 to C-14). The most shielded triplet peaks at 0.86 ppm is as a result of a 3H atoms of a methyl group (Chang *et al*., 2008; Abozid and Ahmed, 2013).

The ^{13}C-NMR spectrum (Figure 4.15) revealed eleven proton de-coupled peaks. The peak at δ179.61 is attributable to a quartenary carbonyl carbon at position 3. A carbonyl group exhibits anisotropic effect on adjacent atoms. This causes the carbonyl carbon and the hydrogens bonded to neighbouring carbon atoms to resonate at the lowest field position because of combined effects of the induced anisotropic field and a nearby electronegative element (Williams and Fleming, 1987; Mohan, 2010). Signals at 130.5 and 129.7 ppm are typical of presence of an sp^2 methylene group at C-1 and methine group at C-2 respectively. The sharp-shielded peak at about 29 ppm confirmed the presence of several methylene carbon atoms resonating at same frequency. The fact that there were about 11 de-coupled visible carbon-NMR signals, with bold lines appearing between 29.1 - 29.4 ppm indicates that there are several methylene carbons in almost similar environments (C-6 to C-15). Deshielded peaks downfield of 29 ppm (33.98 and 31.92) accounts for the effect played by electronegative oxygen attached to a methylene group (Field *et al*., 2005; Kim and Chung, 2009).

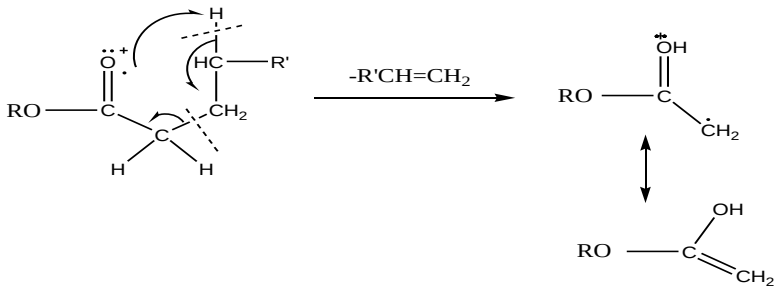
Among the 11 carbon resonances obtained, DEPT-135 (Figure 4.16) revealed that one wasa quaternary (disappeared in the spectrum), fourteen were methylene_(inverted peaks), one was a methine (positive peak) and onlyone was methyl (also normal)carbon atom.

The GC - MS fragmentation patterns (Figure 4.17) revealed the compound to be an aliphatic unsaturated ester, with characteristic peaks at m/z 84, 71 and 55. These peaks are due to bond cleavage next to a carbonyl group and the McLafferty rearrangement as shown in Schemes 5.7 - 5.8. Generally, for straight chain esters, their fragmentation pattern is such that cleavages could occur at C-C bonds to yield positive alkyl

fragments, such as $C_2H_5^+$ (m/z 29), $C_3H_7^+$ (m/z 43) and $C_4H_9^+$ (m/z 57). Cleavages could also occur at C-O bonds to yield oxygen-containing ions having the general formular $C_nH_{2n-1}O_2^+$ (where n= >2) (Silverstein *et al.*, 1991).



Scheme 5.7: Bond cleavage next to carbonyl carbon



Scheme 5.8: McLafferty re-arrangement

From the spectral~~obtained~~ data, it can be concluded~~deduced~~ that the structure of compound **uE-2a2** can be written as shown in Figure 5.5

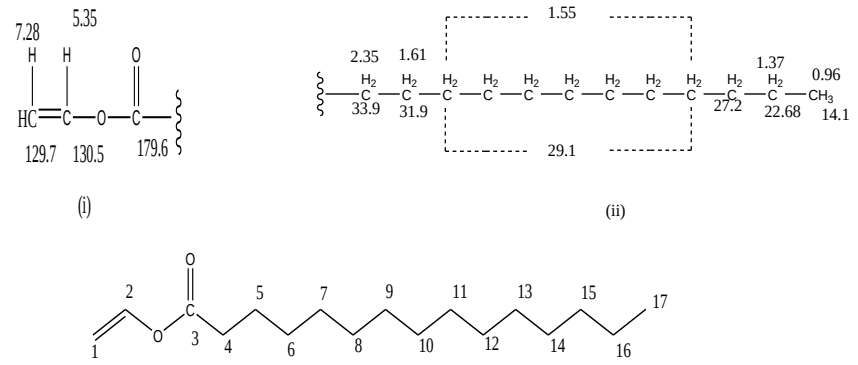


Figure 5.5: Ethenyl pentadecanoate / Pentadecanoic acid, ethenyl ester/ Pentadecanoic acid, vinyl ester/ Vinylpentadecanoate

Correlation of physical, chemical and spectral data of compound **uE-2a2** with literature values revealed almost similar data with an unsaturated fatty acid ester, Ethenyl pentadecanoate. Compound **uE-2a2** was therefore identified as Figure 5.5. The compound has been reported and isolated in several medicinal plants (Chang *et al.*, 2008: Kim and Chung, 2009; Qian *et al*, 2010; Li *et al*., 2011; Abozid and Ahmed, 2013; Csoka, *et al*., 2013; Khan *et al*., 2013; Su *et al*., 2013).

5.7 Compound uE-3a2a

Purification of column sub-fraction **uE-3**, obtained from fractionation of the ethyl acetate-soluble fraction of the defatted methanol extract of the unripe fruits of *N. latifolia* (**uE**) yielded some white crystalline compound, coded **uE-3a2a.** Physical characterization of the compound revealed that it is a homogenous compound on TLC, its positive response to I_2 vapour indicates that it is an organic compound and not a saturated alkane (Kovar and Morlock, 1996), while its positive response to vanillin - H_2SO_4 and anisaldehyde - H_2SO_4 is indicative of its nature as a steroidal compound (Saeidnia *et al*., 2014). Its positive Salkowski and Liebermann-Burchard's test confirms that it is a steroidal compound, possessing a steroidal nucleus (Chaturvedula and

Prakash, 2012), while its positive test to alcohol shows that it is a sterol possessing an alcoholic OH group (Kamboj and Saluja, 2011; Ahmed *et al*., 2013). It also revealed that it is a mid polar, high molecular weight compound with high melting point. Its observed (-0.05) and calculated (-100) optical rotation at wavelength 589 nm shows thatthe molecule is a chiral, levorotatory compound (with likely 9 stereocentres at positions C-2, C-3, C-5, C-8, C-9, C-10, C-13, C-14 and C-17).

Spectral characterization of compound **uE-3a2a** by proton NMR (Figure 4.18) shows peaks that are characteristic of a steroidal system, likely a tetracyclic skeleton (Tripathee *et al.,* 2011). Doublet at δ4.70- 4.69 suggests the presence of a proton at position C-6 (i.e olefinic bond at C-5 and C-6), while proton corresponding to H-3α of a sterol moiety appeared as a tripleof doublet of doublets at δ3.37 - 3.34, δ3.23 - 3.22 and δ3.20 - 3.19, supporting the findings of Chaturvedula and Prakash (2012). Usually, a multiplet at δ3.35 is assigned to the carbinolic proton at C-3. The shielding of this signal indicates its α-orientation (Kumar *et al*., 2014). The spectrum also displayed double of triplets at δ2.44-2.41 and δ2.39-2.37 assigned to methylene protons at positions C-4 and C-7 (i.e neighbours to olefinic proton at C-6). Other triplet and multiplet peaks appeared in the upfield region of between 1.7 and 0.7 ppm (Sen *et al*., 2012). Two angular protons resonating as singlets at δ0.72 and 0.99 are due to quaternary methyl protons at C-18 and C-19 respectively, while; another singlet at δ1.01 was due to primary methyl protons at C-29. The peak integration ratio confirmed that the compound is made up of 50 protons.

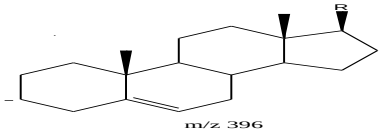
Proton de-coupled Carbon-13 NMR spectrum revealed a total of 29 peaks (Figure 4.19). Of these peaks, signals at 150.48 and 109.70 ppm, were assigned to C_5 and C_6 double

bonds respectively as in Δ^5 spirostene (Agarwal *et al*., 1985), while, a signal at 79.00 was assigned to a carbon bearing an electronegative β-hydroxyl at postion 3 (an oxymethine). Usually, OH at C-3 with a β orientation shows a signal of a carbinolic carbon that appears at 78-79 ppm (Kumar *et al*., 2014). A deshielded peak at ~47.79 ppm was assigned to C-4 because it is a neighbour to C-3 (Ring A, bears the OH group). Another downfield peak at ~60.57 ppm was given to C-17 (Ring D) as a result of the alkyl substituents attached to it. The signals at δ18.3 and 19.1 ppm correspond to angular carbon atoms at C-18 and C-19 respectively, an indication that C-18 is more shielded than C-19 (Pateh *et al*., 2008). Other lowfield peaks were assigned to methine carbons, while highfield peaks were given to methyl carbons.

Among the 29 carbon resonances obtained, DEPT - 135 (Figure 4.20) revealed that three carbon atoms were quaternary (disappeared in the spectrum), nine were methine (above in the spectrum), eleven were methylene (below in the spectrum) while six were methyl (above in the spectrum) carbon atoms (Patra *et al*., 2010).

The GC - MS fragmentation patterns (Figure 4.21) revealed **uE-3a2a** to be a tetracyclic steroidal compound, with characteristic fragment ion peaks at m/z 396, 329, 303, 275 and 273. The intense peak at m/z 396 (Scheme 4.9) indicates loss of water from the molecular ion (M^+-18), which is characteristic for dehydration of steroidal compounds (Gangwal *et al*., 2010). Generally, cyclic alcohols like cyclohexanol (2^o alcohol) undergo fragmentation-involving dehydration by complicated pathways (Silverstein *et al*., 1991). Usually, water is lost by the loose of α-H and β-OH groups (Carey, 2003). Peaks, especially at m/z 329 (M^+- $OC_5H_9^+$), 303 (M^+- $OC_7H_{11}^+$) and 275 (M^+- $OC_9H_{15}^+$)as shown in Schemes 5.10 - 5.11 are diagnostic peaks for sterols possessing Δ^5

– unsaturation. Such compounds fragment readily by a pathway in which the molecular ion looses the ring bearing OH with other rings to form various carbocations (Carey, 2003). Usually, cleavage is favoured at substituted carbon atoms helping to generate carbocations in which degree of stability is cyclic C^+> 3^oC^+> 2^oC^+> 1^oC^+> methyl C^+ , that is; R_3C^+>R_2HC^+> RH_2C^+>H_3C^+(Silverstein *et al.*, 1991). Peak at m/z 273 (414-$C_{10}H_{21}^+$) is attributable to the loss of R substituents on ring D, that is, at position C-17. Usually, saturated rings tend to lose alkyl side chains to form a positive charge on the ring fragment. Usually, cleavage is favoured at alkyl substituted carbon atoms, so that, the more substituted, the more likely is the cleavage (Silverstein *et al*., 1991) as shown in scheme 5.13. Another peak at m/z 255 (M^+- $OC_5H_9^+$ - 18) is likely as a result of loss of side chain at C-17 and further dehydration of such fragment ion as shown in Scheme 5. 14. Another peak at m/z 231 (M^+- $C_{13}H_{27}^+$) is likely as result of loss of R group at C-17 with ring D to form a 3^o carbocation at the position where ring D was as shown in Scheme 5.15



Scheme 5.9: Loss of a water molecule from the molecular ion

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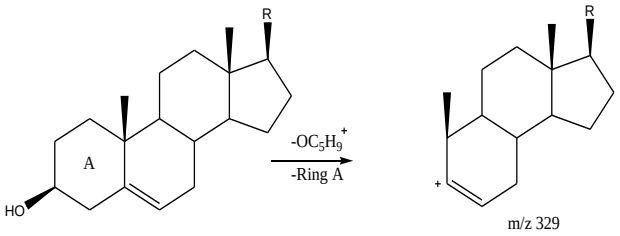
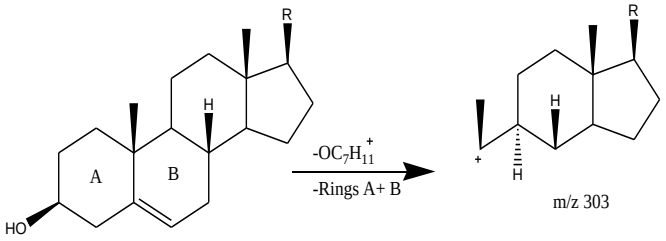
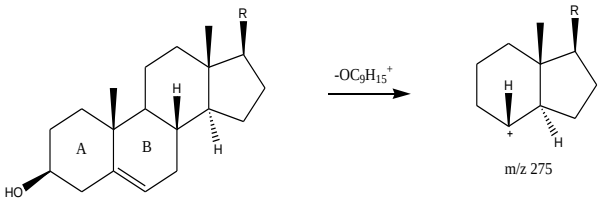


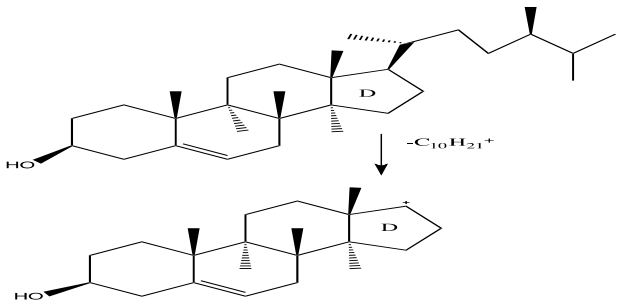
Scheme 5.10: Formation of a resonance-stabilized 3^o C^+ in ring A



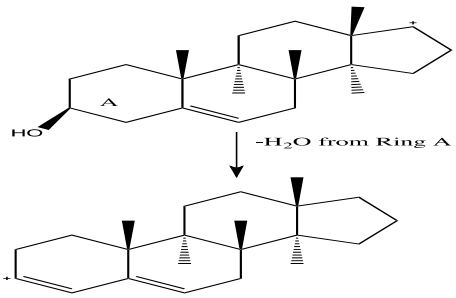
Scheme 5.11: Formation of a resonance-stabilized 3^o C^+ in rings A and B



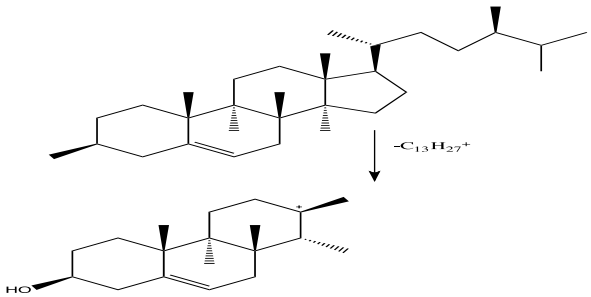
Scheme 5.12: Formation of cyclic resonance-stabilized C^+ on ring C



Scheme 5.13: Retention of positive charge on ring D (m/z 273)

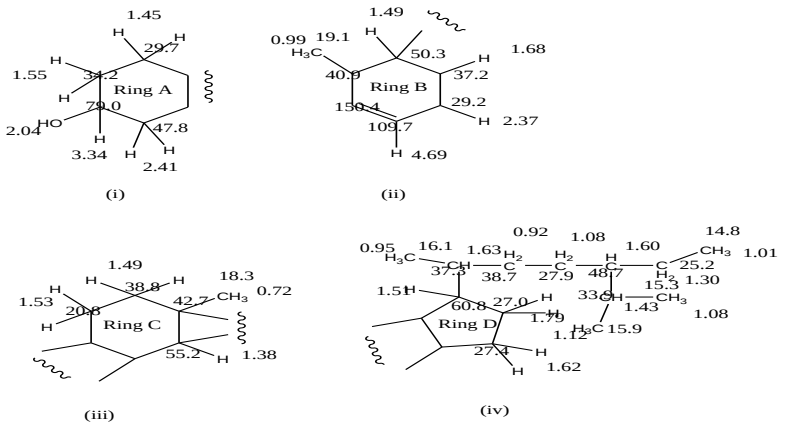


Scheme 5.14: Dehydration of residual ring fragment (m/z 255)



Scheme 5.15: Loss of R group + ring D (m/z 231)

From the spectral ~~obtained~~ data, it can be concluded ~~deduced~~ that the structure of compound of **uE-3a2a**~~,~~ is ~~can be written~~ as shown in Figure 5.6



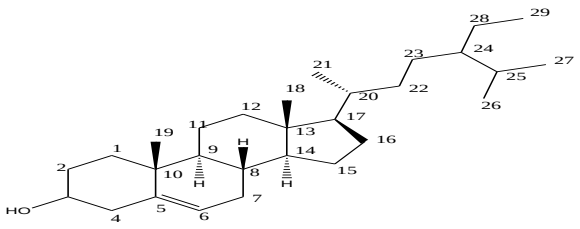


Figure 5.6: β-Sitosterol/ 24β-Ethylcholesterol/ 22, 23-Dihydrostigmasterol/ 5-Stigmasten-3β-ol (24R) / 24S-Stigmast-5-en-3β-ol / β-Dihydrofucosterol

A comparative study of the obtained physical, chemical and spectroscopic data of compound **uE-3a2a** with those published in literature revealed it to be structure Figure 5.6. The assignments are in good agreement with other published works for the structure of the compound. β-sitosterol has been isolated and characterized by several authors from different plants (Habib *et al*., 2007; Pateh *et al*., 2008; Patra *et al.*, 2010; Ahmed *et al*., 2010; Kamboj and Saluja, 2011; Trivedi and Choudhrey, 2011; Chaturvedula and Prakash, 2012; Sen *et al*., 2012; Ahmed *et al.*, 2013; Tripathi *et al*., 2013b). This is the first report of the isolation and characterization of a phytosterol from the unripe fruits of *N. latifolia*, although, Isah *et al* (2014) had earlier reported the isolation of the same compound from the stem bark of the plant (section 2.5).

5.8 Antibacterial Studies

5.8.1 Preliminary antibacterial screening

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The crude petroleum ether **(rP)** and methanol **(rM and uM)** extracts of the ripe and unripe fruits of *N. latifolia* ~~at 10 mg/ml and 50 mg/ml each, exhibited no significant activities against any of the test organisms, while at 100 mg/ml,~~ did not inhibit the growth of any of the test organisms at 100 mg/ml, while the petroleum ether extract of the unripe fruits (**uP**) exhibited moderate inhibitory activity against some Gram-negative organisms withe~~, with~~ diameter of zones of inhibition ranging from 12.1~~0~~ - 15.3 mm. The methanol extract **(uM**), petroleum ether **(rP)** and methanolic (**rM**) extracts of the ripe fruits showed no significant antibacterial activity with zones ranging from ~~0~~ 3.11—13.3 mm (Table 4.28). Although, **uP** revealed the presence of only steroidal nucleus and coumarins (Table 4.2), the moderate activity expressed by this

extract might be as a result of the concentration of these bioactive agentss or the degree of interrelationship between them or other constituents. Generally, the antimicrobial property of a plant extract is correlated with the quantity of bioactive agentss and the interrelationship, which occurs amongst them (Maffei-Facino *et al*., 1990; Hili *et al*., 1997). Plant constituents, even in relatively low concentrations could be responsible for the observed activity (Dall'Agnol *et al*., 2003).

For the organic solvent-water partitioned fractions of the ripe fruits at 50 mg/ml, the ethyl acetate partitioned-soluble fraction (**E**) significantly inhibited the growth of Gram-negative *K. pneumoniae* (17.6 mm) and *S. typhi* (17.3 mm), while the butanol partitioned-soluble fraction (**B**) significantly inhibited the growth of both Gram-positive and Gram-negative bacteria tested, except for *E. coli* (0 mm). The fraction was more active against Gram-negative, with diameter of zones of inhibitions ranging from 15.315.3 - 17.3 mm than Gram-positive bacteria (12.2 – 14.7 mm). The enhanced activity of the partitioned-soluble fractions may be attributed to the presence of some constituents which may be acting synergistically with one another or with other constituents in the fractions (Doughari and Obidah, 2008) or may be the active constituents, which are usually present in trace or dilute amounts become more concentrated with partitioning (Ndip *et al.,* 2009). Partitioning between solvents is an adequate approach for the preliminary separation of complex plant matrices (Mahlke *et al*., 2009).

For the re-extracted soluble fractions at 50 mg/ml, the ethyl acetate-soluble fraction of the unripe fruits **(uE**) displayed significant inhibitory activity against all tested strains, with Gram-positive *B. subtilis* (20.4 mm ± 0.82) and *S. aureus* (21.5 mm ± 0.92) being

the most susceptible. Activity was better than that displayed by chloramphenicol (15.5 mm) and tetracycline (17.2 mm). Sometimes, because plant extracts are complex mixtures, which contain many constituents in which biological activity of a given extract, probably reflect contributions from a number of the constituents (Ndip *et al.*, 2009).

Gram-positive *Staphylococcus aureus* was susceptible to most of the soluble-fractions of both fruits, with zones ranging from 7.33~~16.0~~ – 21.5 mm, especially, the unripe fruits (12.3 - 21.5 mm), while the ripe ranged from 7.33 - 21.2 mm). This is not unusual because *S. aureus* is affected by most antimicrobials (Omwenga *et al.*, 2009). However, this finding is still noteworthy, although, diarrhoea caused by *S. aureus* is usually short and self-limiting (Timbury *et al.*, 2002). All extracts and fractions of the ripe fruits were not active against Gram-negative *Escherichia coli* (0~~0~~ – –11.7 mm), even at higher concentration, while the petroleum ether extract of the unripe fruits (**uP**) at 100 mg/ml (15.0 mm) and some of the soluble fractions of the unripe fruits at 50 mg/ml (17.7 - 18.4 mm) showed significant activity against the organism ~~with zones ranging from 15.0 - 18.4 mm~~. Other Gram-negative bacteria, like *Pseudomonas aeruginosa* and *Salmonella typhi* were also quite susceptible to some of the partitioned fractions (16.4 -17.3 mm) and soluble fractions (14.2 -18.3 mm). Inhibitory activity against *P. aeruginosa* (15.3 - 18.3 mm) is a good development, as it is known as one of the most difficult organisms to manage by commonly used antibiotics because of its cell wall properties (Higgins *et al.*, 2002). *S. typhi* which causes enteric fever, gastroenteritis or food poisoning, is also a bacterial cause of diarrhoea. This probably explains why the plant has found its use as anti dysentery and anti-diarrhoeal in most African countries (Abbiw, 1990; Iwu *et al.*, 1999).

Generally, the extracts and fractions exhibited a concentration-dependent activity against all the organisms used in this study, as evident by their diameter of zones of inhibition. Activity of some of the test compounds (50 mg/ml) compared favourably with those of the standard antibiotics (1 mg/ml) as shown in Table 4.28. For example, the ethylacetate (**uE** 21.5 mm), and diethyl ether (**rD**; 21.2 mm) soluble fractions exhibited comparable activity to erythromycin (22.2 mm), while it exhibited a higherbetter inhibitory activity than chloramphenicol (16.1 mm) and tetracycline (16.6 mm) against *S. aureus.* This shows that some of the fractions of the ripe and unripe fruit of *N. latifolia* are quite active and promising when compared to some of the antibiotics, although, there was generally a higher susceptibilityresponse of the organisms to the standard antibiotics (15.1 – 26.2 mm) than the fractions (14.2 – 21.5 mm). This is probably because the partitioned fractions and soluble fractions being in their crude forms sometimes contain very small amount of the bioactive compound(s), whose biological activity sometimes gets improved with increase in concentration of the fractions. However, as with some drugs, some plant fractions may be more potent *in-vivo* due to metabolic transformation of its components into highly active intermediates that undergoes interaction with the immune system (Ngemenya *et al*., 2006). It is of note, that most plant materials have provided lead compounds for the development of the models for 50 % Western drugs (Robbers *et al*., 2006).

The unripe fruits (14.8 – 21.5 mm) displayed higherbetter activity than the ripe fruits (14.1 – 21.2 mm). This probably explains why .tThe unripe fruit is said to be ethnomedmedicinally better than the ripe fruit (Rajasekaran *et al.*, 2009). The

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difference in the antibacterial activities of ripe and unripe fruit extracts and fractions of *N. latifolia* could probably be due to~~:~~:

(i)

Their compositional/phytochemical differences as shown in Table 4.2.

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~~i)~~ (ii)

The unripe fruit extract was found to be richer in tannins, which are known to be cytotoxic to bacterial cells (Jones *et al*., 1994).

~~ii)~~ (iii)

Ripening or over ripening of fruits could probably have caused distintegration reactions that led to production of some non-active/ not too active phytochemicals, since most ripe fruits are most often prone to microbial attacks (Levey *et al.*, 2007).

~~iii)~~ (iv)

~~iv)~~ Partial/non-solubility of the active constituents of the ripe fruits in methanol (Jigna and Sumitra, 2007).

The observed antibacterial activities of both the ripe and unripe fruits of *N. latifolia* can be attributed to the strong presence of phytochemical constituents such as alkaloids, saponins, flavonoids, tannins, steroidal compounds, coumarins and cardiac glycosides as shown in Table 4.2. These compounds are known to be biologically active and thus aid antibacterial/ antimicrobial activities of medicinal plants. Plants containing them have been reported to posses' antimicrobial, antifungal and other biological activities (Nair *et al*., 2005; Sudharameshwari and Radhika, 2007; Ramya *et al*., 2008a, b). The presence of tannins, saponins and flavonoids in the fruits probably accounted for the observed antidiarrhoeric and antidysenteric property (Kokate, 1988; Dharmananda, 2003), supporting the use of the plant as an antibacterial agent.

All the pathogens used in this study were most susceptible to the fractions of the ripeand unripefruits of *N. latifolia*, -thus supporting the use of these fruits in folklore remedies in the treatment of diseases caused by these microorganisms. The fractions inhibited the growth of both Gram-positive and Gram-negative organisms, showing that the plant possesses a broad-spectrum activity. The plant had earlier been described as one of the plants with promising anti-infective activity/ interesting biological activity (Iwu *et al.*, 1999). Ability of extracts and fractions to inhibit the growth of both Gram-positiveand Gram-negative makes the ripe and unripe fruits of *Nauclea latifolia* good candidates for the isolation of broad-spectrum antimicrobials (Omwenga *et al*., 2009).

5.8.2 Percent antibacterial activity of test compounds (A %) and susceptibility index of test organisms (BSI)

The calculated antibacterial percentual activities (A %) of the ripe and unripe fruits of *N. latifolia* (Figure 4.22) revealed that on the whole, the re-extracted soluble fractions of the unripe fruits gave higher A % value (50 - 100 %) than those of the ripe fruits (0 - 83.3 %), partitioned soluble fractions (0 - 66.7 %) than the crude extracts (0 - 50 %), showing that some of the re-extracted soluble fractions of both the ripe and unripe fruits showed better antibacterial potentials than their partitioned-soluble fractions and extracts, with some of them displaying promising activities similar to those displayed by the standards (83.3 - 100 %). The calculated bacterial susceptible index (BSI) revealed 0-2550 % , 0 - 50 % and 0 - 50% of the organisms tested were susceptible to the extract, partitioned-soluble fractions and ~~and~~ re-extracted soluble fractions of the ripe fruits, while 75 - 100 % were susceptible to the re-extracted soluble fractions of the unripe fruits, an activity better than those exhibited by some of the standards (33.3 -100 %) against some of the organisms as shown in Figure 4.23

Generally, the re-extracted soluble fractions of the unripe fruits exhibited better activity (broad-spectrum) against the test organisms, especially the Gram-positive bacteria, than those of the ripe fruits. This supports the findings of Karou *et al* (2006). A % and BSI are useful tools, which may help to choose the activebetter extract or fraction of a medicinal plant to be studied further (Eloff, 2004; Mahlke *et al*., 2009).

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5.8.3 Minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC) and antibacterial effect (MBC/MIC) of test ——compounds

The active petroleum ether extract of the unripe fruits, **uP** and active partitioned-soluble fractions, **E** and **B** all had an MIC that ranged from 12.5 – 25 mg/ml, while, the soluble fractions of both fruits ranged from 6.25 – 25 mg/ml in comparison with the standards that ranged from 0.20 – 0.50 mg/ml (Table 4.29). MBC of the extract was 100 mg/ml, partitioned fractions (12.5 – 50 mg/ml) and soluble fractions of both fruits ranged from 12.5 – 50 mg/ml in comparison with standards (0.20 – 0.530 mg/ml) as shown in Table 4. 30, an indication that the extracts/fractions of both the ripe and unripe fruits of *N. latifolia* were in their impure states, but they could with further fractionation and purification provide veritable source/sources of active pure antimicrobial agents. Determination of MICs and MBCs using this conventional method gives precise information regarding an organism's susceptibility to a plant extract/fraction. They are used to evaluate the efficacy of an antimicrobial agent (Croshaw, 1983; Morton and Vinks, 2005) or they correspond to the net result of microorganism growth and kill over the selected period of time (Mueller *et al*., 2004), or are described as quantitative

measures of a bacterial strain's susceptibility to anantimicrobial drug (Nester *et al.*, 2007).

The MBC/MIC ratios of the active extract ranged from 4 – 8.00, -partitioned-soluble, 1 - 2.00, soluble fractions of both fruits, 1.00 – 4.00, all in comparison with the standards (0.5 – 1.00) as shown in Table 4.31. The calculated MBC/MIC ratios for the active extract/fractions/antibiotics was used to ascertain if the observed antibacterial effects were bactericidal or bacteriostatic in nature. Generally, the active extract/fractions recorded a ratio > 1, indicative of a bacteriostatic effect. It was previously reported that extracts with MBC/MIC ratio > 1 would indicate a bacteriostatic effect of the extract, while lower than 1 is indicative of a bactericidal effect (Serra and Tessler, 1997; Agnese

et al., 2001; Karou *et al.*, 2005). The standard drugs had an MBC/MIC ratios ranging from 0.50 - 1.00, indicative of a bacteriocidal effect, while the extracts and fractions had ratios –ranging from 1.00 – 8.00, indicative of a bacteriostatic effect. Generally, the lower the MBC/MIC value of a drug/extract/fraction, the better is its potency (Fabry *et al*., 1998; Ndip *et al.*, 2009; Omwenga *et al*., 2009). Generally, with most bactericidal antimicrobials, the MIC and MBC values are often near or equal in value (Hugo and Russell, 1998).

5.8.4 Ethyl acetate partitioned-soluble fraction; its column fractions and column sub-fractions

The crude ethyl acetate partitioned-soluble fraction (**E**) exhibited a significant antibacterial activity at 50 mg/ml against the Gram-negative bacteria (17.3 – 17.6 mm) than the Gram-positive (0 – 8.25 mm), but generally, the fraction, exhibited activity that was lower than that of erythromycin at 1 mg/ml (9.6015.1 – 26.2 mm) as shown in Figure 4. 24). The fraction exhibited its highest activity against Gram-negative *S. typhi* (17.3 mm) and *K. pneumoniae* (17.6 mm), while it displayed low activity against Gram-negative *E. coli* (11.3 mm) which implies that the fraction might probably be useful in the treatment of *S. typhi*-induced diarrhoea, but might not be useful in the treatment of *E. coli*-induced diarrhoea or probably, the activity of the fraction, could be enhanced at higher concentration. Its column fraction, **E-2** expressed a broad-spectrum activity (10.514.8 – 16.73 mm) better than that showed by the soluble fraction, **E** (Figure 4.25). This is probably because the soluble fraction may contain 'inactive substances' which probably antagonized the antibacterial actions of one another (Ebi and Ofoefule, 1997). Its column sub-fraction, **E-2f** expressed a stronger boad spectrum

activity at 20 mg/ml (134.8 – 18.3 mm) than its mother column fraction, **E-2** and its mother fraction, **E** (Figure 4.26). The sub-fraction exhibited significant activity against Gram-positive *S. aureus* (14.8 mm) and Gram-negative *P. aeruginosa* (16.7 mm) and *S. typhi* (18.3 mm), indicating that the sub-fraction probably contributes to the anti-dysenteric and anti-diarrhoeal and other antibacterial property of the fruits. The sub-fraction, **E-2f** that is expectedly a mid polar sub-fraction, may contain more of mid polar/polar constituents, which probably means the observed better activity of **E-2f** could be due to the presence of these mid polar/polar components. This finding is in agreement with previous investigation of antibacterial potentials of mid-polar/polar extracts and fractions. It has been reported that such extracts or fractions or sub-fractions possessing mid-polar/polar components exhibit significant antibacterial/antimicrobial activities against some pathogens (Aljadi and Yusoff, 2003; Thurairajah and Abdulrahim, 2003; Pretto *et al*., 2004; Muskhazli *et al*., 2008; Udobi and Onaolapo, 2009; Nazemi *et al.*, 2010; Udobi *et al.*, 2010).

5.8.5 Acetone-soluble fraction; its column fractions and column sub-fractions

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The acetone-soluble fraction, **rA** expressed no significant activity at 5100 mg/ml (7.000 – 10.1 mm) in comparison with erythromycin at 1 mg/ml (9.60 – 26.2 mm) against any of the test bacterial strains (Figure 4.27), but was further fractionated because of its good yield and promising spots on TLC. Column fraction **rA-5** at 20 mg/ml exhibited a significant activity against Gram-negative *S. typhi* (19.0 mm) as shown in Figure 4.28. Generally, column fractions **rA-3** (10.03.4 - 15.5 mm), **rA-5** (9.0013.4 – 19 mm) and **rA-6** (14.1 – 17.4 mm) displayed moderate activity at 20 mg/ml against test bacteria,

like Gram-positive *S. aureus*, Gram-negative *S. typhi* and *E. coli*, which implies that these column fractions could be useful in the treatment of gastroenteritis and other bacterial infections caused by these organisms, such as, typhoid fever, urinary tract infections and skin infections. Generally, the column fractions were much more active than the acetone-soluble fraction (**rA**), an indication that fractionation improved its antibacterial activity. Sometimes, the amount of active components in crude extracts from medicinal plants may be small or diluted, so that when fractionated, these components become concentrated and therefore exhibit activity (Ndip *et al*., 2009). Fractionation of crude extract/fraction of a plant has reportedly improved the antibacterial/antimicrobial potency of some medicinal plants (Mastelic *et al*., 2005; Walia *et al*., 2007; Khuntong and Sudprasert, 2008; Aiyegoro *et al.*, 2009; Philip *et al*., 2009; Sharifi and Hazell, 2009; Udobi and Onaolapo, 2009; Ullah *et al*., 2009; Chakraborty and Chakraborti, 2010; Nazemi *et al*., 2010; Udobi *et al*., 2010).

Column sub-fraction **rA-5a** significantly inhibited the growth of Gram-negative organisms at 20 mg/ml (15.4 – 18.5 mm) but was less active against *K. pneumoniae* (7.40 mm) than sub-fraction **rA-5b** (17.9 mm) as shown in Figure 4.29. The activity of these sub-fractions were lesser than that of the column fraction, **rA-5**. –An indication that there seems to be a greater degree of synergism amongst the constituents of **rA-5**. This supports the finding of other medicinal plants (Fadipe *et al.*, 2006; Babayi *et al*., 2007; Aliyu *et al.*, 2008; Nazemi *et al.*, 2010).

5. 8. 5. 1 Compound rA-5a1

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The isolated compound, **rA-5a1** at 10~~10~~0 µg/ml displayed moderate antibacterial activity against Gram-positive *B. subtilis* (17.0 mm) and *S. aureus* (15.0 mm), an activity similar to that displayed by erythromycin at 1 mg/ml against these organisms (15.1 and 22.2 mm) as shown in Figure 4.30. This suggests that the compound might be useful in the treatment of infections caused by these organisms. This supports the findings of other authors (Alim Al-Bari *et al.*, 2006; Habib and Karim, 2009; Lyutskanova *et al.*, 2009; El-Sayed, 2012).

5.8.6 Ethyl acetate-soluble fraction; its column fractions and column sub-fractions

Fraction **uE** at 50 mg/ml displayed a significant broad-spectrum activity (17.3 – 21.5 mm) similar to that exhibited by erythromycin at 1 mg/ml (15.1 – 26.2 mm), Figure 4. 31. The column fractions (**uE-1 to uE-5**) at 20 mg/ml displayed moderate broad-spectrum antibacterial activity (8.5~~14.2~~ – 14.8 mm) against the test organisms (Figure 4. 32), an activity lower than that displayed by the soluble-fraction, **uE** at 50 mg/ml. This indicates that either the column fractions contain less active constituents or that the concentration used was too low to have brought about any significant activity. Also, it may be that there is a greater degree of synergism amongst the constituents of **uE**, so that it displayed better activity than its column fractions. This supports the finding of other medicinal plants, where column fractions/sub-fractions display lower activities that their mother extracts or fractions (Fadipe *et al.*, 2006; Babayi *et al.*, 2007; Aliyu *et al.*, 2008; Nazemi *et al.*, 2010). Column sub-fraction **uE-2** (10.4 - 14.6) and its sub-fraction, **uE-2a** (6.6 - 13.8)~~both~~ did not significantly inhibit the growth of any of the test

organisms at 20 mg/ml (Figure 4.33). Again, the role of increased concentration and synergism of combined fractions may have played a role.

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5.8.6.1 Compound uE~~Me2~~-2a1

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Compound **uE-2a1** exhibited moderate antibacterial activity at 100 µg/ml against Gram-positive *B. subtilis* (16.4 mm) and *S. aureus*(15.3mm), while it was not active against the Gram-negative bacteria (10 – 12.7mm), an activity lower than that displayed by erythromycin (9.60 – 26.2 mm) as presented in Figure 4.34. Its activity was similar to that displayed by compound **rA-5a1** (Figure 4.30).

5.8.6.2 Compound uE-3a2a

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The isolated compound at 100 µg/ml displayed low antibacterial activity against both Gram-positive (10.8 – 13.5 mm) and Gram-negative (10.4 – 13.5 mm) organisms in comparison with that displayed by erythromycin at 1 mg/ml (Figure 4.35). The compound only showed moderate activity against *E. coli* (15.1 mm). This suggests that the compound probably at higher concentration might be useful in the treatment of different bacterial infections and could make them useful antibacterial agents. Generally, β-sitosterol has been reported to possess low/moderate antibacterial activity against several bacterial strains as discussed in section 2.5 (Beltrame*et al*., 2002; Sen *et al*., 2012; Woldeyes *et al*., 2012; Rajpoot and Singh, 2014; Yadav *et al*., 2014).

CHAPTER SIX

6.0 SUMMARY, CONCLUSION AND RECOMMENDATIONS

6.1 Summary

Extraction of the secondary metabolites present in both fruits was achieved by maceration (crude extracts), liquid-liquid partition (partitioned-soluble fractions) and further extraction of water-insoluble portion (soluble-fractions of ripe and unripe fruits). Preliminary phytochemical screening of the extracts and fractions of both fruits revealed the presence of steroidal compounds, alkaloids, flavonoids, saponins, coumarins,

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tannins (higher in the unripe fruits) and glycosides. Fractionation, separation and purification of the ethyl acetate partitioned-soluble fraction of the methanol extract of ripe fruits (**E**) using standard chromatographic techniques afforded the isolation and characterization of an ortho-substituted benzanal derivative, which on structural elucidation was identified as 2- (2'-ethyl-3'-tertbutoxypropyl) benzaldehyde (compound **E-2f1a**). From the acetone-soluble fraction of the methanol extract of the ripe fruits (**rA**), a phthalate derivative, identified as di- (2-ethylhexyl) phthalate, DEHP (compound **rA-5a1**) was isolated. Fractionation, separation and purification of the ethyl acetate-soluble fraction of the methanol extract of unripe fruits (**uE**) led to the isolation and characterization of three compounds, identified as (i) di- (2-ethylhexyl) phthalate, DEHP (compound **uE-2a1**); same as was isolated from the ripe fruits, but of lesser quantity; (ii) ethenyl pentadecanoate, an unsaturated fatty acid ethyl ester, FAEE (compound **uE-2a2**) and (iii) β-sitosterol, a phytosterol (compound **uE-3a2a**).

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In-vitro antibacterial investigations of the extracts, partitioned-soluble fractions and soluble-fractions of both fruits using the agar-well diffusion method revealed that the pathogens used in this study were most susceptible to the soluble-fractions of the unripe fruits of *N. latifolia* at 50 mg/ml. The fractions displayed activity that was similar or better than that exhibited by some of standard drugs at 1 mg/ml. Fractionation of partitioned-soluble –fraction **E** yielded column fractions and sub-fractions that significantly inhibited the growth of some of the test organisms. Although, the acetone-soluble fraction of the defatted methanol extract of ripe fruits, **rA** exhibited low antibacterial activity against the test pathogens, its column fractions and sub-fractions exhibited promising antibacterial potentials, while fractionation of ethyl acetate-soluble fraction **uE,** yielded column fractions and sub-fractions that were less active than

fraction **uE**. Compounds **rA-5a1** and **uE-2a1** at 100 µg/ml exhibited moderate antibacterial activity against the Gram-positive pathogens (15.3 - 16.4mm), but less active against the Gram-negative pathogens (10 -12.7 mm) when compared with erythromycin at 1 mg/ml (15.1 -26.2 mm). Compound **uE-3a2a** also at 100 µg/ml exhibited low activity (10.4 – 13.5 mm), but was only moderately active against Gram-negative *E. coli* (15.1 mm) in comparison with erythromycin at 1 mg/ml.

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6.2 Conclusion

The research work carried out on extracts and fractions of the ripe and unripe fruits of *Nauclea latifolia* (Family Rubiaceae) respectively, led to the isolation of (i) 2- (2'-Ethyl-3'-tertbutoxypropyl) benzaldehyde (ii) Di- (2-ethylhexyl) phthalate, DEHP, both from ripe and unripe fruits (iii) Ethenyl pentadecanoate and (iv) β-Sitosterol. The activity exhibited by the fractions of both fruits, their column fractions and sub-fractions, support the use of the fruits of the plant in the treatment of dysentery and diarrhea and other infections caused by organisms used in this study. An indication that the soluble fractions of both fruits, especially, those of the unripe fruits, could make good candidates for the isolation of broad-spectrum antibacterials. The moderate antibacterial activity displayed by DEHP and β-sitosterol against Gram-positive pathogens and Gram-negative *E. coli* respectively, is an indication that may be with increased concentration, both could make good candidates for the treatment of infections caused by these organisms. The isolated compounds and may be others yet to be isolated present in these fruits could probably have acted either individually or synergistically to account for the antidysenteric, antidiarrheric and other antibacterial efficacy exhibited by the extracts, partitioned-soluble fractions, soluble-fractions, their

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column fractions andcolumn sub-fractions of the ripe and unripe fruits of *Nauclea latifolia.*

So far, this is the first report of the isolation of all compounds from the ripe and unripe fruits of the plant.

6.3 Recommendations

Isolation and structural elucidation of more biologically active compounds in other active fractions could not be achieved due to time constraint. It is therefore, recommended that:

i. There should be further isolation/studies of the active principles of other extracts and fractions of both the ripe and unripe fruits, which can provide more biologically active constituents, which may be used to develop more safe and potent antibacterial life-saving drugs.

ii. The antimicrobial potentials of these extracts and fractions against a wider range of bacteria (*Shigella* spps., *Helicobacter pylori*, *Streptococci* spps. and *Enterococci* spps.) fungi, virus and protozoan (*Entamoeba histolytica*) should be studied.

iii. Toxicological studies to determine the safety of the various extracts and fractions of both the ripe and unripe fruits should be carried out, in view of the isolation of phthalates from the fruits of *N. latifolia*.

iv. The isolates from the extracts and fractions of the plant should be tested further against *P. aeruginosa*, since the organism is hardly susceptible to most known antibiotics.

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v. Extracts and fractions of both fruits should be screened for their pharmacological and other biological activities, especially its antimalarial potentials, since the antimalarial potentials of other parts are well documented.

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